Recent Trends in PGPR Research for Sustainable Crop Productivity

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HEAVY METAL RESISTANT PGPR AS A GREEN SOLUTION TO PESTICIDE AND HEAVY METAL POLLUTION

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ABSTRACT

Management of plant diseases through chemical fungicide and contamination of heavy metal ions in agriculture soil is a significant environmental problem affecting human and agriculture health. In addition, these causes decreased soil microbial activity and soil fertility, and yield losses. Plant soil root ecosystem is an important interface of soil and plant; it plays a significant role in controlling plant diseases and in bioremediation of heavy metal contaminated soil. Rhizobacteria are known to affect phytopathogens and heavy metal mobility through release of chelating agents, acidification, phosphate solubilization and redox changes, and therefore, have potential to enhance bioremediation processes. Use of plant growth-promoting rhizobacteria (PGPR) capable of growing in presence of variety of heavy metals offers sustainable and eco-friendly solution as multifaceted bio-inoculant, disease protectant and effective means of bioremediation. In this paper some recent advances played by PGPR as green bio-fungicide, in bioremediation of heavy metal contaminated soils and plant growth promotion in heavy metal contaminated soil are presented. We report two potent PGPR strains capable of growing in varying concentrations of various heavy metal like $MnCl_2.4H_2O$, $NiCl_2.6H_2O$, $ZnSO_4.7H_2O$, $ZnCl_2$, $FeSO_4$, $CuSO_4$, $CuCl_2$, $HgCl_2$, $FeCl_3.6H_2O$, $AgNO_3$ and $CoCl_2$ these strains isolated from local garden soil were identified as Alcaligenes sp. RZS2 and Ps. aeruginosa by polyphasic approach. These strains produced array of antifungal metabolites (AFMs) like siderophore, hydrogen cyanide, chitinase and bacteriocin. AFMs of these strains exhibited superior antifungal activity vis-à-vis commonly used but environmentally hazardous chemical fungicides carbistin and bavistin (organo-chlorine) and copper based fungicide; kitazin and bilcop-50. All pathogens were sensitive to these preparation but expressed complete resistant against copper based fungicide; bilcop-50. These strains in low (25 µl) concentration/MIC inhibited more antifungal activity against A. niger, A. flavus, F. oxysporum, C. arachichola, M. anisopliae, Ps. solanacerum and A. alternate. In addition to the superior antifungal

potential, effective root colonization indicated their rhizospheric potential. We also investigated the possibility of using biodegradable polymer (PHB) as an eco-friendly carrier for sustained release of these bio-fungicides.

Key words: PGPR, Siderophores, Phytopathogen, Heavy metal.

INTRODUCTION

The rhizosphere zone has been defined as the volume of soil directly influenced by the presence of living plant roots or soil compartment influenced by the root (Atlas & Batrah, 1998). It supports large and active microbial population of rhizobacteria (root colonizing bacteria) that exert the beneficial effects on the growth of the host plant. Group of such bacteria is termed as plant growth promoting rhizobacteria (PGPR) (Juanda, 2005, Ahmed et al. 2008).

PGPR have been reported to enhance plant growth directly by a variety of mechanisms: fixation of atmospheric nitrogen, solubilization of phosphorus, production of siderophores, and synthesis of phytohormones. Indirect mechanisms involve the bio-control of phyto pathogens, through the production of antibiotics, lytic enzymes, hydrogen cyanide, and siderophore or through competition for nutrients (Sayyed et al. 2007).

Every year, severe global economic losses to agricultural crops are encountered due to plant diseases caused by pathogens leading to the loss of 30% crop yield amounting Rs. 20,000 million/year (Nehl et al. 1996). Traditionally used chemical fungicide to control these diseases has severely affected agro-ecosystem and has led to the development of pesticide resistance in pests. In this regards, siderophore producing PGPR have been seen as a sustainable means of controlling phytopathogens (Sayyed and Chincholkar, 2009).

A siderophore (Greek = iron carrier) is a low molecular weight (500-1000 d), compound secreted by organisms to chelate the available iron and thereby restrict iron nutrition of respective phytopathogens. This helps in preventing growth and root colonization by phytopathogens. PGPR also provide iron nutrition to roots thereby promote the plant growth (Sayyed et al. 2009).

The present work focuses on detection and estimation and production of biocontrol traits like siderophore, antibiotics and HCN from fluorescent *Pseudomonas* and evaluation of antimicrobial metabolites against common phytopathogens and their comparison carbistin and bavistin (organo-chlorine) and copper based fungicide; kitazin and bilcop-50.

MATERIALS AND METHODS

Sources of cultures and chemical fungicide

Fluorescent *Pseudomonas* was isolated from rhizosphere of banana field from Jalgoan, India. The fungal cultures like *A. niger* NCIM 1025, *A. flavus* NCIM 650, *F. oxysporum* NCIM 1008, were procured from NCIM Pune, and *A. alternata* IARI 715 was procured from Indian Agricultural Research Institute (IARI), New Delhi, India. The bacterial cultures like *Pseudomonas* sp, *Staphylococcus aureus*, *Bacillus subtilis*

and *Proteus vulgaris* were obtained from culture depository of Microbiology laboratory of M. J. College, Jalgaon.

Phenotypic characterization and 16 S rRNA sequencing

Phenotypic fingerprinting was done for utilization of 71 carbon sources and 23 chemical sensitivity assays on GEN III microplates and by BIOLOG system (BIOLOG Microstation™ system), employing tetrazolium redox dye to colorimetrically indicate utilization of carbon sources. The color development was red between 4, 6, 16 and 24 h of incubation, in Microstation Reader and BIOLOG Microlog version 5.1.1 software. Phenotypic fingerprinting profiles were compared and identification acknowledged when similarity index was ≥0.5 with the nearest entry. Genomic DNA was isolated from pure cultures using HiPurA™ Plant Genomic DNA Miniprep Purification Spin Kit (HIMEDIA) (Sambrok et al. 1989). Amplification of 16S rRNA gene sequencing was performed using the primers fD1 (5′-AGAGTTTG ATCCTGGCTCAG-3′) and rP2 (3′-ACGGCTACCTTGTTA-CGACTT-5′). The nucleotide sequencing was done by using Big DyeR Terminator Cycle Sequencing and the sequences were analyzed with gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm. Phylogenetic analysis was performed by evolutionary distance and maximum likelihood with 1,000 bootstrap replicates.

Siderophore production, detection and estimation

In order to screen siderophore production ability, fluorescent *Pseudomonas* was grown in sterile succinic acid medium (SAM) (Meyer and Abdallah, 1978) at 28 ± 2°C at 120 rpm for 24–48 h, followed by centrifugation at 10,000 rpm for 10 min. Siderophore in supernatant was detected by Chrome Azurol Sulphonate (CAS) assay[6] and quantitatively estimated by CAS shuttle assay (Payne 1994).

Detection and production of bacteriocin

Pseudomonas sp was grown in nutrient broth at 28 ± 2°C at 120 rpm for 24–48 h. The biomass was mixed with chloroform (v/v) and centrifuged at 5000 rpm for 20 min. The middle phase was collected and allowed to evaporate in water bath at 40°C.

Determination of antibacterial activity

The spectrum of antibacterial activity of the bacteriocinogenic preparation *Pseudomonas* sp was directed against pathogens like *P. vulgaris*, *S. aureus*, *B. subtilis* and beneficial rhizoflora like *Ps. aeruginosa*, *A. vinelandii, R. meliotii,* and *B. japonicum* by plate assay based on principle of diffusion (Schillingger & Lucke, 1989). Plates were incubated at 28°C for 24-48 h and pattern of growth inhibition was determined with measurement of diameters zone of growth inhibition.

Detection and Production of HCN

HCN production and detection was carried out as per the method of Castric (Castric, 1975) and was observed for color change in HCN indicator paper from yellow to brownish.

Antifungal activity of siderophoregenic preparation against phytopathogen fungi

In-vitro phytopathogen suppression activity of siderophore and siderophor-egenic culture preparations of *Pseudomonas* sp. was directed against *A. niger* NCIM 1025*, A. flavus* NCIM 650*, F. oxysporum* NCIM 1008*, A. alternata* IARI 7152 (Sayed and Chincholkar, 2009). *In-vitro* antifungal activity was based on the principle of diffusion. Inoculated plates after diffusion at 4°C were incubated at 29 ± 1 °C for 48 h and zone of growth was determined.

Determination of minimum inhibitory concentration (MIC)

In order to determine the MIC of the effective preparation, siderophore rich culture broth and cell free supernatant (20-100 µl) were individually added into PDA and NA previously seeded with fungal and bacterial pathogens respectively (one pathogen per plate). Inoculated plates after diffusion at 4°C were incubated at 29 ± 1°C for 48 h and MIC was determined.

Interaction of siderophoregenic Pseudomonas sp with useful soil rhizobia

For the purpose of studying the interaction of siderophore rich broth and bacteriocin rich broth of fluorescent *Pseudomonas* with useful soil rhizobia such as *Ps. aeruginosa*, *A. vinelandii, R. meliotii,* and *B. japonicum*, diffusion assay was performed as stated above, using nutrient agar for *P. aeruginosa,* Ashby's mannitol agar for *A. vinelandii* and *B. japonicum*, and yeast extract mannitol agar for *R. meliotii.*

RESULTS AND DISCUSSION

Phenotypic characterization and 16 S rRNA sequencing

The banana field isolate was Gram negative, motile, rod and fermented various sugars (Table 1). The DNAase, coagulase, catalase and oxidase negativity confirmed their non-pathogenic nature. It also showed a fluorescent pigment on King's B medium; these characteristics very well resembled with the features of *Pseudomonas* sp.

Table 1. Preliminary identification of fluoroscent *Pseudomonas sp.*

Characteristics/Result		Utilization of carbohydrates		Enzyme production	
Gram Characteristics	- ve	Glucose	+ ve	Catalase	- ve
Motility	Motile	Maltose	+ ve	Oxidase	- ve
Siderophore production	+ ve	Mannitol	- ve	Coagulase	- ve
HCN production	+ ve	Lactose	- ve	DNAse	-ve
Bacteriocin production	+ ve	Xylose	+ ve		
		Arabinose	+ ve		

The comparison of the BLAST search of 16S rRNA gene sequences of isolate with the 16S rRNA gene sequences of the NCBI Gene Bank database showed 99.7% identity of the isolate to *Pseudomonas aeruginosa.* The phylogenetic tree based on 16S rRNA gene sequences of the isolate also formed a distinct group with *Pseudomonas* sp. hence the isolate was identified as *Pseudomonas aeruginosa.*

Siderophore detection, production and estimation

In shake flask studies change in color of SAM from colorless to fluorescent green indicated siderophore production. Instant change in the colour of CAS reagent from blue to orange red due to the chelation of Fe^{2+} from HDTMA of CAS confirmed the presence of siderophore. The siderophore content of broth was 71% units. Similar results have been reported by Milagres et al. (Milagres et al. 1999).

Detection and production of bacteriocins

In the preliminary shake flask studies bacteriocin production was noted as production of fluorescent green pigment in nutrient broth. Fluorescence under UV light and absorption spectra of extract within the 245-350 nm confirmed the presence of bacteriocin.

Determination of antibacterial activity

Bacteriocinogenic preparation of fluorescent *Pseudomonas aerugenosa* inhibited growth of *P. vulgaris*, *S.aureus and B. subtilis.* The zone of growth inhibition of these pathogens was 28, 23 and 18 mm respectively while the growth of beneficial rhizobia was unaffected. Similar results have been recorded by Ivanova et al. (Ivanova et al. 2000).

Detection and production of HCN

The modified King's B agar plate inoculated with the isolate, changed the color of HCN indicator paper from initial yellow color to brownish red. This may be due to the fact that HCN when reacts with picric acid solution amended on filter paper strip converts yellow color of paper to brownish red (Castric, 1975).

Antifugal activity of siderophoregenic preparation against phytopathogen fungi

Inhibition of all phytopathogens under test indicated the linear relation obtained with biomass of *Ps. aeruginosa* as antifungal activity. Based upon the degree of inhibition of mycelial growth; different phytopathogenic fungi showed varying sensitivity to the siderophore preparation of *Ps. aeruginosa. A. flavus* appeared as more sensitive phytopathogen as compared to the other fungal pathogens (Table 2a). Sayyed and Chincholkar (Sayyed & Chincholkar, 2009) have reported antifungal activity of siderophore producing *A. feacalis.* Saikia and Bezbruah (Saikia & Bezbruah, 1995) have also reported antifungal activity of *Azotobacter chrococcum* RRL J203.

Table 2a. Antifungal potential of *Pseudomonas* sp. vis-a`-vis chemical fungicide

Fungal sp. Tested	Diameter of zone of inhibition, mm (SD)					
	Control (25 µl)	Culture broth (6×10^6cells ml^{-1}) (25 µl)	Culture supernatant (25 µl)	Bil cop (25 µg)	Kitazin (25 µg)	Carbisitn (25 µg)
A. niger NCIM 1025	–	21.2 (0.66)	20.0 (0.71)	nd	14.1(0.03)	14.2(0.03)
A. flavus NCIM 650	–	28.7 (0.76)	24.1 (0.81)	Nd	15.2(0.05)	16.1(0.05)
F. oxysporum NCIM 108	–	31.0 (0.95)	21.5 (0.07)	nd	16.1(0.01)	17.3(0.01)
A. alternata IARI 715	–	29.5 (0.29)	23.5 (0.07)	Nd	11.7(0.03)	11.8(0.03)

Each value is the average of three replicates. Numbers in parentheses are standard deviations. (—) No inhibition of fungal growth

Determination of minimum inhibitory concentration (MIC)

Siderophore rich broth in less concentration 25 µl exerted more antifungal activity. This preparation was more potent inhibitor of fungal pathogens vis-a-vis chemical fungicides carbistin, bavistin, and kitazin and (Table 2b). All the fungi under test were resistant to copper based fungicide; bilcop-50. More activity of broth indicated the role of other secondary metabolites in the growth inhibition of pathogenic fungi.

Table 2b. MIC of siderophoregenic *Pseudomonas sp.* against common phytopathogenic fungi

Antifungal preparations	Amount	Diameter of zone of reduced growth (mm)			
		A. niger	***A. flavus***	***F. oxysporum***	***A. alternata***
Culture Broth (µl)	20	20.0 (0.71)	26.0 (0.71)	25.0 (0.12)	20.0 (0.70)
	40	20.0 (0.71)	21.0 (0.07)	27.0 (0.64)	18.0 (0.60)
	50	18.0 (0.60)	31.0 (0.61)	21.0 (0.07)	21.0 (0.07)
	75	24.0 (0.12)	24.5 (0.54)	36.0 (0.71)	21.0 (0.07)
	100	23.0 (0.74)	24.5 (0.31)	22.0 (0.56)	20.0 (0.70)
Culture supernatant (µl)	20	10.0 (0.74)	23.0 (0.74)	21.5 (0.07)	14.0 (0.71)
	40	13.0 (0.67)	20.0 (0.71)	18.0 (0.60)	15.0 (0.37)
	50	16.0 (0.71)	25.0 (0.12)	20.0 (0.07)	16.0 (0.71)
	75	15.0 (0.91)	15.0 (0.37)	25.0 (0.12)	23.0 (0.74)
	100	14.0 (0.71)	21.0 (0.07)	15.0 (0.91)	21.0 (0.07)
Chemical fungicide Bilcop 50 (µg)	20	nd	nd	nd	nd
	40	nd	nd	nd	nd
	50	nd	nd	nd	nd
	75	nd	nd	nd	nd
	100	nd	nd	nd	nd
Chemical	20	17.0 (0.37)	21.0 (0.07)	12.0 (0.61)	20.0 (0.70)

fungicide kitazin (µg)	40	25.0 (0.12)	18.0 (0.60)	13.0 (0.67)	24.0 (0.12)
	50	30.0 (0.39)	28.0 (0.57)	20.0 (0.71)	26.0 (0.71)
	75	25.0 (0.52)	31.0 (0.61)	20.0 (0.71)	28.0 (0.57)
	100	26.0 (0.71)	29.0 (0.60)	23.0 (0.74)	27.0 (0.39)
Chemical fungicide bavistin (µg)	20	17.0 (0.37)	21.0 (0.07)	12.0 (0.61)	20.0 (0.70)
	40	25.0 (0.12)	18.0 (0.60)	13.0 (0.67)	24.0 (0.12)
	50	30.0 (0.39)	28.0 (0.57)	20.0 (0.71)	26.0 (0.71)
	75	25.0 (0.52)	31.0 (0.61)	20.0 (0.71)	28.0 (0.57)
	100	26.0 (0.71)	29.0 (0.60)	23.0 (0.74)	27.0 (0.39)

Each value is the average of three replicates. Numbers in parentheses indicate standard deviations. Nd = Not detected i.e. resistance

Interaction of siderophoregenic *Pseudomonas* sp. with useful soil rhizobia

Plate assay revealed that none of the preparations of *Pseudomonas aeroginosa* inhibit the growth of useful soil rhizobia. Sayyed and Chincholkar[6] have reported that *A. fecalis* has no inhibitory effect on useful soil rhizobia.

CONCLUSIONS

Pseudomonas aeroginosa produced antifungal metabolites like siderophore, bacteriocin and HCN. Siderophore and HCN inhibited fungal phytopathogens while bacteriocin exerts toxic effect on bacteria. These metabolites did not inhibit the growth of any of the beneficial rhizobia.

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EFFECT OF MIXED INOCULATIONS OF PLANT GROWTH PROMOTING RHIZOBACTERIA OF CHILLI ON GROWTH AND INDUCED SYSTEMIC RESISTANCE OF *CAPSICUM FRUITESCENCE L.*

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ABSTRACT

In recent years, strategies for development of sustainable agricultural systems of eco-friendly, low input of non-renewable resources and less cost gaining much attention. One such attractive strategy is use of PGPR. Pseudomonas and Bacillus are ubiquitous bacteria in agricultural soils and has many traits that make them well suited as Plant growth-promoting rhizobacteria (PGPR) and mediated biological control indirectly by eliciting induced systemic resistance (ISR). The study was carried out to know the effect of combined inoculations of Plant Growth-Promoting Rhizobacteria (PGPR) on growth and PGP and ISR of chilli. Out of 55 bacterial colonies isolated from chilli rhizosphere, 13 morphologically distinct colonies (AVP1-AVP13) were selected and screened for plant growth promoting traits such as phosphate solubilisation, indole acetic acid, ammonia, siderophore, chitinases and HCN. All 13 isolates exhibited multiple PGP traits and were identified as species of Pseudomonas (AVP1,2,3,4), Bacillus (AVP5,6,7,8,9,10) Achromobacter (AVP22) Klebsiella (AVP23) and Stenotrophomonas (AVP27) based on morphological, biochemical and 16S rRNA gene sequence. AVP 3 showed potential phosphate solubilisation and tolerance to high salt concentration. Only one isolate AVP7 was antagonistic to Colletotrichum gleosporioides and Colletotrichun coccoides. Seed bacterization of chilli by AVP3, AVP7 and mixed inoculation of AVP3+AVP7 resulted in varied growth response and induced systemic resistance (ISR) under greenhouse condition. All inoculations positively influenced chilli growth. ISR response was negative with isolate AVP3 and positive with isolate AVP7 and very high with mixed inoculation of AVP3+AVP7. Results suggested that PGP traits of bacterial isolates were highly specific and application of mixed inoculation of bacterial isolates with varied specificity can influence growth and ISR more efficiently than application of individual PGP isolate.

KEY WORDS: PGPR, ISR, Rhizosphere, Chilli, Mixed inoculation

INTRODUCTION

Plant growth promoting bacteria (PGPB) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms (Glick 1995). Given the negative environmental impact of chemical fertilizers and their increasing costs, the use of PGPB as natural fertilizers is advantageous for the development of sustainable agriculture. The use of plant growth promoting rhizobacteria (PGPR) in sustainable agriculture has been increased in the Last decades in several regions of the world. Various bacteria genus are included in PGPR group, such as *Pseudomonas, Azospirillum, Bacillus, Enterobacter, Xanthomonas*and *Serratia*(Khalid et al. 2004). The mechanisms of action of PGPR may initially linked to inhibition of soil plant pathogens and there by stimulate plant growth indirectly (Gupta et al. 2000). It is often difficult to recognize the mechanisms and relate directly to promotion of plant growth, since more than one mechanism produced by bacteria (Araujo et al. 2005).

During the last two decades knowledge on phosphate solubilising microorganisms increased significantly (Richardson 2001; Rodriguez and Fraga 1999). Several strains of bacterial and fungal species have been described and investigated in detail for their phosphate solubilisingcapabilities (Glick 1995; He et al. 1997). Typically such microorganisms have been isolated using cultural procedures with species of pseudomonas and bacillus bacteria (Illmer and Schinner 1992) and *Aspergillus* and *Penicillium* fungi being predominant (Wakelin et al. 2004). These microorganisms are ubiquitous but vary in density and mineral phosphate solubilising bacteria constitute 1-50% of the total respective population. They are generally isolated from rhizosphere and non-rhizosphere soils, rhizoplane, phyllosphere, and rock P deposit area soil and even from stressed soils using serial plate dilution method or by enrichment culture technique (Zaidiet al 2009). It is very important to find novel strains of bacteria and to know their potential for producing substances that improve plant growth. However there are few studies about evaluation of phosphate solubilising bacteria strains and their efficiency in plant growth promotion in sub-tropical soils. The aims of this paper were to (1) find novel phosphate solubilisingbacteria strains isolated from chillifields (2) to evaluate their plant growth promoting activities and (3) to assess their efficacy on plant growth promotion and ISR.

MATERIALS AND METHODS

Isolation of plant growth promoting rhizosphere bacteria

Soil sampling

Plant growth promoting rhizobacteria were isolated from the chilli rhizosphere soil sample by serial dilution plate methods. Appropriate dilution was spread on nutrient agar plates. Plates were incubated at 28±2ºC for 24 to 48 h. Colonies were picked from these plates and maintained as pure culture in respective media with periodic transfer to fresh media and stocked for further use.

Screening of Phosphate solubilization

All isolates were first screened on Pikovskaya's agar plates for phosphate solubilization (Gaur, 1990). Bacterial cultures were inoculated on centre of agar plate through inoculation loop under aseptic condition. Inoculated plates were incubated for 3 days at 30ºC. Halo zone was obtained on Pikovskaya's agar plates. This halo zone showed positive phosphate solubilization ability.

Production of Indole acetic acid

Bacterial cultures were grown in media with 1mg/ml of tryptone at 30ºC for 48 hours.Fully grown cultures were centrifuged at 8000rpm for 10 min. The supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50ml, 35% perchloric acid, 1ml 0.5 M $Fecl_3$). Development of pink colour indicates IAA production (Brick et al. 1991).

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water.Freshly grown cultures were inoculated in 10 ml of peptone water in each tube and incubated for 72h at 38ºC. Nessler's reagent (0.5ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino & Sharma 1992).

HCN production

Qualitative estimation of HCN production was done by Picrate assay (Lorck, 1948). Nutrient agar medium was amended with 4.4g glycine L^{-1} and bacterium was streaked on plate. A whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed between the base and the lid of the petri dish. Plates were sealed with Para film and incubated at 27±2ºC for 4 days. After incubation, the color change of filter indicates the release of cyanide from bacterial isolate.

Characterization of siderophore

This test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate groups using a strong alkali.The reduction and the release of alkali shows red colour. To a pinch of tetrazolium salt, added 1-2 drops of 2N NaoH and 0.1 ml of the test sample. Instant appearance of a deep red colour indicated the presence of siderophore (Briskot et al. 1986).

Quantitative estimation PGP traits

Inorganic phosphate Solubilization was quantitative estimated by the method (Nautiyal and Jackson 2001). IAA was performed using the method (Loper & Scoth 1986). Ammonia production was estimated by Nesslerization reaction (Demutskaya & Kalinichenko 2010).

Molecular characterization of bacterial isolates

Pure cultures of PGPR were grown until log phase and genomic DNA were isolated (Bazzicalupo et al. 1995).The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) and 27F (5′ AGAGTTTGATCMTGGCTC AG-3′) as per the conditions (Pandey et al. 2005). The PCR product was sequenced by Macrogen Incorporation, Seol Korea. The sequences obtained were compared with those from the GenBank using the BLAST program (Astchul et al. 1990) and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree (Astchul et al. 1990 & Tamura et al. 2007).

Seed treatment and nursery experiments

Seeds of chilli were treated with the 48-h-old culture (approximately 108 CFU/ml) of the selected isolate 30 min and were shade-dried at 28 + 28ºC for 1 h. The treated seeds (100) were sown in pots containing coco peat in a greenhouse. Observations were recorded on germination percentage in the beginning, root length, shoot length and wet weight of the seedlings ever two weeks interval of sowing by removing 10 seedlings from each replication.

Induced systemic resistance

One gram of fresh chilli plant was extracted with 2 ml of sodium phosphate buffer, 0.1M (pH 7.0) at 40ºC. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extracts prepared from chilli plant tissues were used for estimation of defence enzymes. Quantitative estimation of Perooxidase, Poly phenol oxidase and Phenylalanine lyase were carried out as per the procedure (Sudhir et al. 2015).

RESULTS

Screening of plant growth promoting traits

In this study of plant growth promoting traits such as phosphate solubilisation, and production of IAA, Ammonia and Siderophore are screened as shown in Table 1. The selected three isolates AVP3, AVP7 and AVP27 found to be capable of multiple plant growth promoting traits, in which Phosphate solubilization was very high in these isolates AVP3, AVP7 and AVP27 (1640 ppm, 1383 ppm and 1733 ppm). Three isolates are able to produce IAA. IAA production of these isolates after growth optimization reported to be very high (Table 1). Our results showed that these three isolates showed greater efficiency for production of auxin. Production of ammonia also reported to be very high (60-79 $\mu g\ ml^{-1}$) in these isolates (Table 1).

Table 1. Screening of Plant Growth Promoting Traits of AVP3, AVP7 & AVP27 isolated from chillirhizosphere.

Isolate name	Phosphate solubilization (ppm)	IAA µg/ml	Ammonia µg/ml	Siderophore production	HCN production
AVP 3	1640	53	72	POSITIVE	POSITIVE
AVP 7	1383	65	79	POSITIVE	POSITIVE
AVP 27	1733	72	60	POSITIVE	POSITIVE

Molecular characterization of AVP3, AVP7 and AVP27

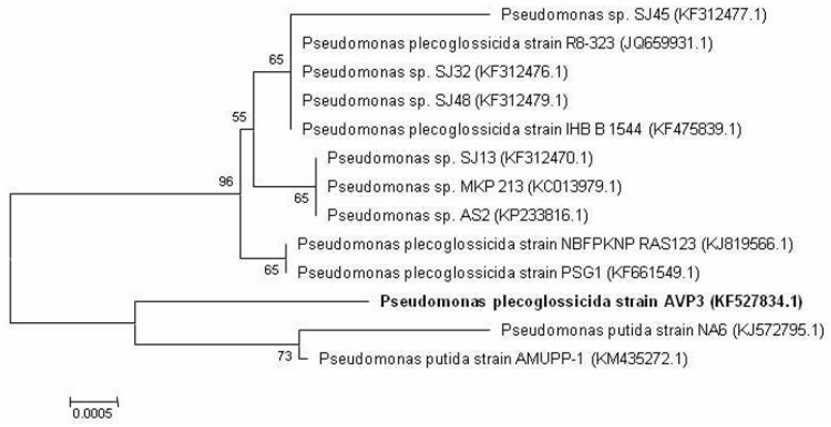
Isolates AVP3, AVP7, AVP27 are tentatively belonged to the genus *Pseudomonas*, *Bacillus* and *Stenotrophomonas* respectively based on Morphological, Biochemical and physiological characterization (Table 2).

Table 2. Characteristics of AVP3, AVP7 and AVP27

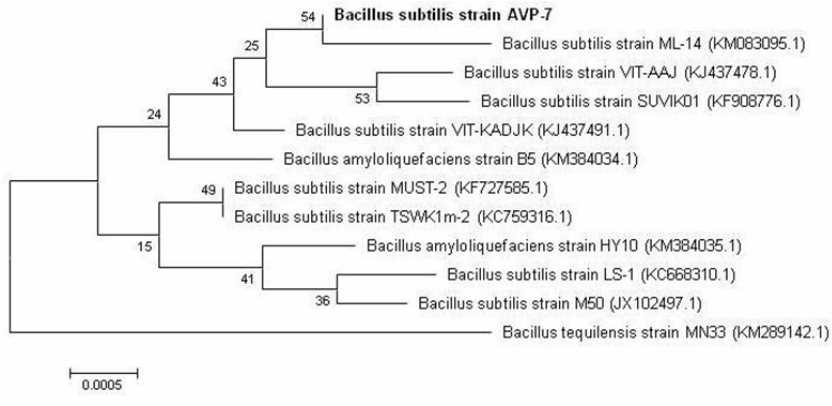
Biochemical Tests	AVP 3	AVP 7	AVP27
Indole	-ve	+ve	+ve
Methyl Red	+ve	+ve	+ve
Vogues proskeur	+ve	-ve	+ve
Citrate test	+ve	-ve	+ve
Catalase	+ve	+ve	-ve
Oxidase	+ve	-ve	-ve
H_2S	-ve	-ve	+ve
Urease	+ve	+ve	+ve
Amylase	-ve	-ve	-ve
Lipase	-ve	-ve	-ve
Glucose	+ve	+ve	+ve
Lactose	+ve	+ve	-ve
Maltose	+ve	+ve	-ve
Sucrose	+ve	+ve	+ve
Tentative genus	*Pseudomonas*	*Bacillus*	*Stenotrophomonas*

AVP3, AVP7 and AVP27 were as characterized by 16S r DNA partial sequence analysis. A 1000 bp PCR product of 16S r DNA gene was amplified from genomic DNA of AVP3, AVP7 and AVP27. Sequence similarity showed AVP3 16S r DNA sequence had 99% similarity 16S r-DNA to *Pseudomonas plecoglossicida strain* PSG1 stain and phylogenetic analysis revealed that it is closely related *Pseudomonas plecoglossicida*. The sequence was submitted in GEN BANK NCBI with a name *Pseudomonas plecoglossicida* AVP3 (Fig. 1). Sequence similarity showed AVP7 16S r DNA sequence had 99% similarity 16S r-DNA to *Bacillus subtilis* strain ML-14n and phylogenetic analysis revealed that it is closely related *Bacillus subtilis.* The sequence was submitted in GEN BANK NCBI with a name *Bacillus subtilis* AVP7 (Fig. 1). Sequence similarity showed AVP27 16S r DNA

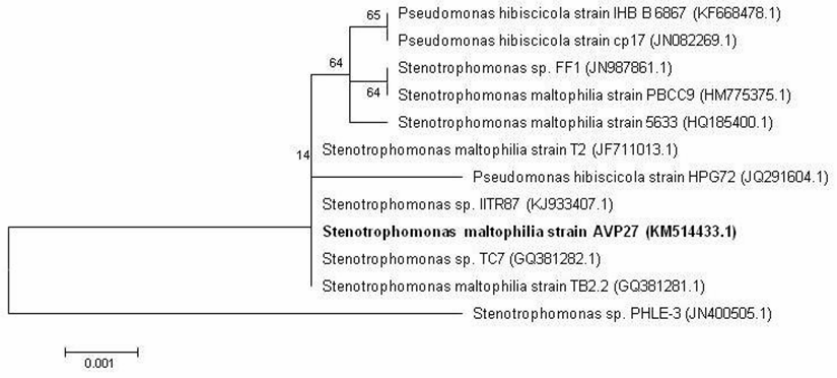
sequence had 99% similarity 16S r-DNA to *Stenotrophomonas* sp TS5 and phylogenetic analysis revealed that it is closely related *Stenotrophomonas* sp. The sequence was submitted in GEN BANK NCBI with a name *Stenotrophomonas maltophilia* strain AVP27 (Fig. 1). Phylogenetic relation of AVP3, AVP7 and AVP27 were shown in Fig. 1.



AVP3



AVP7



AVP27

Fig. 1. Dendrograms ofAVP3, AVP7 and AVP27 showing phylogenetic relation in Neighbor joining mode**.**

Growth optimization for maximum production

AVP3, AVP7 and AVP27 are subjected to optimization of growth for maximum production at three different temperatures (25ºC, 35ºC and 50ºC), pH ranging from pH 1 to pH 5% of NaCl (0.3%, 0.5%, 0.7%, 0.9% and 1%) and carbon sources (Lactose, Sucrose, maltose and dextrose). All three isolates showed maximumgrowth (<1.0) at 35ºC. The growth of AVP7 & AVP27 much higher than AVP3 (Fig. 2a). All the three bacterial isolates showed maximum growth at pH5. Though AVP3, AVP7 and AVP27 are native to tropical soils of chilli field with pH ranging from 6.8-7.8 these isolates can tolerate extreme acidic pH conditions (Fig. 2b). Three isolates showed diversity to their growth pattern at different concentr-ations of NaCl. AVP3 is tolerant to salinity up to 0.9% of NaCl. At 0.9% NaCl, AVP7 and AVP27 showed sensitivity to a level below the optimum but showed maximum growth at 1% NaCl (Fig. 2c). Results indicating that AVP7&27 showed tolerance to 1% NaCl. All the isolates showed diversity in their growth in response to carbon source. AVP27 showed maximum growth at lactose, AVP7 showed maximum Growth at dextrose and AVP3 is dextrose sensitive (Fig. 2d).

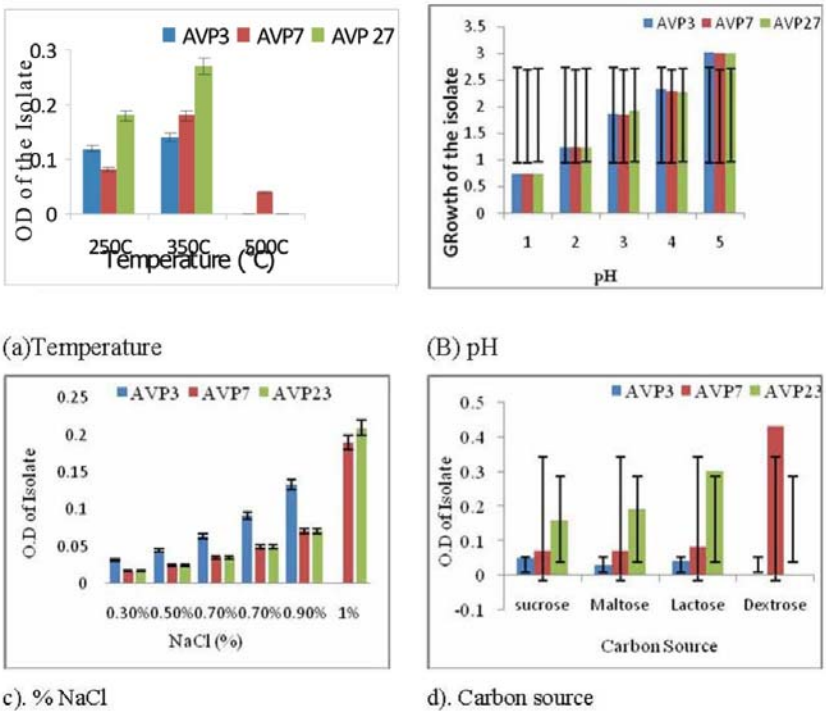


Fig. 2. Growth optimization parameters

In-vitro plant growth promotion

Seed bacterization of chilli by four different inoculations such as AVP3, AVP7, AVP27 and mixed inoculation of AVP3+AVP7 resulted in a significant increase in root length and shoot length.The growth of the seedling was progressively increased

from 1st week to 10th week. However the isolates showed diversity in their efficiency to promote the growth of the seedlings. Seed bacterization of chilli by AVP3, AVP7 and mixed inoculation of AVP3+AVP7 resulted in varied growth response Growth of the root was apparently increased from 3rd week on wards in seedlings treated with AVP3+AVP7 and exponentially enhanced in the 8th week. Where as in the growth of shoot length was triggered significantly from 7th week onward in seedlings treated with AVP3+AVP7 when compared to seedlings treated with individual isolates and control (Fig. 3).

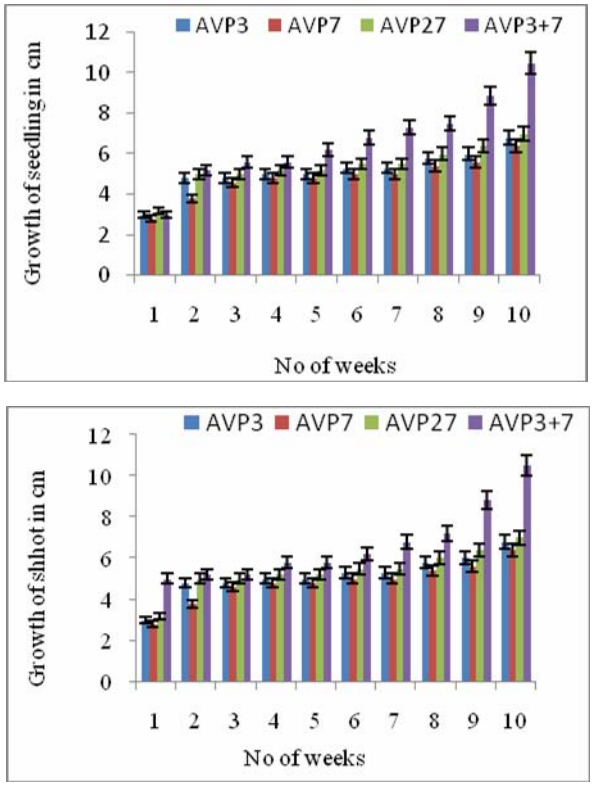


Fig. 3. (A) Root length and (B) shoot length measured in chilli seedlings inoculated with AVP3, AVP7 and AVP27 and the measurements are taken in weekly interval and in triplicates (P is <0.04))

Induced systemic resistance

In the present study, increased PO activity has been recorded in treated chilli plants treated with AVP3, AVP7, AVP27 and AVP3+ AVP7. The maximum PO activity was observed on the third day after challenge inoculation and the activity was maintained at higher level throughout the experimental period (Fig. 4a). A similar pattern of increased activity of PPO and PAL was also observed in chilli plants treated with mixed inoculum AVP3+ AVP7 (Fig. 4b;4c). PO and PAL activity was recorded to be very high (3 fold) in treated chilli plants treated with mixed inoculums AVP3+ AVP7 when compared to control and individual inoculations (AVP3, AVP7,

AVP27). All inoculations positively influenced ISR in chilli seedlings by the expression of oxidative stress enzymes (Fig. 4).

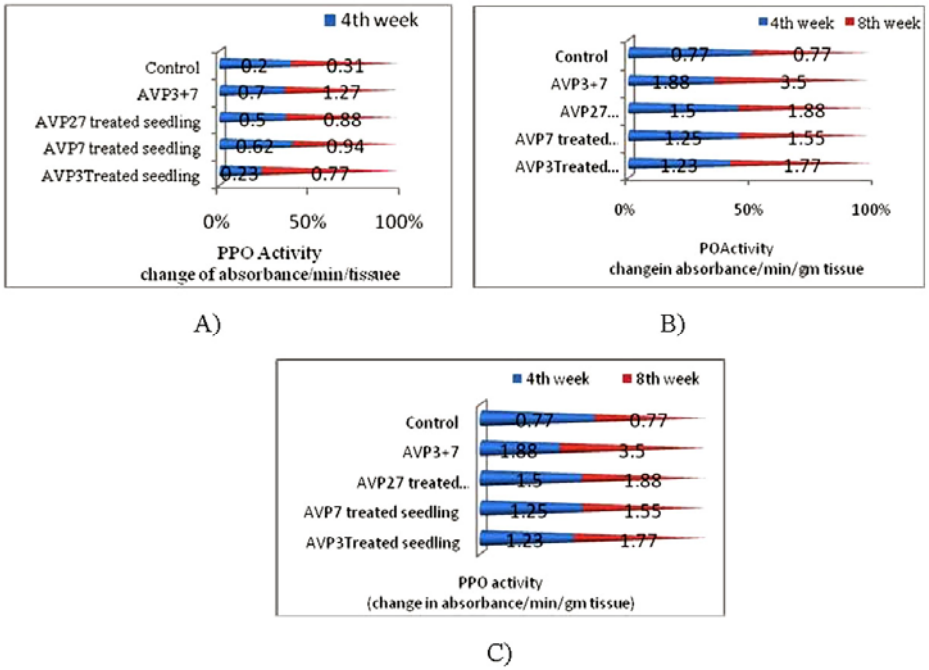


Fig. 4. Oxidative stress enzymes (a) Phenyl Alanine Ammonia lyase (B) Peroxidase and (C) Polyphenol Oxidase activities as markers of induced systemic resistance in PGPR treated seedlings in comparision with control at 4th week and 8th week

DISCUSSION

The ability of bacteria to solubilise phosphate is important for agriculture as it may enhance the availability of phosphorus for plants (Beneduzi et al. 2008). Whereas Araujo et al. (2005) reported bacillus isolates 2B,4B and 6B produced more than 10 µg ml^{-1}. In particular, the isolate 5A was the most efficient IAA producer, with 21.310 µg ml^{-1}. IAA production by PGPR can vary among different species and isolates, and it is also influenced by culture condition, growth stage and substrate availability. In addition to IAA production these three isolates are positive to the production of Ammonia, Siderophore and HCN. The ability bacteria to produce ammonia are also a significant trait for agriculture as it enhances the availability of nitrogen. These three isolates are positive to siderophore and HCN production may be linked to inhibition of soil borne plant pathogens (Gupta et al. 2000). This activity is important for biological control of plant pathogens in rhizosphere and indirectly promotes plant growth. The isolates AVP3, AVP7, AVP27 are considered as multiple potential PGPR bacteria and characterized further.

Three bacterial isolates are grown in respective culture broth with suitable carbon source, NaCl concentration and pH at 37ºC. The cell suspension culture grown in optimized conditions is used for further investigation. Only one isolate

AVP7 was antagonistic to *Colletotrichum gloeosporioides* and *Colletotrichum coccoides*. The response of AVP3 and AVP27 are more significant on growth of chilli seedlings when compared to AVP7. Significant increase in the root length of seedlings were observed from second week onwards. Whereas, a gradual increase in shoot length was recorded in response to all three isolates. No significant increase in shoot dry mass and plant height but increases in root growth by application of Bacillus isolates 2B and 4B as PGPR are reported as an exception (Holguin & Glick, 2001; Silva et al. 2007). PO is a key enzyme in the biosynthesis of lignin and other oxidized phenols. PO catalyzes the oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates, which subsequently are coupled into lignin polymers.Besides the oxidized phenols, the oxidation of which is mediated by PPO and PO they are also highly toxic to the Pathogens (Joseph et al. 1998). Induction of defense enzymes like PAL is one of the hosts for treatment with biocontrol agents. Increase in mRNAs encoding for PAL and chalcone synthase could be recorded in the early stage of the infection between bean roots and various rhizobacteria. PAL activity could be induced in plant- pathogen interactions and fungal elicitor treatments. ISR enzymes response was moderate in AVP3 and AVP27 treated seedlings and very high specific with mixed inoculation of AVP3+AVP7. Results suggested that PGP traits of bacterial isolates were highly specific and application of mixed inoculation of bacterial isolates with varied specificity can influence growth and ISR more efficiently than application of individual PGP traits.

ACKNOWLEDGEMENTS

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3

BIOLOGICAL METHOD FOR IMPROVING GERMINATING AND PRODUCTIVITY OF MELILOT

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ABSTRACT

The strain of cellulolytic bacteria Bacillus cytaseus 21N was selected from the laboratory collection of cellulolytic bacteria, which improves seed germination and stimulates growth of melilot. Molecular genetic studies have shown that the strain belongs to the genus Bacillus, species Bacillus cytaseus. It was established that the strain synthesizes cellulolytic enzymes, biologically active substances (B-group vitamins), and fixes atmospheric molecular nitrogen. Field trials have shown a high efficiency of the strain. Presowing seed inoculation with B. cytaseus 21N increases the germination of the melilot seeds up to 70-75%, green mass yield by 8-10 t/ha. On the basis of the strain, a biological method for improving germinating and productivity of melilot has been developed, and a new biological preparation "Fitobatsirin" for agriculture created.

INTRODUCTION

Plant cultivation in Kazakhstan is one of the most important sectors of agriculture, which provides more than 55% of the gross production; it is considered to be the major direction for the development of the country. Soil salinity is a limiting factor for the plant cultivation in most parts of Kazakhstan; there are no non-saline soils in some regions. The reasons for this lie in both natural and anthropogenic factors (Bilgili 2013; Zhou et al. 2013). The principal anthropogenic factor is the secondary salinization of irrigated lands. Many years' experience has shown that the irrigated agriculture causes a variety of soil disturbances: leaching, destruction of the structure, salinization, swamp formation and, ultimately, complete degradation (Chen et al. 2013; Beverly et al. 2005; Foster & Chilton 2003). It was established that even a weak soil salinization sharply reduced the crop yields (Khan & Panda 2008). The area of solonets and saline soils in Kazakhstan is 94 million hectares; it is almost

40% of the total area *(*Aidarov & Pankova 2007). Selection of cultivated crops that can grow and produce high yields in saline soils is one of the ways to solve this problem.

Melilot is one of the most promising crops for cultivation in saline soils of Kazakhstan. Melilot is capable of growing in the fields with a high level of soil salinity where other legumes cannot germinate. Furthermore, this culture is an active phytomeliorant and has desalinization effect on the soil. After cultivating melilot, salt content in the soil is decreased by 5-15% (Sarsenova 2008). Under influence of the melilot root exudates, an activity of soil microorganisms increases, there is an accumulation of organic substances, which improves soil fertility. The feed of melilot is not worse in quality than that of alfalfa and annual legumes. Cultivation of this crop in saline soils will create a nutritive base for livestock farming on saline soils and organize food supply to the population (Izrailson 1997).

However, when cultivating the melilot, there is a serious problem of poor seed germination because of their hard coat. Frequently, the fields sown with melilot for 1/4 remain empty because of the seeds sown only 30-40% germinates and even less in some regions - 15-20% (http://lib.znate.ru/docs/index-7882.html?page=22).

There are different ways to improve germinating capacity of plant seeds: physical, chemical and mechanical. Scarification is the most commonly used way, that is, a violation of the seed coat integrity by mechanical means. This method often results in damaging the seed embryos, which leads to their infection with a variety of phytopathogens and reduces germinating capacity. In addition, scarification is an energy-consuming process and requires a significant amount of energy and labor (Behlyarova 2009).

Biological methods to improve germination of plant seeds, based on the use of microorganisms, are the most economically sound eco-friendly and meet the requirements of environmental protection.

Previously, it was shown that cellulolytic bacteria may contribute to improving seed germination and stimulate further growth of various crops (Smirnova & Saybenova 1998; Smirnova et al. 2012; Smirnova et al. 2013). The purpose of this study was to select and examine the bacterial strains that improve germinating and productivity of the melilot and to develop on their basis a biological preparation for agriculture.

MATERIALS AND METHODS

In experiments, melilot of the "Kaldybansky" variety was used which is cultivated on saline soils in Kazakhstan. To carry out experiments, melilot seeds were cultivated on the Kovrovtsev's medium in the laboratory conditions. Prior to sowing, the seeds were inoculated bycellulolytic bacteria in a concentration of 10^6-10^8 cells ml^{-1} for 2 h at a temperature of 20-25ºC. The treated seeds were sown, protecting from drying out. In the control, the seeds were soaked in tap water. After 7-10 days, the germinating capacity was determined, and the aerial parts and roots measured.

Bacterial cellulase activity was determined on the solid medium with 0.1% Na-CMC (Na-soluble salt of carboxymethyl cellulose) and expressed in units/ml

(Tolchenov, 2009). To carry out the quantitative assessment of the cellulase activity, the Mandels-Weber method was used (Mandels & Weber 1996).

Composition of the cellulase bacterial complex was determined at the A.N. Bach Institute of Biochemistry, the Russian Academy of Sciences (Moscow, Russia).

Nitrogen-fixing ability of the bacteria was evaluated using the Agilent 6890 Gas Chromatograph, USA (Wang et al. 1994).

B-group vitamins were determined by microbiological methods (Tolysbaev & Bisembayev 1996). Experiments were carried out with 5 replicates, the data obtained were statistically processed, and measurements were considered to be significant at $p \leq 0.05$ (Serensova 2008).

RESULTS

From the laboratory collection, including more than 350 cultures, 37 strains presumably with the ability to improve the germination of the melilot seeds were selected. Of those, 18 cultures were selected for further studies. After testing cultures for phytotoxicity in relation to melilot, 15 strains were selected. These strains are characterized by the absence of phytotoxicity and have growth stimulating activity.

The experiments were conducted under the laboratory conditions to study the effect of cellulolytic bacterial strains on germinating capacity of melilot. To this end, prior to planting, the seeds were inoculated by bacteria at a concentration of 10^7-10^8 cells/ml. The experiments were performed on the Kovrovtsev's medium in Petri plates.

It was found that the tested strains improved the germinating capacity of melilot by 15-40% as compared to the control. However, only ten cultures improved seed germination by 35-40%. The further work was continued with these cultures.

For select the most promising strains of bacteria, a model experiment was carried out in the laboratory conditions to study their effect on the germination seeds and stimulation of plant growth. Experiments were conducted with the soil in the 250 ml containers; 7-10 days later, the plant germination was examined, and growth data taken. In the experiments soil with higher degree of salinity (salt amount of 0.9-1.2%) was used. The data obtained are presented in Table 1.

Table 1. Effect of cellulolytic bacteria strains on the germination and growth of melilot

Strains of the bacteria	Germination,%	Length of the stem, cm	Rootlength, cm
Control	48,2±1,1	3,6±0,2	5,9±0,1
150	75,1±3,1	4,4±0,1	8,3±0,1
21N	95,3±2,0	4,5±0,1	7,2±0,2
60(5)4	85,4±2,3	4,2±0,2	8,1±0,1
21	75,0±2,1	4,2±0,1	7,6±0,2
60CS	80,3±2,4	4,3±0,1	8,1±0,1
160	76,3±2,0	4,0±0,2	8,0 ±0,3

21(2)AS	70,6±1,9	4,3±0,2	7,6±0,1
21(8)	63,5±1,3	3,9±0,1	6,5±0,2
266	65,6±1,2	3,9±0,1	6,1±0,1
614	64,8±1,0	3,8±0,1	17,7±0,2

It was found that all strains had the ability to improve germination of the melilot seeds by 23-45%. At the same time there was a significant stimulation of growth of seedlings. Thus, the stem length increased by 5-25%, root length - by 3-40%, number of roots per plant - by 12-35% as compared with the control. Six strains of bacteria with high ability to improve seed germination and stimulate the growth of melilot were selected.

Since use of the bacteria strains on saline soils is being planned, the plot field experiments have been conducted in the Aral Sea region of Kazakhstan on soils with a salt content of 0.7-1.2%

Presowing inoculation of the melilot seeds with bacterial strains had a positive effect on the germination. Thus, germinating capacity of seeds inoculated with the strain 21N was 79%, strain 21 - 78%, and strain 21(2)AS - 76%, in the control - 30%. The plant height increased 1.4-1.5 times, fresh weight of one plant 1.5-2.0 times in comparison with the control. The plants themselves were characterized by good physiological condition, differed in a darker color, much greater height, leaf mass, and formed a well-developed compact root system. According to the research data, the strain of cellulolytic bacteria 21N was selected that had the ability to effectively improve the germination of the melilot seeds and actively promote the plant growth.

Molecular genetic studies have indicated that the strain belongs to the genus *Bacillus*, species *Bacillus cytaseus* 21N.

The positive effect of the seed treatment with the strain *B. cytaseus* 21N is associated with the ability to synthesize cellulase enzymes that partially degrade the hard coat of the melilot seeds. In connection with this, the strain enzyme complex and cellulase activity were studied. It was shown that the cellulase complex of *B. cytaseus* 21N consists of three enzymes: endo-1,4-β-glucanase, cellobiohydrolase and β-glucosidase.

Effective growth stimulating effect of the strains is due to the ability of the bacteria *B. cytaseus* 21N to fix the atmospheric molecular nitrogen and supply the plants with it (Table 2).

Table 2. Nitrogenase, cellulase activity and productivity of nitrogen fixation by cellulolytic bacteria *B. cytaseus* 21N

Nitrogenaseactivity, mkmolC_2H_4/ml/h	**Fixed nitrogen, mg/100 mlmedium**	**Nitrogen fixation, mg/g of substrate**	**Cellulaseactivity, unit/ml**
3,87±0,01	12,5±0,2	13,75±0,3	4,7±0,02

Table 2 summarizes the data obtained in testing nitrogenase, cellulase activity and nitrogen fixation efficiency of cellulolytic bacteria *B. cytaseus* 21N when grown

on the medium without a nitrogen source; native wheat straw was used as a carbon and energy source.

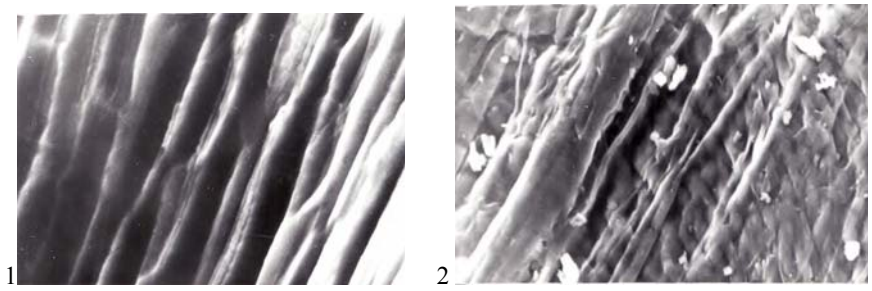
It was also found that the strain is able to synthesize biologically active substances such as B-group vitamins, which provide an additional stimulating effect on the melilot growth (Table 3).

Table 3. Biosynthesis of group-B vitamins by cellulolytic bacteria *B. cytaseus* 21N

Vitamins	**Content of vitamins, mkm/gADB (absolutely dry biomass)**
Thiamin (B_1)	16,4±0,2
Pantothenicacid(B_3)	20,6±0,2
Nicotinicacid (Pp)	180±2,4

Based on these results the mechanism of action of cellulolytic bacteria on thehard seed coat was established:

1. After inoculation of seeds with bacteria, the bacteria colonize on a and begin to produce cellulase enzymes;
2. Cellulase enzymes partially degraded seed coat and micro cracks appeared.
3. Fig. 1 shows the formation of microcracks and microholes on the surface of the seed coat of the melilot;
4. Partly degraded seed coat becomes less hard and does not interfere with seed germination;
5. Through the microcracks accelerates the access of water and dissolved minerals and nutrients to the seed endosperm;
6. The biologically active substances synthesized by bacteria and additional nitrogen, fixed them and stimulate the further development of plants;
7. Biological scarification of seeds by the action of cellulolytic bacteria increases germination and stimulate plant growth.



1- Control; 2 - Coat seed with inoculation cellulolytic bacteria *B. cytaseus 21N*

Fig. 1 - Electron micrograph of a hard coatmelilot seeds

Therefore, the mechanism of action of cellulolytic bacteria strain *B. cytaseus* 21N on a hard seed coat of melilotwas established. The possibility of replacing energy-intensive and time-consuming process of mechanical scarification plant seeds low-cost and cost-effective biological process has been shown.

To examine the effectiveness of the strain under production conditions, the field trials of the strain were carried out during three years.

Soil in the trial plot was meadow-swamp, medium loamy, the content of humus was 1.07%, biogenic elements: -NH_4 – 27.5; N-NO_3 – 30.2; P_2O_5 – 10.6-14.3 mg/kg, the degree of soil salinity was above average - 1.0-1.2% of total salts. According to the content of humus and biogenic elements, the soil was attributed to lowfertility.

The seeding rate was of 18-20 kg/ha, depth of planting reached 4-5 cm. The untreated seeds served as the control. The strain 21N was used for presowing inoculation of seeds. The conventional for the zone farming techniques were applied in the experiments.

The results showed high potential of the use of this bacterial strain of for melilot (Table 4).

Table 4. Effect of inoculation seeds by strain *B. cytaseus 21N* on germination, structure and productivity of green mass melilot

Variants	Germin-ation, %	Plant height, cm	Wet weight per plant, g	Number of branches, pcs	Productivity of green mass,t/ha
Control	32,2±1,1	43,5±2,1	4,21±0,3	7,3±0,1	17,2
21N	74,9±2,0	66,9±2,4	8,64±0,4	12,2±0,9	28,1

The data in Table 4 show that inoculation seeds by cellulolytic bacteria *B. cytaseus* 21N, the germinating capacity improved up to 75% (control - 32%). Plant height increased 1.5 times, wet weight plantincreased to 2.0 times. Productivity of the melilot green mass increased as compared to the control by 8-10 t/ha.

DISCUSSION

Therefore, the strain of cellulolytic bacteria *Bacillus cytaseus* 21N was selected from the laboratory collection of cellulolytic bacteria, which improves seed germination and stimulates growth of melilot. Molecular genetic studies have shown that the strain belongs to the genus Bacillus, species Bacillus cytaseus. It was established that the strain synthesizes cellulolytic enzymes, biologically active substances (B-group vitamins), and fixes atmospheric molecular nitrogen. The mechanism of action of cellulolytic bacteria strain *B. cytaseus* 21N on a hard seed coat of melilot was established. The possibility of replacing energy-intensive and time-consuming process of mechanical scarification plant seeds low-cost and cost-effective biological process has been shown.

Field trials have shown a high efficiency of the strain. Presowing seed inoculation with *B. cytaseus* 21N increases the germination of the melilot seeds up to 70-75%, green mass yield by 8-10 t/ha. On the basis of the strain, a biological

method for improving germinating and productivity of melilot has been developed, and a new biological preparation "Fitobatsirin" for agriculture created.

The results obtained in the field experiments show a high efficiency of the strain effect on germinating capacity and productivity of the melilot green mass.

From independent farmers in Kazakhstan were obtained results of use of the strain under field conditions over an area of 7000 hectares. These results showed the high efficiency of the strain. Improved germination of the melilot and alfalfa seeds up to 80-89% was established as compared to untreated seeds.

A new domestic biological preparation on the basis of the strain *B. cytaseus* 21N for increase germinating and productivity of melilot was developed. This biopreparation was named as "Fitobatsirin". Patents of the Republic of Kazakhstan on the strain *Bacillus cytaseus* 21N, on a method of producing biopreparation and Patent on the trademark "Fitobatsirin" have been obtained.

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4

PRODUCTION AND CHARACTERIZATION OF *TRICHODERMA* METABOLITES: A NEW APPROACH FOR SELECTIVE BIOREMEDIATION

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ABSTRACT

At last decade Trichoderma sp. has been used as biocontrol agent as it produces a plethora of inhibitory secondary metabolites with biological activities, which includes volatile, non volatile and diffusible types. The process of invasion and infection of the ground nuts (Arachis hypogaea L.) by Aspergillus species and subsequent production of aflatoxin is quite complex, but in such situation, the biological control method has been successfully utilized. In the present study 10 species of Aspergilli were isolated and purified from the rhizosphere of infected A. hypogaea. Finally the selection of aflatoxigenic A. flavus and A. parasiticus were done assuming their higher aflatoxin production observed at 365nm under UV on coconut agar medium.Similarly fourspecies of Trichoderma viz. T. viride, T. harzanium, T. flavofuscum and T. virens were screened for their antagonistic properties based on co-culture (Dual culture, pathogen at centre and pathogen at periphery techniques) and inverted colonyplate technique against selected seven Aspergillus species. It has been observed that T. virens and T. harzanium were more effective in their action of diffusible metabolites, while, T. flavofuscum shows the significant results in case of volatile metabolites against aflatoxin producing Aspergillus species. In the co-culture system for bioremediation of aflatoxins, T. harzanium superiorly control the growth of A. flavus (20.94%) with aflatoxin B1 (75%).While in case with A. parasiticus inhibition observed as growth (47.91%), Aflatoxin-B1 (86.66%) and G1 (77.77%). The integrated management of aflatoxin was established in field experiment. It has been found out that the use of Trichoderma culture for twice, 1) in soil and 2) during flowering could provide a better control of A. flavus as T. harzanium reduces B1 concentration from31.35068 to 4.8274176 µg/100g) and A. parasiticus (from 10.33538 to 1.15804 µg/100g) as analysed by UV and HPLC technique. The trichodermal metabolite production was carried out on the PDA fermentation medium for three weeks at 28°C. The fungal

metabolites extracted using the solvent partition fractionation method. The extract was dried and separated by optimizing solvent system for TLC. The antifungal activities of purified metabolites were performed by TLC bioautography method against A. flavus and A. parasiticus. The bioactive band was purified andcharact-erized by UV, HPLC and LC-MS, showing harzianic acid and 6-n-pentyl pyrone as antifungal metabolites.

INTRODUCTION

Mycotoxins contamination inagricultural products causes health hazards to people and animals, which lead to the economic problem. Aflatoxins are the most potent hepatocarcinogen and mutagen among mycotoxins. Therefore, the contamination of mycotoxins should be minimized by designing a series of measures for prevention and control.

Aflatoxins are found in a number of foods and feed, particularly in peanuts, cereals, cottonseed, tree nuts, meat, milk products, eggs and different oilseeds (Brown et al. 2001; Papp et al. 2002). Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is very difficult to avoid since fungal growth in foods is not easy to prevent (CAST, 1989).

Trichoderma is a well-known antagonist fungi, extremely common in agricultural, prairie, forest, salt marsh and desert soil in all climatic zones (Wardle et al. 1993), can be easily detected by coconut odour associated with it. *Trichoderma* is very strong for competition in the soil, for that antibiotics may be an important in the development of microbial population and colonize for food, plant site Weindling (1934) and volatile inhibitory compounds (Dennis & Webter, 1971b).

In this context, the production of secondary metabolites by *Trichoderma* may be an inexhaustible source of antibiotics, from the acetaldehyde's gliotoxin and viridin (Dennis & Webster, 1971), to alpha-pyrones (Keszler et al. 2000), terpenes, polyketides, isocyanide derivatives, piperacines, and complex families of peptaibols (Sivasithamparam & Ghisalberti, 1998). All these compounds produce synergistic effects in combination with CWDEs, with strong inhibitory activity to many fungal plant pathogens (Lorito et al. 1996a).

In general, the direct use of antimicrobial compounds produced by fungal BCAs, instead of the whole ‘‘live’’ organism, is not only advantageous in industrial and agricultural applications, but it may also be more compliant to public opinion because these biological products do not reproduce and spread. Moreover, the selective production of active compounds may be performed by modifying the growth conditions, i.e. utilizing different culture substrates, temperature of incubation, speed of agitation and pH, etc. (Woo & Lorito, 2007). Thus the present study focuses on the production of specific secondary metabolites by *Trichoderma harzianum* having aflatoxigenic bioremedial ability.

MATERIALS AND METHODS

Aspergillus species were isolated from infected roots of *A. hypogea* L. (groundnut) plants by serial dilution method of soil sample. The diluted sample was spread on the Rose Bengal Agar medium containing bacteriostatic agent (ampicillin) to restrict the

wide spreading of fungi. Plates were then incubated for 72 h at 27°C for fungal growth. Morphologically distinct colonies were purified and maintained by single spore isolation method. *Aspergilli* isolates were again screened for their ability to produce aflatoxins based on the detection of UV-induced fluorescence and specific orange-yellow pigmentation on coconut agar medium (Davis et al. 1987).

Trichoderma species were isolated from the agriculture field where the pathogen (s) is known to exist but the disease occurrence is low. The soil samples from different agroclimatic ecosystems of peanut rhizosphere were collected at a 15 cm depth in the upper profile (Broadbent et al. 1971). The soil-tube methods for assaying soil for isolation of antagonistic *Trichoderma* species were used (Baker et al. 1967). The mixed populations of isolated antagonist were additional grown on selective *Trichoderma* medium. The colonies were successively purified and were maintained on Rose Bengal Agar plates for further use.

Genus and species-level identifications were done assuming colony morphology (macroscopic) and microscopic observation (smear made in lacto phenol cotton blue stain). The confirmations of species-level identification of *Trichoderma* species and *Aspergillus* species were carried out with the support of IARI, Indian Agriculture Research Institute, New Delhi (India).

The competitive interactions between aflatoxigenic Aspergilliand *T. harzianum* were studied using dual culture technique (Gachomo & Kotchoni, 2008), pathogen at center (Asalmol et al. 1990), pathogen at periphery technique (Ortiz & Orduz, 2000), antibiotic effect (volatile metabolites) (Dennis & Webster (1971b)) and slide culture interaction (Laing & Deacon, 1991).

Bioremediation of aflatoxin by *T. harzianum* was evaluated in the co-culture system. Spore suspension ($1x10^6$ spores ml^{-1}) of aflatoxigenic *A. flavus* and *A. parasiticus* were co-cultivated with *T. harzianum*in the Richard's broth media (pH 5.5) for 8 days at 25°C.Qualitative assay of aflatoxin by TLC was done as per method (Basappa, 2009), quantitative estimation by UV-Spectroscopy (Nesheim & Stack, 2001) and the level of aflatoxins B_1, and G_1 groundnut samples was estimated by running samples through HPLC according to the AOAC International (2005) procedure with some modifications.

The integrated management of aflatoxin by *Trichoderma harzianum,* a field trial experiment was conducted to evaluate the biocontrol efficiency against *A. flavus* and *A. parasiticus*. The mass multiplication of antagonist and pathogen was done on sorghum seeds soaked in 2% sucrose solution for overnight. The local peanut variety susceptible to *Aspergillus* infection was used as test crop. The biocontrol agent was added two times during the experimental period. First application was given during sowing, and the second application was given after 35 days of trial. The effect on seed germination (without infection), height, yield and protein content of seed was considered as a parameter to assess its biocontrol ability.

The fermentation of fungal culture for induction of bioactive metabolites was done by inoculating log phase fungal culture ($1x10^6$ spore ml^{-1}) in 250 ml of Richards broth (pH 5.5), incubated at 28°C for 10 days. After incubation, broth was filtered twice with Whatman No. 1 filter paper to eliminate fungal mycelia. The 25

ml of cell free extract extracted with 10 ml of chloroform and further passed through a bed of anhydrous sodium sulphate and then evaporated in water bath at 60°C.

Extraction of fungal metabolites was done using n-hexane, chloroform, ethyl acetate, n-butanol and water as dual solvent systems. As ethyl acetate extract showed higher biological activities were selected for the extraction scheme of both extracellular (excreted into the medium) and intracellular metabolites. About 250 ml ethyl acetate was added into each fungal culture flask and kept overnight ensuring complete fungal cell death.

The ethyl acetate phase was separated from water phase (medium) using separation funnels. After evaporation, the ethyl acetate extract was diluted in 90 % methanol and extracted with n-hexane to remove fatty acids and other nonpolar constituents. At the same time, the water phase (medium) was extracted with water saturated n-butanol to collect the polar constituents.

The most active fractions were combined and purified by HPLC column. All the compounds were detected at λ_{max} 254 nm. UV absorption spectrum of the antifungal substance was also measured by the detector at the retention time of the active fraction during HPLC. Thin layer chromatography was performed in various solvent systems. The compound spotted on the TLC plates was sprayed with 2% H_2SO_4. LC-MS analysis of pure fungicidal substance was performed on Binary Nano HPLC system with Mass as the detector (Agilent Technologies, USA).Assessments of the antifungal spectrum of purified fractions obtained were tested for their fungicidal activity on TLC plates (TLC bioautography).

RESULTSAND DISCUSSION

The interaction of *Trichoderma* species with plants confers several benefits for the associated host that include (1) the suppression of phytopathogens using for direct antagonistic mechanisms (i.e. antibiosis, mycoparasitism, competition for nutrient and space); (2) plant growth promotion; (3) enhanced nutrient availability and uptake and (4) induction of plant host resistance (Harman et al. 2004).

Along with these, some *Trichoderma* strains produce compounds that can cause substantial changes in the metabolism of the host plant. Involvement of secondary metabolites in the ability of *Trichoderma harzianum* spp. to activate plant defence mechanisms and regulate plant growth has been investigated (Vinale et al. 2008b). Harzianic acid is one of these natural products that showed antifungal, plant growth promoting activities (Vinale et al. 2009b) and aflatoxin bioremediation ability. In the present study, Harzianic acid was purified, structurally characterized and analysed for its aflatoxin bioremediation ability.

The filamentous fungus *T. harzianum* is one of the most potent agents for the biocontrol of plant pathogens. The antagonistic mode of action of the fungus has been proposed for the production of antibiotics and fungal cell wall degrading enzymes such as chitinases, glucanases and proteases. The effects of *T. harzianum* on radial growth inhibition against groundnut microflora in the co-culture system are interpreted in Table 1.

Table 1. Effect of *T. harzianum* on radial growth inhibition of peanut mycoflora by the production of metabolites on RBA medium in co-culture methods

Sl. No.	Test pathogen	Percentage inhibition (I) in mm			
		Dual culture technique	pathogen at centre technique	pathogen at periphery technique	Volatile metabolites
1	*A. sydowi*	51.56%	62.50%	59.37%	68.18%
2	*A. nidulans*	66.66%	63.88%	65.27%	40.74%
3	*A. terreus*	63.33%	70.00%	56.66%	47.61%
4	*A. ochraceus*	63.41%	70.73%	61.80%	33.33%
5	*A. fumigatus*	77.53%	79.71%	72.46%	63.26%
6	*A. flavus*	78.46%	81.53%	70.76%	57.14%
7	*A. parasiticus*	64.00	76.00%	38.00%	44.11%

It has been found out that *T. harzianum* grow fast towards the pathogen, interact to inhibit their growth of test pathogen promisingly in variable culture techniques ranging from 51.56 - 78.46% in dual culture, 62.50-81.53% in pathogen at center and 38.00-72.46% in pathogen at periphery and 33.33% - 68.18% in presence of volatile metabolites as shown in Table 1. It was observed that growth of all pathogens was maximally suppressed in pathogen at center technique as compare to other culture techniques. The inhibition by volatile metabolites secreted by *T. harzianum* showed highest inhibition against *A. sydowii* and *A. fumigatus* as compare to all other test pathogens. *T. harzianum* inhibits the growth of the target organism, especially *A. fumigates, A. flavus* and *A. parasiticus* through its ability to grow much faster than the pathogenic fungi, thus competing efficiently for space and nutrients shown in Fig. 1. The growth was also restricted due to the presence of toxic metabolites secreted by *Trichoderma* species.

Microscopic observations of the interaction region showed the mycelia of *T. harzianum* grew on the surface of the *Aspergilli*, always coiling around their mycelia and later penetrate into their cell walls directly without formation of appresorium structures (Fig. 2). Hajieghrari et al. (2008) also documented the similar results using *T. virens* T523 and *T. harzianum* T969, suggesting the secretion of diffusible non-volatile inhibitory substances of *Trichoderma* isolates. Earlier studies confirmed that *T. harzianum* could be useful as a biological control agent for toxigenic fungi *A. flavus* in stored maize grains and has its role in mycotoxin B_1 reduction Aguero et al. (2008). Patil et al. (2012) also reported volatile metabolites gave the most acceptable and significant results as compared to the diffusible metabolites, against tomato root rot causing *pythium.*

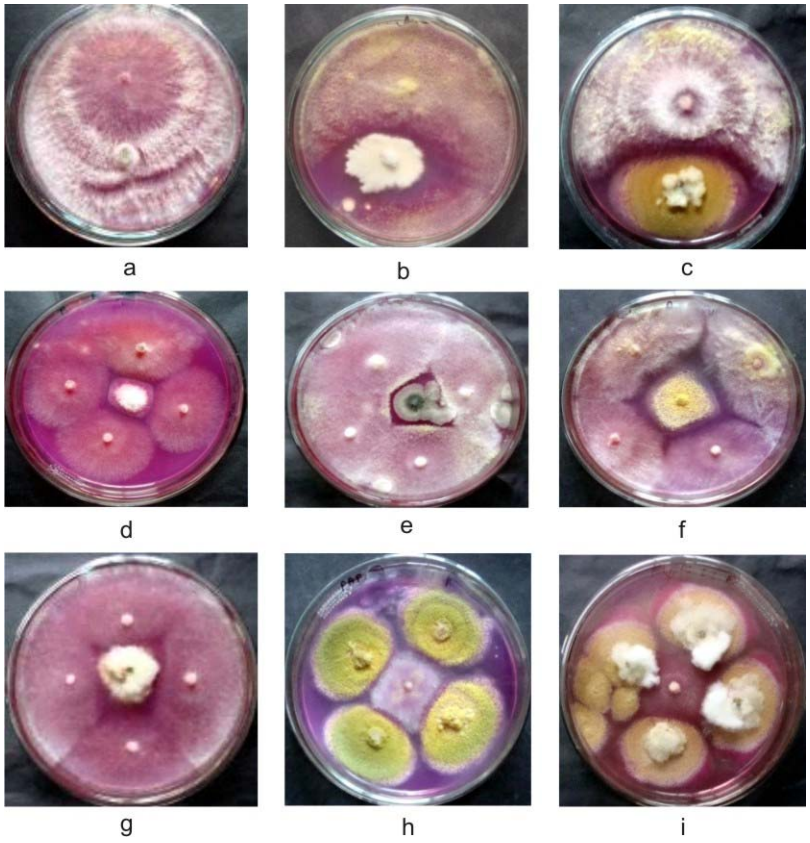

Fig. 1. Antagonism of *T. harzianum* against (a) *A. sydowii;* (b) *A. nidulans;* (c) *A. flavus* in dual culture technique,(d) *A. sydowii;* (e) *A. nidulans;* (f) *A. flavus* and (g) *A. parasiticus* in pathogen at centre culture technique and (h) *A. flavus;* (i) *A. parasiticus* in pathogen at periphery.

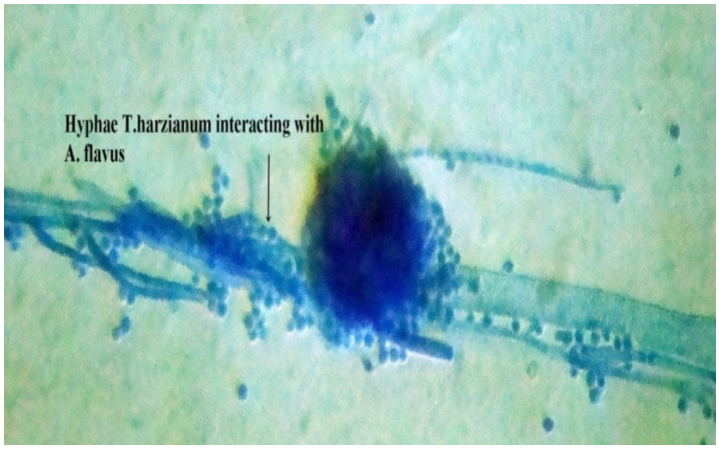

Fig 2. Microscopical view in which hyphaeof *T. harzianum* interacting with aflatoxigenic *A. flavus*

Our results are also similar to the earlier reports by (Gachomo & Kotchoni, 2008), which confirms the role of lytic enzymes in growth suppression as well as aflatoxin B_1 reduction. It is important to mention that *Trichoderma* species are known to produce a number of antibiotics such as Trichodermin, Trichodermol, Harzianum A and Harzianolide (Dennis & Webster, 1971c; Kucuk & Kivanc, 2004) as well as some cell wall degrading enzymes such as chitinases, glucanase that break down polysaccharides, chitins and glucanase thereby destroying cell wall integrity (Elad, 2000). These may also play a major role in mycoparastism because of change in cell wall integrity prior than penetration.

Bioremediation ability of aflatoxin by Trichoderma can be assessed in capabilities to resist the production of aflatoxins as well as to biotransformation and/or biodegradation of these compounds into other metabolites that are either none or less toxic than the original aflatoxin (El-Shiekh et al. 2007). The study confirmed that *T. harzianum*was able to significantly reduce the aflatoxin concentration when co-cultured with aflatoxigenic test pathogens viz. *A. flavus* and *A. parasiticus*. Aflatoxin reduction was thought to be an indirect result of the suppressed in growth of aflatoxigenic pathogen by Trichoderma species, leading to the assumption that smaller colonies produced fewer toxins (Gachomo & Kotchoni, 2008).

For the determination of aflatoxin concentration in co culture and experimental pods, cell-free culture filtrates extract (5ml) was extracted thrice with 10 ml of chloroform in a separating funnel. The chloroform extracts were passed through a silica gel (60-120 mesh) for aflatoxin purification (Mbah & Akueshi, 2009) and pure aflatoxin was eluted with 15 ml of chloroform: methanol (3:1) (Basappa, 2009).

The residues were reconstituted in 100 µl of toluene: acetonitrile (9:1), about 5 µl of sample was spotted on TLC plates along with standard aflatoxin (B_1 and G_1, 10 µg/ml, Hi Media). The plates were developed in chloroform: acetone (9:1) solvent system, air dried and observed under UV trans illuminator for the presence of aflatoxins.

The detected bands of aflatoxins based on color fluorescence from the silica gel coated plates were marked under UV light. The toxin containing band was scrapped in 3 ml of toluene: acetonitrile (9:1) to the silica gel portion and centrifuged at 1000 rpm for 10 minutes. The diluted samples of extract were analysed in a UV-spectrophotometer (Shimadzu) at 350 nm wave length for aflatoxin B_1 and G_1 determination (Nesheim & Stack, 2001). The readings were extrapolated to standard curve of pure aflatoxin B_1 and G1. The HPLC equipment was a Jasco series, HPLC-PU-20801 using C18 reverse phase column. Detection of aflatoxin was carried out at 360 nm and 460 nm excitation. For aflatoxin, B_1 and G_1 fluorescence detector was set at 360 nm. The mobile phase of water: methanol: acetonitrile was used for the separation of aflatoxins (6:3:3). The flow rate was 1.0 ml/min for each mobile phase and the injected volume of working standard was 20 µl. The column temperature was maintained at 30°C and concentration of the sample was determined using calibration curve.

In the co-cultivation of *A. flavus* and *A. parasiticus*in mixed culture with *T. harzianum* showed the promising growth retardation and efficient reduction of B1 and G1 concentration. *T. harzianum* was found to superiorly control the growth (20.94%) as well as aflatoxin B1 conc. (75%) in *A. flavus* and growth (47.91%), B1

(86.66%) and G1 (77.77%) in *A. parasiticus.* The aflatoxin reduction was thought to be an indirect result of suppression in growth of aflatoxigenic pathogen by *T. harzianum.*

When the groundnut seeds pretreated with *T. harzianum* the growth of plant was positively affected along with the aflatoxin bioremediation. In case of UV analysis in the seeds infected with *A. flavus* shows the initial B1 conc. 26.00 µg/ 100g. While in case of *A. parasiticus* shows B1 conc. 9.21µg/100g and G1 8.79 µg/ 100g, while in combination with *Trichoderma* in *A. flavus* the B1 reduced to 0.39 µg/ 100 gas compared to single infection. Whereas in *A. parasiticus* in combination with *T. harzianum* B1 reduced 0.980 µg/100g to and G1 upto 0.312 µg/100g.

In case with HPLC analysis When the groundnut seeds pretreated with *T. harzianum*, growth of plant was positively affected along with aflatoxin bioremediation. In case of seeds infected with *A. flavus* the B1 was reduced from 31.35068 to 4.8274176 µg, while in *A. parasiticus*, aflatoxin B_1 was found to be reduced to 1.15804 µg and G_1 up to 0.62755 µg as compared to control (B_1- 10.33538 µg and G_1- 10.0057 µg) quantified from the pods (seeds) that were only treated with *A. parasiticus*.

Table 2. Estimation of Total level of aflatoxin (µg/100g) from UV-spectroscopic and HPLC analysisin field trail experiment

Sr. No.	Sample	UV analysis		HPLC analysis	
		B_1 (µg/100g)	**G_1 (µg/100g)**	**B_1 (µg/100g)**	**G_1 (µg/100g)**
1.	*A. flavus*	26.00	-	31.35068	-
2	*A. parasiticus*	9.21	8.79	10.33538	10.0057
3.	TH+AF	0.39	-	4.8274176	-
4.	TH+AP	0.980	0.312	1.15804	0.62755

* TH+AP- T. harzianum + A. parasiticus & TH + AF - T. harzianum+ A. flavus

In addition, spots with Rf values higher than that of AFB_1 (the fastest moving aflatoxin) were obtained on the TLC plates of most of the seeds. These spots did not respond positively to the confirmatory test for aflatoxins. This implies that they were non-aflatoxin spots. They probably must have been products of either thermal decomposition of the aflatoxins or its reaction. In the case of chemical reaction involving the aflatoxins, it is anticipated that the G series would be more reactive than their B counterparts. This could be predicted from the structural differences between these compounds. In the B series, there is just one Ether linkage while in the G series, there are two Ether linkages.

In order to see the effect of aflatoxigenic *Aspergillus* sp on growth of *A. hypogaea* L., it has been observed that the pathogen alone was inoculated into the soil, reduces the germination of seeds as compare to control treatment. Pathogenecity might cause damage to plant results in reduced growth parameters and protein content. The seeds pre-treated with *T. harzianum*, showed results in high germination (81%) and growth parameters along with pathogen combination as shown in Table 3.

Table 3. Effect of antagonistic potential of *T. harzianum*on the growth promotion factors of groundnut plant infected with *A. flavus* and *A. parasiticus*

Treatment	Germination (%)	Height (cm)	Fresh weight of plant (gm)	Dry weight of plant (gm)	Protein content in seeds (mg/ml)
Control (Plain soil)	55	27.5	43.120	16.650	0.2646
A. flavus	19	14.5	21.420	10.120	0.1889
A. parasiticus	24	21.5	29.530	12.020	0.1745
T. harzianum	81	51.3	210.300	79.50	0.513
TH +AF	74	49.02	123.76	61.214	0.4978
TH+AP	77	49.30	126.35	62.453	0.4502

It has been demonstrated that *T. harzianum* not only has the direct effect on the pathogen but also induces systemic resistance in plants. The least kernel contamination occurred due to reduced *Aspergillus* population in the rhizosphere of peanut indicating the competitive survival ability and growth potential biocontrol agent over *A. flavus* and *A. parasiticus*. Considerable increase in kernel yield and no adverse effect on plant growth in general suggest the safe use of *Trichoderma* species as a biocontrol agent in peanut crop as shown in Fig. 3. Pod discoloration also had significant positive correlation with total aflatoxin content in pods. *Aspergillus* infection to pods was related to visible damage and pod discoloration, but still no significant correlation existed between visual fungal contamination and total aflatoxin content in pods.

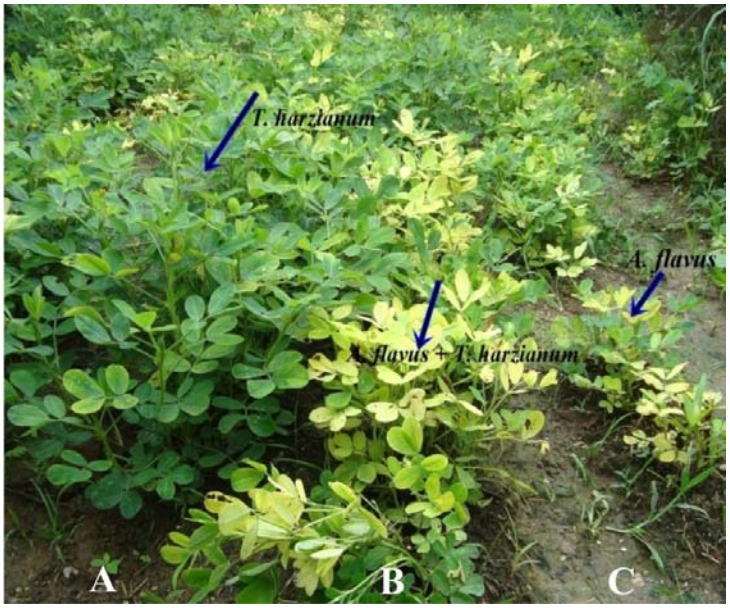

Fig. 3. Effect of test pathogen and biocontrol agent on the growth of groundnut plant. Lane A: plant infected with *A. flavus*, lane B: with *A. flavus* and *T. harzianum* and lane C: with *T. harzianum.*

For the purification of newer metabolites by TLC about 20µl of the ethyl acetate crude extracts of *Trichoderma* species was applied at the edge of TLC plate. Further the plate was "developed" by immersing the loaded edge in a mobile phase vapor-saturated chamberas shown in Table 4. Separated plate was examined under UV trans illuminator for Rf measurements and presence of secondary metabolites as shown in Fig. 4.

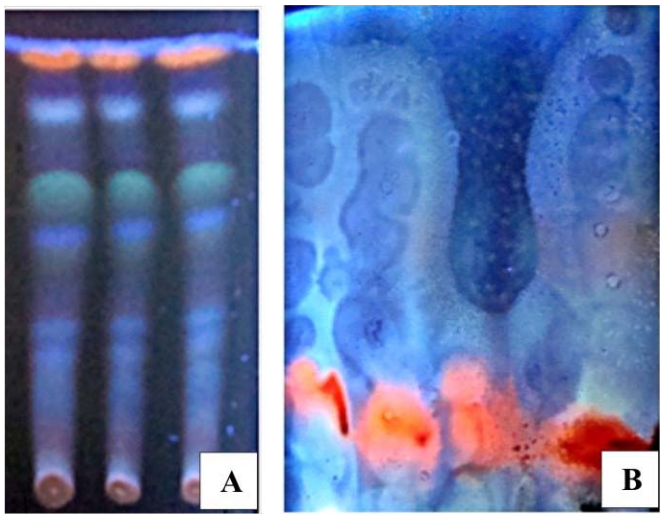

Fig. 4: TLC Chromatogram of combined fractions of *T. harzianum* ethyl acetate extract separated by column chromatography(A) *T. harzianum* (B) after spraying the plate with conc. H_2SO_4 spray reagent detecting the presence of terpenoid

Table 4. Mobile phases and spraying agents used for the detection of class of compounds

Sl. No	Compound to be identified	Solvent system used	Spraying reagent used for identification
1	Sesquiterpene	Mobile phase, Benzene; Acetone (4:1), Benzene: Ethyl acetate (45%), Benzene: Acetone (9:1) and Chloroform:Methanol(2:1)	Plate was sprayed with conc. H_2SO_4.
2	Sulphur compound	Chloroform: Methanol (17:3), Ethyl acetate: Ethanol: H_2O (9:1:2)	Spray solution I: 0.035% solution of N-bromosuccinimide in 1,1,1-trichloroethane. Spray solution II: Fill up 3 ml 0.33% solution of fluorescein in sodium hydroxide solution (c = 0.1 mol/L) to 100 ml with ethanol. Procedure: Spray with I, dry at room temperature and spray with II.
3	Alkaloids	Chloroform: Methanol: Ammonium hydroxide NH_4OH(60 : 10 : 1)	Solution (a): Dissolve 0.85 g basic bismuth nitrate in 10 ml glacial acetic acid and 40 mlwater under heating. If

			necessary, filter. Solution (b): Dissolve 8 g potassium iodide in 30 ml water. Stock solution: (a) I (b) are mixed to 1. Spray reagent: 1 ml stock solution is mixed with 2 ml glacial acetic acid and 10ml water. 1% Ethanolic vanillin (solution I). 10% ethanolic sulphuric acid (solution 11).
4	Terpenoids	Benzene: Chloroform (1:1); Benzene: Ethyl acetate (19:1)	The plate is sprayed with 10 rnl solution T, followed immediately by 10 ml solution II. After heating at ll0°C for 5-10min under observation, the plate is evaluated visibly.
5	Polyacet-ylenes	Dichloromethane: Ethyl acetate (10:1)	Plate was sprayed with 1% $KMNO_4$ in aqueous 2% Na_2CO_3.
6	Saponins and Sapogenins	Chloroform : Glacial acetic acid : Methanol Water (64:32:12:8)	Spray of antimony chloride in conc. HCl was used.

For the extraction of secondary metabolites form *Trichoderma* species, ethyl acetate was found to be the best extraction solvent as it shows more number of bioactive bands on the TLC plate. Especially crude extract of *T. harzianum* shows variety of bands with dark intense blue, brown to faint golden color.

The purified compound purified from TLC plate has colourless sticky oily nature with the UV absorption maximum at 210 nm. The Rfvalue was in range from 0.55 to 0.9 depending on the developing solvents, which indicates that antifungal compound is of hydrophobic nature. The purified fractions were tested for their antifungal activity on TLC plates (Bioautography). The bioautography confirm that extraction with ethyl acetate extract has many more compounds with fungicidal activity then crude extract as shown in Table 5.

Table 5. Antifungal activity of culture filtrates from *T. harzianum* separated by organic solvent.

Sr. No.	**Solvents**	**Inhibition zone (mm)**	
		A. flavus	***A. parasiticus***
1.	n-Hexane	9	10
2	Ethyl acetate	14	12
3.	Chloroform	-	-
4.	n-butanol	8	6

The active fractions were combined and subjected to column chromatography, yielding 9 fractions. Among these fractions, the most active fractions were from 5^{th} to 7^{th} in order of elution, which were combined, concentratedand subjected to HPLC.

The sample was dissolved in 500 µl of ethyl acetate. The 50 µl sample was spiked in 1000 µl methanol. The mobile phase was acetonitrile: water (7:3v/v) solvent system and the flow rate of 0.5 ml/min and injection volume of the sample were 20 µl.

A main compound was obtained and itshowed a single absorption peak at retention time of 13.11 min at 370nm (Fig. 5). Another compound obtained, shows a single absorption peak at retention time 7.1 min at 135nm.

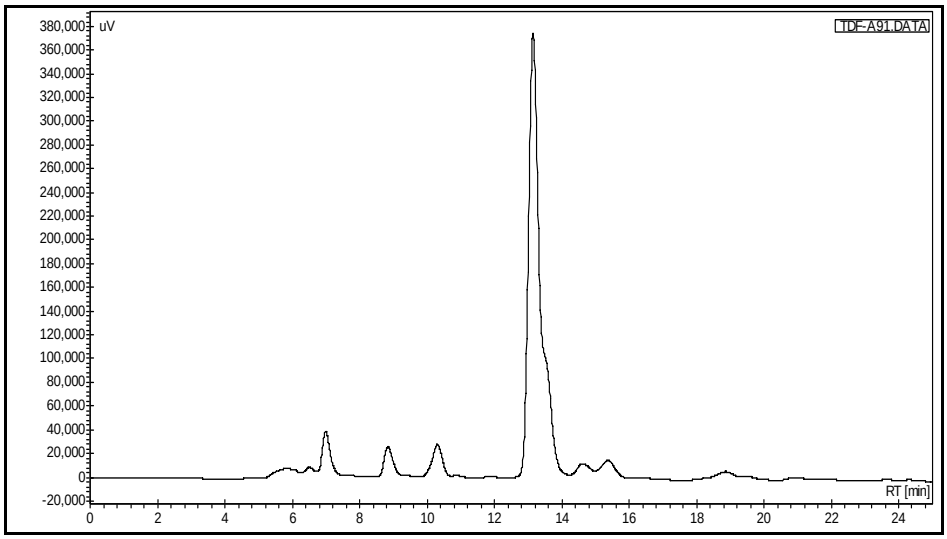

Fig. 5. HPLC chromatogram of the antifungal substance TH1 isolated from the culture filtrate of *T. harzianum*

The molecular mass of 365 Da for purified bioactive compound, was determined by electrospray ionisation–mass spectrometry and its pattern corresponded to that of 2-hydroxy-2-[4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]- 3-methyl-butyric acid (Fig. 6) described by Sawa et al. (1994) and Vinale et al. (2009b). The identification of significant metabolites noted in this study demonstrates that LC-MS analysis adequately determined the characteristic metabolites of *Trichoderma* species.

The confirmation of harzianic acid in purified extract, plates were sprayed with conc. H_2SO_4, in which presence of harzianic acid (Fig. 6a) gives brown colored spot when heated at 100°C for 10 min. Furthermore, the other metabolite 6 pentyl alpha pyrone (Fig. 6b) were purified and identified in ethyl acetate extract as described above.

Using column chromatography and HPLC, six compounds were isolated from ethyl acetate extract of *Trichoderma* species. All the compounds were reacted with p-anisaldehyde- sulphuric acid spray reagent. Many other compounds were present, but the quantity of fractions was too small to enable the characterization of these compounds.

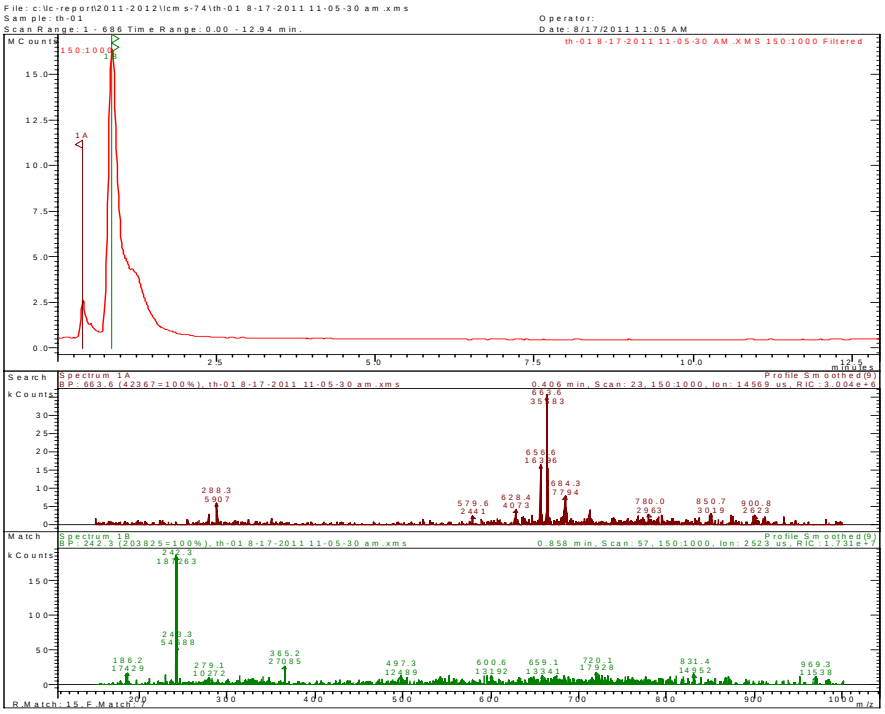

Fig. 6. LC/MS of purified fraction obtained from *T. harzianum* showing harzianic acid (365.4 molecular weight)

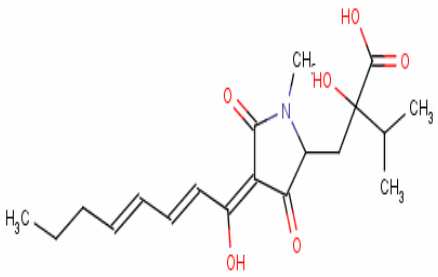

Fig. 6a Structure of harzianic acid

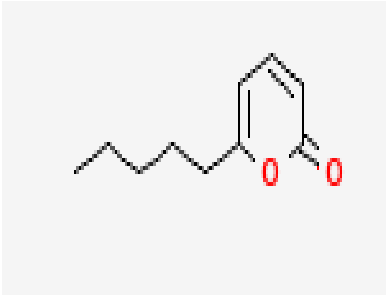

Fig. 6b Structure of 6 pentyl alpha pyrone

Assessment of antifungal spectrum of purified bioactive compound on TLC plates can be done by bioautography. This method employed for localizing antifungal activity present in the purified sample to an inoculated agar plate by diffusion (Rahalison et al. 1991). The developed TLC plates were first over layered with thin layer of potatoes dextrose agar medium (PDA) at approximately 50°C containing spore suspension of *Aspergillus* (10^{-6} spores ml^{-1}). The inoculated plates were then transferred to the sterile plastic boxes, sealed with parafilm to maintain 100% relative humidity and incubated at 25°C in dark for 3-4 days. Mycelial growth

inhibition around the separated band indicated the antifungal potential of the individual fractions. Zones of inhibition are next visualized by a dehydrogenase activity detecting reagent, a tetrazolium salt, which is converted by the bacteria into the intensely colorful product. The antibacterial compounds appear as colorless spots against a coloured background (Hamburger, 1987).

After isolation and purification of the antifungal compound, six fractions from *T. harzianum* were obtained by column chromatography and preparative TLC. Among these fractions two produced by *T. harzianum* showed considerable fungicidal activities according to bioautography test. Fraction with Rf (0.64) exhibited the most distinct inhibition zoneagainst *A. flavus* (70% of the total antifungal activity) by the purified fraction. The presence of *T. harzianum* affects the growth of fungal pathogen. The formation of halos is an indicator of production of antibiotic substances either by the pathogenic fungi to prevent itself from the attack of antagonistic fungus or vice versa as shown in Fig. 7.

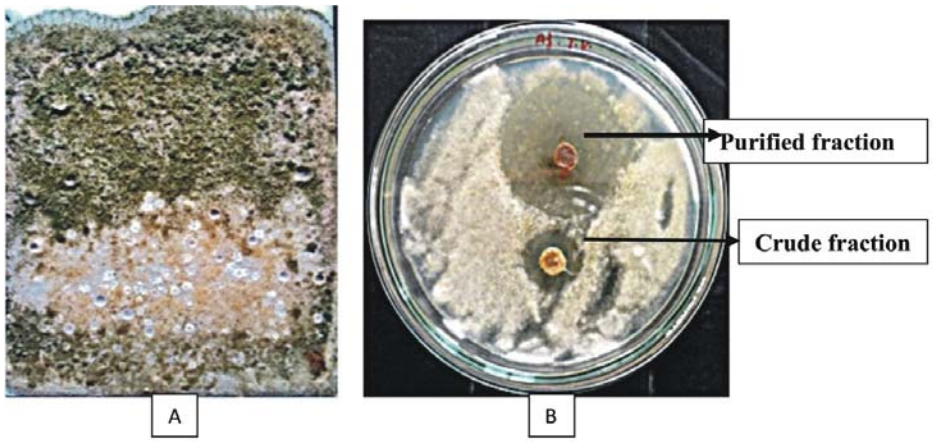

Fig. 7: Antifungal activity of purified extract (50mg/ml) of *T. harzianum* against *A. flavus* (A) TLC bioautography (B) Disc diffusion method

CONCLUSION

The study concluded that *T. harzianum* treatments are able to control the aflatoxin level and growth of *Aspergillus* species by their secondary metabolites. The work, confirm the involvement of harzianic acid and 6-n-pentyl pyrone in antagonistic activity. Chemical analysis of these metabolites will broaden the spectrum of substances known to control the aflatoxin production and possible other fungal growth too. The derived metabolites from the *Trichoderma* species may be the useful future tool for both disease and fungicide's resistance management.

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5

ANALYSING THE PLANT MICROBIOME FOR CONTROL OF PATHOGENS

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ABSTRACT

The plant microbiome is a key determinant of plant health and productivity. Plant microbiome discoveries could fuel progress in sustainable agriculture, such as the development of microbial inoculants as biofertilizers, biocontrol, or stress protection products. Although we recognize a growing market for these bio-products, they still have their problems, e.g., short shelf-life, inconsistent effects under field conditions, and risk predictions. The application of "omics"-technologies has allowed for an enormous progression in the development of so-called next-generation bio-products. New tools may have an impact on (i) the detection of new bio-resources for biocontrol and plant growth promoting agents, (ii) the optimization of fermentation and formulation processes for biologicals, (iii) stabilization of the biocontrol effect under field conditions and (iv) risk assessment studies for biotechnological applications. Advances in these aspects could open new perspectives for sustainable agriculture by the development of high impact next-generation bio-products.

INTRODUCTION

The importance of microbial root inhabitants for plant growth and health has been recognized already 100 years ago. Since that time, much has been learned about microorganisms and their close symbiotic relationship with plants (Phillipot et al. 2013; Berg et al. 2014). Comparable to humans and other eukaryotic hosts, plants also may be realized as meta-organism that harbours a "second genome". These advances in knowledge were driven by both "omics"-technologies guided by next-generation sequencing and microscopic insights. Collectively known as the plant microbiome, plant-associated microbes can help plants fend off disease, stimulate growth, occupy space that would otherwise be taken up by pathogens, promote stress resistance, and influence crop yield and quality. Therefore, the plant microbiome is a key determinant of plant health and productivity. Plant microbiome discoveries could

fuel progress in sustainable agriculture, such as the development of microbial inoculants as biofertilizers, biocontrol, or stress protection products (Berg 2009).

Although we recognize a growing market for these bio-products, they still have their problems, e.g., short shelf-life, inconsistent effects under field conditions, and risk predictions. The application of "omics"-technologies has allowed for an enormous progression in the development of so-called next-generation bio-products (Köberl et al. 2012). New tools may have an impact on (i) the detection of new bio-resources, e.g. medicinal plants of mosses for biocontrol and plant growth promoting agents (Köberl et al. 2011, Bragina et al. 2014), (ii) the optimization of fermentation and formulation processes for biologicals, (iii) stabilization of the biocontrol effect under field conditions and (iv) risk assessment studies for biotechnological applications (Alavi et al. 2013). Advances in these aspects could open new perspectives for sustainable agriculture by the development of high impact next-generation bio-products (Berg et al. 2013).

The control of microbial growth is an important area of microbiology, which resulted in significant advances in agriculture, medicine, and food science. Biological control is an environmentally sound and effective means of reducing pathogens and pests and their symptoms through the use of natural antagonists or enemies. While in the past mainly single organisms were used, often correlated with inconsistent effects, it is now possible to develop predictable microbiome-based biocontrol strategies (Berg et al. 2013).

In many cases, diseases are associated with microbiome imbalances (dysbiosis) or shifts, which make it promising to control the whole microbiome. I predict that analyzing microbiome connections as well as the microbial interplay opens new doors for advanced biocontrol technologies (ABT). Moreover, ABTs can not only be used to suppress pathogens, they can also be effectively used to establish microbiomes in a desirable, beneficial composition. It should be possible to develop "microbiome design" strategies for particular purposes in the future. Due to the impact of the microbiome on health, growth, size, height, weight, reproduction as well as development of their host, microbiome controls are an attractive goal. Two principles should be considered for the development of ABTs: i) microbial diversity is an important factor determining the invasion of pathogens (Elsas et al. 2012), and ii) synthetic ecology can support the selection of microbes (Dunham, 2007). In all three examples mentioned, biocontrol approaches are applied. Plant health has been the target of biological control for more than 100 years, and now safe and predictable control strategies are making the development of next generation biocontrol products possible (Berg et al. 2013). Since we identified the origin and function of rhizosphere microorganisms, indigenous endophyte consortia are showing promise as effective biological control agents. Moreover, in metagenomic approaches to ancient plant-associated microbiomes such as Sphagnum mosses, stress protection is identified as the main function (Bragina et al. 2014). This could be a valuable response against climate change. In comparison, biological control of the gut microbiome is a new but extremely promising development that has been supported by the enormous success of fecal transplantations (De Vrieze, 2013). Many more applications are however possible, e.g. beneficial food microbiomes promoting plant and human health. Further investigation into the impact of the vegetable microbiome on our health

seems to be especially important and needs more attention in the future (Berg et al. 2014). While functional food can sustain human health, targeted microbial treatment of liquid diets could be used as an additional therapy in hospitals. Last but not least, the indoor microbiome needs our attention and also biological solutions for control.

Currently, especially in hospitals and clean rooms, the microbiome is chemically and UV treated allowing only resistant microorganisms to survive. Additionally, hospital acquired infections are permanently increasing, and are especially caused by (multi) resistant pathogens. In 2014, the World Health Organization (WHO) produced a global map of antimicrobial resistance and issued a warning that a 'post-antibiotic' world could soon become a reality. Woolhouse and Farrar (2014) realized that this phase has already started. They have also emphatically called for a dedicated, coordinated plan of action investigating the root causes of resistance, e.g. the misuse of antimicrobials especially in agriculture, and the development of new drugs and alternative therapies. In those areas ABTs can make significant contributions to the development of new sanitary measurements and alternative therapies.

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6

COMMERCIAL TRANSFER OF TECHNOLOGY FOR SUSTAINABLE AGRICULTURE – TO FEED A WORLD POPULATION OF OVER 7 + BILLION PEOPLE (USING PGPR AND RHIZOBIUM, IN SUSTAINABLE AGRICULTURE)

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INTRODUCTION

Humanity is facing its toughest challenge every day, our planet wakes with 200,000 more mouths to feed. Every night, more than 870 million people go to bed hungry. The need to produce more food is already acute, and the world's land, water and energy resources are under unprecedented strain. Nearly 7 million hectares of farmland are lost to soil erosion every year. Many people who produce the world's food are living in poverty. Biodiversity is disappearing fast. And the challenge won't get any easier: by 2050, for example, 4 billion people will be living in countries with water scarcity. Something needs to change we only have one planet, and we're using its resources 50 percent faster than it can take. What we're asking to provide is simply not sustainable. We can't go on like this.

Rhizobia are symbiotic diazotrophs (prokaryotic organisms that carry out dinitrogen fixation) that rhizobia benefit. Biological fixation of nitrogen was the leading form of annual nitrogen input until the last decade of the 20th century (Russelle, 2008). It is gaining attention once again as sustainability becomes a central focus to feed a world population of over 7 billion people. The plant supplies the rhizobia with energy in the form of amino acids and the rhizobia fix nitrogen from the atmosphere for plant uptake. The reduction of atmospheric dinitrogen into ammonia is the second most important biological process on earth after

photosynthesis (Sylvia, 2005). The actual process of dinitrogen fixation can only be carried out by diazotrophs that contain the enzyme dinitrogenase. Nitrogen is the most critical nutrient needed to support plant growth. Unfortunately, atmospheric dinitrogen (78% of air we breathe) is extremely stable due to triple bonds which can only be broken by energy intensive ways. These include electrical N_2 fixation by lightning where oxides of N come to ground with rain, the Haber-Bosch process in industrial fertilizer production, and biological N_2fixation in legumes by bacterial symbionts such as *Rhizobium etli*. Biological fixation of nitrogen was the leading form of annual nitrogen input until the last decade of the 20th century (Russelle, 2008).

Fig. 1. The rhizobia (prokaryotic organisms) supplies plants energy in the form of amino acids and the rhizobia fix nitrogen from the atmosphere for plant uptake

As sustainability becomes a central focus to feed a world population of over 7 + billion people Professor (Dr.) Mahabal Ram, Wheat Scientist at SHIATS Allahabad India took this novel innovative scientific approach to break the yield barrier in wheat by developing, for the first time, a new source of dwarfing gene (MRD, with long sink size by application of gamma rays (Fig. 2).

Fig. 2. Showing dwarfing gene (MRD, with long sink size by application of gamma rays.

Development of semi dwarf wheat varieties at CIMMYT Mexico in the nineteen sixties (1960s) brought a turning point in the wheat production in about 40 spring wheat growing countries of the world because these varieties were non lodging and responsive to higher nitrogen doses as a result wheat production moved up tremendously where 'inputs' and irrigation were not the limiting factors. Though intensive cultivation of semi dwarf wheat varieties in rice-wheat cropping system brought self-suffering in food grain production in many countries including India and the period of 1970-80 was celebrated as Era of Green Revolution, but its impact in general was corrosive to the soil fertility, ground water, environment, germplasm of wheat and rice etc.

However, after a quantum jump in the wheat production, genetic ceiling in the yield of semi dwarf wheat varieties has surfaced once more as a result wheat production moving very slow; at the same time, population in the world in general and India in particular is growing fast which may lead to the Malthusian nightmare in the food grain production (food famine) by mid 21st century.

Wheat breeders at CIMMYT Mexico and other wheat growing countries of the world have failed to break the present ceiling in the productivity of dwarf wheat varieties using conventional and Molecular breeding techniques. According to Professor (Dr.) Mahabal Ram, wheat specialist, working at the Sam Higginbottom Institute of Agriculture, Technology and Sciences (deemed to be University) Allahabad, "Continuous use of Daruma" source of dwarfing genes (Rht1 and Rht2) in wheat for the past 60 years (1955-2015) is responsible for present stagnation in the genetic potential of the current wheat varieties due to genetic uniformity as well as pleiotropic effect of dwarfing genes.

For the first time, Dr. Ram has developed a new source of dwarfing gene (MRD) with long sink size by application of gamma rays. By use of this new dwarf genotype in hybridization program with other Varieties, he has evolved new lines of wheat with enhanced sink size viz 20-25 cm long spike with 120-130 grains/spike and 36-40 gm 1000 grains weight as compared to 8-10 cm. spike length with 40-45 grains/spike of the standard semi dwarf wheat varieties under cultivation in India such as PBW-343, PBW-502 and HD-2733 (Fig.3-shows graded improvement in the sink size of new wheat lines). Dr. Ram is expecting these new wheat lines will be able to give 8-10 tons yield/ha on normal fertility and irrigation management, without raising inputs and irrigation level, thereby dawning the foundation of second wheat revolution.

Due to climate change, global warming has been forecasted by UNO (2014) which may reduce the wheat production in India and China by 30%, Therefore Dr. Ram is incorporating genetic basics of tolerance to higher terminal temperature (35-40 degree Cen.) of February-March to mitigate the adverse effect of global warming on wheat yield. So far, high yielding varieties of wheat having tolerance to higher temperature (35-40 degree Cent.) have been already released by State government of U.P. for commercial cultivation which will serve as Genetic Shield to protect wheat crop grown on about 10 million hectares (1/3 of total wheat growing area in India) against global warming in Uttar Pradesh (U.P). About 90% farmers in Uttar Pradesh U.P are marginal (having 5-1 h.) and small (1-2 h.) land holders will be most benefited for the first time because these varieties require low inputs and limited

irrigation besides having tolerance to higher terminal temperature. In addition, work is in progress by Dr. Ram to improve the protein content and nutritive values of the new elite lines of wheat by genetic manipulation.

Fig. 3. Showing graded improvement in the sink size of new wheat lines

Breeding wheat for enhanced micronutrients

Low concentrations or deficiencies of bioavailable iron (Fe), zinc (Zn) and other essential micronutrients in human food afflict a large proportion of the world's population. Plant biofortification, to improve the mineral concentrations in the edible portions of crop plants by conventional breeding or modern transgenic approaches, is regarded as the most economical and sustainable strategy. Many researchers have demonstrated that there are significant differences in grain mineral element concentrations among wheat (*Triticum aestivum* L.) and its relatives. Compared with cultivated wheat, wild wheat are potential genetic resources for enhancing micronutrient in wheat grain. An ancestral wild tetraploid wheat (*T. turgidum* ssp. *dicoccoides*) carrying the allele *Gpc-B1*, which is associated with increased Fe, Zn, and protein concentrations in grain, was cloned using a positional cloning strategy. Combining conventional breeding with modern genetic engineering approaches, such as introgression of genes from wild relatives into wheat, synthetic hexaploid wheat, quantitative trait locus (QTL) analysis, and even gene cloning and genetic transformation, are important for developing wheat cultivars higher in micronutrients (Xu, Y., An, D., Li, H. and Xu, H. 2011, Canadian Journal of Plant Science, 2011, 91(2): 231-237, 10.4141/CJPS10117).

Reducing poverty

Reducing poverty is one of IRRI's primary goals. Through the coordinated efforts of IRRI and over more than 900 partners worldwide, it has made headway toward achieving this seemingly insurmountable objective.

The Australian Centre for International Agricultural Research (ACIAR), for instance, reported that IRRI's work resulted in rice yields of up to 13% between 1985 and 2009 across three Southeast Asian countries.

Meanwhile, the Swiss Agency for Development Cooperation (SDC) reported that the US$ 12 million investment in rice research by the Swiss government has yielded $70 million in benefits to rice farmers and national economies in four Asian countries.

Increasing food security

Rice and wheat are the most important human food, eaten by more than half of the world's population every day. In Asia, where 90% of rice is consumed, ensuring there is enough affordable rice for everyone, or rice security, is equivalent to food security. In Africa and Latin America, rice is becoming a more important staple too. Much of IRRI's work is around helping increase rice production to ensure food security - particularly for those people most at risk of not getting enough food.

Food security is also recognized as being more than just providing people with enough calories to live on, but ensuring people have enough nutrients for optimal health too. IRRI is working on developing healthier rice varieties to help those who mostly depend on rice can get more nutrients into their diet to reduce malnutrition.

This is what food security is all about. Learn more about IRRI's success stories on increasing food security:

Tackling climate change

Whether it's through the alternative wetting and drying management practice that's being implemented in the Philippines or the development of drought-tolerant rice, IRRI's research is focused on adapting rice to the effects of climate change. Moreover, our scientists are cognizant are constantly looking for ways that rice production can reduce greenhouse gases.

Through IRRI's efforts, farmers from various countries across Asia are being taught to adopt methods of dealing with changes in the climate. IRRI has provided (and continue to provide) farmers with effective tools and knowledge to help them achieve higher yields despite the threatening nature of climate change.

Protecting the environment

A healthy environment is essential to a healthy rice production system and to the health of rice producers and workers. Rice production depends on natural resources like water and nutrients and needs to be protected from pollutants. Moreover, any negative impact of rice production on the environment has to be minimized to ensure rice production is sustainable in the long term.

IRRI works to reduce the impact of rice production on the environment through smart crop management that optimizes inputs like fertilizer, and by reducing greenhouse gas emissions from rice fields.

Making wheat healthier

Two billion people suffer from what is known as "hidden hunger," or micronutrient malnutrition. They get enough macronutrients (carbohydrates, protein, and fat) from their diet, but not enough micronutrients (vitamins and minerals) that are essential to good health. Hidden hunger can result in more frequent and severe illness and complications during pregnancy, childbirth, infancy, and childhood. Many people in Asia rely heavily on wheat and rice for most or their entire calorie needs because they cannot afford or do not have access to a full range of nutritious food such as fruits, vegetables, and foods from animal sources (e.g., meat, dairy products, and eggs). As a result, lack of iron, zinc, and vitamin A has become prevalent micronutrient deficiencies in wheat/rice-consuming countries. The cost of these deficiencies in terms of lives and quality of life lost is enormous, and women and children are most at risk.

Because wheat is the dominant cereal crop in most Asian countries and is the staple food for more than half of the world's population (including many of those living in poverty), even a small increase in the micronutrient content of wheat and rice grains could have a significant impact on human health. Healthier wheat and rice varieties have the potential to reach many people because wheat as well as rice is already widely grown and eaten. IRRI is developing rice varieties that have more iron, zinc, and beta carotene content to help people get more of these important micronutrients. These healthier varieties can complement current strategies to reduce micronutrient deficiencies.

Engaging women

Women play an important role in the global sector as both paid and unpaid family labor. In many parts of Asia, women contribute at least half of the total labor input in wheat and rice production, performing backbreaking tasks such as transplanting and weeding. After harvest, it is usually the women who take care of seed storage and processing of (drying) for home consumption. In Africa, women do much of the work in wheat and rice production systems and play an important role in the value chain after harvest. Yet, these women face many constraints because of the prevalence of gender stereotypes and social restrictions that hamper their access to technical knowledge and technologies.

IRRI as well as SHIATS, Allahabad acknowledges that increased participation of women in agricultural research for development and extension will accelerate the realization of development goals, such as reducing poverty and increasing food security. IRRI is working with women to empower them and strengthen their role in the design, experimentation, and evaluation of agricultural research for development, as well as improved access to resources and control over output.

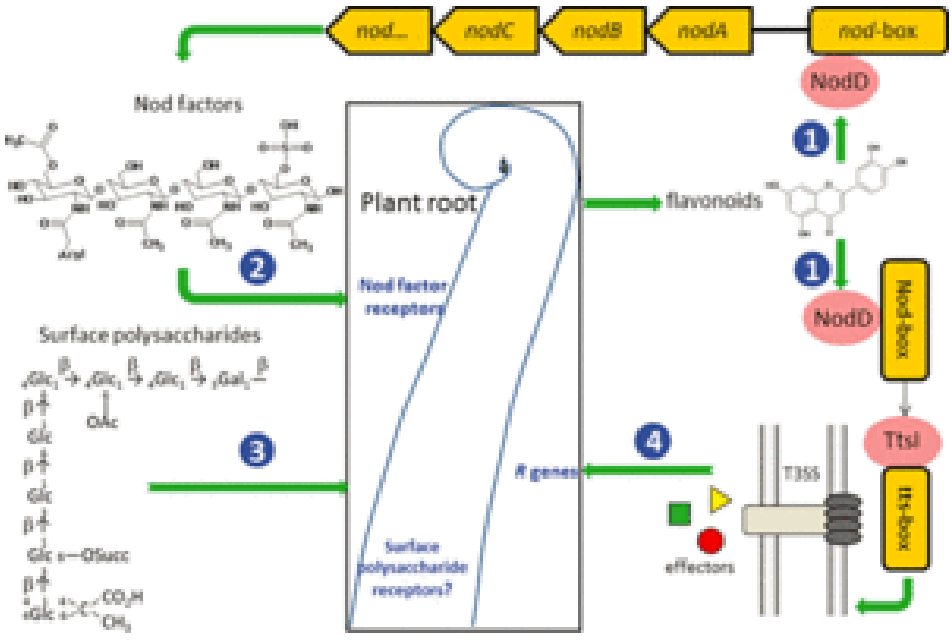
Biological interaction

Biological N_2 fixation takes energy which comes at the expense of photosynthate (sucrose). Generally, legumes gain extra nitrogen for plant growth to offset the loss of photosynthate in this mutualistic association. The rhizobia invade plant roots and induce a nodule in which the bacteria reduce atmospheric nitrogen to ammonia and

supply the plant with nitrogenous compounds (Young, 1989). The plant gains the ability to grow in nitrogen poor soils, and the bacteria gain a protected niche where they multiply and eventually escape back into the surrounding soil when the nodule senesces (Young, 1989). Because biological N_2 fixation requires such a large amount of energy, it is important to understand the energy transfer in the process. The stepwise reaction of energy transfer is characterized by the following steps: N_2 yields diamine which yields hydrazine which yields NH_4^+. Each yield requires 2 e- for a total of 6 electrons needed. Electrons come in via Fe protein and are donated by ferredoxin. ATP is used to reduce N_2 to NH_4^+ whereby ATP hydrolysis takes place and the Fe protein reduces the MoFe protein which reduces the nitrogen (Dixon and Wheeler, 1986). 4 ATPs are used per electron in the N_2 fixation process. N_2 + 6e- + 8H+ yields 2 molecules NH_4^+. So a total of 24 ATPs would be required to make 2 molecules of NH_4^+ from one molecule of N_2.

Nodulation

The actual process of nodulation is a very coordinated effort between the legume and the *Rhizobium* bacteria in the soil. Infection typically occurs in root hairs of legumes. Many rhizobia and host plants are highly specific and legumes can either attract rhizobia to root hairs directly by excretory compounds or by induction of *nod* gene activity in the bacteria.



Molecular determinants of host specificity during nitrogen-fixing symbiosis. http://onlinelibrary.wiley.com/doi/10.1111/j.1462-5822.2011.01736.x/pdf

Communication between legume and *Rhizobium*

1. Flavonoids are released by the host root. The flavonoid is at the highest concentration at the root and interacts with the product of bacterial *nodD* gene. The *nodD* gene produces the protein, *nodD*, which is the sensor that recognizes chemicals excreted by host plant roots (Russelle, 2008).
2. Rhizobia colonize the soil in the vicinity of the root hair in response to the flavonoids. This process is autoregulated where favonoids stimulate Nod factor production, which stimulates flavonoid secretion (Russelle, 2008).
3. Response to Nod factors is extremely rapid and the disruption of cell wall happens very quickly. Disruption of crystallization of cell walls takes place, thereby allowing entrance by the rhizobia. At the same time Rhizobia multiply in the rhizosphere. The root hair is then stimulated and curls to the side where the bacteria are attached which stimulates cell division in the root cortex.
4. A "shepherd's crook" is formed and entraps the rhizobia which then erode the host cell wall and enter near the root hair tip. An infection thread is formed as rhizobia digest the root hair cell wall. Free-living *Rhizobium* bacteria are converted to bacteroids as the infection elongates by tip growth down root hair and toward epidermal cells.
5. Infection thread branches and heads toward the cortex and a visibly evident nodule develop on the root as the plant produces cytokinin and cells divide. Nodules can contain one or more rhizobial strains and can be either determinant (lack a persistent meristem and are spherical) or indeterminate (located at the distal end of cylindrically shaped lobes) (Russelle, 2008). Many infections are aborted due to a breakdown in communication between rhizobia and the host plant leaving nodule number strictly regulated by the plant.
6. Once inside the nodule, rhizobia are released from the infection thread in a droplet of polysaccharide. A plant-derived peribacteroid membrane, which regulates the flow of compounds between the plant and bacteroid, quickly develops around this droplet via endocytosis. This process keeps the microbes "outside" the plant where the rhizobia are intracellular but extracytoplasmic (Russelle, 2008). The loss of the ammonium assimilatory capacity by bacteroids is important for maintaining the symbiotic relationship with legumes.

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7

ENVIRONMENTAL AND ECOLOGICAL CONSTRAINTS ON PGPR FUNCTIONING

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ABSTRACT

One of the major limitations of PGPR technology is the variation in the benefits obtained upon their field application. This is also true for biocontrol strains of Pseudomonas which inhibit phytopathogens by production of different antifungal molecules such as 2,4-diacetyl phloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT) and phenazines (PHZ). In the present work we have studied the effect of C source availability on the manifestation of PGPR trait for biocontrol in different fluorescent pseudomonads strains. Antifungal metabolites production depended on nature of C source. Interactions among two groups of PGPR, biocontrol pseudomonads and root nodulating rhizobia showed that metabolites from Pseudomonas strains inhibited certain rhizobial cultures using ethyl acetate extracts as well as by pure antibiotics. Rhizobium sp. ST1 was sensitive for DAPG, PRN and PLT, followed by R. leguminosarum which was inhibited by DAPG and PHZ while strain IC3123 did not get inhibited by any of the compounds. The extracts containing the antifungal metabolites of Pseudomonas strains showed varying levels of inhibition of rhizobial cultures. R. leguminosarum was found to be the most sensitive rhizobial strain, followed by Mesorhizobium loti which was also inhibited effectively by extracts from several Pseudomonas biocontrol strains. The Bradyrhizobium japonicum strain tested did not get significantly inhibited by extracts of most fluorescent Pseudomonas strains. Plant inoculation experiments with selected strains showed that combinations of multiple strains are more effective in plant growth promotion. This work signifies that certain bio-control strains may have adverse effect on the rhizobial populations present in rhizosphere and hence this criterion should be checked to successfully develop effective consortia. The finding that the ecological interactions may be further controlled by environmental factors is important for understanding the potential field performance of bio-control strains.

INTRODUCTION

PGPR inoculants in practice occasionally fail to survive or do not execute their specific function in soil environment and hence bring about inconsistent results in field. Fluorescent pseudomonads are well known for the biological control of several fungal plant pathogens (Weller, 2007). For many pseudomonads, production of metabolites such as antibiotics, siderophores and hydrogen cyanide (HCN) is the primary mechanism of biocontrol. These organisms areideally suited as inoculants, because they can aggressively colonize the roots and are versatile in terms of their growth requirements (Lugtenberg et al. 2001). Production and regulation of antibiotics by fluorescent *Pseudomonas* isolates under different carbon sources and other nutrients could reveal their ecological performance.

Ecological interactions between rhizobia and other PGPR have been of interest in recent years because of the agronomical implications of co-inoculation with a battery of efficient strains and development of consortia that are more effective than individual inoculants (Antoun & Prevost, 2006).Certain PGPRs can improve nodulation and N_2-fixation in legume plants (Mishra et al. 2012) while some are not effective (Garcia et al. 2004). Although most of the times PGPR may interact synergistically with root nodulating rhizobia, some antagonistic effects are also seen. Fluorescent *Pseudomonas* strains promote plant growth by suppressing plant pathogenic microorganisms through production of antibiotics but the action of these antibiotics is non-specific and thus may also harm the other plant beneficial microorganisms in the vicinity. With this in view, the present study was aimed at studying the effect of antagonistic metabolites produced by fluorescent pseudomonads on the growth of rhizobia. In this work the interaction between rhizobia and *Pseudomonas* and PSB was also investigated in plant inoculation studies.

MATERIALS AND METHODS

Rhizobial strains used were Bradyrhizobiumjaponicum 61A152, *Rhizobium cleguminosarumc* bv. Viciae 384, *Mesorhizobium loti, Sinorhizobium meliloti*, and *Rhizobium* sp. ST1, IC3109, IC3123 and IC3169. *Pseudomonas fluorescens* strains used were *P. fluorescens* (protegens) CHA0, *P. fluorescens* Q287, *P. fluorescens* Pf-5 along with several local isolates of fluorescent pseudomonads. The PSB used in the plant inoculation experiments was *Enterobacter asburiae* PSI3 (Sharma et al. 2005). For the study of the effect of pure antifungal metabolites known to be produced by fluorescent Pseudomonas on rhizobial cultures, Yeast Extract Mannitol Agar (YEMA) plates were spread with 0.1 ml of overnight grown rhizobial culture and various dilutions of pure antifungal compounds- 2,4 Diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyrrolnitrin (PRN) and phenazine (PHZ) were loaded in the wells. Plates were incubated in upright positions overnight at 28ºC and zones of inhibition noted. In experiments with ethyl acetate extracts of fluorescent pseudomonas were used, 80 µl of the extracts were loaded in the well. Production 2,4 DAPG by bacterial isolates on King's B medium was quantified by HPLC analysis of ethyl acetate extracts.

Plant study for the co-inoculation effect of *P. fluorescens (protegens)* CHA0, *E. asburiae* PSI3, and *Rhizobium* sp. ST1 on *Cajanus cajan* were carried out in

locally collected soil (alluvial, loamy sand, pH 7.7 with moisture holding capacity 40%) obtained from fields of Pulse research center, Model farm, Anand Agriculture University, Vadodara. About 1kg air-dried and double sterilized soil was placed in plastic autoclavable bags (2 kg capacity) and cell suspensions of bacterial strains or water (control) were added in different combinations as mentioned in results. The soil was mixed thoroughly and filled in 10-cm diameter plastic pots at 400 g per pot. For the double inoculums combination, 200 g of each treatment from 1 kg master mix was mixed thoroughly and for triple combination 133 gram of each treatment from master mix was mixed properly and filled in the plastic pots at 400 g per pot. Six germinated mungbean seeds sown. Each treatment was replicated three times and randomized. Inoculations resulted in 10^7- 10^8 CFU of introduced bacteria per gram of dry soil. The soil was maintained at 30ºC with sufficient moisture content throughout the experiment. The plants were harvested after 32 d.

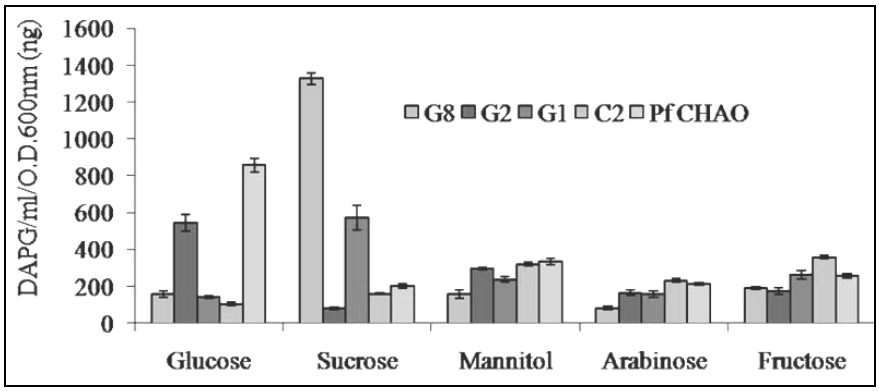

Fig. 1. 2, 4- DAPG production by fluorescent pseudomonads under different carbon sources

RESULTS AND DISCUSSION

Production of DAPG by different strains of fluorescent pseudomonads depends on the C source and variation among different strains for C source preference (Fig. 1). Fig. 2 shows the effect of DAPG, PLT, PRN and PHZ on rhizobia. Rhizobial strains were inhibited different extents for e.g. *Rhizobium* sp. ST1 was found to be sensitive to DAPG, PRN and PLT while *R. leguminosarum* was inhibited by DAPG and PHZ. The effect of partially purified secondary metabolites produced by *Pseudomonas* strains on rhizobia was studied using ethyl acetate extracts of culture supernatants (Fig. 3). *R. leguminosarum* was found to be the most sensitive rhizobial strain and was inhibited by extracts of most fluorescent *Pseudomonas* strains. Other rhizobial strains were inhibited to different extents. Some pseudomonas strains showed inhibition against few rhizobial strains.

Co-inoculation studies of different combinations of biocontrol strain *P. fluorescens* CHA0, PSB strain *E. asburiae* PSI3 with *Rhizobium* sp. ST1 was performed to understand the effect of dual and triple combinations of PGPR with different PGP traits. Fig. 4 shows a positive effect of bacterial inoculations on above ground parts of the plants. Plant shoot dry mass increased in the order of triple inoculation > dual inoculation > single inoculation. *E. asburiae* PSI3 singly

promoted extensive root growth while *Rhizobium* sp. ST1 singly promoted shoot growth. In triple combinations shoot and root biomass was increased significantly.

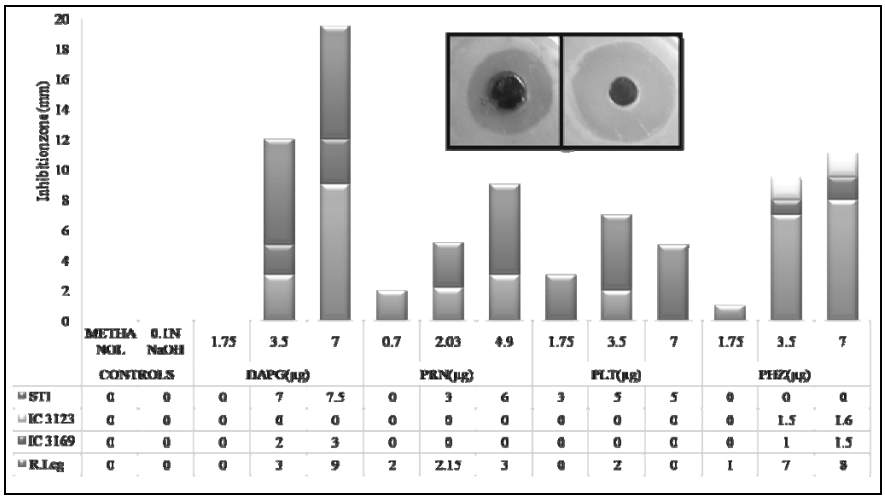

Fig. 2. Effect of antifungal metabolites on rhizobial strains. (Inset shows representative zones of inhibition).

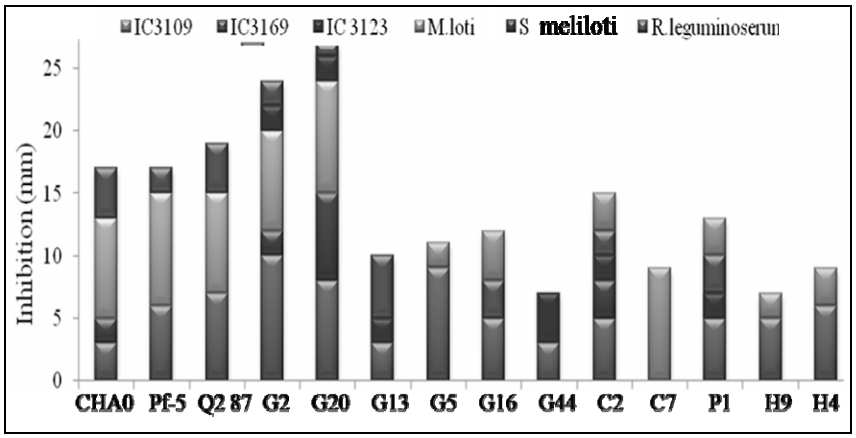

Fig. 3. Inhibition of rhizobial strains by the ethyl acetate extracts of culture supernatants of fluorescent *Pseudomonas* strains. Y axis labels denote names of fluorescent *Pseudomonas* strains.

Overall results show that consortia of different PGPR strains may be an effective strategy to provide plants with the benefits of different PGPR traits. The compatibility of the biocontrol strains with other members of consortium should be checked before their application in the fields for maximum economic gains. Dual combinations of *Rhizobium* and PSB showed good effect while the triple combination with *Pseudomonas* was found to be best. An understanding of ecological and mutual interactions between putative inoculants in a consortium can result in maximum benefits to the host plant.

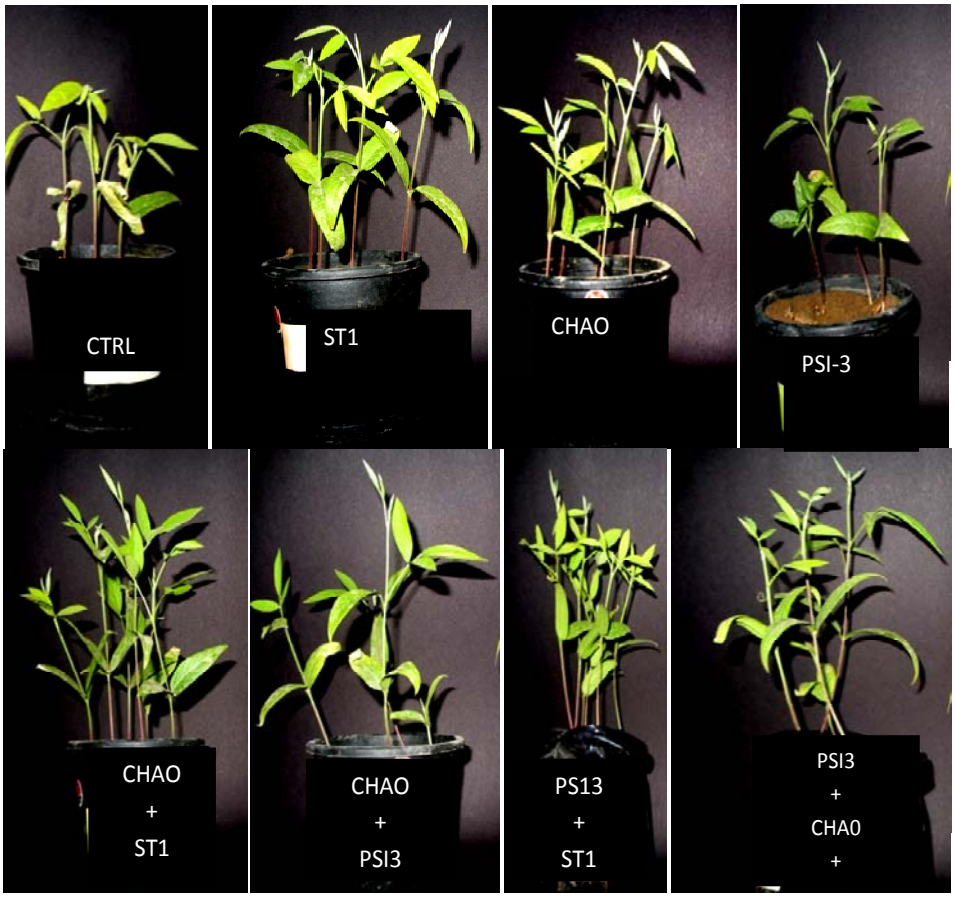

Fig. 4. Pot study for the effect of different combinations of *Rhizobium* sp. ST1, *P. fluorescens* CHA0 and *E. asburiae* PSI3 on *C. cajan*

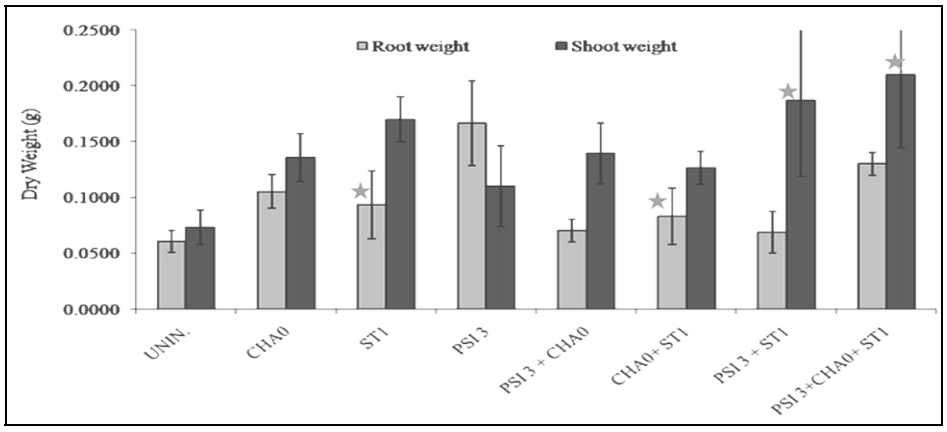

Fig. 5. Plant growth promotion effects of different combinations of *Rhizobium* sp. ST1, *P. fluorescens* CHA0 and *E. asburiae* PSI3 on *C. cajan.* (*P<0.05)

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8

UTILIZATION OF ACTINOMYCETES HAVING BROAD-SPECTRUM OF PLANT GROWTH-PROMOTING AND BIOCONTROL TRAITS IN CHICKPEA, SORGHUM AND RICE

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ABSTRACT

Plant pathogens such as Sclerotium rolfsii (causes collar rot), Fusarium oxysporum (causes wilt) and Macrophomina phaseolina (causes charcoal rot/dry root rot) have a broad host range, affecting several agriculturally important crops including chickpea, pigeon pea, groundnut and sorghum, which are grown under rainfed conditions, leading to significant yield losses. Due to the broad host range of these fungal pathogens, it has become very difficult for the farmers to grow these crops profitably. Hence, there is a need to have broad-spectrum plant growth-promoting (PGP) and biocontrol organisms for use in different cropping systems for the control of multiple diseases in a single crop and there by the crop productivity can be enhanced in the dry-land agriculture. The main objective of the present study was to identify and evaluate broad spectrum PGP and biocontrol agents and their metabolites with multiple actions against different pathogens so that one biological treatment controls more than one problem apart from promotion of plant growth in chickpea, sorghum and rice.

INTRODUCTION

Agricultural sector is facing burden from many ways via lower soil nutrients, attack of pathogen, pest and weeds and fluctuating climatic conditions which leads to economic consequences. Among them, pathogenic microbes are one of the major threat for food production and also ecosystem stability; because a single crop is affected by multiple pathogens, and vice versa i.e., many phyto-pathogens have broad host range and hence affect multiple crops. Ex Chickpea is affected by multiple pathogens including (i) bacteria: *Xanthomonas campestris* - bacterial blight, (ii) fungi: *Ascochytar abiei* - *Ascochyta* blight; *Botrytis cinerea* - *Botrytis* gray mold;

Alternaria alternata – Alternaria blight; *Colletotrichum dematium* - *Colletotrichum* blight, *Sclerotinia sclerotiorum* - *Sclerotinia* stem rot; *Fusarium oxysporum* - *Fusarium* wilt; *F. solani* -black root rot; *Sclerotium rolfsii* - collar rot; *Rhizoctonia solani* - wet root rot, and (iii) virus: Stunt - leaf roll virus; Narrow leaf - yellow mosaic virus; Necrosis - necrotic yellows virus (Nene et al. 2012). Pathogens like *Aschochyta* and *Botrytis* have the host range of around 50 (Skoglund et al. 2011) and 200 (Williamson et al. 2007) plant species, respectively. To overcome this multi-pathogen attacks, farmers are in a situation to increase the use of chemical inputs which further leads to pathogen resistance against the agents and other non-target environmental impacts (Compant et al. 2005). Besides this, increasing human population requires higher productivity of food crops since plants are the basis for every food chain.

In view of the above status, strategies involving plant productivity in an environmentally sustainable manner is necessary in which biological options are of great importance. The antagonistic or plant growth-promoting (PGP) rhizobacteria and their bioactive antimicrobial compounds are considered as environmentally safe and easily biodegradable (Morrissey et al. 2004). PGP microbes are those colonizing the root surfaces and the closely adhering soil interface i.e., the rhizosphere or exist in internal tissues as endophytes and helps in promoting plant growth by various mechanisms. This includes direct (nitrogen fixation, phosphate solubilization, iron chelation and phytohormone production) or indirect (suppression of pathogens, induction of resistance in host plants against pathogens and abiotic stresses) mechanisms. Some of the representatives of PGP microbes are *Bacillus, Pseudomonas, Erwinia, Caulobacter, Serratia, Arthrobacter, Micrococcus, Flavo-bacterium, Rhizobium, Bradyrhizobium, Sinorhizobium,* and *Streptomyces* (Vessey 2003; Ma et al. 2011).

The present study was designed to evaluate PGP microbes isolated form vermicompost for their role in growth promotion on chickpea, rice and sorghum and their antagonistic potential on plant pathogens.

MATERIALS AND METHODS

Microorganisms

Five strains of *Streptomyces* sp., CAI-24 (NCBI accession: JN400112), CAI-121 (NCBI accession: JN400113), CAI-127 (NCBI accession: JN400114), KAI-32(NCBI accession: JN400115), and KAI-90 (NCBI accession: JN400116) form Microbial germ plasm collection at ICRISAT were selected for the study.

In vitro plant growth-promoting traits

The isolates were tested for their PGP traits by the estimation of indole acetic acid (IAA) (Patten & Glick 1996), siderophore (Schwyn & Neilands 1987), phosphate utilization (Nautiyal 1999) and lytic enzymes such as cellulase (Hendricks et al. 1995), protease (Bhattacharya et al. 2009), chitinase (Hirano & Nagao 1988), β-1,3-glucanase production (Singh et al. 1999) and other molecules such as hydrocyanic acid (HCN) (Lorck, 1948). The isolates were also tested for their antagonistic activity

against *Fusarium oxysporum* f. sp. *ciceri* (FOC) and *Macrophomia phaseolina* (Tassi) Goid., (MP) for checking their broad-spectrum potential.

Evaluation of Streptomyces for PGP properties

Chickpea

The five actinomycetes isolates CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 were evaluated for their PGP properties on chickpea variety ICCV2 under field conditions at 2012-2013 at ICRISAT, Telangana, India. The seeds were treated with *Streptomyces* culture (10^8 CFU ml^{-1}) for 45-50 min and sown immediately. Plants were inoculated with respective *Streptomyces* strains (1000 ml; 10^8 CFU ml^{-1}) once every 15 days on the soil close to the plant until the flowering stage. All the agronomic practices were done as and when required. Growth parameters were measured at regular intervals. The details of this trial can be found at Gopalakrishnan et al. (2015).

Rice

Effect of *Streptomyces* sp., CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 on growth performance of rice was performed in the 2011 Kharif (rainy) season at ICRISAT under system of rice intensification (SRI) method (Uphoff 2003). The 11-day-old single seedlings were uprooted from the nursery; their roots were dipped in respective *Streptomyces* culture (containing 10^8 cfu ml^{-1}) for 60 min and transplanted in to the field. Plants were inoculated with respective *Streptomyces* strains (1500 ml; 10^8 cfu ml^{-1}) once in every two weeks until the flowering stage. All the agronomic practices were done as and when required. Growth parameters were measured at regular intervals. The details of this trial can be found at Gopalakrishnan et al. (2013).

Sorghum

Growth promoting ability of the five *Streptomyces* isolates on sorghum variety ICSV112 was evaluated under greenhouse conditions. The seeds were treated with *Streptomyces* isolates (10^8 CFU ml^{-1}) for 45-50 min and sown immediately. Booster doses of *Streptomyces* strains (5 ml per seedling, 10^8cfu ml^{-1}) were applied at 15days interval by the soil drench method. Growth parameters were evaluated at regular intervals. The details of this experiment can be found at Gopalakrishnan et al. (2013).

Characterization for colonization and gene expression

Colonization ability of *Streptomyces* isolates on chickpea roots were done as per the protocols of Bozzola and Russell (1998) and the roots were examined under scanning electron microscope (JOEL-JSM5600). Gene expression profile of these isolates were tested for IAA, β-1,3-glucanase and siderophore genes and their transcription levels were measured as per the protocols of Gopalakrishnan et al. (2014).

Evaluation of Streptomyces for biocontrol properties

Fusarium wilt of chickpea

The five *Streptomyces* sp., CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 were evaluated for biocontrol potential against FOC under greenhouse conditions on chickpea variety JG-62. FOC inoculum was added at the concentration of 20% of pot weight and subjected to the following treatment methods: M1 - inoculation of thepotting mixture with respective actinomycete culture along withFOC two weeks before sowing; M2 - inoculation of the seeds by soaking in the respective actinomycete culture for 1 h; M3 - inoculation of the sprouted seeds by soaking in the respective actinomycete culture for 1 h; M4 - inoculation of the potting mixture with actinomycete culture at the time of sowing (10 ml of actinomycete culture [10^8 CFU ml^{-1}] was applied on the seed and covered with soil) and M5 - inoculation of the seedlings after emergence with actinomycete culture (10 ml of actinomycete culture [10^8 CFU ml^{-1}] was applied on the seedlings after emergence). Similarly, isolates were also tested in *Fusarium*-infested field at ICRISAT during 2009-10 cropping seasons with all the inoculation methods except M1. Disease symptoms and growth parameters were evaluated at regular intervals. The details of this trial can be found at Gopalakrishnan et al. (2011).

Charcoal rot of sorghum

The five *Streptomyces* sp., CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 were evaluated for biocontrol potential against MP under greenhouse conditions on sorghum. The seeds were treated with *Streptomyces* isolates (10^8 cfu ml^{-1}) for 45-50 min and sown immediately. Booster doses of *Streptomyces* strains (5 mlper seedling, 10^8cfu ml^{-1}) were applied at 15days interval by the soil drench method. The disease was induced by inserting toothpick infested with MP inoculum in the second internode of stalk at 10-15 days after flowering (Das et al. 2007). After 25-30 days of disease induction, the symptoms were measured at 1-5 scale.

RESULTS AND DISCUSSION

Plant growth promoting traits - in vitro

All of the *Streptomyces* sp., CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 were invariably found to produce siderophore, HCN, IAA, β-1,3-glucanse and lipase *in vitro* (Fig. 1). Other PGP properties such as cellulase (only in KAI-32 and KAI-90), protease (only in CAI-24 and CAI-127) and chitinase (only in CAI-24, KAI-32 and KAI-90) were also observed. Inhibitory activity of these isolates was documented against FOC and MP. Further details of this can be found at Gopalakrishnan et al. (2011 and 2013).

HCN, hydrocyanic acid; IAA, indole acetic acid; Values on primary (left) axis are rating scales for in vitro PGP traits of (i) siderophore, chitinase, cellulase, lipase and protease production as: 0, no halo zone; 1, halo zone of <1 mm; 2, halo zone of 1–3 mm; 3, halo zone of 4–6 mm and 4, halo zone of7 mm and above; (ii) chitinase production as: 0, no halo zone; 1, halo zone of 1–5 mm; 2, halo zone of 6–10 mm, 3,

halo zone of 11–15 mm; 4, halo zone of 16–20 mm and 5, halo zone of 21 mm and above; (iii) HCN production as: 0, no color change; 1, light reddish brown; 2, medium reddish brown and 3, dark reddish brown; (iv) β-1,3-glucanase (U) – one unit is an amount of enzyme that liberated 1 µmol of glucose hour^{-1} at defined conditions. Values on secondary (right) axis indicate IAA production.

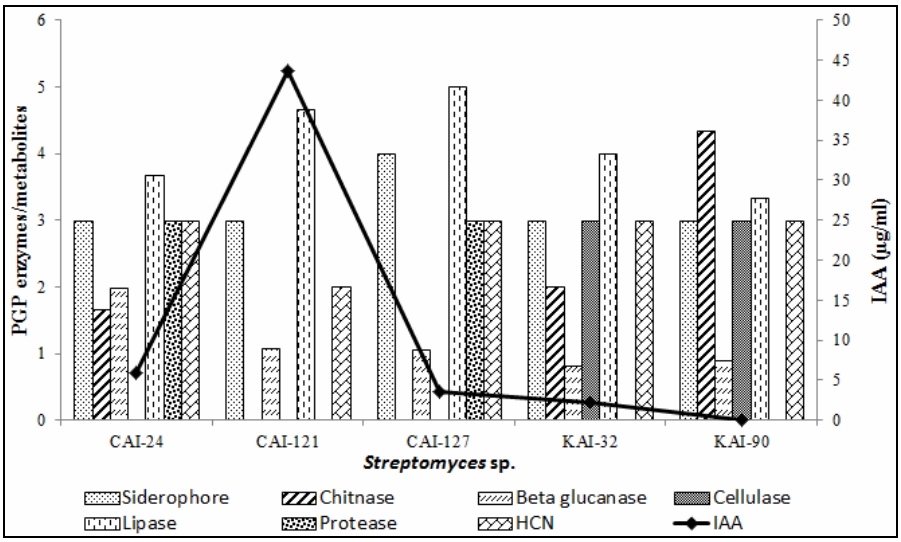

Fig. 1 Plant growth promoting and biocontrol traits of *Streptomyces* sp.

Plant growth promoting properties of Streptomycessp.- Greenhouse and field conditions

Chickpea

All the *Streptomyces* strains were found to enhance agronomic performance of chickpea (Table 1). After 30 days of sowing, the nodule number, nodule weight and root weight was increased up to 70, 82 and 6%, respectively over the control plots. At 60 days after sowing, pod number and pod weight was increased up to 51 and 85%, respectively than control. At harvest, 39 and 12% increase was observed for stover yield and grain yield over the control treatments.

Table 1. Effect of PGP *Streptomyces* sp. on agronomic performance of chickpea

Isolates	At 30 days of sowing			At 60 days after sowing		At harvest	
	Nodule number (plant $^{-1}$)	Nodule weight (mg plant^{-1})	Root weight (mg plant $^{-1}$)	Pod number (plant $^{-1}$)	Pod weight (g plant $^{-1}$)	Stover yield (t ha^{-1})	Grain yield (t ha^{-1})
CAI-24	14	58	175	65	3.05	1.24	1.83
CAI-121	25	53	176	63	4.16	1.67	1.87
CAI-127	17	31	176	59	2.89	1.53	1.8

KAI-32	17	48	184	68	4.44	1.42	1.8
KAI-90	31	75	196	68	4.25	1.82	2.07
Control	12	29	171	43	2.04	1.11	1.68
Mean	19	48	180	61	3.47	1.46	1.84
SE	1.5***	4.1***	3.5***	2***	0.206***	0.03***	0.013***
LSD	4.7	12.8	10.9	6.3	0.64	0.095	0.042
CV%	13	14	3	6	10	4	1

LSD – Least Significant Difference; CV – Co-efficient of Variation;
*** - Significant at $p < 0.001$

The highest increase of agronomic traits was observed in *Streptomyces* sp., KAI-90 treated plot. This might be due to their plant growth promoting traits which were proved *in vitro*; on the other hand, the isolates also increased soil parameters like total N (5%), available P (37%) and organic carbon (9%) than control treatments (Table 2). Microbial action on these influences was proved by higher microbial biomass carbon (55%) and dehydrogenase activity (17%) in rhizospheric soil of *Streptomyces* treated chickpea cultivation fields than control treatments (Table 2). Microbial action on agronomic performance was further revealed by up-regulation on gene expression of PGP genes including IAA (10 fold) and siderophore (12.6 fold) and also biocontrol genes, β-1,3-glucanse (2.4 fold) (Fig. 2). They also documented for colonization capacity on chickpea roots (Fig. 3), which helps in competing against pathogens and hence provide protection against fungal pathogens (Gopalakrishnan et al. 2015).

Table 2. Effect of PGP *Streptomyces* sp. on rhizospheric soil health at harvest of chickpea

Isolates	**MBC (µg g^{-1} soil)**	**DA (µg TPF g^{-1} soil 24 h^{-1})**	**Total N (ppm)**	**Available P (ppm)**	**OC (%)**
CAI-24	1041	71.1	668	10.3	0.5
CAI-121	1157	58.9	667	11.1	0.54
CAI-127	1070	59.1	644	19.3	0.52
KAI-32	1430	58.8	642	17.8	0.5
KAI-90	1134	62.7	701	10.9	0.49
Control	752	53.1	632	10.1	0.47
Mean	1097	60.6	659	13.2	0.5
SE	63.5**	0.66**	7.6**	0.24***	0.005**
LSD	231	2.4	27.7	0.88	0.019
CV%	8	3	2	3	2

MBC - Microbial biomass carbon; DA - Dehydrogenase activity; OC - Organic carbon;
LSD – Least Significant Difference; CV – Co-efficient of Variation; ** - Significant at $p < 0.01$; *** - Significant at $p < 0.001$

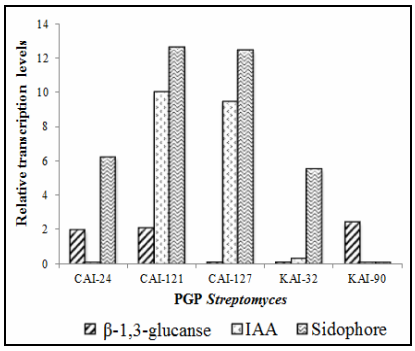

Fig. 2 Expression profile of PGP genes of *Streptomyces* sp.

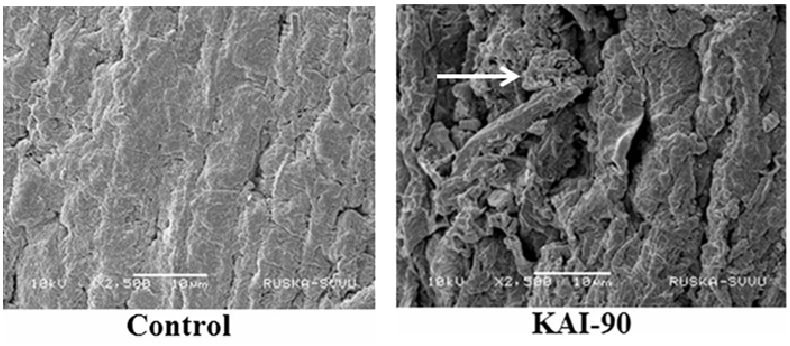

Fig. 3. Scanning electron microscopy images of the control & and *Streptomyces* sp., KAI-90 treated chickpea roots. Arrow indicates colonization of KAI-90 on chickpea roots.

Rice

All the *Streptomyces* sp., were found to enhance rice growth tested under SRI cultivation method (Table 3). During the harvest period, an increase of up to 28%, 18%, 25% and 10% was observed on tiller numbers, panicle numbers, stover yield and grain yield over the control treatment. Root development parameters including root length (up to 15%), root volume (up to 35%) and root dry weight (up to 55%) were influenced by the PGP strains.

Table 3. Effect of PGP *Streptomyces* sp. on agronomic performance of rice, at harvest

Isolates	Tiller numbers (m^{-2})	Panicle number	Grain yield (g m^{-2})	Stover yield (g m^{-2})	Root length (m m^{-2})	Root volume (cm^{-3} m^{-2})	Root dry weight (g m^{-2})
CAI-24	586	41.7	587	584	2087	396	30.5
CAI-121	506	38.3	583	637	3263	513	36
CAI-127	501	43.1	619	754	3470	692	49.5
KAI-32	532	40.5	640	754	3652	627	54.3
KAI-90	589	39.5	587	693	3562	581	44.8

Control	459	36.4	582	601	3182	507	35.1
Mean	529	39.8	600	671	726	88	5.9
SE	15.6***	0.29***	9.7**	27.8**	81.6***	16.8***	1.89***
LSD	47.1	1.06	30.5	87.8			
CV%	7	2	3	7	7	7	10

LSD – Least Significant Difference; CV – Co-efficient of Variation; ** - Significant at $p < 0.01$; ** - Significant at $p < 0.001$

In addition, soil properties were influenced by PGP microbes which were shown up to 122%, 53% and 13% increase of total N, available P and organic carbon over the control treatment (Table 4). Role of microbes in increasing these soil parameters is evidenced by higher concentration of microbial biomass up to 41%, microbial biomass nitrogen up to 52% and dehydrogenase activity up to 75% over the un-inoculated plots. The highest increase of agronomic traits was observed in *Streptomyces* sp., KAI-32 treated plot (Gopalakrishnan et al. 2013).

Sorghum

The five *Streptomyces* sp., strains significantly enhanced PGP parameters of sorghum by increasing plant height (up to 51%), stem weight (up to 39%) and root length (up to 18%), root volume (up to 9%) and root dry weight (up to 25%) over the control treatment (Table 5; Gopalakrishnan et al. 2013).

Table 4. Effect of PGP *Streptomyces* sp. on rhizospheric soil health, at harvest of rice

Isolates	**MBC ($\mu g\ g^{-1}$ soil)**	**MBN ($\mu g\ g^{-1}$ soil)**	**DA (μg TPF g^{-1} soil24 h^{-1})**	**Total N (gKg^{-1}soil)**	**Available P ($mg\ g^{-1}$ soil)**	**OC (%)**
CAI-24	1715	60	94	2.456	0.133	1.49
CAI-121	3293	65	113	1.992	0.117	1.47
CAI-127	2875	88	194	2.644	0.115	1.52
KAI-32	4020	65	135	2.142	0.122	1.66
KAI-90	2946	62	136	2.16	0.129	1.62
Control	2861	58	111	1.19	0.087	1.47
Mean	2952	66	131	2.231	0.117	1.53
SE	150.5***	3.7**	9.3***	0.063*	0.005*	0.032*
LSD	520.7	12	29.7	0.229	0.019	0.116
CV%	9	10	12	4	6	3

MBC - Microbial biomass carbon; MBN - Microbial biomass nitrogen; DA - Dehydrogenase activity; OC - Organic carbon; LSD – Least Significant Difference; CV – Co-efficient of Variation; * - Significant at $p < 0.05$; ** - Significant at $p < 0.01$; ** - Significant at $p < 0.001$

Table 5. Effect of PGP *Streptomyces* sp. on growth performance of sorghum under greenhouse conditions

Isolates	Plant height (cm)	Stem weight (g)	Root length (m plant^{-1})	Root volume (cm^3 plant^{-1})	Root dry weight (g plant^{-1})
CAI-24	114	5.42	90	10.2	0.92
CAI-121	122	5.55	80	9	0.84
CAI-127	100	5.09	88	9.3	0.87
KAI-32	124	6.39	82	10.5	0.89
KAI-90	130	7.03	78	10.5	1.01
Control	86	5.06	76	8.8	0.81
Mean	113	5.76	82	9.7	0.89
SE	4.9***	0.471*	3.4*	0.42**	0.036*
LSD	14.9	1.421	10	1.28	0.109
CV%	9	16	8	9	8

LSD – Least Significant Difference; CV – Co-efficient of Variation; * - Significant at $p < 0.05$; ** - Significant at $p < 0.01$; ** - Significant at $p < 0.001$

Biocontrol potential of PGP Streptomyces

Fusarium wilt of chickpea

All the 5 PGP *Streptomyces* sp., showed inhibitory activity against FOC under laboratory conditions. Under green house conditions, up to 76% reduction in disease incidence was observed at 29 days after sowing (DAS) with highest reduction of 76% in *Streptomyces* sp., CAI-24 which showed good antagonistic activity on M1, M2 and M5 methods of inoculation (up to 76%).

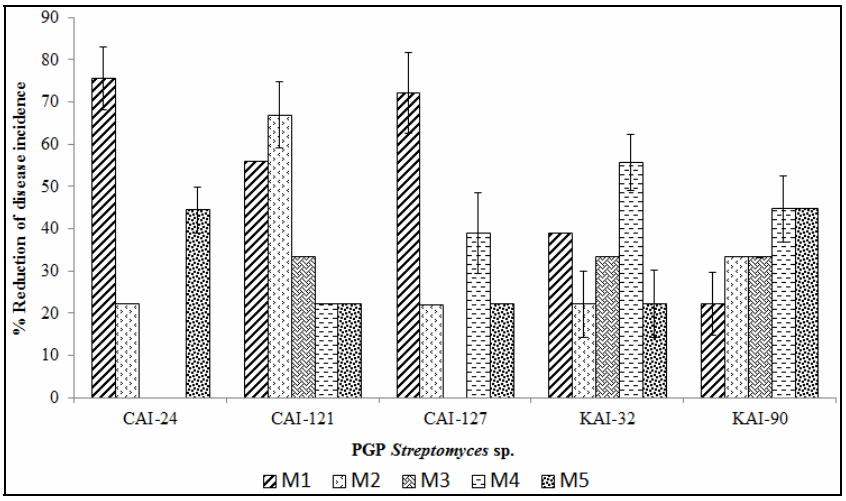

Fig. 4 Effect of PGP *Streptomyces* sp., on disease incidence of *Fusarium*wilt of chickpea under greenhouse conditions at 29 DAS

M1 - inoculation of the potting mixture with respective actinomycete culture along with FOC two weeks before sowing; M2 - inoculation of the seeds by soaking in the respective actinomycete culture for 1 h; M3 - inoculation of the sprouted seeds by soaking in the respective actinomycete culture for 1 h; M4 - inoculation of the potting mixture with actinomycete culture at the time of sowing (10 ml of actinomycete culture [10^8 CFU ml^{-1}] was applied on the seed and covered with soil) and M5 - inoculation of the seedlings after emergence with actinomycete culture (10 ml of actinomycete culture [10^8 CFU ml^{-1}] was applied on the seedlings after emergence).

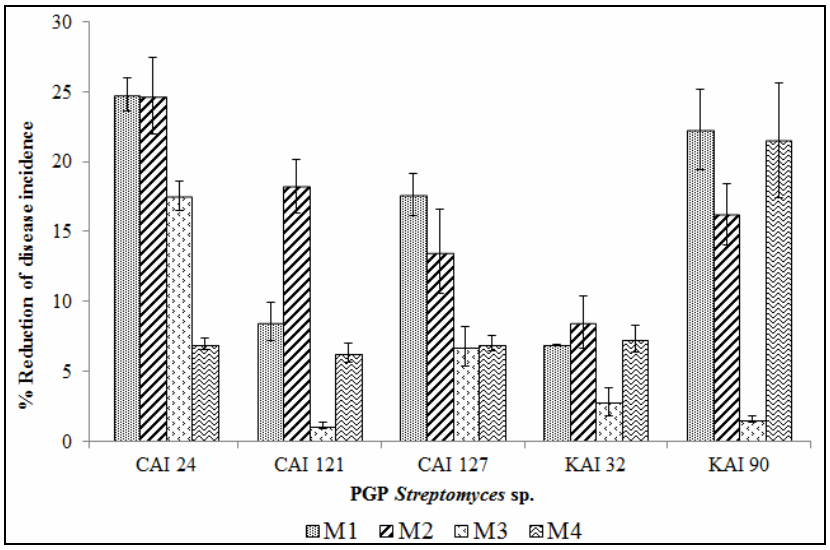

Fig. 5 Effect of PGP *Streptomyces* sp., on disease incidence of *Fusarium* wilt of chickpea under wilt sick plots at 24 DAS

M1 - inoculation of the seeds by soaking in the respective actinomycete culture for 1 h; M2 - inoculation of the sprouted seeds by soaking in the respective actinomycete culture for 1 h; M3 - inoculation of the potting mixture with actinomycete culture at the time of sowing (10 ml of actinomycete culture [10^8 CFU ml^{-1}] was applied on the seed and covered with soil) and M4 - inoculation of the seedlings after emergence with actinomycete culture (10 ml of actinomycete culture [10^8 CFU ml^{-1}] was applied on the seedlings after emergence).

Remaining isolates CAI-121, CAI-127, KAI-32 and KAI-90 reduced the disease incidence on all the 5 methods of actinomycete inoculation up to 67%, 72%, 56% and 45%, respectively, whereas in control plots all the plants got killed by 21 days (Fig. 4). The same isolates also reduced the disease incidence on chickpea under wilt sick plots up to 19% on 28 DAS with the maximum degree of protection by CAI-24 followed by KAI-90. In un-inoculated control plots 100% disease incidence was noticed earlier by 20 DAS.

Among the methods of inoculation, M1 i.e., seed inoculation was found to best (Fig. 5). This might be due the production of inhibitory metabolites such as HCN and hydrolytic enzymes such as chitinase, cellulase, lipase and β-1,3-glucanse which were observed by laboratory analysis and/or gene expression (Gopalakrishnan et al. 2011).

Charcoal rot of sorghum

All of the PGP *Streptomyces* sp., documented biocontrol traits against *M. phaseolina* on sorghum under greenhouse conditions with 20-81% reduction in charcoal rot extension. The highest reduction of 81% was noticed on KAI-90 treated sorghum plants (Fig. 6). These results are yet to be published and the field evaluation trials are in progress.

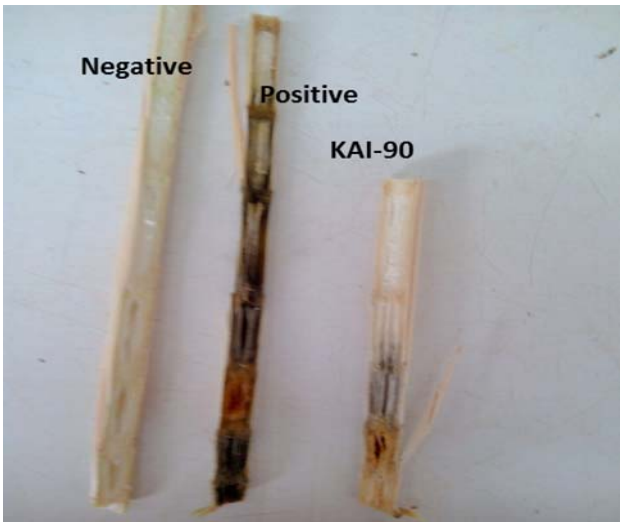

Fig. 6 Charcoal rot reduction of *Streptomyces* sp., KAI-90 treated sorghum

CONCLUSION

The five *Streptomyces* sp., evaluated in the present study documented plant growth promoting traits *in vitro.* They also enhanced the growth and yield in chickpea and rice under field conditions and sorghum under greenhouse conditions. The isolates also possess biocontrol potential against FOC and MP. This was evidenced by the reduced disease incidence on chickpea and sorghum under wilt sick plots and greenhouse conditions, respectively. Colonization ability on chickpea roots and up-regulation of PGP gene such as IAA, siderophore and β-1,3-glucanases are the other identified evidences for their role in both plant growth promotion and biocontrol potential. This study reveals the importance of PGP actinomycetes as broad spectrum growth promoting and biocontrolagents as they proved their effect on multiple crops and pathogens. So, research and development of potential broad spectrum PGP microbes will provide greater understanding of the multiple facets of disease suppression and offer significant strategies for disease management and crop productivity for sustainable agriculture in the near future.

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PLANT GROWTH STIMULATION AND BIO CONTROL OF FUSARIUM WILT (*FUSARIUM OXYSPORUM* F. SP. *CUBENSE*) BY CO INOCULATION OF BANANA (*MUSA* SPP.) PLANTLETS WITH PGPR AND ENDOPHYTES

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ABSTRACT

In banana (Musa spp.), enhanced interest in the use of in vitro produced planting material has revolutionized its cultivation by the way of producing homogenous and clean planting material. However, loss of beneficial microorganism such as endophytes through the axenic production of tissue culture plants may probably make them more vulnerable to disease attack in the field than plants derived from suckers. Although tissue culture plants may offer temporary solutions to disease problems in banana, there is a need to develop affordable, sustainable and environmentally friendly management strategies that complement the benefits of clean planting material offered by tissue culture. These problems could be overwhelmed by reintroduction of microorganisms or their consortia at the rooting medium under in vitro conditions. Plant growth and disease development were tested on the disease susceptible cultivar 'Red Banana' (AAA) after Fusarium infection in the in vitro (by co culturing with the bacterium) bacterized and non-bacterized plantlets. Results revealed that significant differences in both disease suppression and plant growth were obtained between in vitro bacterized and non-bacterized plantlets. Among the treatments, banana plantlets treated with mixture of bacterial strains viz., EPB 10 + EPB 56 + Pf1 was significantly effective in reducing Fusarium incidence under glasshouse and field conditions. It also increased the leaf nutrient status and enhanced the growth, bunch yield and the quality of the fruits compared with untreated plants. Since banana, unlike most other seed plants, is solely dependent on propagation by tissue culture for industrial purposes and thus may lose the multiple natural endophytes through sterilization during the micropropagation process. Hence, enrichment of endophytic communities in tissue

culture plantlets under in vitro conditions would probably benefit the host upon exposure to environmental stress in field plantings.

Keywords: *Musa* spp. - micropropagated plantlets - *Fusarium* wilt - *in vitro* bacterization - economic yield

INTRODUCTION

Bananas and plantains (*Musa* spp.) are grown as staple food, significant cash crops and major export crops in many tropical and subtropical countries. Its cultivation is not free from problems, constraints and challenges, especially pests and diseases which cause huge economic losses to the farming community. Among these, *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) is one of the most important production constraints and is widespread in susceptible cultivars with 80-90% severity in many banana growing states in India.

Of late, tissue culture bananas are increasingly being used by the Indian banana industry because they are the best source of disease and pest free planting material and productivity on the farm can be greatly improved with their use. However, loss of beneficial microorganisms such as endophytes through the axenic production of tissue culture plants, including beneficial plant growth promoting rhizobacteria and fungi may probably make them more vulnerable to disease attack in the field than plants derived from suckers. Survival and performance of *in vitro* bacterized plantlets are found to perform better than non bacterized plantlets. Hence, as a management strategy the strains of PGPR and endophytes were *in vitro* co-cultured with tissue culture plantlets in the present study for containing *Fusarium* wilt effectively.

MATERIALS AND METHODS

Bio-control agents

To investigate *in vitro* bacterized tissue-culture banana plantlets against *Fusarium* wilt, a pot experiment was conducted with different combinations of rhizosphere and endophytic bacteria. The rhizobacterial strain *Pseudomonas fluorescens* Pf1 was obtained from the Department of Plant Pathology, TNAU to study their efficacy against *Fusarium* wilt in banana plantlets. The endophytic bacterial strains *Bacillus* sp., EPB10 and EPB56 were isolated from different parts of banana plants, such as root, leaf and pseudostem. The experiment consisted of the following ten treatments replicated three times with five plants per replication: 1) Pf1; 2) EPB56; 3) EPB10; 4) Pf1+ EPB56; 5) Pf1 + EPB10; 6) EPB56 + EPB10; 7) Pf1+EPB56+EPB10; 8) Carbendazim (0.2%); 9) healthy control; 10) inoculated control.

In vitro co-culturing of banana plantlets with microbial consortium

From multiple clump stage in micropropagation, four-week-old plantlets were removed from the vessels, root tips were trimmed (1-2 mm) to facilitate uptake of bacteria and plantlets were inoculated by soaking the roots in a suspension containing approximately 3×10^8 cfu ml^{-1} bacterial cells in PBS for 5 min. Control plantlets were treated with PBS only. The plantlets were then transferred to rooting medium (MS with agar and sucrose only + 1.0 mg L^{-1} IBA). After removing the agar from roots

under water, plants were shifted to pots having mixture of sterilized sand and clay in 1:1 ratio. These pots were covered with polythene bags to maintain the humidity. These plantlets were acclimatized in greenhouse for eight weeks. Then they were transferred to pots and kept in an glasshouse at the Department of Fruit Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Coimbatore for further studies.

Bioefficacy of bioagents against plant growth and Fusarium wilt under glasshouse conditions

Observations on morphological characters such as pseudostem height (cm), pseudostem girth (cm) and phyllochron (interval between leaf productions) were made at glasshouse conditions. When the plantlets reached their second month of growth, they were inoculated by corm injection of a spore suspension of the pathogen (3 ml/plant, 10^6 cfu ml^{-1}) (*F. oxysporum* f. sp. *cubense* Race 1) and sand maize inoculum (10% w/w/kg soil). Treatments were replicated three times in Completely Randomized Design. Each replication consisted of five plantlets. Observations on the incidence of wilt disease were scored based on a 1-5 scale (Ploetz et al. 1999). The percent wilt index was worked out using Mc Kinney's (1923) formula.

Field experiment

A field experiment was conducted with *in vitro* bacterized tissue culture banana cv. Red Banana (*Musa* spp. AAA) plantlets to test the efficiency of the microbial consortia on banana growth, yield, quality and leaf nutrient status besides disease incidence. The experiment was laid out in a randomized block design (RBD) with nine treatments replicated three times. In each treatment, there were thirty plantlets per replication comprised of bio-formulations of *P. fluorescens*, Pf1 and *Bacillus* strains EPB 56, EPB10 and 2 m × 2 m spacing was adopted. For chemical control, healthy banana plantlets were dipped in carbendazim (0.2%) solution for 5 minutes. Observations on the incidence of wilt disease were scored based on a 1–5 scale (Ploetz et al. 1999). The percent wilt index was worked out using Mc Kinney's (1923) formula.

Growth and yield characters

An observation on morphological characters such as pseudostem height (cm) and leaf area index (leaf area per plant / area occupied per plant) (Watson, 1952) were recorded. Weight of the bunch was recorded including the peduncle up to first bract leaf node above the first hand and expressed in kilogram (kg). The percent total soluble solids (TSS) were determined by using Carl-Zeiss hand refractometer. Leaf samples collected from both the sides of the midrib of the third youngest leaf (Hewitt, 1955) at shooting stage was dried and used for leaf nutrient estimation (Piper, 1966; Jackson, 1973).

Statistical analysis

The data were analyzed as completely randomized design (CRD) using the IRRISTAT version 92-1 programme developed by biometrics unit at International

Rice Research Institute, The Philippines and Probit regression analysis and ANOVA was carried out by SPSS.

RESULTS AND DISCUSSION

The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in agricultural and horticultural crops. In the present study, the effect of *in vitro* bacterization of microbial consortia in tissue culture banana plantlets on wilt incidence and growth parameter under glasshouse conditions was assessed. Among the various treatments, combination of Pf1 + EPB56+ EPB10 reduced the wilt incidence from 73.68 (inoculated control) to 19.12 per cent. Considering the plant growth promotion, Pf1 + EPB56 + EPB10 combination increased the plant growth parameters such as height, pseudostem girth and reduced phyllochron as compared to healthy control (Table 1). Similarly, improved plant growth and reduced disease damage after microbial inoculation was reported earlier in banana (Kavino et al. 2007, 2008, 2010, 2014).

Table 1. Efficacy of *in vitro* bacterization on *Fusarium* wilt incidence in banana cv. Red banana (AAA) under glasshouse conditions

Sl.No	Treatments	Pseudo stem height (cm)	Pseudo stem girth (cm)	Phyllochron (leaf days^{-1})	Per cent wilt index (%)	Per cent reduction over control
T_1	Pf1	82.58 ^{d}	13.30 ^{cd}	8.16^{f}	40.64^{d}(39.60)	44.84
T_2	EPB56	76.62 ^{d}	13.00 ^{cd}	8.26^{e}	45.73^{c}(42.54)	37.93
T_3	EPB10	69.28 ^{e}	12.20 ^{de}	8.56^{d}	50.18^{b}(45.10)	31.89
T_4	Pf1 + EPB56	95.30 ^{c}	13.70 ^{c}	8.09^{g}	30.98^{f}(33.81)	57.95
T_5	Pf1 + EPB10	102.00 ^{bc}	14.20 ^{c}	7.89^{h}	26.56^{g}(31.01)	63.95
T_6	EPB56 + EPB10	106.67 ^{b}	15.65 ^{b}	7.68^{i}	23.25^{g}(28.82)	68.44
T_7	Pf1 + EPB56 + EPB10	115.76 ^{a}	17.00 ^{a}	7.56^{j}	19.12^{h}(25.92)	74.05
T_8	Carbendazim (0.2%)	65.37 ^{ef}	11.50 ^{ef}	9.03^{c}	35.82^{e}(36.75)	51.38
T_9	Healthy Control	60.20 ^{f}	10.40 ^{f}	9.12^{b}	0.100^{i}(1.45)	100.00
T_{10}	Inoculated control	46.20 ^{g}	7.67 ^{g}	9.19^{a}	73.68^{a}(59.17)	0.00

Values are the means of three replicates. Data in parentheses are arcsine transformed values. Means in a column followed by same superscript letters are not significantly different according to Duncan's Multiple Range Test at P=0.05. EPB, endophytic bacteria.

In the field experiment, among different treatments, plants treated with Pf1 + EPB56 + EPB10 showed the highest values for almost all the characters that reflect plant growth. The association of microbial consortium, Pf1 + EPB56 + EPB10 with banana plants resulted in the highest growth values for pseudostem height (435.26 cm) relative to inoculated control at shooting stage. Concerning other characters, plants inoculated with Pf1 + EPB56 + EPB10 showed significant improvement for leaf area index (20.83) as compared to inoculated control in plant crop. Overall, the results from plant crop suggested that the bacterial strain inoculation was generally able to promote growth of banana plants (Table 2).

Table 2. Efficacy of bacterial strains on growth, yield, quality and *Fusarium* wilt incidence in banana cv. Red banana (AAA) under field conditions

Sl.No	Treatments	Plant height (cm)	LAI	Bunch weight (kg)	TSS (%)	Acidity (%)	Per cent wilt index (%)	Per cent reduction over control
T_1	Pf1	368.40 ^{e}	15.87 ^{e}	17.19 ^{e}	18.75 ^{e}	0.20 ^{d}	46.72^{d}	40.97
T_2	EPB56	364.25 ^{f}	15.32 ^{f}	16.60 ^{f}	18.60 ^{f}	0.21 ^{c}	52.12^{c}	34.15
T_3	EPB10	363.65 ^{f}	14.69 ^{g}	16.08 ^{g}	18.21 ^{h}	0.21 ^{c}	59.06^{b}	25.38
T_4	Pf1 + EPB56	392.10 ^{c}	17.65 ^{c}	21.55 ^{c}	19.47 ^{c}	0.19 ^{e}	36.15^{f}	54.33
T_5	Pf1 + EPB10	380.75 ^{d}	16.82 ^{d}	19.26 ^{d}	19.15 ^{d}	0.19 ^{e}	31.06^{g}	60.76
T_6	EPB56 + EPB10	414.50 ^{b}	18.56 ^{b}	22.08 ^{b}	20.39 ^{b}	0.18 ^{f}	26.13^{g}	66.99
T_7	Pf1 + EPB56 + EPB10	435.26 ^{a}	20.83 ^{a}	23.42 ^{a}	21.25 ^{a}	0.17 ^{g}	18.50^{h}	76.63
T_8	Carbendazim (0.2%)	345.70 ^{g}	13.14 ^{h}	13.43 ^{h}	18.34 ^{g}	0.22 ^{b}	41.20^{e}	47.95
T_9	Control	332.90 ^{h}	11.55 ^{i}	10.67 ^{i}	17.35 ^{i}	0.23 ^{a}	79.15^{a}	0.00

Values are the means of three replicates. Means in a column followed by same superscript letters are not significantly different according to Duncan's Multiple Range Test at P=0.05. EPB, endophytic bacteria.

The application of EPB56, EPB10 and Pf1 either alone or in their combinations significantly enhanced the yield characters in plant when compared to untreated control. The increase in bunch yield (23.42 kg) with Pf1 + EPB56 + EPB10 treatment was significantly different from inoculated control plants. Single application of bacterial strains or their combinations, induced benefits in terms of higher values for finger quality characters compared to control (Table 2). From this study, it is understood that application of PGPR was effective in improving the bunch weight substantially compared to the untreated plants.

Table 3. Efficacy of bacterial strains on leaf nutrients content in banana cv.Red banana (AAA) at shooting stage in field conditions

Sl. No	Treatments	At shooting		
		Leaf N (%)	Leaf P (%)	Leaf K (%)
T_1	Pf1	2.78 ^{e}	0.28 ^{d}	2.33 ^{e}
T_2	EPB56	2.76 ^{f}	0.27 ^{e}	2.30 ^{f}
T_3	EPB10	2.75 ^{f}	0.27 ^{e}	2.31 ^{f}
T_4	Pf1 + EPB56	2.86 ^{c}	0.31 ^{c}	2.40 ^{c}
T_5	Pf1 + EPB10	2.82 ^{d}	0.30 ^{c}	2.36 ^{d}
T_6	EPB56 + EPB10	2.91 ^{b}	0.32 ^{b}	2.43 ^{b}
T_7	Pf1 + EPB56 + EPB10	2.97 ^{a}	0.35 ^{a}	2.48 ^{a}
T_8	Carbendazim (0.2%)	2.70 ^{g}	0.24 ^{f}	2.22 ^{g}
T_9	Healthy Control	2.64 ^{h}	0.21 ^{g}	2.14 ^{h}

Values are the means of three replicates. Means in a column followed by same superscript letters are not significantly different according to Duncan's Multiple Range Test at P=0.05. EPB, endophytic bacteria.

Influence of PGPR in improving the yield of perennial crops like fruits has been documented by many groups (Kavino et al. 2007, 2010, 2014). Similarly, the application of consortia of bacterial strains improved the TSS and reduced acidity besides reduction in the wilt index under field conditions. This indicates that bio-formulations were able to accumulate more of carbohydrates during its pre-climacteric phase which at the time of climacteric phase were converted into disaccharides leading to higher sweetness. Artificial inoculation of banana tissue culture plantlets with indigenous endophytes significantly reduced the *Fusarium* wilt disease besides enhance the plant's vigour (Lian et al. 2009). Similarly, consortia of bacterial strains Pf1 + EPB56 + EPB10 promoted the N, P and K uptake of banana cv. Red banana (*Musa* spp. AAA). The highest N (2.97), P (0.35) and K (2.48) contents were obtained from combination of microbes *viz*., Pf1, EPB56 and EPB10 compared to control treatment (Table 3). The suitable management of these microbes represents a feasible strategy for the micro-propagation process in the banana producing regions: micro-propagated plants would be carried to the field with an established microbial rhizosphere which would contribute to an easier adaptation to field conditions.

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COMPOSITE EFFECT OF VARIOUS BIOFERTILIZERS AND NEEM CAKE ON EFFECTIVENESS AND EFFICIENT GROWTH OF *CITRUS LIMONIA* (RANGPUR LIME) SEEDLINGS

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ABSTRACT

In Indian citrus industry, Rangpur lime has a major role in production of export quality Nagpur mandarin. As it is fairly resistant to Phytophthora infections, it is highly recommended and popular rootstock in Indian conditions. It has been observed that growth of Rangpur lime seedling in nursery stage is very slow and need much time to attain buddable size. In present study, pot culture experiment was performed to study the combined effect of biofertilizers on growth of the Rangpur lime seedlings. In present study the eleven combinations of inoculums prepared from Glomus fasciculatum (Gf) (50g and 100g), Glomus mosseae (Gm) (50g and 100g), Phosphate Solubilizing Bacteria (PSB) 3 g and neem cake 20g per seedling were given at the time of transplanting of Rangpur lime seedling in polythene bags. The experiment was replicated thrice and laid out in Randomized Block Design. The growth of Rangpur lime seedlings was assessed in terms of seedling height, stem diameter, number of leaves, leaf area, root growth, biomass accumulation, bud take percentage and final survival. We observed significant results in treatment with Gm-50 g + Gf- 50 g+ PSB- 3g and Gf- 50 g+ PSB- 3g + Neem cake 20 g in all respects during pot experiment. The data recorded at 135 days after transplanting shown that maximum height (58.30 cm), significantly highest stem diameter (0.70 cm) root length (42.50 cm), Number of fibrous roots (236.33) and root density (101.0 ml), highest bud take (95.33%) and final survival (99.17%) of Nagpur mandarin on Rangpur lime were obtained in Gm-50 g + Gf- 50 g+ PSB- 3g which is closely followed by Gf- 50 g+ PSB- 3g + Neem cake 20 g/pot.

KEY WORDS: Biofertilizers, Neem cake, Rangpur lime, growth.

INTRODUCTION

Rangpur lime rootstock is reasonably satisfactory for the yield and quality of Nagpur mandarin. The rootstock is healthy, semi-vigorous, productive, tolerant of salt, exchangeable for sodium percentage in soil, greening disease and resistant to tristeza virus. The Nagpur mandarin with rangpur lime rootstock is excellent drought tolerant because of their deep and vigorous root system. Rangpur lime, therefore, holds good promise for its commercial utilization as rootstock for future mandarin and sweet orange plantation.

The seeds of Rangpur lime take about 20-40 days to germinate and seedling growth in nursery stage are also very slow and hence it takes longer time near about 18-24 months to attained buddable size. In order to make nursery practice efficient, Rangpur lime seedlings must attain good health, vigour and better size for budding.

In last decade, the large-scale seedling production has been achieved by application of chemical fertilizers without considering the crop requirement, which leads to adverse effect on soil health. Phosphorous and other nutrients like zinc, copper, potassium, sulphurs are important elements, essentially required by plant may be unavailable, due to low mobilization, which effects on the growth of the plant, especially in nursery.

The screening of commercial PGPR strains like PSB and AM for better growth of Rangpur lime are the important alternatives or as the supplement to existing chemical fertilizers.

In the present study, the *Glomus fasciculatum*, *Glomus mosseae,* Phosphate Solubilizing Bacteria (PSB) and neem cake were used to assess the effect on growth promotion of Rangpur lime in nursery. In this study the effect of biofertilizers in alone, five different combinations and then compared with control set in secondary nursery stage. The growth parameters were recorded after 30 days of intervals up to 135 days of transplanting in polythene bags.

MATERIALS AND METHODS

The experiment was conducted under 50% shade net conditions at Department of Horticulture, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during August 2012-13. For the experimental purpose, the uniform, healthy Rangpur lime seedlings were transplanted in polythene bags. There were eleven treatments of inoculums of *Glomus fasciculatum* (50g and 100g), *Glomus mosseae* (50g and 100g), PSB 3 g and neem cake 20g per seedling of Rangpur lime and its combination were given at the time of transplanting of selected Rangpur lime seedlings and replicated thrice. The experiment was laid out in Randomized Block design. The Absolute Growth Rate was calculated based on total dry matter accumulation and period of accumulation in the experimental system.

$$AGR = \frac{W_2 - W_1}{T_2 - T_1}$$

Where, W_2 and W_1 indicate dry matter of the seedling at T_2 and T_1 times, respectively.

Root density of the seedlings was calculated by dipping the roots of the seedlings in 1 lit measuring cylinder. Water displaced by the roots was measured in ml. Total chlorophyll content was estimated by the procedure given by Sadasivam and Manickam (1992).

RESULTS AND DISCUSSION

In present study, the *Glomus fasciculatum*, *Glomus mosseae* phosphate solubilizing bacteria (PSB) and neem cake were used to assess the effect on growth promotion of Rangpur lime in nursery, It is observed that, inoculation of *Gm*-50 g + *Gf*- 50 g+ PSB- 3g and *Gf*- 50 g+ PSB- 3g + Neem cake 20 g enhanced the growth of Rangpur lime seedlings in all respects and reduced the duration of seedling to attain buddable size. The experimental data were analyzed by the standard method for statistical analysis of variance. The standard error for the treatment and critical difference at 5 per cent level of significance was worked out. Table 1 represents the growth of rangpur lime seedlings in terms of height, stem diameter and average growth rate. In general, all the treatments improved the growth of the Rangpur lime seedlings. After135 days of transplanting, the maximum height (58.30 cm) was recorded by *Gm*-50g+ *Gf*-50+PSB-3g/pot followed by *Gf*-50g+ PSB-3g+ NC-20g/pot (54.63 cm) combinations of biofertilizers. The diameter of stem represented in Fig.1.

Table 1. Effect of bio-fertilizers and neem cake on the growth of Rangpur lime seedlings

Treatment	Treatment No.	Height (cm)	Stem diameter (mm)	AGR
Gm-50g /pot	T_1	44.66	0.51	4.47
Gm-100g /pot	T_2	43.33	0.49	4.30
Gf-50g /pot	T_3	47.16	0.56	5.50
Gf-100g /pot	T_4	47.38	0.54	5.30
PSB-3g/pot	T_5	42.00	0.48	2.80
Gm-50g+ *Gf*-50g/pot	T_6	49.83	0.65	6.20
Gm-50g+ *Gf*-50+PSB-3g/pot	T_7	58.30	0.70	9.00
Gm-50g+ PSB-3g+ NC-20g/pot	T_8	51.83	0.66	6.90
Gf-50g+ PSB-3g+ NC-20g/pot	T_9	54.63	0.68	7.60
PSB-3g+NC-20g/pot	T_{10}	48.00	0.59	6.00
Control	T_{11}	35.30	0.31	2.30
SE (m)±		1.50	0.05	0.46
CD at 5%		4.38	0.14	1.42

Increase in seedling height of Rangpur lime might be due to mycorrhizal inoculation. Auxin, cytokine, gibberellins and vitamins are shown to be produced by mycorrhizal fungi (Slankis, 1976). Due to production of gibberellins by mycorrhiza, the increase in height of seedling could be attributed to increase in intermodal length. The increase in cell wall plasticity, their by creates diffusion pressure deficit which

leads to increase water uptake causing cell elongation. In presence of gibberellins large amount of new proteins are syntheses and such newly synthesized protein bring about increase in height and girth of Rangpur lime seedling (Filner & Varner, 1967). It also attributed to the beneficial synthesis of these hormones and growth factors by AM fungi through increasing the cell multiplication and cell division (Azcon & Bago, 1994). These results are in line of Cardoso et al. (1986) in Rangpur lime, Vinayak & Bhagyaraj (1990) and Reddy et al. (1999) in acid lime seeding.

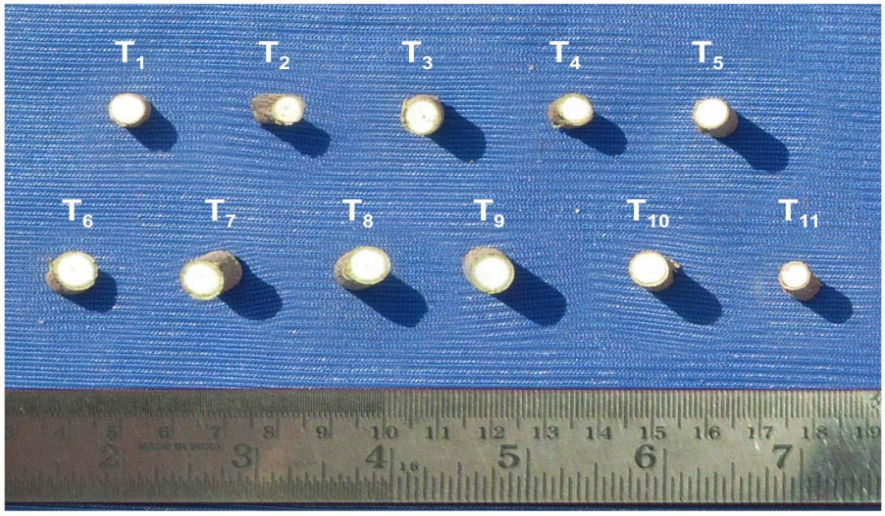

Fig. 1. Stem diameter of the Rangpur lime influenced by the different treatments. The treatment *Gm*-50g+ *Gf*-50+PSB-3g/pot (T_7) showed (0.70 mm) higher diameter of the seedling.

Treatment *Gm*-50g+ *Gf*-50+PSB-3g/pot showed the significantly maximum stem diameter (0.70 cm) over rest of the treatments. Similar findings were reported by Chandrababu & Shanmugam (1983) in citrus, Shaban & Mohsen (2009) in sour orange. This could be attributed due to beneficial effect of microbes present in rhizosphere leading to higher mobilization of solute to the stem (Singh et al. 2000) and Barea (1997).

AGR and leaf area

During 105-135 days after transplanting, maximum AGR (9.0) against (2.30) and leaf area (29.3 cm^2) against (8.6 cm^2) in control were recorded by *Gm*-50g+ *Gf*-50+PSB-3g/pot which was significantly superior at all the treatments except *Gm*-50g+ PSB-3g+ NC-20g/pot, shown in Table 2. These results are in accordance with Reena and Bhagyaraj (1990) in Tamarind and Sharma et *al.* (2009) in citrus seedlings. The increase in AGR and leaf area might be due to significantly higher synthesis of total chlorophyll content as well as higher accumulation of various metabolites (Reducing sugar, total phenols and amino nitrogen) might have resulted from enhance plant growth and biomass production of combine treatment of organic and mycorrhizae (Kohler et al. 2007).

Fresh and Dry weight

The maximum fresh (20.87g) and dry (6.99g) weight of the seedlings were recorded by *Gm*-50g+ *Gf*-50+PSB-3g/pot application. In the present study, the higher fresh and dry weight of shoot and root with mycorrhiza can be correlated with higher all-inclusive growth in corresponding treatment of AMF, this can be stated that increase in overall growth of seedlings led to overall assimilation and redistribution of food material with seedling (Brian et al. 1955). This resulted in higher fresh and dry weight, thus increase is a consequence of increase dry matter accumulation. Similar results also observed in Kagzi lime by Bankar et al. (2009), Dixon (1988) and Manjunath et al. (1983) in *Citrus jambhiri* seedlings.

Table 2. Effect of bio-fertilizers and neem cake on leaf area, fresh and dry weight of Rangpur lime seedlings

Treatment	Leaf area (cm^2)	Fresh weight (g)	Dry weight (g)
Gm-50g /pot	13.50	10.93	3.18
Gm-100g /pot	12.50	9.97	2.90
Gf-50g /pot	17.00	13.70	4.47
Gf-100g /pot	15.00	12.50	3.65
PSB-3g/pot	10.60	9.07	2.74
Gm-50g+ *Gf*-50g/pot	22.50	17.18	5.68
Gm-50g+ *Gf*-50+PSB-3g/pot	29.30	20.87	6.99
Gm-50g+ PSB-3g+ NC-20g/pot	24.00	19.20	6.34
Gf-50g+ PSB-3g+ NC-20g/pot	25.30	20.25	6.70
PSB-3g+NC-20g/pot	21.30	15.84	4.98
Control	8.60	6.67	3.04
SE (m)±	0.15	0.16	0.026
CD at 5%	0.43	0.45	0.077

(Brian et al. 1955) and hence resulted in higher fresh and dry weight, thus increase is a consequence of increase dry matter accumulation. Similar results also observed in Kagzi lime by Bankar et al. (2009), Dixon (1988) and Manjunath et al. (1983) in *Citrus jambhiri* seedlings.

Root growth

Perusal of data in Table 3 revealed that, the maximum root length (42.50 cm), number of fibrous roots (236.33) and root density (101.0 ml) were observed in *Gm*-50g+ *Gf*-50+PSB-3g/pot application which was closely followed by *Gm*-50g+ PSB-3g+ NC-20g/pot and *Gf*-50g+ PSB-3g+ NC-20g/pot as shown in Fig. 2. The results are conformity with those of Onkaraya & Mohandas (1993) in citrus, Panja & Chaudhari (2007) in Darjeeling mandarin and Shaban & Mohasen (2009) in Volkamer lemon. Increase in root growth of seedlings in terms of root length, root density and root weight as observed in present investigation may be attributed to the beneficial synthesis of Auxin like IBA, gibberellins like GA compounds by AMF,

though increasing cell multiplication and cell division leading to overall increase in root growth (Azcon & Bago, 1994).

Table 3. Effect of bio-fertilizers and neem cake on the root growth of Rangpur lime seedlings

Treatment	Root length (cm)	No. of fibrous roots	Root density (ml)
Gm-50g /pot	30.60	107.00	64.55
Gm-100g /pot	28.30	98.33	53.48
Gf-50g /pot	33.60	178.50	79.67
Gf-100g /pot	32.30	112.67	75.50
PSB-3g/pot	26.60	89.67	53.76
Gm-50g+ *Gf*-50g/pot	37.86	183.33	89.00
Gm-50g+ *Gf*-50+PSB-3g/pot	42.50	236.33	101.00
Gm-50g+ PSB-3g+ NC-20g/pot	39.30	210.30	94.51
Gf-50g+ PSB-3g+ NC-20g/pot	41.09	224.00	99.50
PSB-3g+NC-20g/pot	35.60	179.00	86.00
Control	21.30	65.33	40.18
SE (m)±	0.498	9.20	3.30
CD at 5%	1.46	27.05	9.70

Fig. 2. Root development of the Rangpur lime influenced by the different treatments. The treatment *Gm*-50g+ *Gf*-50+PSB-3g/pot (T_7) showed maximum root length, number of fibrous root and root density of the seedling.

Bud-take (%), final survival and chlorophyll content

An appraisal of data in Table 4 revealed that the highest bud-take (95.33%) and final survival (99.17%) of Nagpur mandarin on Rangpur lime were obtained in *Gm*-50g+ *Gf*-50+PSB-3g/pot which is closely followed by *Gf*-50g+ PSB-3g+ Neem Cake-

20g/pot. These results are supported by Barman et al. (2007). It could be attributed to the fact that AMF increased nutrient concentration including phosphorus in citrus (Usha et al. 2004), which increased photosynthetic rate, which might be an indirect effect of RuBP carboxylase activity (Barman et al. 2007).

Chlorophyll content was also significantly higher (2.79 mg/g) in *Gm*-50g+ *Gf*-50+PSB-3g/pot which is closely followed by *Gf*-50g+ PSB-3g+ Neem Cake-20g/pot, while least chlorophyll content was recorded in control (1.15 mg/g). These observations are in accordance with Aseri et al. (2009) in Aonla, Ashokan et al. (2000) in Custard apple and Khan and Hameedunnisa Begum (2007) in acid lime. Increase in chlorophyll content in Rangpur lime seedlings might be due to Neem cake, a source of nitrogen and absorbed nitrogen plays an important role in pigment synthesis.

Table 4. Effect of bio-fertilizers and neem cake on bud-take (%), final survival and chlorophyll content of Rangpur lime seedlings

Treatment	Bud-take (%)	Final survival (%)	Chlorophyll content (mg/g)
Gm-50g /pot	65.83 (54.21)	88.40 (70.09)	1.75
Gm-100g /pot	62.33 (52.12)	87.58 (69.30)	1.55
Gf-50g /pot	72.67 (58.50)	90.42 (71.95)	2.10
Gf-100g /pot	70.90 (57.35)	90.02 (71.76)	1.95
PSB-3g/pot	55.00 (47.87)	85.60 (67.70)	1.35
Gm-50g+ *Gf*-50g/pot	79.00 (62.72)	92.30 (73.89)	2.60
Gm-50g+ *Gf*-50+PSB-3g/pot	95.33 (77.48)	99.17 (84.87)	2.79
Gm-50g+ PSB-3g+ NC-20g/pot	87.20 (69.04)	94.20 (76.06)	2.66
Gf-50g+ PSB-3g+ NC-20g/pot	89.00 (70.63)	98.02 (81.87)	2.73
PSB-3g+NC-20g/pot	77.25 (61.48)	90.82 (72.34)	2.48
Control	45.33 (42.30)	72.58 (58.37)	1.15
SE (m)±	2.33	2.40	0.18
CD at 5%	6.86	7.20	0.53

Over all the result of the experiment indicated that, the growth of Rangpur lime seedlings enhanced significantly by the application of *Gm*-50 g + *Gf*-50 g+ PSB-3g and *Gf*-50 g+ PSB-3g + Neem cake 20 g per pot against rest of the treatments.

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11

EXOPOLYSACCHARIDES BASED BIOFORMULATION FROM *PSEUDOMONAS AERUGINOSA* COMBATING SALINE STRESS

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ABSTRACT

Salt tolerating strain of fluorescent Pseudomonas possessing plant growth promoting activity was screened for the production of exopolysaccharides (EPS). EPS was purified and taken to design talc based bioformulation. The efficacy of the formulation was checked taking sunflower (Hellianthus annuus) as a test crop, under in vitro and in vivo saline conditions (soil irrigated with 125 mM of saline water). Application of EPS bioformulation significantly enhanced the growth attributes of the plant in comparison to control (untreated seeds) under saline and non-saline conditions. Germination rate, plant length, dry weight, seed weight and seed yield increased remarkably in comparison to untreated seeds. The above findings suggest the application and benefits of utilizing talc based EPS formulation in boosting early seedling emergence, enhancing plant growth parameters, increasing seed weight and mitigating stress in saline affected regions. Such bioformulation may enhance root adhering soil (RAS) to root tissue (RT) ratio, texture of the soil, increase porosity, improve uptake of nutrients, and hence may be considered as commercially important formulation for renovation of stressed saline sites along with increasing growth and production of sunflower crop in salinized soil.

Keywords: Bioformulation, *Pseudomonas,* exopolysaccharides sunflower

INTRODUCTION

Oil seed crops, including sunflower (*Helianthus annuus* L.) have served as a backbone of Indian economy since time immemorial, but now-a-days the production is declining due to several abiotic stressors. Sunflower has a great importance all over the world because of its oil bearing seeds but its production is decreasing in different areas where saline toxicity is rapidly increasing (Sadak & Mostafa 2015). The total salt – affected land worldwide is estimated to be 900 million hectares, 6%

of the total global land mass. In India about ten-million hectares of land is suffering from the constraint of salinization stress (Tewari & Arora 2014). The need of the day is sustainable agriculture without harming the delicate balance of soil ecology. Soil borne fluorescent pseudomonads have received particular attention as plant growth promoting rhizobacteria (PGPR) throughout the globe because they produces wide range of metabolites that can perform well under salinized conditions. Metabolites like exopolysaccharides (EPS) produced by fluorescent pseudomonads are instrumental in imparting saline tolerance to plants, but relatively very less attention has been paid on EPS producing pseudomonads and bio formulations developed from them.The aim of the present study was to develop stress tolerating bioformulation utilizing fluorescent pseudomonads for enhancing growth of sunflower crop in salinized soils.

MATERIALS AND METHODS

Pseudomonas aeruginosa PF10 was taken from the Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, India. Strain was monitored for its saline tolerance capability under diverse NaCl concentrations. Effect of salinity on EPS production was recorded according to Titus et al. (1995). Purified EPS was collected and talc based formulation was prepared according to Vidhyasekaran and Muthailan (1995), similarly cell based formulation was also developed and the efficacy of both the formulation's was checked under *in planta* (tube study) and *in vivo* (pot study) under non-saline (soil irrigated with 0 mM NaCl) and saline conditions (soil irrigated with 125 mM NaCl) taking sunflower as test crop. Germination percent was calculated from *in planta* tube study, whereas other plant growth parameters including root length, shoot length, dry weight, root adhering soil/root tissue (RAS/RT) and seed yield were determined from *in vivo* study. For statistical analysis, the data generated during quantitative evaluation of EPS and seed germination or plant growth promotion values were analyzed by means of ANOVA, and means were compared by the Duncans Multiplicity Test Range (DMRT) using the SPSS software (ver. 10.1, SPSS Inc., www.spss.com). The significance level for all analysis was $P = 0.05$.

RESULTS AND DISCUSSION

Pseudomonas aeruginosa PF10 displayed high salt tolerance and EPS production with progressive increase in salinity from 100 to 1800 mM NaCl. There was increase in EPS production recorded by about 27, 39, and 60% at salinity level of 1,000, 1,500, and 1800, respectively in comparison to control (0 mM NaCl). No growth of PF10 was observed beyond 1800 mM NaCl, hence further EPS production was also restricted. Thus, a strong correlation could be observed between EPS production, PF10 population, and salinity tolerance. Increase in EPS production with increase in salinity suggests that under stress condition energy flow of *P. aeruginosa* is directed toward protective mechanism, and synthesis of EPS is opted as a defensive strategy for maintaining its survivability and ameliorating salt stress (Ashraf et al. 2006). Discharge of EPS results in aggregation/flocculation and sheath formation around the cells, which significantly improves survival of bacterial cells under saline stress. *In planta* study showed that seed biopriming with *P. aeruginosa* displayed significant

increase in germination by 25 and 50% in comparison with unprimed seeds under non-saline and saline conditions, respectively. Bioprimed seeds with EPS formulation emerged more rapidly than control (untreated seeds). Biopriming with EPS formulation brought increment in germination by 65 % and 70% in comparison to control under non-saline and saline conditions. *In vivo* study showed that treatment of seeds with EPS formulation brought significant increase in plant growth parameters including root length, shoot length, dry weight, seed yield and RAS/RT ratio both under non-saline and saline conditions, respectively, in comparison to control (untreated seeds). Treatment of seeds with EPS formulation enhanced seed yield by 46.76% and 61% respectively under non - saline and saline conditions, in comparison to control (Table 1). Whereas, cell based bioformulation of *P. aeruginosa* PF10 brought enhancement in seed yield by 33.33% and 35.66% respectively under non-saline and saline conditions in comparison to control. The most conceivable reason for such heightened yield may be due to the fact that introduction of EPS formulation and EPS producing strain brought significant increase in mass of RAS/RT ratio in comparison with uninoculated control as was clearly observed. Increased RAS/RT upsurges adhesion of soil particles, intensify soil aggregation, enhances soil texture, increase water holding capacity of soil and reduce water loss during stress conditions. Enhancement in RAS/RT after applying formulation showed increase in hydrophilization of soil that leads to improved supply of nutrients that is responsible for plant growth promotion. Microbial EPS also assisted in minimizing the effect of salt stress by functioning as an ameliorating agent. EPS formulation when introduced in soil releases EPS that acts as slimy, mucilaginous glue or cementing adhesive and assists in soil aggregation (Alami et al. 2009). Aggregation of soil, influences organic matter storage, soil aeration, water infiltration, and mineral supply to plant thus playing significant role in fertility recapitalization and contributes to plant growth promotion (Kussainova et al. 2013). Hence formulation developed from EPS can go a long way in enhancing sunflower yield in salinity-affected soils and can be involved in reclamation of salinized habitats. Such bioformulation can serve as useful tool for decreasing salinity stress and enhancing the yield of sunflower crop in salt-affected soils.

Table 1: Influence of bioformulation treatment on growth of sunflower plants in under non-saline (control) and saline (*in vivo*) condition

	Non-saline conditions (0 mM NaCl)					**Saline conditions (125 mM NaCl)**				
Treatments	**Root length**	**Shoot length**	**Dry weight**	**Seed yield**	**RAS/ RT**	**Root length**	**Shoot length**	**Dry weight**	**Seed yield**	**RAS/ RT**
Control	08.00±0.01[a]	50.3±0.01[a]	1.51±0.02[a]	10.50±0.02[a]	0.553±0.01[a]	6.31±0.02[a]	45.1±0.03[a]	1.31±0.04[a]	9.00±0.01[a]	0.321±0.02[a]
P.aeruginosa PF10	11.12±0.02[b]	56.7±0.02[b]	1.73±0.03[b]	13.89±0.03[b]	0.661±0.03[b]	10.01±0.03[b]	54.8±0.01[b]	1.46±0.01[b]	12.21±0.02[b]	0.532±0.01[b]
EPS formulation	14.43±0.03[c]	59.4±0.03[c]	1.97±0.04[c]	15.41±0.01[c]	0.798±0.02[c]	14.36±0.01[c]	58.6±0.02[c]	1.89±0.02[c]	14.49±0.03[c]	0.777±0.03[c]

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replications

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PLANT-RHIZOBACTERIA INTERACTIONS MITIGATES DROUGHT STRESS

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ABSTRACT

Drought stress is one of the major agricultural problems limiting crop productivity in most of the arid and semiarid regions of the world. This form of abiotic stress affects the plant–water relations at both the cellular and whole-plant level, causing both specific and non-specific reactions and damage. The conventional approaches such as breeding for stress-tolerant cultivars is a time and labor intensive process. Beneficial plant-microbe interactions are frequent in nature, improving plant nutrition or helping the plant to overcome abiotic and biotic stresses. Inoculation of plants with drought tolerant native plant growth promoting rhizobacteria (PGPR) may increase the drought tolerance of plants growing in arid or semiarid areas. Reports suggest that PGPR mitigate the impact of drought stress on plants through production of hormones like abscisic acid, gibberellic acid, cytokinins, and auxin, production of enzyme 1-aminocyclopropane -1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants, induced systemic tolerance by bacterially-produced compounds, formation of bacterial biofilms containing sugars and oligo-polysaccharides that play important roles in bacteria-plant interactions by improving water availability in root medium. Our investigation on the effect of sunflower seeds inoculated with Pseudomonas sp. Increased the survival, plant biomass, and RAS/RT of sunflower seedlings subjected to drought stress. The inoculated bacteria could efficiently colonized the root adhering soil, rhizoplane, and increase the percentage of stable soil aggregates. At the same time inoculation also increased the compatible solutes and antioxidant status of maize plants under water stress conditions. In a similarly type of study rhizobacteria inoculation changed the elasticity of the root cell membranes which helped the pants to tolerate water deficiency. In recent years, studies have attempted to identify drought stress genes in plants whose expression level were altered upon treatment with the PGPR. These studies indicate that plant-rhizobacteria interactions provides an effectual platform for mitigating drought stress and novel way to improve plant water use efficiency. These new advancements importantly contribute towards solving food security issues in the present scenario of climate change.

INTRODUCTION

Drought stress is one of the major agricultural problems limiting crop productivity in most of the arid and semiarid regions of India (Sandhya et al. 2009). In the context of climate change, the irrigation requirement of arid and semiarid agro-ecosystems are estimated to increase by 10% with every 1^0C rise in temperature (Grover et al. 2011). Drought stress affects plant hormone balance by reducing the endogenous cytokinin level and increasing the levels of abscisic acid (ABA) content in the leaves, thereby eliciting stomatal closure (Figueiredo et al. 2008; Selvakumar et al. 2012). Drought reduces the availability of CO_2 for photosynthesis, which can lead to the formation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals, cause lipid peroxidation of membranes (Sgherri et al. 2000; Mittler & Zilinskas 1994). Drought stress is also known to affect many biochemical activities such as nitrate reductase, which catalyses the rate-limiting step in the nitrate assimilation pathway (Caravaca et al. 2005) and also increases the vulnerability to nutrient losses from the rooting zone through erosion (Gupta 1993). Under drought situations, roots are known to extend their length, increase their surface area, and alter their architecture in an effort to capture less mobile nutrients such as phosphorus (Lynch & Brown 2001). Drought also disrupts root–microbe associations that play a major role in plant nutrient acquisition. Drought stresses also accentuate the biosynthesis of ethylene, which in most cases inhibits plant growth through several mechanisms at molecular level.

Breeding for suitable varieties, improved crop management, and changes in planting dates, etc., to some extent would help in overcoming the effects of climate change (Vanaja et al. 2007). However, evolving low cost methods, which can be easily adopted by small farmers, is a major challenge. Microorganisms are known to enhance the tolerance of plants to abiotic stresses like drought (Timmusk & Wagner 1999), chilling injury (Ait Barka et al. 2006), salinity (Han & Lee 2005), and metal toxicity (Dell'Amico et al. 2008). In one of the study, Timmusk & Wagner (1999) reported that inoculation with the plant growth promoting rhizobacterium (PGPR) *Paenibacillus polymyxa*enhanced the drought tolerance of *Arabidopsis thaliana*. By using RNA display, they concluded that mRNA transcriptions of a drought-response gene, *early response to dehydration 15 (ERD15)*, were augmented in inoculated plants compared to uninoculated controls. Similarly Sandhya et al. (2009) reported that *Pseudomonas putida* GAP-P45 to improve the plant biomass, relative water content, leaf water potential, proline sugars, free amino acids and decreased levels of antioxidant enzymes of maize plants exposed to drought stress.

Based upon the research results, an attempt was made to isolate and characterize ACC deaminase from *Pseudomonas* strains from different crop productions syste of natural habitat as a means to provide the best benefit to drought-stressed plants.

MATERIALS AND METHODS

Isolation

A total of six rhizosphere soil samples were collected from different crop production system covering arid and semiarid regions and used for isolation of *Pseudomonas* spp. using serial dilution spread plate method.

Screening for plant growth promoting traits

All the isolates were tested for plant growth promoting traits like production of ammonia (Dey et al. 2004), siderophore (Schwyn & Neilands 1987), hydrogen cyanide (HCN); (Bakker & Schipper 1987), indole acetic acid (IAA); Gordon & Weber 1951), and for P-solubilization (Mehta & Nautiyal 2001). All the tests were replicated three times at ambient temperature (28°C).

Screening for ACC deaminase activity

The ACC deaminase activity of the isolates was screened (Honma & Shimomura 1978; Penrose & Glick 2003) based on the ability of the respective isolate to use ACC as a sole nitrogen source using DF (Dworkin & Foster 1958) minimal salts medium.

Plant growth promotion studies

The *Pseudomonas* spp. strain PF1-ABF, isolated from maize rhizosphere soil was an efficient ACC deaminase producing isolate and also possessed all of the plant growth promoting traits (Table 1). Therefore, it was selected for plant studies using maize as a model crop. Seeds of maize (var. DHM-117, Maize Research center, ARI, Rajendranagar) were surface sterilized with 0.1% $HgCl_2$ and 70% ethanol, washed with sterile distilled water, and coated with talc based formulation (10^9 cells/g) of PF1-ABF using 1% carboxy methyl cellulose as adhesive. For the control treatment, the seeds were treated with plain talc. The coated seeds were shade dried and sown in plastic cups (surface sterilized) filled with 500g of sterile soil (sterilized for three consecutive days). Both inoculated and uninoculated treatments were replicated six times, and each treatment had one plants per cup. The cups were incubated under greenhouse conditions. The soil moisture was adjusted at 75% of water holding capacity (WHC). Soil moisture was maintained constant during the experiment by daily sprinkling with sterile distilled water. Shoot, root length and dry biomass were determined by harvesting fifteen-days-old seedlings.

Plant biochemical parameters

Fifteen days old seedlings were harvested and the contents of total sugars (Dubois et al. 1956), chlorophyll (Barnes et al. 1992), proline (Bates et al. 1973) and protein (Bradford 1976) content of seedlings was determined.

Molecular characterization of the isolate

For molecular characterization, bacterial genomic DNA was isolated (Chen & Kuo 1993) and the 16SrDNA gene was amplified by PCR using universal forward (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse (5′ AAG GAGGTGATCCAGCCGCA 3′) primers under standard conditions of PCR. The PCR product (~1,500 bp) was purified and sequenced (Xcelries Genomics, Ahmedabad, Gujarat, India). The sequence obtained was compared with the existing database of 16SrDNA gene and submitted to NCBI GenBank.

RESULTS AND DISCUSSIONS

Plants are constantly exposed to abiotic stresses, such drought. The introduction of native ACC deaminase-producing microorganisms in drought-stressed soils can alleviate this stress in crop plants by lowering stress-induced ethylene production. ACC deaminase microorganisms can survive in these habitats and bind to the seed coat or root of developing seedlings, resulting in the deamination of ACC, which is the immediate precursor of ethylene, in plant cells through the production of ACC deaminase (Glick et al. 1998). In present study we isolated and characterized the ACC deaminase producing *Pseudomonas* spp. strain PF1-ABF. A total of five fluorescent *Pseudomonas* spp. grown under different crop production system were isolated. PGPR that have ACC deaminase activity help plants to withstand stress (biotic or abiotic) by reducing the level of the stress hormone ethylene through the activity of enzyme ACC deaminase, which hydrolyzes ACC into α-ketobutyrate and ammonia instead of ethylene (Glick et al. 1998; Arshad et al. 2007). In our study, we screened all the five isolates for ACC deaminase activity and multiple PGP traits. Plant growth promoting (PGP) traits of five isolates was studied. All isolates produced IAA, ammonia whereas variation was observed for siderophore, HCN and P solubilization (Table 1). Isolate PF1-ABF produced maximum amount of IAA (26.4± 0.077 µg/mg protein) production, closely followed by isolate MZ2-ABF (19.2±0.091) and MB1-ABF (12.2±0.071). Amount of P-solubilized was also the maximum in isolate PF1-ABF (41.2±0.12 µg/ml) followed by FTB2-ABF (35.6±0.09). Hydrogen cyanide production was observed in two isolates (PF1-ABF and FTB2-ABF) and siderophore production was only observed in PF1-ABF. In case of ACC deaminase activity we found that one (PF1-ABF) of the five strains screened showed ACC deaminase activity similar to that of different soil bacteria under both non-stress and abiotic stress conditions (Klee et al. 1991; Campbell and Thomson 1996; Hontzeas et al. 2005; Rodríguez-Díaz et al. 2008; Jha et al. 2009; Onofre-Lemus et al. 2009). ACC deaminase-producing bacteria are known to facilitate the growth of a variety of plants, especially under stressful conditions such as flooding, heavy metals, high salt and drought. Isolate PF1-ABF was selected for plant growth studies since it showed the best plant growth promoting traits (Table 1).

Table 1. Plant growth promoting traits of *Pseudomonas* spp.

Isolate	IAA (µg/mg protein)	Phosphate solub-ilization µg/ml	Amm-onia	Sidero-phore	HCN	ACC deam-inase activity
MZ2-ABF	19.2±0.09	21.2±0.22	+	-	-	-
PF1-ABF	26.4± 0.07	41.2±0.12	+	+	+	+
FTB2-ABF	8.22±0.08	35.6±0.19	+	-	+	-
MB1-ABF	12.2±0.07	16.2±0.09	+	-	-	-
MZ7-ABF	4.65±0.06	32.5±0.21	+	-	-	-

IAA, indole acetic acid; HCN, hydrogen cyanide; ACC, 1-aminocyclopropnae-1-carboxylic acid; +, positive; -, negative

The effect of maize seedlings inoculation with *Pseudomonas* spp. strain PF1-ABF was studied under sterile soil conditions. Inoculation improved shoot, root

length, and dry biomass of plant with respect to values of the uninoculated plants which showed lower values (Fig. 1). Dell' Amico et al. (2008) showed that inoculation with cadmium resistant strains *Pseudomonas tolaasi* and *Pseudomonas fluorescens* enabled *Brassica napus* to grow under cadmium stress because of the production of indole acetic acid, siderophores, and ACC deaminase, which protected the plants against cadmium stress and enhanced the shoot and root length of *Brassica napus*. Rhizosphere colonization by the inoculated strain was studied by plating appropriate serial dilutions of rhizosphere soil sample on King's B medium. The inoculated strain colonized the maize rhizosphere as indicated by plate count.

The effect of inoculation on biochemical status of maize plant was studied. Bacterial inoculation significantly enhanced the contents of chlorophyll, total sugars, proline and protein in the shoots of maize seedlings (Table 2).

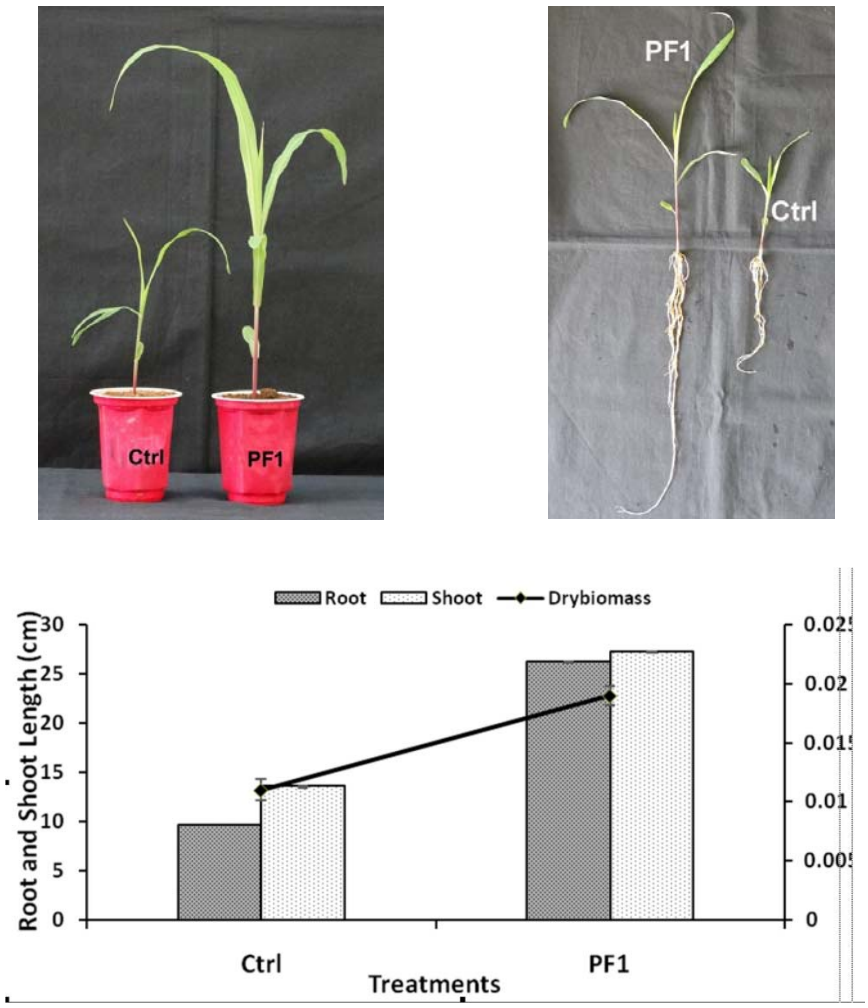

Fig. 1. Effect of *Pseudomonas* spp. strain PF1-ABF inoculation on maize seedlings.

Table 2. Effect of inoculation of *Pseudomonas* spp. strain PF1-ABF on biochemical parameters of maize seedlings.

Isolate	Chlorophyll (mg gFW^{-1})	Total sugars (mg gDW^{-1})	Proline (µmol gFW^{-1})	Total protein (mg gDW^{-1})
PF1-ABF	12.4±0.12	85.2±0.04	6.25±0.21	51.2±0.16
Control	10.9±0.14	76.5±0.09	4.25±0.19	49.6±0.14

In our study inoculation enhanced proline production compared to uninoculated seedlings. Increased proline content in treated seedlings, which may be due to up regulation of proline biosynthesis pathway to keep proline in high levels, which mayhelp in maintaining cell water status, protects membranes and proteins from stress (Yoshiba et al. 1997; Sandhya et al. 2010). In the present study inoculation with strain PF1-ABF also increased soluble sugar content in maize seedlings over than uninoculated seedlings indicating *Pseudomonas* spp. strain PF1-ABF helps in more biosynthesis by degrading the starch content for osmotic adjustment to alleviate stress effect (Sandhya et al. 2010; Deka´nkova´ et al. 2004; Enebak et al. 1997). Soluble sugars are key osmolytes contributing towards osmotic adjustment. The chlorophyll content in the inoculated seedlings was higher as compared to respective control treatments indicating better physiological health of inoculated plants. High chlorophyll content has been linked with drought tolerance in many plants such as pea, maize, wheat (Arunyanark et al. 2008; Zaeifizade and Goliov 2009; Khayatnezhad et al. 2011). Molecular characterization of the strain was done on the basis of 16SrDNA gene sequence that showed 99% homology with that of *P. aeruginosa* strain NCGM257 in the existing database. The sequence was submitted to GenBank under the accession no KC847087.

Our results suggest that the selection and use of native ACC deaminase-producing rhizobacteria with multiple plant growth promoting activities for the facilitation of plant growth in drought environments will be a highly important area for future research. Hence, *Pseudomonas* spp. strain PF1-ABF isolated in the existing study can be further tested under drought stress condition and the mechanism/s behind microorganism induced drought stress tolerance in plant need to be studied at molecular level.

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13

ENDOPHYTIC BACTERIA AS PLANT GROWTH PROMOTERS

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ABSTRACT

There is a growing interest in utilizing endophytic bacteria as plant growth promoting biofertilizers or biological control agents. Beneficial plant–endophyte interactions that promote plant health and development is the subject of study. In order to reduce inputs of pesticides and fertilizers and add value to eco-friendly agriculture, it is important to develop inocula of biofertilizers, stress protection and biocontrol agents. Recent work has investigated the potential microorganisms for the enhanced plant growth. Most of these studies have focused on microorganisms from the rhizosphere/rhizoplane of plants promoting plant growth by free-living rhizobacterial strains, and much still remains to be learned from endophytic bacteria. The bacterial endophytes are believed to enhance the host plant growth and health through mechanisms proposed for plant growth promoting rhizobacteria (PGPR). In comparison with rhizosphere and rhizoplane bacteria, endophytic bacteria are likely to interact more closely with their host plant which live inter and intra-cellularly in plants without inducing pathogenic symptoms. Therefore endophytes could lead to their large scale applications and minimize the risk to the farmers. The objective of this study is to assess,

1. Whether endophytic bacteria stimulate effective plant growth. In the present study endophytic bacteria inside the maize root tissue was isolated. Isolate was screened based on colony characteristic and fluorescence as well as non-fluorescence pigmentation. Bacterial endophyte was tested for plant growth promoting traits as well as biocontrol activity against different plant pathogens and tested for plant growth promotion on maize. The result suggests the possible role of endophytic bacteria in plant growth and protection which may lead to development of microbe based - ready technology.

INTRODUCTION

Endophytic bacteria live in plant tissues without harming or gaining benefit. Bacterial endophytes can be isolated from surface-disinfected plant tissue or

extracted from internal plant tissue (Zinniel et al. 2002). Over the last few years, there is a growing interest in utilizing endophytic bacteria as plant growth promoting biofertilizers or biological control agents (Rosenblueth & Martinez-Romero 2006; Ryan et al. 2008). Many plant species are being tested for the diversity of endophytic bacteria within their tissues (Hallmann & Berg 2006; Rosenblueth & Martinez-Romero 2006; Ryan et al. 2008). Plants are associated with endophytic bacteria and fungi, which live inter and intra cellularly in plants without inducing pathogenic symptoms, while interacting with the host biochemically and genetically. Endophytic microorganisms may function as plant growth and defence promoters by synthesizing phytohormones, enzymes or precursors for secondary plant metabolites, fixing atmospheric nitrogen and CO_2, or controlling plant diseases. As these endophytes are adapted to the presence and metabolism of complex organic molecules, they can show useful properties in plant defence and growth. Being aware of the ecological uniqueness of the rhizosphere, scientists realize the importance of manipulation of rhizosphere microorganisms to improve plant growth and health. One potential way to achieve that goal is the use of bacterial endophytes, bacteria that reside inside the plant roots without causing any apparent damage, to enhance plant growth. Considering the important features of bacterial endophytes, there is increasing interest over the last few years in utilizing these bacteria as plant growth promoters and biological control agents (Rosenblueth & Martinez-Romero 2006; Ryan et al. 2008).

Endophytic bacteria are believed to enhance their host plant growth and health through similar mechanisms proposed for Plant growth promoting rhizobacteria (PGPR) (Vessey 2003). Many of the direct and indirect mechanisms of plant growth have focused on free-living rhizobacterial strains, and much still remains to be learned from endophytic bacteria (Compant et al. 2005). Plant growth-promoting bacteria are either rhizospheric or endophytic and are being exploited for biotechnological applications in the agricultural industry as biofertilizer and/or biopesticide agents to improve plant growth and fitness (Lucy 2004; Banerjee et al. 2006). Both gram-positive and gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species. Furthermore, several different bacterial species have been isolated from a single plant. Endophytes enter plant tissue primarily through the root zone; however, aerial portions of plants, such as flowers, stems, and cotyledons, may also be used for entry (Kobayashi & Palumbo 2000). Specifically, the bacteria enter tissues via germinating radicles, secondary roots, stomata's, or foliar route, stimulate their host plant growth (Compant et al. 2005) or show biocontrol mechanisms and reduced abiotic stresses (Gary & Smith 2005).

MATERIALS AND METHODS

For isolation of endophytic bacteria from different crop plants plant root samples were washed with tap water for removing adhering soil. Roots were surface sterilized using 70% ethanol for 1 min and 2% sodium hypochlorite for 5 min, followed by 5 washings with sterile distilled water (Jasim et al. 2014). The sterilized roots were aseptically cut into 1-2cm sections macerated with 0.8%saline and then decimally diluted spread on Kings B media and Trypticase soya agar media and incubated at

30˚C for 24 hours. The sterility check test was done to ensure that the isolated strains were from the root tissue. Screening of different isolates was based on different colony characters and pigmentation.

The isolates were screened for plant growth promoting (PGP) properties like production of IAA, P-solubilization, and production of HCN, ammonia, and siderophores according to Sandhya et al. (2009). Antagonistic activity was performed using dual culture method (Meera & Balabaskar, 2012). Per cent inhibition over control = {(C-T)/C} x 100 (1).Where, C- l growth of test pathogen in control; T-growth of test pathogen in dual plate.

The endophyte FMR28 isolated from maize root tissue grown in semi-arid region of Anantapur was efficient in plant growth properties and biocontrol activity. Therefore, it was selected for inoculation of maize seedlings. Seeds of Maize (*var*. DHM117) were surface sterilized with 0.1% $HgCl_2$ and 70% ethanol, washed with sterile distilled water, and coated with talc based formulation (10^8 cells/g) using 1% carboxy methyl cellulose as adhesive. For the control treatment, the seeds were treated with plain talc. The coated seeds were shade dried and sown in plastic cups (surface sterilized) filled with 550 g of sterile soil (sterilized for three consecutive days). After 15 days of germination (after 4 days of water stress), the seedlings were harvested and measured for plant growth promotion.

RESULTS AND DISCUSSION

Microscopic studies revealed isolateFMR28 as Gram-negative, motile, having pale yellow, entire, convex, opaque, and mucoid colony morphology. The isolate showed oxidase, catalase and urease activity, and could utilize citrate as carbon source. Based on the biochemical characterization isolate is identified as pseudomonas sp. Further confirmation is needed by molecular analysis. Isolate FMR28 exhibited plant growth promoting properties like P-solubilization, produced of ammonia, siderophore, HCN, and IAA (Table 1).

PGPR have a high potential for application in agriculture because they can improve plant growth through phytohormones (IAA, GA) production, solubilization of mineral phosphate, antagonism of plant pathogens etc.

Table 1. Plant Growth Promoting Properties of Isolate FMR28

Plant growth promoting traits	
Ammonia Production	+
Phosphorous Solubilization	225 (Index)
Siderophores Production	+
Hydrogen cyanide	-
Indole Acetic Acid	219 (±6.07) µg mg−1 protein

Endophytic bacteria are believed to stimulate their host plant growth and development through mechanisms similar to those proposed for PGPR, either directly via increasing the plants ability to acquire nutrients or indirectly by preventing the proliferation of pathogenic microorganisms that suppress plant growth

(Ryan et al. 2008; Glick 1995). The antagonistic potential of the isolate FMR28 was observed against the soil borne fungi under *in vitro* conditions. The results indicate varying degrees of antagonism against different plant pathogenic fungi like *Macrophomina phaseolina; Rhizoctonia solani; Sclerotium rolfsii; Fusarium oxysporum* (Table 2).

Table 2. Percent of Radial Growth Inhibition of Isolate FMR28

Percent of Radial Growth Inhibition	
Macrophominaphaseolina	81.01
Sclerotiumrolfsii	70.23
Fusariumoxysporum	52.12
Rhizoctoniasolani	84.48

The FMR28 showed greater inhibition percentage against *Rhizoctonia solani*. This type of variation in antagonistic potential of isolateFMR28 might be due to their genetic variations, type of root tissue from which it has been isolated. Three bacterial antagonists i.e., *Pseudomonas fluorescens, P. putida and P. aureofaciens* inhibited the mycelial growth of *Rhizoctonia solani* (Lee et al. 1990). *Pseudomonas* strain PS1A12 had a phytohormone like influence on the wheat plants which solubilized phosphates and also inhibited the soil borne root pathogens *Gaeummanomyces graminis* var. *tritici, F. oxsporum* and *F. solani* (Holflich 1992). Similarly *P. fluorescens* was more effective than carbendazim in suppressing the groundnut root rot caused by *Macrophomina phaseolina* (Shanmugham et al. 2002). After 15 days of germination, Inoculation of plant tissue endophyte FMR28 significantly increased total root, shoot length and dry biomass in maize seedlings compared to control. Higher population of bacteria of inoculated plants may have stimulated plant growth (Sandhya et al. 2009, 2010). Growth promotion by the PGPR may be attributed to mechanisms such as production of PGP hormones and other PGP activities (Glick 1995). PGP substances can be helpful in maintaining better nutritional status thus influencing plant-microbe interactions (Sandhya et al. 2010). Bacterial plant growth promotion is achieved by more than one PGP property by the associated bacterium (Yang et al. 2009). In uninoculated seedlings, due to the absence of bacterial population's plant growth effect was less. Moreover, a higher population of strains at roots of inoculated plants may have stimulated growth of inoculated seedlings. Endophytes enter plant tissue through the root zone (Kobayashi & Palumbo 2000). Specifically, the bacteria enter tissues via germinating radicles, secondary roots, stomata's, or foliar route, stimulate their host plant growth (Compant et al. 2005) or show biocontrol mechanisms.

These results show that inoculation of endophytic PGP FMR28 improved plant growth promotion of maize seedlings to a higher level as compared to uninoculated seedlings. By this way a most effective endophyticbacteria can be identified for managing crop yield and crop diseases.

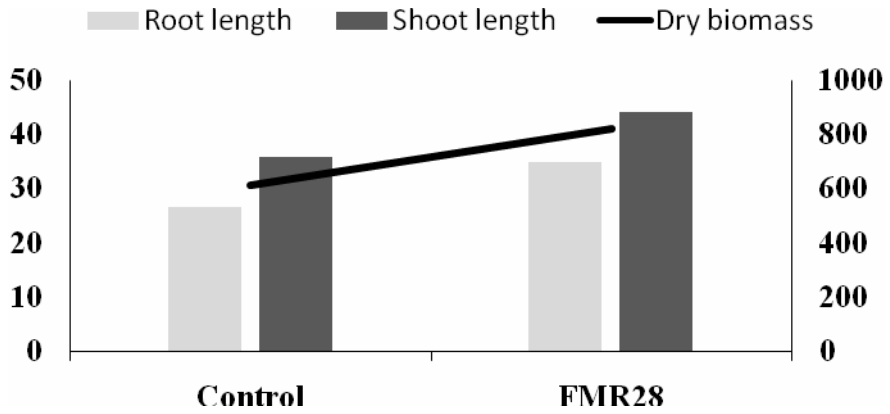

Fig. 1. Plant growth promotion in maize inoculated with root entophyte FMR28

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14

NANONUTRIENTS WITH LACTO-GLUCONATES BASED NUTRITIONAL BIOFERTILIZER'S FOR SUSTAINABLE AGRICULTURE

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ABSTRACT

In context to environmentally benign technology, present study was made for biosynthesis and commercialization of nanonutrients with lactogluconate which were used as nanofertilizer for sustainable agriculture. The present invention of nanonutrients biosynthesis and their uses as nanofertilizer is a green ecofriendly approach to enhance crop production. In order to address issues of low fertilizer use efficiency, imbalanced fertilization, multi-nutrient deficiencies issues and decline of soil organic matter, it is important to evolve a nano-based fertilizer formulation with multiple functions. However, the present biosynthesis and commercialization of nanofertilizer is the new way and first time in globe by Prathista Industries as a fourth generation (4 G) technology.

INTRODUCTION

In current situation, globally uprising in population and rapid urbanization, agronomists and biofertilizer industrialist are left with the critical duty of feeding more people from agricultural fields which are decreasing correspondingly. The world population is expected to be 9.1 billion people by 2050, to meet the requirement total food consumption will have to rise by 50–70% (Keeney, 1997; Jaggard et al. 2010). Fertilizers have an axial role in enhancing the food production in world especially after the introduction of high yielding crop varieties. Investigations show that, a fertilizer contributes to the tune of 35-40% of the productivity of any crop (Pallabi & Dash 2014).

Among these, nanotechnology based biofertilizer's has the potential to revolutionize the agricultural systems and numerous other areas. Nanoparticles are

atomic or molecular aggregates with at least one dimension between 1 and 100 nm, which can drastically modify their physicochemical properties compared to the bulk material. Owing to its high surface area to volume size ratio, exhibit significantly novel and improved physical, chemical, and biological properties, phenomena, and functions (Lengke et al. 2007).

Nanostructured formulation through mechanisms such as targeted delivery or slow/controlled release mechanisms and conditional release, could release their active ingredients in responding to environmental triggers and biological demands more precisely. The use of nanofertilizers can increase in nutrients use efficiency, reduces soil toxicity, minimizes the potential negative effects associated with over dosage and reduces the frequency of the application. Hence, nanotechnology has a high potential for achieving sustainable agriculture, especially in developing countries (Tarafdar et al. 2014).

The objective of this study is giving a brief overview on Prathista Industires 4G technologies based on Nano-nutriets with Lacto-gluconate and its role in sustainability of agriculture. This technology will provide the theoretical backgrounds that are needed for applied agricultural research and practices in this field.

Prathista Industries: A glance

Prathista Industries Limited (www.prathista.com) is a Research and Technology driven company promoted and incorporated by a Young Technocrat - Dr. KVSS Sairam in 1996. Since then, Prathista is in the business of Manufacturing and Marketing of Eco-Friendly Bio Technology products viz., Bulk Drugs, Food Preservatives & Additives, Organic Agri In Puts, Bio-fertilizers, Bio-Pesticides & Bio-control Agents, Botanical Crop Protectors, Animal Health Care (Cattle / Poultry feed supplements) and other value added products based on Carbohydrates which are being produced through Industrial Fermentation Process. Prathista Industries Limited is a largest Industrial Fermentation establishment dealing with various Microbes / Fungus for production of eco-friendly products to cater the needs of Multi segments like Pharma & Food, Animal Health Care and Agro Bio-Technology etc.

Prathista bio-tech innovations

- First Generation (1G)– Cow Manures / Natural farming
- Second Generation (2G)– Nutritional Chemical Fertilizers to enhance agricultural productivity for growing population, Bio Fertilizers & EM
- Third Generation (3G) – Proteino Lacto-gluconate based plant nutrients manufactured through fermentation technology in bio-available form.
- Fourth Generation (4G)- Nanotechnology based biofertilizer.

Fourth generation (4G) innovation

Biofertilizer's based on nanotechnology; it is the matter at nanoscale (1 -100 nm) dimensions. Bio-materials when reduced to the nanoscale show some properties which are different from what they exhibit on a macro scale, enabling unique

applications. Application of plant micronutrient's as a nanoformulation through the smart delivery systems of lacto-gluconates for soil borne and foliar application.

MATERIALS AND METHODS

ICAR (Indian Council for Agricultural Research), a national body under Government of India developed revolutionary nanonutrients technology through biological process for the first time in entire Globe after extensive research both in lab and fields, involving consortium of ICAR institutions and Agricultural universities (ICAR-Jodhpur - Rajasthan, ICAR – Bhopal- MP, Potato Research Institute in HP and Punjab Agricultural University and Indian Agricultural Research Institute, Delhi). Prathista is the first company to commercialize the ICAR Nanonutrients innovative technology.

In order to synthesis of nanonutrients, microorganism was grown over selected nutrient source and provides necessary growth conditions. After the complete growth the biomass was separated. The filtrate was used for isolation of extracellular specific proteins and these were used for nanoparticle synthesis. The selection of microorganism and optimum parameter are specific for synthesis for desired type of nanonutrients.

RESULT AND DISCUSSION

Nanonutrient biosynthesis

Microbial extracellular secreting enzymes are produced which reduce the metal salt of macro or micro scale into nano scale diameter through catalytic effect. Extracellular secretion of enzymes offers the advantage to obtain pure, monodispese nanoparticles, which are free from cellular components, associated with downstream processing (Fig 1). Biosorption and bioreduction were carried out by negative electro kinetic potential of microorganisms enables them to attract the cations and act as trigger of the procedure for biosynthesis of nanoparticles (Fu et al. 2000). These nanoparticles get into plant cells through either stomatal or vascular system which may enhance plant cell metabolic activities that lead to higher crop production. Eichert et al. (2008) suggest that the stomatal pathway is highly capacitive because of its large size exclusion limit and its high transport velocity. Such biologically synthesized, very tiny functional nanoparticles are economically chief, relatively stable, easy downstream processing and environmentally safe as they are encapsulated by fungal protein which is water soluble (Fig. 1).

Commercialization and product development

Prathista has successfully commercialized and develop product according to the ICAR innovation. Prathista incorporated the nano-nutrients with their present 3G lacto-gluconates technology and commercialized different product like NEW SURYAMIN®, AISHWARYA®, BIOZINC®, BIOPHOS®, BIOPOTASH® and MEGACAL®, and in the form of liquids and granules.

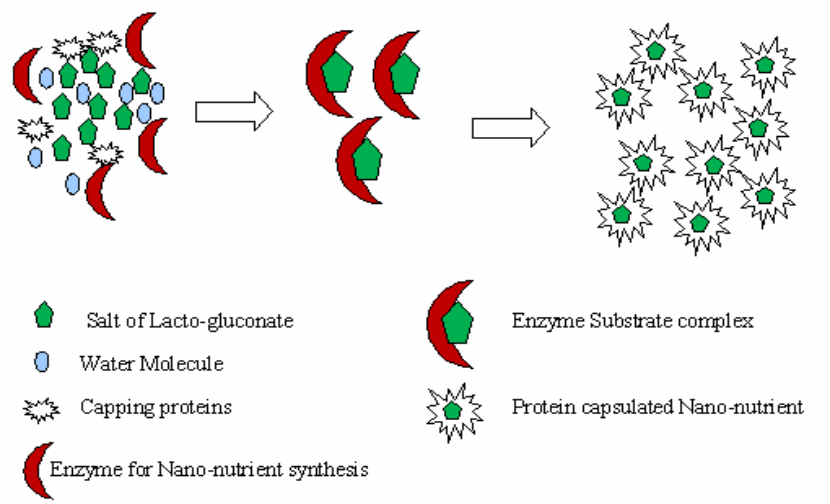

Fig. 1. Diagrammatic representation of Nano-nutrient synthesis

New Suryamin®

It is Prathista's growth enhancer biofertilizer substitute for any soluble fertilizers and is nano protein-lactogluconate with combination of Humic acid / Sea Weed Extracts (Fig. 2).

Aishwarya® (Organic N-P-K)

It is a Prathista's complex biofertilizer fulfils the Nitrogen, Phosphorus and Potassium need (Fig. 3). It is NPK nanonutrients formulation which promotes photosynthesis, development of healthy root system and makes plant more resistant to various climatic conditions.

Fig. 2. Prathista nanotechnology based product New Suryamin® in liquid and granule product

Fig. 3 Prathista nanotechnology based Aishwarya® in liquid and granule

Biozinc®

It is a Prathista's zinc biofertilizer (Fig. 4). In plant systems, zinc plays significant roles in a wide variety of metabolic processes such as carbohydrate, lipid, nucleic acid, and protein synthesis as well as their degradation. Zinc is one of the essential nutrients required for plant growth. Its important role can be adjudged as it controls the synthesis of indole acetic acid, a phytohormone which intensely regulates the plant growth. Besides, it is also necessary for the chlorophyll synthesis and carbohydrate formation (Vitosh 1994). Zinc also enables the plants to withstand lower air temperatures and helps in the biosynthesis of cytochrome, a pigment that maintains the plasma membrane integrity and the synthesis of leaf cuticle.

Biophos®

It is a Prathista's DAP replacement biofertilizer (Fig. 5). In plant system phosphorous is one of the essential required elements, major component of many cell constituents and plays a vital role in several key processes, including photosynthesis, respiration, energy storage and transfer, cell division, membrane synthesis and stability, enzyme activation and inactivation, redox reactions, cellular transporters, cellular signal cascade pathway, nitrogen fixation and cell enlargement (Vance 2011)

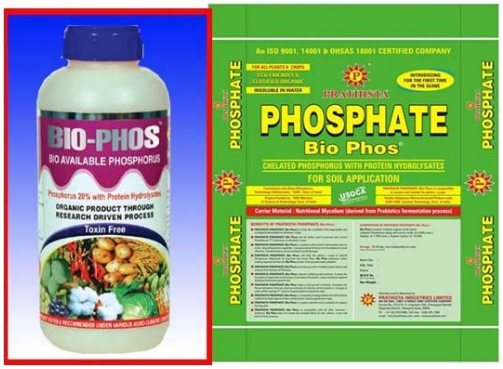

Fig. 4. Prathista nanotechnology based product Biozinc® in liquid and granule

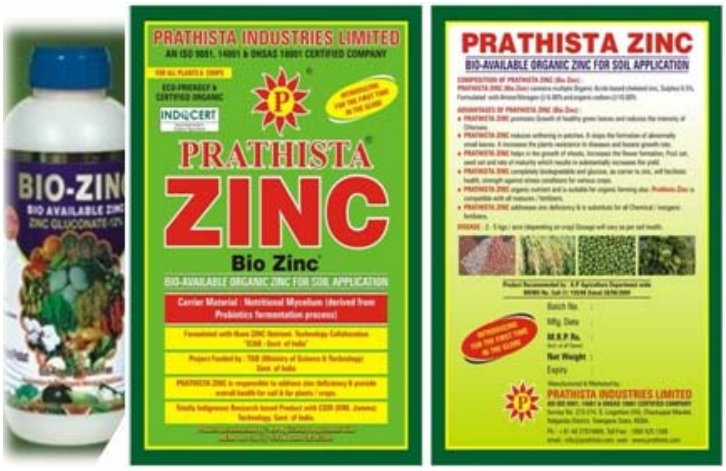

Fig. 5 Prathista nanotechnology based product Biophos® in liquid and granule

Biopotash®

It is a Prathista's murate of potash (MoP) replacement biofertilizer (Fig 6). It is a nanonutrient's of Potash with lactogluconate's. It promotes growth of healthy green leaves and increases plants resistance to diseases and boosts growth rate.

Megacal®

This is Prathista's secondary nutrients biofertilizer substitute for commonly available calcium, magnesium and sulpher based fertilizers (Fig 7). It is a complete plant nutritional product, which contains bio-available nanonutrients of Calcium, Magnesium, Potassium, Zinc, Manganese, ferrous, copper, Boron and all other macro and micronutrients except nitrogen in organic form. This helps size of fruits and gives higher yields.

Fig. 6 Prathista nanotechnology based product Biopotash® in liquid and granule

Fig. 7. Prathista nanotechnology based product Megacal® in liquid and granule

CONCLUSION

In order to have acceptance of ICAR innovation, Prathista industries incorporated the nano-nutrients technology with their present 3G lacto-gluconates technology. The findings reveals that, the nano nutrients doses are just in ppm level to meet nutrient requirement for crops, against to 150 to 200 kgs traditional fertilizer dose per acre. Very interestingly, the cost of these nutrient fertilizers is as par with subsidized fertilizers and computable to use with all traditional fertilizers. Another innovative concept is that, the scalability of technology is commercially and economically feasible and nano-nutrients are 100% safe to human / live stock and 100% eco-friendly. Nanotechnology could provide the much needed trigger for a second green revolution and sustainable agriculture

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IMPORTANCE OF INOCULATION TIME AND METHODS OF THE FUNGAL ANTAGONISTS ON BIO-ENHANCEMENT OF RICE AGAINST THE RICE ROOT-KNOT NEMATODE *MELOIDOGYNEGRAMICOLA*

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ABSTRACT

The use of micro-organisms in controlling plant parasitic nematodes is receiving increasing attention as an important alternative for chemical control and even traditional cultural practices. The efficacy of a biological control agent against plant parasitic nematodes not only depends on the particular endophyte but also on the inoculum density, time and type of application. Studies on the long term biocontrol activity of a non-pathogenic and endophytic Fusarium moniliforme isolate Fe14 against Meloidogyne graminicola have demonstrated a significant reduction in nematode infection when the nematode was introduced to rice plants pre-inoculated with Fe14 four weeks earlier. In the present study, Fe14 and two other endophytic fungal isolates, F. moniliforme Fe1 and F. oxysporum Fo162 and two rhizosphere associated isolates Fusarium F28 and Trichoderma T30 were also tested for their ability to provide early protection of rice seedlings against M. graminicola infection using seed treatment technology. The results showed that the endophytic isolates Fo162, Fe1 and Fe14 did not protect rice seedlings from root-knot nematode infestation while the rhizosphere isolates F28 and Trichoderma T30 slightly reduced galling severity by 7% and 19%, respectively. Growth of rice was slightly affected by fungal applications in the presence or absence of the nematode. Application of the isolate Fe14 either by seed coating and/ or soil drenching gave similar levels of biocontrol. However, it was clearly shown that seed treatment using the endophytic mutualistic Fe14 worked well in biocontrol of the rice root-knot nematode when the plants had reached a more matured stage.

KEY WORDS: endophytic fungi, rhizosphere fungi, seed coating, soil drenching

INTRODUCTION

Biocontrol efficacy is usually inconsistent due to various abiotic and biotic factors and the complex interactions between these two after application. Biocontrol of various plant parasitic nematodes depends on the inoculum density, time and forms of application (Vu, 2005; Dababat & Sikora, 2007). Fungal and bacterial inoculum range from 10^5 to 10^8 colony forming units (cfu) per gram soil (Vu, 2005; Padgham & Sikora, 2007; Dababat & Sikora, 2007; Mendoza & Sikora, 2009). Regarding application, the antagonist can be added by soil drenching, soil incorporation (Hallmann & Sikora, 1994; Vu, 2005; Dababat & Sikora, 2007; Mendoza & Sikora, 2009) or by seed coating (Padgham & Sikora, 2007). With respect to time, the protection of plant at the seedling stage from nematode infection is important for subsequent production and yield because seedlings are generally vulnerable for biotic and abiotic elements. Consequently, many agrochemicals or biological control agents are developed to protect plants in this early stage. Cabanillas & Barker (1989) reported that *Paecilomyces lilacinus* was more effective in protecting tomato against *M. incognita* when it was delivered before transplanting or at transplanting than after plants were infected by nematodes. Similar results were obtained when tomato or banana plants were treated with the mutualistic endophyte *Fusarium oxysporum* Fo162 at transplanting (Vu, 2005; Mendoza et al. 2006; Dababat & Sikora, 2007). In comparison, post-planting application of biocontrol agents, especially in the case of endophytes does not always lead to high levels of biocontrol since the establishment of a biocontrol agent in the endorhiza or the rhizosphere is a prerequisite for the control of endoparasitic nematodes (Vu, 2005; Mendoza et al. 2006; Dababat & Sikora, 2007).

Not all biocontrol agents are however able to protect seedlings, most likely due to lack of rhizosphere competence. Therefore, not only finding the suitable organisms, but also the optimal application form and time is essential for a successful biocontrol approach. The aim of the present study was to study the ability of fungal antagonists to protect plants in the seedling stages and compare soil drenching and seed coating methods with respect to biological control efficacy.

MATERIALS AND METHODS

Fungal and nematode inoculation at sowing

Three endophytic fungal isolates, *F. moniliforme* Fe1 and Fe14, *F. oxysporum* Fo162 and two rhizosphere isolates *Fusarium* F28 and *Trichoderma* T30, which were previously demonstrated biocontrol potential against *Meloidogyne graminicola* and *M. incognita* in tomato and rice (Dababat et al. 2007, Le et al. 2009), were tested for their ability to provide early protection of rice seedlings against *M. graminicola* infection. Rice seeds of the lowland variety BR11 from Bangladesh were first surface sterilized and pre-germinated on wet tissue paper for 3 days.

All fungal isolates were cultured on PDA (Potato Dextrose Agar, Difco) for two weeks to obtain fungal spores. Seed coating method was applied in this study. To do this, the fungal biomass of each isolate was mixed with 2% (w/v) methyl cellulose and then coated to the germinating seeds for 2 hours. Seeds coated with methyl

cellulose alone served as the control. The coated seeds were then sown in experimental pots measuring 7x7x8 cm filled with 250 g autoclaved sandy soil. Additionally, two seeds of each treatment were analyzed for inoculum density obtained by the seed coating approach. The seeds were therefore shaken vigorously in 10ml sterilized tap water and 50 µl of this suspension was plated on PDA using a Jetset spiral plater. This showed a coating efficiency of approximately 10^6cfu per seed. Immediately after sowing, fresh 250 second stage juvenile (J2) of *M. graminicola* per pot were drenched onto the soil (equaling approx. one J2/ g soil). Three weeks after sowing, the rice seedlings were uprooted and carefully rinsed under tap water to remove the adhering soil. Fresh root weight was recorded and the number of galls was counted. The experiment was repeated in the same manner with only two isolates, Fe14 and T30, based on the results obtained in the first experiment.

Drenching versus seed treatment

Control efficacy of Fe14 was evaluated using different forms of fungal inoculation: seed coating, soil drenching or a combination of both. The experiment was designed with 5 treatments, namely: coating (Cg), drenching (D), coating and soil drenching (Cg+D), drenching twice (2xD) and the control treatment (C). All fungal isolates were cultured on PDA for two weeks to obtain fungal inocula. Similar seed coating technique (Cg) was carried out in this experiment. For the soil drenching treatment (D), fungal spores were drenched to the experimental pot at sowing at a dose of approximately $5x10^6$ spores per pot. For the double soil drenching treatment (2xD), the first drench of fungal spores was made at sowing and then repeated three weeks later at a dose of $5x10^6$ spores per seedling each time. For the combination of seed coating and soil drenching (Cg+D), the seeds were coated as described for Cg and three weeks later, additional fungal spores were drenched to the experimental pots at a dose of $5x10^6$ spores per seedling. Four weeks after sowing (i.e. one week after the second fungal application), all rice seedlings were inoculated with *M. graminicola* at a density of 500 J2 per plant. The experiment was harvested 6 weeks after nematode inoculation. Roots were washed and stained with 1% Fuchsine acid and the number of nematodes inside the root was counted under binoculars.

RESULTS

Fungal and nematode inoculation at sowing

Inoculation of rice seeds at sowing with the endophytic isolates Fo162, Fe1 and Fe14 provided no protection from root-knot nematode infestation. The rhizosphere isolates F28 and *Trichoderma* T30 reduced galling severity slightly by 7% and 19% respectively. However, there was no significant difference between treatments (Fig. 1).

Moreover, growth of rice seedling was slightly reduced in treatments with both nematode and fungal inoculations. Fungal inoculation alone did not influence the rice shoot weight and root weight significantly. The height of rice plants was not affected by fungal or nematode inoculation (Table 1).

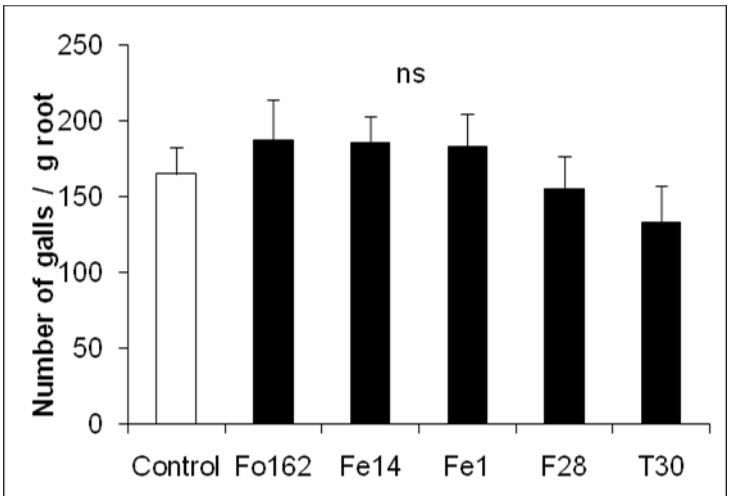

Fig. 1. Effect of the fungal endophytes *F. oxysporum* Fo162, *F. moniliforme* Fe1 and Fe14 and rhizosphere antagonists *Fusarium* F28 and *Trichoderma* T30 on galling severity caused by *M. graminicola* in rice root when the fungi and nematode were applied at sowing. Bars represented standard errors of the mean, ns: not significantly different according to the LSD test (P≤0.05; n=7).

Table 1. Effect of seed treatments with the endophytes *Fusarium oxysporum* Fo162 and *F. moniliforme* isolates Fe1 and Fe14 and the rhizosphere antagonists *Trichoderma* T30 and *Fusarium* F28, and nematode inoculation at sowing on growth of rice 3 weeks after sowing.

Treatment	With *M. graminicola*			Without *M. graminicola*		
	Root weight (g)	Shoot weight (g)	Shoot height (cm)	Root weight (g)	Shoot weight (g)	Shoot height (cm)
Control	0.26 a	0.17 ab	20.5 ab	0.22 a	0.17	20 a
T30	0.27 a	0.19 a	21.5 a	0.21 ab	0.18	21 a
F28	0.22 ab	0.16 ab	20.0 ab	0.24 a	0.16	20 a
Fo162	0.18 bc	0.14 bc	18.0 bc	0.18 b	0.16	20 a
Fe14	0.15 c	0.10 ce	16.0 c	0.20 ab	0.16	20 a
Fe1	0.14 c	0.09 e	16.0 c	0.17 b	0.15	18 b
P-value	0.000	0.000	0.005	0.038	ns	0.001

Means in the same column followed by the same letters are not significantly different according to the LSD test (P ≤ 0.05, n = 7). ns: not significantly different.

In the second experiment, only Fe14 and T30 were repeated for their ability to provide early protection for the rice plant. Seed treatment of Fe14 did not lead to a decrease in galling whereas the *Trichoderma* isolate T30 reduced galling severity significantly when compared with the control and Fe14 treatments (Fig. 2).

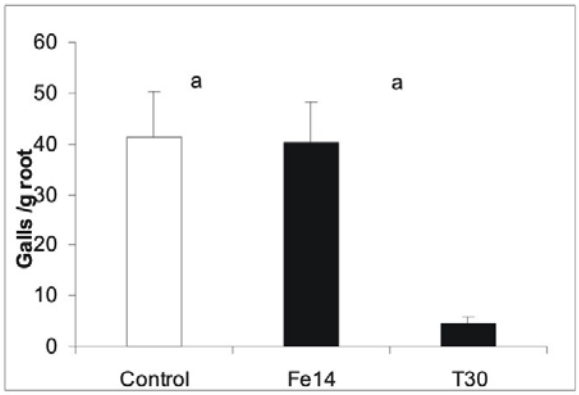

Fig. 2. Effect of the endophyte *Fusarium moniliforme* Fe14 and the rhizosphere *Trichoderma* T30 applied as seed treatment at sowing on *Meloidogyne graminicola* infestation in rice. Means with the same letters are not significantly different based on LSD test ($P \leq 0.05$; n=7). Bars represented standard errors of the mean.

Seed treatment with Fe14 or T30 slightly decreased root weight whereas the shoot weight was similar amongst all treatments (data now shown).

Drenching versus seed treatment

The form of inoculation affected the level of biocontrol toward the rice root-knot nematode. In general, all methods of application reduced nematode infestation significantly by 19-35% when compared to the control treatment. Seed coating or soil drenching alone protected the plants from infection better than when the fungus was applied to rice by a combination of seed coating and soil drenching (Fig. 3).

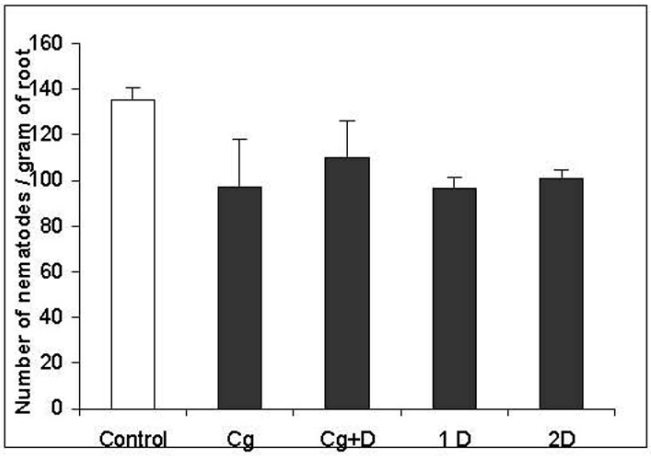

Fig. 3. Effect of application form of the fungal isolate *F. moniliforme* Fe14 as a seed coating (Fe14Cg), coating and soil drenching (Fe14 Cg+D), drenching (Fe14 D), drenching twice (Fe14 2D) and the control (C), on *Meloidogyne graminicola* population densities in rice root. Means with the same letters are not significantly different based on LSD test ($P \leq 0.05$; n=7). Bars represented standard errors of the mean.

DISCUSSION

Fungal and nematode inoculation at sowing

All of the fungal isolates, except the *Trichoderma* T30 did not protect rice seedling from *M. graminicola* infestation when introduced simultaneously at sowing. The slightly higher gall number in the endophytic fungi treated plant was probably due to the low level of the endophytes in the endorhiza when the nematode was penetrating. The results of this experiment are in agreement with that of Vu (2005) and Dababat & Sikora (2007) who demonstrated that the endophytic fungus *F. oxysporum* Fo162 expressed no immediate protection to the plant when exposed to nematode simultaneously at inoculation time. As endophytes, the fungi may need time to establish inside the root tissue and interact with the rice roots before they can express their biocontrol activity.

Inoculation of rice with the fungal isolates F28 and T30 slightly reduced the nematode infestation to the rice root. This could be explained by the fact that these two isolates were rhizosphere fungi whose mode of action is probably different than the endophytic fungi which need time to colonize the root for biocontrol activity. The isolates F28 and T30 may be pathogenic to the J2 or produce antimicrobial substances, which disrupted the host finding of the nematode as previously demonstrated by Windham et al. (1989), Meyer et al.(2001), Sharon et al. (2001) and Dababat and Sikora (2007).

Drenching versus seed treatment

Forms of application are important in biological control. Depending on the antagonists, such as fungi, bacteria or the place where they reside, the forms of application may vary accordingly. In the present study, it was shown that application techniques by seed coating, combined coating and soil drenching or drenching twice gave similar levels of biocontrol.

For biocontrol strategies, seed treatment is regarded as the most economical method for several reasons. First of all, it can provide immediate protection to the seed and germinating seedling, even before the plant is exposed to various biotic and abiotic stresses in the field. Secondly, seed treatment requires small amounts of inoculum and thereby reduces the overall cost of application (Harman, 1991; Sikora et al. 2003; Elzein et al. 2006) provided the shelf life is sufficient. In the present experiment, it was clearly shown that seed treatment using the endophytic mutualistic Fe14 did work in the biocontrol of the rice root-knot nematode.

Similarly, soil drenching is also a common method of biocontrol application in more intensive production systems, such as greenhouses or nurseries. The present study showed that single soil drenching was also an effective method of fungal application. Studies conducted on the soil drenching method using the endophytic *F. oxysporum* Fo162 showed that the technique was adequate to give high level of biocontrol against *Radopholus similis* in banana or against *M. incognita* in tomato if the endophyte has time to establish before being exposed to the nematode in the field (Vu, 2005; Dababat & Sikora, 2007).

Multiple applications of Fe14, either combining seed coating and soil drenching or drenching twice did not significantly increase the overall level of nematode control over that obtained with a single inoculation at sowing. The results demonstrated that the biocontrol activity of endophyte only obtained when it has up to 4 weeks time to colonize the roots before nematode inoculation. Similar results were reported by Dababat & Sikora (2007) when they studied the effect of single or dual applications of the mutualistic endophytic *F. oxysporum* Fo162 for biocontrol of *M. incognita* on tomato. They demonstrated that a single application of the antagonist at sowing was adequate to obtain high level of biocontrol. In comparison, Mendoza & Sikora (2009) suggested a dual application of the nematode egg pathogen *Paecilomyces lilacinus* PL 251 and the endophyte *F. oxysporum* Fo162 was necessary for effective biocontrol of the burrowing nematode *R. similis* on banana. The egg pathogen reduces nematode infection potential in the soil whereas the endophyte affects the infection of the plants. The effect of single or multiple inoculations on biocontrol efficacy is probably dependent on the modes of action of the antagonists. For instance, Fo162, being an endophyte, requires a period of time to establish and express its biocontrol activity inside the host plant whereas PL 251 must be present in the rhizosphere with a substantial amount in order to infect eggs. Therefore, multiple applications can be important when used strategically. In the present study, dual inoculation of only one biocontrol agent, Fe14, did not lead to higher level of biocontrol. Such an approach would then only add to the overall costs of the treatment and therefore is not recommended. The results suggest that seed treatment with our fungal endophyte would be relevant for rice sown in seed beds before transferring to the field. This would give the endophyte time to establish in the endorhizathus being able to exert its biocontrol activity against root knot nematodes.

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RHIZOSPHERE BACTERIA *BACILLUS* STRAINS IN MITIGATION OF BIOTIC AND ABIOTIC STRESSES IN RICE UNDER OXIC AND ANOXIC CONDITIONS

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ABSTRACT

Rice is the single most important staple crop in the world, especially in Asian countries where it accounts for more than 90% (450.6 million tons) of the world production (FAO, 2013). However, rice production systems suffer from a multitude of constraints. The rice root-knot nematode, Meloidogyne gramicola is an important pest in several rice growing areas in Asia while iron toxicity, caused by the excessive ferrous iron (Fe^{2+}) in the soil, is one of the major environmental factors limiting production of lowland rice worldwide. The use of microorganisms to tackle pest and disease problems or nutrient disorders in crop production is not new. However, studies on microbes that have mitigation effects on both abiotic and biotic stresses are not well documented. Furthermore, the rice plant, with its intermittent growth stages under both anoxic and oxic condition requires specific antagonists that can survive and thrive under both conditions.

Several Bacillus strains, isolated from seeds and roots of rice have demonstrated antagonistic activities against the rice root-knot nematode Meloidogyne graminicola. Treatment with Bacillus bacteria under oxic conditions in greenhouse reduced galling severity caused by of M. graminicola by up to 30%. Studies on the modes of action of the isolate Bacillus megaterium against M. graminicola revealed that the bacteria reduced nematode penetration and host finding ability.

When subjected to high iron concentration (Fe^{2+}, 1000 mg/L) in the hydroponic solution, rice seedlings inoculated with Bacillus strains showed lower level of iron toxicity compared to non- treated plants. Application of Bacillus bacteria also reduced leaf Fe content and altered Fe partitioning in plant tissues. The bacterial

isolates clearly showed a differentiated interaction with the individual rice genotypes. The mechanisms of the bacteria and their metabolites alone or in combination with specific rice genotypes that lead to the observed positive effects are being investigated by a series of morphological (growth rate, visual scoring, iron plague) and physiological (phytohormone synthesis, enzyme activities and antioxidant levels) assessments.

Key words: Root-knot nematode, *Meloidogyne graminicola*, iron toxicity, leaf scoring, iron partitioning.

INTRODUCTION

Rice is the single most important staple crop in the world providing food for more than half of the world population. Irrigated and rainfed low land rice are the most common production systems in Asia, covering more than 90% of rice cultivation area while upland rice constitutes 60% of the rice area in Africa (FAOSTAT, 2009).

These rice production systems suffer from a multitude of constraints that are often related to the cropping system as such, climatic, and geographic conditions. Many species of nematodes are associated with rice but only a few are considered as economically important including the rice root-knot nematode *Meloidogyne graminicola* (Bridge et al. 2005) that has been reported to cause significant yield losses of 20-50% in many upland rice production areas. The nematode possesses the capacity to infect, survive, and re-infect the rice root as soils fluctuate between oxic and anoxic states (Bridge et al. 2005). Different management strategies have been used to control the nematode but the degree of success is usually limited and may vary substantially due to site specific and management and resource limitations (Bridge et al. 2005). In the past few decades, biological control has been considered as a promising alternative to expensive and toxic nematicides, limited and inadequate cultural control practices and the lack of resistant varieties (Sikora 1992).

Iron toxicity is a major nutrient disorder of lowland rice which affects about 55% of rice growing areas worldwide. It is frequently reported to cause significant yield loss ranging between 15-30% and can lead to complete crop failures under severe conditions (Becker & Asch 2005). Iron toxicity occurs when rice takes up excess amounts of ferrous iron (Fe^{2+}). This can damage cell components and cell membrane and impair essential physiological processes due to the accelerated production of free radicals (Thongbai & Goodman 2000).

The rice plant has developed several adaptation strategies to cope with and to survive severe iron toxic environments namely 1) exclusion/ avoidance; 2) inclusion/ avoidance and 3) inclusion/ tolerance (Becker & Asch 2005). However, these strategies are dependent on genotypes, plant developmental stages and climatic and geographic conditions. Among the currently available management options, employing ferrous iron-tolerant cultivars is considered the most cost-effective measure but the genotypic mechanisms of iron toxicity tolerance remain unclear up to date (Engel et al. 2012).

The beneficial effects of root-associated bacteria in biologically controlling soil borne pathogens have been well established. Many bacterial species have been evaluated for their antagonistic activity against a wide range of plant parasitic nematodes and their modes of action have been demonstrated: parasitism,

interference with nematode-host recognition, competition for nutrients and induced systemic resistance (Sikora & Hoffmann-Hergarten 1993; Hasky-Günther et al. 1998; Siddiqui & Shaukat 2002). In many cases, antibiotics or the toxic secondary metabolites produced during fermentation processes show nematicidal activity.

Conversely, little is known about how these beneficial microorganisms affect responses of plants to iron stresses as well as their activity under anoxic conditions. During the course of biological studies of the rhizobacteria against *M. graminicola*, the *Bacillus* strains endemic to rice displayed an ability to grow under anaerobic conditions (Padgham & Sikora 2007). This finding, in combination with the current knowledge on the multiple effects of root associated bacteria on plant health (see reviews from Dimpka et al. 2009 and Rejeb et al. 2014) suggested an evaluation to see whether these root-associated bacteria could be used to mitigate the effects of iron toxicity symptoms in lowland rice. The present paper aims to demonstrate the multiple effects of the rhizosphere *Bacillus* strains on the rice root-knot nematode and the tolerance of rice under iron toxicity conditions.

MATERIALS AND METHODS

Biocontrol of Bacillus strains toward M. graminicola under oxic conditions

Four bacterial strains, *Bacillus megaterium*, *B. subtilis* and two isolates of *Bacillus* sp. isolated from seminal roots of Bangladesh and Taiwanese *Oryza sativa indica* lowland rice genotypes were tested for their biocontrol activity against *M. graminicola*.

The rice variety BR11, an irrigated and susceptible rice variety to *M. graminicola* from Bangladesh was used in this experiment.

The *Bacillus* strains were cultured on TSA (Tryptic Soy Agar) for 24 hours. The bacteria were collected by scraping bacterial biomass with a sterile Drisgalski spatula and mixed with 1 ml sterilized methyl cellulose (2%). Surface sterilized seeds (95% ethanol, 30 s and 2.5% NaOCl, 10 min) were coated with the bacterial substrate for 30 min and then planted in experimental pots containing 250 cm^3 sterilized sandy soil (v/v=2:1) with Yoshida nutrient solution at pH 5 as a nutrient source (Yoshida 1976). The average concentration of *Bacillus* strains for inoculation was approximately 10^7cfu per seed, as determined by dilution plating. The seedlings were grown under greenhouse conditions (temperature $27^oC \pm 5$, 15h light period) in the Section of Nematology in Soil Ecosystems, Phytomedicine, INRES, University of Bonn.

Newly hatched second stage juveniles (J2) of *Meloidogyne graminicola* were introduced around the root zone at a density of 500 J2 per seedling at 2 weeks after planting. Ten days later, the experiment was harvested and the number of galls per gram of root was determined.

To investigate the modes of action of the rhizobacteria toward *M. graminicola*, only the isolate *B. megaterium* was selected. The experiment was carried out in the same manner as the first experiment, but the infection with nematodes was evaluated by both galling severity and nematode penetration, which was determined by staining

the rice roots with acid fuchsin 1% and then counting the nematode inside the root using a binocular.

Mitigation of Bacillus strains against iron toxicity

Three *Bacillus* strains (*B. megaterium* and two *Bacillus* strains) were investigated for the interaction with six contrasting rice genotypes ITA 306, ITA 320, TOX 4004 -8-1-2-3, IR 31785-58-1-2-3-3, WITA 7, I Kong Pao under iron toxicity conditions. The genotypes TOX 4004 -8-1-2-3 (in short TOX 4004), IR 31785-58-1-2-3-3 (in short IR 31785) have been reported as tolerant and sensitive cultivars to iron respectively (Engel et al. 2012).

Rice was grown in a hydroponic system consisting of PVC tubes of 3.6cm of diameter and 9 cm length, assembled in a unit of 6x10 by melting the contact-side borders with a soldering iron. These assemblies were placed in a rectangular box measuring 26.5x37cm (6.5 L). In the upper part of each 9 cm long pipe, a half-split ceapren plug (3.5 cm diameter x 3 cm long from Greiner, Germany) was inserted in order to fix each plant individually. The rice roots protruded from the ceapren plug, allowing only the root to be in contact with the nutrient solution.

Rice seeds were pre-germinated on wet tissue paper before planting on washed fine sand for 7 days. The rice seedlings were then transferred to the hydroponic system containing quarter strength Yoshida nutrient solution. Bacteria were applied to the nutrient solution when plants were 3 weeks old as described by Padgham and Sikora (2007). The final concentration of bacteria in the nutrient solutions was approx. $5x10^6$cfu/ml.

Seven days after bacterial inoculation, the nutrient solution was replaced by the respective Fe-treatments (Fe^{2+}, 0 and 1000 mg L^{-1}) for 6 days. Two gas diffusers (3x25cm from Hobby Dohseaquari stik KG) were placed on the bottom of each box. Nitrogen gas was percolated through the nutrient solution for 15 minutes every 2 hours in order to prevent the oxidation of Fe^{2+} to Fe^{3+}.

Iron toxicity assessments

Five days after Fe application, plants were visually scored for the iron toxicity level by assessing the percentage of leaf area affected by bronzing. The leaf scoring ranged from 0 to 10 which 0 means no symptom while 10 means 100% affected, based on the "Standard Evaluation System" for leaf blast (*Pyricularia oryzae*" lesion from IRRI (Engel et al. 2012).

The experiment was harvested after 6 days and leaf tissues were destructively sampled for tissue iron analyses using high pressure acid digestion and AAS (Atomic Absorption Spectroscopy) (Engel et al. 2012).

Statistical analysis

All data were subjected to analysis of variance and mean treatment differences were estimated using a t-test. Mean comparisons were analyzed by Duncan Multiple Range Test when there were more than 2 treatments.

RESULTS

Biocontrol of Bacillus toward M. graminicola under oxic conditions

All four *Bacillus* isolates reduced galling severity caused by *M. graminicola* from 15-30% compared to the non-treated rice roots (Table 1).

Table 1. Effect of *Bacillus* application on root galling of rice seedlings caused by *M. graminicola.*

Treatment	Percent galling reduction (from control)
Bacillus sp. 1	15
Bacillus sp. 2	19
B. megaterium	26
B. pumilus	30

Further investigation revealed that the isolate *B. megaterium* reduced galling severity and nematode penetration significantly by 41% and 45% respectively (Fig. 1).

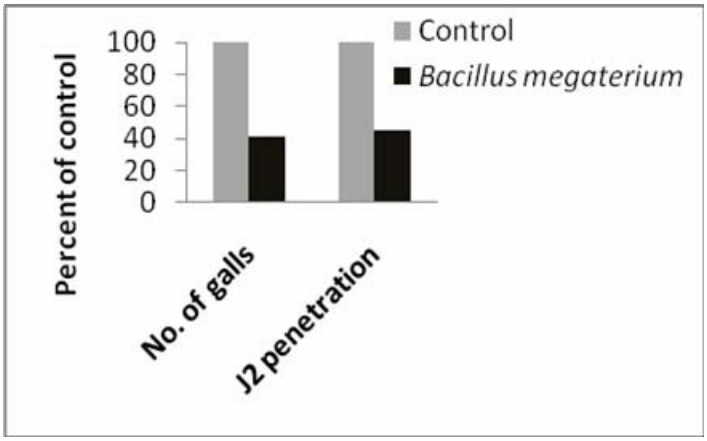

Fig 1. Effect of *Bacillus megaterium* application on root galling and J2 penetration of *Meloidogyne graminicola* in three weeks old rice seedlings.

Mitigation of Bacillus strains against iron toxicity under anoxic condition

On average, three bacterial strains significantly reduced leaf symptom score in all rice varieties. However, total Fe uptake was increased in all bacterial treated plants under induced iron toxic conditions (Fig. 2). There was a clear interaction between some bacteria and rice genotypes in this experiment. For example, Fe uptake increased in all bacterial treatments with two rice genotypes I Kong Pao and IR 31785 while the bacteria *Bacillus* sp. 2 only reduced total Fe uptake in the variety ITA 320 (Fig. 2).

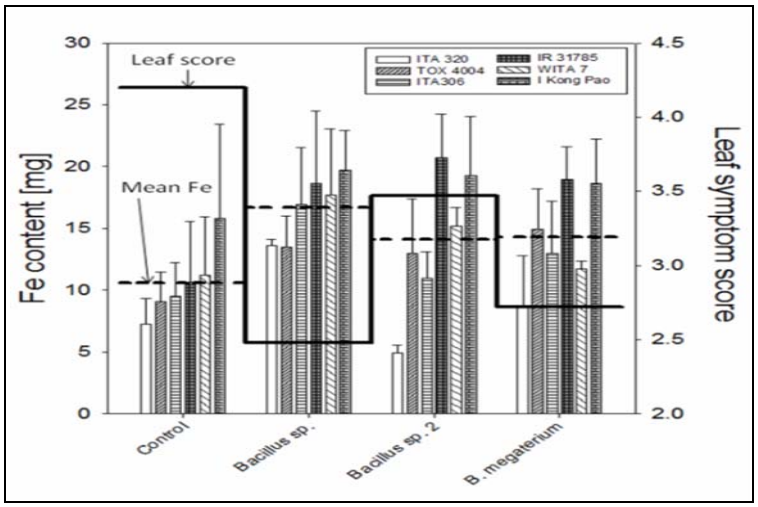

Fig 2. Leaf symptom score and total Fe uptake of 6 rice genotypes subjected to 1000 mg L^{-1} Fe treatment in combination with the application of four rhizobacteria *B. megaterium* and two *Bacillus* strains. The solid line indicates the mean symptoms score and the dotted line indicates the mean tissue Fe content across varieties. Error bars=Standard error of mean with n = 5.

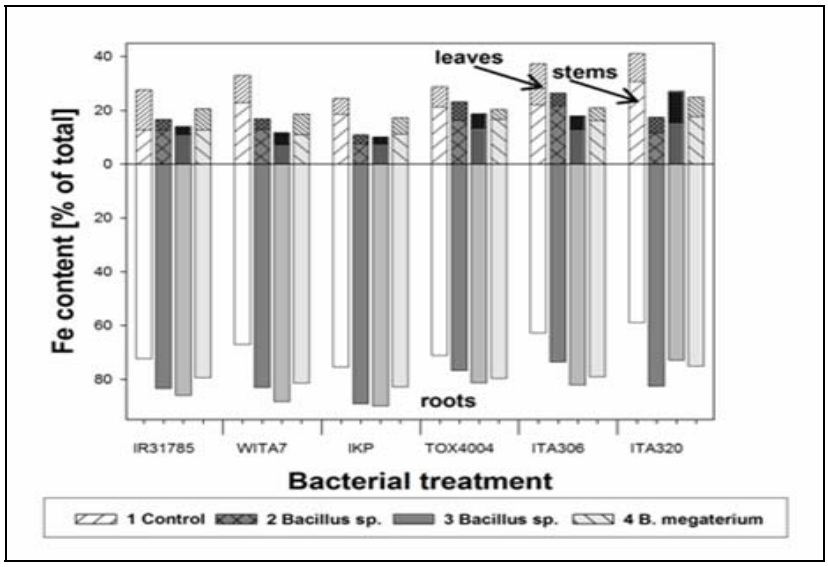

Fig 3. Iron partitioning of rice seedlings subjected to three *Bacillus* treatments under induced iron toxicity conditions (0 and 1000 mg $Fe^{2+}L^{-1}$).

It was shown that the bacterial inoculations strongly altered iron partitioning in rice plant tissues. In general, bacterial applications increased root Fe retention while reduced iron uptake in the upper plant tissues (stem and leaf). Importantly, leaf Fe concentration was reduced in all rice varieties treated with the isolate *B. megaterium* (Fig. 3).

DISCUSSION

Treatment of rice plants with the rhizobacteria *Bacillus* resulted in reduction of galling formation by up to 30% and penetration by 45% compared with non-treated rice roots. The results demonstrated a biological potential of these bacteria against *M. graminicola*. Mode of action studies of the isolate *B. megaterium* revealed its ability to reduce nematode mobility and inhibit egg hatching (Padgham & Sikora 2007). Similar biocontrol activity and modes of action of the rhizobacteria *Bacillus* strains were also demonstrated in Basmati 370 rice cultivar (Pankaj et al. 2010). Moreover, some *Bacillus* strains displayed induced systemic resistance of plant when colonized the rhizoplane (Siddiqui & Shaukat 2002). These studies underline the great biological control potential of the rhizobacteria against the root-knot nematode in rice.

In the present iron toxicity study, all *Bacillus* isolates significantly reduced iron toxicity symptoms, indicating an overall migration effect. The *B. megaterium* strain also reduced leaf Fe content significantly in all cultivars. It has been known that high concentration of Fe (II) in the leaf enhances production of ROS (reactive oxygen species) which leads to irreversible destruction of cell membranes and cell functions and thus adversely affects photosynthesis (Thongbai & Goldman 2000). Therefore, leaf symptom score and leaf Fe content can be combined to identify avoidance mechanism as illustrated by Engel et al. (2012).

Next to reduction in leaf symptom scores, the rhizobacteria also caused changes in total iron uptake and altered iron partitioning. With the two sensitive rice cultivars IR 31785 and I Kong Pao, bacterial colonization resulted in significantly higher Fe uptake compared to the other varieties received the same bacterial treatments. Although iron uptake was increased in all treatments, the symptom of iron toxicity was milder in bacterial treated plants because most of the iron uptake was retained in root tissue, suggesting an inclusion/avoidance mechanism mediated by the bacterial inoculation (Becker & Asch 2005; Engel et al. 2012).

Rhizobacteria, including *Bacillus* species have been frequently reported to promote plant growth, increase water and nutrient uptake as well as enhance plant tolerance or resistance against pests and pathogens. The beneficial effects are enabled through production of secondary metabolites containing phytohormones, antioxidant enzymes and antioxidant molecules, changes in gene expression or induced systemic resistant of the host plant (Glick 2012; Dimpka et al. 2009) suggested that induction of systemic resistance in the host plant may be essential for the cross-tolerance between abiotic and biotic stress.

Similar to plants, iron plays an important role in metabolic activities of bacteria and iron homeostasis must be maintained. Rhizobacteria also produce siderophores that can chelate iron (III) in iron deficient environments or ferritin-like proteins (bacterioferritins, miniferritin and DNA protection) for iron sequestration under iron toxic conditions. In addition, important oxidative stress enzymes such as catalase, superoxide dismutase (SOD), peroxidase (POD) and polyphenol oxidases are found in bacterial metabolites and their roles are believed to be similar to those in plant (Cabiscol et al. 2000). Therefore, research on the physiological, morphological and molecular changes mediated by the rhizobacteria in rice under iron toxic

environment is being undertaken in the Crop Water stress Management in the Tropics and Subtropics (University of Hohenheim, Germany) to understand the mechanisms through which the bacteria positively affect the rice plant. The present research not only demonstrated a potential of using rhizobacteria for the mitigation of both abiotic and biotic stresses in plants but also suggested that these root associated bacteria can be effectively used under anoxic environments.

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17

BOTANICALS: THE GREEN PESTICIDES

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ABSTRACT

Indiscriminate use of pesticides has generated problems, like pest resistance, resurgence of pests, eco toxicity and elimination of natural enemies. These glitches attract the attention of scientific world to develop an ecofriendly and human safe alternative method of pest control. In this regard use of phytochemicals (botanicals), biopesticides and biocontrol agents (natural enemies) offer a good alternative to manage the different pests and diseases in an ecofriendly manner. Over the last four decades there many useful phytochemicals have been identified and isolated with specific and general application to pest control. Of the known about 4,00,000 plant species only about 3,000 plant species have been screened to possess pesticidal potential. Till now more than 500 pesticidal compounds have been identified from various plant species and few have achieved the commercial status worldwide. Present day biopesticide market in the world includes pyrethrins, rotenone, nicotine, ryanodine, sabadilla and azadirachtins. During last few years' plant essential oils comprising mono and sesquiterpenoids are being developed as green pesticides. Some of these oils are well known insect toxins, repellents and deterrents. Currently, ecofriendly pest management strategies are being popular worldwide to enhance crop production while maintaining and contributing to agriculture sustainability. In context the relevancy of ongoing researches on botanicals and their applications, here we have compiled the national and international bibliography for scholars of bioactive compounds.

Keywords: Botanicals, Allelochemicals, Biopesticides, IPM, Phytochemicals

INTRODUCTION

The FAO defines food security as: "When all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life." Globally more than one billion people are undernourished, hungry, and living without adequate daily calories. The people most severely affected by food crises are those already living in poverty. The number of undernourished people in the world was projected

to decline in 2010 as the global economy revived following the 2008 financial crisis, but 16 percent of the population in developing countries remains undernourished (FAO, 2010).

Several workers have attempted to assess crop losses caused by pests and pathogens distributed in India and other countries. Accurate estimates of agricultural losses caused by insects are difficult to obtain because the damage caused by these organisms depends on a number of factors related to environmental conditions, the plant species being cultivated, the socioeconomic conditions of farmers, and the level of technology used. Literature studied for losses in agriculture reveals that food production is hampered greatly by diseases, animals, and weeds which varies crop to crop and country to country according to their habitat and climatic conditions (Savary et al. 2012). Recently Oliveira et al. (2014) observed 7.7% annual losses in Brazil due to insect pests. Crops infected by nematodes especially vegetables such as tomato record yield losses of up to 80 % on heavily infested soils (Kaskavalci, 2007, Singh & Kumar, 2015). Van Berkum & Seshadri (1970) were the first to have calculated monetary losses to crops caused by nematode parasites in India. They estimated annual losses of $10 million on wheat due to "ear cockle disease" caused by *Anguina tritici*, $ 3 million on coffee due to *Pratylenchus coffeae* and $ 8 million due to "Molya disease" caused by *Heterodera avenae* in Rajasthan province alone. Plant- parasitic nematodes cause estimated annual crop losses of $8 billion in the United States and $78 billion worldwide (Barker et al. 1998). Damage caused by plant- parasitic nematodes on 24 vegetable crops in the USA was estimated to be 11% by Feldmesser et al. (1971).

Achieving food sufficiency in a sustainable manner is a major challenge for farmers, agro-industries, researchers and governments. The intensification of agriculture to fulfil food needs has increased the number of insect pest species attacking different crops and as a result the annual production losses of the standing crops. In the past, synthetic pesticides have played a major role in crop protection programmes and have immensely benefited mankind. Due to environmental side effects and health concerns, many synthetic pesticides have been banned. As a result, currently there are only few nematicides left in use, and their limited number makes the repeated applications of the same formulation, inevitable. All the above facts necessitate the urge for new and alternative pest control methods (Chitwood, 2002; Ntalli & Spiroudi, 2012). The need for discovering less toxic and environmentally acceptable substitutes for commercial nematicides is amplified, creating a significant market opportunity for alternative and bioactive products such as botanical nematicides. Plants, as long-lived stationary organisms, must resist attackers over their lifetime, so they produce and exude constituents of the secondary metabolism, playing an important role in their defense mechanisms. Research on biopesticides of plant origin was actively pursued again throughout the second half of the 20th century in order to improve their stability or to discover new molecules and new sources of molecules. The development of pyrethrinoids, synthetic molecules analogous to pyrethrum, and neem products (Meliaceae) are characteristic examples of commercial plant protection products based on botanical sources. Organization for Economic Cooperation and Development (OECD), France defined the botanicals (allelochemicals) as all chemicals involved in species communication (pheromones,

but also plant extracts, plant volatiles, and natural oils) and exhibiting pest control activities. Use of botanicals, biopesticides and biocontrol agents (natural enemies) offer a good alternative to manage the insect pests and diseases in an ecofriendly way. Because, mostly they are- naturally occurring, have high specificity to target pests, no or little adverse effect on beneficial fauna, resistance development to them is slow or less common, have no unknown environmental hazards, less residual activity and are effective against insecticide resistance species of insects. Due to the above reasons the role of biopesticides and bioagents is considered as a potent and reliable tool in Integrated Pest Management Programme (IPM) to manage insect pests. Till now thousands of plants and their parts have been screened for isolation of phytochemicals which are found to be potent against different pests and are in commercial use and formulation (Schillhorn van Veen, 1999; Singh, 1999; Isman, 2008; Ntalli & Caboni, 2012; Singh et al. 2015). Chemically following major isolates are found in botanicals.

Aldehydes and Ketones

Aldehydes like phenols and alcohols induce cytotoxicity, damage the cellular and organelle membranes, act as pro-oxidants on proteins and DNA, and produce reactive oxygen species. These allelochemicals are found to be in rich amount in *Melia azedarach.* Rodriges-Kabana (1993) has reported on the strong nematicidal fumigant activity of furfural against *M. incognita*. Singh (1999) tested aqueous extract of *Melia azedarach* against second stage juvenile of *M. incognita.* Ntalli et al. (2010) observed nematicidal potential of carboxylic acids and aldehydes obtained from *Melia azedarach* fruits.

Maximum number of pesticidal plants belongs to family Meliaceae. Among this neem, *Azadirachta indica* has been found to be promising. Neem based pesticides are marketed in India in different trade names containing different concentrations of azadirachtin. Some of them are Ozoneem Trishul, Margocide OK, Godrej Achook, Nimbicidine, Bioneem, Neemark, Neem gold, Neemax, Rakshak, Econeem, Limnool and Repelin containing 300ppm of azadirachtin. Besides neem seed kernel extract (NSKE) 5%, neem leaf extract, neem cake powder is also used for pest and nematode control. In addition to neem products currently efforts are being made to develop phytochemicals based pesticides from annonin (*Annona reticulata* L.), citrus limnoids (*Citrus* spp.), Karanj (*Pongamia pinnata*) and Mahua (*Madhuca latifolia*).

Almost all parts of neem tree, viz., leaf, drupes, bark and seed contain a pool of biologically active constituents, including the triterpenoids azadirachtin, salanin and meliantriol (Schmutterer et al. 1983; Javad et al. 2006; Ntalli et al. 2009). These compounds give protection against more than 100 species of insects, mites and nematodes including economically important pests like desert and migratory locusts, rice and maize borers, plant hoppers of rice, pulse beetle and rice weevil, root knot and reniform nematodes, and citrus red mite (Kareem et al. 1989; Akhtar, 2000; Orisajo et al. 2007).

Pyrethrum

Pyrethrum is extracted from the flowers of *Chrysanthemum cinerariaefolium*, two formulations of Pyrethrum i.e. Pyrethrum 0.2% dust and Pyrethrum 1% EC are registered for use against insect pests in vegetables and Pyrethrum is also used in combination with other insecticides as synergists for the control of household pests. Eyal et al. 2006 screened the aqueous extract of *Chrysanthemum coronarium* against root knot nematode and found the nematicidal potential. Pérez et al. (2003) confirmed nematicidal efficacy of essential oils of *C. cinerariaefolium in-vitro*. Chatterjee et al. (1982), Chandravadana et al. (1994), Sangwan et al. (1985) and Soler-Serratosa et al. (1996) also identified nematicidal potential in essential oils extracted from several plants including *Chrysanthemum cinerariaefolium.*

Alkaloids

Alkaloids are secondary metabolites of plants containing nitrogen atoms and are derived from various botanical families among which is Solacaneae. 2,5-Dihydroxy-methyl-3,4-dihydroxypyrrolidine is a pyrrolidine alkaloid contained in the genera *Loncocarpus* and *Derris*, exhibiting nematicidal activity. It is found to move downwardly in plant phloem, and its applications on plant foliar appendages decrease galling in roots (Chitwood, 2003). Thoden et al. (2009), Thoden & Boppre (2010) and Odeyemi & Adewale (2011) found sustainable tools in the alkaloids obtained from *Chromolaena odorata, Ageratum houstonianum, Borago officinalis* and *Crotalaria, Senecio bicolor* and *Symphytum officinalis* in reducing the population of plant parasitic nematodes.

Glycosides

Glycosides are derived from amino acids present in different plants. These metabolites play an important role in plant defense as these release of toxic hydrogen cyanide. Glycosides facilitate a strong nematicidal properties, through the action of cyanide (Zagrobelny et al. 2004; Bjarnholt, 2008). Cyanogenic plants have potential as nematicidal green manure (Widmer & Abawi, 2000). Magalhaes et al. (2000) studied the biochemical basis of toxicity of cassava root against insects and nematodes. Soybean greenhouse experiments were conducted by Soler-Serratosa et al. (1996) to evaluate the nematicidal activity of thymol in combination with benzaldehyde against *M. arenaria*. Recently Du et al. (2011) studied the effect of ethanol extract of *Arisaema erubescens* (Wall.) for nematicidal activity against the root-knot nematode (*M. incognita*) as this extract was containing flavone-C-glycosides, namely, schaftoside and isoschaftoside.

Glucosinolates and Isothiocyanates

Glucosinolates metabolites are produced by mustards (*Brassica* and *Sinapis* sp.) and some another genera. They contain sulfur and nitrogen and β-D-thioglucose as well as sulphonated oxime moieties. These include thioglucosides, characterized by a side chain with varying aliphatic, aromatic and heteroaromatic carbon skeletons. Glucosinolates get converted into various degradation products (isothiocyanates, thiocyanates, and indoles) after cutting or chewing of the plant parts that contain

them because through this process, they come in contact with the vacuolar enzyme myrosinase (Mari et al. 2008). Biofumigation is a practice by which nematicidal isothiocyanates (ITCs) are released in soil after incorporating glucosinolate-containing plant material. This practice is considered an ecological substitution of the soil fumigation with toxic fumigants such as methylbromide because these substances are fully biodegradable and less toxic (Vig et al. 2009; Schlaeppi et al. 2010). Henderson et al. (2009) were found mustard bio fumigation as an ecofriendly option to control plant parasitic nematodes.

Limonoids, Quassinoids, and Saponins

Limonoids are metabolically altered triterpenes and have a prototypical structure either containing or derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton. About 300 limonoids are known, one-third of which (meliacins) is obtained from Meliaceae species (*Azadirachta indica* and *Melia azedarach*) corresponding to structurally rather complex substances (Roy & Saraf, 2009). Azadirachtin, a tetran or triterpenoid limonoid found in the Indian Neem tree (*Azadirachta indica* L., Meliaceae), is used for the production of a wide range of commercial formulations registered for nematode control (Javed et al. 2007& El-Din et al. 2012). In India, neem (*Azadirachta indica*) extracts have been introduced in pest management as a part of traditional practice for many years. It is of high importance that limonoids do not have direct negative effects on beneficial insects and for this reason, they can be used in integrated pest management. Azadirachtin is classified as highly toxic to insects and mildly toxic to nontoxic to mammals due to the ability of mammalian cells to remove azadirachtin from the body (Sengottayan & Sehoon, 2006; Morgan, 2009).

Organic Acids

Organic acids are saturated or unsaturated fatty acids, with medium to long esterified carbon chains, and esters of fatty acids with high molecular weight. They develop toxicity against insects by inhalation and contact, suffocating by forming an impermeable film upon the cuticle. Some organic acids penetrate through the cuticle, disrupt the cellular membrane, and uncouple oxidative phosphorylation (Regnault-Roger & Philogène, 2008). Ntalli et al. (2011) reported organic acids also act as nematicidals. *Lantana camara* Linn. var. aculeata is a poisonous plant, containing among others 11-oxo triterpenic acid. This compound was found to be active against root knot nematode *M. incognita*. They have isolated seven components from the aerial parts of *L. camara*, namely, pomolic acid, lantanolic acid, lantoic acid, camarin, lantacin, camarinin and ursolic acid, were effective against the root-knot nematode *M. incognita*. Moreover, Barbarosa et al. (1999) isolated the nonessential amino acid L-3,4-dihydroxvphenylalanine (L-Dopa) from the seeds of *Mucuna* spp and found the nematicidal properties against the phytonematodes *M. incognita* and *H. glycines*.

Phenolics, Flavonoids, and Quinones

Wuyts et al. (2006) evaluated broad spectrum effects of phenylpropanoids on the behavior of *M. incognita*. The nematicidal activity of phenolics from root leachates

of a tropical weed, *L. camara*, used in combination with the plant growth-promoting Rhizobacterium *Pseudomonas aeruginosa* against *M. javanica*, significantly reduced root-knot infection, and enhanced plant growth. The root leachate of *L. camara* was found to contain phenolic compounds, including p-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, and a quercetinglycoside, 7-glucoside (Shazaukat et al. 2003; Simmonds, 2003). Organic extract of the branches of *Magnolia tripetala* showed nematicidal behaviour against *Bursaphelenchus xylophilus*,*Panagrellus redivivus*, and *Caenorhabditis elegans*. Two nematicidal phenolic compounds magnolol and honokiol were isolated from the extract based on bioassay-guided fractionation (Li et al. 2009).

Piperamides

The secondary metabolites produced by *Piper* are called piperamides among which capsaicin is obtained from the genus *Capsicum*, such as chili peppers (*Capsicum frutescens*, Mill.). Djian-Caporalino et al. (2007) and Neves et al. (2009) reported strong nematicidal properties in allelochemicals isolated from pepper plants. As an emerging biocide, very little data are available on the environmental fate of capsaicin, but initial assessment suggests that it will bind to sediments.

Polyacetylenes and Polythienyls

Polyacetylenes and polythienyls are bioactive compounds. These have been isolated from *Tagetes* (marigold) species. Several workers confirmed that the botanicals obtained Tagetes are highly effective against plant parasitic nematodes and insects pests (Wat et al. 1981; Adekunle, 2011). The response of *T. patula* L. against rot knot nematode was studied in order to determine the races and virulence groups of different *Meloidogyne* species and the resistance of marigold characterized (Piedra Buena et al. 2008). The antagonistic effect of the nematode suppressive crops is carried over to reduce the infestation of plant-parasitic nematodes by crop rotating marigold with other ornamental plants or rotating nematode-suppressive cover crops, such as sunn hemp, with field or cash crops may strongly suppress plant-parasitic nematode populations and benefit the following crop (Wang et al. 2007).

Terpenes

Terpenes are natural, volatile compounds with a strong odor, present in aromatic plants. They are isolated by hydrodistillation. Terpenes are formed structurally by coupling different numbers of isoprene units (5-carbon-base; C5), and they may or may not contain oxygen (terpenoids and terpenes). Several factors can affect the chemical composition, toxicity, and bioactivity of the extracts such as the phenological age of the plant, percent humidity of the harvested material, and the method of extraction (Lahlou, 2004). Terpenes are known for insecticidal, antifeedant, repellent, oviposition deterrent, growth regulatory, and antivector activities, along with their wide availability from the flavor and fragrance industries, can make possible the commercialization of essential oil-based pesticides, particularly for organic food production (Mohan et al. 2011). Essential oils have been mostly studied for their activities against agricultural pests like insects, fungi and

nematodes. Essential oils of *Carum carvi, Foeniculum vulgare, Mentha rotundifolia, Origanum, Coridothymus capitatus* and *Mentha spicata* showed nematicidal potentials against plant parasitic nematodes (Isman, 2000; Oka et al. 2000). Pandey et al. (2000) confirmed nematicidal potentials in essential oils extracted from *Eucalyptus citriodora, Eucalyptus hybrida*, *Ocimum basilicum, Pelargonium graveolens*, *Cymbopogon martinii, Mentha arvensis, Mentha piperita,* and *Mentha spicata* against root parasitic nematodes.

CONCLUSION

In conclusion, the above discussion suggests that the bioactive allelochemicals assayed from different plants and their parts have been characterized for biocidal properties against different pests and pathogens. Over a four decade of researches and findings are still awaiting a novel botanical pesticide which will replace the chemical pesticide from every nuk of agriculture sector. Still some new researches are required to understand the molecular behaviour isolated compounds and their interaction with host cells. But the efforts made by science and society for awareness of hazardous effect of synthetic pesticides, discourage and eliminations of such chemicals are pleasing.

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18

CHEMICAL PROPERTIES OF LOCALLY COMPOSTS PRODUCED IN SAUDI ARABIA AND THE NEED FOR REGULATIONS

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ABSTRACT

Most of the soils in Saudi Arabia, located in aired and semiarid region of Asia, it has a very low amount of organic matter. To improve the organic matter in these soils, organic fertilizers are used, which ameliorate the chemical, physical and microbiological characteristics of these soils. Application of compost, as a rich source of organic nutrition, is favorable. With regard to the importance of this issue; our research was aimed to evaluate the quality and stability of compost locally produced in Saudi Arabia, in the term of chemical characteristics included electrical conductivity (EC), pH, C/N ratio, nitrate and ammonium concentrations, organic matter (OM) and heavy metals levels. The results showed variation in the chemical characteristics of all types of compost under study. Final EC values ranged from 0.6 dSm^{-1} to 25.4 dSm^{-1} and about 93% exceeded the upper limit set by CCQC and PAS-100. Most pH values were above 7. Ammonium contents were between 178 mg kg^{-1} and 2650 mg kg^{-1} in the final product with 44.4% above the recommended level, while nitrate contents were between 69.7 mg kg^{-1} and 1157.8 mg kg^{-1} with 22.2% above the recommended. Concentrations of Zn, Cu, Co, and Pb were in the accepted range. However, Cd and Ni concentrations were exceeded the suggested limit in about 42.8% and 33.3% respectively.

INTRODUCTION

Soils in Saudi Arabia have two major problems, low fertility and inadequate water retention. Wind erosion, water erosion, drought, loss of irrigation water and plant nutrients are also expected (El-Hady et al. 2002). Many studies have been conducted for improving the conductivity of sandy soils using synthetic and natural soil fertilizers. Composting is a natural microbiological process of organic matter to stable compost by aerobic action of microorganisms (Adegunloye et al. 2007; Briancesco et al. 2008). This process has the potential of managing organic material in the waste stream such as leaves, farm wastes, cow manure, chicken manure and

plant residues. It provides benefits for soil biological activity; nutritive to plant growth; remediates contaminated soil and suppresses some of the soil borne disease (Straatsma et al. 1994; Baberis & Nappi, 1996; Benito et al. 2003; Herwijnen et al. 2008; Chitravadivu et al. 2009; Hartley et al. 2009). The compost must comply with certain national and international standards and quality grading (Anonymous, 2002; Keeling et al. 2003; Alvarenga et al. 2007). The qualities of the compost include physical, chemical and biological properties such as moisture content, odor, carbon and nitrogen contents, phytotoxic substances, harmful elements, nutrient contents, plant pathogens and effectiveness to plant growth and soil amendment (Harada et al. 1993). As composting is essentially a microbial process, the chemical composition of waste materials define microbial activity and therefore compost quality. Quality control during compost production should insure adequate chemical and physical properties (Inbar et al. 1993), as well as an adequate degree of stability and maturity (Benito et al. 2003). The beneficial effects on crop production and soil quality are directly related to the physical, chemical and biological properties of the compost (He et al. 1995). The aim of this study was to evaluate the main chemical characteristics, as paper part two of locally produced compost in Saudi Arabia and comparing it with the local and international standards of compost quality such as CCQC (2002), PAS100-2005, in order to assess quality and maturity.

MATERIALS AND METHODS

Preparation of compost samples

Different commercial compost samples produced in Saudi Arabia were obtained during summer of 2011 from the facilities in bags of 20-50L. They were identified, registered and a code system was used instead of brand names to ensure confidentiality (Table 1). Composts were immediately brought to the laboratory of soil analysis at college of Agriculture and Veterinary Medicine, Qassim University.

Compost samples were prepared according to the method described by Al-Turki (2010)

Table 1. Code system for different kind of compost locally produced in Saudi Arabia

Type of compost	Compost code
Chicken manure	A, B, C, D, E, F, G, H and I
Cow manure	J, K, L, M, N, O and P
Mixed manure (Chicken manure +cow manure + plant residues)	Q, R, S, T, U, V, W, X and Y

Chemical properties of composts

Compost pH

Compost pH was measured in distilled water using 10g of compost sample and 50 ml distilled water (ratio 1: 5). The suspension was agitated for 3-5 min and placed for 1 hour before measuring the pH value using pH meter.

Electrical conductivity

Compost electrical conductivity (EC) of the compost samples were determined using Electrical conductivity Meter according to TMECC method 04-10-A.

Organic matter

The composting samples were analyzed for organic matter (OM) according to the method of Wakely and Black which described by Nelson & Sommer (1996). Organic matter content was determined by the method of $K_2Cr_2O_7$-H_2SO_4 oxidation. 0.1 g compost was mixed with $K_2Cr_2O_7$-H_2SO_4 solution and heated at 180°C. The solution was kept boiling for 5 min. After cooling, the residual $K_2Cr_2O_4$ was titrated by $FeSO_4$ standard solution using O-phenenthroline hydrate as indicator. Organic matter consumed was calculated based on the amount of $K_2Cr_2O_7$ consumed.

Total nitrogen

Total nitrogen was determined using the micro-Kjeldahl distillation method according to Chapman & Partt (1961).

Ammonium and nitrate nitrogen (NO_3^- and NH_4^+)

Ammonium nitrogen and nitrate nitrogen were determined using micro-Kjeldahl distillation method. NO_3^- and NH_4^+ were determined according to the method of AOAC, 1990.

Total phosphorus

Total phosphorus of compost samples were determined according to the method of Olsen & Dean (1965).

Total potassium

Total potassium of compost samples was determined according to the method of Chapman & Partt (1961).

Heavy metals determination

The dried and ground samples were digested by HNO_3 and $HCLO_4$ (5:1 ratio v/v) to determine heavy metals concentrations in the compost, using AAS (Shimazu 6200 Atomic Absorption Spectrophotometer) according to Chapman & Pratt, 1961. A 1.000 ± 0.01 g sample was digested after adding 36 mL of acid mixture solution. Pb, Ni, Zn, Cd and Cu were determined in the resulting solution using Shimazu 6200 Atomic Absorption Spectrophotometer.

RESULT AND DISCUSSION

A number of parameters can significantly affect compost chemical properties. They include the source and nature of the raw materials or feedstock, pre-treatment, and

the composting method (He et al. 1992). Moreover, the chemical composition of the compost will vary widely with the variation in raw input.

Electrical conductivity (EC)

Large variations were observed in initial and final EC values of compost samples. Initial values of EC ranged from 0.6 dSm^{-1} (M and N facilities) to 22.5 dSm^{-1} (H facility), while the final EC values were between 0.6 dSm^{-1} (N facility) and 25.4 dSm^{-1} (F facility) Table (2). Generally, compost made from chicken manure or compost made from cow manure composts recorded a higher EC values (with mean of 13.8 dSm^{-1} and 11.7 dSm^{-1} respectively) compared with those of compost made from mixed organic materials (with mean of 7.4 dSm^{-1}). Chicken or cow manure usually contains more soluble salts which depend on animal food. Mixed organic materials contain plant residuals that contain low amount of soluble salts.

When we compared between means of initial and final EC values of composts taken from facilities in three different seasons, significant differences ($p < 0.01$) between those EC means were observed in some facilities such as C, J, Q, T, U, V, W, X, Y (Table 2). This finding indicates real variations in EC values of composts produced in these facilities and thus EC values of composts must measured each season.

Table 2. Electrical conductivity (EC) of different compost samples

Compost Facility code	Type of raw Materials	Initial value of EC	Final value of EC	F- test season
A	Chicken Manure	15.3	14.1	Ns
B		15.6	9.1	Ns
C		9.4	12.4	*
D		4.9	12.1	Ns
E		14.4	7.6	Ns
F		17.3	25.4	Ns
G		15.0	15.0	Ns
H		22.5	12.5	Ns
I		10.1	12.4	Ns
Mean		13.8	13.4	-
J	Cow manure	10.9	12.2	**
K		14.4	11.6	Ns
L		1.4	17.6	Ns
M		0.6	4.9	Ns
N		0.6	0.6	Ns
O		20.8	11.9	Ns
P		13.4	23.3	Ns
Mean		8.9	11.7	-

Q		14.8	12.8	*
R		2.6	8.5	Ns
S		17.5	2.7	Ns
T		8.9	9.0	*
U	Mixed organic materials	13.3	12.9	**
V		7.4	7.0	**
W		3.7	6.6	**
X		2.2	2.8	**
Y		7.9	4.7	**
Mean		8.7	7.4	-

During manufacturing composts, we observed fluctuations in EC values of organic materials used for compost production in some facilities such as A and N facilities as shown in Fig. 1a & b. However, changes in EC values were less in some facilities such as Q facility (Fig. 1 c).

The upper limit of EC set by CCQC and PAS-100 for compost used for agricultural application is 4 dSm^{-1}. Accordingly, 93% of studied composts have recorded EC values higher than this limit. Gulf system (GCST, 2006) is more lenient and proposed the value of 10 dSm^{-1} to be the upper limit of compost EC. Only Twelve composts comply with GS. It seems the high concentration of dissolved salts in the compost is the most problematic characteristics faced by agricultural application. It is of importance to find out ways to reduce EC values to the accepted levels for sustainable plant production.

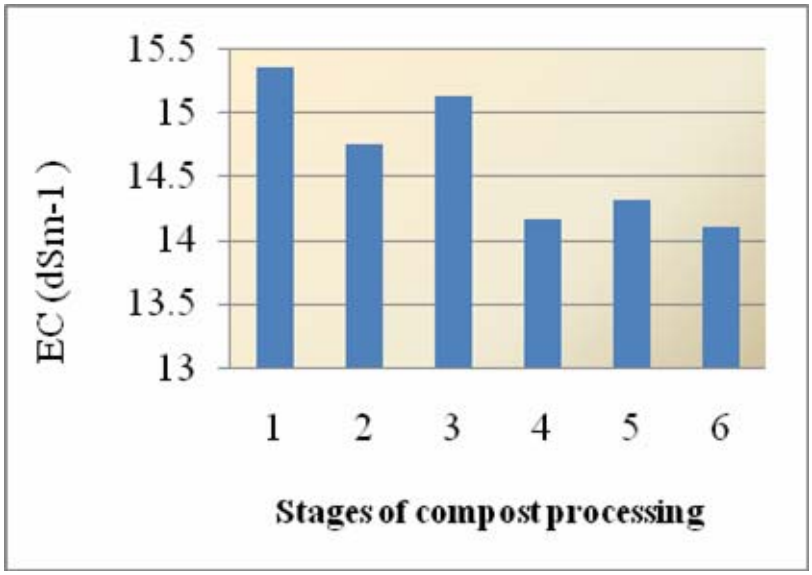

Fig 1a. Electrical conductivity of chicken compost during manufacturing (A facility)

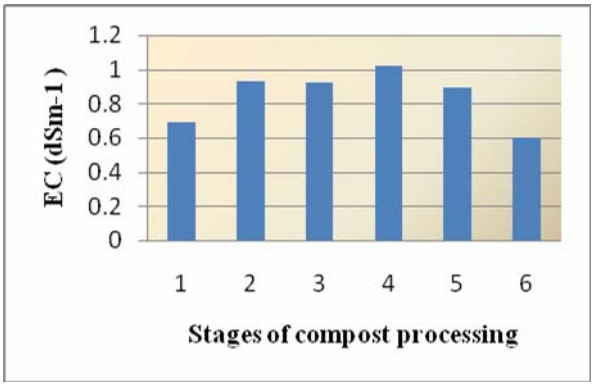

Fig 1b. Electrical conductivity of cow compost during manufacturing (N facility)

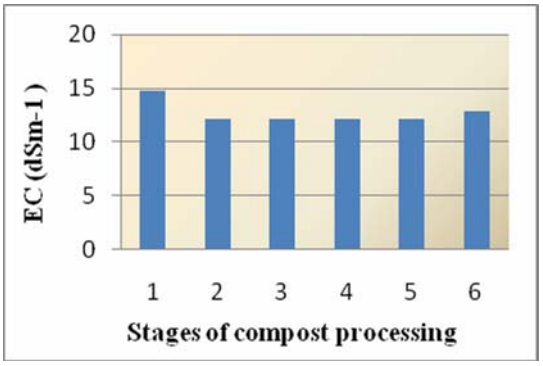

Fig 1c. Electrical conductivity of mixed organic materials compost during manufacturing (Q facility)

pH

The final pH Values of the compost facilities ranged between 6.9 (Y facility) to 8.9 (Q facility).The pH of the composts was considerably varied with the variation in the composted materials. pH in chicken manure compost facilities had acceptable pH value with the range from 6.9 (F facility) to 8.4 (A facility) with the average of 7.46 and in cow manure composts the pH varied from 7.3 (K facility) to 8.5 (M facility) with the average of 8.05, while in mixed organic materials the pH varied from 6.9 (Y facility) to 8.9 (Q facility) with the average of 8.25 (Fig. 2a, b and C). In the mature compost, during the initial stages of decomposition, organic acids are formed. The acidic conditions are favorable for the growth of fungi which breakdown the lignin and cellulose. As composting proceeds, the organic acids become neutralized, and mature compost generally has a pH between 6 and 8. Composts with very low pH (<4.0) should be used with caution since the low pH can be an indication of poor composting practices which result in the formation of potentially toxic organic acids. Composting process leads to major changes in materials and their pH as decomposition occurs. For example, release of organic acids may, temporarily, lower the pH (increase acidity), and production of ammonia from nitrogenous compounds may raise the pH (increase alkalinity) during early stages of composting. Most pH

values (pH) of the types of compost tend to be alkaline. The results also showed that in 63.3% of the types of the compost, the proportion of organic matter in final product is less than the recommended limit of most compost bodies (40%).

Chen & Inbar (1993) showed that the pH of the animal compost initially decrease from 8.3 to 6.2 and then increased gradually to 7.6 at the end of composting process. This decrease resulted from the formation of organic acids and volatilization of ammonia. In a comparative study of composts varying in source materials, the pH was found to range from 5.27 to 8.36 (Zmora-Nahum et al. 2007). A value of pH in the range 6.5 to 7 is considered as an indicator of a proper and full composting process (Alexander, 1977; de Bertoldi et al. 1987).

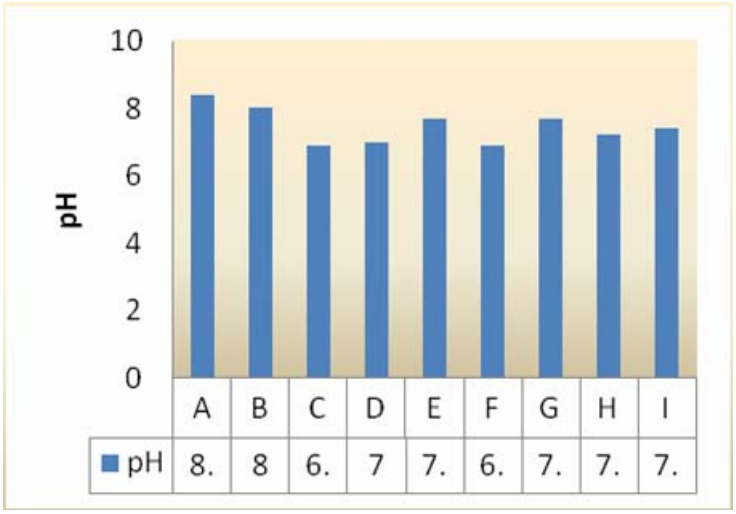

Fig 2a. pH of the chicken manure compost

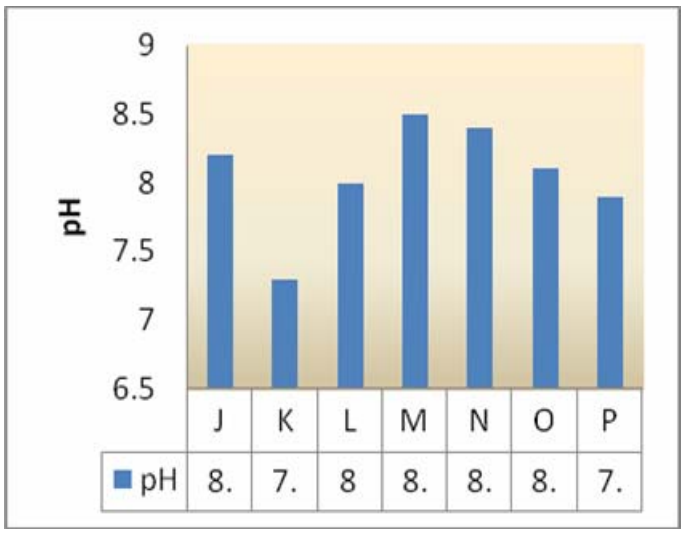

Fig 2b. pH of the cow manure compost

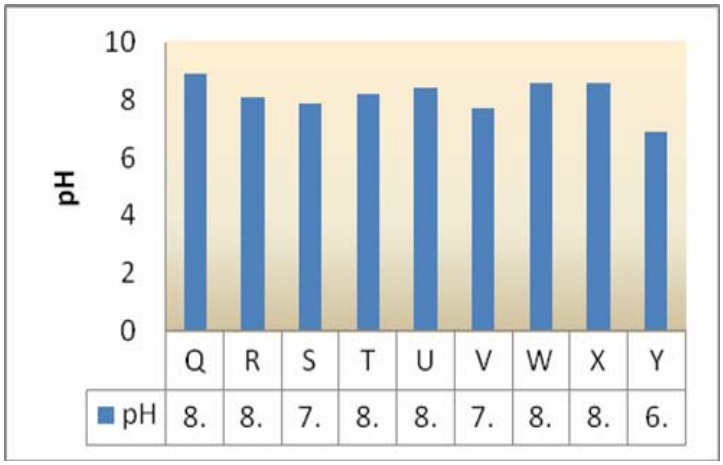

Fig 2c. pH of the mixed manure compost

pH in the n final compost product according to CCQC and PAS-100 should be between 6 to 8.5, while the upper limit of the pH in the final product should be 7.5 in the gulf system (GCST). The pH in the final product of chicken manure composts and cow manure composts, showed acceptable pH value, while in the mixed organic materials four facilities showed pH value upper than 8.5 (Q, W and X facilities).

Lugtenberg (2009) found that in the old compost samples, pH values were found to be higher than the normal range which implies that, during different degradation stages decrease in pH has been observed from 10 to 60 days which can attributed to the produce CO_2 from organic acids and loss of nitrogen.

Ammonium concentration

As shown in Table 3, initial contents of ammonium in organic materials used for compost production varied from 24 mg kg^{-1} (B facility) to 4199 mg kg^{-1} (G facility), while the final ammonium contents were between 17 mg kg^{-1} (facility V) and 2650 mg kg^{-1} (facility I). Initial and final ammonium contents were the highest in chicken manure then in cow manure and then in mixed organic materials.

Table 3. Ammonium concentration (NH_4^+) of different compost samples

Compost Facility code	Type of raw materials	NH_4^+		F- test Season
		Initial	Final	
A	Chicken manure	1274	1761	Ns
B		24	660	Ns
C		2007	1416	*
D		1470	523	Ns
E		2768	1052	Ns
F		1743	1285	Ns
G		4199	178	Ns

H		978	1913	Ns
I		1303	2650	Ns
mean		**1752**	**1271**	**-**
J		330	272	**
K		374	427	Ns
L		252	404	Ns
M	Cow manure	71	106	Ns
N		233	286	Ns
O		53	1672	Ns
P		228	770	Ns
mean		**220**	**562**	**-**
Q		24	90	*
R		30	330	ns
S		141	135	ns
T		199	314	*
U	Mixed organic materials	165	118	**
V		47	17	**
W		65	94	**
X		67	146	**
Y		651	346	**
mean		**154**	**312.1**	**-**

It is well documented that chicken manures contains up to 6% N (Harada et al. 1993). In the present study, compost made from chicken manure had the higher ammonium concentration with mean 24 mg kg^{-1} (B facility) to 4199 mg kg^{-1} (G facility) with mean of 1752 mg kg^{-1} followed by the cow manure compost with mean 53 mg kg^{-1} (O facility) to 374 mg kg^{-1} (K facility) with mean of 220 mg kg^{-1}, while in the mixed organic material compost the initial ammonium concentrations varied from 24 mg kg^{-1} (Q facility) to 651 mg kg^{-1} (Y facility) with mean of 154 mg kg^{-1}. Also, the chicken manure compost and cow manure compost had the higher ammonium concentrations (with mean of 1271 mg kg^{-1} and 562 mg kg^{-1} respectively) compared with those the compost mad from mixed organic materials (with mean of 312 mg kg^{-1}). It is well documented that chicken manure contain up to 6 % nitrogen (Harada et al. 1993; Krupa, 2003). The initial and final mean of ammonium concentration in the composts made from chicken manure, cow manure and mixed organic materials for different compost facilities in three different seasons had significant differences ($P < 0.01$) in some facilities such as C, J, Q, T, U, V, W, X and Y (Table 3).

The concentration of ammonium limit set by the CCQC and PSA- 100 (500 mg kg^{-1}) was exceeded in 50% of the types of compost under study. The ammonium concentrations in some composts facilities such as A, B, D, E, F, G, H and I were significantly higher than the acceptable limits.

Nitrate Concentration

The initial nitrate concentrations ranged from 5 mg kg^{-1} (Q facility) to 1366 (F facility), while the final nitrate concentration ranged from 4 mg kg^{-1} (Q facility) to 2999 (F facility). The chicken manure compost had the higher final nitrate concentration with mean of 659 mg kg^{-1} followed by the compost made from mixed organic materials with mean of 290.5 mg kg^{-1}, while the cow manure compost had the lowest nitrate concentration with mean of 162 mg kg^{-1} Table 4. The nitrate concentration increases in the last steps of the composting process as a result of aerobic conditions. Also, the final value of nitrate concentrations in compost depends on the source material (Gracia et al. 1991; Hue and Liu, 1995).

Some of the compost facilities such as B, F, J, U and W had significant differences ($P < 0.01$) in the nitrate concentrations when we compared between means of initial and final nitrate concentration of composts taken from facilities in three different seasons.

The results in Table 4 showed that, the concentration of nitrate were relatively higher than the standard limit set by the CCQC (150 mg kg^{-1}) in 68% of the types of compost under study. Our results were similar to those reported by (Bernal et al. 2008; Wang et al. 2004).

Table 4. Concentration of nitrate (NO_3^-) in different compost samples

Compost Facility code	Type of raw materials	NO_3^-		F- test Season
		Initial	Final	
A	Chicken manure	37	225	ns
B		34	86	*
C		119	614	ns
D		284	25	ns
E		343	156	ns
F		1366	2999	*
G		260	68	ns
H		487	409	ns
I		379	1345	ns
mean		368	659	-
J	Cow manure	57	28	*
K		43	20	ns
L		522	277	ns
M		24	33	ns
N		82	107	ns
O		48	260	ns
P		107	409	ns
mean		126	162	-

Q		5	4	ns
R		81	644	ns
S		651	651	ns
T	Mixed organic materials	635	57	ns
U		370	134	*
V		65	173	ns
W		24	25	*
X		22	21	ns
Y		341	132	ns
mean		243	290.5	-

Organic matter

Organic matter is the measure of carbon based materials in the compost. The initial percentage of organic matter (OM) in compost samples were varied widely from 19.7% (V facility) to 80.4% (A facility), while the final OM percentage were between 19.3 (V facility) to 68.1 (A facility) Table (4). The chicken manure composts had the higher OM in the final product with mean of 44.1% followed by the cow manure composts with mean of 39.5% and the he composts made from mixed organic materials with mean of 38.1%.

Some facilities such as F, H, M, N, P, Q, V, X and Y had significant differences ($P < 0.01$) when we compared between means of initial and final OM % of composts taken from facilities in three different seasons. The organic matter is important in all composts and has an important role to play in maintaining soil structure, nutrient availability and water holding capacity About 87% of compost samples contain more than 25% OM, the minimum percentage of OM recommended by CCQC (2002) and PAS-100 was 25%, while, only 37% of compost samples contain more than 40% OM, the minimum percentage of OM recommended by GCST was 40%. The OM content in chicken manure composts were significantly higher than in cow manure composts and mixed organic materials composts.

Inorganic nitrogen, $N\text{-}NH_4$ and $N\text{-}NO_3$ are usually affected by the action of proteolytic bacteria and bacteria are partly incorporated into stable organic forms such as amide and heterocyclic nitrogen. Organic matter is decomposed and transformed to stable humic compounds. Humic compounds had a capacity to interact with metal ions, buffer pH, and to act as a potential source of nutrients for plants.

Table 5. Organic matter (%) of different compost samples

Compost facility code	Type of raw material	OM%		F- test season
		Initial	Final	
A		80.4	68.1	ns
B		74.4	33.4	ns
C		45.1	35.3	ns

D	Chicken manure	37.1	58.3	ns
E		62.1	55.6	ns
F		64.1	39.8	*
G		61.8	36.6	ns
H		72.6	36.6	ns
I		41.6	33.4	*
Mean		59.9	44.1	-
J	Cow manure	51.1	28.0	ns
K		44.0	44.3	ns
L		56.7	60.5	ns
M		45.9	44.9	*
N		30.6	21.1	**
O		60.8	37.4	ns
P		61.4	38.9	*
Mean		51.1	39.5	-
Q	Mixed organic matter	29.8	24.3	**
R		46.7	29.9	ns
S		74.7	26.4	ns
T		49.2	26.8	ns
U		41.2	21.3	ns
V		19.7	19.3	**
W		21.9	24.3	ns
X		23.7	20.7	*
Y		49.6	30.8	**
Mean		47.6	38.1	-

Nitrogen (N), phosphorus (P), and potassium (K) are the primary nutrients required by microorganisms involved in composting, as well as the primary nutrients for plants, influencing the value of compost (Dick & McCoy, 1993; Wilkinson, 2007; Saebo & Ferrini, 2006). Almost all organic materials used for composting contain all of these nutrients at various levels which microorganisms use for energy and growth. Barker & Bryson (2006) reported that the beneficial effects of the composts on plant growth were associated with increased supply of nutrients for the plant.

Total nitrogen (TN)

Results in Table (5) showed that the initial total nitrogen contents in compost samples were between 0.43% (X facility) and 5.81% (A facility), while the final total nitrogen in compost samples were between 1.00 (X facility) and 4.1 (A facility). The rise in nitrogen level during maturation phase could be possibly due to concentration effect caused by strong degradation of labile organic carbon compounds which reduces the weight of composting materials (Bermal et al. 1998). The increasing of

total nitrogen during composting was caused by the decrease of substrate carbon resulting from the loss of CO_2 (because of the decomposition of the organic matter which is chemically bound with nitrogen). The chicken manure composts had the highest level in nitrogen content followed by mixed organic materials and then cow manure compost with the mean of 3.70%, 2.55% and 1.77% respectively for the initial nitrogen contents and 2.83, 2.63 and 1.49 respectively for the final nitrogen contents.

Total phosphorus (TP).

The initial total phosphorus contents were between 0.16% (S facility) and 0.53% (A facility), while the final total phosphorus contents were between 0.10% (E facility) and 0.83% (Q facility). The total phosphorous content gradually increased during composting process and water solubility of phosphorous decreases with humification, so that phosphorous solubility during the decomposition was subjected to further immobilization factor (Elango et al. 2009). Chicken manure composts had the highest level in phosphorus contents with mean of 0.33% and 0.39% for the initial and final phosphorus contents respectively, followed by cow manure composts with the mean of 0.24% and 0.38% for the initial and final phosphorus contents respectively, while the mixed organic materials had the lowest phosphorus content with mean of 0.24% and 0.32% for the initial and final phosphorus contents.

Total potassium content (TK).

The initial total potassium contents varied from 0.32% (M facility) to 2.71% (I facility), while the final total potassium contents varied from 0.60 % (N facility) to 2.52% (G facility) Table (5). Potassium is not known to have harmful or toxic effect on human beings and it helps in plant growth as an essential nutritional element. The chicken manure composts had the highest potassium contents followed by cow manure compost, while the compost made from organic mixed materials had the lowest potassium contents with the mean of 1.62%, 1.39% and 1.2% respectively for the initial contents and 1.89%, 1.41% and 1.3% respectively for the final contents of potassium.

Table 6. TN, TP, and TK contents in different compost samples

Compost Facility code	Type of raw materials	TN%		TP %		TK %	
		Initial	Final	Initial	Final	Initial	Final
A	Chicken manure	5.81	4.1	0.53	0.61	1.34	1.34
B		2.95	1.43	0.25	0.36	0.80	1.99
C		2.42	2.39	0.46	0.39	1.90	1.69
D		3.67	3.14	0.25	0.53	1.16	1.14
E		5.13	3.94	0.26	0.10	1.60	2.32
F		3.83	3.15	0.52	0.50	2.13	1.63
G		3.02	2.07	0.25	0.31	1.77	2.52
H		3.03	2.52	0.25	0.37	1.15	2.33

I		3.44	2.77	0.20	0.35	2.71	2.03
mean		3.70	2.83	0.33	0.39	1.62	1.89
J		1.80	1.29	0.18	0.62	1.76	1.48
K		1.3	1.62	0.26	0.31	1.70	1.02
L		1.69	1.67	0.40	0.41	1.13	1.73
M	Cow manure	1.08	0.66	0.16	0.24	0.32	0.86
N		0.44	0.64	0.22	0.30	1.00	0.60
O		3.00	2.12	0.24	0.28	1.97	2.26
P		3.08	2.42	0.27	0.47	1.88	1.92
mean		1.77	1.49	0.24	0.38	1.39	1.41
Q		1.16	1.04	0.36	0.83	1.46	1.25
R		1.03	1.71	0.34	0.59	0.62	1.13
S		0.93	1.64	0.16	0.18	0.91	0.93
T	Mixed organic materials	1.69	1.31	0.13	0.34	0.83	1.07
U		1.49	1.36	0.38	0.42	2.19	1.72
V		0.50	1.05	0.24	0.19	0.87	0.99
W		1.03	1.01	0.29	0.44	0.91	1.33
X		0.43	1.00	0.48	0.42	1.03	1.61
Y		2.58	1.89	0.29	0.43	2.15	1.67
mean		2.55	2.63	0.24	0.32	1.2	1.3

Heavy metals

The heavy metals, as harmful elements, are one of the determinant factors for compost quality (Harada et al. 1993). In our study, there were significant variations in the heavy metals concentration. The concentration of heavy metals (Mn, Cu, Zn, Pb, Ni, Cd and Fe) in compost samples are presented in Table (6, 7 and 8). The initial concentration of Fe varied from 1048 mg kg^{-1} (E facility) to 1962 mg kg^{-1} (J facility), whereas the final concentration of Fe varied form 1008 mg kg^{-1} (I facility) to 2484 mg kg^{-1} (Y facility) Table (6). The cow manure composts had the highest Fe concentration with the mean of 1489 mg kg^{-1} and 1913 mg kg^{-1} for the initial and final Fe concentrations respectively followed by cow manure composts with the mean of 1547 mg kg^{-1} and 1533 mg kg^{-1} for the initial and final Fe concentration, while the chicken manure composts had the lowly Fe concentration with the mean of 1375 mg kg^{-1} and 1340 mg kg^{-1} for the initial and final Fe concentration. The initial Mn concentration varied from 143 mg kg^{-1} (S facility) to 363 mg kg^{-1} (C facility), where the final Mn concentration varied from 145 mg kg^{-1} (D facility) to 439 mg kg^{-1} (Y facility) Table (6). The chicken manure composts had the highest Mn concentration with the mean of 255 mg kg^{-1} and 276 mg kg^{-1} for the initial and final Mn concentration respectively and then the cow manure composts with the mean of 224 mg kg^{-1} and 262 mg kg^{-1} for the initial and final Mn concentration respectively, where the compost made from mixed organic materials had the lowest Mn

concentration with mean of 205 mg kg^{-1} and 240 mg kg^{-1} for the initial and final Mn concentration respectively.

The initial Zn concentration varied from 126 mg kg^{-1} (W facility) to 453 mg kg^{-1} (S facility), while the final Zn concentration varied from 116 mg kg^{-1} (D facility) to 332 mg kg^{-1} (A facility) Table (6). The chicken manure compost had the highest Zn concentration followed by cow manure compost when compared with the compost made from mixed organic materials with the mean of 266, 219 and 194 mg kg^{-1} respectively for the initial Zn concentration and 251, 234 and 180 mg kg^{-1} respectively for the final Zn concentration.

Table 8. Initial and final concentration of Zn, Mn and Fe in different compost samples

Compost Facility code	Type of raw materials	Zn mg kg^{-1}		Mn mg kg^{-1}		Fe mg kg^{-1}	
		Initial	Final	Initial	Final	Initial	Final
A	Chicken Manure	258	323	310	273	1580	1641
B		226	332	146	272	1510	1410
C		387	238	363	327	1573	1486
D		190	116	177	145	1446	1176
E		263	132	205	226	1048	1158
F		321	303	310	312	1708	1494
G		235	215	274	245	1120	1387
H		182	281	163	349	1184	1304
I		329	318	349	334	1209	1008
mean		266	251	255	276	1375	1340
J	Cow manure	311	226	273	418	1962	1762
K		292	312	242	307	1730	1638
L		221	205	250	239	1643	1455
M		151	176	194	191	1292	4803
N		139	144	185	184	1356	1305
O		178	273	185	173	1111	1124
P		243	300	242	323	1327	1304
mean		219	234	224	262	1489	1913
Q	Mixed organic materials	183	152	279	213	1858	1488
R		174	298	226	318	1508	1423
S		453	158	143	199	1544	1530
T		156	206	218	264	1644	1585
U		197	149	238	196	1588	1401
V		153	131	120	171	1309	1421
W		126	139	147	148	1389	1197

X		129	188	149	216	1173	1270
Y		178	205	325	439	1911	2484
mean		194	180	205	240	1547	1533

The initial Cu concentrations were between 17.43 mg kg^{-1} (E facility) to 86.6 mg kg^{-1} (I facility), while the final Cu concentrations were between 17.63 mg kg^{-1} (S facility) and 79.1 mg kg^{-1} (G facility) Table (7). The initial mean concentration of Cu in the cow manure composts was higher than the mean Cu concentration of chicken manure composts, while the composts made from mixed organic material was the lowest with mean of 63.69, 56.32 and 43.1 mg kg^{-1} respectively, whereas, the final mean Cu concentration in the chicken manure compost was the highest followed by the cow manure compost when compared with the compost made from mixed organic materials with mean of 62.58, 53.13 and 45.6 mg kg^{-1} respectively.

The initial Cd concentrations were between 0.49 mg kg^{-1} (I facility) to 6.00 mg kg^{-1} (V facility), where the final Cd concentrations were between 0.83 mg kg^{-1} (X and Y facilities) and 5.7 mg kg^{-1} (K facility) Table (7). The initial mean of Cd concentration of the compost made from mixed organic materials was the highest followed by chicken manure compost, where the cow manure compost had the lowly initial mean of Cd concentration (3.7, 3.30 and 3.05 mg kg^{-1} respectively).

The final mean Cd concentration of chicken manure compost was higher in the final mean Cd concentration than cow manure compost, while the compost made from mixed organic materials had the lowly final mean Cd concentration (3.9, 3.82 and 3.5 mg kg^{-1} respectively)

Table 8. Initial and final concentration of Cd and Cu in different compost samples

Compost Facility code	**Type of raw materials**	**Cd mg kg-1**		**Cu mg kg-1**	
		Initial	**Final**	**Initial**	**Final**
A	Chicken manure	3.9	4.7	51.83	55.07
B		5.7	5.3	50.93	64.30
C		4.4	4.0	70.27	72.33
D		3.2	4.3	38.60	43.43
E		2.39	2.6	17.43	45.97
F		4.95	3.1	75.17	61.97
G		2.63	3.9	47.03	79.10
H		1.94	4.1	69.00	74.70
I		0.49	3.1	86.60	66.33
mean		3.30	3.9	56.32	62.58
J	Cow manure	2.99	4.0	85.47	45.87
K		4.05	5.7	72.23	55.67
L		4.35	3.5	54.93	60.27
M		2.95	2.61	53.53	49.10
N		2.16	3.27	34.30	19.20

O		2.98	3.75	75.83	71.60
P		1.89	3.87	69.57	70.23
mean		3.05	3.82	63.69	53.13
Q		4.03	4.24	60.83	43.67
R		3.10	4.25	55.40	77.07
S		3.44	5.01	24.27	17.63
T	Mixed organic materials	3.51	4.23	55.97	55.70
U		4.33	4.03	45.50	57.33
V		6.00	5.37	23.30	32.73
W		5.04	2.71	49.13	28.87
X		2.23	0.83	26.87	52.50
Y		1.40	0.83	46.80	45.00
mean		3.7	3.5	43.1	45.6

The initial Ni concentration varied form 12.2 mg kg^{-1} (G facility) to 45.8 mg kg^{-1} (B facility), while the final Ni concentration varied from 15.90 mg kg^{-1} (E facility) to 62.8 mg kg^{-1} (C facility) Table (9). The initial mean concentration of Ni in the compost made from mixed organic materials was higher than the mean Ni concentration of chicken manure, while the cow manure compost had the lowest Ni mean concentration (38.1, 27.98 and 24.61 mg kg^{-1} respectively). The final mean concentration was highest in the compost mad from mixed organic materials followed by chicken manure compost and the cow manure compost (43.57, 35.0 and 26.29 mg kg^{-1} respectively).

The initial Pb concentration varied from 11.2 mg kg^{-1} (N facility) to 38.5 mg kg^{-1} (R facility), while the final Pb concentration varied from 7.8 mg kg^{-1} (N facility) to 31.6 mg kg^{-1} (R facility) Table (9). The cow manure compost had the higher initial mean Pb concentration followed by chicken manure compost, while the compost made from mixed organic compost had the lowest initial Pb concentration (23.24, 21.41 and 20.50 mg kg^{-1} respectively). The final mean concentrations were 23.8, 19.08 and 19.05 for mixed organic compost, cow manure compost and chicken manure compost respectively.

Table 9. Initial and final concentration of Pb and Ni in different compost samples

Compost Facility code	**Type of raw materials**	**Pb mg kg^{-1}**		**Ni mg kg^{-1}**	
		Initial	**Final**	**Initial**	**Final**
A		28.33	28.40	26.13	31.17
B		22.53	20.33	45.80	50.17
C	Chicken manure	32.90	26.80	38.97	62.8
D		16.23	10.46	25.50	20.33
E		18.79	13.87	21.53	15.90

F		26.57	19.23	36.17	27.90
G		15.50	20.07	12.2	36.33
H		12.97	15.37	19.80	42.97
I		18.90	16.93	25.67	27.37
mean		21.41	19.05	27.98	35.00
J		32.53	21.40	38.83	28.69
K		28.60	21.20	35.77	35.10
L		29.43	28.13	19.53	33.07
M	Cow manure	25.17	21.23	20.63	16.93
N		11.2	7.8	15.04	18.43
O		13.87	17.27	19.07	26.60
P		21.93	16.57	23.43	25.23
mean		23.24	19.08	24.61	26.29
Q		35.80	26.67	44.57	42.57
R		38.5	31.6	42.03	51.40
S		21.27	25.03	33.67	47.10
T	Mixed organic materials	28.13	26.40	25.20	27.80
U		21.67	28.50	29.20	41.77
V		21.23	29.67	31.10	43.57
W		15.44	13.53	23.27	23.80
X		16.37	12.32	21.47	19.89
Y		20.50	23.80	38.10	43.57
mean		24.3	24.2	32.1	38.0

Composting can concentrate or reduce heavy metals. Reducing the amounts of heavy metal depends on metal loss through leaching. The increase of heavy metal level is due to weight loss in the course of composting following organic matter decomposition, release of carbon dioxide and water and mineralization processes.

The results of the heavy metal concentration in our study were similar to or somewhat higher than the results obtained by (Webber et al. 1983; Tiquia et al. 1996). The differences of metal concentrations in compost samples can be explained by the results from manure variability, in homogeneity in compost preparation and mixing, the metal concentrations increase as composting process (Gomez, 1998). Compost quality is regulated by GCST, 2006; CCQC, 2001 and BSI, 2005. With respect to heavy metals, maximum permissible concentrations in the compost are set for Zn, Cu, Cd, Ni and Pb, with values of 350, 150, 3, 25 and 50 mg kg^{-1} respectively. The concentration levels of heavy metals in the compost samples were lower than the acceptable level recommended by the CCQC and the Gulf system (GCST).

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19

BIOCONTROL POTENTIAL OF ACTINOMYCETES AGAINST *RHIZOCTONIA SOLANI*

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ABSTRACT

The banded leaf and sheath blight is one of the significant fungal diseases causing major economic losses to corn cultivation. It is caused by Rhizoctonia solani Kuhn, a soil-borne fungal pathogen. The fungus is controlled by use of fungicides. However, due to health and environmental implications, utilization of microorganisms for control is a promising alternative The actinomycete with the ability to synthesize a wide variety of bioactive compounds are good candidates as biocontrol agents against this pathogen. One hundred actinomycetes isolated around mangrove areas in Quezon, Zambales and Bataan, Philippines were initially screened against R. solani by agar plug assay. The R. solani used as test organism was previously confirmed to cause the sheath blight disease in vivo. Results showed that among the actinomycetes tested, 20 actinomycetes showed the highest biocontrol activity, with AR1, AR2, AR3 and AR4 inhibiting R. solani with 28.8 mm, 23.65 mm, 23.30 mm and 23.15 mm zones of inhibition, respectively. Greenhouse experiment is presently being conducted to evaluate the efficacy of these actinomycetes as preventive control of R. solani.

INTRODUCTION

Maize ranks as second most important crop produced in the Philippines. White maize is used as rice substitute during times of scarcity, and yellow maize is used for feed formulation (Gerpacio et al. 2004). According to the Bureau of Agricultural Statistics, the present area utilized for corn plantation is 2.61 million hectares.

To meet the increasing demand and consumption, corn production and productivity should be maximized. However, corn production is threatened by pest

and diseases. One of the major concerns in corn production is the banded leaf and sheath blight (BLSB) caused by a soil-borne fungal pathogen the *Rhizoctonia solani* and is now becoming one of the serious threat to the production of corn in different countries in Asia. (Sharma et al. 2001). Losses caused by the disease could be direct- that is, through an early death of the plant, or through damage to the plants' stalks or ears- or indirect, where amount and quality of harvests decrease (Sharma et al. 2001). In China, BLSB was regarded as the reason for yield losses that are close to 100% (Singh & Shahi, 2012).

Sharma et al. (2001) elaborates that massive chemical pesticides namely Carbendazim, Benodanil, Thiobendazole, Validamycin, Topsin M, Rhizolex are good at controlling further growth of *R. solani in vitro,* However, the continued use of excessive chemical pesticides in modern agriculture has led to environmental pollution/degradation and hazards to humans.

Thus, there is a need to develop alternative control methods against *R. solani*. Actinomycetes are good candidates as biocontrol agents against this pathogen. Actinomycetes are the most abundant soil microorganisms and are well known for the production of extracellular enzymes and a range of antimicrobials (Crawford et al. 1993). Moreover, they can promote plant growth by atmospheric nitrogen fixation (Chang & Yang, 2009) and production of siderophores or phytohormones (Dimkpa et al. 2008).

This study presents the potential of actinomycetes as biocontrol agents against *R. solani* in corn.

MATERIALS AND METHODS

Sources of Isolates

Actinomycetes were isolated from soil taken from the different mangrove areas in Quezon, Bataan and Zambales, Philippines. Yeast Malt Agar (YMA) was used in the isolation supplemented with 1.5% sodium chloride. Sterile soil was used in stock preparation of the isolated pure actinomycetes.

Pathogenicity test

A newly isolated *Rhizoctonia solani* (c/o Dr. Teresita U. Dalisay, Crop Protection Cluster) from rice was mass produced using Rice Hull Rice Grain (RHRG) medium as was described in Muis & Quimio (2006). The mass produced pathogen was inoculated into the soil of three-week old corn plants (Syngenta Sweet Corn Sugar 75) grown in pots. Development of the symptoms was observed after a month of inoculation.

Screening of potential antagonists by agar plug assay

A five-day old culture of *R. solani* in a test tube was suspended in sterile distilled water with Tween 80. Mycelia was scraped and an aliquot of 2ml of the culture was added to 50 ml Potato dextrose agar (PDA) Pronadisa®, Madrid, Spain) top agar.

Five ml of the seeded agar was overlaid onto plates previously poured with 10 ml of PDA base agar.

Agar plugs (8 mm dia.) were obtained randomly from 7-day old actinomycete isolates using alcohol-flamed cork borer with 8mm dia. These agar plugs were then deposited in an inverted position on the seeded assay plates with approximately 10^6cells/ml of *R. solani*. Diameter of zones of inhibition was measured using a digital Vernier caliper after 5 days of incubation at ambient room temperature. Three replications were done. The data obtained was subjected to statistical analysis using Analysis of Variance (ANOVA) in Completely Randomized Design (CRD) and the Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSIONS

Pathogenicity Testing

A pathogenicity testing was done to ensure that the test organism used for the agar-plug assay was the pathogen of interest. Mycelia of the *Rhizoctonia solani* isolate was mass produced and inoculated into the soil where corn seeds were planted. After weeks of incubation, symptoms that are seen in the sheaths of the plants resemble that of the description of the banded leaf and sheath blight (Fig 1).

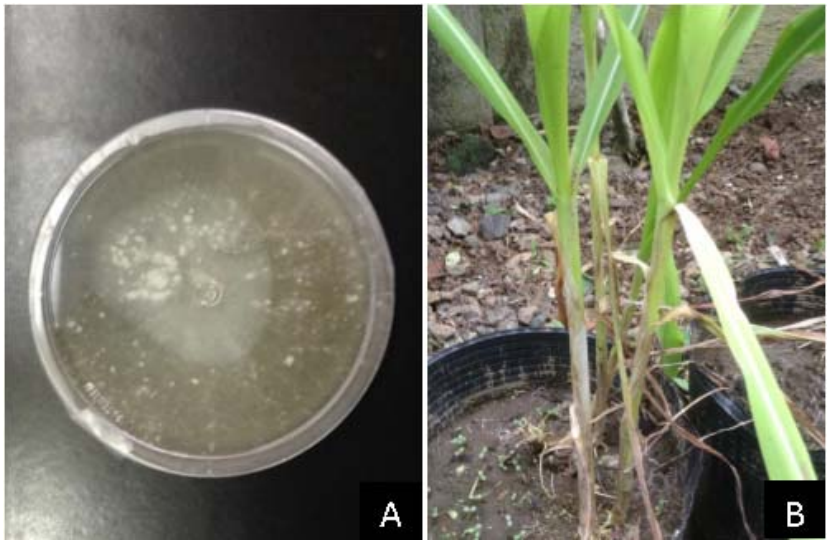

Fig 1. *Rhizoctonia solani* grown in Potato Dextrose Agar (PDA) for three days (A) and corn plant showing symptoms of banded leaf and sheath blight disease (B).

Preliminary screening of antagonists by agar plug assay

A total of 100 actinomycetes isolated from mangrove areas in the provinces of Quezon, Zambales, and Bataan, Philippines were screened for their biocontrol activity against *R. solani* using the agar-plug assay method. After incubation, the diameter of the zones of inhbition (ZOI) formed by the actinomycete agar plugs (Fig.

2) was measured using a digital Vernier caliper, and 20 among the isolates screened had the highest diameters ranging from 11.40 mm to 28.80 mm (Fig. 3).

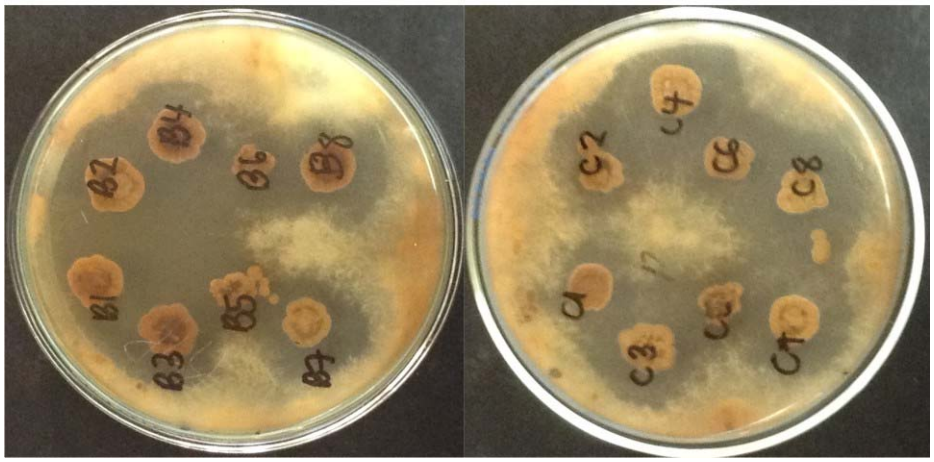

Fig. 2. Growth inhibition of actinomycetes against *Rhizoctonia solani* by agar plug assay.

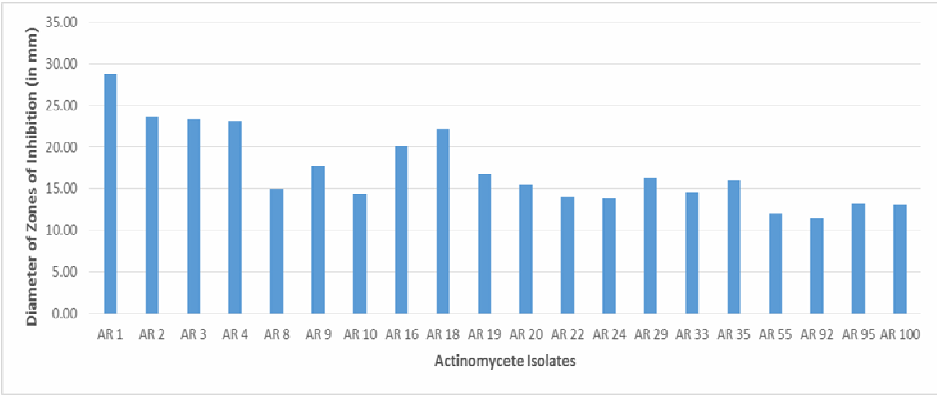

Fig 3. Diameter of the zones of inhibition (mm) of the 20 best isolates screened by agar plug assay.

Among the 20 isolates, the highest growth inhibition recorded were seen in isolates AR 1, AR 2, AR 3, and AR 4 with values 28.8mm, 23.65mm, 23.30mm, and 23.15mm, respectively.

The zones of inhibition formed in the media indicates that *R. solani* did not grow within a particular distance from the actinomycete isolate, indicating that the isolate had an effect on the growth of the pathogen. The higher the ZOI formed, the more effective the isolate is as biocontrol. Thus, the four highest isolates have the highest potential of being used as biocontrol agent against *R. solani*.

According to El-Tarabily & Sivasithamparam (2005), fungal plant pathogens are antagonized by different mechanisms, namely, antibiosis, hyperparasitism, and production of cell-wall degrading enzymes.

The isolates still need to be tested in a greenhouse set-up that is, testing the isolates if they can protect plants from infection if they are planted into soil infected with the pathogen to further validate their potential to be used as biocontrol.

In the studies of Muis & Quimio (2006) and Moussa (2002), they tested the efficacy of their biocontrol by using it as treatment to the seeds before they are planted in the soil. Aside from using biocontrol as seed treatment, El-Tarabily & Sivasithamparam (2005) also elaborated that they can be used as root dips.

Testing the actinomycetes as seed treatment, or as root dip can then be the next step to further validate their efficacy as biocontrol agents.

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USE OF A *XANTHOMONAS*/PEPPER TRANSLOCATION SYSTEM FOR CHARACTERIZATION OF RHIZOBIAL TYPE 3 EFFECTORS

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ABSTRACT

The type 3 protein secretion system (T3SS) of nitrogen-fixing rhizobia delivers type 3 (T3) effector proteins into legume host cells. Here, we have modified an effector translocation system that is based on cell death of pepper (Capsicum annuum cv. ECW-10R) induced by the Xanthomonas campestris avirulence protein AvrBs1. The results show that this system is suitable for future identification and characterization of rhizobial effectors.

INTRODUCTION

Rhizobial infection of legume roots culminates in formation of nodules harboring nitrogen-fixing bacteroids. Nodulation depends on molecular communication between the invading rhizobia and the host legume. Nod factor receptors (LysM domain receptor kinases) of legumes perceive rhizobial Nod factor signals (lipo-chitooligosaccharides) to initiate nodulation signaling that culminates in expression of plant genes required for nodule formation. In addition to Nod factors, rhizobia secrete additional molecules that promote nodulation in specific strain-host combinations. These symbiotic determinants include rhizobial T3 effectors secreted by the T3SS, a complex protein export machinery that delivers T3 effectors into legume host cells (Staehelin and Krishnan, 2015). In our laboratory, we have characterized T3 effectors of *Sinorhizobium* (*Ensifer*) sp. NGR234, namely NopT (Dai et al. 2008), NopL (Zhang et al. 2011) and NopM (Xin et al. 2012).

T3 effectors possess non-cleavable secretion signal sequences at their N-termini. The mechanisms of substrate recognition by the T3SS remain poorly

investigated, however. Computational programs have been developed to predict T3SS substrates based on N-terminal amino acid sequences (e.g. Arnold et al. 2009; Samudrala et al. 2009). To confirm such predictions, experimental approaches are required. For example, T3 effector candidates can be expressed in rhizobia as fluorescence fusion proteins (Lorio et al. 2004) or as FLAG-tagged proteins (Sánchez et al. 2009). Comparative analysis of culture supernatants from the parent strain and a T3SS-deficient mutant provides then information on T3SS-specific secretion of the examined protein. Other methods have been used to confirm T3 effector translocation into host cells. Rhizobial T3 effectors fused to adenylate cyclase were expressed in a given rhizobial strain and cAMP formation in inoculated legume roots or nodules was measured (Schechter et al. 2010: Wenzel et al. 2010). Furthermore, previous work showed that rhizobial T3 effectors expressed as fusion proteins in *Pseudomonas syringae* can be translocated into *Arabidopsis thaliana* cells (Schechter et al. 2010; Kimbrel et al. 2013), indicating that the N-terminal secretion signals are interchangeable among *Sinorhizobium* and *Pseudomonas* bacteria.

To study rhizobial T3 effector translocation, we have modified a *Xanthomonas*/ pepper translocation system (Jiang et al. 2009) that is based on plant cell death (hypersentitive reaction, HR) induced by the translocated reporter protein AvrBs1 (avirulence protein Bs1 of *Xanthomonas campestris*).

MATERIALS AND METHODS

Pepper seeds (*Capsicum annuum* cv. ECW-10R) and the *X. campestris* pv. *campestris* strains 8004, 8004Δ*avrBs1* and 8004Δ*hrpF* were kindly provided by Dr. Wei Jiang (Guangxi University, China). The mutant 8004Δ*avrBs1* does not produce AvrBs1 and 8004Δ*hrpF* cannot translocate T3 effectors into pepper due to deficiency of the tranlocon protein HrpF. Accordingly, both strains do not induce HR on cv. ECW-10R (Xu et al. 2008; Jiang et al. 2009; Fig. 1). For expression of constructs in *X. campestris*, the RK-derived vector pFAJ1702 (Dombrecht et al. 2001) was used. All constructs had a constitutive promoter (from plasmid pHP45 containing an Ω interposon with a spectinomycin resistance gene; Prentki & Krisch, 1984). The coding region of the constructs contained an N-terminal 174-bp fragment of a given gene fused to *avrBs1* without N-terminal secretion signal sequence [encoding AvrBs1(Δ58); AvrBs1 lacking 58 amino acid residues]. The N-terminal sequence of the T3 effector gene *nopM* from *Sinorhizobium* sp. NGR234 (Xin et al. 2012) was used as test sequence. A corresponding N-terminal sequence of the glycanase gene *exoK* of NGR234 (Staehelin et al. 2006) served as negative control. The pFAJ1702 derivatives encoding NopM(1-58)-AvrBs1(Δ58), ExoK(1-58)-AvrBs1(Δ58) or full-length AvrBs1 were mobilized into the different *X. campestris* strains by tri-parental mating. Strain 8004Δ*avrBs1* carrying the empty vector pFAJ1702 was included into the study. Finally, the bacteria were used for infiltration of pepper leaves using needleless syringes. The infiltrated plants were kept at 25°C in a temperature-controlled green house with a 16/8-h light/dark cycle. Formation of HR was observed at different time points post bacterial infiltration.

RESULTS AND DISCUSSION

Rhizobial T3 effectors have been recently characterized by using a *Pseudomonas/Arabidopsis* translocation system that is based on HR induced by the avirulence protein AvrRpt2 (Kimbrel et al. 2013). Here, we employed a *Xanthomonas*/pepper system (Jiang et al. 2009), which depends on cell death elicited by the avirulence protein AvrBs1. An HR occurs in leaves of pepper (cv. ECW-10R) after infiltration with the wild-type strain *X. campestris* 8004, but not in response to 8004Δ*avrBs1* or 8004Δ*hrpF*. Complementation analysis with pFAJ1702 containing an *avrBs1* expression construct indicated that expression of AvrBs1 in 8004Δ*avrBs1* causes HR in the pepper cells. In contrast, expression of AvrBs1 in 8004Δ*hrpF* showed no effects, indicating T3SS-dependent translocation of AvrBs1 into pepper cells (Fig. 1, left panel).

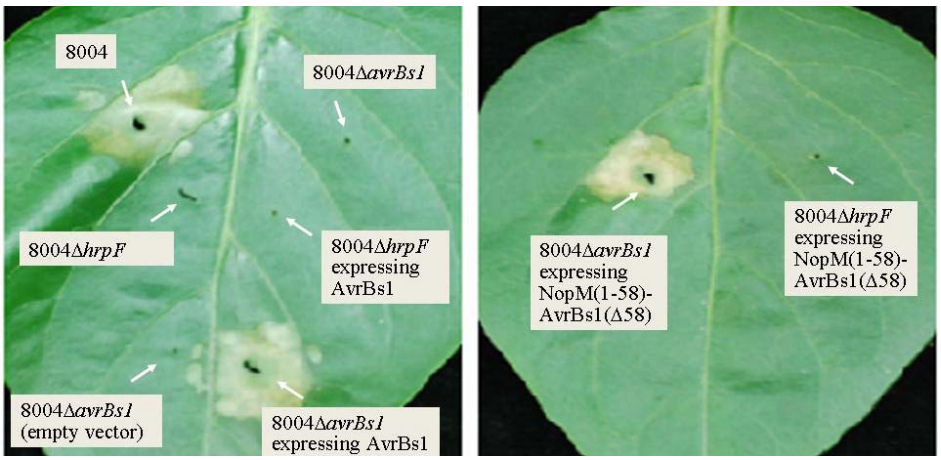

Fig. 1. The *Xanthomonas*/pepper translocation system used in our laboratory. Chimeric proteins [here NopM(1-58)-AvrBs1(Δ58) as an example] consisting of a secretion signal sequence from a rhizobial T3 effector candidate and AvrBs1(Δ58) are expressed in the *X. campestris* mutant strains 8004Δ*avrBs1* and 8004Δ*hrpF*. Once infiltrated into ECW-10R leaves, T3SS-dependent translocation of the fusion protein expressed in strain 8004Δ*avrBs1* results in AvrBs1-induced HR. Such an HR does not occur when the fusion protein is expressed in strain 8004Δ*hrpF*. The white arrows indicate the sites where strains expressing indicated proteins were infiltrated. An HR was observed in response to strain 8004 (wild-type expressing AvrBs1), the complemented mutant 8004Δ*avrBs1* expressing AvrBs1 and 8004Δ*avrBs1* expressing the fusion protein NopM(1-58)-AvrBs1(Δ58). The picture was taken 48 h after bacterial infiltration.

Fusion proteins consisting of a given test sequence and AvrBs1(Δ58) were expressed in 8004Δ*avrBs1* and 8004Δ*hrpF* in a similar way. The chimeric protein NopM(1-58)-AvrBs1(Δ58) expressed in 8004Δ*avrBs1* induced a strong HR in pepper leaves (Fig. 1, right panel). The HR induced by NopM(1-58)-AvrBs1(Δ58) was visible as early as 18 h post bacterial infiltration, indicating a rapid cell death response (not shown). In contrast, expression of NopM(1-58)-AvrBs1(Δ58) in 8004Δ*hrpF* did not cause HR in pepper leaves (Fig. 1, right panel). These results indicate that NopM(1-58)-AvrBs1(Δ58) was translocated into pepper cells and thus

confirm that NopM is a T3 effector of *Sinorhizobium* sp. NGR234 (Xin et al. 2012; Kimbrel et al. 2013). As expected, expression of the control protein ExoK(1-58)-AvrBs1(Δ58) in 8004Δ*avrBs1* did not result in HR (not shown). In conclusion, we demonstrate that the used *Xanthomonas*/pepper translocation system is suitable for characterization of rhizobial T3 effectors. We currently test secretion signals from various candidate effectors in this way.

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We thank Dr. Wei Jiang (Guangxi University, China) for pepper seeds and *X. campestris* strains (8004, 8004Δ*avrBs1* and 8004Δ*hrpF*). Dr. Jan Michiels (Katholieke Universiteit Leuven, Belgium) kindly provided pFAJ1702.

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ACTINOMYCETES, PROMISING RHIZOBACTERIA FOR BIOLOGICAL CONTROL OF PLANT DISEASES

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ABSTRACT

Actinomycetes are aerobic Gram-positive bacteria, which are present in high population densities in soil environments, where they play an important role in reducing inoculum of plant pathogens. In this study on biological control of plant diseases using actinomycetes, a total of 187 strains were isolated from the rhizosphere of sesame and vegetable crops. Several of these strains had antagonistic potential against various fungal pathogens such as Fusarium oxysporum f.sp. sesami (Fos), causing vascular wilt on sesame; Phytophthora nicotianae, causing Phytophthora blight or black shank disease on sesame; P. capsici, causing fruit rot in watermelon, Colletotrichum gloeosporioides, causing anthracnose in chilli and Rhizoctonia solani in cabbage. For each pathogen, we selected strains showing large inhibition zones in dual cultures tests for evaluation of their ability to control the diseases in planta under greenhouse conditions. In experiments with Fos on sesame, soil drenching with suspensions of four individual actinomycete strains (3, 6, 25, 79) or a mixture of these four strains at ten days intervals, resulted in significant reductions in disease incidence (72, 80, 68, 68 and 64% diseased plants, respectively) compared to the control (96% diseased plants) at 25 days after inoculation. For P. nicotianae on sesame, suspensions of actinomycete strains 8, 25, 29, 51 and 89 were applied individually by soil drenching before sowing and combined with collar spraying at one day after pathogen inoculation. All strains significantly reduced the percentage of plant infection, with strains 8, 51 and 89 consistently expressing high levels of disease reduction similar to treatment with the fungicide mancozeb. With respect to P. capsici on watermelon, spraying individual suspensions of strains 51, 22 and 120 twice before and after pathogen inoculation

significantly reduced fruit rot compared to the untreated control. Similarly, for C. gloeosporioides on chilli, spraying individual suspensions of five actinomycete strains one day before pathogen inoculation gave significant reductions in disease with lesion lengths on fruits ranging from 1.3 to 2.4 mm compared the untreated control (9.1 mm) at four days after inoculation. For R. solani in cabbage, soil drenching with suspensions of individual strains 4RM, 54 or 54RM significantly reduced fungal infection compared to the untreated control. Thus, percentages of infected plants were 33, 61 and 56, respectively, compared to 100% in the control treatment. In conclusion, these results clearly show that actinomycetes are promising agents for controlling fungal plant pathogens and in the future, the mechanisms by which the actinomycetes reduce disease will be investigated.

KEYWORDS : Actinomycetes, biological control, plant diseases

INTRODUCTION

Fungal and oomycete pathogens are very important in plants, causing significant yield losses. In sesame, Fusarium wilt and Phytophthora blight are the most important diseases, often causing devastating losses. Fusarium wilt or vascular wilt caused by *Fusarium oxysporum* f.sp. *sesami* has been reported to cause serve yield losses in tropical countries (El-Bramawy *et al.* 2009, Tien *et al*. 2012). Likewise, Phytophthora blight also causes severe yield losses and the disease spreads very fast to cause epidemics under favourable conditions, *e.g*., in India and the Mekong Delta of Vietnam (Gemawat & Prasad 1964; Nguyen Thi My Khuyen 2011).

Severe diseases, caused by fungi or oomycetes, are also found in other important crops. Examples are fruit rot disease in cucurbit plants caused by *Phytophthora capsici*, which commonly occurs under warm and humid conditions, where the disease can cause serve losses of up to 100% (Babadoost 2001). Furthermore, in vegetable crops such as brassicas, Rhizoctonia solani is an important pathogen, causing damping off and root rot (Agrios 2005). Likewise, Colletotrichum gloeosporioides, causing anthracnose of chilli, attacks leaves, stems and fruits as well as during post-harvest (Agrios 2005).

Chemical control of diseases is the main method employed by farmers: However, this method sometimes fails to protect the plants from disease due to development of fungicide resistance in the pathogens. In addition, fungicides pose a hazard for human health and the environment. Biological control based on the use of beneficial microorganisms for controlling plant diseases is a promising disease control strategy, which is considered an important aspect of integrated pest management in agricultural production (Raupach & Kloepper 1998; Yuliar et al. 2015). Actinomycetes are rhizobacteria present in high population densities in soil and they possess different mechanisms to reduce pathogen inoculum. Actinomycetes can produce various types of antibiotics, inhibiting different types of pathogens. They also secrete extracellular hydrolytic enzymes such as chitinases, glucanases and proteinases, which can degrade components of pathogen cell walls as well as nematode eggs (Boukaew et al. 2013; Choudhary et al. 2014; Evangelista-Martinez 2014).

In this study, we isolated actinomycetes from the rhizosphere of vegetable crops and screened them for antagonistic ability against the pathogens F. oxysporum

f.sp. sesami and Phytophthora nicotianae on sesame, against P. capsicum on watermelon, against Rhizoctonia solani on cabbage, against Colletotrichum gloeosporioides on chilli. Initially, in vitro tests were performed and promising strains with high antagonistic potential were further evaluated for ability to control the diseases caused by the pathogens under greenhouse conditions.

MATERIALS AND METHODS

Isolation of actinomycetes

Actinomycetes were isolated from rhizosphere soil of vegetables and sesame from different field in the Can Tho and An Giang provinces in Vietnam. Four 4 g soil was added into a centrifuge tube containing 40 ml sterile distilled water. The tube was shaken for 30 min to release microorganisms and 50 ml suspension from dilution of 10^{-3} and 10^{-4} were spread on plates containing chitin agar medium, a selective medium for actinomycetes (1 g colloidal chitin extract from 10 g crude chitin, 0.7 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4x7H_2O$, 0.01 g $FeSO_4$, 0.001 g $ZnSO_4$). Plates was incubated for 3 days and single actinomycete colonies were transferred to tubes containing mannitol soya flour (MS) medium (20 g soya flour, 20 g D-mannitol, 15-20 agar, 1000 ml distilled water, pH 7.0).

Dual culture tests of effect of isolated actinomycetes on fungal pathogens

The antagonistic ability of 187 strains of actinomycetes was tested in vitro against a range of fungal pathogens: *Fusarium oxysporum* f.sp. *sesami* (Fos), causing vascular wilt on sesame; Phytophthora nicotianae causing Phytophthora blight or black shank on sesame; *P. capsici* causing fruit rot in watermelon; *Colletotrichum gloeosporioides* causing anthracnose in chilli and *Rhizoctonia solani* causing damping off in cabbage. Plates containing ISP2 medium (4g yeast extract; 4g glucose; 10g malt extract; 17g agar; 1000ml distilled water, pH 7.0) were inoculated in the middle with a 5 mm plug from a 7 day old culture of the individual fungal pathogens. Subsequently, the actinomycete strains were inoculated in the margin of the plates, two strains on each plate at opposite sides of the fungal pathogen. The plates were incubated at room temperate for 4-5 days and inhibition zones of the fungal pathogen for each actinomycete strain were measured. Initially, only one test was performed for each strain and for those resulting in inhibition zones, a second test was performed with four replications (Table 1).

Disease reducing ability of actinomycetes in planta

F. oxysporum f.sp.sesami (Fos) on sesame

The experiment followed a completely randomized design with seven treatments: four treatments with individual actinomycete strains (3, 6, 25 and 79); a mixture of the four strains; the fungicide benomyl and a non-treated control. There were five replications for each treatment, one replication constituting a pot with five plants. *Fos* was cultured on potato dextrose agar (PDA) medium for 7-10 days and conidial suspensions for inoculation of $5x10^5$ conidia/ml were prepared. Actinomycetes were

cultured on MS medium for 7 days. Cell suspensions were harvested and the concentrations determined by diluting and spreading on MS medium to give a final concentration of 10^8 cfu/ml. Actinomycetes were applied by soil drenching with 5 ml suspension/plant at 10 day intervals, starting at 10 days after sowing. Pathogen inoculation took place by soil drenching at 30 days after sowing with 5 ml conidial suspension/plant. Autoclaved soil was used for the experiment. Disease was recorded when symptoms started to appear as percentage of infected plants in each pot at 5 days intervals. Furthermore, the area under the disease progress curve (AUDPC) was calculated.

Phytophthora nicotianae on sesame

The experiment followed a completely randomized design with seven treatments: five treatments with individual actinomycete strains (98, 25, 29, 51 and 89); a mixture of the five strains; spraying with the fungicide mancozeb and a non-treated control. There were five replications for each treatment, one replication constituting a pot with 10 plants. Actinomycetes were applied twice: initially by soil drenching before sowing with 10^7 cfu/g soil (each pot contained 3 kg of soil) and subsequently by spraying cell suspensions at the collar position at one day after inoculation (dai) with the pathogen, with 100 ml/pot. Pathogen inoculation was conducted by pipetting 5 ml zoospore suspension of *P. Nicotianae* ($5x10^4$ zoospores/ml)onto each plant at 20 days after sowing. As a fungicide treatment, mancozeb was sprayed with 100 ml/pot at one day before pathogen inoculation. Disease was recorded as percentage of infected plants at 3, 7 and 11 dai.

Phytophthora capsicion watermelon

The experiment followed a completely randomized design with five treatments and was conducted on detached fruits: three treatments with spray application of three individual actinomycete strains (22, 51 and 120, with 10^8 cfu/ml. Application took place twice: at one day before and one day after pathogen inoculation); spraying with the fungicide dimethomorph; spraying with the fungicide ethaboxam (both fungicides were applied twice: one day before and one day after inoculation) and a non-treated control. The pathogen was inoculated by wounding the fruits at their middle with a bundle of needles, followed by placing a plug of *P. capsici* (5 mm in diameter) at the wounded site. The fruits were incubated at room temperature (25^oC) in nylon bags with a plug of wet cotton. Disease was recorded by measuring the diameter of fruit lesions at 48, 60 and 72 hours after inoculation (hai).

Colletotrichum gloeosporioides on chilli

The experiment followed a completely randomized design with six treatments and was also conducted on detached fruits: five treatments with spray application of five individual actinomycete strains (4RM, 21RM, 54RM, 55RM or 58RM, with 10^8 cfu/ml. Application took place at one day before pathogen inoculation and a non-treated control treatment. The pathogen was inoculated by wounding the fruits at their middle with a bundle of needles, followed by spraying with a conidial suspension ($5x10^5$ conidia/ml) at the wounded site. The fruits were incubated at room

temperature (25^{o}C) in nylon bags with a plug of wet cotton. Disease was recorded by measuring the diameter of fruit lesions at 4, 5 and 6 dai.

Rhizoctonia solani on cabbage

The experiment was conducted under greenhouse condition following a completely randomized design with four treatments and seven replications: three treatments with individual actinomycete strains (4RM, 54 and 54RM) and a non-treated control. Actinomycetes were applied by soil drenching with cell suspensions (10^8 cfu/ml) and the pathogen was inoculated by mixing well the soil with a plate of *R. solani* (3 day-old culture), in which the cabbage seeds were sown. Disease was recorded as percentage of infected plants with damping off symptom at 6,10 and 14 dai.

Data analysis

Treatment mean in each experiment were compared using Duncan's Multiple Range Test. Data were analysed by the statistical software MSTATC.

RESULTS

Isolation and screening for antagonistic ability of actinomycetes against fungal pathogens.

A total of 187 actinomycetes were isolated from the rhizospheres of sesame and vegetable crop, with 12.0-15.6% of the strains possessing antagonistic ability against the tested fungal pathogens, i.e., *Fusarium oxysporum* f.sp. *sesami* (*Fos*), causing vascular wilt on sesame; *Phytophthora nicotianae*, causing Phytophthora blight or black shank disease on sesame; *P. capsici*, causing fruit rot in watermelon, *Colletotrichum gloeosporioides*, causing anthracnose in chilli and *Rhizoctonia solani* in cabbage. For each pathogen, several promising actinomycete strains showed large inhibition zones against individual fungal pathogens (Table 1) and these were chosen for testing of their ability to control diseases *in planta*. Thus, four strains (3, 6, 25 and 79) were tested for their ability to control *Fos* under greenhouse conditions. Five strains (8, 25, 29, 51 and 89) were tested against *P. nicotianae* in sesame. Three strains (51, 22 and 120) were tested against *P. capsici* in detached watermelon fruits. Five strains (4RM, 21RM, 54RM, 55RM and 58RM) were tested for ability to control anthracnose (caused by *Colletotrichum gloeosporioides*)in detached chilli fruits and three strains (4RM, 54 and 54RM) were tested against *R. solani* (causing damping off) in cabbage under greenhouse conditions.

Disease reducing ability of actinomycetes in planta

F. oxysporum f.sp. sesami (Fos) on sesame

Soil drenching with individual actinomycete strains or a mixture of four strains at 10 day intervals could significantly reduce percent plants with vascular wilt compared to the control and almost to the same level as treatment with benomyl at 25 dai (Table 2). AUDPC of each treatment was also significantly lower than the control and

benomyl treatments. Fig. 1 shows examples of the amount of disease for each of the treatments.

Table 1. Antagonistic ability of promising actinomycetes against fungal pathogens.

Strain	Inhibition zones (mm) of individual fungal pathogens				
	Fusarium oxysporu **(4 dai)[1]**	***Phytophthora nicotianae*** **(4 dai)**	***Phytophthora capsici*** **(4 dai)**	***Colletotrichum gloeosporioides*** **(4 dai)**	***Rhizoctonia solani*** **(1 dai)**
3	8.3	6.8	9.8	-[2]	--[3]
6	8.0	-	7.8	-	--
8	-	13.5	10.5	-	--
22	1.0	10.3	12.5	-	--
25	6.3	12.0	-	-	--
29	-	10.8	-	-	--
51	-	12.5	15.5	-	--
54	-	-	-	-	5.6
79	7.3	-	-	-	--
89	-	11.3	-	-	--
120	-	7.5	11.8	-	--
4RM	2.1	-	-	7.4	7.5
21RM	6.4	6.4	11.5	8.0	--
54RM	-	-	-	8.4	6.3
55RM	-	-	-	8.2	--
58RM	-	-	-	7.8	--

1 dai: days after inoculation; 2 -: no inhibition zone; 3--: not tested

Table 2. Effect of actinomycete strains on vascular wilt of sesame, caused by *Fusarium oxysporum* f.sp. *sesami*, under greenhouse conditions.

	Per cent infected plants (%)					AUDPC
Actinomycete	**10 dai**	**15 dai**	**20 dai**	**25 dai**	**30 dai**	
Strain 3	20.0 a	44.0 abc	60.0 ab	72.0 bc	88.0 ab	1150 b
Strain 6	20.0 a	28.0 bc	60.0 ab	80.0 b	88.0 ab	1110 b
Strain 25	20.0 a	36.0 abc	56.0 b	68.0 bc	92.0 a	1080 b
Strain 79	28.0 a	40.0 abc	52.0 b	68.0 bc	92.0 a	1100 b
Mixture (3,6,25,79)	44.0 a	52.0 ab	52.0 b	64.0 bc	80.0 ab	1150 b
Benomyl	12.0 a	24.0 c	40.0 b	52.0 c	60.0 b	760 c
Control	28.0 a	56.0 a	76.0 a	96.0 a	96.0 a	1450 a

Means in a column marked with the same letters are not significantly different at $P< 0.05$.

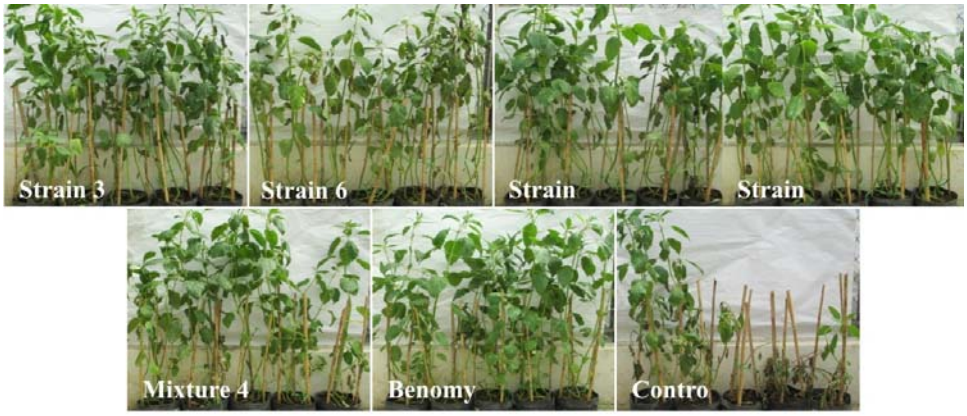

Fig 1.Vascular wilt of sesame caused by *F. oxysporum* f.sp. *sesami* after treatment with the individual actinomycete strains 3, 6, 25 or 79 or a mixture of them. Benomyl and an untreated control treatment were included for comparison. Images recorded at 25 dai.

Phytophthora nicotianae on sesame

Soil drenching before sowing and spraying the collar with suspensions of individual actinomycete strains (8, 25, 29, 51 or 89) one day after pathogen inoculation could reduce disease incidence significantly compared to the control (Table 3). Three strains (8, 51 and 89) showed the largest disease reductions, with an effect comparable to the fungicide mancozeb. Fig. 2 shows examples of the disease levels for the strains 8, 51 and 89 in comparison to treatments with mancozeb and the non-treated control.

Table 3. Effect of actinomycete strains on Phytophthora blight of sesame caused by *Phytophthora nicotianae* under greenhouse conditions.

	Percent infected plants (%)			
Actinomycete	**3 dai**	**7 dai**	**11 dai**	**13 dai**
Strain 8	10.0 abc	21.7 c	23.3 c	23.3 d
Strain 25	10.0 abc	33.3 abc	38.3 c	41.6 cd
Strain 29	8.3 abc	26.6 bc	38.3 bc	43.3 bcd
Strain 51	3.3 bc	21.6 c	33.3 c	36.6 cd
Strain 89	0.0 c	15.0 c	21.6 c	23.3 d
Mixture (8,25,29,51,89)	23.3 a	50.0 ab	68.3 a	70.0 ab
Mancozeb	5.0 abc	20.0 c	30.0 c	35.0 cd
Control	23.3 a	60.0 a	75.0 a	80.0 a

Means in a column marked with the same letters are not significantly different at $P< 0.05$.

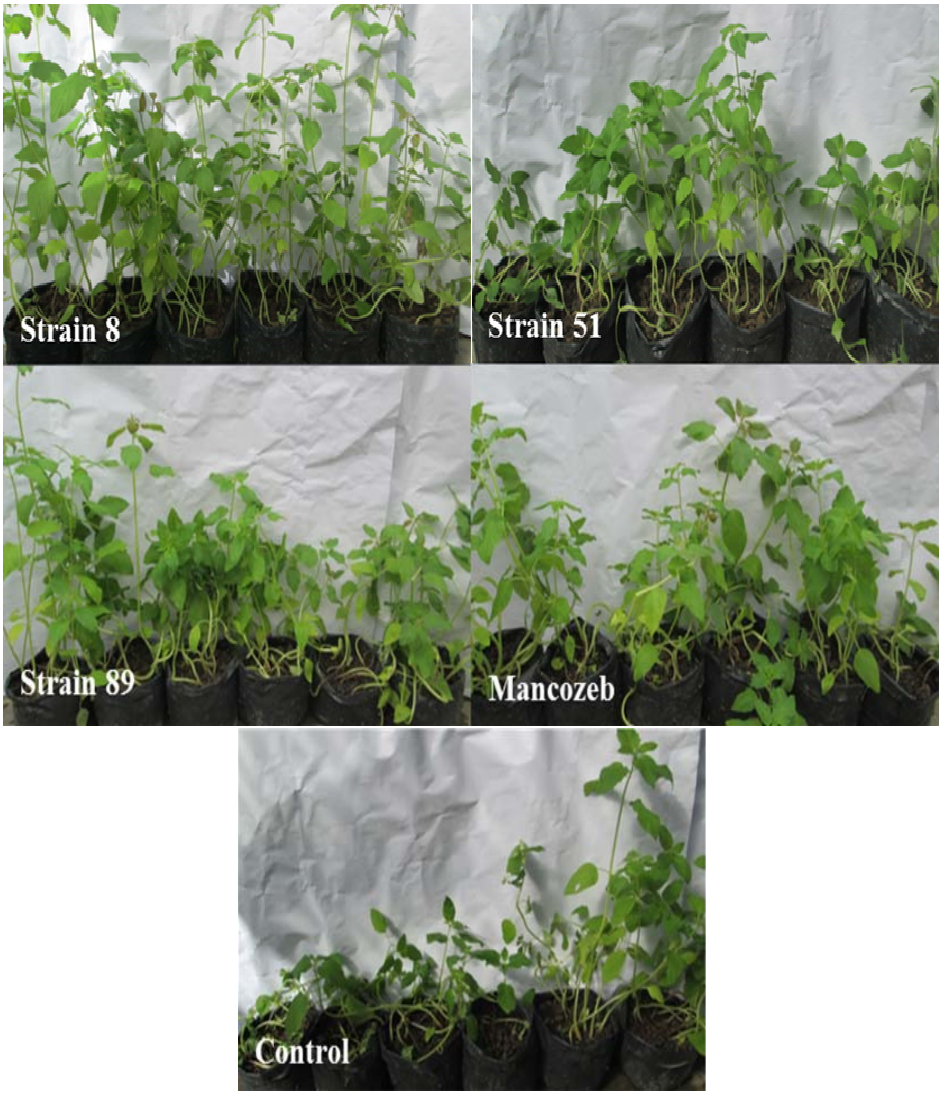

Fig 2. Phytophthora blight, caused by *Phytophthora nicotianae*, after treatment with the individual actinomycete strains 8, 51 or 89. The fungicide mancozeb and an untreated control treatment were included for comparison. Images recorded at 11 dai.

Phytophthora capsici on watermelon

Spraying actinomycete suspensions (10^8 cfu/ml) of three strains (22, 51 or 120) twice at one day before and one day after inoculation significantly reduced infection by *P. capsici* on detached watermelon fruits at the three time points recorded (Table 4). Spraying with the fungicides dimethomorph and ethaboxam twice at one day before and one day after inoculation completely protected the fruits from fungal infection. Fig. 3 shows fruit symptoms for actinomycete strains 22, 51 and 120 in comparison to treatments with dimethomorp and ethaboxam as well as a non-treated control.

Table 4. Effect of actinomycete strains on fruit rot, caused by *Phytophthora capsici*, on detached watermelon fruits

Actinomycete	Diameter of fruit lesions (mm)		
	48 hai	**60 hai**	**72 hai**
Strain 22	12.8 c	29.2 d	36.8 d
Strain 51	6.1 d	37.1 c	44.6 c
Strain 120	22.9 b	47.3 b	53.4 b
Dimethomorph	0.0 e	0.0 e	0.0 e
Ethaboxam	0.0 e	0.0 e	0.0 e
Control	31.8 a	52.6 a	64.3 a

Means in a column marked with the same letters are not significantly different at P< 0.05.

Fig 3. The effect of three different actinomycete strains (22, 51 or 120) on reduction of fruit rot caused by *Phytophthora capsici* in detached watermelon fruits at 60 h after inoculation. The fungicides dimethomorp and ethaboxam and an untreated control treatment were included for comparison.

Colletotrichum gloeosporioides on chilli

Spraying actinomycete suspensions (10^8cfu/ml) of five strains (4RM, 21RM, 54RM, 55RM and 58RM) at one dai on detached fruits reduced anthracnose severity (fruit lesion diameters) significantly compared to the control at 4,5 and 6 dai (Table 5). Fig. 4 shows fruit symptoms for actinomycete strains 58RM, 21RM, 4RM, 54RM and 55RM in comparison to a non-treated control.

Table 5. Effect of actinomycete strains in reducing anthracnose, caused by *Colletotrichum gloeosporioides* in detached chilli fruits.

Actinomycete	Lesion length on chilli fruits (mm)		
	4 dai	**5 dai**	**6 dai**
Strain 4RM	1.3 b	4.3 b	9.6 b
Strain 21RM	1.8 b	4.8 b	10.9 b
Strain 54RM	2.4 b	6.2 b	11.0 b
Strain 55RM	2.0 b	6.3 b	9.9 b
Strain 58RM	1.3 b	5.3 b	11.7 b
Control	9.1 a	15.4 a	24.5 a

Fig 4. Fruit rot, caused by *Colletotrichum gloeosporioides*, in detached chilli fruits after treatment with the individual actinomycete strains 58RM, 21RM, 4RM, 54RM or 55RM in comparison to a non-treated control. Images recorded at 4 dai.

Rhizoctonia solani on cabbage

Soil drenching of actinomycete suspensions (10^8 cfu/ml) of three strains (4RM, 54 and 54RM) significantly reduced damping off in cabbage. Thus, after actinomycete treatment, there were 32.9-60.8% diseased plants compared to 100% for the untreated control at 14 dai (Table 6 and Fig. 5).

Table 6. Effect of actinomycete strains in reducing damping off in cabbage, caused by *Rhizoctonia solani*, under greenhouse conditions.

Actinomycete	Percentage of infected plants (%)		
	6 dai	10 dai	14 dai
Strain 4RM	7.9 c	22.9 c	32.9 c
Strain 54	16.0 b	38.7 b	60.8 b
Strain 54RM	13.5 bc	33.9 b	56.2 b
Control	27.4 a	66.9 a	100.0 a

Means in a column marked with the same letters are not significantly different at P< 0.05.

Fig 5. Damping off in cabbage, caused by *Rhizoctonia solani*, after treatment with the individual actinomycete strains 4RM, 54RM or 54, compared to an untreated control treatment. Images recorded at 14 dai.

Collectively, the results presented here show that actinomycetes expressing antagonistic ability against fungal plant pathogens can also reduce disease incidence and severity in planta. Furthermore, several of the tested actinomycete strains were able to inhibit infection by at least one or more fungal pathogens. Previously, a versatile effect of actinomycetes in reducing fungal diseases has been reported (Evangelista-Martinez 2014; Kanini *et al*. 2013). In addition, to their potential for producing metabolites inhibiting fungal infection, they could also be envisioned to

induce host plant resistance (Zhao et al. 2012). Furthermore, actinomycetes possess an important characteristic, *i.e*., they can survive better in the phyllosphere than other types of biocontrol organisms since they belong to a group of bacteria with a high G+C content, which will help them to survive the UV irradiation in the phyllosphere (Gnanamanickam & Immanuel 2007).

CONCLUSION

Actinomycetes are beneficial bacteria present commonly in soil and rhizosphere. They constitute a promising group of potential biological control agents for fungal plant pathogens. Among 187 actinomycete strains isolated from the rhizosphere of vegetables and sesame crops, several strains showed efficacy in reducing infection of *Fusarium oxysporum* f.sp. *sesame* and *Phytophthora nicotianae* in sesame, *P. capsici* in watermelon, *Colletotrichum gloeosporioides* in chilli and *Rhizoctonia solani* in cabbage. These results clearly show that actinomycetes are promising agents for controlling soil- and air-borne fungal pathogens. The effect of these promising actinomycete strains in controlling the diseases under field conditions needs to be investigated as well as the precise mechanisms responsible for the disease reductions.

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22

APPLICATION OF INDUCE SYSTEMIC RESISTANCE ON VEGETABLES BY PLANT GROWTH-PROMOTING RHIZOBACTERIA, *BACILLUS VALLISMORTIS* EXTN-1 IN VIETNAM

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ABSTRACT

Treatment of Bacillus vallismortis EXTN-1 showed a broad spectrum of resistance to multiple plant pathogens caused by fungal, bacterial and viral pathogens as well as plant growth production. Mechanisms of ISR by EXTN-1 have been reported to oxidative burst, HR, lignifications, production of cyclo-dipeptide derivatives for ISR elicitor and activation of pathogenesis proteins when bacterial endospore suspension of EXTN-1 was treated to various plants. In sub tropical region, Viet Nam, plant diseases caused by Ralstonia solanacearum and Fusarium solani are major problems on important vegetables of Tomato and Potato. Bacterial wilt, Fusarium wilt and Foot rot caused by Ralstonia solanacearum, Fusarium oxysporum, and Phytophthora capsici respectively, continue to be severe problems to tomato, potato and black pepper growers in Vietnam. EXTN-1 (Bacillus vallismortis) was the most effective, providing a mean level of disease reduction 80.0 to 90.0% against Bacterial wilt, Fusarium wilt and Foot rot diseases under greenhouse conditions substilis) also significantly reduced bacterial wilt, Fusarium wilt under greenhouse conditions. Bio-prouduct, EXTN-1 with the greatest efficacy under greenhouse condition was tested for the ability to reduce bacterial wilt, fusarium wilt and foot rot under field condition at Song Phuong and Thuong Tin locations in Ha Tay province, Vietnam. In field condition, EXTN-1 provided a mean level of disease reduction more than 45.0% against all three diseases compared to water treated control. Besides, EXTN-1 treatment increased the yield in tomato plants 17.3% than water treated control plants.

KEYWORDS: Biological control, Bacterial wilt, Fusarium wilt, EXTN-1, PGPR-mediated ISR and Tomato, Foot rot

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is one of the economically important diseases and this disease is of world-wide distributed, having been reported in at least 32 countries. It causes 80% loss in severe cases (McGrath *et al.* 1987; Malthotra *et al.* 1993). *Ralstonia solanacearum* is another important soilborne bacterial plant pathogen with a worldwide distribution and a wide host range of more than 200 species in 50 families (Hayward, 1991). It causes a lethal bacterial wilt disease of diverse plants viz., tomato, potato, eggplant, pepper, tobacco, banana, chilli, and peanut (French and Sequeira, 1970. In Vietnam, tomato, potato and black pepper among economically important crops are high value export crops. However, the production of these crops is remarkably being reduced by economically important soilborne bacterial and fungal pathogens. The weather conditions also influence the pest development in Vietnam (Truong *et al.* 2008; Doan and Nguyen, 2006). In Vietnam, potato is, as a winter crop, the second most important food crop after maize and some 30,000-35,000 hectares of potatoes are cultivated. Control of these soilborne diseases is mainly through chemical soil fumigation and resistant cultivars. However, the broad-spectrum biocides used to fumigate soil before planting are environmentally damaging. Thus, the management of these diseases with chemical measures has proved impractical mainly because of the appearance of fungicide-resistant strains (Hide *et al.* 1992).

Plant growth promoting rhizobacteria that have been successful in promoting the growth of crops such as canola, soybean, lentil, pea, wheat and radish have been isolated (Kloepper *et al.* 1988; Glick *et al.* 1997; Timmusk *et al.* 1999). In this study, three bio-products, EXTN-1 (*Bacillus vallismortis*), ESSC (*Bacillus* sp. and *Paenibacillus* sp.) and MFMF (*Bacillus substilis*) were evaluated against soilborne diseases of Bacterial wilt, Fusarium wilt (tomato and potato) and Foot rot (black pepper) under greenhouse and field conditions at Song Phung, Hoai and Thanh Tin, Ha Tay province of Vietnam.

MATERIALS AND METHODS

Microorganisms and Experimental site

Greenhouse and field experiments were conducted at Hoai Duc and Thanh Oai of Ha Tay province (located at 21º09' N Lat105º 25' E Long 16.4 m) in Vietnam. Three bio-products, EXTN-1 (Park, *et al.* 2001), ESSC (*Bacillus* sp. and *Paenibacillus* sp. at concentration of $1x10^7$ cfu/g) and MFMF (*Bacillus substilis* at concentration of $1x10^7$ cfu/ml) were used in greenhouse and field experiments. *Fusarium oxysporum* and *Phytophthora capsici* causal agents of bacterial wilt, fusarium wilt and foot rot diseases, respectively. The bacterial and fungal pathogens were maintained on sucrose peptone agar (SPA) plates at 28ºC and potato dextrose agar (PDA) plates at 24ºC, respectively.

Preparation of pathogen inoculum

Bacterial pathogen, *R. solanacearum* was grown in sucrose peptone broth (SPB) at 30ºC for 24 h at 150 rpm. Bacterial suspension of *R. solanacearum* was adjusted to 2 x 10^7 cfu/ml by counting with hemacytometer. Fungal pathogens, *P. capsici* and *F. oxysporum* were grown on V8 juice agar and potato dextrose agar (PDA) medium for two weeks, respectively. Zoosporangia of *P. capsici* and conidial spores *F. oxysporum* were scrapped from medium by adding sterile water and they were adjusted to 10^4 sporangia/ml and 10^6 conidia/ml, respectively, by counting with hemacytometer.

Greenhouse experiment

Tomato seeds were surface-sterilized with 2% sodium hypochlorite for 2 min, washed thoroughly with sterilized water, and planted into pots of sterilized soil. After 4 weeks, before transplanting, seedling roots were dipped into diluted bacterial suspensions (10^6 cfu/ml concentration) of EXTN-1, ESSC, MFMF and water for 1 h. Then, seedlings were transplanted into pots containing experimental seedbed soil and grown in the greenhouse at 25-35ºC. After 2 days of transplanting, each 30 ml of bacterial suspension of *R. solanacearum* (2 x 10^7 cfu/ml) and conidial suspension of *F. oxysporum* (10^5 spores/ml) were soil drenched separately. In the experiments on potato, seed tubers of Ackersegen and Diamond varieties were given treatment as described above to tomato seedlings. In the black pepper experiment, disease-free single node (approximately 8 cm length) cuttings were surface-sterilized with 2% sodium hypochlorite for 2 min, washed thoroughly with sterilized water. Before planting, black pepper cuttings were dipped into bacterial suspension of EXTN-1, ESSC, MFMF and water for 1h. Then, they were planted into pots containing experimental seedbed soil and grown in the greenhouse at 25-35ºC. After 2 days, a 30 ml of zoosporangia suspension of *P. capsici* (10^4 sporangia/ml) was soil drenched around the stem. Subsequent applications of bacterial suspension of EXTN-1, ESSC, MFMF and water were given as foliar spray at 7, 15 and 30 days after planting.

Field experiment

For tomato and potato experiments, each plot was 5m in length and 2m in width in one hectare and a number of sixty seedlings were planted in each plot. The bacterial suspension of EXTN-1 was diluted (10^6 cfu/ml concentration) with water, mixed evenly into the organic fertilizer, and applied to the soil. Tomato and potato transplantings were conducted 7 days later.

Statistical analysis

In this study, all data were analyzed with JMP (a PC-version of SAS) (SAS Institute. 1995) software. Significant differences in treatments were determined using Student's T test as LSD at $P = 0.05$.

RESULTS

Root drench and foliar application of bio-products significantly reduced severity of bacterial wilt on tomato and potato, fusarium wilt on tomato and foot rot on black

pepper under greenhouse conditions (Table 1-3). There were upto 91% and 85.5% reduction in severity of bacterial wilt was observed in PT 18 and Poland varieties, respectively treated with EXTN-1, ESSC and MFMF. In potato, bacterial wilt disease was significantly reduced on potato varieties Ackersegen (81.9 to 87.3%) and Diamand (87.0 to 92.8%) treated with three bio-products compared to control treatment under greenhouse condition (Table 2). Application of three bio-products on black pepper reduced the disease incidence of foot rot by 26.6 to 40% under greenhouse condition (Table 3). In greenhouse study, reduction in intensity of disease provided by three bio-products varied with pathogen tested. In field, EXTN-1 treatment significantly reduced disease incidence of bacterial wilt, fusarium wilt and foot rot as well as it enhanced the yield in tomato plants. Furthermore, EXTN-1 treatment increased yield (17.3%) in tomato compared to control (Table 4). By EXTN-1treatment, disease reduction of bacterial wilt and fusarium wilt on potato variety, Diamand was observed to 48.7 and 53.2%, respectively (Table 5).

DISCUSSION

The results indicated that bio-products of PGPRs provided significant disease protection against specific tested soilborne bacterial and fungal pathogens under greenhouse and field conditions. While bio-products, ESSC and MFMF afforded the greatest protection against *R. solanacearum, F. oxysporum* and *P. capsici*, EXTN-1 was the best at controlling diseases. Bio-product, EXTN-1stood out in terms of performance since it reduced the intensity of three diseases tested and thus it was chosen for field study. In previous studies, EXTN-1 has been reported to be involved in induction of systemic resistance on tomato, potato and rice against bacterial, fungal and viral pathogens (Park *et al*. 2006a, b; Park, *et al*. 2007). Moreover, significant disease control on tomato varieties, potato varieties and black pepper by EXTN-1 treatment indicates that the effects of genotype of different varieties did not influence on EXTN-1.

Table 1 Tomato plant protection against *R. solanacearum* and *F. oxysporum* by selected PGPRs under greenhouse condition

[a] Treatment	[b] Diseased plants (%)			
	Ralstonia solanacearum		***Fusarium oxysporum***	
	PT 18	**Poland**	**PT 18**	**Poland**
Control	89.5	98.3	92.0	98.2
EXTN-1	8.0*	14.2*	7.5*	13.4*
ESSC	10.2*	17.0*	8.5*	18.2*
MFMF	11.3*	20.2*	10.0*	20.0*
LSD(p=0.05)	4.6	3.6	3.5	3.7

[a] Treatment included EXTN-1, ESSC, MFMF bio-products and control (water treated);
[b] mean of disease incidence of three replications in percentage; Each replication consisted twenty plants
* = significantly different (p=0.05) according to Student's Least Significant Difference (LSD) test

Table 2. Potato plant protection against Bacterial wilt by selected PGPRs under greenhouse condition

[a] Treatment	[b] Diseased plant (%)	
	Ackersegen	**Diamand**
Control	96.3	84.5
EXTN-1	12.2*	6.1*
ESSC	15.2*	9.1*
MFMF	17.4*	11.0*
LSD (p=0.05)	4.0	3.8

[a] Treatment included EXTN-1, ESSC, MFMF bio-products and control (water treated)

[b] mean of disease incidence of three replications in percentage; Each replication consisted twenty plants

* = significantly different (p=0.05) according to Student's Least Significant Difference (LSD) test

Table 3. Black pepper protection against *P. capsici* by selected PGPRs under greenhouse condition

[a]Treatment	[b] Diseased plant (%)
Control	60.0
EXTN-1	36.0*
ESSC	40.0*
MFMF	44.0*
LSD (p=0.05)	5.6

[a] Treatment included EXTN-1, ESSC, MFMF bio-products and control (water treated)

[b] mean of disease incidence of three replications in percentage; Each replication consisted twenty plants

* = significantly different (p=0.05) according to Student's Least Significant Difference (LSD) test

Table 4. Yield increase and tomato plant production against two major plant pathogens by treatment of *Bacillus vallismortis* EXTN-1 at Song Phuong, Hoai Duc, Ha Tay province, Vietnam

Treatment	**Diseased plant (%)**				**Yield (Ton/ha)**
	Ralstonia solanacearum		***Fusarium oxysporum***		
	60 days	**120 days**	**60 days**	**120 days**	
Control	11.3	15.3	6.7	10.7	41.890
EXTN-1	5.3*	8.0*	3.3*	6.0*	50.645
LSD (p=0.05)	2.6	2.6	2.0	2.6	-

[a] Treatment included EXTN-1and control (water treated); [b] mean of disease incidence of three replications in percentage; Each replication consisted sixty plants; * = significantly different (p=0.05) according to Student's Least Significant Difference (LSD) test

Table 5. Potato plant protection against two major plant pathogens by selected PGPRs under field condition during winter season in Vietnam

[a] Treatment	[b] Diseased plant (%)	
	Ralstonia solanacearum	***Fusarium oxysporum***
Control	15.6	21.8
EXTN-1	8.0*	10.2*
LSD (p=0.05)	1.3	1.4

[a] Treatment included EXTN-1and control (water treated)

[b] mean of disease incidence of three replications in percentage; Each replication consisted sixty plants

* = significantly different (p=0.05) according to Student's Least Significant Difference (LSD) test

Advancing from greenhouse trials to field trials is an important step toward the goal of practical applications of ISR elicited by PGPR Kloepper, *et al*. 2004). There are many examples over the control of soilborne disease by 56% using Bacillus strains in greenhouse (Lemessa & Zeller, 2007) or 30 to 65% reduction at field level with inconsistence performance (Larkin & Fravel, 1998). In a study, Jubina & Girija (1998) reported the highest diseases suppression of foot rot of black pepper in the *in vivo* biological control assay using *Bacillus* sp. in nursery plants.

In this study, we estimated plant growth promotion on tomato along with bio control activity by treatment with EXTN-1 under field condition. Impact of root inoculation and foliar spray with EXTN-1 explored on some quality parameters in field experiments on tomato viz., increased fruit size, fleshy part of tomato, green pigmentation in leaf and age of the plant along with yield. The yield increase was recorded 17.3% compared to water treated control. Similarly, Guo *et al*. (2004) has reported the increased yield in tomato with formulated PGPR products in field trials. In PGPR formulations, the shelf life of a biocontrol product is mainly depended on the characteristics of the biocontrol agent itself. In this case, bioproduct, EXTN-1 has assumed to have long shelf life due to accounted for protection of crops upto harvesting stage. And also, one of the most important problems in biocontrol using microbial products is the storage time of living microbes. In a previous study, Vidhyasekaran, et al. (1997) has reported the problems in storing unformulated bacterial suspensions. By EXTN-1 treatment, tomato plant survival time was recorded to be increased (data not shown) and this feature might account for increased yield in tomato. Violante & Portugal (2007) reported that Bacillus strains have positive effects on tomato fruit quality attributes, particularly on size and texture. And, in field experiments, plants were supplied with the proper amounts of all nutrients since facilitating plant nutrition could be the mechanism by which PGPR enhance crop yield and fruit size (Bar-Ness et al. 1992; Richardson, 2001).

In early study, EXTN-1 (*B. vallismortis*) had been observed to induce systemic resistance against various pathogens (Park *et al*. 2001). Ahn *et al*. (2001) has reported that the major mechanisms by which EXTN-1 bring about disease suppression in crops is by the induction of systemic resistance to the host plant. And, these resistance mechanisms of EXTN-1 have been proven to be effective against

bacterial, fungal and viral pathogens of different crops (Ahn *et al*. 2002; Park *et al*. 2006a; 2006b). In this study, EXTN-1 treatment has played significant role on disease control of soilborne bacterial and fungal pathogens as well as plant growth promotion under both greenhouse and field conditions in Vietnam. The results from our studies suggests that application of *B. vallimortis* EXTN-1 can provide non-specific disease resistance as well as plant growth promotion on different host plants.

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INDUCTION OF SYSTEMIC RESISTANCE AND TOLERANCE AGAINST BIOTIC AND ABIOTIC STRESS IN CHINESE CABBAGE BY CYCLIC PEPTIDES PRODUCING *BACILLUS VALLISMORTIS* STRAIN BS07M

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ABSTRACT

Cyclic peptides (CPs) from plant growth promoting rhizobacteria (PGPR) and their effect on growth and disease control of plants have been in current research trends. In this study, we presented the Bacillus vallismortis strain BS07M (BS07M) as a potential biocontrol agent for disease control and alleviation of abiotic stress such as heat and salinity in Chinese cabbage. Treatment of BS07M improved plant growth in terms of fresh weight and leaf size, and elicited induced systemic resistance (ISR) against soft rot disease in Chinese cabbage. Treated plants showed increased tolerance against salt and heat stresses under greenhouse conditions. One of the CPs derived from BS07 was iturin derivatives. Application of BS07M in combination with another potential dipeptide Q3 showed an enhanced affect on disease resistance and abiotic stress tolerance. Real-time PCR analysis showed an enhanced expression of defense-related gene PR1 in BS07M and Q3 treated plants when challenged with soft rot pathogen Pectobacterium carotovorum. Similarly, a combination treatment of BS07M and Q3 in plants subjected to heat and salt stress showed anup-regulation of expression of abscissic acid (ABA) responsive genes RD29A and KIN1. Expression of hyperosmotic salinity response gene P5CS was significantly higher in a combination treated plants under salt stress. The results from this study indicated that BS07M is a potential bio-agent for ISR against pathogens as well as tolerance against abiotic stress with CPs in Chinese cabbage.

INTRODUCTION

Biological control of plants by microorganisms is one of the promising and alternative ways to the use of pesticides, which are often expensive with adverse effects on humans. Plant growth promoting rhizobacteria (PGPR) are potential biocontrol agents for disease control and induced systemic resistance (ISR). Several elicitors are reported for ISR against various phytopathogens. Among the elicitors, cyclic peptides play a crucial role in establishment of ISR. Antimicrobial cyclic lipopeptides (CLPs) including fengycin, iturin, bacillomycin and surfact in from *Bacillus* spp. play a key role in biocontrol of pathogens (Arguelles-Arias et al. 2009; Kim et al. 2010). Iturins are broad-spectrum antifungal and antibacterial heptapeptides (Pecci et al. 2010; Zeriouh et al. 2011). It is divided into several classes based on the variation of amino acids in peptide moiety and iturin A is secreted by most bacilli strains (Pecci et al. 2010). It is one of the most popular antimicrobial lipopeptides used for the biocontrol of fungal and bacterial plant diseases (Kim et al. 2010; Pecci et al. 2010; Zeriouh et al. 2011). Apart from displaying strong antimicrobial activity, iturin is reported to affect cell spreading, swarming and biofilm formation thus, favoring root colonization of *Bacillus* strains (Hofemeister et al. 2004; Leclere et al. 2006). Although the importance of surfactin and fengycin in ISR has been demonstrated, iturin was rendered ineffective in inducing resistance against cucurbit powdery mildew in melon (Garcia-Gutierrez et al. 2013; Ongena et al. 2007). The present study investigated whether iturin producing *Bacillus vallismortis* strainBS07M alone or in combination with another CP designated as a Q3 for ISR against soft rot disease caused by *Pectobacterium carotovorum* subsp. carotovorum SCC1 (SCC1) and induced systemic tolerance (IST) against salt and heat stress in Chinese cabbage.

MATERIALS AND METHODS

To study the effect of BS07M on growth of Chinese cabbage, three-week-old seedlings were treated with 50 mL of the BS07M suspension (1×10^6 cfu/mL) by soil drenching in plastic pots (15 × 12 cm) containing soilless potting mix (TKS2, Flora Gard Ltd., Germany). The plants were kept for 4 weeks in greenhouse and fresh weight and leaf size were recorded. Distilled water and 0.1 mM Benzothiadiazole (BTH) treatments were taken as negative and positive controls; respectively.Leaf samples were collected and checked for ISR against SCC1 in square plate. SCC1 suspension ($1x10^8$cfu/ml) was loaded in sterile paper discs and placed on leaf samples in moist tissue paper. The disease severity was recorded after incubation at 28°C for 24h as percentage lesion area (%).For IST, plants were exposed to salinity stress (high concentration of NaCl) and heat stress (40°C). Mortality rate was assessed in treated versus control plants. For real time PCR analysis of gene expression, Chinese cabbage was grown in MS media at 22°C for 7 days after which roots were treated with fusaricidin (1.0 ppm) and grown for another 5 days. For biotic stress, plant roots were challenged with SCC1 suspension ($1x10^8$cfu/ml) and samples collected after 6h. For abiotic stress, plant roots were treated with 250mM NaCl (salt stress) or incubated at 40°C (heat stress). Samples were collected after 6h of treatment for gene expression study. Total RNA was isolated and expression of

gene *PR1* (biotic stress), *P5CS, RD29A,* and *KIN1* (abiotic stress) were studied by RT-PCR.

RESULTS AND DISCUSSION

Treatment of BS07M in combination with dipeptide Q3 improved plant growth (Fig. 1). As compared to untreated control, BS07M alone and a combination treatment increased fresh weight of plants (Table 1).

Table 1. Plant growth promotion by combination treatment of BS07M with dipeptide Q3

Treatment	Fresh weigh (kg)
Control	2.00 ±0.86
0.1mM BTH	1.60±0.44
BS07M	2.50±1.07
Q3	3.30±0.58
BS07M+Q3	3.80±0.60

Fig. 1. Plant growth promotion by combination treatment of BS07M with dipeptide Q3

Fig 2. Soft rot disease control by combination treatment of BS07M and Q3

In cubic plate assay, ISR was observed in leaves from plants treated with BS07M+Q3 (Fig. 2). The disease control ability of *Bacillus* is related to the production of a large array of biologically active non-ribosomal peptide synthesases (NRPS) and polyketide synthases (PKS) secondary metabolites (Arguelles-Arias et al. 2009; Ongena & Jacques 2008). Among these, antimicrobial peptides have been implicated in the biocontrol of several plant pathogens and in the promotion of plant growth (Ongena & Jacques 2008). In the present study, BS07M produced seven

different iturin A analogs. Iturin A is one of the most popular antifungal lipopeptides used for the biocontrol of fungal and bacterial plant diseases (Pecci et al. 2010; Qian et al. 2012;Yu et al. 2002). Treated plants showed increased tolerance against salt and heat stresses under greenhouse condition (Fig. 3).

Real-time PCR analysis showed enhanced expression of defense-related gene *PR1* in BS07M and Q3 treated plants when challenged with soft rot pathogen SCC1 (Fig. 4). Treated plants when subjected to abiotic stresses such as heat (Fig. 5) and salinity (Fig. 6) showed an increase in the expresiion of ABA-responsive genes *KIN1, P5CS* and *RD29A* in plants treated with BS07M and dipeptide Q3.Enhanced expression of PR-protein PR1 and RAB18 in fusaricidin treated plants as compared to control when challenged with SCC1 indicated its ability for ISR (Park et al. 2009). Increased expression of marker genes *P5CS, RD29A* and *KIN1* under salt stress and *RD22* under heat stress denoted the triggering of ABA-responsive defense mechanism in fusaricidin treated plants to survive under abiotic stress.

Fig. 3. IST to high temperature by treatment of BS07M and Q3 on Chinese (38ºC 12h)

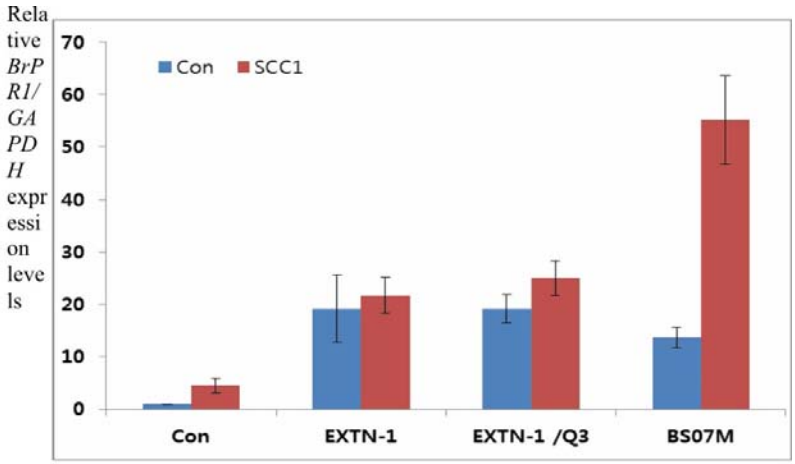

Fig. 4. Enhanced expression of defense-related gene *PR1* in response to SCC1 in plants treated with BS07M alone or combined with Q3

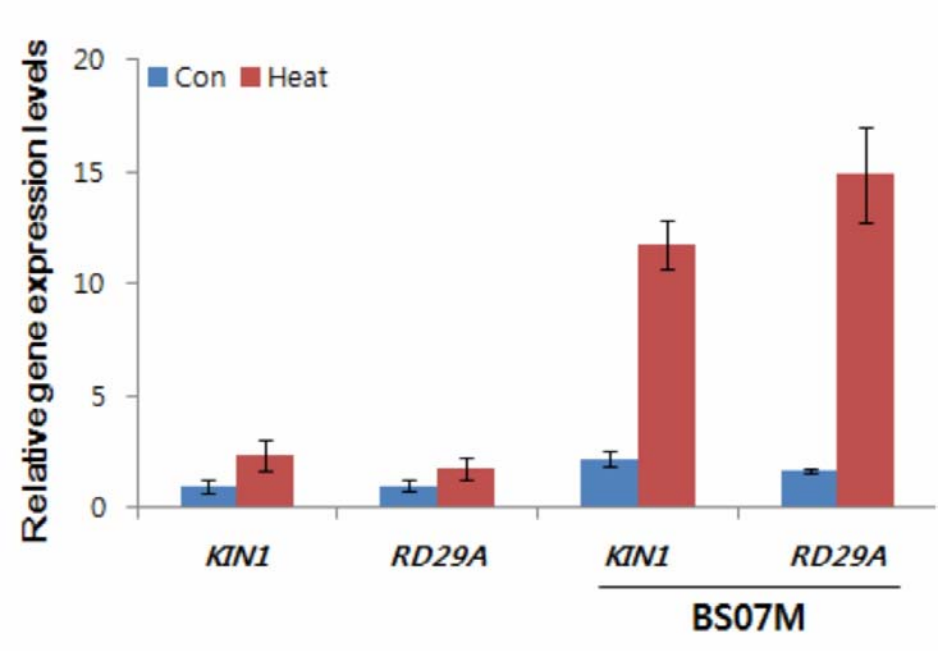

Fig. 5. Enhanced expression of ABA responsive genes*KIN1*and *RD29A* in plants treated with BS07M when subjected to heat stress

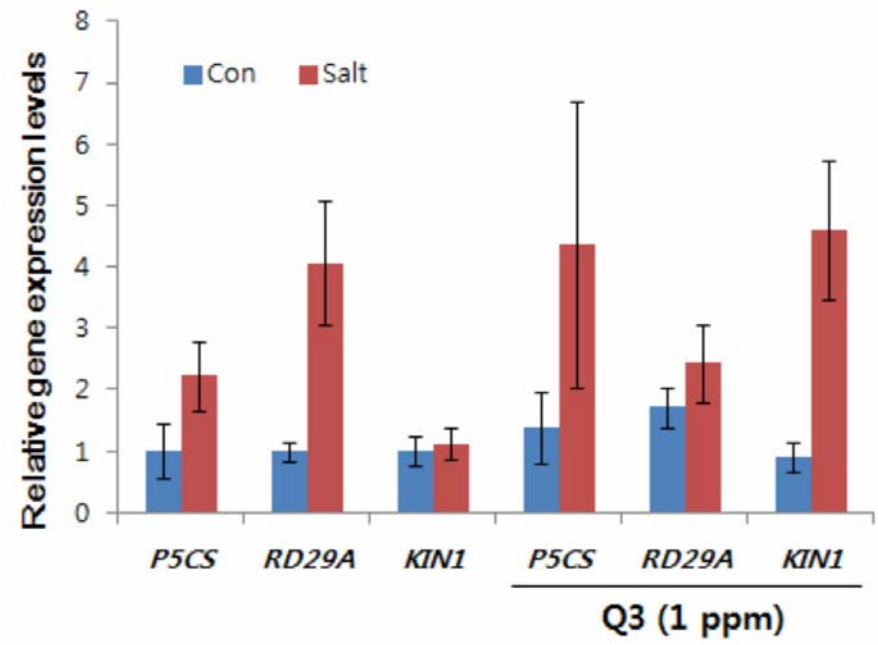

Fig. 6. Enhanced expression of ABA responsive genes KIN1, P5CS and RD29A in plants treated with dipeptide Q3 when subjected to high salinity

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POTENTIAL USE OF RHIZOBACTERIA IN GROUNDNUT GROWN IN SANDY SOIL IN BINH DINH PROVINCE OF VIETNAM

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ABSTRACT

Groundnut (Arachis hypogaea L.) is an important food crop in Vietnam. Binh Dinh province in Vietnam has large area of cultivated groundnut in sandy soil which is low in fertility and drought prone area. Biocontrol using plant growth promoting rhizobacteria (PGPR) may represent a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops. PGPR are generally considered a heterogeneous group of bacteria that live in the plant rhizosphere, where they contribute to plant growth and improve stands under stress conditions. PGPR can improve growth through various mechanisms and have been introduced to soil, seeds or roots to enhance plant growth and health. The present study was conducted to investigate nitrogen fixing bacteria (Bradyrhizobium japonicum), phosphorous solubilizing bacteria (Bacillus megaterium), silicate bacteria (Paenibacillus castaneae) and polysaccharide synthesized yeast (Lipomyces starkeyi). These microorganisms were isolated from rhizosphere and nodules of groundnut. Under greenhouse condition, our results showed that use of mix microorganisms and reduced 10 - 20% NPK for groundnut increased content of total N, P, K in the stems, leaves, increased the plant height, enhanced green biomass and increased the yield pod compared with control (using 100% NPK and no microorganisms). In the field condition, use microbial inoculants for groundnut has a positive effect on the content of absoluble NPK, humidity and indensity of beneficial microbes in the soil. Use microbial inoculants and 100% basal NPK mineral for groundnut increased the humidity of soil (18.7%), increased the plant height (17.0%), enhanced green biomass (16.7%), more yield pod (14.0%) and increased the benefit 8.1 milion VND/ha compared with control (using 100% basal NPK and no microbial inoculants). Also using microbial inoculants and reduced 10 - 20% NPK for groundnut increased the humidity of soil (8.0 - 9.8%), increased the plant height (8.2 - 12.5%), enhanced green biomass (11.9 - 12.4%), more yield pod (8.2 - 11.3%) and increased the benefit 4.61 - 6.48 million VND/ha compared with control (using 100% NPK and no microbial inoculants). Our studies

clearly show that the use of combination of microorganisms tested has the potential to commercialize microbial inoculants for use in groundnut in sandy soil in Binh Dinh province of Vietnam.

KEYWORD: Groundnut, microbial inoculants, nitrogen fixing bacteria, phosphate solubilizing bacteria, polysaccharide synthesized yeast, sandy soil, silicate bacteria.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important food crop in Vietnam. Binh Dinh province in Vietnam has large groundnut cultivated area in sandy soil which is low in fertility and drought prone area.

Biocontrol using plant growth promoting rhizobacteria (PGPR) may represent a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops. PGPR are generally considered a heterogeneous group of bacteria that live in the plant rhizosphere, where they contribute to plant growth and improve stands under stress conditions. PGPR can improve growth through various mechanisms and have been introduced to soil, seeds or roots to enhance plant growth and health.

The present study were conducted to investigate nitrogen fixing bacteria (*Bradyrhizobium japonicum*), phosphate solubilizing bacteria (*Bacillus megaterium*), silicate bacteria (*Paenibacillus castaneae*) and polysaccharide synthesized yeast (*Lipomyces starkeyi*) and evaluate the commercial potential of microbial inoculants used for groundnut in sandy soil in Binh Dinh province of Vietnam.

MATERIALS AND METHODS

Nitrogen fixing bacteria (RA18), phosphate solubilizing bacteria (P1107), silicate bacteria (S3.1), polysaccharide synthesized yeast (PT5.1) isolated from rhizosphere, groundnut nodules and groundnut varieties LDH01 provided by Institute for Southern Coastal Central of Vietnam were used for the study.

The nitrogenase activity of N-fixing bacteria was determined by using the acetylene reduction method (Vietnam standard, 2010). The phosphate or potassium solubilizing capability of the bacteria was described by the diameter of solubilizing zone on the Pikovskaya or Aleksandrov agar medium (Vietnam standard, 1996, Hu et al. 2006). The polysaccharide synthesized capacity of the yeast was determined by the viscosity of the suspension by Ostwald viscometer (Nguyen Kieu Bang Tam, 2009). The Indole Acetic Acid (IAA) producing activity of the bacteria was evaluated by Salkowski modified method (Misra et al. 1989). Taxonomic classification of microbes was based on the partial sequence of 16S rDNA or 28S rDNA. All sequences were blasted against the type strains in the ribosomal database to identify taxa of each strain.

To evaluate the effect to the nutrient uptake of groundnut, the greenhouse trial were conducted in completely randomized design with 5 replications. Groundnut was growth on pot with 10 kg soils and fertilized with different NPK doses depend on the biological activity of selected microbes. As control 100% NPK (urea 0.64 g pot^{-1}, super phosphate 2.5 g pot^{-1}, potassium chloride 0.5 g pot^{-1}) without inoculant was

used. Treatments were the reductions of 10%, 20% and 30% recommended dose of N, P or K with the inoculant of single or mixed strains.

Field trial were also conducted in randomized block design with 3 replications. The recommended dose of NPK (30.60.90/ha) was used as control without inoculation. The treatments were same the greenhouse trial. The density of beneficial microbes were 10^8 CFU/g and 20 kg inoculant applied for ha. Evaluated parameters were the nutrition uptake, the growth and yield groundnut and the economical benefit of groundnut production. IRRISTAT was used for statistical analysis.

RESULTS AND DISCUSSTION

Evaluation of beneficial microbes

Microorganisms was isolated from rhizosphere and nodules of groundnut and classified by sequencing the 16S rDNA or 28S rDNA as *Bradyrhizobium japonicum* for strain of RA18, *Bacillus megaterium* strain of P1107, *Paenibacillus castaneae* for strain of S3.1 and *Lipomyces starkeyi* for strain of PT5.1.The bioactivity of selected microorganisms presented in Table 1.

Table 1. The bioactivity of selected microorganisms

Microbial strains	Bioactivity
RA18	Nitrogen fixation: 3,458 nmol ethylene/plant
	Plant growth promoting (μg IAA/ml): 46.18 μg
P1107	Phosphate solubilization (diameter of solubilizing zone): 18 mm
	Plant growth promoting (μg IAA/ml): 26,13 μg
S3.1	Silicate solubilization (diameter of solubilizing zone): 12 mm
	Polysaccharide production (viscosity): 12.8 x $10^{-3}\eta$ N.s/m^2
PT5.1	Polysaccharide production (viscosity): 37.6 x $10^{-3}\eta$ N.s/m^2

Effect of selected microorganisms on the nutrien uptake and growth, yield of groundnut in greenhouse condition were presented in tables 2, 3 and 4.

Table 2. Effect of nitrogen fixing bacteria (RA 18) on the N uptake and growth, yield of groundnut (greenhouse condition).

Treatment	N content of sterms and leaves (g/pot)	Plant height (cm)	Effective nodules (nodules/ plant)	Dry biomass (g/pot)	Pod yield (g/pot)
Control (No bacteria, 100% NPK)	1.55 a	60.4 a	185.9 a	81.8 a	25.50 a
N-fixing bacteria, reduction of 10% N	1.98 b	66.1 b	216.0 b	88.4 b	27.82 b
N-fixing bacteria, reduction of 20% N	1.80 ab	63.5 ab	207.1 b	86.8 b	27.61 b
N-fixing bacteria, reduction of 30% N	1.59 a	62.6 a	200.6 a	83.5 a	26.38 ab

Means with the same letters in a column do not differ significantly at 0.05 level.

Table 3. Effect of phosphate solubilizing bacteria (P1107) on the P uptake and growth, yield of groundnut (greenhouse condition)

Treatment	P content of sterms and leaves (g/pot)	Plant height (cm)	Dry biomass (g/pot)	Pod yield (g/pot)
Control (No bacteria, 100% NPK)	0.18 ab	60.4 a	81.8 c	25.50 ab
P-solubilizing bacteria, reduction of 10% P	0.20 b	64.6 b	85.9 c	26.77 b
P-solubilizing bacteria, reduction of 20% P	0.18 ab	63.2 ab	82.5 b	26.62 ab
P-solubilizing bacteria, reduction of 30% P	0.17 a	62.1 a	78.3 a	25.70 a

Table 4. Effect of silicate bacteria (S3.1) on the K uptake and growth, yield of groundnut (greenhouse condition)

Treatment	K content of sterms and leaves (g/pot)	Plant height (cm)	Dry biomass (g/pot)	Pod yield (g/pot)
Control (No bacteria, 100% NPK)	1.04 a	60.4 a	81.8 b	25.50 ab
Silicate bacteria, reduction of 10% K	1.14 b	64.9 b	84.4 b	27,63b
Silicate bacteria, reduction of 20% K	1.12 ab	63.1 ab	83.8 b	26,83ab
Silicate bacteria, reduction of 30% K	1.04 a	61.7 a	81.0 a	25,15a

The results showed that using separately strains and reduced 10 - 20% of N, P, K has positive effect to the N, P, K uptake of plant and also the growth, yield of groundnut compared with controls (using 100% NPK and without microorganisms). In treatments, using separately strains and reduce 30% of N, P, K mineral fertilizer, total N, P, K of green biomass has no significant difference compared to control (using 100% N, P, K).

Table 5. Effect of mix strains on the NPK uptake and growth, yield of groundnut (greenhouse condition)

Treatment	N content of sterms and leaves (g/pot)	P content of sterms & leaves (g/pot)	K content of sterms & leaves (g/pot)	Plant height (cm)	Dry biomass (g/pot)	Pod yield (g/pot)
Control (No bacteria, 100% NPK)	1.55 a	0.18 a	1.04 a	60.4 a	81.8 a	25.50 a
Mix trains, reduction of 10% NPK	2.12 c	0.24 b	1.19 b	67.9 b	89.7 b	29.20 b
Mix trains, reduction of 20% NPK	1.95 b	0.21 b	1.15 b	66.7 b	88.3 b	28.69 b
Mix trains, reduction of 30% NPK	1.70 ab	0.19 a	1.07 a	65.1 b	87.3 b	27.15 a

According to Pham Van Toan et al. (2007), if the microorganisms in combination exist together and not limit biological activity of each other, the effect of microorganisms on nutrition, growth and crop yields under combination condition is higher than single condition. The effect of mix microorganisms on the NPK

uptake, growth and yield of groundnut in greenhouse condition have showed in Table 5.

Results in Table 5 showed that using of mixed trains increased 30% NPK uptake of plant. Using of mixed strains and reduce 10%, 20% of NPK increased the total NPK of plant, green biomass and pod yield compared to control (using 100% NPK).

Effect of *microbial* inoculant on the soil fertility, humidity and density beneficial microbes on the soil

Microbial inoculation include nitrogen fixing, phosphate solubilizing and silicate bacteria has produce. Effect of microbial inoculant on the nutrien content, humidity and density beneficial microbes in field trial were presented in Tables 6 and 7.

Table 6. Effect of microbial inoculation on the nutrient content and humidity of soils (field trial, Cat Trinh, Phu Cat, Binh Dinh)

Treatment	Total (%)			Available (mg/100 g soil)		Humidity (mm)
	N	P_2O_5	K_2O	P_2O_5	K_2O	
Control (basal fertilization: 100% NPK)	0.101	0.045	0.023	8.96	6.78	8.36
Microbial inoculant and basal fertilization	0.112	0.040	0.024	10.18	8.56	9.92
Microbial inoculant and reduction 10% of basal fertilization	0.101	0.040	0.024	10.04	8.44	9.18
Microbial inoculant and reduction 20% of basal fertilization	0.112	0.040	0.022	10.04	7.90	9.03
Microbial inoculant and reduction 30% of basal fertilization	0.101	0.033	0.018	8.55	7.63	8.09

The results in table 6 showed that the microbial inoculant has no significant changing in the total NPK content of sandy soil. Using microbial inoculant and reduce 10 - 20% of NPK, the available P, K and humidity in the soil has higher than control (using microbial inoculant and 100% NPK).

Table 7. Effect of microbial inoculation on the to the density of beneficial microbes (field trial, Cat Trinh, Phu Cat, Binh Dinh)

Treatment	Nitrogen fixing	Phosphate solubilizing	Silicate solubilizing	Polysaccharide synthesized
Control (basal fertilization: 100% NPK)	1.2×10^4	1.3×10^4	1.9×10^4	2.0×10^3
Microbial inoculant and basal fertilization	2.4×10^5	4.6×10^5	2.4×10^5	1.5×10^4
Microbial inoculant and reduction 10% of basal fertilization	2.5×10^5	3.4×10^5	2.3×10^5	1.9×10^4

Microbial inoculant and reduction 20% of basal fertilization	2.1 x 10^5	3.4 x 10^5	7.0 x 10^4	1.6 x 10^4
Microbial inoculant and reduction 30% of basal fertilization	2.3 x 10^5	2.1 x 10^5	1.3 x 10^5	4.9 x 10^3

The result in table 7 showed the positive effect of microbial inoculant on the beneficial microbes in the soil compared with control (no microbial inoculant and 100% NPK). The result showed that the density of beneficial microbes in the soil has no significant difference compared to treatment reduced NPK.

Effect of microbial inoculant on growth, yield of groundnut and economical benefit in field trial

Table 8. Effect of microbial inoculant on growth and yield of groundnut (field trial, Cat Trinh, Phu Cat, Binh Dinh)

Treatment	Plant height (cm)	Dry Biomass		Pod yield	
		Yield (qu/ha)	% Increase to control as basal fertil.	Yield (qu/ha)	% Increase to control as basal fertil.
Control (basal fertiliz-ation: 100% NPK)	35.2	45.00	-	37.02	-
Microbial inoculant and basal fertilization	41.2	52.50	16.7	42.21	14.0
Microbial inoculant and reduction 10% of basal fertilization	39.6	50,60	12.4	41.20	11.3
Microbial inoculant and reduction 20% of basal fertilization	38.1	50.35	11.9	40.06	8.2
Microbial inoculant and reduction 30% of basal fertilization	35.7	46.60	3.6	37.39	1.0
CV (%)	3.5	*2.4*		*2.4*	
$LSD_{0,05}$	2.44	*2.15*		*1.70*	

Table 9. Economical benefit of microbial inoculant for groundnut in sandy soil (field trial, Cat Trinh, Phu Cat, Binh Dinh)

Treatment	Total input (1,000 VND/ha)	Total output (1,000 VND/ha)	Benefit/ (1,000 VND/ha)	Increase to control as basal fertil. (1,000 VND/ha)	Rate of profit
Control (basal fertilization: 100% NPK)	25,204.5	74,040.0	48,835.5	-	-
Microbial inoculant and basal fertilization	27,484.5	84,420.0	56,935.5	8,100.0	0.29

Microbial inoculant and reduction 10% of basal fertilization	27,087.5	82,400.0	55,312.5	6,477.0	0.24
Microbial inoculant and reduction 20% of basal fertilization	26,676.0	80,120.0	53,444.0	4,608.5	0.17
Microbial inoculant and reduction 30% of basal fertilization	25,993.5	74,780.0	48,786.5	(49.0)	-

The result in table 8 and 9 shows that in the field experiment, using microbial inoculant and 100% NPK for groundnut increased the plant height 17.0%, enhanced green biomass 16.7%, more yield pod 14.0% and increased the benefit 8.10 million VND/ha compared to control (no microbial inoculant and 100% NPK).

Also results showed that using microbial inoculant and reduced 10 - 20% of NPK in groundnut increased the plant height (8.2 - 12.5%), enhanced green biomass (11.9 - 12.4%), more yield pod (8.2 - 11.3%) and increased the benefit 4.61 - 6.48 million VND/ha compared with control (no microbial inoculant and 100% NPK).

Using microbial inoculant and reduced 30% of NPK in groundnut has the plant height, green biomass, yield pod no significant difference compared to control (no microbial inoculant and 100% NPK).

CONCLUSION

In the greenhouse experiment, using of mix microorganisms and reduced 10-20% NPK for groundnut increased content of total N, P, K in the stems, leaves, increased the plant height, enhanced green biomass and increased the yield pod compared with control (using 100% NPK mineral and no microorganisms).

Microbial inoculation of mixed beneficial microbes has the positive effect on the fertiliy of sandy soil and growth, yield of groundnut growing in sandy soil of Binhdinh province. It brings benefit for the farmer.

In the field experiment, using microbial inoculant and 100% NPK for groundnut increased the plant height 17.0%, enhanced green biomass 16.7%, more yield pod 14.0% and increased the benefit 8.10 million VND/ha compared to control (no microbial inoculant and 100% NPK). Also results showed that using microbial inoculant and reduced 10-20% of NPK in groundnut increased the plant height (8.2-12.5%), enhanced green biomass (11.9-12.4%), more yield pod (8.2-11.3%) and increased the benefit 4.61-6.48 million VND/ha compared with control (no microbial inoculant and 100% NPK).

Microbial inoculant has potential to commercialize for sandy soil cultivated grounut in Binh Dinh province.

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EVALUATION OF A PLANT GROWTH-PROMOTING RHIZOBACTERIA, *AZOSPIRILLUM LIPOFERUM* WITH REDUCED DOSE OF FERTILIZER ON GROWTH AND YIELD OF A LOCAL RICE VARIETY CULTIVATED IN MEKONG DELTA OF VIETNAM

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ABSTRACT

The application of mineral fertilizers is the most advantageous and the fastest way to increase crop yields. In the last few decades the rate of nitrogen (N), phosphorous (P) and potassium (K) or NPK fertilizer application has tremendously increased in crop production. The excessive use of synthetic agrochemicals in crop production and in soil fertility management causes residue toxicity and environmental pollution. This is due to low use efficiency of externally applied fertilizers by the plants, long-term application, leaching, and evaporation to atmosphere. Therefore, the reduced use of synthetic agrochemicals in crop production and to maintain soil fertility by alternative means is the subject of investigation. The challenge is to continue sustainable agricultural crop production through minimization of harmful effect of fertilization. Among the different alternatives, researchers hypothesized that plant growth-promoting rhizobacteria (PGPR) could be a substitute to these. A field trial was carried out in acid sulphate paddy soil to study the effect of Azospirillum lipoferum as a PGPR on the growth of a local rice variety cultivated in Mekong Delta, Vietnam. The results showed that rice inoculated with A. lipoferum with combination of 50% N/ha of inorganic fertilizer improved color leaf index, plant height, length of panicle, number of panicle/m^2, dry weight of straw and rice yield equivalent to those of rice grown with 100%N/ha of inorganic fertilizer without A. lipoferum inoculation. Especially, root length of inoculated rice with A. lipoferum with 50% N/ha was significantly longer than those of uninoculated rice applied only

with 100% N/ha.. Our results suggest that application of A. lipoferum with reduced dose of N/ha could promote rice growth and enhance yields compared to higher dose of N/ha without A. lipoferum. This gives us an idea about the potentiality of these PGPR strain and their application in rice cultivation to get a better harvest index. Their use will also possibly reduce the nutrient runoff or leaching and increase in the use efficiency of the applied fertilizers. Thus, we can conclude that the NPK uptake and management can be improved by the use of PGPR in rice cultivation, and their application may be much more beneficial in the agricultural field in Mekong Delta of Vietnam.

KEYWORDS: Azospirillum lipoferum, inorganic fertilizer, inoculate, local variety of rice, plant growth-promoting rhizobacteria

INTRODUCTION

Mekong Delta is the biggest area for the production of rice in Vietnam. Total area for rice production was approximately 4,4 million ha (Vietnam Statistical, 2013). In the year 2013, total production of rice in MD was nearly 25 million tons. Framers have widely used chemical nitrogen fertilizer to get high yield. As a result, the excessive use of chemical fertilizers causes residue toxicity and environmental pollution. Several studies have been carried out to improve soil fertility, minimize the production cost and reduce the environmental pollution in rice field such as rotation system rice legume rice using Rhizobial inoculants (Hiep et al. 2001). However, due to the need of exporting rice, paddy rice is grown all year round. Study on Plant growth promoting rhizobia (PGPR) with non legume crops such as rice have got beneficial effects through biological nitrogen fixation (Malik et al. 1997; Bashan & Levanony, 1990), increased root growth (Mia et al. 2012), production of phytohormones (Tan et al. 2014), phosphate solubilizing (Charbot et al. 1996) and disease control (Ramamoorthy et al. 2001). In order to have a sustainable agricultural system, PGPR is a promising solution for the production of rice in Mekong Delta, Vietnam. Therefore, a field trial was carried out in Soc Trang province, Vietnam to study the effect of a PGPR *Azospirillum lipoferum* on the growth and yield of local rice variety.

MATERIALS AND METHODS

The field experiment was carried out in My Xuyen district, Soc Trang province. Some chemical and mechanical characteristics of experimental site was shown in Table 1.

Table 1. Chemical and mechanical characteristics of experimental site soil* in 2013

Characteristics	**Chemical analysis**	**Mechanical analysis**	**(%)**
pH (H_2O)	5.2	Clay	60.8
Organic C (%)	1.83	Loam	38.4
Total N (%)	0.071	Sand	0.68
Total P_2O_5(%)	0.076		
Available P_2O_5(%)	11.4		

Exchangeable K_2O	1.46
EC (mS/cm)	2.83

*Soil sample was analysed by Department of Soil Science, College of Agriculture and Applied Biology, Cantho University,Vietnam.

Treatments

The soil was divided into plots (4m x 5m). The experiment included 6 treatments with four replicates using the randomized complete block design as follows

Treatment 1: Uninoculated seeds and 0N + 30 P_2O_5 +30 K_2O

Treatment 2: Uninoculated seeds and applied 100N (100%N) + 30 P_2O_5 +30 K_2O

Treatment 3: Azospirillum +0N +30 P_2O_5 +30 K_2O

Treatment 4: Azospirillum +25N + 30 P_2O_5 +30 K_2O

Treatment 5: Azospirillum +50N + 30 P_2O_5 +30 K_2O

Treatment 6: Azospirillum +75N + 30 P_2O_5 +30 K_2O

Chemical nitrogen fertilizer was used in the form of Urea (46%N), phosphorus (Supper phosphate, 16% P_2O_5) and potassium (KCl, 60% K_2O). Peat based inoculants *Azospirillum lipoferum* ($2.6x10^9$CFU/g) was used to coat germinated seeds 1 hour before sowing at the dose of 5kg of inoculant/ha. Pesticides were applied when needed.

Measurements

Plant samples were taken at two stages of growth. Color leaf index, plant height, root length at 50 days after sowing (DAS) was recorded. The second sampling was taken at harvesting. Plant height, length of panicle, number of panicle/m^2, 1000- seed weight, dry weight of straw, and rice yield were determined. Data of the experiment were analysed using Stahgraphics Centuron XV.II software.

RESULTS

At 50 day after sowing (DAS), the leaf color index of rice inoculated with Azospirillum and combined with 50% or 75% inorganic nitrogen fertilizer was not significantly different with those of non inoculated one and applied 100%N inorganic nitrogen fertilizer. Plants either inoculated with Azospirillum without inorganic nitrogen fertilizer or non inoculated and applied no inorganic nitrogen fertilizer had the same leaf color index (Table 2).

Plant height of rice either inoculated with *Azospirillum lipoferum* combined with 25%, 50% and 75% or applied 100%N of inorganic nitrogen fertilizer only were significant higher than those of control (none inoculated and no nitrogen applied) and those of inoculated plants without inorganic nitrogen fertilizer supplied. Interesting ly, root length of inoculated rice combined with 50%N and 75%N of inorganic nitrogen fertilizer was significantly different with other treatments including non inoculated rice or applied 100%N or inoculated one with the combination of 25%N.

Compared to the non inoculated rice, root length of inoculated plants combined with 50% and 75N increased 56.2% and 61.4%, respectively.

Table 2. Some agricultural characteristics of rice plant at 50 DAS.

Treatments	Leaf color index	Plant height (cm)	Root length (cm)
- Azo + 0N + 30 P_2O_5 +30 K_2O	3.09d	72.8d	19.2c
- Azo +100N + 30 P_2O_5 +30 K_2O	4.31ab	82.5a	25.0b
+Azo +0N +30 P_2O_5 +30 K_2O	3.08d	74.5cd	21.8c
+Azo +25N +30 P_2O_5 +30 K_2O	4.12b	79.5ab	25.1b
+Azo +50N +30 P_2O_5 +30 K_2O	4.30ab	81.6ab	30.2a
+Azo +75N +30 P_2O_5 +30 K_2O	4.40a	82.1ab	31.0a
CV (%)	4.73	3.56	8.12

Means followed by the same letters in the same collum was not significantly different (P<.05)

At harvesting stage, plant height of inoculated rice combined with either 50% N, 75%N or uninoculated rice applied 100%N was significant higher than those of inoculated rice combined with 0%N, 25%N or the control. This result showed that Azospirillum could not promote plant height without supplement of at least 50%N of inorganic nitrogen fertilizer (Table 3). Length of panicle and number of panicle /m^2 of inoculated rice combined with 50%N or 75%N of inorganic nitrogen fertilizer was not significantly different with those of non inoculated rice applied up to 100%N. Azospirillum inoculants increased the length of panicle of inoculated rice 20.5% compared to the control without inoculation. Number of panicle /m^2 of inoculated rice increased 17.3% compared to the control without inoculation.

Table 3. Effects of inorganic nitrogen and Azospirillum inoculants on rice yield components

Treatments	Plant height (cm)	Length of panicle (cm)	Number of panicle/m^2	1000-seed weigth (g)	Dry weigth of straw (tons/ha)
- Azo + 0N + 30 P_2O_5 +30 K_2O	76.3d	18.0d	300b	22.3e	2.14c
- Azo +100N + 30 P_2O_5+30 K_2O	96.6a	22.4ab	593a	24.9ab	5.41a
+Azo +0N +30 P_2O_5 +30 K_2O	78.5d	19.1cd	352b	23.1d	2.35c
+Azo +25N +30 P_2O_5 +30 K_2O	82.2cd	20.7abc	513a	24.4bc	4.20b
+Azo +50N +30 P_2O_5 +30 K_2O	92.1ab	21.7ab	592a	24.9ab	5.30a
+Azo +75N +30 P_2O_5 +30 K_2O	96.5a	22.4a	610a	25.1a	5.42a
CV (%)	4.65	5.85	14.10	1.87	13.34

Means followed by the same letters in the same collum was not significantly different (P<.05)

1000-seed weigth of inoculated rice and combined 50%N, 75%N was not significantly different with those of uninocultaed appled 100%N inorganic nitrogen.

1000-seed weigth of inoculated rice was higher than those of uninoculated one 3.54%.

Dry weigth of straw of inoculated rice combined with 50%N, 75%N and uninoculated rice applied 100%N was not significantly different. This showed that inorganic nitrogen or biological nitrogen played an important role in promoting plant biomass.

The yield of rice inoculated and combined 50%N or 75%N was not significantly different with those of uninoculated rice apllied 100%N (Fig. 1). Compared to the control rice without inoculation, Azospirillum could increase 9.83% yield of inoculated rice. When inoculated rice combined with 50N/ha, the yield increased 202% compared to those of the control rice.

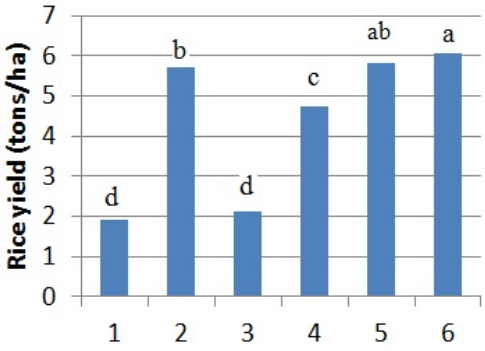

Fig. 1. Effects of *Azospirillum lipoferum* and inorganic nitrogen fertilizer on rice yield
1. No Azo + 0N + 30 P_2O_5 +30 K_2O; 2: on Azo +100N + 30 P_2O_5 +30 K_2O; 3: Azo +0N +30 P_2O_5 +30 K_2O; 4: +Azo +25N +30 P_2O_5 +30 K_2O; 5: +Azo +50N +30 P_2O_5 +30 K_2O; 6: +Azo +75N +30 P_2O_5 +30 K_2O

Symbiotic nitrogen contributed to rice inoculated with Azospirillum combined with 50%N iorganic nitrogen fertilizer with a relative yield increase of 388%.

DISCUSSION

Nitrogen is an important element for plant growth. Several PGPR can support plant growth because they can fix nitrogen from the air and provide this nitrogen source to the plants. In this study, PGPR *Azospirillum lipoferum* could enhance the plant height of rice plant. Similar result was also found by Tan et al. (2014) when they applied local variety of PGPR for rice cultivated in Malaysia. Besides, PGPR can also synthsized various substances such as auxin. These substances can support the elongation of root. In this study, inoculated rice plants had longer roots. This might be because of the phytohormone synthesized by *Azospirillum lipoferum* helped increase the root length of inoculated rice (Tan et al. 2014). According to Fulchieri et al. (1991), *Azospirillum lipoferum* could synthesize auxin, GA_3 and Iso-GA_3 and they could stimulate the root length of inoculated plants. When applied Azospirillum to wheat, Kapunik and Okon (1983) found that this bacteria could increase root length of inoculated 20.6%.

When applied Azospirillum to rice, Sharief et al. (2006) found that this PGPR could increase number of panicle/m^2 2.8% when they carried out the field experiment in Egypt. The result from the study showed that this PGPR *Azospirillum lipoferum* could increase the number of panicle/m^2 better than those found by Sharief et al. (2006). 1000-seed weight is usually belong to the variety of rice, In some cases, PGPR can also support the increase of this parameter. Summer (1990) also found that Azospirillum could increase the 1000-seed weight of inoculated one.

PGPR can also support good biomass of plants due to the increase of plant height, number of panicle/m^2. PGPR *Azospirillum lipoferum* used in this study could increase the rice biomass up to 147.66% compared to the control plant. Dobbelare et al. (2001) found that PGPR significantly increased the biomass of wheat grown in the field.

Kannaiyan et al. (1983) showed that when inoculation *Azospirillum* sp. and combined with 90N/ha, inoculated rice increased 25.4% compared to those of applied 90N/ha without inoculation. The result of the study confirmed that Azospirillum could fix nitrogen and provided it to the rice and enhanced good yield of inoculated rice (Bashan & Levanony, 1990). The same results were also found by Peoples & Cranswell (1992) when they studied the effects of PGPR on the growth and yield of maize, rice, wheat and sorghum.

In conclusion, plant growth promoting bacteria *Azospirillum lipoferum* could support good growth of plants, enhance root length, increase length of panicle, number of panicle/m^2, support rice biomass, and especially increase rice yield. Thus, we can conclude that the NPK uptake and management can be improved by the use of PGPR in rice cultivation, and their application may be much more beneficial in the agricultural field in Mekong Delta of Vietnam.

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ROLE OF PUBLIC-PRIVATE PARTNERSHIPS IN BIOPESTICIDES AND BIOFERTILIZERS RESEARCH AND DEVELOPMENT FOR SUSTAINING AGRICULTURE PRODUCTION

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ABSTRACT

Public-Private-Partnerships (PPPs) are important in involving private sectors for harnessing their efficient and enhanced mass production and delivery of consumer products and services. In agriculture, the rationale for these bilateral or multilateral collaborations is to achieve sustainability in agriculture production. In agriculture research and development (R&D), these PPPs are effective in overcoming public sector institutions limited ability in taking research products and technologies effectively to farmers. This paper emphasizes the role of PPPs in biopesticides and biofertilizers research towards attaining sustainability in agricultural production. The challenges faced by the smallholder farmers in Asia-Pacific region; the benefits of integrating modern and indigenous technologies and materials for increased food production arediscussed in the paper. Particularly, the role of Plant Growth-Promoting Rhizobacteria (PGPR) in sustainable agriculture production, their mechanisms of action in controlling plant diseases and promoting crop yields were highlighted. The role of Asian PGPR Society in building fruitful collaborations among scientific institutes, private enterprises, industries and academic institutions, and thus promoting PPPs in biopesticides and biofertilizers research are discussed. Lessons learnt from PPPs such as the Hybrid Parents Research Consortia (HPRC) model established by ICRISAT; and the Bioproducts Research Consortium (BRC) partnership with ICRISAT were elaborated. The future of PGPR research and the scope of PGPR as biofertilziers and biopesticides with commercial potential in Asia-Pacific region are discussed. The role of Governments in forging PPPs in R&D for biofertilizers and biopesticides as in case of is emphasized. The future role of Asian PGPR Society in accelerating and revitalizing the existing PPPs and facilitating the future partnerships in biopesticides and biofertilizer sector are discussed.

KEY WORDS: Public-Private Partnerships, Biofertilizers, Biopesticides, PGPR and Sustainable Agriculture

INTRODUCTION

The global population is expected to reach~8.9 billion by 2050 (United Nations Report 2004), from the current level of ~7.3 billion in 2015. Food and nutritional security becomes all the more important with the certainty of climate change scenario and the ever increasing human population. Approximately 1 billion people are poor and hungry. Nearly three quarters of the world's poor and 70% of hungry people live in rural areas, where smallholder farming prevails and nearly 2 billion people depend on them for livelihoods. These small holder farmers produce about80% of the food consumed in Asia and sub-Saharan Africa. It is estimated that about 87% of the world's 500 million smallholder farms (with less than 2 ha) are in the Asia-Pacific Region (Nagayets, 2005).The dryland farming systems in many developing countries produce low and unstable crop yields, coupled with fragile ecological balance keeping the farmers in subsistence mode. The smallholder farmers in these regions face several challenges, such as (i) high cost of chemical inputs (fertilizers and pesticides); (ii) poor soil fertility and irregular rainfall; (iii) lack of access to output markets; and (iv) difficulties in mechanization to reduce labor costs. These farmers need to be empowered to move away from subsistence to market-oriented farming (Dar, 2008).However, majority of the smallholder farmers are resource-poor, and cannot afford high-cost inputs. On the other hand, we cannot achieve food and nutrition security without external inputs for increasing agriculture productivity and sustainability in the small-scale sector. This will need supportive policies and technologies to ensure that farmers have access to appropriate technologies and inputs that are affordable and sustainable.

Modern science and technology has made significant progress in the past 4-5 decades, and has provided technologies and inputs for the farmers to increase food production. These technologies include: (i) improved crop varieties and hybrids; (ii) better options for management of soil, water and other natural resources; (iii) technologies for managing pests and diseases; and (iv) improved food processing and storage technologies. Farmer-led and farmer-participatory research and development interventions have played a major role in increasing food production by the smallholder farmers. More specifically, farmers in the third-world countries have many indigenous technologies that have been fine-tuned with modern science to manage the crops better, such as zero or minimum tillage; use of crop residues as organic soil amendments; green manuring; crop rotations; and biological control of pests and diseases. Asian region has a long history of developing and using locally available materials (local medicinal herbs, cow urine, cow dung, milk, butter milk, animal flesh and bones, etc.) to prepare concoctions that were used to manage pests and diseases in crops and also to treat sick animals (Choudhary et al. 2007). Many of the recommendations to control pests and diseases using herbs and animal products can be followed even today with good results.

PGPR, Biopesticides and Biofertilizers

One of the major drawbacks of modern agricultural technologies (sometimes referred to as Green Revolution technologies) has been the excessive and inappropriate use of chemical fertilizers and synthetic pesticides which has been reported to lead to unsustainable agriculture and environmental degradation. Biofertilizers and biopesticides (both botanical and microbial origin) are an important alternative to manage pests and diseases, when used strategically (Rupela et al. 2005). However, a major bottleneck has been the timely availability of standard quality biopesticides and biofertilizers at affordable prices, and in remote rural areas.

Plant Growth Promoting Rhizo-bacteria (PGPR) and other beneficial microbes have gained worldwide acceptance in sustainable agriculture production. More specifically, they have been exploited to a limited extent to support organic agriculture in many developing countries. Many Asian countries such as China, India, Japan, Korea, Nepal, Philippines and Vietnam are advocating use of biofertilizers and biopesticides for sustainable agriculture. Plant growth-promoting (PGP) microbes are soil bacteria that colonize rhizoplane or rhizosphere and enhance plant growth. PGP bacteria can directly or indirectly affect plant growth through various mechanisms which includes fixation of atmospheric nitrogen (Soares et al. 2006), solubilization of minerals (Basak & Biswas 2009; Panhwar et al. 2012), synthesis of various enzymes and phyto-hormones (Patten & Glick 2002), and inhibition of phyto-pathogens (Hao et al. 2011; Gopalakrishnan et al. 2011a, b). ICRISAT has a collection of over 1,500 microbes including bacteria and actinomycetes, isolated from various composts and soil rhizosphere, in which at least one out of six has documented either single or multiple agriculturally favorable traits.

Some of the actinomycetes in the germplasm collection such as *Streptomyces* spp., (such as *S. caviscabies, S. globisporus* sub sp. *caucasicus*, and *S. griseorubens)* have registered *in vitro* PGP traits such as IAA and siderophore production and positive effect on the up regulation of PGP genes such as IAA and siderophore-producing genes. *In vitro* trials have shown potential of enhanced growth in rice under field conditions via increased tiller numbers, panicle numbers, filled grain numbers and weight, stover yield, grain yield, total dry matter, root length, root volume, and root dry weight. In addition, they significantly enhanced rhizospheric total nitrogen, available phosphorous, organic carbon, microbial biomass carbon, microbial biomass nitrogen, and dehydrogenase activity over the un-inoculated control. Apart from the PGP traits, they also have the capacity to act as biocontrol agents due to the production of hydrogen cyanide and enzymes such as lipase, chitinase, and β–1,3 glucanase (Gopalakrishnan et al. 2012, 2013, 2014a, b). Other PGP actinomycetes such as *Streptomyces tsusimaensis, S. caviscabies, S. setonii,* and *S. africanus* have shown inhibitory activity against *Fusarium oxysporum* f. sp. *ciceri* (FOC) under green house and *Fusarium* wilt-sick fields (Gopalakrishnan et al. 2011b). They have also shown inhibitory action on *Macrophomina phaseolina*, a causative agent for the charcoal rot of sorghum (Gopalakrishnan et al. 2011a) under greenhouse conditions.

Role of Asian PGPR Society in promoting PPPs and Sustainable Agriculture

The "Asian PGPR Society for Sustainable Agriculture" has been making sincere efforts in this direction. However, more proactive and concerted efforts will be needed to ensure that Asian PGPR Society remains relevant in the future. Most important will be the task of ensuring that high quality PGPR and other bioproducts (including biopesticides and biofertilizers) are available to the small holder farmers in rural areas. Although a few products are available in the market, the quality of these is inconsistent and unreliable. By bringing scientists, researchers, entrepreneurs and progressive farmers on to a common platform to exchange the ideas, Asian PGPR Society can influence future directions on PGPR research for sustainable agricultural production. However, much has still to be done to visualize the biopesticide and biofertilizer research and the product application in farmer's fields on a large scale. Strong public-private partnerships are therefore essential for achieving these goals. Asian PGPR Society has also been a platform for enabling fruitful interactions among various scientific institutes, private enterprises at the international level and is leveraging the concept of establishing linkages between academic institutions and industry. It is envisaged that this will also enable in promoting the integrity of research in PGPR related areas.

Efforts should be to enhance access of reliable, high quality and affordable bioproducts in the market so that smallholder farmers can benefit from these technologies. After analyzing past successes, failures and lessons learnt in the R&D community globally, we consider that a strong Public-Private Partnership Model, similar to the highly successful ICRISAT-Private Sector Hybrid Parents' Research Consortia (HPRC), will be essential.

Hybrid Parents' Research Consortia (HPRC) Model

Public-Private partnerships in agriculture R&D are increasingly viewed as an effective means of conducting advance research, commercializing new technologies, and deploying new products for the benefit of resource-poor farmers (Gowda et al. 2009). Multilevel, strategic partnerships mobilizing science and technology for the poor is at the heart of CGIAR research. The CGIAR Centers recognize that building capacity of partners is a two-way process, where private sector partners benefit from IARC's expertise and technologies, and Centers benefit from the market experience of private sector partners. The synergies gained by a combination of social equity of the public sector research and the efficiency in product delivery of the private sector companies creating the linkages in the supply chain for delivery of inputs (in this case the seed of hybrid cultivars) to smallholder farmers (Gowda et al. 2009). Pooling of resources minimizes the risks associated with R&D, for mutual benefit and sharing of costs leads to lower product costs, thus benefiting the consumers (in this case farmers as primary consumers).

The HPRC was established by ICRISAT and a few interested Private Sector Seed Companies in 2000. The HPRC enabled ICRISAT to work synergistically and support the private sector seed companies (and also profit making public sector seed corporations) to ensure availability of quality seed of high performing hybrids (of sorghum, pearl millet and pigeonpea crops) to smallholder farmers, through

partnership-based approach. Using the vast genetic resources available in its genebank, ICRISAT conducts strategic and applied research to develop improved breeding lines and hybrid parental lines (of sorghum, pearl millet and pigeonpea), and shares these with both public sector and private sector (who are members of the HPRC) plant breeders. The PS Seed Companies select good parental lines adapted to the ecological niches and with traits that are preferred by the farmers, in their own market segments. Test Hybrids are evaluated widely in the target areas to select the best performing hybrids. Seed companies then mass produce the seed, process, pack, and market the hybrid seeds using the vast network of agro-dealers in the rural areas (Gowda et al. 2009). Involvement of a large number of companies in the consortium (at one time, more than 50 seed companies were members of one or more of the consortia) increases competition and reduces the monopolistic behavior of seed companies. Cost of hybrid seed is kept at reasonable levels by competitive forces, hence within the reach of the resource-poor smallholder farmers. Private sector seed companies are able to make good profits from the sale of improved hybrid seeds, while ICRISAT is able to show the impact of its crop improvement research in the farmers' fields.

Promoting Biopesticides and Biofertilizers through PPP

As mentioned earlier, biopesticides are important alternatives to chemical pesticides, with good track record of biosafety and efficacy when these are used strategically. Similarly, the biofertilizers have shown their merit in sustaining crop yields and improving soil fertility and health. In many cases biopesticides and biofertilizers are highly affordable, especially when these are prepared by farmers themselves or locally at the community or village level (Rupela et al. 2005). However, these locally produced products are not pure and hence their efficacy is not high nor consistent. Farmers and scientists should work together to blend modern science with traditional knowledge and practices to produce high quality products for the rural markets. On the other hand, methodological breakthroughs in molecular biology and biotechnology have strengthened microbiologists' capacity to mass multiply microbes in large quantities that was not possible in the previous decades. With the demand for organically grown foods in many countries, and in view of sustainability of agriculture in general, the demand for biofertilizers and biopesticides (including both botanicals and microbial pesticides) is increasing globally. However, supply of good quality bioproducts is not able to meet the demand. Hence, there is a need for involvement of the private sector in joining hands with the public sector researchers to ensure that the farming community is able to get quality bioproducts at affordable prices.

In view of the highly successful public-private partnership in the Hybrid Parents Research Consortium (HPRC), ICRISAT and a few private sector biopesticide manufacturing companies initiated the ICRISAT-Private Sector *Biopesticide Research Consortium (BRC)* in January 2005, that was later renamed as Bioproducts Research Consortium to include PGPR and biofertilizers (Rupela et al. 2005). Eleven biopesticides/biofertilizers companies joined the consortium as its founding members. The overall goal of BRC was to make quality biopesticides and other bioproducts to the farming community at affordable price. ICRISAT had a collection of

>1500 microbial germplasm (many with PGPR and biopesticidal properties); a few on-the-shelf technologies [such as Helicoverpa nuclear polyhedrosis virus (HNPV), and a few proven biopesticidal microbial strains], fermentation technologies, small-scale fermenters, and expertise in policy issues related to biopesticide testing and registration. On the other hand, the biopesticide/ biofertilizer companies had medium to large-scale capacity factories to manufacture bioproducts, and also the needed market linkages with a network of agro-dealers. The BRC Phase I was implemented with good success (2005-07), and Phase II was started in 2008. Unfortunately, only 3 out of 11 companies continued their membership. Without the critical mass of partners needed for a viable R&D consortium, the BRC became inoperative in 2010.

Lessons learnt from BRC experience

- Private sector companies demand and need "ready-to-use", on-the-shelf technologies that can be mass produced and launched within 1 or 2 years to maximize their profits,
- Most companies were unwilling to invest in long-term strategic and basic research at ICRISAT to develop potential future technologies,
- The CGIAR policy did not allow PS companies to use institution's (ICRISAT) name in marketing of products,
- Currently available PGPR strains were unable to show large and significant effects under varied on-farm situations to convince the farmers to use these bio-products,
- Most manufacturers were willing to produce and provide high quality products (PGPR, inoculants, biopesticides, etc.), but not willing to invest in research to ascertain why the products do not work in the real world (on farmers' fields) situations,
- Most companies wanted BRC to facilitate government clearance and lobby for favorable policies. This was initiated in Phase 2, but was not fully pursued as very few companies remained in the consortium, and
- Success of spinosad-like products indicate that purified forms of biopesticidal components do have future potential, but need large R4D investments.

The Future

PGPR microbes have multiple functions and features that promote plant growth, aid in controlling insect pests and diseases, and also in influencing soil health. However, the extent of success in realizing the benefits of PGPR tends to diminish as it moves from laboratory to greenhouse, and eventually to the farmers' fields, which reflects the scarcity of research on the effectiveness of PGPR microbes under field conditions. Therefore, generation of comprehensive knowledge on screening technologies for selection of best rhizobacterial strain for rhizosphere competence and survival is critical to enhance the field level successes. Inoculant strains that survive and are effective in varied agro-ecologies need to be promoted more aggressively so that farmer acceptance and adoption increases.

Chemical fertilizers have received government support, including subsidies, for many decades in many countries resulting in the over-use or inappropriate use of fertilizers. In order to provide a level playing field, governments should either stop subsidies to chemical fertilizers, or provide similar support to biopesticides and biofertilizers. Preferably, governments should incentivize farmers (through direct money transfers) to promote sustainable agriculture, including ecosystem services such as rainwater harvest for charging aquifers, enhancing the population of beneficial insects (natural enemies of pests) and pollinators, and beneficial microbes in the rhizosphere.

The role of governments is also critical in forging PPPs in biofertilizers and biopesticides research and development. For example, in India, Biotechnology Industry Research Assistance Council (BIRAC) is one such entity which brings together multiple stakeholders in public and private sector and work towards converting agricultural technologies into products that can reach farmers. A product in reality will always convince the policy makers in a much better way.

It is evident that there is a huge potential for PGPR microbes in biopesticide and biofertilizer industry. The market for trade in "Bioproducts" is large, both in domestic and international markets. However, what is needed is a change in mindset and attitudes of people in both public and private sector, and they should start a strategic partnership model on the lines of the ICRISAT-Private Sector Hybrid Parents Research Consortium. The Asian PGPR society should facilitate the formation of a synergistic "platform" or a "consortium" to encourage interested and committed entities and institutions to come together for mutual benefit, and to serve the farming community. The next steps of Asian PGPR Society should be to revitalize the existing public-private partnerships (PPPs); encourage and envisage the scope of future partnerships that can benefit the biopesticide and biofertilizer research exploiting the PGPR microbes. However, the models that are formulated for establishing these partnerships should be viable and mutually beneficial, besides maintaining transparency. With the overall goal of improving the livelihoods of smallholders, promoting PPPs for biopesticide and biofertilizer research and development in Asian countries is vital in the agriculture and allied sectors.

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PREVENTING MOKO, PANAMA, AND BLACK SIGATOKA DISEASES IN BANANAS USING RHIZOBACTERIA FROM MANGROVE RHIZOSPHERES AS BIOLOGICAL CONTROL AGENT

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ABSTRACT

Banana is considered as one of the most important crops being exported around the globe. In terms of gross value of production, bananas are the world's fourth most important food crop after rice, wheat and maize. Presently, there are five major diseases threatening the global banana production. These are Panama disease caused by a fungus Fusarium oxysporum f.sp. cubense (Foc), black Sigatoka caused by the fungus Mycosphaerella fijiensis, bacterial wilt caused by Ralstonia solanacearum together with recently emerged Xanthomonas campestris pv. musacearum, and banana bunchy top disease caused by Banana bunchy top virus (BBTV). As these diseases caused serious and expanding threat to banana plantations, it is imperative to address this problem. This study determines the potential use of marine bacteria as biological control against Ralstonia solanacearum, Fusarium oxysporum, and Mycosphaerella fijensis.

Marine Rhizobacteria were isolated from three randomly selected mangrove rhizosphere collected from Lianga Bay, Philippines. The rhizobacteria were purified and screened using agar plug assay against R. solanacearum, F. oxysporum, and M. fijiensis. The best isolates (based on its bioactivity in vitro) were selected for inoculation to tissue cultured Cavendish banana seedling. In the in-vivo testing, 60 tissue cultured banana seedlings were used to test the potential of the Bacillus and Actinomycetes isolates as biological control agents against R. solanacearum, F. oxysporum, and M. fijiensis.

A total of 76 Bacilli and 48 Actinomycetes were isolated and screened. Of these isolates, 31 bacilli and 15 actinomycetes showed inhibition against R.

solanacearum, 10 bacilli and 8 Actinomycetes against F. oxysporum, and 9 Actinomycetes against M. fijiensis. The isolates which showed most potential are YS3B1, YS4A5, and YS3A4 for R. Solanacearum; YS4B1, YS1A3, YS2A5 for F. Oxysporum; and YS1A3 and YS2A1 for M. fijiensis. Results of the In vivo testing showed that the isolates mitigated the infection in tissue cultured bananas. Results suggest that the isolates have protected the plants from these banana diseases by inhibiting bacterial and fungal growth of the pathogen in the soil.

This paper reports the use of mangrove rhizobacteria in controlling banana diseases.

Keywords: Panama disease, Black Sigatoka Disease, *Actinomycetes, Bacillus*, Rhizobacteria

INTRODUCTION

Bananas (*Musa* spp. L.) are not only considered as the most popular of all the fruits because it serve as a representation of major dietary sources of important nutrients but also it is one of the most valuable agricultural crops that are being exported around the globe (Ploetz 2001). In some countries, banana serves as the people's staple food; and most importantly, it plays a role in the nation's gross income when it comes to its agricultural sector.

In the Philippines, a total area of 1,200 hectares of banana plantation was already wiped out in Mindanao and the Philippine government has already sought assistance in the outbreak that involves research that spend hundreds of millions (Ng, 2011). Globally, banana plantations devastation has gone far enough to consider it as a serious matter. Records have shown that problems pertaining to banana industries have been already spotted also in Indonesia, Central America, and South America; in which if not solved, would surely result serious losses to commercial and subsistence farmers (Sequeira, nd). In recent reports, the production damage of banana could rise to a number of billion dollars that would greatly affect the smallholders. This issue also threatens the more than 70 million people in Africa who depend on banana in their food maintenance and livelihood (FAO, 2009). The commotion about banana plantation problems is mainly caused by variety of fungal and bacterial diseases. Factors that are responsible for the inhibition of these diseases include animal and human factors, and an addition of natural factors like contaminated tools, insect vectors, browsing animals and diseased planting materials (Mbaka et al. 2008). This shows that spreading of these diseases is inevitable and finding technique to fight against it is imperative.

Three of the most common microorganisms that cause disease to bananas are *Fusarium oxysporum, Mycosphaerella fijiensis,* and *Ralstonia solanacearum*. These organisms, along with the others, have already declined banana production in some countries, particularly in Kenya (Mbaka et al. 2008). *F. oxysporum* is the cause of a disease commonly called Fusarium wilt or Panama disease which caused millions of damaged in the Philippines. On the other hand, *M. fijiensis* causes Black Sigatoka disease in banana. It causes over 50% yield losses of all banana plantations through premature ripening which is a serious defect in the fruit (Ploetz, 2001). Black Sigatoka is found in all major banana exporting countries. This disease is broadly spread in South East Asia, India, China, the southern Pacific islands, East and West

Africa, USA (Hawaii), Grenada (Caribbean), Trinidad, Central and South America (Horlock, 2011). On the other hand, disease caused by *R. solanacearum* is considered as the most threatening bacterial disease in banana and plantain industries all over the world (Sequeira, nd). The disease caused by this microorganism is commonly called as Moko Disease. This disease is a very dangerous bacterial infection that causes the fruits to ripen not in time and it can terminate infected plants in just weeks. Moreover, the National Federation of Banana Producers president Silverio González described Moko as more deadly than the Sigatoka fungus, which has wreaked havoc in plantations in the Dominican Republic (Gonzales, 2004 as cited by Toomey, 2004).

Mangroves are a very adaptive group of plants. It can adapt to harsh environment, thriving normally in a saline, acidic, and nutrient-poor area (Conroy, 2000). Despite the nutrient-deficiency of these areas, mangrove still look as healthy forests which suggests that there exists a good plant-microbe relationship in Mangrove (Espinoza, 2012). Mangroves contain a large diversity of organisms which includes microorganisms with beneficial roles in nutrient cycling and availability. As a unique habitat, Mangrove areas promise to hold diverse communities of microbes which help the mangrove to grow in limiting environmental conditions. Furthermore, mangroves are believed to associate with rhizobacteria that are capable of functioning as biological control agent against plant diseases. These bacteria have the ability of suppressing a spectrum of bacterial, fungal, and nematode diseases (Shakilabanu et al. 2012). However, the benefits of microorganisms (in particular those associated with roots) to the productivity of mangroves and the roles they play in plant fitness, survival and overall ecosystem resilience have been scarcely studied (Gomes, et al. 2011). Thus, this project is conceptualized to search for local microbial isolates which can be used in controlling diseases in tissue cultures bananas. It hoped to find rhizobacteria which can function as biological control against *Fusarium oxysporum, Mycosphaerella fijiensis,* and *Ralstonia solanacearum*.

METHODOLOGY

Mangrove Rhizospheres (Soil and Root samples) from three randomly selected Mangal sites along the coast of Lianga Bay, Philippines were collected. The samples were air-dried and homogenized using mortar and pestle. Using 10-fold dilution, 10 gram of each of the samples were serially diluted with 90 mL NSS (prepared by dissolving 15% NaCl in Distilled Water and 0.1% Peptone). 10^{-1} to 10^{-4} Dilutions were made. 0.1 mL aliquots from10^{-3} and 10^{-4} dilutions were spread on Nutrient Agar (NA) and Yeast Malt Agar (YMA) plates and incubated overnight at 30^{o}C. Pure colonies were selected, purified, and screening against *Fusarium oxysporum, Mycosphaerella fijiensis,* and *Ralstonia solanacearum* using agar plug assay as described by Raymundo (1991). Zones of Inhibitions (in mm) were then measured after 24hrs using a digital Vernier Calliper. The ZOI were recorded and analyzed for the selection of the Best Isolates. The criteria for selecting the best isolates were based on bioactivity against the test organisms. The selected isolates were then inoculated to Tissue-Cultured Banana Seedlings. Inoculated seedlings were infected with the test pathogens by administering fresh culture to injured roots before it was transplanted to potting soil. The plants were then grown in the nursery under

controlled conditions. The extent of the bacterial and fungal damages to the plants was then noted after 3 weeks observation.

RESULTS AND DISCUSSIONS

Panama disease caused by *Fusarium oxysporum* is characterized by the yellowing of the banana leaves. The *F. oxysporim*- mediated wilting starts from older leaves and spreads to younger leaves. In addition the Foc-infected leaves snap at the leaf petioles. The start of infection can be noticed starting from the leaf petioles instead of from the tip like in Xanthomas infection. Fig. 1 shows the tissue culture banana infected with *Fusarium oxysporum* and left untreated for 3 weeks incubation period. The Seedlings showed infection and are starting to wilt in 3 weeks while the Tissue Cultured Banana Seedlings inoculated with the Bacterial Isolates (Fig 2, 3, and 4) showed more resistance to the Panama Wilt Disease compared with the infected and untreated samples. Compared with the uninfected seedlings, seedlings treated with the bacterial isolates are healthier.

A. Fusarium Wilt in Banana (Panama Disease)

Fig 1. Bananas infected with *F. oxysporum*

Fig 2. Treated with *Bacillus YS4B1*

Yellowing of the leaves are symptoms of *Ralstonia* Wilt .The attack of bacterial wilt *Ralstonia solanacearum* is shown in Fig. 5-8. The young leaves change color, and on the midrib of banana leaves there are yellow brown lines towards the margins. Untreated banana seedlings showed wilting after 3 weeks inoculation of the test pathogens. *Ralstonia* Wilt in young bananas is often fatal in young seedling in the actual field. Compared with the untreated seedlings, those which are treated with the bacterial showed reduced degree of infection after three weeks incubation.

Fig 3. Treated with Actinomycete YS2A5

Fig 4. Treated with Actinomycete YS1A3

B. Ralstonia Infection (Moko Disease) in Banana

Fig 5. Tissue Cultured Bananas infected with *R. solanacearum*

Fig 6.YS3B1 on *R. solanacearum*-infected bananas

Fig 7. Treated with Actinomycete YS4A5

Fig 8. Treated with Actinomycete YS3A4

Black Sigatoka symptoms start as tiny, yellow broken streaks followed by the development of brown spindle shape lesions in the streaked areas with a yellowish halo. Slowly lesions darken and become elliptical brown spots with a grayish centre demarcated by a well defined brown or black border. Bananas infected and are untreated (Fig. 9) showed symptoms of Black Sigatoka disease. Its leaf blades developed brown lesions with yellow halo around it. This lesions will eventually

grown darker giving the disease its characteristic black streaaks in the leaf. This will cause eventual death to the banana as it decrease photosynthetic activities. The treated samples showed tolerance to the disease.

C. *Mycosphaerella fijiensis* Wilt (Black Sigatoka Disease)

Fig 9. Tissue Cultured Banana infected with *M. fijiensis*

CONCLUSIONS AND RECOMMENDATIONS

This study is designed to search for a potential biological control agents against banana plant disease-causing microorganisms: *Ralstonia solanacearum*, *Fusarium oxysporum,* and *Mycosphaerella fijiensis*. Generally, the result suggests that Mangrove Rhizobacteria can be potential biological control agents against *Ralstonia solanacearum*, *Fusarium oxysporum,* and *Mycosphaerella fijiensis* which mainly cause most of the banana disease outbreaks worldwide. Specifically, *Bacillus* Isolate YS4B1 and *Actinomycete Isolates* YS2A5 and YS1A3 have the highest potential among isolates as Bio Control Agent of *R. solanacearum*; the potential Biocontrol Agent against *Fusarium* Wilt are *Bacillus* Isolate YS4B1 and *Actinomycete* Isolates YS1A3 and YS2A5; and for controlling Black Sigatoka in Bananas, *Actinomycetes* Isolates YS4A5 and YS3A4 were selected as best biological control agents against *M. fijiensis.*

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28

INFLUENCE OF *CELLULOMONAS FLAVIGENA, AZOSPIRILLUM SP.* AND *PSEUDOMONAS SP.* ON RICE GROWTH AND YIELD GROWN IN SUBMERGED SOIL AMENDED WITH RICE STRAW

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ABSTRACT

Organic toxicity is a common phenomenon in soils with continuous rice grown.. Under these conditions, the growth of rice plant is often stunted and resulted with reduced yields. In our study, we evaluated three plant growth-promoting rhizobacteria (PGPR), such as a cellulolytic bacteria Cellulomonas flavigena, nitrogen-fixing bacteria Azospirillum sp. and a phosphate-disolving bacteria Pseudomonas sp. on growth and yield of rice cv: IR50404. Experiments were conducted under greenhouse conditions in a randomized complete block design. There were 10 treatments and each treatment was replicated five times. The replicated pot was filled with submerged soil collected from rice field amended with rice straw for rice cultivation in autum-winter season. Rice seeds treated with PGPR (Azospirillum sp., Pseudomonas sp.) as a seed soaking and planted in the pots. Microbiological fertilizers containing cellulolytic bacteria Cellulomonas flavigena (pellets) was sprayed in the pots. Our results showed that rice plant treated C. flavigena + inorganic fertilizer (75N-45P_2O_5-30K_2O kgha-1) significantly increased plant height, number of tillers, root weight, 1000 – grain weight, panicles and rice yields compared with untreated controls (100 N-60P_2O_5-30K_2O kgha-1), reduced 25% inorganic fertilizer (25 kg N and 15 kg P_2O_5).

KEYWORDS : IR50404 rice varieties, cellulolytic bacteria *Cellulomonas flavigena*, nitrogen-fixing bacteria *Azospirillum* sp. and phosphate-disolving bacteria *Pseudomonas* sp, rice straw.

INTRODUCTION

Organic poisoning usually occurs on intensive farming land, due to the fact that organic material such as straw, and floral decompose fastidiously in wetlands, creating toxins such as organic acids, H_2S, ... (Ponnamperuma, 1984; Gao et al. 2004; Kyuma, 2004).In order for decomposition of straw to takes place fast, Nguyen Thanh Hoi *et al.* (2014) fertilized straw by incubating with *Trichoderma* sp. significant yield increase compared to unfertilized control. However, the straw compost can take a lot of effort, difficult to encourage farmers to implement. Therefore, to reduce the labor of farmers in the straw incubation, the hypothesis is to spread straw on the field then use the bacterial decomposition of straw to help fine straw decomposition under submerged conditions, in a short time. In Vietnam, the study by Van Phuoc Vo and Cao Ngoc Diep (2011) have isolated and multiplied the success Q4 strains from bovine rumen fluid which were capable of producing cellulose enzyme, cell analysis molecular-based 16S rRNA sequences that strains Q4 isomorphic with the *Cellulomonas flavigena*, and recomposed effectively photocopy paper and straw. Besides, the current use of nitrogen-fixing bacteria and bacterial soluble phosphorus can reduce chemical fertilizer, save costs, reduce environmental pollution. According to Cao Ngoc Diep *et al.* (2009), two strains of nitrogen-fixing bacteria *Azospirillum lipoferum* and dissolved phosphorus *Pseudomonas stutzeri* help increase rice yield reached 4.6 to 4.7 tons / ha and 50% reduction in the amount of chemical fertilizers, while fertilizer chemistry was only 4.6 tonnes / ha.

Currently, high-yielding rice varieties like short IR50404 is promising, good tolerance to some insects, diseases and is growing in popularity in the Mekong Delta provinces. However, studies using the strain *Cellulomonas flavigena*, *Azospirillum* sp., *Peudomonas* sp on the rice IR50404, to reduce the toxicity of organic, reduce chemical fertilizer, increasing yields on land rice straw fresh buried in flooded conditions, are still limited. Therefore, the subject was conducted to assess the effect of target microbial fertilizer *Cellulomonas flavigena* bacteria, microbes and bacteria *Azospirillum* sp., *Peudomonas* sp. on the growth and yield of rice straw buried IR50404 with fresh conditions in wetlands.

MATERIALS AND METHODS

The experiment was conducted from May 03-06 / 2014 in the Summer and Autumn in Crop Science Department, College of Agriculture and Applied Biology, Can Tho University.

IR50404 is selected from the same group imported varieties of IRRI. Recognized by Decision No. 126 of Agriculture Science and Technology / QD, dated 05/21/1992, IR50404 has the following characteristics: growing period: 85-90 days, height 80 to 90 cm tree, resistance to lodging poorly adapted to a wide variety of alluvial lands to the alkaline medium, cultivated in all 3 cases, and slightly brown rice blast infection Slim, lightweight infected with gold leaf disease, infected with both diseases sheath, yield: winter-spring season 6-8 tons / ha, Autumn season 5-6 tons / ha. cellulolytic bacteria *Cellulomonas flavigena* $1x10^8$ CFU/g, nitrogen-fixing bacteria *Azospirillum* sp. and phosphate-disolving bacteria *Pseudomonas* sp $1x10^9$ CFU/ml; Nitrogenous fertilizer (urea) [CO (NH_2) 2], 46% N; Super Long Thanh Lan Ca(H_2PO4) 2 16% P_2O5; Potassium chloride (KCl) 60% K_2O, fresh straw, plant

protection chemicals, soil pots and some other tools such as knives, hoes, weight clock, electronic scales, water pumps ...

The experiment was arranged in a randomized form, a factor with five replications and ten treatments (T): T1: no use of microbiological fertilizer + no NPK; T2: no use of microbiological fertilizer + 100N-60P_2O_5-30K_2O kg/ha; T3: use of *C. flavigena* bacteria + no NPK kg / ha; T4: use of bacteria (*Azospirillum* sp., C. *flavigena*, *Pseudomonas* sp.) + No NPK kg/ha; T5: use of bacteria *C. flavigena* + 0N-0 P_2O5-30K_2O kg/ha; T6: use of bacteria (*Azospirillum* sp., *C. flavigena, Pseudomonas* sp.) + 0N-0P_2O_5-30K_2O kg / ha; T7: use of bacteria *C. flavigena* + 50N-30 P_2O_5-30K_2O kg/ha; T8: using bacteria (*Azospirillum* sp., *C. flavigena, Pseudomonas* sp.) + 50N-30P_2O_5-30K_2O kg / ha; T9: use of *C. flavigena* bacteria + 75N-45P_2O_5-30K_2O kg/ha; T10: use of bacteria (*Azospirillum* sp., *C. flavigena, Pseudomonas* sp.) + 75N-45P_2O_5-30K_2O kg/ha. Fertilizers provided under the weight of 1 ha of paddy land is 2 million kg of dry and natural land and are divided into 3 times.

The monitored indicators include: plant height (cm), number of branches /pots, number of seeds/ flowers, grains ratio (%), 1000 – grain weight (W14%, g), actual yield (W14%, g / pot), economic factors (HI), root length (cm), root weight (W14%, g). Statistical data is calculated by SPSS 16.0 soft ware and Duncan test is used to compare differences among treatments at 5% significance level.

RESULTS

Rice plant height, number of tillers, root weight

The results in Table 1 show that in treatments using *Cellulomonas flavigena* bacteria associated with fertilizer formula 75N-45 P_2O_5 (kg / ha) there is an effective increase in the height and number of branches rice, root weight equivalent treatments done with 100% urea nitrogen and phosphorus chemistry (100N-60P_2O_5).

Table 1. Rice plant height, Number of tillers pots, root weight at harvest stage of IR50404

Treatment	Harverst		
	Rice plant height	Number of tillers/ pot	Root weight
T 1	45,31 ^{d}	5,00 ^{c}	5,50 ^{d}
T 2	51,36 ^{abc}	12,40 ^{a}	13,24 ^{a}
T 3	45,55 ^{d}	5,20 ^{c}	5,23 ^{d}
T 4	47,31 ^{cd}	6,00 ^{c}	7,38 ^{bcd}
T 5	47,80 ^{cd}	5,00 ^{c}	6,36 ^{cd}
T 6	48,52 ^{bcd}	6,20 ^{c}	5,38 ^{d}
T 7	49,45 ^{bcd}	11,40 ^{ab}	11,21 ^{abc}
T 8	51,96 ^{abc}	9,00 ^{b}	8,85 ^{abcd}
T 9	53,08 ^{ab}	12,80 ^{a}	12,64 ^{ab}
T 10	55,22 ^{a}	12,00 ^{a}	12,22 ^{ab}
F	**	**	*
CV (%)	6,51	18,38	45,77

The yield components

The statistical analysis results in Table 2 show that the number of grains/ panicle, grains ratio in all treatments were not statistically different. The number of fully grains on panicle fluctuates from 53.12 to 71.43 (grain/panicle), the grain ratio ranged from 71.48 to 79.41%. However, treatments using *C. flavigena* bacteria + 75N-45P_2O_5-30K_2O kg / ha (kg / ha) for some number of panicles/ pot (12.80 flowers/ pot) and 1000 - grain weight (33.16 g / pot) are equivalent to the ones with no use of microbiological fertilizer + 100N-60P_2O_5-30K_2O.

Grain yield (g/pot)

The results in Table 2 show the actual yield in all the different treatments significant statistical difference at 1%, ranging from 5.11 to 15.28 (g / pot). Treatments using C. flavigena bacteria + 75N-45P_2O_5-30K_2O (kg / ha) have the highest grain yield (15.28 g / pot), and they are equivalent to the ones not using microbiology fertilizer + 100N-60 P_2O_5-30 K_2O (kg / ha); using bacteria (*Azospirillum* sp., *C. flavigena*, *Pseudomonas* sp.) + 75N-45P_2O_5-30K_2O (kg / ha) and different from the others. Thus, using *C. flavigena* bacteria and reducing 25% nitrogen and phosphorus chemistry (75N-45P_2O_5-30K_2O kg / ha) help increase rice yield equivalent to using 100% nitrogen and chemical phosphorus (100N-60 P_2O_5-30 K_2O kg / ha) fertilizer.

Table 2. Yield and yield components of IR 50404 rice varieties according to the degree of chemical fertilizers combined bacterial stains in the Summer - Autumn season in 2014

Treatment	Yield components				
	Number fully grains on panicle	**Number of panicles/ pot**	**1000 – grain weight**	**Grain ratio (%)**	**Grain yield**
T 1	53,12	5,00 ^{c}	24,69 ^{bcd}	71,74	5,11 ^{c}
T 2	56,41	14,40 ^{a}	28,65 ^{abc}	72,16	14,79 ^{a}
T 3	59,33	5,20 ^{c}	24,89 ^{bcd}	77,16	6,03 ^{c}
T 4	56,04	6,00 ^{c}	23,55 ^{cd}	72,35	6,68 ^{c}
T 5	62,56	5,00 ^{c}	23,10 ^{d}	75,64	6,09 ^{c}
T 6	60,26	6,20 ^{c}	25,12 ^{bcd}	71,48	6,27 ^{c}
T 7	53,26	11,40 ^{ab}	28,16 ^{bcd}	72,94	12,08 ^{b}
T 8	56,90	9,00 ^{b}	31,41 ^{a}	75,29	11,93 ^{b}
T 9	67,07	12,80 ^{a}	29,84 ^{ab}	79,41	15,28 ^{a}
T 10	71,43	12,00 ^{a}	33,16 ^{a}	74,08	15,25 ^{a}
F	ns	**	**	ns	**
CV(%)	15,57	18,43	13,33	6,00	12,40

DISCUSSION

Overall, using *C. flavigena* bacteria combine with the reduction of 25% nitrogen and phosphorus chemistry (75N-45P_2O_5-30K_2O kg/ha) on the IR50404 rice variety grown under submerged and buried straw help increase plant height, number of tillers, root weight, 1,000-grain weight, panicles and the high yield is equivalent to treatments using entirely 100% nitrogen and phosphorus chemistry (100N-60 P_2O_5-30 K_2O kg / ha). Reduction of 25% nitrogen and phosphorus chemistry (25 kg N and 15 kg P_2O_5). According to Sami *et al.* (1988) *Cellulomonas flavigena* bacteria are capable of decomposing straw in 7 days by cellulases and hemicellulases secretion. Besides, *C. flavigena* bacteria helps rice roots not to be organically poisonous and adds organic ingredients to provide nutrients for the growth of roots and activities of the microorganism in the root (Yang *et al*. 2004; Sidiras et al. 2002).

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BIOSYNTHESIS OF NANONUTRIENTS: A FUTURE PROSPECTIVE FOR HIGHER CROP PRODUCTION

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ABSTRACT

The present study demonstrate an eco-friendly and low cost protocol for synthesis of P, Zn, Fe and Mg nanoparticles using cell free filtrate of identified fungi when supplied with aqueous salt solutions. Identification of the fungal isolate was based on nuclear ribosomal DNA internal transcribed space (ITS) identities. Average particle size measurement (Particle size analyzer) was found to be 1.3 to 20.3nm of different nanonutrients used. Transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS) revealed the shape and purity of the particle. With the application of recommended doses (10 ppm for Zn, 20ppm for Mg, 30ppm for Fe and 40 ppm for P) on four different crops (clusterbean, mung bean, pearl millet, wheat) in four different seasons (two kharif and two rabi) at critical growth stage (6weeks old plant), a significant improvement in microbial biomass (13.2-45.8%), which was more under nano-P, was noticed.The beneficial enzyme activities (acid phosphatase, alkaline phosphatase, phytase, dehydrogenase, and esterase) enhance between 9.1 and 90.9% over control. The effect was more under nanonutrients as compared to bio-nutrients. The increase in grain yield over control was varied between 12.0 to 27.6% for cereal (pearl millet and wheat) and between 15.9 to 24.7% for legumes (clusterbean and mung bean). The increase in dry matter yield was observed between 4.5 to 19.6 % for bio-nutrients as compared to 12.6 to35.2% by nanonutrients over control. The results clearly demonstrated better prospective of biosynthesized nanonutrients for higher crop production and the effect of bio-nutrients can be enhanced by mixing with the nanonutrients in equal proportion for the foliar spray, which was more effective on legumes.

Key words: biosynthesis, nanoparticles, bio-nutrients, crop production, beneficial enzymes.

INTRODUCTION

Nanotechnology has started increasing applications to change our lives. It is considered as a generic technology that offers better-built long-lasting, cost-effective and smart products that will find wide applications in agriculture and food industry (NAAS 2013). Taking inspiration from natural biological systems, we were able to develop an alternative strategy for nanoparticle synthesis using microorganisms (Tarafdar, 2012; Tarafdar et al. 2014). Well adapted microbes isolated from native soil conditions can be a better source for bio-inspired synthesis of nanoparticles which may be relatively stable due to natural encapsulation by mother protein (Tarafdar & Raliya, 2011). Phosphorus, zinc, iron and magnesium are the essential nutrient required for plant growth. The elements also are the structural component of beneficial enzymes (Tarafdar et al. 2014; Erdal et al., 2002), which plays crucial role in native nutrient mobilization in the rhizosphere. The application doses also already standardized (Tarafdar et al. 2012) for these elements. The entire characterisation of the nanoparticles was carried out. The aim of the present work was to demonstrate the effect of these biosynthesized nanoparticles to the crops under field condition and compare their effect with the already available bio-nutrients in the market produced by Pratistha Industries Limited in India.

MATERIALS AND METHODS

Biosynthesized nanoparticles of P, Zn, Mg, and Fe were prepared following the procedure of Tarafdar and Raliya (2012). The fungi used for the production of nanoparticles were presented (Table 1). Size distributions of nanoparticles were estimated using DLS measurements that determine particle size by measuring the rate of fluctuations in the laser light intensity scattered by particles as they diffuses through solvent. Particle size analyzer (Beckman Delsa Nano C, USA) was used for size measurements and confirmation of nanoparticle size distribution. For confirmation of size and shape, TEM measurements was carry out using drop coating method in which a drop of solution containing nanoparticles was placed at the carbon- coated copper grids and kept under vacuum desiccation for overnight before loading them on to a specimen holder. The TEM micrographs of the sample were taken using the JEM-2100F TEM instrument. The instrument was operated at an accelerating voltage of 200 KV. For EDS used particularly for elemental composition analysis, samples were prepared on a carbon-coated copper grids and kept under vacuum desiccation for 3 hrs before loading them on to a specimen holder. Elemental analysis on single particles was carried out using Thermo Norman EDS attachment equipped with TEM (JEM-2100F). It was performed for determination of the elemental composition and purity of the sample by atom % of metal.

The seed of pearl millet (cv.HHB67), clusterbean (cv. RGC 936), mung bean (cv. K851) were obtained from seed house of CAZRI, Jodhpur and sown at 3-cm depth, and were grown under rainfed condition during *kharif* 2013 & 2014, whereas wheat (cv. Raj 4083) was procured from the market and sown at 3 cm depth and were grown under irrigated condition during *rabi* (2013 & 14) at arid zone agricultural field ($26^0$18' N $73^0$01 E) of CAZRI to evaluate the effect of biological synthesized nanonutrients as compared to bio-nutrients and in combination, recommended doses of fertilizer under arid condition and control. The bio nutrients

of P, Mg, and Zn were obtained from Pratistha Industries to compare their effect with the nanonutrients as well as control. There were 12 treatments (control, Nano- P, Nano-Zn, Nano–Mg, Nano-Fe, Bio-P, Bio-Zn, Bio-Mg, Nano-P+Bio-P, Nano-Zn+Bio-Zn, Nano-Mg+Bio-Mg, N_{60} P_{40} K_{40}).

On an average 324mm of rainfall was received during the *kharif* season. There were four replicate of each treatments of 20 square meters. The bio nutrients (3 mL L^{-1} as recommended on bottle) and nanonutrients (nano-P 40 ppm, nano-Zn 10ppm, nano-Mg 20 ppm and nano-Fe 30 ppm as recommended) was applied as foliar spray on two weeks (14 days) old plants. The combined treatment received equal amount of (1:1) nano and bio-nutrients. The NPK treatment (N60, P40, K40), was applied in the soil as recommended before sowing of seed. The experiment was conducted in a randomized block designed for two years (four seasons). No other fertilizer and irrigation was received by the crops except wheat crops have received five irrigations during the entire growing periods.

At critical growth stage (6 weeks old plant), four plant of each replicate with interact roots were carefully uprooted and rhizosphere soil was collected for biochemical parameters. The crops were finally harvested at maturity. The rhizosphere enzyme activity was assayed by the method given by Tabatabai (1982) and Tarafdar and Yadav (2013) .

RESULTS AND DISCUSSION

The extra cellular synthesis of Zn, P, Fe and Mg nanoparticle was carried out by exposure of precursor salt of 0.1 mM concentration to fungal cell free filtrate of respective particles (Table 1) in an aqueous solution. The reaction was carried out for 72 hrs.

Table 1. List of organisms used for preparation of nanoparticles, NCBI Accession number size distribution and polydispersity index (PDI) value

Nano- - particle	Name of organism	NCBI GenBank Accession No.	Average Size (nm)	PDI Value
Fe	*Aspergillustubingensis*TFR 3	JN126255	11.7	0.471
Mg	*Aspergillusbrasiliensis*TFR 23	JX999490	1.3	0.387
P	*Emericullanidulance* TFR15	KC175550	5.8	0.435
Zn	*Aspergillusflavus* TFR19	KC175554	20.3	0.463

The results clearly showed that 100% of particles are in nano-form with the average size ranges between 1.3 and 20.3 nm and polydispersity index varies from 0.387 (Mg) to 0.471 (Fe), which also clearly showed that particles are monodispersed. Particle size of bio transformed nanoparticles was analysed by DLS using particle size analyser. The size was further confirmed by TEM. The TEM images of the particles are shown in Fig. 1-4, indicated well distribution of nanoparticles, which was encapsulated by thin layer of protein at the measurement scale bar of 50 nm with 100 KV applied voltage.

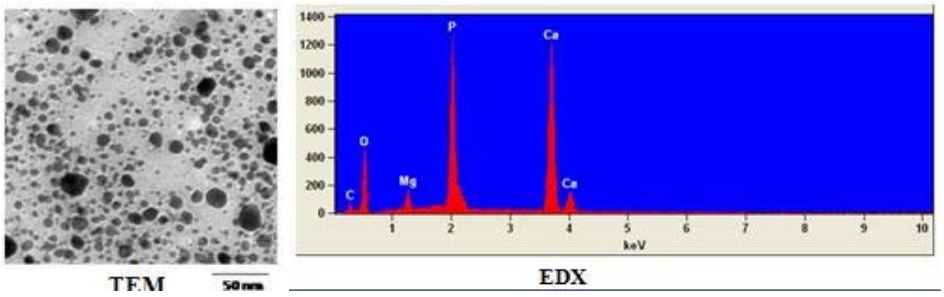

Fig. 1. TEM and EDX images of Phosphorous Nanoparticles used for the study

The EDX analysis confirmed 67% purity of P nanoparticles (Fig. 1) used for the study while 97% purity of Mg nanoparticles (Fig. 2), 98% purity of Zn nanoparticles (Fig. 3) and 93% purity of Fe nanoparticles.

The results demonstrated a significantly higher plant growth in field owing to the application of nano and bio- nutrients as compared to control. At critical growth stage a significant improvement in beneficial enzymes (Table 2) was obtained.

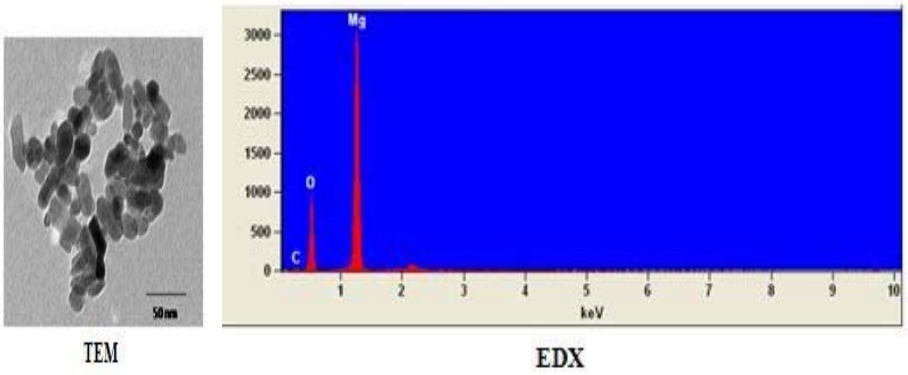

Fig. 2.TEM and EDX images of Mg Nanoparticles used for the study

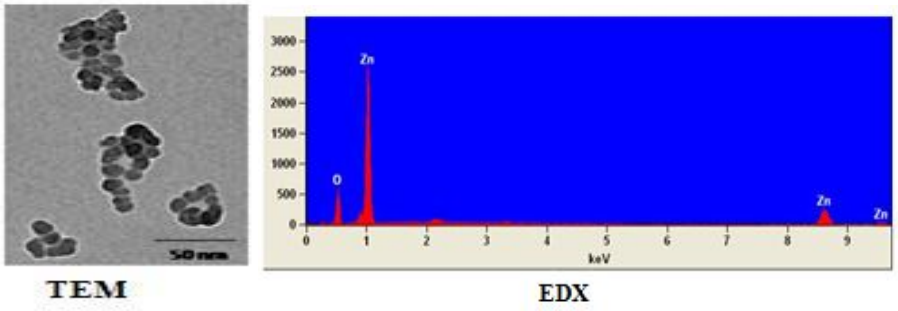

Fig.3. TEM and EDX images of Zn nanoparticles used for the study

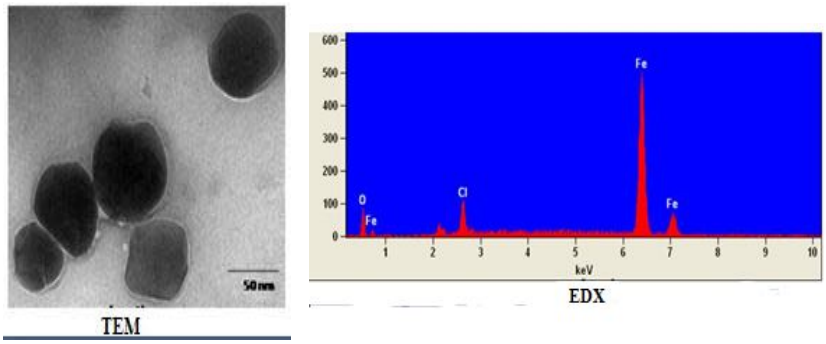

Fig.4. TEM and EDX images of Fe nanoparticles used for the study

Table 2. Effect on beneficial enzyme activities at critical growth stage (% change over control); average of four crops and two years

Treatment	Dehydrogenase	Esterase	Acid Phosphatase	Alkaline Phosphatase	Phytase
Bio-Mg	+8.2	+1.4	+16.1	+11.5	+6.3
Bio-P	+8.9	+27.5	+10.9	+20.1	+6.9
Bio-Zn	-3.3	-6.7	+10.3	+14.9	+7.0
Nano-Fe	+15.7	+19.9	+17.0	+23.2	+9.1
Nano-Mg	+37.9	+43.9	+33.3	+21.7	+19.9
Nano-P	+42.8	+90.9	+28.7	+41.1	+30.7
Nano-Zn	+11.9	+23.8	+27.2	+19.7	+13.7
Nano-Mg+Bio Mg	+32.3	+19.8	+28.6	+18.7	+15.2
Nano-P+bioP	+37.6	+63.7	+20.9	+35.5	+14.2
Nano-Zn+bio Zn	+4.3	+6.2	+18.7	+17.6	+11.0
N60P40K40	+29.1	+77.4	+6.0	+10.2	+4.7

+,- shows % increase and decrease over control, respectively

In general, positive influence on beneficial enzyme activities was noticed over control in all the treatments except the treatment of bio-Zn on dehydrogenase and esterase activities where 3-6% decline in activities was observed. The dehydrogenase, alkaline phosphatase, phytase and esterase activity improvement was more with nano-P treatment and acid phosphatase activity was more in nano-Mg treatment. The more dehydrogenase and esterase activities suggest that enhancement in microbial population in the rhizosphere that may also add in enhancement of nutrient mobilization and availability of nutrients for plant uptake (Tarafdar et al. 2014). Phosphatasesand phytase are P mobilizing enzymes, therefore, increase in their activities indicate more P mobilization in the rhizosphere.

The effect on microbial population also reveals the enhancement over control with the application of both bio and nanonutrients except of bio-Zn. The improvement was much higher with the application of nanonutrients. The microbial biomass also improved significantly (Table 3), which was almost 11 times more in

case of nano-Mg as compared to the recommended NPK fertilisers. Even bio-P showed three times more microbial biomass as compared to the recommended doses of fertilizers while nano-P showed 10 times improvement at critical growth stages of crops.

Table 3. Effect on microbial population and microbial biomass in the rhizosphere at critical growth stage (% change over control, average of four crops and two years)

Treatment	Microbial population			Microbial biomass
	Fungi	Bacteria	Actinomycetes	
Bio-Mg	+2.0	+1.5	+10.7	+5.8
Bio-P	+43.8	+7.2	+7.7	+11.7
Bio-Zn	-1.2	-4.6	+2.3	0.0
Nano-Fe	+39.7	+49..4	+11.9	+13.2
Nano-Mg	+70.7	+67.2	+39.8	+45.8
Nano-P	+75.5	+59.4	+16.6	+39.8
Nano-Zn	+27.6	+3.2	+18.4	+18.7
Nano-Mg+BioMg	+67.7	+63.4	+31.2	+40.1
Nano-P+Bio-P	+71.1	+50.5	+14.2	+33.4
Nano-Zn+Bio-zn	+18.8	+3.1	+16.9	+15.6
N60, P40, K40	+7.9	+11.1	+1.6	+3.9

+,- shows % increase and decrease over control, respectively

The effect of bio- and nanonutrients on yield of cereals at crop maturity was presented as Table 4. The result suggested that nano P was most effective on pearl millet and wheat crops where 27.6% and 18.8% respectively yield increase were noticed. The dry matter yield also increased by 26.5% and 21.2% which was much higher than recommended doses of fertilizers as well as bio-nutrients. In general, more positive effect on pearl millet crop than wheat between the two cereals tested for the study.

Table 4. Effect of bio- and nanonutrients on yield of cereals (pearl millet & Wheat) under field condition at crop maturity (average of two seasons)

Treatment	Grain yield(Kg/ha)		Dry matter yield(Kg/Ha)	
	Pearl Millet	Wheat	Pearl Millet	Wheat
Control	782	3856	2320	6131
Bio-Mg	852(9.0)	4025(4.4)	2598(12.0)	6714(9.5)
Bio-P	861(10.1)	4118(6.8)	2610(12.5)	6801(9.9)
Bio-Zn	841(7.5)	4221(9.5)	2482(7.0)	6728(9.7)
Nano-Fe	889(13.7)	4318(12.0)	2712(16.9)	6902(12.6)
Nano-Mg	938(19.9)	4489(16.4)	2885(24.4)	7215(17.7)

Nano-P	998(27.6)	4582(18.8)	2935(26.5)	7428(21.2)
Nano-Zn	941(20.3)	4502(16.8)	2930(26.3)	7381(20.4)
Nano-Mg+ Bio-Mg	908(16.1)	4421(14.7)	2871(23.8)	6925(13.0)
Nano-P+ Bio-P	982(25.6)	4576(18.7)	2905(25.2)	7380(20.4)
Nano-Zn+Bio-Zn	945(20.8)	4480(16.2)	2900(25.0)	7385(20.5)
N60P40K40	885(13.2)	4312(11.8)	2718(17.2)	6900(12.5)

*Fig.s in parenthesis indicate the percent improvement over control

The effect on legumes showed more enhancement of yield on mung bean than clusterbean (Table 5). The maximum effect also noticed due to application of nano-P. In general, two times more effect with the application of nanonutrients as compared to bio-nutrients was observed. The effect of nanonutrients was also much higher than the recommended doses of fertilizer. The results clearly showed that the effect of bio-nutrients can be magnified after mixing with equal quantity of nanonutrients for foliar spray.

Table 5. Effect of bio and nano nutrients on yield of legumes (cluster bean & mung bean) under field condition at crop maturity (average of two seasons).

Treatment	**Grain yield (Kg ha-^{1})**		**Dry matter yield(Kg ha-^{1})**	
	Clusterbean	**Mungbeam**	**Clusterbean**	**Mungbean**
Control	765	860	1836	1580
Bio-Mg	834 (9.0)	929(8.0)	1918(4.5)	1862(17.8)
Bio-P	842 (10.1)	942(8.7)	1979(7.8)	1890(19.6)
Bio-Zn	837(9.4)	912(6.0)	2000(8.9)	1834(16.1)
Nano-Fe	891(16.5)	982(14.2)	2138(16.4)	2001(26.6)
Nano-Mg	887(15.9)	996(15.8)	2217(20.8)	2016(27.6)
Nano-P	930(21.6)	1072(24.7)	2232(21.6)	2136(35.2)
Nano-Zn	916(19.7)	999(16.2)	2244(22.2)	2011(27.3)
Nano-Mg+Bio-Mg	880(15.0)	945(9.9)	2200(19.8)	1895(19.9)
Nano-P+Bio-Zn	901(17.8)	1025(19.2)	2162(17.8)	2051(29.8)
Nano-Zn+Bio-Zn	894(16.9)	994(15.6)	2235(21.7)	2005(26.9)
N_{60},P_{40},K_{40}	861(12.5)	932(8.4)	2109(14.9)	1864(18.0)

Fig. under parenthesis indicate % increase are control.

The results clearly indicate the positive effect of nano-nutrients over bio-nutrients and recommended doses of fertilizer which will generate new knowledge in nanoparticle farming and shall address to the current emerging issue of decreasing resource use efficiency with emphasis of nutrients this approach would also enable in breaking of existing barriers in utilisation of native nutrient and reduce dependence on fertilizers.

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ISOLATION OF *BACILLUS LICHENIFORMIS* MH48 WITH ANTAGONISTIC ACTIVITY AGAINST PLANT PATHOGENS

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ABSTRACT

This work presents the evaluation of the capacity of Bacillus licheniformis MH48 against plant fungal pathogens, and establishes its role as a biocontrol agent. In this study, we isolated an bacterial strain MH48 from rhizosphere soil, identified the strain as Bacillus licheniformis MH48 by the 16S rRNA gene sequence analysis and demonstrated significant antifungal activity against Rhizoctonia solani, Colletotrichum gloeosporioides, Botrytis cinerea, Phytophthora capsici, Pestalotiopsis karstenii, Pestalotia diospyri, Glomerella cingulata. MH48 produced lytic enzymes such as chitinase, β-1,3-glucanase, protease. MH48 showed strong antagonistic activities against the fungal pathogens in dual culture assays. In addition, bacterial culture filtrate of B. licheniformis MH48 was highly antifungal activity in inhibiting growths of the fungal pathogens at 50% concentration. As a result, we worked that evidences for antagonism were established by production of antifungal metabolites from B. licheniformis MH48. Bacterial crude extract of B. licheniformis MH48 with 10mg amount significantly inhibited the fungal pathogens growth. The antifungal crude extract was purified by silica gel column chromatography, Sephadex LH-20 column chromatography and ODS column chromatography. This work is in progress and the final purification step. This study suggests that our results demonstrated B. licheniformis MH48 as a potential biological control agent for various fungal pathogens management.

Keywords : Antagonistic activity, *Bacillus licheniformis*, Enzyme activity, Plant fungal pathogen, Crude extract.

INTRODUCTION

Fungal plant pathogens are among the most important biotic agents causing serious losses and damages to agricultural products. Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced by growers around the world. Plant diseases are mostly controlled by the use of chemical pesticides and in some cases by cultural practices. But the widespread use of chemicals has been a subject of public concern and scrutiny due to the potential harmful effects on the environment, their unwanted effects on non-target organisms and possible carcinogenicity of some chemicals (Agrios, 1988; Cook, 1993; Heydari, 2007; Heydari et al. 2007). The need for the development of non-chemical alternative methods to control plant diseases is therefore clear, i.e. the use of microorganisms or their metabolites (Ronel et al. 1986; Montesinos, 2003).

During the past 50 years, many studies were reported about bacterial and fungal plant pathogens as well as the application of different microbes as biocontrol agents (Hoda et al. 2011). Antagonism could be considered as d method of inhibiting phytopathogenic fungi through secretion of substances that interrupt with the life cycle of the target microbe (Pliego et al. 2010).

The microorganisms of *Bacillus* genus are known to be one of the most important biocontrol agents, avirulence to humans and this can be genetically modified as Gram positive bacteria the endospores. Also, Several *Bacillus* species produce various antibiotics some of which have antifungal activity (Korzybski et al. 1978).These antifungal antibiotics play a major role in biological control of plant pathogens (Phae et al. 1990; Potera 1994; Leifertet al. 1995).

This study focused on isolation, selection and characterization of *Bacillus licheniformis* MH48 for the biological control efficacy against various plant pathogens as well as their role as biocontrol agents.

MATERIALS AND METHODS

Soil samples collected from the reclaimed land around the Gunsan, Korea in April, 2014. Soil were diluted and inoculated on chitin yeast medium (CY) containing colloidal chitin 0.2%, yeast extract 0.1% and agar 2% and incubated at 30º for 5 days. After several colonies possessing strong chitin clearance zones were selected and sub-cultured on the same medium for more purification. All isolates were tested by a dual culture assay against various plant pathogens. One isolate having the strongest antifungal activity was selected and stored in glycerol solution 25% at 70ºC for further experiments.

To examine cell growth, *B. licheniformis* MH48 was cultured on Fertilizer medium containing ($(NH_2)_2$ CO 0.27%; KH_2PO_4 0.24%; KCl 0.06%; K_2SO_4 0.01%, Ca-Mg fertilizer 0.02%; sucrose 0.8%; yeast extract 0.1% and crab shell powder 0.1%) at 30ºC on a rotary shaker at 140rpm for 7days.Colony forming unit (CFU) has been counted from 1 to 7days after inoculation using serial dilution technique on TSA plates.

To examine chitinase, β-1,3-glucanase and protease activity, MH48 was cultured on Fertilizer medium at 30ºC on a rotary shaker at 140 rpm for 7days. The

supernatant was daily collected and assayed by method of Tabatabai (1982) and Yedidia et al. (2000), separately. Also, pretease activities were determined by method of Kembhavi et al. (1993).

MH48 was cultured in fertilizer medium on a rotary shaker at 140rpm for 5days. Supernatant colleted by centrifugation at 8000rpm for 20min at 4ºC was rendered cell-free by filtration through a sterile membrane with a 0.45μm pore size to obtain bacterial culture filtrate(BCF). BCF was added to potato dextrose agar (PDA) at around 50^0C to the final concentrations of 0, 10, 30 and 50% (v/v). PDA plates without BCF (0%) were used as controls. A mycelial plug from a culture of *Rhizoctonia solani* (RS), *Phytophthora capsici* (PC), *Collectotrichum gloeosporioides* (CG), *Botrytis cinerea* (BC) was placed in the center of the PDA plate and incubated at 28^0C in the dark. Mycelial growth was measured at 3days for *R. solani* and 7days for *P. capsici*, *C. gloeosporides* and *B. cinerea*. Growth inhibition percentage = (R-r)/R*100; 'R' is the radius of the fungal colony in the control plates, and 'r' is the radius of the fungal cology in the treatment plates.

For preparation of antifungal metabolites, the bacterial culture filtrate obtained as described above was acidified with concentrated HCl to pH 2.0 and extracted with an equal volume of chloroform. The soluble organic fraction was concentrated by a rotary evaporator. The antifungal metabolites was dissolved in 3ml methanol and subjected to silica gel column chromatography with stepwise elution of Chloroform: MeOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60 and 0:100; v/v). All fractions of the elution were concentrated, and each fraction dissolved in MeOH was tested for antifungal activity against *R. solani*. *C. gloeosporides* by paper disk method. The fraction (100:0, 1.87g) having the highest antifungal activity was purified by gel filtration on Sephadex LH-20 column chromatography using running phase of 100% MeOH (100ml). Fractions (2ml) were collected separately and tested for the antifungal activity by paper disk method. Fractions (710mg) showing strong antifungal activity was further purified by ODS column chromatography, and eluted with a stepwise gradient of H_2O:MeOH (100:0, 95:5, 90:10,…, 5:95 and 0:100; v/v). One fraction (30:70, 52mg) showing strong antifungal activity was further purified by C18 column chromatography using the stepwise elution of H_2O: MeOH (5:0, 4.75:0.25, 4.5:0.5,…, 0.25:4.75 and 0:5; v/v). Each fraction was separately collected and tested for antifungal activity against *R. solani*. Finally, a purified antifungal compound (2.25:2.75, 6mg) confirmed by showing one major peak in high performance liquid chromatography (HPLC) analysis using a C18 reversed-phase column. The mobile phase of H_2O: MeOH (35:65; v/v) at a flow rate of 0.5ml/min was used and the peak was detected at 254nm by a SPD-10 UV-VIS detector.

Chemical structure of the purified antifungal compound was mainly determined by ^{1}H-NMR analysis. The purified antifungal compound was dissolved in 0.5ml methanol-*d*4 (CD_3OD) and subjected to spectral analysis. NMR spectra were recorded on a Bruker (Rheinstetten, Germany) DRX 500 NMR instrument operating at 600 MHz for ^{1}H at room temperature. Chemical shifts were reported in ppm (δ) using CD_3OD as a solvent.

Molecular weight of the purified compound was identified by gas chromatography-mass spectrometry (GC-MS) and confirmed by comparing to the standard compound.

RESULTS AND DISCUSSION

This isolate was identified as *Bacillus licheniformis* based on 16S rRNA gene sequence analysis. The septate isolate sequence showed high identity when compared with other matching sequences, and designated as *Bacillus licheniformis* MH48 with GenBank accession No. KP099612 and BLAST analysis revealed strong homology with *Bacillus licheniformis* JF62 (KF053203) data not shown.

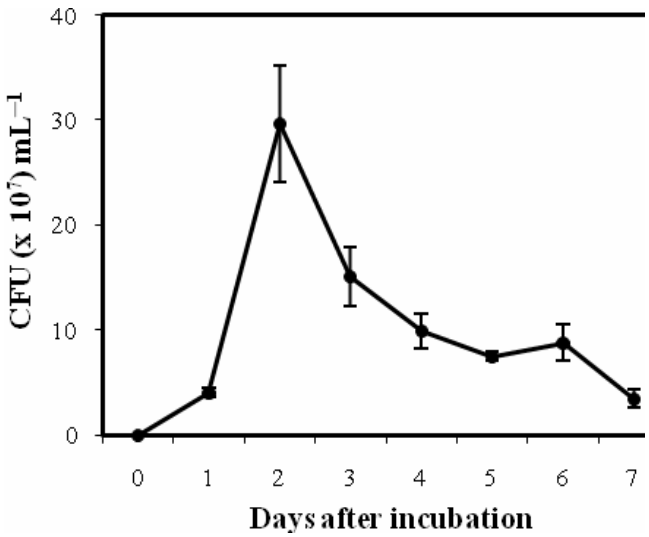

Fig 1. Cell growth curve for *Bacillus licheniformis* MH48 on fertilizer medium at 30ºC for 7 days. Calculated mean values are from three replicates. Error bars represent standard error of the mean.

B. licheniformis MH48 colony growths in Fertilizer medium were evaluated by counting CFU in TSA plates (Fig. 1). Growth of MH48 rapidly increased from 1 to 2days. After that it gradually decreased to 7 days. The highest cell growth was found to be 29.7x 10^7 CFU mL^{-1} after 2 days of incubation.

Production of lytic enzymes, chitinase, β-1,3-glucanase and protease, was examined from the culture supernatant of MH48. Chitinase activity gradually increased in the time period of 3 days, and eventually reached a maximum value of 0.57 unit/ml (Fig. 2A). Also, β-1,3-glucanase activity rapidly increased in the time period of 2 days, a maximum value of 6.04 unit/ml (Fig. 2B). Thereafter, they gradually decreased until 7 days. Similarly, Protease activity was a gradual increase unil 3day (0.79 unit/ml) and then it declined (Fig. 2C).

The various concentrations of BCF inhibited the growths of tested pathogenic fungi. The inhibitory effects were significantly increased with the increase in concentrations of BCF from *B. licheniformis* MH48 (Fig. 3). At 30% concentration, BCF clearly showed visible inhibitions against tested pathogenic fungi (Fig. 3A). BCF concentration of 50% inhibited 96.1%, 100%, 63.1% and 100% growth of *R. solani*, *P. capsici*, *C. gloeosporides* and *B. cinerea*, respectively (Fig. 3B).

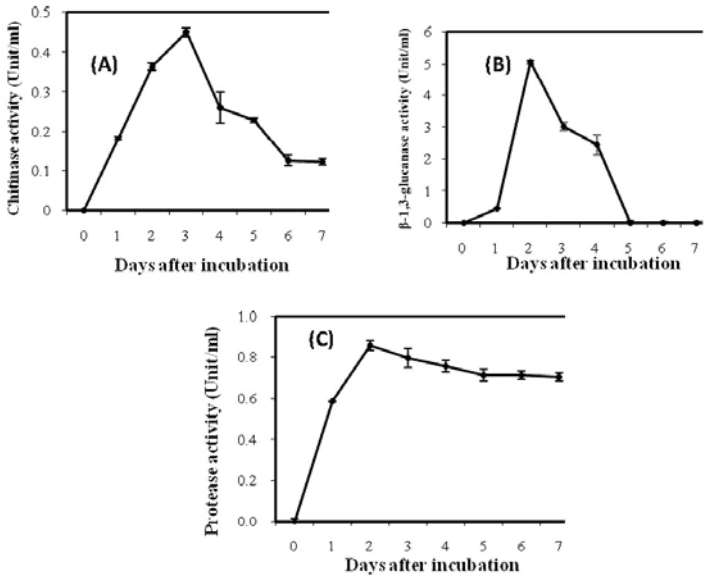

Fig. 2. Changes in Chitinase (A), β-1,3-glucanase (B) and protease (C) activity of *Bacillus licheniformis* MH48 culture broth at 30ºC for 7 days. Calculated mean values are from three replicates. Error bars represent standard error of the mean.

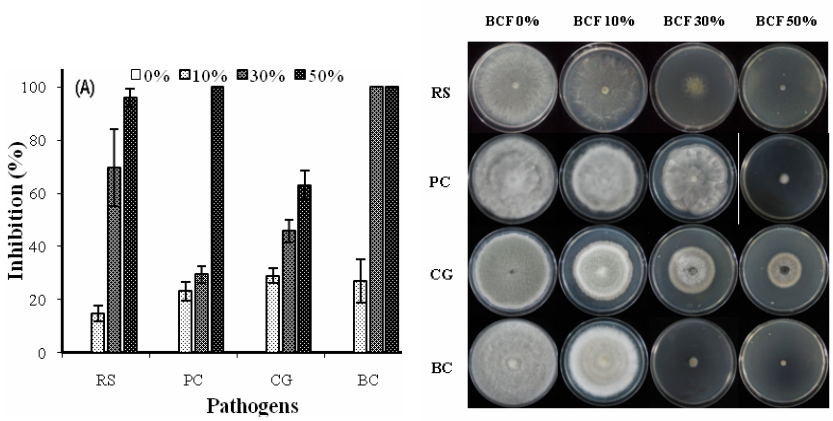

Fig 3A, 3B. Growth inhibition of *R. solani* (RS), *P. capsici* (PC), *C. gloeosporioides* (CG) and *B. cinerea* (BC) by bacterial culture filtrate (BCF) from *B. licheniformis* MH48. In Fig (A) and pictures (B). Calculated mean values are from triplicates. Error bars represent standard error of the mean.

Though organic extraction and different chromatography techniques, 6mg of a Purified antifungal compound was isolated from 40L culture broth of *B. licheniformis* MH48. The purified antifungal compound as yellowish solid obtained from a distinct fraction showed HPLC retention time of 18.18min (Fig. 4).

The chemical property of purified antifungal compound was identified according to its ^{1}H-NMR spectra: 600MHz, CD_3OD (Fig. 5). When the purified

compound was analyzed by GC-MS (by trimethyl silylation), it was confirmed as benzoic acidwith 194 *m/z* molecular weight (Fig. 6).

In this study, we purified and identified an antifungal compound from *B. licheniformis* MH48 designated benzoic acid, and determined its structure based on spectral data. Benzoic acid derivatives are known to be the best inhibitors of some major postharvest pathogens including *Alternaria* spp, *B. cinerea, P. digitatum, S. sclerotiorum* and *F. oxysporum* (Barkai Golan, 2001). However, until this report, there has been no information of benzoic acid derived from strains of *B. licheniformis* MH48.

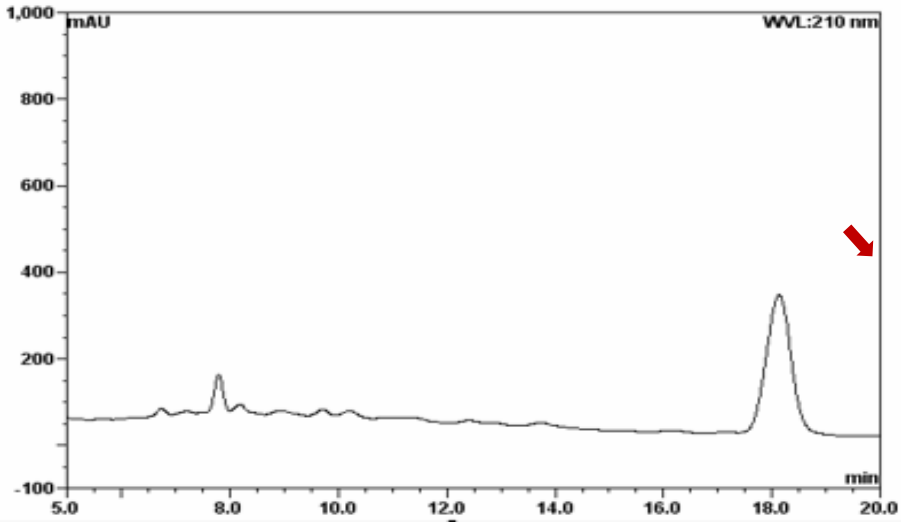

Fig. 4. High performance liquid chromatography spectrum of purified antifungal compound isolated from culture filtrate of *B. licheniformis* MH48.

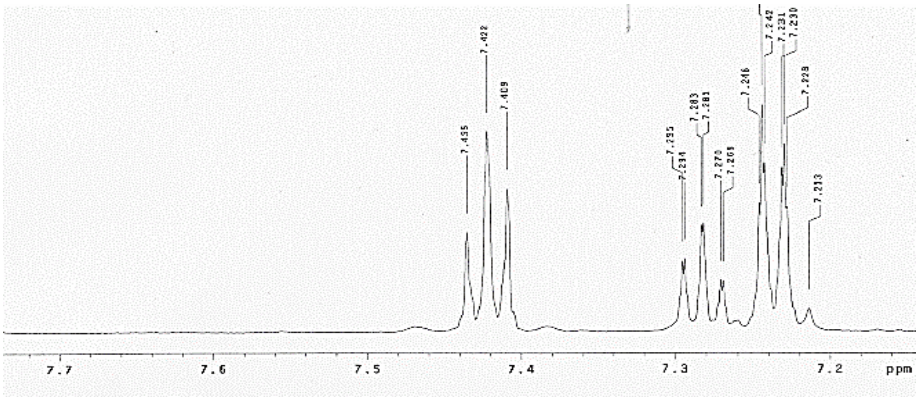

Fig 5. 1H nuclear magnetic resonance (NMR) spectra of the purified antifungal compound from *B. licheniformis* MH48.

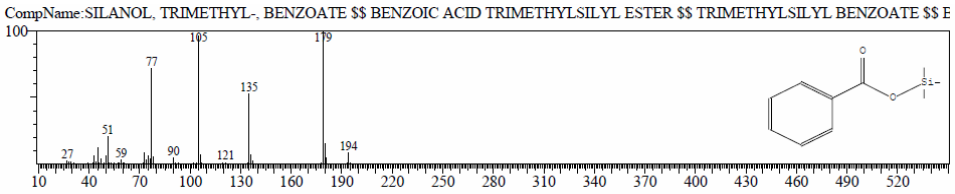

Fig 6. Gas chromatography-mass spectrometry (GC-MS) spectra of trimethyl silylated the purified antifungal compound from *B. licheniformis* MH48.

Further studies are needed to evaluate its antifungal effects under in vitro and in vivo. In additions, the possible role of benzoic acid in the antagonism of *B. licheniformis* MH48 towards several phytopathogens should be further studied for consideration of this bacterium as a promising biocontrol agent.

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