# PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) FOR SUSTAINABLE AGRICULTURE

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# Preface

#### Prof. Joseph W. Kloepper

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The Second Asian PGPR Conference continues and expands upon an important series of international scientific meetings. Beginning in the 1980s, the International PGPR Workshop has been held every three years.

The original reason for convening the PGPR Workshop was to create a single forum to bring together people engaged in research and development of beneficial plant-associated bacteria. These bacteria, called plant growth-promoting rhizobacteria (PGPR) and also PBPB (plant growth-promoting bacteria) are typically studied in widely diverse disciplines within universities, including departments of faculties of agronomy, forestry, horticulture, microbiology, plant pathology, environmental microbiology, crop science, soil science, and agricultural ecology. Hence, there was not a specific national or international organization for PGPR/PGPB.

Over the years, these international workshops have been held in North America, Europe, Australia, South America, and Asia. The 8<sup>th</sup> International PGPR Workshop was held in 2009 in Portland, Washington, USA and the 9<sup>th</sup> International will be held in Medell ń, Colombia in 2012. In addition to providing a forum for scientific exchange focused on PGPR, the International Workshops typically spawned interest in the field of beneficial bacteria and microbial inoculants for plants in countries that hosted the workshops. This was particularly true in Asia. Following the 6th International PGPR Workshop in Kerala, India, there was an increasing national commitment to funding R & D of bacterial inoculants, which resulted in a marked increase in the numbers of researchers and graduate students engaged in studies of PGPR.

Asia is now the continent with the largest number of researchers engaged in R & D related to PGPR. To me, this is a logical development, given the importance of agriculture to the peoples and economies of the Asian nations. While it is certainly true that more human beings on the planet need more food, the growth in agriculture in Asia during the 21<sup>st</sup> century is about much more than simply maintain the caloric intake of citizens. The rapidly growing middle class in India and China are now demanding more food choices. Fresh fruits and vegetables are equally important to middle class consumers world-wide as are the traditional "staple" crops of the country, such as wheat, rice, corn, and soybeans. At the same time, increases in meat consumption by the middle class result in increased demand for field crops used in animal feed. China alone produces nearly 450 million pigs each year, compared with 65 million in the U.S.

In recognition of the many researchers in Asia working on PGPR, the International PGPR Workshop agreed to encourage development of "Regional PGPR Workshops." The First Asian PGPR Congress was held in Hyderabad, Andhra Pradesh, India in

2009. Given the success of that meeting in bringing together PGPR workers from all parts of India and many other Asian countries, the decision was made to convene an Asian PGPR Conference every two years within Asian countries. Our Asian PGPR colleagues should be very proud of the leadership they have shown the world community on forming regional PGPR meetings as a way to synergize national and regional R & D. Researchers in Latin America hope to replicate the success of the Asian PGPR group by holding the First Latin American PGPR Workshop in conjunction with the 9<sup>th</sup> International Workshop in 2012.

Finally, it has been said that this century will be the century where India and China become world leaders in many economic sectors. I am very pleased to see that both countries have already assumed leadership in the area of PGPR for sustainable agriculture. I offer my sincere thanks to the hundreds of people who made the Second Asian PGPR conference a great success. I especially thank my colleague and friend, Prof. M.S. Reddy, Department of Entomology and Plant Pathology, Auburn University, USA whose vision and tenacity led to the Asian PGPR Conference series.

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The conference would not have been a successful event without the support of the students, faculty and staff of Department of Plant Pathology, China Agricultural University, Beijing who smoothly coordinated various committees. The organizers are specially thankful to the untiring efforts of Dr. Yan Li and Shashi KR. Yellareddygari to make this conference a great success. The organizers also thank all the delegates of the 2<sup>nd</sup> Asian PGPR Conference for sharing their research experiences. We would like to place on record special thanks to Mr. Mark Bransby, our Webmaster, College of Agriculture and AAES /ACES/Ag IT Department, Auburn University for his help in designing Asian PGPR website and for online help to make this conference a great success.

Finally, the organizers gratefully acknowledge the generous financial support offered by our co-organizers: National Natural Science Foundation of China, Jining Sanhuan Chemical Industry Co., Ltd., Xinjiang Tianwu Science & Technology Development Co., Ltd., KIWA Bio-Tech Products Group Corporation, China Green Health Agriculture (Beijing) Biotechnology Co., Ltd., and Prathista Industries Limited, Secunderabad, Andhra Pradesh, India.

Prof. M. S. Reddy Chairman, Asian PGPR Society Department of Entomology and Plant Pathology Auburn University Auburn, AL, USA

# Section -I

# **Plenary Session Lectures**

# Commercial potential of biofertilizers and biofungicides (PGPR) for sustainable agriculture in Asia and the scope of Asian PGPR Society

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#### Abstract

Research collaborators throughout Asia have conducted work on various crops that include: legumes, row crops, vegetables, rice, ornamentals, forestry, spices etc. The rationale for our research is that there is a great need for environmentally-friendly microbial technologies (PGPR) in Asian agriculture. Asian agriculture is plagued by two main constraints. The first is the depletion of nutrient supply in the agricultural soils, and consequently, the sizable gap between achievable and actual yields in various crops. The second main constraint is crop protection. Devastating pathogens lead to  $15\% \sim 30\%$  average crop losses annually. Despite the constraints, the Asian

continent has made great strides in agricultural production. Sustainable approaches are those that are not aimed solely at maximizing short-term production but rather those that consider long-term production gains, the ecology of agricultural systems, and profitability of farmers. Further, sustainable solutions result in empowerment of women, farm laborers, and rural communities. Our research deals with restructuring the crop rhizospheres for improving and sustaining the nutrient supply in the soils and enhancing the health and yield of crops through sustainable practices based on microbial technologies. By microbial technologies, we mean the principles of microbial ecology, which encompass inoculation of crops with beneficial microorganisms and the use of cultural practices that enrich indigenous beneficial microorganisms in individual agricultural fields. There are two main outcomes or effects from beneficial microorganisms: enhanced plant growth and crop protection, both of which represent the two main constraints to Asian agriculture. Our network partnership in Asia has correctly identified the use of microbial inoculants to provide holistic health and sustainable crop yields. Each partner in the Asian network has made substantial contributions to Asian agriculture in research with plant-associated rhizobacteria. These research leaders bring their expertise together in building a US-Asia Partnership in Higher Education. Several large development projects in agriculture failed after initiation because of new pest and soil health problems. There is an urgent need to promote integrated pest/disease management at a faster rate and it is driven by emphasizing organically-produced food, conservation of biodiversity, unpolluted environment, and sustainable agriculture. However, the adoption of biopesticides by farmers in Asia is still in its infancy. While already threatened by the unforeseen drought spells, crops suffer the frequent outbreaks of pests that lead to total crop failure. Because of such crop failures, the resource poor farmers cannot afford expensive crop protection technologies, and remain poor. Development and propagation of low-cost technologies would certainly help in the improvement of these farmers' economic situations and thereby eliminate the cycle of poverty through collaborative efforts within the scope of Asian PGPR Society.

# Research and application of Bacillus in China

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#### Abstract

Environmental problems have raised great interest in environmental friendly sustainable agricultural practices. The use of growth promoting rhizobacteria (PGPR) is a promising solution for sustainable, environmentally friendly agriculture. The research on PGPR started in China in 1970's, when much work on biological control of soil-borne plant pathogens had been done. In 1980s, much research has been done on *Bacillus*. "YIB" (Yield Increasing Bacterial) was a successful example of PGPR which was used on a large scale in China. Since then, the research on *Bacillus* PGPR increased sharply as it could form endospore and has high resistance to adverse conditions.

The research on *Bacillus* PGPR in China included isolating and screening antagonists targeted different diseases, evaluating their effectiveness in greenhouse and field, dissecting their mechanisms, improving the fermentation art, and expanding their application. Research on improvement of *Bacillus* by genetic engineering is also conducted in order to increase effectiveness. Some of them showed stable and devastating activity and were widely used in field in a quite large area. So far, seven species of *Bacillus* have been registered as biopesticides in China. Some strains were registered as biofertilizers.

Plant microecology is a complex system with all members interrelated. It is a good source to obtain *Bacillus* PGPR strains and develop biopesticides and/or biofertilizers because their intimate relationship with plants. High throughput screening system and large *Bacillus* PGPR library has been built in China.

*Bacillus* PGPR control the damage to plants from phytopathogens and promote the plant growth by a number of different mechanisms. Successful colonization on/around the host plant is the precondition for their effective functions. Biological assays showed that attenuation of the chemotaxis-encoding gene *cheA* or the flagellin-encoding gene *flaA* reduced bacterial populations in the rhizosphere. Attenuation of SOD gene(s) resulted in reduced populations of *B. cereus* 905 in the wheat rhizosphere. Antagonists can reduce plant diseases by various mechanisms. Nutrient and space competition, production of enzymes that inhibit the phytopathogen, stimulation of the systemic resistance of the plant, and growth substrate production is another angle to disclose their action mode.

# Impact of induced systemic resistance on the bacterial rhizosphere microflora

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#### Abstract

Induced systemic resistance (ISR) has been described for several strains of fluorescent pseudomonads in a variety of crop plants and is effective against a wide range of pathogens. Using the model plant *Arabidopsis thaliana*, progress has been made in understanding signal transduction pathways involved in induced resistance. Perception of ethylene and jasmonic acid is important for ISR. The traits of *Pseudomonas* spp. that can trigger ISR appear to be diverse, and include iron regulated metabolites, antibiotics, and lipopolysaccharides. Since pathogen growth is restricted on plants that are in the state of ISR, we hypothesized that the indigenous microflora could also be affected by ISR. Using cultivation dependent and independent techniques, effects of plant defense signaling on the total bacterial and the *Pseudomonas* spp. microflora of Arabidopsis were studied and related to susceptibility of Arabidopsis genotypes to bacterial speck caused by *Pseudomonas syringae* pv. *tomato*.

Key words: fluorescent *Pseudomonas* spp.; induced systemic resistance; phyllosphere; root colonization

# Introduction

Induced systemic resistance (ISR) triggered by selected plant growth-promoting *Pseudomonas* bacteria is effective against a wide range of plant pathogens (Bakker *et al.*, 2007). Activation of ISR in plants leads to an enhanced defensive capacity enabling plants to respond faster and/or more effectively to microbial attackers (Van Loon *et al.*, 1998; Conrath *et al.*, 2002; Verhagen *et al.*, 2004). ISR requires an intact response to jasmonic acid (JA) and ethylene (ET), although it is not associated with increased production of these hormones, and neither with increased expression of known defense-related genes (Van Wees *et al.*, 1999; Pieterse *et al.*, 2000; Verhagen *et al.*, 2004). Arabidopsis mutants defective in expression of ISR and/or altered in salicylic acid (SA) signaling, and exogenous application of methyl-JA and SA were used to study the possible impact of defense signaling on the rhizosphere microflora. Denaturing gradient gel electrophoresis (DGGE) revealed that mutants of *Arabidopsis thaliana* affected in the JA and/or SA responsive signal transduction pathway developed a bacterial rhizosphere microflora that differed from the one on the wild-

type control, however, application of JA or SA did not affect the bacterial community structure (Doornbos *et al.*, 2011). In the present study possible effects of selected Arabidopsis mutants that differ in their susceptibility to *Pseudomonas syringae* pv. *tomato* DC300 (*Pst*) on rhizosphere and phyllosphere bacterial and *Pseudomonas* spp. populations were evaluated.

## **Material and Methods**

## Cultivation of plants

Arabidopsis genotypes used are listed in table 1. Seeds were sown in autoclaved sand in shallow plastic containers. The containers were covered and kept at 4 °C in the dark for 2 days, after which the seeds were allowed to germinate in a greenhouse conditioned as described below, at 100% relative humidity. Routinely, 2–week-old seedlings were transferred individually to 60 ml pots containing a potting-soil/sand mixture (12:5 v/v) that had been autoclaved twice for 20 min with a 24 h interval. However, plants used for analysis of abundance and diversity of indigenous *Pseudomonas* populations in the rhizosphere were transplanted into non-autoclaved potting soil-sand mixture. After transplanting, plants were grown in the greenhouse with an 8 h-day (200  $\mu$ E m<sup>-2</sup> sec<sup>-1</sup>) at 24 °C and 16 h night cycle at 20°C and 70% relative humidity, and watered with half-strength Hoagland nutrient solution once a week and with tap water as required.

#### Disease induction and assessment

Five-week-old plants were inoculated with *Pst* as described by Pieterse *et al.* (1996). Briefly, plants were placed at 100 % relative humidity one day before inoculation. *Pst* was cultured overnight in liquid KB medium (King *et al.* 1954) at 28 while shaken at 180 rpm. Bacterial cells were washed by centrifugation for 5 min at  $1.200 \times g$  and resuspended in 10 mM MgSO<sub>4</sub>. Leaves were dipped in a bacterial suspension of  $2.5 \times 10^7$  cfu/ml *Pst* supplemented with 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands), and kept at 100% relative humidity. Four days after inoculation, disease severity was quantified by determining the fraction of leaves per plant showing necrotic lesions and/or chlorosis. From ten plants of each genotype, leaves of five replicates consisting of two pooled plants were ground in a mortar and pestle in 10 mM sterile MgSO<sub>4</sub>. Population densities of *Pst* were quantified by plating appropriate dilutions on KB agar (King *et al.* 1954) supplemented with 100 µg/ml natamycin and 50 µg/ml rifampicine. Numbers of colony forming units (cfu) were determined after incubation for 48 h at 28 °C (Pieterse *et al.*, 1996).

Genotype	Relevant characteristics
Col-0	Wild-type accession Colombia-0
cpr1-1	constitutive expressor of PR genes 1, SA overproducer
etr1-1	ethylene response 1, ET insensitive
jar1-1	jasmonate resistance 1, JA insensitive
NahG	Transformant expressing bacterial SA hydroxylase, does not accumulate SA
npr1-1	non-expressor of PR-genes, impaired in SA and JA/ET-dependent defense responses

Table 1 Listing and relevant characteristics of Arabidopsis lines used

#### Quantification of bacterial populations in the rhizosphere

Roots with tightly adhering soil were harvested and shaken three times in 10 mM MgSO<sub>4</sub> with glass beads ( $0.6 \sim 0.8$  mm) on a vortex at maximum speed for 1 min. Population densities of aerobic heterotrophic bacteria were determined by plating serial dilutions on 1/10 strength TSA<sup>+</sup> [3 g/L tryptic soy broth (Difco Laboratories, Detroit, Mi, USA), 13 g/L agar technical (Oxoid Ltd, Basingstoke, UK) and 100 µg/ml natamycin (Delvocid, DSM, Delft, NL)] and counting colony forming units (cfu) after 7 days incubation at 20 °C. Quantification of *Pseudomonas* spp. was performed by plating appropriate dilutions on KB<sup>+</sup> agar [KB agar (King *et al.* 1954), supplemented with 13 µg/ml chloramphenicol, 40 µg/ml ampicillin and 100 µg/ml natamycin (Delvocid, DSM, Delft, NL) and counting cfu after incubation for 48 h at 28 °C.

#### Analysis of bacterial phyllosphere communities

From ten 5-week-old plants, three leaves were removed with sterile scissors and placed in an Eppendorf vial. Weighed samples were ground in 10 mM sterile MgSO<sub>4</sub> with a sterile Eppendorf pestle (Eppendorf, Hamburg, Germany). Population densities of aerobic heterotrophic bacteria were assessed by plating serial dilutions on 1/10 strength TSA<sup>+</sup> [3 g/L tryptic soy broth (Difco Laboratories, Detroit, Mi, USA), 13 g/L agar technical (Oxoid Ltd, Basingstoke, UK) and 100 µg/ml natamycin (Delvocid, DSM, Delft, NL)]. Numbers of cfu per gram of leaf were determined after incubation for 7 days at 20 °C . Quantification of predominantly *Pseudomonas* spp. was performed by plating appropriate dilutions on KB<sup>+</sup> agar [KB agar (King *et al.*, 1954), supplemented with 13 µg/ml chloramphenicol, 40 µg/ml ampicillin and 100 µg/ml natamycin (Delvocid, DSM, Delft, NL). Numbers of cfu were determined after incubation for 48 h at 28 °C.

#### **Results and Discussion**

# Disease severity and Pst population densities in Arabidopsis mutants affected in defense signaling

A significantly higher percentage of diseased leaves was observed in the mutants *etr1* and *npr1*, and in the NahG transformant as compared to the wild type. Population densities of *Pst* were determined by selective plating and varied between  $10^4$  and  $10^6$  cfu per gram of leaf. Although no significant differences in *Pst* populations were observed, their numbers were approximately 10-fold lower in the *cpr1* mutant, which constitutively expresses SA-dependent defenses. In line with this observation, the genotypes unable to express SA-dependent defense responses, *npr1* and NahG, displayed 8- and 6-fold higher population densities of the pathogen, respectively. However, a significant correlation between disease severity and population densities of the pathogen was not apparent for the different genotypes.

# Populations of culturable bacteria and Pseudomonas spp. in the phyllosphere and rhizosphere of Arabidopsis genotypes differing in Pst susceptibility

To assess the effect of altered defense signaling on the indigenous bacterial microflora in the phyllosphere, the same genotypes were assayed for their bacterial microflora in the absence of *Pst*. Population densities of aerobic bacteria and *Pseudomonas* spp. were quantified by selective plating. Compared to Col-0, higher population densities of culturable bacteria were found in the phyllospheres of *etr1*, *npr1*, and NahG. For the *Pseudomonas* spp. it was observed that not only *etr1*, *npr1*, NahG, but also *jar1* harbored significantly higher population densities. Apparently, increased susceptibility of a genotype to *Pst* is correlated with higher population densities of indigenous bacteria in the phyllosphere.

Population densities of total culturable bacteria in the rhizospheres of the different Arabidopsis genotypes ranged from  $2 \times 10^7$  to  $1 \times 10^9$  cfu per gram of rhizosphere soil. The JA-response mutant *jar1*, the ET-response mutant *ein2* and the constitutive SA-producing *cpr1* showed significantly lower numbers of culturable bacteria compared to the Col-0 wild type. Numbers of cfu of *Pseudomonas* spp. in the rhizosphere were between  $5 \times 10^5$  and  $5 \times 10^7$  per gram root and demonstrated tendencies similar to total bacteria, except for *ein2*. However, *Pseudomonas* populations seemed more sensitive to SA-dependent defenses, indicated by a decreased abundance in *cpr1* and a tendency of increased bacterial numbers in the NahG rhizospheres.

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# Do cropping practices and systems influence fluctuations in PGPR populations or vice versa? *Is it a chicken or egg situation?*

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#### Abstract

Rhizosphere and soil bacteria are important drivers in nearly all biochemical cycles in terrestrial ecosystems and participate in maintaining health and productivity of soil in agriculturally managed systems. However, the effect of agricultural management systems on bacterial communities is still poorly understood. 454- Pyrosequencing based analysis of the V1-V3 16S rRNA gene region was used to identify shifts in soil and rhizoshpere bacterial diversity and community composition under different cropping systems in Manitoba, Canada. This included monoculture vs. rotation, zero tillage vs. conventional tillage, organic farming vs. conventional farming. The generated dataset composed of 215,000 high quality sequences, which were affiliated to bacterial taxonomy by comparing sequences to the 16S rRNA database. In order to assess the effects of agriculture management on bacterial communities, the data were further analyzed using generalized linear mixed-model methodology (GLIMMIX) of SAS by fitting normal, Poisson and negative binomial distributions. Results showed that different cropping systems did not influence the diversity of bacterial communities. However, a significant variation in relative abundances of bacterial communities at both phylum and genus level was observed among different cropping systems. Compared to conventional farming systems, organic farming system had higher percentage of phylum Proteobacteria (many PGPR) and lower percentage of phylum Actinobacteria. When canola monoculture was compared to wheat-oatcanola-pea rotation, significantly higher percentage of Proteobacteria and lower percentage of Actinobacteria were found in rotation system. Wheat monoculture shared similar bacterial communities with wheat-oat-canola-pea rotation. Zero tillage did not change bacterial communities profile except for an increase in Firmicutes (many PGPR) compared to conventional tillage. At the genus level, significant differences were found for the dominant genera Pseudomonas, Rhizobium, Stenotrophomonas, Brevundimonas, Burkholderia, Marmoricola, Microlunatus, and Solirubrobacter. The bacterial distribution correlated with soil pH and Carbonate C (lime) content. This comprehensive study provided fundamental information about how different cropping systems affect soil and rhizosphere bacterial communities, which can be used to guide Manitoba farmers to choose proper farming systems to maintain soil health and increase PGPR populations in soil.

### Introduction

Over the last decades, world agriculture experienced high increase in crop yield. This was achieved through high input of inorganic fertilizers and pesticides, and mechanization driven by fossil fuel. Over the years this led to serious environmental problems such as depletion of soil quality and health, ocean and ground water pollution, and emergence of resistant pathogens. It is a big challenge to feed the increasing world population on decreasing farmland areas without damaging environment. It is well known that rhizosphere and soil microorganisms (PGPR) play an important role in maintaining crop and soil health through versatile mechanisms: nutrient cycling and uptake, suppression of plant pathogens, induction of resistance in plant host, direct stimulation of plant growth (Kloepper et al., 2004; Haas and Defago 2005). Maintaining biodiversity of PGPR in soil could be an important component of environment-friendly sustainable agriculture strategies. Some studies have demonstrated that agricultural practices affecte the diversity and function of rhizosphere and soil microorganisms (Mader et al., 2002; Esperschutz et al., 2007; Sugiyama et al., 2010). Organic farming differs from conventional agriculture in the production process and it relies on techniques such as crop rotation, green manure, and biological pest control to maintain the soil productivity instead of chemical fertilizer and pesticides (Zhengfei, 2005). Tillage is a common practice in modern agriculture that involves mechanical manipulation of soil to enhance decomposition of crop residues to prepare seedbeds for planting. It also serves as a method of postemergence weed control and a management strategy to reduce the incidence of diseases and pests. However, extensive tillage leads to soil erosion and environemtal pollution. There are two types of tillage systems: conventional tillage and conservation tillage [CT] (at least 30% residue left on the soil surfaces). It was found that CT can reduce soil erosion and increase soil organic matter and microbial biomass compared to conventional tillage (Drijber *et al.*, 2000; Kabir, 2005). One drawback of CT is that some soilborne plant diseases can reach to damaging levels as pathogens survive on crop residues left on the soil surface (Guo et al., 2005). An approprate agricultural strategy such as crop rotation can prevent this problem by excluding pathogen hosts.

The objective of this study is to explore bacterial structure including PGPR consortium changes under different cropping practices and systems, and get better understanding how to build soil holistic ecology to maintain the health and productivity of plants.

The methods used to investigate microbial structure and composition include culture-dependent and molecular methods. Culture-dependent method only can assess less than 1% microorganisms dwelling in soil. Moelecular methods are powerful tools to explore microbial structure and composition. Pyrosequencing is a high-throughput DNA-sequencing technique based on sequencing-by-synthesis and has the potential to detect, cost effectively, low abundant unculturable microbial species (Roesch *et al.*, 2007).

# **Materials and Methods**

**Experimental design and soil samples collection:** The experiments were conducted at Ian Morrison Research Station located in Carman and Glenlea Research Station in Glenlea, Manitoba, Canada, Canola monoculture trial, wheat monoculture trial, wheat-canola-oat-pea rotation trial as well as zero tillage and conventional tillage trials were set at Ian Morrison Research Station. A randomized complete block design with three replicates was used for the monoculture and rotation trials. Conventional and zero tillage practices were conducted on the canola monoculture trial. At Glenlea Research Station, conventional and organic farming systems trials were investigated. The experiment design was randomized completed block design in a split-plot arrangement with three replicates. Two crop rotations including flax-oat-faba beanwheat (grain only rotation) and wheat-alfalfa-alfalfa-flax (grain-forage rotation) were main plots, and certificated organic and conventional methods served as subplots. In 2006, bulk soil and rhizosphere soil were randomly collected at Ian Morrison Research Station. In 2008, bulk soil and rhizosphere soil were sampled throughout wheat plots at Glenlea Resereach Station. Each sample was mixed well in plastic bags and kept at -20  $^{\circ}$ C until DNA extraction.

**DNA extraction from soil:** The total soil DNA was extracted from pre-washed soil samples (He *et al.*, 2005) using the Powersoil DNA isolate kit according to the manufacturer's specifications (Mobio Labs, Solana Beach, CA). The bulk soil and rhizoshpere soil DNA under the same treatment were pooled before pyrosequencing.

**Pyrosequencing:** DNA samples were pyrosequenced using bacterial tag-encoded GS FLX-Titanium amplicons as described by Dowd *et al.*, 2008. A mixture of Hot Start, HotStar high fidelity taq polymerases and Titanium reagents were used to perform a one-step PCR (35 cycles) with primer 27F, which covered the variable regions V1 to V3 of the bacterial 16S rRNA genes. Mothur software package (Schloss *et al.*, 2009) was utilized to perform sequence quality control. All sequences shorter than 200 bp, having an ambiguous base, or containing a homopolymer length equal or greater than 8 bp were removed from the dataset. Sequences were then aligned against a database of high quality 16S bacterial sequences derived from Silva database. The furthest neighbor algorithm with a cutoff of 95% similarity was used to assign sequences to operational taxonomic units (OTU). Chao1 richness indices, Shannon diversity estimators and Good's non-parametric coverage index were calculated based on the OTU data. Representative sequences from each OTU were taxonomically classified with a confidence level of 60% using RDP Baysian approach (Wang *et al.*, 2007).

**Statistical analysis:** Percentage data approach was used to evaluate statistical differences among treatments at phylum and genus taxonomical level. In this approach, the raw data for each taxon was first transformed to the percentage of that taxon in an individual sample. Normal distribution of percentage data of each phylum and genus was tested using UNIVARIATE procedure of SAS (2004). For data that were not normally distributed, Poission and negative binomial distribution model in

GLIMMIX procedure of SAS (ver9.2; 2004) were then used to assess the effect of treatments. The differences between treatments were considered significant at P<0.05. To identify treatment effect (JMP ver8; SAS Institute Inc., Cary, NC), Principal component analysis (PCA) of genus data was performed on the genus taxon level (percentage >0.5). To test the effect of soil edaphic properties on core bacterial phylum, canonical community ordination (CANOCO) was used (Plant Research International BV, Wageningen, the Netherlands).

#### **Results and Discussion**

Generally, pyrosequencing data revealed that different cropping practices and systems did not influence the diversity of bacteria in our tested trials (Table1, Table 2). However, they altered the relative abundance of bacterial communities at phylum (Fig.1) and genus levels (Table3, Table4). Compared to conventional farming systems, organic farming systems have similar OTU, richness and diversity except for Good's coverage estimator, which only reached 76% (average of grain only organic and forage-grain organic) meaning 76% of total species presented in samples (Table1). Wheat monoculture had significant highest OTU and Chao1 richness estimator, with 2755 and 4870.79, respectively, when compared with wheat rotation, canola monoculture and canola rotation. Compared to conventional tillage, Zero tillage had relatively higher OTU and Chao1, though statistically non-significant (Table2). It was argued that diversity parameters only based on OTU without taxonomic identity of different bacteria were not sensitive enough to investigate the effect of treatment on the bacterial structure because changes in some bacterial groups could be compensated by changes of other groups (Hartmann and Widmer, 2006).

When sequences were affiliated to different taxonomic phylum and genus level, significant variation of bacterial structure was observed (Fig. 1, Table 3, Table 4). At Ian N. Morrison Research Station, Canola rotation had higher percentage of Proteobacteria (49.17%) while canola monoculture, wheat monoculture and wheat rotation had an average of 31% Proteobacteria. However, a relatively higher percentage of Actinobacteria was found under canola and wheat monoculture (43.8%) compared to canola and wheat rotation (33.37 %) (Fig.1A). Within phylum the percentages of genera Rhizobium, Proteobacteria, Variovaorax, and Pseudomonas were higher in canola rotation compared to canola monoculture (Table 3). Canola releases compounds such as glucosinilate, which are inhibitory to some microorganisms (Garyston and Germida, 1990). When canola is rotated with other crops the concentration of these inhibitors in the soil could be diluted. Principal component analysis revealed wheat and canola supported different bacterial genus (Fig.2A). These crops produce different exudates, which attract different bacterial populations. Compared to conventional tillage, zero tillage shared similar bacterial phyla profile, except for an increase in percentage of Firmicutes (5.16%) (Fig.1B). At genus level, PCA analysis also gave the similar pattern of bacterial composition under conventional and zero tillage farming practices (Fig.2B). From this point, we

assumed that zero tillage would not change PGPR consortium dwelling in soil, which was important for controlling soil born pathogens.

At Glenlea Research Station, it was found that percentage of phyla Proteobacteria was significantly high under organic farming system (44.45%) while it was only 27.25% under the conventional farming system. Interestingly, the percentage of Actinobacteria showed the opposite pattern, which reached 43.12% under conventional farming systems and 32.48% under organic system (Fig.1C). When genus level of bacteria was analyzed, the relative abundances of different genera belonging to phyla of Proteobacteria (many PGPR) and Actinobacteria varied among the samples. The genera belonging to phylum Proteobacteria, such as Pseudomonas, Stenotrophomonas, Brevundimonas, and Burkholderia were more frequently found in Many PGPR isolates belong to genus organic farming systems (Table4). Pseudomonas, Stenotrophomonas, and Burkholderia (Haas and Défago, 2005; Ryan et al., 2009). It was found that conventional farming systems associated with higher percentage of Actinobacteria. Some studies have showed that actionbacterial fraction in bacterial community become greater in cultivated soil compared to pasture and forest soils (Lauber et al., 2008). Actinobacteria is able to degrade a variety of organic compounds including some herbicides and pesticides. The presence of pesticides and fertilizers might stimulate Actinobacteria population. It was reported that human activities including cultivation and urbanization encouraged the actinobacterial communities (Hill et al., 2010). Our results are consistent with these previous findings. Principal component analysis of pyrosequencing patterns for bacterial genus taxon variability revealed that samples from organic farming system scattered along the PCA1, while samples from the conventional farming system clustered along PCA2 (Fig.2C). Canonical correspondence analysis tested the effect of soil edaphic properties on samples by using an unconstrained analysis (RDA) (Fig. 3). pH accounted for 24% variance (P = 0.06), and CaCO<sub>3</sub>C for 19% variance (P =0.02). The C: N ratio only explained less than 5% variance (P = 0.52). Compared to conventional farming systems, organic farming systems lead to neutral soil pH (data not shown). It was reported that soil pH is main factor to influence bacterial communities (Lauber et al., 2009). Neutral soil pH could create friendly environment for survival and reproduction of beneficial bacterial consortia.

Rotation	Management Number of trimmed sequence	Number of	Mean (SEM) results for indicated varible					
		trimmed sequences	OTU <sup>1</sup> (95% distance)	Coverage (%)	Chao1	NpShannon		
Grain only	Organic	30,482	1917 (236.5)	78.77 <sup>a,b</sup> (2.72)	3659.96 (512.14)	7.21 (0.22)		
	Conventional	20,473	1628 (236.5)	84.53 <sup>a</sup> (2.72)	2889.74 (512.14)	6.83 (0.22)		
Forage-grain	Organic	23,923	2118 (236.5)	73.22 <sup>b</sup> (2.72)	4308.71 (512.14)	7.60 (0.22)		
	Conventional	31,552	1860 (236.5)	80.34 <sup>a.b</sup> (2.72)	3494.36 (512.14)	7.21 (0.22)		
R(P-Value)			0.35	0.11	0.20	0.2		
M(P-Value)			0.30	0.04	0.13	0.2		
RxM( <i>P</i> - Value)			0.95	0.83	0.96	0.97		

#### Table 1 Summary of pyrosequenced 16S rRNA sequences from the Glenlea samples

a,b - Means with different letters are significantly different (for management at P<0.05). Standard error of means is displayed in parentheses.;10TU=operational taxonomic units; R - Rotation; M - Management.

Treatment	Number of trimmed		Mean (SEM) results from indicated variable					
	sequences	OTU distance)	(95%	Coverage (%)	Chao1		NpShannon	
Canola monoculture	15,116	1581.67 (249.97)	b	83.2 (0.84)	2928.75 (379.36)	b	6.78 (0.28)	
Canola rotation	14,604	1540.67 (249.97)	b	82.4 (0.84)	3047.44 (379.36)	b	6.75 (0.28)	
Wheat monoculture	13,254	2755.00 (249.97)	а	81.1 (0.84)	4870.79 (379.36)	a	7.78 (0.28)	
Wheat rotation	16,025	1702.00 (249.97)	b	82.7 (0.84)	3081.89 (379.36)	b	6.82 (0.28)	
Conventional tillage	15,116	1581.67 (271.77)	b	83.2 (0.01)	2928.75 (469.13)	b	6.78 (0.34)	
Zero tillage	23,208	2141.67 (271.77)	a,b	85.1 (0.01)	3947.21 (469.13)	a,b	6.99 (0.34)	

#### Table 2 Summary of pyrosequenced 16S rRNA sequences from the Ian Morrison samples

a,b,c - Means with different letters are significantly different for treatment at P < 0.05.

This research is one of the first to exam how different cropping practices and systems influence PGPR and bacterial consortia. Organic farming systems, zero

tillage and crop rotation are beneficial for some PGPR consortia. Further studies are needed to find out other factors, such as bacteria: fungi ratio and soil borne pathogen survey, which affect PGPR fluctuation. This will be essential in developing more sustainable and eco-friendly farming systems.



Figure1 Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA sequences under different cropping systems

Phylum; Family; Genus	Canola Rotation	Canola Monoculture	Wheat Monoculture	Wheat Rotation	SEM	P-Value
Proteobacteria; Rhizobiaceae; Rhizobium	3.48 <sup>a</sup>	0.26 <sup>b</sup>	0.25 <sup>b</sup>	0.29 <sup>b</sup>	0.5	0.04
Proteobacteria; Comamonadaceae; Variovorax	1.53 <sup>a</sup>	0.44 <sup>b</sup>	0.33 <sup>b</sup>	0.48 <sup>b</sup>	0.18	0.006
Proteobacteria;Pseudomonadaceae; Pseudomonas	1.52	0.48	0.39	2.39	0.75	0.32
Actinobacteria;Geodermatophilaceae; Blastococcus	0.44 <sup>b</sup>	0.70 <sup>b</sup>	1.95 <sup>a</sup>	1.68 <sup>a</sup>	0.25	0.007
Actinobacteria;Pseudonocardiaceae; Pseudonocardia	0.69 <sup>b</sup>	0.65 <sup>b</sup>	1.36 <sup>a</sup>	$1.60^{a}$	0.15	0.004
Actinobacteria;Solirubrobacteriaceae; Solirubrobacter	0.49 <sup>b</sup>	0.69 <sup>b</sup>	1.9 <sup>a</sup>	1.27 <sup>a,b</sup>	0.24	0.01

# Table 3Phylogenetic composition (percentage) of bacteria fluctuation at genus level under monoculture and rotationfarming practices at Ian Morrison Station

and conventional faithing practices in Gremea Research Station							
Phylum; Family; Genus	Organic	Conventional	SEM	P-value			
Actinobacteria; Kineosporiaceae; Quadrisphaera	0.2	0.41	0.05	0.007			
Actinobacteria; Intrasporangiaceae; Lapillicoccus	0.25	0.46	0.06	0.008			
Actinobacteria; Nocardioidaceae; Aeromicrobium	0.26	0.41	0.06	0.08			
Actinobacteria; Nocardioidaceae; Marmoricola	0.71	1.06	0.14	0.08			
Actinobacteria; Propionibacteriaceae; Microlunatus	1.13	2.03	0.21	0.008			
Actinobacteria; Pseudonocardiaceae; Pseudonocardia	1.33	2.1	0.19	0.009			
Actinobacteria; Rubrobacteriaceae; Rubrobacter	0.53	1.21	0.18	0.01			
Actinobacteria; Solirubrobacteriaceae; Solirubrobacter	0.83	1.62	0.16	0.002			
Bacteroidetes; Sphingobacteriaceae; Pedobacter	0.61	0.16	0.56	0.13			
Chloroflexi; Chloroflexaceae; Roseiflexus	0.92	1.88	0.22	0.007			
Proteobacteria; Caulobacteraceae; Brevundimonas	6.01	0.1	0.58	0.0004			
Proteobacteria; Caulobacteraceae; Phenylobacterium	0.51	0.31	0.08	0.11			
Proteobacteria; Methylobacteriaceae; Microvirga	0.21	0.42	0.06	0.02			
Proteobacteria; Burkholderiaceae; Burkholderia	3.4	0.07	1.1	0.02			
Proteobacteria; Comamonadaceae; Variovorax	1.02	0.39	0.38	0.11			
Proteobacteria; Pseudomonadaceae; Pseudomonas	2.58	0.97	0.79	0.18			

Table 4Phylogenetic composition (Percentage) of bacteria at genus level under organic<br/>and conventional farming practices in Glenlea Research Station



Figure 2 Principal components analysis of samples under different cropping practices and systems



Figure 3 Redundancy analysis ordination plots of pyrosequencing corephylum

for Glenlea Research Station samples

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# Antibiotic production and quorum sensing regulation involved in biocontrol capacity in *Pseudomonas fluorescens* 2P24

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#### Abstract

Pseudomonas fluorescens 2P24 is a biocontrol agent isolated from the wheat takeall decline soil in China. This strain produces several antifungal compounds, such as 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide and siderophore(s). 2,4-DAPG is the major determinant in antibiosis and biocontrol activity. Genetic mutant of strain 2P24 defective in 2,4-DAPG production could not protect plants against soilborn plant diseases. The production of 2,4-DAPG is regulated by a series of transcriptional and translational factors in strain 2P24, including the pathway-specific repressor PhIF, the transcriptional regulator PsrA, the two-component system GacS/GacA, regulatory RNAs RsmX/Y/Z and their binding proteins RsmA/E, RNA chaperone Hfq, H-NS family regulators MvaT and MvaV, sigma factor RpoS, and a multidrug efflux pump EmhABC. These elements constitute a complicated and delicate regulatory network controlling the environmental adaption and biocontrol capacity in *P. fluorescens* 2P24. Another functional character involved in biocontrol of strain 2P24 is a LuxR/LuxI family quorum-sensing (QS) system, PcoR/PcoI. Mutation on signal biosynthase gene *pcol* did not detectably affect the production of 2,4-DAPG, but significantly influenced biofilm formation, colonization on wheattomato rhizospere and biocontrol ability against wheat take-all and tomato bacterial wilt. We further investigated the upstream regulators that influenced the transcription of the pcoI gene using a chromosomal pcoI::lacZ fusion reporter strain. The twocomponent system GacS/GacA, sigma factor RpoS and RNA chaperone Hfq, which have been known to be involved in antibiotic regulatory pathway, were identified to affect QS regulon. In addition, a special two-component system PhoP/PhoQ, which responds to environmental  $Mg^{2+}$  starvation, was also identified as a negative regulator of OS system. Our results revealed that antibiotic 2.4-DAPG and OS in biocontrol strain 2P24 shared some common upstream regulatory factors, but involved in biocontrol via different action models.

# Molecular signalling of bacteria based on the quorum sensing compounds N-acylhomoserine lactones and its PGPR effects on different plants

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#### Abstract

Roots are colonized by a high density of micro-organisms which are attracted and well nourished by plant borne organic nutrients. On the other hand, this microhabitat is characterized also by high competition and stress. Autoinducer signalling (e.g. Nacylhomoserine lactone, AHL) compounds have high importance for optimizing physiological efficiency as well as communication amongst bacterial populations in the rhizosphere and on the root surface. In addition, cross-kingdom interactions of bacteria with eucaryotes (fungi and plants) based on AHL-type molecules do occur which trigger different plant responses. In some plants (e.g. Arabidopsis or barley), these compounds can be taken up by roots and are distributed even to the shoot, while in other plants (like most legumes) plant derived lactonases degrade them efficiently. Examples for altered hormonal regulation or systemic resistance acquirement in different plants which are induced by AHL-compounds will be presented. Thus, the plant microbiome has very important consequences for the overall performance of the plant in the hologenome sense.

### Use of Bacillus amyloliquefaciens subsp. plantarum for biocontrol

### and plant growth promotion

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#### Abstract

The whole genome sequenced rhizobacterium FZB42 (Chen et al., 2007) and other plant associated Bacillus strains either designated as Bacillus amyloliquefaciens or Bacillus subtilis are used commercially to promote growth and health of crop plants. Previous investigations revealed that the strains represent an ecotype related to B. amyloliquefaciens, however its exact taxonomic position remained elusive (Reva et al., 2004). Here we have demonstrated ability to colonize Arabidopsis roots for a group of Bacillus strains, closely related to FZB42. According to their phenotypic traits the strains were similar to Bacillus amyloliquefaciens DSM7T, but differed considerably in DNA sequences of the genes encoding 16S rRNA, gyrase subunit A (gyrA) and histidine kinase CheA (cheA) from the type strain. Phylogenetic analysis performed with partial gyrA and cheA sequences revealed that plant-associated Bacillus strains including FZB42 are forming a lineage which can be discriminated from the cluster of strains closely related to Bacillus amyloliquefaciens DSM7T. DNA-DNA hybridization (DDH) performed with genomic DNAs from DSM7T and FZB42 yielded 63.7% to 71.2% homology. As complementary approach, we used several genomic methods, as direct whole genome comparison, digital DDH, and microarray-based comparative genomic hybridization (M-CGH). Plant-associated strains were discriminated from DSM7T and B. subtilis type strain by their different potential to synthesize nonribosomally antimicrobial lipopeptides and polyketides. According differences found in marker gene sequences and in whole genomes, we propose the two *B. amyloliquefaciens* subspecies "plantarum†for plantassociated, and amyloliquefaciens, for their non-plant-associated representatives. This is in line with results of DDH, MCGH, and the MALDI TOF mass spectrometry of cellular components justifying that both ecovars represent two different subspecies.

# Plant social networking system: Leaf insect feeding recruits beneficial root-associated bacteria and fungi in pepper

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#### Abstract

Plants have evolved general and specific defense mechanisms to protect themselves from diverse enemies, including herbivores and pathogens. To maintain fitness in the presence of enemies, plant defense mechanisms are aimed at inducing systemic resistance: in response to the attack of pathogens or herbivores, plants initiate extensive changes in gene expression to activate "systemic acquired resistance" against pathogens and "indirect defense" against herbivores. Recent work revealed that leaf infestation by whiteflies, stimulated systemic defenses against both an airborne pathogen and a soil-borne pathogen, which was confirmed by the detection of the systemic expression of pathogenesis-related genes in response to salicylic acid and jasmonic acid-signaling pathway activation. Further investigation revealed that plants use self-protection mechanisms against subsequent herbivore attacks by microorganisms growth-promoting beneficial recruiting called plant rhizobacteria/fungi, which are capable of reducing whitefly populations. Our results new evidence that plant-mediated aboveground belowground to provide communication and vice versa are more common than expected.

#### Introduction

*Rhizoctonia solani*, the causal As sessile organisms, plants are unable to actively avoid the attack of predators. To overcome this, plants have evolved a multilayer immune system against herbivores and pathogens (Jones and Dangl, 2006). Plants, unlike animals, lack adaptive immunity. Instead, plants are dependent on a heritable, innate immunity based on the recognition by receptors of the presence of microbial triggers (cues) including effector proteins and microbe-associated molecular patterns (Jones and Dangl, 2006). The perception of microbial cues leads to the induction of a broad spectrum of plant defenses called systemic acquired resistance (SAR) (Bostock, 2005). Until recently, SAR was thought to be limited to the induction of plant defenses against foliar microbial pathogens. However, recent results suggested that plants can activate signal exchanges between aboveground (AG) and belowground (BG) responses (Bezemer and van Dam, 2005). Three phenomena indicate that plants

can make use of cues that are systemically indicative of future enemy attack: (i) induced resistance against AG pathogens by BG microbes and vice versa, (ii) indirect defenses against AG insects by AG herbivore infestation, and (iii) BG pathogen infection leading to root exudate-mediated recruitment of BG bacteria. First, many strains of rhizosphere microbes referred to as plant growth-promoting rhizobacteria/fungi (PGPR/PGPF) have beneficial effects by positively affecting plant growth and resistance against foliar plant pathogens – a process known as induced systemic resistance (ISR) (Kloepper and Ryu, 2006). Inducible defense responses triggered by the foliar pathogen Pseudomonas syringae pv. tomato DC3000 included the induction of root secretions such as L-malic acid that effectively recruited a PGPR strain, Bacillus subtilis FB17, in Arabidopsis roots (Rudrappa et al., 2008). Second, herbivore attacks on plants trigger the induction of distinct resistance responses referred to as "indirect defenses" (Baldwin et al., 2006). In addition to the "direct defense" reaction mediated by the *de novo* production of toxic secondary compounds against enemies, plants also defend themselves by releasing volatile organic compounds (VOCs) or extrafloral nectar (EFN) to attract natural enemies (carnivores) of the herbivores AG (Heil and Ton, 2008). Third, as plant root exudates function as BG signaling molecules that affect the composition of rhizosphere microbial populations, (Badri and Vivanco, 2009) certain rhizobacteria express antifungalassociated genes such as the 2.4-diacetylphloroglucinol biosynthesis gene phlA. The expression of these genes is in turn influenced by root exudates, which are modulated by soil-borne fungal infections (Jousset et al., 2011).

In prior studies, only one-way signal transduction was considered, such as AG to BG, AG to AG, or BG to BG (Baldwin et al., 2002; Bezemer et al., 2004; Heil, 2008; Yi et al., 2009). The above three examples provide evidence of induced resistance against the same or a similar group of organisms, such as resistance against insects by insects, or against microbes by microbes. However, there are few studies addressing insect-microbe combinations during the elicitation of induced resistance. More specifically, indirect defenses by symbiotic root interactions AG were found, such as the volatile blends released by plants with arbuscular mycorrhizal fungi, which were more attractive to aphid parasitoids than the blends from plants without mycorrhiza (Guerrieri et al., 2004). The BG to AG defense responses of plants are not limited to arbuscular mycorrhizal fungi against herbivores. In addition to mycorrhiza-altered insect feeding preferences, a combination of *Pseudomonas* spp. strains affected the development of leaffolder pest and actively enhanced resistance against leaffolder attack by triggering the synthesis of systemic defense enzymes such as chitinase and proteinase inhibitors in rice plants (Saravanakumar et al., 2007). Bacillus sp. PGPR strain treatment of tomato triggered ISR to Tomato mottle virus under natural conditions by reducing the population of the silverleaf whitefly vector (Murphy et al., 2000).



# Figure 1 . A model of plant-mediated aboveground to belowground communication and *vice versa* during the induction of systemic resistance *via* tritrophic (insect-plant-rhizobacteria) interactions.

Whitefly infestation elicits plant systemic defenses against leaf and root pathogens. Chemical cues from root exudates secreted from AG whitefly infestation trigger the recruitment of beneficial microbes including saprophytic fungi, Gram-positive bacteria, and actinomycetes. The induction of systemic resistance by colonization by beneficial microbes confers plant self-protection against subsequent herbivore attacks.

Recently, we found another type of induced resistance response: bidirectional signal exchanges between AG and BG (Yang et al., 2011). Our study demonstrated that the phloem feeding whiteflies can induce systemic resistance against both a leaf bacterial pathogen and a soil-borne bacterial pathogen. A similar study using the whitefly as an AG feeding insect to test the induction of plant defenses only observed its effects against conspecific insect herbivore competitors AG.(Mayer et al., 2002) However, in our study, foliar attack by the whitefly not only elicited AG resistance against a leaf pathogenic bacterium, *Xanthomonas axonopodis* pv. vesicatoria, but also enhanced resistance against the soil-borne pathogenic bacterium, *Ralstonia solanacearum*. The induction of systemic resistance was confirmed by significant upregulation of the SA and JA defense signaling pathway marker genes, *Capsicum annuum pathogenesis-related protein (CaPR)1, CaPR4, CaPR10* and *Ca protease inhibitor (CaPIN)* in both leaves (AG) and roots (BG) after whitefly feeding.

Interestingly, AG whitefly feeding significantly increased the population density of beneficial BG microflora including Gram-positive bacteria, actinomycetes and saprophytic fungi that may induce systemic resistance.(Kloepper and Ryu, 2006) Among BG microbial groups, several Gram-positive *Bacillus* sp. strains significantly elicited plant systemic defenses against the whitefly population in the tomato field.(Murphy et al., 2000) Our studies provide a new understanding of tritrophic (insect-plant-PGPR) interactions and their role in the induction of defense mechanisms. In the near future, it will be important to define plant defense signaling molecules from AG to BG and to dissect the signaling transduction pathways using "omics" technology to reveal the mechanisms by which plants protect themselves against enemy attacks.

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Yi, H.S., Heil, M., Adame-Alvarez, R.M., Ballhorn, D.J., and Ryu, C.M. 2009. Airborne induction and priming of plant defenses against a bacterial pathogen. Plant Physiology 151:2152.

# Cyclic lipopeptides of *Pseudomonas* sp. CMR12a are involved in biocontrol, swarming motility and biofilm formation

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#### Abstract

The fluorescent biocontrol strain Pseudomonas sp. CMR12a was isolated from cocoyam roots in Cameroon and shows an unique adaptation to humid tropical soils such as the ability to switch to methylotrophic and anaerobic growth. The strain has two quorum sensing systems and produces several interesting metabolites, including phenazine-type antibiotics and two different types of cyclic lipopeptides (CLPs). CLPs are composed of a partially cyclised peptide, linked to a fatty acid of variable length, and can exhibit powerful toxicity towards a wide range of organisms. Their synthesis is governed by non-ribosomal peptide synthetases, which are encoded on large gene clusters. So far, simultaneous production of two CLPs by one strain has only been demonstrated for plant pathogenic pseudomonads. The structure of both cyclic lipopeptides was elucidated by combining two approaches. Firstly, the biosynthesis gene clusters for the CLPs were identified and analyzed, which provided an in silico structure prediction. Secondly, chemical structure identification was performed, involving purification of the compounds by liquid chromatography followed by mass spectrometry, and amino acid hydrolysis and analysis by HPLC. It appeared that CMR12a produces two distinct CLPs, which were designated sessilin and motilin, respectively. Sessilin is structurally related to the toxin tolaasin, produced by the bacterial mushroom pathogen *Pseudomonas tolaasii*. The structure of motilin on the other hand is very similar to that of orfamide, which was recently described in biocontrol strain *Pseudomonas fluorescens* Pf-5. Sessilin is important for biofilm formation and is, together with the phenazine antibiotics, essential for the biocontrol capacity of CMR12a towards Rhizoctonia solani root rot on bean (Phaseolus vulgaris) and Chinese cabbage, while motilin is indispensable for swarming motility of CMR12a.

# Bioactive metabolites involved in the microbiological control of tomato foot and root rot

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#### Abstract

The disease tomato foot and root rot (TFRR) is caused by the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl). Application of some microbes, such as *Pseudomonas fluorescens* or *Serratia plymuthica*, can efficiently reduce the disease. The microbes can be applied by coating on the seeds or by drenching. Using studies with auto fluorescent proteins, we observed that the pathogen and the biocontrol bacteria occupy the same niches on the root surface. It was also observed that the *Pseudomonas* biocontrol strain, applied on the seed, reaches these niches earlier than the pathogen.

In this paper, the roles of many molecular signals which play a role in the microbiological control of TFRR will be discussed.

The major mechanisms used by microbes for the control of root diseases are the following. (i) Antibiosis, (ii) Predation and Parasitism (P&P), (iii) Competition for Nutrients and Niches (CNN), (iv) Induced Systemic Resistance (ISR), and (v) Inhibition of fungal spore germination, and (vi) Combinations of the above.

Root colonization by the beneficial microbe is a process which is required for all mechanisms of biocontrol. Roots secrete up to 20% of the bound carbon as root exudate. Microbes are attracted chemotactically by certain components secreted by the root.

Weapons used by beneficial microbes to attack the pathogen include lytic enzymes such as chitinase, and antibiotics such as phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, cyclic lipopeptides, 2-hexyl-5-propyl resorcinol and D-gluconic acid. Antibiotic production occurs at the end of the exponential growth phase and usually requires quorum sensing, mediated by *N*-acyl homoserine lactones (AHLs), as a mechanism. Biocontrol strain *Pseudomonas chlororaphis* PCL1391 is chemo-attracted to fusaric acid (FA) secreted by *Forl*. The bacterial cells subsequently attach to the fungal hyphae and kill the fungus by secreting the anti-fungal metabolite phenazine-1-carboxamide (PCN). Its synthesis is subject to quorum sensing.

Key words: Fusarium oxysporum; Pseudomonas fluorescens; tomato foot and root rot

#### Introduction

Crop plants are threatened by pathogens, many of which are fungi. Disease reduction can be brought about by chemicals, crop rotation and resistant cultivars. A modern and increasingly successful method to reduce disease pressure is the use of certain microbes. Applied microbes include the bacteria *Pseudomonas fluorescens*, *Serratia plymuthica*, and *Bacillus subtilis*, and the fungus *Trichoderma*. The microbes can be applied by coating on the seeds or by drenching. I will use the disease tomato foot and root rot (TFRR), caused by the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl), as an example. Approaches used by us to study the mechanisms involved in the disease were (i) visualisation of interactions at the cellular level, (ii) genetics, and (iii) the role of metabolites in disease and disease suppression.

#### **Materials and Methods**

The most important used methods and the corresponding references are the following. Visualisation of interactions at the cellular level: Lagopodi *et al.*, 2002; analysis of root exudates: Kamilova *et al.*, 2006; chemotaxis towards root exudate components: De Weert *et al.*, 2002; chemotaxis towards FA: De Weert *et al.*, 2004; selection of CNN bacteria: Kamilova *et al.*, 2005; inhibition of spore germination: Kamilova *et al.*, 2008; identification of colonization traits: Lugtenberg *et al.*, 2001.

#### **Results and Discussion**

**Visualisation of biocontrol** (Lagopodi *et al.*, 2002) Using auto-fluorescent proteins to mark organisms, we observed that *Forl* hyphae attack the root by attaching to the root hairs. The next step is colonization of the root, which starts with attaching to the junctions between plant cells. Finally, the hyphae enter the root and destroy it completely.

The biocontrol agents *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391, coated on the seed, attach to the germinated plant also by occupying the junctions between plant cells. We assume that exudate is present at these junctions and that the microbes are attracted to these sites. It was also observed that the biocontrol cells, applied on the seed, reach these niches earlier than the pathogen coming from the soil.

**Metabolites** This rest of this paper will focus on the metabolites which play a role in the microbiological control of plant diseases caused by fungi. Metabolites can be involved in many processes such as growth, chemotaxis and defence reactions, and in the actual biocontrol process. In this presentation, I will discuss mainly the roles of molecular signals which play a role in the microbiological control of TFRR.

**Root exudate** As argued before, both pathogen and BCA are attracted by the root. Roots can secrete up to 20% of the bound carbon as small soluble root exudate components. In tomato root exudate the major components are organic acids (such as citric acid, malic acid, lactic acid and succinic acid), sugars (such as glucose, xylose, maltose and sucrose), and amino acids (such as aspartic acid, glutamic acid, isoleucine and leucine). It should be noted that this analysis was based on searching specifically for these groups of components. It is feasible that several root exudate components were not detected because we did not search for them. For example, putrescine was discovered by accident as an important root exudate component, because the analysis of a colonization mutant suggested that it could be present (Kuiper *et al.*, 2001). This suggestion was confirmed by subsequent analysis.

**Chemotaxis** Microbes are attracted chemotactically by certain components secreted by the root. Chemotaxis assays using *P. fluorescens* WCS365 showed that amino acids, especially L-leucine, are good attractants, that sugars have no such activity and that organic acids are also good attractants. Based on the concentrations estimated to be present in the rhizosphere, we suggest that citric acid and malic acid are the major attractants during biocontrol (De Weert *et al.*, 2002). The same BCA is also attracted to the *Forl* hyphae by chemotaxis towards FA secreted by *Forl* (De Weert *et al.*, 2004).

**Root colonization** by the beneficial microbe is a process which is required for all mechanisms of biocontrol. Using a competitive root colonization assay, we isolated a number of root colonization mutants. By analysing the impaired genes and operons, we identified many root colonization traits (Lugtenberg *et al.*, 2001).

**Mechanisms of biocontrol** The major mechanisms used by microbes for the control of root diseases are (i) Antibiosis, (ii) Predation and Parasitism (P&P), (iii) Competition for nutrients and niches (CNN), (iv) Induced systemic resistance (ISR), (v) Inhibition of fungal spore germination, and (vi) Combinations of the above.

Strains acting through the production of antibiotics can be isolated by screening on a plate inoculated with the target pathogen. Antibiotics used by beneficial microbes to attack the pathogen include phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, cyclic lipopeptides such as surfactin, 2-hexyl-5-propyl resorcinol and D-gluconic acid (Haas and Defago, 2005). Antibiotic production occurs at the end of the exponential growth phase and usually requires quorum sensing, mediated by *N*-acyl homoserine lactones (AHLs).

Strains acting through Predation and Parasitism can produce enzymes (such as chitinase, cellulase, B-1,3 glucanase, and protease) which lyse the fungal cell wall.

CNN microbes have recently been isolated (Kamilova *et al.*,2005). This mechanism has the advantages that a) it can be selected for, and b) it can act without the action of antibiotics which makes the BCA safer than strains acting through antibiosis. Pliego *et al.* (2007) used the same approach except that they isolated those

strains which are not only good competitors but also produce antibiotics. In this way they isolated BCAs which use two mechanisms.

Microbes acting through ISR (such as some strains of *Bacillus, Pseudomonas* and *Trichoderma*) colonize the root where they send signals to the plant which prime the plant into a stage in which it quickly reacts on attack by a pathogen. Priming occurs by a combination of signals. Individual components shown to be able to induce ISR are flagella, lipopolysaccharies, *N*-acyl homoserine lactones, siderophores, antibiotics (such as phloroglucinol and some cyclic lipopeptides such as surfactin) and volatiles such as 2,3-butanediol produced by *Bacillus* spp. (Ryu *et al.*, 2004). Signalling is systemic i. e. protects all plant parts. Moreover, signalling is dependent of the plant hormones jasmonate and ethylene. ISR can protect against a variety of pathogens such as bacteria, fungi, viruses and even insects (Van Wees *et al.*, 2008).

*P. fluorescens* WCS365 inhibits the germination of spores of the *Fusarium* fungus (Kamilova *et al.*, 2008).

It has advantages to use more than one mechanism to suppress diseases. Firstly because this mechanism may not be active under the tested conditions, e.g. antibiotic synthesis is very dependent on environmental conditions. Secondly, in case resistance would occur against one mechanism, the resistant microbe will be attacked through the second mechanism. Some strains can use a variety of mechanisms. For example, *P. fluorescens* WCS365 is an enhanced root coloniser and can also use ISR and inhibition of spore germination.

**Resistance against BCAs** is only known for the mechanism of antibiosis (Duffy *et al.*, 2003). It can occur through at least three different mechanisms. (i) Inhibition of antibiotic production. Biocontrol strain *P. chlororaphis* PCL1391 is chemo-attracted to the pathogen *Forl* because *Forl* secretes FA. The bacterial cells subsequently swim towards the fungal hyphae and kill the fungus by secreting the anti-fungal metabolite phenazine-1-carboxamide (PCN). However, if sufficient amounts of FA acid are present, this compound inhibits the synthesis *N*-AHL. Since this compound is required for PCN synthesis, antibiotic synthesis is inhibited. (ii) Detoxification of the antibiotic 2,4-diacetylphloroglucinol to the mono-acetyl form, thereby inactivating the antibiotic. (iii) Active efflux of the antibiotic. Some *Botrytis* strains are resistant towards phenazine because they have an efflux pump which keeps the intracellular phenazine concentration low.

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# *Section - II* Oral Session 1 – Lectures

### **PGPR** in Groundnut: opportunities and challenges

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#### Abstract

Groundnut (Arachis hypogaea L.) is an important food legume in Asian countries earning a lot of revenue. In the semi-arid tropics, the production levels of groundnut are hampered majorly by foliar and soilborne diseases incited by fungal pathogens. Present management of these groundnut diseases using chemical fungicides has environment related concerns. More over the labeled fungicides are very expensive and can not affordable by resource poor small and marginal farmers. Use of bacterial antagonists such as plant growth-promoting rhizobacteria (PGPR) as a viable alternative to chemical management of these groundnut diseases is an emerging concept. In this paper, the scope, potential and future prospects of using PGPR as antagonists to manage these foliar and soilborne diseases is discussed in detail. Elaborate review on the challenges encountered by native and introduced PGPR strains in the micro climate of groundnut ecosystem was presented. Current trends on PGPR based disease management, success stories on suppression of foliar and soilborne diseases in groundnut are elaborated. The current research activities at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India in the management of these foliar and soilborne diseases with emphasis on early and late leaf spots induced by Cercospora spp., stem rot (Sclerotium rolfsii), collar rot (Aspergillus niger) and aflatoxin contamination problem are presented.

### Potential of PGPR application for seed spices with special reference to coriander and fenugreek in India

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#### Abstract

The seed spices such as coriander (Coriandrum sativum L.), cumin (Cuminum cyminum L.), fennel (Foeniculum vulgare M.) and fenugreek (Trigonella foenumgraecum L.), are cultivated in India predominantly in the states of Rajasthan and Gujarat. Major constraints of seed spice cultivation include low fertility of soils, poor input management, low germination, slow initial growth and high susceptibility to diseases and frost. The plant growth promoting rhizobacteria (PGPR) are reported to alleviate both biotic and abiotic stress in several crops. About one hundred and seventy rhizosphere and endophytic bacteria were isolated from coriander, cumin, fennel, fenugreek and Eruka sativa. From these collections, twenty five isolates with beneficial traits were shortlisted. From the shortlisted strains, two promising isolates were selected based on greenhouse evaluation. These were identified as *Pseudomonas* putida (FK14), and Microbacterium paraoxidans (FL18) using biochemical and molecular tools. The isolate P. putida is from rhizosphere of fenugreek and M. paraoxidans is an endophyte from fennel roots. Talc formulation of these two isolates were evaluated both as a seed treatment and soil application on four seed spices namely coriander, cumin, fennel and fenugreek. The results obtained from different parts of india, representing South (Coimbatore-Tamil Nadu, Guntur-Andhra Pradesh), West (Jagudan-Gujarat and Jobner-Rajasthan) North (Hisar-Haryana) and Central (Raigarh-Chhattisgarh, Dholi-Bihar, and Kumarganj-Uttar Pradesh) regions for coriander and fenugreek are presented in this paper. The soil type in these regions varied from sandy loam to black loam with a soil pH ranging from 6.5 to 9.0. The total rain fall ranged from 400mm to1100mm in these regions and the crops were raised both as rain fed and irrigated conditions. In all the locations, PGPR application resulted in enhanced growth and yield. The increased yield ranged from 10 to 20 per cent under experimental plots for two consecutive years. Based on this result the PGPR technology was demonstrated in farmers' plots for two years in eight locations representing as many states. The population of the introduced organisms in these locations was monitored during the crop season. Application of PGPR at the time of planting lasted throughout the crop season and the population was found both in rhizosphere and rhizoplane. The farmers were convinced about the beneficial effects not only in terms of growth and yield but also reduced incidence of pests and diseases.

#### Introduction

Coriander (Coriandrum sativum L.), cumin (Cuminum cyminum L.), fenugreek (Trigonella foenum-graecum L.), and fennel (Foeniculum vulgare M.) known as seed spices are cultivated in the Indian states of Gujarat, Rajasthan, Harvana, Punjab, Uttar Pradesh, Bihar, Madhya Pradesh, Andhra Pradesh and Tamil Nadu. Major production constraints of these crops includes low fertility of soils, less use of inputs, poor germination, slow initial growth, competition from weeds and high susceptibility to diseases and frost (Agrawal et.al., 2001, AICRPS 2009, 2010). Plant growth promoting rhizobacteria (PGPR) are a wide range of root colonizing bacteria with the capacity to enhance plant growth by increasing seed emergence, plant growth and crop yield (Kloepper, 1992). Soil or seed application of PGPR have been used to enhance growth of the several crops (Glick et al., 1995) as well as to suppress the growth of the plant pathogens. Germinating seeds and growing plants influence the activities of soil microorganisms in the adjoining volumes of soil known as the spermosphere and the rhizosphere, respectively (Lynch, 1990.). The microorganisms that colonize the rhizosphere profoundly affect root and plant biology in relation to nutrition, development and health. PGPR, promote plant growth and yield either directly or indirectly (Kloepper et al., 1989; Glick et al., 1995). Seed spices are cultivated in soils in cool dry climate during winter months in lands that are fallow during hot summer months. There is absolutely no work done on the association of rhizosphere microorganism in seed spices in India. This work was undertaken to isolate screen, evaluate and utilize the potential of rhizobacteria for seed spices (AICRPS, 2009).

#### **Materials and Methods**

#### Isolation of bacteria

Healthy plant samples for isolation of bacteria were collected from Jagudan in the state of Gujarat and Jobner, Rajasthan. Samples of four seed spice crops namely coriander, cumin, fennel, fenugreek and an oil yielding plant Taramera (*Eruka sativa*) that grows luxuriantly in that area were used for the isolation. Bacteria were isolated from the rhizosphere, rhizoplane and endophytic bacteria from internal tissues of roots and stem of apparently healthy plants using standard protocol. Nutrient agar and tryptic soy agar were used as isolation media. The individual bacterial colonies from each sample were selected and sub cultured on nutrient agar. The representative isolates were cryopreserved at -80°C in 20% glycerol filter sterilized for further studies. The isolates were tentatively grouped and documented based on phenotypic characteristics such as colour, form, elevation, margin, diameter, surface, opacity and texture. Motility, cell morphology, size, Gram reaction, spore formation and production of UV-fluorescent pigments were also recorded using standard procedures. Routine biochemical tests such as Indole, methyl red, Voges-Proskauer, succinic acid and hydrogen cyanide production were done for each endophyte as described by Zvyagintsev (1991).

#### Screening for growth promotion

Based on the colony morphology, five distinctly different bacterial isolates from each crop namely coriander, cumin, fennel, fenugreek and taramera were selected. These twenty-five bacterial isolates were used in green house screening for growth promoting ability using fennel as test crop in complete randomized design (CRD) with three replications. Observations on germination, growth of the plant, number of leaves, root weight, total biomass and dry weight were recorded for 3 months and statistically analyzed. After repeating the trial second time five isolates were short listed. These five isolates were further screened and selected two promising isolates FK14 and FL18. The identity of the isolates was confirmed by 16S rDNA sequences and BIOLOG based identification.

#### Evaluation in the field

The effect of selected two PGPR on seed germination, seedling growth and yield of major seed spice crops were evaluated in seed spice growing states of India through All India Coordinated Research Project on Spices (AICRPS) that has centres all over the country. The talc formulation of these efficient PGPR strains namely FK 14 and FL 18 were used for the evaluation. Their efficiency on seed spice crops namely coriander, cumin, fennel and fenugreek were evaluated for two years 2007 and 2008. The treatments included seed treatment and soil application either singly or in combination of these two strains as given below.

T1 Rhizobacteria FK 14 (seed treatment)

T2-Rhizobacteria FK 14 (seed treatment + soil application)

T3-Rhizobacteria FL 18 (seed treatment)

T4-Rhizobacteria FL 18 (seed treatment + soil application)

T5-Rhizobacteria FK 14 + FL 18 (seed treatment)

T6-Rhizobacteria FK 14 + FL 18 (seed treatment + soil application)

T7-Trichoderma MTCC 5179

#### T8-Control

All the treatments received the recommended dose of chemical fertilizers at each location. The observations included, germination per cent, growth parameters and the yield.

#### Technology demonstration in farmers' fields

Based on the encouraging results in the research stations, best two treatments along with traditional practices followed at each location were demonstrated in farmers plots. Talc formulations of these two strains were distributed to ten farmers under selected AICRP centers during 2009 and 2010. Interactive meeting was conducted in farmer's forum in these areas to popularize the technology. Survival of the introduced rhizobacterial strains was confirmed by collecting soil samples from the plot and population monitoring throughout the crop period through specific antibiotic media developed for this purpose.

#### **Results and Discussion**

A total of 172 bacteria were isolated from rhizosphere, rhizoplane and endophytic regions of seed spice crops (Table, 1). These isolates were tested in the laboratory for their growth promotion traits such as IAA production, phosphate mobilization, solubilization of zinc, silica, potash and other beneficial characters (Table2). The shortlisted five strains were evaluated in green house trial for their plant growth promoting efficacy of seed spices. The identity of these were confirmed through 16S rDNA sequences and studies with Biolog as Pseudomonas putida (FK 14) and Microbacterium paraoxidans (FL 18). Only yield data is presented here and data collected on various parameters are not presented here. The yield of coriander and fenugreek varies from place to place based on the geographical location. In Tamil Nadu the maximum yield is about 570kg/ha whereas it is about 1700kg/ha at Hisar in Haryana and in the rest of the locations it is between these two levels. Irrespective of the yield potential of the soils in these locations, treatment with PGPR has recorded higher yield when compared to the local control under experimental plots (Fig.1). Similarly, fenugreek yield varies from about 250kg/ha at Guntur, Andhra Pradesh to about 1600kg/ha in Hisar, Haryana. Here also Application of PGPR has recorded higher yields under experimental conditions (Fig.2). When the best treatments under experimental plots were demonstrated in farmers' plots, the yield increase ranged from 10%~35% (Fig. 3).

The rhizosphere and endophytic microbial flora play an important role in crop productivity and growth promoting abilities of rhizobacteria are well documented (McInroy and Kloepper 1995, Hallmann et al., 1997, Cartieaux et al., 2003, Hong et al., 1991, Sturz 2000, Whitelaw, 2000). Besides growth promotion the plants are also protected against pathogen by induction of induced systemic resistance (Liu et al., 1995, Haas and Keel, 2003, Hammerschmidt, R. 1999, Harman et al., 2004). The enhanced growth and increase in yield was so apparent that farmers were willing to adopt the technology in all the locations. In order to reduce the burden of treating seeds with talc formulations, a simple seed coating method was developed so that farmers could directly sow the coated seeds (Fig.4). The summer ambient temperatures in the northern plains of India goes very high to about 45 °C and also the surface soil gets heated and the land looked parched. The microbial populations are also very low. After the crops are grown the microbial population builds up gradually (Data unpublished). Perhaps, this is the reason for initial slow growth of seed spices and it was thought to be growth character of seed spices. When treated with rhizobacteria, plants grow vigorously from the beginning resulting in increased number of branches and the difference is perceptible from the beginning. The germination percentage is also higher and the farmers need to invest less on seeds. As the growth of the crop is vigorous, weed growth is also suppressed thereby saving money on herbicides. The increased yield brings higher returns and the profitability of farmers is enhanced. In the farmers' demonstrations, they also claimed fewer incidences of pests and diseases. At research stations there was increased nodulation in fenugreek (Data not presented).

Since this is an applied research, not much of scientific data could be collected on the mechanism of growth promotion. The primary objective was to enhance initial growth and get higher yield that is achieved in this study, further studies are in progress and there is ample scope to collect scientific data to explain the growth promotion and yield increase in seed spices.

	Locations								
Crop	Jobner			Jagudan	No.of strains				
	Rhizosphere	Rhizoplane	Endophytic bacteria	Rhizosphere	Rhizoplane	Endophytic bacteria	isolated		
Cumin	2	7	6	5	8	4	32		
Coriander	6	6	3	7	7	4	33		
Fennel	6	8	6	4	5	9	38		
Fenugreek	9	6	7	8	7	4	41		
Taramera	5	8	10	-	-	-	23		
Total	28	35	32	23	27	21	172		

#### Table 1 Isolation of bacteria from seed spices

		Growth parameters							
Si. No	Isolate	Height	No. of. Leaves	Leaf length	Shoot weight	Root weight			
1	CN 1	3.67	7.67	14.33	1.30	0.46			
2	CN 2	3.50	6.33	13.83	1.42	0.24			
3	CN 3	2.83	6.33	12.33	0.96	0.28			
4	CN 4	4.12	6.67	13.33	1.35	0.35			
5	CN 5	3.58	7.00	14.67	0.96	0.38			
6	CR6	3.00	5.17	11.33	0.99	0.17			
7	CR 7	3.25	5.83	11.00	0.86	0.25			
8	CR 8	3.10	6.00	12.33	1.07	0.42			
9	CR 9	1.58	5.17	7.67	0.58	0.20			
10	CR10	3.42	5.83	13.00	1.13	0.35			
11	FK 11	1.42	2.50	4.33	0.33	0.10			
12	FK 12	4.50	6.17	12.67	1.13	0.35			
13	FK 13	3.25	4.67	11.33	1.03	0.23			
14	FK 14	4.67	7.00	14.67	1.15	0.52			
15	FK15	3.25	6.33	13.33	0.87	0.25			
16	FL 16	2.67	3.67	8.33	0.80	0.17			
17	FL 17	1.58	3.83	5.50	0.62	0.10			
18	FL 18	6.25	6.33	13.00	1.33	0.27			
19	FL 19	2.90	4.67	10.17	1.00	0.35			
20	FL 20	3.57	6.67	13.67	1.38	0.27			
21	TR 21	3.25	6.17	14.67	1.00	0.18			
22	TR 22	0.92	2.00	4.17	0.30	0.08			
23	TR 23	2.50	4.50	9.00	0.73	0.27			
24	TR 24	2.97	5.83	12.33	0.90	0.23			
25	TR 25	2.17	4.33	9.17	0.60	0.18			
26	Control	2.33	4.67	8.67	0.75	0.28			
LSD 0.05	5	2. 525	3.612	7.524	0.7752	0.2839			

 Table 2
 Screening for growth promotion using fennel as test crop

Table 3 Second level of screening of short listed PGPR with fennel as test crop

	Isolata			Growth Parameters				
Si. No	Isolate	Height	No. of. Leaves	LF length	Shoot weight	Root weight	Dry Shoot weight	Dry Root weight
1 2 3 4 5	CN 4 CR 8 FK 14 FL 18 TR23	1.47 b 3.0 ab 3.80 a 3.60 a 4.05 a	2.40 b 3.90 ab 3.90 ab 5.00 a 4.20 ab	5.20 b 9.85 ab 9.95 ab 11.00 a 11.60 a	0.265 a 0.538 a 0.518 a 0.440 a 0.440 a	0.087 a 0.127 a 0.164 a 0.143 a 0.134 a	24.50 b 55.16 ab 58.82 a 60.50 a 65.66 a	8.82 c 13.0 bc 15.66 bc 17.16 bc 29.66 a
6	Control	2.2 ab	4.40 a	8.60 ab	0.378 a	0.123 a	52.50 ab	13.00 bc



Figure 1 Yield increase in coriander with rhizobacterial treatment



Figure 2 Vield increase in fenugreek with rhizobacteria treatment



Figure 3 Performance of PGPR in fenugreek at farmers plot. *Kalakh, Ethwa juncia, Pachar* (Rajasthan), *Hisar* (Haryana), *Guntur* (Andhra Pradesh)



Figure 4 Seed coating with rhizobacteria; a) Coated fenugreek seeds, b) Normal seeds, c) Coated corainder seeds, d) Nomal coriander seeds

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# Effects of biological seed coating by PGPR against soybean cyst nematode in the northeast of China

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#### Abstract

Soybean Cyst Nematodes is important pathological organisms in the world, but it is difficult to control in the field. Many natural enemies can attack nematodes in soil and reduce SCN population, and the most-studied natural enemies of nematodes are bacteria and fungi. In order to research new biological seed coating on controlling SCN, we screened some nematicidal activity microbes in vitro and fields. Then, basing on principle of microbial biodiversity in soil, a few PGPR stains were screened and combined as virtual components together with micro-element, macro-element and different adjutants. It was used coating on Liaodou 15 soybean variety and assigned to experimental plots in Liaoning and Heilongjiang Provinces.

Basing on the results of treatments in 2007, we designed another experiments with other treatments plots of soybean field in Wangjia and Kangping village of Shenyang city in Liaoning province, and Anda city of Heilongjiang province in 2008 and in 2010. The results showed that the numbers of SCN outside and inside roots of soybean in treatment bio08-1(use PGPR strains) was significantly less than that in CK. The yield componentwere better than that of CK, especially 100-seed weight of soybean. The biological seed coating had the potential in increasing yield, and it could be an effective method that the biological seed coating prevents and controls SCN in the future.

Results from this stduy suggested that using effective PGPR isolated from soil ecosystem as biological seed coating could be an important way to control soybean cyst nematode without destroying the environment and soil. These effective microorganisms mixture as the enemies of phytoparasitic nematodes are worth further study.

# Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates

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#### Abstract

A series of microarray experiments were performed in order to elucidate the transcriptomic response of Bacillus amyloliquefaciens FZB42, a well-studied Grampostive plant growth-promoting rhizobacterium (PGPR), in response to maize root exudates. A total of 302 genes representing 8.2% of FZB42 transcriptome were significantly altered in transcription by the presence of root exudates. Whereas only 41 genes were down-regulated, the majority of the genes (261) were up-regulated in expression by the root exudates. Several groups of the genes which were strongly induced by the root exudates are involved in the metabolisms relating to nutrition utilization, bacterial chemotaxis and motility, as well as antibiotic production. This result not only provided a profile of how plant root exudates orchestrate gene expression of Gram-postive PGPR, but also helped to identify the genes involved in plant-microbe interaction. The effect of soil extract on gene expression of FZB42 was also discussed. Moreover, the "interaction exudates" collected from the maize roots inoculated with FZB42 were compared with the common exudates collected in a gnotobiotic system, although little difference was found in their effects on FZB42 gene expression.

Key words: transcriptomic profiling ; *Bacillus amyloliquefaciens* ; plant root exudates

# LCO Applications provide improved responses with legumes and non-legumes

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#### Abstract

LCOs (Lipo-chito-oligosaccharides, a.k.a. Nod factors) are signal molecules produced by rhizobia that are essential for the nodulation process in legumes. Formulations for soybeans, peanuts, alfalfa, and peas/lentils combining the respective LCO and rhizobia for each legume have been developed and commercialized. LCO seed application of non-legumes such as corn, cotton, and wheat also demonstrate increased growth parameters, including root and shoot dry biomass in greenhouse studies, and enhanced yields in field trials. Root examination with the WinRHIZO scanner proved data demonstrating an increase in root length, surface area and volume. In addition, foliar applications of LCOs on legume (soybean) and non-legume (corn) in greenhouse and field trials improved plant growth parameters, including yield. LCO treated soybean and wheat seeds were rinsed with sterile water and this rinsed-off water was used to investigate LCO on-seed longevity by root hair deformation of Siratro (*Macroptilium atropurpureum*). LCO applications with legumes and non-legumes applied as seed treatments, in-furrow, and foliar spray have provided improved plant growth and yield responses.

Key words: LCO; signal molecule; seed treatment; foliar treatment

#### Introduction

During the mutualistic symbioses with legume plants, rhizobia secret lipochitooligosaccharides (LCOs) or Nod factors which act as signal molecules. While LCOsignaling is a prerequisite for successful rhizobial infection, nodulation-induction and atmospheric nitrogen fixation by legume plants, it is very intriguing how LCO exhibits plant growth-promoting effect in non-legume crop plants such as corn, rice, canola, potato, cucumber, cantaloupe, melon, apple, grape, beet and *Arabidopsis* (Prithiviraj *et al.*, 2003; Smith *et al.*, 2007). The plant growth promotions reported in such crops include enhanced seed germination, seedling or plant biomass increase or photosynthesis increase in greenhouse trials. In our current text, we represent trials conducted in the greenhouse and field to elucidate LCO's growth promoting effects on soybean, corn, wheat and cotton.

#### **Materials and Methods**

For all greenhouse and field trials LCO's was used to formulate the treatment solutions (10<sup>-7</sup> to 10<sup>-9</sup> M LCO) for seed and foliar treatments. All seed treatments were applied as slurry where LCO solutions were added with common fungicides for the specific crop. The LCO seed and foliar application rates were 10 ml/kg of seeds and 2 ml/plant, respectively. Most treated seeds were planted 24 h after (otherwise mentioned) treatment in greenhouse plastic pots containing peat-perlite mix. Foliar applications in the greenhouse were performed at V4 growth stage with seedlings grown from non-treated seeds. Greenhouse trials were conducted following completely randomized design having 5 replicates per treatment. Field trials were conducted as small plots trails following randomized complete block design. The data were analyzed using ANOVA and means were separated by least significant difference (LSD) at 5% leve.

Table 1. Measurement of plant dry weight from LCO treated seeds grown in g	greenhouse.
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Crops	Shoot dry weight (g)		Root dry weight (g)		Total dry biomass (g)	
_	Control	LCO 10 <sup>-8M</sup>	Control	LCO 10 <sup>-8M</sup>	Control	LCO 10 <sup>-8M</sup>
Corn	3.281	3.47	1.135	1.256	4.416	4.721
Cotton	1.031	1.118*	0.283	0.321	1.314	1.439*
Wheat	0.420	0.448*	0.617	0.652	1.038	1.100

\*denotes significance at 0.05

#### **Results and Discussion**

LCO treated Corn, cotton and winter wheat seeds were harvested 5 weeks after planting in greenhouse. In corn, there was a consistent increase in shoot and root dry biomass in all plants from LCO treatment but the increase was not significant. Cotton and wheat exhibited significant above ground plant dry biomass increase and non-significant root dry biomass increase. Overall, plant dry biomass increase for cotton and wheat was significant (Table1). Field trials from multiple sites showed increases in yield per hectare for corn, cotton and wheat (Table2). Positive response for yield increase compared to a control was between 65 to 100% for 4 crops. Seven (7) days old seedlings grown from treated corn and wheat seeds showed significant increase in root length, surface area and volume when measured by a WinRHIZO<sup>TM</sup> scanner (Table3). This early enhancement in seedling root growth may explain yield improvement in a later stage as shown in Table2. Seed wash liquid from LCO treated 1 yr old soybean, 6.5 months old *Lotus japonicus* and 3.8 months old spring wheat seeds induced Siratro seedling root hair deformation indicating that LCO can remain as an active molecule on the seed coats for these periods of time (Fig.1).

Crops	Number of	Country	Yield (Kg/Ha)		Yield increase	%	(%)Positive
	trials				(Kg/ha)	increase	Response
Corn	N=5	USA	11,992	12,390	398	3.3	100
	N=10	Argentina	12,675	13,144	469	3.7	80
Cotton	N=26	USA	181.74	191.86	10	5.57	69
Wheat	N=3	USA	4609.6	5333.2	723.6	15.7	100
Soybean	N=424	USA	3,198	3,318	120	3.7	65
-	N=399	Argentina	3,012	3,253	241	8.0	79

Table 2. Effect of LCO seed treatment on non-leguminous crops grown in field.

Table 3. Measurement of plant growth parameters from LCO treated seeds grown in greenhouse.

Crops	Length(cm)		Surface area (cm <sup>2</sup> )		Volume (cm <sup>3</sup> )	
	Control	LCO 10 <sup>-8M</sup>	Control	LCO 10 <sup>-8M</sup>	Control	LCO 10 <sup>-8M</sup>
Corn	40.68	73.19*	10.5	19.37*	0.21	0.41*
Wheat	18.41	21.18*	3.62	4.0*	0.058	0.06

\*denotes significance at 0.05

Table 4. Effect of LCO foliar application on soybean and corn.

Crops	Shoot dr	y weight (g)	Root dry	weight (g)	Total dry	y biomass (g)	Yield (	(Kg/Ha)
	Control	LCO 10 <sup>-9M</sup>	Control I	LCO 10 <sup>-9M</sup>	Control	LCO 10 <sup>-9M</sup>	Control	LCO
Soybean	4.226	4.698	1.494	1.546	5.72	6.244*		
Greenhouse								
Corn Field:							11,172	11,526
USA -	· <b>-</b> · <b>-</b> · <b>-</b> · <b>-</b>		- · - · - · - · -	• = • = • = • = •	- · - · - · - · ·	• - • - • - • 🌩		
Argentina -		- · - · - · - · - ·	- · - · - · - · -		- · - · - · - · ·	•••••	10,500	10,941

\*denotes significance at 0.05

Table 5. Effect of LCO foliar application on soybean leaf greenness and carbohydrate content

Treatment	Leaf Chlorophyll	Leaf carbohydrate content (ug sugar/gram of leaf tissue)					
	(SPAD reading)	Pentose	Hexose	Total sugar			
Control	30.1	238	671.3	909.3			
LCO 10 <sup>-8</sup> M	33.3	402.3*	960.0*	1362.3*			

\*denotes significance at 0.05



LCO foliar application on greenhouse-grown soybean significantly increased aboveground dry biomass leading to overall plant dry biomass increase (Table 4). In a separate greenhouse experiment, foliar LCO application increased leaf chlorophyll and carbohydrate contents in soybean leaves 10 days after foliar application (Table5) which may explain LCO-induced increased photosynthesis as indicated by Smith et al., 2007. As compared to control, average yield increase of corn foliar field trials from five USA and five Argentina sites was about 400 kg/hectare (Table 4). We have reported here the observed effect of pea LCO signal molecules on non-host specific corn, wheat and cotton seeds and their subsequent growth and yield promotions. Earlier studies reported enhanced germination and early seedling growth with LCO on non-legumes (Prithiviraj et al., 2003; Smith et al., 2005). This was probably mediated through either LCO-induced increase in photosynthesis (Smith et al., 2007) or enhanced nutrient uptake (Supanjani et al., 2006) or LCO-stimulated mycorrhizal colonization (Xie et al., 1995). These may help infer the mechanism of LCO activity with non-legumes. However, this work mostly evaluated the LCO responses with corn, wheat and cotton and did not study the mode of action.

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# Investigations on the role of organic fertilizers in increasing crop productivity: a case study with commercial products of Prathista Industries Ltd, India

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#### Abstract

The importance of organic fertilizers in present day agriculture is increasing due to growing concerns on the use of chemical fertilizers. Application of organic amendments to soil enhances soil health besides reducing the leaching, volatilization and other losses due to chemical fertilizers. This paper presents the research results on the beneficial effects of organic fertilizers manufactured and supplied by Prathista Industries Ltd (PIL), Hyderabad, India. Field studies conducted on rice with an organic product, Aishwarya® (macro & micro nutrient complex of plant origin) as soil application enhanced seedling growth and tillers/plant. Further, the grain and straw yields were maximum with Aishwarya® and are at par with recommended chemical fertilizers (RDF). The other organic products of PIL such as Bio-zinc (Zinc Proteino Lacto Gluconate), Bio-Phos (organic phosphorus) and Bio-potash (organic potash) as soil application also enhanced tillers/plant, grain and straw yields in rice over that of control. The performance of these commercial products is at par with that of RDF. All these biotech manures including Suryamin® (organic nitrogen and other nutrient complex) individually and in combination at different rates yielded positive results in field evaluation studies on sugarcane. Higher cane yields and superior juice quality were reported with these commercial products over control and RDF. In another study, significant increase in biometric characteristics and yield were reported when Bio-Phos was applied to vegetable crops such as Brinjal and Onion. These commercial organic products also improved soil health and water holding capacity of soil especially near the root zone of crops.

Further in Vitro studies indicated that Prathista Organic products are highly degradable, increases Organic Carbon and Microbial activity of the soil and does not contaminate ground water. Studies on Rice-Pulse cropping system revealed that the Organic products increased the enzymatic activity of Ureases, Phosphotases and dehydrogenises in the soil resulting in more microbial activity and greater uptake of nutrients from the soil. Experiments conducted at Mekerere University, Kampala, Uganda on Bio-efficacy and field performance of Organic products resulted in increase in yield (20%) and quality in Rice, Corn and Vegetables. The efficacy results of these commercial products and other products supplied by PIL as nutrient

supplements in the leafy vegetable, Pechay (Brassicaceae) were also discussed in the paper. Our results indicate that the commercial organic products of PIL are highly effective in ameliorating soil health, enhancing yields of various crops under the conditions evaluated. Further, these products can have potentially beneficial effects on these crops. The scope of application of these PIL commercial products on other important vegetable crops is also discussed.

#### Introduction

Prathista industries limited (PIL) a bio-technology driven and professionally managed organization having ISO 9001, 140001, and OHSAS certifications. The organic inputs are certified by internationally accepted agencies as per National Programme for Organic Production (NPOP) and National Organic Programme (NOP) standards. Some of its products were reviewed and listed in Organic Materials Review Institute (OMRI), Oregon, USA. PIL manufactures these Organic Agricultural inputs through Bio-technology process by utilizing different strains like *Aspergillus niger, Lactobacillus delbrukii. fraturiaarentia, Pseudomonas flouroscence, Trichoderma viridae* etc.

Aspergillus niger has a capacity to convert glucose under certain specifically defined physico-chemical conditions to produce nutrient based Gluconate salts, this process involves several steps. Initially the culture (*Aspergillus niger*) is stored and maintained in its spore form on a specific solid medium. The spores of the organism are germinated in another defined liquid medium. The germinated spores act as preseed for raising a seed culture under submerged conditions at constant temperature, which is aerated and agitated at determined rates for certain period. On completion of fermentation process, the broth is there after filtered through filter press to separate dead biomass and to obtain a clear solution containing specific nutrient based Gluconates and will be evaporated to required concentration through vacuum evaporation. The final solutions will be formulated as per the specified requirements.

Effectiveness of any nutrient depends on its bioavailability, which means how well the living body absorbs and assimilates it for the ultimate utilization in their biochemical processes. Gluconic acid usually utilized by the soil microbial flora and thus further synergies other nutritional availability to plants. Nutrients uptake may be more efficient from naturally occurring chelates than from synthetic chelates. The significance of chelating process of gluconates in soil increase the availability of nutrients, prevent mineral nutrients from forming insoluble precipitates, reduces toxicity of some metal ions to plants, prevents nutrients from leaching, increases the mobility of plant nutrients and suppresses the growth of plant pathogens.

#### **Case Studies**
Series of experiments were conducted in both national and International Institutions to test the bio-efficacy and field performance of bio-tech products of Prathista Industries and the results are reviewed with specific reference to crops like Rice, pulses, sugarcane and vegetables.

Several long term experiments all over India indicated a decrease in rice productivity due to continuous use of chemical fertilizers. Integrated nutrient management involving certified organic manures aims to improve soil health and sustain high level of productivity and production. The experiment was conducted with Prathista organic manures (i.e. New Suryamin, Bio phos and Bio potash) and different types of vermicomposts prepared from various organic wastes i.e. cane trash, market waste, weeds and paddy straw along with recommended dose of chemical fertilizers under rice-pulse cropping system at Regional Agricultural Research Station, Anakapalle, India. Rice (Oryza sativa) and green gram (Phaseolus aureus) were taken as test crops. Results of two years experimentation revealed that the treatment receiving at 30 kg each of Suryamin, Bio phos and Bio-potash as basal and foliar sprayings of Bio-Zinc, Organic NPK, New Suryamin and Bio-potash @ 625 ml/ha at the time of 20, 40 and 60 days after transplanting (DAP) recorded significantly higher grain and pod yields in both the seasons consecutively for two years. However this was on par with the treatment received 75% RDF + 50% N through market waste vermicomposts in an integrated manner. The values of nutrient uptake by Rice and succeeding Pulse crop also showed significant differences among Prathista Organic manures and different vermicomposts. Addition of different organic manures resulted in the higher Organic Carbon content and available nutrient status of the soil compared to chemical fertilizers. An appreciable change in soil chemical properties with an enhancement in nutrient uptake was observed with the application of Prathista Organic manures. Enzymatic activities were analyzed consequent of application of Organic inputs to assess the revival of soil fertility. Urease, Phosphatase and dehydrogenase were analyzed at different stages of crop growth. Results showed that significantly maximum enzyme activity was recorded in the Prathista Organic manures added plots. Maximum dehydrogenase activity was observed in plots which received Prathista Organic manures might be due to more biological activity in this plot. The study revealed that the sustainability of rice-pulse cropping system can be enhanced by applying Prathista Organic manure with better nutrient uptake and soil fertility status compared to Inorganic Fertilizers. (Rao. P.C, Ramalakshmi Ch. S. and Sreerama Reddy N, 2011).

The efficacy of bio-potash formulation was investigated in paddy in comparison to muriate of potash an inorganic source of Potassium and control (No potash application). The experiment comprising of five treatments was laid out in a randomized block design with four replications and observations were recorded for grain yield, yield component characters and pest incidence. The results revealed a significant increase in grain yield, ear-bearing tillers/m<sup>2</sup> and filled grains/panicle with

the application of bio-potash compared to control and RDF. Application of 50kg /ha bio-potash as basal followed by bio-potash spray 625 ml in 200 lit of spray fluid per hectare at panicle initiation stage has resulted in a significant in grain yield (36% over control and 14.41% increase over RDF). Further cost of the bio-potash treatment was observed to be on par with muriate of potash application and hence may be recommended for use in paddy.

A field study was conducted to evaluate Bio-zinc on the grain yield of rice during kharif. 2008 at Regional Agricultural Research station, Warangal, India. The results indicated that soil application of Bio-zinc @12.5kg/ha along with the recommended dose of fertilizers (RDF) was statistically at par with the application of ZnSO<sub>4</sub> 25kg/ha along with the RDF.

Prathista organic manures were tested on sugarcane crop at Regional Agricultural Research Station, Anakapalle, India during 2009~2010. Four different combinations of biotech manures (Suryamin, Biophos, Biopotash and organic NPK), farmers practice and recommended dose of chemical fertilizers (112 kgN-100 kg P<sub>2</sub>O<sub>5</sub>+120 kg K<sub>2</sub>O/ha.) were tested in randomized block design with four replications. The treatmental details are as follows: T1: Recommended Dose of chemical Fertilizers, T2: Farmers Practice, T3: Suryamin+Biophos+Biopotash (each 75 kg/ha) as basal and sprayings of Org. NPK 45 DAP, Megacal 60 DAP and Biopotash 90 DAP (each 625ml/ha) T4: Suryamin+Biophos+Biopotash (each 75 kg/ha) as basal and sprayings of Org. NPK 30 DAP, Megacal 60 DAP and Biopotash 90 DAP (each 625ml/ha), T5: : Suryamin+Biophos+Biopotash (each 114 kg/ha) as basal and sprayings of Org. NPK @ 625ml/ha at 45, 60 and 90 DAP, T6: Biophos + Biopotash (each 75 kg/ha) as basal and spraving of Org. NPK at 45 & 65 DAP and Biopotash at 90 DAP each @625ml/ha. The Experimental soils are neutral in reaction with normal conductivity. Plant and soil samples were collected at the end of grand growth stage (270 days after planting) and after harvest of the crop. Chemical analysis of soil and plant samples were done. Juice analysis was carried at harvest. Similarly stalk population counts were recorded at the end of grand growth stage (270 days after planting). The results revealed that the treatment (T5) which received Suryamin + Bio-phos+Bio-potash each 115 kg/ha as basal and sprayings of Organic NPK 650 ml/ha at 45,60 and 90 DAP recorded highest cane and sugar yields of 84.5 and 10.41 t/ha. An increase of 23% of cane yield and 33% sugar yield were recorded over farmers practice. The same treatment when compared with RDF also recorded 7.6 and 11.7% increase in cane yield and sugar yield, respectively. However, this treatment was on par with Suryamin + Biophos + Biopotash each 75 kg/ha as basal and sprayings of Org. NPK 30 DAP & 60 DAP, Megacal 60 DAP and Bio-potash 90 DAP 625ml/ha (80.5 t/ha cane yield and 9.78 t/ha sugar yield) whereas recommended dose of chemical fertilizers recorded 78.5 and 9.32 t/ha of cane and sugar yields respectively. (Ratna Kumari and Ramalakshmi Ch. S, 2010).

A field experiment was conducted to study the comparative performance of different Organic manures (Farm yard manure, vermicompost and press mud cake) and Prathista organic manures (Suryamin, Bio-phos, Megacal, Bio-potash and Organic NPK) on soil health and yield of sugarcane at Agricultural Research Station, Anakapalle, India with a variety 93 A 145 during 2009. Result revealed that the application of organic manures significantly increased the soil fertility over initial status. Prathista organic manures slightly improved soil fertility status in organic manures treated plots than Prathista organic manures add plots, Maximum cane and sugar yield with better stalk population and number of millable canes were recorded in the Prathista organic manure treated plots. Maximum cane yield (102.41t/ha) and sugar yield (13.28t/ha) were recorded in Prathista organic manures treatment followed by vermicompost 5 t/ha (86.12 & 10.73 t/ha), farm yard manure 25 t/ha (85.17and 10.93 t/ha and press mud cake 7.6t/ha (72.71 and 9.01t/ha). Highest Benefit Cost Ratio (BCR) of 2.42 was recorded in Prathista organic Manures treatment. The study showed that Prathista organic manures are better substitute for chemical fertilizers in sugarcane crop for substained productivity and fertility in sugarcane. (Ratna Kumari and Ramalakshmi Ch. S, 2010).

The grain yield was significantly more in all the zinc applied treatments either through soil application or spraying treatments than the control. This might be due to direct contribution of nitrogen in terms of synthesis of chlorophyll, protein and amino acid and phosphorus by stimulating root system that directly helped for greater absorption and translocation of nutrients. Significantly higher yield (7790 kg/ha) was obtained with soil application of ZnSO<sub>4</sub> 10 kg/acre + spraying 3 ml/lt at 45 DAT along with recommended dose of fertilizers and soil application 5 kg/acre + spraying of bio zinc 3 ml/lt at 45v DAT along with recommended dose of fertilizers (7248 kg/ha) than control (4319 kg/ha) and RDF alone (6032 kg/ha) also influenced the grain yield of rice over RDF alone (6032 kg/ha). The favourable effect of zinc along with recommended dose of fertilizers on yield might be due to tryptophnn, the precursor of growth promoting substance led to better growth and higher yield.

Effect of organic agri inputs on soil nutrient availability and increase in yield parameters in green gram (*Phaseolus aureus*) was studied at AGNR. Agricultural University (ANGRAU). Bio-Phos Granules and Bio-potash Granules 75 kg/ha along with Bio-Zinc Granules 12.5 kg /ha + FYM 5 t/ha was compared with RDF. Highest soil organic carbon content of 1.44% was record in the treatment (20N-40  $P_2O_5$  and 20  $K_2O$ ) where "K" was applied through Bio-Potash compared to inorganic fertilizer application (1.00). Bio-phos alone incomparision with Bio potash Granules + Bio Zinc Granules resulted in higher "K" in soil. Highest Number of effects in nodules (12.0) was record in the treatment where P, K and Zn was Substituted by organic sources. Highest seed yield (1425.6 kg/ha) was recorded by treatment with organic inputs (Anuradha Ch. and Ramesh, 2010).

Field experiments were conducted separately to study the effect of Bio-phos granules of Prathista Industries 50 and 62.5 kg/ha as basal application and Bio-phos liquid 2 ml/lt (1, 2 and 3 sprays at 20 days after transplanting, pre-flowering and flowering stage) as foliar application on growth and yield of Egg Plant (Solanum melangila) and to study the effect of Bio-phos granules 50 and 62.5 kg/ha as basal application in two split doses at  $20 \sim 25$  and  $45 \sim 50$  days after planting and 2 sprays of biophos liquid 3 ml/lt at  $20 \sim 25$  and  $45 \sim 50$  days after planting on growth and yield of onion (Allium cepa) during summer 2008 and rabi 2008~2009, respectively at Vegetable Research Station, Rajendranagar, India. In Egg plant among different treatments, recommended farmers practice excluding P<sub>2</sub>O<sub>5</sub> + basal application of Biophos 50 kg/ha + 2 sprays of Biophos liquid 2 ml/lt of water at two critical stages of crop duration was found significantly superior with regard to plant spread, number of fruits per plant and yield over other treatments with application of generally available P<sub>2</sub>O<sub>5</sub> fertilizer. Application of Biophos granules is easy compared to Single Super Phosphate and is labour saving. With regard to the efficacy of Biophos granules, it is observed that 1 kg of Biophos granules will be equivalent to about 2 kg of single super phosphate to supplement the required  $P_2O_5$  and can be alternative to phosphatic fertilizer. Cost Benefit Ratio (1:5.01) of the treatment of Bio-phos 20 kg/acre + 2 sprays of Biophos liquid 2 ml/lt of water at two critical stages of crop growth and development compared to the control (1:4:2) is highly encouraging for economic benefits to the farmer. In onion, among different treatments, 50% recommended dose of DAP + two foliar application of Biophos liquid 3ml/lt was found significantly superior with regard to average bulb weight, yield per hectare, polar and equatorial diameter of bulb (bulb size) over other treatments with  $P_2O_5$ from other sources of fertilizer (SSP, TSP and Biophos granules) followed by the treatment with 50% recommended dose of SSP + two foliar applications of Biophos (3ml/lt). Highest cost ratio benefit of 1:2.69 was recorded with the treatment with 50% recommended dose of DAP + two foliar applications of Biophos (3 ml/lt) when compared to all the treatments and seems to be encouraging for the benefit of the farmer. Biophos as foliar spray is effective when applied with other phosphatic fertilizers at half of the recommended dose (RVSK.Reddy. RVSK 2010).

Pathological analysis, quality verification and field valuation of different Prathista Products (New Suryamin, Push, Megacal, Biozinc, Biopotash, Biophos) was done at Makerere University, Kampala Uganda. All the products were tested for their field evaluation in different crops viz., Rice, Maize, Banana, Cotton, Vegetables etc and an increased yield of about 15%~20% was reported in all the crops. (Herbert Talwana, 2008). Prathista Organic inputs for (Newsuryamin, Aishwarya, Bio-phos, Bio-zinc, Megacal, Push, Safe) were evaluated for their Bio-efficacy and field performance at The University of Phillippines, Los Banos, the results were positive in terms of Plant growth & yield compare to conventional chemical fertilizers. The products Megacal, Bio-Zinc, Bio-Potash and Newsuryamin were tested DASGAN Ltd., Turkey on Green pepper, Cereals and Banana. The performance was found to be satisfactory on yield which has recorded an increase of 20% over the usage of conventional fertilizers.

Organic fertilizers are associated in general with slow release of nutrients. Nutrients in organic fertilizers are usually bound up in organic matter and are gradually released over a period a period weeks, months, or even years as they are processed by soil microbial organisms. Studies were conducted at ANGRAU to evaluate the environment impact of Prathista organic fertilizers, Bio-Phos, Bio-Potash, Bio-Zinc, Mega Cal, Bio Magnesium, Bio-Sulphur and New Suryamin in comparison with recommended doses of inorganic fertilizers. Laboratory soil column experiments were conducted simulating the field condition to study the leaching losses / for probable contamination of groundwater. A crop experiment was also conducted to evaluate impact of the organic fertilizers on growth and yield of green gram (*Phaselolus aureus* Wilczek).

It has been determined that the organic fertilizers contain more than one essential nutrient. Thus these organic fertilizers could be a good source for increasing the nutrient pool of the soil. Application of these organic fertilizers to soils improved physico-chemical properties and nutrient status of the soils. The readily available carbon fraction of the organic fertilizers supported the development of microbial biomass in the soil. The microbial biomass in soil was higher by 3 log cycles in organic fertilizer treatments compared to inorganic fertilizers. The results revealed that bacterial population was higher compared to fungi. Analysis of leachates equivalent to three pore volumes of water recorded nutrients at higher concentration initially than inorganic but with time there was decrease in nutrient content, indicating the availability and slow release of nutrients. The leaching study revealed that the leachates were within the permissible limits of ground water quality. The benefits were also reflected in growth and yield of green gram. An increase of 51% in leaf area, 20% in dry matter and effective nodulation were recorded wherein in P, K and Zn was applied through organic fertilizers. The yield attributing characters and seed yield was recorded wherein in P, K and Zn was supplied through organic fertilizers. The yield attributing characters and seed yield were recorded high by application of Bio-Phos + Bio-Potash + Bio-Zinc. Organic fertilizers resulted in higher uptake of respective nutrients and also showed complementary effect on the nitrogen content, compared to inorganic fertilizers. Thus, present investigation clearly indicated that organic fertilizers could be a good option to replace the indiscriminate use of inorganic fertilizers (Muttulakshmi. D and Prabhuprasadini, 2010).

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# PGPR bioinoculants for ameliorating biotic and abiotic stresses in crop production

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## Abstract

Worldwide, salinity is one of the most important abiotic stresses that limit crop growth and productivity. Ion imbalance and hyperosmotic stress in plants caused by high concentrations of salt often lead to oxidative stress conditions for plants. Salinization may be due to natural causes, and is common in the hot and dry regions of the world, or it may be a consequence of inadequate irrigation management practices. Our study focused on evaluation of plant growth promoting rhizobacteria (PGPR) for induction of growth promotion and enhancement of tolerance to salt in wheat under pot conditions. The interaction between PGPR strains reduced total root length but enhanced root hair length. The mechanism of PGPR elicitation of growth promotion may involve the enhancement of root hair development and therefore increase nutrient and water uptake. Single and dual inoculations of PGPR strains showed variations in their effect to enhance the crops tolerance to salt. The bacterial consortium was effective for wheat plants as an acceptable and ecofriendly technology to improve plant performance and development.

# Introduction

The growth of plants in the field is determined by the numerous and diverse interactions among its physical, chemical and biological components of soil as modulated by the prevalent environmental conditions. In particular, the varied genetic and functional activities of the extensive microbial populations have a critical impact on soil functions and plant growth, based on the fact that microorganisms are driving force for fundamental metabolic processes involving specific enzyme activities (Nannipieri *et al.*, 2003). The crop production in general and productivity in particular is inhibited by a large number of both biotic and abiotic stresses. These stresses include extremes of temperature, high light, flooding, drought, the presence of toxic metals and environmental organic contaminants, radiation, wounding, insect predation, high salt, and various pathogens including viruses, bacteria, fungi and nematodes (Abeles *et al.*, 1992). As agricultural production intensified over the past

few decades, management of these biotic and abiotic stresses on crop plants with increased use of chemical inputs caused several harmful effects on the environmental quality.

A major strategy to counteract the rapid decline in environmental quality is to promote sustainable agriculture with the gradual reduction in the use of fertilizers and pesticides and greater use of the biological and genetic potential of plant and microbial species, which may help to sustain high production in agriculture. In addition to the plants ability to modify its physiology and metabolism, certain soil microorganisms present in the rhizosphere can help the plants to either avoid or partially overcome these environmental stresses (Govindasamy *et al.*, 2008). The application of plant growth promoting rhizobacteria as biofertilizers and biocontrol agents is being considered as an alternative or supplemental way of reducing the use of chemicals in crop production (Kloepper *et al.*, 1989; Vessey 2003; Maheshwari, 2011).

In this paper we review the work done in our laboratory using plant growth promoting rhizobacteria (PGPR), for combating biotic and abiotic stresses in plant growth and production.

# **Material and Methods**

# Bacterial isolates

Rhizospheric soils samples from field grown soybean, mustard, wheat and rice were collected and stored in -20 °C till use. Serial dilutions were performed from these soils and plated on appropriate media. Bacterial colonies were picked based on their dominance and variations. These were purified and cryopreserved in -80 °C as glycerol stocks.

# PGPR traits

All isolates were screened for their PGPR activities under *in vitro* conditions viz; IAA production, siderophore production and ACC deaminse activity. The antagonistic ability of the bacterial isolates against *Rhizoctonia bataticola*, *Slerotium rolfsii* and *R. solani* was tested on potato dextrose agar (PDA) plates.

Detection of antibiotic genes and nifH

PCR amplification was carried out with a set of gene specific primers of *phlD*, *phz* and *nifH*. PCR protocol and conditions used was as per the procedures published (Senthilkumar *et al.*, 2009).

Seed bacterization and in vivo screening for biocontrol activity and cadmium stress

Pots with 2kg of soil were sterilized, inoculated with *R. bataticola* (grown on sand: maize grain [4:1] medium for 7 days), and incubated for 1 week. Surface sterilized seeds of soybean susceptible to charcoal rot were treated with bacterial isolates and sown in the pots. Plant were grown under controlled conditions and observed for percent germination and disease symptoms after 10 day and 21 days of sowing respectively.

For *in vivo* screening for cadmium and salinity stress, wheat seeds were surface sterilized as given above. These were imbibed with bacterial suspension (CFU  $1 \times 10^6$  ml seed<sup>-1</sup>) along with 0.1% CMC for 4h. Bacterized seeds were placed in conical flasks containing moist filter papers and incubated for 8 days. Half of the seedlings were subjected to Cd and another half to NaCl stress after 8 days by adding 2ml of 150mg/kg CdCl<sub>2</sub> and NaCl respectively. After 36h, stress ethylene evolved was measured. Effect of salinity on seed germination and root elongation was measured on seeds treated to different NaCl concentrations (80mM, and 160mM) with and without bacterization.

# Data analysis

Data from completely randomized design experiment on in vivo biocontrol acitivity was arc sine transformed prior to statistical analysis. Results were analyzed by using one way ANOVA.

# **Results and Discussion**

We investigated some physiological traits related to biocontrol and plant growth promoting activity of rhizospheric isolates from different crop species (Fig 1.). Our aim was to select bacterial isolates with potential for ameliorating biotic and abiotic stress to the selected crop plant. Five hundred bacterial isolates were screened and studied individually for some selected PGP traits like IAA production, siderophore production, nitrogen fixation, ACC deaminase activity and biocontrol activity.

Forty six percent of the bacterial isolates showed potent antifungal and antibacterial activity against the test pathogens under *in vitro* conditions (Fig.2). However, only one isolate was able to inhibit all the test pathogens under these conditions. This bacterium was identified based on the 16S rDNA sequencing as *Paenibacillus polymyxa*. The same was also tested for its efficacy to control the charcoal rot disease incidence under pot conditions. Treatment with *R. bataticola* caused severe charcoal rot of soybean seedlings, and a PDI as high as 71.0 was recorded (Table1). Typical symptoms were noted; i.e the presence of a reddish-brown lesion on emerging hypocotyls, silvery to gray areas on the lower portion of the stem and tap root and the presence of microslerotia in the pith and outer surface. However, with the seed bacterization, the PDI was lowered by almost 80%. Percent seed germination also improved in *P. polymyxa* treated seeds. Mutants (which had lost their antifungal activity) were unable to stem the disease (Table1).

ACC deaminase positive isolates stimulated root length and its growth (Fig.3). Under heavy metal stress these isolates lowered the ethylene production in the vicinity of the growing seedlings and prevented them from stunted/reduced growth (Fig.4).

On screening all the bacterial isolates for growth in different NaCl concentrations, we found ten potential isolates growing well in 10% salt concentration. These were screened for their effect on seed germination of wheat under different salt regimes. One visible effect of both salt stress on wheat seedlings was the lower shoot size. In

fact the relative shoot elongation rate (RER) of shoots diminished significantly in wheat seedlings grown at 160mM and 320mM NaCl.

Our results clearly demonstrate the ability of bacteria with such traits to be of potential use as bioinoculants for crops growth and productivity under adverse environmental conditions.

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Fig. 1 Functional diversity in rhizobacteria



Fig 2 In vitro bioassay against Rhizoctonia bataticola



Fig 3 Effect of selected bacterial isolates on root elongation of wheat seedlings under gnotobiotic conditions



Fig 4 Effect of selected bacterial isolates on reduction in ethylene synthesis of wheat seedlings under cadmium stress

Treatments	Seeds germination (%)	Mean disease ratio (MDR)	Percent·disease-incidence- (PDI)+
<sup>L</sup>			
NRC·12*	72.50(58.38)b	1.25	31.25 (33.98)cd
NRC·12			
(control)	37.50(37.75)c	2.84	71.00 (57.42) c+
PK-262	67.50(%%.25)b	1.35	33.75 (35.50)c+
Carbendazine	72.50(58.38)b	1.17	29.25 (32.72)ad+
P. polymyxa	80.00(63.54)a	1.08	27.00 (31.29)a
Mutant-1	22.50(28.31)d	2.77	69.25 (56.32)de+
Mutant-2	25.00 (29.92)d	2.68	67.00 (54.93)d.

Table 1 In vivo biocontrol activity of Paenibacillus polymyxa against R. bataticola in soybean

NRC 12 = susceptible variety; PK 262= tolerant variety

Figures in parenthesis are arcsine transformed values

Treatment values followed by same superscript letter do not differ significantly at P=0.05\*Except this treatment; the pot mixture of all treatments were challenged with *R. bataticola* 

# An improved selection strategy of *Ralstonia solanacearum* biocontrol agents based on rhizocompetence and antagonistic activity towards genetically diverse isolates

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## Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is a serious threat to crop production in China. A collection of 319 R. solanacearum strains isolated from 14 different diseased host plants collected in 15 Chinese provinces was investigated by BOX fingerprints in order to test the influence of the site and the host plant on the genetic diversity. Phylotype, *fliC*-RFLP patterns and biovar were determined for all strains. BOX-PCR fingerprints generated from the genomic DNA of each strain revealed a high diversity of the phylotype I strains. Many 28 BOX clusters comprised isolates from different provinces and several host plants but some plant- or sitespecific BOX groups were also identified (Xue et al., 2011). Eight antagonistic bacterial strains were tested for their inhibition towards a range of genetically diverse R. solanacearum strains and for their efficiency to control Ralstonia wilt of tomato under greenhouse conditions. The results indicated that the same antagonists showed remarkable differences in *in vitro* antagonistic activity towards different R. solanacearum strains, and biocontrol efficacy. Serratia marcescens XY21 was selected for further study because of its best in vitro inhibition of all ten R. solanacearum strains and its good rhizocompetence (> $10^7$  CFU/g rfw) on tomato plants. Furthermore, the biocontrol efficacy of XY21 towards seven R. solanacearum strains that were pathogens of tomato ranged from 19% to 70%. A good correlation of in vitro antagonistic activity and the actual biocontrol efficacy towards different R. solanacearum strains was found. The results suggest that potential biocontrol strains of *R. solanacearum* should be selected not only based on their rhizocompetence but also on their ability to antagonize a range of genetically diverse R. solanacearum isolates.

# Root exudates/metabolite profile are differently altered in host/nonhost plant growth promoting rhizobacteria (PGPR) interactions

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#### Abstract

Interaction of plant growth promoting rhizobacteria (PGPR) with plant roots is crucial for the successful establishment of PGPR and their beneficial effect. Root exudates (RE) are critical for sufficient colonization of roots and compatibility of the PGPR with the plant host. Changes that occur both in the plant and bacterial systems would provide valuable information regarding the involvement of different metabolites in plant-PGPR interaction. Attenuated Total Internal Reflection-Infrared (ATR-IR) spectroscopic studies of two PGPR, namely Bacillus cereus (MTCC 430) and Paenibacillus elgii, when grown in minimal medium and/or in presence of host and non-host RE show distinct variation in the functional groups. Difference in peaks at wavenumbers 1050, 1660 and  $2700 \sim 3000 \text{ cm}^{-1}$  suggested changes in cell wall polysaccharides, lipids/fatty acid components and proteins in membrane/cell wall. Split amide I peak in presence of host RE suggested a change in protein conformation in the cell wall due to nutritional variation. The changes in the host and/or non-host RE compounds such as organic acids, phenols, flavonoids and sugars were studied in presence or absence of *B. cereus*. Organic acids and sugars did not differ in host RE in response to B. cereus however, qualitative changes occurred in non-host RE at the initial stages of interaction affecting colonization by B. cereus in non-host plant resulting in no growth promotion. Similar changes in phenols and flavonoids during the initial stages of interaction substantiated the early response of the plant to PGPR.

# **Evaluation of rhizosphere bacteria for biological control of** *Fusarium* foot and root rot of tomato (TFRR) in salinated soil

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# Abstract

Crop cultivation in salinated soils is one of the major agricultural challenges worldwide. The aim of the present work was to improve tomato fruit yield by protecting these plants against soil-borne pathogens and to promote their growth in salinated soil. Twenty one percent of the tomato plants grown in salinated Uzbek soil showed disease symptoms, indicating the presence of tomato pathogens in this soil. The dominant pathogen, which causes tomato foot and root rot, was isolated and was identified as F. solani. Fifty two bacterial strains from strain collections of five different institutes were evaluated for their ability to protect plants against tomato foot and root rot after bacterization of the seeds and infestation of soil with the isolated F. solani pathogen. Infestation of the soil with F. solani resulted in an increase of the percentage of diseased plants from 21 to 46. Priming of seedlings with the eight selected bacterial strains, reduced this proportion to as low as 19 %. In addition, in the absence of an added pathogen, three strains namely P. putida 1T1, P. extremorientalis TSAU20 and Stenotrophomonas rhizophila ep10 showed a significant stimulatory effect on tomato plant growth, increasing the dry weight of whole tomato plants up to 27% in comparison to the non-bacterized control. The strains also increased tomato fruit yield in a greenhouse varying from 14% to 22%. We conclude that many beneficial bacteria isolated from plants growing on nonsalinated soil are perfectly able to promote plant growth and control plant diseases in salinated soil. In other words, salination caused by a possible future climate change does not seem to be a threat for the application of presently used plant-beneficial bacteria. In addition, our results show that the dogma that beneficial strains should be isolated from the plant and climate on/in which they should be applied is not valid: all our strains were isolated from plants other than cucumber and came from cold or moderate climates.

Key words: biological control; tomato; tomato foot and root rot; *Fusarium* solani; rhizobacteria

# Introduction

Tomato (Lycopersicon esculentum) is the second most important vegetable crop in the world. The present world production is about 100 million tons, of which Uzbekistan produces 1,583,571 tonnes (FAOstat, 2007). Soil salinization is reducing the area that can be used for agriculture by  $1\% \sim 2\%$  every year, hitting hardest in the arid and semi-arid regions (Shirokova et al. 2000). Uzbekistan, located in Central Asia, is an example of a country in which soil salinity is a major concern in that it results in degradation of agricultural land. Substantial areas on which vegetables are cultivated are affected by soil salinity. As a result of soil salinization, plants are under saline or water unbalance stress and become more vulnerable to diseases, often caused by pathogenic fungi. For example, soil borne pathogens such as Fusarium, Pythium, Rhizoctonia, Botrytis and Verticillium cause significant losses in tomato fruit yield (Kita et al., 2005; Sharma and Nowak, 1998). Tomato foot and root rot seedling disease is a serious problem in saline soils of Uzbekistan and often results in a substantial stand loss. One of the possible measures to improve crop health is to use salt tolerant bacterial inoculants which can control diseases (biological control agents) and/or which promote plant growth (Lugtenberg and Kamilova 2004; Mayak et al., 2004). In this study we describe experiments in which a series of salt tolerant plantbeneficial bacterial strains was tested for their ability to control tomato foot and root rot caused by *Fusarium solani* and stimulate growth of tomato in salinated soils.

## **Material and Methods**

The eight most successful microbial strains of the original 52, were selected and appeared to be *P. trivialis* 3Re27, *P. putida* 1T1, *Serratia plymuthica* RR-2-5-10 and *Stenotrophomonas rhizophila* e-p10 from Germany, *Pseudomonas fluorescens* SPB2145 and *P. chlororaphis* RRj228 from Russia, *P. extremorientalis* TSAU20 from Uzbekistan and *P. fluorescens* PCL1751 from Netherlands. For biological control experiments, sterilized tomato seeds were coated with bacteria (1x10<sup>8</sup> CFU/ml) in sterile PBS buffer. For soil infestation, *F. solani* spores were mixed with salinated soil to  $3.0 \times 10^7$  spores/kg soil. The used soil has an EC value of 659 mS m<sup>-1</sup> soil. Each treatment contained four groups of twelve plants. The plants were grown under open natural conditions at  $21 \sim 24$  °C. The number of diseased plants was determined usually four weeks after sowing. The dry weight of the whole plants was also determined.

For competitive root tip colonisation assay cell suspensions of overnight grown strains to compete were mixed equally. In the case of competition the test strain versus WCS365 or PCL1285, (a Tn*5luxAB* derivative of WCS365) ten seedlings were used. Inoculated seedlings were sown in sterile glass tubes with sand. Plants were grown for 7 days in climate-controlled chambers with 16 h of daylight at 24  $^{\circ}$ C. Subsequently 1 cm of root tip from plantlets was collected. Bacterial cells were

removed from the root tip by vortexing in PBS and plated on KB medium and KB medium supplemented with kanamycin to distinguish between the strains put in competition. Algorithm of data was used for statistical analysis. The salinated soil of the greenhouse in the province of Tashkent that was used in 2009 has an EC value of 659 mS/m<sup>-1</sup> soil. *Pseudomonas* was grown in KB medium and *Stenotrophomonas* in Corn-molasses medium. The tomato seeds were coated as described previously. When seedlings had reached the 2-4 leaf stage, they were transplanted to a non-heated greenhouse in six rows in each experimental plot  $(3 \text{ m} \times 2.5 \text{ m})$ . The temperature ranged between  $24 \sim 27 \text{ °C}$  during the day and between  $14 \sim 17 \text{ °C}$  at night.

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98. Comparisons were done using Student's *t*-test. Mean comparisons were conducted using a least significant difference (LSD) test (P=0.05).

#### **Results and Discussion**

Salt stress not only causes a decline in the metabolic activity of plant cells, it also results in an increased susceptibility of the plant towards phytopathogens (Werner and Finkelstein, 1995). Fifty two bacterial strains were evaluated twice for their ability to promote plant growth and to control TFRR in preliminary experiments in salinated soil. Based on the results of the experiments, the eight most efficient strains, *P. fluorescens* PCL1751, *P. trivialis* 3Re27, *P. putida* 1T1, *P. chlororaphis* RRj228, *P. fluorescens* SPB2145, *P. extremorientalis* TSAU20, *S. rhizophila* e-p10 and *S. plymuthica* RR2-5-10 were screened for their ability to suppress TFRR caused by the identified *F.solani* isolate (Table1). Twenty one percent of the plants which had grown in soil to which no *Fusarium solani* spores had been added were diseased, whereas in the presence of the added pathogenic fungus 46% of the plants had disease symptoms (Table1). All selected bacterial isolates, with the exception of *P. trivialis* 3Re27, *P. fluorescens* SPB2145 showed statistically significant disease control in comparison to the *Fusarium*-infected control plants (Table1).

All bacterial strains increased the shoot or root growth of tomato in comparison with the untreated control (Table1). This effect is not necessary the result of plant growth stimulation by the bacteria, it may also be caused by their biocontrol properties since the natural level of pathogens is high.

The best performer strains were *P. putida* 1T1, *P. extremorientalis* TSAU20 and *S. rhizophila* e-p10 which increased the root, shoot length and dry weight by 39%.

Treatments <sup>a</sup>	F. solani	Diseased	Shoot length	Root length	Dry weight
		$(\% \pm SD)$	length	lengui	
Positive control	-	$21 \pm 4.8$	100	100	100
Negative control	+	$46 \pm 10.8$	$(7.65)^{b}$	$(5.64)^{b}$	$(0.14)^{c}$
P. fluorescens PCL1751	+	$21 \pm 4.8*$	105	122*	101
P. trivialis 3Re27	+	$33 \pm 9.6$	109	120*	110
<i>P. putida</i> 1T1	+	$20 \pm 4.2*$	125*	134*	124*
P. chlororaphis RRj228	+	$25 \pm 6.8*$	95	121*	100
P. fluorescens SPB2145	+	$33 \pm 6.8$	112	105	101
P.extremorientalis TSAU20	+	$25 \pm 9.6^{*}$	128*	130*	127*
S. rhizophila e-p10	+	$19 \pm 4.2*$	119*	139*	120*
S. plymuthica RR2-5-10	+	$33 \pm 6.8$	106	116	106

 Table 1
 Control of tomato foot and root rot and stimulation of plant growth in salinated soil by selected bacterial isolates

<sup>a</sup> Plants were grown under open natural conditions in pots containing salinated soil infested with *F. solani* spores, except for the positive control in which no spores were added to the soil. <sup>b</sup> Expressed as cm of root and shoot, <sup>c</sup> Expressed as g per plant, \* Significantly different from the control at P < 0.05

Mechanisms by which bacteria are able to prevent damage caused by plant pathogens and promote plant growth include antagonism, induction of systemic resistance, competition for nutrients and niches, mobilization of nutrients and production of phytohormones (Lugteberg and Kamilova, 2004).

It is known that unfavourable environmental factors such as salinity lead to sharp changes in the balance of phytohormones, associated with a decline in the level of growth activating hormones such as IAA (Jackson, 1997). We propose that bacteria which are able to produce IAA under saline condition may supply additional phytohormone to the plants, thus may help stimulate root growth and reverse the growth inhibiting effect of salt stress to a certain extent in both shoot and root growth. The bacterial strains *S. plymuthica* RR2510, *S. rhizophila* e-p10 and *P.fluorescens* SPB 2145 produce auxin, were antagonistic against *F.solani*.

Best strains *P. putida* 1T1, *P. extremorientalis* TSAU20, *S. rhizophila* e-p10 which stimulated plant growth and control root rot disease of tomato were selected for greenhouse experiments. All three bacterial strains caused statistically significant increases in plant height and yield of tomato (Table2). The bacterial treatments increased the yield of tomato fruit up to 22%, and plant height up to 27% compared to the uninoculated control plants. The results presented here indicate that plant growth-promoting rhizobacteria (PGPRs) can have a substantial beneficial effect on tomato yield in salinated soil. To our knowledge this is the first report illustrating an increase in fruit yield of tomato growing in salinated soil caused by bacterization of seeds.

Treatment	Plant height % (cm)		Fruit yield (kg/m <sup>2</sup> )	%
None	$125 \pm 3.7$	100	13.9 ±0.9	100
P. putida 1T1	159* ±3.4	127	$16.6^* \pm 1.2$	119
P.extremorientalis TSAU20	$157* \pm 4.2$	126	$17.0^{*} \pm 0.7$	122
S. rhizophila e-p10	$150^* \pm 4.7$	120	$15.9* \pm 1.0$	114

#### Table 2 Effect of bacterial strains on tomato (cv. Belle) germination, height and

fruit yield in greenhouse experiments

<sup>a</sup> Tomato seeds were sown on 1.03.2009 and fruits were harvested on 20.06.2009; the temperature range was: day  $22\sim24$ °C, night  $12\sim14$ °C

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# Use of root endophytic bacteria for bio-control of soil-borne diseases in *Atratylodes macrocephala*

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#### Abstract

Atractylodes macrocephala (Compositae) is an important traditional Chinese medicinal plant. However, the soils they grow often suffer the pressure of continuous cropping, which leads to an increase in *disease* incidence caused by some soil-borne phytopathogenic fungi including *Fusarium oxysporam, Sclerotium rolfsii* and *Rhizoctonia solani*. The demand for fungicide-free herbs necessitates the development of novel microbial agents to suppress the diseases. Indigenous root mutualistic endophytic bacteria have recently been recognized for their multiple beneficial effects for plant health and physiology in a variety of ways. In this work, we aim to assess their biotechnological value to enhance the tolerance of plants to pathogen infection. A wide range of bacteria were recovered from healthy root samples. In vitro (on medium) bioassay indicated that some of them (especially *some* Pseudomonas fluorescens) showed strong anti-pathogens activity. In vivo (in the rhizosphere) test would be performed to evaluate the bio-control efficiency of these candidate bacterial strains.

# Genetic diversity of siderophore-producing bacteria in rice

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# Abstract

Microbes have developed high-affinity ingestion mechanisms to assimilate iron (Fe) and other metals. Effective plant growth-promoting rhizobacteria (PGPR) including siderophore-producing bacteria were used to enhanced the growth of plant. However, endophytic siderophore-producing bacteria in plant were competitive advantages to colonize plant tissues and to exclude other microorganisms from the same ecological niche. This work shows that the community of endophytic siderophore-producing bacteria (SPB) isolated form rice in Yunnan province is diverse. The genetic diversity of SPB was studied by amplified ribosomal DNA restriction analysis (ARDRA), 16S rDNA sequence homology and phylogenetics analysis methods. These results show that 42% of the total 220 strains produced siderophores on CAS medium. Less than 15% of the heterotrophic bacteria produced siderophores in leaves and stems, but most of them were siderophore-producers in roots. Bacteria belonging to the genera Bacillus, Burkholderia, Agrobacterium and Pantoea were predominant in roots. The genus Bacillus, Pseudomonas and Sphingomonas appeared in leaves and stems. However, Bacillus was permanently associated to any of the plant tissues. The genetic diversity of strains showed that a total of 21 ARDRA patterns were identified among the 92 siderophore-producing bacterial strains. On the basis of multiple correspondence analyses, the 21 ARDRA patterns constituted four putative genetic lineages. Lineage 1 consisted of 12 and 9 stains isolated from roots and leaves respectively, but no strains from stems. Lineage 2 contained 42 strains that formed a dominant lineage; this lineage was widely distributed in different part of the plant. Many strains isolated from root were in lineage 3. Lineage 4 was less-strains lineage sharing two ARDRA patterns, but strains isolated from stems were not found.

Key words: rice; genetic diversity; siderophore-producing bacteria; PGPR

# Pseudomonas aurantiaca PB-St2 - a bio-fungicide and

# a bio-fertilizer for the future

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# Abstract

Sugarcane is an important cash crop grown over one million hectare in Pakistan. Although production in the country has increased over time, the increase in productivity per unit area has been very low. Red rot disease caused by the fungus Colletotrichum falcatum is one of the production constraints. It is also responsible for the deterioration of sugarcane cultivars in other countries such as USA, Australia, Taiwan, Thailand, India and Bangladesh. As plant protection chemicals are currently not available for this disease, one key to overcome this situation is the use of biocontrol agents to contain this disease. A new strain PB-St2, of Pseudomonas *aurantiaca* has been isolated from the stem of a local sugarcane variety. Our studies showed that it produces antifungal compounds which are active against isolates of *Colletotrichum falcatum*, Fusarium Fusarium oxysporium and lateritium. Investigation of its secondary metabolites showed the production of phenazines, a cyclic lipopeptide, a pyoverdine, a hydroxamate type siderophore and a homoserine lactone. In addition to that it also produces indole acetic acid, HCN and has ability to survive at alkaline pH, high salt concentration and a range of low to high temperature. The isolate was also positive for the production of ACC deaminase, lipase and protease enzymes. Based on reports in the literature and the production of antifungal compounds by this strain, a formulation of bio-fungicide and a bio-fertilizer, for sugarcane in Pakistan can be envisioned.

#### Introduction

Red rot is one of the oldest known diseases of sugarcane caused by the fungus *Colletotrichum falcatum*. It occurs in most cane-growing countries. In Pakistan it is the most common disease of sugarcane which affects the cane yield and sugar recovery. The average sugarcane production in Pakistan is 35 tons/ha whereas India with almost similar soil and climatic conditions is obtaining 66 tons/ha (www.pakkissan.com). When the production is already low, red-rot disease aggravates the situation. It decreases the cane yield and sugar recovery from 25 to 75% depending on crop variety. Farmers are recommended to use healthy seeds but it

is difficult to diagnose the dormant fungal infection in seed canes. Chemical fungicides to suppress this disease are not available.

In order to increase the yield of sugarcane, commonly chemical fertilizers are applied. However, the usage these substances results also in environmental and ground water pollution. Furthermore, most of the concerned farmers in developing countries experience shortage of the recommended fertilizers during crop season due to a supply bottleneck. The use of plant growth promoting rhizobacteria (PGPR) as a biological control agent and a bio-fertilizer can help to solve both issues.

Fluorescent pseudomonads have received the most prominent attention as candidates for biocontrol agents and biofertilizers because of their ability to colonize the surfaces and internal tissues of roots and stems (endo- and exorhizosphere) at high densities. These bacteria can compete successfully with soil microorganisms. Two species, P. fluorescence and P. putida are well known to promote the growth of several plants including sugarcane due to their ability of indole acetic acid production and phosphate solubilization (Mehnaz et al., 2009 b; Rodriguez and Fraga, 1999). Pseudomonads also have a tremendous capacity for production of antifungal secondary metabolites. Over twenty Pseudomonas species are known to synthesize more than 100 aromatic antibiotics and antibiotic-like compounds (Feklistova and Maksimova, 2008). Some well known antibiotics are phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN),2,4-diacetyl phloroglucinol (Phl). pyocyanine, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, viscosinamide and tensin (Liu et al., 2007). A number of *Pseudomonas*-based biocontrol inoculants have now been commercially developed (Mark et al., 2006).

As part of our ongoing assay-based screening program for new rhizobacteria, indigenous to sugarcane plants with antagonistic activity against phytopathogens, we isolated a novel strain of fluorescent pseudomonad, *Pseudomonas aurantiaca* PB-St2 (Mehnaz *et al.*, 2009a), which exhibited potent inhibition of the red-rot causative agent, the fungus *C. falcatum*. The current study describes the isolation and characterization of its secondary metabolites and assessment of its potential as a biofertilizer and biocontrol agent.

## **Material and Methods**

## Assays for the identification of PGPR traits

Agar plate assays were performed to check the phosphate solubilization, production of ACC deaminase, extracellulase enzymes, HCN, siderophores, and homoserine lactones, as described by Mehnaz *et al.* (2010). Indole acetic acid production was confirmed by HPLC analysis (Mehnaz and Lazarovits, 2006). Survival of PB-St2 at pH  $3 \sim 10$ ,  $4 \sim 41$  °C and NaCl concentration,  $0 \sim 5\%$  was

checked on LB medium. Antifungal activity by agar diffusion assay was checked against *Colletotrichum, Fusarium* and several other pathogenic strains (see Table1).

# General analytic procedures

High-performance liquid chromatography (HPLC) was carried out using a Merck-Hitachi system consisting of a L-6200 pump, a L-7420 UV-VIS detector and a Knauer interface box, controlled by Knauer EuroChrom software. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 DPX spectrometer. Spectra were referenced to the residual solvent signals with resonances at  $\delta_{H/C}$ 3.35/49.0 ( $d_4$ -MeOH) and  $\delta_{H/C}$  7.26/77.0 (CDCl<sub>3</sub>), respectively. HR-ESIMS were recorded on a Bruker Daltonic's micrOTOF-Q instrument.

# **Isolation of phenazine analogs**

PB-St-2 was grown with shaking (140 rpm) at 26°C in four 5,000 ml Erlenmeyer flasks, each containing 1 liter LB medium. After 72 h of incubation, cell and supernatant fractions of the cultures were separated by centrifugation at 3000 rpm for 20 min. Portions of the supernatant were extracted four times with 1 liter chloroform at pH3 to yield 1.6 g of crude extract. The extract was fractionated by vacuum liquid chromatography (VLC) over reversed-phase silica gel (Macherey-Nagel Polygoprep 50-60) using a stepwise gradient of methanol-water and finally dichloromethane to give six fractions. <sup>1</sup>H NMR profiling of these fractions indicated the 20% H<sub>2</sub>O in MeOH fraction and the 100% MeOH fraction to be of further interest due to a large number of aromatic resonances in its <sup>1</sup>H NMR spectrum. Separation of the (80:20)fraction by RP-HPLC by using a linear gradient of  $55:45 \sim 100:0$  MeOH-H<sub>2</sub>O (0.05% TFA) over a period of 40 min, followed by isocratic elution at 100% MeOH for an additional 20 min (Phenomenex Luna C18-100A, 250mm × 10 mm, 5 µm column in combination with a Phenomenex SecurityGuard Luna-C18  $10 \text{mm} \times 10 \text{ mm}$ precolumn; 1.9 ml/min flow rate; UV monitoring at 215 nm) produced nine subfractions. Subfraction 4 showed typical resonances for phenazine-like compounds in its <sup>1</sup>H NMR spectrum and appeared to be a mixture of at least two phenazines. The chloroform-soluble parts of subfraction 4 represented already pure 2.0 mg phenazine-1-carboxylic acid, while rechromatography of the methanol-soluble parts by RP-HPLC applying a linear gradient of 65:35~80:20 MeOH-H<sub>2</sub>O (0.05%TFA) over a period of 30 min (Phenomenex Luna C18-100A, 250mm×10mm, 5µm column in combination with a Phenomenex SecurityGuard Luna-C18  $10mm \times 10mm$  precolumn; 2.0 ml/min flow rate; UV monitoring at 215 nm) provided 3.5 mg of pure 2-OH phenazine.

**Phenazine-1-carboxylic acid (PCA)** yellow needles; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  8.00 (1H, brt), 8.03 (1H, brt), 8.05 (1H, brt), 8.32 (1H, brd), 8.37 (1H, brd), 8.54 (1H dd, J = 1.5; 8.8 Hz), 8.99 (1H, dd, J = 1.5; 7.1 Hz); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):

δ 124.9 (s), 128.0 (d), 130.1 (d), 130.3 (d), 131.7 (d), 133.2 (d), 135.1 (d), 137.4 (d), 139.8 (s), 140.1 (s), 143.4 (s), 144.1 (s), 165.9 (s); HR-ESIMS [M+Na]<sup>+</sup> *m*/z 247.0475 (calc. for C<sub>13</sub>H<sub>8</sub>N<sub>2</sub>NaO<sub>2</sub>, 247.0483).

**2-OH-Phenazine** orange powder; <sup>1</sup>H NMR (300 MHz,  $d_4$ -MeOH):  $\delta$  7.31 (1H, d, J = 2.6 Hz), 7.62 (1H, dd, J = 2.6; 9.5 Hz), 7.87 (1H, td, J = 1.5, 7.6 Hz), 7.97 (1H, td, J = 1.5, 7.6 Hz), 8.10 (1H, brd, J = 8.7 Hz), 8.12 (1H, brd, J = 9.5), 8.21 (1H, brd, J = 8.7); <sup>13</sup>C NMR (75 MHz,  $d_4$ -MeOH): 105.7 (d), 127.0 (d), 128.3 (d), 2 x 130.6 (d), 132.2 (d), 133.5 (d), 141.3 (s), 142.0 (s), 142.5 (s), 144.0 (s), 163.6 (s); HR-ESIMS [M+H]<sup>+</sup> m/z 197.0709 (calc. for C<sub>12</sub>H<sub>9</sub>N<sub>2</sub>O, 197.0715).

# **Isolation of cyclic lipopeptides**

P. aurantiaca PB-St2 was grown in Davis Minimal Medium without dextrose (Difco, USA), supplemented with glycerol (1.33 ml/L) with shaking (140 rpm) at 26℃ in six 5,000 ml Erlenmeyer flasks, each containing 1.5 liter medium, at room temperature. After 72 hours, the pH of the culture was adjusted to 2.0 with hydrochloric acid and subsequently the broth extracted twice with equal volumes of ethyl acetate. The crude extract (730 mg) was subjected to RP-vacuum liquid chromatography (Macherey-Nagel Polygoprep  $50 \sim 60$ ) using a stepwise gradient of methanol-water and finally dichloromethane to give four fractions. <sup>1</sup>H NMR profiling of these fractions indicated the 100% MeOH fraction to be of further interest due to characteristic lipopeptidic resonances. Separation of the this fraction by RP-HPLC, using a linear gradient of  $60:40 \sim 100:0$  MeOH-H<sub>2</sub>O over a period of 30 min, followed by isocratic elution at 100% MeOH for an additional 30 min (Phenomenex Luna C18-100A, 250mm $\times 10$ mm, 5 µm column in combination with a Phenomenex SecurityGuard Luna-C18 10mm × 10mm precolumn; 2.0 ml/min flow rate; UV monitoring at 215 nm) produced two subfractions. Subfraction 1 showed typical resonances for lipopeptides compounds in its <sup>1</sup>H NMR spectrum, but appeared to be a mixture of natural products. Purification of subfraction 1 by RP-HPLC applying a linear gradient of 60:40~100:0 MeOH-H<sub>2</sub>O (0.05%TFA) over a period of 30 min, followed by isocratic elution at 100% MeOH for an additional 30 min (Phenomenex Synergi Max-RP, 250mm  $\times$  4.6mm, 4  $\mu$ m column; 0.9 ml/min flow rate; UV monitoring at 215 nm) provided 2 mg of pure viscosin.

**Viscosin** brownish film; LR-ESI-MS  $[M+H]^+ m/z$  1127,  $[M-H]^- m/z$  1125.

## White-line-in-agar (WLA) test

The WLA bioassay was performed on King's B medium. Bacterial strains were streaked at 1 cm distance next to *P. tolaasii* LMG  $2342^{T}$  and the formation of a white precipitate in the agar was evaluated after 24 to 72 h growth at 26 °C. *P. reactans* LMG 5329 (WLIP producer) and *P. fluorescence* SWB 25 (viscosin producer) were

included as positive and negative controls, respectively. Assays were carried out in duplicates and repeated twice.

## Results

## Assays for the identification of PGPR traits

On the basis of agar plate assays, it was concluded that PB-St 2 produces ACC deaminase, lipase, protease, HCN and hydroxamate type siderophores. It was negative for phosphate solubilization, cellulase, pectinase and chitinase production. PB-St2 grew well at 4°C to 37°C but growth was not observed at 41°C. Growth at pH 5 to 10 and in the presence of 3% NaCl was observed but inhibited at 4% NaCl. Indole acetic acid production was 0.15  $\mu$ g/ml in the presence of 100 mg/L L-tryptophan in the medium. A bioautography-assay with *Chromobacterium violaceum* CV026 confirmed the production of homoserine lactones (Fig. 1). Antifungal activity was observed against 20 isolates of *Colletotrichum, Fusarium* and *Pythium* spp. (Table 1, Fig. 2).

## Isolation and identification of secondary metabolites

*P. aurantiaca* Pb-St2 produced several phenazines in LB medium. Based on NMR data, two of them were already identified as phenazine-1-caboxylic acid (PCA) and 2-hydroxy phenazine (2-OH-Phz). Characterization of the remaining phenazines is currently underway.

In addition, a cyclic lipopeptide belonging to the viscosin group was isolated from *P. aurantiaca* Pb-St2, using a minimal medium. The molecular mass of 1126 m/z and 1D- and 2D Nuclear magnetic resonance experiments proved the compound to be either viscosin or white line inducing principle (WLIP), as both compounds are chemical identical in every respect except for the chirality of Leu5, which possesses D configuration in WLIP and L configuration in viscosin. However, these compounds can be distinguished by the white-line reaction, in which only WLIP producing strains are capable of forming a white line in agar when grown in association with *P. tolaasii*. Since the cyclic lipopeptide, obtained from *P. aurantiaca* Pb-St2 showed no white-line reaction, the compound was identified as viscosin.

# Discussion

It has long been recognized that there are many naturally occurring bacteria and fungi that are antagonistic to crop pathogens and consequently have the potential to provide an alternative to chemical fungicide. Isolation of the bacteria from the target crop is essential for successful identification of potential biocontrol agents. Consequently, rhizobacteria, indigenous to sugarcane plants with antagonistic activity against the *C. falcatum* were screened and selected. In this report, we described the PGP traits of a newly isolated strain PB-St2 of *P. aurantiaca*, and its major secondary

metabolites. This strain showed antifungal activity against local strains of *C. falcatum* and *Fusarium* spp., phytopathogens of agricultural significance. Two phenazines, phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ) and the cyclic lipopeptide, viscosin has been isolated and identified. Identification of further compounds including phenazine analogs and pyoverdines is the subject of future investigations.

Several studies described the use of fluorescent pseudomonads as effective biocontrol agent against plant diseases. The antifungal or antibacterial activity of pseudomonads is traced back to the production of phenazines, 2-4-diacetyl phloroglucinol, pyrrolnitrin, pyoluteorin, cyclic lipopeptides (Liu et al., 2007), and rhizoxin (Loper et al., 2008). They play a vital role in biological control. In addition PCA was shown to play a role in ecological fitness (Chin-A-Woeng et al., 2003). Earlier studies have reported the production of various antifungal compounds by P. aurantiaca including phenazine and its derivatives, i.e., 1-oxyphenazine, phenazine-1, 6-dicarboxylate, and 2-hexyl-5-propyl-alkylresorcinol (Feklistova and Maksimova. 2008; Nowak-Thompson et al., 2003). Recently, Mandryk et al. (2007) reported new antimicrobial compounds produced by this bacterium. Rovera et al. (2008) communicated the production of 2,4-diacetyl phloroglucinol by P. aurantiaca SR1. Reports about PCA production by P. aurantiaca could not be found although Peix et al. (2007) used the production of phenazine-1-carboxylate as a characteristic feature of P. aureofaciens and P. aurantiaca when they re-classified these two bacteria as a sub-species of P. chlororaphis.

Cyclic lipopeptides (CLPs) are produced by several plant-associated *Pseudomonas* spp., including pathogenic *P. syringae*, *P. tolaasii*, *P. fuscovaginae*, *P. corrugata* and *P. fluorescens* and by multiple strains classified as antagonistic *P. fluorescens* and *P. putida* (Raaijmakers *et al.*, 2006). CLPs are versatile molecules with antimicrobial, cytotoxic and surfactant properties. For the antagonistic *Pseudomonas* spp., CLPs play a key role in antimicrobial activity, motility and biofilm formation. The antifungal activity has been studied for many different CLPs and for a wide variety of plant and human-pathogenic fungi and yeast. Viscosin was first described in 1951 and was isolated from *Pseudomonas viscosa* (Kochi *et al.*, 1951). Production of viscosin has also been reported by *P. fluorescens* and *P. libanensis* (Neu *et al.*, 1990; Saini *et al.*, 2008) but *P. aurantiaca* is not reported earlier as a viscosin producer.

Several *Pseudomonas* strains have already been marketed as commercial biocontrol products such as 'Mycolytin', an antifungal biopesticide formed by *P. aurantiaca* M-518 (Omel'yanets and Mel'nik, 1987) or 'Cedomon' (BioAgri AB, Upsala, Sweden), a seed treatment based on a *Pseudomonas chlororaphis* strain providing protection against seed-borne diseases in barley. As *P. aurantiaca* has a very strong homology with *P. chlororaphis*, it supports the use of this strain on commercial basis as well. In addition to the biocontrol potential, PB-St2 also showed the ability to produce indole acetic acid and ACC deaminase, which supports its use

as a biofertilizer agent as well. Rovera *et al.* (2008) reported the significant growth promotion of alfalfa and wheat plants when inoculated with *P. aurantiaca* SR1. Higher yield of wheat and maize are reported when plants were inoculated with *P. aurantiaca* with fertilizers (Rosas *et al.*, 2009). Carlier *et al.* (2008) observed a significant increase in several parameters of wheat crop under field conditions. Although any of the authors did not perform plant experiments yet, reports in the literature make the potential of *P. aurantiaca* to become a commercial PGPR-strain already apparent.

In this study, for the first time we report about PB-St2 as a new strain of *P. aurantiaca* from the sugarcane stem, with the production of viscosin, HCN, PCA, and 2-OH PHZ and we suggest that in future PB-St2 could be use as an effective biocontrol agent and a bio-fertilizer, to decrease the incidence of plant diseases and promote the plant growth.

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Fungal isolates	Disease/ plant	Anti-fungal activity
Colletotrichum falcatum BF166	Red rot/ sugarcane	++
C. falcatum C01148	Red rot/ sugarcane	+++
C. falcatum CP77400	Red rot/ sugarcane	++++
C. falcatum SPF 234	Red rot/ sugarcane	+++
C. acutatum	Anthracnose/ blue berries	+++
C. coccodes JAT2241	Anthracnose/ tomato	++
C.lindemuthanium 2221	Anthracnose/ common bean	++
C. orbiculare 2195	Anthracnose/ melons & cucumbers	++++
Cylindrocarpan destructans 1378	Anthracnose	++
Fusrium lateritium 543	Shisham root	++
F. graminearum V20251	Headblight/ corn	++
F. graminearum V14435	Headblight/ corn	++
F. graminearum RS29B01	Headblight/ corn	++
F. graminearum 212698	Headblight/ corn	++
F. oxysporium	Wilt	++++
F. oxysporium 540	Shisham root	++
F. oxysporium.lycopersicum. FOL 1835	Wilt/ tomato	++++
F. oxy. Radicis lycopersici 1833	Wilt/ tomato	+++
F. solani 120	Rose root	-
F. solani 1888	Wilt/ cucumber	++
F. solani 1891	Wilt/ pepper	++
F. solani 1892	Canker/ pepper root	++++
Pythium aphanidermatum 2102	Damping off, rot & blights/ cucumber	++
P. aphanidermatum 2190	Damping off, rot & blights	++
P. capsici	Fruit rot/ peppers	++
P. ultimum	Damping off	-
Rhizoctonia solani	Damping off	+
Phytophthora cactum	Root rot/ Ginseng	-

Table 1 Anti-fungal activity of P. aurantiaca PB-St2 against pathogenic fungal isolates

-, no activity; +, weak activity; ++, moderate; +++, strong; ++++, very strong.



Fig. 1 Bioassay for the identification of homoserine lactones produced by *Pseudomonas aurantiaca* PB-St2



Figure 2 Antifungal activity of *Pseudomonas aurantiaca* PB-St2 against local isolates of *Colletotrichum falcatum* 

# Field evaluation of P solubilising bacteria on the growth and yield of wheat and maize grown in rock phosphate amended soils

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#### Abstract

Phosphorus is an important nutrient for plant growth and development. Despite its wide distribution in nature, it is a deficient nutrient in most soils. Phosphorus is added in the form of phosphatic fertilizers, part of which is utilized and the remainder converted into insoluble fixed forms. Phosphate fertilizers are expensive and there is a need for alternative sources. Natural phosphate rocks (RP) have been recognized as a valuable alternative for P fertilizers. In India, it is estimated that there are almost 260 million tons of phosphatic rock deposits and this material should provide a cheap source of phosphate fertilizer for crop production. Unfortunately, rock phosphate is not plant available in soils with a pH greater than  $5.5 \sim 6.0$ . We have isolated P solubilising bacteria from the mine landfills of rock phosphate and inoculated to RP amended alkaline soils and studied the growth and yield of wheat and maize sown in two consecutive seasons. The results showed that inoculation of bacteria along with RP fertilization significantly increased yield and nutrient uptake of wheat and maize plants compared to the control soil. The P uptake by wheat and maize plants and the available P increased significantly in the RP amended soil inoculated with these bacteria as compared to control. These results suggest that inoculation of P solubilizing bacteria along with RP can substitute the chemical fertilizers in alkaline soil and help in improving the crop production.

# Isolation and characterization of plant growth promoting bacteria and their effect on chilli (*Capsicum annuum*) seedling growth

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# Abstract

Microorganisms are known to suppress soil borne plant diseases, promote plant growth and cause changes in texture of soil and vegetation. A total of 388 bacteria were isolated from various plant rhizosphere soils and from active Mud Volcano soils during  $2008 \sim 2010$ . Present study deals with the efficacy of ten bacterial isolates on their influence on growth of chilli. Ten potential bacterial strains showing antagonistic and PGP activities were selected for characterization. Of the total, seven isolates belonged to Gram negative and three isolates belonged to Gram positive. The isolates were identified using Microbial Identification System (Biolog) which revealed four isolates of Bacillus spp., two each of Pseudomonas spp. and Enterobacter spp whereas one each of Alcaligenes faecalis and Klebsiella spp. In vitro test of all the ten isolates showed antagonistic activity against Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum capsici, Rhizoctonia solani, Macrophomina spp. and Pythium spp. In the context of plant growth promoting properties, all isolates exhibited production of indole acetic acid whereas five isolates produced siderophore and solubilized inorganic phosphate. The plant growth promotion properties were demonstrated through the chilli based bioassay under greenhouse conditions. The isolates BM8, LC6, OM10, PfR8, PfS1 and PfR5 showed significant plant growth promotion with respect to increase in root and shoot length and number of secondary roots as compared to control. Hence, these isolates can be further formulated and used for field applications.

Key words: *Bacillus* sp.; *Pseudomonas* sp.; Biolog; PGPR activity; Andaman and Nicobar Islands

# Introduction

Bacteria of several taxonomic classes are found in crop rhizosphere and soil, and many of them can increase plant growth and productivity. Attempts to introduce beneficial bacteria into rhizosphere of agricultural crops have generally met with varying degrees of failure due to the difficulties of incorporating nonresident bacterial species into established and acclimated microbial communities. Native bacteria isolated from a specific plant rhizosphere may contain highly efficient genotypes to promote plant growth and perform this function better than exotic strains.

Plant growth promoting bacteria (PGPB) can promote plant growth directly or indirectly, via biocontrol of host plant diseases, production of phytohormones, or improvement of plant nutritional status (Glick, 1995). Rhizobia are perhaps the best known beneficial plant associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis. The co-inoculation of other PGPB with rhizobia is becoming a practical method in the development of sustainable agriculture, because of yield increases seen compared with inoculation with rhizobia alone. PGPB that have been tested as co-inoculants with rhizobia include strains of the following well-known rhizobacteria: *Azospirillum, Azotobacter, Bacillus, Pseudomonas, Serratia* and *Streptomyces* (Baudoin *et al.*, 2010; Biari *et al.*, 2008; Ahmad *et al.*, 2008). PGPB have been isolated by screening the rhizosphere, phyllosphere, and the tissues of plants showing particularly vigorous growth in the field. In this study we isolated the effective plant-growth-promoting bacterial strains from various soil samples collected from tropical Island of Andaman and Nicobar, India.

## **Materials and Methods**

#### **Isolation of bacteria**

The soils used for bacterial isolation was collected from rhizosphere soils of vegetables growing areas, plantation crops and Mud Volcano soils of tropical regions of Andaman and Nicobar Islands, India. The processed sample was serially diluted, spread plated on nutrient agar and incubated at  $28^{\circ}$ C for 48 h. A total of 388 colonies were isolated on nutrient agar, purified with repeated culturing and maintained in 20% glycerol at -20°C. Potential isolates were selected on the basis of results of screening for antagonistic and plant growth promoting properties.

# Antagonistic activities against plant pathogenic fungi

The antagonistic effects of bacterial isolates were tested against six fungal plant pathogens viz., *S. rolfsii*, *Pythium* sp., *Macrophomina* sp., *R. solani*, *F. oxysporum* and *C. capsici*. For this the bacterial isolates were streaked at a distance of 3.5 cm from rim of individual Petri plate containing potato dextrose agar (PDA) medium. Six mm mycelial disc from a 7-day old PDA culture of fungal pathogens were then

placed on the other side of the Petri dish and the plates were incubated at  $28 \degree C$  for 4 days. The per cent inhibition was calculated by using the formula, I = (C - T)/C \* 100, where I is per cent inhibition of mycelial growth over the control, C is mycelial growth of fungal pathogen in control plate and T is mycelial growth of fungus in bacteria inoculated plate. The experiment was carried out in three independent replicates.

# Production of secondary metabolites IAA (Indole 3-Acetic Acid) production

IAA production by the isolates was detected by qualitatively by (Naik *et al.*, 2008). Fresh cultures were streaked onto LB medium amended with L-tryptophan (5 µg/ml) then plates were overlaid with sterile Whatman No 1 filter paper and incubated at  $28\pm2$ °C for 72 h. After incubation 2~3 drops of O-phosphoric acid was added to the filter paper and then soaked with Salkowskis reagent (1ml of 0.5M FeCl<sub>3</sub> in 50ml of 35% HClO<sub>4</sub>). Production of IAA was interpreted by the formation of red colour on the filter paper immediately.

# Siderophore production

Bacterial culture (2 days old) was streaked on King's B medium amended with an indicator dye. The tertiary complex chrome-azurol-S (CAS)/Fe<sup>3+</sup>/hexadecyl trimethyl ammonium bromide served as an indicator. Change of blue color of the medium surrounding the bacterial growth to fluorescent yellow indicated production of siderophore. The reaction of each bacterial strain was scored either positive or negative to the assay (Schwyn and Neilands, 1987).

# Phosphate solubilization

All bacterial isolates were screened for inorganic phosphate solubilization according to the method developed by Verma *et al.*, (2001). A loopful of fresh bacterial culture was streaked onto Pikovaskaya's medium amended with inorganic phosphate and plates were incubated at  $28 \pm 2^{\circ}$ C for 4 d. A clear halo around the bacterial colony indicated solubilization of mineral phosphate.

# **Biolog identification**

Preliminary biochemical characterization of potential bacteria was carried out as per standard methodologies (Collins and Lyne, 1980). The identities of the bacterial isolates were revealed on the basis of Biolog carbon source utilization. Bacterial suspensions were inoculated into Biolog GENIII Micro plates as described in instruction manuals and incubated at 30°C for 24 h. The results were interpreted with Biolog Micro Log<sup>TM</sup>, Release 4 software (Biolog, Hayward, CA).

# Plant growth promotion ability on chilli

The chilli seeds were sterilized with 70% ethanol for 2 min and in 2% sodium

hypochlorite for 2 min, followed by ten times washing in sterile tap water. For this experiment, pure cultures were grown in nutrient broth at  $28^{\circ}$ C and diluted to a final concentration of  $10^8$  colony-forming units (CFU) ml<sup>-1</sup> in sterile saline water (0.85%). The surface sterilized seeds were immersed in appropriate PGP bacterial suspension for 1 h, air-dried and sown immediately. The following treatments with three replicates were investigated with two individual experiments: For (1) control (without bacterial inoculation), (2) Klebsiella sp BM8 (3) A. faecalis PfL7 (4) P. aeruginosa LC6 (5) B. pumilus OM10 (6) E. asburiae OM5 (7) E. cloacae PfR8 (8) B. lichiniformis BL5 (9) P. aeruginosa LC5 (10) B. subtilis PfS1 and (11) B. pumilus PfR5. Pots were sterilized with 20% sodium hypochlorite solution and filled with sterile loam soil. The chilli seeds (50 seeds in each pot) were sown in plastic pots filled with 1 kg sterile field soil. On 18<sup>th</sup> day after sowing, the chilli seedlings were thinned to one plant per hole. The pots were arranged in a completely randomized factorial design and were placed on a platform in the greenhouse. The seedlings were grown at a temperature of  $28 \sim 32^{\circ}$  C and 85% relative humidity in a greenhouse under a day-night cycle of  $13 \sim 14$  h natural light. The pots were watered to 50% water-holding capacity and were maintained at this moisture content by watering to weight every day. For each species and treatment, the plants of three pots were harvested 3 weeks after the emergence of seedlings, washed and morphological characteristics viz., root length, shoot length, dry and wet weight of stem and root and total number of secondary root of each plant was recorded.

#### Statistical analysis

Data were statistically analyzed by analysis of variance using the general linear model software Agres and Agdata and means were compared using the least significant difference (LSD) method;  $P \le 0.05$  was considered significant.

# Results

## Isolation and characterization of bacterial isolates

A total of 388 bacteria were isolated from various plant rhizosphere soils and from active Mud Volcano soils from 2008 to 2010. These isolates were evaluated for their antagonistic and plant growth-promoting traits. Ten potential bacterial strains showing antagonistic and PGP activities were selected for characterization. Among 10 isolates six were Gram negative and four were Gram positive. Phenotypic and biochemical characterization were done using Biolog based carbon source utilization and identified four species as *Bacillus*, species of *Pseudomonas* and *Enterobacter* were two isolates each and one isolate each of *Klebsiella* sp. and *Alcaligenes faecalis*.

# Anti-phytopathogenic activity and metabolites production

The all isolates exhibited most obvious antagonistic activity in vitro against the tested pathogens. pathogens and showed significant growth inhibition activity against
*S. rolfsii*, *C. capsici*, *R. solani*, *Pythium* sp., *Macrophomina* sp.. The isolates PfL7 (*A. faecalis*) and BM8 (*Klebsiella* sp.) failed to inhibit the growth of *S. rolfsii*. All isolates found positive to IAA production and five isolates were found to positive to siderophore production and phosphate solubilization. The isolates LC6, LC5, PfS1 and PfR5 were found positive to all plant growth promoting properties tested. (Table 1).

#### **Plant yield parameters**

The isolates from various niches were tested for their influence on growth parameters showed considerable influence on the chilli crops. The isolates BM8 (73.2% and 68.6%), LC6 (25.6% and 70.3%), OM10 (30.2% and 51.7%), PfR8 (38.4% and 50.0%), PfS1 (60.5% and 51.7%) and PfR5 (23.2% and 48.3%) showed increased shoot and root length. A corresponding significant increase in the root and shoot biomass was also observed in bacterized seeds. Seed bacterization resulted in greater enhancement of the shoot growth, as compared with the root growth and also the dry biomass weight. The number of secondary roots was increased in the bacterized treatment compared to control (Table 2).

#### Discussion

Plant hormones are central endogenous regulators of many aspects of plant growth and development. Auxin, one of the most extensively studied hormones regulates cell division, cell elongation, cell differentiation and pattern formation in plants (Berleth and Sachs, 2001). The isolates from the tropical regions of Andaman and Nicobar Islands, where the climate is with different range of rainfall, humidity and number of diseases encounter in number of crops.Such temporal and climatic selection processes are bound to help in the evolution of a variety of living organisms that are highly adapted to changing extremities of weather and are known to be a rich source of microbial diversity (Greenland and Losleben,2001). Bacterial plant growth promotion is a well-established and complex phenomenon and is often achieved by the activities of more than one plant growth-promoting traits exhibited by the associated bacterium (Lifshitz *et al.*, 1987). In this study, isolates of tropical crops possessed multiple plant growth traits, like P-solubilization, IAA production and siderophore production. All strains exhibited the most obvious antifungal activity in vitro and showed significant growth inhibitory activity against tested phyto-pathogenic fungi.

In this study, investigation on the plant growth promotion potential of strains OM10, PfS1 and PfR5 member of the genus *Bacillus* spp. BM8 member of the genus *Kelbsiella* sp and LC6 member of the genus *Pseudomonas* has been demonstrated. The plant growth promoting phenomenon can be attributed to the ability of the isolate to produce IAA, as IAA positively influences root growth and development, thereby enhancing nutrient uptake (Khalid *et al.*, 2004). It is a well-established fact that improved phosphorous nutrition influences overall plant growth and root development (Jones and Darrah, 1994). Siderophore production by the isolate

assumes significance for iron nutrition of plants grown under iron deficient conditions (Pieterse *et al.*, 2001) as well inhibition of phytopathogens. Worldwide, there is a profound need to explore varied agro-ecological niches for the presence of native beneficial micro-organisms. Many studies have been undertaken to understand the nature and properties of these unique microbes which harbor potential plant growth promoting traits. With increasing awareness about the problems of chemical-fertilizers-based agricultural practices (Ahmed, 1995), it is important to search for region-specific microbial strains which can be used as a potential plant growth promoter to achieve desired product. In this study, all of the ten isolates stimulated the growth of chilli seedlings under pot culture conditions. The increased nutrient uptake parameters could be attributed to the enhancement of the root growth and development. The differences in plant growth promotion among the isolates are attributed to their individual competencies. Future studies are required to prove the nature of these isolates and to harness their potential as bio-inoculants in agriculture.

#### Acknowledgment

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Isolate	Biolog	*Growth inhibition of pathogen over control (%)					PGP properties			
Name	identification	Macrophomina sp	S. rolfsii	C. capsici	R. solanai	Pythium sp.	Siderophore	Phosphate	IAA	
								solubilization	production	
BM8	<i>Klebsiella</i> sp	25.8	19.2	ND	43.3	26.7	-	-	+	
PfL7	A. faecalis	ND	6.6	40.0	ND	ND	+	-	+	
LC6	P. aeruginosa	52.5	40.2	ND	57.2	67.2	+	+	+	
OM10	B. pumilus	21.7	30.0	ND	8.3	25.0	-	-	+	
OM5	E. asburiae	30.6	43.3	ND	32.5	33.3	-	-	+	
PfR8	E. cloacae	ND	22.2	53.3	ND	ND	-	+	+	
BL5	B. lichiniformis	ND	37.8	66.7	ND	ND	++	+	+	
LC5	P. aeruginosa	60.0	38.3	ND	60.0	65.4	+	+	+	
PfS1	B. subtilis	ND	46.7	56.7	ND	ND	+++	+	+	
PfR5	B. pumilus	ND	40.0	56.7	ND	ND	-	-	+	

 Table 1 Antagonistic and Plant growth promoting activities of bacteria and its Biolog identification

\*Values are mean of three replications; ND- Not determined; - No activity; + 0.3–0.5 cm; ++ 0.6–0.9 cm; +++ >1 cm

S. No	Isolate	seed germination(%)	Root		Stem				
NO	Name		Primary root length (cm)	Secondary Root Numbers	Wet weight (g)	Dry weight (g)	Length (cm)	Wet weight (g)	Dry weight (g)
1	BM8	69.7	14.9	31.0	0.61	0.32	19.9	0.59	0.32
2	PfL7	84.0	8.7	36.0	0.61	0.34	18.3	0.59	0.16
3	LC6	78.0	10.8	23.0	0.75	0.16	20.1	0.44	0.22
4	OM10	86.0	11.2	23.3	0.57	0.16	17.9	0.66	0.18
5	OM5	86.0	9.1	22.0	0.53	0.18	15.4	0.41	0.10
6	PfR8	73.0	11.9	21.7	0.76	0.12	17.7	0.44	0.09
7	BL5	92.7	6.1	24.3	0.48	0.15	15.1	0.49	0.17
8	LC5	85.3	6.6	24.3	0.55	0.10	15.1	0.64	0.37
9	PfS1	84.0	13.8	26.0	0.60	0.22	17.9	0.90	0.33
10	PfR5	78.0	10.6	29.3	0.44	0.10	17.5	0.67	0.14
11	Control	74.3	8.6	21.7	0.24	0.07	11.8	0.23	0.07
CD (.05)		5.63	3.91	5.53	0.16	0.13	2.58	0.25	0.14
SEd		2.70	1.89	2.65	0.08	0.06	1.24	0.12	0.07

Table 2 Effect of inoculation of bacteria on the growth parameters of chilli seedlings

Results obtained were of mean of triplicates. Data were analyzed using one-way analysis of variance and treatment means were compared (PB  $\leq$  0.05%).

# Paenibacillus polymyxa strain PKPB1: a PGPR and an impressive bio-fungicide candidate

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#### Abstract

Paenibacillus polymyxa is a well-known PGPR that occurs naturally in soil. We isolated the strain P. polymyxa PKPB1 from canola roots and confirmed its plant growth promoting traits and documented its inhibitory action on several fungi that cause economically important diseases on several vegetable crops. The purified antibiotic produced by PKPB1 was chemically characterized and shown to have an inhibitory effect on several economically important disease-causing fungi such as Leptosphaeria maculans, Sclerotinia sclerotiorum, Pythium spp., Rhizoctonia solani, Fusarium spp., Alternaria brassicae, Ascochyta spp., and Marasmius oreades. Commercial Application: Strain PKPB1 or its purified metabolite can be used for various applications in the agriculture, home garden and greenhouse markets. It is a protective biocontrol agent for Pythium root rot in greenhouse cucumbers, and is capable of increasing crop yields, inhibiting growth of fungal pathogens, and fairy ring in turfgrass. The bacterium or the antibiotic metabolite can be applied as a seed treatment, soil amendment, or as a foliar spray. PKPB1 can be stored as dried spores for long periods with little loss of viability. PKPB1 bacterial cells can be added to nutrient solutions for disease control in greenhouse crops, or mixed with compost for application to field- or horticultural crops and its presence can be confirmed using Bioassay and DNA molecular methods. The Alberta Innovates-Technology Future's BioResource Technologies unit develops biological agents as alternatives to existing chemical controls for weeds and diseases, and works with the private sector to market these quickly. We are seeking a commercial partner to develop P. polymyxa strain PKPB1, into a marketable product as growth promoter, bio-pesticide, and food preservative. A United States Patent (No. 6,602,500) and an Australian Patent (No 758577) have been issued for this bacterium.

## The Critical role of IR-4 in specialty crop pest management in the U.S.

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#### Abstract

The IR-4 project (Interregional Research project No.4.) is a cooperative government and industry effort whose mission is to provide safe and effective pest management solutions for growers of specialty crops. Specialty crops include most vegetables, fruits, nuts, herbs, nursery and flower crops whose production areas encompasses less than 300,000 acres (as defined by the US EPA, Environmental Protection Agency). The total value of these crops in the United States is approximately \$43 billion which represents about 46% of the total U.S. farm crop value. Despite the high value of these minor acreage crops, the financial incentive for the agro-chemical companies to pursue registering pest management tools for these crops is largely absent. The USDA along with State Agricultural Experiment Stations (SAES) recognized this problem in 1963, and out of their efforts the IR-4 project began. This report will focus on the critical role that IR-4 plays in securing sustainable pest management tools for its large group of stakeholders, with focus on the IR-4 Southern Region. In addition to a general overview of the program, specific highlights of the Ornamentals, Biopesticides and Public Health Aspects of the program will be presented.

## Physiochemical and structural characterization of biosurfactant from fluorescent *Pseudomonas* with biocontrol activity against *Macrophomina phaseolina*

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#### Abstract

Plant growth promotory fluorescent Pseudomonas EKi from the rhizosphere of tomato was screened for the production of biosurfactant. The biosurfactant production was monitored as a function of emulsification activity and cell hydrophobicity. Extracted biosurfactant was purified by thin-layer chromatography. Cell-free culture supernatant and purified biosurfactant were checked for biocontrol activity against Macrophomina phaseolina. Purified biosurfactant was characterized biochemically and by fourier transform infrared spectroscopy (FTIR) analysis. EKi showed 66.73% of cell adherence with crude oil and emulsification activity of 67.65% after 4 hours of cultivation, thereby confirming the biosurfactant-producing ability. Cell-free supernatant and crude biosurfactant caused 42 mm and 36 mm zone of inhibition of M. phaseolina. The positive rhamnose test indicates that the purified biosurfactant is a glycolipid. FTIR analysis showed the presence of asymmetric and symmetric C-H stretching vibrations of aliphatic chain and ester group, like those represented in the hydroxydecanoic acid chain tails of rhamnolipid. The fingerprint region of the spectrum is typical for carbohydrates. The presence of band at 1559 to 1577 cm<sup>-1</sup> clearly showed the presence of amine group. FTIR analysis clearly reported that biosurfactant is composed of high percentages of lipid and carbohydrate in addition to a minor fraction of protein, a characteristic new for the structure of rhamnolipids.

## Key words: Biocontrol; biosurfactant; Macrophomina phaseolina; Pseudomonas; rhamnolipid

#### Introduction

A biosurfactant is defined as a surface-active agent produced by living cells, mostly by microorganisms (Fiechter, 1992). The molecule of biosurfactant, which has both water-soluble and water insoluble parts, balance the hydrophilic and hydrophobic moieties, imparts unusual properties including an ability to lower the surface tension of water (Cooper,1986). Basically, there are six major types of biosurfactant; hydroxylated and crosslinked fatty acids (mycolic acids), glycolipids, polysaccharide-lipid complexes, lipoproteins-lipopeptides, phospholipids and the

complete cell surface (Kosaric *et al.*, 1987). Among these biosurfactants, glycolypids show various chemical structures including, rhamnose, trehalose, mannose and sophorosa as hydrophilic moieties. In addition to surface activity, microbial glycolipids are shown to have biological activities to induce cell differentiation (Isoda *et al.*, 1997), antimicrobial activities for plant disease control (Staghelli and Miller, 1997) and even some immunological activities (Piljac and Piljac, 1996).

Numerous plant-associated pseudomonads are known to produce biosurfactants with versatile functions (Tran *et al.*, 2007). For plant pathogenic pseudomonads, biosurfactants are important virulence factors and for antagonistic *Pseudomonas* strains, they contribute to motility, biofilm formation, root colonization, antimicrobial activity and biocontrol of plant disease (Kruijt *et al.*, 2009). *Pseudomonas* spp. are well known for its ability to produce rhamnolipid, first described by Jarvis and Johnson (1949). The biosurfactant activity of rhamnolipids makes them excellent candidates for assisting in the breakdown and removal of oil spills. Rhamnolipids also demonstrate antibacterial and antifungal activities, suggesting possible roles in the medical and agricultural fields (Desai and Banat, 1997).

The aim of the study is to evaluate the biocontrol ability of biosurfactant produced by *Pseudomonas* EKi against *Macrophomina phaseolina*, and to identify key components of the crude biosurfactant mixture, including its molecular characterization by Fourier transform infrared spectroscopy.

#### **Materials and Methods**

#### **Microorganisms**

Fluorescent *Pseudomonas* EKi with biocontrol activity against *Macrophomina phaseolina* was taken in the study (Khare *et al.*, 2011). The surfactant production capacity was determined on medium 5 cultivated at 28°C by shaking at 160 rpm for four days (Turkovskaya *et al.*, 2001).

The plant pathogenic fungus, *M. phaseolina* ARIFCC257 procured from Agharkar Research Institute, Pune, was grown and maintained on potato dextrose agar (PDA) media (Himedia, Mumbai) at  $28^{\circ}$ C and  $4^{\circ}$ C, respectively.

#### **Biosurfactant Production**

Cell hydrophobicity was measured by BATH according to a method similar to that described by Rosenberg *et al.* (1980). The emulsification activity was determined accordingly to Cooper and Goldenberg (1987).

#### Antimicrobial Action

Antimicrobial activity of cell free supernatant and extracted biosurfactant was

determined. Biosurfactant was extracted in chloroform-methanol (Carvalho, 2005) and extract was evaporated to dryness then dissolved in 1 ml sterile distilled water (SDW). Biocontrol activity was checked following well diffusion method. The experiment was conducted in triplicates.

#### Chemical Nature

Biosurfactant compound was separated on TLC plate (Silica gel 60  $F_{254}$ , Merck). Chloroform-methanol-water (65:15:2v/v/v) was used as the solvent system. Biosurfactant spot was revealed by spraying with distilled water and heating at 110 °C for 5 min, for detection of hydrophilic compounds (Tahzibi *et al.*, 2004). The compound was expressed by retardation factor. Band developed on TLC plate by distilled water was scraped and compound re-extacted in chloroform-methanol (1:2) then evaporated to dryness. Dried powder was dissolved in DW (1 ml) used as sample. The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose (Dubois *et al.*, 1956).

The molecular characterization of dried biosurfactant compound from TLC spot was performed by Fourier transform infrared (FTIR) spectroscopy. The infrared (IR) spectra were recorded on the Bruker IFS113vFTIR-spectrometer, in the  $4000 \sim 400$  cm<sup>-1</sup> spectral region.

#### Results

#### **Biosurfactant Production**

After 4 h of cultivation there was 66.73% cell adherence with crude oil in the BATH assay and an emulsification activity of 67.65% was detected, thereby confirming the biosurfactant-producing ability of the EKi.

#### **Antimicrobial** Action

After two days of incubation inhibition zone of 42 mm and 36 mm appeared around the well filled with cell-free broth and biosurfactant respectively (Fig.1).

#### **Chemical Nature**

Thin-layer chromatography revealed a spot (Rf =0.36) on water spray. The positive rhamnose test indicates that this separated biosurfactant compound from TLC is of glycolipid type. The molecular composition of the surfactant compounds was evaluated by FTIR spectroscopy (Fig. 2). The presence of amide N–H streching was indicated by wavenumbers 3394 to 3427 cm<sup>-1</sup>. The double bands at 2955 to 2921 and 2850 to 2953 cm<sup>-1</sup> are derived from symmetric C-H stretching vibrations of aliphatic groups. A band near 1246 to 1379 cm<sup>-1</sup>, 1559 to 1577 cm<sup>-1</sup>, 1730 cm<sup>-1</sup> was present in FTIR spectrum of biosurfactant compound. In the fingerprint region of the spectrum,

the area between  $1200 \sim 1460 \text{ cm}^{-1}$  and lower range of the fingerprint region below  $1200 \text{ cm}^{-1}$  were also present.

#### Discussion

Fluorescent *Pseudomonas* strain EKi showed high cell surface hydrophobicity. In *P. aeruginosa*, lipopolysaccharides (LPS) in the outer membrane contain carbohydrates and high levels of 3-hydroxydecanoic and 3-hydroxydodecanoic acids (Rivera *et al.*, 1988). It seems therefore that biosurfactant are synthesized as a part of the outer membrane leading to their accumulation in the culture medium. The extract of biosurfactants showed antifungal activity against *M. phaseolina*. There are reports on antifungal properties of rhamnolipids produced by *Pseudomonas* sp. against various fungi (Abalos *et al.*, 2001).

TLC results also suggested that the isolated surface-active product from *Pseudomonas* EKi was rhamnolipid. Christova *et al.* (2004) reported that commercial rhamnolipid sample had two predominant characteristic spots. The lower spot of dirhamnolipids (Rf = 0.16) and the higher spot of monorhamnolipids (Rf = 0.37). The TLC results indicated that spot (Rf = 0.36) consisted of mono-rhamnolipid.

The product from TLC spot showing positive rhamnose test was next submitted for FTIR analysis confirmed the presence of rhamnolipid. This showed the presence of asymmetric and symmetric C-H stretching vibrations of aliphatic chain and ester group, like those represented in the hydroxydecanoic acid chain tails of rhamnolipid. The fingerprint region of the spectrum is typical for carbohydrates as in the rhamnose units of the molecule (Leitermann *et al.*, 2008). The presence of band at 1559 to 1577 cm<sup>-1</sup> clearly showed the presence of amine group (Rodrigues *et al.*, 2006) a characteristic new for the structure of rhamnolipids. Earlier, Lotfabad *et al.* (2009) reported biosurfactant from *P. aeruginosa* composed of high percentages lipid and carbohydrate in addition to a minor fraction of protein. The FTIR analysis clearly showed that the compound from TLC spot is a form of rhamnolipid. Identification of newly-found biological entities, such as bacterium strains and their various biological attributes may provide very useful information for both basic research and drug discovery (Chou, 2004). This study will have major impact on biosurfactant industry and agriculture technology.

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Figure 1 Inhibition of *M. phaseolina* by (a) Cell free supernatant and (b)

b



#### **Biosufactant from EKi**

Figure 2 FTIR spectrum of biosurfactant compound

### Phenotypic variation in *Acidovorax radicis* N35 influences plant growth promotion

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#### Abstract

Acidovorax radicis N35, isolated from surface-sterilized wheat roots (Triticum *aestivum*), showed irreversible phenotypic variation when grown in NB medium, resulting in a differing colony morphology. In addition to the wild-type form (rough colony type), a phenotypic variant form (smooth colony type) appeared at a frequency of  $3.2 \times 10^{-3}$  per cell per generation on NB agar plates. In contrast to the N35 wildtype, the variant N35v showed almost no cell aggregation and had lost its flagella and swarming ability. Following the inoculation of soil-grown barley plants, only the wild-type N35 significantly promoted growth in barley based on increased root and shoot dry weight. After co-inoculation of dixenically-grown barley seedlings with differentially fluorescently-labeled N35 and N35v cells, decreased competitive endophytic root colonization in the phenotypic variant N35v was observed using confocal laser scanning microscopy. In addition, 454 pyrosequencing of both phenotypes revealed almost identical genomic sequences. The only stable difference noted in the sequence of the phenotype variant N35v was a sixteen-nucleotide deletion identified in the *mutL* gene, which encodes for the mismatch repair protein MutL. The deletion resulted in a frameshift which revealed a new stop codon resulting in a truncated MutL protein missing a functional MutL C-terminal domain. The mutation that occurred was consistent in all investigated phenotype variant cultures. These results suggest that MutL might be responsible for the observed phenotypic variation in A. radicis N35.

#### **Review of PGPR research and products in Europe**

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#### Abstract

Fundamental research on PGPR in Europe has a long history. In the 1970's the main interest was in Bradyrhizobia and symbiotic nitrogen fixation. Presently, scientific interests are focused on mechanisms of action, active compounds and genetic regulation in the major PGPR groups as Azospirillum, Bacillus and Pseudomonas. Europe is among the leaders in topics such as microbial triggering of Induced Systemic Resistance in plants, root colonization, biocontrol mechanisms, the role of endophytes in plant growth stimulation, and the role of siderophores and bacterial biosurfactants in biological control. Leading academic centers are in Austria, Belgium, Ireland, Italy, Germany, France, the Netherlands and Switzerland. Despite the unquestionable success of Europe in the academic PGPR research, the European market of microbials is rather limited in comparison with Asia and the USA. Products containing bacilli, Mycorrhizae and various pseudomonads are present on the market as plant strengtheners, plant health improvers, soil improvers, microbial fertilizers etc. Since they are not covered by uniform European legislation, it is impossible to evaluate their market share. Major microbial fungicides sold in Europe are formulated Glyocladium, Coniothyrium and Trichoderma strains which are registered as Plant Protection Product (PPP). Just two Pseudomonas-based products and only one Bacillus-based product are sold as PPP. The share of microbial fungicides in the European biopesticide market in 2008 was estimated at about \$ 12 mln (approximately 22%), while the total pesticide market is estimated at as much as \$ 12850 mln. This difference reflects the strong competition with chemical products. Only in situations in which chemical alternatives do not exist, a microbial product has a chance to penetrate the market. Presently, the biocontrol industry is focusing on products which demonstrate stable records in efficiency and are compatible with existing fertilizing and chemical fungicidal schemes applied in horti- and agriculture.

## Genetic diversity of PGPR *Pseudomonas* associated with rhizosphere of different crops cultivated in the saline agri-ecosystem

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#### Abstract

Increasing salinity due to extensive use of chemical fertilizer is an imperative threat in coastal, arid and semi-arid regions, and is also a major limiting factor for plant growth. Salinity is predicted to affect >50% of all arable land by the year 2050. Use of PGPR's with biofertilizer and biopesticides traits suitable to the agriecosystem to promote Good Agriculture Practices (GAP) is the need of the hour. PGPR *Pseudomonas* are common inhabitants of the rhizospheres and are reported to exhibit many beneficial properties such as production of plant growth regulators like indoleacetic acid (IAA), gibberellic acid (GA) and cytokinins, nitrogen fixation, denitrification, solubilization of mineral phosphates and other nutrients, degradation of aromatic compounds, biosynthesis of polyhydroxybutyrate, protection against production phytopathogenic microorganisms by of siderophores, 2.4diacetylphloroglucinol (2,4-DAPG), phenazines (Phz), pyrrolnitrin (Prn), pyoluteorin surface-active antibiotics, biocides such as hydrogen cyanide and lytic (Plt), enzymes. In addition Pseudomonads produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which lowers the stress ethylene levels during biotic and abiotic stress and environmental stresses. With this background the genetic and functional diversity of the Pseudomonas associated with paddy, finger millet, pearl millet, pepper, cotton etc. capable of surviving in the saline conditions was extensively studied. It was interesting to note that the percentage of occurrence of P. pseudoalcaligenes and P. alcaligenes was highest in the pearl millet and cotton rhizospheres, while their numbers declined in the paddy rhizosphere and the occurrence of P. flourescens strains increased. Fluorescent pseudomonads dominated at non-saline sites whereas salt-tolerant species, in particular P. alcaligenes and P. pseudoalcaligenes dominated the saline sites. The diversity of Pseudomonas associated with finger millet was also studied in-depth. Of the total 102 strains genotyping patterns analyzed through BOX-PCR using BOX primer 5'-ACGGCAAGGCG ACGCTGACG- and saline tolerant Pseudomonas spp. with tolerance to salinity up to 2M was observed. These saline tolerant *P. aeruginosa*, *P.* alcaligenes, P. fluorescens and P. pseudoalcaligenes controlled Bacterial blight (X. oryzaea) of rice in ADT-36 and increased yield upto to  $20\% \sim 30\%$  and reduced disease upto 50%. Thus identifying appropriate strains for specific ecological niches is the need of the hour to address issues related to the use of PGP for GAP.

Key words: Pseudomonas; PGPR; salinity stress

#### Introduction

Soil salinity in arid regions is an main limiting factor for cultivating agricultural crops in addition to drought which limits the growth and productivity of crops, particularly in arid, semi-arid and coastal areas as reported by Kramer and Boyer, (1995). In coastal regions, the problem of increasing salinity and consequently, decreasing crop productivity, are major concerns.Soil plays a prominent role in the microbial selection process as environmental stress has been shown to reduce bacterial diversity (Borneman *et al.*, 1996). However, fewer reports have been published on PGPR's as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess. The term 'induced systemic tolerance' (IST) for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress has been proposed by Yang *et al.* (2008). The genus *Pseudomonas* is widespread in soil, its ability to colonize the rhizosphere of host plants and its capacity to produce a large number of compounds antagonistic to various serious plant pathogens has been reported by Thomashow *et al.* (1997).

*Pseudomonas* spp. are common inhabitants of the rhizospheres and phyllospheres and survive in a broad range of environmental conditions, because of their competitive ability for nutrients and suitable niches on the root surface as well as their ability to respond rapidly to environmental modifications. Different groups of *Pseudomonas* are reported to be predominant in the rhizosphere regions of different crops. *Pseudomonas* strains exhibit a wide range of PGPR property by producing indoleacetic acid (IAA), gibberellic acid and cytokinins; phosphate solubilization and other nutrients (Vyas and Gulati, 2009); Pseudomonas exhibit antagonistic activity against phytopathogens by the production of siderophore, antibiotics such as 2,4diacetylphloroglucinol (2,4-DAPG), phenazines, pyrrolnitrin and pyoluteorin, surface-active antibiotics, biocides such as hydrogen cyanide (HCN) (Raaijmakers et al., 2002) and cell wall lytic enzymes (Haas and Defago, 2005). In addition, pseudomonads are reported to produce the enzyme, 1-aminocyclopropane-1carboxylic acid (ACC) deaminase, which plays an important role in maintaining ethylene levels in plant tissues under biotic and abiotic stresses (Penrose and Glick, 2003). The phytohormone producing ability is widely distributed among bacteria associated with soil and plants. Studies have demonstrated that the PGPR can stimulate plant growth through the production of auxins; indole acetic acid (IAA) (Spaepen et al., 2008), gibberellins (Bottini et al., 2004) and cytokinins (Timmusk et al., 1999), or by regulating the high levels of endogenous ethylene in the plant (Glick et al., 1998).

Baxter and Gibbons, way back in 1956 studied the diversity of salt-tolerant bacteria present in the rhizosphere of *Oryza sativa* and reported the occurrence of *Pseudomonas aeruginosa* strains tolerant to 3% salinity. The production of isocitric, succinic, malic, and lactic dehydrogenases and cytochrome oxidase from *Pseudomonas salinaria*, an extreme halophile, were present in high conc. of sodium or potassium chloride.

*Pseudomonas extremorientalis* TSAU20 and *Pseudomonas chlororaphis* TSAU13 possessed the ability to colonise and survive in the rhizosphere of common bean under saline conditions. Of the four salinity levels (5.0, 7.5, 10.0, and 12.5 dS/m) maintained in the gnotobiotic system using NaCl proved that increasing salt content impacted the root-tip colonization of both the bacterial strains. The results indicated that TSAU20 and *P. chlororaphis* TSAU 13 survived in ecologically stressed conditions, such as saline and nitrogen deficient soils but high salinity inhibited their colonization in the rhizosphere and thus their stimulatory effect on plants was also reduced. (Egamberdieva, 2011). Asghari Bano and Mussarat Fatima, (2009) observed that *Rhizobium* spp. were was more effective than *Pseudomonas* in plant growth promotion under normal conditions but under salt stress the *Pseudomonas* performed better.

#### **Materials and Methods**

#### Isolation and screening for saline tolerant Pseudomonas spp.

About ten grams of each soil sample was dispersed in 30 ml of sterilized 1% phosphate-buffered saline (PBS) in falcon tube and vortexed for 15 min and made up with 70 ml of sterilized 1% PBS in a conical flask and kept in a shaker at 150 rpm for 2 h. Then, the sample were serially diluted up to 10–6 in 1% PBS in triplicates and then plated on King's B agar (KBA) medium and incubated at  $37^{\circ}$ C for 48 h at room temperature. These strains were then tested for their ability to grown in different conc.of NaCl ranging from 100mM- 2M.

#### Genotypic fingerprinting analysis

Genotyping patterns of the isolated strains were analyzed through BOX-PCR, Rep-PCR as described by Rademaker *et al.* (1998). The BOX-PCR genetic patterns were visualized under UV illuminator and documented using Bio-Rad Gel Doc system. Normalization, recognition and assignment of bands on the gel were performed with the FINGERPRINTING II (Bio-Rad) program by the Dice coefficient. The cluster analysis of similarity matrices was performed by the unweighted pair group with mathematical average (UPGMA) algorithm for generation of Dendrograms.

#### **Result and Discussion**

#### Diversity of Pseudomonas spp. associated with different rhizosphere

The diversity of the *Pseudomonas* populations assessed from three different plant rhizospheres, namely pearl millet, cotton and paddy, grown in saline soils along the coastline of Southern India showed that increasing salinity caused a predominant selection of salt tolerant species, in particular P. pseudoalcaligenes and P. alcaligenes, irrespective of the host rhizosphere. (Rangarajan et al., 2001). The presence of salt tolerant *Pseudomonas* spp. in the cotton and pearl millet rhizosphere suggests that natural selection has occurred due to increasing salinity. Earlier reports also indicate that bacteria isolated from saline environment are more likely to survive inhibitory salt concentrations than their counterparts from non-saline habitats (Hua et al., 1982). The total genomic DNA was isolated using standard procedures (Graves and Swaminathan 1993) and the 16S rDNA region amplified using Pseudomonas 16S rRNA genus-specific primers confirmed that the strains belonged to *Pseudomonas*. The 20-mer forward primer (5¢-GGTCTGAGAGGATGATCAGT-3¢) and 18-mer reverse primer (5¢- TTAGCTCCACCTCGCGGC-3¢) (Alm et al., 1996) were used (Widmer et al., 1998) and one hundred and twenty four isolates showing a single distinct amplified product of size 986 bp, confirmed that these isolates belong to the genus Pseudomonas. The diversity was determined by RAPD analysis and also by biochemical tests using NEFERM plates (Boeufgras et al., 1987) All these tests showed that most of them belonged to either P. pseudoalcaligenes or P. alcaligenes and the other species such as P. flourescens, P. stutzeri and P. aeruginosa had minimum representation. It was interesting to note that the percentage of occurrence of *P. pseudoalcaligenes* and *P. alcaligenes* was highest in the pearl millet and cotton rhizospheres, while their numbers declined in the paddy rhizosphere, instead, the occurrence of *P. flourescens* strains increased. Strains that work very well in one environment have failed to elicit any plant response in others due to the stresses that operate there. This is because abiotic stresses, in particular, are limiting factors for the efficiency of many strains (Miller and Woods, 1996). Glick et al. (1998) have proposed that some PGPR function as a sink for 1-aminocylopropane-1-carboxylate (ACC), the immediate precursor of ethylene in higher plants, by hydrolyzing it to aketobutyrate and ammonia, and in this way, promote root growth. They have suggested that the ACC-deaminase trait of rhizobacteria could be used for efficient isolation/selection of effective PGPR.

Rangarajan *et al.* (2002) explored the microbial diversity of rice rhizosphere and isolated a total of 256 *Pseudomonas* strains from five sites cropped with rice with varying salinity levels from the coastal regions of Tamil Nadu. All the strains screened for tolerance to NaCl exhibited varying levels of salt tolerance viz., 44 (36.0%) and could grow upto 0.5 M NaCl conc. 20 (16.4%) in 1.0 M NaCl and 4 (3.3%) in 1.5 M NaCl conc. None of the strains tolerated NaCl concentrations higher

#### than 1.5 M.

The functional and genetic diversity of *Pseudomonas* spp. associated with finger millet rhizosphere grown in semiarid regions was explored (Jegan et al., 2008) unpublished data) using rpoD primers more specific for Pseudomonas population. Screening for salt tolerance showed that 94 (92%) strains could grew in 0.5 M NaCl; 18 strains (17%) grew in 1.0 M NaCl and 1 (1%) strain in 1.5 M. All the 102 isolates failed to grow in 2 M NaCl. The soil samples used in the study had an average EC value of  $0.2 \sim 0.3 \text{ m}\Omega^{-1} \text{ cm}^{-1}$ . This may be the reason that the isolates failed to grow in 2M and 1.5M NaCl conc. In this study, more number of P. putida and P. aeruginosa and few strains of P. alcaligenes and P. pseudoalcaligenes tolerant to 1 M NaCl conc. were observed. Bacterial strains adopt to salinity stress by the de novo synthesis of osmolytes (Alanine, glycine, Glutamine, serine, Thr and Aspartic acid) in the cytosol. PAGE protein profiling of total cell proteins revealed the induction and repression of salt stressed proteins were upregulated and down regulated which was analyzed by Differential display (DD). (Diby Paul et al., 2005). The expression of different proteinsunder salt stress had molecular weights and isoelectric points between 12 and 77 kDa compared to the cultures grown in non saline conditions. The functions of seven proteins were not determined as there were no significant matches in the protein database. The majority of proteins identified were homologous to stress proteins in prokaryotes (Diby Paul et al., 2006).



The application of saline tolerant PGPR *Pseudomonas* strains MSSRFA3, MSSRFC9, MSSRFC10, MSSRFD6, MSSRFE12 and MSSRFG1 displayed better growth when compared to those inoculated with non saline tolerant strains MSSRFA8, MSSRFC8, MSSRFD1 and MSSRFG2. The control seedlings without the inoculation of PGPR strains failed to grow after a week, while the seedlings treated with the above mentioned PGPR remained healthy for more than a week which may be due to the microbial inoculation which induced tolerance in the plant. (Ananthi *et al.*, 2011, unpublished).

#### Conclusion

The research outcome of the laboratory findings has proven that the association of the PGPRs with the rhizosphere different crops is very much influenced by the abiotic factors such as salinity drought etc. The diversity of the Pseudomonas varied with increasing salinity levels. Saline tolerant strains such as *P. alcaligenes*, *P. pseudoalcaligenes*, *P. auregenosa* and *P. putida* were predominantly present under saline conditions compared to the non saline conditions were the *Pseudomonas fluorescence* group was predominant. Thus it can be concluded that salinity of the soil determined by the EC influences the microbial population of the plant rhizosphere. Bacteria isolated from saline environment are more likely to survive inhibitory salt concentrations than their counterparts from non-saline habitats and salinity levels caused a predominant selection of salt tolerant species, in particular *P*. *pseudoalcaligenes* and *P. alcaligenes*, irrespective of the host rhizosphere.

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## *Section - III* Oral Session 2 – Lectures

## Protection to initial infection of agave wilt on *Agave tequilana* Weber var. *azul* using *Trichoderma* sp.

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#### Abstract

Agave is the raw material to produce tequila, the most traditional Mexican alcoholic beverage. This crop takes six to eight years to get industrial maturity. Agave has been reproduced by centuries to new commercial plantations using small cloned plants that grownup adjacent to mother plants called "hijuelos" (lateral buds), and recently by *in vitro* produced plantlets, low genetically variability is present. Agave wilt is a very important soil-borne disease in this crop and is caused by combined action of a vascular wilt induced by Fusarium oxysporum a stem rot induced by F. solani pathogenic stains. Now, an incidence average of 23% of dead plants is very important, especially when low prices reduce the area of new plantations for future production. Chemical control is ineffective because cropland is rain fed and by the pathogen long incubation period of at least two years. In 2005 naturally grown agave root associated *Trichodema* spp stains were sought in different commercial crops. Bar 331 and ZAP6-1M Trichoderma spp strains shown an endorhizosphere colonization capacity superior to 90% with good in vitro antagonistic ability against Fusarium oxysporum and F. solani pathogenic to agave. Increase of PRs proteins quitinase, glucanase and peroxidase activities on agave leave tissue were recorded when *Trichoderma* spp. strains were inoculated in sterile soil, next to agave root in greenhouse trials. Reduction of incidence of xylem vessel injury to 41 and 38% were recorded when Thrichoderma strains Bar 331 and ZAP6-1M were inoculated with BAS1 F. oxysporum strain on in vitro reproduced agave plants, means similar to no inoculated control, that were inferior than 63% mean observed when BAS1 strain was applied alone. The incidence of root rot in root fragments near crown root was reduced from 81% in plants treated with Z15 F. solani strain alone, to 47% when inoculated with ZAP6-1M or at 10% when it was inoculated with Bar331 Trichoderma spp strain. This biotechnology will be a good option to protect new *in vitro* produced plants to establish new agave commercial crops.

Key words: Fusarium oxysporum; Fusarium solani; PR proteins

#### Introduction

Agave crop is the raw material of the Mexican tequila beverage. This crop spent six to eight years to get the maturity necessary to the industry. A hectare had around 3,000 plants and Tequila industry needs around 35 million plants at year to produce tequila for national and international commerce. This crop has different parasitic restrictions to their productivity, where pest and diseases have a predominant position. The agave wilt called "marchitez" is an important disease that kills 25% to 35% of agave plants because a severe wilt that starts since the third year in average (Aceves, 2003). Fusarium oxysporum induce a vascular wilt and F. solani is frequently isolated from reddish crown and root rot tissue in agave plants (Beckman, 1987; Avila et al., 2010; CRT, 2005 and Luna, 1996) ). Generally, to establish new commercial agave crops, farmers transplant in new fields, vegetative plantlets called "hijuelos" that grown beside the mother plant from lateral buds, increase the possibility of use asymptomatic but diseased vegetative material. Because visual selection of "hijuelos" before transplant and chemical control are ineffective to reduce incidence or severity of marchitez the use of in vitro reproduced agave plantlets is a strategy to produce vegetative material free of diseases. This management activity is efficient if low inocula level is present in soli, but if not, the susceptibility of this genetically homogeneous crop (Eguiarte and Gonz alez, 2007), induce severe epidemics in infected fields transplanted with this material. In 2007, roots of several agave plants from different production regions in Jalisco and Nayarit states were sampled to isolate Trichoderma spp. strains in close relation to agave roots. Low number of Trichoderma strains were found colonizing the agave endorhizosphere, strains Bar331 and Zap61M, were found in mother plants from Santa Mar a del Oro, Nayarit and Tepatitlán, Jalisco. This work had the objectives of determinate the root colonization capacity of both Bar331 and Zap61-M Trichoderma strains on in vitro produced agave plantlets. Where determined levels of protection induced by Trichoderma strains when BAS1 Fusarium oxysporum or Z15 Fusarium solani pathogenic strains were inoculated. Induction of proteins related to pathogenicity (PRs) were recorded on agave plantlets inoculated Trichoderma and/or Fusarium spp. and compared with their basal expression in agave non inoculated plants.

#### **Materials and Methods**

Two groups of *in vitro* produced plantlets of *A. tequilana* with 3 and 6 months old respectively of *ex vitro* adaptation period were used like hosts. Sterile plastic pots were added at half of their capacity with sterile soil-peat moss 2:8 mix, *Trichoderma* Zap61-M or TrBar3-3-1strains like active mycelia growing in a wheat bran-peat moss mix, were added to the pot at a dose of 5g, then agave plants where planted, adding substrate mix to complete the pot capacity and irrigated. Filtrated water was used to formulate nutrient solution used for irrigation. Later of 15 days of transplant pots were inoculated in the substrate into the root system with 4 ml of a conidial suspensions of N9 *F. oxysporum* or Z15 *F. solani* strains with a concentration of  $4 \times$ 

 $10^4$  ml. These strains of fungi were previously isolated from stem and root respectively of field agave plants with wilt symptoms. Five treatments including control un-inoculated plants were evaluated. At dates of 15, 30, and 60 days after transplant, groups of 5 plants of each treatment were selected to evaluate. *Trichoderma* strains root colonization capacity was evaluated plating ten 1 cm root segments on Trichoderma Selective Media, recording the percent of root segments of each plant with *Trichoderma* growth. The evaluation the quitinase, peroxidase and glucanase enzymatic activity was determined in the same dates using protocols reported by. The incidence of root rot at the crown area was recorded like percent of segments with internal necrosis in the different treatments; Incidence in percent of xylem vassel injury was determined counting total an injured xylem vassels in stem tissue observed in microscope at 400X. SAS statistics software ver 8.0 was used to analysis (SAS Institute Inc., 2000).

#### **Results and Discussion**

The *Trichoderma* strain TR ZAP6-1M had a 100% of root colonization since 15 days evaluation date , and TrBar3-3-1get superior to 95% percent of colonization in the  $15\sim60$  days period. This high capacity of colonization probably was the reason of the early over-induction foliar of PR's proteins, compared with non-inoculated agave plants for quitinase, glucanase and peroxidase enzymatic activity (Yedidia *et al.*, 1999 and Yedidia *et al.*, 2000).

Table 1 Peroxidase, β,1-3 Glucanase and Quitinase enzimatic activity recorded in foliar tissue of agave (Agave tequilana Wever var azul) plants at three different evaluation dates later of inoculation with TrBar3-3-1 and ZAP 61-M *Trichoderma* spp strains combined or not with BAS1 *Fusarium oxysporum* strain or Z15 *F. solani* strain

	Quitinase activity (U/mg FW)			Glucanase activity (U/mg FW)			Peroxidasas Activity ∆ absorbance 490nm min <sup>-1</sup> mg <sup>-1</sup> FW		
Evaluation date Treatments <i>F. oxysporum</i> strain	15 days	30 days	60 days	15 days	30 days	60 days	15 days	30 days	60 days
Control	55.24c	47.2c	50.7 b	0.01b	0.0b	0.01c	0.09b	0.06c	0.03e
TrZAP6-1M	71.3 b	69.9b	86.1a	0.0ab	0.1a	0.13a	0.12a	0.10b	0.06d
TrBAR3-3-1	86.5a	78.03a	74.8ab	0.03a	0.2a	0.1b	0.14a	0.18a	0.07d
BAS1+TrZAP6-			70.8ab			0.1b			0.12c
BAS1+ TrBAR3- 3-1			58.4ab			0.1ab			0.37a
BAS1			65.7ab			0.1ab			0.2 b
Treatments F. solani strain									
Control	12.2b	75.9b	54.3c	0.01b	0.0b	0.02c	0.31b	0.25c	0.2bc
TrZAP6-1M	63.32a	81.0b	87.24a	0.01b	0.0c	0.12b	0.31b	0.39b	0.18b
TrBAR3-3-1	52.21a	92.62a	54.56c	0.07a	0.1a	0.12b	0.43a	0.71a	0.19b
Z15+TrZAP6- 1M			69.3b			0.2ab			0.36a
Z15+TrBAR3-3- 1M			53.90c			0.16a			0.12d
Z15			58.18c			0.2ab			0.1cd

Means with the same letter are not different (Tukey  $P \leq 0.05$ )

Like benefit of the induction of PR's proteins, we observed that reduction of the means of incidence of the symptom of stem xylem vassals with occlusions that is regularly observed in plants affected with vascular wilt (Beckman, 1987) in that plants inoculated with the Trichoderma strains plus BAS1 F. oxysporum strain, this means were significantly minor than the mean of 63% determined in plants inoculated only with the BAS1 strain. Root fresh weight in plants inoculated with Trichoderma strains plus Z15 F. solani strain were similar to un-inoculated agave plants; and Bar3-3-1+ Z15 treated plants were superior statistically in fresh weight to Z15 treated plants. The mean of incidence of root rot induced by Z15 F. solani stain was 81%, that was statistically superior to 46.6% and 10% registered in plants inoculated with this pathogen plus ZAP6-1M and TrBar331 Trichoderma strains respectively. These results support that an efficient root colonization of agave plants, and the positive induction of PR's proteins, could be favorable conditions that explain the reduction of symptoms induced by pathogenic strains of F. oxysporum and F. solani . This biotechnology, combined with in vitro reproduction of agave plants, could be an efficient strategy to reduce effectively of initial inocula for disease management of agave wilt (Madden et al., 2007).

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### Influence of arbuscular mycorrhizal Fungi on plant growth

### and nutrition of sorghum

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#### Abstract

The growing use of chemical fertilizers has been witnessing the grave consequence of global economic imbalance as well as environmental pollution. In this context of increased awareness of the benefits of biofertilizers, the potential of Arbuscular Mycorrhizae(AM) as a biofertilizer can play a significant role in the future agroeconomic scenario as a partial substitute to chemical fertilizers. The attractions of mycorrhizae are being naturally symbiotic, they act as biofertilizers and they are nonpolluting to the environment. The most important factor stimulating interest in mycorrhizae was the overwhelming published evidence indicating the growth promoting aspects of mycorrhizal fungi. Mycorrhizal fungi with their extramatrical hyphae increased the absorption of relatively immobile elements in soil especially phosphorus by substantially extending the area of absorption beyond the root hairs. Mycorrhizal fungi increase the uptake of mineral nutrition with the consequent increase in all the growth parameters. Therefore, keeping these factors in view an attempt has been made to study the role of indigenous AM fungi G. fasciculatum as a bioinoculant in seedling establishment, plant growth, grain yield and nutrient uptake(N, P, K) in sorghum. Pot and field experiments were conducted by selecting 4 different genotypes E.36-1, RS-29, M.35-1 and CSV-8R. All these four selected genotypes responded favourably to mycorrhizal inoculations showing positive response to plant growth, grain yield and nutrient uptake. ANOVA analysis of the data for plant growth parameters revealed that the mycorrhizal treated plants were more efficient and significantly superior over the corresponding uninoculated controls in enhancing plant growth, nutrient uptake, grain yield and 100 seed weight indicating that all the genotypes studied responded favourably to G. fasciculatum. The root and shoot tissue analysis of AM inoculated plants showed enhanced levels of N, P, K over the corresponding uninoculated nonmycorrhizal control plants in all the genotypes tested. The impact has been more pronounced with reference to phosphorus. A definite stimulation of plant growth response to AM fungi is attributed to improved phosphorus uptake from the soil. The experimental study has revealed not only the efficiency of indigenous strain G. fasciculatum but also increased level of phosphorus in these most popular genotypes of sorghum commonly used by the farmers in the phosphorus deficient soils. The genotype E.36-1 has been more responsive to nutrient uptake, plant growth, grain yield and 100 seed weight over the other genotypes studied. Thus, the indigenous strain *G. fasciculatum* employed in the present investigation study has proved to be a potential benefactor to all four selected genotypes of *Sorghum bicolor*.

Key words: Arbuscular Mycorrhizae; biofertilizers; sorghum

#### Introduction

The growing use of chemical fertilizers has been witnessing the grave consequence of global economic imbalance as well as environmental pollution and side effects felt from the use of agrochemicals have become more concern. A novel bio-technological solution, using the application of beneficial microorganisms to improve plant health and productivity has therefore been extensively researched. The most common type is arbuscular mycorrhizal fungi (AMF), a beneficial microorganism, the presence of which also generally stimulates plant growth by increasing the capabilities of root system to absorb and translocate nutrients through extensive mycelia. AMF play an ecologically important role in enhancing nutrient uptake (Giri et al., 2005; Cavagnaro et al., 2006) particularly Phosphorus (Gerdemann and Nicholson, 1963, Mosse, 1973), water acquisition, improve soil structure, reclamation, rehabilitation and establishment of micropropagated plantlets. AMF enable plants to cope up with abiotic and biotic stresses (Chao et al., 2006), may protect plants against several soil borne fungal root pathogens (Singh et al., 2000; Azcon et al., 2002; Xavier Biozetchko, 2004; St. Arnaud and Elson, 2005). AMF might alleviate nutrient deficiencies, improve drought tolerance, overcome the detrimental effects of salinity and enhance tolerance to pollution (Brundrett, 1991; Turmen et al., 2005).

AMF association is considered an important component, the use of this microbial inoculants play an important role in sustainable agriculture (Bagyaraj, 2006). The importance of AMF as a tool for improving the growth and productivity in diverse groups of plants was recognized only after the work of Gerdemann (1968) and Mosse (1972). Mycorrhizal fungi are sustainable, remaining in the soil as spores or in association with the roots, while fertilizers can get leached from the soil (contributing to pollution) and need to be replenished.

Considerable reported evidences on wide variety of plants among and within the different species of AMF in their ability to enhance plant growth led to the concept of host preference by AMF (Mosse, 1973). Hence it is always better to select predominant indigenous AMF to prove its efficiency and harness maximum benefits.

The genus sorghum is an annual or perennial usually robust grass, distributed in the tropics and sub-tropics of both the hemispheres, with a few species extending into the temperate regions. The genus includes a large number of economically important sorghum, some of which are extensively cultivated in many countries and grown chiefly for their grain, commonly called sorghum or sometimes great millet. It is used chiefly as a feed for poultry, cattle and other animals. However for evoking better

plant growth response and for enhancement of nutritional and yield components of the inoculation of AMF in fodder crops are an untapped resource, therefore, they merit study.

Therefore, the present study is aimed to use predominant native AMF *Glomus fasciculatum* (Fig. 1a & b) and their multiplication in the root of compatible host (sorghum) in pot cultures for use in the inoculation experiments and to assess the effect of native AMF on the growth, nutrient uptake and grain yield of *Sorghum bicolor* L. Moench under controlled glass house conditions.

#### **Materials and Methods**

The pot experiments were conducted during rabi/post rainy season using four cultivars of Sorghum bicolor L. Moench viz. E.36-1, M.35-1, RS-29 and CSV-8R. The alfisol soil (pH 6.8, 926 mg of N, 4.2 mg of phosphorus, 560 mg of potassium/kg soil) sterilized by autoclaving for three consecutive days at 20 lbs pressure for 1hr. was used for pot experiments. Plants were maintained in sterile chambers of glasshouse conditions in order to avoid contamination. The treatments used included, test pathogen Macrophomina phaseolina (MP), AM inoculum (G. fasciculatum), Dual inoculation consisting of pathogen and AM endophyte (MP + Gf) in the ratio of 1:1 and uninoculated control. The test pathogen M. phaseolina isolated from diseased sorghum plants was multiplied on sorghum meal sand medium in 250 ml flasks (100g medium in each flask) inoculated with 5 mm disc of mycelium taken from 7 days old culture and then incubated for 7 days in a BOD incubator. Soil root-cultures of G. fasciculatum maintained on sorghum host using a mixture of soil and sand in1:1 ratio, grown for 90 days. The cultures containing extramatrical chlamydospores (20 spores/gm soil) and root segments (70% colonization) were used as mycorrhizal inoculum. Inoculum of each treatment was mixed in upper 2 inches soil layer of the pot, incubated for 2 days and then surface sterilized seeds of selected cultivars were sown in each pot. The experiment was arranged in randomized block design with three replicates each. Hoagland nutrient solution (Hoagland and Arnon, 1950) without phosphorus was given to the plants at regular intervals equally to all the treatments and water holding capacity of the soil was maintained by adding sterilized distilled water to the pots whenever required and were grown for 120 days.

All the treatments were sampled 30, 60, 90 and 120 days growth period after emergence until maturity.

Random samples of three plants from each treatment were uprooted carefully, roots washed thoroughly. The root samples were processed, stained with cotton blue (Phillips and Hayman, 1970) and per cent root colonization was calculated by morphometric technique (Toth and Toth 1982). Biomass increment (gm/plant) and rate of dry matter production (gm/day) as an index of growth character (Sestak *et al.*, 1971) was recorded in terms of shoot length, shoot and root fresh weights, shoot and

root dry weights. Dry weights were determined after drying to a constant weight at 70 C for 92 hrs. The oven dried shoot and root samples were estimated for nitrogen using micro-kjeldahl method (AOAC 1950), phosphorus by Vando- molybdate phosphoric acid yellow color method outlined by Jackson (1973) and potassium with the help of an atomic absorption spectro-photometer (Isaac and Kerber, 1971). Estimation of potassium was done at 766.5 nm wavelengths. Nitrogen, phosphorus and potassium content of the shoot and root were estimated in terms of grams/plant. Grain samples were oven dried at moisture content of about 10 % and yield (grams) per plant was recorded.

#### **Statistical analysis**

The data under the present study were subjected to Analysis Of Variance (ANOVA) to determine significance of variances among the treatments and comparisons of treatment means were accomplished by least significance difference (LSD) test at 5% level of significance.

#### **Results and Discussion**

The effect of AM inoculation on growth performance of four selected sorghum genotypes was evaluated in pot experimental conditions. The results obtained after completion of the experiment showed a positive effect of AMF inoculation on different growth parameters viz., per cent root colonization, shoot length, fresh and dry weights of shoots and roots, total biomass, dry matter production, nutrient (N, P, K) uptake is summarized in Table 1. All the varieties selected for the present study exihibited AM fungal association. AM fungal root colonization was characterised by the presence of hyphae, hyphal coils, arbuscules and vesicles Figs. 2a and 2b. Percentage of root colonization showed gradual increase from 30 days crop growth recording maximum at harvest in mycorrhizal inoculated plants of all the four selected varieties. The AM fungal root colonization in dual inoculated plants was more or less at par with AM inoculated plants. Uninoculated control plants showed no root colonization. This indicates that AMF used, as bioinoculant was effective in colonizing the selected sorghum varieties. These results are in agreement with the earlier findings of Krishna and Bagyaraj, 1982. During the active growth period the mycelium, vesicles and arbuscules were observed to be abundant. Among the genotypes tested E.36-1 showed maximum root colonization (89%) whereas, M.35-1 recorded least colonization (70%) at harvest stage. In the present investigation study all the genotypes proved to be responsive to G. fasciculatum inoculation. The present results showed significant differences between the sorghum varieties as regard to their ability to form AM association.



Fig.1 a.Sporocarp of G. fasciculatum



Fig.1b Single spore of G.fasciculatum



**Fig.2a.** Root colonization showing mycelium vesicles



**Fig. 2b.**Root colonization showing mycelium and arbuscules

The degree of root colonization by indigenous AMF varied significantly in four cultivars and this variation can be attributed to the differential preference of AMF towards the varieties. These results support the findings of various authors who reported that root colonization of AMF is genetically controlled (Kesava Rao *et al.*, 1990; Kade and Rodrigues, 2009). The degree of mycorrhizal colonization indicates the dependency of host plant to mycorrhizal infection.

In general, all the parameters recorded showed gradual increase parallel to the increase in plant age 30, 60, 90 and 120 days. The data of shoot height in terms of length in centimetres (Table 1) showed significant increase in mycorrhizal inoculated plants compared to uninoculated control plants. Among the genotypes tested CSV-8R showed maximum shoot height (150.60 cm) followed by M.35-1, RS-29 recording minimum in E.36-1 (116.0 cm) in mycorrhizal inoculated plants. Mycorrhizal inoculated plants showed better shoot height differing significantly from other treatments and the least being in pathogen inoculated plants. However, in dual inoculated plants (pathogen + *G. fasciculatum*) shoot height was more or less at par with mycorrhizal (*G. fasciculatum* alone) inoculated plants. The data on analysis of variance for shoot growth of different genotypes tested showed significant differences

between treatments, plant growth period and among the genotypes. Mycorrhizal inoculated plants significantly enhanced shoot length in comparison to uninoculated controls may probably be due to uptake of more nutrients, which increased vegetative growth. These results are in agreement with earlier findings of Boby and Bagyaraj, 2003 and Tharun *et al.*, 2006 who has reported increased plant height by AM fungal inoculation.

The data on shoot and root dry weights, biomass in terms of grams per plant and dry matter production in grams per day of the four genotypes tested presented in the table reveals that inoculation with *G. fasciculatum* resulted in growth parameters significantly different from that of uninoculated plants. There was a gradual increase in all the growth parameters from  $30 \sim 120$  days crop growth period. The mycorrhizal inoculated treatments were significantly superior in all the growth parameters followed by dual inoculated treatments which were then followed by uninoculated controls recording minimum in the pathogen inoculated plants. Among the genotypes tested E.36-1 showed maximum growth parameters followed by RS-29, M.35-1 recording the least in CSV-8R. Shoot dry weights were maximum in plants inoculated with *G. fasciculatum* followed by dual inoculation, which did not differ significantly, from each other. The same trend was observed in root dry weights. AM inoculated plants showed a significantly increase in respect to root fresh weight and root dry weight as well as total biomass. Significantly highest total biomass production observed in mycorrhizal inoculated plants when compared to uninoculated control.

The biomass production was significantly high in mycorrhizal inoculated plants. The results indicated a positive effect on mycorrhizal inoculation on fresh and dry weights of shoots and roots. Tarafdar and Praveen, 1996 reported that shoot biomass was significantly improved in mycorrhizal plants. The results of the present study support with the findings that root colonization of most of the *Glomus* isolates significantly increased plant shoot dry weights. The significant increase in plant height, shoot and root dry weights due to inoculation with AMF in other words mean AM fungal treatment resulted in better growth (Tharun *et al.*, 2006; Giri *et al.*, 2005). Plant biomass was enhanced due to *G. fasciculatum* inoculated compared to uninoculated control plants. The plant biomass is an important parameter for selecting a fungus for its symbiotic efficiency (Gupta and Janardhanan, 1991; Oliveira *et al.*, 2007).

The data on analysis of variance for the growth parameters showed highly significant differences among the treatments, different stages of growth period and among the genotypes tested. Therefore the mycorrhizal treated plants were more efficient and significantly superior over other treatments enhancing growth parameters clearly indicating that all the genotypes tested responded favourably *G. fasciculatum*.
Table 1
 Effect of soil inoculation with AM fungi on growth parameters, per cent root colonization, nitrogen, phosphorus and potassium uptake of four cultivars of Sorghum bicolor L.Moench

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Treatmen	SL ts (cm)	DWS ←	DWR gr	BMS BN n	AR DMS → ←-gr	S DMR n/day>	NS ←	NR F	PS PR	KS	KR →	PC(%)		
E.36-1						30 DAY	s							
с	10.10	0.66	0.2	1 -		-		10.9	3.93	1.1	0.47	2.9	1.03	-
М	12.10	0.70	0.2	0 -	-	-	-	7.2	2.57	0.6	0.29	2.1	0.70	-
MP+Gf	20.90	1.61	0.89	) -	-	-	-	13.9	4.60	1.6	0.60	3.5	1.30	20.8
AM	29.80	2.91	1.33	3 -	-	-	-	18.5	6.87	2.0	0.77	4.2	1.5	22.4
60 DAYS														
с	25.10	0.98	0.26	0.313	0.053	0.0104	0.0018	12.6b	4.50	1.6	0.67	4.1	1.47	-
MP	18.20	0.79	0.23	3 0.030	0.041	0.0030	0.0013	8.9	3.4 3	1.2	0.52	3.5	1.17	-
MP+Gf	33.10	2.32	1.09	0.711	0.302	0.1237	0.0097	16.6	5.87	2.5	1.97	5.0	1.77	26.4
AM	39.40	4.97	1.76	2.662	1.531	0.1687	0.0477	20.6	8.17	3.8	1.37	5.9	2.07	29.6
					90	DAYS								
с	50.20	1.44	0.31	0.461	0.047	0.0153	0.0015	17.1	6.30	2.2	0.90	5.2	1.90	_
MP	31.80	1.06	0.30	0.293	0.070	0.0089	0.0033	13.0	4.57	1.7	0.63	4.3	1.43	-
MP+Gf	73.10	5.03	1.98	2.706	0.748	0.0902	0.0243	18.5	6.80	3.2	1.20	6.1	2.20	49.6
Gf	78.60	8.63	2.14	3.657	0.876	0.1919	0.0825	25.2	9.53	5.8	2.10	7.2	2.63	50.7
				120	) DAYS									
с	61.10	1.97	0.37	1.003	0.557	0.0176	0.0018	23.0	8.23	3.2	1.13	6.3	2.17	-
MP	43.50	1.60	0.36	0.534	0.160	0.0178	0.0050	15.8	5.40	2.4	0.87	5.5	1.83	-
MP+Gf	100.60	7.30	2.33	1.775	1.450	0.0958	0.0716	24.1	8.10	4.5	1.63	7.2	2.63	85.5
Gf	118.00	12.35	3.55	2.911	2.233	0.1700	0.1947	38.4	10.40	7.8	3.20	9.1	3.03	88.6
RS-29					30 DAY	S								

С	12.40	0.75	0.25	5 -	-	-	-	11.1	3.77	7 1.9	0.	53 2	2.8	0.93	-			
М	14.60	0.73	0.24	1 -	-	-	-	7.	4 2.63	3 0.6	0	.37	2.0	0.77	-			
MP+Gf	26.40	1.48	0.73	-	-	-	-	10.8	4.67	1.4	0.0	53 3	3.2	1.27	18.	4		
AM	25.10	2.82	1.03	-	-	-	-	18.2	6.70	1.8	0.	80 4	4.0	1.53	20.	6		
					60	DAYS												
С	28.90	0.93	0.27	0.180	0.021	0.0060	0.007	12.2	4.77	1.3	0.70	3.8	1.	.40	-			
MP	18.50	0.78	0.28	0.056	0.046	0.0018	0.0015	9.2	3.23	1.1	0.57	3. (	0 1	1.03	-			
MP+Gf	40.30	2.59	0.99	1.110	0.255	0.0370	0.0085	14.0	5.63	2.0	0.93	4.6	1.	.60	26.3			
AM	41.40	4.09	1.98	1.274	0.947	0.1424	0.0315	20.1	8.06	3.6	1.30	5.4	2.	.13	28.9			
				9	0 DAYS												-	
	49.60	1.14	0.30	0.209	0.030	0.0069	0.0019	16.8	6.07	2.0	0.87	5.0	1	.97	_			
MP	40.30	1.09	0.30	0.311	0.022	0.0103	0.0027	12.9	4.53	1.5	0.63	4.5	1	.40	-			
MP+Gf	91.40	4.73	1.53	2.145	0.543	0.0715	0.0181	15.4	6.90	3.0	2.01	5.8	2	2.07	50.4			
Gf	81.30	7.83	2.18	3.739	0.604	0.1246	0.0668	24.5	9.37	5.3	2.20	7.0	2	.53	52.8			
				12	0 DAYS													
с	60.80	2.16	0.34	0.897	0.034	0.0340	0.0011	22.6	8.27	2.9	1.17	6.2	2.3	30	-			
MP	54.30	1.7	0.33	0.641	0.081	0.0991	0.0020	15.5	5.30	2.2	0.80	5.2	1.7	77	-			
MP+Gf	124.10	6.24	1.93	1.603	1.363	0.0801	0.0581	20.6	8.13	4.2	2.52	6.9	2.1	7 7	5.5			
Gf	127.70	10.73	3.04	2.700	1.743	0.1590	0.0947	28.2	10.80	7.4	2.80	8.6	3.0	77	8.7			
M.35-1					30 DAYS													
 с	20.10	0.70	0.24			 -			2.1 4.0	)3 1.4	0.4	40 2		1.03	_			
MP	14.50	0.55	0.20	-	-	-	-	1	7.8 2.	37 0.4	0.	30 2	2.1	0.63	-			
MP+Gf	26.30	1.24	0.69	-	-	-	-	1	1.9 4.	.40 1.5	i 0.	40	3.6	1.17	15.4			
AM	26.00	) 2.3	57	1.00	-	-		-		-	19.3	6	5.87	1.	9	0.83		4.5

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60 DAYS

С	32.30	0.89	0.26	0.695	0.028	0.195	0.001	12.8	4.63	1.8	0.8	7	4.1	1.30	)	-
MP	25.70	0.65	0.24	0.101	0.040	0.003	0.001	9.9	3.03	1.0	0.4	0	3.5	0.87	7	-
MP+Gf	39.60	2.63	0.92	1.396	0.228	0.047	0.008	15.1	5.20	1.8	0.7	3	4.2	1.40	0 2	3.2
АМ	44.50	4.50	1.31	2.223	0.312	0.074	0.010	21.0	8.37	3.2	1.2	7	5.9	2.00	0 2:	5.4
				90 E	DAYS											
С	62.10	1.13	0.41	0.007	0.087	0.145	0.0036	17.5	6.17	2.2	1.0	03	5.4	1.87	7	-
MP	47.30	0.85	0.28	0.201	0.033	0.0067	0.0011	11.8	4.30	1.2	0.6	i3 4	4.9	1.27		-
MP+Gf	90.20	4.93	1.42	2.294	0.500	0.0764	0.0166	18.2	6.37	2.8	1.2	20	5.9	2.00	4	1.3
Gf	102.90	7.89	1.93	3.398	0.654	0.1132	0.0218	22.6	9.50	) 5.0	) 2	2.27	6.5	2.3	37	42.7
				120 D.	AYS											
с	64.20	1.93	0.45	0.766	0 .045	0.0299	0.0015	24.8	8.37	2.8	1.1	27 (	5.0	2.20		-
MP	58.10	1.69	0.32	0.845	0.041	0.0281	0.0013	14.9	4.70	2.0	0.9	93 (	5.2	1.57		-
MP+Gf	124.90	5.31	1.73	1.583	1.305	0.0629	0.0501	22.5	7.63	3 3.2	2 1	.63	6.5	2.4	0	55.4
Gf	132.60	9.85	2.89	2.501	1.586	0.1484	0.0528	26.8	10.	77 3	7.0 2	2.77	8.9	3.1	3	70.4
CSV-8R				30	DAYS											
с	19.83	0.73	0.32						13.2	4.10	1.0	0.:	 37 2	2.5	0.98	-
М	13.30	0.62	0.20	-	-	-	-		8.2	2.27	0.2	0.2	20 2	2.0	0.37	-
MP+Gf	23.90	1.37	0.80	-	-	-	-	1	2.5	3.97	1.3	0.3	3 3.	.4	0.88	16.4
AM	23.30	2.47	1.00	-	-	-	-		19.0	6.30	2.0	0.1	70	4.6	1.63	19.7
				60 DAY	/S											
с	30.17	0.83	0.35	0.093	0.026	0.0034	0.0008	14	1.2 4	.60	1.2	0.90	4.0	) 1	.20	-
MP	23.60	0.71	0.24	0.086	0.043	0.0026	0.0014	10	.1 2.	.73	0.9	0.33	3.1	0	.70	-
MP+Gf	39.30	3.01	1.06	1.642	0.261	0.0547	0.0087	16	6.0 4	1.93 1	.9	0.67	4.4	4 1.	.27	20.5
AM	43.70	5.04	1.74	2.520	0.738	0.0856	0.0246	2	2.5	8.40	3.2	1.3	5.	.5 1	.93	23.5

	90 DAYS													
С	45.87	1.13	0.39	0.292	0.042	0.0097	0.0014	17.8	6.20	2.0	1.10	5.3	1.83	-
MP	30.30	0.81	0.28	0.100	0.032	0.0033	0.0011	12.4	3.47	1.2	0.53	5.2	0.97	-
MP+Gf	87.00	4.33	1.20	1.319	0.147	0.0439	0.0049	18.9	5.63	2.1	0.97	5.6	1.63	40.5
Gf	96.30	7.73	2.33	2.693	0.593	0.0897	0.0197	24.3	9.77	5.6	2.30	6.0	2.47	54.9
			12	20 DAYS										
С	75.60	1.89	0.43	0.530	0.044	0.0255	0.0014	23.9	8.27	2.9	1.17	5.8	2.07	-
MP	60.00	1.53	0.34	0.714	0.064	0.0238	0.0019	16.1	3.73	1.8	1.16	6.5	1.31	-
MP+Gf	140.00	5.07	1.30	0.445	1.206	0.0618	0.0432	23.6	6.13	3.2	1.42	6.6	2.2	60.6
Gf	150.60	9.38	2.37	1.646	1.407	0.0548	0.0469	28.2	10.53	7.2	2.67	8.2	3.07	72.8

SL- Shoot Length; DWS- Dry Weight of Shoot; DWR- Dry Weight of Root; BMS- Biomass Shoot; BMR-Biomass Root; DMS-Dry Matter Shoot; DMR- Dry Matter Root; NS-Nitrogen uptake in Shoot; NR-Nitrogen Root; PS-Phosphorus Shoot; PR- Phosphorus Root; KS- Potassium Shoot; KR- Potassium Root; PC- Per cent Root Colonization (%)

All the values are means of three replicates; C- uninoculated control, MP-Macrophomina phaseolina; Gf- Glomus fasciculatum.

The inoculum of AMF in response to nutrient uptake (N, P, K) by shoot and root is presented in Table 1 reveals that mycorrhizal fungi have positive role in response to the nutrient uptake. AM fungal inoculated treatments significantly enhanced nutrient uptake by the shoots of all genotypes in comparison with the other treatments. A similar trend was observed for root nutrient uptake being maximum in treatment with AMF and least in pathogen inoculated plants. The highest nutrient uptake was recorded in *G. fasciculatum* (alone) treatment followed by dual inoculated treatment (pathogen + *G. fasciculatum*), which was then followed by uninoculated control treatment. The lowest nutrient uptake was found in pathogen-inoculated treatments. Results of this experiment showed that with the increase of plant growth period nutrient uptake increased.

	E.3	6-1	Rs-29		M.3	5-1	CSV-8R		
Treatments	Grain yield	Wt. of 100							
	(gm/plt)	Seeds (gm)	(gm/plt)	Seeds (gm)	(gm/plt)	Seeds (gm)	(gm/plt)	Seeds (gm)	
С	30.23	3524	40.5	2.782	32.6	3.036	36.5	3.092	
MP	25.4	2.537	30.6	1.925	24.3	3.371	26.8	2.526	
MP + Gf	39.7	3.686	48.0	3.060	38.6	2.952	42.1	3.226	
Gf	42.5	3.980	61.4	3.284	46.9	3.510	50.3	3.634	

Table 2 Effect of G.fasciculatum on grain yeild in four cultivars of Sorghum bicolor in pot experiments

All the values are means of three replicates;- uninoculated Control; MP- Macrophomina phaseolina; Gf- Glomus fasciculatum.

Significantly higher levels of three elements viz., N, P, K in AM inoculated plants over uninoculated control indicates that these elements were efficiently transported in mycorrhizal inoculated plants. AM fungal inoculation had their most significant effect on P and N uptake compared to K which were statistically on par with nutrient uptake in all the genotypes studied. Among the genotypes tested E.36-1 showed significantly highest nutrient uptake compared to the other genotypes studied. The maximum percent increment in phosphorus uptake in mycorrhizal plants was more than two folds. The increased 'P' content may be attributed to increase in uptake of P facilitated due AM fungal colonization through various mechanisms. AM fungi are known to improve P s cavenging a larger volume of soil with extensive hyphae (Ortas et al., 2002; Kothari, et al., 1990). Therefore, higher availability of nitrogen, significantly increased uptake, dry matter accumulation, translocation of nutrients during reproductive stage which inturn improved yield attributes that affect the grain and straw yield at the end (Patel et al., 1988; Kumar et al., 2002; Sharma et al., 2003). The genotype E.36-1 has been more responsive to nutrient uptake particularly P agreeing to the earlier observations.

The yield performance in terms of yield per plant and 100 seed weight presented in Table 2 indicates that AMF inoculated plants showed highest yield in the genotype RS-29 followed by CSV-8R, M.35-1 and lowest grain yield was recorded in E.36-1. The statistical data on yield performance showed significantly better yield per plant and improved 100 seed weight in AM inoculated plants. Highly significant differences were observed in the treatments of inoculated plants over uninoculated controls. Weight of 100 seeds has contributed significantly to the yield data.

The grain yield per plant also showed almost similar trend, highest yield being in the plants inoculated with *G. fasciculatum*, followed by dual inoculation, which were

on par with each other. Yield of plants inoculated with *G. fasciculatum* alone and uninoculated control plants did not differ significantly.

The present results confirm various reports on plant growth enhancement by AM inoculations on crop plants (Iyer *et al.*, 1988; Boby and Bhagyaraj, 2003). In the present experiment constant increase in intensity of mycorrhizal infection with the age of the plant could be attributed to the ability to stimulate plant growth, nutrient uptake and yield. The data obtained in the present study is of conclusive evidence on the positive role of AM fungi as a benefactor and biofertilizer on all the genotypes under study.

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## Cyanobacteria as novel plant growth promoting and biocontrol options - metabolites, genes and "cross talk" with plants

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## Abstract

Cyanobacteria represent a remarkable group of photosynthetic prokaryotes, whose ubiquity and diversity is unparalled in the microbial world. Although the role of cyanobacteria is well established as nitrogen supplements for rice, their taxonomic and functional diversity in the rhizosphere of rice and wheat crops is less explored. Generally considered to be obligate phototrophs, our investigations with samples from the rhizosphere of rice and wheat plants from diverse agroecologies of India, revealed interesting facets regarding their genetic diversity and production of bioactive metabolites. Efforts were made to utilize these cyanobacterial isolates (Anabaena, Calothrix and Nostoc species) with Providencia, Alcaligenes, Bacillus as PGPR consortia for rice-wheat cropping system, which revealed synergistic interactions, intracellular colonization in roots and production of diverse metabolites, leading to enhanced crop biomass, yields and soil fertility. Selected cyanobacteria and consortia also showed biocontrol activity against phytopathogenic fungi, besides enhanced plant defense responses and reduced disease severity, which led to an improved understanding of the tripartite interactions among cyanobacteriaphytopathogenic fungi-plants. Genes encoding novel *cho* gene (chitosanase) associated with GH3-like family and  $\beta$ -1,4 (end 1 and 2) and  $\beta$ -1,3 (end 2) endoglucanase and fungicidal activities have been identified, purified and characterized from Anabaena strains, which represent first reports in cyanobacteria. The intracellular colonization of cyanobacteria in wheat and rice roots and their potential as PGPR agents for wheat has also been reported for the first time. Novel formulations of cyanobacteria and agriculturally useful bacteria as biofilms and disease suppressive composts have been developed, which have shown promise as environment friendly options in selected crops.

**Key words:** biocontrol; chitosanase; cyanobacteria; endoglucanases; fungicidal; rice; wheat; tomato

## Introduction

Cyanobacteria are a unique group of organisms, exhibiting cell structure similar to bacteria, but employ plant-like photosynthesis, and several forms exhibit independence for C and N nutrition. They play diverse roles in the environment, as nutrient supplements (biofertilizers) and soil compaction agents in agriculture, besides having tremendous ecological significance as carbon sequestering and bio remediating agents (Venkataraman, 1972; Prasanna et al., 2008a; Prasanna et al., 2009b; 2010a). The role of cyanobacteria as biofertilizers has undergone a major change in recent years, with their utility as not only diazotrophs, but also as plant growth promoting inoculants in a diverse range of crops (Karthikeyan et al., 2007; Nain et al., 2010; Manjunath et al., 2011). Plant growth promoting rhizobacteria (PGPR) enhance plant biometric parameters/yields and aid in biological control of soil borne pathogens, which has been intensively investigated by several workers. During the last couple of decades, a significant increase in growth and yield of agronomically important cereal crops and improved soil fertility, in response to inoculation with PGPR has been reported (Asghar et al., 2002; Richardson et al., 2009). Most of the work in this area has focused on strains of *Pseudomonas* and related genera. Wheat, together with rice, represent the most important staple food crops used to sustain humanity, as they provide more calories and proteins in the diet than any other crop. Rice (Oryza sativa L.) is one of the most prominent food crops globally, and represents the staple diet for almost half of the human population of the world. Our research efforts (Nain et al., 2010; Prasanna et al., 2011a, b, c) have been directed towards employing synergistic combinations of rhizospheric isolates of cyanobacteria and bacteria and evaluating their utility for enhancing plant growth/yields and improving the nutritional status of crop and soil in the rice-wheat cropping sequence.

In recent years, cyanobacteria are also being explored as sources of bioactive molecules having pharmaceutical and agricultural significance (Jaiswal *et al.*, 2008; Prasanna *et al.* 2008a,b; Prasanna *et al.* 2009a,b, 2010b; Manjunath *et al.*, 2010). Such bioactive compounds are known to be important determinants of allelopathic activity in water and soil. With the increasing concern to health and environment due to fungicides and resistance development in target pathogens it has become necessary to intensify research towards development of novel, more effective and sustainable fungal disease control solutions. The excretion of hydrolytic enzymes is known to be a common trait of plant pathogens/symbionts, which promotes a closer association with plant roots/target organisms and improve the stability of such associations. Prasanna *et al.* (2008b, 2009a) and Gupta *et al.* (2010; 2011) revealed for the first time, the activity and homologues of hydrolytic enzymes in several *Anabaena* strains and their correlation with fungicidal activity.

Tomato is one of the most important vegetables grown and consumed worldwide. Its popularity is due to its high nutritive value, diversified use, and nutritional significance as a source of vitamins A and C. Damping off and related soil borne diseases are a serious problem as they cause high seedling mortality in nurseries and fields (Kloepper *et al.*, 1999). Effective soil fungicide treatments for this disease are also unavailable, and this has stimulated the search for biological options. Our efforts were directed towards evaluating the biocontrol potential of cyanobacterial formulations (amended composts, biofilms) in phytopathogenic fungi-challenged vegetable crops, including tomato and understanding the cyanobacteria-plant- fungi interactions. Therefore, this compilation describes our significant research achievements, on the less investigated aspects of cyanobacteria, and their combinations with bacteria, as PGPR for rice-wheat cropping system and biocontrol agents for tomato.

## **Materials and Methods**

### Organisms used in this study

A set of cyanobacterial strains belonging to the germplasm of *Anabaena* isolates from diverse agro ecologies of India (Prasanna *et al.*, 2009a) available in the germplasm of the Division of Microbiology, IARI, New Delhi and bacterial strains which had shown promise in preliminary screening under *in vitro* conditions (Dukare *et al.*, 2011; Chaudhary *et al.*, 2010; Nain *et al.*, 2010) were used in this study. They were maintained under optimal conditions of light and temperature  $(27 \pm 2^{\circ}C)$  and a light intensity of  $52 \sim 55 \,\mu$  mol photon m<sup>-2</sup> s<sup>-1</sup> and 16L: 8D Light: dark cycles).The fungal strains *Pythium debaryanum* (ITCC 95), *Rhizoctonia solani* (ITCC 4578), *Fusarium moniliforme* (ITCC 4223) and *Fusarium oxysporum lycopersici* (ITCC 4998) were obtained from the Indian Type Culture Collection, Division of Plant Pathology, IARI, New Delhi.

## Growth and maintenance

The axenised cyanobacterial strains were grown and maintained in nitrogen free BG 11 medium (Stanier *et al.*, 1971), under  $27 \pm 1^{\circ}$ C L: D (Light: Dark cycles 16:18), white light (50~55 µmol photons m<sup>-2</sup>s<sup>-1</sup>). For biocontrol experiments, the cultures were grown under optimized conditions (Manjunath *et al.*, 2010). The fungal strains were grown and maintained on potato dextrose agar medium (CAB, 1968), at  $28 \pm 2^{\circ}$ C in a BOD incubator. The bacterial strain was grown at 30°C and maintained in nutrient broth.

### Experimental set up

All experiments conducted in IARI field, with rice and wheat crop, involved fertilizer controls and microbial formulations were made with compost as carrier. The biocontrol formulations were prepared using compost: vermiculite (1:1) as carrier and

the experiments conducted in pots under the controlled environmental conditions of National Phytotron Facility, IARI, New Delhi.

## In vitro fungicidal assay of cyanobacterial cultures

Sterile 5mm discs were placed on the lawn of respective fungal strains. Fifty  $\mu$ l of the leachate were dispensed on the sterile discs and incubated under optimal conditions. Nystatin and sterile water were used as positive and negative controls. Observations on the development of inhibition zone were taken regularly up to 72 h incubation period.

## Plant Enzyme assays

The plants were sampled for estimating the defense enzymes (PAL, PPO and PO activity) and hydrolytic enzymes, using the optimized protocols (Ramamoorthy *et al.*, 2002; Prasanna *et al.*, 2008b).

## Soil microbiological and nutrient analyses

Samples were collected from root region of soil, in triplicates from each treatment, for assessing the microbiological parameters. A minimum of three plants were taken for analyzing the plant related parameters. Dehydrogenase Activity Microbial biomass carbon, nitrogen was estimated using protocols standardized in earlier experiments (Nain *et al.*, 2010).

## Molecular and Bioinformatic analyses

The purified PCR products for chitosanase and endoglucanase (Prasanna *et al.*, 2009a; Gupta *et al.*, 2010, 2011) were subcloned into pIVEX GST fusion vector (Roche) and transformation was carried out using *E. coli*-JM109 (DE3) strain (Promega). Purification of the specific Cho and End proteins was done using Ni-NTA kit (Qiagen). BLASTN and BLASTP were used to identify nucleotide identity and amino acid sequence similarity, respectively. ORFs were identified using GENDB and GENMARK. Nucleotide and amino acid sequences were aligned using Clustal W2. The prediction of signal peptide and cleavage site in the translated sequence was obtained using Neural Network and HMM (Hidden Markov Model) algorithms.

## Experimental design and statistical analyses

The experiments were designed as completely randomized design and the data was recorded in triplicate for the selected parameters and subjected to ANOVA (analysis of variance) in accordance with the experimental using SPSS statistical software to quantify and evaluate the sources of variation. Duncan's Multiple Range Test (DMRT) was employed to compare the mean performances of different strains for the specific parameters under study and the critical difference between treatments, calculated at 5% level of significance, denoted as C.D. (Critical Difference) /LSD (Least Significant Difference) in the tables. The rankings are denoted by superscripts in the relevant tables and graphs, with a denoting the highest rank. Standard deviation is denoted by the bars in the graphs.

## **Results and Discussion**

Blue green algae or cyanobacteria are among the most primitive of organisms on earth which combine in themselves the photosynthetic ability of algae along with the morphology and physiology of both bacteria and green plants. They exhibit a wide range of morphological diversity, ranging from unicellular to branched filamentous organization and can differentiate heterocysts for N<sub>2</sub> fixation, akinetes for surviving and hormogonia for taxis. This structural – functional plasticity confers great versatility, enabling cyanobacteria to adapt and inhabit a wide range of environments and niches, including the rhizosphere (Prasanna *et al.*, 2009b). Cyanobacteria are prokaryotic phototrophs of evolutionary significance which have mainly exploited as diazotrophic inoculants. They exist as free-living forms or as symbionts with diverse members of plant kingdom and endogenous pools of cyanobacterial phytohormones, such as IAA are known to improve the growth and yield of wheat (Karthikeyan *et al.*, 2007, 2009; Hussain and Hasnain 2011).

Studies were undertaken to characterize the abundance, genera-wise diversity and metabolic capabilities of cyanobacteria isolated from the rice rhizosphere, for the first time, revealed that the genera Nostoc and Anabaena comprised 80% of the rhizosphere isolates (Prasanna et al., 2009b). Rhizosphere samples from rice cultivars grown at Aduthurai exhibited low generic richness i.e. only two genera Anabaena and Nostoc were observed (Prasanna and Nayak, 2007; Prasanna et al. 2009b). As rhizosphere isolates can be better competitors, due to their direct linkage with roots, therefore, their inclusion in biofertilizer/biocontrol consortia can improve the effectiveness of these inoculants. The rhizosphere dynamics and plant growth promoting ability of a set of inoculated strains in rice crop revealed the persistence of strains on roots upto harvest stage, entry into roots (Karhikeyan et al., 2009) and enhancement in plant growth and yield attributes, besides improved soil fertility/microbiological parameters and plant growth parameters (plant height, dry weight, grain yields) carbon (Prasanna et al., 2009a).Our reports on the influence of rhizocyanobacteria (isolated from the rhizosphere of rice and wheat plants from diverse agroecologies of India) and evaluation of their synergistic interactions with

other PGP bacteria for their agronomic efficiency as PGPR represent first reports. Field evaluation of promising cyanobacterial and bacterial strains (Table 1; Figure 1) revealed the promise of combinations of cyanobacteria-bacteria (T7, T8) in enhancing crop biomass and yields. A similar trend was recorded in terms of nutrient uptake and soil fertility parameters, including dehydrogenase activity and carbon and nitrogen sequestration.



Figure 1 Effect of PGPR inoculation on microbiological and nutritional parameters in rice and wheat crop

S.No	Treatment No.	Grain yield (t ha <sup>-1</sup> )	Total biomass (t ha <sup>-1</sup> )
Wheat	Сгор		
T1	Absolute control	4.18	14.15
T2	FD NPK	4.70	15.72
T3	½ N + PK	4.37	14.54
T4	2/3 N + PK	4.66	14.54
T5	<sup>1</sup> / <sub>2</sub> N + PK+ Anabaena sp.	4.15	15.33
T6	<sup>1</sup> / <sub>2</sub> N + PK + Providencia sp.	4.77	13.76
T7	<sup>1</sup> / <sub>2</sub> N + PK + Anabaena sp. + Providencia sp.	4.87	13.56
T8	<sup>1</sup> / <sub>2</sub> N + PK + Anabaena sp.+ Calothrix sp. + Anabaena sp.	4.66	14.94
	SEm ±	0.11	0.41
	LSD ( $P \le 0.05$ )	0.31	1.14
Rice C	rop		
T1	Absolute control	8.06	22.80
T2	FD NPK	8.65	24.57
T3	½ N + PK	8.65	22.96
T4	2/3 N + PK	8.45	24.17
T5	2/3 N+ PK + Providencia sp.+ Brevundimonas sp.+ Ochrobacterium	9.63	26.34
T6	<i>2/3</i> N+ PK + <i>Nostoc</i> sp.+ <i>Brevundimonas</i> sp. + <i>Ochrobacterium</i> sp.	8.84	25.16
T7	2/3 N+ PK + Brevundimonas sp.+ Anabaena sp.+ Providencia sp.	9.83	27.67
T8	2/3 N+ PK + Providencia sp.+ Anabaena sp.	8.20	24.76
	SEm ±	0.39	1.03
	LSD ( $P \le 0.05$ )	1.07	2.84

 Table 1
 Influence of PGPR inoculation on crop growth and yields

Cyanobacteria produce a diverse range of secondary metabolites, exhibiting various bioactivities such as inhibitory properties against microorganisms (bacteria, cyanobacteria, algae, viruses, fungi) and toxicity to invertebrates and vertebrates (Wiegand and Pflugmacher, 2005). These metabolites may be used for the development and application as algaecides, fungicides, herbicides, insecticides.. Lytic enzymes such as chitinase (or chitosanase),  $\beta$ -1,3-glucanases are known to be involved in the antagonistic potential and hyperparasitism against fungal pathogens, and thereby in effective biocontrol. These enzymes are widely distributed in a large group of chitin degrading organisms, including fungi, bacteria, insects, plants and animals and more recently in cyanobacteria (Prasanna *et al.*, 2008b, 2009a).

Novel antifungal chitosanase (cho) and endoglucanase genes were identified from Anabaena fertilissima and Anabaena laxa, respectively. A. fertilissima along with A. sphaerica (negative control) showed amplified products of 1.086kb and 1.101 kb, respectively using chitosanase specific primers (Gupta et al., 2010). The sequence analyses showed *cho* from both *Anabaena* strains belonged to glycoside hydrolase family 3 (GH3) (EC: 3.2.1). An open reading frame of 362 and 367 amino acids with a predicted molecular mass of 40 kDa and 40.6 kDa in A. sphaerica and A. *fertilissima*, respectively. Pair-wise alignment of the corresponding protein sequences identified a 23 amino acids long putative signal peptide and 5 insertions and 5 substitutions in the amino acid sequence of A. fertilissima which may be responsible for function of the chitosanase and the observed antifungal activity.. The data obtained after the HPLC analyses of Cho protein from A. fertilissima revealed its ability to degrade (GlcN)<sub>5</sub> into (GlcN)<sub>2</sub> and (GlcN)3, thus confirmed the presence of chitosanase activity in A. fertilissima. Site directed mutagenesis revealed that Glu-121 and Glu-141 positions are essential for the antifungal activity in A. fertilissima. The importance of glutamic/aspartic acid residues in regulating the activity of chitinases and chitosanases is also proposed by previous reports (Yun et al., 2005). The absence of these residues in A. sphaerica further supported the hypothesis. The expression level of *cho* was also found to increase significantly under the condition of long dark phase in A. fertilissima by quantitative real-time RT-PCR (qRT-PCR). Anabaena laxa was characterized in terms of both  $\beta$ -1,3 and  $\beta$ -1,4 endoglucanase activities responsible for fungicidal activity (Gupta *et al.*, 2011). The plate assays for both  $\beta$ -1,3 and  $\beta$ -1,4 endoglucanase activities were confirmed by evaluating both these activities in terms of quantitative analyses using cell free recombinant proteins from the positive clones screened from the genomic library of Anabaena laxa. SDS-PAGE of the recombinant purified proteins revealed 38 and 74 kDa molecular weight from End 1 and 2, respectively. The putative end 1 and end 2 encoding endoglucanase associated with Peptidase M20 family and glycoside hydrolase family 5 (GH5), respectively similar to the earlier isolated endoglucanases from Bacillus and Pseudomonas sp. (Ledger et al., 2006; Wong et al., 2009). End2 revealed high expression against CMC and low against laminarin in the Zymograms; while End1 showed high expression only against CMC.

Several mechanisms have been proposed to explain the suppression of plant diseases by compost; including beneficial chemical components in compost, stimulation of microbial communities antagonistic to pathogens (Chen *et al.*, 1987; Dukare *et al.*, 2011), and induction of systemic acquired resistance have been implicated in the suppression of plant disease. It is well known that plants possess a range of active defense responses that contribute to resistance against a variety of pathogens. They respond to pathogen attack by activating various defense responses including enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (PO), Polyphenol oxidase (PPO), lipoxygenase, superoxide dismutase, and  $\beta$ -1,3 -glucanase.

Prasanna *et al.*, (2010b) and Chaudhary et al. (2010) observed that the cyanobacterial strains producing hydrolytic enzymes caused mycelial lysis, vacuolation and granulation of cytoplasm, branching and other hyphal deformities in the fungal structures may be due to the presence of chitosanases and  $\beta$ -1,3-glucanase production. The level of defense related enzymes is known to play a crucial role in the degree of host resistance. Increase in activity and accumulation of these enzymes depends mainly on the inducing agent but also on the plant genotype, physiological conditions and the pathogen.

The cyanobacterium/bacterium amended formulations were evaluated in pot culture experiments with tomato seeds challenged with inoculated with fungal consortium, which revealed the promise of *Anabaena* sp. amended formulation , especially T4 (*Anabaena variabilis*) as fungicidal agents and enhancing plant defense enzymes (Figure 2). A significant enhancement in the hydrolytic and plant defense enzymes was recorded in the root tissues in the amended compost inoculated treatments (T1-4), emphasizing the significant role of cyanobacterial in biocontrol. A positive correlation was recorded between disease severity and PAL and PPO which helps us to conclude from our study that PPO and PAL activity could be useful indices for elucidating response of tomato to fungal challenge.

Plant pathogen interactions are mediated by a complex network of molecular and cytological events that determine a range between susceptibility and resistance. To date, no published information is available on cyanobacterial inoculation induced defense enzymes and their role in plant growth /disease suppression, it can be hypothesized that the cyanobacteria amended formulations employ a combination of mechanisms, including induction of defense enzymes, activity of hydrolytic enzymes for reducing disease severity. Testing under different agro-climatic conditions will help to further evaluate their agronomic efficiency and utility in integrated nutrient management of rice-wheat cropping sequence.



**Figure 2** Modulation of Chitosanase,  $\beta$ - 1,3 - glucanase, PAL and PO activity in tomato roots at different time intervals; IN: Inoculation with *Fusarium* sp.; UN: Uninoculated with *Fusarium* sp. [T1: *Anabaena laxa* Control Condition (C); T2: *Anabaena laxa* Optimized Condition (O); T3: *Anabaena variabilis* Control Condition(C); T4: *Anabaena variabilis* Optimized Condition (O); T5: *Anabaena* sp. (Negative Control); T6: *Bacillus subtilis*; T7: Thiram + Carbendazim (Chemical control); T8: *Trichoderma* (Biological Control); T9: Negative Control (Soil + Carrier Mixture]; Fungicidal activity of formulations against *Fusarium* sp..

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## Multi-pathogen disease caused by *Didymella bryoniae* and bacteria on Styrian oil pumpkin: microbial ecology and biocontrol

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## Abstract

The Styrian oil pumpkin, Cucurbita pepo L. subsp. pepo var. styriaca Greb., is a crop of cultural, medical and commercial importance in Austria but also in other regions of the world, e.g. in China. In the recent years, fruit rot and black rot caused by the ascomycete Didymella bryoniae (Fuckel) Rehm has lead to dramatic yield losses. In the field, the fungal disease was usually associated with characteristic symptoms of bacteriosis. Bacterial pathogens include Erwinia carotovora, Pseudomonas viridiflava, Pseudomonas syringae and Xanthomonas cucurbitae. The high coincidence of fungal and bacterial disease suggests mutualistic effects in pathogenesis. In this study, Styrian oil pumpkin-associated microbial communities with focus on bacterial endophytes were analyzed by microbial fingerprints performed by PCR-Single Strand Conformation Polymorphism analysis (SSCP) and Fluorescence in situ hybridisation (FISH) in combination with confocal laser scanning microscopy. Computer-assisted comparisons of community profiles revealed microhabitat-dependent community structures for Pseudomonas, whereas *Bacillus* communities were more influenced by the plant development stage. By cultivation dependent methods, the fraction of Styrian oil pumpkin inhabiting in vitro antagonists against D. bryoniae and bacterial pathogens was determined: 9% (= 199 strains) of bacterial and fungal isolates showed an antagonistic potential against the fungus. From these in vitro Didymella antagonists, 43 bacterial strains inhibited growth of at least two of the three tested bacterial pathogens. Based on genotypic characterization of these isolates, five potential broad-spectrum antagonists were identified: strains of Lysobacter spp., Pseudomonas chlororaphis, Paenibacillus *polymyxa* and *Serratia plymuthica*. They were successfully evaluated in field trials. On their basis, a biological product to protect the Styrian oil pumpkin against multipathogen disease is currently under development.

## Introduction

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a pumpkin variety that bears its name according to its origin of cultivation that is the Austrian district Styria. The specialty of this cultivar is the absence of a wooden seed shell that facilitates the production of pumpkin seed oil. Beside the culinary aspect of this dark green oil it is famous as a very healthy nutritional supplement containing high levels of polyunsaturated fatty acids, antioxidants, vitamins A, B1, B2, B6, C, D, E and counteracts diseases of bladder and prostate. Nowadays, Styrian oil pumpkin is mainly cultivated in China and Russia; however it is also economically and culturally important for Styria and Austria.

In recent years, dramatic yield losses of Styrian oil pumpkin were reported in Styria due to black rot of pumpkins caused by *Didymella bryoniae* (Auersw.) Rehm, anamorph *Phoma cucurbitacearum* (Fr.) (Huss *et al.*, 2007). The ascomycete has a broad host range within the Cucurbitaceae and causes symptoms on vegetative plant parts as well that are known as gummy stem blight (Keinath *et al.*, 1995). Styrian oil pumpkins are also affected by bacterial pathogens *Pectobacterium carotovorum*, *Pseudomonas* spp. and *Xanthomonas cucurbitae* causing soft rot of pumpkins and leaf diseases (Huss, 2011).

To understand the structure and function of pumpkin-associated microorganisms, endophytic microbial communities from roots-, fruits-, and flower-associated microfloras from three different pumpkin cultivars were analyzed by a multiphasic approach based on microbial fingerprints of 16S rRNA genes, FISH-CLSM studies and cultivation-dependent methods to obtain oil pumpkin associated microbial antagonists against D. bryoniae and bacterial pathogens. Selected antagonists were tested for their efficacy under practical conditions at the field and are subjected to the development of biological product oil а for Styrian pumpkin.

## **Materials & Methods**

#### Experimental design and sampling

Plant samples of roots, flowers and fruit pulp from three different oil pumpkin cultivars, cv. Gleisdorfer Ölkürbis, Gleisdorfer Diamant and GL Maximal, from always 4 different individual plants from always 4 different sites at a field located in Gleisdorf, Austria, were collected. Roots were washed with tap water and then surface sterilized in 0.54 M NaOCl for 5 min. and subsequently washed three times with sterile water. The different plant materials were homogenized in sterile 0.15 M NaCl with mortar and pestle. For microscopic analysis of the female oil pumpkin flower, samples were taken from petals and pistils from the oil pumpkin cultivar GL Opal.

#### Total community DNA isolation and fingerprint analysis

Suspensions of homogenized plant parts (as described above) were centrifuged for 20 min. at  $10.000 \times g$ . From corresponding pellets DNA was extracted using the FastDNA®Spin Kit for Soil.Bacterial fingerprints of Pseudomonas and Bacillus from roots, flowers and fruits were analyzed by single-strand conformation polymorphism (SSCP) analysis (Schwieger and Tebbe 1998). 16S rRNA genes from Pseudomonas and Bacillus were amplified using Taq-&GOTM PCR Mastermix (Qbiogen BIO 101 Systems, Carlsbad, USA) by a nested PCR design: for the first amplification of Pseudomonas we used 0.4 µM of primers F311 Ps (5'-CTG GTC TGA GAG GAT GAT CAG T-3') and 1459 rPs (5'-AAT CAC TCC GTG GTA ACC GT-3'), MgCl<sub>2</sub> (1.5 µM) and 1 µl of DNA in a 20 µl reaction mix. PCR conditions were: initial denaturation at 95°C for 5 min., followed by 26 cycles consisting of 95°C for 20 s,  $63^{\circ}$ C for 30 s and  $72^{\circ}$ C for 60 s, and a final elongation step at  $72^{\circ}$ C for 10 min. Obtained PCR products were applied in a nested PCR reaction for that we used Unibac-II-515f/phosphorylated Unibac-II-927r as primers (Lieber et al., 2002). For Bacillus-specific PCR we used 0.5 µM of primers Bspez 3f (5'-AGA CTG GGA TAA CTC CG-3') and BACr833 (5'-CTA ACA CTT AGC ACT CAT-3') and 1 µl of DNA in a 20 µl reaction mix. For the seminested PCR we used 0.5 µM of primers Bspez 6f (5'-CGA CCT GAG AGG GT-3') and phosphorylated BACr833. Conditions for both PCRs were: 95  $^{\circ}$ C for 5 min. initial denaturation, 30 cycles of 95  $^{\circ}$ C for 45 s, 54°C for 30 s, 72°C for 45 s and final elongation at 72°C for 10 min. PCR products were purfied with the peqGOLD MicroSpin Cycle Pure Kit and DNA fragments were separated with a TGGE Maxi apparatus at 400 V and 26°C. Silver staining of gels was applied for visualization of the bands (Bassam et al., 1991).

## Microscopic analysis of bacterial communities on female flower

Samples of flowers parts were fixed in paraformaldehyde and then used for fluorescence *in situ* hybridization by applying the following probes: EUB338MIX (Cy3 labelled) for detection of the overall bacterial community, GAM42a (Cy5

labelled) for sensing Gammaproteobacteria, ALF968 (Cy5 labelled) for visualization of, Alphaproteobacteria, BET42a (6-FAM labelled) for detection of Betaproteobacteria and LGC354MIX (FITC labeled) for visualization of Firmicutes. Stained samples were analyzed with a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with argon and helium/neon lasers.

## Isolation of oil pumpkin associated microorganisms

Dilution series with obtained suspensions from plant samples (as described above) were prepared and 0.1 mls of the dilutions were plated out onto R2A (for bacteria) and SNA (for fungi) medium. Agar plates were incubated at 20°C until colonies were countable that were then transferred onto LB (for bacteria) and PDA (for fungi) plates after assessment of CFUs.

In addition, seed borne microbial strains were obtained from aforementioned varieties by the isolation from roots, stems and leaves from plants that seeds were surface sterilized and grown under gnotobiotic conditions.

## Selection, characterization and identification of antagonists

The pathogens *Didymella bryoniae* A-220-2b, *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4 were provided by Herbert Huss (Bundesanstalt für alpenländische Landwirtschaft, Gumpenstein, Austria) and Athanassios Mavridis (University of Göttingen, Germany). Isolates (1,748 bacteria and 572 fungi) were characterized *in vitro* in dual culture assays. Broad spectrum antagonists that had the potential to suppress *D. bryoniae* as well as at least 2 bacterial phytopathogens of oil pumpkin were characterized genotypically by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and BOX PCR (Rademaker & de Bruijn, 1997). Selected isolates were identified by partial sequencing of 16S rRNA genes and BLAST analysis (Altschul *et al.*, 1997).

## Evaluation of broad-spectrum antagonists in a field trial

Seeds of the variety GL Opal were primed with suspensions of 5 selected broad spectrum antagonists separately. Additionally a control treatment was performed with 0.15 M NaCl. 16 seeds per treatment were sown on always three replicate plots that were organized in a completely randomized plot design. Health statuses of plants were monitored and furthermore harvest yields and 100 corn weights were evaluated.

## Statistical analysis

Band patterns from ARDRA and BOX-PCR gels were normalized and subjected to cluster analysis based on the unweighted pair group method using average linkages to the matrix of similarities obtained (UPGMA) using the Gel ComparII software (Version 5.1, Applied Maths). Analysis of Variance (ANOVA) in addition with Duncan's multiple range test (P<0.1) was performed with Predictive Analysis

SoftWare (PASW, Version 18.0.0) for evaluating data obtained from the field experiment.

## **Results and Discussion**

## Oil pumpkin associated community structures of Pseudomonas and Bacillus

To observe community structures of *Pseudomonas* and *Bacillus* from oil pumpkin, SSCP profiles were generated from specific plant organs (roots, flowers, fruit pulp). Investigated communities were strongly influenced by the microhabitat and by the plant stage. No significant effect on microbial communities by the oil pumpkin cultivar was noticed.

## FISH-CLSM analysis

The female oil pumpkin flower as an underexplored and a spatially very heterogeneous microhabitat offering various niches for bacterial colonization was analyzed in more detail by FISH-CLSM studies. Beta- and Gammaproteobacteria were observed on petals and pistils. Furthermore, pollen grains were mainly colonized by Gammaproteobacteria. This result suggest pollen acts as a dissemination vehicle for bacteria between plants and shape by this means the flower- and pumpkin-associated bacterial community of oil pumpkin.

## Selection, characterization and identification of broad-spectrum antagonists

Broad-spectrum antagonists, which have the potential to suppress *D. bryoniae* as well as at least two bacterial phytopathogens, were characterized genotypically by ARDRA. This resulted in a grouping of 43 bacterial isolates into four different genera: *Pseudomonas, Paenibacillus, Serratia* and *Lysobacter*. As a relative high number of isolates belonged to *Paenibacillus* and *Lysobacter* they were further analyzed by BOX PCR to get insight into the intraspecific diversities. Within the group of *Paenibacillus*, a negligible variability between BOX patterns was observed in contrast to strains of *Lysobacter* which were divided into five groups. Finally, five potential broad-spectrum antagonists were chosen for further analysis: one representative for *Pseudomonas, Paenibacillus* and *Serratia* and representatives for two *Lysobacter* clusters. Partial sequencing of 16S rRNA genes with subsequent BLAST analysis was performed for their identification, and the following species could be affiliated to respective strains: *Pseudomonas chlororaphis* P34, *Paenibacillus polymyxa* PB71, *Serratia plymuthica* S13, *Lysobacter antibioticus* L175 and *L. gummosus* L101.

## Evaluation of broad-spectrum antagonists ad planta

Selected broad-spectrum antagonists were tested in a field study for their antagonistic efficacy against *D. bryoniae* and their impact on plant health and growth of the Styrian oil pumpkin cultivar GL Opal. Germination rate was highest for *S. plymuthica* S13 (95.8%), and was even higher than seed emergence by a chemical stripper. Germination rates observed for treatments with *L. gummosus* L101 and *P.* 

*chlororaphis* P34 (91.7%) were significantly higher compared to the control treatment as well. As an important parameter for the production of pumpkin seed oil, harvest yields obtained from 100 seeds were compared that revealed significant highest values after treatment with *P. polymyxa* PB71. Due to an extensive infestation by mildew, degree of leaf area covered with the pathogen was assessed and compared between treatments: *P. polymyxa* PB71 and *L. gummosus* L101 suppressed this pathogenic pressure significantly.

In further field studies, the selection of bacterial strains will be evaluated as single or combined applications for the development of a biological strengthener for Styrian oil pumpkin.

#### Acknowledgements

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## Interactions between PGPRs and crops with special reference to chilli (*Capsicum annuum* L.)

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#### Abstract

Chilli (Capsicum annuum L.) is an indispensible spice crop and suffers from several pests and diseases. In recent years, Plant Growth Promoting Rhizobacteria (PGPR) have gained worldwide importance and acceptance for agricultural benefits. These microorganisms have the potential tools for disease control and growth promotion in various crops and patho-systems which involve multidisciplinary approaches to understand adaptation of PGPR to the rhizosphere, mechanisms of root colonization, effects on plant physiology and growth, bio-fertilization, induced systemic resistance, bio-control of plant pathogens, production of determinants etc. Considering the seriousness of pest and diseases in chilli, investigations were carried out to utilize indigenous PGPR isolates of Fluorescent Pseudomonads for ecofriendly management of diseases of chilli. These isolates of Fluorescent Pseudomonads were evaluated for bio-control activity and induction of systemic resistance in chilli. Isolates with high defence related enzymes (peroxidase, polyphenly oxidase, phenyl alanine lyase) and  $\beta$ -1,3-glucanase as well as production of antibiotics (phenazine), antimicrobial compounds (hydrogen cyanide) and salicylic acid were used in this study. Molecular characterization of antibiotic coding genes (DAPG) in Fluorescent Pseudomonads, their shelf-life and compatibility with other insecticides, fungicides and bio-agents were undertaken before its use as a component in Integrated Pest Management (IPM) trial. The IPM trial was conducted on farmers' field of 25 acres. The IPM strategies were followed by regular monitoring of pests and diseases throughout the chilli growing season. The results showed that the incidence of powdery mildew, leaf spot, wilt, die-back/anthracnose, as well as sucking pest and fruit borer was significantly reduced in IPM plot treated with PGPR compared to control. The number of pesticide sprays was significantly lower (30%) in IPM plots as compared to non IPM plot in adjacent region. Overall, the average yield in IPM plot was 31.46 q/ha compared to 24.60 q/ha in non IPM plot. In terms of monetary value, Rs. 2,03,243 (US \$ 4,418) per ha was recorded in IPM plot as against Rs. 1,48,581(US \$ 3,230) in non IPM plot, which is a net gain of Rs. 54,662 (US \$ 1,188) per ha.

## Introduction

India is the largest producer of chilli in the world contributing 25% to the world production. Many factors influence successful production and quality of chilli in which diseases and pests play a very important role. Chilli crop suffers from diseases such as damping-off, leaf spot, powdery mildew, anthracnose, die back, Fusarium wilt during growing season and aflatoxin contamination as a post harvest problem. Among the insect pests, sucking pest, defoliator and fruit borer play a crucial role. Farmers were solely dependent on pesticides and often the number of sprays exceed 25 to 30 under irrigated conditions which consequently increase the cost of production apart from extreme damage to eco-system. PGPR such as Fluorescent *Pseudomonads* play a crucial choice for its versatility and ability to control large number of plant pathogens in diverse target environments. Hence, we have focused biological and molecular characterization of indigenous on Fluorescent Pseudomonads, development of efficient formulation, shelf-life, compatibility to various pesticides and their efficacy at the field level using IPM strategies. In this paper we have presented a series of judicious and supervisory interventions using eco-friendly bioagents such as Fluorescent Pseudomonas and botanicals as well as other pesticides against diseases and pests of chilli.

## Material and Methods

Indigenous fluorescent *Pseudomonas* isolates were collected and characterized and then subjected to dual culture technique to determine the wide spectrum of plant pathogens. The selected indigenous PGPR isolate of Fluorescent *Pseudomonads* developed and formulated in talcum powder and that was used in IPM trial.

The genomic DNA was isolated and the molecular detection of DAPG gene was carried out using PCR reaction with specific primers. The amplification of 16s rDNA was carried out from the genomic DNA using specific primers. The procedure for isolation of DNA was followed as per Rosales *et al.* (1995). The siderophore production among *Pseudomonas fluorescens* isolates was determined according to the procedure given by Schwyn and Neilands (1987).

The superior performing *s* Fluorescens *Pseudomonas* isolates (Pf4 and RPf13) were used for the study of induction of systemic resistance in chilli crop and these were integrated in IPM schedule. The IPM trial was conducted on 25 acres of farmers' field for three consecutive seasons from  $2008 \sim 2010$  in Tungabhadra Project Area. The selected farmer beneficiaries from the region were provided with bio-inputs and other IPM interventions as and when necessary.

The team of entomologists and plant pathologists supervised the IPM demonstrations by regular visit and intervened judiciously with bio-agents, fungicides, insecticides and plant products wherever required. The observations on incidence of

diseases such as leaf spot, powdery mildew, anthracnose, wilt and rot and insect pests such as thrips, mites, borer, aphids and foliage and fruit damage.

## **Results and discussion**

A total of 92 isolates of *Pseudomonas fluorescens* were collected and evaluated for bio-control and growth promotion activity. Among them two most promising strains *Pseudomonas fluorescens* and *Pseudomonas putida* (Pf4 and RPf13) were identified on the basis of dual culture test and induction of systemic resistance. The isolates were tested against broad spectrum of pathogens such as *Macrophomina phaseolina*, *Rhizoctonia bataticola*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Colletotrichum gleosporioides*, *Fusarium solani*, *Sclerotium rolfsii*, *Cercospora capsici*, *Alternaria sesami*, and a bacterial pathogen, *Xanthomonas axonopodis* pv. *punicae*.

The PGPR isolates were evaluated for formulation and carrier material. Out of three carrier materials studied for survival, talc maintained the highest population of PGPR even after 365 days of storage. Vidyasekaran and Muthamilan (1995) observed the shelf life of talc based formulation up to eight months after storage. Talc being inert material having more surface area with neutral pH will help for the survival of *Pseudomonas fluorescens* than any other carrier material like farm yard manure (FYM) and vermicompost. Talc based formulation has been used and tested against fusarium wilt and *Alternaria* blight of sesame (Naik *et al.*, 2009). *Pseudomonas fluorescens* was compatible with carbendizm and thiram among fungicides, imidacholoroprid, choloropyriphos, carbofuron and endosulfan among insecticides , neem seed kernel extract (NSKE), garlic extract and nimbicidine among plant products. The compatibility of *Pseudomonas fluorescens* with imidacholoroprid, carbofuron and neem cake have been reported by Kumar *et al.*, (2008).

Among 92 fluorescent pseudomonads, six isolates produced higher siderophore production. Siderophores chelate with the ferric ion and serve as vehicle for transport of ferric ion into the cells (Neilland, 1981). The siderophore production has been determined by Sharma *et al.*,(2007) in Fluorescent *Pseudomonads*.

Among 92 isolates, phID gene was detected only in one isolate and the 16S rDNA of the isolate was sequenced and deposited in the NCBI Gen Bank Maryland, USA (Manjunath *et al.*, 2010). Detection of 2, 4, DAPG gene among the Fluorescent *Pseudomonads* is the first report from Tungabhadra project area (TBP) and Upper Krishna project areas (UKP) of cultivation. This particular investigation will go a long way in TBP and UKP areas since it has the potential for managing wide range of diseases and pests (Jay Kumar *et al.*, 2004). Vellu Swamy (2006) reported the detection of 2, 4, DAPG gene from Tamil Nadu, India. The potential isolate was used as one of the bio-control and PGPR input in the series of IPM interventions made in chilli fields to manage diseases and pests, during IPM demonstrations. Along with a series of IPM interventions made in farmers' field, a regular monitoring of pests and

diseases by team of plant pathologists and entomologist throughout the season was conducted for three years. The judicious use of pesticides, bio-agents, botanicals and other IPM inputs helped to reduce the indiscriminate use of pesticides significantly in the last three years.

In the IPM demonstration trial, the average per cent incidence of leaf spot, powdery mildew, and fruit rot/wilt and also aflatoxin contamination came down from 26.16 to 8.95, 17.25 to 15.95, 14.32 to 9.65, and 17.91 to 11.89 respectively in the three years trials conducted (Table 1). The damage caused by insect pests such as sucking pests, defoliators and fruit borer also came down (Table 2) significantly. As a result, the number of pesticides sprays and the cost of protection was reduced significantly by 25% to 30% with an average net profit of Rs 2, 03,243/- per ha in IPM plot as against Non-IPM plot with a net profit of Rs 1,48,581/- (US \$ 3,230) per ha which is a (US \$ 4,418) gain of Rs.54,6620/- (US \$ 1,188) per ha (Table 3). In addition, untold ecological damage which is inevitable in Non-IPM practice, as a result of indiscriminate use of pesticides was minimized in IPM practice. There is need to expand the IPM practices with bio-agent and PGPR as major input for realizing India's vision of 2020 for sustainable food and nutritional security.

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#### Table 1 The Incidence of various diseases of chilli in IPM plots compared to

Sl.No	Diseases	Range of incidence			
		IPM (%)	Non –IPM (%)		
1	Leaf Spot	08.95~26.16	10.62~36.73		
2	Powdery Mildew	15.95~17.25	18.58~28.00		
3	Fruit Rot	09.65~14.32	16.69~17.48		
4	Wilt	11.89 ~17.91	13.40~26.33		

#### Non-IPM plots during 2008-2011

## Table 2 Incidence of insect Pests of chilli in IPM plots compared

Sl.No	Insect Pests	Range of incidence	
		IPM	Non IPM
1	Thrips/ Leaf	1.77~2.20	2.59~3.18
2	Mites/ Plant	1.16~1.61	1.79~2.06
3	Aphids/ Plant	$0.00 \sim 0.00$	0.41~1.81
4	S. litura	0.35~0.73	0.62~1.50
5	H. armigera	0.29~0.41	0.63~0.93
6	Foliage damage (%)	2.29~2.66	5.89~5.73
7	Fruit damage (%)	1.63~1.92	5.17~7.65

#### to non IPM plots during 2008-2011

## Table 3 Economics of chilli production in IPM and Non IPM plots during2008-2011

			Cost of cultivation	on (Rs/ha)		
Location	Dry chilli yield* (qt/ha)	Total income (Rs/ha)	Production cost	Protection cost	Total cost (Rs/ha)	Net Profit (Rs/ha)
Nelahal						
IPM plots	31.26	236797	20950/-	12604/-	33554/-	203243/-
Non IPM plots	24.6	186941	18950/-	16410/-	38360/-	148581/-

## Effects of plant growth-promoting rhizobacteria on

## controlling tobacco mosaic virus

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## Abstract

Viral disease is an important factor limiting the tobacco leaf production in China. In this study, we have screened plant growth-promoting rhizobacteria (PGPR) isolates with antagonistic activities to a broad spectrum of plant pathogenic fungi and then evaluated their growth-promoting effects on tobacco and inhibitory effect to tobacco mosaic virus (TMV). There were 190 bacterial isolates from 80 soil samples were obtained and then tested their antagonistic activities to Fusarium sp., Alternaria alternate. Exserohilum turcicum, Verticillium dahliae, Cytospora mali. Trichothecium roseum, Rhizoctonia solani, Colletotrichum sp. using plate bioassays. Twenty isolates showed significant inhibitory activity to the tested fungi and isolates YHN and YHL showed the best effects. According to the morphological, physiochemical and molecular characteristics, isolates YHL and YHN were identified as Paenibacillus polymyxa and Bacillus amyloliquefaciens, respectively.

Tobacco seeds were dipped in YHL and YHN at a concentration of 10<sup>7</sup>cfu/ml and incubated in Petri dishes at room temperature. Twenty days later, the averaged tobacco root lengths increased by 26.88% with YHL and 21.97% with YHN over control. The averaged shoot lengths increased by 33.41% with YHL and 41.40% with YHN over control. When four to five-leaf stage tobacco seedlings were sprayed with YHL and YHN at the same concentration for three times at 5-day interval in greenhouse, the fresh weights increased by 39.17% and 30.08%, respectively, over the control 30 days after the final spray.

Tobacco leaves of *Nicotiana tabacum* cv. Samsun were inoculated with TMV after spayed with YHL and YHN. The number of local lesions induced by TMV of untreated Samsun was  $30.4 \pm 4.5$  per leaf, while those of treated with YHL and YHN were  $18.1 \pm 1.1$  and  $17.3 \pm 3.6$ , respectively, which mean that the inhibition rate were reduced by 40.46% and 43.09%, respectively. In field plot experiment, the tobacco plants were sprayed with YHL and YHN at a concentration of  $10^7$  cfu/ml for three times at 10days interval from the first week after transplanting. The relative control

efficiency of treatment with YHL and YHN were 28.37% and 24.80%, respectively, at 70 days after final spray. The dry weights of tobacco leaves increased by 10.42% (YHL) and 8.13% (YHN), respectively. These data suggest that the two bacterial isolates have a great potential in promoting tobacco leave production.

# *In vitro* studies on efficacy of *Pseudomonas* spp. for plant growth promoting traits and biocontrol of diseases in tea plants

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#### Abstract

Tea is one of the major non-alcoholic beverages in the world. Fungal pathogens are important factor limiting the productivity and quality of tea. There is a major environmental concern in the use of fungicides as they increase toxic level in the tea soil. Plant growth-promoting rhizobacteria (PGPR) are precious bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR will be alternative to manage several fungal diseases that occur in tea plants to replace chemical fertilizers. In our present study, a total of 250 bacterial isolates were obtained from different agroclimatic zone of southern Indian tea rhizospheric soils by using King's B medium. Out of which nine strains belonging to Pseudomonas fluorescens was selected for further studies based on various morphological, biochemical and physiological screening methods. In vitro screening was done in search of efficient Pseudomonas fluorescens strain for their plant growth promoting traits and antagonistic activities against tea pathogens such as *Cercospora theae*, Phomopsis theae and Poria hypolateritia. The result indicated that VP5 exhibited innate potential of plant growth promotion and biocontrol activities in vitro. Also all the isolates were capable of producing phytohormones and other PGP traits. The present study confirmed the presence of various antifungal metabolites in the bacteria which are involved in the growth inhibition of fungal tea pathogen. However field application of this biocontrol agent as soil inoculant in tea plants is required to confirm its real efficacy.

## Key words: Pseudomonas fluorescens; PGP traits; Cercospora theae; Phomopsis theae; Poria hypolateritia

## Introduction

Bacteria that colonize the rhizosphere and plant roots and enhance plant growth by any mechanism are referred to as plant growth-promoting rhizobacteria (PGPR). PGPR have been applied to various crops to enhance growth, seed emergence and crop yield and some have been commercialized (Herman *et al.*, 2008). PGPR have shown positive effects in plants on such parameters as germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Kloepper *et al.*, 2004). Production of indole acetic acid (IAA) by *Pseudomonas* and its role in the development of root system is also evidenced (Patten and Glick, 2002). For many *Pseudomonads*, production of metabolites such as antibiotics, siderophores and hydrogen cyanide (HCN) is the primary mechanism of biocontrol (Weller and Thomashow, 1993).

Tea is the most popular and inexpensive beverage produced from the young shoots of commercially cultivated tea plant (Camellia sinensis (L) O.Kuntze). Tea gardens of South India are spread in the Western Ghats in the three states of Karnataka, Kerala and Tamil Nadu. Tea is a perennial woody plant having a single main stem from which numerous branches are developed to a crown of leaves to get a bushy appearance. Perennial habit of tea plant, particularly cultural conditions and warm humid climate of the tea growing areas are highly conducive for disease development. There are many prominent diseases which are caused by fungi. In a recent report on tea diseases, Chen and Chen (1990) described nearly 400 pathogens. Phytopathogens causes more economic losses in tea plantation crops. The most effective means of managing phytopathogens are chemical means of fungicides which are already in use. Several such products have been found implicated in contamination of ground water, soils and food products. Currently at present, the most effective means are biological methods found to be efficient, protective and biosafety in the area of economically important plantation crops. In tea, plant growth activities are affected by leaf, stem and root disease causing soil borne fungal pathogens, which results in yield loss (Sharma, 1960). Soil drenching with systemic fungicides are found to be effective in controlling a few stem diseases (Ponmurugan and Baby, 2005; Chandramouli and Baby, 2002). Soil drenching of fungicides leads to deleterious effect on beneficial micro-organism, especially plant growth promoting rhizobacteria. Biological control of managing leaf, stem and root disease is a viable alternative.

Therefore, isolation and identification of beneficial rhizobacteria from tea soil is going to be effective means of eradicating disease causing pathogen in case of tea plants growing areas. A major group of predominant common rhizobacteria namely *Pseudomonas* spp. has been found to be beneficial bacteria for economically important plantation crops such as rice, cotton, sugarcane, turmeric, lemon, chilli, etc. In this present scenario soil samples were collected in different tea growing areas of southern India. In our current study this symbiotic bacteria was screened for biocontrol agent by eliciting both plant growth promoting trait and antagonistic activity in tea plants.

### Materials and methods

### Collection of soil samples

Soil samples were collected from tea growing areas of southern India such as Tamil Nadu, Kerala and Karnataka. These samples were allowed to air dry at room temperature and various parameters like soil pH, total organic carbon (Walkley and Black, 1934), total nitrogen (AOAC, 1990), available phosphorous (Jackson, 1973) and exchangeable potassium (Murphy and Riley, 1962). About 1gram of soil was taken from each place and subjected to serial dilution and plated on King's B specific medium to isolate PGPR strain namely *Pseudomonas fluorescens*.

#### Morphological and biochemical characterization of isolates

The various morphological parameters such as colony appearance, colony shape and cell shape using Gram's reaction were determined by using King's B agar medium to identify the isolated bacterial strain. Biochemical test such as IMVIC test, Catalase test, Oxidase test, Triple sugar iron test, Urease test etc performed using standard methods (Cappucino and Sherman, 1992). The colony morphology and cell size were recorded periodically. Nutrient factors such as carbon sources and nitrogen sources on growth of *Pseudomonas* spp. were studied.

### Screening of bacterial isolate for their plant growth promoting (PGP) activities

## Assay for IAA and GA<sub>3</sub>

IAA production was detected by following the modified method of Brick et al. (1991). Quantitative analysis of IAA production by P. fluorescens was performed by the method of Gupta et al. (1999) with slight modifications. Bacterial cultures were grown at 100 µg/ml concentrations of tryptophan for 48h in King's B broth at 28±2°C. 5 ml of each bacterial culture were centrifuged at 7,000 rpm  $(2191 \times g)$  for 15 min at room temperature. The supernatant was collected and finally passed through the millipore filter of 0.2  $\mu$  pore size. The supernant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the salkowski reagent (50 ml of 35% of perchloric acid and 1ml of 0.5M FeCl<sub>3</sub> solution). Development of pink colour indicated IAA Optical density was measured at 530nm by using UV-Vis production. spectrophotometer (Hitachi, Japan). Concentration of IAA produced by cultures was measured with the help of standard standard graph of IAA (Himedia) obtained in the range of  $10 \sim 100 \ \mu g/ml$  and expressed as  $\mu g$  per ml. For extraction of Gibberellic acid (GA<sub>3</sub>), culture media was filtered for elimination of bacterial biomass, then the pH value of supernant was adjusted to 2.5 using stock (37%) HCl. Acidified supernant was extracted three times with ethylacetate (1:3 medium solvent ratio) by following the method of Cho et al. (1979). Aqueous phase of all three stages was discarded and solvent portion was pooled, dried and residue collected for estimation of GA<sub>3</sub>. In this method, GA<sub>3</sub> is converted to gibberellenic acid, which was detected at 254 nm by using UV spectrophotometer.
# Ammonia production

Bacterial isolates were screened for the production of ammonia in peptone water. Freshly grown culture were inoculated in 10 ml peptone water in each tube and incubated for  $48 \sim 72$ h at  $28 \pm 2$ °C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive for ammonia production (Cappucino and Sherman, 1992).

# Hydrogen cyanide (HCN) production

Production of HCN was determined by the modified method of Miller and Higgins (1970). *Pseudomonas* isolates were inoculated into 250 ml flasks containing nutrient broth and were incubated at  $32 \pm 1$  °C. These isolates were separately streaked on nutrient agar medium supplemented with 4.4 g glycine/L with simultaneous addition of whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate and the Petri plates were sealed with Parafilm. After incubation for  $2\sim3$  days at  $32 \pm 1$ °C, change in colour was observed. Development of orange to red colour indicated HCN production.

# Phosphate solubilization

All isolates were screened on Pikovskaya's agar plates for phosphate solubilization as described by Gaur (1990). Pikovskaya broth was prepared (Pikovskaya, 1948), inoculated with *Pseudomonas* isolates separately and incubated at  $28 \sim 30^{\circ}$ C on a shaker for  $3 \sim 4$  days. Test bacterium was centrifuged; 1 ml aliquot from supernatant was taken and mixed with 10 ml of chloromolybdic acid. After shaking, it was diluted with 0.25 ml chlorostannous acid and final volume was made up to 50 ml. Intensity of blue colour was read at 600 nm. Standard curve was prepared with KH<sub>2</sub>PO<sub>4</sub> to find out the amount of solubilized phosphorus in µg per ml (Saxena *et al.*, 2002).

# Siderophore production

Siderophore production was determined by using Chromeazurol (CAS) agar assay (Schwyn and Neilands, 1987) and was modified by Silva-Stenico *et al.* (2005). Comparative account of siderophore production was done in terms of percent siderophore units. Cell free supernatant was subjected to estimation of siderophore as per Chrome Azurol S (CAS) liquid assay (Payne, 1994). Briefly, 1 ml of culture supernatant was mixed with 1 ml of CAS assay solution. Uninoculated medium was used as reference. The optical density (OD) at 630 nm was measured for loss of blue colour resulting from siderophore production.

#### Salycylic acid production

The King's B broth cultures were centrifuged (8,000 rpm for 10 min). Cell free supernatant was subjected to estimation of salycylic acid (SA) (Visca *et al.*, 1993). The SA was extracted from the acidified culture supernant. The filtrate was adjusted to pH 2 with 1M HCl and extracted twice with double volume of ethyl acetate. To 1 ml of the extraction, 2 ml of 2M FeCl<sub>3</sub> and 1 ml of distilled water were added. The SA reacts with 2 M FeCl<sub>3</sub> to form a purple iron + SA complex in the aqueous phase with a maximum absorbance at 527 nm using UV spectrophotometer. The SA concentration in extraction was determined using a calibration curve of standard SA.

# Dual culture technique

The strains were tested for antifungal activity against tea pathogens by dual culture technique (Rabindran and Vidyasekaran, 1996). *P.fluorescens* were streaked at one side of Petri dish (1 cm away from the edge) containing PDA medium. A 5 mm mycelial disc from seven days old tea pathogens such as *Cercospora theae*, *Phomopsis theae* and *Poria hypolateritia* were placed on the opposite side in the Petri dish perpendicular to the bacterial streak and plates were incubated at room temperature ( $28 \pm 20^{\circ}$ C) for  $3 \sim 7$  days. At the end of incubation period, the zone of inhibition was recorded by measuring the distance between the edges of the fungal mycelium and the antagonistic bacterium. Plates inoculated with fungus only served as control. Three replications were maintained for each isolate.

# Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and the significant means were segregated by critical difference (CD) at various levels of significance (Gomez and Gomez, 1984).

#### Results

# Population density of Pseudomonas fluorescens and nutrient status of southern Indian tea soils

The population density of *P.fluorescens* in different tea soils of southern India are presented in the Table 1. It was observed that there was a significant difference on the population density in which it was found to be in the range of  $3.3 \sim 8.7$  cfu/g soil dry wt. It was found to be highest (8.7) in the soil samples of Coonoor and least in Coorg (3.3) areas. The population density was generally found to be more in Tamil Nadu followed by Kerala and least in Karnataka state. It was positively coincided with the result of soil nutrient factors such as soil pH, organic carbon, total nitrogen, available phosphorous and exchangeable potassium. The total nitrogen content was recorded in the range of 3715 and 3887 mg/kg followed by phosphorus (15.6 $\sim$ 17.7 mg/kg) and potassium (54.0 $\sim$ 67.3 mg/kg). A total of 250 isolates of *P.fluorescens* were obtained

from the soil samples from which only one strain from each agroclimatic zone was selected for further studies based on various morphological, biochemical and physiological screening methods. The strains were designated based on name of the agroclimatic zone and tea estate field numbers.

# Isolation and biochemical characterization of different strains of P.fluorescens obtained from southern Indian tea soils

The tea rhizospheric soil samples were collected from different agroclimatic zones from which different strains of *P.fluorescens* were isolated using King's B medium by following serial dilution techniques (King et al., 1954). All the strains were fluorescenced under UV light at 260 nm wavelength. The fluorescent Pseudomonas spp. showed a positive reaction for catalase, oxidase, citrate, starch and gelatin hydrolysis tests. Further all the isolates showed strictly a negative reaction for Gram's staining, indole, methyl red, voges proskauer, triple sugar iron test and hydrogen sulphide production (Table 2). Cell size, morphology and colony appearance of strain were assessed as described previously (Kotouc Kova *et al.*, 2004). The cell size range from  $3.5 \sim 1 \,\mu\text{m}$  and  $0.4 \sim 0.2 \,\mu\text{m}$ , appearance of colony range from  $1 \sim 5 \,\text{mm}$  in diameter. In King's B medium the colonies of all strains were observed circular and smooth. Effect of different carbon and nitrogen sources were tested to find out the optimum growth of *P.fluorescens*. Among the carbon sources tested, glycerol followed by glucose, peptone and yeast extract were found to be the best. On the basis of cultural, morphological and biochemical characteristics, all the soil isolates grouped into Pseudomonas spp.

# Production of phytohormone by various fluorescent Pseudomonas

A great variation was observed in the IAA and GA<sub>3</sub> production by *P.fluorescens* tested. All the inoculated strain of *P.fluorescens* were able to produce phytohormone namely IAA and GA<sub>3</sub> wherein the production was varied from 5.4 to 13.3 and 7.0 to 25.3  $\mu$ g/ml of IAA and GA<sub>3</sub> respectively by the strains Table 3. There was an increase in the level of IAA with the increasing concentration of L-tryptophan (500~2500  $\mu$ g/ml). The data were not shown. Similar trend of IAA production with the increasing concentration of the respective precursors was also reported by Barazani and Friedman (2000). The above data in Table suggeted that these strains may have field application in tea rhizosphere and also the intrinstic ability of bacteria to produce growth hormone in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant (Arshad and Franken Berger, 1993). The maximium gibberellic acid production was obtained after 72 h of incubation. previously it was demonstrated that *Pseudomonas* spp. can be used a potent bacterial source to obtain high yields and provide a good alternative for gibberellic acid biosynthesis (Safak and Nilufer, 2005).

#### In vitro screening of bacterial strains for plant growth promoting traits

The yellowish green pigment was observed when the strains of *P.fluorescens* were grown on King's B medium which indicated the presence of siderophore production. When the strains were grown in CAS liquid medium, VP5 strain produced 50.8% of siderophore units when compared to other strains. Moreover, the strains isolated from Tamil Nadu tea soils were produced more amount of siderophores in the range of  $41.2\% \sim 50.8\%$  than the strain obtained from Kerala and Karnataka states tea plantations. Bano and Musaurat, 2003 reported that P. aeruginosa NJ15 strain produced hydroxamate type of siderophore which had good activity. Screening of phosphate solubilization was carried out to identify the capacity to solubilize insoluble rock phosphate into soluble form. The phosphate solubilization results under liquid assay indicated that CGP1 strain efficiently solubilize 82.70 µg/ml followed by VP5 and MPP2 strains which accounted 78.43 µg/ml and 76.00 µg/ml (Table 4). The selection of phosphate solubilizing *Pseudomonas* strains is considered as possible inoculation tools for phosphate deficient soils may focus on the integral interpretation of laboratory assays, green house experiments as well as field trials (Frankem Henri, 2008). Similarly this was previously reported that *Pseudomonas* spp. have high potential to solubilize phosphorus which in turn useful to supply soluble form of phosphate to plants (Rodriguez and Fraga, 1999).

Nearly 90% of *P.fluorescens* were able to produce hydrogen cyanide (HCN). In our study, all the *Pseudomonas* isolates showed a positive response upon HCN production in which two types of colours such as reddish orange and brick red were recorded. Several studies have demonstrated that production of siderophore and other bioactive secondary metabolites including lytic enzymes by *P.fluorescens* was found to be most effective in controlling many plant root pathogens (Thomashow *et al.*, 1990). Similarly all the strains of *Pseudomonas fluorescens* were able to produce salycylic acid under *in vitro* condition. Among the different strain, MP7 strain produced (18.0  $\mu$ g/ml) followed by VPP3 (16.7  $\mu$ g/ml) and VP5 (16.3) strains. It has been reported that salycylic acid has been found synthesized in the culture supernants of *P. aeruginosa* and *P. capacia* (Viska *et al.*, 1993).

### Antifungal activity of Pseudomonas isolates

Among the different *P.fluorescens* tested for biocontrol activities, the maximum growth inhibition was recorded by VP5 strain followed by GP4 and CP2 strains. In the case of *Cercospora theae*, the growth inhibition over control was 47% by VP5 strain where the linear growth of this pathogen on 12<sup>th</sup> day was 24.0 mm. In *Phomopsis theae*, the inhibition of colony growth was 26% by the same strain where the linear growth on 7<sup>th</sup> day was 33.7 mm (Table5). On the other hand, growth inhibition of *Poria hypolateritia* due to *Pseudomonas* strains was found to be higher followed by *Cercospora theae* and *Phomopsis theae*. It may be due to sensitivity and tolerance nature of the tea pathogens. Among the several *Pseudomonas* strains tested

for biocontrol activity VP5 strain showed the maximum antibiotic potential to control tea pathogens. According to Demain and Fancy (1995) the most widely accepted theory is that antibiotics are used to compete with other organisms in nutrient depleting environment. The growth inhibition of fungus is due to antifungal and antibiotic properties of *P.fluorescens*. Moreover, *Pseudomonas* strains are generally able to secrete hydrolytic enzyme, siderophore and hydrogen cyanide production.

# Discussion

The population density of *P.fluorescens* as well as soil nutrient factors were least in Karnataka tea soils due to low elevated hills with high influencing environmental factors (Sanjay *et al.*, 2008). In general the total rainfall is always least in Karnataka tea plantation districts which coincided with high temperature and low relative humidity. Further the result is coincided with the report of Baby *et al.* (2002) and Ponmurugan *et al.* (2007) wherein beneficial microorganisms and actinomycetes diversity was positively correlated with the nutrient factors and environmental factors of tea plantations. This was similarly reported in *Azospirillum* and phosphobacteria diversity in southern Indian tea soils by Baby *at al.* (2002). The variation in the population of *P.fluorescens* might be attributed to many soil factors such as pH, temperature, moisture content, water holding capacity, organic carbon, total nitrogen, available phosphorous and exchangeable potassium elements. The selected isolates were biochemically characterized into *Pseudomonas* spp. as per the standard method (Cappuccino and Sherman, 1992).

It has been reported that IAA production by PGPR can vary among different species and strains and it is also influenced by culture condition, growth stage and substrate availability (Mirza *et al.*, 2001). Production of plant growth hormones particularly IAA has long been considered as an important attribute of PGPR strains (Patten and Glick, 1996). At present, GA<sub>3</sub> is produced using the fungus *G. Fujikuroi* on an industrial scale. A comparison of *Pseudomonas* spp. and *G. Fujikuroi* for GA<sub>3</sub> production shows best yields of GA after 12 or 18 days of incubation. It is observed that *Pseudomonas* spp. has a shorter incubation period and simple cultural conditions compared to *G. fujikuroi* for GA<sub>3</sub> production (Safak and Nilufer, 2006).

*Pseudomonas fluorescens* is one of the fluorescent pseudomonads that secrete siderophores called pyoverdins which assist in sensing and eventual uptake of iron from the medium. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to plants that represent a possible mechanism of plant growth promotion under field conditions (Verma *et al.*, 2001). Meyer and Hofte (1997) stated that some plant growth promoting bacteria (PGPB) do trigger a salycylic acid dependent signalling pathway by producing small amount of salycylic acid in the rhizosphere. The present study indicated that all the strains were salycylic acid producing organism which have the merits to explore for defense mechanism during host pathogen interactions in tea plantations. Based on these results obtained

from the study, it can be inferred that *P.fluorescens* can be used as a soil inoculant to prevent the growth of tea pathogens as well as supress the soil-borne pathogenic microrganism. Moreover the plant metabolism may be improved by absorbing phytohormone secreted by these bioinoculant.

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Name of agroclimati c zone	Design ation of strains	Population Density#	Soil pH	Organic carbon (%)	Total nitrogen (mg/kg)	Available phosphorus (mg/kg)	Exchangeable potassium (mg/kg)
Valparai <sup>*</sup>	VP5	8.4	4.8	3.5	3825	15.6	67.3
Coonoor <sup>*</sup>	CP2	8.7	5.0	3.3	3887	16.2	54.0
Gudalur <sup>*</sup>	GP4	8.3	4.6	3.4	3715	17.7	54.7
Munnar <sup>**</sup>	MP7	5.2	4.5	3.7	3408	15.3	51.3
Vandiperiy ar <sup>**</sup>	VPP3	4.9	4.7	2.7	2689	13.4	53.7
Meppadi <sup>**</sup>	MPP2	5.5	4.9	3.6	3071	16.3	67.3
Coorg <sup>***</sup>	CGP1	3.3	5.2	2.3	2918	12.6	43.3
Koppa <sup>***</sup>	KP6	4.9	5.1	2.7	2945	12.8	45.0
Chickmagal ur <sup>***</sup>	CMP3	4.7	4.8	2.8	2837	13.2	45.7
SE± CD at P=	0.05	0.66 1.24	0.42 0.85	0.03 0.50	35.47 48.48	1.28 2.44	5.38 8.36

# Table 1 Enumeration of population density of Pseudomonas fluorescens and analysis of edaphic parameters in southern Indian tea soils

\* located at Tamil Nadu, \*\* located at Kerala, \*\*\* located at Karnataka # cfuX10<sup>7</sup> cfu/g soil dry wt

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Biochemical	Strains of <i>P.fluorescens</i>								
tests	VP5	CP2	GP4	MP7	VPP3	MPP2	CGP1	KP6	CMP 3
Gram's staining	-	-	-	-	-	-	-	-	-
Fluorescence In UV	++	++	+	++	+	+	+	+	+
Indole test	-	-	-	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-	-	-	-
Voges proskauer Test	-	-	-	-	-	-	-	-	-
Catalase test	++	+	+	++	+	+	+	++	+
Oxidase test	++	++	+	++	+	+	++	++	+
Citrate test	++	++	+	++	++	++	++	++	++
Urease test	++	+	-	++	+	+	+	-	+
Triple sugar iron Test	-	-	-	-	-	-	-	-	-
H <sub>2</sub> S production	-	-	-	-	-	-	-	-	-
Nitrate reduction	++	++	-	+	++	++	-	+	-
Starch hydrolysis	++	+	+	++	+	+	++	+	+
Gelatin hydrolysis	++	+	+	++	++	+	++	++	++
++ Hi	+ Medium reaction, - Negative reaction					ction			

Strains of <i>P.fluorescens</i>	IAA production (µg/ml)	GA <sub>3</sub> production (µg/ml)
VP5	9.3	13.7
CP2	8.7	10.7
GP4	8.7	11.3
MP7	13.3	08.3
VPP3	5.7	11.7
MPP2	8.3	07.0
CGP1	7.4	14.3
KP6	5.4	25.3
CMP3	7.8	09.0
SE± CD at P=0.05	2.85 3.34	02.47 03.89

Table 3 Quantitative assay of IAA and GA<sub>3</sub> production by selected isolates

Table 4 Plant growth promoting activities of *Pseudomonas* isolates

P.fluorescens strains	Siderophore production (%)	Phosphate solubilization (µg/ml)	Hydrogen cyanide production	Ammonia production	Salicylic acid production (µg/ml)
VP5	50.8	78.43	RO	+	16.3
CP2	41.4	74.50	BR	++	13.0
GP4	41.2	70.37	BR	+	8.7
MP7	41.1	66.50	RO	++	18.0
VPP3	29.1	64.27	BR	+	16.7
MPP2	34.0	76.00	BR	++	15.3
CGP1	38.9	82.70	RO	++	14.7
KP6	27.7	74.63	RO	+	11.0
CMP3	20.7	68.00	BR	++	13.3
SE± CD at P=0.05	8.38 12.44	8.94 13.16	-	-	2.48 4.16

RO Reddish orange, BR Brick red, ++ High, + low

	Cercopso	ra theae <sup>*</sup>	Phomops	is theae <sup>**</sup>	Poria hypolateritia <sup>***</sup>		
Pseudomonas strains	Linear growth on 12 <sup>th</sup> day (mm)	Inhibition over control (%)	Linear growth on 7 <sup>th</sup> day (mm)	Inhibition over control (%)	Linear growth on 21 <sup>st</sup> day (mm)	Inhibition over control (%)	
VP5	24.0	47	33.3	26	15.0	67	
CP2	25.3	44	34.6	25	20.7	60	
GP4	24.7	45	33.7	26	16.2	64	
MP7	35.3	21	37.0	18	20.3	55	
VPP 3	31.0	32	36.0	20	29.7	34	
MPP 2	36.0	20	35.7	21	25.3	44	
CGP 1	28.7	36	35.0	22	21.0	43	
KP 6	30.7	32	34.3	24	16.7	43	
CMP 3	30.7	32	39.7	15	31.7	30	
Control	45.0	_	45.0	-	45.0	-	
SE± CD at P=0.05	4.34 9.08	-	6.37 12.34	-	6.78 11.66	-	

 Table 7 Effect of antibiotic potential of *P.fluorescens* on the growth of tea pathogens

\* Leaf pathogen, \*\*

\*\* Stem pathogen,

\*\*\* Root pathogen

# Rapid assessment of the antagonistic potential of *Bacillus* strains against the infection with *Phytophthora capsici*

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# Abstract

Evaluation of antagonistic Bacillus strains against phytopathogens is a timeconsuming and laborious work. Here we report a rapid method to assess the antagonistic potential of Bacillus strains by analysis of their biofilm formation and lipopeptide encoding genes. Of 93 Bacillus strains tested, 48 strains had ability to form biofilm on polypropylene tube walls, and were subjected to PCR amplification as the DNA sources by use of the primers specific for lipopeptide encoding genes (srfAA, srfAB, ituC and fenD). The antagonistic ability of the strains forming biofilm and/or harboring lipopeptide coding genes against the infection with Phytophthora capsici was evaluated by radicle assays on agar plates. Fifteen strains had great antagonistic efficacy of above 50%, of which 8 strains varying in biofilm formation, antagonism and lipopeptide encoding genes were further tested in the greenhouse. Our results indicated that the high-level biofilm formers (4 strains) harboring srfAA, srfAB and fenD that showed significant antagonism on plates had control efficacy of above 50% in greenhouse. The efficacy of middle-level biofilm formers (2 strains) harboring srfAA, srfAB, ituC and fenD in greenhouse was similar with that on plates. By combining the analysis of biofilm formation, lipopeptide coding genes and radicle assays on agar plates, this method was revealed to be rapid and efficient to screen *Bacillus* strains against phytophthora disease in pepper.

Key words: biofilm; lipopeptide encoding genes; phytophthora disease; Bacillus sp.

# Introduction

Evaluation of antagonistic *Bacillus* strains against phytopathogens is a timeconsuming and laborious work. Generally antagonistic *Bacillus* strains are selected based on the production of antibiotics. Till now, more than 12 kinds of lipopeptide antibiotics have been found to be synthesized by *Bacillus*. Among them, iturin, surfactin and fengycin are well studied (Nagórska *et al.*, 2007). Recently, the specific primers to amplify genes encoding three lipopeptide antibiotics were designed (Joshi & Gardener, 2006), and have been used for rapid screening of antagonists *in vitro*  (Giacomodonato *et al.*, 2001; Ramarathnam *et al.*, 2007). However, colonization of antagonists in the rhizosphere is the first and important step to suppress soil-borne pathogenic fungi. Recent research revealed that surfactin played an important role in bacteria colonization and inhibition against infection by phytopathogenic bacteria, while iturin and fengycin strongly inhibited hyphae growth of pathogenic fungi (Ongena & Jacques, 2008). In this study, we presented a method to assess the antagonistic potential of *Bacillus* strains by analysis of biofilm formation, lipopeptide encoding genes and antagonism on agar plates. Further greenhouse assay confirmed that this method was rapid and effective to select antagonistic *Bacillus* strains against phytophthora disease in pepper.

#### **Materials and Methods**

Ninety-three *Bacillus* strains isolated from the rhizosphere of soybean and vegetable and conserved in our laboratory were selected for the experiment. Biofilm formation of these strains on polypropylene tube walls were assessed according to the method described by Fall *et al.* (2004), and the 5-level of index was used as follows: 0, no purple color on PP tube walls; (1) faint purple film; (2) obvious purple film; (3) clear and circle purple film; (4) clear and thick circle purple color.

Those strains which could form biofilm on polypropylene tube walls were further subjected to PCR amplification as the DNA sources by use of the primers specific for lipopeptide encoding genes (*srfAA*, *srfAB*, *ituC* and *fenD*) (Joshi & Gardener, 2006). *Bacillus* sp. B006 and B010 were used as positive reference (Guo *et al.*, 2010). PCR reactions were carried out with a thermocycler (PTC-200; MJ Research,Waltham, MA, USA) under the following conditions: 5 min at 95 °C, 1 min at 94 °C, 33 cycles of 45 s at 62 °C, 2 min at 70 °C and one final step of 8 min at 70 °C. The PCR products were analyzed by electrophoresis on 1% agarose gel.

Antagonism of 48 biofilm producing strains against the infection with *Phytophthora capsici* was evaluated by pepper radicle assays on agar plates (Kim *et al.*, 2008). The strains varying in biofilm formation, lipopeptide encoding genes and antagonism assay were further tested in the greenhouse to evaluate their biocontrol efficacy. Bacteria were incubated in nutrient broth at 30°C for 12 h with constant agitation at 180 rpm. Cells were collected by centrifugation at 10,000 rpm for 2 min, and suspended in 0.1% MgSO<sub>4</sub> to OD600 of 0.5 ( $10^8 \text{ ml}^{-1}$ ). Pepper seeds were sterilized with 1% NaClO, and seeded into autoclaved vermiculite. Pepper seedlings with 4~6 leaves were dipped in bacterial suspension for 5 min, and transplanted to the soil infested with *P. capsici* at the density of 4×10<sup>3</sup> spore ml<sup>-1</sup>. The seedlings treated with 0.1% MgSO<sub>4</sub> were taken as controls. Each treatment had 6 replicates, and each replicate had 5 seedlings. The greenhouse tests were conducted during the summer ( $30\sim34^{\circ}$ C) and autumn ( $26\sim30^{\circ}$ C). The disease index was determined at  $7\sim10$  days after transplantation, and was analyzed by SPSS 10.0.

# Results

Of 93 *Bacillus* strains tested, 48 strains had ability to form biofilm on polypropylene tube wall, 5 strains at level 4, 16 at level 3, 21 at level 2 and 6 at level 1, respectively.

The 48 strains were subjected to PCR amplification specific for lipopeptide encoding genes (*srfAA*, *srfAB*, *ituC* and *fenD*) and antagonism assay against *P*. *capsici*. Fifteen strains had great antagonistic efficacy of above 50%, of which 4 strains harbored 4 tested lipopeptide encoding genes (*srfAA*, *srfAB*, *ituC* and *fenD*) and 7 strains had 3 lipopeptide encoding genes (*srfAA*, *srfAB* and *fenD*), respectively. Those strains only harboring *ituC* and *fenD* genes or only harboring *ituC* gene had no ability to suppress *P. capsici* (Table 1). This result indicated that biofilm formation played an important role in bacterial antagonism.

Eight strains varying in biofilm formation, antagonism and lipopeptide encoding genes were further tested in the greenhouse. The results indicated that the high-level biofilm formers (4 strains) harboring *srfAA*, *srfAB* and *fenD* that showed significant antagonism on plates had control efficacy of above 50% in greenhouse. The efficacy of middle-level biofilm formers (2 strains) harboring *srfAA*, *srfAB*, *ituC* and *fenD* in greenhouse was similar with that on plates (Table 2). By combining the analysis of biofilm formation, lipopeptide encoding genes and antagonism on agar plates, this method was revealed to be rapid and efficient to screen *Bacillus* strains against phytophthora disease in pepper.

### Discussion

Some *Bacillus* strains have been found to be efficient biocontrol agents, and are registered in many countries (Copping, 2001). Wild strains having ability to suppress pathogens via more than one type of antagonistic mechanisms are more attractive for application in the field. Successful colonization in the rhizoplane and rhizosphere soil is the first step of biocontrol agents. Following that, the agents compete for nutrient and space with pathogenic microorganisms such as bacteria and fungi, and produce some antibiotic metabolites to suppress the growth and infection of pathogens. For example, surfactin not only involves in colonization of *B. subtilis* on *Arabidopsis* rhizoplane (Bais *et al.*, 2004), but also suppresses bacterial infection through direct cell killing and inducing plants to produce systemic resistance (Ongena & Jacques, 2008).

Biofilm level	Amount of strains		Lipopepti	de encoding	genes	
	Forming Biofilm	Showing antagonism on agar plate	srfAA	srfAB	ituC	fenD
4	3	1	+	+	-	+
3	9	4	+	+	-	+
2	4	2	+	+	_	+
1	1	0	+	+	_	+
4	1	0	+	+	+	+
3	6	2	+	+	+	+
2	10	2	+	+	+	+
2	3	1	+	+	+	-
1	2	1	+	+	_	-
2	2	0	-	-	+	+
2	1	0	-	-	+	-
4	1	0	-	-	-	+
3	1	0	-	-	_	+
1	2	1	-	-	_	+
2	1	0	-	-	_	-
1	1	1	-	-	_	-
Total	48	15				

 Table 1
 Evaluation of antagonism of Bacillus strains against Phytophthora capsici infection on agar plates

Biofilm level: *0* no purple film on tube walls; *1* faint purple film; *2* obvious purple film; *3* clear circle purple film; *4* clear thick circle purple film;+ positive amplification; – negative amplification.

Strains	Biofilm level	Lipopeptide encoding genes				Biocontrol efficacy on	Biocontrol efficacy in	
		srfAA	<i>srfAB</i>	ituC	fenD	agar plates (%)	greenhouse (%)	
B006	3	+	+	-	+	60.0	60.0	
CS-4-1	3	+	+	_	+	60.0	52.0	
C5-4	2	+	+	+	+	100.0	40.0	
C2-3	2	+	+	+	+	15.0	5.0	
СК	0	-	-	_	-	0	0	
C11-1*	3	+	+	_	+	53.0	73.0	
C4-3*	3	+	+	_	+	50.0	70.0	
C14-3*	2	+	+	+	+	52.5	57.0	
WS-1-2*	2	+	+	+	+	20.0	23.0	
CK*	0	-	_	_	_	0	0	

# Table 2 Evaluation of antagonism of Bacillus strains against Phytophthora capsici infection on agar plates and in greenhouse

Biofilm level: *0* no purple film on tube walls; *1* faint purple film; 2 obvious purple film; 3 clear circle purple film; 4 clear thick circle purple film. \*Strains were tested in greenhouse during the autumn.; + positive amplification; – negative amplification

Our previous study that used SWA medium to screen *Bacillus* strains to suppress the growth of *Fusarium* also indicated that surfactin might play an important role in *Bacillus* colonization (Guo *et al.*, 2010). In this study, we developed a rapid method to screen *Bacillus* strains which could suppress crop diseases via several antagonistic ways by combining the analysis of biofilm formation, lipopeptide encoding genes, and biotic antagonism on agar plates. The screening time for *Bacillus* strains was greatly shortened to four months, and the screening efficacy from radicle assay on agar plates to seedling assay reached up to 33.3%, much higher than the sequential screening method (17.0%; Kim *et al.*, 2008). The results in greenhouse test were consistent with that on the agar plates except for strain C5-4, which demonstrated that radicle assay was very effective, similar to previous reports (Chang *et al.*, 2001; Kim *et al.*, 2008). This rapid and efficient screening method based on several antagonistic mechanisms could be expanded to screen more effective biocontrol antagonists against other plant diseases.

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# The effect of inoculation by cellulolytic bacteria *Bacillus cytaseus* on wheat productivity

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# Abstract

From rhizosphere of wheat plants isolated and selected a strain of cellulolytic bacteria Bacillus cytaseus IMV B-52 with high antagonistic activity against a broad spectrum of pathogenic fungi. It was found that the strain is capable of stimulating the germinating capacity of cereals and further development of their germs. The growthstimulating strain activity is associated with its ability to synthesize biologically active substances such as vitamins  $(B_1, B_6)$ , nicotinic acid, free amino acids, as well as the fixation of atmosphere molecular nitrogen. At presowing treatment of wheat seeds, the variety "Akmola-2", by cellulolytic bacteria B.cytaseus IMV B-52 their germinating capacity increased on average by  $30\% \sim 32\%$ . The attack of seeds by mold and plantlets by root rot was reduced to 7.3 times, at simultaneous biomass accumulation up to 19.2% for raw biomass, and 7.7% for dry one. Application of cellulolytic bacteria strain B.cytaseus IMV B-52 in field conditions showed that bacterial treatment of wheat seeds increases the thickness of young growth, layering capacity, ear graining, and exerts positive influence on factors of yield structure. Yield in areas where the treated with cellulolytic bacteria seeds were applied was 2.3 cn/ha higher than in control, and 2.6 cn/ha higher than following their treatment by chemical preparation "Raksil 6".

Key words: cellulolytic bacteria *Bacillus cytaseus;* pathogenic fungi; cereals; wheat

# Introduction

Currently in the Republic of Kazakhstan the problem of ecological advancement is acute. In this regard, a great attention is focused on issues related to agricultural production. Application of chemical fungicides and agents for plant protection results in some negative consequences: pollution of soil, water and air, development of resistance by pathogen to fungicides, agrobiocenosis violation, crop reduction and quality losses due to the accumu-lation of toxic substances in the final products (Reddy et al., 2009, Grishechkina, 2002) Elimination of these effects is possible by introduction of new highly efficient and environmentally safe methods of plant protection. One of the tendencies is the development and use of purpose-made biological preparations based strains-antagonists of fungal on and bacterial pathogens. The main strategically important crops of the Republic

of Kazakhstan are cereals (wheat, barley and oats), at the same time in 2008  $\sim$  2009 due to various diseases in the fields of the country over 30% of the wheat crop was lost.

An alternative to the chemical agents for protection and promoting the growth of cultivated plants is the use of biological preparations, the effective agents of which are micro-organisms (Labutova, 2011. Zang al. 2008. et Monastyrsky, Pershakova, 2009, Kulikov et al., 2006). In this regard, the most promising is the use of cellulolytic bacteria of the genus Bacillus, physiological and biochemical properties of which (the high growth rate. nonfastidiousness, simplicity of cultivation, etc.) ensure their high technological effectiveness in biomass production. In addition, in nature, in the real situation due to the higher growth rate, population stability and ability of cellulolytic bacteria to excrete antifungal metabolites they sustain competition with fungi for food source cellulose, and are one of the major groups of microorganisms capable of cellulose decomposition in aerobic and anaerobic conditions.

The objective of the study was an isolation and selection of the strain adapted to the local conditions to protect cultivated plants against pathogenic fungi that cause diseases of cereals, as well as stimulating plant growth and development.

# Materials and Methods

A strain of cellulolytic bacteria Bacillus cytaseus IMV B-52 was isolated from decaying wood. It was shown that the strain has a number of valuable production properties (high growth rate, non-fastidiousness, simplicity of cultivation, population stability). It was established that culture is nonpathogenic and nontoxic to humans and animals. The strain antagonist activity was evaluated by zones of growth inhibition for phytopathogenic fungi (Egorov, 1996). As test organisms the following phytopathogenic fungi were used: Alternaria alternata (Fr.) Keissler (4 strains), Bipolaris sorokiniana Shoem (3 strains), Fusarium solani Schleht., F.oxysporum var.orthraceras (App.et Wr.) Bilai, F. oxysporum var.solani Raillo, F. oxysporum Schleht. f. melonis, F. sporotrichiella Sherb., F. heterosporum Nees, F. graminearum Schw., Verticillium dahliae Kleb, causing diseases of cereal crops such as glume mold, yellow leaf blotch, seedling blight (including both typical and isolated in the fields of North Kazakhstan, the main place of cultivation of grain crops in Kazakhstan).

The research results were statistically processed using Student's test, the measurements considered significant at  $P \le 0.05$ .

# Table 1 The antagonist activity of Bacillus cytaseus IMV B-52 relative to

# phytopathogenic fungi

Test cultures	Diameter zones of bacterial growth inhibition of test cultures, mm
Alternaria alternata	48± 2.1
A. alternata 101	19± 0.7
A. alternata 1120	18±0.6
A. tenuis № 6	39±1.3
Fusarium solani	48± 2,2
F. oxysporum	13±0.5
F. oxysporum var. orthraceras	29±1.2
F. oxysporum var. solani	$16 \pm 0.5$
F. oxysporum forma melonis	40± 2.4
F. sporotrichiella	25±1.3
F. heterosporum № 8	22± 1.0
F. graminearum	34± 2.4
Bipolaris sotokiniana ET	36± 2.5
B. sotokiniana № 8	32± 1.2
B. sotokiniana Nch	41± 2.1
Verticillium dahliae	37± 2.3

# **Results and discussion**

The study of antagonist activity of cellulolytic bacteria *B.cytaseus* IMV B-52 against some pathogenic fungi demonstrated that the strain has not only high antagonist activity, but a broad action spectrum as well (Table 1).

Data from Table 1 demonstrate that the strain B. cytaseus IMV B-52 has a pronounced antagonist activity against pathogenic agents of alternariosis, fusariosis and helminthosporiosis as evidenced by the large-diameter zones of growth and development inhibition for these fungi (up to  $41 \sim 48$  mm). It should be noted that the zone sizes vary considerably, but they are of the distinguished and well-defined boundaries. When extending the exposure time the duration long of which zone conservation is observed (more than two months), is а significant valuable production factor (Figure 1).



Fusarium oxysporum var. orthraceras

F. oxysporum



#### causing wheat diseases

To study the growth-stimulating activity of the strain *B. cytaseus* IMV B-52 the seeds of cereals-wheat, barley, oats, maize-were used, which prior to sowing were treated with a suspension of cellulolytic bacteria in a concentration of  $1 \times 10^6$  cells per 1g of seeds. In the control group grain seeds were soaked in tap water. The results are given in Table 2.

	G( 1 (1							
Experiment modification	Stem length, cm	Root length, cm	Germinating, %					
Wheat								
Control	14.6±0.2	8.9±0.1	93.1±2.1					
Treatment with <i>B. cytaseus</i> IMV B-52	18.9±0.1	14.2±0.2	98.9±2.3					
	Barley							
Control	16.6±0.3	15.1±0.4	67.5±1.8					
Treatment with	21.2±0.4	22.3±0.3	88.2±2.1					
B. cytaseus IMV B-52	,							
	Oat							
Control	15.7±0.2	8.5±0.1	58.8±1.7					
Treatment with	18 3+0 3	10.9+0.2	89 5+1 9					
B. cytaseus IMV B-52	10.5±0.5	10.9±0.2	09.5±1.9					
Maize								
Control	13.3±0.2	6.4±0.1	64.3±1.6					
Treatment with <i>B. cytaseus</i> IMV B-52	16.3± 0.3	10.2±0.2	84.7±2.0					

Table 2 The effect of Bacillus cytaseus IMV B-52 on plant germinating ability and growth

Data in Table 2 demonstrate that the root length of plants treated with bacterial strain *B. cytaseus* IMV B-52 has increased by  $28\% \sim 59\%$  as against the control, the stem length – by  $17\% \sim 29.5\%$ . It was shown that the strain is capable of stimulating the germinating ability of cereals seeds and further development of seedlings. Previously it was established that the strain growth-stimulating activity is associated with its ability to synthesize biologically active substances such as vitamin *B. complex* (B1, B6), nicotinic acid, free amino acids, as well as with fixation of atmosphere molecular nitrogen. At presowing treatment of wheat seeds, the variety "Akmola-2", by cellulolytic bacteria *B. cytaseus* IMV B-52 their germinating capacity increased on average by  $30\% \sim 32\%$ . The attack of seeds by mold and plantlets by root rot was reduced to 7.3 times, at simultaneous biomass accumulation up to 19.2% for raw biomass, and 7.7% for dry one.

The estimate of the strain efficiency in field conditions was carried out jointly with the Kazakh Research Institute of Plant Protection and Quarantine, Ministry of Agriculture of Republic of Kazakhstan. Seeds of spring wheat "Omsk 18" were inoculated with bacterial suspensions and sown on the fields of Kostanai region of Kazakhstan. Seeds treated with chemi-cal agent Raksil 6 were used as a reference, untreated seeds – as a control. The data are shown in Table 3.

Experiment	Consump-	Seed parameters,	%	Sprout	Stem	Grain
mouncation	l/t	germinating	molding	pcs/m <sup>2</sup>	cm	cn/ha
<i>B. cytaseus</i> IMV B-52	0.1	99.8	0.3	269	75.6	22.6
Raksil 6						
(reference)	0.4	100.0	0.2	267	73.1	20.0
Control	-	99.0	0.6	262	72.8	20.3

# Table 3 The effect of seed inoculation by strain B. cytaseus IMV B-52 on germinating ability and contamination by phytopathogens and wheat crop

Application of cellulolytic bacteria strain *B. cytaseus* IMV B-52 in field conditions demonstrated that bacterial inoculation of wheat seeds increases sprout density, plant layering capacity, ear graining, positively affects the crop structure. Twofold reduction in seed moulding was observed under strain application in comparison with the control group. The average stem length of wheat has increased by 3 cm. The yield at fields where the treated with cellulolytic bacteria seeds were used was by 2.3 t/ha higher than in the control and 2.6 t/ha higher than following the treatment with the chemical agent Raksil 6.

Thus, the strain of cellulolytic bacteria *B. cytaseus* IMV B-52, adapted to local conditions, was isolated and selected, allowing efficient plant protection from pathogenic fungi that alternariosis, fusariosis and helminthosporiosis and stimulating plant growth and development. Presowing inoculation of wheat seeds by the strain results in the raising the level of wheat yield by an average of 2.3 kg/ha, as compared with untreated seeds, and by 2.6 kg/ha against the application of the chemical agent. The strain patent of the Republic of Kazakhstan,  $N_{\text{P}}$  16831 was obtained.

The using of this method for crop protecting is environmentally safe and meets the requirements of environment control, as it is based on the interaction of organisms in nature, does not lead to soil contamination and disturbance of biological balance because cellu-lolytic bacteria are the necessary representatives of soil microflora, ensuring its fertility. Produc-tion of cellulolytic bacteria could be established on basis of simple nutrient media con-taining cellulose as a carbon source. The strain capacity to sporogenesis provides the agent preservation for a long time and can improve soil quality, not only following application, but in subsequent years. Laboratory regulations for production of biological preparation named "Batsirin" were worked out on basis of the strain.

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# Effect of *Bacillus cereus*, a plant growth promoting rhizobacterium (PGPR) on *Fusarium* root and stalk rot pathogen of sorghum

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# Abstract

Sorghum bicolor (L.) Moench is an important cereal crop and is grown in 44 million ha, in tropical, semi-arid and temperate regions of the world with remarkable range of adaptability under diverse environmental conditions. India ranks second largest producer of grain sorghum after USA. In spite of high yield potential and excellent adaptability, it is prone to various diseases and pests. Fusarium root and stalk rot disease complex involving *Fusarium moniliforme* affects sorghum plants at all stages of growth. Plant growth promoting rhizobacteria (PGPR) forms an ideal biocontrol agent which helps crops by stimulating plant growth and/or by reducing the damage from soil-borne diseases. An indigenous gram positive, spore producing plant growth promoting rhizobacterium (OU N 21) isolated from sorghum rhizosphere from Andhra Pradesh, India, showed 99 per cent 16S rDNA homology with Bacillus cereus. The isolate was assessed for its growth promotion and biological control ability against F. moniliforme. The PGPR isolate was antagonistic to the pathogen with maximum inhibition as revealed by the dual culture and filter paper disc methods. The isolate produced chitinases and phosphate solubilizing enzymes and reacted positive for many biochemical characteristics including urease production while it reacted negative to phenyl alanine deamination. It utilized glucose and esculin as a sourse of carbon. The PGPR isolate produced indole acetic acid at low concentrations besides several antifungal compounds including pyrrolnitrin. We report a new metabolite with mono- or di- substituted benzene ring with aliphatic chain containing 23 protons. Seed bacterization nullified the impact of the pathogen in pot culture experiments. Disease control was over 95% compared to control. The growth parameters such as root and shoot length and root and shoot biomass were significantly enhanced by PGPR treatment. Increment in growth was statistically significant in sterile and non sterile soils suggesting its potential in the management of Fusarium root and stalk rot disease in sorghum.

#### Introduction

Sorghum bicolor (L.) Moench, is an important cereal crop and is grown in 44 million ha, in tropical, semi-arid and temperate regions of the world. It has a remarkable range of adaptability and grows under diverse environmental conditions. Sorghum ranks fifth in among major cereals in area sown following wheat, rice, maize and barley and an important cereal crop in India after rice, wheat and maize. It is generally grown as a dry land crop or as an inter crop with some legume, or as a rotated crop in the states of Maharashtra, Karnataka, Andhra Pradesh and Tamilnadu and also in certain parts of north Indian plains. Millions of people in semi arid regions of Africa and Asia use sorghum as the most important staple food, serving as the main source of energy, protein, minerals and vitamins. Inspite of high yield potential and excellent adaptability, sorghum is prone to various diseases and pests. Fusarium root and stalk disease complex of sorghum caused by *Fusarium moniliforme* affects sorghum plants at all stages of growth causing seedling blight, root and stalk rot, pokkah boeng, seed mold and head blight.

Biocontrol of soil- borne diseases is complicated because of the dynamic nature of soil root interface known as rhizosphere. Plant rhizosphere under the immediate influence of growing root provides a unique space for plant-microbe interactions and rhizobacteria at this soil-root interface play either a positive growth promoting or negative deleterious role in influencing the plant growth. Brown (1972) reported bacteria as most abundant group of microorganisms producing plant growth substances from rhizosphere and rhizoplane of wheat plants. Kloepper and Schroth (1978) coined the term plant growth promoting rhizobacteria (PGPR) to designate the rhizobacteria which benefit crops by stimulating plant growth or by reducing the damage from soil- borne plant pathogens hence are ideal biocontrol agents.

A variety of antibiotics involved in growth promotion and biocontrol have been identified which include 2,4-Diacetyl phloro glucinol (DAPG), Hydrogen cyanide, Oomycin A, Phenazine, Pyoleuteorin, Pyrrolnitrin, cyclic lipopeptides produced by Pseudomonads and Kanosamine, Zwittermycin A produced by *Bacillus, Streptomyces* spp. The role of antibiotics in the biocontrol has been demonstrated using mutants lacking production of antibiotics or over producing mutants (Bansall *et al.*, 1997; Chin A Woeng *et al.*, 1998; Nowak-Thompson *et al.*, 1999). In this paper we explored the sorghum rhizosphere for potential PGPR and report the presence of anti fungal compounds from *B. cereus* and the efficacy of this isolate in growth promotion and boiological control of *Fusarium moniliforme* in pot culture.

#### **Materials and Methods**

Plant growth promoting *Bacillus cereus* was isolated from the Sorghum rhizosphere soils by soil dilution plate method and screened for its ability to produce ammonia, indole acetic acid (IAA) and HCN (Dye, 1962; Gordan and Weber, 1951; Bakker and Schippers, 1987). *In vitro* antibiosis against the pathogen *F.moniliforme* was assessed using dual culture and filter paper disc methods.

#### Molecular characterization

DNA of *B.cereus* was isolated from the cells harvested from the overnight grown cultures. 16S rDNA fragment was amplified by PCR using 518 forward primer and 800 reverse primer. Details of primers used are: 518F-CCAGCAGCCGCGGTAATACG; 800R: TACCAGGGTATCTAATC. The 16S rDNA amplified fragments were analyzed (16S rRNA genes) using bioinformatics tools. The sequences were compared with available reference sequences taken from nucleotide sequence libraries in the NCBI BLAST database.

### HPLC analysis of antifungal compounds

Antifungal compounds produced were extracted from cultures and quantified with high performance liquid chromatography (HPLC). Metabolites were identified in comparison with the UV spectra of reference compounds. Liquid cultures of 20ml were acidified to pH 2 with 400 to 700  $\mu$ l of 1N HCl and extracted with 20ml of ethyl acetate for 30 min with vigorous shaking at 150 to 200 rpm. Phase separation was accelerated with 25 min of centrifugation at 4,500 rpm. The organic phase was flash evaporated and the residue was dissolved in 1ml of HPLC grade methanol. Aliquots of 10  $\mu$ l were injected into a reverse phased column (4 by 100mm) packed with Nucleosil 120-5-C18 and thermostatically controlled at 50°C (Duffy 1999). Proton NMR spectra measured on Avamce 500 spectrometer using TMS as internal standard and DMSO as solvent. Spectroscopic conditions - spectral width 7523.04, acquisition time 1.089, data points 16384.

#### Seed bacterization

The method of Weller and Cook (1983) was followed. Seeds of sorghum (CSH14) were surface sterilized using 2% sodium hypochlorite solution for  $2\sim3$  min, rinsed with sterile distilled water. Bacteria were grown in nutrient broth for 48h at  $28\pm2^{\circ}$ C. The surface sterilized seeds were soaked in broth culture for 30 min. Seeds were removed and air dried. Uninoculated nutrient broth served as control. Bacterized seeds were sown in pots containing sterilized/unsterilized soil in triplicate and the pots were randomly placed in glass house. Biocontrol efficacy of *B. cereus* isolate OU N21 was tested against the sorghum stalk rot pathogen *F. moniliforme*. The pathogen was mass cultured on sterilized seeds by inoculating 5mm mycelial discs in

polyethylene bags containing 150g of sorghum seeds. Bags were incubated for 15 days at  $30^{\circ}$ C. Inoculum was mixed at the rate of 30g/1kg soil.

# **Results and Discussion**

The 16s rDNA analysis using primers 518F and 800R revealed that the indigenous Gram positive bacterial isolate from sorghum rhizosphere is *B.cereus* as it exhibited 99% homology with the *B.cereus* strain YYW-8. Antagonistic activity was assessed by dual culture and filter paper disc methods and the bacterium suppressed the growth of *F.moniliforme*. Inhibitory effect was at its maximum in two-day old culture and the metabolites produced are effective at low concentrations and are thermo stable (Table1).

The rhizosphere isolate produced indole acetic acid at low concentrations while tested positive for ammonia production, nitrate reduction and negative for HCN. It was tested positive for several enzymes like urease, lysine decarboxylase, ornithine decarboxylase and the most important extra cellular enzyme involved in biocontrol, the chitinase. The isolate has the phosphate solubilizing ability and utilized esculin and glucose as sole source of carbon (Table2).

Table 1	Effect of	quantity	and	incubation	time of	on antagonistic
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	Colony diameter (mm)					
Days	Control	20(µl)	30(µl)	40(µl)	50(µl)	
Day 1	30.0	21.3 (21.3)	24.3 (24.3)	24.0 (24.0)	26.3 (26.3)	
Day 2	30.0	17.6 (18.6)	21.0 (19.0)	21.0 (21.0)	21.3 (20.3)	
Day 3	30.0	24.0 (24.3)	24.0 (25.0)	24.0 (27.6)	28.6 (28.3)	
Day 4	32.0	21.6 (22.0)	24.6 (24.3)	25.3 (24.0)	26.3 (26.6)	
Day 5	30.0	25.0 (24.6)	25.3 (26.0)	29.0 (29.3)	30.3 (31.0)	
Day 6	31.0	26.6 (25.0)	27.3 (27.3)	29.6 (29.3)	29.6 (32.6)	
Day 7	32.0	29.0 (29.0)	30.0 (30.0)	30.6 (31.3)	33.0 (32.3)	

activity of Bacillus cereus OU N21 against Fusarium moniliforme

\*values in parentheses are for autoclaved soil

Enzyme/Metabolite	Activity		
Chitinase	+		
Phosphate solubilization	+		
Protease	-		
Lipase	-		
Cellulases	-		
IAA	+		
NH3	+		
HCN	-		
ONPG	+		
Lysine Decarboxylase	+		
Urease	+		
Phenyl alanineDeamination	-		
Nitrate Reduction	+		
H2S Production	+		
Citrate	+		
Voges Proskauer's	-		
Methyl Red	-		
Malonate	+		
Ornithine Decarboxylase	+		

 Table 2 Biochemical Characteristics of B. cereus isolate OU N21

Up on HPLC analysis for antifungal compounds, Pyrrolnitrin was consistently present. <sup>1</sup>H NMR analysis was characterized by the following chemical shifts  $\delta$ , 7.67(H,q),  $\delta$ , 7.59 (1H,s),  $\delta$ , 7.59(1.5H,q),  $\delta$ , 7.26(1H,q),  $\delta$ , 4.16(3H,m),  $\delta$ , 0.93(12H,m),  $\delta$ , 3.3(2H,s),  $\delta$ , 2.56(4H,T)  $\delta$ , 2.14(2H,s). NMR results reveal another important metabolite from *B. cereus* which is having a mono or disubstituted benzene ring with an aliphatic chain containing 23 protons.



Table3 Effect of seed bacterization by B. cereus on sorghum

Age	C1	T1	T2	T3	T4
Week1	16.26	6.46	23.33	19.3	20.6
Week2	18.30	13.9	28.53	25.73	23.0
Week3	22.0	16.06	32.6	28.13	28.8
Week4	24.8	16.63	32.9	28.6	29.73

 $({\bf root \ length}) \ {\bf in \ } Fusarium \ moniliforme \ {\bf inoculated \ soils}$ 

C1- sterile control, T1- F. moniliforme, T2-B.cereus, T3- B. cereus+ F. moniliforme, T4- B. cereus+ F. moniliforme (unsterile soil).

Seed bacterization with *B. cereus* OU N21 isolate controlled the sorghum stalk rot by 95% in CSH14. The PGPR was equally effective in sterilized and unsterilized soils in pot culture experiments. Growth promotion was significant with reference to root and shoot length (Table3 & 4, Fig. 1) and the biomass. Root and shoot biomass increased gradually and the difference in the increment was significant at 5% level (Fig. 2).

#### Table 4 Effect of seed bacterization by B. cereus on

Age	C1	T1	T2	T3	T4
Week1	8.13	5.26	12.46	11.2	10.3
Week2	17.36	11.43	25.06	22.83	20.5
Week3	19.3	15.16	31.3	26.86	25.6
Week4	22.23	16.76	37.3	32.23	26.5

#### sorghum (shoot length) in Fusarium moniliforme inoculated soils

C1-sterile control, T1- F. moniliforme, T2-B.cereus, T3- B. cereus+ F. moniliforme, T4- B. cereus+ F. moniliforme (unsterile soil).



Figure 1 Effect of seed bacterization by *B. cereus* on sorghum CSH 14 plant growth (root and shoot length in cm) in *Fusarium moniliforme* inoculated soils



Figure 2 Effect of seed bacterization by B. cereus on sorghum CSH14 biomass

#### in Fusarium moniliforme inoculated soils

C1-sterile control, T1- *F. moniliforme*, T2-B.cereus, T3- *B. cereus*+ *F. moniliforme*, T4- *B. cereus*+ *F. moniliforme* (unsterile soil).

Growth promoting rhizobacteria have shown positive effect in many annual crops including wheat, maize and lettuce. PGPR influence plant growth in different ways by producing phytohormones like IAA and gibberellins, siderophore production, phosphate solubilization, synthesis of antibiotics, enzymes and/or antifungal compounds (Ahmad *et al.*, 2006; Bharathi *et al.*, 2004).

Up to 80% of rhizobacteria can synthesize indole-3-acetic acid (IAA) (Loper and Schroth 1986). Root growth promotion by free living PGPR, e.g., *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus*, species of Azospirillum, *Pseudomonas* and *Xanthomonas* as well as by symbionts such as *Bradyrhizobium japonicum* and *Rhizobium* spp., has been attributed to low levels of IAA secretion (Patten and Glick 1996). In contrast the inhibitory effect of some deleterious rhizobacteria (DRB) has been related to their high amounts of IAA excretion, e.g., *Enterobacter taylorae* (Sarwar and Kremer, 1995) and *Pseudomonas putida* (Xie *et al.*, 1996). Barazani and Friedman (1999) reported high levels of IAA (76.6 µM) by four deleterious rhizobacteria *viz.*, *Micrococcus leteus*, *Streptoverticillium* sp., *Pseudomonas putida* and *Gluconobacter* sp. They stated that high concentration of IAA released by DRB accounted for the suppression of root growth. We have observed the presence of both high and low IAA producers in sorghum rhizosphere

from the state of Andhra Pradesh, India and the *B. cereus* isolate OU N21 produced IAA in low concentrations ( $5.0 \mu g/ml$ ). In addition to pyrrolnitrin, it has produced mono or disubstituted benzene ring with an aliphatic chain containing 23 protons consistently which may account for the growth promotion and biocontrol of *F. moniliforme*. However, the antibiotics Kanosamine and Zwittermycin A reported from *B. cereus* WU85 (Milner *et al.*, 1996, Silo-suh *et al.*, 1994) were not encountered with this isolate, therefore it unique indigenous PGPR.

PGPR may directly influence plant growth through enhanced provision of nutrients and the production of phytohormones and indirectly suppress deleterious rhizobacteria and pathogens to affect biological control by the production of antibiotics and iron chelating siderophores and the induction of plant resistance mechanisms (Persello- Cartieaux 2003). Phosphorous is a major essential macronutrient for biological growth and development. The most efficient phosphate solubulising microbes belong to genera *Bacillus* and *Pseudomonas* amongst bacteria and *Aspergillus* and *Penicillium* amongst fungi. The reported bacilli include, *B. brevis*, *B. circulans*, *B. cereus*, *B. licheniformis*,

*B. megaterium*, *B. polymyxa*, *B. pumilis* and *B. subtilis* from the rhizosphere soils of legumes, cereals, oat, jute and chillies (Gaur 1990). The ability of PGPR isolate *B. cereus* OU N21 from sorghum rhizosphere soils to solubilize phosphates is a contributing factor for its growth promotion.

Chitinolytic activity, as shown by the present isolate might have played a role in curtailing the stalk rot pathogen, *F. moniliforme* and brought the biocontrol. In pigeon pea *Bacillus subtilis* AF1 controlled fungal pathogens and increased seedling emergence, dryweight (Manjula and Podile 2005). Antibiotic and enzyme producing abilities of this isolate makes it a promising biocontrol and growth promoting agent of grain sorghum in Andhra Pradesh, India.

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# Plant-specific selection of drought-resistant biological control agents

# against soil-borne pathogens

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### Abstract

Desert agriculture is a strongly growing field of land use. However, emerging problems with soil-borne pathogens limit the yield. Biological control agents (BCAs), which are able to suppress soil-borne pathogens, are promising candidates for plant protection but for desert application specific, drought-resistant strains are required. Here we report a stepwise selection procedure of BCAs from one of the most prominent organic desert farms SEKEM in Egypt. In a first step, we characterized the bacterial and fungal communities of the target habitat – the rhizospheres and endorhiza of medical plants (Matricaria chamomilla L., Calendula officinalis L. and Solanum distichum Schumach. & Thonn.). The bacterial communities were highly different – for the plant species as well as for both investigated microenvironments. The fungal community was less discriminative but characterized by phytopathogens. In a cultivation-dependent approach, isolates from all parts were obtained and characterized by their anti-phytopathogenic potential against fungi (Fusarium Rhizoctonia solani. Verticillium dahliae). bacteria culmorum. (Ralstonia solanacearum) and nematodes. In parallel, genotypic diversity was analysed by ARDRA and BOX-PCR. Both procedures resulted in the selection of 46 unique, broad-spectrum antagonists. However, their diversity was low: 89% of the selected strains belonged to the Bacillus/Paenibacillus cluster. Bacillus subtilis (subsp. subtilis and *spizizenii*) was the main species identified in cultures and also a dominant band in soil, rhizosphere and endorhiza in microbial fingerprints. Furthermore, using a metagenomic approach, it was shown that Firmicutes and especially *Bacillus* was enriched in SEKEM soil in comparison with the surrounding desert. In contrast, from the original desert soil, diverse antagonistic *Streptomyces* strains were selected.

Key words: desert farming; medical plants; antagonists

# Introduction

On the Sekem farms in Egypt, desert land was converted into arable land, and organic (biodynamic) agriculture is operated for over 30 years now (www.sekem.com). Today, Sekem is carrying out organic agriculture on more than 6,000 hectares and has the largest market for organic products outside Europe and North America. They produce organic foods, spices, tea, cotton textiles and natural remedies.

Crop production, especially cultivation of medical plants is more and more affected by soil-borne phytopathogens, which lead to significant yield losses. The major problems are caused by the soil-borne pathogenic fungi Verticillium dahliae Kleb., Rhizoctonia solani Kühn and Fusarium culmorum (Wm.G. Sm.) Sacc. as well as by the soil-borne pathogenic bacterium Ralstonia solanacearum. Although grown in organic agriculture, which aims to minimise the impact on the environment by practices such as crop rotation, using pathogen resistant cultivars, and the use of organic manure (compost) instead of synthetic fertilisers (Schmid et al., 2011), they have an increasing importance. One reason is an intensive growing of a limited number of crops in short rotations. Here, BCAs should solve these problems and help to suppress soil-borne pathogens on a natural and sustainable way. The objective of the study was to develop a specific biocontrol product, which will be optimised for desert farming-regarding soil, weather, pathogen species, etc. Thus, autochthonous bacteria isolated from rhizosphere and endorhiza of medical plants as well as from bulk soil collected in Egypt were evaluated for their potential for biocontrol (Köberl et al., 2011).

Beside soil-borne pathogens, contamination and colonisation of plants with *Escherichia coli* pathovars caused infections in humans (van Elsas *et al.*, 2011). For example, in 2011 Germany reported one of the largest outbreaks of haemolytic uremic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC), also commonly referred to as verocytotoxin-producing E. coli (VTEC) and enterohaemorrhagic E. coli (EHEC) (Frank et al., 2011; Struelens et al., 2011). Between 2<sup>nd</sup> May and 15<sup>th</sup> June, 3,351 STEC cases, including 821 cases of HUS, were reported from 13 European Union (EU)/European Economic Area (EEA) Member States and 37 patients have died. Over 97% of STEC cases have been reported from Germany (ECDC, 2011). The search for the source and vehicle of the outbreak has been a long and arduous process. Initial epidemiological findings pointed to raw vegetables and salads as likely vehicles of infection, but extensive investigations identified sprouts produced at an organic farm in Lower Saxony. Also for the target pathogen E. coli, biocontrol is a possible and environmentally friendly alternative. To avoid the colonisation of crops and medical plants with E. coli, the introduction of naturally occurring microorganisms with antibacterial activity towards

those intestinal bacteria is a promising strategy (van Elsas *et al.*, 2011). For this reason, *E. coli* was additionally included in the selection procedure of drought-resistant BCAs.

# **Materials and Methods**

# Experimental design and sampling

Samples from agriculturally used soil were taken at the Sekem farm Adleya, located in the north-eastern desert region of Egypt near Bilbeis (30°22'88"N; 31°39'41"E). Plant growth completely depended on irrigation water coming from the Nile or from local ground water drillings; sprinkler and drip irrigation systems were used. The soil at Sekem was fertilised with compost that was produced on their own composting facility, where rice straw, water hyacinth, wood chips, organic waste, clay, chicken and cow manure was mixed to receive a final C/N ratio between 20 and 30. The compost was applied twice a year (May and September), during the preparation of the fields for the cropping seasons. The soil texture at the Sekem farm was classified by Luske & van der Kamp (2009) as loamy sand (pH 8.5) with an organic carbon content of 0.5% and a clay content of 3%. For microbial analysis, desert soil was collected in the Sinai desert (1<sup>st</sup> sampling; 30 35'01"N; 32 25'49"E) and in the desert of Saqqara near Cairo (2<sup>nd</sup> sampling, 35 59'0"N; 41 2'0"E). The soil was characterised by a low moisture level; plants were very scarce (Luske & van der Kamp, 2009). At each site, four composite samples of soil in a horizon of  $0 \sim 30$  cm depth were collected. Regarding agricultural soil, roots with adhering soil were obtained from three different species of medical plants (German chamomile [Matricaria chamomilla L.], pot marigold [Calendula officinalis L.] and Solanum distichum Schumach. & Thonn.) planted on the Adleya farm (30°22'88"N; 31°39'41"E). From each plant four independent composite samples consisting of  $5 \sim$ 10 plants were taken. At the first sampling time (October 2009), Matricaria chamomilla and Calendula officinalis have been in the seedling stage, whereas the samples from the perennial *Solanum distichum* were taken from lignified plants. At the second sampling time (April 2010), all medical plants were in the flowering stage.

#### Isolation and selection of bacteria

To isolate bacteria from soil and rhizosphere 5 g of soil or roots with adhering soil were added to 45 ml of 0.85% NaCl and vortexed. For isolation from the endorhiza, 5 g of roots were surface-sterilised with 4% NaOCl for 5 min, then the roots were washed three times with sterile Aqua dest. After 10 ml sterile 0.85% NaCl was added the roots were homogenised using mortar and pestle. Cell suspensions were used for dilution and plating on R2A (Roth, Karlsruhe, Germany) in duplicates. Plates were incubated for 4 days at room temperature (RT) and colony forming units were counted to calculate the means of colonies (log<sub>10</sub> CFU) based on fresh weight (fw). If
possible, for each replicate 24 bacterial isolates were selected and subcultured on nutrient agar (NA). The isolates were purified and then stored at -70 °C in nutrient broth (NB) (Sifin, Berlin, Germany) containing 15% glycerol. Isolates were encoded using a combination of letters and numbers indicating: (1) soil type or plant species (Wb = desert soil; Sb = Sekem soil, Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*), (2) replicate (1-4), (3) microenvironment (Re = endorhiza, rhizosphere and soil have no further designation), and (4) consecutive number of the isolate per replicate.

#### Screening for in vitro activity towards pathogens

Altogether, 1,212 bacterial isolates obtained were screened in dual-culture *in vitro* assays on Waksman agar (WA) (Berg *et al.*, 2002) for their antagonistic potential towards the phytopathogenic fungi *Verticillium dahliae* Kleb. V25, *Rhizoctonia solani* Kühn AG4, and *Fusarium culmorum* (Wm.G. Sm.) Sacc. E1. For *R. solani* and *F. culmorum* agar disks of 5 mm diameter with mycelia were directly cut out from PDA plates (Roth, Karlsruhe, Germany) and placed between the streaks of four bacterial isolates. *V. dahliae* was grown in liquid culture in Czapek Dox broth (Duchefa, Haarlem, Netherlands) at 20°C. 200 µl of the suspension containing hyphal fragments were plated onto WA and after surface drying the bacterial isolates were placed on the same plate. Inhibition zones were measured after  $4 \sim 7$  days of incubation at 20°C. Each isolate was tested twice independently.

A selection of 45 promising biocontrol strains with antagonistic activity towards pathogenic fungi was tested for antibacterial activity towards *Escherichia coli* OP50 and *Ralstonia solanacearum* 1609 and B3B. The activity of all isolates against both *R. solanacearum* strains was identical. *E. coli* was grown in tryptic soy broth (TSB) (Roth, Karlsruhe, Germany) at 30 °C and the bacterial suspension ( $OD_{600} = 0.6 \sim 0.8$ ) was mixed with 1.5% LB agar (Roth, Karlsruhe, Germany) at a ratio 1:10. Bacterial antagonists were streaked onto those plates and after an incubation time of 3 days at RT presence or absence of inhibition zones surrounding the antagonists were assessed. For the screening towards *R. solanacearum* yeast peptone glucose (YPG) medium was used and Tetrazolium Violet (Sigma-Aldrich, Saint Louis, USA) was added to the medium prior pouring (Adesina *et al.*, 2007).

#### In vitro screenings for direct plant growth promoting abilities

The 45 selected bacterial *in vitro* antagonists were tested for nitrogen fixation, ACC deaminase activity and phosphate solubilisation. For detection of N<sub>2</sub> fixation and ACC deaminase activity bacteria were transferred to Brown & Dilworth (BD) minimal medium (Brown & Dilworth, 1975) with and without the addition of 0.07% ACC solution as unique nitrogen source. After 2 weeks at RT their extent of growth was assessed as described by Fürnkranz *et al.* (2009). As positive control for the

bacterial growth BD medium with 0.07% NH<sub>4</sub>Cl solution was used. P solubilisation was tested on National Botanical Research Institute's phosphate growth agar (NBRIP) (Fürnkranz *et al.*, 2009). Formation of clear halos was recorded 5 days after incubation at 20°C.

#### **Results and Discussion**

#### Cultivation and selection of strains

The rhizosphere of all three investigated medical plants was highly colonised by bacteria:  $\log_{10}$  CFU ranged from 7.8 ± 0.3 to 8.0 ± 0.2 g<sup>-1</sup> fw. In contrast, in the endorhiza significantly lower CFUs were detected:  $\log_{10} 2.0 \pm 0.2$  to 3.7 ± 0.8 g<sup>-1</sup> fw. The highest colonised rhizosphere as well as endorhiza was detected for *S. distichum*, followed by *M. chamomilla*, and the lowest population density in both microenvironments was found for *C. officinalis*. The determined bacterial abundance was significantly higher in agricultural soil ( $\log_{10} 7.7 \pm 0.4 \text{ g}^{-1}$ ) in comparison to desert soil ( $\log_{10} 4.6 \pm 0.6 \text{ g}^{-1}$ ). From each of the four replicates per plant and microenvironment, 24 bacterial colonies were randomly selected for further characterisation.

#### Characterisation of the indigenous antifungal potential in vitro

All 1,212 selected bacterial strains were screened by dual testing regarding their antagonistic activity towards *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum*, which are known as main soil-borne fungal pathogens. In general, isolates obtained from *M. chamomilla* and *S. distichum* showed a higher *in vitro* antagonistic potential towards soil-borne phytopathogenic fungi than those from *C. officinalis* (Table1). Especially isolates from the endorhiza from *M. chamomilla* harboured a high proportion of antagonists. Whereas in the soil and in the rhizosphere bacterial antagonism was mainly directed towards *F. culmorum*, in the endorhiza of the medical plants antagonism was mainly found towards *V. dahliae*. Bacterial isolates obtained from the soil of the farm exhibited a higher antagonistic potential in comparison to the bacteria isolated from the desert soil (agricultural soil 21.6%  $\pm 0.8\%$ ; desert soil 12.4 % $\pm 0.7\%$ ).

#### **Detection of antibacterial activity**

According to unique genotypic patterns ascertained by employing amplified ribosomal RNA gene restriction analysis (ARDRA) with the restriction endonucleases *Hha*I and *Pst*I and BOX-PCR genomic fingerprinting (data not shown), 45 genotypically different strains with antagonism towards fungal pathogens were selected to test them regarding their antibacterial activity towards *Escherichia coli* and *Ralstonia solanacearum*. Of these isolates 15.6% were able to inhibit *in vitro* the growth of *E. coli*. These were all isolates of the genus *Paenibacillus* (identified as *P*.

*brasilensis*, *P. polymyxa* and *P. kribbensis*). The growth of *R. solanacearum* was inhibited by 33.3% of the selected antagonists: most isolates of *Streptomyces* (3 of 4 isolates) and some strains of the *Bacillus subtilis* group (12 of 30 isolates). Interestingly, the antibacterial activity was very specific, i.e. there was no isolate with suppression of both bacterial species (Fig.1).

		Relative abundance of antagonists (%) <sup>a</sup>		
Origin	Microenvironment	V. dahliae	R. solani	F. culmorum
Matricaria chamomilla	Rhizosphere	$12.5\!\pm\!2.9$	8.3 ±0.7	$13.0 \pm 1.8$
	Endorhiza	$19.9 \pm 1.8$	$16.4 \pm 2.3$	$18.8 \pm 2.6$
Calendula officinalis	Rhizosphere	9.0 ±0.5	$7.1 \pm 0.1$	10.1 ±3.1
55	Endorhiza	$4.2 \pm 2.9$	$0.0\ \pm 0.0$	$1.4 \pm 1.0$
Solanum distichum	Rhizosphere	$13.7 \pm 2.3$	$13.8 \pm 3.8$	$15.7 \pm 0.0$
	Endorhiza	$13.5 \pm 5.1$	$10.4 \pm 5.8$	$12.5 \pm 5.8$
Soil	Agricultural Soil	$20.0 \pm 1.6$	$21.9 \pm 2.2$	$22.6 \pm 1.4$
	Desert Soil	$11.1\pm1.8$	$12.8 \pm 0.6$	$13.4 \pm 0.1$

 Table 1 Proportions of bacterial isolates antagonistic towards the soil-borne fungal pathogens

 Verticillium dahliae, Rhizoctonia solani and Fusarium culmorum

<sup>a</sup>Data are averages of  $1^{st}$  and  $2^{nd}$  sampling  $\pm$  confidences.



Figure1 Relative abundance and diversity of antagonists with additional antibacterial activity towards *E. coli* and *R. solanacearum*. Out of all strains, 45 genotypically different isolates with antifungal properties were tested and identified by partial 16S rRNA gene sequencing.

#### Determination of plant growth promoting abilities

The selected 45 genotypically different antagonists were screened for direct PGP properties. Among all tested strains, 24.4% were declared as diazotrophs, all *Paenibacillus* spp. and *Streptomyces* spp. were able to grow on N-free medium. ACC degradation could be shown only for 4.4% strains (two isolates of *Bacillus subtilis* 

subsp. *spizizenii*). No isolate of the selected antagonists was able to solubilise phosphate.

#### Assessment of antagonistic strains

From 1.212 isolates, 45 selected antifungal strains were characterised more in detail. For half of the strains additional antibacterial activity was detected. Whereas the majority of strains showed antifungal activity towards all three target pathogens, antibacterial activity was exclusively found against one of the pathogens. According to the results, specific antagonists against pathogens but also broad spectrum antagonists were found. Two outstanding specific antagonists were identified towards the main soil-borne fungal pathogen Verticillium dahliae: Paenibacillus kribbensis Sb3-1 and Brevibacillus limnophilus Mc6-4. Activity against E. coli was exclusively found by *Paenibacillus* isolates from various origins, whereas *Streptomyces* isolates from desert soil but also Bacillus strains were active against the phytopathogen R. solanacearum. Beside some broad spectrum antagonists, which could be isolated from plant-associated environments from different plant species, also plant-specific BCAs were found. Surprisingly, isolates selected from desert soil belong to the most active isolates. One isolate of this group - Streptomyces subrutilus Wb2n-11 - was the most promising biocontrol candidate by rating all antagonistic properties. Further interesting candidates were Bacillus subtilis subsp. subtilis Co1-6 and Streptomyces peucetius Wb2n-2.

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### Microbial diversity and molecular signals controlling plant-microbe interaction in the rhizosphere of grasses including wheat

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#### Abstract

Microbial communities are influenced by number of factors in the rhizosphere environment hence are distributed in a characteristic manner in the soil. Rhizotrophs (a distinct group of rhizospheric bacteria) have some kind of affinity with the roots based on several factors including bacterial cross-talk, molecular signaling and quorum sensing (QS). These quorum sensing components from bacteria have been documented to switch certain genes for use in a variety of plants.

Analysis of bacterial inoculation on plant growth revealed that bacteria inoculation in the soil has increased shoot length as well as root length in plants inoculated with *Azospirillum brasilense* and *Pseudomonas* sp. Shoot dry weight, leaf and root area were significantly increased in both plants inoculated with *Azospirillum brasilense* and Pseudomonas sp. but maximum root biomass was increased in plants inoculated with *Azospirillum brasilense*.

Rhizospheric bacteria were isolated from root zones of wheat grown in designed rhizobox. These were cultured in laboratory conditions and investigated for plant beneficial traits i.e. IAA production and solubilization of insoluble phosphate and quorum sensing. There were 14 strains found to produce IAA, with QTS4 strain producing highest amount (8.34  $\mu$ g/ml) and WR-5 having minimum production(0.03  $\mu$ g/ml) while 16 strains appeared to solubilize the phosphate. In Gram-negative bacteria, quorum sensing is mediated by *N*-acylhomoserine lactones (AHL). Out of 43 screened rhizospheric bacterial isolate of wheat for AHL production 6 isolates were found to be positive.

Metagenomic study from soil was carried out by sampling thin sliced rhizospheric soil at increasing distance from root of wheat in a model rhizobox system. DNA was extracted and the 16S rRNA amplified, cloned and sequenced. Phylogenetic analysis of these sequences with those held on-line, revealed that 2.5% of the clones fell within the *Nocardioides* sp. 7.5% of them were *Duganella* sp. 2.5% of *Bacillus* sp. 2.5% *Marmoricola* sp. 2.5% *Stenotrophomonas* sp. 5.0% *Acidobacterium* sp. 2.5% *Balneimonas* sp. Additional 75% clones were uncultured microorganisms and may represent novel bacterial lineages.

## Plant growth promoting rhizobacteria and agricultural

## research in Iran

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#### Abstract

Increasing use of fertilizers and highly productive systems has created environmental problems. In recent years, biofertilizers have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields.

Beneficial rhizobacteria that stimulate plant growth are usually referred as Plant Growth Promoting Rhizobacteria or PGPR. These bacteria vary in their mechanisms of plant growth promotion but generally influence plant growth via -Nitrogen fixation, -P solubilization, -Nutrient uptake -Phytohormons production, -decrease environmental Stress, -Biocontrol, -Production of Antibiotics, and also -Induced systemic resistance. Today these bacteria are widely used. The aim of this study was evaluation of papers that presented in Iranian congress related to this subject. The results showed that although papers presented in this area has increased, but a small percentage of the total research are included. The effects of these bacteria on nutrient absorption, reduce the stress effects and improve the germination process are the main project area. Application of these bacteria as a biological control factor has a small portion of research in this field.

Key words: PGPR; biocontrol; biofertilizer; agricultural research; iran

#### Introduction

In both natural and man-made agro ecosystems, interactions between plants and soil micro-organisms have a profound effect on biogeochemical cycling of nutrients, soil quality, adaptation of plant to changing environment and plant growth (Kloepper *et al.*, 1989; Bashan *et al.*, 2004). Diverse arrays of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter* and *Bacillus* have been shown to promote plant growth and improve soil structure (El-Komy, 2005; Yasari and Patwardhan, 2007).

The Stimulatory effects of plant growth promotion by these bacteria have not been completely elucidated, but they may result from synthesis of antibiotics (Ge *et al.*, 2006) or siderophores (Kloepper *et al.*, 1980), synthesis of phytohormones(Bashan *et al.*, 2004), N<sub>2</sub> fixation (Boddey *et al.*, 2001), reduction of membrane potential of the roots (Bashan , Levanony., 1991), synthesis of some enzymes (such as ACC deaminase) that modulate the level of plant hormones (Shaharoona *et al.*, 2006), as well as the enhancement of availability of some minerals(Roesti *et al.*, 2006)

Stimulation of different crops by *Azospirillum* spp. and *Pseudomonas* spp. inoculation has been demonstrated by many studies (Salanture *et al.*, 2006). At now agricultural research in the areas of ecological aspects is expanding. Application of PGPR is rapidly spreading, although it is not common in the farmer's field and is in the early stages.

The aim of this study was evaluation of papers that presented during Iranian congress related to PGPR application and show the situation in field research.

#### **Materials and Methods**

In this research evaluation of congress Key Words include: - 10 iranian agronomy and crop science congress which was held 20 years. In this way the total number of 16000 key words derived from congress proceedings. -19<sup>th</sup> Iranian Plant Protection Congress (2010), the first national conference on sustainable agriculture and healthy product (2010) and 12<sup>th</sup> Congress of Soil Science in Iran (2010).

#### Results

#### Iranian agronomy and crop science congress

Among the research conducted, wheat 23%, corn 10.57%, rice 7.99%, Canola 7.2%, barley 5.85%, sugar beet 5%, soybean 4.35%, cotton 4.1%, peas 3.74% and finally with Sunflower 3.45% of the research formed.

In issues related to agroecology, topics such as rotation, planting pattern, the use of beneficial bacteria and fungi, mulch, cover crops and... were selected. Only 3% of total papers presented during 20 years congress activity were in the section of agroecology. the term of intercropping systems with 20.4 % of ecological key words was in the first place and then plant growth promoting rhizobacteria include 14.22% from agroecological research was in second place. The number of paper related to agro ecology increased from 17 papers in first congress to 112 in 10<sup>th</sup> congress. This indicates that more attention to ecological areas take place. All articles presented about the use of PGPR discuss the impact of these bacteria on seed germination, phytohormones production and finally improvement of crop growth.

#### Iranian Plant Protection Congress

In 19<sup>th</sup> Iranian Plant Protection Congress, that was held every two years. All papers presented in three main sections include: plant disease, insects and weed. In the plant diseases section only 19 papers related to application of PGPR. One paper related to symbiosis ability of these bacteria on of some growth factors of wheat under greenhouse conditions. The other papers related to the biocontrol effects of PGPRs. Briefly include: biocontrol of potato wilt caused by Fusarium oxysporum and F. solani, Biocontrol of rhizome soft rot in valeriana by Pseudomonas spp., antagonistic mechanisms in Pseudomonads fluorescent against Fusarium oxysporum, antagonistic activity of *Pseudomonades fluorescent* and their efficacy mechanism disease. Screening on *Phytophthora* damping-off of some isolates of Pseudomonas fluorescens against Sclerotinia sclerotiorum on sunflower, effect of epiphytic bacteria in biocontrol of wheat head blight, Biological control of common root rot of wheat by *Pseudomonads fluorescent*, Development of formulation of Bacillus subtilis for management of root and foot rot of cucumber caused by *Pythium aphanidermatum*, antagonistic mechanisms of *Bacillus* spp. in biocontrol of cucumber root and foot rot caused by *Pythium aphanidermatum*. In vitro and in vivo biological control of blue mold of apple fruit by *Bacillus subtilis*, Antagonistic activity of Bacillus and Pseudomonas isolates against Fusarium oxysporum, interaction between *Glomus fasciculatum* and *Bacillus subtilis* on control of common root rot of wheat, effect of *Bacillus subtilis* on cucumber root and stem rot, caused by Fusarium oxysporum. -Mode of action of Bacillus subtilis on aflatoxin control, effects of *Bacillus* species on biological control and induced resistance in bean seedling roots against the root rot fungus, Induction of systemic resistance to bacterial blight caused by Xanthomonas axonopodis in cotton by Pseudomonads fluorescent.

#### National conference on sustainable agriculture and healthy product

The first national conference on sustainable agriculture and healthy product was held in 2010. Total 511 articles accepted at the conference only 11 articles related to the bacteria growth stimulant. 8 articles related to increasing yield, fertilizer management and nutrient uptake. 2 cases related to the effects of PGPR on better seed germination and 1 case related to improve drought stress resistance

#### Iranian Congress of Soil Science

At 12th Congress of Soil Science in Iran, 1100 paper presented in various field of soil science (soil biology, soil fertility, soil physics and soil erosion...) only 85 papers had subject of PGPR. 75 cases related to the effects of these bacteria on water relation,

nutrient absorption and stress resistance (abiotic), 9 cases about phytohormone production and 1 case about environmental pollution.

Agricultural research in iran had a significant role in the development of this sector. With a review of past research done we can recognize research trend and on the other hand it can be the future direction of research. The aim of this paper reviews was evaluation of research conducted during different Congress Crop Science.

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## Development and application of a new antifungal pesticide "Shenqinmycin" by genetically modifying the melon rhizosphere-originating strain *Pseudomonas* sp. M18

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#### Abstract

The development of "green" pesticides with high efficiency and low toxicity has aroused wide public interest in recent years. Phenazine-1-carboxylic acid (PCA) produced by pseudomonads has proven effectively again a range of soil-borne fungal phytopathogens and has great potential for development as a new kind of fungicide. *Pseudomonas* sp. M18, which was isolated from the melon rhizosphere, produces two different antibiotics, PCA and pyoluteorin. During the last decade, our lab has identified several signaling systems which are involved in the regulation of PCA biosynthesis in M18. Four rounds of genetic modifications, including inactivation of the quorum sensing repressor QscR and the global regulator GacA, and increasing the copy number of PCA biosynthesis cluster, have been conducted and PCA yield in the engineered strain has been significantly increased. Through optimizing the culture medium components, PCA yield was achieved as high as 5,000 mg/L, which is economically applicable for large-scale commercial purposes. Recently we have sequenced the genome of M18 and we found that seven genomic islands and six biocontrol-related gene clusters probably contribute to its biocontrol activities and living abilities in rhizosphere niches. We are currently investigating the mutual relationship of two PCA biosynthesis cluster, and the global regulatory network of PCA biosynthesis. PCA was commercially renamed as "Shenqinmycin" in China. The control effects of "Shenqinmycin" on rice sheath blight disease have been tested in field during  $2008 \sim 2009$  in 10 provinces of China. "Shenqinmycin" has been officially registered as a new pesticide against rice sheath blight disease in March of 2011.

### Potential of PGPR in the management of nematodes and as bionematicides-research initiatives - national and international

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#### Abstract

Nematodes such as *Meloidogyne incognita* and *Rotylenculus reniformis* were found to inflict huge losses in important horticultural crops in the world. Management of nematodes using chemicals is expensive, results in toxic residues in edible products and environment. So it was thought to investigate the efficacy of Pseudomonas fluorescens, against nematodes. We found that this PGPR help very much in the management of above mentioned nematodes and reduce the populations of these nematodes in the root-systems. This PGPR reduced populations of R. reniformis by 64% in tomato and 69% in capsicum rhizosphere, M. incognita by 72% in tomato and 68% in capsicum. A significant increase was observed in the yields of these crops to the tune of  $19\% \sim 26\%$ . We developed the effective strain of P. fluorescens as bio-nematicide. Developed delivery systems of this bio-nematicide in these crops grown under field conditions. These technologies of bio-management of nematodes using above mentioned PGPR were transferred to the farmers in various regions of India. *Phl D* gene producing DAPG was found to be responsible for the nematicidal action of *P. fluorescens*. Commercialized the effective strain of *P.* fluorescens by generating toxicological data, bio-efficacy data against Radopholus similis, Tylenchulus reniformis, M. incognita and R. reniformis on certain important horticultural crops, by transferring the technology to 45 entrepreneurs in India.

Key words: Bio-nematicide; P. fluorescens; horticultural crops; disease complex

#### Introduction

Excessive chemicalization of horticultural eco-systems is affecting adversely soil natural bio-diversity and biological suppression. This has resulted in the increase of populations of nematodes in the soil. Nematodes such as *Meloidogyne incognita* and *Rotylenchulus reniformis* significantly reduce the yields of many horticultural crops (Rao *et al.*, 2004; Rao and Shylaja, 2004). Surveys also show that these nematodes are widespread and together cause significant losses in yields of in most of the horticultural crops (Rao, 2007, 2008; Rao *et al.*, 2004; Rao and Shylaja, 2004).

Keeping in view the hazardous consequences of the use of chemicals for the control of nematodes, it was thought to evolve strategies to utilize PGPR (Plant Growth Promoting rhizobacteria) for the management of nematodes. *P. fluorescens* has been reported to be effective against various pathogenic bacteria and fungi (Bloemberg and Lugtenberg, 2001; Kishore *et al.*, 2003; Shouan Zhang *et al.*, 2003). Similarly, various researchers have reported the bio-control potential of *P. fluorescens* against the root-knot and other nematodes (Parveen, *et al.*, 1998; Shanthi and Sivakumar, 1995; Siddiqui *et al.*, 1999; Rao *et al.*, 2004; Rao, 2007, 2008).

In these investigations, we thought to develop strategies for the effective utilization of *P. fluorescens* by developing the suitable delivery systems, generate toxicological data, bio-efficacy data against *M. incognita* and *R. reniformis* on certain important horticultural crops and develop it as a bio-nematicide for its commercialization among entrepreneurs in India.

#### **Materials and Methods**

Talc based PGPR formulation of *P. fluorescens* (IIHR-PF-2) of Indian Institute of Horticultural Research (IIHR), Bangalore, was used for enriching the substrates. 5 kg of talc based formulation of *P. fluorescens* ( $2 \times 10^8$  CFU/g) was mixed in 200 kg of de-oiled neem cake or 1000 kg vermi-compost. The enrichment process was done under shade. The enriched substrates were covered and optimum moisture was maintained for 15 days. After every 5 days the heap was turned around for aeration.

The enriched formulations of *P. fluorescens* were used to develop delivery systems and evaluate them in the field infested with *M. incognita* and *R. reniformis* at Indian Institute of Horticultural Research, Bangalore, India, and standardized a strategy for nematode management in tomato (Cv. Arka Vikas) and capsicum (Cv. Indra). Neemcake and vermi-compost enriched with *P. fluorescens* were applied 20 g/m<sup>2</sup> and 100 g/m<sup>2</sup> respectively. These enriched organic inputs were applied in the field before transplanting and also after 45 days after transplanting. The plot size maintained was of  $3 \times 3$  m<sup>2</sup>. Simultaneously the seedlings of tomato and capsicum were also treated by *P. fluorescens* by root dip treatment before planting. The root dip treatment was given to the seedlings of by tomato and capsicum dipping the roots for 5 minutes in a tray containing uniform suspension of *P. fluorescens* in water (CFU  $2 \times 10^8$ /ml).

The treatments were - T1-seedlings of tomato or capsicum treated with *P*. *fluorescens* (CFU  $2 \times 10^8$ /ml); T2 - untreated seedlings sown in the plots mixed with neem cake enriched with *P*. *fluorescens* 20 g/m<sup>2</sup>; T3 - untreated seedlings sown in the plots mixed with vermi-compost enriched with *P*. *fluorescens* 100 g/m<sup>2</sup>; T4-

seedlings treated with *P. fluorescens* sown in plots mixed with neem cake enriched with *P. fluorescens*; T5- seedlings treated with *P. fluorescens* sown in plots mixed with vermi-compost enriched with *P. fluorescens*; T6- Untreated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T7- Untreated seedlings transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T8- seedlings treated with *P. fluorescens* were transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T8- seedlings treated with *P. fluorescens* were transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T10 - untreated seedlings transplanted in the plots with out any treatment, served as control. All the treatments were replicated five times in a completely randomized block design.

Observations on root populations of *M. incognita* and *R. reniformis*, yield per plot and percentage increase in the yield of tomato and capsicum at harvest were recorded.

Colonization of roots by *P. fluorescens* was also recorded at random by uprooting plants after 90 days from a plot. Root colonization by *P. fluorescens* was assessed by following the standard serial dilution technique. One gram of root sample was washed, grounded and the CFU was checked by the serial dilution followed by pour plate method. Serial dilutions up to  $10^{-6}$  concentration were prepared. One ml from each of  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  dilutions were pipetted into the Petri dishes and spread completely in the plate. Freshly prepared King's B agar (*P. fluorescens*) media was poured into each plate and made to spread evenly by pour plate method and allowed for solidification. Three replicates for each dilution were maintained with controls and incubated at  $27\pm1^{\circ}$ C.

After 24 h, *P. fluorescens* colonies on King's B media (King, Ward, and Raney, 1954) emitting a pale green fluorescent light under UV at 302 nm were counted and recorded CFU (Colony Forming Unit).

Root populations of both nematodes were estimated by taking 10g of root (at the rate of 2g of root sample/ plant, which was stained using acid fuchsin) collected at random from 5 plants from each plot homogenized using a homogenizer and counting under stereo-microscope. The data related to the reduction in the nematodes density due to the application of *P. fluorescen* were computed. The data were analyzed using ANOVA.

#### **Results and discussion**

It is interesting that seedling treatment with *P. fluorescens* itself, was able to reduce the number of *M. incognita* and *R. reniformis* in roots of tomato and capsicum to some extent (Table 1 & 2). However it is essential develop the delivery systems in such a way that the bio-agents are augmented more and more in rhizosphere of the crop in the field conditions so that the biological suppression can happen in a sustainable manner. Hence we thought of evaluating two organic inputs (vermicompost and neem cake) to deliver the bio-agents to the field. In our preliminary studies we observed that these organic inputs support the growth and multiplication of *P. fluorescens*. Hence they were enriched with this PGPR (method has been explained in materials and methods) and applied to the beds. Seedling treatment with *P. fluorescens* and application of neem cake enriched with *P. fluorescens* was found to be significantly effective in reducing the population of nematodes. This treatment reduced root-populations of *R. reniformis* by 64% in tomato and 69% in capsicum rhizosphere, *M. incognita* by 72% in tomato and 68% in capsicum (Table 1 & 2). There was significant increase in the yield of tomato by 26% and capsicum by 19%. (Table 3).

*P. fluorescens* has been reported to be effective in the management of root-knot nematodes (Siddiqui *et al.*, 1999, Parveen *et al.*, 1998, Rao *et al.*, 2004; Rao and Shylaja, 2004; Shanthi and Sivakumar, 1995). In our earlier studies we observed that *P. fluorescens* strain producing 2,4 Di Acetyl Phloro Glucinol (DAPG) was found to be more effective in reducing the population of *M. incognita* in vegetable crops (Dhananjay Naik and Rao, 2004). *P. fluorescens* is known to control the pathogen by the mechanism called siderophoric effect. It was also found very effective in the control of various soil borne fungi causing root rot in certain vegetables crops (Mukhopadhaya, 1987). Induction of growth promoting substances from the PGPR (Plant Growth Promoting rhizobacteria) and induction of systemic resistance against pathogenic fungi was reported by Ramamoorthy *et al.*, 2001 and Rao and Shylaja, 2004.

In general the growth of tomato and capsicum plants treated with *P. fluorescens* was better. This could be due to the plant growth promoting activity of *P. fluorescens* which is very well documented (Bloemberg and Lugtenberg, 2001; Kishore *et al.*, 2003; Shouan Zhang *et al.*, 2003).

TREATMENTS	No. of <i>M. incognita</i> in 10 g root of tomato	reduction of <i>M. incognita</i> (%)	No. of <i>R. reniformis</i> in 10 g root of tomato	R. reniformis of M. incognita(%)
T1	24.03	46	21.23	39
T2	15.58	65	14.62	58
T3	17.80	60	17.05	51
T4	12.46	72	12.52	64
T5	16.47	63	16.10	54
T6	23.35	47	21.23	39
T7	29.37	34	26.10	25
Τ8	20.47	54	18.79	46
Т9	21.36	52	19.84	43
T10	44.5		34.8	
CD - 5%	3.46		2.84	_

 
 Table 1
 Effect of application of P. fluorescens enriched neem cake or vermi-compost on root population densities of M. incognita and R. reniformis in Tomato

Values are mean of 5 replicates; T1-seedlings of tomato or capsicum treated with *P. fluorescens*; T2 - untreated seedlings sown in the plots mixed with neem cake enriched with *P. fluorescens* 20 g/m<sup>2</sup>; T3 - untreated seedlings sown in the plots mixed with vermi-compost enriched with *P. fluorescens* 100 g/m<sup>2</sup>; T4- seedlings treated with *P. fluorescens* sown in plots mixed with neem cake enriched with *P. fluorescens*; T5- seedlings treated with *P. fluorescens* sown in plots mixed with vermi-compost enriched with *P. fluorescens*; T5- seedlings treated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T7- Untreated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T10 - untreated seedlings transplanted in the plots mixed with vermi-compost alone  $20g/m^2$ ; T10 - untreated seedlings transplanted in the plots mixed with vermi-compost alone  $20g/m^2$ ; T10 - untreated seedlings transplanted in the plots without any treatment (control).

TREATMENTS	No. of <i>M. incognita</i> in 10 g root of capsicum	reduction of <i>M. incognita</i> (%)	No. of <i>R. reniformis</i> in 10 g root of capsicum	reduction of <i>R. reniformis</i> (%)
T1	22.54	41	19.83	39
T2	15.28	60	13.65	58
T3	16.43	57	15.93	51
T4	12.32	68	10.07	69
T5	14.13	63	14.95	54
Τ6	22.17	42	19.83	39
Τ7	26.74	30	24.38	25
Τ8	18.72	51	17.55	46
Т9	19.86	48	18.53	43
T10	38.2		32.5	—
CD-5%	3.24	_	2.68	_

Table 2	Effect of application of P. fluorescens enriched neem cake or vermi-compost on Capsicum root
	population densities of <i>M. incognita</i> and <i>R. reniformis</i>

Values are mean of 5 replicates; T1-seedlings of capsicum treated with *P. fluorescens*; T2 - untreated seedlings sown in the plots mixed with neem cake enriched with *P. fluorescens* 20 g/m<sup>2</sup>; T3 - untreated seedlings sown in the plots mixed with vermi-compost enriched with *P. fluorescens* 100 g/m<sup>2</sup>; T4- seedlings treated with *P. fluorescens* sown in plots mixed with neem cake enriched with *P. fluorescens*; T5- seedlings treated with *P. fluorescens* sown in plots mixed with vermi-compost enriched with *P. fluorescens*; T6- Untreated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T7- Untreated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T10 - untreated seedlings transplanted in the plots without any treatment (control).

Seedling treatment followed by substrate treatment using the combination of *P*. *fluorescens* and neem was more effective followed by *P*. *fluorescens* enriched vermicompost than either of the treatments individually in both the crops (Table 1, 2 & 3). Application of neem cake was reported to be very effective in the management of nematodes through variety of mechanisms including the production of antimicrobial compounds and induction of systemic resistance (Alam and Khan, 1980; Mankau, 1962; Muller and Gooch, 1982).

Application of *P. fluorescens* with neem cake also did not affect the root colonization. Rather neem cake helped in the increased root colonization of *P. fluorescens* in both crops (Table 3). Root colonization of *P. fluorescens* was more when the PGPR was applied along with neem cake in comparison to its application

along with vermi-compost, in both the crops (Table 3). Both neem cake and vermicompost when enriched with *P. fluorescens* are able to support the growth and multiplication of this PGPR. However when it is applied along with neem cake it had an increased bio-efficacy and we assume that neem cake application is changing the root surface phenomenon and making it more favorable for higher colonization of this PGPR. We always observed that higher the root colonization of *P. fluorescens*, higher was its bio-efficacy. It was our objective to develop an effective delivery method which helps in the rapid augmentation of this PGPR in the crop rhizosphere. Through these investigations, we could standardize a cost effective delivery system for the effective use of this PGPR under the field conditions for the sustainable management of nematodes on these important vegetable crops.

After evaluating the nematicidal potential of *P. fluorescens* against *M. incognita* infecting egg-plant, tuberose, gerbera, carnations and gladioli, we also developed bio-efficacy data on *Radopholus similis* infecting banana and *Tylenchulus semipenetrans* infecting acid lime in more than 4 seasons. Simultaneously generated bio-efficacy data from different agro-climatic regions of India.

Further we generated toxicology data of primary culture and formulation, container content compatibility data and commercialized this bio-nematicide among 45 bio-pesticide industries in India.

TREATMENTS	Root colonization of <i>P. fluorescens</i> $(CFU/g \times 10^{6})$ in tomato	Root colonization of <i>P. fluorescens</i> (CFU/g $\times 10^6$ ) in capsicum	increase in yield of tomato (%)	increase in yield of capsicum (%)
T1	3.0	1.7	08	06
T2	6.3	4.0	10	14
T3	4.1	2.3	15	12
T4	8.3	6.4	26	19
T5	5.3	3.6	21	16
T6	0	—	06	05
Τ7	0	_	04	04
Т8	3.6	2.5	12	08
Т9	4.5	2.0	10	05

 Table 3 Effect of application of P. fluorescens enriched neem cake or vermi-compost on

 PGPR root-colonization and yield Tomato and Capsicum

T10	0	_	_	
CD-5%	0.76	0.45	_	_

Values are mean of 5 replicates; T1-seedlings of capsicum treated with *P. fluorescens*; T2 - untreated seedlings sown in the plots mixed with neem cake enriched with *P. fluorescens* 20 g/m<sup>2</sup>; T3 - untreated seedlings sown in the plots mixed with vermi-compost enriched with *P. fluorescens* 100 g/m<sup>2</sup>; T4- seedlings treated with *P. fluorescens* sown in plots mixed with neem cake enriched with *P. fluorescens*; T5- seedlings treated with *P. fluorescens* sown in plots mixed with vermi-compost enriched with *P. fluorescens*; T6- Untreated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T7- Untreated seedlings transplanted in plots mixed with neem cake alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T9- seedlings treated with *P. fluorescens* were transplanted in plots mixed with vermi-compost alone  $20g/m^2$ ; T10 - untreated seedlings transplanted in the plots without any treatment (control).

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## Bioremediation of Pentachlorophenol (PCP)-Polluted Soil by Plant Growth promoting Rhizobacteria (PGPR)

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#### Abstract

Pentachlorophenol (PCP) is used as an insecticide and a leather and wood preservative as well. It is present in tannery effluents and also formed unintentionally in effluents of paper and pulp industries. Microbes and plants are among the most important biological agents that remove and degrade waste materials to enable their recycling in the environment. Rhizosphere interactions between plants and microbial communities benefit both. The bacteria receive nutrients from root exudates and the plants enhance nutrient uptake and reduction in the toxicity of soil contaminants. In the present investigation, a number of plant species were evaluated for their ability to tolerate different concentrations of pentachlorophenol (PCP) in the soil. The toxic effect of PCP on plants was studied by monitoring seed germination, plant growth and biomass. Although PCP had a negative effect on all the plant species tested, maize and groundnut showed the maximum tolerance to PCP. Other tolerating crops included wheat, safflower, sunflower, and soybean. From the rhizosphere soil of the tolerant seedlings, as many as 27 PCP tolerant rhizobacteria and 19 endophytic bacteria were isolated and assessed for PCP degradation efficiencies. The efficient PCP degrading isolates were evaluated *in vitro* for plant growth promoting activities such as P- solubilisation, IAA production, HCN production and Antibiosis. Most of the isolates solubilized TCP. Many rhizobacteria and endophytes showed good results for IAA production, HCN production and Antifungal activity against Rhizoctonia solani, indicating the overall suitability of PCP degrading bacteria in sustainable agriculture.

Key words: PCP; bioremediation; PGPR; plant growth promotion

#### Introduction

Pentachlorophenol (PCP) is a widely used biocide. It is highly substituted aromatic compound and widely used as the herbicide, fungicide, and wood preservative. Due to continuous use of PCP as pesticides, appreciable quantities of pesticides and their degraded products may accumulate in the soil ecosystem. Biodegradation of organic compounds is affected by the plant-microbial interactions in the rhizosphere, which apparently offers a favorable environment for co-metabolism of soil-bound and recalcitrant chemicals. The microbial transformation of organic compounds is probably helped by the abundance of energy provided in root exudates. Rhizosphere interaction between plants and microbial communities, including PGPB, benefits both organisms. The bacteria receive nutrients from root exudates and the plants have enhanced nutrient uptake and reduction in the toxicity of soil contaminants. Soil microorganisms are also known to produce biosurfactants that may facilitate removal of organic pollutants.

The present study evaluated the PCP impact on the germination and growth of different crops over a different concentration and *in vitro* evaluation of plant growth promoting substances by rhizobacteria and endophytic bacteria of PCP tolerating crops.

#### **Materials and Methods**

#### Screening of crop species to tolerate PCP

Ten different crop species viz., Green gram, Black gram, Soybean, Sunflower, Safflower, Bengal gram, Maize, Ground nut, Wheat and Horse gram were tested for tolerance to different concentrations of Pentachlorophenol (PCP) ie., 0 mg/kg, 25 mg/kg, 50 mg/kg. At 30 days after sowing, effect of PCP on seed germination, root and shoot length and biomass were measured and tabulated.

#### Sampling and isolation of predominant bacteria

Soil samples collected from the rhizosphere of crops grown in the PCP polluted soils as per the treatments were appropriately diluted and plated out on Nutrient Agar plates. After 48 h of incubation at 37 °C, the predominant bacteria were picked up and purified. Root samples of PCP tolerant plant species were collected and the predominant endorhizosphere bacteria were isolated and purified. Similarly, the endophytic bacteria were isolated from the leaves of PCP tolerant plant species. All the bacterial isolates of rhizosphere and endophytic were point inoculated on mineral salts medium containing PCP 25 mg/kg and incubated at 37 °C for 4 $\sim$ 6 days and checked for their growth. BTB was added to the medium (0.5% in ethanol) as the pH

indicator. And, the isolates were initially tested for their growth on PCP at 25 mg/kg and later on 50 mg/kg.

#### Production of growth promoting substances by the isolates

Qualitative assessment of the PCP degrading bacteria

All the bacterial isolates were subjected to qualitative analysis for P- solubilisation using Pikovasky's medium amended with TCP, IAA production (Bric and Bastock, 1991), HCN production using King's B with 4.4 % glycine as per the method of Wei *et al.*, (1991) and *in vitro* screening of the isolates for antagonistic inhibition of *Rhizoctonia solani* following the dual culture assay (Ganesan and Gnanamanickyam, 1987).

#### **Results and Discussions**

Table 1 shows the effect of PCP on seed germination and relative lengths of shoot and root at 30 DAS. Seed germination had a downward trend at 25 mg/kg against the control. While 50 mg/kg concentration showed further inhibitory influence on seed germination as compared to their relevant controls. Although PCP had negative effect on all plant species tested, maize and groundnut showed maximum tolerance to PCP at 25 mg/kg compared to 50 mg/kg. The root and shoot length of these two plant species were dramatically increased at  $0 \sim 25$  mg/kg but decline was observed at 50 mg/kg. As compared to total length the mean total length of maize crop showed significantly higher at 0 and 25 mg/kg compared to 50 mg/kg. Ferro et al., (1994) used 100 mg PCP/kg soil in an experiment with hycrest crested wheatgrass [Agropyron desertorum (Fisher ex Link) Schultes]. Other tolerating crops included wheat, safflower, sunflower, and soybean. The effects of different PCP concentration on fresh shoot, root and total biomass are listed in Table 2. It was observed that different concentration of PCP posed inhibitory effects on shoot and root biomass on most of the plant species tested. It revealed that maize and ground nut were significantly different (P>0.01) as compared to their respective controls. A significant (P > 0.01) shoot and root inhibition was seen at 50 mg/kg PCP in all the plant species tested. Overall as PCP levels increased total biomass production in all the crops dropped significantly. Mills et al. (2006) illustrated the influence of PCP and its cocontaminants such as Cr, As, Cu and B on plant growth. They also showed as PCP levels increased total biomass production in both poplar and willow dropped significantly.

In the present investigation, totally 27 bacteria were isolated from rhizosphere soils of different plant species grown in presence of 0mg/kg, 25mg/kg and 50

mg/kg.From soybean, 8; sunflower, 3; safflower 8; maize 2; groundnut and wheat, 3 each isolates were made on 25 mg/kg. Chu and Kirsch (1972) isolated a bacterial strain, KC- 3, from industrial waste- water capable of mineralizing PCP. Haggblom *et al.* (1988) isolated two isolates with the ability to mineralize PCP. The degradation efficiency of each isolate was monitored by its growth in the medium. The efficient isolates produced yellow colored colonies on a mineral salt medium containing PCP and Bromothymolblue. Totally 18 endophytic bacteria were isolated from roots and leaves of different plant species grown in presence of 0 mg/kg, 25 mg/kg and 50 mg/kg. From soybean, 2 each from leaves and root; 2 from sunflower root; 1 from leaves and 4 from root of safflower; 1 from leaves and 4 from root of groundnut and one each from leaves and root of wheat. Results here suggest that the endophytic community composition, size and response to contamination, is plant species depend.

Table3 shows the qualitative assessment of rhizobacteria for plant growth promoting substances. Most of the isolates showed positive for P-solubilisation. Almost seventeen rhizobacteria and ten endophytic isolates could solubilize phosphate supplemented with TCP (Table4). Both rhizobacteria and endophytes showed overall good results for IAA production and HCN production. Almost twenty three rhizobacterial and seven endophytic isolates showed antibacterial activity against the pathogen *Rhizoctonia solania*. These plant growths promoting rhizobacteria (PGPR) that increase the plant tolerance can vigorously promote plant growth, resulting in more rapid and massive biomass accumulation (Glick 2003). They work by preventing stress ethylene synthesis and providing auxins to the roots .The result is much greater biomass (especially roots) and therefore faster remediation (Glick and Holguin, 1998).

Hence both plants and soil microorganisms have some limitations in their individual abilities to remove and break down organic compounds. For example, even plants that are relatively tolerant of various environmental contaminants often remain underdeveloped, or slowly grow in the presence of the contaminants. A technology combining plant and microorganisms, mainly PGPB, may promote a synergistic action, leading to improved plant growth. This may overcome many of the limitations of single organisms, and provide a useful and more powerful approach for enhancing remediation of contaminated environments.

Crear	Treatmen	Germination	Shoot length	Root length	Total length
Crops	ts	(%)	(cm/plant)	(cm/plant)	(cm/plant)
D11-	0 mg/kg	44.98 d-h	4.12 g-k	26.00 a-e	31.68 c-g
Black	25 mg/kg	34.99 f-i	4.76 g-k	8.50 g-l	29.10 c-g
gram	50 mg/kg	10.00 ij	3.6 h-k	4.93 j-l	8.53 g-i
	0 mg/kg	74.97 a-c	12.18 с-е	39.22 a	60.83 b
Soybean	25mg/kg	29.99 g-i	8.00 e-i	12.17 e-l	31.33 c-g
	50 mg/kg	0.00 j	0.00 k	0.00 1	0.00 i
	0 mg/kg	69.97 a-d	9.67 d-g	7.00 i-l	23.86 d-h
Sunflower	25 mg/kg	54.98 b-g	8.00 e-i	6.39 j-l	20.72 d-i
	50 mg/kg	39.98 e-h	7.63 e-i	4.89 h-l	15.00 e-i
	0 mg/kg	64.97 a-e	8.56 e-h	11.12 f-l	26.71 c-h
Safflower	25 mg/kg	34.99 f-i	5.63 f-j	8.10 g-l	13.73 e-i
	50 mg/kg	29.99 g-i	2.67 i-k	2.13 kl	5.10 hi
Damaal	0 mg/kg	39.98 e-h	14.08 cd	13.33 e-l	30.83 c-g
Beligai	25 mg/kg	24.99 h-j	8.00 e-i	6.50 i-l	14.50 e-i
gram	50 mg/kg	10.00 ij	4.67 g-k	4.67 k-l	9.33 f-i
	0 mg/kg	89.96 a	17 abc	39.00 a	98.33 a
Maize	25 mg/kg	79.97 ab	16.94 a-c	34.72 ab	86.67 a
	50 mg/kg	49.98 c-h	16.22 bc	31.19 ac	47.42 bc
	0 mg/kg	89.96 a	7.08 e-j	20.25 c-i	36.75 с-е
Groundnut	25mg/kg	59.98 b-f	7.06 e-j	18.92 c-j	41.48 b-d
	50 mg/kg	49.98 c-h	7.00 e-j	21.78 b-g	28.78 с-д
	0 mg/kg	69.97 a-d	21.83 a	24.08 b-f	82.17 a
Wheat	25 mg/kg	39.98 e-h	19.33 ab	29.00 a-d	61.17 b
	50 mg/kg	24.99 h-j	10.5 d-f	20.92 c-h	25.61 c-h
Horse	0 mg/kg	69.97 a-d	5.58 f-j	15.42 d-k	32.33 c-f
gram	$2\overline{5} \text{ mg/kg}$	10.00 ij	1.73 jk	2.67 kl	4.40 hi
gram	50 mg/kg	0.00 j	0.00 k	0.001	0.00 i

### Table I Effect of different concentrations of PCP on seed germination and shoot

#### and root length of different crop species

Values are the means of three replications  $\pm$ SE. Variants possessing the same letters (a-j) are not statistically significant at P < 0.01

	Fresh Biomass (g/plant)				
		Shoot		Total biomass	
Crops	Treatments	biomass	Root biomass		
	0 mg/kg	0.47 kl	0.37 f	0.83 fg	
	25 mg/kg	0.40 kl	0.17 f	0.57 fg	
Black gram	50 mg/kg	0.20 kl	0.13 f	0.33 fg	
	0 mg/kg	2.97 c-f	0.63 ef	4.60 cd	
	25mg/kg	1.30 h-k	0.17 f	1.47 fg	
Soybean	50 mg/kg	0.001	0.00 f	0.00 g	
	0 mg/kg	2.67 d-g	1.03 ef	3.70 de	
	25 mg/kg	1.73 g-j	0.43 f	2.17 ef	
Sunflower	50 mg/kg	0.93 j-l	0.27 f	1.20 fg	
	0 mg/kg	1.03 i-l	0.30 f	1.33 fg	
	25 mg/kg	0.67 j-l	0.20 f	0.87 fg	
Safflower	50 mg/kg	0.20 kl	0.10 f	0.30 fg	
	0 mg/kg	2.07 f-i	0.30 f	2.37 ef	
	25 mg/kg	0.93 j-l	0.40 f	1.33 fg	
Bengal gram	50 mg/kg	0.33 kl	0.13 f	0.47 fg	
	0 mg/kg	3.57 cd	5.83 a	9.40 a	
	25 mg/kg	3.13 с-е	3.97 b	7.10 b	
Maize	50 mg/kg	2.33 e-h	2.33 cd	4.67 cd	
	0 mg/kg	7.23 a	3.00 bc	10.23 a	
	25mg/kg	5.90 b	3.33 bc	9.23 a	
Groundnut	50 mg/kg	3.77 c	2.33 cd	6.10 bc	
	0 mg/kg	1.70 g-j	4.00 b	5.70 bc	
	25 mg/kg	0.63 j-l	1.67 de	2.30 ef	
Wheat	50 mg/kg	0.27 kl	1.00 ef	1.27 fg	
	0 mg/kg	1.67 g-j	3.33 bc	5.00 cd	
Horse gram	25 mg/kg	0.071	0.33 f	0.40 fg	
-	50 mg/kg	0.001	0.00 f	0.00 g	

#### Table II Effect of different concentrations of PCP on fresh biomass of different crop species

Values are the means of three replications ±SE. Variants possessing the same letters (a-j)

re not significantly different at P < 0.01.

		Plant growth promoting activities of the isolates					
S1.	Code No. of the						
No.	Isolates	P- solubilization	IAA -Production	HCN Production	Antibiosis		
1	ASOY1	-	-	-	+		
2	ASOY2	+	+	+	+		
3	ASOY3	-	+	+	+		
4	ASOY4	+	+	+	-		
5	ASOY5	+	+	+	-		
6	ASOY6	-	+	+	+		
7	ASOY7	+	-	+	+		
8	ASOY8	+	+	+	+		
9	ASUN1	+	+	-	+		
10	ASUN2	+	-	-	+		
11	ASUN3	+	-	-	-		
12	ASAFF1	-	+	+	+		
13	ASAFF2	+	+	+	+		
14	ASAFF3	-	+	+	+		
15	ASAFF4	-	-	-	+		
16	ASAFF5	-	-	+	+		
17	ASAFF6	-	-	+	+		
18	ASAFF7	-	+	+	+		
19	ASAFF8	+	+	+	+		
20	AGRN1	-	-	-	+		
21	AGRN2	+	-	+	+		
22	AGRN3	+	+	+	+		
23	AMAZ1	+	+	+	+		
24	AMAZ2	+	+	+	+		
25	AWHT1	+	+	+	-		
26	AWHT2	+	+	+	-		
27	AWHT3	+	+	+	-		

#### Table 3 Plant growth promotional activities of the PCP degrading rhizobacterial isolates

		Plant growth promoting activities of the isolates						
S1.	Code No. of the							
No.	Isolates	P- solubilization	IAA -Production	HCN Production	Antibiosis			
1	SOY <sub>rt</sub> 1	+	+	-	-			
2	SOY <sub>rt</sub> 2	-	+	+	-			
3	SAF <sub>rt</sub> 1	-	-	+	-			
4	SAF <sub>rt</sub> 2	+	+	+	-			
5	SAF <sub>rt</sub> 3	+	+	+	+			
6	SAF <sub>rt</sub> 4	+	+	+	+			
7	SUN <sub>rt</sub> 1	+	+	+	+			
8	SUN <sub>rt</sub> 2	+	-	-	+			
9	SUN <sub>rt</sub> 3	-	-	-	+			
10	GRN <sub>rt</sub> 1	+	-	+	-			
11	GRN <sub>rt</sub> 2	-	-	+	-			
12	GRN <sub>rt</sub> 3	-	+	-	-			
13	GRN <sub>rt</sub> 4	+	+	+	+			
14	WHT <sub>rt</sub> 1	+	+	+	-			
15	SOY <sub>lf</sub>	+	+	+	-			
16	SOY <sub>lf</sub>	+	+	+	+			
17	SAF <sub>lf</sub>	-	+	+	-			
18	GRN <sub>lf</sub>	-	-	-	-			
19	WHT <sub>lf</sub>	+	-	+	-			

Table 4 Plant growth promotional activities of the PCP degrading Endophytic isolates

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# The use of coconut fiber and antagonistic *Pseudomonas* CMR12a for biological control of *Rhizoctonia* damping-off on

#### chinese cabbage (*Brassica rapa*)

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#### Abstract

Isolated from cocoyam roots in Cameroon, *Pseudomonas* CMR12a has been explored as a promising antagonistic agent due to its capacity to produce phenazines and biosurfactants. CMR12a and mutants of this strain impaired in phenazine and/or biosurfactant production, were applied at a concentration of  $10^7$  CFU g<sup>-1</sup> soil to control damping-off caused by *Rhizoctonia solani* AG 2-1 on chinese cabbage. Data recorded 14 days after inoculation indicated that disease severity had decreased from 3.5 to 1.42 in CMR12a-treated seedlings. In soil inoculated with phenazine- or biosurfactant-deficient mutants, seedlings were partially protected and disease score was 2.29 and 1.96, respectively. However, the disease suppressive effect was completely lost in soil inoculated with a mutant impaired in the production of both compounds. These data suggest that both phenazines and biosurfactants were responsible for the profound effects of *Pseudomonas* CMR12a.

In another experiment, coconut fiber (CF) was incorporated into soil at the concentration of 0.1, 1 or 5% to reduce viability of sclerotia produced by this fungus. After 4 weeks of incubation with 5% CF, the viability of *R. solani* sclerotia was dramatically decreased from 75% to 20% while the mean number of sclerotia killed by mycoparasitism was significantly raised from 4% to 66%. The incorporation of 5% CF into soil also resulted in a steady increase in population of fluorescent pseudomonads and *Trichoderma* spp. which might contribute to the death of sclerotia.

Generally, it can be concluded that both phenazines and biosurfactants are involved in the biocontrol ability of *Pseudomonas* CMR12a towards *Rhizoctonia* damping-off on chinese cabbage. In addition, viability of *Rhizoctonia* sclerotia could be effectively reduced by incorporating 5% CF into soil.

Key words: *Rhizoctonia solani*; sclerotia; phenazines; biosurfactants; coconut fiber; biocontrol

#### Introduction

Chinese cabbage (*Brassica rapa*) is a vegetable which is universally available in almost all countries and cultures. Besides being known for its great taste, chinese cabbage is an excellent source of vitamins and minerals. In Asia, this type of vegetable has been grown on a large scale and it is present in the diet of local inhabitants. However, the increase in production of this vegetable also results in the spread of *Rhizoctonia solani*, a soil-borne pathogen which is widely distributed among soils all over the world. Young or newly emerged cabbage seedlings are highly susceptible to damping-off disease induced by several different anastomosis groups (AGs) of *R. solani* (Sneh *et al.*, 1991). Infected plants have dark, water-soaked to brown discoloration on their stem before they collapse (Koike *et al.*, 2006).

*R. solani* can survive for a long period in plant debris, contaminated seeds, or infested soils as mycelium or sclerotia. Due to the presence of melanin, sclerotia of this fungus are protected from a number of adverse impacts and may serve as a source of primary inoculum in soil (Jacobson, 2000). It has been shown that the incorporation of lignin-rich organic amendment into soil can reduce the persistence of *Rhizoctonia* sclerotia (Soltani *et al.*, 2002; Debode *et al.*, 2005; Van Beneden *et al.*, 2010). In addition, actively growing hyphae of this fungus can be effectively controlled when *Pseudomonas* CMR12a, a rhizospheric bacterium which has capacity to produce phenazine and biosurfactant antibiotics, is applied to soil (D'aes *et al.*, 2011). Therefore, the objectives of our study were to investigate the biocontrol effect of *Pseudomonas* CMR12a towards Rhizoctonia damping-off on chinese cabbage and the potential of coconut fiber to reduce the viability of *Rhizoctonia* sclerotia.

#### **Materials and Methods**

## Screening Pseudomonas CMR12a and CMR12a-mutants for biocontrol capacity towards damping-off disease on chinese cabbage

Chinese cabbage seeds *Brassica rapa* cv. Excellent (Het Vlaams Zaadhuis, Waarschoot, Belgium) were germinated on wet filter paper in Petri dishes one day before they were sown in substrate containing 50% sand and 50% soil (Structural; Snebbout, Kaprijke, Belgium) (w/w).

*R. solani* BK008-2-1 used in this study was one of the most pathogenic isolates collected from Belgian cauliflower fields (Pannecoucque *et al.*, 2008). This isolate belongs to anastomosis group (AG) 2-1 and its virulence towards chinese cabbage was confirmed under *in vitro* and *in vivo* conditions (unpublished data). Inoculum of *R. solani* was produced as previously described by Scholten *et al.* (2001). Briefly, actively growing hyphae from a 3-day-old PDA culture of *R. solani* AG 2-1 were

used to inoculate sterile wheat kernels kept in 250 ml Erlenmeyer flasks. These flasks were incubated at  $28^{\circ}$ C for 14 days before they were used for inoculation.

Bacterial inoculum used in this study included *Pseudomonas* CMR12a and its mutants deficient in the production of phenazines (CMR12a- $\Delta$ Phz), biosurfactant type 1 (CMR12a-CLP1) or both metabolic compounds (CMR12a- $\Delta$ Phz-CLP1) (D áes *et al.*, 2011). These bacteria were collected from overnight cultures on King ś B plates (King *et al.*, 1954) and applied to soil at a concentration of 10<sup>7</sup> CFU g<sup>-1</sup> soil before sowing. Four days after, six wheat kernels colonized by *R. solani* AG 2-1 were used to infect a group of three cabbage seedlings grown in a plastic box containing 600 g soil substrate. All plants were incubated in a growth chamber (18°C, RH = 60%, 16 h photoperiod) and disease severity was evaluated 14 days after inoculation based on disease severity rating scale ranging from 0 to 4 where 0 = healthy, no symptoms observed; 1 = small brown, water-soaked lesion covering less than 25% of stem and/or hypocotyl; 2 = large lesion covering less than 50% of stem or hypocotyl; 4 = hypocotyl is completely decayed and seedling dead.

A completely randomized design was employed with 4 replications per treatment. Data were subjected to non-parametric Kruskal-Wallis tests to compare mean scores of all treatments. Then, the Mann-Whitney analyses were used to compare means pairwise at P = 0.05. All statistic tests were conducted in SPSS 15.0 (SPSSinc, Illinois, USA).

#### Pot assay with coconut fiber and sclerotia of Rhizoctonia solani AG 2-1

Soil used in this experiment was collected from a commercial field in Bottelare (Belgium). Before amendment with coconut fiber at the concentration of 0.1%, 1% and 5%, moisture content of this soil was measured and adjusted to 16.5% (w/w) by adding sterile water. Soil without coconut fiber served as control.

Sclerotia of *R. solani* AG 2-1 were produced according to Manning *et al.* (1970). Batches of five sclerotia were placed inside one nylon mesh bag and five bags were buried in each glass jar containing 200 g soil substrate.

The jars were kept at 22 °C for four weeks and sclerotia were removed for surface sterilization in 1% sodium hypochlorite followed by rinsing two times with sterile water. Then, each surface-disinfected sclerotium was cut in half. The viability of the sclerotia was tested by plating one half on PDA amended with streptomycin (100 mg L<sup>-1</sup>) and prochloraz (4 mg L<sup>-1</sup>). The other half of the sclerotium was placed on PDA amended with streptomycin (100 mg L<sup>-1</sup>) to evaluate mycoparasitism. Then, plates were incubated for two weeks at 22 °C. The growth of *Rhizoctonia* hyphae and the identification of mycoparasites based on their morphological characteristics started from day six after incubation.

Changes in *Pseudomonas* and *Trichoderma* population in soil were determined using a soil dilution technique. From each jar described, two replicate soil samples were taken. For *Pseudomonas* counting, serial dilutions of soil samples in sterile physiological solution were spread on Pseudomonas Isolation Agar (PIA, Difco). After 24 h of incubation at 28°C, colonies were counted and transferred to KB to check their fluorescence under UV light. On the other hand, in order to determine *Trichoderma* population, sterile water was used to prepare soil suspensions. The plating was carried out on Trichoderma selective medium (Williams *et al.*, 2003) and plates were incubated at 22°C for four days before counting.

Statistical analysis of data was done with the software package SPSS 15.0 (SPSSinc, Illinois, USA). Viability and mycoparasitism of the sclerotia were analysed using binary logistic regression (P=0.05). Population density data of *Pseudomonas* and *Trichoderma* were log<sub>10</sub> transformed and analysis of variance was performed.

#### **Results and Discussion**

## *In vivo* biological control of root rot caused by *Rhizoctonia solani* AG 2-1 on chinese cabbage by *Pseudomonas* CMR12a and its mutants

Each treatment in this *in planta* experiment was conducted with 24 plants grouped into 4 replications. Data collected 14 days after inoculation showed that the disease severity varied among treatments (Figure1). Disease severity on plants was significantly reduced when soil was treated with either *Pseudomonas* CMR12a, CMR12a- $\Delta$ Phz or CMR12a-CLP1. CMR12a appeared to be the most powerful biocontrol agent. The average disease score of seedlings grown in soil inoculated with CMR12a was 1.42, whereas the mean score in plants belonging to diseased control was 3.5. Due to the loss in capacity to simultaneously synthesize phenazines and biosurfactants, protection by mutants impaired in the production of one of these metabolites was lower than that of the wild type although the difference was not statistically significant in the treatment inoculated with the biosurfactant-deficient mutant. Compared to the parental strain, biocontrol capacity of a mutant deficient in both biosurfactant and phenazine production, was completely lost.



Figure 1 Effect of *Pseudomonas* strain CMR12a and its mutants on the severity of Rhizoctonia damping-off on chinese cabbage. Control treatment was treated with sterile physiological solution and diseased control treatment was infested with *R. solani* AG 2-1 strain BK008-2-1. Different letters indicate significant differences among treatments (*P*<0.05).

From the obtained data, it seemed that *Pseudomonas* CMR12a can effectively control *R. solani* on chinese cabbage. The biocontrol capacity of *Pseudomonas* CMR12a can be explained by the production of phenazine and biosurfactant antibiotics. These results are similar to the investigation of D'aes *et al.* (2011) about the role of these metabolic compounds in biocontrol of Rhizoctonia root rot disease on bean.

#### Effect of coconut fiber on viability of Rhizoctonia sclerotia

The effectiveness of coconut fiber incorporation in inhibiting the germination of *R*. *solani* AG 2-1 sclerotia was evaluated. After 4 weeks of incubation, the difference in percentage of viable sclerotia corresponding to the variation in the amount of coconut fiber added to soil was observed. Only 20% viable sclerotia were detected when they were buried in soil amended with 5% CF. Soil containing 1% CF also showed a slight

decrease in sclerotial viability (58% compared to 75% of non-amended control treatment) but this reduction was not statistically significant (Table 1).

It has been suggested before that the addition of lignin-rich organic materials might favor the growth of indigenous lignin composers (Debode *et al.*, 2005). Ligninolytic enzyme activities of these microorganisms can also destroy melanin, a stable component present in the cell wall of *Rhizoctonia* sclerotia (Butler and Day, 1998). When melanin is degraded, sclerotia become more susceptible to both biotic and abiotic stress (Gomez and Nosanchuk, 2003; Butler et al., 2005). Analysis of the quantity of sclerotia parasitized by fungi in our study showed that there was a significant raise in the mean number of parasitized sclerotia when soil was supplemented with either 1% or 5% CF. Due to the difference in morphology, these colonizing fungi could be classified into 3 groups including Trichoderma spp., Fusarium spp. and other fungi (data not shown). The incorporation of 5% CF into soil also favored population growth of *Trichoderma* spp. and fluorescent pseudomonads (Table 1). Therefore, we assumed that the growth of lignin degrading fungi is stimulated in coconut fiber-amended soils and these fungi are responsible for melanin degradation of sclerotia. When melanin is broken, sclerotial bodies of R. solani can be easily attacked by other microorganisms such as mycoparasites and *Pseudomonas* spp. and they lose their viability.

Traatmants	Viable	Total non-viable	Population density (log CFU g <sup>-1</sup> soil)	
ricatilients	(04)	sclerotia (%) <sup>a,b</sup>	Pseudomonas	Trichoderma
	(%)		spp.	spp.
Control	75.0 a	4.0 c (21.0)	2.43 b	0.79 b
0.1% coconut fiber	75.0 a	22.0 c (2.0)	2.53 b	0.74 b
1% coconut fiber	58.0 a	30.0 b (12.0)	2.67 ab	0.91 b
5% coconut fiber	20.0 b	66.0 a (14.0)	3.09 a	3.17 a

Table 1Percentage of viable and non-viable sclerotia and population density of *Pseudomonas* spp. and<br/>*Trichoderma* spp. in non-amended soil (control) and in soil amended with 0.1%, 1% and 5%<br/>coconut residue after 4 weeks of incubation at 22℃

<sup>a</sup> Percentage of sclerotia that were killed due to mycoparasitism

<sup>b</sup> Values between brackets represent percentage of sclerotia dead naturally after introducing into soil

Obviously, the incorporation of CF at a concentration of 5% might decrease the viability *R. solani* AG 2-1 sclerotia. These results are in agreement with previous findings of Soltani *et al.* (2002), Debode *et al.* (2005) and Van Beneden *et al.* (2010) about the degradation of melanized structures such as sclerotia of *R. solani* due to the addition of lignin-rich organic material to soil.

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### Paenibacillus polymyxa M-1, a potential anti-bactericide against Erwinia

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#### Abstract

The plant growth promoting rhizobacterium (PGPR) *Paenibacillus polymyxa* M-1 isolated from surface sterilized wheat roots can suppress the growth of two bacterial phytopathogens, *Erwinia amylovora* Ea273 and *Erwinia carotovora, in vitro*. By MALDI-TOF mass spectrometry (MALDI-TOF MS) and reversed-phase high-performance liquid chromatography (RP-HPLC), two antibacterial compounds were detected in M-1 culture supernatant. The active principle acting against the two *Erwinia* strains was isolated from TLC plates and identified by structural analysis using postsource decay (PSD)-MALDI-TOF mass spectrometry (PSD-MALDI-TOF MS). Whole genome sequencing revealed the presence of a gene cluster encoding the synthetase of the antibacterial compounds detected. Identical morphological changes of the bacterial phytopathogens treated with either antibacterial compounds or culture supernatant of M-1 were observed by scanning electron microscopy (SEM). In a word, our results showed that *P. polymyxa* M-1 was a potential bio-bactericide candidate which might be used to control *E. amylovora* and *E. carotovora*.
## Diversity analysis of culturable bacteria in the root of

## tree peony (Paeonia ostii)

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## Abstract

The distribution of culturable bacteria in the bulk soil, rhizosphere, and rhizoplane of tree peony plants was investigated in this study. A total of 507 bacterial strains with different colony characteristics were isolated. There were 46 operational taxonomic units (OTUs) which were identified by amplified ribosomal DNA restriction analysis (ARDRA). Representatives of each group were selected for partial 16S rRNA gene sequencing and phylogenetic analysis. Results showed that the major bacteria group in the bulk soil, rhizosphere and rhizoplane were Firmicutes (63.2%), Actinobacteria (36.3%) and Betaproteobacteria (53.0%), respectively. The most common genus in the bulk soil were Bacillus (49.6%), in the rhizosphere were Microbacterial community was more diverse than the rhizoplane and bulk soil bacterial communities. Our results show that obvious differences exist among the culturable bacterial communities in the bulk soil, rhizosphere and rhizoplane. It is obviously that a selective pressure of tree peony plants on their associated bacterial populations occurred.

Key words: Culturable bacteria; diversity; ARDRA; tree peony (Paeonia ostii)

## Introduction

Plant-associated bacteria reside in the rhizosphere, phyllosphere, and inside the tissues of healthy plants, which are diverse in their ability to affect plant health, their genotypic and phenotypic characteristics, and their phylogeny (Beattie, 2006). Some of them are designated as plant-growth promoting bacteria (PGPB). Plant-associated bacteria have been isolated from many crop plant species (Rosenblueth and Martinez-Romero, 2006), including rice (Engelhard, *et al.*, 2000), soybean (Kuklinsky-Sobral, *et al.*, 2004), potato(Asis and Adachi, 2004), wheat (Coombs and Franco, 2003), maize (Zinniel, *et al.*, 2002), and some strains have developed as biofertilizers (Podile and Kishore, 2006). But, only a little of researches focus on ornamental plant-associated bacterial community and their applications. Until now, relatively little

information was available regarding the bacterial community associated with tree peony.

The tree peony is an important ornamental plant indigenous to China, which belongs to the section Moutan in the genus *Paeonia*, Paeoniaceae. In China, tree peony has been cultivated since the Dongjin Dynasty 1600 years ago. In spite of these, many cultivars with good ornamental traits can't grow well in some specific areas because of the bad soil and climate conditions. For example, some Zhongyuan and Xibei cultivars can't grow very well in the south of Yangtze River in China. Based on the characteristics of plant grow-promoting bacteria strains, we think it's a good way to screen and apply these strains on the tree peony cultivation. So, it's the beginning to investigate the plant-associated bacterial community for the application of the PGPR strains.

## **Materials and Methods**

Soil samples were obtained from Luoyang National Peony Garden (Luoyang, Henan Province, China), according the methods described by Han (Han, *et al.*, 2009) with some modifications. Rhizospheric and rhizoplane soil samples from the root domain of variety 'Fengdan' were collected randomly at a depth of 5cm to 15 cm from the stem base, with each plant at least 50 m from each other.

Rhizospheric, rhizoplane, and soil bacteria were isolated according to the previous procedures (Courchesne and Gobran, 1997; Han, *et al.*, 2005), with LB, TSA, YG, R2A, 0.1LB and KB plates. In all cultivation experiments, the agar plates were incubated in the dark for 3 days at 28 °C. Based on the colony characteristics, single colonies were selected and stored in 15% glycerol at -80 °C for further study.

The DNA of bacterial isolates was prepared according to the procedures of Park (Park, *et al.*, 2005). The 16S rRNA genes were amplified from genomic DNA by PCR using the primers 27F and 1378R (Watanabe, *et al.*, 1998). Amplification products were digested using the four base-cutting restriction enzyme *Msp*I and *Alu*I (1 U) at 37 °C for ARDRA analysis (Costa, *et al.*, 2006). The restricted products were electrophoresed in 2.5% agarose gel and then the patterns in the gels were compared. Representative phylotypes were sequenced on an ABI 3100 DNA sequencer by Chinese National Human Genome Center (Shanghai, China). An operational taxonomic unit (OTU) was defined as a 16S ribosomal DNA (rDNA) digestion group in a same profile in ARDRA. Phylotype richness (S) was calculated as the total number of OTUs. The Shannon-Wiener index was calculated as (Martin, 2002). CHIMERA-CHECK program of the Ribosomal Database Project II (RDP II) (Maidak, *et al.*, 1999). The most similar sequences were searched within the NCBI database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search

## Tool (BLAST) and the sequences obtained in this study were deposited in GenBank. **Results and Discussion**

507 isolates obtained from the root of 'Fengdan' plants were subjected to ARDRA analysis by digestion of the amplified 16S rRNA gene with *Rsa*I and *Alu*I. Same banding patterns obtained after the double digestions were grouped and defined as an OTU (Operating Taxonomy Unit). 161 isolates from rhizosphere were grouped into 21 OTUs; 66 isolates from rhizoplane were grouped into nine OTUs; 280 isolates from the bulk soil were grouped into 18 OTUs (Table 1). Phylotype Shannon-Wiener index (H) of the bacterial communities in the bulk soil, rhizosphere and rhizoplane of tree peony plants were calculated as 2.41, 2.71 and 1.87, respectively.

Representatives of each group were selected for partial 16S rRNA gene sequencing so as to retrieve sequence similarity and bacterial identity from sequence databases. All of the bacterial isolates were assigned to five phyla within the domain Bacteria, namely Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, and Actinobacteria. The bulk soil isolates were represented by three phyla, which were Firmicutes (63.2%), Betaproteobacteria (17.2%) and Actinobacteria (19.6%). All the isolates from bulk soil were assigned to nine genera. The members of the genus Bacillus were the major taxon among them, which was 49.6%. The rhizospheric isolates were represented by five phyla, which the majority of the isolates falls within the Actinobacteria groups with 36.3%. All the rhizospheric bacterial isolates were assigned 10 genera. The four bacterial genera, Microbacterium (21.1%), Bacillus (15.5%), Variovorax (18.6%), and Pseudomonas (16.8%), represented 72% of the isolates from the rhizospheric of tree peony plants. The phylogenetic analysis indicated that the isolates from the rhizoplane could also be grouped into four phyla, Betaproteobacteria (53.0%), Actinobacteria (19.7%), Alphaproteobacteria (16.7%) and Gammaproteobacteria (10.6%), respectively. All the bacterial isolates assigned rhizoplane were eight genera. Members of Betaproteobacteria were predominant (53.0%) and all isolates in this group were *Variovorax*, which also was the major genera (Table 2).

Media	Bulk soil	Rhizospheric soil	Rhizoplane soil	Total OTUs
R <sub>2</sub> A	15	18	6	37
LB	8	11	4	23
0.1LB	10	11	5	26
YG	13	20	5	37
KB	9	9	4	22
TSA	9	11	4	22

Table 1 OTUs of bacterial populations in the root domain of tree peony with different media

Phylogenetic group	Genus	Species	Bulk soil (280)	Rhizosphere (161)	Rhizoplane (66)
	Agromyces	aruantiacus allii italicus	11	18	2
	Arthrobacter	pascens sulfonivorans	10	8	
	Agrococcus	jenensis			
	Cellulosimicrobi um	cellulans			
		arborescens insulae	10 1	8	9
Actinobacteria		thalassium	6		
	Microbacterium	hominis		21	
		trichotecenolyti cu		5	
		flavescens			
		phyllosphaerae			2
	Mycobacterium Nocardioides	neoaurum hankookensis	14		2
	Streptomyces	gulbargensis	3		
	Agrobacterium Bosea Ensifer Nitratireductor	tumefaciens eneae adhaerens basaltis			5
	Phenylobacteriu m	koreense			
Alphaproteobacteria	Sphingopyxis	ginsengisoli witflariensis		5	5
	Sphingobium	estrogenivorans			1
	Sinorhizobium	meliloti			
	Cupriavidus	respiraculi	48		
	cuprurraus	taiwanensis		1	20
Deterrete che starie		koreensis soli		30	20
Бегаргогеобастепа	Variovorax	paradoxus			15
Gammaproteobacteri		gummosus		6	
a	Lysobacter	daejeonensis			

# Table 2 Distribution of representative bacterial taxa in the bulk soil and rhizosphere, rhizoplane of tree peony plants<sup>†</sup>

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	Pseudomonas	niabensis brassicacearum chlororaphis fluorescens migulae putida resinovorans sp. thivervalensis		1 8 18	
	Xanthomonas	campestris			7
Phylogenetic group	Genus	Species	Bulk soil (280)	Rhizosphere (161)	Rhizoplane (66)
		bataviensis		8	
		cereus		6	
		drentensis	34		
		firmus	4		
		horneckiae	1		
		indicus	5		
	Racillus	megaterium			
	Ducinus	niabensis	9	6	
		niacini		3	
Firmicutes		pumilus	14		
		psychrodurans	9		
		pichinotyi		1	
		soli		1	
		sp			
	Paenibacillus	pabuli	6		
	Sporosarcina	psychrophila		2	
	Sporosurcina	soli		5	
	Staphylococcus	epidermidis			
	Terribacillus	saccharophilus	32		

<sup>\*</sup>Numbers indicate strains assigned to each species, and those in parentheses are the total numbers of isolates.

In total, the bacterial isolates comprised 18 genera, among which 9 were presented in the bulk soil, 10 in the rhizosphere, and 8 in the rhizoplane roots. Although isolates of *Agromyces*, and *Microbacterium* were found in all three root domains, many isolates were found only in a single domain. For example, strains of *Streptomyces*, *Nocardioides*, *Paenibacillus* and *Terribacillus* were only found in the bulk soil; strain of *Sporosarcina* was only found in the rhizosphere and strains of *Xanthomonas*, *Agrobacterium*, *Mycobacterium*, and *Sphingobium* were only found in the rhizoplane (Table 2).

*Pseudomonas* and *Bacillus* are considered as important constituents in the rootassociated microbial community, and their ability to colonize the root surface, preventing the development of plant pathogens and improving plant growth, is well known (Park, *et al.*, 2005). However, we were surprised that no members of this genus were found in the rhizoplane of tree peony variety plants. In fact, no members of Firmicutes were isolated in the rhizoplane. As of now, it is unknown whether the absence of Firmicutes in rhizoplane of tree peony plants is a common phenomenon. But what's worth mentioning is that tree peony is not only a kind of ornamental plant, but also has been used in traditional Chinese medicine as an antimicrobial or anti-inflammatory, which the main effective components are paeonol and paeoniflorin (Yan, *et al.*, 2004). At present, we don't know whether and how the plant-associated bacterial community is influenced by these antimicrobial components in tree peony plants.

This study provides basic information about the diversity of bacteria associated with tree peony, a traditional famous ornamental plant species in China. While this study of bacterial diversity, based on a culture-dependent approach, has its own limitations, future work is needed to compare these results with those obtained with culture-independent approaches.

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## Using metagenomic tools for biocontrol: analysis of moss- and lichenassociated microbial communities

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## Abstract

Bryophytes and lichens represent interesting bio-resources; their intense interaction with microorganisms is only partly understood. Recently, it was shown that both groups of cryptogams are colonized at high abundances by specific microbial communities. They are involved in important functions for the moss plantlets as well as for the lichen symbiosis. To characterize biodiversity of associated microbial communities, we applied a combination of methods: phylogenetic diversity, spatial organization and specificity were assessed using clone libraries, fluorescent in-situ hybridization (FISH) and SSCP fingerprinting. These data were placed into an ecological context by multivariate analysis using CANOCO software. Furthermore, metagenomic and metaproteomic data sets were explored. In addition, isolates were investigated regarding their interaction with plants. According to our results, cryptogam-associated microbial communities comprise a unique pool of highly diverse, mostly uncultivable and host-specific microorganisms. Structure of the communities is strongly dependent on a-biotic and biotic factors. Associated bacteria have a function for nutrient supply and pathogen defence. Knowledge about diversity and function was used to isolate bacteria for biotechnological applications. A high proportion of isolates showed antifungal activities and a remarkable plant growth promotion by nitrogen fixation, phosphate solubilisation and production of phytohormons as well as an extraordinarily high biocontrol potential.

**Key words:** metagenomics ; environmental proteomics ; *Sphagnum* mosses ; lichens; biocontrol; plant growth promotion

## Introduction

Sphagnum bog ecosystems belong to the oldest vegetation forms with more or less constant conditions for thousands of years. By coverage of four millions square kilometers they contribute to approx. 3% of the Earth surface. They have a high value for biodiversity conservation serve as a reservoir of fresh water, for human welfare and for our world climate due to its extraordinary role in carbon sequestration (Farrell & Feehan, 2008). The latter resulted in a net cooling effect on the global radiation balance (Dise, 2009). Sphagnum mosses form a unique habitat for microorganisms. High acidity (pH 3.5 to 5.0), low temperature, water saturation together with oxygen deficiency and extremely low concentration of mineral nutrients are characteristic abiotic factors. Furthermore, Sphagnum leafs are highly specialized: they form a special tissue of living, chlorophyll-containing chlorocytes and dead, noncytoplasmatic hyalocytes, which are responsible for the huge potential to store water. Recently, we could show that *Sphagnum* mosses are colonized at high abundances with specific microorganisms, which fulfill important functions for moss growth and health (Opelt et al., 2007a, b). These bacterial communities show a contrasting biodiversity in comparison to the surrounding plant communities (Opelt et al., 2007c).

Lichens represent another form of life of so-called cryptogams and represent symbioses of fungi with a photosynthetic partner (algae and/or cyanobacteria). Recent studies based on molecular analyses showed that bacteria form a third functional aspect of the lichen symbiosis (Cardinale *et al.*, 2008, 2011). All investigated lichens contain a highly diverse bacterial community, which prompted us to expand the traditional concept of lichens (Grube *et al.*, 2009).

The objective of this study was to assess the potential of *Sphagnum*- and lichenassociated bacterial communities for biocontrol and plant growth promotion in agricultural concepts. Therefore, we used a polyphasic approach to study the ecology of bacteria including metagenomics and environmental proteomics. New approaches were included to isolate bacterial strains for biotechnological approaches.

## **Material and Methods**

The samples of two species of *Sphagnum* (*S. fallax* and *S. magellanicum*), which play different roles in bogs ecosystem and spread widely enough were collected in three geographical points in Austria (Rotmoos, Waasenmoos, Pürgschachen Moor). The bacteria used in this study were isolated from the lichens species *Cladonia arbuscula*, *Umbilicaria cylindrica, Lecanora polytropa* and *Lobaria pulmonaria*. The lichens specimens were sampled in Austria (Styria), Portugal (Madeira), Switzerland (St. Gallen) and Norway (Bergen).

Fresh material  $(2 \sim 4g)$  was washed with sterile sodium chloride solution (0.85%) and transferred into a sterile stomacher bag. The samples were suspended in NaCl (0.85%) and homogenised for 3 min by agitation. The samples were serially diluted and plated onto R2A agar medium (Difco, Detroit, USA) for 5 days at 20°C (Opelt and Berg, 2004). After incubation in R2A medium, the colonies were counted for calculation to determine the mean number of colonies (CFU) based on fresh weight. The total-community DNA of bacterial-cell consortia was extracted using FastPrep Instrument (Qbiogene, BIO101, Carlsbad, USA) for 30 s at speed 5.0. The extracted DNA was purified by using the Geneclean Spin Kit (Qbiogene, Bio 101, Carlsbad, Calif.) according to the manufacturer's protocol.

Bacterial isolates were screened for their activity toward phytopatogenic fungi according to the protocol of Berg *et al.* (2002) and for their plant growth promotion activity on radish. The seeds of radish cv. "Red"("Dom semyan" Company, St.Petersburg, Russia) were surface sterilized as follow: 2 min. with 70% ethanol and three washes with sterile water for 3 min. Sterile seeds were inoculated with bacterial suspension in 0.85% NaCl solution adjusted to10<sup>7</sup> cfu/ml for 15 minutes. As a negative control, seeds were treated with sterile 0.85% NaCl solution. The seedlings were incubated under sterile conditions at 28°C for 5 days, and length of roots and stems was measured after incubation.

To analyse the taxonomic composition of the soil bacterial community, the hypervariable V4-V5 region of the 16S rRNA gene (*E. coli* positions 515 to 927) was amplified in a nested PCR approach for pyrosequencing. For taxonomy-based analysis, the web server SnoWMAn 1.7 (http://snowman.genome.tugraz.at) was employed.

A metaproteomic approach was then used to analyze structure and function of the symbiotic consortium. Proteins extracted from two lichens samples of *L. pulmonaria* (replicate I and II; sampled in April 2009 in Switzerland) were analyzed by onedimensional gelelectrophoresis (1D-SDS-PAGE) combined with LC-MS/MS and the resulting MS and MS/MS data were searched against a database consisting of protein sequences obtained from the public UniRef100 database (Schneider *et al.*, 2011).

## **Results and discussion**

#### Diversity of Sphagnum-associated bacterial communities

In a first step, the bacterial community composition of *Sphagnum fallax* and *Sphagnum magellanicum* was analyzed by establishment of 16S rRNA gene clone

libraries. Bioinformatic analysis of the obtained clones was followed by reconstruction of the phylogenetic trees. 16S rRNA gene fragment clone libraries were created using total microbial DNA isolated from both S. fallax and S. magellanicum. Up to 97% of all cloned sequences were affiliated to uncultivable microorganisms. Taxonomic position and ratio of the most abundant groups differ species. S. fallax-associated community is dominated between moss by Verrucomicrobia, Planctomycetes and Alphaproteobacteria, whereas S. magellanicum is mostly colonized by Alpha-, Deltaproteobacteria and Bacteroidetes, followed by Betaproteobacteria and Planctomycetes. Bacterial clones and closely related microorganisms from the databases represent wide spectra of physiological functions, environments and geographical locations; especially remarkable are nitrogen-fixers and clones isolated from the acidic Sphagnum peat bogs of Siberia. To get a deeper insight into the Alphaproteobacteria community, a pyrosequencing approach was used. Both mosses were colonized by members of 24 different genera of Alphaproteobacteria, several of them well-known for their beneficial effect on plants, e.g. Azospirillum, Caulobacter, Methylobacterium and Rhizobium.

## Study of the spatial organization of the microbial communities associated with moss plantlets in situ

FISH-CLSM was established to study the occurrence of two taxonomical groups abundant in both *Sphagnum* species – Alphaproteobacteria and Planctomycetes. Bacteria vary in morphology from coccoid, vibrio to rod-shaped-cells, forming microcolonies, clusters or chains. Several conglomerates detected in the basal cells of stem leaves were formed by specifically stained cells and cells that hybridized only with a general eubacterial probe. Bacteria were also found to be associated with fungal hyphae and algal cells. Species-specific colonization were confirmed by bacterial colonization patterns.

## Screening for antagonistic isolates with antifungal and antibacterial activity

The screening of isolates for antagonistic activity against phytopathogenic fungi resulted in 60% positive isolates but a lower number of isolates with antibacterial activity against phytopathogenic bacteria ( $15\% \sim 30\%$ ). For study of ability of moss associated bacteria to colonize the roots of cultivated plants the isolates with maximum antagonistic activity were selected (Shcherbakov *et al.*, 2011). The inoculation of sterile seeds of radish was performed and seedlings were incubated under sterile conditions. It was shown that about 15 cultivable isolated strains of *Sphagnum*associated bacteria can colonize the roots of radish and promoted the growth of seedlings. Increased length of roots and stems of inoculated radish seedlings has been observed. Furthermore, it was shown that four isolates inhibited the growth of seedlings which can be explained by high number of cells in the inoculum or the physiological and biochemical properties of tested strains.

## Bacteria have important functions in lichens

Investigations on bacterial isolates and functional genes suggested that they are involved in nitrogen fixation, antibiosis and nutrient cycling (Grube *et al.*, 2009). Environmental proteomics was used to gain a first insight into other bacterial functions (Schneider *et al.*, 2011). A major proportion of bacterial proteins appeared to be involved in posttranslational modifications and protein turnover. Furthermore, several proteins were involved in synthesis of secondary metabolites. Although lichens are well-known for the richness of secondary metabolite production, almost only products of fungal origin were studied so far. We are convinced that bacteria associated with lichen are a rich source for pharmaceutical research with new lead compounds.

## Lichens are an interesting reservoir for biotechnologically relevant bacteria

A set of 263 selected bacterial strains were tested for i) lytic abilities (chitinases, proteases, glucanases and lipases), ii) solubilization of phosphate, iii) mobilization of iron, and vi) production of auxin. The analyzed strains showed a high hydrolytic activity: proteolytic (47%), chitinolytic (29.2%), glucanolytic (29.3%), and lipolytic (20%). In addition, numerous bacteria demonstrated phosphate-solubilizing activity (16.7%), the excretion of siderophores (48%), or production of auxin (21%). One third of the lichen-associated bacteria have the potential to produce PHA biopolymers (Gasser *et al.* 2011). Interestingly, the strains showed a remarkable high antagonistic potential against plant pathogens; up to 100% were antagonistic especially against leave pathogens such as *Alternaria alternata*. This suggests that the long-living lichens protect themselves against parasites, a potential, which can be used for biocontrol.

## Prospects for application of cryptogam-associated bacteria in agricultural biotechnology

Cryptogams, such as the studied mosses and lichens are often long-time adapted to special, sometimes extreme, environments and present in these habitats interesting and long-living ecological niches for bacteria. The bacteria are adapted to beneficially interact with their host. Owing to these interactions in a living habitat, these bacteria are an interesting bio-resources for agricultural biotechnology. New molecular and microscopic tools help to identify these bacteria-to a large proportion unculturable bacteria-and to find new isolation and enrichment procedures (Köberl *et al.*, 2011). For example, using an environmental proteomic approaches, we found that bacteria are involved in secondary metabolite production, which can be use as indicator of antagonistic bacteria. For mosses we used a metagenomic approach, which revealed diverse plant growth promoting bacteria within the Alphaproteobacteria group.

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## Resuscitation of indigenous endophytic bacteria in Eucalyptus urophylla after inoculation of *Bacillus subtilis* strain CN030

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## Abstract

Endophytic bacteria have been found widely in plant tissues and most of them can not be cultured yet. Some of these bacteria can even survive in harsh conditions with high osmotic pressure. In our previous studies, endophytic bacteria were discovered in seedlings germinated from surface sterilized seeds of *Eucalyptus urophylla*. In this experiment, the culturability of indigenous endophytic bacteria was tested by inoculation of in vitro grown seedlings of E. urophylla with the endophytic Bacillus subtilis strain CN030, isolated from Pinus elliottii. Strain CN030 was inoculated at a population density of 10<sup>8</sup> CFU/ml and its fermentation solution. The results showed that, some endophytic bacteria became culturable from the 5<sup>th</sup> day onward after inoculation, and bacterial species isolated were different with times. More than 6 dominant genera were obtained, most of which were Gram positive. Four of them were identified by 16S rDNA gene sequencing and homology analysis in database RDP (Ribosomal Database Project). Among them, strain No.1 had a similarity of 99.0% to Brevibacterium linens and Br. aureum; strain No.2 had a similarity of 99.5% to Pantoea agglomerans; strain No.3 a similarity of 99.5% to Bacillus subtilis subsp. subtilis; and strain No. 4 a similarity of 99.1% to B. subtilis subsp. subtilis and B. amyloliquefaciens. The fermentation solution of CN030 could not induce the resuscitation of indigenous endophytic bacteria. We conclude that living cells of strain CN030 can stimulate the culturability of indigenous endophytic bacteria in E. urophylla.

## Section – IV Poster Session – Lectures

## Efficacy and crop tolerance evaluation of Ato Cide for control of bacterial leaf spot of lettuce and angular leaf spot of cucumber

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## Abstract

Agri Neo is the Canadian company located in Montreal, Canada and is planning to develop several products to combat plant pathogens against various vegetable crops. One of their primary product named as Ato Cide has been evaluated for control of bacterial leaf spot (BLS) disease in greenhouse grown lettuce and angular leaf spot (ALS) in cucumber. Interest in developing new effective fungicides has augmented with the increase in development of advanced disease resistant pathogens to existing fungicides. The costs to discover, develop and market the new fungicide are currently around 23 to 25 million U.S. Dollars. In order for the new developed fungicide to be profitable it needs to be protected by patent rights until the manufacturer gets reasonable returns on the investment. Therefore, economic and environmental considerations play a bigger role in developing and testing the fungicide. Besides, the fungicide being safe for user, consumer and environment the new product should have an edge over other existing alternative treatments. Hence, the research objective was to evaluate the efficacy and crop tolerance of Ato Cide for control of BLS in greenhouse grown lettuce and ALS in cucumber. BLS is an economically important disease caused by bacterial pathogen Xanthomonas campestris py. vitans in lettuce and ALS caused by *Pseudomonas syringae* pv. *lachrymans* in cucumber. Greenhouse experiments were conducted at Plant Science Research Center, Auburn University, and Auburn, USA. Experiments with vegetables lettuce cv. Iceberg and cucumber cv. Burpless were conducted using randomized complete block design (RCBD). The following treatments were included such as healthy control, pathogen control, standard (KOCIDE), and Ato Cide 0.5, 1, 2, 5 and 10 g/L of water on Lettuce and healthy control, pathogen control, standard (KOCIDE), and Ato Cide 2, 5, 10 and 20 g/L of water on Cucumbers. Overall, there was a significant control of BLS and increase of plant vigor of lettuce and ALS control in cucumber with Ato Cide compared to pathogen control. Our results suggest that the use of Ato Cide has a commercial potential for suppression of BLS on lettuce and ALS in cucumber under greenhouse conditions. More work is warranted under field conditions before Ato Cide is being released for commercial use both in Canada and USA.

## Introduction

Bacterial Leaf Spot (BLS) of lettuce caused by *Xanthomonas campestris* pv. *Vitans* is a widespread disease and can result in significant losses to the growers. Substantial economic losses were reported in California, Ohio, and Quebec, Canada. Contaminated seed have been suggested as a possible source of these temporally associated outbreaks in various lettuce-growing areas around the globe. So far, only lettuce has been reported to be a natural host of this bacterium in the field. Symptoms of BLS initially consist of water-soaked leaf lesions that rapidly become black, followed by tissue collapse. Later, lesions become dry and papery in appearance. BLS generally does not reduce plant size, but renders lettuce unmarketable due to the unsightly leaf blemishes. Early symptoms appear on the outer older leaves as small ( $^{1}/_{8}$  to  $^{1}/_{4}$  inch) water soaked lesions (Koike, 2009). The diagnostic feature of the disease is black angular lesions. Lesions are typically angular in shape because the pathogen does not penetrate or cross the veins in the leaf. In severe disease conditions, numerous lesions may coalesce, leading to the collapse of the leaf.

During warm, wet weather, bacterial angular leaf spot can cause serious yield and quality loss to cucumbers. The bacterium, Pseudomonas syringae pv. lachrymans (*Pseudomonas lachrymans*), also infects other cucurbits including zucchini, squash, honeydew melon, muskmelon, and watermelon. Heavy infections of cucumber can occur during extended rainy periods when plant tissues become filled with water. On leaves the bacterium causes small, angular, water soaked areas which later turn brown or straw-colored. Leaf lesions are delimited by the veins, hence the angular appearance of the lesions. Under humid conditions, a white, milky exudate, consisting of bacteria, forms on the lesions and dries to form a thin, white crust (Delahaut and Stevenson, 2004). Sometimes dead tissue falls away, giving the leaf a ragged shothole appearance. Infections on fruits are first small, circular, water-soaked and soft but older lesions are chalky and cracked. These spots often crack open and turn white in color. Rot may extend internally and predispose infected fruit to secondary bacterial soft rot. If attacked when very young, the fruit may fall off the plant. The bacterium can overwinter in seed and on diseased plant debris in the field. Splashing rain spreads bacteria from the soil to plant parts and from plant to plant. The organism is easily spread in the field by cultivation equipment, harvesters, and by wind-blown rain. Angular leaf spot is most active between  $75 \sim 82 \,\text{F} (24 \sim 28 \,\text{C})$  and is favored by high humidity (Hansen, 2008).

Wet and cool conditions are ideal for infection and disease development by both the pathogens either on lettuce or cucumber. Overhead irrigation and rain water splashing disperses the pathogen in the field and enables to infect significant number of plants. Bacterium can survive in seed, soil and weeds. Residual bacterial inoculum, left in the soil following an infected crop, will potentially infect a subsequent planting. Therefore, timely crop rotations are recommended if the bacterial leaf spot is chronic in heavily planted lettuce and cucumber fields. Elimination or minimizing the use of overhead sprinkler irrigation system will significantly reduce the disease. Use of identified resistance varieties is clearly an option for disease control. However, chemical control is the most cost effective. The objectives of this study were to evaluate the efficacy and crop tolerance of Agri-Neo Company's "Ato Cide" for control of BLS in greenhouse lettuce and ALS in cucumber.

## **Materials and Methods**

Two separate greenhouse experiments for lettuce and cucumber vegetables were conducted at the Plant science Research Center, Auburn University, Auburn, USA. Leaf lettuce cultivar Iceberg and Cucumber cultivar Burpless was used to produce seedlings. Seeds were planted in seedling trays, one seed per cavity and allowed to grow for 4 weeks under greenhouse conditions. After 4 weeks the seedlings were transplanted in six inch sized pots filled to the top with soilless media (premier peat), one seedling per pot. The experimental layout was randomized complete block design (RCBD), consisting of eight treatments (lettuce) and seven treatments (cucumber), 10 replications per treatment, with one pot per replication. Replicated pots were arranged on a greenhouse bench in a RCBD fashion. Standard fertigation (NPK) was used during the growth of the seedlings and normal agronomic conditions were maintained throughout the experiment. The pots were maintained at  $26\pm2$ °C, relative humidity (RH) of 90% and photoperiod of 16 h and grown for 45 days.

BLS pathogen, *Xanthomonas campestris* pv. *vitans* and ALS pathogen, *Pseudomonas syringae* pv. *lachrymans* were obtained from infested leaves of lettuce and cucumber, respectively. The pathogens were maintained for long term storage at  $-80^{\circ}$ C in a tryptic soy broth (TSB) containing 20% glycerol (NB, Difco Laboratories, Detroit, MI, USA) until needed. For both experiments, the strains were grown in individual flasks of NB on a shaker at 200 rpm for 48 h. Cultures were centrifuged for 10 min at 7,000 rpm, the supernatant was decanted, the bacterial pellets were resuspended in sterile distilled H<sub>2</sub>O, and the suspension was centrifuged again as described. The resultant bacterial pellets were adjusted approximately to  $3.79 \times 10^{11}$  CFU/gallon ( $1 \times 10^9$  CFU/ml) spectrophotometrically. Bacterial concentrations were confirmed by dilution plating. Approximately 0.03 fluid of inoculum was applied to each plant using a handheld sprayer (Model D, R & D Sprayers, Opelousas, LA) at 7 and 10 days after transplanting (DAT) for cucumber and lettuce, respectively. To

enhance disease development, 5 min of overhead irrigation was applied immediately prior to bacterial inoculations.

Treatments for Lettuce	Treatments for Cucumber
TRT 1: Untreated, uninoculated control	TRT 1: Untreated, uninoculated control
TRT 2: Untreated, inoculated control	TRT 2: Untreated, inoculated control
TRT 3: Ato Cide 0.5 g/ L of water	TRT 3: Ato Cide 2 g/ L of water
TRT 4: Ato Cide 1 g/ L of water	TRT 4: Ato Cide 5 g/ L of water
TRT 5: Ato Cide 2 g/ L of water	TRT 5: Ato Cide 10 g/ L of water
TRT 6: Ato Cide 5 g/ L of water	TRT 6: Ato Cide 20 g/ L of water
TRT: 7 Ato Cide 10 g/ L of water	TRT 7: Standard treatment - KOCIDE
TRT 8: Standard treatment - KOCIDE	

Table 1 Treatment description and number of treatments applied for lettuce and cucumber

Immediately after inoculation the pathogen challenged plants were placed in a humid chamber for 48 hours and then moved to controlled growth rooms maintained at  $28\pm2$ °C,  $80\% \sim 90\%$  relative humidity (RH) and photo period of  $10\sim14$  h day or night, respectively. Within 3 days after the first bacterial inoculation, the first treatments were applied. Thereafter each treatment was applied on a bi-weekly basis. For treatment application, calculated amount of Ato Cide was mixed in water and each treatment was applied using a handheld sprayer. DuPont<sup>TM</sup> Kocide® 3000 was used for the standard treatment of BLS and ALS. Treatment application rates were listed in Table 1 for both experiments.

Lettuce plants were harvested 45 DAT, earlier than standard harvesting time due to notice of bolting symptoms. Cucumber plants were harvested 51 DAT. Disease incidence and disease severity were assessed in each pot one week before harvest. Disease incidence was determined by counting the number of bacterial spot lesions present on the leaves in each treatment replicates. A mature outer leaf from each replicate pot was separated and the number of lesions were counted and recorded. Disease severity was evaluated by rating the most severely damaged area on the plant on a scale of 1 to 9 with 1 being no disease and 9 being completely blighted plant tissue. The numbers 2 and 3 represent few angular lesions, with less than 5% leaf tissue affected; 4 and 5 have big lesions with  $10\% \sim 15\%$  tissue damage; 6 and 7, the lesions coalesce causing up to 25% tissue damage; 8 being more than 40% tissue injury due to coalesce of lesions. Plant vigor was also visually analyzed and rated based on the photosynthesis characteristic leaf color. Vigor was ranked on a scale of "1 to 5" where, 1= very poor; 2= poor; 3= good; 4= very good; and 5= Excellent. The recorded data was analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means and standard error (S.E) were differentiated by a Tukey's honest significant difference (HSD) at P = 0.05 using PROC-GLM.

### **Results and Discussion**

There was significant difference among the treatments applied for the control of BLS in lettuce, with P value 0.0001. Among different treatments applied, disease (lesion) count was maximum (32.50) in untreated and inoculated treatment (T2). No, BLS symptoms were noticed in healthy control (T1). There was a significant reduction of BLS by the Ato Cide treatments irrespective of its concentrations. But, the highest reduction in BLS lesion count (4.60) was observed with Ato Cide 10 g/L of water treatment (T7) (Table 2). This was followed by standard treatment (T8) with mean value of 4.80 (Table 2). In severe disease conditions numerous small lesions coalesced to form big necrotic lesions, sometimes leading to the death of partial/whole leaf. Figs. 1 and 2 represent BLS symptoms and treatment comparison during different crop growth stages.

Table 2Mean number of bacterial leaf spot lesions per leaf  $\pm$  S.E in lettuce leaf sampled before harvest

Treatment <sup>1</sup> description	BLS lesions per leaf <sup>2</sup>
TRT 1: Untreated, uninoculated control	$0.70^{d}$
TRT 2: Untreated, inoculated control	32.50 <sup>a</sup>
TRT 3: Ato Cide 0.5 g/ L of water	14.50 <sup>b</sup>
TRT 4: Ato Cide 1 g/ L of water	9.10 <sup>bc</sup>
TRT 5: Ato Cide 2 g/ L of water	7.70 °
TRT 6: Ato Cide 5 g/ L of water	8.70 <sup>bc</sup>
TRT 7: Ato Cide 10 g/ L of water	4.60 <sup>cd</sup>
TRT 8: Standard treatment - KOCIDE	4.80 <sup>cd</sup>

<sup>1</sup>Treatments were applied bi-weekly three days after the pathogen applied until harvest except healthy and pathogen control. Rate applied refers to one application and not the over-all amount applied. <sup>2</sup>Mean number of bacterial leaf spot lesions (BLS) per mature leaf  $\pm$  S.E. Means followed by the same letters in the columns is not significantly different at  $P \le 0.05$  according to HSDs for mixed model. There were 10 replicates in each treatment. BLS lesions are mean values from 10 mature leafs per treatment.

Fungicide rates (treatments) had a significant effect (P= 0.0001) on disease severity and plant vigor of lettuce plants. The BLS affected plants, applied with Ato Cide have disease severity highest (4.90) on plants treated with Ato Cide 0.5 g/L of water (T3) and lowest (1.50) in plants treated with Ato Cide 10 g/L of water (T7) (Table 3). This indicates that increase in Ato Cide rate have significantly reduced the disease severity (Table 3). Ato Cide provided excellent control of BLS under the conditions tested in lettuce. There was no phytotoxicity observed in any of the Ato Cide treated plants.

Table 3 Mean disease severity per treatment rated on 1 to 9 scale in lettuce plants sampled before harvest

Treatment <sup>1</sup> description	Disease severity <sup>2</sup>
<b>TRT 1:</b> Untreated, uninoculated control	$0.90^{d}$
TRT 2: Untreated, inoculated control	$8.00^{a}$
<b>TRT 3:</b> Ato Cide 0.5 g/ L of water	4.90 <sup>b</sup>
<b>TRT 4:</b> Ato Cide 1 g/ L of water	3.60 <sup>bc</sup>
<b>TRT 5:</b> Ato Cide 2 g/ L of water	2.20 <sup>cd</sup>
<b>TRT 6:</b> Ato Cide 5 g/ L of water	1.70 <sup>cd</sup>
<b>TRT 7:</b> Ato Cide 10 g/ L of water	1.50 <sup>d</sup>
TRT 8: Standard treatment - KOCIDE	$1.40^{d}$

<sup>1</sup>Treatments were applied bi-weekly three days after the pathogen applied until harvest except healthy and pathogen control. Rate applied refers to one application and not the over-all amount applied. <sup>2</sup>Mean disease severity per treatment in lettuce plants. Means followed by the same letters in the columns is not significantly different at  $P \le 0.05$  according to HSDs for mixed model. Disease severity was evaluated (1= no disease; 2 and 3 represent few angular lesions, with less than 5% leaf tissue affected; 4 and 5 have big lesions with 10% ~15% tissue damage; 6 and 7, the lesions coalesce causing up to 25% tissue damage; 8 being more than 40% tissue injury due to coalesce of lesions; 9= completely bighted tissue), then per plant treatments were averaged across all plants within each treatment replicate.

Plant vigor has shown an upward trend with the increase of Ato Cide rate with the maximum vigor identified was  $(4.80 \pm 0.26)$  for standard treatment (T8) and closely followed by T7 (4.70) (Table 4). It is very interesting to note, most of the lettuce plants treated with Ato Cide were very healthy and greenish compared to healthy control (Table 4).

There was significant difference among the treatments applied for the control of BLS in cucumber, with P value 0.0001. Among different treatments applied, disease (lesion) count was maximum (25.80  $\pm$  1.48) in untreated and inoculated treatment (T2). No, BLS symptoms were noticed in healthy control (T1). There was a significant reduction of BLS lesions by Ato Cide treatments irrespective of its concentrations. But, the highest reduction in BLS lesion count (2.40  $\pm$  1.48) was observed with standard treatment (T8) (Table 5). This was followed by Ato Cide 20 g/L of water treatment with mean value of 2.80  $\pm$  1.48 (T6) (Table 5). In severe disease conditions numerous small lesions coalesced to form big necrotic lesions, sometimes leading to the death of partial/whole leaf. Figs. 3, 4 and 5 represent BLS applied treatment comparisons during the time of harvest.

 Table 4
 Mean plant vigor per treatment rated on 1 to 5 scale in lettuce plants sampled before harvest

Treatment <sup>1</sup> description	Vigor rating <sup>2</sup>
TRT 1: Untreated, uninoculated control	4.50 <sup>ab</sup>
TRT 2: Untreated, inoculated control	$1.10^{d}$
TRT 3: Ato Cide 0.5 g/ L of water	3.10 <sup>c</sup>
TRT 4: Ato Cide 1 g/ L of water	3.40 <sup>bc</sup>
TRT 5: Ato Cide 2 g/ L of water	$4.00^{ m abc}$
TRT 6: Ato Cide 5 g/ L of water	4.20 <sup>abc</sup>
TRT 7: Ato Cide 10 g/ L of water	$4.70^{a}$
TRT 8: Standard treatment - KOCIDE	$4.80^{a}$

<sup>1</sup>Treatments were applied bi-weekly three days after the pathogen applied until harvest except healthy and pathogen control. Rate applied refers to one application and not the over-all amount applied. <sup>2</sup>Mean plant vigor rated on a scale of 1 to  $5 \pm S.E$  in lettuce plants. Means followed by the same letters in the columns is not significantly different at  $P \le 0.05$  according to HSDs for mixed model. Plant vigor was evaluated (1= very poor; 2= poor; 3= good; 4= very good; and 5= Excellent), then per plant treatments were averaged across all plants within each treatment replicate.

Table 5 Mean number of angular leaf spot lesions per leaf on cucumber leaf sampled before harvest

Treatment <sup>1</sup> description	ALS lesions per leaf <sup>2</sup>
TRT 1: Untreated, uninoculated control	3.60 <sup>c</sup>
TRT 2: Untreated, inoculated control	$25.80^{a}$
TRT 3: Ato Cide 2 g/ L of water	17.00 <sup>b</sup>
TRT 4: Ato Cide 5 g/ L of water	8.30 <sup>c</sup>
TRT 5: Ato Cide 10 g/ L of water	5.40 <sup>c</sup>
TRT 6: Ato Cide 20 g/ L of water	$2.80^{\circ}$
TRT 7: Standard treatment - KOCIDE	$2.40^{c}$

<sup>1</sup>Treatments were applied bi-weekly three days after the pathogen applied until harvest except healthy and pathogen control. Rate applied refers to one application and not the over-all amount applied. <sup>2</sup>Mean number of angular leaf spot lesions (BLS) per mature leaf .Means followed by the same letters in the columns is not significantly different at  $P \le 0.05$  according to HSDs for mixed model. There were 10 replicates in each treatment. BLS lesions are mean values from 10 mature leafs per treatment.

Fungicide rates (treatments) had a significant effect (P=0.0001) on disease severity and plant vigor of cucumber plants. The ALS affected plants, applied with Ato Cide have disease severity highest (4.40) in plants treated with Ato Cide 2 g/L of water (T3) and lowest (2.20 ±0.50) in plants treated with Ato Cide 10 g/L of water (T5) (Table 6) . This indicates that increase in Ato Cide rate have significantly reduced the disease severity (Table 6). Ato Cide provided excellent control of ALS under the conditions tested in cucumber. There was no phytotoxicity observed in any of the Ato Cide treated cucumber plants. Plant vigor has shown an upward trend with the increase of Ato Cide rate with the maximum vigor identified was (4.30) for standard treatment (T7) and closely followed by T1 (4.20) and T6 (4.10), respectively (Table 7).

Table 6	Mean di	sease severity	per	treatment	rated	on 1	to 9	scale i	in cucumb	er
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Treatment <sup>1</sup> description	Disease severity <sup>2</sup>
TRT 1: Untreated, uninoculated control	$0.80^{\circ}$
TRT 2: Untreated, inoculated control	7.70 <sup>a</sup>
TRT 3: Ato Cide 2 g/ L of water	$4.40^{b}$
TRT 4: Ato Cide 5 g/ L of water	2.80 <sup>bc</sup>
TRT 5: Ato Cide 10 g/ L of water	$2.20^{\circ}$
TRT 6: Ato Cide 20 g/ L of water	2.30 <sup>bc</sup>
TRT 7: Standard treatment - KOCIDE	$2.20^{\circ}$

#### plants sampled before harvest

<sup>1</sup>Treatments were applied bi-weekly three days after the pathogen applied until harvest except healthy and pathogen control. Rate applied refers to one application and not the over-all amount applied. <sup>2</sup>Mean disease severity per treatment in cucumber plants. Means followed by the same letters in the columns is not significantly different at  $P \le 0.05$  according to HSDs for mixed model. Disease severity was evaluated (1= no disease; 2 and 3 represent few angular lesions, with less than 5% leaf tissue affected; 4 and 5 have big lesions with 10% ~15% tissue damage; 6 and 7, the lesions coalesce causing up to 25% tissue damage; 8 being more than 40% tissue injury due to coalesce of lesions; 9= completely bighted tissue), then per plant treatments were averaged across all plants within each treatment replicate.

### Conclusion

In conclusion, *X. campestris* pv. *vitians* can survive as an epiphyte on lettuce plant without showing symptoms. Consequently, it is difficult for seed manufacturers to produce uncontaminated seeds without a very sensitive detection test. In cucumbers, ALS disease pressure was high up to 80% indicating that the cucumber cultivar used might be susceptible to the disease. Disease progress differed significantly in cucumber plants with application of different Ato Cide treatments. Hence, like most bacterial diseases, prophylactic measures using Ato Cide and integrated pest management must be favored for the disease control.

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Treatment <sup>1</sup> description	Vigor rating <sup>2</sup>
TRT 1: Untreated, uninoculated control	$4.20^{a}$
TRT 2: Untreated, inoculated control	1.10 <sup>b</sup>
TRT 3: Ato Cide 2 g/ L of water	$3.30^{a}$
TRT 4: Ato Cide 5 g/ L of water	3.30 <sup>a</sup>
TRT 5: Ato Cide 10 g/ L of water	$3.70^{a}$
TRT 6: Ato Cide 20 g/ L of water	$4.10^{a}$
TRT 7: Standard treatment - KOCIDE	$4.30^{a}$

<sup>1</sup>Treatments were applied bi-weekly three days after the pathogen applied until harvest except healthy and pathogen control. Rate applied refers to one application and not the over-all amount applied. <sup>2</sup>Mean plant vigor rated on a scale of 1 to 5 ± S.E in cucumber plants. Means followed by the same letters in the columns is not significantly different at  $P \le 0.05$  according to HSDs for mixed model. Plant vigor was evaluated (1= very poor; 2= poor; 3= good; 4= very good; and 5= Excellent), then per plant treatments were averaged across all plants within each treatment replicate.

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T2: Necrotic BLS symptoms on mature lettuce leaf treated with only pathogen.

T7 @ 10 g/L: Few BLS symptoms on Ato Cide treated lettuce leaf

Fig. 1 Comparison of typical BLS lesions on the lettuce leaves between pathogen control and treated with Ato Cide 10 g/L bi-weekly after the pathogen inoculation



Fig. 2 Comparision of Ato Cide treated lettuce plants to healthy control, pathogen control and standard at the time of harvest under greenhouse conditions



Fig. 3 Comparison between healthy control (T1) and pathogen control (T2) treated cucumber plants



Fig. 4 Comparision of Ato Cide 2 g/L of water (T3) treated cucmber plants to healthy control (T1) and pathogen control (T2) at the time of harvest under greenhouse conditions



Fig. 5 Comparision of Ato Cide treatments to healthy (T1) and pathogen control (T2)

## Evaluation of combined efficacy of *Bacillus subtilis* MBI 600 and Azoxystrobin in managing rice sheath blight caused by *Rhizoctonia solani*

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### Abstract

Among various biotic stresses affecting rice, sheath blight (ShB) is an important fungal disease causing significant yield losses. For a PGPR strain to be effective under field conditions, its compatibility with commonly used fungicides is a prerequisite. Among various PGPR strains tested in our previous studies, strain MBI 600 of Bacillus subtilis (MBI 600) showed consistent efficacy against ShB in rice. Strobilurin group of fungicides are commonly used in rice ShB management. Among them, Azoxystrobin is recommended in the United States. The objective of this paper was to evaluate the combined efficacy of MBI 600 and Azoxystrobin against rice ShB by adopting micro-chamber inoculation method. Greenhouse experiments were conducted at the Plant Science Research Center, Auburn University, Auburn, using 5 ×7 factorial randomized complete block design (RCBD). A high-yielding, very early maturing long-grain rice cultivar Cocodrie developed at LSU Ag Center was used in this experiment. A multinucleate and virulent R. solani anastomosis groupAG-1 IA (Arkansas isolate) isolated from ShB infected rice seedlings was inoculated on rice for disease symptoms. Strain MBI 600 was evaluated at concentrations of 0,  $10^3$ ,  $10^6$ ,  $10^9$ ,  $10^{11}$  CFU/ml (5 factors); in combination with azoxystrobin at 0, 1/6, 2/6, 3/6, 4/6, 5/6, and at recommended rate of 2378 mg/kg (7 levels). In total, including healthy control, there were 36 treatments, four replications per each treatment, and one plant per replication. Disease assessment was calculated by percent relative lesion height method (RLH) with a formula of (% RLH =  $100 \times \text{Total height of lesions}$ ) and ShB

disease severity was rated on 0 to 9 scale with 0 being no disease and 9 having 80% tissue affected. Overall, azoxystrobin at recommended rate (R) when used in conjunction with any of the concentrations of strain MBI 600 resulted in complete reduction of ShB lesions (0% severity by RLH). In a similar way, strain MBI 600 at a concentration of  $10^{11}$  CFU/ml when applied in conjunction with any of the concentrations of azoxystrobin resulted in complete control of ShB lesions (0% severity by RLH). Also, the results from other treatments tested suggest that combined application of *B. subtilis* MBI 600 ( $10^9$  CFU/ml) and Azoxystrobin (3/6 of recommended rate) is an ideal dose of PGPR and fungicide in controlling ShB disease.

## Introduction

Among various biotic stresses affecting rice, sheath blight (ShB) is an important fungal disease causing significant yield losses. Strong sources of genetic resistance are not available against ShB and so the disease is managed mostly through use of chemical fungicides. Indiscriminate use of fungicides has several disadvantages such as ground water and environmental pollution, escalated costs, and pathogen resistance. As an alternative to the use of fungicides, biocontrol of ShB is gaining popularity and has potential benefits. Among different biocontrol agents, use of plant growthpromoting rhizobacteria (PGPR) is in practice especially in rice disease management. Continuous application of PGPR to rice fields is effective against ShB on a long term basis and can be an alternative or supplement to fungicides.

Of different PGPR, *Bacillus subtilis* is an endospore producing Gram positive bacterium that is widely used. For a PGPR strain to be effective under field conditions, its compatibility with commonly used fungicides is a pre-requisite. Strobilurin group of fungicides are commonly used in rice ShB management, and of them, Azoxystrobin is recommended in the United States. Compatibility of PGPR to Azoxystrobin is a pre-requisite for devising biocontrol based IDM strategies against rice ShB. Our earlier studies on screening different PGPR strains under laboratory and greenhouse conditions proved that *B. subtilis* strain MBI 600 was highly efficacious in reducing ShB pathogen and lesion spread. In the present study, the combined efficacy of MBI 600 and azoxystrobin was evaluated at different rates of application to determine the optimum dose of bioagent and fungicide. The objective is to reduce the fungicidal application and to minimize the environmental hazards and costs incurred in plant protection.

## **Materials and Methods**

## Source of Rhizoctonia solani

A multinucleate and virulent isolate of *R. solani* anastomosis group AG-1 IA (Arkansas isolate) was obtained. The isolate was originally isolated from ShB

infected rice seedlings. The culture was maintained on potato dextrose agar (PDA) or on rye kernels for further use.

## Source of B. subtilis MBI 600

The *B. subtilis* MBI 600 was obtained from Dr. J. W. Kloepper, Department of Entomology and Plant Pathology, Auburn University, AL, USA., and used in this study. The purified and identified strain was grown for 48 h at 25 °C in 20 ml sterile tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) on a reciprocating shaker (80 rpm). Bacteria were pelletted by centrifugation for 20 min at  $10,000 \times g$ . Bacterial cells were then washed (twice) in 0.1 M phosphate buffer (PB) (pH 6.8), resuspended in TSB amended with 20% sterile glycerol, and frozen in vials at -80°C for long term storage.

## Source of rice cultivar

A high-yielding, very early maturing long-grain cultivar of Cocodrie developed at Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA, was used. The seeds were stored at  $4^{\circ}$ C prior to use.

## Studies on combined efficacy under greenhouse conditions

The combined efficacy of MBI 600 and azoxystrobin was determined against rice ShB by adopting micro-chamber inoculation method. The experiment was conducted in  $5 \times 7$  factorial RCBD under greenhouse conditions. Strain MBI 600 was evaluated at concentrations of zero,  $10^3$ ,  $10^6$ ,  $10^9$ , and  $10^{11}$  CFU/ml (5 factors); in combination with azoxystrobin at zero,  $1/6^{\text{th}}$ ,  $2/6^{\text{th}}$ ,  $3/6^{\text{th}}$ ,  $4/6^{\text{th}}$ ,  $5/6^{\text{th}}$ , and at recommended rate (7 levels). The recommended dose (R) of azoxystrobin against ShB is 2378 mg/kg. Rice seeds of CV. Cocodrie were surface sterilized with 2% sodium hypochlorite for 5 min and washed with sterile distilled water two times. For seed treatment, surface sterilized seeds were soaked in different concentrations of MBI 600 prepared by multiplying the strain on Tryptic Soy Agar (TSA) media in Petri dishes and adjusting to final concentrations. Later, seeds were soaked for 24 h, separately. Seeds were later removed from the bacterial soaked solutions and air dried in a laminar flow hood for 30 min. Seeds were sown in 6-inch plastic pots containing sterilized local soil (1 part) and peat-vermiculite mixture (2 parts). At 3 leaf stage (30 days after sowing), plants were sprayed until run off with MBI 600 and azoxystrobin at different concentrations according to treatment combinations described previously. After 24 h of imposing the foliar spray with MBI 600 and azoxystrobin, immature sclerotium of R. solani was inoculated at the base of treated seedlings near the soil line to produce ShB disease to evaluate the efficacy of combined application of MBI 600 and azoxystrobin. Inoculated seedlings were covered with Coca-Cola transparent bottles for maintaining inside moisture and for optimum ShB lesion development. Seedlings inoculated with *R. solani* sclerotia and with no seed treatment and foliar spray with MBI 600 served as pathogen control. Seedlings produced by soaking seeds in water, and later sprayed with water and not inoculated with sclerotia served as healthy control. There were 36 treatments, four replications per each treatment, and one pot per replication.

Replicated pots were arranged on a greenhouse bench in RCBD with factorial arrangement of treatments (Fig.1). The seedlings in pots were maintained at  $26\pm2^{\circ}$ C, RH of 90%, and photoperiod of 16 h and grown for 45 days. Ten days after post inoculation, length of ShB lesions and culm length were measured.

## **Disease** Assessment

ShB disease was calculated by Relative Lesion Height method (RLH) by using the formula:

RLH (%) =  $100 \times$  Total height of lesions/Total plant height

ShB disease severity grade was given as follows:

Disease severity is on a "0 to 9 scale" where 0 = plants healthy, no symptoms, 1 =restricted dark brown oval lesions at waterline or infection points; 2= few oval or coalesced lesions with broad borders on lower sheaths or at infection points, 5% or less of tissue affected; 3=lesions on lower leaf sheaths or at infection points, lesions coalescing, less than 10% of tissues affected; **4**=lesions mainly restricted to sheaths on lower third of plant, lowest leaves, or other infection points, lesions discrete or coalescing with narrow red-brown border, 10% to 15% of leaf and sheath tissues affected; 5=lesions mainly restricted to sheaths and leaves of lower half of plants, lesions usually coalescing with large necrotic centers and narrow red-brown borders, 15 to 25% of tissue affected; 6=lesions usually coalescing and affecting lower twothirds of sheath area of plant, lesions extending to blades of lower leaves or lower leaves killed by injury to sheath, 25% to 40% of tissues affected; 7=lesions usually coalescing and affecting lower three-fourths of sheath area of plant, lesions extending to leaf blades of lower two-thirds of plant, 40% to 60% of tissues affected; 8=lesions reaching to flag leaf, lower sheaths with coalesced lesions covering most of tissue, lower and middle leaves dead or dying, 60% to 80% of tissues affected; and 9=lesions reaching to flag leaf, lower leaves mostly dead, sheaths dried, culms brown, collapsing, most tillers lodged, over 80% of tissue affected.

## Statistical analysis

The data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA), and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

## **Results and Discussion**

## Efficacy of azoxystrobin

Among different concentrations of azoxystrobin evaluated, ShB lesions were significantly reduced over control with 4R/6, 5R/6, and R (recommended rate) concentrations of the fungicide (Table 1). Of them, highest reduction of ShB was obtained with recommended rates (R) (4% RLH) (Fig. 2) and is significantly superior to ShB lesions at 4R/6 and 5R/6 concentrations. No significant differences were noted between ShB severity at these 4R/6 and 5R/6 concentrations, and ShB severity ranged from 6.8% to 7.2% by RLH. However, azoxystrobin was not effective at concentrations up to 3R/6 of the recommended rates when compared with control seedlings. ShB severity in control seedlings was about 14.4%.

## Efficacy of B. subtilis MBI 600

Of different concentrations of MBI 600 evaluated, the PGPR strain was effective at concentrations of  $10^9$  and  $10^{11}$  CFU/ml in reducing ShB severity. At a concentration of  $10^{11}$  CFU/ml of MBI 600, ShB lesions were completely arrested (ShB severity was 0% by RLH). At  $10^9$  CFU/ml of MBI 600, the ShB severity by RLH was about 5.9% and is significantly superior to control seedlings (ShB severity of 14.4%, Figs. 3a & 3b). The strain MBI 600, however, was not effective at concentrations of  $10^3$  and  $10^6$  CFU/ml against ShB.

## Combined efficacy of B. subtilis MBI 600 and azoxystrobin

Azoxystrobin at recommended rate (R) when used in conjunction with any of the concentrations of strain MBI 600 ( $10^3$ ,  $10^6$ ,  $10^9$ . and  $10^{11}$  CFU/ml resulted in complete reduction of ShB lesions (0% severity by RLH) (Table1). In a similar way, strain MBI 600 at a concentration of  $10^{11}$  CFU/ml when applied in conjunction with any of the concentrations of azoxystrobin under study (R/6 through R) resulted in complete control of ShB lesions (0% ShB severity). However, MBI 600 at  $10^{11}$  CFU/ml alone could completely inhibit ShB lesion development. The combined application of MBI 600 at any of the concentrations under study with azoxystrobin at recommended rate was significantly superior (0% ShB severity) to the application of azoxystrobin alone at recommended rate, R (4% severity by RLH).

ShB lesion size was significantly less than control (pathogen control) in other treatments involving combinations of MBI  $600 \times 10^3$  and azoxystrobin at different concentrations. However, complete control of ShB lesions was seen in the following treatments that do not involve MBI 600 strain at  $10^{11}$  CFU/ml and azoxystrobin at recommended rate (R) as factors.

- 1. MBI 600  $\times 10^9$  CFU/ml + azoxystrobin 3/6 of the recommended rate, R.
- 2. MBI 600  $\times 10^9$  CFU/ml + azoxystrobin 4/6 of the recommended rate, R.
- 3. MBI 600  $10^9$  CFU/ml + azoxystrobin 5/6 of the recommended rate, R.
- 4. MBI 600  $\times 10^9$  CFU/ml + azoxystrobin recommended rate, R.

Based on the above facts, it can be inferred that *B. subtilis* strain MBI  $600 \times 10^9$  CFU/ml when used in combination with azoxystrobin 3/6 and above the recommended rate provided complete control of ShB lesion spread under GH conditions. Of them, the combined application of MBI 600 with 3/6 of recommended rate is the optimum dose since the combination involved less quantities of azoxystrobin. Hence, it can be concluded that combined application of *B. subtilis* MBI 600 ( $\times 10^9$  CFU/ml) and Azoxystrobin (3/6 of recommended rate) is an ideal Dose of PGPR and fungicide.

Treatment Relative Lesion Height Trt. No Severity grade (%) (Bacillus subtilis MBI 600 + Azoxystrobin) 14.4<sup>abc</sup> 6.3<sup>abc</sup> 1 MBI 600 0 cfu/ml + Azoxy zero mg/kg (Pathogen ctrl 2 MBI 600  $\times$ 0 cfu/ml + Azoxy R/6 mg/kg  $14.4^{abc}$ 6.3<sup>abc</sup> BB 13.7<sup>bcde</sup> 3 BA MBI 600  $\times$  0 cfu/ml + Azoxy 2R/6 mg/kg 6.8<sup>a</sup> 6.3<sup>abc</sup> 4 14.8<sup>ab</sup> BA MBI 600  $\times$  0 cfu/ml + Azoxy 3R/6 mg/kg 5  $6.8^{\text{gh}}$ BA MBI 600  $\times$  0 cfu/ml + Azoxy 4R/6 mg/kg 3.3<sup>f</sup>  $3.5^{f}$ 7.2<sup>gh</sup> 6 BA MBI 600  $\times$  0 cfu/ml + Azoxy 5R/6 mg/kg 7 Ba MBI 600 @ 0 cfu/ml + Azoxy R mg/kg  $4.0^{i}$ 3.8<sup>f</sup>  $6.3^{abc}$ 8 13.6<sup>bcde</sup> MBI  $600 \times 10^3$  cfu/ml + Azoxy zero mg/kg  $14.0^{abcd}$ 9 MBI 600 @  $10^3$  cfu/ml + Azoxy @ R/6 mg/kg 5.5<sup>de</sup> BB 13.4<sup>cde</sup> 10 MBI 600  $\times$  10<sup>3</sup> cfu/ml + Azoxy 2R/6 mg/kg 6.0<sup>bcd</sup> 12.3<sup>ef</sup> 11 MBI  $600 \times 10^3$  cfu/ml + Azoxy 3R/6 mg/kg 5.3<sup>e</sup> 12 MBI 600  $\times$  10<sup>3</sup> cfu/ml + Azoxy 4R/6 mg/kg 12.9<sup>def</sup> 5.5<sup>de</sup> 5.8<sup>cde</sup> 13 MBI  $600 \times 10^3$  cfu/ml + Azoxy 5R/6 mg/kg 11.8<sup>f</sup> 14 MBI  $600 \times 10^3$  cfu/ml + Azoxy R mg/kg  $0^{j}$  $0^{g}$ 15 MBI 600  $\times$  10<sup>6</sup> cfu/ml + Azoxy zero mg/kg 15.2<sup>a</sup> 6.8<sup>a</sup> 16 *BB* MBI 600 @  $10^6$  cfu/ml + Azoxy R/6 mg/kg 14.3<sup>abcd</sup> 6.5<sup>ab</sup> MBI 600 imes 10<sup>6</sup> cfu/ml + Azoxy 2R/6 mg/kg 17  $14.9^{ab}$ 6.5<sup>ab</sup> 7.3<sup>gh</sup> 18 MBI 600  $\times$  10<sup>6</sup> cfu/ml + Azoxy 3R/6 mg/kg 3.8<sup>f</sup> 19 3.3<sup>f</sup> MBI 600  $\times$  10<sup>6</sup> cfu/ml + Azoxy 4R/6 mg/kg 7.4<sup>g</sup> 7.2<sup>gh</sup>  $3.3^{f}$ 20 MBI 600  $\times$  10<sup>6</sup> cfu/ml + Azoxy 5R/6 mg/kg 21 MBI 600  $\times$  10<sup>6</sup> cfu/ml + Azoxy R mg/kg  $0^{j}$  $0^{g}$  $3.3^{\overline{f}}$ 22 MBI 600  $\times$  10<sup>9</sup> cfu/ml + Azoxy zero mg/kg 5.9<sup>h</sup> 6.5<sup>gh</sup> 3.5<sup>f</sup> 23 BB MBI 600 @  $10^9$  cfu/ml + Azoxy R/6 mg/kg 24 5.9<sup>h</sup> 3.3<sup>f</sup> MBI 600  $\times 10^9$  cfu/ml + Azoxy 2R/6 mg/kg 25 MBI  $600 \times 10^9$  cfu/ml + Azoxy 3R/6 mg/kg 0<sup>j</sup> 0<sup>g</sup> MBI 600  $\times$  10<sup>9</sup> cfu/ml + Azoxy 4R/6 mg/kg  $0^{j}$  $0^{g}$ 26 27 MBI  $600 \times 10^9$  cfu/ml + Azoxy 5R/6 mg/kg  $0^{j}$  $0^{g}$ 0<sup>j</sup> 0<sup>g</sup> 28 MBI 600  $\times$  10<sup>9</sup> cfu/ml + Azoxy R mg/kg 29 MBI 600  $\times$  10<sup>11</sup> cfu/ml + Azoxy zero mg/kg oj  $0^{g}$ MBI 600  $\times$  10<sup>11</sup> cfu/ml + Azoxy R/6 mg/kg 30 BB  $0^{j}$  $0^{g}$  $\overline{\text{MBI}}$  600 × 10<sup>11</sup> cfu/ml + Azoxy 2R/6 mg/kg 0<sup>j</sup>  $0^{g}$ 31 MBI 600 $\times$ 10<sup>11</sup> cfu/ml + Azoxy 3R/6 mg/kg 32  $0^{j}$  $0^{g}$ 33 MBI 600  $\times$  10<sup>11</sup> cfu/ml + Azoxy 4R/6 mg/kg 0<sup>j</sup>  $0^{g}$ 34 MBI 600  $\times$  10<sup>11</sup> cfu/ml + Azoxy 5R/6 mg/kg  $0^{j}$ 0<sup>g</sup> 35 MBI 600  $\times$  10<sup>11</sup> cfu/ml + Azoxy R mg/kg  $0^{j}$  $0^{g}$ 36 Healthy control 0<sup>j</sup>  $0^{g}$ 

 Table 1 Combined efficacy of Bacillus subtilis MBI 600 and Azoxystrobin in controlling rice sheath blight disease under greenhouse conditions
Values are means of four replications Means followed by a common letter in the column are not significantly different at  $P \le 0.05$ .

Disease severity is on a "0 to 9 scale" where 0= Plants healthy, no symptoms, 1= restricted dark brown oval lesions at waterline or infection points ; 2= few oval or coalesced lesions with broad borders on lower sheaths or at infection points, 5% or less of tissue affected ; 3=lesions on lower leaf sheaths or at infection points, lesions coalescing, less than 10% of tissues affected; 4=lesions mainly restricted to sheaths on lower third of plant, lowest leaves, or other infection points, lesions discrete or coalescing with narrow red-brown border, 10% to 15% of leaf and sheath tissues affected : 5=lesions mainly restricted to sheaths and leaves of lower half of plants, lesions usually coalescing with large necrotic centers and narrow red-brown borders, 15 to 25% of tissue affected; 6=lesions usually coalescing and affecting lower twothirds of sheath area of plant, lesions extending to blades of lower leaves or lower leaves killed by injury to sheath, 25 to 40% of tissues affected; 7=lesions usually coalescing and affecting lower three-fourths of sheath area of plant, lesions extending to leaf blades of lower two-thirds of plant, 40% to 60% of tissues affected; 8=lesions reaching to flag leaf, lower sheaths with coalesced lesions covering most of tissue, lower and middle leaves dead or dying, 60% to 80% of tissues affected; and 9=lesions reaching to flag leaf, lower leaves mostly dead, sheaths dried, culms brown, collapsing, most tillers lodged, over 80% of tissue affected.

In our GH studies, the optimum combination of *B. subtilis* MBI 600 ( $\times 10^9$  CFU/ml) and Azoxystrobin (3/6 of recommended rate) was selected as it provided complete inhibition of ShB lesions. Although several other combinations of MBI 600 and Azoxystrobin resulted in ShB lesion reduction significantly over control, complete inhibition at an optimum dose under GH is desirable. This is because the pathogen inoculum of *R. solani* under field conditions is manifold and the chances of disease outbreak are more compared to under GH conditions. Further studies are needed that evaluate these combined applications of *B. subtilis* MBI 600 and Azoxystrobin at various combinations to determine the efficacy and optimum dose for ShB reduction under field conditions.



Fig. 1 Evaluation of different treatment combinations of *Bacillus subtilis* MBI 600 and Azoxystrobin at different concentrations in a Randomized Complete Block Design with factorial arrangement of treatments under greenhouse conditions



Fig. 2 Sheath blight lesion development on rice seedlings in different treatment combinations under greenhouse conditions



Fig.3a, (Control)

Fig. 3 (Azoxystrobin Recommended rate)



Fig.3b, (Control)

# Screening of PGPR strains for suppression of rice sheath blight caused by *Rhizoctonia solani* under greenhouse conditions

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# Abstract

Sheath blight (ShB) of rice causes significant grain loss, to an extent of 50% under optimum disease development conditions. Reliable and effective disease management strategies are needed for control rice ShB disease caused by Rhizoctonia solani. Selection of elite plant growth-promoting rhizobacteria (PGPR) strains is a vital step in ShB disease management at field level. The objective of this paper is the screening of numerous PGPR strains (MBI 600, S8-G6, SE-52, 99-101, EXTN-1, GB03, 17A-3, E681, and, 33B-9) which have shown high efficacy in inhibiting R. solani mycelial growth under *in-vitro* assays for control of ShB under greenhouse conditions. Greenhouse experiments were conducted at the Plant Science Research Center, Auburn University, Auburn, using a randomized complete block design (RCBD). In this experiment a high-yielding, very early maturing long-grain cultivar of Cocodrie developed at LSU AgCenter, Louisiana, USA, was used. A multinucleate and virulent R. solani anastomosis group AG-1 IA (Arkansas isolate) isolated from ShB infected rice seedlings was used to inoculate rice seedlings for disease development. Disease assessment was calculated by percent relative lesion height method (RLH) formula (% RLH =  $100 \times$  Total height of lesions) and ShB disease severity was graded on 0 to 9 scale with 0 being no disease and 9 having 80% tissue affected. Overall, there was a significant difference among the strains screened for ShB lesion spread, with MBI-600 showing highest reduction in lesion spread (1.6% RLH) with a concomitant disease severity grade of 1.3. Also, closely followed strains were 33B-9 (4.2% & 3.0) and S8-G6 (6.8% & 3.3) with RLH and disease severity grades, respectively. These results suggest that the strains MBI-600, 33B-9, and S8-G6 were superior to other PGPR strains in reducing ShB lesion spread and in lessening ShB severity.

#### Introduction

Sheath blight (ShB) of rice caused by *Rhizoctonia solani* is an economically significant disease in all crop growing areas of the world. Grain losses to an extent of 50% are reported under optimum conditions of disease development. Chemical control of ShB is a common practice in present day rice cultivation and has several disadvantages such as escalated costs, resistance gain by the pathogen to fungicides, and environmental pollution. Biocontrol of ShB is a viable alternative to presently used chemical fungicides. Among different biocontrol agents, plant growth-promoting rhizobacteria (PGPR) offer an eco-friendly, economically viable option that is sustainable in the long run. These PGPR also produce certain growth promoting effects on rice besides reducing ShB.

Screening and selection of PGPR strains is a vital step in ShB disease management at field level. An ideal PGPR strain should control the ShB lesion development at an early stage of infection process. For selecting an elite PGPR strain, screening of different PGPR is mandatory under greenhouse conditions. In the present study, nine PGPR strains that have shown high efficacy in inhibiting *R. solani* mycelial growth under *in-vitro* dual culture assays were screened under greenhouse conditions. The long term goal of the study is to formulate a biocontrol-based IDM strategy against rice ShB using PGPR.

# **Materials and Methods**

#### Source of Rhizoctonia solani

A multinucleate and virulent isolate of *R. solani* anastomosis groupAG-1 IA (Arkansas isolate) was obtained. The isolate was originally isolated from ShB infected rice seedlings. The culture was maintained on potato dextrose agar (PDA) or on rye kernels for further use.

#### Source of PGPR strains

Nine PGPR strains were obtained from Dr. J. W. Kloepper, Department of Entomology and Plant Pathology, Auburn University, AL, USA., and used in this study. The selected strains possessed one or several of the following characteristics: (i) *in-vitro* antibiosis against various fungal pathogens, (ii) promotion of rhizobial root nodulation, (iii) enhancement of root and shoot growth of various crops and vegetables, and (iv) capacity to produce plant growth regulators. Purified and identified strains were grown for 48 h at 250 °C in 20 ml sterile tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) on a reciprocating shaker (80 rpm). Bacteria were pelletted by centrifugation for 20 min at  $10,000 \times g$ . Bacterial cells were then

washed (twice) in 0.1 M phosphate buffer (PB) (pH 6.8), resuspended in TSB amended with 20% sterile glycerol, and frozen in vials at  $-80^{\circ}$ C for long term storage. *Source of rice cultivar* 

A high-yielding, very early maturing long-grain cultivar of Cocodrie developed at Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA, was used. The seeds were stored at 4°C prior to use.

#### Screening of PGPR under Greenhouse conditions

The efficacy of different PGPR on ShB severity was tested under GH conditions by adopting micro-chamber inoculation method. Seeds of rice were sown in 6-inch plastic pots containing sterilized local soil (1 part) and peat-vermiculite potting mixture (2 parts). Antagonistic PGPR strains prepared at a concentration of  $10^9$  CFU/ml were sprayed onto seedlings at 3 leaf stage (30 days after sowing) until run off. After 24h of bacterial inoculation, immature sclerotium of *R. solani* was inoculated at the base of treated seedlings near soil line to produce ShB disease to evaluate the efficacy of different PGPR. Treated seedlings were covered with Coca-Cola transparent bottles for maintaining inside moisture and for optimum ShB lesion development. Seedlings inoculated with *R. solani* sclerotia and sprayed with water served as inoculated control. Seedlings sprayed with water and not inoculated with sclerotia served as healthy control. There were 11 treatments, four replications per treatment, with one pot per replication.

Replicated pots were arranged on a greenhouse bench in a RCBD fashion. The pots were maintained at  $26\pm2$ °C, RH of 90%, and photoperiod of 16 h and grown for 45 days. Ten days after inoculation, length of ShB lesions and culm length were measured.

Bacillus subtilis MBI 600 Bacillus subtilis subsp. subtilis S8-G6 Bacillus safensis SE-52 Bacillus subtilis subsp. subtilis 99-101 Bacillus subtilis subsp. subtilis EXTN-1 Bacillus amyloliquefaciens GB03 Bacillus subtilis subsp. subtilis 17A-3 Paenibacillus peoriae E681 Bacillus subtilis subsp. subtilis 33B-9 Pathogen Control Healthy Control

#### **Disease Assessment**

ShB disease was calculated by Relative Lesion Height method (RLH) by using the formula:

 $RLH(\%) = 100 \times Total$  height of lesions/Total plant height

ShB disease severity grade was given as follows:

Disease severity is on a "0 to 9 scale" where 0 = plants healthy, no symptoms, 1 =restricted dark brown oval lesions at waterline or infection points; 2= few oval or coalesced lesions with broad borders on lower sheaths or at infection points, 5% or less of tissue affected; 3=lesions on lower leaf sheaths or at infection points, lesions coalescing, less than 10% of tissues affected; **4**=lesions mainly restricted to sheaths on lower third of plant, lowest leaves, or other infection points, lesions discrete or coalescing with narrow red-brown border, 10% to 15% of leaf and sheath tissues affected; 5=lesions mainly restricted to sheaths and leaves of lower half of plants, lesions usually coalescing with large necrotic centers and narrow red-brown borders, 15 to 25% of tissue affected; 6=lesions usually coalescing and affecting lower twothirds of sheath area of plant, lesions extending to blades of lower leaves or lower leaves killed by injury to sheath, 25% to 40% of tissues affected; 7=lesions usually coalescing and affecting lower three-fourths of sheath area of plant, lesions extending to leaf blades of lower two-thirds of plant, 40% to 60% of tissues affected; 8=lesions reaching to flag leaf, lower sheaths with coalesced lesions covering most of tissue, lower and middle leaves dead or dying, 60% to 80% of tissues affected; and 9=lesions reaching to flag leaf, lower leaves mostly dead, sheaths dried, culms brown, collapsing, most tillers lodged, over 80% of tissue affected.

#### Statistical analysis

The data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

#### **Results and Discussion**

Among different PGPR strains screened, ShB lesion spread up to 12.2%. Highest reduction in ShB lesion spread (1.6% by RLH) with a concomitant ShB severity grade of 1.3 was obtained when seedlings were sprayed with *Bacillus subtilis* MBI 600 (Fig 1). This was followed by strain *Bacillus subtilis* subsp. *subtilis* 33B-9 with a ShB lesion spread of 4.2% (RLH) and a ShB severity grade of 3.0. The strain *Bacillus subtilis* subsp. *subtilis* S8-G6 also effectively reduced ShB lesion development with a ShB spread of 6.8 with a concomitant ShB severity of 3.3. Among the 9 PGPR strains

screened, ShB lesion spread was effectively reduced by six strains with a range of 1.6% to 10.3%. Control seedlings recorded a ShB lesion spread of 12.0% (pathogen control) (Fig 2). No ShB incidence was observed in healthy control seedlings. ShB severity grade was least in MBI 600 as against pathogen control that recorded a severity grade of 6.5 (Table 1).

In our present study, the strains MBI 600, 33B-9, and S8-G6 were found superior to other PGPR strains in reducing ShB lesion spread and in lessening ShB severity. Of these superior strains, MBI 600 was found to perform best with a ShB lesion spread of 1.6% and a ShB severity grade of 1.3. Our earlier studies also indicated that MBI 600 strain was highly antagonistic to mycelial growth and sclerotial germination of *R. solani* under laboratory conditions. The strain MBI 600 was further selected for pesticide compatibility studies under greenhouse conditions to determine the optimum dose of bioagent as well as fungicide in order to develop a biocontrol-based IDM strategy for rice ShB.

Trt.No	PGPR	Relative Lesion Height (%)	Severity grade
1	Bacillus subtilis MBI 600 (AP 301)	1.6 <sup>g</sup>	1.3 <sup>d</sup>
2	BBBacillus subtilis subsp. subtilis S8-G6(AP- 52)	6.8 <sup>e</sup>	3.3°
3	BABacillus safensis SE-52(AP-7)	10.9 <sup>abcd</sup>	5.3 <sup>b</sup>
4	BABacillus subtilis subsp. subtilis 99-101 (AP- 136)	10.3 <sup>cd</sup>	5.8 <sup>ab</sup>
5	BABacillus subtilis subsp. subtilis EXTN- 1(AP-295)	10.6 <sup>bcd</sup>	5.3 <sup>b</sup>
6	BABacillus amyloliquefaciens GB03 (AP-305)	12.2 <sup>a</sup>	5.8 <sup>ab</sup>
7	BaBacillus subtilis subsp. subtilis 17A-3 (AP- 188)	9.6 <sup>d</sup>	5.3 <sup>b</sup>
8	PaPaenibacillus peoriae E681 (AP-294)	11.3 <sup>abc</sup>	6.3 <sup>a</sup>
9	BBBacillus subtilis subsp. subtilis 33B-9 (AP-209)	$4.2^{\mathrm{f}}$	3.0 <sup>c</sup>
10	Pathogen control	12.0 <sup>ab</sup>	6.5 <sup>a</sup>
11	Healthy control	$0.0^{\rm h}$	0.0 <sup>e</sup>

 
 Table 1 Efficacy of various PGPR strains in controlling rice sheath blight disease under greenhouse conditions

Values are means of four replications; Means followed by a common letter in the column are not significantly different at  $P \leq 0.05$ .



# **MBI 600**

Fig 1 Sheath blight lesion development on rice seedlings sprayed with Bacillus subtilis MBI 600 on rice CV. Cocodrie under greenhouse conditions



Fig 2 Sheath blight lesion development in control rice seedlings on rice

CV. Cocodrie under greenhouse conditions

# PGPR: a Potential Management Option for Sheath Blight and Bacterial Panicle Blight of Rice in the United States

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#### Abstract

Sheath blight (Rhizoctonia solani) and bacterial panicle blight (Burkholderia glumae or B. gladioli) are the two most important rice diseases in the southern United States. None of the leading high yielding cultivars have acceptable levels of resistance to either disease. While effective fungicides are available for control of sheath blight, there are no chemicals registered for bacterial panicle blight in the U.S. Studies have been initiated to develop a strategy of utilizing plant growth-promoting rhizobacteria (PGPR) as a management option for sheath blight and bacterial panicle blight in rice. Separate field experiments were established in Texas to evaluate the efficacy of PGPR strains for suppression of each disease. The three strains MBI-600, EXTN-1 and 99-101 of *Bacillus subtilis* were tested in comparisons with chemicals. These strains were among the most effective strains in reducing either disease in over 70 PGPR strains that were screened in the in vitro and greenhouse evaluations. MBI-600, the active ingredient in the biopesticide Integral, was effective in reducing sheath blight; combined use of MBI-600 with azoxystrobin at <sup>1</sup>/<sub>2</sub> the full recommended rate further reduced the disease, resulting in a yield comparable to azoxystrobin at the full rate. EXTN-1 and 99-101 reduced bacterial panicle blight severity by up to 50% compared to the untreated control and had yields similar to oxolinic acid. Use of these PGPR may offer a new option for managing sheath blight and bacterial panicle blight in rice.

Key words:Rice; sheath blight; bacterial panicle blight; *Rhizoctonia solani*; *Burkholderia glumae*; *Burkholderia gladioli*; PGPR

#### Introduction

Sheath blight, caused by *Rhizoctonia solani*, occurs in rice worldwide and is the most economically important rice disease in Texas and other southern rice-producing United States. The disease affects leaf sheaths, leaf blades, culms (stems) and sometimes panicles. Attacks to leaf sheaths and culms weaken rice plants, frequently resulting in lodging. Sheath blight can cause total yield losses of as much as 50% and whole head milling yield reductions of up to 31% (Marchetii, 1983). Due to the lack

of complete sheath blight resistance in the most commonly planted cultivars, southern U.S. rice farmers apply more than 1 million pounds of fungicides annually to control sheath blight. Hence, there is a need to develop alternative or complementary management options to reduce fungicide use.

Bacterial panicle blight, caused by *Burkholderia glumae* or *B. gladioli*, poses an increasing threat to rice production in the southern United States and other ricegrowing countries of Central and South America and Asia. The estimated yield loss can be as great as 50%. In 2010, the disease occurred in all the southern riceproducing United States and was epidemically severe in Texas, Arkansas and Louisiana (Zhou *et al.*, 2010c). Currently, there are no rice cultivars with high levels of bacterial panicle blight resistance and registered chemicals to use in the U. S. Rice farmers are in an urgent need to have an effective management option for bacterial panicle blight.

The development and use of biocontrol agents, especially plant growth-promoting rhizobacteria (PGPR), may be a new management option for sheath blight and bacterial panicle blight in the U. S. Biocontrol bacterial agents including Bacillus subtilis and Pseudomonas fluorescens have been used in managing sheath blight in rice in Asia (Kumar et al., 2009a; Mew et al., 1986, 1992, 2004; Vasantha et al., 1989). Use of PGPR in rice disease management is of a particular interest because of their abilities to promote plant growth. PGPR formulations have been reported to suppress sheath blight, promote plant growth, and boost grain yield in rice (Nandakumar et al., 2001). In our recent studies, some PGPR strains including B. subtilis strain MBI-600 showed strong activities in inhibiting mycelium growth and sclerotium germination of R. solani and reducing sheath blight severity in the in vitro and greenhouse evaluations (Kumar et al., 2011; Zhou et al., 2010a). Strain MBI-600 has also been shown to be effective in reducing sheath blight in rice under field conditions in India (Kumar et al., 2009b). MBI-600 is the active ingredient in the commercial biopesticide Integral. In addition, our recent research also demonstrated the *in vitro* and greenhouse antibiosis of some PGPR strains such as *B. subtilis* strains 99-10 and EXTN-1 against B. glumae (Zhou et al., 2010b).

The objectives of the present study were to: 1) evaluate the efficacy of *B. subtilis* strain MBI-600 and its combined use with rate-reduced fungicide for managing sheath blight under field conditions; and 2) determine the efficacy of *B. subtilis* strains 99-101 and EXTN-1 and their combination for bacterial panicle blight control and yield increase in the field. Preliminary results of this study have been published previously (Zhou *et al.*, 2011a, 2011b).

#### **Materials and Methods**

The sheath blight control experiment was established in a field of League-type soil (3% sand, 32% silt, and 64% clay) at the Texas A&M University System's AgriLife Research and Extension Center, Beaumont (AgriLife REC-Beaumont), TX. The following five treatments were ranged in a randomized complete block design with four replications: 1) *Bacillus subtilis* strain MBI-600, 2) Combination of MBI-600 with azoxystrobin (Quadris) at 0.08 kg a.i./ha, 3) azoxystrobin at 0.08 kg a.i./ha, 4) azoxystrobin at 0.16 kg a.i./ha, and 5) Untreated control. Plots consisted of seven 5.2m rows, and spaced 18 cm between rows. Seeds of the rice cultivar Cocodrie were treated with MBI-600 at approximately 10<sup>9</sup> cfu/ml prior to planting. Rice was drill seeded at 90 kg/ha. Plots were managed as locally recommended. All plots were inoculated with the sheath blight pathogen at the time of panicle differentiation. After 1 week of inoculation, plots were sprayed with MBI-600 at 10<sup>8</sup> cfu/ml and azoxystrobin at 0.08 or 0.16 kg a.i./ha. At maturity of rice, severity of sheath blight was rated on a scale of 0 to 9, where 0 represents no symptoms, and 9 represents most severe. Plots were harvested, and grain yield adjusted to 12% grain moisture.

The bacterial panicle blight control trial was also established at AgriLife REC-Beaumont, TX. There were five treatments: 1) *Bacillus subtilis* Strain 99-101, 2) *Bacillus subtilis* Strain EXTN-1, 3) Mixture of the strains 99-101 and EXTN-1, 4) Oxolinic acid (an antibiotic), and 5) untreated control. These treatments were arranged in a randomized complete block design with four replications. Seeds of Cocodrie were treated with the *Bacillus* strains at approximately  $10^9$  cfu/ml prior to planting. Seeding method and rate as well as plot size were the same as the 1<sup>st</sup> trial. Plots were sprayed with the *Bacillus* strains at  $10^8$  cfu/ml or oxolinic acid at 200 mg/kg at the flowering stage. All plots were spray inoculated with *Burkholderia glumae* at  $10^8$  cfu/ml the following day. Near maturity, bacterial panicle blight severity on rice panicles was assessed on a scale of 0 to 9, where 0 represents no symptoms and 9 represents most severe. Plots were harvested at maturity and grain yield adjusted to 12% grain moisture. Disease severity and yield were analyzed with the procedure PROC in SAS. Means were separated using Fisher's protected least significant difference (LSD) test.

#### **Results and Discussion**

When applied to both seed and the foliage, MBI-600 resulted in a significant reduction in sheath blight severity over the untreated control (Fig.1). The combined use of MBI-600 with azoxystrobin at 0.08 kg a.i./ha for foliar spray further reduced disease severity. The efficacy of this combined treatment was comparable to that of azoxystrobin at 0.18 kg a.i./ha (the full recommended rate). The combined treatment

tended to have numerically higher grain yield than the untreated control and have similar yield to azoxystrobin at 0.18 kg a.i./ha although there were no statistically significant differences among treatments.

Both *Bacillus* strains 99-101 and EXTN-1 significantly reduced the severity of bacterial panicle blight and increased yield compared to the untreated control (Fig. 2). These strains decreased disease severity by 41 to 50% and increased rice yield by 11 to 17% (equivalent to 458 to 715 lb/A). Mixture of these two strains did not further increase yield compared with the strains alone. Treatment with oxolinic acid had lowest bacterial panicle blight severity and highest yield. Application of oxalinic acid reduced disease severity by 86% and increased yield by 21%.

The results of this study suggest that the combined use of the *Bacillus* strain MBI-600 with a rate-reduced fungicide is an effective means to control sheath blight while reducing the usage of fungicide. Application of *Bacillus* strains 99-101 and EXTN-1 at the heading or flowering stage can protect rice panicles from infection by *B. glumae* effectively. Use of PGPR strains may offer a new management option to minimize the damages caused by sheath blight and bacterial panicle blight in rice in the U. S.



Fig.1 Effects of *Bacillus subtilis* strain MBI-600 and azoxystrobin (Quadris) on sheath blight (ShB) severity and rice yield in the field. Bars with the same letter are not significantly different according to Fisher's protected LSD at P = 0.05. Values are means of four replicated plots



Treatment

# Fig.2 Effects of *Bacillus subtilis* strains 99-101 and EXTN-1 and oxolinic acid on bacterial panicle blight (BPB) severity and rice yield in the field. Bars with the same letter are not significantly different according to Fisher's protected LSD at P = 0.05. Values are means of four replicated plots

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# Induced resistance of banana seedlings to *Fusarium* Wilt with antagonistic bacterial extract

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# Abstract

Banana wilt is one of the most destructive diseases on banana production worldwide, caused by Fusarium oxysporum f. sp. cubense (Foc). No effective control measure for Fusarium wilt has been found other than the use of resistant cultivars We report here studies on induced resisance of banana seedlings CV. Raja to Fusarium wilt with antagonistic bacterial extracts. The following treatments were included to test their effect on Fusarium wilt disease severity, growth of the seedlings and levels of phenolic compounds in the banana seedlings. Treatments included: Healthy control, Fusarium wilt fungus infested control, extracts of extract of Pseudomonas flourescens P60, P. flourescens P32, or Bacillus subtilis and with the fungus. Each treatment was replicated six times and were arranged in a completely randomized block design. Following application of the treatments, seedlings were incubated and measured the following observations: phenolic compound content qualitatively (glycoside, saponin, tannin), disease severity, infection rate, antagonistic effectivity, Fusairum late density, number of infected roots, germination inhibition, the fungus inside the crop, crop height, number of leaves, and wet weight of root. Our results showed that the extract of P. flourescens P60 and P. flourescens P32 on banana seedlings protected from Fusarium wilt disease compared to non-bactetized extract. The bacterial extracts significantly suppressed the disease development and the incubation period from 76 days to 75 days after inoculation. Also, the antagonistic effectivity of *P. flourescens* P60 and P. flourescens P32 in the seedling corm was 31.51% and 25.88%, respectively, and in the seedling leave was 19.67% and 20.58%, respectively. The treatments did not have any influence on growth of banana seedlings under the conditions tested.

Key words: Induced resistance; Fusarium wilt; Antagonistic bacteria

#### Introduction

Among diseases of banana, Panama wilt disease caused by the fungus *Fusarium* oxysporum f.sp. cubense (Foc) has been a major cause of loss (Pegg et al., 1995; 1996). In Indonesia, incidence of Panama wilt is widespread and in some districts the disease incidence is as high as 80%~90% (Hermanto and Setyawati, 2002; Nasir et al., 2003; 2005). This disease kills susceptible banana plants and there is no cure. Fusarium wilt is the preferred name for what was first called Panama disease because it became prominent in that Central American country early last century. The fungus infects banana plants through the roots and invades the plant's water conducting tissues (Saravanan et al., 2003). Once Foc is introduced into banana gardens, it remains in the soil making it impossible to grow susceptible bananas in the same location for up to several decades (Nasir et al., 2005). The disease becomes the most dangerous disease in the world. In Indonesia, the most severe attact of the pathogen (together with *Ralstonia solanacearum*) was found in West Sumatera barat (Nasir and Jumjunidang, 2004).

Banana is one of high value horticulutral crops and used as raw processed material food. Banana contains high enough nutrition such as carbohydrate, protein, fat, and vitamins of A, B1, B2 and C (Directorate of Processing and Marketing of Horticultural Products, 2005). Banana production in Indonesia is mainly concentrated in West Java, Central Java, Esat Java, Lampung, West Sumatera, Jambi, Kalimantan, Sulawesi, Maluku, Bali, and NTB (Ditjen PPHP, 2005).

There are no effective chemical mesuares of Panama wilt of banana and a currently practised corm-injection procedure with the locally available fungicides as tedious and also expensive. Over the years, there are many reports on the possibilities of using antagonistic Actinomyctes, Artrobacter, Agrobacterium and other bactreria for suppression of Panama wilt disease caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (Foc). In recent years there has been much success in obtaining biological control of plant pathogens by bacterization with fluorescent *Pseudomonads* and *Bacillus* sp. Many of these Pseudomonads and Bacillus have been shown potential to enhance plant growth and yields. These isolates have been called as plant growthpromoting rhizobacteria, PGPR. Moreover, biological control of soilborne plant pathogens environmental friendly. The use of bacterial antagonists have been reported (Leisinger and Margraff, 1979; Knudsen and Spur, Jr., 1987; Nuryani et al., 2001). Pseudomonas fluorescens and Bacillus subtilis are antagonistic bacteria for several plant pathogenic fungi or bacteria (Knudsen and Spur, Jr., 1987; Soesanto and Termorshuizen, 2001). Pseudomonas fluorescens P60 is one of antagonistic P. fluorescens strains tested towards several plant diseases such as to suppress F. oxysporum causing gladiolus wilt (Soesanto et al., 2008) and shallot wilt (Santoso et al., 2007).

Induced resistance mechanisms is plant pre-inoculation with various physical, chemical, and biological agents causing changing disease reaction resulted from subsequent inoculation with target pathogen (Misaghi, 1982). Besides that, biochemical reaction happened in plant cell or tissue could produce toxin substances to pathogen or create unfavourable condition inhibiting pathogen growth in plant (Agrios, 2005). Chemical resistance is showed by formation of chemical substances that could protect pathogenic growth and development (Chairul, 2003) and generally with higher concentration than in unresistant plant (Mansfield, 2000; Agrios, 2005).

Based on that, increasing plant resistance to disease was needed and should be applied. One of them could be done by induced resistance mechanism to Fusarium wilt on Raja cultivar banana seedlings using extract of several antagonistic bacteria. This research was aimed at knowing the effect of the bacterial antagonistic extracts on increasing resistance of the banana seedlings to the disease, on the disease development, and on the seedlings growth.

#### **Materials and Methods**

The research wa scarried out at the Laboratory of Plant Diseases and the screen house, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto for six months.

# Source of Fusariun wilt isolate and pathogen preparation

The fungus *F. oxysporum* f.sp. *cubense* (Foc) isolate was obtained from Cavendish banana corm (Haryono, 2007) and maintained on potato dextrose agar (PDA) at 5 °C. The actively growing culture of *F. oxysporum* f.sp. *cubense on* PDA was inoculated into 500 ml of potato dextrose broth and incubated on a *Orbital shaker* for five days at a speed of 150 rpm at room temperature. Then the suspension was fileterd into muslin cloth. The supernatant was used as the extract and measured using haemacytomter.

#### Source of bacterial antagonists and preparation of bacterial extracts

Three bacterial antagonists belong to *Pseudomonas fluorescens* isolate P60 and *P. fluorescens* isolate P32 (Soesanto, 2000) were obtained from the culture collection of Loekas Soesanto, Department of Plant Pests and Diseases, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia. These isolate were originally isolated from wheat rhizosphere. Another isolate of *Bacillus subtilis* was obtained from the culture collection of Darini Sri Utami, Department of Plant Pests and Diseases, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia. This isolate was originally isolated from peanut rhizosphere. Both isolates of *Pseudomonads* were grown in liquid King's B (Schaad, 1980) on a shaker at a

speed of 150 rpm at room temperature for 24 hours. Isolate of Bacillus was grown in nutrient broth for 48 hours at speed of 150 rpm at room temperature. Those suspensions were fileterd into muslin cloth. The supernatant was used as the extract and measured using haemacytomter.

# Production of banana seedlings

Banana CV. Raja seedlings were raised from two week-old tissue culture of source material produced by Hortucultural Seddlings Institute, Salaman, Purworejo, Indonesia. These seedlings were later transferred to pots containing 250 g of sterile sand mixed with pasteured compost (1:2).

# Testing of bacterial antagonistic extracts for induced resistance against Fusarium wilt

To test the effect of three bacterial extracts against Fusarium wilt, the following treatments were used. They are: healthy control (KO); pathogen conrol (K1); pathogen + extract of *P. fluorescens* P60 (P1); extract of *P. fluorescens* (P2); and extrcat of *B. subtilis* (BS). Banana seedlings were soaked in these extracts for 5 min and transplanted into pots containing the media. Fusarium wilt pathogen was applied to the root are of banana seedlings wounding with sterile knife. Inoculated seedlings were maintained by placing the pots in a screen house for about four months with temperature of  $26 \sim 27^{\circ}$ C and RH of  $80\% \sim 90\%$  with double trays technique (Mak *et al.*, 2008). The seedlings were fertilized with Hoagland solution (Lo, 2002) given 10 ml pot<sup>-1</sup> once a week.

Following inoculation of the banana seedlings with the above treatments, and after two weeks period was the symptoms begin and the following observations were made. They are incubation period measured from inoculation of the fungus pathogen to rise the first symptom in days after inoculation (dai), disease severity using attack category or damage scale according to Mak et al. (2008), i.e., for leaves symptom, scale 1 = no infection (healthy plant), scale 2 = slight streaking and/or yellowing oflower leaves, scale 3 = streaking and/or yellowing of most of the lower leaves, scale 4 = extensive streaking and/or yellowing on most of all of the leaves, and scale 5 =dead plant; for corm or rhizome, scale 1 = no discoloration of tissue of steller region of rhizome or surrounding tissue, scale 2 = no discoloration of steller region of rhizome; discoloration at junction of rrot and rhizome, scale 3 = trace of 5% of steller region discolored, scale 4 = 6%  $\sim$  20% of steller region discolored, scale 5 = 21%  $\sim$ 50% of steller region discolored, scale 6 = more than 50% of steller region discolored, scale 7 = discoloration of the entire rhizome stele, and scale 8 = dead plant. The overall disease severity index (DSI) for leaf symptoms and rhizome discoloration for each treatment was calculated as follows:

 $\sum$  (Number on scale x Number of seedlings in that scale)

DSI = -----

 $\sum$  (Number of tretated seedlings)

The DSI consists of four designations, i.e., resistant, tolerant, susceptible, and highly susceptible (Table 1).

Scale of DSI for LSI	Scale of DSI for RDI	Translation
1	1	Resistant
1, 1 - 2	1,1-3	Tolerant
2, 1 - 3	3,1-5	Susceptible
3,1-4	5, 1 - 8	Highly susceptible

Table 1 Translation of DSI scales

Infecation rate was calculated according to formula of van der Plank (1963):

$$r = \frac{2,3}{t} \left( \log \left( \frac{1}{1 - Xt} \right) - \log \left( \frac{1}{1 - Xo} \right) \right)$$

Which r = infection rate, Xo = the proportion infected begin, Xt = the proportion infected at time t, and t = time observation. Antagonist effectivity was measured according to Djaya *et al.* (2003):

$$Ea = \frac{(IPk - IPp)}{IPk} x100\%$$

Where Ea = antagonist effectivity, IPk = disease intensity at control, and IPp = disease intensity at treatment; number of infected roots was calculated at the end of the observation. Plant growth components consisted of plant height, number of leaves, and root wet weight. Plant tissue analysis was carried out qualitatively for knowing phenolic content. Phenolic compound analyzed was glycoside and saponin according to Chairul (2003) and tannin according to Farnsworth (1996) as followed. Glycoside was analyzed using Keller-Killiani reagent: 10 g of plant materials was extracted with 80% ethanol, filtered with muslin cloth and dried on water heater. Hexana was used several times for washing plant lipids until plant pigment were disappeared then dried on water heater for hexana excess. 3 ml of FeCl reagent was added, homogenized and tranferred into a tube. 1 ml of concentrated sulphate acid through inner tube wall then observed color change. Saponin was analyzed using the froth test, extracted 1 g of *Sapindus rarak* fruit with ethanol 10 ml. 2 ml of the extract was put into a tube as a

control. 10 ml of plant material extract in 80% of ethanol was put into another tube. Both tubes were added 10 ml of water, shaked strongly for 30 min and kept for another 30 min. When froth formed was higher than 3 cm, the plant materials contained positively saponin. Tannin was analyzed using protein precipitation principle from gelatine by tannin. 10 g of plant materials was extracted with 80% of ethanol, filterd as above and then dried on water heater. The extract was solved with 20 ml of hot water and added 5 drops of NaCl. A tube containg 5 ml of the extract was added with 3 drops of FeCl<sub>2</sub> reagent. When the plant extract contained tannin, blue or dark blue colour would be formed compared to control without addition of the reagent.

Inhibition of conidia growth was tested according to Wirianata (2004) modified. Observation of fungal pathogenmic growth in plant tissue was done by isolation of plant roots and corms. Either roots or corms were cut and soaked into 1% of sodium hypochlorite solution for  $\pm 5$  min, washed twice in running tap-water, and dried on sterile filter paper. Both plant materials were cut into pieces ( $\pm 1$  cm), isolated on PDA substituted with streptomycin, and then incubated at room temperature ( $\pm 26^{\circ}$ C) for at least five days to be observed. Observation was carried out by identified the growing isolates using microscope after treated with lactophenol cotton blue. The plant materials were also observed directly after treated with lactophenol cotton blue by using intersection method.

#### Data analysis

Data were analyzed by F-test followed by Duncan Multiple Range Tests at 5% when significant diffetent was found.

# **Result and Discussion** *Phenolic compound analysis*

Result showed that there was increase of glycoside, saponin, and tannin content after tretaed with the antagonist extrcats (Table 2).

Treatments	Glycoside	Saponin	Tannin
K0	+	+	+
K1	+	++	+
P1	+++	+++	+++
P2	+++	++	+
Bs	+++	+++	+

Table 2 Qualitative analysis result of phenolic compound

Note: - = no phenolic compound, + = small phenol, ++ = moderate phenol, +++ = high phenol. K0 = without the antagonistic extract, + without *F. oxysporum*, K1 = *F. oxysporum* without the antagonistic extract, P1 = extract of *P. flourescens* P60 + *F. oxysporum*, P2 = extract of *P. flourescens* P32 + *F. oxysporum*, and Bs = extract of *B. subtilis* + *F. oxysporum*.

Both banana roots and corms after induced with the antagonist extract showed different glycoside content compared to control. Glycoside, saponin, and tannin of banana increased after treated with the extract of P. fluorescens P0 and P32 and B. subtilis (Table2). Increasing these compound contents in banana plants by the extracts was caused by phenolic compound. In accordance with Nio (1989) and Chairul (2003), glycoside is an intermediate compound between carbohydrate and another substance called aglicon radical, found in varioud plants and affected plant physiological characters. As glycoside, saponin played important role in activation of plant defence system to pathogen. Nio (1989) stated that saponin was found in all plants with high concentration in certaint part of the plants influenced by plant variety and growth level. The extract of P. fluorescens P60 could induce the highest tannin content compared to other extracts. The extract could actively induce plant defence mechanisms, in accordance with Soesanto and Rahayuniati (2009). Phenolic compound and tannin was found in leaves and young fruit cells with high concentration and responsible in these tissue resistances to pathogenic microbes (Meera et al., 1995; Agrios, 2005).

#### The effect of treatments on pathosystem components

The antagonists extract could significantly affected incubation period, antagonistic effectivity on leaves and corm, and late population density of *Fusarium*, but not for disease incidence, infection rate, and number of infected roots (Table 3).

#### **Incubation period**

Incubation period of the disease was in the range of  $44 \sim 76$  days after inoculation. There was significantly difference of inter-treatments on incubation perid. Treatment of K1 (only *F. oxysporum*) differ significantly with K0 (control), P1 (with *P. fluorescens* P60 extract), and P2 (with *P. fluorescens* P32 extract), but not with *B. subtilis* extract (P2). The average value showed that K1 treatment had the fastest incubation period as 44.67 days after inoculation (dai). This was caused by faster adaptation of the pathogen and infection to banana roots compared to another treatment. The longest incubation period was at P1 or gave slowness incubation period of 41.49%.

The differences on incubation period were affected by phenolic compound resulted from banana seedlings treated with the antagonist extract. The banana seedlings induced by *P. fluorescens* P60 extract (P1) showed that content of glycoside, saponin, and tannin was higher that other treatments (Table1). *P. fluorescens* P60 extract could induce banana resistant systemically and the extract could be tranlocated in the banana tissue. This was in accordance with Weller and Cook (1983), Pieterse *et al.* (1996), van Loon *et al.* (1998), and Zehnder *et al.* (2001).

#### **Disease severity**

Disease severity closely related with incubation period. Based on LSI and RDI, the average of LSI was lower than RDI. This was caused by pathogen did not enter all part of banana plants yet and still infect the root and corm or rhizome. The secondary symptoms appeared at another plant location is resulted from damage of primary symptoms part. The symptoms were slow because disease expression usually takes 4~5 months (Mak *et al.*, 2008).

The banana seedlings induced by *P. fluorescens* P60 (P1) and *P. fluorescens* P32 (P2) extract appeared more resistance to Fusarium wilt grouped into tolerant. P1 treatment caused the lowest either LSI or RDI as 1.23 and 1.99, respectively, or decreasing disease severity as 19.07% and 41.12%, respectively. This was caused by low pathogenic virulence and environmental factor effect that could promote or inhibit growth of the pathogen. According to Agrios (2005), disease establishment related closely to pathogen virulence, susceptible host, and suitable environment condition.

Treatments	Incubation period	Disease	Severity <sup>ns</sup>	Infectio n rate	Late Fusarium	Antagonisti effectivity (	c %)	Number of infected	Severity level
	(dai)	Leaves	Corms	(unit day <sup>-1</sup> ) <sup>ns</sup>	density (n $\times$ 10 <sup>7</sup> g <sup>-1</sup> soil)	Leaves	Corms	roots (%) <sup>ns</sup>	
K0	72.50 b	1.31	3.05	0.003	0.19 b	0.0b	0.0 b	66.4	Toleran
K1	44.66 a	1.52	3.38	0.004	1.47 a	0.0 b	0.0 b	81.9	Susceptib
									le
P1	76.33 b	1.23	1.99	0.002	1.23 a	19.6 a	31.51 a	60.5	Toleran
P2	75.16 b	1.26	2.94	0.003	0.82 ab	20.5 a	25.8 ab	64.7	Toleran
Bs	52.5 ab	1.51	3.33	0.005	1.49 a	14.0 ab	12.0 ab	67.7	Susceptib le

Table 3 The effect of treatments on pathosystem component

Note: K0 = without the extract + without *F. oxysporum*, K1 = *F. oxysporum* without the extract, P1 = extract of *P. flourescens* P60 + *F. oxysporum*, P2 = extract of *P. flourescens* P32 + *F. oxysporum*, and Bs = extract of *B. subtilis* + *F. oxysporum*. Value followed by same word in the same column did not differ significantly according

to DMRT at 5 %. Data of infection rate were transformed to  $\sqrt{(x+0,5)}$ . ns = no significant difference and dai = days after inoculation.

#### Infection rate

Low infection rate was assiciated with low disease severity caused by virulent pathogen so that resistat mechanisms in the plant were failing. According to van Loon *et al.* (1998), all plants have active resistant mechanisms against pathogen attact. The mechanisms were failing when the plant was infected by virulent pathogen because the pathogen could prevent the resistant reaction or escape the effect of activated resistance. When the mechanisms could be promoted earlier before infection, disease could be decreased.

#### Late Fusarium density

Early pathogenic population density was  $1.04 \times 10^8$  conidia ml<sup>-1</sup> solution which decreased to 85.86% at the end of observation. Statistical analysis of conidia density showed that K1 was significantly difference toward P2 but not toward P1 and Bs. This pointed out that *P. fluorescens* P32 extract (P2) could decrease the population density. Epidemiology of disease development including Fusarium wilt, is depent on number of earlier inoculum and infection rate at time (Zadock and Schein, 1979; Boff, 2001). To control the disease, the pathogen inoculum has to be reduced or lost.

#### Antagonists effectivity

*P. flourescens* P60 (P1) and *P. flourescens* P32 (P2) extracts could induce the high effectivity on leaves part as high as 19.67 and 20.58%. At corm part, the most effective treatment was P1 as 31.51%. Plant infected cell wall increased composition substances formation especially lignin, suberin, cutine, and other phenolic compounds as self-protection, according to Maryani and Kasiamdari (2004).

# Number of infected roots

*P. fluorescens* P60 (P1) and *P. fluorescens* P32 (P2) extract could show the smallest infection average of root numbers compared to control. This was caused by the environmental effect during observation, i.e., average of temperature and relatif humidity as  $34^{\circ}$ C and 80%, respectively, so that distrubution and development of the disease were affected. According to Smith (2007), the disease caused by *Fusarium* sp. could be spread by water or contaminated agricultural equipments. As soil-borne plant pathogen, *F. oxysporum* need some special advantages in order to make it to the plant rhizosphere such as specific soil temperature and relative humidity to grow and develop (Ioannou *et al.*, 1977).

#### The effect of treatments on seedlings banana growth

Data analysis of plant growth components did not differ (Table 4). Observation of plant height resulted in between  $40 \sim 46$  cm and the lowest one was on Bs treatment as 40.9 cm and the highest one was on P1 as 46.6 cm. Number of leaves was almost the same as  $5 \sim 6$  leaves and also root wet weight as  $5 \sim 6$  g. This was caused by *F. oxysporum* did not enter the plant root to infect so that plant height and leaves number were relative the same. The pathogen infected all plant parts need a longer time to get started at a site of infection. According to Smith (2007) and Mak *et al.* (2008), soilborne pathogens do not have opportunity to spread as rapidly as do the above-ground organisms.

Treatments	Crop height (cm) <sup>ns</sup>	Number.of leaves <sup>ns</sup>	Wet weight of root (g) <sup>ns</sup>
K0	44.70	5.40	5.763
K1	42.10	5.30	5.333
P1	46.60	5.90	6.127
P2	45.50	5.60	6.470
Bs	40.90	5.00	5.022

Table 4	The effect	of treatments	on plant growth
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Note: K0 = without the extract + without *F. oxysporum*, K1 = F. oxysporum without the extract, P1 = extract of *P. flourescens* P60 + *F. oxysporum*, P2 = extraxt of *P. flourescens* P32 + *F. oxysporum*, and Bs = extract of *B. subtilis* + *F. oxysporum*. ns = no significant difference.

Treatments	Conidia germination (%)	Establishment of Foc (%) <sup>th</sup>	
		Roots	Corms
K0	15.87	10.00	10.00
K1	47.00	50.00	50.00
P1	15.59	30.00	30.00
P2	29.19	30.00	30.00
Bs	42.13	20.00	20.00

Table 5 Conidia germination and Fusarium establishment

Note: K0 = without the extract + without *F. oxysporum*, K1 = F. oxysporum without the extract, P1 = extract of *P. flourescens* P60 + *F. oxysporum*, P2 = extract of *P. flourescens* P32 + *F. oxysporum*, and Bs = extract of *B. subtilis* + *F. oxysporum*. ns = no significant difference.

# The effect of treatments on fungal conidia germination and *Fusarium* establishment in plant

#### Germination of fungal conidia

Either root or corm extract was different in percentage of pathohenic fungal conidia germination inter-treatment (Table5). At control (K0), the highest conidial germination was found as 47% and the lowest one was found at P1 as 15.59% or decrease as high as 31.41%. Low germination at P1 resulted in increasing resistant reaction showed by increasing the phenolic compound. In accordance with Edreva (2004) and Firmansyah (2008), increasing mechanism activity of induced plant resistance was affected by biotic and abiotic factors. Antagonistic bacteria played role as biotic factor inhibiting pathogenic infection and phenolic compound reaulted from banana seedlings as abiotic factor.

#### **Fungal establishment in plant**

Isolation of the Fusarium fungus was done at infected root and corm resulted in no significantly difference. Based on the result, the highest number of pathogen population was showed by control (K0) either from root or corm as 50% and 60%, respectively.

#### Conclusions

Antagonist bacterial extract of *P. flourescens* P60 and *P. flourescens* P32 potentially induced resistance to Fusarium wilt in banana cv. Raja seedlings showed by increasing phenolic compound, i.e., glycoside, saponin, and tannin.

The extract of *P. flourescens* P60 and *P. flourescens* P32 could suppress Fusarium wilt development in banana cv. Raja seedlings showed by slowness of incubation period as 76.333 and 75.167 days after inoculation, respectively, and with antagonist effectivity in corm as 31.51% and 25.88%, respectively; while in leaves as 19.67% and 20.58%, respectively.

The extract could not able to increase growth of banana cv. Raja seedlings.

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# Induction of systemic resistance by rhizobacteria for the management of root-knot nematodes in tomato

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#### Abstract

Pseudomonas fluorescence and Bacillus subtilis as Plant Growth Promoting Rhizobacteria (PGPR) isolated from root rhizosphere of tomato plant were utilized for growth promotion and induction of systemic resistance in tomato against root knot nematodes. Present study was aimed to study the ability of two rhizobacterial isolates i.e., Pseudomonas fluorescence and Bacillus subtilis to induce systemic resistance and their effect on growth promotion The influence of rhizobacteria *Pseudomonas* fluorescence and Bacillus subtilis on growth parameters of the tomato plant and mortality of nematodes was studied in growth chamber and in net house conditions. More increase was observed in case of Pseudomonas fluorescence and results elucidated an increase in plant height (12.4%), fresh weight of shoot (45.6%), dry weight of shoot (37.03%), fresh weight of root (68.75%) and dry weight of root (114.28%) in growth chamber in case of *Pseudomonas fluorescence* while in net house plant height was observed to be increased by 25.6%, 66.66% increase in fresh weight of shoot, fresh weight of root and dry weight of root were also increase by 47.6% and 40% respectively while 7% decrease was observed in dry weight of shoot. 33.40% decreased population of nematodes was achieved by *Pseudomonas* fluorescence in growth chamber while Bacillus subtilis decreased nematode population up to 31.59%. The production of siderophores with nematicidal properties by inducing systemic resistance isolate of Pseudomonas fluorescence is an essential step for its efficacy but not for Bacillus subtilis. We concluded that Pseudomonas fluorescence and Bacillus subtilis are potential biocontrol agents and has induced systemic resistance against root knot nematodes in tomatoes.

# Introduction

Among the vegetable crops grown in Pakistan, the importance of tomatoes (*Lycopersicon Lycopersicum* Karst) as a food crop cannot be over emphasized. It contains the highest amount of vitamins among all the vegetables, possesses anticancerous properties, and is good for diabetic patients. Among the 15 vegetable grown in the world, Tomato ranks seemed in terms of total production. In Pakistan, its cultivated area is 47100 hectares with a total production of 502300 tones (Anonymous, 2007). The yield is quite low as compared to other developing countries as Iran, India and Bangladesh. General biotic factors that affect the productivity and fruit quality of tomato are plant pathogens (viruses, bacteria, fungi, and nematodes), insects and weeds but the main reason for this low production of tomatoes in Pakistan are pathogenic nematodes especially Root knot nematodes.

Root knot nematodes (*Meloidogyne* spp.) are more widely distributed throughout the world than any other group of plant parasitic nematodes. However the degree of geographical adaptation to continuous existence is evident among the species. Tomato plant affected by root knot nematodes exhibit slow development and stunted appearance. If the infestation appears early it gets severe, leaves become yellow green to yellow; tend to droop and the plants wilt. The roots become galled and the presence of galls is the most characteristic symptom of infection. In case of severe infection there may be complete loss of plant vigor resulting into heavy yield losses.

Biological control of nematodes has been developed successfully during the last few years. PGPR belonging to *Pseudomonas* and *Bacillus* spp. have also been used in bioantagonism (Ismail *et al.*, 1997, Aksoy *et al.*, 2004). There has been large body of literature describing potential uses of PGPR against control of plant parasitic nematodes of different crops. Initial investigations on antagonistic rhizobacteria against nematodes include work by (Zavaleta-Meija and van Gundy, 1982; Becker *et al.*, 1988; Oostendorp and Sikora, 1989; Kloepper *et al.*, 1992) and latest investigations include (Insunza *et al.*, 2002, Hamid, *et al.*, 2003. Their investigations show satisfactory results for nematodes control. Keeping these facts in mind it was planned to isolate bacterial strains from rhizosphere which are bioantagonistic to root knot disease of tomato caused by *Meloidogyne* spp.

#### **Materials and Methods**

#### Sampling and extraction of root knot nematodes

Sampling from tomato plants was done by uprooting the whole plant from soil the using spade. One hundred grams of soil and roots from each sample were processed for the isolation of nematodes. Nematodes were extracted by using Whitehead and Hemming tray methods (Whitehead, 1986) and sieving method.

Egg sacs from galled roots of sampled plants were obtained by teasing washed roots in water and recovering the egg sacs on a 60 mesh sieve. Egg sacs were added to 100 ml of tap water, 500 ml of 1% sodium hypochlorite macerated in an electric blender for 40 sec. The suspension of egg and root debris was poured through 100 and 400 mesh sieves over a container. The material retained on the 100 mesh sieve was discarded while the material retained on the 400 mesh sieve (37 mm) was washed several times with distilled water and the filtrate were collected in a beaker. More eggs were recovered by repeated sieving and rinsing (Mecheal *et al.*, 1973). The

number of nematode eggs in an aqueous suspension was determined by using a counting dish in which 5 cm diameter glass Petri dish was used as a counting dish. Counting of each sample was repeated four times in this manner. The mean number of nematode eggs per ml was determined by averaging the counts taken.

#### Quantitative estimation of nematodes in infested roots

For quantitative assessment of second stage juvenile nematodes in infested roots, the stained and cleaned roots were cut into 1 cm pieces and put into a 7 cm vial using forceps. The roots were immersed in a sufficient amount of water and macerated by a laboratory homogenizer for  $20 \sim 30$  seconds, depending on the age of the roots. Water was added to this suspension and a 2 ml aliquot was taken by pipette for counting. The number of juveniles per root system was determined from counts of four replicates.

After counting, nematodes were picked by using bamboo needle followed by killing and fixing of nematodes. Nematodes were identified on the basis of perennial pattern.

To multiply the culture of root-knot nematodes, the most susceptible variety of egg plant (Black beauty) was used. Three weeks old nursery was transplanted in earthen pots containing 2.5 kg, 1:2 sandy loam soil sterilized with formalin one plant/pot was planted. One week after transplanting, these plants were inoculated with approximately 2000 freshly hatched second stage juveniles of *Meloidogyne incognita*. Tap water was used to irrigate young seedlings throughout out the period of study. The temperature range  $20 \sim 30$  °C was recorded.

# Plant growth promoting rhizobacterial isolates

Principally, rhizobactreial isolates were obtained from of Department of Soil Science, Pir Mehr Ali Shah Arid Agriculture University Rwalpindi, and they were multiplied and maintained at  $20 \sim 30$  °C. PGPRs were identified on the bases of cell morphology, colony morphology, and gram staining.

#### Siderophore production

Siderophore production was assessed by suspending bacterial cells in a 10 mM MgSO<sub>4</sub> which was spot-inoculated (5  $\mu$ l) at two points on NA plates supplemented with 100  $\mu$ M FeCl<sub>3</sub>and incubated at 27 °C for 48 h.

# Antagonistic activity of PGPRs against Meloidogyne javanica in-vitro

Rhizobacterial strains were maintained at on King's B medium at  $6^{\circ}$ C before use. The inoculums were produced by transferring two loopful of the bacteria from a 5 day old culture to 100 ml KB liquid medium and incubated at room temperature on a shaker (150 rpm) for 48 h. The bacterial cells were centrifuged at (4500 ×g) for 15 min. Supernatant was discarded and pellet was suspended in sterile MgSO<sub>4</sub> (0.1M). For preparation of cell free culture filtrate, the bacterium was grown at 30 °C in KB Liquid medium for 48 h in dark and centrifuged twice at 2800 rpm for 20 min. Pellets were discarded and culture filtrate was collected in a sterilized beaker prior to use. The filtrate was spread over KB medium for the presence or absence of bacterial cells.

No bacterial growth was observed on any of the plate. *Meloidogyne javanica* was maintained on egg plants in a glass house. Egg masses were handpicked from infested roots and kept in autoclaved distilled water. Juveniles were collected three days after incubation. Nematicidal activity of bacterial culture filtrate was determined by transferring 2 ml of filtrate in a 3 cm cavity glass slide along with  $25 \sim 30$  surface sterile juveniles per ml. Number of dead juveniles were counted after 24 and 48 h. The mean percentage of dead larva was estimated. The juveniles were considered dead when they do not move on probing with a fine needle (Cayrol *et al.*, 1987).

# Development of induced systemic resistance

Two root system each having bacteria (*Pseudomonas fluorescence* and *Bacillus subtilis*) and nematodes were inoculated separately. The seedlings were uprooted from sterilized soil after three weeks, then seedlings were washed with tap water and their roots were splitted into two halves with dissecting scalpel. Each of root system was transplanted into separate plastic pots. One root system was treated with cell suspension of bacterial strain prepared in distilled water. The Ringer solution was used as a controlled treatment. After one week the other half root system was treated with freshly hatched with juveniles of *Meloidogyne* spp. The Nematode in vision was observed after 21 days of inoculation from the nematode treated one of the root system.

# Efficacy of *Pseudomonas fluorescence* and *Bacillus subtilis* against *Meliodogyne javanica* under growth chamber and in net house conditions

The two bacteria, *Pseudomonas fluorescence* and *Bacillus subtilis* were selected for evaluation in growth chamber and in net house. The bacteria were grown on liquid KB medium for 48 h, as shake culture at room temperature. Tomato seed were surface sterilized in 1% Ca(OCl)<sub>2</sub> for 3 min, then rinsed several times with distilled water, and was then treated with an aqueous cell suspension of the bacteria using 1% gum Arabic as a sticking agent.

The process yielded  $4 \times 10^9$  cfu/seed. Twenty four pots were used. Three treated seeds were sown in each pot and after germination, one seedlings retained per pot. For root dip treatment, also 24 pots were used. Roots were dipped in bacterial suspension and one seedling was planted in each pot. Seed and root treated with sterile MgSO<sub>4</sub> (0.1 M) without the bacterium served as control. For application of the

bacteria as a soil drench, a 25ml aqueous cell suspension prepared in sterile MgSO<sub>4</sub> (0.1M) was drenched onto the soil and three seeds sown into the pot. One seedling per pot was retained after emergence.24 pots were also used. Soil drenched with 25ml sterile MgSO<sub>4</sub> (0.1M) served as controls.

One week after seedling emergence, each pot was inoculated with 1000 freshly hatched juveniles of *M. javanica* that were added to a hole made between the seedlings in the soil of each pot. Treatments and controls were replicated four times and the pots were arranged in randomized complete block design. Eight weeks after nematodes addition, the root system was washed and number of galls induced by *M. javanica* on entire root system recorded. The root-knot nematodes in bacteria treated and untreated pots were extracted using a modified Baermann funnel technique and following treatments were used.

- T1 = *Pseudomonas fluorescence*
- T2 = Pseudomonas fluorescence+ Meloidogyne javanica
- T3 = Bacillus subtilis
- T4 = Bacillus subtilis + Meloidogyne javanica
- T5 = *Meloidogyne javanica*
- T6 = No bacteria + No nematode

# **Results and Discussion**

Tomato samples were collected from different sites. The frequency of infection from these sites ranged between 35% to 69%. Both *Meliodogyne javanica* and *Meloidogyne aeruginosa* were found. But at most locations, *Meloidogyne javanica* was found to be most destructive species.

# Antagonistic activity of bacterial strains

All tested strains produced compounds that resulted in the death of *Meloidogyne javanica* ranging from  $(3\% \sim 53\%)$ . Maximum mortality was observed by *Pseudomonas fluorescence* Rh37 which gave 53% juvenile mortality after 48 hrs as compared to control. The lowest mortality (3%) was observed with strains Asr14 and R.E.4. Strains 7NSK2, Mr53, Asr38, Asr28 and Rh17 showed mortality of *Meloidogyne javanica* 39%, 28%, 25%, 24% and 23% respectively. Mortality of *Meloidogyne javanica* by the other strains ranged from 9%~23%. The six most active strains (Rh37, 7NSK2, CHA0, Asr38, Asr28 and Rh17) were chosen for further analysis of their metabolite products.

# Antagonistic activity of PGPRs against Meloidogyne javanica in- vitro


# Siderophore production

During the tests for siderophore production, Orange discoloration of the bacterial colonies occurred after 48 h of incubation with coloured zones of varying widths around the colonies indicating positive siderophore production.

# Development of induced systemic resistance

Application of two strains, there transforments to one half the split root section reduced nematode invasion to the other (non-bacterized nematode treated) half the root system in both strains and tomato seedling however wild type strains induced systemic resistance against *Meloidogyne* spp.

# Efficacy of *Pseudomonas fluorescence* and *Bacillus subtilis* against *Meloidogyne javanica* under growth chamber

# Effect on Growth parameters

Analysis of variance of data regarding plant fresh weight was significant (Table1). For treatment effect comparison of treatment means shows that maximum fresh weight of shoot was observed with *Pseudomonas fluorescence* treatment which increased weight by 45.65% over control. The lowest fresh weight of shoot was observed with nematode alone as showing 45.65% decrease over control. In case of *Bacillus subtilis*, a 26.8% increase over control was seen. *Pseudomonas fluorescence* + *Meloidogyne javanica* and *Bacillus subtilis* + nematode gave 6% and 21.73% decrease over control. However *Bacillus subtilis* with nematode showed 26.08% increase in plant height over control. Therefore it was concluded that *Pseudomonas* 

*fluorescence* improved the weight of plants to greater extent both alone and in the presence of nematodes.

**Effect on dry weight of Shoot** F. value in ANOVA for this value was highly significant as shown in the Table1. Comparison of treatment means showed that dry weight of shoot was maximum with *Pseudomonas fluorescence* which gave 37 % increase over control and was maximum with nematode alone which showed 88% decrease over control. Dry weight of shoot in case *Bacillus subtilis* was enhanced and it shows 18.51 increase of control. Other treatments *Pseudomonas fluorescence*+ *Meloidogyne javanica* and *Bacillus subtilis* + *Meloidogyne javanica* showed 37% and 40.74% decrease over control. Although *Pseudomonas fluorescence* increased weight of shoot to a greater degree than *Bacillus subtilis*, both gave the same reduced yield in the presence of nematodes.

**Fresh weight of root** F- Value in respect of fresh weight of root was highly significant as shown in Table1. Treatment with nematode alone shows maximum increase (243%) over control due to gall formation. Fresh weight of root was maximum with *Bacillus subtilis* only which shows 31.25% increase over control. Other treatments *Pseudomonas fluorescence+ Meloidogyne javanica* and *Bacillus subtilis* + *Meloidogyn javanica* showed 125% and 187% increase over control. *Pseudomonas fluorescence* showed better results in reducing gall formation.

**Dry weight of root** Analysis of variance in respect of dry weight of root was highly significant as shown Table1. For treatment effect comparison of treatment means indicated that dry weight of root was maximum with nematode alone which gave 400% increase over control because of gall formation. Dry weight of root with *Pseudomonas fluorescence* was more (214%) than that of *Bacillus subtilis* due to hormone production. However in nematode inoculated treatments *Bacillus subtilis* showed better performance in reducing gall formation.

**Effect on nematode population** F- Values regarding number of juveniles in soil, roots and number of egg masses in roots showed that treatment with *Pseudomonas fluorescence* + *Meloidogyne javanica* showed 33.40%, 47% reduction in juvenile population and 45% decrease in egg mass production as compared to control, respectively. While *Bacillus subtilis* + *Meloidogyne javanica* gave 32% reduction in nematode population in soil, 37% reduction in nematode penetration in roots and 25% decrease in egg mass production under growth chamber conditions.

Treatments	Plant height (cm)	Fresh wt. of shoot (g)	Dry wt. of shoot (g)	Fresh wt. of root (g)	Dry wt. of root (g)	No. of juveniles /250 gm of soil	No. of juveniles /g of fresh root	No. of egg masses/ root	Galling index
Pseudomonas fluorescence	8.86a	0.66a	0.4a	0.2d	0.1d	0.0c	0.0d	0.0d	0.0c
Pseudomonas fluorescence + nematode	7.81c	0.46c	0.2c	0.4b	0.2b	293b	50c	11c	3.1b
Bacillus subtilis	8.11b	0.56b	0.3b	0.2e	0.1c	0.0c	0.0d	0.0d	0.0c
Bacillus subtilis + nematode	7.61c	0.36d	0.2c	0.4c	0.2b	339b	59b	15b	3.3a
Nematode alone	7.06d	0.26e	0.03e	0.5a	0.4a	482a	93a	20a	3.6a
No bacteria + no nematode	7.88c	0.46c	0.2d	0.2e	0.1e	0.0c	0.0d	0.0d	0.0c

 Table 1 ANOVA showing the effect of Induced Systemic Resistance by Rhizobacteria against Meloidogyne javanica in tomatoes when rhizobacterial isolates were applied in growth chamber

Treatments	Plant height (cm) %increase /decrease over Control	Fresh weight of shoot(g) %increase /decrease over Control	Dry wt. of shoot (g) %increase /decrease over control	Fresh wt of root (g) %increase /decrease over control	Dry wt. of root (g) %increase /decrease over control	No. of juveniles /250 gm of soil %increase /decrease over control
Pseudomonas fluorescence	12.43	45.65	37.03	68.75	114.28	0.0
Pseudomonas fluorescence + nematode	-0.08	-6.52	37.03	125	214.28	33.40
Bacillus subtilis	2.91	26.09	18.51	31.25	28.57	0.0
Bacillus subtilis+ nematode	-3.42	-21.77	-40.74	187.5	257.14	31.59
Nematode alone	-10.40	-45.65	-88.88	243.75	400	440
No bacteria + no Nematode	7.88	0.46	0.27	0.16	0.07	0.0

 Table 2
 Percentage change in parameters when rhizobacterial isolates were applied in growth chamber

# Efficacy of *Pseudomonas fluorescence* and *Bacillus subtilis* against *Meloidogyne javanica* in net house conditions

#### Effect on plant height

Effect of *Pseudomonas fluorescence* and *Bacillus subtilis* on the growth parameters of tomato and development of root knot nematode disease was measured in pot experiment in net house (Table3 and Table4).

The results obtained regarding plant height was analyzed and presented in the (Table 3 and 4). F-value in ANOVA for treatments was found to be highly significant. Comparison of treatment means showed that height was maximum with *Pseudomonas fluorescence* treatment which showed 26% increases over control and was lowest with nematode alone which gave 13% decrease over control. Treatment with *Bacillus subtilis* enhances plant height and gave 13% increase over control. Both the treatments *Pseudomonas fluorescence* + *Meloidogyne javanica* and *Bacillus subtilis* + *Meloidogyne javanica* 19.2% increased plant height over control and gave 6% decrease in plant height.

It means that *Pseudomonas fluorescence* is considered as much significant for plant growth.

## Effect on fresh weight of Shoot

Results regarding this parameter showed that F. value in ANOVA was significant as shown (Table3). Comparison of treatment means shows that maximum fresh weight of shoot was observed with *Pseudomonas fluorescence* treatment which shows 67% increase over control. The lowest fresh weight of shoot was observed with nematode alone. This showed 20% decrease over control. Other treatments *Pseudomonas fluorescence* + *Meloidogyne javanica* and *Bacillus subtilis* + *Meloidogyne javanica* gave 5% and 9% decrease over control. However *Bacillus subtilis* showed 40% increase in plant height over control. It was concluded that *Pseudomonas fluorescence* improved weight of plant shoot to a greater extent both alone and in the presence of *Meloidogyne javanica*.

## Effect on dry weight of Shoot

F- value in ANNOVA for dry weight of shoot was highly significant as shown in the Table3. Comparison of treatment means showed that dry weight of shoot was maximum with *Pseudomonas fluorescence* treatment which showed 116 % increase over control and was minimum *Meloidogyne javanica* which showed 28% decrease over control. Dry weight of shoot in case of *Bacillus subtilis* was enhanced and it showed 84% increase over control both the treatments *Pseudomonas fluorescence*+ *Meloidogyne javanica* and *Bacillus subtilis* + *Meloidogyne javanica* showed 8% and 16% decrease over control. *Pseudomonas fluorescence* increased weight of plants shoot to a greater extent.

# Fresh weight of root

F- Value in respect of fresh weight of root was highly significant as shown in the Table3. Treatment with nematode alone showed maximum increase (209%) over control due to gall formation. Fresh weight of root was lowest with *Bacillus subtilis* only which shows 67% increase over control. Both the treatments *Pseudomonas fluorescence+ Meloidogyne javanica* and *Bacillus subtilis + Meloidogyne javanica* showed 119% and 171% increase over control. *Pseudomonas fluorescence* showed better results in reducing gall formation.

**Dry weight of root** Analysis of variance in respect of dry weight of root was highly significant as shown in the Table3. For treatment effect comparison of treatment means indicated that dry weight of root was maximum with *Meloidogyne javanica* alone which showed 213% increase over control because of gall formation. Dry weight of root with *Pseudomonas fluorescence* was more than that *Bacillus subtilis* which gave 40% and 33% increase over control respectively due to hormone production. However in nematode inoculated treatments *Pseudomonas fluorescence* + *Meloidogyne javanica* and *Bacillus subtilis* + *Meloidogyne javanica* 213 % and 140% increase in dry weight of root was observed as compared to control. So the treatment *Pseudomonas fluorescence* + *Meloidogyne javanica* showed better performance in reduction of nematode gall formation.

 Table 3 ANOVA shown the effect of Induced Systemic Resistance by Rhizobacteria on plant

 growth and *Meloidogyne javanica* on tomatoes when rhizobacterial isolates were applied in net house

Treatments	Plant height (cm)	Fresh wt. of shoot (g)	Dry wt of shoot (g)	Fresh wt of root (g)	Dry wt of root (g)	No. of juvenile s /250 gm of soil	No. of juvenile s /g of fresh root	No. of egg masse s/root	Gallin g index
Pseudomonas fluorescence	10.04 a	0.67a	0.37a	0.3d	0.15d	0.0	0.0d	0.0c	0.0c
Pseudomonas fluorescence + nematode	7.81 c	0.43c	0.17c	0.3b	0.22b	293	293c	14b	3.2b
Bacillus Subtilis	8.11 b	0.58b	0.32b	0.2e	0.09e	0.0a	0.0d	0.0c	0.0c
Bacillus subtilis+ nematode	7.61d	0.36d	0.16d	0.4c	0.25c	301	339b	17b	3.5a
Nematode Alone	7.06 f	0.25e	0.03e	0.5a	0.35 a	1482a	440	26a	3.8a
No bacteria + no nematode	7.88 e	0.46c	0.27d	0.1e	0.07e	0.0d	0.0d	0.0c	0.0c

**Effect on nematode population** F- Value regarding nematode population in 250gm of soil showed significant results as shown in the Table3. The treatment with *Pseudomonas fluorescence+ Meloidogyne javanica* showed 39% reduction of juveniles in soil population, 40% reduction in nematode penetration in roots, 46% decrease in egg mass production as compared to control while *Bacillus subtilis + Meloidogyne javanica* showed 30% reduction in nematode population in soil, 35% reduction in nematode penetration in roots and 35% decrease in egg mass production over control. *Pseudomonas fluorescence+ Meloidogyne javanica* showed significant degree in reducing number of nematodes in soil than that of *Bacillus subtilis + Meloidogyne javanica*.

Treatments	Plant height (cm) %increase /decrease over control	Fresh weight of shoot(g) %increase /decrease over Control	Dry wt. of shoot (g) %increase /decrease over Control	Fresh wt. of root (g) %increase /decrease over control	Dry wt. of root (g) %increase /decrease over control)	No. of juveniles /250 gm of soil) (%increase /decrease over control
Pseudomonas fluorescence	25.65	66.66	-7	47.61	40	0.0
Pseudomonas fluorescence + nematode	19.02	-4.44	-8	119	113.33	-39.21
Bacillus subtilis	13	4	84	66.66	33.33	0.0
Bacillus subtilis+ Nematode	-5.63	-8.	-16	171.42	=140	- 29.66
Nematode alone	-13	-20	-2	209.5	213.33	482
No bacteria + no Nematode	7.99	0.45	0.25	0.21	0.15	0.0d

#### Table 4 The percentage change in parameters when rhizobacterial

isolates were applied in net house conditions

Increase in the growth parameters of the tomato plant was as a result of increased synthesis of auxin which is a growth promoter. There is always found a positive correlation of growth parameters and IAA production by the rhizobacterias. Production of auxin by rhizobacteria has been reported by many species and *Pseudomonas* spp. has always been found to produce more auxin thus increased plant height, root and shoot weights. This may imply that auxin produced by PGPR isolates caused improvement in root weight, shoot weight, plant height and more biomass production. However, other mechanism through which PGPR plays role in the growth of plant should also be considered. Glick (1995) viewed that mechanism most commonly invoked to explain the various effects of PGPR on plants is the production of phytohormones, and IAA may play the most important role in growth promotion.

Treatment with *Pseudomonas fluorescence* showed greatest increase in plant height likely it is because indole acetic acid producer. Phosphate solubilization and indole acetic acid production by *Pseudomonas* spp. has also been reported (Debora *et al.*, 2007).

Nematode mortality up to  $17\% \sim 96\%$  was recorded after 48 hours when seaweed at the concentration 2mg/ml was used against *Meliodogyne javanica* (Gehan *et al.*,

1996). The reason is that microorganisms antagonistic to nematodes secrete different metabolites which would be responsible for nematode mortality. In our study proteases were detected and may be responsible for mortality of *Meloidogyne javanica*.

*Pseudomonas fluorescence* suppressed root-knot nematode greater than *Bacillus subtilis* both in growth chamber and in net house. Moreover nematicidal activity of *pseudomonas* was enhanced in the growth medium containing iron (Siddiqui, 2004) and it was found that tomato has good contents of iron that may be the reason that *Pseudomonas* spp. activity was more pronounced than *Bacillus* spp. due to more affinity to iron. The secondary metabolites such as DAPG, pyoluteorin and hydrogen cyanide secreted by *Pseuodomonas* spp. which act as biocontrol factor against nematodes (Cronin *et al.*, 1997; Gallagher and Manoil, 2001). Biosynthesis of hydrogen cyanide is affected by environmental factors including iron, phosphate and oxygen concentrations (Knowles and Bunch, 1986) this may be the cause of difference in different antagonistic activity of different strains. Based on the results presented here it could be concluded that *Pseudomonas fluorescence* and *Bacillus subtilis* has induced the systemic resistance against root-knot nematode in tomato.

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# Investigation on indigenous bacterial diversity and population succession dynamics in maize spermosphere

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#### Abstract

The spermosphere as an important habitat in plant micro-ecosystem has a unique significance of seed microbial ecology, while the related reports has been scarce. In this study, the mature seeds of reciprocal cross maize (*Zea mays* L., Nongda108) were collected to investigate the diversity and population succession dynamics of spermosphere indigenous bacteria in 12 h, 24 h and 36 h of seeds germination by 16S rDNA library construction. It was found that the spermosphere indigenous bacteria of Nongda108 included *Pseudomonas*, *Burkholderia*, *Bacillus*, *Paenibacillus*,

Stenotrophomonas and so on. In the spermosphere of one plant sample of the reciprocal cross maize, Nongda108A (Huang  $C \times 178$ ), the first and second dominant bacteria genera identified were Pseudomonas and Burkholderia respectively. In the time course of seeds germination, the kinds of dominant genera of Nongda108A were not changed, and with seeds germinating, the abundance of Pseudomonas was increased with the order of 59.596%, 75.000% and 82.609%; while Burkholderia was decreased with the order of 39.394%, 25.000% and 15.217%. The kinds of dominant bacteria genera of Nongda108B (178×Huang C) changed among the time course of seeds germination. Bacillus, Paenibacillus and Stenotrophomonas as the dominant genera from the other plant sample, Nongda108B. The population succession dynamics of indigenous bacteria in Nongda108B was more apparent than the other. The abundance of Paenibacillus as the same dominant genus after 12h, 24h and 36h of seeds germination was decreased with the order of 68.000%, 46.154% and 13.265%; while the abundance of Stenotrophomonas (32.000%), Bacillus (53.846%) and Burkholderia (77.551%) as dominant genera after 12 h, 24 h and 36 h increased respectively. Some dominant bacteria genera identified from maize spermosphere were the common PGPR.

# Evaluation of PGPR for biocontrol and nutrition mobilization in black pepper (*Piper nigrum* L.)

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#### Abstract

Black pepper (*Piper nigrum* L.) is one of the important spice crops of India. A large number of PGPR were isolated from major black pepper growing areas in Kerala and Karnataka. These rhizobacterial strains were grouped based on morphological characters into nine genera namely, Arthrobacter, Bacillus, Burkholderia, Curtobacterium, Enterobacter, Klebsiella, Micrococcus, Pseudomonas and Serratia. All the isolates were screened against *Phytophthora capsici* from black pepper by dual culture and for the production of secondary metabolites such as DAPG, Phenazine, hydrogen cyanide and biosurfactant. Three isolates BRB21 (Burkholdera), BRB 28 (Psedomonas) and BRB 49 (Serratia) were shortlisted for biocontrol studies and BRB 3 (Micrococcus), BRB 13 (Enterobacter), BRB 23 (Micrococcus) were shortlisted for nutrition mobilization. The green house trials on biocontrol indicated that isolate BRB 49 was found to reduce root rot incidence. The nutrition mobilization studies included applying varying levels of major nutrients, nitrogen, phosphorus and potash (NPK) along with PGPR strains. After about Eight months of the experiments growth parameters and nutrition mobilization were recorded. Analyses of soils for physicochemical properties revealed that soil pH varied little between the treatments, while soil mineral N, Bray P and exchangeable K levels were significantly lower in the control and the treatments with only PGPR. Increasing N, P and K application rates significantly enhanced the levels of mineral N, Bray P and exchangeable K in soils. However, combined application of inorganic fertilizers and PGPR markedly increased their availability in soil. For instance, mineral N and Bray P levels were greatest in the treatment 100% NPK + BRB 23 (189.6 mg kg<sup>-1</sup> and 7.0 mg kg<sup>-1</sup> respectively) and exchangeable K level was greatest in the treatment with 100% NK + 75% P + BRB 3  $(517.4 \text{ mg kg}^{-1}).$ 

# Effect of seed biopriming with *Trichoderma viride* and *Pseudomonas* fluorescens in chickpea (*Cicer arietinum*) in Andhra Pradesh, India

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#### Abstract

Every year, mankind relies on the miraculous transformation of seeds into plants and back into seeds again. About 60% of all food crops are grown from seed each year, producing more than 2.3 billion tonnes of grain. To get higher yields in any crop, seeds must germinate and seedlings emerge, quickly and uniformly throughout the field so that light, water and soil nutrients may be used with maximum efficiency. Giving crops a good start is of crucial importance for strong and healthy growth with resistance to pests and diseases. The easiest way to get good crop stand is to soak seeds in water before sowing. In Andhra Pradesh, India, majority of pulse crops were grown under rainfed situation. Among them, chickpea is a rabi sown crop, often suffers with drought during early stages leads to poor growth and low yields. A rapid establishment of healthy seedlings can be achieved by seed priming. In order to study the effect of seed priming with water, urea and native isolates of Trichoderma viride and *Pseudomonas fluorescens* in chickpea, experiments were conducted at Regional Agricultural Research Station at Lam near Guntur city of Andhra Pradesh, India during rabi season of 2008-2009. The experiments were laid out in a randomized complete block design with four replications. The seeds of chickpea variety JG-11 was primed in water (100 seeds/100 ml of water), 0.2% urea, 10<sup>9</sup> cfu/ml of T. viride and P. fluorescens. Seeds without priming were used as control treatment. Our results showed that seed priming with the above treatments significantly increased seed germination and seedling vigor compared to non primed control. Seeds primed with T. viride and P. fluorescens significantly improved seedling emergence of 96 and 98% respectively. Seed priming also reduced incidence of dry root rot, 28% with T. viride35% and 35% with P. fluorescens. Higher yields were obtained with priming than non priming. Based on our results, we conclude that seed priming with T. viride followed by P. fluorescens improves improved grain yield of chickpea due to their growth promoting and biocontrol activities.

# Introduction

Chickpea (Cicer arietinum) has gained world wide economic importance as a source of protein for human and animal nutrition. It is an important rabi season food legume currently grown in over 50 countries and consumed over in 120 countries. The global chickpea area is about 11.08 m ha with a production of 9.8 m tonnes (FAOSTAT 2009). South and South East Asia contribute about 77.7% to the global chickpea production and India is the principal chickpea producing country with a share of 84% in this region. The yield potential of present day chickpea cultivars exceeds 5.0 tonnes/ha, however, average yield is about 0.8 tonnes/ha (Haware 1998). This gap between the potential yield and average yield is mainly due to various biotic and abiotic stresses which affect chickpea productivity. There is a growing realization that poor crop stand establishment is a major constraint on crop production in semi-arid areas. Fields without a reasonable number of well-spaced, vigorous plants cannot be expected to produce good yields. There are many reasons for poor stand establishment in crops inadequate seedbed preparation (Joshi, 1987), low untimely sowing (Oosterom et al., 1996), poor sowing technique quality seed. (Radford, 1983), inadequate soil moisture (Harris, 1996), adverse soil properties such as a propensity to form surface crusts (Soman et al., 1992) and high temperatures (Peacock et al., 1993). The conditions after sowing had a large influence on emergence and seedling vigor in sorghum and speeding of germination and emergence was an important determinant of successful establishment (Harris et al., 1996). Rapidly germinating seedlings could emerge and produce deep root systems before the upper layers of the soil dried out, and hardened. To overcome all these difficulties and to accelerate germination and improve seedling uniformity in many crops, the available economical option is seed priming. The simple procedure of seed is soaking seeds in water followed by air-drying before sowing priming and substantial improvement of yields of chickpea (C. arietinum) and mungbean (Vigna radiata) were observed (Amanda and Whipps 2008). Seed priming with Pseudomonas fluorescens decreased tomato damping-off (Harris et al., 2005), and collar rot (S. rolfsi) (Jayaraman et al., 2007). In bio-priming, the inoculum applied as antagonist is in close proximity to the sites of pathogen and much less antagonist inoculum is needed than for soil treatment, which will reduce crop production costs (Musa et al., 2001). In view of this, the current research was conducted to study the effect of biopriming in chickpea to control dry root rot incidence and improve seed germination, vigor, good stand establishment and yield with T. viride and P. fluorescens.

# Materials and methods

**Seed germination bioassays** Germination tests in Chickpea were carried out by paper towel method. Twenty five seeds for each treatment viz., 0.2 % urea,  $10^9$  cfu/ml of *T. viride* and *P. fluorescens*, water alone and untreated dry seeds with four replications in completely randomized design and incubated in growth chamber at

28°C. After 7 days seed germination, root length, and shoot length were measured to determine the vigor index with following formula: Vigor index = (mean root length + mean shoot length)×germination (%) (Abdul Baki and Anderson, 1973).

**Pot bioassay** One hundred chickpea seeds were soaked in 100 ml of water for  $5 \sim 6$  h in 0.2% urea,  $10^9$  cfu/ml of (Abdul Baki and Anderson, 1973). separately and airdried before sowing. Seed soaked in water alone and dry seeds were used as control treatments. Five seeds were sown in 15 cm diameter plastic pots using non-sterilized sick soil containing dry root rot pathogen (population  $9 \times 10^2$  cfu/g of soil ) collected from Lam field at Regional Agricultural Research Station, Lam, Guntur, Andhra Pradesh. Thinning was done to three plants per pot on fifth day of emergence. Plants were irrigated once every 2 days with water. The treatments were arranged in a randomized complete block design with four replications and repeated twice. The parameters such as plant height, plant biomass (dry weight), number of nodules, and nodule weight were measured at 45 days after sowing by destructive sampling method. At the time of harvest, dry root rot disease intensity, yield and 100 seed weight were recorded.

#### **Results and Discussion**

Our results showed that seed priming significantly improved the germination and seedling vigor in Chickpea compared to non-priming seeds. Seeds primed with biocontrol agents resulted in high seedling emergence of 98% with *P. fluorescens* treatment and 96% with *T. viride* treatment. Urea primed seeds resulted 93% germination followed by 89% in water soaked and 72% in control treatment. Maximum vigor index was recorded with *P. fluorescens* (2143.75) followed by *T. viride* (2054.40). Least vigor index was obtained in control treatment (539.93).

Seed biopriming have shown more positive growth responses under pot bioassay conditions compared to urea, water primed and untreated controls. Specifically, both *P. fluorescens* and *T. viride* treatments increased the plant height to a maximum extent (56.25 cm and 53.75 cm, respectively). Urea priming increased the height by 16% and by 13.3% in water primed seeds. Number of nodules were maximum (9 nodules/plant) both in *T. viride* and *P. fluorescens* treatments. Control treatment yielded minimum of 5.5 nodules/plant. Where as the nodule weight was highest (11.375 mg) in *P. fluorescens* followed by *T. viride* (10.175 mg). Remaining priming treatments of 2% urea and water alone have shown nodule weight of 5.975mg and 5.55 mg, respectively (Table 1).

Significant reduction of dry root rot disease was observed in bioprimed treatments when compared with Urea, water primed and untreated control. Priming with *P. fluorescens* reduced the incidence of dry root rot disease to a greater extent 45% followed by *T. viride* (38%).Urea priming and water priming also reduced the disease incidence by 24%,13% and in control treatment, the disease was 81.75%.All

the treatments increased yield when compared to the untreated control (2123 kg/ha) (Table 2). Among the different treatments biopriming treatments increased the yield by 18.4% (*P. fluorescens*) 13.3% (*T. viride*) and 30.4% in urea primed and 22% in water priming treatment.

Effect of different treatments on 100 seed weight, which is the indicator of yield of grains, was shown in Table 2. There was a significant increase in 100 seed weight in all the treatments with maximum (27.7g) in *P.fluorescens* followed by *T.viride* (26.9 g). Urea and water priming treatments increased 100 seed weight by 21.5% and16.5%, respectively.

Priming treatments have shown significant effect on increase of plant biomass. The increase in plant biomass was 15% in *P. fluorescens* followed by 11% in *T. viride*. It was observed that 0.2% urea seed priming method increased the plant biomass by 7% against 5% in only water priming.

The findings of the present study are in conformity of the earlier studies of on-farm participatory trials in Western India by ICRISAT. They found that seed priming increased yields of chickpea, maize, wheat and upland rice (Harris *et al.*, 1999). Co-inoculation of fluorescent *Pseudomonas* and Rhizobium improved plant growth of *Pisum sativum* (Dileep Kumar *et al.*, 2001). In this study *Pseudomonas* sp. CDB 35 suppressed *S. rolfsii* and decreased disease incidence. Seed bio-priming with *P. fluorescens* and *T. viride* showed enhancement in growth and reduced disease intensity in chickpea (Hameda *et al.*, 2010), induce resistance against downy mildew and enhance growth of pearl millet (Niranjan *et al.*, 2004). Bio-priming resulted in enhanced germination, increased microbial population which inhibit the pathogen propagules on seeds thereby protecting the plant (Van Dijk and Nelson 1998). Finally it may be concluded that seed biopriming is an effective method for resource poor farmers to increase the yields and decrease the dry root rot disease in chickpea.

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Table1 Effect of different priming treatments on seed germination, seedling vigor, plant

		7 days af	fter sowing	At the time of harvest			
Treatment	Seedling emergen ce	Shoot Length (cm)	Root length (cm)	Seedling vigor index	Plant height (cm)	Nodul es/pl	Wt. of nodules (mg)
Seeds primed with 2% urea	93	6.475	9.3	1467.08	52.25	6.75	5.97
Seeds primed with Pseudomonas fluorescens	98	10.62	11.25	2143.75	53.75	9	11.37
Seeds primed with Trichoderma viride	96	9.15	12.25	2054.40	56.25	9	10.17
Seeds primed with water only	89	4.75	8.325	1163.68	51	7	5.55
Dryseed (control)	69	3.575	4.25	539.93	45	5.5	4.27
SEM	0.72	0.15	0.18		0.97	0.63	0.14
CD	2.24	0.46	0.56		2.99	1.96	0.43

#### height and nodulation in Chickpea

# Table 2 Effect of different priming treatments on plant biomass, dry root rot disease incidence

#### and yield of chickpea

Treatment	Plant biomass (mg)	Percent disease incidence	Seed yield (kg/ha)	100 grain weight (g)
Seeds primed with 2% urea	735.50	62.5	1952.5	25.40
Seeds primed with Pseudomonas fluorescens	840.00	45.25	2326	27.78
Seeds primed with Trichoderma viride	846.75	50.75	2116.5	26.90
Seeds primed with water only	676.25	76.25	1790	24.35
Dry seed (control)	496.00	81.75	1725	20.90
SEM	8.429	1.05	49.1	0.28
CD	25.97	3.28	151.24	0.88

# Induced systemic resistance against *Pseudomonas syringae* pv. *maculicola* by a long chain bacterial volatile emitted from *Paenibacillus polymyxain Arabidopsis thaliana*

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#### Abstract

Some strains of plant growth-promoting rhizobacteria (PGPR) elicit induced systemic resistance (ISR). Previously, volatile organic compounds (VOCs), including acetoin and 2,3-butanediol, emitted from PGPR were identified as bacterial determinants of ISR. We screened for ISR using a microtiter plate and Iplate bioassay in which seedlings were challenged with the pathogen Pseudomonas syringae pv. maculicola ES4326 in the presence of bacterial volatiles. To investigate the induction of ISR signaling by the VOCs emitted from the reference strain, GB03, and test strain, *Paenibacillus polymyxa* E681 which showed strong capacity on ISR and plant growth promotion under in vitro and field conditions. To identify plant signaling pathways involved, we screened Arabidopsis plants transformed with PR1::GUS and PDF1.2::GUS, indicators of salicylic acid and ethylene signaling, respectively. GB03 elicited ISR via ethylene-dependent signaling, as indicated by elevated PDF1.2 expression in exposed seedlings, while E681 increased SA-dependent signaling, as indicated by elevated *PR1* expression suggestive that E681 and GB03 strains activate different signaling transduction pathways The efficacy of induction was also strain-specific, with stronger protection against P. syringae in plants exposed to VOCs released from E681 versus plants exposed to GB03. Among more than thirty low molecular weight identified volatile compounds, including methanethiol, isoprene, and an acetic acid-butyl ester, hexadecane, a C16 hydrocarbon was found to be released exclusively from only strain E681 and can prime transcriptional levels of *PR1* defense gene. These results provide the first evidence for the existence of a novel E681 signal molecule that can serve as a bacterial determinant in ISR against *P. syringae*.

#### Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of root-colonizing bacteria in the rhizosphere of many plant species that exert beneficial effects on plants, such as enhancing plant productivity and eliciting induced systemic resistance (ISR) against multiple plant pathogens (Kloepper and Metting, 1992; Ryu et al., 2004a; Ryu et al., 2006). These PGPR have been found to produce many bacterial determinants that promote plant growth and ISR (Van Loon et al., 1998; Kloepper et al., 2004). Ryu and coworkers (2003) demonstrated significant growth promotion of Arabidopsis by volatile organic comounds (VOCs) emission of Bacillus subtilis strain GB03 and B. amyloliquefaciens strain IN937a. Exposure of Arabidopsis to VOCs from strains GB03 and IN937a resulted in a significant reduction in the severity of disease caused by Erwinia carotovora subsp. carotovora. The signaling pathway was found to be ethylene-dependent but salicylic acid (SA) -independent, using PDF1.2 and Jin14, indicator genes for ethylene/jasmonate and jasmonate signaling, respectively (Ryu *et al.*, 2004b). Using the *PR-1* indicator gene, no role was found for SA signaling in volatiles from strain GB03 during ISR. An analysis of the bacterial volatiles produced by strain GB03 indicated that 2,3-butanediol elicits plant growth and ISR in a dose-dependent manner.

Here, our objective was to evaluate whether VOCs from *Paenibacillus polymyxa* strain E681 that reported plant growth promotion and induced systemic resistance in several crops (Ryu *et al.*, 2005ab) can also promote induced resistance against *P. syringae* pv. maculicola ES4326 in *Arabidopsis*. We further elucidated the mechanisms of ISR using hormonal mutant lines of *Arabidopsis*, and transgenics transformed with *PR-1a::*GUS and *PDF1.2::*GUS promoter fusions. In addition, a comparison of the VOC profiles of bacilli strains GB03, IN937a, and E681 revealed that a C16 hydrocarbon, hexadecane, is produced only by strain E681. Our results led to identify a novel hydrocarbon-based signal molecule from strain E681 as the bacterial determinant(s) involved in ISR in plants.

#### **Materials and Methods**

#### Bacteria and plant preparation

PGPR strains, *Bacillus subtilis* GB03, and *Paenibacillus polymyxa* E681 were streaked onto tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA) plates and incubated for 24 hours in darkness at 28°C. Strain E681 was previously isolated from the roots of winter barley in the southern part of Korea. For long-term storage, bacterial cultures were maintained at -80°C in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) that contained 20% glycerol. *Arabidopsis thaliana* plants were prepared as described previously (Ryu *et al.*, 2003; 2004ab).

#### Assessment of induced systemic resistance by bacterial volatiles

To determine if exposure of *Arabidopsis* seedlings to bacterial volatiles elicited ISR against *Pseudomonas syringae* pv. *maculicola* ES4326, we developed a 24-well, microtitre-based disease assay system. Twenty microliters of bacterial suspensions of PGPR strains *B. subtilis* GB03, and *P. polymyxa* E681 at  $OD_{600} = 1$  ( $10^{8-9}$  cfu/ml) were drench-applied to two-week-old *Arabidopsis* seedlings. Seven days after PGPR inoculation, 2.5 ml of a bacterial suspension (at  $OD_{600} = 1$ ) of *P. syringae* pv. *maculicola* ES4326 grown on King's B medium was added each well, whole seedlings were soaked in the suspension for 5 min, and the bacterial suspension was removed. Then, the 24-well microtiter plates were placed in a growth chamber that was maintained at 21°C in 12/12 day and night condition. Disease severity was measured four to seven days after pathogen challenge.

#### Analysis of bacterial volatiles

Bacillus subtilis strain GB03 and P. polymyxa strain E681 were grown in 20 ml vials on MS medium containing 1.5% (w/v) agar, 1.5% (w/v) sucrose, and 0.4% (w/v) TSA for 24 h at 37  $^{\circ}$ C before volatiles were collected. Vials were sealed with a steel crimp cap fitted with a Teflon/silicon septum that was previously conditioned at 100°C for 30 minutes. (Z)-3-Hexenyl acetate, which is absent from bacterial cultures, was used as an internal standard (IS), and was injected at a concentration of 1  $\mu$ g per vial.Solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) analysis were performed as previously described (Farag et al., 2006). Arabidopsis GUS fusion assays GUS activity was measured in seedlings 10 days after exposure to PGPR VOCs or water treatment, using a fluorometric assay. The GUS assay followed a previously described procedure (Ryu et al., 2004b; Ryu et al., 2004a).

#### Statistical Analysis

Analysis of variance for experimental data sets was performed using JMP software version 4.0 (SAS Institute Inc., Cary, NC). Significant treatment effects were determined based on the magnitude of the *F* value (P = 0.05). When a significant *F* test was obtained for treatments, separation of means was accomplished by Fisher's protected least significant difference (LSD) at P = 0.05. Bioassays were conducted at least twice, with eight replicates per treatment, and one seedling per replicate. For VOC analyses, four replicates per bacterial culture measured were performed.

#### Results

#### Bacterial volatile analysis of P. polymyxa

We recently reported that solid-phase micro extraction (SPME) combined with gas chromatography-mass spectrometry (GC-MS) provides better tool for profiling of volatile blends in strains of PGPR (Farag et al., 2006). Results from this analysis provided a comprehensive compositional profile of the volatiles released by strain IN937a. We used this method in the current study to profile the volatiles released by E681; strain GB03 was included as a positive control. High levels of acetoin were consistently released from strains E681 and GB03 (74 and 200 µg/24 h, respectively) released only 0.1  $\mu$ g /24 h of acetoin (data not shown). Marginal detection levels of 2,3-butanediol were only detected in VOCs released by the E681 strain. Besides 2,3butandiol and acetoin, other volatiles released from three bacilli strains GB03, IN937a, and E681 included methanethiol, isoprene, and acetic acid-butyl ester (data not shown). A comparison of the VOC profiles of strains GB03 and E681 revealed that hexadecane, a C16 hydrocarbon, is released exclusively only from strain E681. Hexadecane, which called as cetane is an alkane hydrocarbon with the chemical formula C16H34 (MW = 226.44 daltons). The production of a volatile hexadecane at the headspace of *P. polymyxa* was first report.

#### The effect of bacterial volatiles on PR-1 and PDF1.2 gene expression in plant

To compare previous result obtained with strain GB03, we evaluated expression from *PR1* and *PDF1.2* genes GUS construct in *Arabidopsis*. A GUS assay revealed that exposure to VOCs released by strain E681 increased the expression of the *Arabidopsis PR-1* promoter 7.3 fold, but did not affect that of the *PDF1.2* promoter (Fig. 2A). In contrast, treatment with strain GB03 stimulated the expression of the *Arabidopsis PDF1.2* promoter 27 fold relative to the control.A comparison of the VOC profiles of strains GB03, IN937a, and E681 revealed that hexadecane, a C16 hydrocarbon, is released exclusively only from strain E681 (Table 1; Fig. 3A). Hexadecane, which called as cetane is an alkane hydrocarbon with the chemical formula C16H34 (MW = 226.44 daltons). The production of a volatile hexadecane at the headspace of *P. polymyxa* was first report.

#### Hexadecane-elicited Induction of systemic resistance against P. syringae



application of hexadecane in the I-plate system protected *Arabidopsis* seedlings against a biotrophic pathogenic bacteria *P. syringae* pv. maculicola ES4326 at seven days after soaking-inoculation.



**Fig. 1.** GUS activity in *Arabidopsis* plants transformed with *ProPR1::GUS* or *PDF1.2::GUS* that exposed to VOCs and its VOC contents released from *P. polymyxa* E681 and *B. subtilsis* GB03 (A) and induced systemic resistance against *Pseudomonas syringae* pv. maculicola ES4326 by a bacterial volatile hexadecane (B) A. Induction of *PR1* and *PDF1.2* promoters fusion GUS by *P. polymyxa* E681 and *B. subtilsis* GB03. Different letters (a, b for *PR-1a*; x, y for *PDF1.2*) indicate significant differences between treatments within each *Arabidopsis* line, according to least significant difference at P = 0.05. The error bars indicate S.E.M. **B.** Induced systemic resistance against *P. syringae* pv. maculicola ES4326 elicited by hexadecane and 0.33 mM BTH using the I-plate system. Disease severity (0 = no symptom, 5 = severe chlorosis) was recorded seven days after pathogen challenge at  $10^8$  cfu/ml. Different letters indicate significant differences at P = 0.05.

Previously, indirect application (physical separation between plant and treatment) of any bacterial volatiles did not induce systemic resistance against any biotrophic pathogenic bacteria. In this study, we obtained the first evidence that release of

hexadecane can significantly induce systemic resistance against *P. syringae* pv. *maculicola* ES4326 (Fig. 1B). Highest concentration of hexadecane (10 mM) did not induce ISR against *P. syringae*. (Fig. 1B). However, disease severity in *Arabidopsis* seedlings exposed to 100  $\mu$ M and 1  $\mu$ M hexadecane significantly differed from 10 mM hexadecane treatment. The indirect treatment of 0.33 mM BTH reduced symptom development when compared to control (Fig. 1B). It is noteworthy that any centration of hexadecane did not affect plant growth (data not shown) indicating that hexadecane out of VOCs from strain E681 plays a role only in ISR.

#### Discussion

Our results presented here demonstrate that the rhizobacterium, Paenibacillus polymyxa E681, produce a volatile blend that elicits ISR against P. syringae, in the absence of physical contact with plants. In this current study, we further demonstrated that ISR resulting from exposure to a unique VOC released by strain E681 depends on salicylic acid signaling (Fig. 1A). Previously, an analysis of the VOCs released from three bacteria, including Paenibacillus polymxa from potato tubers, showed that dimethylformamide, pentadecene, and hexadecane are unique volatiles generated by P. polymxya (De Lacy, 1999). This result is in agreement with our data (data not shown). A comparison with our previous data (Farag et al., 2006) revealed that hexadecane was emitted exclusively from P. polymyxa, but not from B. subtilis and B. *amyloligefaciens*. To the best of our knowledge, only other bacterial species known to produce hexadecane is the cyanbacterium Oscillatoria perornata (Tellez et al., 2001). To date, microbial production of this hydrocarbon has not been extensively studied, and the plant's response to hexadecane has not been thoroughly assessed. Hexadecane is a novel candidate signal molecule that can induce *PR-1* expression. How plants perceive and respond to hexadecane has yet to be fully elucidated using large scale gene expression techniques.

Production of cytokinin by *P. polymyxa*, or by the plant exposed to *P. polymyxa* was proposed to be a bacterial determinant in growth promotion (Timmusk *et al.*, 1999). Our data presented here indicate that bacterial volatiles emitted from *P. polymyxa* E681 play an important role in promoting the growth of *Arabidopsis* seedlings. Moreover, the same strain produce hexadecane out of a blend of volatiles that elicit ISR against *P. syringae* pv. *maculicola* strain ES4326. Interestingly the hexadecane did not show any effect on the plant growth promotion (data not shown) indicating that individual VOC from a bacterium play a different role on plant growth promotion and ISR in plant. VOCs released by strain E681 elicited only ISR and induced expression of the *PR-1* gene to a greater extent than from those released by strain E681 elicited plant defense mechanism mostly depending on SA and JA signaling by measuring *PR1* and PDF1.2 gene expression respectively (data not shown). Our results provide evidence that strain E681 emits a long chin C16 volatile

triggering ISR response stronger than 2,3-butanediol or acetoin, suggesting for the presence of unidentified molecules that need to be identified and examined in large-scale experiments.

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# Application of bacterial volatiles in pepper roots primes systemic resistance against *Xanthomonas axonopodis* and *Cucumber mosaic virus* under field condition

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#### Abstract

*Bacillus amyloliquefaciens* strain IN937a which is previously known as thriving inside plant tissues referred to as an endophyte was reported to be stimulated plant growth and induced systemic resistance (ISR) via its emission of volatile organic compound (VOC). We investigated ISR capacity of the VOCs collected from the strain IN937a against bacterial spot disease caused by *Xanthomonas axonopodis* pv. vesicatoria on pepper in the greenhouse. Among 18 bacterial VOCs, 3-pentanol was selected for further experiment. 3-pentanol was drenched onto the four week-old pepper before transplanting in the field. Disease severity was assessed at seven days after pathogen challenge when infiltrated in the pepper leaves at 10, 20, 30 and 40 days post transplant (dpt). 3-pentanol treatment significantly increased ISR compared to control treatment at 30 dpt. Taken together; bacterial VOC 3-pentanol can be utilized as a trigger of ISR against broad spectrum of pathogens under field condition.

#### Introduction

In 2003, volatile organic compounds (VOCs) emitted by two *Bacillus* spp. were established as being the primary determinants of both plant growth promotion and elicitation of ISR in *Arabidopsis* (Ryu *et al.*, 2003; Lucy *et al.*, 2004; Ryu *et al.*, 2004). Ryu and coworkers (2003) demonstrated significant growth promotion of *Arabidopsis* by *Bacillus subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a. Among the VOCs from strain GB03, 2,3-butanediol was the major compound that promoted plant growth and ISR against *Erwinia carotovora* subsp. *carotovora*. Several mutant lines of *Arabidopsis*, including brassinosteroid- and gibberellic acid-insensitive mutants, as well as auxin-transport-deficient and cytokinin receptor-deficient mutants, were used to elucidate the signaling pathways that promote growth.

The VOCs tested did not promote plant growth in *cre1*, a cytokinin receptor-deficient mutant, suggesting that cytokinin signaling is essential for the promotion of plant growth in response to bacterial volatiles (Ryu *et al.*, 2004). Furthermore, a study using The Affymetrix *Arabidopsis* AG GeneChip revealed that volatile emissions from strain GB03 differentially up-regulated more than 600 transcripts, which encoded proteins with various functions, such as cell wall modification, primary and secondary metabolism, stress responses, and hormone regulation (Zhang *et al.*, 2007) Exposure of *Arabidopsis* to VOCs from strains GB03 and IN937a also resulted in a significant reduction in the severity of disease caused by *Erwinia carotovora* subsp. *carotovora*. The signaling pathway was found to be ethylene-dependent but salicylic acid (SA) -independent, using *PDF1.2* and *Jin14*, indicator genes for ethylene/jasmonate and jasmonate signaling, respectively (Ryu *et al.*, 2004).

In this study, we assessed effect of bacterial volatiles from different bacterial species under greenhouse and field conditions. Through 18 bacterial volatiles was evaluated ISR capacity against bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* in pepper in the greenhouse. From the pre-screening, 3-pentanol was selected and drench-applied into pepper seedlings under field condition. The ISR capacity of 3-pentanol was measured by symptom development following leaf-infiltration of bacterial suspension and naturally occurring disease symptom including bacterial spot and *Cucumber mosaic virus*.

# Materials and Methods

#### Induced resistance against X. axonopodis pv. vesicatoria

To screen a promising candidate of bacterial volatiles, 18 bacterial volatiles that reported the production from *Bacillus subtilis* strain GB03, *B. amyloliqefaciens* IN937a, and *P. fluorescens* strain 89B61 (Farag *et al.*, 2006). 5 ml of 18 bacterial volatiles was drench-applied in the crown part of 3 week old pepper seedling at 10  $\mu$ M and 100 nM. Bacterial suspensions of 10<sup>6</sup> cfu/ml *X. axonopodis* pv. *vesicatoria* were forced to penetrate pepper leaves backside using the needleless syringe method at 7 days after volatile application in the greenhouse. The disease severity was measured at 7 days after pathogen challenge. For ISR test in field, Ten days after dipping application of BTH and 10  $\mu$ M 3-pentanol into 6 week old pepper seedling before transplanting, *X. axonopodis* pv. *vesicatoria* was challenged with same method described as above. Seven days after the pathogen challenge, the severity of symptoms on the leaf was scored from 0 to 5; 0=no symptoms, 1 = mild chlorosis, 2 = chlorosis only, 3 = chlorosis and mild necrosis, 4 = necrosis, and 5 = severe necrosis of the inoculated area. The experiment was repeated four times with 10 replications (one plant per replication).

#### **Statistical Analysis**

Analysis of variance for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., Cary, NC). Significant effects of treatment were determined by the magnitude of the *F* value (P = 0.05). When a significant *F* test was obtained, separation of means was accomplished by Fisher's protected LSD at P = 0.05.

#### **Results and Discussion**

In this study, we tested 18 bacterial volatiles that previously identified from different genera including pseudomond and bacilli. To screen any promising volatile candidate, we infiltrated bacterial suspension at relatively high concentration such as10<sup>6</sup> cfu/ml X. axonopodis pv. vesicatoria. Such high disease pressure allowed us to select a strong candidate to apply ISR capacity of bacterial volatile in the field. Among 18 volatiles, only 3-pentanol treatment showed significant induction of systemic resistance against bacterial spot in the greenhouse (Fig.1). Furthermore, 3pentanol did not affect plant growth (data not shown). Normally, strong induction of systemic acquired resistance by chemical inducer or avirulent pathogen treatments resulted in reduction of plant growth referred to as "allocation fitness cost" (Heil and Baldwin, 2004). Based on the consistent results of 3-pentanol in the greenhouse, 10 µM 3-pentanol was finally selected for further field trial. The pre-treatment of 3pentanol elicited ISR against subsequent challenge of X. axonopodis pv. vesicatoria at 10, 20, 30, and 40 days after 3-pentanol treatment (data not shown). The pathogen population in the plant treated with 3-pentanol was significantly reduced compared to control (Fig. 2A). At the end of season, bacterial spot and abnormal virus symptom were outbreak cross the field. Further identification revealed X. axonopodis pv. vesicatoria for the bacterial spot and Cucumber mosaic virus for virus symptom. Interestingly, typical CMV symptoms appeared significantly lower frequency in the 3-pentanol treated plants (Fig. 2B). Our results suggest that 3-pentanol will be a strong candidate for a chemical trigger of ISR against bacterial and viral diseases under field conditions.



**Fig. 1.** Effect of 18 bacterial volatiles on ISR against *X. axonopodis* pv. vesicatoria in the greenhouse; 18 bacterial VOCs with two concentrations (10  $\mu$ M and 100 nM). As a positive control plants were treated with 0.5 mM BTH. Disease severity (0 – 5) was measured 7 days after pathogen challenge. \* indicates statistically significant differences (*P* = 0.05) compared to control. Data represents the means the standard error of the mean.



**Fig. 2.** Induced systemic resistance against naturally occurring diseases by 3-pentanol under field condition.; A) bacterial spot disease caused by *X. axonopodis* pv. vesicatoria, B) Viral disease symptom (Representative photographs taken 3 months after occurrence of natural disease.), C) bacterial spot disease for 3 months after treatment. As a positive control, plants were treated with 0.5 mM BTH. Disease severity (0 - 5) as an indicator of ISR was measured 3 months after transplant. 0, no symptoms; 5, severe necrosis. Different letters indicate statistically significant differences (P = 0.05). Data represents the means the standard error of the mean.

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# Bio-management of disease complex in Gladiolus by using PGPR

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#### Abstract

*Gladiolus grandiflorus* is seriously hampered by disease complex caused by Meloidogyne incognita and Fusarium oxysporum Schelecht. f.sp. gladioli (Masey) Snyder and Hans. These two pathogens reduce the productivity to the tune of  $40\% \sim 60\%$ . Indiscriminate use of chemical pesticides proved ecologically hazardous. Hence it was thought to standardize a eco-friendly approach to manage the disease complex in gladiolus using Plant Growth Promoting Rhizobacetria (PGPR). Single bio-agent cannot be very effective in the management disease complex in different soil types and agro climatic regions. Hence an attempt was made to evaluate the combined effect of a PGPR Pseudomonas fluorescens Migula and Pochonia chlamydosporia (nematophagous fungi) against disease complex at Indian Institute of Horticultural Research, Bangalore. Formulations of *P. fluorescens* and *P. chlamydosporia* were prepared separately. Two hundred kgs of de-oiled neem cake was enriched for 15 days by mixing 5 kg of *P. fluorescens* or P. chlamydosporia formulation. Optimum moisture was maintained for better enrichment of neem cake with bio-pesticides. These enriched neem cake with either P. fluorescens or P. chlamydosporia was applied 20g/m<sup>2</sup>. Corms of Gladiolus (cv. White prosperity) were also treated with the 10% suspension of P. fluorescens and P. chlamydosporia enriched neem cake for 5~10 min. Results indicated that combination treatment of P. fluorescens and P. chlamydosporia was more effective when compared with either of P. fluorescens and P. chlamydosporia treatments individually. This treatment reduced the incidence of *M. incognita* and *F. oxysporum* f.sp. *gladioli* by 66% and 58% respectively. There was also a significant increase in the yield of the crop which was to the tune of 23%.

Key words: Gladiolus; bio-management; Meloidogyne incognita; Fusarium oxysporum f. sp. Gladioli; disease complex; Pochonia chlamydosporia; Pseudomonas fluorescens

## Introduction

*Gladiolus grandiflorus* is most popular for cut flowers, due to their different sizes, shades and excellent vase life (Bose *et al.*, 2003). This crop is gaining popularity with

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small scale growers for both local and export markets (HCDA, 2000). The volume of cut-flower exports increase from 60,980 MT in 2003 to 91,200 MT in 2007 (MoA, 2008). Productivity of Gladiolus is seriously hampered by corm rot disease caused by the fungal pathogen *Fusarium oxysporum* Schelecht. f.sp. *gladioli* (Masey) Snyder and Hans., resulting in extensive financial loss to the growers (Chandel and Bhardwaj, 2000). And, most of the time it is associated with root-knot nematodes.

Root-knot nematodes are sedentary endoparasites of roots, attacking a wide range of crops worldwide. The infection starts with root penetration of second stage juvenile ( $J_2$ ) hatched in soil from eggs laid by females. Infection of roots by root-knot nematodes (*Meloidogyne incognita*) predisposes plants to infection by soil-borne root-infecting fungi- *F. oxysporum* f.sp. *gladioli* (Armstrong *et al.*, 1976) which results in the manifestation of disease complex. The disease complex damage plants more severely and render the disease control more difficult than single pathogens alone.

During last few decades, disease control has been largely dependent on the use of chemicals, which has resulted in adverse environmental effects, disturbing the ecological balance and making plants even more susceptible to pests and diseases (Bhanti and Taneja, 2007; Mancini *et al.*, 2008). Hence, it is required to develop an alternative disease management systems without using pesticides (Singh *et al.*, 2003; Cuthbrtson and Murchie, 2005). So, it was thought to develop an eco-friendly approach in the management of the disease complex caused by *M. incognita* and *F. oxysporum* f.sp. *gladioli* in gladiolus by using PGPR- *Pseudomonas fluorescens* and *Pochonia chlamydosporia*.

*P. fluorescens* has been reported to be an potential bio control agent (Vidyasekaran *et al.*, 1999). It was found to be effective in the control of various soil borne fungi causing root rot in certain vegetables and ornamental crops. But in most of the cases, single bio-agent cannot be very effective in the management disease complex in different soil types and agro climatic regions. Hence an attempt was made to combine

PGPR with *Pochonia chlamydosporium* Zare *et al.*, a nematophagous fungi. It has been reported as very promising bio control agent of nematodes by various researchers (De Leij and Kerry, 1991; Kerry *et al.*, 1993; Rao *et al.*, 1997). However, there are no reports till now on the use of *P. fluorescens* with nematophagous fungi *P. chlamydosporia* for the management of disease complex. Hence, it was thought to study the combined effect of these two bio-agents for the management of disease complex caused by *M. incognita* and *F. oxysporum* f.sp. *gladioli* on Gladiolus.

#### **Materials and Methods**

The local isolates of *P. fluorescens* (IIHR-PF-2) and *P. chlamydosporia* (IIHR-PC-3) maintained in the Indian Institute of Horticultural Research (IIHR), Bangalore, were mass produced as talc based formulations. These bio-pesticide formulations were used after the enrichment in de-oiled neem cake. The enrichment was done by mixing 5 kg of either of *P. chlamydosporia* or *P. fluorescens* or 2.5 kg each of *P. fluorescens* and *P. chlamydosporia* together in 200 kg of neem cake by maintaining optimum moisture for about 15 days under the shade.

# Evaluation of effect of application of Combi-formulation of *P. fluorescens* and *P. chlamydosporia* in the management of disease complex in gladioli under field conditions

An experiment was conducted using these biopesticides enriched neem cake in the field with plot size of  $4 \times 2.5$  sq. mts. 200 kg of neem cake was enriched with 5 kg of either of P. chlamydosporia or P. fluorescens or 5 kg of combination formulation containing P. fluorescens and P. chlamydosporia. Corms of uniform size of a F. oxysporum f.sp. gladioli susceptible variety, Gladiolus grandiflorus (cv. White prosperity) were used in the field studies. The treatments were as follows: T1 -Corms were treated with 10 % suspension of P. fluorescens (CFU  $2 \times 10^8$ /ml) and sown in the plots PF - ST; T2 – Untreated corms of gladioli were sown in the plots mixed with neem cake (20g/sq.mt) enriched with P. fluorescens. PF - Neem; T3 -Corms were treated with 10% suspension of *P. chlamydosporia* (CFU  $2 \times 10^{6}$ /ml) and sown in the plots. PC - ST; T4 -Untreated Corms were sown in the plots mixed with neem cake (20g/sq.mt) enriched with P. chlamydosporia PC - Neem; T5 - Corms were treated with combi-formulation of P. fluorescens and P. chlamydosporia, sown in the plots. PF + PC - ST; T6 - Untreated Corms were sown in the plots mixed withneem cake enriched with combi-formulation of P. fluorescens and P. chlamydosporia 20g/sq. mt PF + PC - Neem; T7 - Corms treated with P. fluorescens were sown in

plots containing neem cake (20g/sq.mt) enriched with *P. fluorescens* PF - ST + Neem;T8 – Corms treated with *P. chlamydosporia* were sown in plots containing neem cake enriched with *P. chlamydosporia* PC - ST + Neem; T9 – Corms treated with combi-formulation of *P. fluorescens* and *P. chlamydosporia*, sown in the plots mixed with neem cake enriched with combi-formulation of *P. fluorescens* and *P. chlamydosporia* 20g/sq. mt PF + PC (ST + Neem); T10 – Untreated corms were sown in the plots mixed with neem cake (20g/sq.mt) Neem alone; T11 – Untreated corms were sown in the plot with out any treatment (Control).

Observations were made on the plant height (after 90 days), rhizospheric density of both the bio-agents in soil, root colonization by *P. fluorescens* and *P. chlamydosporia*, no. of *M. incognita* in 5g of the root, percentage of disease incidence by *F. oxysporum* f.sp. *gladioli* and percentage reduction in *M. incognita*. Yield per plot (number of spikes per plot) and percentage increase in the yield at harvest were also recorded.

Root populations of root-knot nematode were estimated from 5g samples of roots from each plant. The root samples were stained using acid fuchsin following the method of Bridge *et al.*, (1982), homogenized and the numbers of nematodes in the roots were recorded.

Colonization of roots by *P. chlamydosporia* and *P. fluorescens* were recorded at random by uprooting 45 days old plants from a plot. Root colonization by *P. fluorescens* was assessed by following the standard serial dilution technique. One gram of root sample was washed, grounded and the CFU (Colony Forming Unit) was checked by the serial dilution followed by pour plate method. Serial dilutions up to  $10^{-6}$  concentration were prepared. 1ml from each of  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  dilutions were pipetted into the Petri dishes and spread completely in the plate. Freshly prepared King's B agar *P. fluorescens* media was poured into each plate and made to spread evenly by pour plate method and allowed for solidification. Three replicates for each dilution were maintained with controls and incubated at  $27 \pm 1^{\circ}$ C. After 24 h, *P. fluorescens* colonies on King's B media emitting a pale green fluorescent light under UV at 302 nm were counted for recording CFU. Also estimated the rhizospheric density of *P. fluorescens* in soil after the harvest of the crop.

Similarly, the root colonization and rhizospheric density of *P. chlamydosporium* were estimated using semi-selective medium (Kerry *et al.*, 1993). The data were analyzed using ANOVA.

#### **Results and discussions**

Results indicated that treatment of corms with both *P. fluorescens* and *P. chlamydosporia*, along with application of neem cake enriched with *P. fluorescens* and *P. chlamydosporia* proved significantly effective in the management of disease complex caused by *F. oxysporum* f.sp. *gladioli* and *M. incognita* on gladiolus under field conditions (Table2). There was a significant reduction in the *M. incognita* population hatched from the roots and the percentage reduction was about 66%. There was also significant reduction of disease incidence by *F. oxysporum* f.sp. *gladioli* of about 58% (Table2). There was also significant increase in the yield of the crop of about 23%. This indicates that combinations of more than one bio-agent provided more protection against the disease than a single bio-agent. The similar observations were also made by other researchers (Ehteshamul-Haque *et al.*, 1995; Izhar *et al.*, 1995; Rao *et al.*, 2004).

Rhizospheric density of P. fluorescens and P. chlamydosporia in the treatment T9 - PF + PC (ST + NEEM) were found to be  $7.3 \times 10^6$  and  $6.6 \times 10^5$  respectively. which was at par with the third and sixth treatment T3 - PF(ST + NEEM) and T6 -PC (ST + NEEM). Root colonization of P. fluorescens and P. chlamydosporia (enriched in neem cake) was also high in the ninth treatment T9 - PF + PC (ST + NEEM), compared to its application individually. CFU of P. fluorescens and P. chlamydosporia per gram of root was found to be  $6.6 \times 10^6$  and  $5.9 \times 10^5$  (Table 1). This indicates that rhizospheric density and root colonization by *P. fluorescens* and P. chlamydosporia were more when applied together in comparison to individual treatments (Table1). This indicated that root colonization of either of P. chlamydosporia or P. fluorescens was not affected by the presence each other when combined together. This also indicates that both bio-agents co-exist without affecting the root colonization of either of the bio-agents. Application of neem cake was also proved to be very effective in the management of nematodes as it is reported to be controlling the entry of pathogens through variety of mechanisms including the production of antimicrobial compounds (Mankau, 1962; Alam et al., 1980; Muller and Gooch, 1982).

Treatments	Plant height (53-130)	Density of <i>P.fluorescens</i> (CFU/g) in soil ( $\times 10^6$ )	Root colonization (CFU/g) of <i>P.fluorescens</i> $(\times 10^{6})$	Density of <i>P.chlamydosporia</i> (CFU/g) in soil $(\times 10^5)$	Root colonization (CFU/g) of <i>P.chlamydosporia</i> $(\times 10^5)$
T1 - PF ST	82	3.8	2.4	0	0
T2 - PF NEEM	107	5.8	4.5	0	0
T3 - PC ST	73	0	0	2.7	2.2
T4 - PC NEEM	95	0	0	5.3	4.1
T5 – PF + PC ST	89	4.0	3.3	4.3	3.3
T6 – PF + PC NEEM	121	6.2	5.8	5.8	4.9
T7 – PF (ST + NEEM)	114	6.9	6.0	0	0
T8 - PC (ST + NEEM)	101	0	0	6.5	5.5
T9 - PF + PC $(ST + NEEM)$	130	7.3	6.6	6.9	5.9
T10 - NEEM alone	68	0	0	0	0
T11 - CONTROL	53	0	0	0	0
CD - 5%	8.43	0.45	0.26	0.72	0.34

 Table 1
 Effect of combi-formulation of P. fluorescens and P. chlamydosporia with neem on plant height of gladiolus and their rhizospheric density and root colonization

There was significant increase in the plant height (Table 1). This could be due to the plant growth promoting activity of *P. fluorescens* which was very well documented by many researchers (Bloemberg *et al.*, 2001; Shouan Zhang *et al.*, 2003; Kishore *et al.*, 2003). *P. fluorescens* also has been reported effective in the management of root-knot nematodes (Parveen *et al.*, 1998; Siddiqui *et al.*, 1999; Rao, 2007) and other fungal pathogens.

It can be concluded that use of neem cake enriched with *P. fluorescens* and *P. chlamydosporia* would be highly useful in the sustainable management of nematode induced disease complex. This strategy can help in the sustainable production of gladiolus.

Treatments	No of <i>M. incognita</i> in 5g root	Percent reduction in <i>M. incognita</i> population (%)	Disease incidence (%)	Percent reduction in disease incidence (%)	Yield(No of spikes) Per plot of $4 \times 2.5$ m	Percent increase in yield (%)
T1 - PF ST	12.54	34	33.7	23	41.2	6
T2 - PF NEEM	9.88	48	27.2	38	43.6	12
T3 - PC ST	13.3	30	35	20	40.5	4
T4 - PC NEEM	11.02	42	29.3	33	42.8	10
T5 - PF + PC ST	11.78	38	31.5	28	42	8
T6 – PF + PC NEEM	7.22	62	20.6	53	47.1	21
T7 – PF (ST + NEEM)	7.98	58	22.3	49	45.9	18
T8 – PC (ST + NEEM)	8.93	53	25.4	42	44.7	15
T9 – PF + PC (ST + NEEM)	6.46	66	18.4	58	47.8	23
T10 - NEEM alone	13.68	28	35.9	18	39.7	2
T11 - CONTROL	19.00	_	43.8	_	38.9	_
C.D – 5%	2.72	_	_	_	3.48	_

 Table 2
 Effect of combi-formulation of P. fluorescens and P. chlamydosporia with neem in the management of disease complex in gladioli under field conditions

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## Biological control of *Phytophthora capsici* and *Colletotrichum acutatum* by a PGPR strain *Bacillus subtilis* KP07 on red-pepper

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#### Abstract

Plant growth-promoting rhizobacteria (PGPR) can reduce the disease severity and help to increase the productivity in many crop plants either directly or indirectly by the production of antagonistic compounds or through the elicitation of a plant defense response. Antibiotic-producing Bacillus subtilis KP07 was tested for elicitation of induced systemic resistance (ISR) in control of Colletotrichum acutatum, Phytophthora capsici and Pectobacterium carotovorum SCC1 on red-pepper (*Capsicum annum* L.) in green house and field conditions based on its antibiosis. The elicitation of ISR by KP07 against C. acutatum revealed minimum disease severity (7.9%) when compared to chemical control (17.0%), whereas the disease severity of P. capsici was recorded 60% in KP07 treated plants when compared to chemical control (80%) in field condition. Additionally, the study also revealed the significant increase in fruit yield by soil drenching of KP07 strain (10<sup>8</sup> cfu/ml) under field conditions. The maximum fruit yield (102.4 kg/plot) was recorded with KP07 followed by 93.8 kg/plot with chemical control. It has also been observed that KP07 treated plants showed higher chlorophyll content in the fresh leaves compared to untreated control. Hence the results of this study identify B. subtilis KP07 as a promising biocontrol agent against multiple diseases and also growth promotion on various crops as well as red-pepper plants.

## Antibiosis and induced systemic resistance against major plant pathogens on red-pepper by PGPR strain *Bacillus subtilis* KP07

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#### Abstract

Bacillus species are possessing broad spectrum activities in biological control. Various cyclodipeptidal antibiotics or biosurfactants were produced by these strains. The strain *Bacillus subtilis* KP07 was isolated from rhizosphere of red-pepper plants in Korea, and it has strong promising biocontrol activity in inducing systemic resistance (ISR) by producing antibiotics as a mode of action. In the present study, we investigated the role of Plant growth-promoting rhizobacterial (PGPR) B. subtilis KP07 as one of the potential biocontrol agents against several phytopathogens such as Colletotrichum acutatum, Phytophthora capsici and Pectobacterium carotovorum SCC1 on red-pepper (Capsicum annum L.). Methanol fractions from the culture filtrate of KP07 showed significant in vitro antibiosis against major fungal pathogens such as Colletotrichum acutatum, Fusarium oxysporum, Alternaria alternate, Sclerotinia sclerotiorum, Botrytis cinerea, Penicillium italicum, Penicillium expansum, Penicillium expansum and Rhizoctonia solani. Among various fractions (0%, 10%, 20%, 40%, 60%, 80% and 100%) tested for antagonistic activity, only the higher methanol fractions at 80% and 100% inhibited the growth of the pathogens. In the cases of S. sclerotiorum and B. cinerea the fraction at 60% also inhibited the fungal growth. In systemic induction studies, the bacteria and culture fractions had lower but significant suppression of the above said diseases. In RT-PCR analysis, PR1 and PDF1.2 gene expression pattern was determined on Arabidopsis thaliana mutant lines. This study suggests that the induction of systemic resistance is by salicylic acid dependent regulatory pathway.

## Isolation and selection plant growth promoting microorganism from the soil of rubber in Son La, Dien Bien and Lai Chau Provinces-Vietnam

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#### Abstract

To increase crops productivity the farmer usually many kind of chemical medicines to plant growth promoting. However, this medicine is very bad for health and pollution to environment. So replacing it by bio-products contained a lot of microorganisms that have capacity creating the plant growth promoting substances is needed meter. We isolated and selected isolates KT1, KT2, KT3, KT6, KT7, KT8, KT9 and, KT12 from the soil planted rubber in Son La, Dien Bien and Lai Chau province in Vietnam. This isolates is *Bacillus* and *Azotobacter*, that living aerobic, at  $28 \sim 30^{\circ}$ C, pH 6.5  $\sim$  7.5. Among them isolate KT2, KT9, KT12 have capacity creating the plant growth promoting substance is highest and these will be used to produce bio-fertilizer for planting rubber in Vietnam.

#### Introduction

In the actual production, growth of plant in agricultural soils is influenced by a myriad of abiotic and biotic factors, to increase crop yields farmers use many kinds of chemical drugs to stimulate plant growth. However, many drugs contain chemicals that can affect human health and causing environmental pollution, the application of microbial products for this purpose is less common. So replacing them with biological products containing microorganisms capable of generating growth stimulants for plants to improve productivity and quality of agricultural products is a very practical job<sup>[4,5]</sup>.

Rubber trees are considered prospective, open a new shift in the direction of plant structure in the area some northwestern provinces of Vietnam. However, all the fertilizers used for rubber care mainly mineral fertilizers, so not really meet the actual needs of current production. Effectiveness of microorganisms in increasing plant growth and development, fertilizer savings and increased productivity, quality of agricultural products has been confirmed in many research projects in many countries around the world <sup>[2,3]</sup>.

Within the scope of this paper will present some results for the selection of microorganism's active plant growth stimulants for use in the production of fertilizer for rubber in Vietnam.

#### **Materials and Methods**

#### Materials

- Soil samples were collected from rubber plantation in the province of Dien Bien, Son La and Lai Chau.

- Green peas seeds.

- King B, SPA, AT medium: to isolate and biomass reproduction of microorganisms that stimulate plant growth for the groups *Bacillus, Pseudomonas* and *Azotobacter* respectively<sup>[1]</sup>.

#### Methods

Method of determining probable biosynthesis IAA

- Determining qualitative on ability biosynthesis IAA of microorganisms by Salkowski improved method (Misra *et al.*, 1989).

- Determining on IAA content generated by colorimetric method at a wavelength of 530 nm with standard graph IAA.

Assessment methods germination stimulation, roots and long body of the green peas of the selected microorganisms

- Experimental formulas: Seeds were sterilized with alcohol for 2 minutes; rinse the seeds with sterilized distilled water. Then seeds soaked in distilled water sterilized about 3 hours. After the seed bloated then soaked into solution of micro-organisms were centrifuged (3000 rpm) and solution of micro-organisms after 4 days culture  $(10^7 \text{ CFU/ml} \text{ densities})$  approximately 15 minutes. Seeds were incubated on petri dishes steriled. Germination rate of seeds were monitored after 2 days for root lenght and after 5 days for shoot length, respectively.

- Control formula: carry out the same as the experimental formulas, only changing the solution of micro-organisms was centrifuged and solution of micro-organisms by distilled water.

Determination of some biological characteristics and effects of culture conditions on ability biosynthesis IAA of microorganisms

determined according to the research methods common microorganisms<sup>[1]</sup>.

#### **Results and Discussion**

## Selection strains of Azotobacter, Bacillus capable of generate IAA from rubber plantations in Dien Bien, Son La and Lai Chau

From soil samples collected in rubber cultivation in Dien Bien, Son La, Lai Chau have selected the eight strains of microorganisms capable of generate IAA belong to the *Bacillus* and *Azotobacter*. Results are presented in Table 1.

-						
No Strain		Second of a cit	Morphological characteristics of colonies			
		Source of son	Shap	Colour		
1	KT1	Chieng an –Muong La –Son La	Wrinkled, flattened, with round convex	white		
2	KT2	Cheng Ban - Co Noi – Son La	Circular, convex	flowing white border, gold		
3	KT3	Chieng an –Muong La –Son La	Round, flat, slightly mucilaginous	opaque white		
4	KT6	Tuan Giao – Đien Bien	Round, flat, convex in middle, white label	white		
5	KT7	Muong Nhe – Đien Bien	Circular, convex, mucilaginous T	pale yellow		
6	KT8	Tuan Giao – Đien Bien	Flat, very opaque white mucous	opalescent		
7	KT9	Xin Ho - Lai Chau	Circular, convex, mucus	yellow		
8	KT12	Xin Ho - Lai Chau	Circular, convex, very yellow	white mucous borders		

Table 1 Origin and biological characteristics of microorganisms isolated





Figure 1 Images of colonies of some strains of microorganisms



The *ability* to synthesize IAA of bacterial strains was conducted by the method of quantitative determination. When adding 8ml Salkowski reagent in 2 ml broth culture of selected microorganisms that was centrifuge, depending on the amount of IAA generated that will give the mixture a pink to red colour. The results were 8 microorganisms capable of biosynthesis of IAA, which is a symbol strains: KT1, KT2, KT3, KT6, KT7, KT8, KT9 and KT12.

Figure 2 shows that, the tubes containing strain of microorganisms KT2, KT8, KT9 had deep red colour. This indicated that IAA value generated by strains KT2, KT8, KT9 is more than other strains. The tubes containing micro strains remain as KT1, KT3, KT6, KT7, KT12 had lighter in color due to IAA content produce by them less. Thus, initially identified strains KT2, KT8, KT9 have strong IAA biosynthesis.

#### Some biological characteristics of selected microorganisms

Biological characteristics and culture conditions affect to ability IAA biosynthesis of the microorganism were determined by conventional methods. Results are presented in table 2:

No		Temperature (°C)				Reaction		
	Strain	20-25	25-30	30-35	5.0-6.5	6.5-7.5	7.5-8.0	Gram
1	KT1	*	***	*	*	***	*	-
2	KT2	*	***	*	*	***	*	+
3	KT3	*	***	*	*	***	*	+
4	KT6	*	***	*	*	***	*	+
5	KT7	*	***	*	*	***	*	-
6	KT8	*	***	*	*	***	*	+
7	KT9	*	***	*	*	***	*	+
8	KT12	*	***	*	*	***	*	+
<u>Caption:</u> *: Poorly developed + : Gram positive ***: Strongly developed - : Gram nagative								

Table 2 Biological characteristics of se	elected microorganisms
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All of the selected microorganisms are bacteria, mainly belong to the genera Azotobacter and Bacillus. They developed appropriate in aerobic conditions, the medium AT and King B. Temperature of about  $28 \sim 31^{\circ}$ C, pH of about  $6.5 \sim 7.5$  is the most appropriate conditions for the growth and development of them, IAA generated is strongest. If outside the range of temperatures and pH above

#### **Determination of IAA formated according to time**

decreased.

Depending on the characteristics of individual selected strains that produced IAA contents in the same time are different between different strains and levels of IAA generated each time of microorganisms are also different.

microorganisms will growth and development weaker, active generate IAA also

No	Strains	IAA contents produced in the culture fluid of microbial strains ( $\mu g / ml$ )						
		2 day	3 day	4 day	5 day			
0	Con trol	0.86	0.86	0.86	0.86			
1	KT1	24.55	25.12	25.49	21.67			
2	KT2	80.19	123.90	145.20	154.02			
3	KT3	15.33	15.78	27.56	31.50			
4	KT6	19.65	19.34	22.10	69.69			
5	KT7	30.61	40.06	43.33	40.13			
6	KT8	98.52	112.90	169.70	170.20			
7	КТ9	91.45	117.68	145.05	164.28			
8	KT12	49.30	65.20	80.24	91.11			

 Table 3 Content of IAA generated by the selected microorganisms

Table 3 showed that, the microbial strains KT2, KT8, KT9 were capable to generate IAA very strong (after 5 days of culture shaking, IAA concentration in the solution > 150  $\mu$ g/ml), the microbial strains remaining were weaker (IAA concentration in solution <100  $\mu$ g/ml). KT8 and KT9 strains have high levels of IAA generated the highest on day 4 and day 5. IAA content of KT8 strain have increased rapidly in the first 4 days of culture and almost unchanged after 5 days. Comparison capable of generated IAA through different times of strains KT2, KT8, KT9 represented by figure 3:





## The assessment results stimulating germination ability, to root and the body length of green peas in the laboratory

In the experimental formula then green peas are soaked, incubated with broth culture of strains KT2, KT9, KT8 (strains generated high levels of IAA) on steriled petri dishes. Peas in the control formula was soaked with distilled water. Peas germination ability was tested after 2 days incubation, root length and body length was measured after 5 days incubation. Results are showed in Table 4.

No	Strains	Germination Rate (%)	Length of root (mm)	Length of body (mm)					
0	Control	73.3	4.1	19.2					
1	KT2	86.7	5.4	21.0					
2	КТ9	95.5	5.8	22.4					
3	KT8	93.3	5.9	23.1					

 
 Table 4 Ability to stimulate germination, roots and body length of green peas of selected microorganisms



Figure 4 Peas germination ability after 5 days experiment

Table 4 showed that, when green peas planted in the greenhouse, the percentage of germination in the plots that incubated with bacterial strains was higher than the control sample, showing the formula 2 (infected with strains KT9) for germination rate the highest was 95.5 %, while this rate in the control formula was 73.3 %. After 3 days of germination of the plants infected with selected strains of microorganisms, the length of root in the formula 3 has the highest root length was 5.9 mm, whereas this size in the control formula is 4,1 mm, the body length in the formula 3 is 23.1 mm, higher than 19.2 mm in the control formula.



Figure 5 Ability of the roots and body length of peas after 5 days experiment

From the above results showed that the selected microorganisms are able to produce IAA, especially including 3 strains KT2, KT8 and KT9 is superior to all. This is the strains has a great potential in agricultural production.

#### Conclusion

Has selected the 8 strains of microorganisms have the ability to generate growth stimulants (IAA) from soil samples rubber plantation at Dien Bien, Son La, Lai Chau,

which are KT1, KT2, KT3, KT6, KT7, KT8, KT9, KT12. These strains are belonging to genus *Bacillus* and *Azotobacter*, aerobic growth when cultured in standard environment at the temperature  $28 \sim 30^{\circ}$ C, neutral pH (pH 6.5 to 7.5).

Has selected the 3 strains KT2, KT9, KT8 have higher ability to generate growth stimulants (IAA) and stimulates root, long stem of green peas.

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## Bio-control efficacy of some plant extracts against pulse beetle Callosobruchus chinensis

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#### Abstract

We loose about 35% of the annual crop due to the feeding by insect larvae and adults which accounted to be billions of rupees every year. The insects damage that much amount of food which could have fed millions of people for a year. Thus insects are in neck to neck competition with man. Hence for survival of man to control the agricultural and food grain pest by using safer methods has become important. The effect of extracts of onion Urginea indica (Roxb.), fruit of desert date Balanites aegyptiaca (L.) and seeds of Castor Ricinus communis (L.) on fecundity, fertility, POP, S.I. and Longevity of Pulse beetle Callosobruchus chinensis (L.) were studied. The % decrease in fecundity has been affected and found to be highest in 2% castor seeds, less in 1.5% desert date and least in 1.5% onion extracts. The % hatching is found to be least in 2% onion and 2% castor seeds and maximum in 2% desert date while 1.5% lies in between. Sterility Index (S.I.) showed effective results 2% castor seeds and 2% onion show highest relative index while lowest in case of 1.5% onion and 1.5% desert extracts. Pre-Oviposition Period (POP) found to be highest in 2% castor seeds, less in 1.5% castor seeds, lower in 2% onion and lowest in 1.5% onion extracts. Longevity was observed highest in 1.5% onion, less in 1.5% desert date, lesser in 2% onion and least in 2% castor seeds extracts.

## Key words: Callosobruchus chinensis; Urginea indica; Balanites aegyptiaca;Ricinus communis; fecundity and Sterility Index

#### Introduction

From the total gain of agricultural products we loose near about 35% of annual crop because of the feeding by insect larvae or adults because of that we loose billions of rupees every year. The insects damage that much amount of food, which could have fed millions of people for a year. Thus the insects are in close association and competition with man for food, fiber and shelter.

So, it is important for survival of man to control the agricultural as well as food grain pest. Now a days man has invented many highly effective organic chemicals for the control of pest. As Yadav *et al.* (1980) tested the effect of organophosphorus

insecticides against stored product beetles, Mahabubarraheman and Yadav (1987) also studied the effect of toxicity of different solutions and dusts of synthetic pyrethroids against the *Callosobruchus maculatus* (L.) and *Callosobruchus chinensis* (L.).

Malathion has been used as a contact insecticide in storage since 1954, but recent data indicates that many strains of stored products insects have developed resistance to Malathion (Champ and Dyte, 1976).

The available information on pyrethrins, rotenone and nicotine shows that these insecticides of plant origin are comparatively safer to mammals and higher animals (Feinstien, 1952) and suggests possibility of occurrence of such insecticides in other plant resources hither to unexplored hence man has to find out alternative methods to reduce these hazards.

Scientists are of the view that instead of chemical pesticides, the naturally occurring pesticides are safer. So, more attention is to be paid towards the pesticides of plant origin.

Today more than 2000 species of plants have reported to have insecticidal properties (Caius, 1986). There are many plants present in nature which have the different chemical substances which act as a controller for the pests. The best examples of these are the work of Jacobson (1975), Bernays and Chapman (1977), Smelyanez (1978). Insecticidal properties of rhizomes of sweetflag *Acorus calamus* against rice weevil were observed (Mukerjee and Ram Govind, 1959). The lactone extracted from number of species of plant family Asteraceae is known for its action as insect feeding deterrent, particularly for beetles (Picman *et al*, 1978). Patel and Bhadane (2006) also noted gustatorepellent activity of ethanolic extracts of different parts of neem *Azadirachta indica* in case of Red Cotton Bug *Dysdercus koinigii*. Research on seeds as protectant has been conducted on *Sitophilus* species and stored product Lepidoptera (Koona and Njoga, 2004).

Swella and Mushobozy (2007) observed that coconut seed provided the best protection of the natural products against *Callosobruchus maculatus*.

Schluter (1984) has shown disturbance of epidermal and fat body tissue after feeding Azadirachtin and its consequence on larval moulting. Petroleum ether extracts of neem seed protect gram seed at least up to 135 days against *Callosobruchus chinensis* (Pandey *et al*, 1976).

Tapondjou *et al.* (2002) showed that the dry ground leaf of *Chenopodium ambrosioides* inhibit  $F_1$  progeny production and emergence of *Callosobruchus chinensis*.

Yadav and Pant (1977) studied the food consumption character of both species of *Callosobruchus* and reported that consumption rate of *Callosobruchus maculatus* and *Callosobruchus chinensis* in 5:2 against the body weight ratio of 2:1. According to Swella and Mushobozy (2009) the order of ovipositional preference for all legume seeds found to be almost the same irrespective of the host on which *C. maculatus* had been reared, also there was no association between the seeds prefer for oviposition and culture on which the bruchid was reared.

#### **Material and Methods**

The test insect pulse beetle *Callosobruchus chinensis* (Bruchidae: Coleoptera) were reared in laboratory by usual culture method (Md. Aslam *et al.*, 2006). Longevity, Oviposition, fecundity and development of *Callosobruchus chinensis* was studied by Seddiqi (2009).

#### **Collection of Plant parts**

Onion, *Urginea indica* (Roxb.), Fruit of desert date *Balanites aegyptiaca* (L.) and Castor seeds *Ricinus communis* (L.) collected locally.

#### **Preparation of Plant Extracts**

The plant parts of onion, desert date and castor seeds were chopped into small pieces with sharp knife, dried in the oven at  $50^{\circ}$ C and fine powder was made in electric mixer grinder. The cold alcoholic extracts were prepared from these powders. 5 gram of each dried powder was soaked with 10ml of 80% ethyl alcohol in a reagent bottle & allowed to stay over for 24h it was then filtered and washed with solvent till filtrate measured 20ml.

These extracts were further diluted and following concentrations were worked out with glass distilled water. 1.5 And 2 percent extracts used, in which dried chickpea seed were soaked for 24 hrs at room temperature and dried again and offered to adults of pulse beetle *Callosobruchus chinensis* (L.) which were used for stock culture for different experiments.

#### **Experimental Designs**

#### Fecundity, Fertility and Sterility index

For this assay three pairs for control and 3 pairs for each concentration were kept in fiber bottles covered with muslin cloth fastened by rubber or elastic bands. The number of eggs laid by 3 females, in each bottle counted during each oviposition and immediately kept in separate containers used as hatching chambers. The incubation period was noted for each concentration as well as percent hatching was calculated. From these observations, cumulative sterility index (S.I.) for each concentration was calculated by the following formula (Koul, 1983).

S.I. =100 - 
$$\frac{\text{Test number of eggs X \% hatch}}{\text{Control number of eggs X \% hatch}} \times 100$$

Numbers of ovipositions were recorded, time interval between two successive ovipositions designated as pre-oviposition period (POP) or inter-oviposition period (IOP) is determined. Numbers of eggs laid per oviposition were recorded.

#### Longevity

To study the longevity, freshly moulted adult pulse beetles were pooled from our laboratory culture and 3 pairs were kept in each bottle. For control, normal seeds and

for experimental treated seeds with respective concentrations were offered for feeding the beetles. Five seeds were offered to each set. The longevity was calculated from the day of emergence to the day of death. Deaths were recorded and mean of 3 pairs was taken in each case as an average longevity in days.

#### Observation

Effect on fecundity and fertility (hatchability) of different concentrations of various extracts were studied.

Table 1Effect of different extracts of Urginea indica (Roxb.), Balanites aegyptiaca (L.) and Ricinuscommunis (L.) on Fecundity, POP and Longevity of pulse beetle Callosobruchus chinensis (L.)

Percent Extract fed	Pre- oviposition Period (POP) in Hrs.	Total no. of eggs laid	Average no. of eggs per female	Percent decrease in fecundity	Longevity in days
Control	18	110	36.6	00	32
1.5% U. indica	25	40	16.6	54.644	20
2% U. indica	27	32	10.6	71.038	0
1.5% B. aegyptiaca	37	12	04	89.071	17
2% B. aegyptiaca	40	08	2.6	92.896	15
1.5% R. communis	42	06	02	94.535	10
2% R. communis	45	02	0.6	98.360	0

The fecundity, in these fed with seeds treated with castor seed extract of 2% concentration was found lowest (0.6 eggs per female). Slightly more in castor seed of 1.5% concentration (2 eggs per female) and still more in desert date of 2% (2.6 eggs per female). Desert date of 1.5 concentration shows 4 eggs per female, more in onion of concentration 2% (10.6 eggs per female) and in onion of 1.5% concentration (16.6 eggs per female). In case of control the average number of eggs laid by female is 36.6 (Table 1).

The percent decrease in fecundity is also calculated with reference to control. From Table 1, it is highest in 2% castor seed (98.360) less in 1.5% desert date (89.071) and least in onion (54.644).

Fertility (hatchability) of eggs has been assessed in terms of percentage (Table 2). In control the fertility in terms of percent hatching is 90.90. It is least in 2% onion and 2% castor seed (0), maximum in 2% desert date and 1.5% desert date, 1.5% onion extracts lies in between.

Percent Extract fed	Total No. of eggs laid	No. of eggs hatched	No. of eggs unhatched	Hatching (%)	Ratio hatched/ unhatched	Sterility Index (SI)
Control	110	100	10	90.90	10	9.1
1.5% U. indica	40	5	35	12.5	0.142	94.99
2% U. indica	32	-	32	0	0	100
1.5% B. aegyptiaca	12	5	7	41.66	0.7	95.00
2% B. aegyptiaca	08	4	4	50	1	95.99
1.5% R. communis	06	1	5	16.66	0.2	99.00
2% R. communis	02	-	2	0	0	100

Table 2Effect of different extracts of Urginea indica (Roxb.), Balanites aegyptiaca (L.) and<br/>Ricinus communis (L.) on Fertility and Sterility Index (S.I.) of pulse beetle<br/>Callosobruchus chinensis (L.)

The sterility index (S.I.) was calculated by formula already mentioned in material and method (Koul, 1983). The ratio of hatched eggs to unhatched eggs was determined. It was 10 in control. In 2% onion and 2% castor seed it is minimum (0), maximum in desert date (1) and other concentrations and all between these two end values.

The treated and control females were studied for 3 ovipositions and average number of eggs laid per female successively were noted. Average numbers of eggs laid during first oviposition were compared. In control the number was 36.6, in 1.5% onion extract 16.6 that is highest and in 2% castor seed 0.6 eggs that is lowest while in case of 2% onion, 1.5% desert date, 1.5% onion and 2% castor seed.

When second oviposition was studied female treated with 2% castor seed could not oviposit second time. In remaining concentration 2% onion and 2% desert date average eggs laid per female (4 and 1) and highest in 1.5% onion and 1.5% desert date (5 and 2) while in control it was 84. The third oviposition was shown in 1.5% onion extract very low number of eggs (5) and highest oviposition was shown by control female while all experimental females fail to oviposit 3<sup>rd</sup> time. When Pre Oviposition Period (POP) is studied the average POP in control is 18 hrs. in onion 1.5% and 2% onion it is lowest (25 and 27 hrs.), higher in 1.5% and 2% desert date (37 and 40 hrs.) while, highest in 1.5% and 2% castor seed (42 and 45 hrs.), (Table 1). Thus POP is affected considerably.

The incubation period is calculated in control and in experimental. For control it is 8 days. For 1.5% castor seed it is 16 days and for 1.5% onion, 1.5% desert date, 2% desert date it is 10, 11 and 12 days respectively. Longevity of insects was calculated in experimental and compared with the control (15 days). Longevity has been found to be reduced with 1.5% castor seed, it has been also found reduced as 2% desert date, 1.5% desert date and 1.5% onion extract (Table 1).

#### Discussion

Ketker (1989) observed that castor oil acts as surface protectant on green gram to check the pulse beetle. Apart from repellent action of castor seed, more serious damage could be caused if feeding inhibited. It could also be expected that a disruption in the cellular component in reproductive organs leading to a derangement of metabolic machinery causing depletion in fecundity and fertility (hatchability) have been studied in several insect species following castor seed (Talukder and Howse, 2000).

Shrivastava and Gupta (2007) found that there was significant reduction in the number of eggs laid per pair of the *C. chinensis* when treated with different formulations. A very significant reduction was observed in seeds treated with aqueous extract of fruit of *Solanum surratense*.

Dwivedi and Kumari (2000) also observed reduced oviposition when the grains treated with *Ipomoea palmata* leaf extracts in case of *C. chinensis*. Leaf extracts of *Fagonea cretica* were found to bring down egg laying in *C. chinensis* by Mann (1997). According to Dwivedi and Maheshwari (1997) acetone extracts of croton and petroleum ether extracts of *Verbesena encelioides* and *Occidentalis* where found to exhibit ovipositional deterrent activity against *C. chinensis* in stored cowpea.

The efficacy of *Aegle marmelos* oil as fumigant against insect infestations of stored grains and strainghthan the possibility of using it as an alternative to synthetic chemical as it has significantly reduced oviposition and adult emergence *C. chinensis* in treated cowpea seeds (Rajesh Kumar, *et al*, 2008). Rajpakse and Ratnasekera (2008) found the highest bioactivity (90%  $\sim$  100% mortality) by the crude ethanol extracts of *Azadirachta indica* and *Ocimum sanctum* for the control of *Callosobruchus* spp.

Patole (2009) noted ovipositional efficacy of aqueous, alcohol and acetone extracts of seed kernel of neem and flowers of gorakhmundi (*Sphaeranthus indicus* Linn.) on green gram treated seeds for the control of *Callosobruchus chinensis* and found to be effective for egg laying and was depending on dose and exposer time. A maximum adult mortality was observed in *C. chinensis* treated with 10% ether extracts of root of *Withania somnifera* (Gupta and Srivastava, 2008). Maximum % ovicidal action was noted in *Murayya koenigii* (Linn.), *Tabarnaemontana divaricata* Linn. and *Chenopodium album* Linn. As 70%, 75% and 66.66% at 100% dose level respectively (Dwivedi and Venugopalan, 2001). The efficacy of coconut oil in reducing the level of egg production and exit holes by *C. chinensis* was found to be the most effective among Cashew nut oil, Udara nut oil and Neem leaf oil (Dialoke, 2010).

Mummigatti and Raghunathan (1977) reported that seed of castor inhibited the multiplication of *Callosobruchus chinensis* (L.) females were with fewer eggs as compared to females fed with other plant extracts. In present study fecundity of *Callosobruchus chinensis* (L.) is greatly influenced by castor seed. Maximum percent reduction in fecundity was 2% castor seed and least in 1.5% onion extracts.

#### **Findings and conclusion**

The effects were evaluated in terms of reproductive activity following exposure to the alcoholic extracts. Fecundity and fertility of the beetle is adversely affected to a remarkable extent. The fecundity in seeds treated with 2% castor seed was found lowest (0.6 eggs per female), slightly more in 1.5% castor seed (2 eggs per female), still more in desert date of 2% (2.6 eggs per female), 1.5% desert date shows (4 eggs per female), more in 2% onion (10.6 eggs per female) and in 1.5% onion (16.6 eggs per female).

The effect on percent decrease in fecundity also showed the adverse effect of different extracts. It is highest in 2% castor seed (98.360) less in 1.5% desert date (89.071) and least in 1.5% onion (54.644). The fertility (hatchability) of eggs was also calculated in percent. In control the percent hatching is 90.90 which is least in 2% onion and 2% castor seed (0%), maximum in 2% desert date (50%) and 1.5% desert date , 1.5% onion lies between (41.66%, 12.5%). The sterility index was calculated which also showed effective result 2% castor seed and 2% onion extract shows highest sterility index (100%) which is lowest in 1.5% onion and 1.5% desert date extract (94.99 and 95.00).

When the ratio of hatched and unhatched number of eggs was calculated it was 10 in control. It is less in 2% onion and 2% castor seed (0), it was more in 2% desert date (1) and other concentrations were between these two end values as 1.5% onion (0.142), 1.5% desert date (0.7) and 1.5% castor seed (0.2).

When pre-oviposition period (POP) was studied the average pre oviposition period in control was 18hrs. after the treatment of different extracts that result which got shows the highest pre-oviposition period was observed in 1.5% and 2% castor seed (42 and 45 h respectively), 1.5% and 2% desert date (37 and 40 h respectively) and lowest in 1.5% and 2% onion extract (25 and 27 h respectively). At last the longevity was calculated as compared to control (32 days) which has been reduced as in 1.5% and 2% castor seed (20 and 0 days), 1.5% and 2% desert date (17 and 15 days) and 1.5% and 2% onion (10 and 0 days).

The above findings are in conformation with the present study which is found to be effective in bringing down the fecundity, fertility and longevity. Therefore, it can be suggested that plant extracts under consideration are found to be effective enough against the control of *C. chinensis* and chemical pesticides with increase of prevailing prices as well as their hazardous properties can be substituted.

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#### Plant Parasitic Nematodes in Agriculture and Horticulture

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#### Introduction

Nematodes co-exist with plants since the time immemorial, yet they were never considered economically important as in recent years. In ancient times the unusual crop failure were generally attributed to some supernatural powers and soil sickness. But now it has been well established that the plant parasitic nematodes are one of the major limiting factors in the agriculture as well as in the horticulture throughout the world. The hidden enemy of the crops causes economic losses ranging from slight, perhaps less than 1% to total mass. The average annual loss of agricultural crops has been estimated to be 10% or more. Estimated annual yield losses in the world's major crops due to plant parasitic nematodes is about 12.3% and it is about 14% in the developing countries. In India recent estimate showed nematode is responsible for both quantitatively and qualitatively yield losses amount Rs 240 billion every year. Wheat growing regions of Rajasthan, UP, Bihar and Madhya Pradesh particularly in tribal belts where tons of wheat wasted every year. Nematodes are responsible for average losses of 9% global potato amounting to about 40 million tons. Association of high population M. xenoplax has also been found to result in low yield in peach and plum. Nematode associations have been suspected to facilitate crown gall infection in fruit trees and assist in causing root rot and winter injuries in apple. Some studies conducted on ectoparasitic nematodes revealed that P. prunni though less pathogenic than *M.incognita* can cause about 35% yield losses in plum when present together with *Tylenchorhynchus mashhoodi* and *M.xenoplax*. Although nematodes are world wide in distribution but the records indicate that certain genera and species are restricted to particular soil types and climatic conditions. The adverse effects of nematodes on plants become apparent after their population build up attain a particular threshold level. Symptoms like dwarfism, hairy roots, root lesions, root galling, cessation of root growth and wilting are caused by most of the nematodes, whereas in many plants they do not cause visual symptoms. They gradually alter the transport pattern of the host, thereby depriving the plant of its supply of nutrients. These deleterious effects result in the low yield and poor quality of crop plants. The effects become more pronounced and enhanced when roots damaged by nematodes provides an easy access to soil borne pathogens of fungal, bacterial and viral origin into the host. Synergistic relationship between plant pathogenic fungi and nematodes in increasing the severity of plant diseases well established in a number of plant pathogens and in nematode interactions. The task of describing and cataloguing many species of nematodes is incomplete but enough work has been done for several alternatives. Despite these pioneer works it is clear that much more information on the fine structure of these nematodes is necessary before studying their disease effects on a particular plant. If one may be permitted to speculate on future nematode pathology must surely develop one day into an active branch of science.

#### Material and Methods

The soil used in the experiments collected from rhizosphere of particular plant. The soil along with the feeder roots are collected in polythene bags and brought to laboratory for analysis. In laboratory, active nematodes are extracted by using sieve and Baerman's funnel methods. After 48 hours the suspension containing nematodes are collected and transferred to a measuring cylinder. The suspension is stirred and 10% is drawn by a pipette into a counting dish for recording the counts of nematodes. The number of nematodes is multiplied by the dilution factors to give the total nematode population. For nematode population in root system, roots were chopped into small pieces of about 5 cm length. The pieces are thoroughly mixed and 5mg is taken at random and stained with acid fuchsin (Byrd *et al.*, 1983). The total numbers of nematodes are counted. For identification, nematodes are killed and fixed in TAF. Specimens are mounted in anhydrated glycerol and sealed to check the evaporation of glycerine.

#### Nematodes and their Environment

Millions of these organisms may live in 1 square meter of soil, yet only about 10% of them have been fully studied and described. The breadth of nematology facilitates linkages with invertebrate zoology, medicine, parasitology, plant pathology, microbiology, ecology and many other disciplines. The developmental biology of the bacterial- feeding soil nematode *Caenorhabitis elegans* has become the best characterized of multicellular organisms. This nematode is a prime model organism in molecular and developmental biology. The importance of nematode research in protecting the nation's supply of food and fibre can be illustrated by the nematode impact on soyabean production in the United States.

#### Crop losses by nematodes in India

In previous years several fields of crops were visited and soil and root samples were processed and examined for the association of various nematodes associated with food and fruit crops. On the world basis, crop losses to the extent of 5% have been estimated due to root knot nematodes. In Haryana 90% loss in okra, 45% loss in tomato and 27% in brinjal was estimated due to *M.incognita* at initial population density of  $2800 \sim 3460/\text{kg}$  soil. In india cyst nematode is most wide spread in Rajasthan and Haryana. Its occurrence has been recorded in Delhi, Himachal Pradesh, J&K, Madhya Pradesh, Punjab and Uttar Pradesh. Wheat and barley are the main host of *H.avenae*. During a survey of fruit growing localities in Himachal Pradesh,

northern India, conducted between  $1981 \sim 1983$ , 9 important plant parasitic nematodes were found. Peach trees had the greatest populations of nematodes while apricot trees had the lowest. New records for this State and/or India are: Meloidogyne sp. on apple and almond; Paratylenchus nainianus on apple, almond, apricot and plum; Pratylenchus penetrans on apple, apricot, peach and plum; Helicotylenchus on almond, apple, apricot and plum; Xiphinema sp. on almond, apple and plum; *Tylenchorhynchus* sp. on apple, apricot and plum; *Criconemoides* sp. on apricot and plum and Hoplolaimus sp. On apricot. Aphelenchoides fragariae was found infecting strawberry. Investigations were carried out to find out the distribution of Tylenchulus penetrans and other plant parasitic nematodes associated with citrus in H.P. The survey conducted in eight districts (Bilaspur, Hamirpur, Mandi, Kullu, Kangra, Sirmour, Solan and Una) revealed the prevalence of 13 nematode genera associated with citrus plantations and occurrence of Tylenchulus was highest (228 out of 254 samples and from 12 out of 18 orchards surveyed). The highest population recorded for this nematode was 24,750/ 250 ml soil) at Dhaulakuan. The second important genus found associated with citrus was Helicotylenchus (recorded from 8 out of 18 localities, with maximum population of 216/250 ml soil) at Dhaulakuan. Out of eleven other genera (Tylenchorhynchus, Hoplolaimus, Pratylenchus, Hemicycliophora, Hemicriconemoides, Xiphinema, Meloidogyne, Longidorus, Rotylenchus, Leptonchus and Isolaimus), occurrence of Leptonchus and Isolaimus is being reported for the first time. The interaction studies between T. semipenetrans and Fusarium solani showed that T. semipenetrans was more pathogenic to C. jambhiri than F. solani and when together, their effect remained as severe as by nematode alone.

#### **Recommended Action Strategies and Resources**

Basic and applied nematology research and education need new and increased resources. To meet needs, we must replace at least one half of the nematologists lost over the 15 years in order to increase our knowledge of nematodes and to use this increased understanding to develop new environmentally safe and cost effective management strategies. We also need to expand awareness among the people who are engaged in horticulture and agriculture with modern telecommunication technology, so that we can have a better crop at national and internationally.





Data from Mitchell et al., 1993

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## Current status of biofertilizers development, farmers acceptance, utilization and future perspective in Andhra Pradesh, India

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#### Abstract

Andhra Pradesh climate is generally hot and humid. The major role in determining the climate of the state is played by south west monsoons. Agriculture plays a pivotal role in the economy of the state. In recent years, indiscriminate use of chemical pesticides and fertilizers lead to increased cost of cultivation and environmental pollution. Due to, energy crisis and increased fertilizer cost have necessitated to use of cost effective organic sources of nutrients to maintain soil fertility, productivity and health for sustainable crop production. Use of organic manures and biofertilizers could offer a safe and best option for reduction of currently used agrichemicals. Beneficial microbes such as Rhizobium, associative and free living nitrogen-fixing bacteria, phosphate solubilizing bacteria, arbuscular-mycorrhiza, and other beneficial fungi are considered as bio-fertilizers. The extensive research program on beneficial bacteria and fungi in our lab has resulted in the development of a wide range of biofertilizers, which satisfied the nutrient requirements of crops, suppression of diseases and increased the crop yields. Our experiments in the greenhouse and under field conditions showed that different crops responded positively to microbial inoculations. In particular, Rhizobial inoculants when applied to leguminous crops increased crop yield and quality. An attempt was made to review the research on biofertilisers and their acceptance by farmers in Andhra Pradesh. Our review showed that limited usage of biofertilizers and biopesticides in Andhra Pradesh. This is due to lack of awareness, availability, cost effectiveness, delivery systems, proper handling etc. Our talk will focuses on reasons for the non adoption and bottlenecks in the production, marketing as well as challenges facing the more widespread utilization of biofertilizers and biopesticides by Andhra Pradesh farmers of India.

#### Introduction

Biofertilizers, more commonly known as microbial inoculants, that are artificially multiplied certain soil beneficial organisms that can improve soil fertility and crop productivity through varied mechanisms. Although the beneficial effects of legumes in improving soil fertility was known since ancient times and their role in biological nitrogen fixation was discovered more than a century ago, and their commercial exploitation of recent interest and in practice.

The commercial history of biofertilizers began with the launch of 'Nitragin' by Nobbe and Hiltner, with a laboratory culture of Rhizobia in 1895, followed by the discovery of Azotobacter and then the blue green algae, Azospirillum and Vesicular-Arbuscular Micorrhizae (VAM). In India, the first study on legume Rhizobium symbiosis was conducted by N.V. Joshi and the first commercial production started as early as 1956. Although biofertilizers have been promoted as supplement or complement of chemical fertilizers, in reality they are two alternative means of accessing plant nutrients. Commonly explored biofertilizers in India are mentioned below along with some salient features.

**Rhizobium (RHZ)** These inoculants are known for their ability to fix atmospheric nitrogen in symbiotic association with plants forming nodules in roots (stem nodules in sesabaniamrostrata). RHZ are however limited by their specificity and only certain legumes are benefited from this symbiosis.

**Azotobacter** (**AZT**) This has been found beneficial to a wide array of crops covering cereals, millets, vegetables, cotton and sugarcane. It is a free living and non-symbiotic nitrogen fixing organism that also produces certain growth promoting substances and secondary metabolites that suppress many soil borne pathogens.

**Azospirillum (AZS)** This is also a nitrogen-fixing micro-organism beneficial for non-leguminous plants. Like AZT, and the benefits transcend nitrogen enrichment through production of growth promoting substances.

**Blue green Algae (BGA) and Azolla** *BGA* are free living, photosynthetic nitrogen fixers abundance in Indian soils. They too add growth-promoting substances including vitamin B12, improve the soil's aeration and water holding capacity and add to bio mass when decomposed after life cycle. Azolla is an aquatic fern found in small and shallow water bodies and in rice fields. It has symbiotic relation with BGA and can help rice or other crops through dual cropping or green manuring of soil.

**Phosphate solubilizing (PSB)/Mobilizing biofertilizer** Phosphorus, both native in soil and applied in inorganic fertilizers becomes mostly unavailable to crops because of its low levels of mobility and solubility and its tendency to become fixed in soil. The PSB are life living and can help in improving phosphate uptake of plants in different ways. The PSB also has the potential to make utilization of India's abundant deposits of rock phosphates possible, much of which is not enriched.

#### **Responses and Limitations**

Nitrogen equivalence indicates that biofertilizers are cheap and convenient to use relative to chemical and organic fertilizers (FYM) and moreover they have

considerable promise for all crops especially cereals, oilseeds, vegetables and cotton. Although, biofertilizers have been promoted as supplement for chemical fertilizers but not as total substitute for chemical fertilizers because, they were only indirectly approximated through controlled experiments in greenhouse. However, while positive responses have been observed in a wide range of field trials, there was a remarkable inconsistency in responses across crops, regions and other conditions because of varied reasons.

#### Research conducted in ANGRAU under different schemes

## All India Network project on Biofertilizers: Mixed microbial consortium of rice fallow blackgram

A combination of RH1 (RBG 31) with PSB 1 or PSB 2 (AMT 1001 or AMT 1005) gave significantly higher grain yields of blackgram in the both rice fallows and upland conditions. The results are interesting and indicated that the microbial insolates of rice fallow blakgram fields are suitable for upland conditions also. There is a grain yield increase of 40% in rice fallows blackgram and 28% of upland blackgram over 100% RDN by the inoculation of compatible microbial inoculants.

By using plant-growth promoting rhizobacterial (PGPR) isolates *Pseudomonas fluorescens* (PF), *Bacillus polymyxa* (BP), and *Bacillus subtilis* (BS) along with *Rhizobium* (RBG 314) and PSB (AMT 1001) as a mixed microbial consortium was formulated and used. Inoculation of the consortia improved nodulation and plant DM significantly. Maximum response was observed with consortium of *Rhizobium* + PSB + PF.

It could be inferred that the microbial consortium of *Rhixobium* (RBG 314), PSB (AMT 1001) and *Pseudomonas fluorescens* was best for enhancing the rice fallow blackgram yields in the experimental fields as well as in the farmers fields.

#### Mixed biofertilizers consortium for chilli

In order to reduce the usage of chemical fertilizers, a mixed microbial consortium was developed using *Azospirillum* SCH 18, *Azotobacter*, PSB (AMT 1003), PGPR (PF) and AMT (Table 1). The nitrogen content in the shoots at harvest was highest (2.2%) when both nitrogen fixers were applied along with PSB, PGR and AM in the presence of 75% recommended dose of fertilizers (RDF). Soil microbial populations were increased and the highest dry chilli yield which was 14.5% more than in 100% RDF was recorded by saving 25% chemical fertilizers.

Table 1 A mixed microbial consortium model for chilli using Azospirillum SCH 18, Azotobacter,

Treatment	Total Bacteria $\times$ $10^7/g$ soil	PSB $\times 10^4$ /g soil	AM propagules 10 <sup>7</sup> /g soil	Dry chilli yield (kg/ha)
Azoto+Azosp+PSB+PGPR+AM+ 75%RDF	73	58	670	3410

PSB (AMT 1003), PGPR (PF) and AMT

#### Mixed biofertilizers consortium for cotton

In cotton growing areas of Andhra Pradesh, farmers apply very high doses of chemical fertilizers and neglect the use of biofertilizers. By using native isolates of *Azospirillum, Azotobacter and* PSB a mixed microbial consortium was developed (Table 2). Inoculation of mixed microbial consortium along with 75% RDF enhanced the rhizosphere soil microflora to maximum level, along with highest nitrogen accumulation and kapas yield (3420 kg/ha) which significantly higher (+13.9) than 100% RDF (3003 kg/ha) while saving of 25% chemical fertilizers.

Table 2 A mixed microbial consortium model for cotton using native Azospirillum,

#### Azotobacter and PSB

Treatment	N in plants at harvest (%)	Kapal Yield (kg/ha)	
Azoto+Azosp+PSB+PGPR+AM+75%RDF	2.26	3420	

#### Rice – Maize – Mung; Lentil – Maize (RAU)

CB-Tobacco waste base applied after 5 days of transplanting and the integration of all the nutrient resources cyanobacterial biofertilizer dose 1 kg/ha +50 kg N + dipping of rice seddling in *Azispirillum* liquid culture for 1 hr. before transplanting + EM5 t/ha) resulted in highest grain yield (42 q/ha) by saving 50% N fertilizers.

## Research conducted at Agricultural Research station, Amaravathi, Guntur, ANGRAU

#### 2007

Application of PGPR organisms, either *Bacillus Polymixa* (BP) or *Pseudomonas flurescens* (PF) enhanced the nodulation of Rhizobium in rice fallow black gram.

The mixed microbial consortium of Rhizobium + PSB + PF could enhance the population of soil Rhizobium, PSB and total cultural bacterial populations to the highest levels under field conditions.

#### 2008

Three arbuscular mycorrhiza fungal isolates were purified which can enhance the phosphorus uptake along with other micronutrients from the deeper layers of soil.

#### 2009

Microbial inoculants enhanced groundnut pod yields by 18.8% when they were applied along with 100% of RDF. When released three differently formulated media for Rhizobium, Azospirillum and PSB, supports the self- life of microorganisms for more than 360 days. Liquid inoculants coated seed can be stored up to 24 h for sowing. The carrier based inoculam coated seed should sow within 4 to 8 h.

#### 2011

Developed a medium (MGM3) to grow three biofertilizer organisms – Azospirillum, PSB and PGPR together for farmers benefit and launched as new biofertilizers called VA Mycorrhiza fungi from ARS, Amaravathi.

Application of liquid inoculants with 50% RDF (1886kg/ha) gave 15% extra grain yield over 100% RDF treatment (1640kg/ha) in pigeonpea. Liquid Rhizobium biofertilizer significantly enhanced the growth and nodulation over solid carrier based biofertilizres in pigeon pea.

Liquid inoculants of Azospirillum + PSB along with 75% RDF gave significantly higher grain yield (4621kg/ha) over the treatment of solid carrier based inoculants with 75% RDF in maize (3986kg/ha).

#### Biofertilizers production and supply by ANGRAU

By reviewing the production details (Figs.1 and 2) it can be found that the production of bioinoculants was increased over years. The percent increase is gradual and in the tune of 60 % to 215% from 2004 to 2010. Supply of bio-inoculants also fallowed the same trend as production. The production and supply were increased to 215 % and 219 %, respectively, during the year  $2009 \sim 2010$ .

#### Supply by department of agriculture

The use of rhizobium over years as seed treatment was maximum in the soybean followed by groundnut. PSB usage was more in oil seed crops compared to pulses. The biofertilizers PSB, azotobacter, azospirillum were used both for seed treatment

and soil application for increased yield by enhancing the seed and seedling vigour (http://agri.ap.nic.in2007,2008,2009).

### Survey report of farmers awareness, adoption of PGPR

A quick survey was conducted and the findings reveal that usage of PGPR almost at minimal level. Among the products, *Pseudomonas fluorescens and Trichoderma viride* are widely known (92%) and adopted product (54%) followed by Rhizobium with adoption of 12 percent and aware by 72 percent. In case of other products even awareness was quiet less and adoption was almost negligible.

### **Reasons for non adoption**

- 1. Availability: quality product availability was also a major problem expressed by the farmers during survey.
- 2. Quality: Quality of the product was a big question. Many players in the market without much name and fame.
- 3. Observability: the efficacy of the product was comparatively less observable in comparison to the chemical ones.

### Constraints in bio-fertilizer use

**Production Constraints** The progress in the field of BF production technology is not upto the mark because of the varied reasons:

- Unavailability of appropriate and efficient region specific native strains
- Unavailability of suitable carrier due to shelf- life of bio-fertilizers is short
- Mutation during fermentation of biofertilizers tend to mutate during fermentation and thereby raising production and quality control cost.

### Market level constraints

- Lack of awareness of farmers
- Inadequate and Inexperienced staff
- Lack of quality assurance
- Seasonal and unassured demand.

### **Resource constraint**

• Limited resource generation for BF production. The investment in biofertilizer production unit is very low. But keeping in view of the risk involved largely because of short-shelf life and no guarantee of off take of bio-fertilizers, the resource generation is very limited.

#### Field level constraints

- Soil and climatic factors among soil and climatic conditions, high soil fertility status, unfavorable PH, high nitrate level, high temperature, drought, deficiency of P, Cu, Co, Mo or presence of toxic elements affect the microbial growth and crop response.
- Native microbial antagonistic microorganism already present in soil competes with microbial inoculants and many times do not allow their effective establishment by out competing the inoculated population.
- Faulty inoculation techniques. Majority of the marketing sales personals do not know proper inoculation techniques. Biofertilizers being living organisms required proper handling, transport and storage facilities.

#### **Conclusion and future strategies**

- Identification/ selection of efficient location/ crop/soil specific strains for Nfixing, P, Zn- solubilizing and absorbing (mycorrhizal) to suit different agro climatic conditions.
- Improvement of strains through biotechnological methods.
- Developing suitable alternate formulations viz., liquid inoculants / granular formulations for all bioinoculants, to carrier based inoculants.

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		2007-08		2008-2009			2009-2010				
Biofertlizer	Crop	Kha rif	Rab i	Khari	f	Rabi		Kharif		Rabi	
		ST	ST	ST	SA	ST	SA	ST	SA	ST	SA
	Groundnut	33	145	120	0	300	0	380	0	132	0
	Soybean	0	0	300	0	0	0	610	0	305	0
D1:1:	Blackgram	0	27	5	0	70	0	31	0	0	0
Knizobium	Greengram	0	8.5	25	0	0	0	65.5	0	0	0
	Pigeonpea	0	0	32.5	0	0	0	132.25	0	0	0
	Bengalgram	0	0	0	0	180	0	0	0	0	0
DCD	Oilseeds	33	76	420	123.5	300	415	289	135.6	437	45.15
FSD	Pulses	0	50	62.5	34	250	330	110.75	0	0	0
	Paddy	0	0	31	0	0	0	84.375	0	0	0
Agogninillum	Maize	0	0	0	0	0	0	1.25	0	0	0
Azospirinum	Sunflower	0	0	0	45	0	0	0	0	0	49
	Oilseeds	0	0	0	0	0	0	0	34.4	0	0
Azeteheeter	Cotton	0	0	0	0	0	0	6.5	0	0	0
Azatobacter	Oilseeds	0	0	0	0	0	0	0	11.5	0	16
	Total	66	307	996	202.5	1100	745	1710.6	181.5	874	110.2

 Table1 Biofertlizers supplied under different schemes by Department of Agriculture, Government of Andhra Pradesh, India (2007 to 2010)

ST- seed treatment, SA soil application

Azospirillum: Seedling dip treatment 1.25 kg per ha of Paddy and Seed treatment 0.5 kg per ha of Maize and

Soil application 1kg per ha

Azatobacter: Seed treatment 0.5 kg per Ha of Cotton and Soil application 1kg per ha

Rhizobium: Seed treatment 200g per kg of seed of Blackgram, Greengram, Pigeonpea, Bengalgram

PSB: Seed treatment 200g per kg of seed and Soil application 1kg/ha

S No	PGPR	Awareness (%)	Adoption (%)
1.	Rhizobium	72	12
2.	Azospirillum	24	4
3.	Azatobacter	28	3
4.	Phosphorous solubulising bacteria	37	-
5.	Pseudomonas fluorescens /Trichoderma viride	92	54



Fig. 1 Percent increase in production of bioinoculants In ANGRAU



Fig. 2 Percent increase in supply of bioinoculants in ANGRAU



Fig. 3 K and Zn solubilizing Bacillus sp. from Amaravathi (New organisms)



Fig. 4 Native *Pseudomonas fluorescens* isolated from soils of Amaravathi, Guntur district, Andhra Pradesh, India

## PGPR interactions with nematodes of horticultural crops

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#### Abstract

Nematodes such as Meloidogyne incognita, Rotylenchulus reniformis, Radopholus similis and Tylenchulus semipenetrans were found to inflict huge losses in important horticultural crops in the world. Management of nematodes using chemicals is expensive, results in toxic residues in edible products and environment. So it was thought to investigate the interactions of *Pseudomonas fluorescens*, *Bacillus subtilis*, *B. pumilis* and nematodes. We found that these PGPR interact very much with the above mentioned nematodes and reduce the populations of these nematodes in the root-systems to the tune of  $65\% \sim 74\%$  in the crops mentioned here. Also reduced were soil populations of R. reniformis by  $64\% \sim 69\%$  in tomato and capsicum rhizosphere, M. incognita by  $70\% \sim 76\%$  in okra and brinjal rhizosphere, R. similis by  $62\% \sim 68\%$  in banana rhizosphere and T. semipenetrans by  $64\% \sim 69\%$  in acid lime rhizosphere. A significant increase was observed in the yields of these crops to the tune of  $19\% \sim 26\%$ . Developed delivery systems of these bio-agents in certain transplanted, non-transplanted vegetable, ornamental crops grown under poly-house/ protected conditions, in the open fields and also in the perennial fruit crop ecosystems. These technologies of bio-management of nematodes using above mentioned PGPRs were transferred to the farmers in various regions of India. Phl gene producing DAPG was found to be responsible for the nematicidal action of P. *fluorescens*. This gene was sequenced from the strain and the details will be presented during the conference.

# Commercialization of *Pseudomonas fluorescens* (IIHR Pf-2) as bio-nematicide in India, transfer of technology to the industries

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#### Abstract

Nematodes affect economically important horticultural crops in the world. The loss in the yields of horticultural crops in India due to the nematodes would be to the tune of  $14\% \sim 26\%$ . Nematologists developed methods for the management of nematodes using chemicals, which proved to be expensive, besides being hazardous. Hence evaluated the bio-efficacy of *Pseudomonas fluorescens* against nematodes mentioned here. We documented the bio-efficacy of this PGPR in the management of Rotylenchulus reniformis on tomato, Meloidogyne incognita on capsicum and carnations, Radopholus similis on banana and Tylenchulus semipenetrans on acid lime. These bio-agents were observed to reduce nematodes to the tune of  $62\% \sim 75\%$ in above mentioned crops. Hence developed protocol of mass production of P. *fluorescens*  $(2 \times 10^9 \text{ cfu/g})$ . Developed delivery methods of this bio-nematicide also generated the toxicological data, shelf-life, container content compatibility and other relevant data for the registration of this bio-nematicide. Subsequently, this was registered by Central Insecticide board and Registration Committee, Ministry of Agriculture, India and transferred the technology of this bio-nematicide to 31 industries in India. All these industries also registered this bio-nematicide in their respective trade names. They undertook the commercial production of this bionematicide with the label claim of crops and nematodes mentioned here. During the conference details of commercialization and particulars of industries using the technology and other relevant data will be presented.
# Effect of organic fertilizers on rhizosphere, growth and yield of tomato

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### Abstract

A pot culture experiment was conducted in ANGRAU, Hyderabad using medium textured soil, tomato as test crop, to study the effect of organic fertilizers/manures which are sources of organic carbon, on soil microbial load, crop growth and yield. The experiment was completely randomized with factorial design replicated thrice consisting of twelve treatments. The treatments were:  $T_1$  (control),  $T_2$  (Inorganic N 120 kg ha<sup>-1</sup> + Inorganic P 60 kg ha<sup>-1</sup> + Inorganic K 60 kg ha<sup>-1</sup>), T<sub>3</sub> (New Suryamin 25 kg ha<sup>-1</sup>), T<sub>4</sub> (T<sub>3+</sub>T<sub>2</sub>), T<sub>5</sub> (Aishwarya 125 kg ha<sup>-1</sup>) , T<sub>6</sub> (T<sub>5</sub> + T<sub>2</sub>), T<sub>7</sub> (EM compost 5 t ha<sup>-1</sup>),  $T_8(T_7 + T_2)$ ,  $T_9$  (Vermicompost 5t ha<sup>-1</sup>),  $T_{10}(T_9 + T_2)$ ,  $T_{11}$  (Horse dung 5t ha<sup>-1</sup>) and  $T_{12}$  ( $T_{11} + T_2$ ). Inorganic N, P and K were supplied through urea, single super phosphate and muriate of potash. Soil samples collected from the rhizosphere were analyzed for organic carbon and microbial load. Soil analysis for microbial load showed highest values in the treatment of 'Aishwarya + Inorganic NPK'. It was higher by a log cycle compared to all other treatments. It was followed by 'Horse dung + Inorganic NPK' and 'New Suryamin + Inorganic NPK' combinations. The results also revealed a decline in microbial activity from vegetative stage to harvesting stage wherein only inorganics were applied and showed increased microbial load till harvest stage with the innovative organic inputs. Among organic fertilizers/manures soil organic carbon content was highest when Aishwarya was applied followed by New Survamin > EM compost > horse dung > vermicompost. The results of the analysis of nutrient suppliers revealed that Aishwarya contained 27% and others on average contained 3% organic carbon, which might have enhanced the microbial load. The data on fruit yield revealed the beneficial effect of these organic inputs particularly when they were applied along with inorganic NPK. Tomato fruit yield was recorded higher by 10.5% to 14.8% with organic fertilizers over inorganic NPK treatment, and by 8% to 18% over organic manure treatments. However, yields were further increased by 5% to 15% when these organic fertilizers applied along with inorganic fertilizers. The study concludes were the recommendation of the use of these organic fertilizers as part of INM, either alone or along with inorganics.

## Introduction

The rhizosphere is a hot spot of microbial interactions as exudates released by plant roots are a main food source for microorganisms and a driving force of their population density and activities. Microorganisms that are beneficial include Nitrogen-fixing bacteria, endo and ectomycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) and fungi. Soil organic carbon is very important in the functions of soil, considered as a good indicator of soil quality, because it mediates many of the chemical, physical, and biological processes controlling the capacity of a soil to perform successfully. This is because it provides the principle food for microbes which helps in their rapid multiplication. In India, FYM remains to be the most popular organic carbon supplier/organic manure applied to fields. In modern agriculture, supply of organic manure in large quantities  $(1 \sim 10 \text{ tons per hectare})$  poses a challenge. A different alternate option of organic nutrient suppliers is the need of the hour for sustainable agriculture or organic farming.

## **Materials and Methods**

Pot culture study was taken up in the green house of the Department of Soil Science & Agricultural Chemistry, College of Agriculture, Rajendranagar, 500030 during *kharif*, 2010. The experiment was laid out in completely randomised design replicated thrice. There were twelve treatments consisting of  $T_1$  (control),  $T_2$ (Inorganic N 120 kg ha<sup>-1</sup> + Inorganic P 60 kg ha<sup>-1</sup> + Inorganic K 60 kg ha<sup>-1</sup>), T<sub>3</sub> (New Suryamin 25 kg ha<sup>-1</sup>), T<sub>4</sub> (T<sub>3+</sub>T<sub>2</sub>), T<sub>5</sub> (Aishwarya 125 kg ha<sup>-1</sup>), T<sub>6</sub> (T<sub>5</sub> + T<sub>2</sub>), T<sub>7</sub> (EM compost 5t ha<sup>-1</sup>),  $T_8$  ( $T_7+T_2$ ),  $T_9$  (Vermicompost 5t ha<sup>-1</sup>),  $T_{10}$  ( $T_9+T_2$ ),  $T_{11}$  (Horse dung 5t ha<sup>-1</sup>) and  $T_{12}$  ( $T_{11} + T_2$ ). Inorganic N, P and K were supplied through urea, single super phosphate and muriate of potash. Fertilizers were applied as per the treatments. Soil samples were collected at 30 DAT and 90 DAT after removal of plants and were analysed for microbial load taking care of no moisture loss. The microbial load was estimated using serial dilution technique and plate counting method using nutrient augar. Microbial colonies were estimated using colony counter. Organic carbon content of the soil was estimated by the wet digestion method (Walkley and Black, 1934). Data on drymatter production and fruit yield were recorded at harvest (90DAT). The results were subjected for statistical analysis as per the procedures outlined by Snedecor and Cochran (1967).

## **Results and Discussion**

Data recorded on soil and plant parameters is presented and discussed below.

## Soil organic carbon

The organic carbon content was the highest in  $T_5$  (Aishwarya) with 1.41% and 1.24% followed by  $T_6$  (Aishwarya + Inorganic NPK) with 1.39% and 1.20% at vegetative and harvest stages, respectively. Among the two manufactured organic nutrient suppliers, Aishwarya contained the highest (27.8%) organic carbon content which was reflected in soil organic carbon content till harvest of the crop. The data (Table1) clearly indicated the effect of manufactured organics over naturally available sources. For example, organic carbon content of soil was recorded as 1.13% and 0.87% at vegetative and harvest stages of the crop in  $T_{11}$  (Horse dung) which was still lower by 20% and 42% compared to sole application of Aishwarya. Poornesh *et al.* (2004) also reported increased organic carbon in soil by the application of urban garbage compost.

## Soil bacterial load

During the vegetative stage (30 DAT) highest number of colonies  $(153 \times 10^9 \text{ CFU})$  were recorded by T<sub>6</sub> (Aishwarya + Inorganic NPK) followed by T<sub>12</sub> (Horse dung + Inorganic NPK) with 88 x 10<sup>9</sup> CFU and the lowest number of colonies  $(31 \times 10^9 \text{ CFU})$  was recorded by T<sub>1</sub> (control). Among organic manures, sole application of Horse dung recorded  $70 \times 10^9$  CFU which was statistically on par with Aishwarya alone  $(72 \times 10^9 \text{ CFU})$ . When combinations were applied, among organic manures bacterial load was recorded highest in by Horse dung with  $88 \times 10^9$  CFU which was 73.9 per cent lower when compared to 'Aishwarya + Inorganic NPK'.

The bacterial colonies found at harvest stage (90 DAT) varied from  $200 \times 10^9$  to 24  $\times 10^9$ . The highest colonies were found in the treatment where organic carbon percent was more *i.e.*, in the treatment T<sub>6</sub> (Aishwarya + Inorganic NPK). The lowest number of colonies were found in T<sub>1</sub> (control) with  $24 \times 10^9$  CFU. The data showed that there was significant variation among different treatments at both the stages of the crop.

CFU were significantly increased from vegetative to harvest stage where organic fertilizers were applied and there was a reduction in the treatments where inorganics were applied. There was an increase from  $153 \times 10^9$  CFU to  $200 \times 10^9$  CFU in treatment T<sub>6</sub> (Aishwarya + Inorganic NPK) from vegetative to harvest stage while, there was a decrease from  $68 \times 10^9$  to  $32 \times 10^9$  CFU in treatment T<sub>2</sub> (Inorganic NPK) from vegetative to harvest stage (Table 2). Manqiang Liu Feng *et al.* (2008) showed that organic amendments with low amount of chemical fertilizers enhanced microbial biomass, activity and nutrient availability more than recommended amount of chemical fertilization alone.

## **Drymatter production**

Drymatter recorded in terms of g pot<sup>-1</sup> at harvest stage (90DAT) of tomato crop is presented in Table 3. Compared to control, all the treated soils recorded higher dry matter production. Highest dry matter was produced with integrated approach of application of nutrients as in  $T_{10}$  (Aishwarya + inorganics NPK) with 37.1 g pot<sup>-1</sup> followed by (EM Compost + Inorganic NPK) with 35.5, (Horse dung + Inorganic NPK) with 31.1 and  $T_8$  (New Suryamin + Inorganic NPK) with 30.5 g pot<sup>-1</sup>. Inorganic NPK alone without any organic sources recorded 30.5 g pot<sup>-1</sup> and the lowest was recorded by  $T_1$  (control). Similar increase in dry matter was reported by Sharma and Dayal (2005) in cowpea with inorganic fertilizers application and by Yadav and Malik (2005) with organic sources of fertilizers added along with *rhizobium* inoculation. Marimuthu *et al.* (2003) recorded the highest growth of green gram by the application of 25 kg  $P_2O_5$  ha<sup>-1</sup> as Mussorie rock phosphate along with enriched biodigested slurry.

### Fruit yield

The highest fruit yield of tomato was recorded by the treatment  $T_6$  (Aishwarya + Inorganic NPK) with 212 g plant<sup>-1</sup>. Among the naturally available organic nutrient suppliers, 'EM compost + Inorganic NPK' recorded highest (196.7) followed by 'Horse dung + Inorganic NPK' with 189.9 and the lowest yield (169.3 g pot<sup>-1</sup>) was obtained by  $T_9$  (Vermicompost alone). All organic nutrient suppliers, natural or manufactured, recorded higher yields compared to 'Inorganic NPK' treatment (162.4 g pot<sup>-1</sup>). Application of FYM with C: N ratio 25:1 increased the yield of soyabean by 3q ha<sup>-1</sup> when compared to the application of recommend dose of inorganic fertilizer (Maheshbabu *et al.*, 2009). Tomato yield data indicated that in the sole applications of organic nutrient sources there was no significant difference among organic fertilizers and organic manures, but when applied in combination with Inorganic NPK, Aishwarya recorded the highest, which was 8 and 18% higher compared to EM compost and horse dung, respectively.

## Conclusions

The organic inputs both manufactured and naturally available, tested in this experiment performed well compared to the inorganic fertilizers in terms of fruit yield of tomato crop. However, performance of the manufactured organic fertilizers namely New Suryamin and Aishwarya were on par with the natural organic manures/composts studied. But when these were applied in combination with inorganic fertilizers, Aishwarya recorded  $8\% \sim 18\%$  higher yield compared to naturally available organic manures, suggesting the INM option.

Like organic manures, these organic fertilisers also increased the organic carbon percent in soil. The readily available C fraction of the organic fertilizers supports the development of microbial biomass in the soil. Organic fertilizers improved the fertility status and microbial properties till the harvest of the crop. Aishwarya which contained 27.8% organic carbon content performed best either alone or in

combination with inorganics. Hence, these can be a recommend option against scarce availability of organic manures or indiscriminate use of inorganic fertilisers.

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#### \*original not seen

	Treatments	30 DAT	90 DAT
T <sub>1</sub>	Soil alone	0.68	0.72
T <sub>2</sub>	Inorganic NPK (120-60-60)	0.84	0.74
T <sub>3</sub>	New Suryamin	1.30	0.71
$T_4$	New Suryamin+ Inorganic NPK	1.28	0.68
T <sub>5</sub>	Aishwarya	1.41	1.24
T <sub>6</sub>	Aishwarya + Inorganic NPK	1.39	1.20
<b>T</b> <sub>7</sub>	EM compost	1.20	0.92
T <sub>8</sub>	EM compost + Inorganic NPK	1.14	0.83
T <sub>9</sub>	Vermicompost	0.96	0.76
T <sub>10</sub>	Vermicompost + Inorganic NPK	0.94	0.75
T <sub>11</sub>	Horse dung	1.13	0.87
T <sub>12</sub>	Horse dung + Inorganic NPK	1.11	0.8

#### Table 1 Effect of organic fertilisers on soil organic carbon (%)

CD (5%)	0.194	0.223

	Treatments		90 DAT	
$T_1$	Soil alone	31	24	
T <sub>2</sub>	Inorganic NPK (120-60-60)	68	32	
T <sub>3</sub>	New Suryamin	68	78	
$T_4$	New Suryamin+ Inorganic NPK	87	92	
T <sub>5</sub>	Aishwarya	72	96	
T <sub>6</sub>	Aishwarya + Inorganic NPK	153	200	
T <sub>7</sub>	EM compost	45	52	
T <sub>8</sub>	EM compost + Inorganic NPK	67	80	
T <sub>9</sub>	Vermicompost	17	20	
T <sub>10</sub>	Vermicompost + Inorganic NPK	19	26	
T <sub>11</sub>	Horse dung	70	50	
T <sub>12</sub>	Horse dung + Inorganic NPK	88	76	
	CD (5%)	13.5	23.0	

# Table 2 Effect of organic fertilisers on soil bacterial load ( $\times 10^9$ CFUs)

	Treatments	Drymatter (g plant <sup>-1</sup> )	fruit weight (g plant <sup>-1</sup> )
T <sub>1</sub>	Soil alone	16.6	67.7
T <sub>2</sub>	Inorganic NPK (120-60-60)	30.5	162.4
$T_3$	New Suryamin	25.7	194.1
$T_4$	New Suryamin+ Inorganic NPK	35.6	201.5
T <sub>5</sub>	Aishwarya	28.0	184.6
T <sub>6</sub>	Aishwarya + Inorganic NPK	35.6	212
$T_7$	EM compost	27.4	186.1
$T_8$	EM compost + Inorganic NPK	35.5	196.7
T <sub>9</sub>	Vermicompost	17.6	169.3
T <sub>10</sub>	Vermicompost + Inorganic NPK	25.9	180.6
T <sub>11</sub>	Horse dung	24.5	179.5
T <sub>12</sub>	Horse dung + Inorganic NPK	31.1	189.9
	CD (5%)	5.75	16.40

Table 3 Effect of organic fertilisers on drymatter production and fruit yield at 90 DAT

# Impact of co-inoculation of bacterial and fungal antagonists on bottom rot disease and on indigenous microbial community in the lettuce rhizosphere

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### Abstract

Application of a single biocontrol strain resulted often in inconsistent disease suppression in the field. In contrast to application of a single inoculant, the use of several antagonists with different modes of action may improve the biocontrol efficacy under a wide range of environmental conditions. Therefore, the biocontrol efficacy was evaluated combining a bacterial (*Serratia plymuthica* 3Re4-18) and a fungal (*Trichoderma viride* GB7) antagonist possessing different biocontrol activities against the bottom rot pathogen *Rhizoctonia solani* on lettuce. The effect of co-inoculation of these antagonists in the presence and absence of the pathogen was evaluated on lettuce under controlled conditions and in comparison to the inoculation with the sole antagonists. Moreover, the impact of the co-inoculation was studied two times on the indigenous communities of the lettuce rhizosphere using 16S or ITS-based fingerprinting methods.

Both, *Serratia plymuthica*, 3Re4-18 and *Trichoderma viride*, GB7 were able to suppress the bottom rot disease effectively. The successful control of bottom rot disease was affected by the application especially of the fungal antagonist. However, the combined application of the two antagonists improved significantly the biocontrol efficacy. Moreover, the composition of the bacterial and fungal community was more pronounced by co-inoculation of both antagonists compared to the effect of inoculation of the single agents. The colonization density of the antagonist 3Re4-18 in the rhizosphere was significantly influenced by the pathogen but not by the fungal antagonist. In summary, the used cultivation-independent methods provided insights into the complex interaction in response to the pathogen presence and to the antagonist's inoculation.

# Inhibitory effects and control efficacy of *Paenibacillus polymyxa* WY110 on *Fusarium oxysporum* of Watermelon

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### Abstract

The inhibition and prevention efficiency of *Paenibacillus polymyxa* WY110 to watermelon *Fusarium oxysporum* were studied based on the medium plate inhibition, spore germination, mycelial growth and seedling growing experiments. The results showed that *Paenibacillus polymyxa* WY110 can inhibit mycelial growth of *Fusarium oxysporum* and produce significant inhibition zones on PDA plate medium. The inhibitory effects to hyphal growth, hyphal dry weight, conidia germination and germ tube elongation were 60.7%, 51.1%, 100% and 100% respectively. The control effect of *Paenibacillus polymyxa* WY110 to watermelon fusarium wilt was 52.8% in greenhouse, it was significantly higher than the control effect of carbendazim 22.6%.

# Use of plant growth promoting rhizobacteria strains in tomato (*Lycopersicon esculentum* Mill.) in villaflores, chiapas, M éxico

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### Abstract

The strains GB03 and IN937b of Bacillus subtilis and IN937a of B. *amyloliquefaciens* were evaluated at three rates  $(10^6, 10^7, \text{ and } 10^8 \text{ CFU/ml}^{-1})$  in seed germination and growth promotion in tomato plants. The most probable number was used to determine the bacterial population. In the experiment conducted under laboratory conditions, the germination rate increased in seeds treated with IN937a at 10<sup>8</sup> CFU/ml and GB03 at 10<sup>6</sup> and 10<sup>7</sup>CFU/ml. The root length was increased with GB03 at 10<sup>8</sup> CFU/ml and the caliper diameter with the same strain at 10<sup>6</sup> CFU/ml and the strain IN937b at  $10^8$  CFU/ml. Under greenhouse conditions, seed germination was increased with strains IN937a at 10<sup>6</sup> CFU/ml and GB03 at 10<sup>6</sup> and  $10^8$  CFU/ml. Shoot and root weight was enhanced with the strain GB03 at  $10^7$ CFU/ml while the total weight with the strain GB03 at  $10^6$  CFU/ml and IN937b at  $10^{6}$  CFU/ml. Stem length was increased with IN937a at  $10^{6}$  CFU/ml, GB03 at  $10^{7}$ CFU/ml and IN937b at 10<sup>8</sup> CFU/ml. In general strains IN937a and GB03 increased the caliper diameter. Growth promotion was achieved with the strains IN937a at  $10^8$ CFU/ml, GB03 at  $10^6$  and  $10^7$  CFU/ml and IN937b in all three concentrations. The chlorophyll content was increased with IN937a at  $10^7$  and  $10^8$  CFU/ml.

### Introduction

In horticultural plants nutrient management is based on high inputs of chemical fertilizer, with the potential contamination of ground water by nitrates and of runoff water by phosphates. In addition, the potential damage to human health and environment contamination. In tomato, in Chiapas, Mexico, regularly is applied 250-180-140 kg per ha of nitrogen, potassium and phosphorus, respectively, increasing the production cost. Besides a biological degradation occurs in the soil in which the soil diversity is reduced. Under these conditions, plant pathogens populations may increase and cause disease (Paulitz and Belanger, 2001). Therefore, a potential alternative to chemical fertilization could be the use of PGPR as

biofertilizers. With the use of PGPR plant growth is stimulated (Kloepper *et al.*, 1980, Kloepper *et al.*, 1992; Benchabane *et al.*, 2000), and it is possible increase the plant production (Kloepper *et al.*, 1988; Benchabane *et al.*, 2000). PGPR once established in the rhizosphere may stimulate seed germination, plant health and plant growth (Kloepper *et al.*, 1980; Lynch and Whipps, 1991). Plant growth may occur by synthesizing hormone-like compounds (Lifshitz *et al.*, 1987) or enhancing uptake of nutrients (Frommel *et al.*, 1991) and suppressing some plants pathogens (Smith and Goodman, 1999). However, there is a lack of information due few studies using PGPR as biofertilizer in tomato here in Chiapas, Mexico. In addition, the efficacy of using PGPR is occasionally variable, reason why it is important to try under local conditions, since fluctuating environmental conditions may play a role in efficacy and consistency of the strains by modifying some characteristics of the metabolic process in the plant host (Hannusch and Boland, 1996).

Growth promotion and disease suppression induced by PGPR have been reported with *Azospirillum* spp., *Pseudomonas* spp., *Serratia* spp., *Burkholderia* spp., and *Bacillus* spp. in several plant systems (Kloepper *et al.*, 1988; de Freitas and Germida, 1992; Holguin and Bashan, 1996; Veena and Srivastava, 1999; Chanway *et al.*, 2000).

Production of plant growth regulators was considered one of the modes of action by which PGPR stimulated plant growth (Cattelan *et al.*, 1999). PGPR have the capacity to produce or change the concentration of some plant hormones in the rhizosphere (Glick, 1999) such as indole-3-acetic acid (IAA) (Patten and Glick, 1995; Tang, 1994; Lifshitz *et al.*, 1987), cytokinins (Tien *et al.*, 1979; Salamone *et al.*, 1997), or gibberellins (Tang, 1994). Reduction in ethylene level in plants by PGPR allows enhanced root elongation (Glick *et al.*, 1999; Mayak *et al.*, 1999) through degradation of 1-aminocyclopropane-1-carbxylate (ACC), a precursor of ethylene, by producing ACC deaminase (Glick *et al.*, 1999).

Mechanisms of plant growth promotion by some PGPR in which plant growth regulators are not involved, include enhancement of mineral uptake and N availability in the soil (Okon *et al.*, 1988; Glick, 1999); biocontrol-related effects by protecting plants from diseases caused by root pathogens (Schippers *et al.*, 1987; Kloepper *et al.*, 1992; van Loon *et al.*, 1998); or by triggering and activating plant defense mechanisms called induced systemic resistance (ISR) (Kloepper *et al.*, 1992; van Loon *et al.*, 1998). It was discovered that the production of volatile organic compounds by some PGPR strains constitutes a new mechanism for induction of growth promotion. *Bacillus subtilis* GBO3 and *B. amyloliquefaciens* IN937a released 2,3-butanediol and acetoin that were responsible for growth promotion observed in *Arabidopsis thaliana* (Ryu *et al.*, 2003).

The objective was to evaluate the effect of single strains of GB03 and IN937b of *Bacillus subtilis* and IN937a of *B. amyeloliquefaciens* on plant growth in tomato. Since the benefit and contribution of using such strains here in Chiapas has never been examined this approach could eventually lead to a reduction of the high input of fertilizers while obtaining the best quality of fruits.

#### **Materials and Methods**

Experiments were conducted under laboratory and greenhouse conditions. Seeds of tomato (*Lycopersicum sculentum*) cv "Rio grande 745" (Petoseed Inc). (P.O Box, 4206, Saticoy, CA. 93007-4206) were used, since it is an important crop in Chiapas. *B. subtilis* strains GB03 and IN937b and *B. amyloliquefaciens* strain IN937a obtained from the PGPR culture collection of Auburn University, Alabama, USA, were used in the experiments. These strains were selected based on results of previous experiments.

Prior to use, PGPR from the culture collection were transferred from the freezer in tryptic soy broth (TSB) (Difco, Laboratories, Detroit, MI) amended with 20% glycerol to tryptic soy agar (TSA). Cultures were initiated by streaking PGPR strains onto TSA and then incubating at 27°C. After 72 h of incubation, vegetative cells and bacterial spores were harvested from the TSA plates using sterile distilled water. The resulting concentration of the bacterial suspension was determined by MPN (most probable number) test. Spore concentration was then adjusted to three final densities of  $10^6$ ,  $10^7$ , and  $10^8$  CFU/ml.

Experiments were performed two times and conducted during the Spring and Summer of 2010. Plants were grown in a glass covered greenhouse with an average temperature of  $28^{\circ}$ C, with relative humidity from 65% to 75%, and a 16/8h light day/night photoperiod and were watered as needed.

Tomato seeds were sown in a plug tray (plug size 2.5 by 2.5 cm, 200 plugs/tray) containing soilless Cosmopeat<sup>®</sup> growing medium (Premier Peat Ltd, Riviere-du Loup, Quebec, Canada), to a depth of approximately 1 cm (one seed per cell). Twenty five days after sowing, seedlings were transplanted from the plug trays into 13-cm square pots using the same growing medium. Plants were not chemically fertilized, except one treatment using the formula 250-180-140 (N-P-K) which is applied by local growers.

The experiment was arranged as randomly. Each treatment had three replications and was sampled per each concentration at three sampling times. The statistical analyses were performed with SAS software (SAS Institute, 1985 Inc, Cary, NC. USA). Data collected were subject to analysis of variance using PROC ANOVA and treatments were separated using DMS P= 0.05.

Both experiments were performed two times. *In the first one*, under laboratory conditions, five days after applying the PGPR strains, germination test was carried by paper towel method. Additionally, were measured hypocotyls and root lengths, and seedling vigor was analyzed by measuring the caliper diameter, that was

recorded with a thumbwheel caliper meter (Fred V. Fowler Co. Inc. Newton, MA, 02466).

This experiment, was carried out with three replicates of 25 seeds of tomato each one and were surface sterilized with 0.2% sodium hypochlorite, rinsed thoroughly with sterile distilled water, and then soaked during 12 hours in 15 ml of bacterial suspension.

In the *second experiment*, under greenhouse conditions, growth promotion of tomato was evaluated. There were 50 replications (each consisting of a tray of seeds or pot of seedling). Seeds of tomato were surface sterilized as described previously and later bacterization of tomato seeds was done by submerging seeds for 15 minutes in the bacterial suspension prior to seeding. At seeding time, 5 ml of bacterial suspension was added directly to cosmopeat growing-medium where the seed was placed in trays. Twenty five days after sowing, seedlings were transplanted from the plug trays into 13cm square pots. Prior to transplanting the seedlings were bacterized for a second time by submerging the roots into 10 ml of bacterial suspension for five minutes.

Ten replications were sampled at four different sampling times. The first sample was taken 25 days after sowing and the followings were done every fifteen days after transplanting. The root, shoot and total fresh weight, plant length (only for the first sampling time) and caliper diameter (only for the first sampling time) were measured, as well as the chlorophyll content, by using a SPAD -502 (Spectrum Technologies, Inc. Plainfield, IL 60544).

## Results

Under laboratory conditions, increased germination occurred with strains IN937a, at  $10^8$  CFU/ml, GB03 at  $10^6$  and  $10^7$  CFU/ml and enhanced caliper diameter with IN937b at  $10^8$  and GB03 at  $10^6$  CFU/ml. The root length also was increased with the strain GB03 at  $10^8$  CFU/ml, but not the hypocotyls length (Table 1).

lengths (cm), and canper thankter (mm) of tomato, five days after second							
Treatments	Germination	Root length	Hypocotyl	Caliper			
(cfu/ml)	rate		length	diameter			
IN937a 10 <sup>6</sup>	86.67 <sup>°</sup>	1.491 <sup>e</sup>	2.255 <sup>bc</sup>	0.875 <sup>abcd</sup>			
IN937a 10 <sup>7</sup>	84.00 <sup>d</sup>	2.113 <sup>cd</sup>	2.858 <sup>ab</sup>	0.783 <sup>cde</sup>			
IN937a 10 <sup>8</sup>	92.00 <sup>a</sup>	1.661 <sup>de</sup>	2.511 <sup>abc</sup>	0.775 <sup>de</sup>			
IN937b 10 <sup>6</sup>	78.67 <sup>e</sup>	2.633 <sup>bc</sup>	1.961 <sup>cd</sup>	0.908 <sup>abc</sup>			
IN937b 10 <sup>7</sup>	88.00 <sup>b</sup>	2.091 <sup>cd</sup>	1.622 <sup>d</sup>	0.827 <sup>bcde</sup>			
IN937b 10 <sup>8</sup>	88.00 <sup>b</sup>	1.625 <sup>de</sup>	2.477 <sup>abc</sup>	0.930 <sup>ab</sup>			
GB03 10 <sup>6</sup>	92.00 <sup>a</sup>	1.975 <sup>de</sup>	2.461 <sup>abc</sup>	0.972 <sup>a</sup>			
GB03 10 <sup>7</sup>	92.00 <sup>a</sup>	3.200 <sup>b</sup>	2.830 <sup>ab</sup>	0.700 <sup>e</sup>			
GB03 10 <sup>8</sup>	88.00 <sup>b</sup>	4.302 <sup>a</sup>	2.861 <sup>ab</sup>	0.788 <sup>cde</sup>			
Nontreated Control	88.00 <sup>b</sup>	2.841 <sup>b</sup>	2.916 <sup>a</sup>	0.702 <sup>e</sup>			

 Table 1
 Effect of seed treatment with PGPR strains, on germination rate, root and hypocotyls lengths (cm), and caliper diameter (mm) of tomato, five days after seeding

All values are means of two experiments with 25 replications in each experiment. Means with the same letters in a column do not differ according to Tukey test at P < 0.05 level.

In the second experiment, the chlorophyll content of tomato was not enhanced by all treatments across all bacterial rates compared to the control (data not showed) and we found that the magnitude of growth promotion varied among treatments. For example, in the first sampling time, the root fresh weight was not increased by any treatment, but the shoot fresh weight was significantly increased with the strain GB03 at 10<sup>7</sup> CFU/ml, as well as the total fresh weight with GB03 at 10<sup>6</sup> CFU/ml and IN937b at 10<sup>8</sup> CFU/ml, compared with the control. Plant height was enhanced with the strains IN937a at 10<sup>8</sup> CFU/L, IN937b at 10<sup>8</sup> CFU/ml and GB03 at 10<sup>7</sup> CFU/ml, compared with the caliper diameter of tomato was increased with the strains IN937a and GB03 across all bacterial rates here tested (Table 2).

Treatments (cfu/ml)	Root weight	Shoot weight	Total plant weight	Plant height	Caliper diameter
IN937a 10 <sup>6</sup>	0.463 <sup>ab</sup>	0.470 <sup>b</sup>	0.970 <sup>b</sup>	10.300 <sup>bc</sup>	3.066 <sup>b</sup>
IN937a 10 <sup>7</sup>	0.300 <sup>cd</sup>	0.573 <sup>b</sup>	0.873 <sup>b</sup>	10.720 <sup>abc</sup>	3.480 <sup>a</sup>
IN937a 10 <sup>8</sup>	0.263 <sup>d</sup>	0.573 <sup>b</sup>	0.836 <sup>b</sup>	11.566 <sup>a</sup>	3.420 <sup>a</sup>
IN937b 10 <sup>6</sup>	0.323 <sup>cd</sup>	0.560 <sup>b</sup>	0.783 <sup>b</sup>	9.673 <sup>c</sup>	1.900 <sup>°</sup>
IN937b 10 <sup>7</sup>	0.350 <sup>bcd</sup>	0.560 <sup>b</sup>	0.910 <sup>b</sup>	10.153 <sup>bc</sup>	1.943 <sup>c</sup>
IN937b 10 <sup>8</sup>	0.416 <sup>bc</sup>	0.666 <sup>ab</sup>	1.713 <sup>a</sup>	11.320 <sup>ab</sup>	2.166 <sup>c</sup>
GB03 10 <sup>6</sup>	0.393 <sup>bcd</sup>	0.846 <sup>ab</sup>	1.750 <sup>a</sup>	10.333 <sup>bc</sup>	2.783 <sup>b</sup>
GB03 10 <sup>7</sup>	0.573 <sup>a</sup>	1.380 <sup>a</sup>	1.380 <sup>ab</sup>	11.300 <sup>ab</sup>	3.000 <sup>b</sup>
GB03 10 <sup>8</sup>	0.286 <sup>cd</sup>	0.506 <sup>b</sup>	0.793 <sup>b</sup>	9.766 <sup>c</sup>	2.926 <sup>b</sup>
Chemical Fertilized control	0.583 <sup>a</sup>	0.386 <sup>b</sup>	0.970 <sup>b</sup>	11.286 <sup>ab</sup>	2.016 <sup>c</sup>
Nontreated Control	0.366 <sup>bcd</sup>	0.360 <sup>b</sup>	0.736 <sup>b</sup>	9.773 <sup>c</sup>	1.960 <sup>°</sup>

 Table 2 Effect of seed and seedling treatment with PGPR strains on root weight (g), shoot weight (g), total plant weight (g), plant height (cm) and caliper diameter of tomato, twenty five days after seeding

All values are means of two experiments with 10 replications in each experiment. Means with the same letters in a Column do not differ according to Tukey test at P < 0.05.

At the second sampling, 15 days after transplanting, the strain GB03 at  $10^7$ , significantly increased root weight, compared to the control. At the third sampling, 30 days after transplanting, all concentrations of strain IN937a, IN937b at  $10^8$  CFU/ml and GB03 at  $10^6$  and  $10^7$  CFU/ml, significantly increased the root weight, compared to the both controls. At the fourth sampling, all concentrations of strain IN937b and IN937a at  $10^6$  CFU/ml enhanced the root weight compared to the controls. In the last sampling, all concentrations of strains IN937b and IN937a increased the root weight. Same result was found with GB03 at  $10^6$  (Table 3).

Treatments	Sampling time				
(cfu/ml)	40 dat*	55 dat	70 dat	85 dat	
IN937a 10 <sup>6</sup>	0.463 <sup>ab</sup>	0.9111 <sup>ab</sup>	3.2889 <sup>ab</sup>	3.0889 <sup>ab</sup>	
IN937a 10 <sup>7</sup>	0.300 <sup>cd</sup>	0.8333 <sup>ab</sup>	2.2000 <sup>bcd</sup>	2.8556 <sup>b</sup>	
IN937a 10 <sup>8</sup>	0.263 <sup>d</sup>	0.8333 <sup>ab</sup>	1.9778 <sup>bcde</sup>	3.3222 <sup>ab</sup>	
IN937b 10 <sup>6</sup>	0.323 <sup>cd</sup>	0.7111 <sup>abc</sup>	2.8556 <sup>abc</sup>	3.5111 <sup>ab</sup>	
IN937b 10 <sup>7</sup>	0.350 <sup>bcd</sup>	0.7000 <sup>bc</sup>	3.3556 <sup>ab</sup>	3.2222 <sup>ab</sup>	
IN937b 10 <sup>8</sup>	0.416 <sup>bc</sup>	0.9333 <sup>ab</sup>	3.6889 <sup>a</sup>	3.7444 <sup>a</sup>	
GB03 10 <sup>6</sup>	0.393 <sup>bcd</sup>	1.0000 <sup>a</sup>	1.8222 <sup>cde</sup>	3.2000 <sup>ab</sup>	
GB03 10 <sup>7</sup>	0.573 <sup>a</sup>	0.7777 <sup>ab</sup>	1.7444 <sup>cde</sup>	2.7778 <sup>bc</sup>	
GB03 10 <sup>8</sup>	0.286 <sup>cd</sup>	0.4000 <sup>d</sup>	0.6000 <sup>e</sup>	1.1222 <sup>e</sup>	
Chemical Fertilized control	0.583 <sup>a</sup>	0.4666 <sup>cd</sup>	1.3778 <sup>ed</sup>	1.9889 <sup>cd</sup>	
Nontreated Control	0.366 <sup>bcd</sup>	0.3888 <sup>d</sup>	0.6333 <sup>e</sup>	1.1556 <sup>de</sup>	

Table 3 Effect of PGPR strains treated seed and seedling, on root weight (g) of tomato at40, 55, 70 and 85 days after transplanting

All values are means of two experiments with 10 replications in each experiment.

Means with the same letters in a column do not differ according to Tukey test at P < 0.05 level;

\* dat= Days after transplanting

At the second sampling, 15 days after transplanting, only strain GB03 at  $10^{7}$  increased *shoot weight*, compared with both controls, while at the third sampling, 30 days after transplanting, with GB03 at  $10^{6}$  and  $10^{7}$  CFU/ml, IN937b at  $10^{8}$  CFU/ml and IN937a at  $10^{8}$  CFU/ml (Table 4). At the fourth and fifth sampling all bacterial concentrations here tested significantly increased the shoot weight, compared to the control, except the strain GB03 at  $10^{8}$  CFU/ml (Table 4). At the second sampling, 15 days after transplanting, the strains IN937b a  $10^{8}$  CFU/mL and GB03 at  $10^{6}$  CFU/ml significantly increased the *total plant weight*, compared with the controls. At the third sampling, 30 days after transplanting, this parameter was increased with the strains IN937a at  $10^{7}$  and  $10^{8}$  CFU/mL, IN937b at  $10^{8}$  CFU/ml and GB03 at  $10^{6}$  and  $10^{7}$  CFU/ml (Table 5). In the following samplings we found an inconsistency since the total plant weight was increased in any treatment, compared with the controls.

Treatments	Sampling time	e		
(cfu/ml)	40 dat	55 dat	70 dat	85 dat
IN937a 10 <sup>6</sup>	0.470 <sup>b</sup>	1.955 <sup>cd</sup>	5.1778 <sup>a</sup>	9.378 <sup>b</sup>
IN937a 10 <sup>7</sup>	0.573 <sup>b</sup>	2.833 <sup>bcd</sup>	6.2222 <sup>a</sup>	10.933 <sup>ab</sup>
IN937a 10 <sup>8</sup>	0.573 <sup>b</sup>	2.944 <sup>bc</sup>	5.9556 <sup>a</sup>	9.811 <sup>b</sup>
IN937b 10 <sup>6</sup>	0.560 <sup>b</sup>	2.344 <sup>bcd</sup>	6.1667 <sup>a</sup>	9.889 <sup>b</sup>
IN937b 10 <sup>7</sup>	0.560 <sup>b</sup>	2.655 <sup>bcd</sup>	7.3333 <sup>a</sup>	10.989 <sup>ab</sup>
IN937b 10 <sup>8</sup>	0.666 <sup>ab</sup>	3.422 <sup>ab</sup>	6.6444 <sup>a</sup>	10.700 <sup>ab</sup>
GB03 10 <sup>6</sup>	0.846 <sup>ab</sup>	4.122 <sup>a</sup>	6.3889 <sup>a</sup>	11.333 <sup>ab</sup>
GB03 10 <sup>7</sup>	1.380 <sup>a</sup>	3.200 <sup>ab</sup>	5.6000 <sup>a</sup>	9.544 <sup>b</sup>
GB03 10 <sup>8</sup>	0.506 <sup>b</sup>	0.766 <sup>e</sup>	1.1444 <sup>b</sup>	3.356 <sup>c</sup>
ChemicalFertilized control	0.386 <sup>b</sup>	1.788 <sup>de</sup>	6.7889 <sup>a</sup>	14.156 <sup>a</sup>
Nontreated Control	0.360 <sup>b</sup>	0.766 <sup>e</sup>	0.8667 <sup>b</sup>	3.256 <sup>c</sup>

Table 4 Effect of PGPR strains treated seed and seedling, on shoot fresh weight (g) of tomato, at 40, 55, 70 and 85 days after transplanting

All values are means of two experiments with 10 replications in each experiment. Means with the same letters in a column do not differ according to Tukey test at P < 0.05 level.

Treatments	Sampling time					
(cfu/ml)	40 dat	55 dat	70 dat	85 dat		
IN937a 10 <sup>6</sup>	0.970 <sup>b</sup>	2.866 <sup>cd</sup>	8.467 <sup>a</sup>	13.022 <sup>a</sup>		
IN937a 10 <sup>7</sup>	0.873 <sup>b</sup>	3.666 <sup>bc</sup>	8.422 <sup>a</sup>	13.133 <sup>a</sup>		
IN937a 10 <sup>8</sup>	0.836 <sup>b</sup>	3.777 <sup>abc</sup>	3.777 <sup>abc</sup> 7.933 <sup>a</sup>			
IN937b 10 <sup>6</sup>	0.783 <sup>b</sup>	3.055 <sup>cd</sup> 9.022 <sup>a</sup>		13.400 <sup>a</sup>		
IN937b 10 <sup>7</sup>	0.910 <sup>b</sup>	3.355 <sup>bcd</sup>	10.756 <sup>a</sup>	14.211 <sup>a</sup>		
IN937b 10 <sup>8</sup>	1.713 <sup>a</sup>	4.355 <sup>ab</sup>	10.333 <sup>a</sup>	14.444 <sup>a</sup>		
GB03 10 <sup>6</sup>	1.750 <sup>a</sup>	4.955 <sup>a</sup>	8.211 <sup>a</sup>	14.544 <sup>a</sup>		
GB03 10 <sup>7</sup>	1.380 <sup>ab</sup>	3.977 <sup>abc</sup>	7.344 <sup>a</sup>	12.322 <sup>a</sup>		
GB03 10 <sup>8</sup>	0.793 <sup>b</sup>	1.166 <sup>e</sup>	1.744 <sup>b</sup>	4.478 <sup>b</sup>		
Chemical Fertilized control	0.970 <sup>b</sup>	2.255 <sup>de</sup>	8.167 <sup>a</sup>	16.233 <sup>a</sup>		
Nontreated Control	0.736 <sup>b</sup>	1.155 <sup>e</sup>	1.200 <sup>b</sup>	4.411 <sup>b</sup>		

Table 5 Effect of PGPR strains treated seed and seedling, on total plant weight (g) of<br/>tomato, at 40, 55, 70 and 85 days after transplanting

All values are means of two experiments with 10 replications in each experiment.

Means with the same letters in a column do not differ according to Tukey test at P < 0.05 level.

### Discussion

Results of the present study with tomato confirm the efficacy of PGPR strains in promoting plant growth under our conditions. However, growth promotion capacity varied among strains and bacterial rates. Assessment of growth by PGPR strains under laboratory conditions identified enhancement of emergence rate with the three strains here evaluated from tomato seeds treated with PGPR strains. Similar reports of growth enhancement using different PGPR strains are available in the literature. For example, Amruthesh *et al.*, (2003), when evaluating strains of SE34 and IN937b of *Bacillus subtilis* found enhancement of seed germination and growth promotion in pepper plants. Considerable increase of root length and seedling growth was observed by Lazarovits and Nowak (1997) when they used *Pseudomonas* spp. in potato plantlets.

Results from the experiment conducted under field conditions also confirmed that the magnitude of growth promotion varied among treatments. For example, in the first sampling time, the root fresh weight was not increased by any treatment, but the shoot fresh weight was significantly increased with the strain GB03 as well as the total fresh weight with GB03 and IN937b. However, in the followings samplings the root weight, shoot weight and total plant weight were increased at all sampling times with variable response among strains and bacterial concentrations. All the strains here tested belong to *Bacillus* species. *Bacillus* has the capacity to form endospores that allow them to survive for extended periods under adverse environmental conditions (Balkwill *et al.*, 1997). This capacity and their metabolic diversity are significant factors contributing to wide distribution of the bacilli and they can colonize a wide variety of natural environments (Balkwill *et al.*, 1997; Blanc *et al.*, 1997; Wipat and Harwood, 1999).

The variable response here found may be explained because poor colonization rhizosphere occurred. Rhizosphere colonization which is the process whereby bacteria survive inoculation onto seeds or into soil, multiply in the spermosphere in response to seed exudates (Kloepper *et al.*, 1985), and colonize the developing root system externally and internally. To elicit a host-response such as plant growth promotion microorganisms must first colonize the rhizosphere and establish an adequate density level (Hatzinger and Alexander, 1994). Therefore, extensive colonization of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant to obtain the benefits of bacterial inoculation (Cattelan *et al.*, 1999). In addition, rhizosphere colonization is a complex phenomenon influenced by many biotic and abiotic factors (Benizri *et al.*, 2001; Whipps, 2001).

Inoculation of soil or seeds planted in soil is a practice used for the establishment of plant growth-promoting bacteria and biological control of plant pathogens. However, the addition of an appropriate microorganism does not always lead to the desired outcome (Jjemba and Alexander, 1999). Several reasons may account for the differences in rhizosphere competence among bacteria. The existing bacterial community may influence root colonization by PGPR or their particular interactions in the rhizosphere may or may not affect the introduced PGPR. However, some failure in root colonization by PGPR could be attributed to competition between the inoculated bacteria and pathogenic or saprophytic organisms present in the soil environment (Basham and Holguin, 1997; Benhamou *et al.*, 1996).

Although we did not conducted studies to understand the mechanisms of growth promotion by the PGPR strains here tested, a thorough survey of literature indicate that have the capacity to promote plant growth through biostimulation or biological protection against diseases in plants when inoculated onto seeds or into the rhizosphere (Kloepper *et al.*, 1988; Kloepper *et al.*, 1992; Benchabane *et al.*, 2000). Growth promotion can be evident at early stages of plant development including seed

germination and seed emergence (Kloepper, 1980 a; 1980b) as here in our results showed.

The results here presented demonstrate that single strains increased seed germination and plant growth compared to the control.

In general chlorophyll content of tomato was not enhanced by all treatments across all bacterial rates compared to the control. The effect of these bacteria on growth of tomato plants are reported to be very complex and also the result of various interactions between the plant and the environmental factors (Schippers, 1992) that could explain why results from the experiment varied among strains and bacterial rates.

It can be concluded from the results of the present study that the use of PGPR as seed treatment would prove beneficial effects in tomato plants. Since these PGPRP strains increased plant growth is suitable for any practical agriculture system. It is evident that rhizobacteria could possibly serve as ecofriendly and sustainable alternative to the hazardous chemical fertilizers used for the nutrient management in horticultural systems, in particular in tomato crops in the Frailesca, Chiapas, Mexico.

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# Study on bacterial colonization dynamics in the rhizosphere of three plants by the end-point dilution assay

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### Abstrct

We studied on the colonization dynamics of five bacterial strains (Pseudomonas fluorescence Q2-87, P. fluorescence Pf-5, P. fluorescence 1M1-96, Burkholderia cepacia C23 and Burkholderia xenovorans LB400) in the rhizosphere of three plants (Robinia pseudoacacia, Cucurbita moschata cv. wuyuezaonangua and Cucurbita pepo cv. chaojizaoqing). Two methods were used to inoculate plant rhizosphere with bacteria; the first one was soaking the germinated seeds in the 10<sup>6</sup>CFU/ml bacterial liquid suspension; the second one was dripping quantificationally the 10<sup>6</sup>CFU/ml bacterial liquid suspension onto the radices of the germinated seeds in sewing holes. Robinia pseudoacacia were inoculated by two methods; Cucurbita moschata cv. wuyuezaonangua and *Cucurbita pepo* cy. chaojizaoqing were inoculated only by the second method. The results showed: 1) At the 10<sup>th</sup>, 20<sup>th</sup> and 40<sup>th</sup> day after inoculated, the population density of the inoculated bacteria in the rhizosphere was between 8.7  $\times 10^3$  and  $6.1 \times 10^6$  CFU/g; 2) In the rhizosphere of *Robinia pseudoacacia* inoculated by two methods, only strain Q2-87 among the five strains could be detectable at all of the 10<sup>th</sup>, 20<sup>th</sup>, 40<sup>th</sup> and 60<sup>th</sup> day, and at the 10<sup>th</sup> day the population density of the bacteria inoculated by the second method was higher than that of the bacteria inoculated by the first method; 3)After 40 days since the three plants were inoculated, the population density of inoculated bacteria showed the descending currency; 4) At the 10<sup>th</sup> day after inoculated, the population density of inoculated bacteria in the rhizosphere of *Cucurbita pepo* cv. chaojizaoqing was highest among the three plants. In a word, the colonization comprehensive competence of strain Q2-87 among the five trains in the rhizosphere of three plants was strongest, and that of strain LB400 was weakest; at the time, in the rhizophere of *Cucurbita pepo* cv. chaojizaoqing the colonization comprehensive competence of the inoculated bacteria was strongest among three plants; and two inoculation methods affected differently the population dynamics of the inoculated bacteria in the rhizosphere of Robinia pseudoacacia; the experiments demonstrated that the end-point dilution methods could be applied well in the research on the bacterial population dynamics in the rhizosphere of forest trees (e.g. *Robinia pseudoacacia*)

# Selection for drought tolerance in corn and their response to biofertilizer

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### Abstract

Drought is one of the major problems affecting crops production, including corn, in many parts of Uzbekistan. The need to breed maize cultivars with improved drought tolerance is apparent. Understanding the plant growth promoting properties of bacterial strains affected by plant cultivar is widely recognized as a key to improving the level and reliability of plant growth stimulation by PGPR. The results showed that assessing hybrids according to some selection indices lead to introduce Harinoso de Ocho and Celaja as drought tolerant ones. Our results showed that the growth of four corn hybrids was stimulated by bacterial inoculants in pot experiments. The bacterial inoculants stimulated the dry weight of corn hybrids Harinoso de Ocho (21%). Celaj (27%), Colorado (19%) and Celaja<sup>\*</sup>up to 29%. There was no significant growth stimulation for other corn hybrids. Our results, plus related literature, suggest that a selection index combining these secondary traits and grain yield should result in faster improvement of grain yield under drought stress than selection for grain yield alone. However, their response to bacterial inoculants differs among hybrids and it is recommended to select also for corn hybrids that benefit from association with these bacteria.

# Isolation and characterization of nitrogen-fixing and phosphatesolubilizing bacteria from *Arundo donax* L. (giant reed)

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### Abstract

Arundo donax, giant reed, is a perennial grass that has been extensively evaluated as a source for biofuel. Interestingly, in temperate climate zones Arundo grows very quickly each year, reaching  $4 \sim 6$  m without any fertilization. This rapid growth without fertilizer suggests a possible involvement of biofertilizers. The objective of this project was to isolate and characterize nitrogen-fixing (N-fixing) bacteria and phosphate-solubilizing (P-solubilizing) bacteria from rhizosphere soil and endophytically from inside roots and stems of Arundo. Isolations for N-fixing bacteria were made using JNFb, NFb and LGI semisolid media, and Ashby's, JNFb, and NFb solid media. Isolations for P-solubilizing bacteria were done on modified SRSM medium with rock-phosphate. Results showed that N-fixing bacteria were isolated from the rhizosphere and endophytic samples, while P-solubilizers were only isolated from the rhizosphere. The nitrogenase activity of the isolated N-fixing bacteria was confirmed using the acetylene reduction method. The P-solubilizing activity was determined using the phosphate solubilization index. The largest solubilization index was 2.4, which is very high. DNA was extracted from the isolates, and the 16S rDNA sequence was used for molecular identification. Interestingly, N-fixing isolates represented much larger diversity (14 genera), compared to P-solubilizers (2 genera). P-solubilizers included 6 species of Pseudomonas and one of Microbacterium.

Key word: Arundo; Nitrogen-fixing bacteria; Phosphate-solubilizing bacteria; Acetylene reduction activity; 16S rDNA

### Introduction

Arundo donax, giant reed, is a perennial grass that has been extensively evaluated as a source for biofuel. Interestingly, in temperate climate zones Arundo grows very quickly each year, reaching  $4\sim 6$  m (Figure1) without any fertilization. This rapid growth without fertilizer suggests a possible involvement of biofertilizers. Nitrogenfixing bacteria (N-fixing) and phosphate-solubilizing (P-solubilizing) bacteria are the two main groups biofertilizers of plant growth-promoting rhizobacteria (PGPR). Finding such bacteria will help us evaluate biofuels better, and these bacteria could be used on crops to allow reduced rates of fertilizers. The main objective of this project was to isolate and characterize N-fixing bacteria and P-solubilizing bacteria from rhizosphere soil and endophytically from inside roots and stems of *Arundo*.



Figure 1 Plant height of Arundo in Autumn, 2010

# Methods

# Methods of isolation

*Arundo* rhizosphere soil, root and stem samples were collected in south central Alabama, USA. Collected rhizosphere soil was placed directly on the Ashby's (Lü, 2010) solid agar and incubated at  $28^{\circ}$ C for up to 5 days. The roots and stems were washed with sterile distilled water and were surface disinfected by washing with 70% ethanol, and 50% commercial bleach. Stem and root samples (1.0g) were macerated separately using a Kleco grinder in sterile distilled water and serially diluted to  $10^{-6}$ . One hundred microliters of these dilutions were inoculated into JNFb (Olivares, 1996), NFb (Bashan, 1993), and LGI (Cavalcante & Döbereiner, 1988) semisolid media and plated onto SRSM (Vazquez & Bashan) solid medium.

Different nitrogen-free semisolid media were used in order to isolate different Nfixing bacteria. Pellicles from these semisolid media were transferred to the corresponding solid media to purify the isolates. For example, pellicles (Figure 2) that in JNFb semisolid medium were transferred to JNFb solid medium for further purification. Modified SRSM was used to isolate P-solubilizing bacteria.



Figure 2 Pellicles in NFb semisolid medium (Left=pellicles formed from macerated tissue; Right=pellicles formed from purified N-fixing bacteria)

### Determination of nitrogenase activity

The nitrogenase activity of N-fixing bacteria was determined using the acetylene reduction method. Purified bacteria were grown on LB for 24 hours; 100  $\mu$ l of the cultures was then added to 5 ml of NFb semisolid medium with incubation at 28°C for 24 hours. Acetylene (10%) was injected into the culture tubes, which were incubated for 1h and then analyzed for ethylene by gas chromatography.

## Determiantion of phosphate solubilization index

The capacity of the bacteria to solubilize insoluble phosphate was described by the solubilization index: the ratio of the total diameter (colony + halo) and the colony diameter (Kumar, 1999) (Figure 3).



Figure 3 Phosphate solubilization index

### Molecular characterization and phylogenetic analysis

Taxonomic classification of each strain was based on the partial sequence of 16S rDNA. DNA was extracted and amplified with universal bacterial primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3'). PCR was performed using Lucigen EconoTaq Plus Green 2X master mix (Lucigen Corp.) with cycling parameters: initial denaturation at 95 °C for 5 min; 31 cycles of 94 °C for 1 min, 57 °C for 45 sec, 70 °C for 2 min; and a final extension at 70 °C for 10 min. All sequences were blasted against the type strains in the ribosomal database project to identify bacterial taxa of each strain.

### Results

### Isolation and determination of nitrogenase activity

A total of 15 N-fixing strains were isolated from nitrogen free media. The nitrogenase activity (Table 1) of the total 15 N-fixing strains was confirmed by acetylene reduction activity. The nitrogenase activity varied from 67.2 nmol (N5)  $C_2H_4/h$  L to 145.3 nmol  $C_2H_4/h$  L (N13).

### The phosphate solubilization activity

All the isolated P-solubilizing strains showed clear halos after 24 hours. The largest phosphate solubilization index was 2.4 and the smallest phosphate solubilization index was 1.1(Table 1, Figure 4).



Figure 4 Halozone of phosphate solubilization (48h)

Strain number	Nitrogenase activity (nmolC <sub>2</sub> H <sub>4</sub> /h.L)	Strain number	Phosphate solubilization index (48h)
N1	77.7	P1	1.6
N2	67.4	P2	1.7
N3	81.4	P3	1.5
N4	90.1	P4	1.7
N5	67.2	P5	1.2
N6	79.4	P6	1.2
N7	72.5	P7	1.5
N8	87.4	P8	1.6
N9	78.4	P9	2.4
N10	91.5	P10	1.1
N11	78.4		
N12	139.8		
N13	145.3		
N14	73.6		

Table 1 Nitrogenasse activity and phosphate solubilization index

*Phylogenetic analysis by 16S rDNA sequences;* N-fixing isolates represented a large diversity of 14 genera: *Sphingobacterium, Phyllobacterium, Stenotrophomonas, Flavobacterium, Lysobacter, Pseudomonas, Rahnella, Microbacterium, Sporosarcina, Chryseobacterium, Azotobactor* (Figure 5), *Azospirillium, Rhizobium and Bacillus.* In contrast, P-solubilizers represent only 2 genera, included 6 species of *Pseudomonas* and one of *Microbacterium.* 



Figure 5 Azotobactor

## **Conclusion and discussion**

The results reported here represent the first isolation attempts in April to July, 2011. We expect to find differences in species and population of endophytic bacteria and rhizosphere bacteria during to the different times of the season. More work is planned to test this.

The Acetylene reduction activity for the N-fixing bacteria will be done using a new GC-MS machine. In addition, nitrogen fixation (*nif*) genes will be detected and the universal nif primers will be developed in our laboratory. P-solubilizing activity will be evaluated by the Molybdenum-Anitimony colorimetric method, and pH will be measured at the same time. HPLC will be used to detect different kinds of organic acids. The capacity of the isolated strongest N-fixing and P-solubilizing strains will be assessed by inoculating onto target plants. The <sup>15</sup>N<sub>2</sub> gas incorporation and <sup>15</sup>N dilution techniques will be used for the assessment.

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# Selecting mixtures of PGPR for biological control of multiple plant diseases

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#### Abstract

Many plant pathogens (Pythium, Rhizoctonia solani, Fusarium spp., Xanthomonas spp. and *Pseudomonas* spp.) have a wide host range, a cosmopolitan distribution, and the capacity to cause tremendous economic damage. Biological control using specific PGPR strains has been demonstrated against these pathogens. Typical biocontrol studies evaluate a single PGPR strain against a single pathogen. Our long-term aim is to develop mixtures of PGPR for biocontrol of multiple plant diseases. The underlying hypothesis of this work is that compatible mixtures of PGPR with antagonism to multiple pathogens will provide greater disease suppression than individual PGPR. In this research, we evaluated three collections of PGPR known to have growth promotion and biocontrol potential: Collection 1 = 318 strains from Pathway, collection 2 = 140 bacilli strains from Auburn University, collection 3 = 58strains from Becker Underwood. Nine pathogens (Pythium ultimum, P. aphanidermatum, three isolates of Rhizocotonia solani (each from a different host plant), Fusarium oxysporum f. sp. lycopersici, F. oxysporum f. sp. vasinfectum, Xanthomonas campestris pv. vesicatoria, Pseudomonas syringae pv. tomato) were tested. A novel antibiosis technique with a different agar type for the PGPR and the challenged pathogen was developed. Some strains from two collections inhibited all 9 pathogens: 22 strains (6.9 %) from collection 1, and 7 strains (5.0 %) from collection 2. Inhibition against 8 of the 9 pathogens were inhibited by 25 strains (7.9 %) from collection 1, 20 strains (14.3%) from collection 2, and 1 strain (1.7%) from collection 3. The selected strains were identified using 16s r DNA. Results indicated that strains that inhibited 8 or 9 of the pathogens most commonly belonged to one of three genera: Bacillus (B. amyloliquefaciens, B. cereus, and B. subtilis), Brevibacillus (B. formosus, B. brevis, and B. laterosporus), and Paenibacillus (P. peoriae, and P. jamilae). Future work is planned to test mixtures of the selected PGPR for biocontrol against multiply plant pathogens in bioassays.

Key words: Mixtures of PGPR; biological control; multiple plant diseases

## Introduction

Biological control using specific PGPR strains has been demonstrated against many plant pathogens (*Pythium, Rhizoctonia solani, Fusarium* spp., *Xanthomonas* spp. and *Pseudomonas* spp.) which have a wide host range, a cosmopolitan distribution, and the capacity to cause tremendous economic damage (Kumar *et al.*, 2009; Szczech *et al.*, 2008; Ryu *et al.*, 2007; Domenech *et al.*, 2006).

Typical biocontrol studies evaluate a single PGPR strain against a single pathogen (Zhang *et al.*, 2010; Murphy *et al.*, 2003). Compared with single PGPR, mixtures of PGPR could be used to manage multiple plant diseases which often occur in the field (Domenech *et al.*, 2006; Jetiyanon *et al.*, 2003; Jetiyanon *et al.*, 2002). Our long-term aim is to develop mixtures of PGPR for biocontrol of multiple plant diseases. The underlying hypothesis of this work is that compatible mixtures of PGPR with antagonism to multiple pathogens will provide greater disease suppression than individual PGPR.

## Methods

### Source of bacterial strains

In this research, three collections of PGPR known to have growth promotion and biocontrol potential were tested (Collection 1 = 318 strains from Pathway, collection 2 = 140 bacilli strains from Auburn University, collection 3 = 58 strains from Becker Underwood). The identity of all strains was determined using 16S rDNA sequencing with comparison to sequences of type strains. All strains used in this study were stored in tryptic soybean broth amended with 15% glycerol at -80°C. Each strain from ultra-cold storage was streaked onto tryptic soy agar (TSA) and then incubated at 28°C for 24 h to check for purity. For making bacteria suspensions for test, a half -loop of bacteria was mixed with 9 ml sterilized water.

## Source of pathogens

Nine pathogens were tested. There were two Pythium pathogens (the causal agent of damping-off and root rot disease) -- Pythium ultimum and Pythium aphanidermatum; three Rhizoctonia solani pathogens (the causal agent of damping-off disease) which were isolated from pepper, lettuce, and Zoysia; two Fusarium oxysporum pathogens (the causal agent of wilt disease)—F. oxysporum f.sp. lycopersici and F. oxysporum f. sp. vasinfectum which were isolated from tomato and cotton; one Xanthomonas campestris pv. vesicatoria (the causal agent of bacterial spot disease) which was isolated from tomato; and Pseudomonas syringae pv. tomato (the causal agent of bacterial speck disease) which was isolated from tomato. The pathogens and their sources are list in Table 1. All these pathogens were stored in TSB with 15% glycerol at -80°C. For this experiment use, fungal pathogens were

grown on potato dextrose agar (PDA) except *Pythium* which was grown on corn meal agar (CMA; Difco). Bacterial pathogens were grown on TSA.

Name of Pathogens	Source
Tunie of Fullogens	Bouree
Pythium ultimum	Cotton
Pythium aphanidermatum	Cotton
Rhizoctonia solani	Pepper
Rhizoctonia solani	Lettuce
Rhizoctonia solani	Zoysia grass
Fusarium oxysporum f. sp. lycopersici	Tomato
Fusarium oxysporum f. sp. vasinfectum	Cotton
Xanthomonas campestris pv. vesicatoria	Tomato
Pseudomonas syringae pv. tomato	Tomato

Table 1 Pathogens tested in vitro

### In vitro test for antagonistic activity between bacterial stains and pathogens

A novel antibiosis technique with a different agar type for the PGPR and the challenged pathogen was developed. Tests for antibiosis of bacterial stains against the fungal pathogens were conducted on PDA plates. Three holes were punched into agar of each plate with a sterilized cork borer. Holes (13mm) were filled with TSA, and 10  $\mu$ l of the bacterial suspension was applied to the TSA disc. Plates were incubated at 28°C for 48 hours. After 48 hours, the plates were exposed to UV (1000×100  $\mu$ J/cm<sup>2</sup>) for 2 minutes to stop bacterial growth out of the TSA disc. The test fungus was taken from the edge of a growing culture, and put in the middle of the agar plates. Plates were incubated at appropriate temperature for the target strain and were examined daily for inhibition.

Tests for antibiosis of bacterial strains against the bacterial pathogens were conducted in water agar plates. Holes were cut and filled with TSA as described above. The bacterial pathogen was scraped from TSA in sterilized water to make the pathogen suspension. One ml of this suspension was mixed with 50 ml soft agar (0.4% in 50% TSB) cooled to  $37^{\circ}$ C. After gently swirling, 7 ml of the resulting suspension was added to each plate. The results were evaluated by visual inhibition zones: strong positive (++), positive (+), and negative (-).





TSA





suspension on TSA

Treat with UV for 2 Incubate at28°C



Overlay the bacterial pathogen suspension

the center



Check

results

Check results

Figure1 The novel antibiosis procedure

for 48 hours



Figure 2 Antibiosis evaluation scale

# Results

Results of the antibiosis tests for each PGPR collection are presented in Tables  $2\sim$  4. With the Pathway collection (Table 2), strong inhibition (++) was demonstrated against various pathogens by  $4\% \sim 23\%$  of the PGPR. With the Auburn collection (Table 3), strong antibiosis occurred with  $0\sim 24\%$  of the PGPR, and with the Becker Underwood collection,  $0\sim 25\%$  had strong antibiosis. Overall, the pathogen which was most commonly inoculated by PGPR was *R. solani*, while the least commonly inhibited were the two bacterial pathogens.

Name of Pathogens	Total	++	%	+	%	Total	%
Pythium ultimum	318	30	9.4%	75	23.6%	105	33.0%
Pythium aphanidermatum	318	27	8.5%	51	16.0%	78	24.5%
Rhizoctonia solani	318	42	13.2%	102	32.1%	144	45.3%
Rhizoctonia solani	318	73	23.0%	81	25.5%	154	48.4%
Rhizootonia solani	318	69	21.7%	83	26.1%	152	47.8%
Fusarium oxysporum f.sp. lycopersici	318	54	17.0%	77	24.2%	131	41.2%
Fusarium oxysporum f.sp. vasinfectum	318	69	21.7%	69	21.7%	138	43.4%
Xanthomonas campestris pv. vesicatoria	318	32	10.1%	96	30.2%	128	40.3%
Pseudomonas syingae pv. tomato	318	13	4.1%	29	9.1%	42	13.2%

 Table 2
 Collection 1-Pathway results
Name of Pathogens	Total	++	%	+	%	Total	%
Pythium ultimum	140	22	15.7%	20	14.3%	42	30.0%
Pythium aphanidermatum	140	0	0.0%	3	2.1%	3	2.1%
Rhizoctonia solani	140	17	12.1%	71	50.7%	88	62.9%
Rhizoctonia solani	140	34	24.3%	57	40.7%	91	65.0%
Rhizootonia solani	140	28	20.0%	61	43.6%	89	63.6%
Fusarium oxysporum f.sp. lycopersici	140	18	12.9%	62	44.3%	80	57.1%
<i>Fusarium oxysporum</i> f.sp. vasinfectum	140	18	12.9%	63	45.0%	81	57.9%
Xanthomonas campestris pv. vesicatoria	140	1	0.7%	45	32.1%	46	32.9%
Pseudomonas syingae pv. tomato	140	1	0.7%	35	25.0%	36	25.7%

 Table 3
 Collection 2-Auburn University results

 Table 4
 Collection 3-Becker Underwood results

Name of Pathogens	Total	++	%	+	%	Total	%
Pythium ultimum	58	8	13.8%	20	34.5%	28	48.3%
Pythium aphanidermatum	58	0	0.0%	0	0.0%	0	0.0%
Rhizoctonia solani	58	12	20.7%	27	46.6%	39	67.2%
Rhizoctonia solani	58	22	37.9%	21	36.2%	43	74.1%
Rhizootonia solani	58	25	43.1%	12	20.7%	37	63.8%
Fusarium oxysporum f. sp. lycopersici	58	5	8.6%	23	39.7%	28	48.3%
<i>Fusarium oxysporum</i> f. sp. <i>vasinfectum</i>	58	7	12.1%	19	32.8%	26	44.8%
Xanthomonas campestris pv. vesicatoria	58	0	0.0%	3	5.2%	3	5.2%
Pseudomonas syingae pv. tomato	58	0	0.0%	3	5.2%	3	5.2%

Inhibition of all 9 pathogens was demonstrated by some strains from two collections: 22 strains from collection 1, and 7 strains from collection 2. Inhibition against 8 of the 9 pathogens was demonstrated by 25 strains from collection 1, 20 strains from collection 2, and 1 strain from collection 3. Bacterial identification indicated that PGPR strains that inhibited 8 or 9 of the pathogens most commonly belonged to one of three genera: *Bacillus (B. amyloliquefaciens, B. cereus, and B.*)

*subtilis*), *Brevibacillus* (*B. formosus*, *B. brevis*, and *B. laterosporus*), and *Paenibacillus* (*P. peoriae*, and *P. jamilae*). Future work is planned to test mixtures of the selected PGPR for biocontrol against multiple plant pathogens in bioassays.

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# Production of nematode free, PGPR and mycorrhiza colonized seedlings of capsicum and tomato

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## Abstract

Almost all horticultural crops in seedling stage itself are invariably attacked with one or more species of nematodes. Securing healthy seedlings of any crop is essential to ensure optimum plant population stand, good growth and higher yields. Seedlings infested with the nematodes cannot establish well in the fields. Nematode attack on the root system makes the seedlings vulnerable for the infection of pathogenic fungi and bacteria. Nematode damage also results into the breakdown of resistance against pathogenic fungi and bacteria. Nematodes such as Meloidogyne incognita, M. javanica (root-knot nematodes), Rotylenchulus reniformis (reniform nematode) infect the seedlings capsicum and tomato. As these crops are grown in open field and also protected conditions it is important that the seedlings are free from the infestation of nematodes for their sustainable and profitable production. Seeds were treated with talk based formulation of IIHR - *Pseudomonas fluorescens*  $(2 \times 10^8)$ cfu/g) at the rate of 15g/kg of seed. Treated seeds were grown in portrays filled with coco-peat. Coco-peat was mixed with formulation of IIHR - P. fluorescens and inoculum of *Glomus mossease* (containing  $28 \sim 30$  chlamydospore/g) each at the rate of 10 g/kg of coco-peat before filling in the trays. These treatments helped in the production of  $95\% \sim 97\%$  seedlings of tomato and capsicum, which were free from nematodes. These seedlings were colonized by P. fluorescens and G. mossease. These seedlings carried P. fluorescens and G. mossease to the main field and enriched the soil with these useful eco-friendly components of management, which increased the growth and yield of these crops significantly. These technologies of biomanagement of nematodes using above mentioned PGPR and mycorrhiza were transferred to the farmers.

Key words: seedlings; tomato; capsicum; Pseudomonas fluorescens; Meloidogyne incognita; Glomus mossease

# Introduction

Securing healthy seedlings of any crop is essential to ensure optimum plant population stand, good growth and higher yields. Seedlings infested with the nematodes cannot establish well in the fields. Nematode attack on the root system makes the seedlings vulnerable for the infection of pathogenic fungi and bacteria. Nematode damage also results into the breakdown of resistance against pathogenic fungi and bacteria. Nematodes such as *Meloidogyne incognita*, M. javanica (root-knot nematodes), Rotylenchulus reniformis (reniform nematode) infect the seedlings capsicum and tomato. The occurrence of root-knot nematodes - Meloidogyne incognita and reniform nematodes - Rotylenchulus reniformis were observed on the seedlings of various horticultural crops, during our surveys in India. Hence, it was thought to develop methods to produce nematode free seedlings of tomato and capsicum using *Pseudomonas* fluorescens and Glomus mosseae. Various researchers have reported the bio-control potential of Pseudomonas fluorescens against the root-knot and other nematodes (Parveen et al., 1998; Rao et al., 2004; Siddiqui et al., 1999). Bagyaraj et al. (1979), found that the mycorrhizal seedling were less infected by root-knot nematodes after transplanting. In these studies, we to develop a strategy to using the PGPR and AMF for producing the wanted seedlings of tomato and capsicum free of *M. incognita*.

# Materials and Methods

The experiment was conducted at the Indian Institute of Horticultural Research farm. The coco-peat was used for producing the seedling of tomato and capsicum. Seeds were treated with talk based formulation of IIHR - *Pseudomonas fluorescens* ( $2 \times 10^8$  cfu/g) at the rate of 15g/kg of seed. Treated seeds were grown in portrays filled with coco-peat. Coco-peat was mixed with formulation of IIHR – talc based or neem based formulation of *P. fluorescens* and/ or inoculum of *Glomus mossease*, each at the rate of 10 g/kg of coco-peat before filling in the trays. The inoculum of this Arbuscular Mycorrhizal Fungus (AMF) containing  $28 \sim 30$  chlamydospore/g was used in the experiments. Coco-peat with out adding any PGPR or AMF served as control. The seeds tomato (cv. Arkavikas) and capsicum (cv. California wonder) were sown in portrays.

The treatments were T1- seed treatment with talc based formulation of *P*. *fluorescens*, T2- coco-peat treatment with talc based formulation of *P*. *fluorescens*, T3 - coco-peat treatment with neem based formulation of *P*. *fluorescens*, T4 - coco-peat treatment with *G*. *mossease*, T5 - seed treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with *G*. *mossease*, T6 - seed treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with talc based formulation of *P*. *fluorescens* + coco-peat treatment with

talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T10 - coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T11 - coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with a coco-peat treatment with *G. mossease*, T12 - seed and coco-peat with out treatment served as control.

Experiment was terminated after 35 days. Observations on the plant growth (leaf tip to root tip), number of seedlings with out any galling, number of seedlings having galls on the roots and colonization of *P. fluorescens, G. mossease* on the roots were recorded.

Root colonization by *P. fluorescens* was assessed by following the standard serial dilution technique. One gram root subsample was taken and washed gently to remove the soil. The dilutions were prepared up to  $10^{-5}$  and a 0.1 ml of the  $10^{-4}$  and  $10^{-5}$  dilutions were spread on petriplates containing king's B medium and they were incubated at  $30 \pm 1$  °C. The colonies emitting a pale green fluorescent light under UV at 302 nm were counted and calibrated to  $10^{-6}$  cfu/ml. Root colonization by *G. mosseae* was assessed by clearing the roots with 10% KOH and then staining with trypan blue (Philips and Hayman, 1970).

The data related to the number of nematodes in the roots computed and the data were analyzed using ANOVA.

## **Results and Discussion**

It is interesting that seed treatment with talc based formulation of *P. fluorescens* itself was able to increase the growth of the seedlings of tomato and capsicum (Table 1). However this treatment alone could not reduce the penetration of *M. incognita* in roots of tomato or capsicum and hence we could observe a few seedlings with galls of this nematode (Table 1 & 2). However it is essential that the nursery seedlings produced in any system should be free from pathogenic nematodes/fungi/bacteria. Hence it was required to develop a delivery system for the effective use of this PGPR along with AMF. When we combined simple seed treatment with talc based formulation of *P. fluorescens* and coco-peat treatment with the talc based or neem formulation of *P. fluorescens* it had increased effect of this PGPR.

Treatments	seedling length (tip of the shoot to bottom of root (cm)	nematode free seedlings (%)	Seedlings with 1-2 galls caused by <i>M. incognita</i> (%)	Seedlings with more than 2 galls caused by <i>M. incognita</i> (%)
T1	19.04	87	4	9
T2	20.52	90	5	5
T3	21.60	91	4	5
T4	21.28	85	2	19
T5	23.85	92	3	5
T6	21.35	90	2	8
T7	22.86	92	2	6
Т8	24.27	94	3	3
T9	25.16	97	2	1
T10	22.37	93	2	5
T11	24.46	94	4	2
T12	17.26	86	2	12
CD - 5%	2.66	_		

# Table 1 Effect of application of P. fluorescens and G. mossease on the growth of tomato seedlings and infestation of M. incognita

Values are mean of 5 replicates; T1- seed treatment with talc based formulation of *P. fluorescens*, T2- coco-peat treatment with talc based formulation of *P. fluorescens*, T3 - coco-peat treatment with neem based formulation of *P. fluorescens*, T4 - coco-peat treatment with *G. mossease*, T5 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T6 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens*, T7 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T10 - coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T11 - coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T12 - seed and coco-peat with out treatment served as control.

TREATMENTS	seedling length (tip of the shoot to bottom of root (cm)	nematode free seedlings (%)	Seedlings with 1-2 galls caused by <i>M. incognita</i> (%)	Seedlings with more than 2 galls caused by <i>M. incognita</i> (%)
T1	17.84	84	3	13
T2	19.96	88	6	6
T3	20.74	90	3	7
T4	18.84	81	1	18
T5	19.80	90	4	6
T6	20.49	87	2	12
T7	22.86	89	3	8
Т8	21.52	92	4	4
Т9	24.75	95	3	2
T10	22.39	91	4	5
T11	23.76	93	3	4
T12	15.20	83	3	14
CD - 5%	2.66		_	_

# Table 2 Effect of application of P. fluorescens and G. mossease on their colonization on tomato and Capsicum

Values are mean of 5 replicates; T1- seed treatment with talc based formulation of *P. fluorescens*, T2- coco-peat treatment with talc based formulation of *P. fluorescens*, T3 - coco-peat treatment with talc based formulation of *P. fluorescens*, T3 - coco-peat treatment with *G. mossease*, T5 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T6 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens*, T3 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with a mean based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T10. coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T11 - coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T12. Seed and coco-peat with out treatment served as control.

This resulted in to the production of increased number of nematode free seedlings to tomato and capsicum (Table 1 & 2). *P. fluorescens* has been reported to be effective in the management of root-knot nematodes (Parveen *et al.*,1998; Rao *et al.*, 2004; Siddiqui *et al.*,1999). *P. fluorescens* is known to control the pathogen by the mechanism called siderophoric effect. It was also found very effective in the control of various soil borne fungi causing root rot in certain vegetables crops (Mukhopadhaya, 1987). Induction of growth promoting substances from the PGPR (Plant Growth Promoting rhizobacteria) and induction of systemic resistance against pathogenic fungi was reported by (Ramamoorthy *et al.*, 2001).

Generally coco-peat is pasteurized and many times we have not come across the presence of nematodes in coco-peat. However we did observe  $8 \sim 10$  juveniles of *M*. *incognita* in coco-peat used by a few seedling producers. We used this nematode

infested coco-peat in our experiments. Further we also observed the infestation by these two nematodes in tomato, capsicum, egg-plant and cauliflower seedling produced by certain seedling producers and infestation by nematodes was to the tune of  $12\% \sim 17\%$ .

Coco-peat treatment with G. mosseae did not make that much difference as far as nematode infestation is concerned, but it had increased the growth of seedlings of tomato and capsicum marginally (Table 1 & 2). However, when this treatment was coupled with seed treatment with talc based formulation of P. fluorescens and cocopeat treatment with the neem based formulation of P. fluorescens, there was significant increase in the production of nematode free tomato and capsicum seedlings (Table 1 & 2). Application of neem cake was reported to be very effective in the management of nematodes through variety of mechanisms including the production of antimicrobial compounds and induction of systemic resistance (Alam and Khan, 1980; Mankau, 1962; Muller and Gooch, 1982; Sitaramaiah, 1990). There seem to be synergistic interaction among P. fluorescens, G. mosseae and neem cake. They appear to be complementing each other for their effective use with resultant additive effect on the shoot and root growth. (Table 1 & 2). We do not know their combined effect on the physiology of root-system and it is worth investigating in the future.

Application of neem based formulation of *P. fluorescens* did not affect the root colonization. Rather neem cake helped in the increased root colonization of *P. fluorescens* and *G. mossease* in both crops (Table 3). Through these methods we are able to produce mycorrhizal and PGPR colonized seedlings of tomato and capsicum. Bagyaraj *et al.* (1979), found that the mycorrhizal seedling were less infected by root-knot nematodes after transplanting.

Treatments	Root colonization of <i>P. fluorescens</i> $(CFU/g \times 10^{6})$ in tomato	Root colonization of <i>P. fluorescens</i> (CFU/g×10 <sup>6</sup> ) in capsicum	Root colonization of <i>G. mossease</i> in tomato (%)	Root colonization of <i>G. mossease</i> in capsicum (%)
T1	1.56	1.32	—	
T2	3.42	2.69	—	—
T3	4.82	3.66	_	_
T4	0	0	27	24
T5	2.25	1.79	—	_
T6	4.94	4.14	—	_
Τ7	6.39	5.28	—	—
Τ8	5.68	5.14	34	30
Т9	8.26	8.15	42	38
T10	4.66	4.32	37	33
T11	23.76	93	40	42
T12	15.20	83	—	—
CD - 5%	1.72	2.24	—	—

 Table 3
 Effect of application of P. fluorescens enriched neem cake or vermi-compost

on PGPR root-colonization and yield Tomato and Capsicum

Values are mean of 5 replicates; T1- seed treatment with talc based formulation of *P. fluorescens*, T2- coco-peat treatment with talc based formulation of *P. fluorescens*, T3 - coco-peat treatment with neem based formulation of *P. fluorescens*, T4 - coco-peat treatment with *G. mossease*, T5 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T6 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens*, T7 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with talc based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with a based formulation of *P. fluorescens*, T8 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T9 - seed treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T10 - seed treatment with *G. mossease*, T10. coco-peat treatment with talc based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T11 - coco-peat treatment with neem based formulation of *P. fluorescens* + coco-peat treatment with *G. mossease*, T12. seed and coco-peat with out treatment served as control.

In our studies also, we observed that that, when these seedlings were transplanted in to the main field there was significant reduction in the infestation of *M. incognita*, *M. javanica* (root-knot nematodes), *Rotylenchulus reniformis* (reniform nematode) on tomato and capsicum.

After realizing the potential use of these PGPR and AMF, efforts were made to disseminate the technology to the nursery seedling producers in southern India.

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# The use of coconut fiber and antagonistic *Pseudomonas* CMR12a for biological control of *Rhizoctonia* damping-off on Chinese cabbage, (*Brassica rapa*)

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# Abstract

Isolated from cocoyam roots in Cameroon, *Pseudomonas* CMR12a has been explored as a promising antagonistic agent due to its capacity to produce phenazines and biosurfactants. CMR12a and mutants of this strain impaired in phenazine and/or biosurfactant procution, were applied at a concentration of  $10^7$  CFU g<sup>-1</sup> soil to control damping-off caused by *Rhizoctonia solani* AG 2-1 on chinese cabbage. Data recorded 14 days after inoculation indicated that disease severity had decreased from 3.5 to 1.42 in CMR12a-treated seedlings. In soil inoculated with phenazine- or biosurfactant-deficient mutants, seedlings were partially protected and disease score was 2.29 and 1.96, respectively. However, the disease suppressive effect was completely lost in soil inoculated with a mutant impaired in the production of both compounds. These data suggest that both phenazines and biosurfactants were responsible for the profound effects of *Pseudomonas* CMR12a.

In another experiment, coconut fiber (CF) was incorporated into soil at the concentration of 0.1%, 1% or 5% to reduce viability of sclerotia produced by this fungus. After 4 weeks of incubation with 5% CF, the viability of *R. solani* sclerotia was dramatically decreased from 75% to 20% while the mean number of sclerotia killed by mycoparasitism was significantly raised from 4% to 66%. The incorporation of 5% CF into soil also resulted in a steady increase in population of fluorescent pseudomonads and *Trichoderma* spp. which might contribute to the death of sclerotia.

Generally, it can be concluded that both phenazines and biosurfactants are involved in the biocontrol ability of *Pseudomonas* CMR12a towards *Rhizoctonia* damping-off on chinese cabbage. In addition, viability of *Rhizoctonia* sclerotia could be effectively reduced by incorporating 5% CF into soil.

Key words: *Rhizoctonia solani*; sclerotia; phenazines; biosurfactants; coconut fiber; biocontrol

# Introduction

Chinese cabbage (*Brassica rapa*) is a vegetable which is universally available in almost all countries and cultures. Besides being known for its great taste, chinese cabbage is an excellent source of vitamins and minerals. In Asia, this type of vegetable has been grown on a large scale and it is present in the diet of local inhabitants. However, the increase in production of this vegetable also results in the spread of *Rhizoctonia solani*, a soil-borne pathogen which is widely distributed among soils all over the world. Young or newly emerged cabbage seedlings are highly susceptible to damping-off disease induced by several different anastomosis groups (AGs) of *R. solani* (Sneh *et al.*, 1991). Infected plants have dark, water-soaked to brown discoloration on their stem before they collapse (Koike *et al.*, 2006).

*R. solani* can survive for a long period in plant debris, contaminated seeds, or infested soils as mycelium or sclerotia. Due to the presence of melanin, sclerotia of this fungus are protected from a number of adverse impacts and may serve as a source of primary inoculum in soil (Jacobson, 2000). It has been shown that the incorporation of lignin-rich organic amendment into soil can reduce the persistence of *Rhizoctonia* sclerotia (Soltani *et al.*, 2002; Debode *et al.*, 2005; Van Beneden *et al.*, 2010). In addition, actively growing hyphae of this fungus can be effectively controlled when *Pseudomonas* CMR12a, a rhizospheric bacterium which has capacity to produce phenazine and biosurfactant antibiotics, is applied to soil (D'aes *et al.*, 2011). Therefore, the objectives of our study were to investigate the biocontrol effect of *Pseudomonas* CMR12a towards Rhizoctonia damping-off on chinese cabbage and the potential of coconut fiber to reduce the viability of *Rhizoctonia* sclerotia.

# **Materials and Methods**

# Screening *Pseudomonas* CMR12a and CMR12a-mutants for biocontrol capacity towards damping-off disease on chinese cabbage

Chinese cabbage seeds *Brassica rapa* cv. Excellent (Het Vlaams Zaadhuis, Waarschoot, Belgium) were germinated on wet filter paper in Petri dishes one day before they were sown in substrate containing 50% sand and 50% soil (Structural; Snebbout, Kaprijke, Belgium) (w/w).

*R. solani* BK008-2-1 used in this study was one of the most pathogenic isolates collected from Belgian cauliflower fields (Pannecoucque *et al.*, 2008). This isolate belongs to anastomosis group (AG) 2-1 and its virulence towards chinese cabbage was confirmed under *in vitro* and *in vivo* conditions (unpublished data). Inoculum of *R. solani* was produced as previously described by Scholten *et al.* (2001). Briefly, actively growing hyphae from a 3-day-old PDA culture of *R. solani* AG 2-1 were used to inoculate sterile wheat kernels kept in 250 ml Erlenmeyer flasks. These flasks were incubated at 28°C for 14 days before they were used for inoculation.

Bacterial inoculum used in this study included *Pseudomonas* CMR12a and its mutants deficient in the production of phenazines (CMR12a- $\Delta$ Phz), biosurfactant type

1 (CMR12a-CLP1) or both metabolic compounds (CMR12a- $\Delta$ Phz-CLP1) (D áes *et al.*, 2011). These bacteria were collected from overnight cultures on King ś B plates (King *et al.*, 1954) and applied to soil at a concentration of 10<sup>7</sup> CFU g<sup>-1</sup> soil before sowing. Four days after, six wheat kernels colonized by *R. solani* AG 2-1 were used to infect a group of three cabbage seedlings grown in a plastic box containing 600 g soil substrate. All plants were incubated in a growth chamber (18°C, RH = 60%, 16 h photoperiod) and disease severity was evaluated 14 days after inoculation based on disease severity rating scale ranging from 0 to 4 where 0 = healthy, no symptoms observed; 1 = small brown, water-soaked lesion covering less than 25% of stem and/or hypocotyl; 2 = large lesion covering less than 50% of stem or hypocotyl; 3 = small wilted plant with large lesion covering less than 75% of stem or hypocotyl; 4 = hypocotyl is completely decayed and seedling dead.

A completely randomized design was employed with 4 replications per treatment. Data were subjected to non-parametric Kruskal-Wallis tests to compare mean scores of all treatments. Then, the Mann-Whitney analyses were used to compare means pairwise at P = 0.05. All statistic tests were conducted in SPSS 15.0 (SPSSinc, Illinois, USA).

# Pot assay with coconut fiber and sclerotia of Rhizoctonia solani AG 2-1

Soil used in this experiment was collected from a commercial field in Bottelare (Belgium). Before amendment with coconut fiber at the concentration of 0.1%, 1% and 5%, moisture content of this soil was measured and adjusted to 16.5% (w/w) by adding sterile water. Soil without coconut fiber served as control.

Sclerotia of *R. solani* AG 2-1 were produced according to Manning *et al.* (1970). Batches of five sclerotia were placed inside one nylon mesh bag and five bags were buried in each glass jar containing 200 g soil substrate.

The jars were kept at 22 °C for four weeks and sclerotia were removed for surface sterilization in 1% sodium hypochlorite followed by rinsing two times with sterile water. Then, each surface-disinfected sclerotium was cut in half. The viability of the sclerotia was tested by plating one half on PDA amended with streptomycin (100 mg L<sup>-1</sup>) and prochloraz (4 mg L<sup>-1</sup>). The other half of the sclerotium was placed on PDA amended with streptomycin (100 mg L<sup>-1</sup>) to evaluate mycoparasitism. Then, plates were incubated for two weeks at 22 °C. The growth of *Rhizoctonia* hyphae and the identification of mycoparasites based on their morphological characteristics started from day six after incubation.

Changes in *Pseudomonas* and *Trichoderma* population in soil were determined using a soil dilution technique. From each jar described, two replicate soil samples were taken. For *Pseudomonas* counting, serial dilutions of soil samples in sterile physiological solution were spread on Pseudomonas Isolation Agar (PIA, Difco). After 24 h of incubation at 28°C, colonies were counted and transferred to KB to check their fluorescence under UV light. On the other hand, in order to determine *Trichoderma* population, sterile water was used to prepare soil suspensions. The plating was carried out on Trichoderma selective medium (Williams *et al.*, 2003) and plates were incubated at  $22^{\circ}$ C for four days before counting.

Statistical analysis of data was done with the software package SPSS 15.0 (SPSSinc, Illinois, USA). Viability and mycoparasitism of the sclerotia were analysed using binary logistic regression (P = 0.05). Population density data of *Pseudomonas* and *Trichoderma* were log<sub>10</sub> transformed and analysis of variance was performed.

## **Results and Discussion**

# *In vivo* biological control of root rot caused by *Rhizoctonia solani* AG 2-1 on chinese cabbage by *Pseudomonas* CMR12a and its mutants

Each treatment in this *in planta* experiment was conducted with 24 plants grouped into 4 replications. Data collected 14 days after inoculation showed that the disease severity varied among treatments (Figure1). Disease severity on plants was significantly reduced when soil was treated with either *Pseudomonas* CMR12a, CMR12a- $\Delta$ Phz or CMR12a-CLP1. CMR12a appeared to be the most powerful biocontrol agent. The average disease score of seedlings grown in soil inoculated with CMR12a was 1.42, whereas the mean score in plants belonging to diseased control was 3.5. Due to the loss in capacity to simultaneously synthesize phenazines and biosurfactants, protection by mutants impaired in the production of one of these metabolites was lower than that of the wild type although the difference was not statistically significant in the treatment inoculated with the biosurfactant-deficient mutant. Compared to the parental strain, biocontrol capacity of a mutant deficient in both biosurfactant and phenazine production, was completely lost.

From the obtained data, it seemed that *Pseudomonas* CMR12a can effectively control *R. solani* on chinese cabbage. The biocontrol capacity of *Pseudomonas* CMR12a can be explained by the production of phenazine and biosurfactant antibiotics. These results are similar to the investigation of D'aes *et al.* (2011) about the role of these metabolic compounds in biocontrol of Rhizoctonia root rot disease on bean.

## Effect of coconut fiber on viability of Rhizoctonia sclerotia

The effectiveness of coconut fiber incorporation in inhibiting the germination of R. *solani* AG 2-1 sclerotia was evaluated. After 4 weeks of incubation, the difference in percentage of viable sclerotia corresponding to the variation in the amount of coconut fiber added to soil was observed. Only 20% viable sclerotia were detected when they were buried in soil amended with 5% CF. Soil containing 1% CF also showed a slight decrease in sclerotial viability (58% compared to 75% of non-amended control treatment) but this reduction was not statistically significant (Table 1).



Figure 1 Effect of *Pseudomonas* strain CMR12a and its mutants on the severity of Rhizoctonia damping-off on chinese cabbage. Control treatment was treated with sterile physiological solution and diseased control treatment was infested with *R. solani* AG 2-1 strain BK008-2-1. Different letters indicate significant differences among treatments (P<0.05)

It has been suggested before that the addition of lignin-rich organic materials might favor the growth of indigenous lignin composers (Debode *et al.*, 2005). Ligninolytic enzyme activities of these microorganisms can also destroy melanin, a stable component present in the cell wall of *Rhizoctonia* sclerotia (Butler and Day, 1998). When melanin is degraded, sclerotia become more susceptible to both biotic and abiotic stress (Gomez and Nosanchuk, 2003; Butler et al., 2005). Analysis of the quantity of sclerotia parasitized by fungi in our study showed that there was a significant raise in the mean number of parasitized sclerotia when soil was supplemented with either 1% or 5% CF. Due to the difference in morphology, these colonizing fungi could be classified into 3 groups including Trichoderma spp., Fusarium spp. and other fungi (data not shown). The incorporation of 5% CF into soil also favored population growth of Trichoderma spp. and fluorescent pseudomonads (Table1). Therefore, we assumed that the growth of lignin degrading fungi is stimulated in coconut fiber-amended soils and these fungi are responsible for melanin degradation of sclerotia. When melanin is broken, sclerotial bodies of R. solani can be easily attacked by other microorganisms such as mycoparasites and *Pseudomonas* spp. and they lose their viability.

Treatments	Viable	Total non-viable	Population density (log CFU g <sup>-1</sup> soil)		
	sclerotia (%)	sclerotia (%) <sup>a,b</sup>	Pseudomonas spp	Trichoderma	
			i seudomontas spp.	spp.	
Control	75.0 a	4.0 c (21.0)	2.43 b	0.79 b	
0.1% coconut fiber	75.0 a	22.0 c (2.0)	2.53 b	0.74 b	
1% coconut fiber	58.0 a	30.0 b (12.0)	2.67 ab	0.91 b	
5% coconut fiber	20.0 b	66.0 a (14.0)	3.09 a	3.17 a	

Table 1 Percentage of viable and non-viable sclerotia and population density of *Pseudomonas* spp. and *Trichoderma* spp. in non-amended soil (control) and in soil amended with 0.1%, 1% and 5% coconut residue after 4 weeks of incubation at 22°C

<sup>a</sup> Percentage of sclerotia that were killed due to mycoparasitism

<sup>b</sup> Values between brackets represent percentage of sclerotia dead naturally after introducing into soil.

Obviously, the incorporation of CF at a concentration of 5% might decrease the viability *R. solani* AG 2-1 sclerotia. These results are in agreement with previous findings of Soltani *et al.* (2002), Debode *et al.* (2005) and Van Beneden *et al.* (2010) about the degradation of melanized structures such as sclerotia of *R. solani* due to the addition of lignin-rich organic material to soil.

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# Fertilizer consumption and effectiveness for rice cultivation in Vietnam

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## Abstract

There is more than 70% of population of Vietnam, living on agricultural production. So that economic development in Vietnam is still based on the agricultural production. Agricultural production in Vietnam is multiform with different crop groups. In last 17 years, cultivated lands were increased from 9,040 thousand ha to 13,555.6 thousand ha (1.5 time).

To meet the requirement for agricultural production, fertilizer production and consumption in Vietnam have been being continuously increased. Fertilizer consumption was increased from 3,242 thousand tones in 1995 to 5,182 thousand tons in 2000 and 9,338.5 thousand tons in 2009. Vietnam has to import amount of  $2311 \sim 4395$  thousand tons per year. Domestic fertilizer is promoted to produce in Vietnam, so that domestic fertilizers were increased from no product in 1995 to 1,204.3 thousand tons in 2000 and 4,035.5 thousand tones in 2009. Total consumption for agricultural production is estimated by 469.9 thousand tons of  $(N+P_2O_5+K_2O)$  in 1995, 2,307.5 thousand tones in 2005 and 2,819.5 thousand tones in 2009, equal to 54.8; 173.7 and 208.9kg  $(N+P_2O_5+K_2O)/ha$  cultivated land, respectively.

Farmer practices in rice cultivation are quite differences between regions. Fertilizer rates applied for rice change in range of  $27 \sim 253$  N;  $14 \sim 284$  P<sub>2</sub>O<sub>5</sub>;  $0 \sim 351$  K<sub>2</sub>O kg/ha/crop in the south;  $60 \sim 181$  N,  $0 \sim 138$  P<sub>2</sub>O<sub>5</sub>,  $0 \sim 201$  K<sub>2</sub>O/ha in the central part and  $49 \sim 261$  N;  $24 \sim 151$  P<sub>2</sub>O<sub>5</sub>;  $29 \sim 167$  K<sub>2</sub>O/ha in intensive rice-rice system and  $58 \sim 167$  N,  $22 \sim 146$  P<sub>2</sub>O<sub>5</sub>,  $25 \sim 173$  K<sub>2</sub>O/ha in rice-rice-maize system in the northern part. Economic return resulted that total input/cost (C= labor, fertilizer, irrigation, plant protection, variety) is estimated by  $5.35 \sim 6.10$  million VN D/ha, of which input cost is  $2.08 \sim 2.33$  million VND/ha (39%) for fertilizer and  $18 \sim 33$  for labor. Total benefit (B) is  $11.1 \sim 18.1$  million VN D, interest (B-C) is  $5.77 \sim 12.04$  million VND/ha with B/C ratio of  $1.14 \sim 3.23$  in the South. In the central part, total cost is  $4.34 \sim 6.5$  million VND/ha, of which input consist of  $43\% \sim 46\%$  for fertilizer, and  $16 \sim 28$  for labor. Benefit is  $10.87 \sim 15.54$  million VND/ha, of which input is  $22.8\% \sim 24.\%$  for

fertilizer and 63 $\sim$ 66 for labor. Benefit was obtained by 10.8 $\sim$ 14.3 million VND/ha with B/C ratio is 1.7 $\sim$ 2.3.

Due to deference in technology, in the Southern and Southern central part, fertilizer input is highest, in the Northern part, labor is highest input for rice cultivation. How to reduce the fertilizer cost in the southern part and labor cost in the north should be more study to get advanced technique for farmer and safety environment.

# Plant growth promoting activity of *Enterobacter* sp. C1D in heavy metal contaminated soils

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#### Abstract

Chromium  $(Cr^{6+})$  is a widespread soil pollutant and is released from various industries including tanneries, metal cleaning and processing, chromium plating, wood processing, and alloy formation. At elevated levels  $Cr^{6+}$  is highly toxic to most plants, impairing their metabolism and reducing plant growth. In recent past, free living, metal-resistant native soil bacteria that exert beneficial effects on plants have gained lot of attention at a global scale. Present study was aimed at exploring the potential of plant growth promoting, metal resistant bacteria at alleviation of metal toxicity to plants. Metal resistant bacteria were isolated form soil contaminated with industrial effluent at estuarine zone of Mahi River, Sarod, Gujarat, western India. Studied soil was polluted with toxic heavy metal ions such as Ag<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Cr<sup>6+</sup>  $Ni^{2+}$ ,  $Pb^{2+}$  and  $Zn^{2+}$ . Metal resistant bacteria were isolated and their 16S rRNA based phylogeny revealed that most of the isolates belonged to Proteobacteria and *Firmicutes.* One of the isolates, *Enterobacter* sp. C1D, was found to be multi-metal resistant in nature and showed plant growth promoting traits. Plant inoculation studies with Vigna radiata GM4 in natural soil supplemented with Cr<sup>6+</sup> along with Enterobacter sp. C1D were performed to elucidate the effect of Enterobacter sp. C1D on plant growth promotion in  $Cr^{6+}$  contaminated soils. *Enterobacter* sp. C1D had clear positive measurable effects on root length, shoot length, fresh shoot weight, fresh root weight and chlorophyll content of the V. radiata GM4 in  $Cr^{6+}$  amended soils (up to 350mg/kg). In order to discern the credible mechanisms that are responsible for the plant growth promotion by Enterobacter sp. C1D in Cr<sup>6+</sup> amended soils, all the PGPR traits were determined in presence of Cr<sup>6+</sup> High amount of acidic periplasmic phosphatases and elevated IAA production probably enable *Enterobacter* sp. C1D to enhance plant growth in Cr<sup>6+</sup> contaminated soils.

**Key words:**Metal toxicity; Metal resistant bacteria; Plant growth promotion; *Enterobacter* sp. C1D.

# Introduction

Elevated concentrations of heavy metals in soils and their uptake by plants adversely affect the growth, symbiosis and consequently the yields of crops (Wani et al., 2008). Accumulation of these toxic metals in the soil inversely affects the microbial compositions and poses a major threat to plant beneficial rhizobacteria. Chromium  $(Cr^{6+})$  is a widespread soil pollutant and is released from various industries including tanneries, metal cleaning and processing, chromium plating, wood processing, and alloy formation. At elevated levels, Cr<sup>6+</sup> is highly toxic to most plants, impairing their metabolism and reducing plant growth. Metal resistant plant growth promoting bacteria (MR-PGPB) include a diverse group of free-living soil bacteria that can improve host plant growth by various mechanisms (Fig.1) by mitigating toxic effects of heavy metals on the plants (Belimov et al., 2004). Therefore in recent past, exploring novel Cr<sup>+6</sup> resistant plant growth promoting bacteria for sustainable agriculture has gained attention (Kumar et al., 2009; Wani et al., 2008). Metal resistant plant growth promoting bacteria suitable for escalation of phytoremediation in contaminated systems has also been well established (Khan et al., 2009; Sheng and Xia. 2006; Glick. 2003). Searching novel metal resistant plant growth promoting bacteria has significance in the management of environmental and concurrent agricultural problems in polluted terrestrial ecosystems. The main objectives of the present study were

1. Isolation and characterization of Cr<sup>6+</sup>resistant plant growth promoting bacteria

2. Elucidation of credible mechanisms which are responsible for the plant growth promotion by the isolate in  $Cr^{6+}$  polluted soil.

# **Materials and Methods**

 $Cr^{6+}$  resistant bacterium (MIC- 400 mg/kg) was isolated from a soil contaminated with industrial effluent at estuarine zone of Mahi River, Sarod, Gujarat, western India. The isolate was identified as *Enterobacter* sp. by 16S RNA sequencing. In the present investigation, plant growth promoting traits of the bacterium such as mineral phosphate solubilisation, organic acid production, siderophore production, phosphatase activity (both acidic and alkaline), IAA production and ACC deaminase activity were estimated by standard protocols.



Fig. 1 Likely mechanisms of plant growth promotion (direct and indirect ways) by metal resistant plant growth promoting bacteria (MR-PGPB) in contaminated soils

*Enterobacter* sp. C1D was used in gnotobiotic plant inoculation experiments to evaluate plant growth promoting potential of the isolate under elevated levels of Cr<sup>6+</sup>. Soil samples were collected from *Pulse research station*, Anand Agricultural University., Vadodara (Gujarat), India. The soil was sieved (2 mm) and sterilized by autoclaving at 121°C, 15 psi for 1 h. After cooling the soil was amended with aqueous solution of  $K_2Cr_2O_7$  to achieve the final concentrations of 50 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg, 250 mg/kg, 300 mg/kg, 350 mg/kg and 400 mg/kg. Vigna radiata GM4 seeds were surface sterilized. For inoculation of the seeds, bacterial culture was grown for 18 h, cells were harvested by centrifugation (6000 rpm, 10 min), washed twice with sterile distilled water, and resuspended in saline (0.85% NaCl). The seeds were inoculated by soaking in a bacterial suspension (O.D = 0.6) for 1 h, as described by Burd et al. (2000). Seeds soaked in sterile water were used as control. After 25 days the plants were carefully removed from the plastic bags and the root surface was cleaned several times with distilled water. Growth parameters such as shoot length, root length and fresh shoot weight and fresh root weight were measured. The chlorophyll a and chlorophyll b content of the plants were also been quantified (Xiong and Wang, 1999). In order to know the mechanisms involved in plant growth promotion under elevated metal concentrations, PGPR traits discussed above were studied in the presence of 100 mg/kg of  $Cr^{6+}$ .

# **Results and Discussion**

PGPR traits of *Enterobacter* sp. C1D revealed it to solubilize inorganic phosphate by reducing the pH. It was also proficient at hydrolysis of organic phosphates by various phosphatases (Table 1). In addition to this the organism is able to produce significant amount of gluconic acid. This organic acid is important in solubilization of insoluble mineral phosphates (Gyaneshwar *et al.*, 1999) as well as in assisting the hydrolysis of phytate that is complexed with  $Al^{+3}$ ,  $Fe^{+2}$ , or  $Fe^{+3}$  (Patel *et al.*, 2010; Tang *et al.*, 2006) and has also been implicated in providing metal tolerance to plants (Kavita *et al.*, 2009). It has been well documented that the biosynthesis of IAA along with its excretion into soil makes a major contribution to the bacterial plant growthpromoting effect. It was found that that *Enterobacter* sp. CID produces 38.79 µg/ml IAA (Table 1).

 $Cr^{6+}$  toxicity was noticed in above and below ground biomass of *Vigna radiata* GM4 during plant experiments. Apparently, at 300 mg/kg very poor growth of plants (root length and shoot lengths were 0.91cm & 3.8cm respectively) was observed in uninoculated plants. *Enterobacter* sp. C1D significantly increased root length and shoot length (Fig.2). It had positive effects on root biomass, shoot biomass, fresh shoot weight, and fresh root weight and chlorophyll content of the *Vigna radiata* GM4 in Cr<sup>6+</sup> amended soil (data not shown).

In order to know the probable mechanisms attributing to the plant growth promotion in  $Cr^{6+}$  contaminated site, the PGPR traits were monitored in presence of  $Cr^{6+}$  (100 mg/kg). It was found that the organism exhibited high amount of acidic periplasmic phosphatase (36 fold more), elevated IAA production (41.6µg/ml) and good ACC deaminase activity in the presence of the metal are these may be the probable mechanisms that enable *Enterobacter* sp. C1D to enhance plant growth in  $Cr^{6+}$ contaminated soils (Table 1).

Table 1PGPR traits exhibited by *Enterobacter* sp. C1D in metal free conditions and in the presence of<br/> $Cr^{6+}$  (100 mg/kg)

Bacterial isolate	Siderop- hore production	Mineral phosphate solubilisatio n	IAA produ ction	ACC deami nase	pH* drop	Organic acid production <sup>+</sup>	Phosphatas E.P I.I B A B	$\begin{array}{c} \text{se activity}^{\#} \\ P & P.P \\ \hline B & A \\ A \\ \end{array}$
Enterobac ter sp. C1D (without metal Enterobac	+++	+++	38.7	237.8	5.5	Gluconate <sup>+</sup>	0.13 7.0 0.9 33	)4 9.4 .9 0.18
<i>ter</i> sp. C1D (with 100 mg/kg Cr <sup>6+)</sup>	-	-	41.6	198.8	-	-	0.78 2.2 1.55 32.	3 3.55 9 36.16

Unit for IAA production,  $\mu g/ml^{-1}$ ; unit for ACC demianase activity, nmol  $\alpha$ -ketobutyrate protein mg<sup>-1</sup> h<sup>-1</sup>; \*pH drop from startin pH of 8.0 after 6 days in minimal media amended with rock phosphate; <sup>+</sup> 62.13 mM gluconate; <sup>#</sup> Unit for phosphatase activity,  $\mu g$  P-nitrophenol mg<sup>-1</sup> min<sup>-1</sup>; E.P – Extracellular phosphatase activity; I.P- Intracellular phosphatase activity; P.P- Periplasmic phosphatasea ctivity; A-Acidic phosphatase; B- Alkaline phosphatase

In conclusion, the chromium resistant bacterium *Enterobacter* sp. C1D possesses the ability to produce plant growth promoting substances (IAA & ACC deaminase), solubilize inorganic P, organic P and synthesize siderophores. Therefore, this bacterium is endowed with the ability to mitigate the toxic effects of elevated  $Cr^{6+}$ . The growth promoting activities of *Enterobacter* sp. C1D are of practical importance for plant growth promotion in normal and metal tainted environment. Moreover, phytoremediation of metal accumulating plants could be enhanced by *Enterobacter* sp. C1D for effective remediation of heavy metal contaminated soils (Khan *et al.*, 2009).

# Fig. 2 Plant growth promotion by *Enterobacter* sp. C1D in metal free



# and Cr<sup>6+</sup> amended soil

Enterobacter sp. C1D inoculated Vigna radiata plants

Uninoculated Vigna radiata plants

	Ţ	
5+	Shoot	Root
ncentration	length	length
ppm)	(cm)	(cm)
	25.5±1.18	10.8 ±0.77
	21.5±1.16	10.1±1.16
	17.2 ± 0.90	7.8±0.47
	15.6±1.39	6.7±1.19
	$12.5 \pm 0.67$	4.12 ±1.2
	8.1±1.1	1.9±0.21

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# Optimization of GA<sub>3</sub> biosynthesis by bacteria associated with the rhizosphere of sugarcane

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#### Abstract

A total of 33 bacterial isolates (*Bacillus* sp. 12, *Pseudomonas* sp. 10, *Azotobacter* sp. 5 and *Rhizobium* sp. 6) were isolated from different rhizospheric soil of sugarcane in the vicinity of Surat city, India and characterized as per standard methods. These isolates were tested for the production of gibberellic acid (GA<sub>3</sub>) in a nutrient medium. Out of all these isolates PSG8 (*Pseudomonas* sp.) produce more gibberellic acid as compared with the others. Optimization of various physiological conditions (incubation temperature, pH of the growth media, incubation period, and incubation condition) were carried out to achieve maximum production of gibberellic acid. The highest 290 mg/l of gibberellic acid production was obtained in nutrient broth when the bacteria culture was incubated at  $30^{\circ}$ C for 78 h at pH 6.5 on rotary shaker.

Key words: Gibberellic acid; Sugarcane; Pseudomonas

# Introduction

The gibberellins (GAs) are an important group of phytohormones occurring in higher plants. Gibberellins are a typical example of the production of plant growth regulators by microorganisms, and are important biotechnological products which are increasingly used in agriculture and horticulture. GAs have been identified and isolated from higher plants and microorganisms. It was reported that 136 different chemical structures have been characterized from higher plants & fungi as naturally (http://www.plant-hormones.info/gainfo.asp). occurring gibberellins However. gibberellins are produced not only by higher plants and fungi (MacMillan 2002) but also by bacteria (Atzorn et al., 1988; Bastian et al., 1998; Bottini et al., 1989; Gutierrez-Manero et al., 2001; Janzen et al., 1992; MacMillan 2002). In fungi and bacteria there is no known role for gibberellins, rather they seem to be secondary metabolites that may play a role as signaling factors towards the host plant fixation (Bashan and Levanony 1990; Okon and Laban-dera-Gonzalez 1994, and literature cited therein). The main product of gibberellin biosynthesis by microorganisms is

gibberellic acid (GA<sub>3</sub>), which is formed from GA<sub>4</sub> via GA<sub>7</sub>. GA<sub>3</sub> is used extensively in agriculture, nurseries, greenhouses, tea gardens, viticulture etc. Gibberellic acid has no apparent role in the microbes, it has been found to elicit a variety of responses in higher plants including shoot elongation, fruit growth and seed germination (Bruckner 1992; MacMillan 1997). The current study was carried out to find the gibberellin producing capacity of soil bacteria. Furthermore, the optimization of GA<sub>3</sub> production parameters such as growth-medium-pH, incubation period, incubation temperature and incubation conditions (rotary shaker and static incubation).

## Materials and methods

## Isolation of rhizobacteria

The rhizospheric soil samples (four) were collected from fields growing sugarcane in the vicinity of Surat, India. All bacterial strains were isolated on their respective media; *Rhizobium* was isolated on yeast extract mannitol agar, *Azotobacter* on Jensen's medium *Pseudomonas* on King's B medium and *Bacillus* on nutrient agar. Bacterial cultures were maintained on the respective slants. (Joseph *et al.*, 2007, Cattelan, *et al.* 1999, Rennie *et al.*, 1983)

# **Biochemical characterization of rhizobacteria**

Selected isolates of *Bacillus*, *Pseudomonas*, *Azotobacter* and *Rhizobium* were biochemically characterized by Gram's reaction, carbohydrate fermentation, oxidase test, H<sub>2</sub>S production, IMViC tests, NO<sub>2</sub> reduction, and starch and gelatin hydrolysis as per the standard methods. (Cappuccino, J. C., Sherman, N., 1992)

#### Production of gibberellic acid

Production of gibberellic acid was detected by spectrophotometric method. Bacterial cultures were grown for 72 h (*Azotobacter* and *Rhizobium*) and 48 h (*Pseudomonas* and *Bacillus*) on their respective media at  $36\pm2$  °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The pH value of supernatant was adjusted to 2.5 using stock 3.75 N HCl. Supernatant was extracted using liquid-liquid (ethylacetate/NaHCO<sub>3</sub>) extraction method. The amount of gibberellic acid in the ethylacetate phase was measured by the UV spectrophotometer at 254 nm. (Glick, 1995, Julio et. al., 2004)

#### **Optimization of culture condition**

For the maximization of GA<sub>3</sub> production by the isolated strain, experiments were conducted for the optimization of production parameters such as growth-medium-pH,

incubation period, incubation temperature and incubation conditions (rotary shaker/static incubation).

## Incubation Temperature and Condition (Static/Shaker) for GA<sub>3</sub> Production

Culture flasks were incubated at different temperatures ranging from 20 °C to 40 °C to determine optimum temperature for growth and production of GA<sub>3</sub>. Growth media inoculated with *Pseudomonas* sp. were incubated both on rotary shaker - static conditions at  $30 \pm 1$  °C for 72 h to determine the amount of GA<sub>3</sub> produced.

## Optimal Initial pH for GA<sub>3</sub> Production

For this test, the pH of each growth medium was adjusted to  $5.0 \sim 8.0$  with 1N HCl. Growth media were incubated at  $30 \pm 1^{\circ}$ C for 72 h on a rotary shaker.

#### Optimum Incubation Period for GA<sub>3</sub> Production

To determine the optimal incubation time for GA<sub>3</sub> synthesis, *Pseudomonas sp.* was inoculated into nutrient broth incubated for 12h, 24h, 36h, 48h, 72h, 75h, 78h, 81 h at  $30 \pm 1^{\circ}$ C on a rotary shaker (150 rpm). After incubation, the GA<sub>3</sub> amount was estimated by spectrophotometer.

# **Results and Discussion**

A total number of 33 isolates *were* obtained from the rhizosphere soil of sugarcane of different varieties from different localities of Surat District and they were grouped into *Bacillus, Pseudomonas, Azotobacter,* and *Rhizobium* as based on their morphological, cultural and biochemical characteristics. General features of the test isolates are illustrated in Table 1. In the present investigation 12 isolates of *Bacillus* spp., 10 isolates belonging to *Pseudomonas* spp., 05 isolates of *Azotobacter* spp., and *06 isolates of Rhizobium* spp. were screened for *in vitro* PGP activities.

Sr. No.	Morphological and Biochemical Characterization	Bacillus (12)*	Pseudomonas (10)*	Azotobacter (05)*	Rhizobium (06)*
1	Grams reaction	G+ve	G -ve	G –ve	G –ve
2	Shape	rods	rods	Rods	Rods
3	Indole	-	-	+	+
4	Methyl red	-	-	+	+
5	Vogues Proskauer	+	-	+	+
6	Citrate utilization	+	+	+	-
7	$H_2S$ production	-	-	+	+
8	Nitrate reduction	+	+	+	+
9	Dextrose	+	+	+	-
10	Lactose	+	-	+	W
11	Sucrose	+	+	+	-
12	Mannitol	+	-	+	+
13	Catalase	+	+	+	+
14	Starch Hydrolysis	+	+	+	+
15	Gelatin Hydrolysis	+	+	-	-
16	Oxidase	-	+	+	+

Table 1 Morphological & Biochemical Characteristics

\* Number of isolates present in group

Production of gibberellic acid by isolates as given in Table 2 reveals that many of the isolates produce GA<sub>3</sub>. The production of GA<sub>3</sub> was in the range of 03 mg/L to 94 mg/L, 12 isolates produced less than 25 mg/L, 6 isolates produced in between 25 to 50 mg/L and 7 isolates produced more than 50 mg/L amount of gibberellic acid. The minimum potential was shown by RA3 (3 mg/L) whereas isolate PSG8 produced maximum (94 mg/L) amount of GA<sub>3</sub> which was significantly more than the other isolates.

Genus	Isolates	GA <sub>3</sub> Production
	BG1	38
	BG2	66
	BG3	86
	BG4	-
	BG5	50
sulli us	BG6	09
3acı	BG7	78
1	BG8	08
	BA1	20
	BA2	11
	BA3	12
	BA4	-
	PSG1	-
	PSG2	32
	PSG3	80
as	PSG4	38
iou	PSG5	-
opn	PSG6	18
Pse	PSG7	-
	PSG8	94
	PSA1	10
	PSA2	15
	AG1	-
cter	AG2	06
oba	AG3	-
zot	AA1	58
A	AA2	10
	RG 1	25
u	RG2	08
biun	RG3	-
uizo	RA1	62
R	RA2	40
	RA3	03

The results obtained in the present investigation suggests that the isolate PSG8 (*Pseudomonas* sp.) exhibit more potential for the production of GA<sub>3</sub> compared with the others. Various cultural parameters such as different incubation temperatures (20°C, 25°C, 30, 35 °C and 40 °C), incubation periods (12h to 84 h), pH (ranging from 5.0 to 8.0) and incubation conditions (static and rotary shaker) were studied for their effects on the production of GA<sub>3</sub> by *Pseudomonas* sp.

Various incubation temperatures showed a diverse effect on GA<sub>3</sub> production. The maximum level of GA<sub>3</sub> production (152 mg/L) and bacterial growth was detected at  $30 \pm 1^{\circ}$ C and the level started decreasing at higher temperatures (35~40 °C) (Figure 1).

After the potent microorganism *Pseudomonas* sp. was incubated at different conditions (rotary shaker and static) the  $GA_3$  production was determined. The highest level of yield was 245 mg/L obtained over a rotary shaker (Table 3). This may be due to the rotary shaker provides more oxygen both for bacterial growth and oxidase enzymes in  $GA_3$  biosynthesis.

The effects of different pH levels (ranging from 5.0 to 8.0) were tested on the production of  $GA_3$ . The maximum yield was obtained at pH 6.5, while lower  $GA_3$  production was obtained between pH 5.0 and 6.0 (Figure 2).

Finally, the effects of different incubation periods on the biosynthesis of  $GA_3$  are presented in Figure 1. Increase in  $GA_3$  production started at 12 h of incubation and reached a maximum level (280 mg/L) at 78 h (Figure 3). Thereafter, a gradual decline of  $GA_3$  production was observed up to 81 h of incubation.



Figure 1 Effect of incubation temperature on GA<sub>3</sub> production



# Table 3Effect of incubation conditions<br/>on GA3 production

The results showed that production of  $GA_3$  is highly dependent on the optimization of some cultural parameters. Based on the data presented, the  $GA_3$  yield was maximized (290 mg/L) at 78 h of incubation at pH 6.5 at 30 °C on a rotary shaker. **References** 

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# Efficacy of microbial products for managing bacterial spot of tomato

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### Abstract

Bacterial spot, caused by Xanthomonas spp., is an economically important disease of tomato worldwide. Despite tremendous efforts to manage this disease, it still remains a major challenge in tomato production in regions such as Florida with warm and humid weather conditions. Greenhouse and field experiments were conducted to evaluate the efficacy of four microbial products, i.e. Actinovate® AG, Companion®, BU EXP 1216C and BU EXP 1216S alone or in combination with acibenzolar-Smethyl (ASM; Actigard® 50WG, Syngenta). ASM is a commercial systemic acquired resistance (SAR) inducer labeled on tomato for control of bacterial spot. Each of these materials, alone and in combination with ASM, was applied as a foliar application at weekly intervals. In the greenhouse trials, Companion<sup>®</sup> applied alone significantly (P < 0.05) reduced bacterial spot severity or AUDPC values compared to the nontreated control (CK). Data from two repeated field trials revealed significant disease reduction in the development of bacterial spot by treating tomato plants with Actinovate® AG, BU EXP 1216C and BU EXP 1216S alone. Actinovate® AG and BU EXP 1216C alone significantly increased the marketable vields when compared with the CK in one of the field trials. In both greenhouse and field trials, tomato plants treated with all microbial products in combination with ASM had significantly lower values of AUDPC than the CK, and levels of disease reduction in combined treatments were generally greater than the standard chemicals Kocide 3000 and Manzate. Results from this study indicate a considerable potential for these microbial products with ASM to be incorporated into integrated management programs for control of bacterial spot on tomato in Florida.

# Bio-management of nematode induced wilt disease complex of Gerbera using PGPRs in open field conditions

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#### Abstract

Gerbera is a commercially important cut flower crop, generally grown in protected conditions for the quality flowers which demands very high investments. Attempts were made to cultivate certain cultivars of gerbera in open field conditions by various researchers in India. A few farmers in India are cultivating this crop in open fields. As it is a long standing crop it provides congenial conditions for the rapid multiplication and buildup of pathogenic fungi and nematodes. Sustainable production of gerbera is seriously hampered by Meloidogyne incognita and *Phytophthora* spp., which produce disease complex in this crop. Experiments were conducted for the biological control of these disease complexes using PGPR (*Pseudomonas fluorescens*) with different organic substrates (de-oiled neem cake, FYM, vermicompost and cocpeat). Various dosages (concentrations) prepared from IIHR- PGPR formulations of P. fluorescens  $(2 \times 10^8 \text{cfu/g})$  were used to enrich the organic substrates in different ratios. Organic substrates were enriched for 15 days at optimum moisture levels were for the soil application. P. fluorescens enriched organic substrates were applied in the field  $20g/m^2$  at the time of planting and at an interval of 3 months. Successful bio-management strategies have been standardized in enhancing the sustainable production of gerbera using IIHR formulations of P. *fluorescens* in open field conditions. Observed  $57\% \sim 64\%$  reduction of *M. incognita* population in roots and  $60\% \sim 65\%$  reduction in the disease infestation on gerbera in the beds which were treated with deoiled neem cake enriched with P. fluorescens. This helped in the reduction of the cost of production. These treatments also increased yield by  $20\% \sim 22\%$  with enhanced shelf life enhanced shelf life and quality of the flowers.

**Key words:** Gerbera; *Pseudomonas fluorescens*; *M. incognita*; *Phytophthora* spp.; disease complex and organic substrates and bio-management
## Introduction

Sustainable production of gerbera is seriously hampered by the disease complex caused by *M. incognita* and *Phytophthora cryptogea* (Schlang and Sikora, 1978). Most of the highly fetching exotic cultivars of gerbera have shown 40% to 60% mortality in polyhouse beds due to root-knot nematode infection (Nagesh and Parvatha Reddy, 1996).

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Plant parasitic nematodes are known to predispose some plants to fungal pathogens (Bergeson, 1972; Mai and Abawi, 1987; Sidhu and Webster, 1977). Migratory nematodes also represent a predisposing factor to infection by certain fungi. Mechanical wounding of the root favours the entry of other pathogens. For example, *Pratylenchus* sp. is reported to interact synergistically with *Verticillium* and *Belonolaimus* sp. and *Trichodorus* sp. with *Fusarium* (Faulkner *et al.*, 1970).

Bacterial wilt caused by *Ralstonia solanacearum* is more severe in resistant cultivars of tomato and eggplant in the presence of *M. incognita*. The combination of the two pathogens suppresses the survival rate of wilt-resistant tomato plants to 33-36%. Both capsicum and eggplant are prone to many soil borne diseases, among which the bacterial wilt (*R. solanacearum*) in combination with root-knot nematode (*M. incognita*) takes heavy toll every year all over the world (Naik and Rao, 2004).

*Pseudomonas fluorescens* belongs to Plant Growth Promoting Rhizobacteria (PGPR), the important group of bacteria, which play a major role in the plant growth promotion, induced systemic resistance, biological control of pathogens etc. PGPR are known to enhance plant growth promotion and reduce severity of many fungal and nematode diseases.

Keeping in view the potential of *P. fluorescens* in the management of pathogenic fungi nematodes, it was thought to develop effective delivery methods of this biopesticide in the field conditions using neem cake and Farm Yard Manure (FYM) after their enrichment with *P. fluorescens*.

It was also thought to study the effect of these methods of application on disease complex caused by *M. incognita* and *P. crytogea* in gerbera in open field conditions.

## **Materials and Methods**

#### **Enrichment of organic substrates**

Talc based PGPR formulation of *P. fluorescens* (IIHR-PF-2) available at Indian Institute of Horticultural Research (IIHR), Bangalore, was used for enriching the substrates. 2 kg of talc based PGPR formulation (IIHR-PF-2) of *P. fluorescens* ( $2 \times 10^8$  CFU/g) was enriched in 100 kg of de-oiled neem cake or 1000 kg FYM. The enrichment process was done under shade. The enriched substrates were covered and the optimum moisture (7.5%) was maintained for 15 days. After every 5 days the heap was turned around for aeration.

## Field evaluation of the formulations in gerbera

The enriched formulations of *P. fluorescens* were used to develop delivery systems and evaluate them in the field infested with *M. incognita* and *P. crytogea* at (Indian Institute of Horticultural Research, Bangalore, India) and standardized a strategy for the bio-management of disease complex in gerbera (cv. Arka krishika) caused by *M. incognita* and *P. crytogea*. Neemcake and enriched FYM enriched with PF were applied 20 g/m<sup>2</sup> and 200 g/m<sup>2</sup> respectively at an interval of 3 months. The plot size maintained was of  $1 \times 1$  m<sup>2</sup>. Simultaneously the seedlings (cv. Arka krishika) were also treated by *P. fluorescens* by root dip treatment before planting. The root dip treatment was given to the seedlings of gerbera by dipping the roots for 5 min. in a tray containing uniform suspension of *P. fluorescens* in water (CFU  $2 \times 10^8$ /ml).

The individual treatments were as follows: T1-seedlings of gerbera treated with P. *fluorescens* (CFU  $2 \times 10^8$ /ml) (PF-SD); T2 - untreated seedlings sown in the plots mixed with neem cake enriched with P. fluorescens 20 g/m<sup>2</sup> (PF+NC - SB); T3 untreated seedlings sown in the plots mixed with FYM enriched with P. fluorescens 200 g/m<sup>2</sup> (PF+FYM - SB); T4- seedlings treated with *P. fluorescens* sown in plots containing neem cake enriched with P. fluorescens (PF+NC – SD+SB); T5- seedlings treated with P. fluorescens sown in plots containing FYM enriched with P. fluorescens (T1+T4) (PF+FYM - SD+SB); T6- Untreated seedlings transplanted in plots mixed with neem cake alone 20g/m<sup>2</sup> (NC - SB); T7- Untreated seedlings transplanted in plots mixed with FYM alone 20g/m<sup>2</sup> (FYM - SB); T8- seedlings treated with P. fluorescens were transplanted in plots mixed with neem cake alone  $20g/m^2$  (PF-SD +NC - SB); T9- seedlings treated with P. fluorescens were transplanted in plots mixed with FYM alone  $20g/m^2$  (PF-SD +FYM - SB); T10 untreated seedlings transplanted in the plot with out any treatment, served as Control. All the treatments were replicated five times in a completely randomized block design.

Observations on the shoot and root length, weight, root galling index of M. *incognita* on a 1~10 scale (Bridge and Page, 1980), percentage of disease incidence

by *P. cryotgea*, the yield per plot and percentage increase in the yield of gerbera at harvest (average of 30 times of harvest) were recorded.

Colonization of roots by *P. fluorescens* was also recorded at random by uprooting plants after 90 days from a plot. Root colonization by *P. fluorescens* was assessed by following the standard serial dilution technique. One gram of root sample was washed, grounded and the CFU was checked by the serial dilution followed by pour plate method. Serial dilutions up to  $10^{-6}$  concentration were prepared. 1ml from each of  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  dilutions were pipetted into the Petri dishes and spread completely in the plate. Freshly prepared King's B agar (*P. fluorescens*) media was poured into each plate and made to spread evenly by pour plate method and allowed for solidification. Three replicates for each dilution were maintained with controls and incubated at  $27\pm1^{\circ}$ C.

After 24 h, *P. fluorescens* colonies on King's B media (King *et al.*, 1954) emitting a pale green fluorescent light under UV at 302 nm were counted and CFU (Colony Forming Unit) was recorded. The data were analyzed using ANOVA.

## **Results and discussion**

Seedling treatment with *P. fluorescens* and application of neem cake enriched with *P. fluorescens* proved significantly effective in the management of disease complex caused by *M. incognita* and *P. cryptogea* on gerbera under field conditions (Table 2). There was a significant reduction in the root-knot index of *M. incognita* and percentage of disease incidence by *P. cryptogea* (Table 2).

*P. fluorescens* is known to control the pathogen by the mechanism called siderophoric effect. It was also found very effective in the control of various soil borne fungi causing root rot in certain vegetables and ornamental crops (Mukhopadhaya, 1987). Induction of growth promoting substances from the PGPR (Plant Growth Promoting rhizobacteria) and induction of systemic resistance against pathogenic fungi was reported by Ramamoorthy *et a.l.*, 2001. Application of neem cake 25 g/m<sup>2</sup> was found effective for the management of disease complex in gerbera cv. Debora in polyhouse conditions (Manoj Kumar *et al.*, 2010).

Seedling treatment followed by substrate treatment using the combination of *P. fluorescens* and neem was more effective followed by *P. fluorescens* and vermicompost than either of the treatments individually (Table2). It was also effective in reducing the root-knot index and also disease incidence by *P. cryptogea* (Table 2). Application of neem cake is proved to be very effective in the management of nematodes as it is reported to be controlling the entry of pathogens through variety of mechanisms including the production of antimicrobial compounds (Alam and Khan, 1980; Mankau, 1962; Muller and Gooch, 1982).

Root colonization of *P. fluorescens* was more when applied together with neem cake in comparison to individual treatments (Table 2). Higher the root colonization of *P. fluorescens*, higher the reduction in the root knot index.

There were significant increases in the shoot length, shoot weight and root length, root weight (Table 1). This could be due to the plant growth promoting activity of *P*. *fluorescens* which is very well documented (Bloemberg and Lugtenberg, 2001; Kishore *et al.*, 2003; Shouan Zhang *et al.*, 2003). *P. fluorescens* has been reported effective in the management of root-knot nematodes (Siddiqui *et al.*, 1999; Parveen *et al.*, 1998) and fungal pathogens (Cronin *et al.*, 1997; Johnson *et al.*, 1993; Xu and Gross, 1986).

Application of *P. fluorescens* with neem cake also did not affect the root colonization. Rather neem cake helped in the increased colonization of both *P. fluorescens* (Table 1).

There was significant increase in the yield of spikes per plot and the percentage increase in the yield was about 22.8% (Table 2). The combined application of *P*. *fluorescens* and neem cake has resulted in lesser root knot index which in turn showed reduction in the percentage of disease incidence by *P. cryptogea* (Table 2). As the entry of the fungus is mainly through the wounds, lesser the nematode infestation, lesser the wounds created and there by lesser the bacterial infection. So, ultimately the effect of combined application of *P. fluorescens* enriched in neem could significantly reduce the disease complex and there by increased the yield of the gerbera significantly.

TREATMENTS	Shoot length (tip of leaf to bottom of stem) (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)	Root colonization of <i>P. fluorescens</i> (CFU/g $\times$ 10 <sup>6</sup> )
T1 - PF-SD	15.0	24.1	41.9	22.0	1.03
T2 - PF+NC - SB	23.2	31.2	55.0	28.6	1.35
T3- PF+FYM - SB	19.2	26.6	47.2	25.2	1.31
T4- PF+NC – SD+SB	27.2	37.4	63.0	34.6	2.1
T5- PF+FYM – SD+SB	24.6	33.4	58.6	31.6	1.97
T6- NC – SB	19.4	27.0	51.0	27.0	0
T7 - FYM - SB	18.2	24.6	45.2	24.6	0
T8-PF-SD +NC - SB	22.1	28.0	52.4	28.2	1.78
T9 - PF-SD +FYM- SB	19.3	25.8	46.0	25.2	1.65
T10 - Control	11.6	17.6	36.6	17.6	0
CD - 5%	2.26	1.98	3.42	2.75	0.74

## Table 1 Effect of application of P. fluorescens enriched organic substrates on

**Plant growth parameters** 

CD Values are mean of 5 replicates

Additionally it was also found that the flowers harvested from the treatmental beds with neem cake enriched with *P. fluorescens* showed an increased vase life compared to control. This bio-management strategy helps the farmers to grow better quality flowers of gerbera in open field conditions with minimal losses due to the native soil pathogens.

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TREATMENTS	Root galling index on 1-10 Scale	% Decrease in galling index	Disease Incidence (%)	% Decrease in Disease incidence	% increase in yield
T1 - PF-SD	7.7	18.94	38.06	24.67	15.12
T2 - PF+NC - SB	6.3	3.68	20.04	60.34	21.97
T3- PF+FYM - SB	7.1	25.26	29.93	40.76	20.36
T4-PF+NC - SD+SB	4.1	56.84	18.16	64.06	28.52
T5- PF+FYM – SD+SB	5.3	44.21	25.77	49.00	26.92
T6- NC – SB	8.5	10.52	45.40	10.15	10.78
T7 - FYM - SB	9.3	2.10	49.49	2.05	9.63
T8-PF-SD +NC - SB	6.4	32.63	35.67	29.40	16.22
T9 - PF-SD +FYM- SB	7.4	22.10	35.61	29.52	15.97
T10 – Control	9.5	-	50.53	-	0
CD - 5%	0.44	-	-	-	-

#### Table 2 Bio-efficacy of P. fluorescens enriched organic substrates against

disease caused by M. incognita and P. cryotogea in gerbera

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## The effect of *Pseudomonas aeruginosa* 23<sub>1-1</sub> in protecting watermelon from wilt disease caused by *Fusarium oxysporum* f.sp. *niveum*

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## Abstract

Study the effect of antagonistic rhizobacterium *Pseudomonas aeruginosa*  $23_{1-1}$  in protect watermelon from wilt disease caused by Fusarium oxysporum. f.sp. niveum was conducted in in vitro, greenhouse and field condition. Results found that, in dual test Ps. aeruginosa 23<sub>1-1</sub> could inhibit Fusarium growth with inhibition zone  $6 \sim 12$ mm at 6 days after inoculation on PDA medium, extract of bacterial culture could inhibit the fungal spore germination. By seed soaking and soil drenching with Ps. *aeruginosa* 23<sub>1-1</sub> suspension ( $10^7$  or  $10^8$  cfu/ml) before, or after, or before plus after pathogen inoculation could reduce wilt disease infection in greenhouse condition; and two treatments show edgh disease protection are seed soaking plus soil drenching with Ps. aeruginosa 23  $_{1-1}$  (10<sup>8</sup> cfu/ml) before pathogen inoculation and treatment seed soaking plus soil drenching with *Ps. aeruginosa* 23  $_{1-1}$  (10<sup>8</sup> cfu/ml) before and after pathogen inoculation. In field conditions, all four treatments i.e. seed soaking plus soil drenching with bacterial suspension  $(10^7 \text{ or } 10^8 \text{ cfu/ml})$  with 7 days or 14 days interval showed effective in reduction of wilt disease. Especially, seed soaking plus soil drenching with *Ps. aeruginosa*  $23_{1-1}$  (10<sup>7</sup> cfu/ml) with 7 and 14 days intervals gave high and stable disease protection.

## Sources of resistance to cowpea (vigna unguiculata l.) bacterial leaf blight disease

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## Abstract

Cowpea [Vigna unguiculata (L.) Walp] is an important legume crop grown in tropical and subtropical countries. In addition to using green pods and grains for food, it is also grown as fodder crop. The production and productivity of cowpea is affected by both biotic and abiotic stresses. Bacterial blight of cowpea caused by Xanthomonas campestris py. vignicola is one of the important diseases of cowpea among biotic stresses which significantly reduce the yield and quality of cowpea all over the world. The identification and use of resistant sources against pests and diseases is a pre-requisite for any crop improvement programme. Although appropriate control measures for bacterial leaf blight are available under rainfed agriculture, the use of host plant resistance is the most economical and practicable method. In the present study 196 cowpea genotypes were screened for bacterial leaf blight under field conditions. Fifty genotypes showing resistant reactions under field conditions were selected for further confirmation under glasshouse condition with artificial inoculation. Ten plants of each genotype were artificially inoculated by pin prick method. Disease or symptom development which appeared after  $21 \sim 30$  days on each genotype was recorded at 15 days interval up to maturity and genotypes were evaluated. Among fifty genotypes tested, V-16 showed highly resistant, APC-140, HC-03-02 moderately resistant, GC-4, P-695 moderately susceptible and C-152 highly susceptible reactions. The resistant lines can be used as a donar in the breeding programme in development of cowpea varieties resistant to bacterial blight disease

Key words: Cowpea; Bacterial leaf blight; Xanthomonas campestris pv. vignicola

## Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important arid legume crop grown in tropical and subtropical countries in the world. It is consumed in the form of green pods and grain. In India it is grown on an area of approximately by 3.75 lakh hectares with annual production of 3.09 lakh tonnes and productivity of 824kg/ha (Indiastat.com 2010). The average productivity of cowpea in the existing traditional systems is low due to complexity of biotic and abiotic stresses. The biotic factors

include insect pests, bacterial, fungal and viral diseases. Concerted efforts are being made to develop improved cowpea varieties with resistance to these constraints. Several factors could be considered responsible for low productivity, but mostly grain yield is often affected drastically by adverse environmental conditions and out-break of epidemic diseases. In India four bacterial diseases have been documented to attack cowpea. Two of them are of economic importance they are, bacterial leaf blight induced by Xanthomonas campestris pv. vignicola (Burkholder Dye, 1944) and bacterial pustule (Xanthomonas campestris py. vignaeuguiculatae) (Rachie, 1985; Enechebe and Florins, 1997; Shoaga, 1998). Bacterial blight is the most widespread disease of cowpea, reported from all regions of the world where cowpea is cultivated (Emechebe and Florins, 1997). The other bacterial diseases of cowpea are bacterial wilt (Pseudomonas syringae pv. solanacearum) and halo blight (Pseudomonas syringae py. tabaci) (Emechebe and Florins, 1997). Bacterial leaf blight disease has been reported to induce yield loss of as high as 71% in pod, 68% in seed and 53% in fodder (Okechikwn et al., 2000). The identification and use of resistant sources against pests and diseases is an important component of genetic improvement programme. Although appropriate control of bacterial leaf blight can be achieved through several approaches, but the use of host plant resistance is the most economical and practicable method. In this context, the present study was carried out with the objective to identify resistant genotype to be used in breeding programme to develop bacterial blight resistant cowpea varieties.

## **Materials and Methods**

The experiment was conducted at the Zonal Agriculture Research Station, Gandhi Krishi Vigyan Kendra of University of Agriculture Sciences, Bangalore. Among 196 genotypes evaluated under field condition against bacterial blight, fifty genotypes (Table 2) showed resistant reaction under filed condition which were again evaluated under greenhouse condition against bacterial leaf blight disease with artificial inoculation.

The culture of the blight organism *Xanthomonas campestris* pv. *vignicola* used was isolated from the diseased sample. It was maintained at  $4\sim 6^{\circ}$ C in refrigerator and propagated at room temperature ( $28\sim 30^{\circ}$ C) on YDCA (Yeast Dextrose Calcium Carbonate Agar Medium, consists of Yeast extract 10gm, 20g each of Glucose and Calcium Carbonate, Agar Agar 15gm in one litre of distilled water, with pH of 7.2). The bacterial growth in YDCA after  $24\sim 48$  h was scrapped in a container and used directly as inoculum.

## Pathogenecity test

The bacterial isolate was multiplied separately in nutrient broth taken in Erlenmeyer flask by inoculating a loopful of 48 h old bacterial cultures separately.

The inoculated flasks were incubated for 3 days at 28 °C. Cowpea plants were raised in polythene bags containing sterilized soil. Three weeks old cowpea plants were divided into two sets. One set of plants were mildly injured with an insect mounting pin (pin prick method) and the second set of plants were not injured. The bacterial suspension in which the cell suspension was adjusted to  $7 \times 10^7$  cfu/ml was sprayed on to the plants with the help of an automizer. The sprayed plants were kept in 80% humidity in a humid tent made of plastic sheet for the next 48 hrs. Thereafter the plants were taken out from humid tent and kept in the glass house. The plants similarly sprayed with sterile water served as control. Observations were recorded for the development of symptoms on the plant.

## **Confirmation of the bacterial blight in infected cowpea leaves**

The leaves of cowpea plants inoculated with Xanthomonas campestris pv. vignicola showing the symptoms of bacterial blight i.e. yellow lesions were used for isolation of the bacteria in order to confirm the bacterial blight. The infected leaves were cut with the help of a pair of scissors from plants grown in the glass-house and taken into the lab. These leaves were then washed with autoclaved distilled water for  $2\sim3$  times. After that, the isolation of the bacteria was done by using the following method

- 1. Direct plating of infected leaves
- 2. Plating inoculated cowpea leaves using dilation method

## Table1 Disease scoring scale used in screening of cowpea genotypes

	Scale				
Diseases reaction	Percentage of leaves infected	Per cent leaf area affected			
Highly resistant	0~5	1~5			
Moderately resistant	5~10	5~15			
Moderately susceptible	10.20	15~25			
Highly susceptible	>20	>25			

#### against bacterial leaf blight (Khan, 1989)

After 48 h, plants were observed for presence of *Xanthomonas campestris* pv. *vignicola*. The same procedure was followed for all the cowpea genotypes inoculated with *Xanthomonas campestris* pv. *vignicola* showing the visual symptoms of blight. The disease severity was evaluated for fifty cowpea genotypes on the basis of symptom development and scored according to scale (Table 1) given by Khan (1989).

#### **Results and Discussion**

Since bacterial blight is a potentially destructive disease on cowpea in India, it is imperative, therefore, that sources of resistance to the disease be identified. Usually field screening for disease will not give the true picture of the resistance of disease as there is chance of disease escape. Hence, it is always essential to screen the genotypes with epiphytotic conditions with artificial inoculation.

In the present study YDCA medium was used for the growth of the bacterial strain. The incubation temperature for the bacterial growth was maintained at  $30^{\circ}$ C/48 h. Similarly Patel (1983) used  $28 \sim 30^{\circ}$ C incubation temperature for the growth of *Xanthomonas campestris* pv. *vignicola* on culture medium. After 24 hrs of incubation, yellow, smooth, convex and circular colonies were observed which turned somewhat irregular after 48h due to viscous fluid secreted by the bacteria. Similar types of culture and viscous colonies on PSPA (potato-sucrose-peptone-agar) medium were reported by Patel (1983).

Bacterial culture suspended in nutrient broth was used to inoculate cowpea plants. Similar technique was adopted by Liew and Alvarez (1981) for Xanthomonas campestris strains. Several inoculation techniques are available for inoculating X. *campestris* pv *vignicola* on cowpea plants, but they vary in their level of disease development and ease of application. In this study inoculation of cowpea plants with Xanthomonas campestris pv. vignicola was done using three different methods such as leaf cutting method, seed to plant transmission method and pinprick method. Among three methods followed pinprick method of artificial inoculation was showed better disease expression. Similarly Allen *et al.*(1981) asserted that the stem injection and leaf infiltration methods were reliable in glasshouse while foliar spray with wounding method of inoculation was appropriate for both glasshouse and field experiments. The interaction observed between the cowpea varieties and inoculation technique probably indicates that the mode of infection influences initiation of disease, but subsequent disease development will depend on cultivar susceptibility, method of infection, strain of the bacterium, inoculum concentration, and environmental factors. Similar conclusions were made by Aggour et al. (1989) while working with strains of Xanthomonas campestris pv. phaseoli in beans .

The symptoms produced on different parts of plants were, light yellow, irregular to round spots, measuring 4mm to 10 mm in diameter with necrotic brown centers appeared after  $21 \sim 30$  days of inoculation in susceptible varieties and increased irregularly until the whole leaf was covered. Severely affected leaves became straw colored and dropped at the slightest touch. A similar type of symptom expression was reported on cowpea infected with bacterial blight disease in India by Patel and Divan (1950). To confirm the symptoms showed by the inoculated cowpea plants were due to presence of *Xanthomonas campestris* pv. *vignicola*, diseased leaf bits were plated

on YDCA medium and the incubation temperature for the bacterial growth was maintained at  $30 \sim$  for 48h. The colonies produced were similar to the bacterial colonies used for inoculation, i.e. yellow, smooth and viscous (Fig.2).

The results of pathogenecity test on each genotype are summarized in Table 2. Among fifty genotypes tested under greenhouse condition nineteen genotypes showed highly resistant reaction, six genotypes were moderately resistant, thirteen showed moderately susceptible reaction and remaining twelve were highly susceptible reaction. The incidence and severity of bacterial blight varied with the cowpea genotype. Most of the genotypes showed moderate to severe infection. The highest disease severity was recorded on the line C-152 while genotypes V-16, V-585, APC-841 AND TCM-44 showed zero reaction.

The highly resistant genotypes of cowpea can be very well utilized by the breeders as source of resistance for transferring resistant genes to any susceptible genotype with good agronomic base. However, it is suggested to confirm the nature of resistance for other pathovars if any and also the resistant genotypes can tested for yield and other attributing characters over locations before considering it as a promising genotype

## Acknowledgment

The authors are grateful to Prof. Sir. Ed. Southern of Kirk house Trust, UK for funding a project on Cowpea and also Dr. Robert Koebner for technical support while carrying out the experiment.



## Figure 1 Symptoms of bacterial leaf blight infected cowpea plant. HC-03-02 (Moderately resistant), C-152 (Highly susceptible), V-16 (Highly resistant).

**Note:** HR- Highly Resistant, MR- Moderately resistant, MS-Moderately susceptible, HS- Highly susceptible



Figure 2 Typical colonies of *Xanthomonas campestris* pv. *vignicola* on a)Nutrient Agar b)YDCA media. Note the orange yellow pigmentation of colonies.

Sl.No.	Cultivar	Percentage of leaves infected	Per cent leaf area affected	Disease reaction
1	EC-458489	1.2	4.2	HR
2	NBC-14	5	3.8	HR
3	IC-402180	1	0	HR
4	CPD-19	2.2	4.5	HR
5	IC-202290	0	3.9	HR
6	KBC-2	1.6	0	HR
7	CB-10	5	0	HR
8	EC-394838	3	4.1	HR
9	HBC-51	2	1.2	HR
10	TCM-44	0	2.5	HR
11	APC-81	0	0	HR
12	EC-170584	4	2.8	HR
13	NBC-38	3.7	0	HR
14	HBC-41	4.4	4.5	HR
15	C-1071	2.8	5	HR
16	V-585	3.6	0	HR
17	EC-458440	0	2	HR
18	V-16	0	0	HR
19	NBC-43	1	4.5	HR
20	KM-5	5.1	6	MR
21	EC-170578	8.6	13.2	MR
22	GC-3	9.8	12	MR
23	HC-03-02	5	6.2	MR
24	APC-140	5.2	10	MR
25	IC-330996	6.2	6	MR
26	IC-4506	15	22.6	MS
27	EC-458506	10.42	13.6	MS

 Table 2
 Reaction of cowpea genotypes f0r resistance to bacterial leaf blight disease

28	EC-472252	10	25	MS
29	K5499-38	10.5	24.3	MS
30	NBC-32	11.6	15	MS
31	NBC-18	15.2	17	MS
32	NBC-39	16	19	MS
33	IC-201095(52)	17.2	20	MS
34	IC-402090	13	20	MS
35	EC-458473	10	20.5	MS
36	GC-4	10	19.2	MS
37	P-695	4.7	3.6	MS
38	IT-97	19	22	MS
39	EC-458417	26	30.2	HS
40	IC-402104	26.2	26	HS
41	HBC-9	29.5	28	HS
42	EC-472271	36.8	30	HS
43	EC-458402	25	29	HS
44	NB-12	20.1	26	HS
45	IC-402159	20	38	HS
46	NBC-148	25.8	25	HS
47	TVX-944	32	25	HS
48	IC-202711	30	25.00	HS
49	C-325	42.5	36	HS
50	C-152	3360	44	HS

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#### Efficacy of Bacillaceae and Bucellus against Ginger Rhizome Pythium Rot

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## Abstract

The inhibition effect of three *Bacillaceae* (*P. polymyxa*, *B. vallismortis*, *B.*sp.) and two different molecular weight chitosan on *Pythium* causing ginger rhizome rot was determined in lab culture. The results showed that all of the tested *Bacillaceae* showed strong inhibitory activity against Pythium and the *P. polymyxa* showed the highest activity. At different concentration, the inhibition caused by *P. polymyxa* on pathogens were  $54.23\% \sim 96.76\%$  and  $62.25\% \sim 93.57\%$ , respectively. *B. vallismortis* was less strong than *P. polymyxa*, and *B.sp*.was the weakest. In addition, there was obvious difference in inhibition effect of two different molecular weight chitosan on pathogens. The inhibitive effect of the low molecular weight chitosan was higher than middle molecular weight, and the EC<sub>50</sub> for mycelial growth of the pathogens was 771.8694 mg/L and 487.3678mg/L, respectively.

## Development and application of a new antifungal pesticide "Shenqinmycin" by genetically modifying the melon rhizosphereoriginating strain *Pseudomonas* sp. M18

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## Abstract

The development of "green" pesticides with high efficiency and low toxicity has aroused wide public interest in recent years. Phenazine-1-carboxylic acid (PCA) produced by pseudomonads has proven effectively again a range of soil-borne fungal phytopathogens and has great potential for development as a new kind of fungicide. Pseudomonas sp. M18, which was isolated from the melon rhizosphere, produces two different antibiotics, PCA and pyoluteorin. During the last decade, our lab has identified several signaling systems which are involved in the regulation of PCA biosynthesis in M18. Four rounds of genetic modifications, including inactivation of the quorum sensing repressor QscR and the global regulator GacA, and increasing the copy number of PCA biosynthesis cluster, have been conducted and PCA yield in the engineered strain has been significantly increased. Through optimizing the culture medium components, PCA yield was achieved as high as 5,000 mg/L, which is economically applicable for large-scale commercial purposes. Recently we have sequenced the genome of M18 and we found that seven genomic islands and six biocontrol-related gene clusters probably contribute to its biocontrol activities and living abilities in rhizosphere niches. We are currently investigating the mutual relationship of two PCA biosynthesis cluster, and the global regulatory network of PCA biosynthesis. PCA was commercially renamed as "Shenqinmycin" in China. The control effects of "Shenqinmycin" on rice sheath blight disease have been tested in field during  $2008 \sim 2009$  in 10 provinces of China. "Shenqinmycin" has been officially registered as a new pesticide against rice sheath blight disease in March of 2011.

## New Microbioassay for Discovery of Antagonistic Bacteria against Tobacco Black Shank

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## Abstract

We developed a simple, rapid, small-scale micro-bioassay for infection of tobacco seedlings by *Phytophthora parasitica* var. *nicotanae*. Twelve 14-day-old tobacco seedlings cultivated in the Petri dishes containing filter papers moistened with 5 ml dilution of Hoagland's solution, were inoculated with 10000 zoospores per ml of *P. parasitica*. After 96 h all of the inoculated seedlings of the susceptible cultivar, Honghuadajingyuan, were infected. One hundred and sixty pure bacteria were isolated from rhizosphere soil of tobacco, and screened for the protective ability against tobacco black shank. Result presented that 15 bacteria had high activity against *P. parasitica* on tobacco seedlings; they were identified by Biolog system and were distributed in *Bacillus amyloliquefaciens, Bacillus subtilis, Paenibacillus pabuli, Bacillus atrophaeus, Bacillus licheniformis, Bacillus pumilus and Providencia heimbachae*. This microassay proved to be a rapid, reproducible, and efficient method for screening of potential biological agents or microorganisms and may be useful for studying mechanisms of infection and control of *Phytophthora parasitica* under hydroponic conditions.

## Biological nitrogen fixation associated with sugarcane plants cultivated in Guangxi, China

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## Abstract

Guangxi is the major sugarcane and sugar producing area in China which produces over 60% sugarcane and sugar in China. Present sugarcane mean yields were approximately 70 ton/ha and nitrogen fertilizer inputs are  $500 \sim 700$  kg/ha in Guangxi. Under the high nitrogen fertilization, the number of nitrogen-fixing bacteria in sugarcane tissues is rather low. Using nitrogen-deficient medium to enrich diazotrophs, over 500 isolates were isolated from surface-sterilized roots, stems and leaves of major sugarcane cultivars in Guangxi. Using PCR amplification of *nifH* gene and acetylene reduction assay, 86 isolates were identified as diazotrophs. 16S rRNA gene sequence analysis revealed that the 86 nitrogen-fixing isolates were respectively affiliated to Alpha-, Beta-, and Gammaproteobacteria, and Actinobacteria, and that enterobacteria were prevalent. Inoculation of individual strains belong to Enterobacter, Klebsiella, Burkholderia, or Microbacterium, or the model strain Gluconacetobacter diazotrophicus PAL5 increased biomass and nitrogen content of micropropagated sugarcane cv. ROC22 seedlings grown in pots with one-third or half amount of the current nitrogen fertilization applied in the field in Guangxi. <sup>15</sup>N isotope dilution assays demonstrated that bacterial nitrogen fixation contributed to these sugarcane growth-promotions. Moreover, application of molybdenum increased the growth-promotion and nitrogen fixation. Promisingly, using reduced nitrogen fertilization and biological nitrogen fixation can develop a high benefit-cost ratio and environmentally benign sugarcane production in Guangxi.

## Diversity of endophytic diazotrophs isolated from wild rice and promotion of the growth of crops

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## Abstract

Nitrogen is the most frequent limiting nutrient in rice production. Maximum exploitation of biological nitrogen fixation will significantly contribute to long-term nitrogen nutrient availability to the rice crop. The southern region of China is one of the centers of rice and some wild rice species, diverse in their genetic diversity, hence diverse endophytic bacteria are expected in the wild rice species. The study is aimed to search and explore the diverse world of potential novel endophytic nitrogen-fixing bacteria living in wild rice tissues. Based upon these considerations, we have isolated and characterized some endophytic diazotrophs from different wild rice species. The aim of this study was to search potential novel endophytic nitrogen-fixing bacteria living in wild rice Oryza alta, O. australiensis, O. grandiglumis, O. latifolia, O. officinalis, O. meyeriana, O. punctata, O. rhizomatis grown in Wild Rice Core Collection Nursery, South China Agricultural University in Guangdong Province. A clustering analysis based on SDS-PAGE of whole-cell proteins, IS-PCR, ddT clusering, high resolution melting and 16S rRNA gene sequencing analysis were conducted. The function of the promotion of the plant growth (such as rice, Sonchus *loeraceus*, Sesbania cannabina), the tolerance to low pH soil and the flocculation of the industrial waste water and the waste dyes were tested. The descriptions of the novel nitrogen-fixing species was carried out.

## Diversity, indole-3-acetic acid and siderophore production of endophytic bacterial in peanut growing in four different soils

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## Abstracts

A total of 63 endophytic bacteria of the peanut from different soil types (brown soil, cinnamon soil, moisture soil, Sand soil ginger) were isolated using culture-dependent approaches. 22 IAA-producing strains and 18 siderophore-producing strains were obtained using Salkowski reagent and chrome azurol S (CAS) agar. The diversity of these endophytic bacteria was investigated based on amplified ribosomal DNA restriction analyses (ARDRA). The range of IAA production was 11.4-385µg/ml. The siderophore units range was  $0.2 \sim 1.6$ . The isolates which have the IAA-producing and siderophore-producing function were CS7, CS8, CS11, GS10 and GS12. The ARDRA showed that all the endophytic bacteria of the peanut were clustered into 4 groups at the similarity level of 80%. The strains from the brown soil were divided into 2 groups at the similarity level of 100%. In addition MS4, MS6, MS10, MS11, MS12, MS13 and MS14 isolated from the moisture soil were clustered into one group at the similarity level of 83%, and produces IAA except MS12. Our results revealed that the communities of the endophytic bacteria of the peanut and the agrotype have very big dependency. There is a strong association between the agrotype and the ability of the strains to generate IAA and siderophore. The present study is useful for exploitation of biofertilizer. But more detailed investigation is required for the application of these organisims in agricultural fields.

Key words: peanut; endophytic bacterial; ARDRA; IAA; siderophore

## Effects of PGPR strain PAB-2 on Growth promotion and Control of Fusarium-wilt of Banana Plantlets under Greenhouse Conditions

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## Abstract

Banana require large amount of chemical fertilizers which are costly and is hazardous to the environment when used excessively. Banana growth is seriously affected by the vascular wilt disease known as Fusarium wilt. Plant growth-promoting rhizobacteria (PGPR) are important in achieving a sustainable agriculture, and could play a vital role as substitution to commercially available fertilizer in crop production, to reduce environmental pollution and suppression of banana Fusarium-wilt. Experiments were conducted in the greenhouse at Soil and Fertilizer Research Institute to evaluate the effects of PGPR on growth, nutrient and bio-control of tissuecultured banana plantlets. The results indicated that a remarkable increase in root properties, such as length (47.6%) and weight (69.5%) with a PGPR strain PAB-2 (Bacillus sp.) inoculation, beside a higher shoot height (28.9%), pseudo-stem diameter (18.2%), and shoot biomass (33.9%). The inoculated plants showed higher formation of root hair which was visible within 7 days of inoculation; The total biomass (39.8%) was also increased due to PGPR inoculation. A substantial increase in nutrient accumulation (N 51.1%, P46.1%, K165.2%, CaO7.4% and MgO32.8%) was also observed in banana plantlets inoculated with PAB-2. The strain significantly reduced the impact of Fusarium wilt on banana, resulted in more than 46.9% reduction in foliar symptoms. The overall growth performance of inoculated seedlings was higher compared to un-inoculated control. This study suggests that PGPR strain PAB-2 (*Bacillus* sp.), could be used as crop-enhancer, bio-fertilizer, biocontrol agent to enhance better plantlets production and control of Fusarium wilt disease of banana.

**Key words:** plant growth-promoting rhizobacteria (PGPR); the banana fusarium-wilt disease; growth and nutrient effect; Greenhouse condition

#### Introduction

The banana (*Musa* spp.) production is one of the largest agricultural industries, providing employment and a cheap, nutritious food for thousands of farmers. The industry generates an annual income of hundreds of million. As an important fruit crop, Banana requires large amounts of chemical fertilizers which are costly and can be hazardous to the environments when are used excessively. However, the industry is seriously affected by the vascular wilt disease known as Fusarium wilt (Panama disease), caused by the soil borne fungus Fusarium oxysporum f. sp. cubense (FOC). Currently, no effective control method is available, and banana farmers have been forced to change to other crops. It is an important disease of banana in almost all banana-producing countries of the world. The recent occurrence of a highly virulent form of FOC in Asia, known as 'Tropical Race 4' (TR4), presents an imminent threat to the region's Cavendish-based banana industries. FOC4 has caused severe damage to the Cavendish banana industry of Taiwan since 1967. In 2004, severe FOC infection in banana plantations in Guangdong province, South China, heightened the FOC4 threat. The China government, private banana companies and Biodiversity International are collaborating to manage the disease and try to contain further spread of FOC4.

Plant growth promotion rhizobacteria are important in achieving a sustainable agricultural system. PGPR can play a vital role as substitution to commercially available fertilizer in crop production and reduction of environmental problem to some extent (Saravanan *et al.*, 2003; Hafeez *et al.*, 2006). The aim of this research was to develop an integrated control program, using biocontrol agent against Fusarium wilt of banana (Kang *et al.*, 2010). An experiment was conducted in the green house of Soil and Fertilizer Research Institute under greenhouse condition to evaluate the effects of PGPR (Plant Growth Promoting Rhizobacterial) inoculation on growth and nutrient of tissue-cultured banana plantlets under greenhouse condition.

#### **Materials and Methods**

This pot experiment was carried out at the greenhouse of Soil and Fertilizer Research Institute, GAAS (Guangdong Academy of Agricultural Sciences) from 12th July 2010 to 6th September 2010 for a total of 56 days. A PGPR strains and A banana fusarium-wilt pathogen strains were used in the experiments, namely, PAB-2 (*Bacillus* sp.) and FOC4 (*Fusarium oxysporum* f. sp. *cubense* race 4). The design of the experiment was completely randomized with six replications, one plantlet in each replicate. Four treatments were applied: 1)CK (control, without FOC4 and without PGPR); 2) PAB-2 (without FOC4 and with PAB-2); 3) FOC4 (with FOC4 and without PGPR); 4) FOC4+PAB-2(with FOC4 and with PGPR).

One tissue-cultured banana plantlet cv. "Cavendish" (AAA, *Musa*) (ex-laboratory, about  $10 \sim 11$ cm height of three-leafed stage) was planted in per plastic pot (0.5kg soil) and there after transferred to a larger polyethylene pot (10 kg soil). Before

transplanting, the plantlets were acclimatized for 5 days and the existing roots around the corm of the plantlets were removed gently with a sterile blade. The banana plantlets of transplant were grown under greenhouse conditions with temperature ranging from  $28^{\circ}$ C to  $30^{\circ}$ C for 56 days. Plants were treated with each of the four treatments. A 40-ml broth culture of PAB-2 and A 40-ml PD (Potato and Dextrose Medium) culture of FOC4 was applied to each respective pot prior to transplanting process. The same volume of sterile media (without inoculation) was applied to the control pots.

Measurements of morphological parameters, namely, plant height, pseudo-stem diameter, shoot biomass, primary root length, fresh weight of roots and total biomass were also taken. The separated plant parts oven dried at 70 °C for 48 h. Harvested shoot dry matter were prepared for chemical analyses. The oven-dried samples were ground in a Willey hammer mill, passed through a 2 mm sieve, mixed well and stored in plastic vials. The ground samples were digested by micro-Kjeldahl method (2000). The digested samples were analysed for N, P, K, Ca, Mg were determined by Auto-analyzer (Technicon II, Technicon Ltd.). Disease severity Intensity (DSI) of above-ground symptoms was measured every weeks and rating the symptoms on a scale of 0 to 5 (with 0 = healthy and 5= completely discoloured), Control effect =(Control DSI—Treatment DSI)/DSI×100%.

The collected data were analyzed statistically using the Statistical Analysis System (SAS, version 9.0, 2004). Following the analysis of variance procedure (ANOVA), differences among treatment means were determined using the Least Significant Difference (LSD) and Duncan's New Multiple Range Test (DMRT) comparison method (whenever applicable) at 5% level of significance.

## Results

## **Root Growth**

PGPR strain PAB-2 inoculation stimulated the root hair formation. The plants inoculated with PAB-2, initiated more root hairs compare to control treatments. Inoculation with PAB-2 increased significantly primary root length (47.6%) and the biomass of roots (69.5%); Inoculation with PAB-2 and FOC4 increased significantly primary root length (72.4%) and the biomass of roots (65.3%) compare to FOC4 treatments. However, PGPR inoculation did not increase the total number of primary roots (Table 1).

## Shoot Growth

The effect of PGPR strain PAB-2 inoculation resulted in more shoot growth compared to un-inoculated control plants. Plant height (28.9%), pseudo-stem diameter (18.2%), shoot biomass (33.9%) as well as total biomass content (39.8%)

were significantly increased in inoculated plants. The effect of PAB-2 and FOC4 inoculation resulted in more shoot growth compared to FOC4 treatments plants. Plant height (42.9%), pseudo-stem diameter (23.0%), shoot biomass (42.7%) as well as total biomass content (46.1%) were significantly increased in inoculated plants (Table 1). The number of leaves was not influenced by PGPR inoculation.

|--|

Treatment	Plant height (cm)	Pseudo-stem diameter(mm)	Biomass (g plant <sup>-1</sup> )	Root length (cm)	Biomass of root (g plant <sup>-1</sup> )	Total biomass (g plant <sup>-1</sup> )
СК	50.00 b	19.49bc	72.24 b	24.87 b	14.19 a	86.43 bc
PAB-2	64.43 d	23.04 d	96.76 c	36.70 c	24.05 c	120.82 d
FOC4	38.83 a	16.98 a	68.79ab	20.23 a	12.19 a	80.98 a
FOC4+PA B-2	55.50 c	20.89 c	98.14 c	34.87 c	20.15 b	118.29 d

and FOC4 strains for 56 days

Means having same letter(s) in a column do not differ significantly at 0.05 level by DMRT. The same below.

#### Nutrient accumulation

Plants were harvested 56 days after bacterial inoculation. PGPR strain PAB-2 significantly increased plant nutrition. Inoculated plants showed that shoot mineral content, i.e. N, P, K, CaO and MgO (51.1%, 46.1%, 165.2%, 7.4%, 32.8%) was significantly increased following application of microorganisms than non-treated control bananas. No adverse effect on plant health growth due to *Bacillus* spp. inoculation could be detected (Table 2).

Table 2	Shoot nutrient accumulation of tissue-cultured banana plantlets inoculated
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with PGPR strains PAB-2 and FOC grown for 56 days

Treatment	N (mg plant <sup>-1</sup> )	$P_2O_5$ (mg plant <sup>-1</sup> )	K <sub>2</sub> O (mg plant <sup>-1</sup> )	CaO (mg plant <sup>-1</sup> )	MgO (mg plant <sup>-1</sup> )
СК	203.49 a	32.86ab	195.09 a	312.28 c	15.87 b
PAB-2	307.47 d	48.00 d	517.34 d	335.29 d	21.08 d
FOC4	212.35ab	33.91bc	249.94 b	139.60 a	14.50 a
FOC4+PAB-2	269.69 c	48.60 d	388.81 c	178.71 b	18.07 c

#### Suppression of fusarium-wilt disease

Bananas foliar symptoms and DSI were investigated weekly after FOC and PAB-2 inoculation. PGPR strain PAB-2 significantly reduced the impact of Fusarium wilt on banana (Table 3). Inoculated plants showed that DSI of shoot (2.6%, 12.5%, 29.2%) were significantly reduced following application of microorganisms than FOC4-treated bananas (36.3%, 47.9%, 66.7%). PGPR strains PAB-2 resulted in more than 46.9% reduction in foliar symptoms.

Treatment	25d	35d	45d	55d	Control effects
FOC4	36.3	47.9	54.2	66.7	
FOC4+PAB- 2	2.6	12.5	29.2	35.4	46.9

with PGPR strains PAB-2 and FOC grown for 56 days

## Discussion

Plant growth-promoting rhizobacteria (PGPR) are microorganisms colonized in the plant rhizosphere, which can promote the plant growth and yield, and inhibit plant pathogens. Bacillus spp. is one of the most important groups of PGPR (Klopper et al., 1980, 1991; Hafeez et al., 2006). Our results demonstrate that the application of PAB-2 highly benefits banana plants and therefore could be considered a cropenhancer during the acclimatization stage of banana plantlets. The bacterial isolate PAB-2 (Bacillus sp.) has been identified as a potential biocontrol agent to manage Fusarium wilt disease caused by FOC4. The application of PAB-2 in formulated forms to the field is highly desirable to ensure its continuous survival in the soil despite the presence of unfavourable environmental conditions. Similar results were found in different cereal crops and tomato seedlings where PGPR inoculation enhanced root growth, shoot growth and uptake of mineral nutrient, the growth promoting, effects of PGPR inoculation are mainly derived from morphological and physiological changes in inoculated plant roots and enhancement in water and plant nutrient uptake (Jaizme-Vega et al., 2004; Rodriguez-Romero et al., 2005; Kang et al., 2010).

## Conclusions

The results of the experiment indicated that PGPR strain PAB-2 (*Bacillus* sp.) inoculation significantly increased the root properties (length 47.6% and biomass 69.5%), shoot growth, the plant height (28.9%), pseudo-stem diameter (18.2%), shoot biomass (33.9%) and total biomass content (39.8%) of banana plantlets grown under

greenhouse condition; A substantial increase in nutrient accumulation (N 51.1%, P 46.1%, K 165.2%, CaO 7.4% and MgO 32.8%) was also observed in banana plantlets inoculated with PAB-2; The strain significantly reduced the impact of Fusarium wilt on banana, resulted in more than 46.9% reduction in foliar symptoms. This study suggested that PGPR strains, PAB-2 (*Bacillus* sp.), can be used as crop-enhancer, bio-fertilizer, bio-control agent to enhance better plantlets production, control Fusarium wilt disease of bananas.

#### Acknowledgements

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# *PhoP/phoR* two-components systems sequences as a phylogenetic marker to differentiate the species in genus *Bacillus*

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## Abstract

The genus *Bacillus* contains the closely related taxa among species either from agricultural, industrial, and bio-technological and potential pathogens to humans. Therefore, it is becoming increasingly important for differentiation of isolates with a potential economic importance. However, the high degree of morphological and biochemical similarities and the high percentage of sequence similarity of 16S rDNA between closely related species poses a extreme difficulty for differentiation. In this study, a pair of degenerate primers was designed according to the two relatively conserved nucleotide sequences of phoP and phoR genes in the genus Bacillus, respectively. 28 Bacillus type strains were tested for the PCR amplification, and an expected successful amplification could be obtained in the tested strains. The phoP/phoR sequences similarities between the tested Bacillus type strains were 49.5% to 99.8% and the sequences divergence ranged from 0.3% to 85.6%. compared to the gyrB sequences, the phoP/phoR sequences showed a faster base substitution rate than the gyrB sequences at the interspecies or intraspecies levels, therefore, the phoP/phoR sequences could be used as a phylogenetic DNA marker for identification and phylogenetic studies of *Bacillus* genus. 45 *Bacillus*-like strains were tested for the identification and phylogenetic analysis based on the sequences of phoP/phoR. Results showed that the 45 strains could be clearly delineated into five distinct groups and the most strains belong to the B. subtilis group. These results indicted that the phoP/phoR sequences provided a higher genetic variation than the gyrB sequences and may be an alternative marker for phylogenetic and taxonomic analysis in the species of Bacillus.

Key words: Bacillus; gyrB; phoP/phoR; Phylogenetic

## Purification and properties of β-glucosidase from antifungal Streptomyces spp.R<sub>15</sub>

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## Abstract

Beta-glucosidase is an important component of the cellulose. It not only hydrolyzes cellobiose and cellooligosaccharide, but also prevent accumulation of cellobiose and reduce the inhibition of cellulase. By using of solid fermentation, fractional ammonium sulphate precipitation, ion-exchange chromatography on DEAE-52 and Sephadex G75 chromatography, beta-glucosidase from antifungal Streptomyces spp.R15 which was obtined from the chinese cabbage rhizosphere in the suburb of taian, Shandong province were purified. The results showed that purification folds and yield were 16.28% and 14.59%, respectively. The molecular mass of the enzyme was estimated to be 63.89kDa by 12% SDS-PAGE. To make an analysis of the enzyme reaction by TLC, beta-glucosidase can react with salicin to form D-glucose and salicyl alcohol, which proved that the protein was betaglucosidase. By using of salicin as substrate, the Km and Vmax values for betaglucosidase were 10.644mmol/L and 0.525umol•L<sup>-1</sup>•min<sup>-1</sup>, respectively. The optimum temperature was 65  $^{\circ}$ C, the beta-glucosidase was more stable when temperature was lower than 70°C. The optimum pH was 5, when pH was between 3 and 7, the relatively activity was above 75%.  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Fe^{2+}$  could inhibit the activity of beta-glucosidase, the relatively activity were 0, 3.60%, 62.98% respectively. The inhibitory effect of  $Hg^{2+}$ ,  $Cu^{2+}$  were the strongest of them, the activity was completely lost.  $Ca^{2+}$ ,  $Mn^{2+}$  could stimulate the activity, the relatively activity were 137.18%, 119.52% respectively. Fe<sup>3+</sup>, Ba<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, Al<sup>3+</sup> and Zn<sup>2+</sup> had no significant effect on the activity at the level P < 0.05.

Key words: Streptomyces; beta-glucosidase; purification; property; enzyme reaction

# Cloning and sequence analysis of *rrdA<sub>mgh</sub>* regulatory gene of *Streptomyces roseoflavus* Men-myco-93-63

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## Abstract

Streptomyces roseoflavus Men-myco-93-63 isolated from potato scab decline soil is an antagonistic strain, which has a very strong inhibition to Verticillium dahliae. In the field and greenhouse experiments, the strain showed very good control to many important pathogenic fungi, such as Verticillium dahliae, Sphaerotheca fuliginea, and Botrytis cinerea. The strain has shown a great potential on biological control of plant diseases. rrdA (regulator of redD) gene is a regulator gene, which was first found in S. coelicolor. The gene encoded a TetR family protein negatively regulated the production of prodigiosin. The 393 bp gene fragment sequences of Men-myco-93-63 was amplified by the primer sequences designed from S. coelicolor A3(2). The sequence with the S. coelicolor A3(2) rrdA gene at nucleotide sequence homology reached 100%. The full length gene sequence of rrdA gene of Men-myco-93-63 was amplified with primers of rrdA-1 and rrdA-2, which has a complete open reading frame encoding 221 amino acids, with the S. coelicolor A3(2) rrdA gene at nucleotide sequence and amino acid sequene homology reached 80%. The clone of the gene was named as rrdA<sub>mgh</sub>.

Key words: Streptomyces roseoflavus Men-myco-93-63; regulator gene; sequence analysis

## Genotypic characterization and plant growth-promoting ability of four PGPR strains from mangrove

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#### Abstract

Mangrove forest is known as highly productive ecosystem that provides large quantities of organic matter to adjacent coastal water in the form of debris and live animals. Nevertheless, mangrove forests are nutrient-deficient ecosystems, especially of nitrogen and phosphorus, which are essential for plant growth. Artificial reforestation inoculated with PGPR might be a potential tool for mangrove reforestation. The present study was to identify four PGPR with phosphatesolubilization and nitrogen-fixation capacity, and examine plant growth-promoting ability of strains through inoculation. Four PGPR were genetic analyzed by PCR detection of nifH and 16S rRNA gene. The phosphate-solubilizing ability and nitrogen-fixation capacity were examined by spectrophotometric quantification and acetylene reduction assay, respectively. And strains were also inoculated onto mangrove seedlings to evaluate the effect of plant growth. Phylogenetic analyses based on nifH and 16S rRNA gene sequences indicated that HN011 was mostly related to Vibrio natriegens, and SZ7-1 and SZ7-2 resembled Klebsiella oxytoca. Although similarity of 16S rRNA sequence showed that SZ002 belong to Paenibacillus sp., nifH gene of SZ002 were high level of sequence similarities with Klebsiella genus. This phenomenon could attribute to gene transfer. Phosphate solubilization showed that four PGPR solubilized insoluble phosphate well in liquid medium, and nitrogen-fixation capacity of strains was high. Inoculation showed that strains could benefit plant growth, and the dry weight, total N and total P in some inoculated plants were significantly higher than in noninoculated plants. This is first investigation of PGPR isolated from mangrove with dual ability of phosphate solubilization and nitrogen fixation, and strains could benefit mangrove growth. This work may support the use of PGPR as inoculants for mangrove reforestation.

## Isolation and identification of endophytic bacteria with antipathogenic and nitrogen-fixing functions of Rice

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## Abstract

Inhibition and PCR amplification of *nifH* gene fragments of nitrogen-fixing bacteria which were isolated from roots, stems and leaves of rice collected in Lufeng of Yunnan province were determined in this research.111 isolates were obtained by nitrogen-free medium. It showed that endophytic bacteria in different parts had different density, with peak value in roots and the lowest density in stems. Among the 111 isolates,35 endogenetic strains showed suppression effect on two main bacteria diseases, Xanthomonas oryzae pv. oryzae and Xanthomonas oryzae pv. oryzicola of rice. 16 strains were identified as nitrogen-fixers by using PCR amplification of nifH gene fragments. 3 strains, (N-18, N-64 and N-79), which were isolated from roots ,had both functions. Fungistatic effect of the 3 Strains showed that in addition to the three major diseases in rice(bakanae disease, rice blast and banded sclerotial blight), they also had high suppression effect on some other 15 important plant diseases, such as pepper wilt, tobacco wilt, wheat snow mold etc. 16S rDNA sequence analysis showed that N-18 and N-79 were identified as Paenibacillus polymyxa and N-64 was identified as B. subtilis. The experiment above showed the 3 nitrogen-fixing bacteria isolated and identified in this study had potential value on agriculture.

Key words: nitrogen-fixation; endogeny; nifH gene; 16S rDNA

## Colonisation of *Pseudomonas chlororaphis* TSAU13 and *Pseudomonas extremorientalis* TSAU20 in the rhizosphere of wheat under salt stress

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## Abstract

The aim of the work was to evaluate the effect of salinity on the colonisation of *Pseudomonas putida* TSAU13 and *Pseudomonas extremorientalis* TSAU20 in the rhizosphere of wheat. The study was conducted in pot experiment using a potting soil under controlled greenhouse conditions. Salinity treatments were established by adding 0, 25, 50, 75, 100 and 125 mM of NaCl and the pots were irrigated with NaCl solutions. *Pseudomonas cholororaphis* TSAU13 and *P .extremorientalis* TSAU20 were able to colonize in the rhizosphere of wheat under saline conditions up to 125 mM NaCl. The strains increased wheat growth compared to the control that was exposed to stress at each NaCl (25, 50, 75, 100mM) concentration tested. Our salt tolerant bacterial strains were able to produce IAA even at 3% NaCl, This study demonstrates that salt tolerant bacterial strains could be a new approach to increasing the salinity tolerance of wheat under salinity conditions.

Key words: wheat; colonization; salinity; rhizosphere bacteria

## Distribution pattern of endophytic bacteria in Eucalyptus urophylla

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## Abstract

To understand the role of endophytic bacteria in *Eucalyptus urophylla* in disease resistance to bacterial wilt caused by *Ralstonia solanacearum*, distribution patterns of these bacteria in *E. urophylla* were investigated using light microscopy, haemocytometer counts, scanning electron microscopy, and denaturing gradient gel electrophoresis (DGGE). Light microscopical observation and enumeration by haemocytometer measurements revealed high population densities of endophytic bacteria in root, stem and leaf tissues of E. urophylla seedlings, with an average population density of about 9.3 log CFU/g fresh tissue. The population densities of endophytic bacteria in roots (9.7 log CFU/g) were significantly higher than those in leaves (8.7 log CFU/g), whereas those in the stem were intermediate (9.3 log CFU/g). Scanning electron microscopy showed coccus and rod shaped endophytic bacteria in transverse and longitudinal sections of different organs, but in the stem apex, up to 0.12 mm, no endophytic bacteria were found. DGGE fingerprinting of 16S rDNA amplicons indicated that endophytic bacterial communities in root, leaf and stem are different, suggesting that different bacterial species exist in different organs of E. *urophylla* seedlings. The absence of bacterial cells in the apical stem of *E. urophylla*, establishes a basis for obtaining bacteria-free eucalypt materials.

## Rhizobacteria of sunflower: *In-vitro* Study for their plant growth promoting potentials

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## Abstract

Looking to the global scenario, the problem of environmental pollution is of much concern today. Sunflower being an oleaginous plant, is used in the bioremediation and phytoremediation studies. Here the study of beneficial bacteria of its rhizosphere and their plant promoting activities are studied in-vitro. About 42 bacteria were isolated from different sites i.e. bulk soil, rhizosphere and endorhizosphere regions of the plant. Their morphological, colonial, phenotypic, biochemical and nutritional characteristics were studied. They mainly belonged to the Azotobacter and Pseudomonads group. The phytohormone study showed that indole acetic acid was present in 10 isolates, Gibberellic acid was observed in 8. Another direct mechanism of plant growth promotion is making available insoluble Phosphorous to the plant. 5 isolates showed solubilization of Phosphorous giving an SI of 2.3-3. The production of lytic and detoxification enzymes by the bacteria is an indirect mechanism for stimulating plant growth. Protease production was observed in 4 isolates. Amylase and Lipase was detected in 11 and cellulase was seen to be present in 5. Study of antifungal activity showed that 69% of isolates inhibited the growth of Fusarium, 47% were antagonistic against Aspergillus, 62% inhibited the growth of Curvularia and 57% were antagonistic against *Helminthosporium*. The other antagonistic mechanisms studied were the production of Ammonia  $(NH_3)$  that was detected in 7 and HCN production was observed in 2 of the isolates. Seed germination in plate assays showed that 12 isolates gave 100% germination after 72 h. Bacterized seeds had elongated roots and shoots as compared to control. Among these, 10 isolates were exhibiting several of the plant growth promoting potentials tested. From these a consortium could be selected which would show ideal plant growth promoting activities to be used as bioinoculants.

Key words: PGPR; Sunflower; phytohormone; phytoremediation
# Bio-efficacy studies on Prathista soluble organic fertilizers through fertigation in sugarcane

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#### Abstract

Sugarcane is an industrially important commercial crop grown in India. Yield, sugar, jaggery production and consumption in sugarcane are majorly determined by healthy agronomic practices and the type of fertilizer application plays a pivotal role. In the present study, different soluble organic fertilizers manufactured by Prathista Industries Limited (PIL), Hyderabad, India were evaluated through fertigation. Field studies were taken up during the year, 2010 at Agricultural Research Station, Perumallaple in a Randomised Completely Block Design with three replications per treatment to study their comparative efficacy over the application of chemical fertilisers in sandy loam soils with an early maturing sugarcane variety, 2003V46. Seven treatments were imposed involving organic and inorganic fertilizers and their combinations. In organic fertilizer treatments, nitrogen was supplied through Prathista organic nitrogen, phosphorous through Bio phos and potassium through Bio potash as per the requirement of crop. A common dose of Aishwarya (Prathista organic manure) 100kg/acre was applied to all the treatments except for control where NPK were supplied exclusively (100%) through chemical fertilizers. Fertigation to the crop was scheduled depending on crop growth starting from 20 days of planting upto 180 days. Mean length of canes, mean diameter of canes, millable canes/ha, cane yield t/ha, juice extraction percent, juice quality parameters viz., brix percentage, sucrose *percentage*, CCS *percentage* and CCS yield t/ha and jaggery quality were evaluated. The results indicated that 100% Prathista organic soluble NPK bio-fertilizers through fertigation in sugarcane contributed to higher cane yield, more number of millable canes, high quality juice and jaggery over compare to recommended dose of chemical fertilizers (RCF) (standard check) and also other treatment combinations except the treatment combination of 50% Organic and 50% inorganic .Higher B:C ratio with soluble organic fertilizers through fertigation compared to inorganic chemical fertilizers was observed. Application of organic soluble fertilizers contributed not only to overall increase of yield and quality of sugarcane but also to reduce cost of cultivation.

# Introduction

Sugarcane is a major cash crop in India responsible for the overall socio economic development of the farming community. It is cultivated on 5.15 million hectares providing an annual sugarcane production of 340 million tonnes ( $2008 \sim 2009$ ). Average productivity is thus relatively low, at 66 mt /ha. Currently India consumes about 18.5 million tones of sugar but to meet the demands of an increasing population, there will be a need to produce 28 million metric tones of sugar by 2015. Water and nutrient management play an important role in sugarcane production. Improper management of water and imbalanced nutrition are the main constraints to increased productivity. It is imperative to increase sugarcane productivity through modern and precise methods of cultivation, including fertigation.

Sugarcane is a long duration crop which produces huge amounts of biomass, requiring large quantities of water and nutrients. The crop requires 400 m<sup>3</sup> of water to produce one metric tonne of total dry matter and 200m<sup>3</sup> of water to produce one metric tonne of cane. Nutrient requirement of the crop is also very high. Fertilizer use efficiency is low under conventional method of irrigation. Considerable variations are noted in the absorption and uptake of nutrients and water by sugarcane crop from the soil. Adoption of drip irrigation to sugarcane offers an opportunity for placing fertilizers in a soluble form at the root zone of the crop along with the irrigation water, thus increasing water and fertilizer use efficiency. Fertigation ensures that essential nutrients are supplied precisely at the area of most interactive root activity according to the specific requirement of sugarcane crop and type of soil, there by resulting in higher cane yields and sugar recovery (Schumann, 2000).

During the past few decades, intensive agriculture involving exhaustive high yielding varieties has led to heavy withdrawal of nutrients from the soil. Generally excessive amounts of inorganic fertilizers are applied to sugarcane in order to achieve high yield and maximum growth. However the use of inorganic fertilizers alone may cause problem for human health and environment (Arisha et al., 1999). Long term studies on various crops indicated that the balanced use of NPK fertilizers could not maintain the higher yields over years because of emergence of secondary and micronutrient deficiencies and deterioration of soil physical properties. Use of organic manures alone cannot fulfill the crop nutrient requirement (Kondapa et al., 2009). Bokhtiar et al. (2008) reported that organic manures, when applied with chemical fertilizers gave better yield than individual ones. In recent years, consumers are demanding higher quality and safer food and highly interested in organic products (Ouda et al., 2008). Hence there is urgent need to improve organic fertilizers with natural materials through biological processes. In this context, in order to improve fertilizer use efficiency and soil fertility, Prathista Industries Limited, has developed unique fermentation technology to manufacture various organic inputs/ manures,

which are totally bio available and are capable of supplying all major plant nutrients and micronutrients in readily absorbable form for all agricultural crops meeting their nutrient requirement at lower dosages. The present study is carried out to evaluate bio efficacy of Prathista organic fertilizers through fertigation in sugarcane in sandy loam soils.

# Materials and Methods

Field studies were carried out during  $2010 \sim 2011$  at Agricultural Research Station, Perumallapalle, Andhra Pradesh. The soils of the experimental site are sandy loam in texture, neutral in reaction, non saline in nature, low in organic carbon, low in available nitrogen, medium in available phosphorus and high in available potassium (Table 1). The experiment was laid out in Randomized Completely Block design with three replications per treatment to study their comparative efficacy over the application of chemical fertilizers in sandy loam soils with an early maturing variety, 2003 V 46. Seven treatments were imposed involving organic and inorganic fertilizers and their combinations viz., T1: 100% RDF through fertigation using available inorganic chemical fertilizers; T2: 100% Prathista organic fertilizers through fertigation;T3: 100% N through FYM;T4: 25% RDF through chemical fertilizers + 75% through prathista organic fertilizers through fertigation; T5: 100% P through Prathista Bio Phos (N & K as inorganic);T6: 100% K through Prathista Bio Phos (N & P as inorganic);T7: 50% RDF through chemical fertilizers + 50% through Prathista organic fertilizers through fertigation. A common dose of Aishwarya (Prathista Organic manure) 100 kg/acre was applied to all the treatments except for control. Fertigation to the crop was scheduled depending on crop growth starting from 20 days after planting up to 180 days. Each treatment consisted of 8 rows 8m length with paired row spacing of  $60 \text{cm} \times 120 \text{cm}$  and sowing was done with overlapping method using 40,000 three budded setts /ha. All the agronomic practices like irrigations, weeding, earthing up, propping and other cultural operations were carried out uniformly in all the treatments. Crop was harvested at the age of completion of 10 months.

Data on yield parameters lke number of miilable canes (NMC)/ha, cane length, cane diameter and cane yield were recored at harvest. Juice quality parameters viz., sucrose %, purity % and CCS % were recorded by following standard procedures (Spencer and Meade, 1963). Sugar yield (t/ha) was computed by using the formula, cane yield t/ha  $\times$  CCS % /100. Jaggery was prepared at harvest according to treatments and dried for two days under shade. Samples were collected for analysis of quality parameters. Quality of jaggery in terms of color intensity, hardness, ash content and net rendement value (NRV) was evaluated according to standard procedures (Asokan, 1983). Data on soil beneficial micro flora was recorded by following standard procedures. Data collected were statistically analyzed and the results were presented.

# **Results and discussion**

Results pertaining to effect of application of organic and inorganic fertilizers and their combinations on growth parameters, yield, juice quality, jaggery quality and beneficial micro flora of sugarcane crop are presented and discussed.

# **Growth parameters**

Growth observations including number of millable canes (NMC), cane length, cane and diameter and number of internodes were recorded at harvest and data are presented (Table2) and discussed. Number of millable canes at harvest was significantly influenced by the application of organic fertilizers through fertgation. Significantly highest NMC('000/ha) was recorded with T2 where 100% recommended nutrients are applied in the form of Prathista NPK through fertigation followed by T1,100% RDF in the form of inorganic fertilizers through fertigation over other treatments and on par with combination of organic NPK and inorganic fertilizers, T7 & T4, respectively. Lowest NMC was recorded with T3, nutrients were applied through FYM. The millable cane length at harvest varied from  $270 \sim 301$  cm between treatments. Significant differences between cane length and cane diameter with the application of fertigation treatments were observed except in T3, T4, T5 & T6. Number of internodes followed the same trend that of length of cane. Highest number of internodes was observed with T1 (25.02) and T2 (25.00). Significantly highest number of internodes was observed with FYM applied treatment (19.90).

# **Cane yield**

Cane yield significantly affected by the application organic fertilizers through fertigation (Table 3). Significantly highest cane yield of 118.97 t/ha & 117.85t/ha was recorded with T1 (100% inorganic fertilizers) and T2 (100% Prathista NPK), respectively. These treatments were on par with T7 (50% inorganic & 50% organic NPK) and superior over all other treatments. Lowest cane yield (96.78 t/ha) was recorded in treatment T3, where 100% recommended nutrients were applied through FYM.

# Juice quality

Though there was no significant difference was observed between the treatments with sucrose %, highest sucrose (20.18) was recorded with T2 (100% Prathista NPK) and the similar trend was observed with CCS %. CCS 5 at harvest was highest in the same treatment. However, with the additional cane yield, CCS yield was significantly influenced by the application of organic fertilizers through fertigation. Significantly

highest CCS yield of 16.27 t/ha was recorded in T2 followed by T1 (15.44 t/ha) over other treatments. On par CCS yield was recoded in T7 (50% inorganic & 50% organic NPK). These results show that in terms of gained income, which is strongly related to the CCS yield, farmers can achieve the highest income with the treatment T2, which is significantly higher than the other treatments.

# Jaggery quality

Jaggery quality in terms of color intensity, sucrose% and NRV was analyzed and presented in Table 4. Jaggery yield was highest in T2 (11.3 t/ha) followed by T1 and T7. As jaggery yield is dependent on cane yield, it followed the same trends that of cane yield. Highest sucrose % and lowest EC was recorded in the same treatment, which indicates less hygroscopic nature and good keeping quality of jaggery. Low color intensity, which indicates good color of jaggery was also lowest in T2. High Net Rendement Value (NRV) was recorded in T2 followed by T7 & T4, which can be graded as excellent.

# Effect on beneficial micriflora

Estimations on population levels of beneficial microflora in sugarcane rhizosphere at harvest yielded interesting results. Among different treatments evaluated, the population levels of *Trichoderma* spp. were highest in plots applied with 100% Organic fertilizers supplied by Prathista Industries Limited, Hyderabad India and in plots treated with 100% Farm Yard Manure ( $8.8 \times 10^4$  and  $8.3 \times 10^4$  CFU/g soil respectively). The population levels of *Trichoderma* spp were also highest in plots treated with 100% Farm Yard Manure ( $8.3 \times 10^4$  CFU/g soil). However, no significant differences were noticed between these two treatments. The control plots recorded population levels of  $3.2 \times 10^4$  CFU/g soil. The treatments involving Organic Phosphorus and Organic Potassium (T6 and T5) when applied with other inorganic fertilizers were however not significantly superior over control (Table 5).

The population levels of the bacterial antagonists, *Pseudomonas fluorescens* were also estimated at harvest. Of different treatments, highest population levels of *P*. *fluorescens* were recorded in plots that received 100% Organic fertilizers supplied by Prathista Industries Limited, Hyderabad India  $(6.3 \times 10^6 \text{ CFU/g} \text{ soil})$ . This was followed by in treatment that received RDF as 100% FYM  $(5.2 \times 10^6 \text{ CFU/g} \text{ soil})$ . All the treatments were significantly superior over control. The population levels of P. fluorescens in control are about  $1.9 \times 10^6 \text{ CFU/g}$  soil (Table 5).

# **Economics**

Cost of cultivation of sugarcane for each treatment, including drip irrigation system was calculated. Considering yield levels in these treatments and sugarcane price, the income was arrived treatment wise. Net income was calculated and Cost: Benefit ratio was presented in Table 3. Highest C:B ratio of 1.98 was obtained with 100% Prathista Organic NPK treatment compared to 100% inorganic fertilizers (1.61).

#### Conclusions

The results indicated that 100% Prathista organic soluble NPK bio fertilizers through fertigation in sugarcane contributed to higher cane yield, more number of millable canes, high quality juice and jaggery over other treatments. Higher B:C ratio with soluble organic fertilizers through fertigation compared to inorganic chemical fertilizers was observed. Use of recommended dose of fertilizers through chemicals can be reduced by 50% or by 100% with the application of Prathista organic NPK fertilizers. Application of organic soluble fertilizers contributed not only to overall increase of yield and quality of sugarcane but also to reduce cost of cultivation.

рН	7.22
EC (dS/m)	0.22
Organic carbon(%)	0.21
Available Nitrogen (kg/ha)	196.26
Available Phosphorus (kg/ha)	14.80
Available Potassium (kg/ha)	285.75
Texture	Sandy loam

 Table 1
 Physico-chemical properties of experimental site

# Table 2 Effect of application of Prathista soluble organic fertilizers through fertigation on growth parameters of sugarcane

Treatments	NMC '000/ha	Cane length (cm)	Cane diameter (cm)	No of internodes
T1	107.86*	300.78*	3.35*	25.02*
T2	108.50*	299.54*	3.27*	25.00*
T3	94.75	270.16	2.36	19.90
T4	101.28	297.16*	2.95	22.18
T5	95.68	273.75	2.47	21.12
T6	97.89	274.19	2.53	22.08
T7	106.77*	298.06*	3.31*	24.77*
SE+/-	2.66	2.81	0.07	0.31
CD @ 5%	6.84	8.72	0.21	1.08

\* Statistically significant data as compared to other treatments

 Table 3 Effect of application of Prathista soluble organic fertilizers through fertigation on cane yield and juice quality parameters of sugarcane

Treatments	Cane yield (t/ha)	Sucrose (%)	CCS (%)	CCS Yield (t/ha)	C : B ratio
T1	118.97*	19.78	12.98	15.44*	1.60
T2	117.85*	20.18	13.82	16.27*	1.98*
T3	96.78	19.10	12.78	12.37	1.43
T4	106.55	19.31	12.97	13.82	1.58
T5	98.34	19.78	13.17	12.95	1.56
T6	99.78	20.07	12.99	12.96	1.58
T7	114.98*	19.34	13.29	15.28*	1.88*
SE+/-	2.82	1.92	0.16	0.21	0.022
CD @ 5%	6.98	NS	NS	0.95	0.066

\* Statistically significant data as compared to other treatments

Table 4	Effect of application	of Prathista soluble of	organic fertilizers	throughfertigation
			8	

Treatments	Jaggery yield (t/ha)	Brix (%)	Sucrose (%)	EC (dS/m)	color intensity (%)	NRV	Grade
T1	10.9*	90.92	80.77	0.382	62.33	48.78	Medium
T2	11.3*	92.98	82.56	0.131	48.75	67.64*	Excellent
Т3	8.5	90.11	80.81	0.256	56.35	48.98	Medium
T4	9.7	91.89	81.65	0.184	52.18	64.23*	Good
T5	8.7	90.11	80.81	0.256	56.35	47.15	Medium
T6	8.8	90.35	80.15	0.375	58.90	47.50	Medium
T7	10.5*	91.53	80.96	0.105	50.15	61.33*	Good

#### on jaggery yield and quality

\* Statistically significant data as compared to other treatments

m i i	Population levels of Antagonists in Rhizosphere		
Treatment	$Trichoderma spp^2$	Pseudomonas fluorescens	
	$(\times 10^{4} \text{ CFU/g soil})$	$(\times 10^{\circ} \text{ CFU/g soil})^{\circ}$	
T1: 100% RDF (as inorganic) (Control)	3.2 <sup>ef</sup>	1.9 <sup>g</sup>	
T2: 100% Organic fertilizers	$8.8^{\mathrm{a}}$	6.3 <sup>a</sup>	
T3: 100% RDF as FYM	8.3 <sup>ab</sup>	5.2 <sup>b</sup>	
T4: 25%	6.8 <sup>c</sup>	4 Q <sup>bc</sup>	
RDF through inorganic + 75% Organics	0.8	4.2	
T5: 100% Phosphorus through Biophos	3 2 <sup>ef</sup>	2 1 <sup>ef</sup>	
(100% N & K as inorganic)	5.2	2.1	
T6: 100% Potash through Biopotash	3 1 <sup>e</sup>	2 3 <sup>e</sup>	
(100% N & P as inorganic)	5.4	2.5	
T7: 50% RDF through Inorganic and	1 8d	$2 0^d$	
50% through Organic fertilizers	4.0	2.9	

# Table 5 Effect of Prathista soluble Organic Fertilizer Application in Sugarcane on organisms (*Trichoderma* spp. & fluorescent Pseudomonads) Beneficial Micro

Values are means of five replications

<sup>1</sup>Fertilizers were applied as different combinations of Recommended doses of Fertilizers (RDF) using organic fertilizers manufactured by Prathista Industries Limited, AP, India ; <sup>1</sup>Fertilizers were applied from 20 days after planting up to 180 days after planting at an interval of 10 days depending on crop growth.

<sup>2</sup>Populations of *Trichoderma* spp. were estimated at harvest on *Trichoderma* specific medium and expressed as colony forming Units per gram soil; <sup>3</sup> Populations of Pseudomonas *fluorescens* were estimated at harvest on King's B Medium and expressed as colony forming units per gram soil. Means followed by a common letter in the columns are not significantly different according to LSD (at P<0.05)

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# Disease-preventing and growth-promoting effects of antifungal bacteria against *Phytophthora nicotianae* on tobacco

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#### Abstract

Tobacco black shank is a severe soil spread disease of tobacco caused by *Phytophthora parasitica* var. *nicotiana*. Pot experimental studies were conducted to evaluate the potential of the use of plant growth-promoting rhizobacteria (PGPR) for control of tobacco black shank, and the ability of plant growth-promoting. Strains YC0573 and YC0136 are 2 *Paenibacillus polymyxas* screened from tobacco rhizospheric soil. Both of them can significantly antagonize the *Phytophthora parasitica* var. *nicotiana in vitro*. In addition, YC0136 can also antagonize the *Ralstonia solanacearum* which causes the tobacco bacterial wilt. Potting test under greenhouse has 4 treatments: (1, 2) YC0573 or YC0136 is applied as a soil drench when the seedlings transplanted respectively. For the best effect of disease control, the seedlings' roots are soaked in the suspension of the strain YC0573 or YC0136 for 30 minutes before transplanted, and drench the pathogen the next day; (3)metalaxyl was applied as control; (4) drench the pathogen alone as the disease control.

We found that PGPR strains YC0573 and YC0136 applied for  $1 \times 10^8$  CFU/ml significantly (*P*<0.05) reduced disease severity compared to the disease control. Control efficiencies of YC0573 were 90.9% in 35 days after transplanting while those of YC0136 were 95.5%, both of them are better than metalaxyl control(86.4%).

Studying on agronomic traits of different growth stages of tobaccos, we found that the treatments with YC0573 and YC0136 were better than the treatment of metalaxyl and the disease control, especially at the 60th day after transplanting. The results showed that stem height, stem girth, number of leaves, the biggest leaf length, the biggest leaf width and the area of largest leaf of tobacco increased with the application of PGPR strains YC0573 and YC0136. The differences are significant (P<0.05).

Studying on some of defense enzyme activity of tobacco leaves at the 30th, 60th and 90th days after transplanting, the treatments with PGPR strains demonstrated higher levels of the activities of PAL, PPO, POD, SOD and CAT, the differences are significant (P<0.05) at the 30th and 60th days. This just shows that PGPR strains YC0573 and YC0136 can induce system resistance of tobaccos (ISR), and the effects mainly reflect at the early stage and mid stage of the growth of tobacco.

Besides, the effects of bio-control bacterial strains on the microbial community strucrure in the rhizosphere soils of tobacco were examined by polymerase chain reaction-denaturing gradient gel electrophoresis(PCR-DGGE), the result shows that inoculation with bio-control bacterial had effects on the structure of bacterial community, the two strains had the similar effects. These results indicate that PGPR strains YC0573 and YC0136 are effective against *Phytophthora nicotianae* on tobacco, which has a number of potential applications.

Key words: *Phytophthora nicotianae*; antifungal; PGPR; promote growth; PCR-DGGE

# Isolation and characterization of plant growth promoting diazotrophs from rhizosphere of wheat in saline soils in Northwestern China

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#### Abstract

Characteristics of plant growth promoting diazotrophs strains isolated from rhizosphere of wheat in saline soils in northwestern China were detected. The results showed that there were diazotrophs present in root-soil system, the number of diazotrophs varied with fractions. The maximum number of diazotrophs was appeared in Rhizoplane. Eight high nitrogenase activity strains were obtained, eight out of seven strain secreted indoleacetic acid, and two strains showed property of dissolve calcium phosphate as well. Diazotrophs strains showed strong promoting wheat shoot length and dry weight based on tube experiment in Hoagland N free semi-solid nutrient. Quantification of nitrogen fixed by diazotrophs strains were assayed using <sup>15</sup>N isotope dilution technique. To further confirm the role of diazotrophs stains to wheat, pot experiment was carried out, and the results indicated wheat shoot length and dry weight were increased by stains inoculants and their combination. Stains Zoogloea sp. ChW6, Pseudomonas sp. ChW1, and stains combination (Pseudomonas sp. ChW1: Azotobacter sp. ChW5: Zoogloea sp. ChW6=1:1:1) have a potential for producing biofertilizer for wheat in Northwester China.

**Key words:** wheat; diazotrophs; nitrogenase activity; phosphate-solubilizing; indoleacetic acid (IAA); <sup>15</sup>N isotope dilution

# Construction of multifunction rhizobium engineering strain

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#### Abstract

Rhizobium engineering strain were obtained deriving from rhizobium and bacillus with antagonist to pathogens of soybean root rot and soybean cyst nematode were screened within soybean nodule, after study the system of both strains protoplast preparation regeneration and amalgamation. The characters of this engineering strain were fixing nitrogen, promoting growth and antagonist to pathogens. Its fermentation conditions, action mechanism on fungi and plant parasite nematode, resistance induced were studied systematically. 786 strains were obtained from 138 soil samples collected from more than 20 provinces of china. The result showed that rhizobium L396 and bacillus Snb2 were screened out form strains with effectively nematicide toxin to *heterodera glycined*. After seeking seed with bacteria suspension, both strains promoted growth the seeding. According to traditional taxonomy including morphology and Physio-biochemical, the bio-control bacteria strains L396 and Snb2 were classified as Sinorhizobium sp. and Bacillus sp. The sequences of 16S rDNA gene of L396 and Snb2 were sequenced and analyzed; the results showed that strains L396 and Snb2 belong to Sinorhizobium fredii, Bacillus subtilis, respectively. Take  $40\mu$  g/ml penicilium and  $50^{\circ}$ C, 120min heat treated as fusion marker of parents strain Snb2 and L396. The best optimal protoplast preparation, regeneration and amalgamation conditions were researched.

RH5 was picked out based on colony morphology, size of thalli, effect against pathogens and difference of soluble protein among which could nodule on root of soybean. The mortality of J2 treated with Bacterial culture filtrates of RH5 was 44%. The fermentation conditions of RH5were investigated by single factor and designs of orthogonal experiments, results were as follow: the optimum carbon and nitrogen resource was lactose and urea, respectively. The effect of Bacterial culture filtrates of RH5 on spore germination and mycelia configuration were testified the germination of spore were inhibited, the ratio of germinated spore was malformed. Protoplast agglomerate appeared in hypha treated culture filtrates, hypha were swelled and dissolved gradually. RH5 produced some nematicide: HCN, H<sub>2</sub>S, had ability of amylase and oxygenation hydroxybenzene. It had some effect on total sugar content and protein content of *H. glycines*. The seeding was promoted inoculated with RH5

suspension. The activity defense enzymes were promoted the increase range followed the bacteria concentration; the result indicated the optimum inoculation was  $10^{10}$  cfu/ml.

Key words: Rhizobium; Bacillus; *Heterodera glycines;* soybean root rot; protoplast fusion; multifunctional; engineering strain

# Influence of bio-rationals and Indigenous plant protection measures in enhancing the vitalities of bio-control agents for Induced Systemic Resistance suppressing Asian soybean rust in India

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#### Abstract

Asian Soybean rust, *Phakopsora pachyrhizi* Syd is the economically important disease, which causes significant yield loss in India. Lack of resistant cultivars, growing concern over use of chemical pesticides and increasing area under organic soybean cultivation has lead to exploitation of Indigenous Technology Knowledge in the management of Asian Soybean Rust. The present study comprised of thirteen different treatments comprising of botanicals, bioagents and micronutrients taken up at MARS, UAS, Dharwad during *Kharif* 2009 and 2010. The treatments were applied thrice with first one immediately after appearance of rust symptoms in the field and subsequently at 10 days interval. Among the bio-intensive strategies seed treatment with Trichoderma viride 6g/kg+ Spray with Cow urine10%+T.viride0.5% recorded minimum(39.6) Per cent Disease Index(PDI) followed by 41.8,41.9 PDI in case of spray with Cow urine 10% + Pongamia pinnata oil 0.5% and Cow urine 10% + Cristol 56SL 0.5% respectively. Minimum PDI was recorded in Hexaconazole 1ml/L(32.2) which differed significantly from rest of the treatments. The maximum seed yield of 12.33g/ha was recorded in Hexaconazole 0.1% followed by ST with Trichoderma viride 6g/kg+spray with Cow urine 10% + Trichoderma viride 0.5% (11.82q/ha). The highest disease pressure was in untreated check (86.7 PDI) with seed yield of 8.52q/ha. We also studied role of different enzymes in triggering the host defense by use of these ITK measures by employing Lowry's Method and Poly Acrylamide Gel Electrophoresis(PAGE) for assessing the total protein and estimation peroxidase, polyphenol oxidase and catalase activity. The peroxidase activity ranged between 35 to 50Kda. The maximum peroxidase activity was recorded in cowurine 10% and neem oil 1%. The maximum polyphenol activity was recorded in Cow urine 10% +Adathoda vessica 5%, Cow urine 10% +Trichoderma viride 0.5% and neem oil 1%. The polyphenol activity ranged between 70 to 90 kDa. There was no expression of catalase activity in any of treatments signifying salicylic acid based pathway in inducing defense in the soybean. Thus, the studies opened a new window of opportunity in managing Asian soybean rust in India.

# Management of sheath blight disease in rice by *Pseudomonas* aeruginosa MML2212

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# Abstract

A total of 671 different isolates of FPs were isolated from rice rhizosphere soils collected from seven districts of Tamil Nadu state, India. Among them, 87 showed inhibitory activity against R. solani with the zone of inhibition ranged from 0.5 cm to 2.8 cm. Further screening was resulted in selecting an isolate, which was designated as MML2212 owing to its superior antagonistic activity against R. solani and later it was identified as Pseudomonas aeruginosa. Application of culture and culture filtrate of P. aeruginosa MML2212 increased the seed germination and other growth parameters in rice. This bacterium did not produce 2,4 diacetylphloroglucinol, pyoluteorin and pyrrolnitrin. Phenazine-1-carboxamide (PCN) was purified from culture filtrate of P. aeruginosa MML2212 and its crystal structure was elucidated. The purified PCN decreased the mycelial dry weight of *R. solani* up 48.9% at 5 µg/ml compared to control. Importantly PCN completely (100%) inhibited the sclerotial germination compared to 100% germination in untreated and sterile medium controls. P. aeruginosa MML2212 was grown in KBB, formulated with talc powder and its shelf life was determined up to four months. Application of talc formulation of P. aeruginosa MML2212 significantly reduced the ShB and enhanced the yield parameters compared to PCN, carbendazim treatments and R. solani control in green house and also in field conditions.

# Kew wards: Rice rhizosphere; Sheath blight; *Pseudomononas aeruginosa* MML2212 and PCN

#### Introduction

Sheath blight (ShB) disease of rice is caused by *Rhizoctonia solani* Kühn [Teleomorph: *Thanetophorus cucumeris* (Frank) Donk] is becoming serious concern in rice growing areas of worldwide with maximum disease incidence of 91.2% and yield loss up to 69%. Although several strategies such as cultural practices, host resistance, chemical control, biological control and transgenic approaches have been suggested for the control of ShB disease (Mathivanan *et al.*, 2005; Khan and Sinha,

2007), farmers are still mostly depending on chemical control. However, the environmental pollution caused by constant and indiscriminate use of agrochemicals, as well as the development of resistance in plant pathogens over these chemicals have led to considerable changes towards chemical control. Hence, efforts on developing alternative disease control strategies are gaining momentum in recent past. Among these, biological control is considered the best viable alternative or supplement to chemical control (Jayaprakashvel and Mathivanan, 2008). Plant growth promoting rhizobacteria (PGPR) have drawn much attention at present as they not only promote the plant growth but also effectively control the plant diseases. Therefore, the present work has been initiated to identify efficient plant growth promoting rhizobacterium especially fluorescent pseudomonads (FPs) for the management of ShB.

#### **Materials and Methods**

#### Isolation of FPs from rice rhizosphere soils

Rice plants uprooted along with the soils from the farmers' fields in different districts of Tamil Nadu were kept in sterile polypropylenes bags and brought to the laboratory. The soils attached to the roots were dislodged by gently shaking in sterile conical flasks. To 10 g of soil samples obtained from each rhizosphere in a conical flask, 95 ml of sterile distilled water was added and the flasks were placed on a rotary shaker at 120 rpm for 30 min. The resulted suspension was serially diluted and spread plated on to King's B agar (KBA) (King *et al.*, 1954). The plates were incubated at room temperature ( $28 \pm 2^{\circ}$ C) for 48 h and observed under UV light. Distinct single colonies of fluorescent bacteria were picked, sub-cultured to purity and maintained in KBA slants and also in 30% glycerol vials. Each isolate was designated with a unique accession number prefixed with three letters MML followed by four digit numerical numbers.

#### Screening of FPs against plant pathogens

All the 671 isolated FPs were subjected to primary screening against sheath blight pathogen, *Rhizoctonia solani* uding dual culture assay. Among the 671 FPs, a strain designated as MML2212 isolated from the rice rhizosphere collected from Illanthaikulam village of Virudhunagar district was selected for further studies as it effectively inhibited *R. solani* than other isolates.

#### Effect of plant growth in rice by *P. aeruginosa* MML2212

Seeds of rice cv. IR-50 were washed in tap water and surface sterilized with sodium hypochlorite and then air-dried. They were soaked for 6 h in 24 h old culture suspension (0.5 OD at 600 nm) and also in the culture filtrate of *P. aeruginosa* MML2212. Along with bacterial culture and culture filtrate treatments, sterile KBB was also included as control. The seeds were then sown in plastic cups at 10 seeds in each cup. After 10 days, the root and shoot lengths dry weight and vigour index were

calculated for each treatment. This experiment was conducted in both sterile and nonsterile soils.

# Detection of antifungal metabolites produced by P. aeruginosa MML2212

The strain of *P. aeruginosa* MML2212 was cultivated in 3 L lab scale fermentor (Bioengineering AG, Switzerland) using 2 L of KBB at 30°C. A 10 ml sample was drawn from the fermentation vessel at 4 h intervals up to 52 h and the growth of *P. aeruginosa* was measured at 600 nm in a spectrophotometer. The culture was centrifuged at 10000 rpm and the cell free supernatant was tested for biocontrol activity against *R. solani* by well diffusion method. Further, the culture supernatant of *P. aeruginosa* MML2212 was extracted with two volumes of ethyl acetate and the crude extracts were chromatographed on pre-coated  $F_{254}$  silica gel thin layer chromatography (TLC) plates (Merck, Germany) using ethyl acetate: hexane (40: 60) as solvent system. TLC plates were visualized under UV and after reaction with iodine and ninhydrin.

# **Purification of metabolites**

The antifungal metabolites of produced by P. aeruginosa MML2212 was purified according to the method of Shanmugaiah et al. (2010). Briefly, 32 h old culture of P. aeruginosa MML2212 was harvested, centrifuged at 10000 rpm for 10 min at 8°C and extracted two times using an equal volume of ethyl acetate (EA) each time. All the extracts were pooled, concentrated by rotary evaporator, mixed with  $60 \sim 120$  mesh silica gel (Sigma Chemicals Co., USA) to prepare the metabolite-silica gel slurry and air-dried. A glass column measuring 4.5 cm diameter  $\times$  65 cm height was packed with silica gel up to 45 cm height and washed with 500 ml of hexane. Then the metabolites-silica gel slurry was loaded on to the column and eluted initially with hexane followed by different ratios of hexane: EA [3:1, 2:1, 1:1, 1:2, (v/v)], EA (100%) and finally with acetone. About 150 ml of each solvent system was used for elution and fractions of 5 ml each were collected. The presence of compounds was analyzed by TLC with hexane and EA at 4:2 (v/v) as solvent system. Fractions showing similar spots on TLC were pooled and concentrated. The partially purified metabolites were further fractionated using  $230 \sim 400$  mesh silica gel (ACME, Mumbai, India) column chromatography. The column size was 2.5 cm diameter  $\times$  53 cm height and the silica gel was packed up to 25 cm. Similar elution procedures as described in the above column chromatography were followed. Fractions showing single similar spot on TLC (hexane:EA at 3:2 ratio) were pooled together and concentrated. Three different compounds were separated through the above two steps silica gel column chromatography. Among the three, a major compound appeared as yellow needles was further characterized as it exhibited antifungal activity on R. solani.

#### **Characterization of purified compound**

Various spectral analyses viz., UV, Mass, IR, <sup>1</sup>H NMR and <sup>13</sup>C NMR were performed to characterize the purified compounds. Further, the compound was crystallized using standard method and X-ray diffraction data were collected. Structure elucidation of the purified compound revealed that it is phenazine-1-carboxamide (PCN) and hence, it is deposited with the Cambridge Crystallographic Data Centre (Shanmugaiah *et al.*, 2010).

# Antifungal activity of purified PCN

The minimum inhibitory concentration (MIC) and the effect of PCN on sclerotial germination and mycelia growth of *R. solani* were determined.

#### Evaluation of PCN against ShB disease in greenhouse

The greenhouse experiment was conducted in potted plants using complete randomized block design (CRBD) with three replications. Five treatments viz., seed treatment + root dipping + soil application + foliar spray of *P. aeruginosa* MML2212, foliar spray of PCN, foliar spray of carbendazim, control with any treatment and pathogen (*R. solani*) control were maintained. The purified PCN and carbendazim were dissolved in water at 100  $\mu$ g/ml concentration and made uniform suspensions by adding 0.01% SDS. Sterile distilled water was used in controls. All the treatments were sprayed as prophylactic measure at 25, 45 and 60 days after transplantation. The plant height, number of healthy and infected tillers, ShB incidence and yield parameters were recorded.

#### Formulation of *P. aeruginosa* MML2212

The talc formulation of *P. aeruginosa* MML2212 was developed as described by Vidhyasekaran and Muthamilan (1999). The talc formulated product of *P. aeruginosa* MML2212 was packed in polythene bags, sealed and stored at different temperatures (4°C, 8°C, 23°C and 37°C). One gram of the product was drawn at every 15 days intervals up to 120 days and the population was estimated by serial dilution method on KBA. The population of *P. aeruginosa* MML2212 was expressed as colony forming units (cfu)/g of product.

#### Evaluation of *P. aeruginosa* MML2212 against ShB disease in field conditions

A field experiment was conducted at Maduravoyal Field Research Laboratory, Centre for Advanced studies in Botany, University of Madras, Chennai. The experiment was laid out with randomized block design (RBD) with four replications. All the recommended agronomic practices were followed for the cultivation of rice. After 20 days of transplantation, *R. solani* was inoculated to the selected rice hills for inducing the ShB disease. The methods of seed treatment, root dipping and foliar spray of *P. aeruginosa* MMI2212 were as similar to that of the previous greenhouse experiment. Soil application of *P. aeruginosa* MMI2212 was carried out at 2.5 kg/ha after mixing with 100 kg of farm yard manure (FYM) in the main field before and

after 25 days of transplantation. The plant height, number of healthy and infected tillers, sheath blight incidence and yield parameters were recorded.

#### Statistical analysis

The experimental data were tabulated and analyzed using one-way ANOVA by the Agres statistical software package (Agres, 1994). The least significant difference (LSD) analysis was performed to group the treatment mean values.

#### Results

#### Isolation of fluorescent pseudomonads (FPs) and screening against R. solani

A total of 671 different FP isolates were obtained from 70 different rice rhizosphere soils collected from the farmers' fields in seven southern districts of Tamil Nadu. Interestingly all the soil samples had population of FPs ranged from 76 to 139. Among 671 isolates screened, only 87 showed inhibitory activity against *R. solani* and the zone of inhibition towards the pathogen ranged from 0.5 cm to 2.8 cm (Table 1). Among 87, the isolate designated as MML2212 was selected for further studies owing to its superior performance against *R. solani*.

#### Effect of plant growth in rice by P. aeruginosa MML2212

The seed germination of rice was significantly increased due to treatment with culture and cell free culture filtrate of *P. aeruginosa* MML2212 compared to treatment with control medium in poly pot experiments with sterile and non-sterile soils. However, maximum germination and other growth parameters viz., root and shoot lengths, dry weight and vigour index were observed in seeds treated with the culture of *P. aeruginosa* MML2212 as compared to treatments with culture filtrate and sterile medium (Tables 2 & 3).

#### Detection of antibiotic coding genes by PCR

The genes that encode 2,4 diacetylphloroglucinol (DAPG) (*phl*), pyoluteorin (*plt*) and pyrrolnitrin (*prn*) were not detected in *P. aeruginosa* MML2212 as determined by PCR. The reference *P. fluorescens* CHAO strain exhibited amplification for *phl* (DAPG) and *plt* (pyoluteorin) genes. None of these antibiotic genes were present in *P. aeruginosa* MML2212, but the other isolates of FPs designated as MML2102, MML2156, MML2257, MML2345, MML2420, and MML2512 showed the presence of genes for pyoluterin.

# Detection of antifungal metabolites in the culture filtrate of *P. aeruginosa* MML2212 by bioactivity against *R. solani*

The culture filtrates of all the ages of fermentor cultures of *P. aeruginosa* MML2212 (4 $\sim$ 52 h old) inhibited the mycelial growth of *R. solani* with maximum in 32 h old culture filtrate compared to the culture filtrates of the rest of the ages.

#### Purification of metabolites from the culture filtrate of P. aeruginosa MML2212

Three distinct compounds with  $R_f$  values of 0.46, 0.63 and 0.69 were purified from the culture filtrate of *P. aeruginosa* MML2212. Among the three, 56 mg of a yellow solid compound was obtained. Subsequently, it was crystallized with chloroform.

#### Characterization of the purified compound

The pure compound isolated from *P. aeruginosa* MML2212 was yellow crystal and odourless. The melting point of the compound was determined between 220°C and 222°C. It was freely soluble in most of the organic solvents such as EA, acetone, chloroform and dimethyl sulfoxide and also in water. It exhibited  $\lambda_{max}$  at 256 nm and 366 nm. Analysis of IR, mass, <sup>1</sup>H NMR and C<sup>13</sup> NMR spectra revealed that the purified compound was phenazine-1-carboxamide (PCN). Further, its crystal structure has been elucidated for the first time and hence been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 617344 (Shanmugaiah *et al.*, 2010).

# Effect of PCN on R. solani

# Minimum inhibitory concentration (MIC)

The PCN remarkably decrease the mycelial dry weight of *R. solani* at 5  $\mu$ g/ml in which 0.597 g dry weight/50 ml was estimated, which is 48.9% reduction compared to control (1.168 g dry weight/50 ml). Thus the MIC of PCN against *R. solani* was determined as 5  $\mu$ g/ml. Interestingly, complete inhibition (100%) of mycelial growth of *R. solani* was achieved at 50  $\mu$ g/ml (Table 4).

# Sclerotial germination

The PCN at 5  $\mu$ g/ml caused 20% and 10% inhibition of sclerotial germination of *R*. *solani*, when the sclerotia were soaked for 4 h and 8 h prior to inoculation on PDA. Importantly it completely (100%) inhibited the sclerotial germination after 12 h and 24 h of prior to inoculation compared to 100% germination in untreated and sterile medium controls (Table 5).

#### Mycelial growth

The PCN at 5  $\mu$ g/ml remarkably inhibited the mycelial growth of *R. solani* compared to carbendazim and control. The pathogen was able to grow on PCN and carbendazim amended PDA for 4 days and then its growth was completely ceased.

The mycelial growth was measured from 1.9 cm to 4.3 cm and 2.0 cm to 6.7 cm at different days in PCN and carbendazim, respectively amended medium as compared to 3.0 cm to 9.0 cm in control. Interestingly, the inhibitory effect of PCN on *R. solani* was superior to carbendazim.

# Evaluation *P. aeruginosa* MML2212 and PCN against sheath blight disease in greenhouse

The talc formulation of *P. aeruginosa* MML2212 as seed treatment + root dipping + soil application + foliar spray significantly reduced the sheath blight incidence compared to foliar spray of PCN, carbendazim and *R. solani* control. The plant height in *P. aeruginosa* MML2212 treatment was recorded as 78.8 cm as against 53.6 cm in control. In PCN and carbendazim treatments, it was 72.3 cm and 71.8 cm, respectively. The ShB lesion height was only 8.4 cm following the application of talc formulation of *P. aeruginosa* MML2212 as seed treatment + root dipping + soil application + foliar spray as against 12.6 cm, 13.5 cm and 32.7 cm, respectively in PCN, carbendazim and control. The ShB incidence was significantly reduced due to application of *P. aeruginosa* MML2212, in which only 10.7 2% ShB incidence was recorded, compared to 61.0% disease incidence in control. In PCN and carbendazim treatments, it was 17.4% and 18.8%, respectively.

#### Effect of P. aeruginosa MML2212 and PCN on yield parameters in rice

The talc formulation of *P. aeruginosa* MML2212 as seed + soil + root + foliar applications significantly increased the number of tillers, productive tillers, 1000 grains weight and grain yield compared to PCN, carbendazim treatments and control. The maximum of 20 total tillers/hill and 18 productive tillers/hill, 28.5 g of 1000 grains weight and 38.7 g grain weight/hill was recorded following the treatment of *P. aeruginosa* MML2212 as seed + soil + root + foliar application compared to  $11 \sim 16$  totaltillers,  $6 \sim 14$  productive tillers,  $18.8 \sim 25.9$  g of 1000 g weight and 16.8g to 31.3 g grain weight/hill in PCN, carbendazim treatments and control (Table 6).

#### Formulation of P. aeruginosa MML2212 and its shelf life

The talc formulated product of *P. aeruginosa* MML2212 can be stored up to 4 months with adequate population of at least  $10^7$  cfu/g of product in all the tested temperature of  $4 \sim 37$  °C.

#### Evaluation P. aeruginosa MML2212 against ShB disease in field conditions

The talc formulation of *P. aeruginosa* MML2212 as seed treatment + root dipping + soil application + foliar spray significantly reduced the sheath blight incidence compared to foliar spray of carbendazim and control in the field experiment. The plant height in *P. aeruginosa* MML2212 treatment was recorded as 84.3 cm as against 74.5 cm and 66.7 cm in carbendazim treatment and control, respectively. The ShB lesion height was only 10.6 cm following the application of talc formulation of *P.* 

*aeruginosa* MML2212 as seed treatment + root dipping + soil application + foliar spray as against 13.0 cm and 35.3 cm, respectively in carbendazim treatment and control. The ShB incidence was significantly reduced due to application of *P. aeruginosa* MML2212, in which only 12.5% ShB incidence was recorded, compared to 52.9% disease incidence in control. In carbendazim treatments, it was 17.4%.

The talc formulation of *P. aeruginosa* MML2212 as seed + soil + root + foliar applications significantly increased the number of tillers, productive tillers, 1000 grains weight and grain yield compared to carbendazim treatment and control. The maximum of 26 tillers/hill and 22 productive tillers/hill, 26.2 g of 1000 grains weight, 735 g grain weight/plot and 4.9 t grain yield/ha was recorded following the treatment of *P. aeruginosa* MML2212 as seed + soil + root + foliar application compared to 15, 9, 18.2 g, 540 g and 3.6 t, respectively of total tillers, productive tillers, 1000 grains weight, grain yield/plot and grain yield/ha recorded in control. In carbedazim treatment, 22 total tillers, 18 productive tillers, 24.2 g of 1000 g weight, 615 g grain weight/plot and 4.1 t grain yield/ha were recoded (Table 7).

#### Discussion

Interest in biological control of plant pathogens has increased significantly in recent years, partly due to public concern on the indiscriminate use of hazardous chemical pesticides. Fluorescent pseusdomonads (FPs) were studied extensively for the biological control of various soil borne pathogens (Hass and Defago, 2005). In this scenario, the present study was aimed to investigate the biological control of sheath blight (ShB) disease using FPs, as the disease is becoming more severe and major constrain for rice production all over the world. A total of 671 FPs isolates were obtained from 70 different rice rhizosphere soils, which indicated the richness of rhizobacteria. Similarly, several attempts were already made to isolate diverse FPs from rhizosphere of different crops (Sakthivel *et al.*, 1986; Ramesh Kumar *et al.*, 2002; Velusamy *et al.*, 2005).

In general, the probability to get efficient bacterial antagonists from a diverse pool of bacterial isolates collected from different agroclimatic zones is high. Therefore, researchers around the world accumulate huge microbial strains and eventually select a few of them with single or multiple desirable characteristics. All the 671 isolates were screened against the ShB pathogen, *R. solani* and an effective antagonistic isolate designated as MML2212 was selected. It is a rod-shaped bacterium with unipolar flagella as observed under light microscope and SEM. Later, it was identified as *P. aeruginosa* based on 16S rRNA sequence analysis. Ramesh Kumar *et al.* (2002) isolated different FPs from the rhizosphere of rice and sugarcane, of which, majority of the rice rhizosphere pseudomonads exhibited antifungal activity. Similarly Velusamy *et al.* (2005) isolated 637 strains of FPs from rhizosphere samples of different crops including rice from five states of India. Among them 44%

of the strains showed antagonistic activity against bacterial leaf blight pathogen of rice, *X. oryzae* pv. *oryzae*.

The increase in per cent seed germination and seedling growth in rice following seed soaking with the culture of *P. aeruginosa* MML2212 showed the significance of this bacterium that it can be used as a growth enhancer for plant growth improvement. It is highly appreciable to have both the biocontrol and growth enhancer traits in a single strain as these kinds of phenomenon can effectively be utilized in sustainable agriculture to improve the crop yields. The growth promotion activity of microorganisms is directly correlated to the release of plant growth hormones or substances predominantly auxins (Dey *et al.*, 2004; Compant *et al.*, 2005). In this present study, the production of indole acetic acid (IAA) by *P. aeruginosa* MML2212 was demonstrated. Interestingly the production of IAA was remarkably high with the maximum production of 37  $\mu$ g/ml.

Antibiosis is considered as the prime mechanism extensively exhibited by FPs, as they reported to produce an array of antibiotic compounds such as phenazines, pyrollnitrin, pyoluteorin and phloroglucinols during their interaction with pathogens (Chin A. Woeng et al., 2003; Dwivedi and Johri 2003; Velusamy et al., 2005). However, P. aeruginosa MML2212 did not produce DAPG, pyrollnitrin and pyoluteorin as confirmed by the PCR analysis. The genes that encode DAPG (phl), pyoluteorin (plt) and pyrrolnitrin (prn) were not detected in P. aeruginosa MML2212, which indicated that other than these three different groups of compounds would have involved in biocontrol activity of P. aeruginosa MML2212. Interestingly, phenazine-1-carboxamide (PCN) was purified from the culture filtrate of P. aeruginosa MML2212 as yellow crystals and its crystal structure has been elucidated fore the first time and deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 617344 (Shanmugaiah et al., 2010). Further biological studies showed that the PCN significantly decreased the mycelial dry weight of R. solani at 5 µg/ml, which is 48.9% reduction compared to control. Interestingly, this compound also inhibited the mycelial growth, conidial and sclerotial germination of many other fungal pathogens. Therefore, it can be developed as fungicide for the management of many fungal diseases of crop plants.

Management of the rice pathogens is considered very important in the present scenario because the ShB disease caused by *R. solani* is most prevalent and serious limiting factors for the successful cultivation of rice worldwide next to blast caused by *Pyricularia oryzae (Magnaporthe oryzae)* (Saikia *et al.*, 2006). In spite of the use of many modern chemical fungicides, the control of these diseases in the farmers' fields is still a difficult task. As the PCN showed excellent antifungal activity against *R. solani* under *in vitro* bioassays with low MIC value, it was further evaluated against ShB disease of rice *in vivo* under greenhouse experiment. Application of *P. aeruginosa* MML2212 significantly decreased the ShB and improved the growth and

yield parameters of rice compared to PCN and carbendazim. It is obvious that the effectiveness of purified molecule and the commercial fungicide is lasting for specific periods depend on their half life. Therefore, it is essential to supplement their application many times. However, the live bacterial biocontrol agent, *P. aeruginosa* MML2212 can survive for a longer period in rice rhizosphere and phyllosphere and hence, sustainable production of antifungal compounds like PCN is possible till crop maturity, which could offer significant control of ShB disease due to this treatment. In addition, *P. aeruginosa* MML2212 also significantly improved the growth parameter as it demonstrated to produce growth promoting substance, IAA. Both the disease control and growth promoting characteristics of *P. aeruginosa* MML2212 could make this treatment is the best, which eventually reflected in enhanced grain yield of rice compared to PCN and carbendazim treatments.

The talc formulated product of *P. aeruginosa* MML2212 was developed and its study on the shelf life indicated that the product can be stored up to 4 months with adequate population. Vidyasekaran et al. (1997) have already developed a talc formulation of Pseudomonas fluorescens and tested against many crop diseases including ShB in rice. Similar talc formulation of P. fluorescens of has been reported to control ShB disease of rice (Radjacommare *et al.*, 2002; Mathivanan *et al.*, 2005). Application of *P. aeruginosa* MML2212 as seed treatment + root dipping + soil application + foliar spray significantly improved the plant growth, reduced the sheath blight incidence and eventually grain yield in rice as compared to rest of the treatments. Adequate population of P. aeruginosa MML2212 in all the four possible ways of application would have certainly maintained adequate bacterial population and hence, significant control of ShB was possible as compared to other treatments. This phenomenon has already been clearly demonstrated in many crop plants with different BCAs (Mathivanan et al., 2000) including rice (Nagarajakumar, et al., 2005). Further, Vidhyasekaran and Muthamilan (1999) obtained high level disease control by the application of PGPR strain in both rhizosphere and phyllosphere. Other treatments such as seed treatment, root dipping, soil application and foliar spray either individually or in different combinations also decreased the ShB incidence and increased the plant growth and yield parameters compared to control, however, their effect was not comparable with that of combined application of P. aeruginosa MML2212 in all the four possible ways (seed + root + soil + foliar). This has strongly supported the hypothesis of "adequate population of BCA(s) is essential to achieve effective management of plant pathogens and satisfactory control plant diseases". Finally, the overall analysis of the experimental results revealed that P. aeruginosa MML2212 could be an excellent biocontrol candidate for the management of sheath blight disease of rice.

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District	Designation of FPs	No. of rhizosphere soil	No. of FPs	No. of antagonistic FPs against <i>R. solani</i>
Kanyakumari	MML2001-2080	10	80	11
Tirunelveli	MML2081-2162	10	83	16
Virudhunagar	MML2163-2272	10	108	8
Tutucorin	MML2273-2351	10	76	11
Madurai	MML2352-2453	10	102	15
Dindugal	MML2454-2531	10	83	14
Theni	MML2532-2670	10	139	12
To	Total			87

 Table 1
 Details of FPs isolated from different districts of Tamil Nadu, India

Table 2	Plant growth promotion	in rice by <i>P. aeruginos</i>	a MML2212 in rice in sterile soil
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Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Dry weight (g)	Vigor Index
Culture	100 <sup>a</sup>	23.29 <sup>a</sup>	15.27 <sup>a</sup>	0.335 <sup>a</sup>	3856 <sup>a</sup>
Culture filtrate	100 <sup>a</sup>	15.31 <sup>b</sup>	10.01 <sup>c</sup>	0.403 <sup>b</sup>	2532 <sup>b</sup>
Control	85 <sup>b</sup>	11.82 <sup>c</sup>	11.56 <sup>b</sup>	0.206 <sup>c</sup>	1988 <sup>c</sup>

Values in a column with same letter are not significantly different at 5% level.

Table 3	Plant growth promotion of r	ice by P.	aeruginosa MML2212 in non-sterile soil
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Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Dry weight (g)	Vigor Index
Culture	100 <sup>a</sup>	13.7 <sup>a</sup>	10.87 <sup>a</sup>	0.335 <sup>a</sup>	2484 <sup>a</sup>
Culture filtrate	100 <sup>a</sup>	13.3 <sup>b</sup>	10.56 <sup>b</sup>	0.302 <sup>b</sup>	2394 <sup>b</sup>
Control	90 <sup>b</sup>	10.7 <sup>c</sup>	6.85 <sup>c</sup>	0.219 <sup>c</sup>	1579 <sup>c</sup>

Values in a column with same letter are not significantly different at 5% level.

Concentration (µg/ml)	Dry weight (g)
Control	$1.168 \pm 0.023$
1	$1.108 \pm 0.022$
5	$0.597 \pm 0.011$
10	$0.308 \pm 0.006$
25	$0.109 \pm 0.002$
50	0

#### Table 4 MIC of PCN on R. solani

Values are mean of triplicates with SD.

#### Table 5 Effect of PCN on sclerotial germination of R. solani

Soaking time (b)	Germination (%)			
Soaking time (ii)	Untreated	Sterile medium	PCN treated	
4	$100 \pm 0$	$100 \pm 0$	$20 \pm 0.4$	
8	$100 \pm 0$	$100 \pm 0$	$10 \pm 0.4$	
12	$100 \pm 0$	$100 \pm 0$	-	
24	$100 \pm 0$	$100 \pm 0$	-	

Values are mean of triplicates with SD.

# Table 6 Effect of P. aeruginosa MML2212 and PCN on yield parameters of rice in greenhouse conditions

Treatment	No. of tillers	No. of productive tillers	1000 grains weight (g)	Grain yield (g)
Control (Rs)	11 <sup>c</sup>	$6^{d}$	18.8 <sup>d</sup>	16.8 <sup>d</sup>
Pa (ST + RD + SA + FS + RS)	20 <sup>a</sup>	18 <sup>a</sup>	28.5 <sup>a</sup>	38.7 <sup>a</sup>
PCN + R. solani	16 <sup>b</sup>	14 <sup>b</sup>	25.9 <sup>b</sup>	31.3 <sup>b</sup>
Carbendazim + R. solani	16 <sup>b</sup>	13 <sup>c</sup>	24.6 <sup>c</sup>	29.2 °

Pa: *P. aeruginosa* MML2212; Rs: *R. solani*; ST: Seed treatment; RD: Root dipping; SA: Soil application; FS: Foliar spray

Values are mean of three replications. Values in a column followed by different letter are significantly different at P = 0.05.

#### Table 7 Effect of P. aeruginosa MML2212 application on yield parameters of rice in field conditions

Treatment	No. of tillers/hill	No. of productive tillers/hill	1000 grains weight (g)	Grain yield (g/plot)	Grain yield (t/ha)
Control (Rs)	15 <sup>c</sup>	9°	18.2 <sup>c</sup>	540 <sup>c</sup>	3.6 <sup>c</sup>
ST + RD + SA + FS + RS	26 <sup>a</sup>	22 <sup>a</sup>	26.2 <sup>a</sup>	735 <sup>a</sup>	4.9 <sup>a</sup>
Carbendazim + R. solani	22 <sup>b</sup>	18 <sup>b</sup>	24.2 <sup>b</sup>	615 <sup>b</sup>	4.1 <sup>b</sup>

Rs: *R. solani*; ST: Seed treatment; RD: Root dipping; SA: Soil application; FS: Foliar spray Values are mean of three replications. Values in a column followed by different letter are significantly different at P = 0.005

# Solubilization of rock phosphate and plant growth promotion by rhizobial strain in buffering condition

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#### Abstract

Phosphorus exists in nature primarily in insoluble form leading to unavailability to plants. Rhizobia are well known for their ability to fix nitrogen (N). In addition, their capacity to solubilize phosphate has been receiving world wide attention. Forty two rhizobial isolates from various legumes from Kanpur were screened for phosphate solubilizing ability. Twelve rhizobial strains showed phosphate solubilizing ability in both non-buffered and buffered media (100mM Tris ±HCl pH 8.0) conditions. Strain BPSH1 showed maximum phosphate solubilizing efficiency under non buffering (295 %) and buffering (200 %) conditions. After seven days of incubation BPSH1 showed solubiliztion of 275 µg/ml of phosphorus with simultaneous decreases in pH of media to 3.96. In 100 mM buffering media there was 205 µg/ml solubilization of phosphorus and pH decrease up to 4.60. Strain BPSH1 also showed production of indole acetic acid (IAA) and siderophore. Plant growth promotory activity of BPSH1 was checked in culture tubes 1/3 filled with plant growth media (50 mM buffering condition) deficient for soluble phosphorus taking chickpea (*Cicer arietinum*) as test crop in following sets of experiment: (i) rock phosphate (control), (ii) rock phosphate + BPSH1. Strain BPSH1 showed 96.66% and 66.67% increase in shoot length and root length in comparison to control. Paper chromatography revealed the production of succinic acid by BPSH1. The results indicated that phosphate solubilizing ability of BPSH1 is due to production of succinic acid leading to reduction of pH.

# In vitro activity of exopolysaccharides as biopriming agent

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#### Abstract

Present study was conducted *in vitro* to examine the possibility of improving seed germination and seedling growth in Sunflower achens (Helianthus annuus) under saline conditions by pre-treatment of the seedlings with exopolysaccharides (EPS), produced by stress tolerating strain of Fluoresent *Pseudomonas* PF23. The results showed that, different levels of salinity (0 mM, 50 mM, 75 mM, 100 mM and 125 mM NaCl) have significant effect on seed germination and growth. Germination rate and vigor index in all the salinity levels remarkably improved when bacterial EPS was applied in comparision to control (seeds untreated with EPS).Germination improved significantly by 14.28% to 80%, and vigor index increased by 1.39 to 3.2 times in response to EPS. Results suggested that biopriming of seeds with EPS could serve as a useful tool for alleviating salinity stress in salt-sensitive plants. Salinity alleviation due to bacterial EPS may be attributed to Na<sup>+</sup> removal from the salinized media due to biosorption. Seedling growth in absence of salt indicates growth stimulating factors may be present in the EPS. The above findings suggest the application and benefits of utilizing bacterial EPS, boosting early seedling emergence and mitigating salt stress in salinity affected regions.

# Commercial application of a PGPR product (Azobac) under rain fed land for Low income farmers in a low return on investment region south of the Mexican corn belt where corn is the main income a two year study

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#### Abstract

With high petroleum prices that increase fertilizer prices, the need of low cost fertilizer inputs like PGPR biofertilizers with efficient microorganisms to produce PGRS that induce root growth plant health, induce resistance, fix nitrogen and make phosphorus soluble for plant use is of great use.

This project was established in 3000 hectares in the San Gabriel region south of the Mexican Corn Belt region in Jalisco M éxico. The farmers have a cooperative where you can reach each one to follow up the project though a project leader . the farmer gets technical support through an engineering consultant group and the government supply funds trough JADEFO for the farmer to try new approaches to soil analysis, land preparation, local seeds selection, substitution of fertilizers by PGPR bio fertilizers by 50% and 100% with a comparison in production with the historical values that the same farmers have reported in previous years. In 2009 the corn yield was with no significant difference to the historical corn yield but the cost in traditional farming was \$ 272.00 and the use of a PGPR product Azobac had a cost of \$ 44.00 per hectare. Giving an increase in farmers income of 18.5%. In the 2010 growth season which had a much better rainy season of 920 mm. The results showed an 8% increase in yield With a lower production cost of 21% .

SECRETARIA DE AGRICULTURA, GANADERIA, DESARROYO RURAL, PESCA Y ALIMENTACIÓN.National Agricultural University in Mexico.

# Evaluation of PGPR strains for growth and disease suppression in ginger (*Zingiber officinale* Rosc.)

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#### Abstract

Ginger (Zingiber officinale Rosc.) is one of the major spice crops produces from India. Soft rot caused by *Pythium* spp. and bacterial wilt caused by *Ralstonia* solanacearum are the major production constraints. Use of pathogen free planting material is a major component of integrated disease management. Plant growth promoting rhizobacteria (PGPR) are ideally suited as seed inoculants both for growth promotion and disease suppression. One hundred rhizobacteria were isolated from ginger rhizosphere collected from ginger growing areas of Kerala and Karnataka states of India. From this collection, ten isolates, five each for biocontrol and nutrition mobilization were selected based on in vitro tests. They were evaluated for two years in the green house and later in the field. The selected strains included three Serratia (GRB 38, GRB 58 and GRB68), two *Enterobacter* (GRB 70, GRB 71) one each of Burkholderia (GRB 25), Bacillus (GRB 35), Klebsiella (GRB 36), Curtobacterium (GRB 57), and *Pseudomonas* (GRB 91). The experiment on nutrient mobilization with four isolates namely Burkholderia cepaceae (GRB 25), Klebsiella sp. (GRB 36), Serratia marcescens (GRB 38) and Enterobacter sp. (GRB 70) and graded levels of NPK revealed that combined application of inorganic fertilizers and PGPR markedly increased their availability in soil. For instance, mineral N and Bray P levels were greatest in the treatment 100% NPK + GRB 38 (215.6 mg kg<sup>-1</sup> and 7.8 mg kg<sup>-1</sup> respectively) and exchangeable K level was greatest in the treatment with 100% NP + 75% K + GRB 70 (549.0 mg kg<sup>-1</sup>). The field experiments revealed that B. amyloliquifaciens (GRB 35) and S. marcescens (GRB 68) were effective for disease control and plant growth promotion. Both the isolates (GRB 35 and GRB 68) recorded more than 75% sprouting. Soft rot incidence was also significantly less (<10%) compared to control. The Rhizobacterial treatment recorded significantly higher yield.

#### Introduction

Ginger is one of the widely used spices. The major production constraint includes crop losses caused by diseases. Soft rot caused by *Pythium* spp; bacterial wilt caused by *Ralstonia solanacearum* and yellows disease caused by *Fusarium* are the major diseases (Dake and Edison., 1989; Dohroo, 2005). In the current investigation, we focused on rhizobacteria isolation, characterization and screening for their biocontrol activity against fungal plant pathogens (*Pythium myriotilum* and *Fusarium* sp. of ginger and *Phytophthora capsici* on black pepper) and also for their growth promoting efficacy.

# Isolation and identification of rhizobacteria

Rhizosphere soils were collected from ginger cultivating regions such as Calicut and Wayanad Districts in Kerala and Kodagu District in Karnataka. Ten grams of rhizosphere soil was serially diluted up to  $10^{-8}$ , pour plated on Tryptic Soy Agar (TSA), Nutrient Agar (NA) and Basal medium amended (glucose, mannitol, sorbitol, inositol and sucrose) were used for isolation of rhizobacteria and incubated at 28 °C for 2~3 days. The most suitable dilution was selected for estimating the population of the rhizobacteria, and expressed as number of colony forming units (CFU) per gram of soil. Colonies on each plate were distinguished based on phenotypic characteristics such as shape, motility, colour, rate of growth, culture morphology and Gram staining reaction. The representative isolates were selected cryopreserved at -80°C in glycerol (20%) for further studies. Isolates were tentatively grouped based on phenotypic identification (Zvyagintsev 1991). All the isolates were identified using biochemical and phenotypic characterization. For confirming the identity, modern tools namely Biolog and 16S rDNA sequence analysis were adopted.

# Screening of bacteria for antagonism towards plant pathogens

# **Dual plate assay**

Bacterial isolates were screened for their activity towards plant pathogenic fungi by a dual culture as *in vitro* assay on Potato Dextrose Agar (PDA). Zones of inhibition were measured after 4 days of incubation at 28 °C, according to Dennis and Webster (1971) and Berg *et al.* (2002). All strains were tested in three replicates independently for three plant pathogens namely, with *Pythium myriotilum*, *Phytophthora capsici* and *Fusarium* sp. The percentage inhibition over untreated control was calculated, normalized by angular transformation and statistically analyzed using MSTATC.

# Evaluation of strains for biocontrol in green house and field

A pot experiment comprising of three strains of rhizobacteria (GRB 35- *Bacillus amyloliquifaciens*, GRB 68- *Serratia marcesens* and IISR 51-*Pseudomonas*) in comparison with Streptomycin and Metalaxyl mancozeb was conducted. One set of the treatment were set apart for inoculation with *Ralstonia solanacearum* and another for *Pythium*. The treatments were given at the time of planting and repeated thrice at 30 day interval. Pathogens were inoculated after 30 days of planting. Observations were recorded on sprouting, incidence of bacterial wilt and soft rot and the data were

statistically analyzed. The same treatments were repeated in the field but there was no inoculation with pathogens, but natural incidence of disease and growth parameters were recorded.

#### **Results and discussion**

A total 100 distinctly different rhizobacterial isolates were isolated from different cultivars of healthy ginger from different geographical regions in Kerala and Karnataka. Of the total isolates, 70 were Gram-negative and 30 were Gram-positive organisms. These isolates were tentatively grouped into *Pseudomonas* spp. (12 strains), *Serratia* spp. (17 strains), *Enterobacter* spp. (15 strains), *Klebsiella* spp. (14 strains), *Burkholderia* spp. (5 strains), *Bacillus* spp. (15 strains), *Acinetobacter* (2 strains) *Arthrobacter* spp. (2 strains), *Micrococcus* spp. (2 strains), *Curtobacterium* spp. (1 strain) and 15 unidentified strains based on the keys provided in the Bergey's manual.

All the rhizobacterial isolates were tested *in vitro* for their biocontrol activity against the fungal plant pathogens. Among the rhizobacteria five isolates, GRB 35, GRB 57, GRB 58, GRB 68 and GRB 91 showed strong antagonistic activity with more than 70% inhibition against all the fungal plant pathogens (Table 1).

The green house experiments on sprouting, incidence of bacterial wilt and soft rot reveled that two strains GRB 35 and GRB 68 were superior to chemical treatments (Fig. 1, 2). The same treatments when repeated in the field also recorded better growth promotion and less disease incidence (Fig 3, 4) and were shortlisted as the effective strains. Yield of ginger was also higher in these two treatments. The short-listed isolates of rhizobacteria, GRB 35 and GRB 68 were subjected to Biolog based identification. The isolate GRB 35 was identified as *Bacillus amyloliquifacens* and GRB 68 were identified as *Serratia marcescens*. Partial sequence data for the 16S rDNA gene have been deposited in the GenBank (NCBI) nucleotide sequence data base library under the following accession numbers: *Bacillus amyloliquifaciens* GRB 35 (FJ493539) and *Serratia marcescens* GRB 68 (FJ493540).

Biological control is an attractive optional alternative to toxic pesticides for protection of plants against diseases. In this study there were over hundred isolates obtained belonging to *Pseudomonas, Serratia., Enterobacter, Klebsiella, Burkholderia, Bacillus, Acinetobacter, Arthrobacter, Micrococcus* and *Curtobacterium.* But only two strains namely *Bacillus amyloliquifacien* and *Serratia marcescens* were effective for growth promotion and disease suppression both in green house and field. The studies are underway to test these strains in farmers plots.

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Tuble 1 In this bereening of Thizobacteria				
Isolata Number	Inhibition (%)			
isolate Number	Pythium sp.	Phytophthora capsici		
GRB 35	78.51 (62.41)	77.08(61.43)		
GRB 57	85.9 (67.95)	78.75 (62.51)		
GRB 58	85.92 (67.99)	75.83 (60.56)		
GRB 68	84.07 9 (66.45)	75.00 (60.02)		
GRB 91	73.93 (58.88)	83.52 (66.08)		
Control	0.0 (4.9)	0.0 (4.9)		
LSD	53	43		

 Table 1
 In vitro screening of rhizobacteria

Values in the indices are Arc Sine transformed value.



Figure 1 Effect of rhizobacteria on sprouting in ginger in green house



Figure 2 Disease percentage in ginger in green house



Figure 3 Effect of rhizobacteria on soft rot incidence in field



Figure 4 Effect of rhizobacteria on the yield in ginger
# Genotypic analysis and plant growth-promoting ability of one nitrogen-fixing bacterium associated with sugarcane

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#### Abstract

Guangxi is the major sugarcane and sugar producing area in China and produces over 60% sugarcane and about 70% sugar in China. Nitrogen fertilizer input for sugarcane production in China is very high, compared to some other countries such as Brazil. It is imperative to substantially reduce the nitrogen fertilizer inputs and to substitute nitrogen fertilizers with biological nitrogen fixation to develop a high benefit-cost ratio and environmentally benign sugarcane production in China. In this study, we were engaged in screening and identifying nitrogen-fixing bacteria isolated from sugarcane plants in Guangxi and evaluate their plant growth-promoting ability. A nitrogen-fixing bacterium isolate GXS152 showed bright prospect in application of biological nitrogen fixation to reduce fertilizer input in commercial sugarcane production, protecting and improving the environment, and improving plant stress resistance of sugarcane in China.

The strain GXS152 was isolated by Ashby nitrogen-deficient minimal media from the rhizosphere soil of sugarcane, and was screened out by using acetylene reduction assay and amplification of *nif*H gene. GXS152 was indentified according to 16S rRNA sequence analysis and biologmicrobial identification system, and certified by other biochemical and physiological characteristics. The strain possessed plant growth-promoting traits of siderophore production, phosphate solubilization and ACC-deaminase activity, besides nitrogen fixation. The results of 16S rRNA sequence analysis indicated that GXS152 shared 99.8% homology with *Burkholderia cepacia*, and it was further classified by biologmicrobial identification system which results was contrast to *Burkholderia* sp.. The virulence test for indicated that the strain does not show pathogenicity to tobacco leaves and onion bulbs, and there was no any fragment appearing in the PCR products cloned from the GXS152 genome with specific primers of *B. cepacia* epidemic strain marker (BCESM), which indicated that GXS152 contain no *B. cepacia* epidemic strain marker. GXS152 could be introduced into the micropropagated plants of sugarcane in the one-tenth MS medium. The strain significantly promoted the growth of micropropagated sugarcane plants, increased dry weights and nitrogen content by 46.8% and 45.9%, respectively, compared with the uninoculated controls. <sup>15</sup>N isotope dilution assays showed that associative nitrogen fixation of GXS152 contributed 9.41%nitrogen to sugarcane plants. GXS152 inoculation increased the primary root length of canola growing under normal (25°C) and low (4°C) temperature. Likely, GXS152 contains ACC deaminase and reduces the level of developmental and stress ethylene in canola through degrading ACC.

Key words: nitrogen-fixing bacterium ; plant growth-promoting ability ; sugarcane

# Screening and identification of *Bacillus* strains against cereal cyst nematode in wheat

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# Abstract

The cereal cyst nematode (CCN), Heterodera avenae, has caused economically significant damage to wheat in China. Two hundred ninenty three Bacillus strains were isolated from wheat root and rhizosphere samples collected from middle plain region of China. Antagonistic abilities of these isolates against to the second stage juvenile (J2) of *Heterodera avenae* were carried-out.. The ferment suspension of 15 strains showed strong nematicidal activity to J2. The strains YLT40, BMT8, BMT19, BMT20 and YLT24 gave 100% mortality to J2 in-vitro after 48 h of treatment. Through the pot trials of wheat in the greenhouse we have selected superior. The results of control test showed that 10 strains inhibited formation of the galls. The control efficiency of strain YLT40, BMT20, and BMT8 were 59.09%, 45.45% and 40.91%, respectively, after 60 days. The strain YLT40 showed a significant reduction of disease symptoms and severity, compared to control under field conditions. Under laboratory, greenhouse, and field conditions, the strain YLT40 showed in increased root and shoot weight of wheat plants and shoot length and grain yield. The strain YLT40 was identified as *Bacillus pumilus* by using morphological, physiological, biochemical tests and cellular fatty acids analysis. In addition, 16S rDNA gene analysis illustrated that YLT40 exhibits high levels of similarity to known Bacillus species. Therefore YLT40 was finally identified as Bacillus pumilus.

# Siderophore based heavy metal resistant green fungicides

# for sustainable environment

Sayyed R  $Z^1$  & Redyy  $MS^2$ 

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#### Abstract

The growing cost of pesticides and consumer demand for pesticide-free food has led to a search for substitutes for these products. In this regards siderophore producing PGPR has been recognized as effective biocontrol agent against plant pathogens. Though the siderophores are specific ferric ion chelator, but they can also bind other metals also. Thus heavy metal contaminated soil can be heavily influenced by siderophores. In present study in-vitro phytopathogen suppression activity of siderophoregenic preparations of Ni and Mn resistant Alcaligenes sp. STC1 and Acinetobacter sp. SH-94B isolated from soil were found superior over the chemical pesticide. Siderophore rich culture broth and siderophore rich supernatant exerted antifungal activity against Aspergillus niger NCIM 1025, Aspergillus flavus NCIM 650, Fusarium oxysporum NCIM 1281, Alternaria alternata ARI 715, Cercospora arachichola, Metazhizium anisophilia NCIM 1311 and Pseudomonas solanacerum NCIM 5103. Siderophore rich broth and supernatant exhibited potent antifungal activity vis-àvis oraganophosphorus chemical fungicide; kitazine. The minimum fungicidal concentration required was 25 µl for Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Cercospora arachichola, Metazhizium anisophilia, Pseudomonas solanacerum and 75 µl for A. alternata.

Key words: *Alcaligenes* sp., *Acinetobacter* sp.; siderophores; phytopathogen; biocontrol; PGPR

#### Introduction

Heavy metals are metals with a density above 5 g/cm<sup>3</sup> (Nies, 1999) Presence of heavy metals even in traces is toxic and detrimental to both flora and fauna. PGPR capable of growing in presence of variety of heavy metals are seen as potent inoculants (Nilanjana *et al.*, 2008). Bioabsorption is one of the most important biological mechanisms which involve the ability of microorganisms to accumulate heavy metals from contaminated site through metabolically mediated pathway (Rafia Azmat *et al.*, 2007).

Every year, severe global economic losses to agricultural crops are encountered due to plant diseases caused by more than sixty pathogens leading to the loss of 30% crop yield amounting 416 Million US dollars (Nehl *et al.*, 1996). Since agricultural fields due to the uncontrolled use of chemical pesticides and fertilizers are most contaminated, search for Plant Growth Promoting Rhizobacteria (PGPR) having potential of adsorbing heavy metals from field will have twin advantage of bioremediation and plant growth promotion (Tripathi and Srivastava, 2007). Biocontrol through siderophore-mediated competition for iron have merged as a sustainable approach for Integrated Plant Disease Management (Sayyed *et al.*, 2005. Sayyed and Chincholkar, 2009. Lugtenberg *et al.*, 2002. Thrane *et al.*, 2000. Johri *et al.*, 2003).

Siderophores are also found to complex with heavy metals like Cadmium, Lead, Nickel, Arsenic (III, V), Aluminium, Magnesium Zinc, Copper, Cobalt, and Strontium other than iron (Sayyed RZ and Chincholkar SB, 2010. Nair *et al.*, 2006). Under iron stress conditions, rhizobacteria produce siderophores that chelate the available iron and prevent the iron nutrition of respective phytopathogen (Lemanceau and Albouvette, 1993) and there by restrict the proliferation and root colonization by phytopathogen. Siderophore producing rhizobacteria are also known to impart Induced Systemic Resistance (ISRs) to the plants (Van Wees *et al.*, 2000 Pieterse *et al.*, 2001) and suppressiveness to the soil (Mazzola, 2002) and have been implicated in the biocontrol of several plant diseases (Sindhu *et al.*, 1997). Siderophore based Biological Control Agents (BCAs) are gaining commercial significance as they are safer, do not lead to biomagnification, their self-replication circumvents repeated application and target organisms do not develop pesticide resistance (Sayyed *et al.*, 2005). They also provide iron nutrition to the crops thereby promote the plant growth (Sayyed *et al.*, 2007b).

The present work focuses on the antifungal activity of siderophore producing heavy metal resistant *Alcaligenes* sp., *Acinetobacter* sp., against some common phytopathogenic fungi and bacterial strain.

# **Materials and Methods**

# Sources of cultures

Bacteria were isolated from local soil and were labeled as *Alcaligenes sp. and Acinetobacter* sp. and fungal cultures were procured from National Center for Industrial Microorganisms [NCIM], NCL, Pune, India, All the cultures were preserved at  $4^{\circ}$ C.

#### Heavy metal resistance

To obtain maximum heavy metal resistance level for *Alcaligenes* sp. and *Acinetobacter* sp. they were stepwise inoculated on nutrient agar plate with increasing grades of heavy metal concentrations of MnCl<sub>2</sub> and NiCl<sub>2</sub>.

# Screening for siderophore production

In order to screen siderophore production ability, *Alcaligenes* sp. and *Acinetobacter* sp. were inoculated into sterile succinic acid medium (SAM) containing (gL<sup>-1</sup> in distilled water): K<sub>2</sub>HPO<sub>4</sub>, 6.0; KH<sub>2</sub> PO<sub>4</sub>, 3.0; (NH<sub>4</sub>) <sub>2</sub>SO<sub>4</sub>, 1.0; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.2; C<sub>4</sub>H<sub>4</sub>Na<sub>2</sub>O<sub>4</sub>.6H<sub>2</sub>O, 4.0; and pH.7, at 28  $\pm$ 2 °C at 120 rpm for 24~48 h. (Don *et al.*, 1984).

#### Partial identification and 16s rRNA sequencing

The isolates were subjected to various biochemical tests as per the procedure and protocols of Bergey's manual of systematic bacteriology (Don *et al.*, 1984). The presterilized biochemical kits (Hi Media, Mumbai, India) were used for biochemical's test. Further this partially identified culture was subjected to 16s rRNA gene profile. Standard phenol-chloroform methods were used for genomic DNA extraction (Sambrook *et al.*, 1989). The 16S rRNA genes of the isolate were amplified by PCR (Pidiyar *et al.*, 2002) and sequenced directly on an automated DNA sequencer (ABI377) using the Big Dye terminator kit (Applied Biosystems) (Hauben *et al.*, 1997). Results were compared with the public databases (NCBI) to determine the identity and homology of the isolate.

#### Siderophore production, detection and estimation

Growth and siderophore production was carried out in 500 mL Erlenmeyer flask containing 100 ml of modified SM (Meyer and Abdallah, 1978). For this purpose, *Alcaligenes* sp. and *Acinetobacter* sp.  $[6 \times 10^{6} \text{ cells ml}^{-1}]$  were grown independently in SM at  $28 \pm 2$  °C at 120 rpm for  $24 \sim 48$  h. The detection and estimation of siderophores was performed by using CAS test (Meyer and Abdallah, 1978) and CAS shuttle assay (Schwyn and Neilands, 1987).

#### In-vitro interaction with phytopathogenic fungi

*In-vitro* phytopathogen suppression activity of siderophoregenic culture and supernatant of *Alcaligenes* sp. and *Acinetobacter* sp. and kitazin was directed against *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Alternaria alternata, Cercospora arachichola, Metazhizium anisophilia* and *Pseudomonas solanacerum.* These strains are known to be common phytopathogens capable of causing major damages to the groundnut and other crops. *In-vitro* antifungal activity was based on the principle of diffusion assay. Control was prepared by removing siderophore through the addition of 8 hydroxyquinone. After 48 h incubation at 29 °C plates were observed for the inhibition of fungal and bacterial growth.

#### **Determination of Minimum Inhibition Concentration (MIC)**

In order to determine the MIC of the preparations, various preparations were separately taken in the range of  $20 \sim 100 \,\mu$ l. Each preparation was separately added

into PDA and NA previously seeded with fungal pathogen [one fungus per plate] and bacterial culture, MIC was determined following the 48 h incubation at  $29 \pm 1$  °C.

# Evaluation of safety for useful soil rhizobia

To be an ideal antagonist, the BCA should be effective against a wide range of pathogens at the same time it should not harm the useful soil rhizobia. Safety of these preparation against useful soil rhizobia like *A. vinelandii*, *R. meliotii* and *B. japonicum* was tested by diffusion assay.

# Results

# Heavy metal resistance

Maximum metal resistance level for bacterial *Alcaligenes* sp. and *Acinetobacter* sp. were observed on nutrient agar with different concentrations of MnCl<sub>2</sub> and NiCl<sub>2</sub>. Bacterial *Acinetobacter* sp. showing resistant to MnCl<sub>2</sub> salt uptill 3mg during step by step repeated culturing of bacterial strain on nutrient agar. Similarly *Alcaligenes* sp. resistant to 1mg NiCl<sub>2</sub> was obtained.

# Partial identification and 16s rRNA sequencing

Preliminary phenotypic characterization showed that the isolates were a Gramnegative straight, mobile rod, presented a fermentative metabolism on a wide range of sugars. (Table 1) and synthesized a fluorescent pigments when grown on nutrient agar. The isolates are named as *Alcaligenes* sp. and *Acinetobacter* sp. 16S ribosomal RNA partial gene sequencing of this isolates showed close relationship i.e. 98%, 99% with *Alcaligenes* sp. STC1 and *Acinetobacter* sp. SH-94B respectively therefore, this isolates are named as *Alcaligenes* sp. STC1 and *Acinetobacter* sp. SH-94B.

# Screening for siderophore production

In the shake flask studies, change in the color of SM from colorless to fluorescent green after 24h, indicated siderophore production.

# Siderophore production and detection

Addition of CAS to cell free supernatant changed the blue color of CAS to orange indicated siderophore production. *Alcaligenes sp* excreted highest amount (92.61%) of siderophore while *Acinetobacter* sp. produced less amount (43.22%) of siderophore.

# In-vitro interaction with phytopathogenic fungi

Siderophores produced by rhizobacteria chelate available iron and therefore create artificial shortage of iron to the respective phytopathogens thereby limiting their growth (Lemanceau and Albouvette, 1993). *In-vitro* phytopathogen suppression by *Alcaligenes* sp., *Acinetobacter* sp. indicated their biocontrol potential. Both siderophore rich culture broth as well as cell free supernatant were found to inhibit the

growth of phytopathogenic fungi. However, siderophore rich culture broth (Fig. 1) proved to be potent inhibitor of fungal pathogens than cell free culture supernatant (Fig. 2) and the chemical fungicide kitazin (Fig. 3). The presence of siderophoregenic rhizobacteria around root zone of plants is known to protect the plant from phytopathogen infestations by preventing its iron nutrition ((Estrella and Chet, 1998. Bloemberg and Lugtenberg, 2001. Manwar *et al.*, 2000. Johri *et al.*, 2003).

# **Determination of MIC**

As depicted in Table 1, the MIC of culture broth containing siderophoregenic *Alcaligenes* sp. and *Acinetobacter* sp. respectively was 25  $\mu$ l for *A. niger*, *F. oxysporum*, *A. flavus*, *C. arachichola* and *P. solanacerum* 75  $\mu$ l for *A. alternata*. The MIC of cell free supernatant was 75  $\mu$ l for *A. niger*, *A. alternata*, *A. flavus*, *F. oxysporum*, *C. arachichola* and *P. solanacerum*. As per the Table 1 the MFC of kitazin (fungicide) was 75  $\mu$ l for *A. niger* and *A. flavus* while 100  $\mu$ l for *F. oxysporum*, *A. alternata*, *M. ansisophilia and P. solanacerum*.

# Evaluation of safety for useful soil rhizobia

Siderophoregenic *Alcaligenes* sp. and *Acinetobacter* sp. didi not inhibit the growth of nay of soil rhizobia under test.

# Discussion

# Heavy metal resistance

Most bacterial strain accumulates metal by employing physico-chemical mechanisms and transport system of varying specificity. However, higher accumulation of metals ions than optimal level, prove toxic to organisms. Under such conditions, organisms adapt a mechanism of detoxification to ensure survival. We in this study were successful in developing Ni and Mn resistant strains by step-by-step repeated culture and selection on the medium containing increasing concentration of Ni and Mn.

# Antifungal activity against phytopathogenic fungi

Siderophores produced by heavy metal resistant isolates have been implicated in the biocontrol of several diseases, like vascular wilts caused by Fu. oxysporum and stem rot of pea nut caused by *Rhizoctonia solani* (Sindhu *et al.*, 2007). It have been reported that siderophore producing *Pseudomonas aeruginosa* was capable of inhibiting the growth of *A. niger*, *A. flavus*, *F. oxysporum* and *A. alternata* (Saikia and Bezbruah, 1995). It have been also reported that hydroxamate type of siderophore producing A. chrococcum RRLJ 203 inhibited the growth of *F. oxysporum* and other phytopathogenic fungi (Plessner *et al.*, 1995).

The siderophoregenic culture broth proved to be potent inhibitor of fungal pathogens than kitazin and cell free culture supernatant indicating the role of other secondary metabolites along with siderophores in the growth inhibition of pathogenic fungi.

#### Evaluation of safety for useful soil rhizobia

It have been reported that siderophore producing *P. aeruginosa* did not cause the growth inhibition of *A. vinelandii*, *R. meliotii* and *B. japonicum*(Saikia and Bezbruah, 1995). These results provide evidence for eco-friendly role of siderophoregenic *Alcaligenes* sp. and *Acinetobacter* sp. as a potent BCA.

#### Conclusion

Siderophore rich broth and supernatant are ecofriendly bio-control agents and have greater antifungal potential than chemical fungicide kitazin. Research into the mechanisms of plant growth promotion by *Alcaligenes* sp. and *Acinetobacter* sp. have provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents.

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# Over-view of commercial potential of plant growth-promoting rhizobacteria (PGPR) in the USA

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#### Abstract

Plant Growth-Promoting Rhizobacteria (PGPR) are potential alternatives to chemicals in plant disease management and yield enhancement of many agricultural crops which is an emerging concept worldwide. In the United States, the concept is gaining popularity and has wide applicability in field crops and vegetables. This paper focuses on the commercial availability of different categories of bioinoculants and the companies produced in the USA. The formulation types, delivery of the products, target host and pathogen will be reviewed. The current trends in PGPR research in the United States on important diseases of cereals (Sheath blight of rice) and vegetables (Bacterial leaf spots of pepper, tomato and cucumber) are discussed. Field level applicability and promising results obtained with a commercial PGPR formulation, Integral<sup>®</sup> (Bacillus subtilis MBI 600, from Becker Underwood, Iowa, USA) in rice sheath blight management will be presented. The results on mode of action of Integral in inhibiting mycelial growth, sclerotial germination of sheath blight pathogen, R. solani, and sheath blight lesions determined through scanning electron microscopy; and its colonization potential on rice seeds also forms the text of the present report. The future of PGPR based product marketability in the United States will be highlighted.

Key words: PGPR; Commercial potential; bio-efficacy; USA

# Introduction

Biological control of plant diseases as a potential alternative to fungicidal management is a promising concept gaining worldwide popularity. Of different biocontrol agents, plant growth-promoting rhizobacteria (PGPR) have wide applicability due to their potential antagonistic activities against plant pathogens and abilities to promote plant growth (Glick, 1995; Patten and Glick, 1996). Plant growthpromotion by PGPR is by both direct and indirect mechanisms (Patten and Glick, 1996). Both growth-promoting and pathogen controlling abilities of PGPR strains are well established (Nandakumar et al., 2001; Karpagavalli et al., 2002). Important mechanisms by which these PGPR act in soil include antibiosis and hyperparasitism (Raaijmakers et al., 1997). General characteristics and contributions of PGPR in plant growth-promotion include nitrogen fixation, enhancing phosphorus availability to plants, production of phyto-hormones, and regulation of ethylene levels in plants. Besides, biocontrol of plant diseases through production of antibiotics, HCN, and siderophores are the other major contributions of PGPR (Martinez-Viveros et al., 2010). Some of the PGPR strains with multiple mechanisms include Bacillus spp.: Azotobacter chroococcum; Enterobacter sp. NBRI K28; Pseudomonas fluorescens PSRB21; P. putida PSRB6; and Serratia marcescens (Martinez-Viveros et al., 2010). For example, in *Azospirillum*, plant growth-promotion is attributed to several other functions including phyto-hormone production rather than N fixation (Dobbelaere et al., 1999; Malhotra and Srivastava, 2008).

With increasing demand for organic farming, there is an increased emphasis on the use of chemical free produce. Commercial bio-pesticide usage in agriculture is increasing day by day owing to their advantages over chemicals. Generally, these biopesticides are eco-friendly, less toxic, more target specific, and they decompose faster after application when compared to conventional chemical pesticides (Raudales and Gardener, 2008). The market for commercial biocontrol agents is active and growing rapidly in the USA. The key segments of consumption of bio-pesticides include organic agriculture industry, vegetable farming, orchard crops and berries, federal and municipal programs on forestry, and water resources, etc. Commercial PGPR products are being applied to a wide variety of crops for controlling several plant diseases of fungal and bacterial origin. The common methods of application of these commercial PGPR products are to seed (Mew and Rosales, 1986), seedling dip (Al-Taweil et al., 2009), soil (Wang et al., 2009), foliar spray (Saikia et al., 2006), and by broadcasting (Kanjanamaneesathian et al., 2007). Commonly available PGPR formulations include talc, peat, press mud, vermiculite (Nakkeeran et al., 2005), water-soluble granules, liquid, floating pellets, and empty fruit bunches (Kumar et al., 2011).

In the United States, the field level applicability of commercial PGPR products range from cereals, millets, legumes, fiber crops, oilseeds, horticultural crops, nonbearing plants, vegetables, and orchards. The target diseases in these crops mainly

include soilborne fungal pathogens such as Fusarium, Rhizoctonia, and Aspergillus spp. (Kumar et al., 2011). The commercial PGPR products include different strains of Bacillus and Pseudomonas spp. The details of commonly used commercial PGPR products used in plant disease management in the USA are listed in Table 1. Vegetable farming is one of the major segments that consume commercial PGPR products. Foliar diseases of vegetables induced by bacterial pathogens in cucumber, tomato, and pepper cause significant yield losses. In the U.S., bacterial leaf spot of tomato (Lycopersicon esculentum) and pepper (Capsicum annuum) caused by Xanthomonas campestris pv. vesicatoria is an economically important disease. The pathogen causes defoliation and sun scald symptoms in fruits, thereby reducing market value. In cucumber (Cucumis sativus), angular leaf spot (Pseudomonas syringae pv. lachrymans) causes severe yield and quality losses. This paper elucidates the efficacy of certain commercial PGPR products in managing important diseases of vegetables. The mode of action of *Bacillus subtilis* MBI 600, (Integral<sup>®</sup>) manufactured by Becker UnderWood, Inc., Ames, USA, against rice sheath blight pathogen (Rhizoctonia solani) and colonization potential of Integral® on rice seeds is also presented in the present investigation.

#### **Materials and Methods**

#### i) Foliar diseases of Cucumber, Pepper and Tomato

Seedling plugs of cucumber, pepper, and tomato of the susceptible varieties to bacterial leaf spot disease were produced in seedling trays containing commercial soil-less potting mix in a greenhouse for  $3\sim4$  weeks. The seedlings were transplanted after four weeks into 4" plastic pots filled with same potting mix at two to three leaf stage. The plants were maintained in the greenhouse at 28°C with natural illumination. The plants were spray inoculated with *X. campestris* pv. *vesicatoria* (tomato and pepper) and *P. syringae* pv. *lachrymans* (cucumber) ( $1 \times 10^6$  CFU/ml) at three-to-five leaf stage until run-off. The products were applied at 1 week after pathogen inoculation as foliar spray at recommended doses. A randomized complete block design (RCBD) was used for the evaluation studies. There are 27 different treatments and each treatment was replicated five times. Separate healthy and pathogen inoculated controls were maintained. Transplants were watered as neededand maintained in a greenhouse. Seedlings were monitored for symptom production and also for phyto-toxicity symptoms if any. Plants were assessed for disease incidence and severity after 10 days.

# *ii) Mode of Action of B. subtilis MBI 600 (Integral<sup>®</sup>) on rice sheath blight pathogen* Interaction between *B. subtilis* MBI 600 and mycelia of *Rhizoctonia solani*

The discs of 8 mm mycelial mat of *R. solani* grown on PDA were sprayed with Integral<sup>®</sup> ( $2.2 \times 10^9$  CFU/ml) and incubated for three days at 28 °C. Fungal discs sprayed with sterile distilled water served as controls. Discs of fungal mycelium were

later prepared for SEM examination using standard procedures (Ziedan and El-Mohamedy, 2008). The occurrence of morphological changes in the hyphae of *R*. *solani* was recorded.

# Antagonism of B. subtilis MBI 600 on sclerotia of Rhizoctonia solani

Mature sclerotia of *R. solani* were dipped in 250 ml flasks containing 100 ml of Integral<sup>®</sup> ( $2.20 \times 10^9$  CFU/ml) and incubated for 24 h. Sclerotia dipped in sterile distilled water served as controls. The sclerotia were later dried on filter papers for another 24 h. Sclerotia were later cut and processed for SEM studies. Changes in the structure of sclerotia were recorded.

#### iii) Evaluation of seed colonization potential of B. subtilis strain MBI 600 in rice

Rice seeds (cv. Cocodrie) were surface-sterilized in 2% sodium hypochlorite for 10 minutes and then rinsed twice with sterile distilled water. Seeds were then soaked in cell suspension of MBI 600-rifr-1 (rifampicin resistant mutant of *B. subtilis* MBI 600) and prepared at a concentration of  $1 \times 10^8$  CFU/ml for 30 minutes. The treated seeds were later divided into 8 samples of five seeds each. The seed samples were dried in a laminar flow hood for 15 min and stored at room temperature. Seeds soaked in sterile distilled water served as a control. The samples were designated as "Day Zero through Day Six" serially. The colonizing potential of strain MBI 600 on rice seeds was determined by enumerating the population levels of MBI 600-rifr-1 on seeds in respective seed samples for every 24 h. Two seeds per each sample were taken and placed in 10 mL of sterile distilled water in test tubes and vortexed for 30 sec to resuspend the bacterial cells. Serial dilutions were prepared from the bacterial suspension and the population levels of MBI 600-rifr-1 were enumerated by plating on Tryptic Soy Agar plates amended with 100 mg/kg of rifampicin. Treated seeds from Day Six sample were plated on TSA plates amended with 100 mg/kg of rifampicin and incubated for 48 h to check for the presence of colonies of strain MBI 600. There were seven treatments, five replications for each treatment, and two seeds per replication.

#### **Results and Discussion**

#### i) Foliar diseases of Cucumber, Pepper and Tomato

#### Angular leaf spot of Cucumber

Of all the products evaluated, the effective ones include Cease/Millstop, Citrex, KPX-B2, Kleengrow, Kocide, K-Phite, SC, MBI 10605, SUMM-IR 4, and AMV 4024 Sum (Table 2). The mean disease severities for these products were in the range of 1.7 to 3.0 with no significant differences between them. The pathogen control plants recorded a mean disease severity of 5.0. Correspondingly, the number of bacterial leaf spots for these products ranged from 35 to 115.3 compared to the pathogen control which recorded a mean of 156.3 spots/leaf. Further, no significant differences were noted for these products with respect to the number of spots/leaf.

Ineffective products include Companion, Harpin, HMO 736, Kleengrow, Regalia, MBI 106020, Seacide, NAI 4201, Teagro, Actigard, and Kasumin. Mean disease severity for these products ranged from 3.3 to 5.0 and is on par with pathogen control (a disease severity of 5.0). Correspondingly, for these products the mean number of spots/leaf ranged from 71 to 220.7.

#### **Bacterial leaf spot of Pepper**

The disease severities for these products ranged from 1.0 to 2.7 compared to the pathogen control that recorded a disease severity of 3.7 (Table 3). Correspondingly, the mean number of spots/leaf on pepper plants for these products ranged from 4.7 to 40.0. Certain products were found to be more effective over others. Highly effective products include Citrex, Harpin, KPX-B2, Kleengrow, Kocide, K-Phite, Regalia, Seacide, MBI 10605, SUMM-IR4, NAI-4201, Taegro, Actigard, Kasumin, and AMV4024 Sum. The disease severities for these products were in the range of 1.0 to 1.6 with no significant differences. The other products that were found to be moderately effective include Cease/Millstop, Companion, Harpin, HMO 736, Kleengrow, Kocide, MBI 106020, SC 27, and Taegro.

#### **Bacterial leaf spot of Tomato**

Highly effective products include Cease/Millstop, Citrex, Companion, Harpin, HMO 736, KPX-B2, Kleengrow, Kocide, K-Phite, Regalia, MBI-106020, SC 27, Seacide, MBI-10605, NAI 4201, Taegro, Actigard, and Kasumin (Table 4). The mean disease severities for these chemicals ranged from 1.0 to 2.0 compared to the pathogen control (3.7, disease severity). Correspondingly, the number of spots/leaf for these chemicals was in the range of 7.7 to 24.0. The moderately effective products against tomato bacterial leaf spot include Kleengrow, SC 27, and SUMM-IR4 with a mean disease severity of 2.3.

# ii) Mode of Action of B. subtilis MBI 600 (Integral<sup>®</sup>) on rice sheath blight pathogen

#### Interaction between B. subtilis MBI 600 and mycelia of Rhizoctonia solani

The bacterial growth was seen adhering to and colonizing the hyphae, thus leading to maceration and malformation of hyphal tissues. Hyphal deterioration leading to breakage and lysis was the final step of mycoparasitism (Fig. 1). The hyphal width of *R. solani* was greatly reduced due to bacterial colonization ( $3.429\mu$ m) compared to that of healthy hyphae in control plates ( $5.744 \mu$ m).

Antagonism of *B. subtilis* MBI 600 on sclerotia of *Rhizoctonia solani*. Cross sections of mature, brown sclerotia of *R. solani* dipped in commercial formulation of strain MBI 600 have shown that the sclerotial contents were completely colonized by endospores (Fig. 2). Maceration of cell walls and fragmentation of inner hyphal elements were observed. Cross sections of sclerotia in the control that were dipped in sterile distilled water had intact inner cell walls and the structural integrity was maintained.

#### iii) Evaluation of seed colonization potential of B. subtilis strain MBI 600 in rice

The population of strain MBI 600 that was applied to rice seeds decreased with increase in time. At Day Zero, the population/seed of strain MBI 600 was  $12 \times 10^4$  CFU/ml. The population levels decreased to  $5.8 \times 10^4$  CFU/ml when enumerated on Day One. Significant differences were noticed between population levels of strain MBI 600/seed from Day Zero to Day One. Subsequent enumerations of populations of strain MBI 600 from Day Three through Day Six did not show any significant reductions on seeds. However, significant differences in population levels of strain MBI 600 were noticed for Day Two with Day Five and Day Six. The population levels of strain MBI 600 on rice seeds at the end of Day Six per seed were about 0.8  $\times 10^4$  CFU/ml. Plating of rice seeds at the end of Day Six produced growth of MBI 600-rifr-1 mutant on TSA plates amended with 100 mg/kg of rifampicin.

Overall, these commercial products were found to be promising under greenhouse conditions against foliar diseases of vegetables. Further, Integral<sup>®</sup> is highly antagonistic to rice sheath blight pathogen. The seed colonization potential of Integral<sup>®</sup> was significant in view of its survival on rice seeds up to six days. Our studies on field evaluation of Integral<sup>®</sup> in rice showed that it significantly improved root and shoot lengths of seedlings as seed treatment at  $2.2 \times 10^8$  and  $2.2 \times 10^9$  cfu ml<sup>-1</sup> over untreated control. Integral application on a transplanted crop as seed treatment + seedling root dip + foliar spray at  $2.2 \times 10^9$  cfu ml<sup>-1</sup> significantly reduced sheath blight severity (19.2 vs 69.7 in control) and per cent diseased tillers (25.1 vs 99.4 in control). Significant improvement in plant height (98.1 vs 78.5 cm in control) and number of tillers/plant (12.8 vs 10.0 in control) and grain yields were maximum with Integral application at  $2.2 \times 10^9$  cfu ml<sup>-1</sup> as seed treatment + seedling root dip + foliar spray.

Increased awareness on improving standards of food quality and phasing out of chemical pesticide usage is mandatory. An important factor contributing to biopesticide marketability is increased consumer preference for organic and pesticide free produce. Coupled with emphasis by on sustainable agricultural systems using IPM and increasing numbers of companies in the bio-pesticide business, the market potential for bio-pesticides is growing (Dent and Waage, 2001). Given their efficacy in plant disease management and plant growth-promotion in a wide range of crops, commercial potential and marketability of PGPR based products are expected to increase keeping in view the aforesaid factors.

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# Evaluation of rhizobacterial strains against Bhendi Yellow Vein Mosaic Virus (BYVMV) Disease in Okra

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#### Abstract

As many as fifty rhizobacterial isolates from the culture collection of Departments of Agricultural Microbiology and Biotechnology, UAS Dharwad were screened against Bhendi Yellow Vein Mosaic Virus (BYVMV) disease in bhendi. The bioformulations of rhizobacteria applied to seed, soil and foliage significantly reduced the incidence of BYVMV, with a concomitant increase in plant growth and fruit yields under glasshouse condition. Based on the disease control, five isolates (B-40, 212(1), 212(4), 205(4) and 218(1) were selected and further evaluated for their ability to control BYVMV and promote growth in bhendi. These isolates controlled the disease ranging from 60 to 86.67 percent. The green house experiment revealed that *Pseudomonas* 218(1) was the most promising isolate which controlled BYVMV by 86.67 per cent. The mechanism of virus control was elucidated. All the isolates induced systemic resistance in bhendi plants. Plants inoculated with the lignite based formulation of fluorescent *Pseudomonas* 218(1) recorded the highest phenol content, peroxidase activity and PALase activity (20.83 %, 79.35 % and 47.05 % respectively higher than the diseased control). The plants treated with this strain also showed the highest reduction in the insect population (83.33 per cent less than that of the diseased control). Semi quantitative PCR analysis revealed lower viral load accumulation in plants inoculated with these isolates. The fluorescent Pseudomonas 218(1) recorded maximum plant height, total biomass, chlorophyll content and fruit yield, which were increased by 46, 56, 62 and 132 per cent, respectively over the diseased control.

Key words: rhizobacteria; BYVMV; bhendi; induced systemic resistance; biocontrol

# Introduction

Bhendi or Lady's finger (*Abelmoschus esculentus* (L). Moench) is one of the important vegetable crops grown throughout the tropical, subtropical and warm sections of the temperate zones of the world. It is affected by a number of diseases which cause substantial losses in yields. The yellow vein mosaic virus is the most serious disease of okra in the Indian sub-continent. In Karnataka, South India, the

disease is more serious when sowing is carried out from January to May (Singh, 1980). The yield loss due to this virus ranged from 50 to 90 per cent depending on the stage of the crop growth at which the infection occurs (Sastry and Singh, 1974). It is transmitted through whitefly (*Bemesia tabacii* Gennadius) (Aleyrodidae: Homoptera).

Several approaches have been attempted to manage the bhendi yellow vein mosaic virus. Vector control through spray of chemicals in many instances does not bring about the desired results. Commercially acceptable resistant varieties are not always available. Of late, management of virus diseases has been attempted by many scientists using plant growth promoting rhizobacteria (PGPR) as an alternative strategy for the disease management which is ecologically sound and environmentally safe.

A number of plant growth promoting rhizobacteria have been implicated in the biocontrol of virus diseases in many crop plants such as tomato spotted wilt virus (Kandan *et al.*, 2003), sunflower necrosis virus (Srinivasan *et al.*, 2005), banana bunchy top virus (Kavino *et al.*, 2003) and TMV in tomato (Kirankumar, 2007). These viruses have been controlled essentially through induced systemic resistance by activating defense genes encoding Chitinase, beta-1, 3 glucanase, peroxidase, PALase and other enzymes (Srinivasan *et al.*, 2005, Kirankumar, 2007) and chemicals (M'piga *et al.*, 1997). Hence, the present study was conducted to screen the rhizobacterial collection for the biocontrol of BYVMV and elucidate the mechanism of biocontrol in the selected rhizobacteria.

# Materials and methods

**Raising Bhendi plants:** Bhendi seeds were sown in plastic pots filled with soil and farmyard manure. Ten to fifteen days old seedlings, at two-leaf stage, were used for inoculation purposes.

**Maintenance of the viral inoculum:** The culture of BYVMV was obtained from the field in and around Agricultural College, UAS, Dharwad and inoculated to healthy bhendi plants using whiteflies (*Bemisia tabaci*) as the vector and the plants maintained in the glass house throughout the period of study.

**Vector Culture rearing:** Whiteflies were collected from cotton and Bhendi plants from fields by sucking with the help of an aspirator by slowly turning the leaves slightly upwards. Whiteflies were released on to the BYVMV diseased Bhendi plants grown in insect proof rearing cages and continuously maintained by introducing new younger plants in to the rearing cage. Thus, the insects were made viruliferous.

**Release of viruliferous insects:** The viruliferous insects were sucked from the diseased plants with the help of an aspirator and released on to healthy, PGPR treated bhendi seedlings on the top leaves. Immediately, glass tubes were placed over these seedlings in the inverted position so as to contain these insects for 24 h to bring about

infection by the virus. Two leaf stage seedlings were used for release of the viruliferous insects. Thus, it was ensured that all seedlings were infected with BYVMV.

**Rhizobacterial isolates and its maintenance:** From the culture collection of Department of Biotechnology and Department of Agricultural Microbiology, fifty rhizobacterial isolates, including fluorescent *Pseudomonas*, non fluorescent *Pseudomonas* were randomly picked up and fluorescent *Pseudomonas* were maintained on King's B agar medium and non fluorescent *Pseudomonas* on nutrient agar.

**Greenhouse study:** The rhizobacteria were seed treated to bhendi seeds by mixing them with the lignite-based culture 200 g per acre seed after coating with jaggery solution, air dried for 30 minutes under shade and the seeds were then sown in pots. For soil application, the lignite based culture was applied to soil 5kg/ha before sowing seeds and mixed. For foliar application, the lignite based culture was filtered through muslin cloth and sprayed 1% (w/v) at 20 days after sowing (DAS) and 30 DAS. Control plants in pots without application of rhizobacteria were also maintained. All treatments were replicated three times and arranged in a completely randomized design under glasshouse conditions.

**Preparation of lignite based formulation of rhizobacteria:** Erlenmeyer's flasks (100 ml) containing 50 ml nutrient broth were inoculated with a loopful of bacteria and incubated on a rotary shaker at 150 rpm for 3 days at 30°C. The broth was mixed with sterile lignite powder at 1:3 ratio and the formulation prepared.

**Sample collection, Enzyme and phenol estimation:** Leaf samples were collected at 45 DAS and 75 DAS from both treated and untreated bhendi plants. They were frozen immediately in liquid nitrogen, ground to a powder and stored at -80 °C until determination of phenylalanine ammonia lyase (PALase) enzyme and peroxidase activities. The powder sample was extracted with 10 ml of chilled 0.1M phosphate buffer (pH 7.0) in a pestle and mortar. The mixture was centrifuged at 18,000 rpm at 5°C for 30 min. and the supernatant used as the enzyme source for the estimation of peroxidase enzyme. For the estimation of PALase activity, however, one gram powdered sample was extracted with two ml of Sodium phosphate buffer 0.1M (pH 7.0) at 4°C. The homogenate was centrifuged at 10,000 rpm for 20 min. and the extract prepared. The Peroxidase activity was assayed spectrophotometrically following the method described by Mahadevan and Sridhar (1986). The PALase activity was determined using the method described by Ross and Sederoff (1992). And, the total phenol content in leaves was estimated at 45 and 75 DAS by following Folin Cio-calteau method (Sadasivam and Manickam, 1991).

**Enumeration of the vector**, *Bemisia tabaci* : The number of insects present on the lower surface of leaves were counted by slowly turning the leaf without disturbing the insects. Enumeration was done in all the treatments at 45 DAI and 75 DAI.

**Semi quantitative analysis of rhizobacterial treated leaves:** Semi quantitative PCR was carried out for detection and estimation of the viral DNA accumulation in the leaves. One hundred nanograms of DNA from each treatment were used as template in a 20µl PCR reaction containing PCR ingredients. For the PCR reaction, dNTP (1 mM), Forward (5 pM) and Reverse (5pM) primer, Taq buffer and Taq polymerase were used. ('Eppendorf' make Thermal cycles)The PCR reaction was performed for different reaction cycles of 10, 20, 30 and 40 cycles with the same reaction conditions throughout. After the reaction, the samples were run on 1% agarose gel for comparison. BYVMV specific coat protein (CP) primers - forward sequence 5'-AAGCTTATGTCGAAGCGAGCTGCCCT-3' (25 nt) and reverse sequence 5'-TCAATTCGTTACAGAGTC-3' (18 nt) designed by Jose and Usha (2000) were used for the amplification of the coat protein of BYVMV.

**Growth and Physiological parameters:** To study the influence of rhizobacteria on growth and yield of bhendi, plant height (cm), total biomass (g), yield parameters (fruit number and fruit weight per plant) were recorded. The plant height and total biomass content were estimated at 25, 40, 60 and 75 DAS. Chlorophyll content was measured by using a SPAD (Soil Plant Analysis Device) meter by selecting four leaves randomly at the centre of the branch and the average worked out.

**Statistical analysis:** The data were subjected to Completely Randomized Block Design analysis and interpretation of the data was carried out in accordance with Panse and Sukhatme (1985). The level of significance used in the 'F' and 't' test was P=0.01. The critical difference values were calculated whenever the F test values were significant.

# **Results and Discussion**

The management practices that are available to manage BYVMV in bhendi crops are not economically viable. Excessive chemical application renders soil unproductive. Thus, pest management practices are to be replaced by naturally occurring and introduced biocontrol agents. Towards this end, the biocontrol ability of the five rhizobacterial isolates was evaluated under high virus - vector pressure conditions. Application of rhizobacteria significantly reduced BYVMV incidence under glasshouse conditions. At 30 days after inoculation (30 DAI) of the pathogen, the disease control varied from 33.33 to 100 percent. Based on the percent disease control by the isolates and their effect on visual plant growth, five isolates B-40, 212 (1), 212 (4), 205 (4) and 218 (1) were selected for further characterization and biocontrol studies. Though all the isolates controlled the disease, the maximum disease reduction of 86.6 % was observed in bhendi plants treated with fluorescent *Pseudomonas* 218(1) (Table 1). To our knowledge, this is the first report on the biocontrol efficacy of rhizobacterial isolates against BYVMV in bhendi. Murphy *et al.* (2000) also observed the biocontrol efficacy of *P. fluorescens* against CMV and tomato mottle virus in tomato under field conditions. Kandan *et al.* (2003) showed the biocontrol efficacy of *P. fluorescens* strains against tomato spotted wilt virus (TSWV) in tomato under field conditions.

Recent investigations on the mechanisms of biocontrol by rhizobacteria have revealed that several strains protected plants from various pathogens including viruses by activating defense molecules (Kandan *et al.*, 2002). In the present study, higher level of accumulation of phenolics was observed in plants treated with fluorescent *Pseudomonas* 218(1) compared to untreated plants. This strain recorded 20.83 per cent higher phenol content than the disease control (Fig 1). These findings are in agreement with Kandan *et al.* (2003), who observed increase in phenolics in cowpea due to *P. fluorescens* inoculation which, in turn, protected plants from spotted wilt virus.

Inoculation of bhendi plants with *Pseudomonas* 218(1) also resulted in the highest synthesis of phenylalanine ammonia lyase (47.05 per cent higher than the diseased control) (Fig 2). Phenylalanine ammonia lyase (PALase) activity has been observed to be induced during plant -pathogen and plant - pest interactions (Harish, 2005) and is known to play an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.*, 1997). Though all the strains tested in this investigation induced biosynthesis of peroxidase, *Pseudomonas* 218(1) showed the highest induction of peroxidase which was 79.35 percent higher than the diseased control (Fig 3). These results are in agreement with the previous findings of Harish (2005), who observed control of banana bunchy top virus (BBTV) due to induction of increased peroxidase activity. Thus, the observed induction of enzymes and the corresponding reduction in BYVMV infection in bhendi supports the hypothesis that the resistance induced by isolates is systemic.

The inoculation of the rhizobacteria resulted in significant reduction in the population of the vector. In the present study, *Pseudomonas* 218(1) reduced the population over 80 per cent when compared to the diseased control (Fig 4). This is in conformity with the finding of Sikora and Murphy (2005) who also observed that PGPR controlled cucumber mosaic virus in tomato by reducing the population of its vector, aphids by 80 percent.

The semi quantitative PCR can detect and estimate the viral DNA load in the plants. Hence, PCR reactions using coat protein gene specific primers were performed for different number of cycles *viz.*, 10, 20, 30 and 40. The leaves of rhizobacteria-treated and untreated bhendi plants were tested for the presence of the virus. In general, a slight reduction in the viral inoculum load was observed in all the treated plants. At both 45 DAS and 75 DAS, fluorescent *Pseudomonas* 218(1) showed the least viral

load (Fig.5). However, this needs to be further validated. Zehnder et al (2000) also followed ELISA method to detect the viral load (cucumber mosaic virus) in PGPR treated cucumber plants. Even in field trials, they observed significantly lower ELISA values in all PGPR treatments than in the disease control, with a concomitant decrease in disease severity.

In addition to suppressing the viral disease, all the rhizobacterial isolates greatly improved plant growth, biomass, chlorophyll content and fruit yield (Table 2). This may be due to higher production of phytohormones by the rhizobacteria. *Pseudomonas* B-40 had stimulated growth and yield of tomato in earlier investigations (Earnapalli 2005; Kirankumar 2007). Inoculation of *Pseudomonas* 218(1) significantly improved growth components and fruit yield of bhendi. It increased the plant height by 46.15 percent, biomass by 56.63 percent, chlorophyll content by over 62.69 percent and fruit yield by 132 percent compared to the virus inoculated control. Increased growth, biomass and yield as a result of bacterization with rhizobacterial strains have been previously reported and have been implicated to be due to an increase in the production of plant growth substances (Sivaprasad *et al.*, 2003 and Earnapalli, 2005).

Thus, the study has brought out the biocontrol potential of the selected PGPR in reducing the incidence of BYVMV in bhendi essentially through induced systemic resistance by triggering defense molecules and deterring whiteflies as well. The strains have also promoted plant growth and fruit yield of bhendi.

		45 DAI	75 DAI
Sl. No.	Treatments	Per cent disease control	Per cent disease control
1	Pseudomonas B-40 + BYVMV	80.00	80.00
2	Pseudomonas 212 (1) + BYVMV	60.00	60.00
3	Pseudomonas 212 (4) + BYVMV	60.00	60.00
4	Pseudomonas 205 (4) + BYVMV	80.00	80.00
5	Fluorescent Pseudomonas 218 (1) + BYVMV	86.67	86.67
6	(P. fluorescens NCIM 2099) + BYVMV	86.67	86.67
7	Diseased control (only BYVMV)	0.00	0.00
8	Healthy control (no rhizobacteria, no BYVMV)	-	-

Table 1 Inhibition of BYVMV by the selected rhizobacteria

DAI - Days after inoculation

Sl. No.	Treatments	Plant height (cm)	Total biomass (g/plant)	Chlorophyll (SPAD)	Fruit yield (kg/plant)
1.	Pseudomonas B-40 + BYVMV	43.00 (23.44)	21.46 (42.09)	35.360 (35.16)	0.400 (60)
2.	Pseudomonas 212 (1) + BYVMV	44.26 (33.10)	20.86 (37.79)	34.520 (31.95)	0.350 (40)
3.	Pseudomonas 212 (4) + BYVMV	48.20 (35.96)	22.42 (46.36)	39.840 (52.29)	0.520 (108)
4.	Pseudomonas 205 (4) + BYVMV	50.00 (43.44)	23.22 (53.40)	41.820 (59.86)	0.560 (124)
5.	Fluorescent <i>Pseudomonas</i> 218 (1) + BYVMV	51.22 (46.15)	24.58 (56.63)	42.560 (62.69)	0.580 (132)
6.	Reference strain ( <i>P. fluorescens</i> NCIM 2099) + BYVMV	50.18 (45.09)	23.40 (54.32)	40.900 (55.41)	0.570 (128)
7.	Diseased control (only BYVMV)	35.10 (-)	15.54 (-)	26.160 (-)	0.250 (-)
8.	Healthy control (no rhizobacteria, no BYVMV)	41.64	20.10	31.700	0.390
	SEm <u>+</u>	0.71	0.36	1.04	0.001
	CD 1%	2.75	1.41	4.03	0.006

 Table 2
 Plant growth parameters of bhendi as influenced by inoculation with rhizobacteria (at 60 DAS)

\* Figures in parentheses indicate per cent increase over diseased control DAS – Days after sowing



M:100 bp marker Lane C: BYVMV DNA amplicon after 20 cycles (Positive control)

Lane 1-6: PGPR treated plants after 20 cycles

# Figure 1 Semi- quantitative PCR analysis of BVYMV in bhendi. Experiment for detection of viral load

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# Studies on pre-harvest aflatoxin contamination in peanut and its integrated management using host plant resistance

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# Abstract

Groundnut is an important oil seed crop of Asia earning lot of revenue. Among biotic stresses in groundnut, aflatoxin contamination is a major problem showing significant impact on trade. Aflatoxins are carcinogenic, teratogenic and hepatotoxic metabolites secreted by A. *flavus* and A. *parasiticus*. The present study focuses on characterization of aflatoxin resistant cultivars of groundnut and its integrated management through use of host plant resistance. The objectives include i) Screening of various groundnut genotypes for their resistance to aflatoxin contamination, and ii) Integrated management of aflatoxin problem under greenhouse and field conditions. The methodology involved screening of elite groundnut germplasm lines under laboratory conditions for their resistance to A. flavus colonization, and under field conditions for their resistance to aflatoxin contamination. Ten elite genotypes were evaluated at field level in a factorial Randomized Complete Block Design with irrigation and drought as main treatments, 12 sub treatments and six replications in A. *flavus* sick soil. Resistance in genotypes was determined through aflatoxin content by ELISA, total sugars, total proteins, total phenols and shell wall integrity. In-vitro studies revealed that of different genotypes, highest toxin content was found in Narayani (>5000 ppb/kg) and lowest in TPT-4 and K-134 (2855 ppb/kg) seeds. Field studies indicated that TAG-24 recorded low toxin levels under drought (7.3 ppb/kg)

whereas in irrigated plots, TIR-9 recorded lowest levels (4.82 ppb/kg). The genotypes TPT-4 and TCGS-913 had high phenol content and exhibited high shell wall integrity. Based on our results, we can conclude that TPT-4 can be recommended for drought prone areas under rainfed (*Kharif*) conditions, whereas TCGS-913 is ideal for post rainy season (*Rabi*) under the conditions evaluated. Isolation of fluorescent *Pseudomonads* from groundnut rhizosphere and their laboratory screening against *A. flavus* also significantly inhibited these fungi. Further studies are in progress to identify a potential Plant Growh-Promoting Rhizobacteria (PGPR) against aflatoxin problem through various screening assays.

# Key words: groundnut; aflatoxins; Aspergillus flavus; host plant resistance; management

# Introduction

Groundnut (*Arachis hypogaea* L.) is a major legume and an important oilseed crop in India and in many other developing countries. The crop is a rich source of highquality edible oil, minerals, vitamins A, B and some of B<sub>2</sub> group vitamins for humans. Processed groundnut cake flour and groundnut protein concentrates are being used on a wide scale in diets of infants and children. The crop is affected by several diseases like leaf spots, collar rot, rust, bud necrosis, stem necrosis etc. Apart from these, aflatoxin is one of the major problems, produced in the infected peanut seeds by *Aspergillus flavus* Link ex fries and *A. parasiticus* Speare, particularly at the end of season under drought conditions (Diener *et al.*, 1987). Both pre- and post-harvest aflatoxin contamination are problematic and have significant negative impact on peanut industry besides posing serious health risks (Will *et al.*, 1994). Besides groundnut, aflatoxins are also problematic in cotton and maize, and thus contaminating food and animal feed (Doyle *et al.*, 1982) These aflatoxins are highly carcinogenic, immunosuppressive agents, highly toxic and fatal to humans and animals particularly affecting liver and digestive tract.

The extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of the peanuts to fungus before they are harvested, and during storage, and/or processing periods. Aflatoxin is a naturally occurring mycotoxin produced by two types of mold: *Aspergillus flavus* and *A. parasiticus*. Of them, *A. flavus* is common and widespread in nature and is most often found when certain grains are grown under stressful conditions such as drought. The mold occurs in soil, decaying vegetation, hay, and grains undergoing microbiological deterioration and invades all types of organic substrates whenever and wherever the conditions are favorable for its growth. Favorable conditions include high moisture content and high temperature. At least 13 different types of aflatoxin are produced in nature with aflatoxin B<sub>1</sub> considered as the most toxic. While the presence of *Aspergillus flavus* does not always indicate harmful levels of aflatoxin it does mean that the potential for aflatoxin production is present.

Aflatoxin contamination can be minimized by adopting certain cultural, produce handling, and storage practices. However, these practices are not widely adopted particularly by the small farmers in the developing countries, which contribute about 60% to the world groundnut production. The available management practices are not economical and no effective method has been found to reduce the aflatoxin production in groundnut. Hence, the possibility of managing this disease is through host plant resistance. Moreover, the biochemical studies for disease resistance will give a clear insight into the basis of host-pathogen interaction. The use of electrophoresis to analyze the different proteins and isozymes is an essential feature of the molecular study, which could be used as markers in breeding program. One of the possible means of reducing aflatoxin contamination of groundnut is the use of cultivars resistant to seed invasion by aflatoxin-producing fungi or to aflatoxin production. These cultivars will be of great value to the farmers in both developed and developing countries as there is no cost input. Therefore, breeding for resistance to A. flavus and A. parasiticus and/or aflatoxin production can play a significant role in preventing aflatoxin contamination in groundnut and consequently associated economic losses and health hazards. Therefore, this study was undertaken to screen various groundnut genotypes for their resistance to aflatoxin contamination, and ii) Integrated management of aflatoxin problem under greenhouse and field conditions.

#### **Materials and Methods**

The laboratory, pot culture experiments pertaining to the present investigation on "Integrated Management of *Aspergillus flavus* in Groundnut" were carried out in the Department of Plant Pathology, S. V. Agricultural College, A N G R Agricultural University, Tirupati, India. Field experiments were carried out in dry land, S.V. Agricultural College Farm, Tirupati. The experimental site is located at 130 North Latitude and 790 East Latitude with an altitude of 182.9 m. above mean sea level in the tropical belt of South India.

# **Plant Material**

Ten groundnut genotypes were used in screening for aflatoxin production. They were Narayani, TPT-4, TPT-25, K-134, TAG-24, TCGS-888, TCGS-913, TCGS-341, TIR-9, VG-39 obtained from Regional Agricultural Research Station, Tirupati, AP, India.

# *In vitro* screening of groundnut genotypes

In vitro screening of different groundnut genotypes for seed colonization by *Aspergillus flavus* has done according to the procedure given by Mixon and Rogers (1973). Twenty grams seeds were placed in a 250 ml beaker, soaked for 15 to 20 minutes in sterile water using two changes of approximately 100 ml each in the soaking process. After the second change, the water was drained from the seed, and

the sample was inoculated with a 1 ml of spore suspension  $(4 \times 10^6 \text{ spores/ml})$  obtained from 10 days old culture of *Aspergillus flavus* multiplied on *Aspergillus flavus* and *parasiticus* agar (AFPA) medium. Seeds were placed in petri-plates and sterile water was added to adjust the seed moisture to 30 percent incubated in a humid chamber for 7 days at 25°C, 98% relative humidity. The percentage of seed infection was recorded based on number and color of the spots on the seed. Aflatoxin content of seed samples was estimated by indirect Enzyme Linked Immunosorbent Assay (ELISA).

#### In vivo screening of groundnut genotypes for aflatoxin contamination

#### Mass multiplication of Aspergillus flavus

The virulent strain of *A. flavus* was obtained from Department of Plant Pathology, Regional Agricultural Research Station, Tirupati, AP, India. The fungus was multiplied by organic-matrix method as per the modified procedure given by Will *et al.*, 1994. A cracked Bajra seed was soaked in water for overnight. After that seeds were taken out and sterilized in autoclave at 121°C for 15 minutes. A 5 mm disc of *A. flavus* from actively growing 10 day old culture was transferred to sterilized seed and incubated at  $25 \sim 30$ °C for 7 days.

#### Field Evaluation

The experiment was conducted during, *Rabi* season with two main treatments and 12 sub treatments and 6 replications with factorial RBD design. The two treatments were drought and irrigated conditions each with 3 replications in mini plots with the size of  $1.8 \times 2.5$  m<sup>2</sup>. The field was prepared by ploughing thrice and then leveled by harrowing. The seeds were sown in the plots with a size of  $1.8 \times 2.5$  m<sup>2</sup>. A spacing of  $30 \times 10$  CM was adopted. The groundnut varieties viz., Narayani, TPT-4, TPT-25, K-134, TAG-24, TCGS-888, TCGS-913, TCGS-341, TIR-9, VG-39 were screened in the present study. The field was prepared by ploughing thrice and then leveled by harrowing. The seeds were sown in the plots as per the layout given below along with recommend dose of fertilizers. The mass multiplied inoculum A. flavus was applied at 30 days after sowing (DAS) and second time at 60 DAS. Initially slight furrow was made at one side of the row and then pathogen was applied 2.5 g per one meter length of the plot and then the furrow was closed. Irrigation was given just two days before the inoculum application in order to allow the multiplication of the pathogen in the soil. A total of sixteen irrigations were given to the crop in the irrigation treatment  $(R_1, R_2 \& R_3)$  at an interval of 7 to 10 days. The crop under drought treatment (R<sub>4</sub>, R<sub>5</sub> & R<sub>6</sub>) was given regular irrigations (7 to 10 days interval) up to 60 days after sowing 60 DAS the interval between irrigations were up to 18 days for imposing the stress conditions. The total number of irrigations given to the crop in this treatment was reduced to nine. The crop was harvested after 120 days

and then the genotypes were evaluated for natural seed colonization using standard procedures, and for aflatoxin contamination by ELISA test. Biochemical characters of harvested groundnut seeds such as total phenol content using standard procedures. Similarly, shell wall integrity was determined using as per the standard protocols. Shell wall integrity was determined by using the formula

Shell wall integrity (%) =  $10 \times$  Initial Electrical Conductivity/Final Conductivity

# **Results and Discussion**

# In vitro screening of groundnut genotypes

Of different groundnut genotypes screened under in vitro conditions for colonization and aflatoxin contamination due to *A. flavus*, all of them were susceptible. The seeds of all the genotypes appeared visually green colored due to green colored conidia of the mold. Aflatoxin production was observed in all genotypes and significant differences were observed among genotypes. Highest toxin content was reported in Narayani (>5000 ppb per kg) and lowest in TPT-4 and K-134 (2855 ppb per kg) (Table 1).

# In vivo screening of groundnut genotypes for aflatoxin contamination

Of ten groundnut genotypes screened under field conditions for their natural infection by A. *flavus* and aflatoxin contamination, the results indicated that there was significant difference in aflatoxin content for both treatments. The mean aflatoxin content of all groundnut genotypes were higher under drought (882 ppb) compared to irrigated conditions (24 ppb). Genotypic differences were non significant under both stress and irrigated conditions. The groundnut genotypes, VG-39 (2573.4 ppb per kg kernel) and K-134 (2482.3 ppb per kg) recorded high aflatoxin content; whereas TAG-24 (7.3 ppb per kg) recorded low aflatoxin content under drought conditions. Under irrigated conditions TCGS-888 (90.2 ppb per kg) was observed with the high aflatoxin contamination where as TIR-9 (4.82 ppb per kg) was recorded the low. The interaction between genotypes and treatments was also non significant (Table 2). Results on natural seed colonization of A. flavus on groundnut genotypes also revealed that there was significant difference in seed colonization between the two treatments except for TPT-25 and TPT-4. Further, the seed infection was observed more under drought conditions compared to irrigated conditions (Table 2). It was more in Narayani (56%) and less in TCGS-888 (21%) under irrigated conditions. Under drought conditions less seed infection was observed in TCGS-888 (25%) and more in TPT-4 (80%).

# Total phenol content of groundnut genotypes

The experimental results on total phenols in groundnut seeds after harvesting under drought and irrigated conditions were presented in Table 3. These results indicated

that there is a non-significant difference in total phenolic content in harvested groundnut kernels under irrigated conditions except TPT-4, TCGS-913 and VG-39. The genotypic differences were non-significant under drought conditions for all genotypes except for TPT-25, K-134, TAG-24 and Narayani. Among the genotypes tested TPT-4 recorded highest phenols (3.23 mg per gm of seed), where as TPT-25 and TCGS-341 recorded lowest (2.01 and 2.12 mg per gm of seed) under irrigated conditions. When stress was imposed Narayani reported highest phenolic content (2.932 mg per gm) followed by TAG-24 (2.93 mg per gm) followed by TCGS-888 (2.59 mg per g) and TPT-25 showed lowest (1.46 mg per gm) compared to all other genotypes. The interaction between genotypes and treatments was non-significant.

#### Shell wall integrity of groundnut genotypes

The experimental results were presented in Table 3. These results indicated that there is a significant difference in shell wall integrity in groundnut kernels at the time of harvesting between irrigated and drought conditions. Shell wall integrity was more under irrigated conditions when compared to drought conditions. Under irrigated conditions it was highest in the genotype TCGS-341 (96.3%) followed by VG-39 (94.2 %) and it was lowest in TPT-4 (73.2 %). Under drought conditions also the TCGS-341 showed maximum (89.3 %) followed by minimum in TPT-4 (66.3 %) values respectively. It also indicated that genotypes differed significantly under irrigated and drought conditions. The genotypes TPT-25, TIR-9 and K-134 showed non-significant difference under irrigated conditions. TPT 4 and K-134 are on par with each other in shell wall integrity under drought conditions. The interaction between genotypes and treatments was also significant.

Screening of groundnut germplasm for their resistance to invasion by A. flavus and subsequent aflatoxin contamination is a pre-requisite for identification of resistant lines that can finally be incorporated into the breeding program. This step will finally enable to identify the factor of host plant resistance and its subsequent inclusion in the ambit of IDM of pre-harvest aflatoxin management in groundnut. Mixon and Rogers (1973) developed a new in vitro seed colonization procedure for screening the groundnut genotypes against A. flavus. Their results indicated that Valencia type genotypes viz., PI337394F and PI337409 were resistant to two toxin producing strains of the fungus. In another study, Priyadarshini and Tulpule (1978) studied the reaction of different varieties of maize and groundnuts and stated that there is no direct correlation between fungal growth and aflatoxin production, suggesting that the genotypes produced different amounts of aflatoxin per unit growth of the fungus. Mehan *et al.*, (1981) reported that inoculation of seeds of seven ground nut cultivars with three different toxigenic strains of A. flavus showed marked differences in invasion potential between cultivars. Among them, J-11, PI 337409 and PI 337394 were found to be resistant to invasion and colonization by all three strains and the strain NRRL, 3000 was less virulent than other two on all the cultivars.

Overall, our results indicated that of different genotypes, highest toxin content was found in Narayani (>5000 ppb/kg) and lowest in TPT-4 and K-134 (2855 ppb/kg) seeds under *in vitro* conditions. Field studies indicated that TAG-24 recorded low toxin levels under drought (7.3 ppb/kg) whereas in irrigated plots, TIR-9 recorded lowest levels (4.82 ppb/kg). The genotypes TPT-4 and TCGS-913 had high phenol content and exhibited high shell wall integrity. Based on our results, we can conclude that TPT-4 can be recommended for drought prone areas under rainfed (Kharif) conditions, whereas TCGS-913 is ideal for post rainy season (Rabi) under the conditions evaluated.

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S. No.	Groundnut Genotypes <sup>1</sup>	Aflatoxin (ppb) <sup>2</sup>
1.	Narayani	5000
2.	TPT-4	2855
3.	TPT-25	3987
4.	K-134	2855
5.	TAG-24	2899
6.	TCGS-888	2978
7.	TCGS-913	4172
8.	TCGS-341	3501
9.	TIR-9	4567
10.	VG-39	4200
LSD	240.4	

#### Table 1 In vitro screening of groundnut genotypes for aflatoxin

#### contamination due to Aspergillus flavus

<sup>1</sup>Groundnut seeds were inoculated with *A. flavus* inoculum ( $4 \times 10^6$  cfu/ml) and incubated for 7 days at 25 and 98% RH.; <sup>2</sup>Aflatoxin content of the seeds was estimated by indirect ELISA and expressed in parts per billion (ppb).

S. No	Groundnut	Seed infection		Aflatoxin (ppb)	
	Genotypes	Irrigated	Drought	Irrigated	Drought
1.	Narayani	55.8	73.8	21.92	8.96
		(42.4)	(71.8)	(0.82)	(1.38)
2.	TPT-4	41.2	79.3	12.23	1594.9
		(39.58)	(59.8)	(0.69)	(1.63)
3.	TPT-25	51.3	43.9	12.2	1301.5
		(47.9)	(34.5)	(0.39)	(2.34)
4.	K-134	34.2	44.4	10.59	2482.3
		(31.31)	(36.2)	(0.73)	(1.39)
5.	TAG-24	39.4	52.2	14.02	7.3
		(32.4)	(32.1)	(0.48)	(0.76)
6.	TCGS-888	21.0	24.8	90.2	7.3
		(24.28)	(23.9)	(0.30)	(0.59)
7.	TCGS-913	32.9	38.3	36.3	394.4
		(31.90)	(32.1)	(0.92)	(1.42)
8.	TCGS-341	44.2	44.2	32.3	406.3
		(34.4)	(23.2)	(0.76)	(1.43)
9.	TIR-9	23.5	50.2	4.82	42.1
		(26.39)	(39.4)	(0.32)	(0.58)
10.	VG-39	50.4	42.1	4.89	2573.4
		(41.2)	(25.9)	(0.69)	(1.03)
	Mean	39.39	49.32	23.95	881.85
		(35.18)	(37.89)	(0.61)	(1.26)

 Table 2
 Field evaluation of different groundnut genotypes for natural seed infection and aflatoxin contamination induced by Aspergillus flavus

Seed infection

LSD (5%) Treatments		8.32					
Genotypes	20.4						
Interaction	10.13						
Aflatoxin val	ues	Log aflatoxin values					
LSD (5%)							
Treatments	12.03	NS					
Genotypes	NS	NS					
Interaction	NS	NS					
	Genotypes	Total phenols	Total phenols		Shell wall integrity (%)		
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S. No		(mg/g seed)	(mg/g seed)				
		Irrigated	Drought	Irrigated	Drought		
1.	Narayani	2.19	2.932	81.3	75.6		
				(63.3)	(61.0)		
2.	TPT-4	3.23	1.92	73.2	66.3		
				(59.3)	(62.3)		
3.	TPT-25	2.01	1.46	75.2	77.3		
				(63.4)	(62.8)		
4.	K-134	2.53	1.52	79.7	73.5		
				(64.2)	(62.3)		
5.	TAG-24	2.63	2.93	84.2	79.3		
				(67.5)	(64.6)		
6.	TCGS-888	2.43	2.59	81.3	77.1		
				(66.3)	(64.7)		
7.	TCGS-913	2.99	2.34	87.2	77.7		
				(71.3)	(64.2)		
8.	TCGS-341	2.12	2.32	96.3	89.3		
				(72.5)	(68.0)		
9.	TIR-9	2.72	2.39	84.7	81.3		
				(73.5)	(64.2)		
10.	VG-39	3.07	2.45	94.2	88.3		
				(78.4)	(73.5)		
	Mean	2.59	2.29	83.73	78.57		
				(67.97)	(64.76)		

0.358

0.944

1.335

#### Table 3 Evaluation of groundnut genotypes for total phenol content and shell wall integrity estimated at harvest under irrigated and drought conditions

LSD (5%) Total Phenols Treatments 0.021 0.597 Genotypes 0.0514 0.146 Interaction 0.072 0.206 Shell wall Integrity Treatments Genotypes

Interaction

Normal values	Transformed values
	0.505
	1.237
	1.75

# Screening of *Bacillus cereus* 905 Swarming Null Mutants by Use of TnYLB-1, a mariner-Based Transpson

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#### Abstract

*Bacillus cereus* 905, a biocontrol agent isolated from the wheat on the base of the Plant Micro-ecological System. As an effective plant growth promoting rhizobacteria (PGPR) bacterium, it showed the effective function in controlling the diseases, promoting the crops growth and improving the quality in greenhouse and field. Successful colonization of the bacteria on/around the host plant is required for its effective function. In our study, we used transposon to analysis the role of swarming in the colonization of *B. cereus* 905. TnYLB-1, a *mariner*-based transposon, can generate insertion mutations. Our results showed that TnYLB-1 transposed into the *B. cereus* 905 chromosome with high frequency ( $9.8 \times 10^{-3}$ ) from pMarA plasmid. Southern hybridization analysis of transposants and sequence analysis of insertion sites of 7 of these are consistent with random transposition. One in two thousand transposants showed null swarming activity. In this study, *mariner*-based transposon system of *B. cereus* 905 was set up, which will be potentially applicable to other phenotype screening. Besides, our results will provide theoretical basis for studying of colonization mechanism of *B. cereus* 905.

### Studies on the management of banded leaf and sheath blight disease of maize (*Rhizoctonia solani* f. sp. *Sasaki*) using *fluorescent Pseudomonads*

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#### Abstract

Among different fungal diseases affecting maize production, banded leaf and sheath blight induced by *Rhizoctonia solani f. sp. sasaki* causes significant grain yield losses. The disease is more prevalent in rice fallow maize in deltaic soils of Andhra Pradesh, India. In our present study, we have investigated the biocontrol potentiality of certain fluorescent Pseudomonads under in vitro conditions as an alternative to presently available fungicidal management. Eight strains of P. fluorescens were isolated from different soils under maize cultivation. In vitro studies were carried out to study the effect of these PGPR strains on mycelial growth, sclerotial germination of R. solani, and for the antagonistic effect of volatile and non volatile metabolites. The growth promoting effects of Pseudomonads on maize seedlings was studied by treating seeds with PGPR solutions at log 9 cfu/ml for 24h and later incubated for 7 days at room temperature. All the Pseudomonads have significantly inhibited the mycelial growth and sclerotial germination of *R. solani* ranging from  $48\% \sim 92\%$  and  $29\% \sim 87\%$  respectively over controls. Further, all the strains were found to produce volatile and non-volatile metabolites that were inhibitory to the test pathogen. Studies on growth promoting effects indicated that maize seedling vigor was significantly enhanced by majority of these strains. The root and shoot lengths were enhanced by 25.4% to 55% and 22.2% to 67.79 % over that of control seedlings for the effective strains. Overall, our results suggest that these PGPR have significant potential in reduction of maize banded leaf and sheath blight disease and in growth promotion. Further studies are in progress to elucidate their disease suppressing and growth promoting abilities under greenhouse conditions.

#### Introduction

Maize (Zea mays L.) is one of the most important cereals crop grown in rice fallows in Andhra Pradesh, India. In recent years area under maize crop has increased drastically both in rice fallows and upland because of varied reasons. Sheath blight has been an endemic disease in rice in Krishna zone and succeeding maize crop is effected by the pathogen and this cropping pattern invariably supports perpetuation and survival of R. solani. Due to this the disease intensity in maize increases and able to cause considerable reduction in maize yields. The disease causes direct losses resulting in premature death of the plant, stalk breakage and ear rot besides causing indirect losses by reducing the gross yield. Singh and Sharma (1976) estimated 40.5% loss in grain yield with 71% disease index. However, the magnitude of grain loss may reach as high as 100% if the ear rot phase of the disease predominates. In India, losses in grain yield have been estimated in the range of 23.9% to 31.9% in ten cultivars (Lal et al., 1980). Payak and Sharma (1985) reported that annually around 1% of the total grain yield is reduced by BLSB in India. Presently available methods of control were seed treatment, soil application and foliar spray with systemic fungicides and antibiotics. However, these methods were not cost effective and moreover cause pollution to the environment. In recent years, fluorescent Pseudomonads have drawn attention worldwide because of its antagonistic potential by employing production of secondary metabolites such as siderophores, antibiotics, volatile compounds, hydogen cyanide, enzymes and phytohormones. Keeping in this view, the present research work was carried out to study the potential of native pseudomonas isolates to control banded leaf and sheath blight disease and improve crop yield by growth promotion.

#### Materials and methods

**Isolation of** *Rhizoctonia solani*: Banded leaf and sheath blight diseased plant parts were collected from farmers fields of Guntur district. For isolation of *R. solani*, small pieces of infected sheath and leaf, were washed and surface sterilized with 5% sodium hypochlorite for 10 minutes. The infected tissues were cultured on Potato Dextrose Agar (PDA). The plates were incubated at room temperature  $(28\pm2^{\circ}C)$  for a week. The growing colonies of fungi were transferred to new plates for purification and identification was done based on myceial and sclerotial characteristics (Barnett, 1969).

**Preparation of inoculum of** *R. solani* **and pathogenicity test:** For pathogenicity test, approximately 0.2 g of inoculum (mycelium and sclerotia) placed inside the leaf sheath of maize with a few drop of sterile water invariably induced single discrete, uniform size lesion, irrespective of type of inoculum used (i.e., sclerotium or mycelium).

#### Isolation of fluorescent Pseudomonad strains and identification:

Rhizosphere colonizing fluorescent pseudomonads were isolated from fresh roots of maize grown in RARS Lam farm and farmers fields of Guntur district. After vigorous shaking of excised roots to remove all but tightly adhering soil, root segments (1g) were shaked in 100 ml of sterile distilled water for 25 min. Antagonists were tentatively identified by gram staining (Vincent 1970) and light microscopy. Fluorescent Pseudomonads were isolated on King's medium B (KB) (Todaz,2005)

#### Antagonism

#### Efficacy of P. fluorescens isolates to inhibit R. solani in vitro

**Dual culture:** Efficacy of the *P. fluorescens* isolates in inhibiting the growth of *R. solani* was tested by streaking each bacterial isolate on one side of a petri dish containing PDA medium. One 5 mm mycelial disc from 4 days old culture of *R. solani* was placed at the opposite side of the petri dish and treatments were replicated three times. Growth of *R. solani* was inhibited when it grew toward the bacterial colony and the inhibition zone was measured from the edge of the mycelium to the bacterial colony edge.

**Production of volatile metabolites:** Two hundred fifty micro liter of antagonistic *Pseudomonas* bacterial suspension  $(10^8 \text{ CFU ml}^{-1})$  were placed at the petri dish containing KB medium and a 5 mm disk of a four days old pure culture of *R. solani* was placed at the center of another petri dish containing PDA. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 28 °C for 6 days and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist (mock inoculation with 6mm disk of PDA). Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times. Results are expressed as means of % inhibition of the growth of *R. solani* 

in the presence and absence of any bacterial isolate. Percent inhibition was calculated using the following formula (Vincent 1947).

inhibition(%) = 
$$\underline{C} \cdot \underline{T} \times 100$$

(C- growth of test pathogen in the absence of antagonist T- growth of test pathogen in the prescence of antagonist))  $\times$  100

**Production of diffusible metabolites:** This effect was tested according to Montealegro *et al* (2003). PDA plates, covered with a cellophane membrane, were inoculated in the center with 250 µl of a bioantagonistic bacterial suspension at room temperature. After incubation for 48 h at  $28^{\circ}$ C, membrane with the grown bacterial isolate was removed the and the plate was inoculated in the middle with a 5 mm of a pure culture of *R. solani* plates and incubated at  $28^{\circ}$ C for 5 days and the growth of the pathogen was measured. Control were run with mock inoculated PDA containing plates on the cellophane membrane (replacing the bacterial suspension by sterile distilled water) and future incubated with *R. solani*. Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least two times.

Secretion of extracellular metabolites: These tests were performed in 250 ml Erlenmeyer flasks containing 100 ml of sterile nutrient broth (NB). One milliliter of isolates of bacterial suspension  $(10^8 \text{ CFU/ ml})$  were added to the flasks containing NB. The flasks were then incubated at 28°C for 6 days on a rotary shaker at 175 rpm at room temperature  $(28\pm2^{\circ}C)$ . Bacterial cells were pelleted by centrifugation at 5000 g for 12 min. The supernatants were collected and 15% and 25% (v/v) of culture filtrate were mixed with PDA and a 5 mm disk of a four days old pure culture of *R*. *solani* was placed at the center of petri dish. The experiments were independently repeated two times.

Effect of antagonistic isolates on germination of sclerotia of *R. solani in vitro*: Sclerotia of *R. solani* were collected from the culture grown on PDA and surface sterilized with 2.5% sodium hypochlorite and dipped in the Erlenmeyer flasks containing 50 ml of KB and 1 ml suspension of antagonistics isolates( $10^8$  CFU/ ml) were inoculated. The bacterial cell suspensions in KB medium were placed for 24 h on a rotary shaker at 175 rpm at room temperature ( $26\pm 2^{\circ}$ C) and 5 sclerotia were inoculated in each bacterial isolate and was placed for 24 h on a rotary shaker at 175 rpm. At bacterial suspension ( $10^8$  CFU/ ml) were placed at the petri dish containing KB. After incubation for 72 h at 26°C, sclerotia of *R. solani* were produced, their surface sterilized with 2.5% sodium hypochlorite and were placed for 6 weeks at 26°C, the lysis of the sclerotia pathogen was measured. Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times. Controls were run with inoculated replacing antagonistic isolates suspension by sterile distilled water.

**PGPR strains inoculum preparation and mode of application.:** Bacterial strains were used as maize seed treatments. Seeds of maize (Pioneer 30V92) were surface sterilized with 0.02% sodium hypochlorite for 2 min and rinsed thoroughly in sterile distilled water. For inoculation seeds were soaked in the bacterial suspension of log10<sup>9</sup> cfu over night. Seeds soaked in sterile distilled water was served as check.

**Seed germination bioassays**: Germination tests were carried out by paper towel method. Twenty five seeds for each treatment with three replications in completely randomized design and incubated in growth chamber at 28 °C. After 7 days seed germination, root length, and shoot length were measured to determine the vigor index with following formula (Abdul Baki and Anderson, 1973).

Vigor index = (mean root length+ mean shoot length)  $\times$  germination (%)

#### **Results and Discussion**

Efficacy of *P. fluorescens* isolates to inhibit *R. solani in vitro*: All the tested *Pseudomonas* strains significantly reduced the mycelial growth of the banded leaf and sheath blight pathogen in antibiosis tests.

**Dual culture**: Mycelial growth of *R. solani* was reduced by all isolates with percent inhibition ranging from 0 to 92% (Tables 1). No physical contact was observed between *R. solani* and any of the pseudomonas isolates tested and moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. Change of mycelial color was observed close to the colony end of *R. solani* which was dark brown when compared with the centre of colony. Microscopic observations revealed the cytoplasmic leakage of mycelium resulting in deformation. Four strains exhibited more than 70% inhibition. Of these the highest per cent inhibition was obtained with *P. fluorescens* Lam isolate (76%). The inhibition zones for these strains ranged from 0 to 6.3 mm.

**Volatile metabolitess:** All antagonistics isolates significantly inhibited *R. solani*. Maximum inhibition of 57.6% was recorded with *P. flourescens* L1 followed by *P. flourescens* A (57.3) and *P. flourescens* L2 (55.6) respectively and minimum of 47% was showed by *P. flourescens* T.

**Diffusible metabolites:** Isolate *P. flourescens* L recorded inhibition of 68.3% followed by *P. flourescens* T (68 %) and *P. flourescens* L2 (65.6 %) respectively. Diffusible antibiotics of isolate *P. flourescens* N expressed least inhibition of 58.6% on the growth of *R. solani* (Table 1).

Secretion of extra cellular metabolites: Pathogen could not grow well in the presence of bacterial extra cellular metabolites. All antagonistic isolates have shown significant differences in inhibition of mycelial growth of *R. solani* and ranged from 67.3% to 76% (Table 1).

**Influence of PGPR on the germination of sclerotia of** *Rhizoctonia solani*: Sclerotial germination was reduced to the tune of 67%. Maximum reduction was recorded with *P. fluorescens* L (67%) and minimum of 21.6% with *P. fluorescens* T. Pseudomonas isolates obtained from the rhizosphere of different crops have varying antagonistic potential (Kreit Low 1949 and Hagedron *et al.*, 1989). Members of the genus Pseudomonas are well known antagonistic bacteria and production of secondary metabolites such as siderophore, diffusible, volatile and extracellular metabolites (Compat,2005 and Murphy and Riley1962) resulted in effective biocontrol and production of phytohormones (Park *et al.*, 2005 and Mohsin Tariq *et al.*, 2010) improves seed vigor. The results of dual culture studies showed that *P. fluorescens* isolates significantly inhibited the radial growth and germination of sclerotia in *in vitro*. Similar results were obtained by Montealegro *et al.*, 2003 and Kazempour 2004.

**Influence of PGPR on seedling vigor of maize :** The majority of the screened PGPR strains when applied to maize seeds were found to enhance germination, seedling length and thereby the vigor as observed on 7-day-old seedlings (Table 2). Highly effective strains promoted seedling vigor (2819.96) in comparison to 1378.96 in control seedlings. These superior strains were also found effective in reducing mycelial growth of pathogen. Highest seedling vigor was obtained with PGPR strains *P. fluorescens* L1 followed by *P. fluorescens* L2 with significant differences between them.

Seed inoculation with bacterial strains significantly enhanced seed germination and seedling vigor of maize. However the rate of enhancement varied with bacterial strains. All strains increased seed germination over control. The highest enhancement of vigor indices were obtained from *P. fluorescens* L1 and *P. fluorescens* A strains. The present investigation confirms the earlier works in maize (Gholami *et al.*, 2009) rice (Vijaykrishna *et al.*, 2010), wheat (Shaukat *et al.*, 2006), pearlmillet (Niranjan *et al.*, 2004), sunflower (Burd *et al.*, 2000) and sorghum (Raju *et al.*, 1999). In the present study we concluded that native pseudomonas strains *Pseudomonas fluorescens* L1 and L2 strains were effective in control of *R. solani* pathogen *in vitro* and promoting seed germination and seedling vigor. Further studies were planned to evaluate the efficacy of the superior strains identified in this screening bio-assays for biocontrol of banded leaf and sheath blight disease and improvement of yield under greenhouse and field conditions.

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Isolates	Per cent inhibition of mycelial growth				Inhibitio n zone (mm)	inhibition of sclerotial germination(%)	
	culture	Volatile	Diffusible	Extra cellular	· · ·		
Pseudomonas fluorescens F1	50	47.6	62	71.0	3.3	52.0	
Pseudomonas fluorescens F2	66	54.0	61	67.3	3.3	44.0	
Pseudomonas fluorescens F3	71	52.6	60	68.6	1.3	36.6	
Pseudomonas fluorescens T	48	47.0	68	72.6	0	21.6	
Pseudomonas fluorescens N	63	51.3	58.6	75.0	2	53.6	
Pseudomonas fluorescens A	72	57.3	62	69.3	3.6	61.6	
Pseudomonas fluorescens L2	85	55.6	65.6	71.3	4	55.6	
Pseudomonas fluorescens L1	92	57.6	68.3	76.0	6.3	67	
Control	0	0	0	0	0	0	
SEm	0.995	0.66	0.68	0.59	0.436	1.09	
CD	2.98	1.99	2.05	1.77	1.306	3.293	

#### Table 1 Effect of antibiosis of Pseudomonas fluorescens isolates in inhibition of mycelial growth of Rhizoctonia solani in vitro

Isolates	Seed germinat ion (%)	Root length (cm)	increase of root length over control (%)	Shoot length (cm)	increase of shoot length over control (%)	Seedling vigour index
Pseudomonas fluorescens F1	98.6	15.4	37.61	12.74	54.24	2774.60
Pseudomonas fluorescens F2	91.6	14.0	25.37	13.86	67.80	2551.98
Pseudomonas fluorescens F3	100	17.3	55.00	10.09	22.19	2739.00
Pseudomonas fluorescens T	96.6	14.6	30.74	13.81	67.15	2744.41
Pseudomonas fluorescens N	94.6	16.8	50.44	9.28	12.35	2467.17
Pseudomonas fluorescens A	98	14.7	31.46	11.03	33.57	2521.54
Pseudomonas fluorescens L2	92.6	14.7	31.46	10.20	23.49	2305.74
Pseudomonas fluorescens L1	98.6	16.9	51.63	11.70	41.65	2819.96
Control	88	7.4	0.00	8.27	0.00	1378.96
SEm	1.94	0.72		0.53		
CD	5.81	2.18		1.59		

 Table 2
 Effect of bacterial inoculation on seed germination and seedling vigour

#### Screening of Bacteria against Root Rot Disease of Sugar Beet

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#### Abstract

Sugar beet root rot is one of the most important root diseases on sugar beet, especially in continuous cropping fields in China. In this study, Two hundred and forty bacteria strains were isolated from sugar beet roots and rhizosphere samples collected from Inner Mongolia and Hei Longjiang province, among 240 bacteria isolates, 18 displayed between 30 and 66.3% inhibition of mycelia growth of *F. oxysporum* in vitro. These isolates were further tested for their biocontrol effects under the greenhouse condition. The results showed that 5 bacteria isolates resulted 50 % to 64.9 % disease suppression compared to the control. In the sugar beet continuous cropping field test, the control efficiency of isolate HL26 and BXA4 were 63.24% and 58.5%, respectively. By means of the 16s rDNA sequence analysis and physiological and biochemical reactions, HL26 and BXA4 was identified as *Bacillus subtilis*.



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