International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 6(4): 571-580 December 2013
DOI Number 10.5958/j.2230-732X.6.4.033

Plant Pathology

Antagonistic Activity of Plant Growth Promoting Rhizobacteria Isolated from Tomato Rhizosphere Against Soil Borne Fungal Plant Pathogens

Abhishek Walia[1], Preeti Mehta[2], Anjali Chauhan[3] and C. K. Shirkot[1]*

[1] Department of Basic Sciences (Microbiology Section), Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan – 173230, India
[2]National Institute of Plant Genome Research, Department of Biotechnology, Govt. of India, 110067, New Delhi, India
[3]Department of Basic Sciences (Microbiology Section), Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan – 173230, India

Email: profckshirkot@gmail.com

Paper No. 153 Received: January 17, 2013 Accepted: August 17, 2013 Published: November 29, 2013

Abstract

The use of single strain inoculum with multiple plant growth promoting activities offers a new concept to address mode of action by combined use of multi-strain inoculum of plant growth promoting rhizobacteria (PGPR) each with specific capability and function. In the present work, bacterial isolates with plant growth promoting activities like P-solubilization, IAA production, siderophore production and broad spectrum antifungal activity against *Fusarium oxysporum*, *Rhizoctonia solani and Sclerotinia sclerotiorum* were isolated from the rhizosphere soil of tomato seedlings. *In vitro* antifungal antibiotic study revealed that among eleven isolates N_{11} showed maximum inhibition against *F. oxysporum* (82.85%), *R. solani* (76.45%) and *S. sclerotiorum* (74.71%) after seven days of incubation. The per cent growth inhibition increased with increase in bacterial cell density from O.D 0.25 to 1.50. The novelty of the isolates is that the antibiotic production is induced only in the presence of fungal host and lack of antifungal activity in culture filtrate. Bacterial isolate N_{11} showing maximum plant growth promoting activities and was identified to species level by biochemical characterization and 16S rRNA sequencing. Preliminary identification of bacterial isolate was made on the basis of morphological and biochemical characters and confirmed by partial 16S rRNA gene sequencing, which validated N_{11} as *Bacillus subtilis* CKT1.Thus, the present study concludes that these bacterial isolate could serve as a proficient biocontrol PGPR inoculant in the integrated management of soil borne diseases of tomato.

Highlights

The present study suggests that the use of single strain inoculum of CKT1 as a proficient biocontrol PGPR with multiple plant growth promoting activities offers a recent concept to address multiple mode of action by combined use of multi-strain inoculum of PGPR with each having specific capability and function.

Keywords: Tomato, PGPR, antifungal activity, phosphate solubilization, IAA and siderophore production

Tomato (*Solanum lycopersicum* L.) is most important cash crop grown in mid hills regions of Himachal Pradesh. Since, tomato plays an important role in human health; the quality of the nutritional components of this major vegetable crop is of particular concern to producers and consumers throughout the world. Pathogenic micro-organisms

affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production intensified over the past few decades, producers became more and more dependent on agro-chemicals as a relatively reliable method of crop protection. The use of chemical pesticides has caused soil pollution and harmful effects on human beings. One of the most acceptable and environmentally conscious approaches to solve these problems is the use of naturally occurring microbial inoculants.

PGPR have gained worldwide importance and interest because of their agricultural benefits and are thus potential tools for sustainable agriculture for the future (Kloepper *et al*., 2007). The use of (PGPR) offers an alternative way to replace and /or supplement chemical fertilizers and pesticides. Soil bacteria living in the rhizosphere can enhance plant growth by several mechanisms like antagonism against plant pathogens, solubilization of phosphates (De Freitas *et al*., 1997), production of phytohormones (Frankenberger and Arshad 1995; Arshad and Frankenberger 1998), siderophores production (Kloepper *et al.,* 1980*;* Raaska *et al*., 1993), antibiotic production (Schinder *et al.,* 1994), inhibition of plant ethylene synthesis (Glick *et al.,* 1998) and induction of plant systemic resistance to pathogens (Kloepper *et al.,* 1999). Utilization of these beneficial microorganisms can reduce the use of pesticides and fertilizers that are potential pollutants of the environment.

The concept of using PGPR mixture for disease control and plant growth promotion has already been well demonstrated in several crops. However, in some instances the root colonization of participating member in a consortium was severely affected. Compatibility of individual strains in mixture among themselves, and with commonly used agrochemicals is also some of the constraints in the use of multiple strain mixture of PGPR. Isolation of a PGPR strain with multiple plant growth promoting activities might help to address these problems. Recently, such a PGPR strain of *Bacillus circulans* MTCC 8983 has been isolated from rhizosphere of apple (Mehta *et al*., 2010; Shirkot and Sharma, 2005; Sultana *et al.,* 2004). PGPR has not yet been reported for tomato crop of mid-hill temperate region of Himachal Pradesh, India. Thus, the aim of this study was to isolate and characterize PGPR with multiple plant growth promoting activities and broad spectrum antifungal activity.

Materials and Methods

Isolation of rhizobacteria

Bacteria were isolated from the rhizosphere of tomato plants from Solan (Himachal Pradesh, India). Plants and roots were collected by removing 20 blocks of soil, which were kept in plastic bags at 0°C for 12 hr until processing. Roots were separated from soil, soaked in sterile phosphate-buffered saline (PBS, pH 7.2, 10 mM K_2PO_4-KH_2PO_4, 0.14 M NaCl) for 10 min, chopped into 3 pieces and 1 g suspended in 9 ml PBS. Root samples were blended in a sterile Waring blender at high speed for 1 min and serial dilutions (1/10) made in PBS. Aliquots (0.1 ml) were plated onto nonselective (1/10 strength) nutrient agar (1/ 10 NAM) medium using a spiral platter and plates incubated at 37°C for 3 days. Out of sixty isolates eleven rhizobacteria and endorhizobacterial isolates were selected on the basis of P-solubilization, IAA and siderophore production by modified replica plating technique. Isolates were restreaked on NAM (pH 7.0) of following composition (g/l): Peptone, 5.0; Beef extract, 3.0; NaCl, 5.0; Agar, 20.0, checked for purity, and stored on NAM slants at 4°C. The bacterial culture was maintained in 30% glycerol at -20° C.

Phosphorus solubilization

An aliquot (0.1-1.0 ml) from the culture supernatant was made to final volume of 5 ml with distilled water and 5 ml ammonium molybdate was added. The mixture was thoroughly shaken. The contents of the flasks were diluted to 20 ml. 1.0 ml of chlorostannous acid was added and its volume was made to 25ml in the volumetric flask. The contents were mixed thoroughly and the blue colored intensity was measured after 10 minutes at 660 nm. An appropriate blank was kept in which all reagents were added except the culture (Sundara Rao *et al.,* 1963).

P solubilizaton = T- C

Where,

T = PVK with TCP, inoculated

C = PVK with TCP, un-inoculated

Siderophore production

0.5 ml of cell free supernatant was mixed with 0.5 ml Chrome-azurol-S (CAS) assay solution along with 10 µl of shuttle solution (0.2 M 5-Sulfosalicyclic acid). It was kept at room temperature for ten minutes and absorbance was recorded at 630 nm for change in color from blue to pink. The minimal medium was used as a blank and the

reference (r) was prepared using exactly the same components except the cell free extract of culture supernatant (Schwyn *et al.*, 1987).

The siderophore units were calculated using the equation:

$$\text{Per cent siderophore unit} = \frac{Ar - As}{Ar} \times 100$$

Where,

Ar is the absorbance at 630 nm of reference.

As is the absorbance at 630 nm of the test.

Indole acetic acid (IAA) production

Quantitative measurement of auxin was done by colorimetric method (Glick 1995) with slight modification. 2-3 drops of orthophosphoric acid was added to 2 ml of supernatant and 4ml of Salper reagent (2ml of 0.5M $FeCl_3$) in 98 ml of 35% $HClO_4$). This mixture was incubated at room temperature in dark for 25 minutes. Absorbance was measured at 535nm for the development of pink color. Concentration of Indole-3-acetic Acid was estimated by preparing calibration curve using Indole-3-acetic Acid (IAA, Hi-media) as standard (10-100µg/ml).

Production of Hydrogen Cyanide (HCN)

To determine the production of HCN, bacteria were streaked onto Kings'B agar plates supplemented with glycine (4.4 g l^{-1}). After this, the Petri dish was inverted and a piece of filter paper impregnated with 0.5% picric acid (yellow) and 2% sodium carbonate was placed on the lid. Petri dish was sealed with parafilm and incubated at 28ºC for 96 h. Discoloration of the filter paper from orange to brown after incubation was considered as microbial production of cyanide (Kumar *et al.*, 2005).

In vitro compatibility assessment

Dual culture compatibility assessment

A culture of bacterial isolate was spot inoculated on nutrient agar medium. The plates were incubated for 48 hr at 35±2°C. The incubated plates were over- layered with 2 ml of half strength molten nutrient agar (sterilized) containing culture of another bacterial isolate. Similarly, culture of another bacterial isolate was spot inoculated on nutrient agar medium, followed by 48 hr incubation at 35±2°C and over-layering with 2 ml of half strength molten nutrient agar (sterilized) containing culture of bacterial isolate. The plates were observed for inhibition zone after 48 hr of incubation at 35±2°C and experiment was replicated thrice.

***In vitro* evaluation of the antagonistic activity of isolated bacteria against soil-borne fungi by agar streak method**

The rhizospheric bacteria isolated were evaluated by agar streak method for their *in vitro* antagonistic activity against *F. oxysporum, S. sclerotinia, R. solani*. Bacterial isolates were incubated in broth medium (NB based on the medium from which they were isolated) at 35°C. Fungal pathogens were grown on malt extract agar (MEA). In order to test the efficacy of the rhizobacterial antagonists, a loop full of 48h old culture of each isolate and their mixture were streaked a little below the centre of the pre poured petriplates (MEA) separately. Mycelial disc of 5 days old culture of the test fungal pathogen was placed simultaneously on one side of the streak (three replicate plates per each biocontrol agent–pathogen). A check inoculated with the test pathogen only was kept for comparison. Isolates that formed zones of inhibition (haloes without mycelial growth or distorted hyphae) over 2 mm were selected. Per cent growth inhibition was calculated according to Vincent 1947 using the equation:

$$I = \frac{C - T}{C} \times 100$$

Where, I = per cent growth inhibition; C = growth in control; T = growth in treatment.

Antifungal antibiotic activity by using agar diffusion assay

Agar diffusion assay was used for *in vitro* screening of PGPR for antifungal antibiotic activity. Inhibition of the fungi was studied by adding bacterial cell suspension of different population density (OD 0.25, 0.5, 1.0 and 1.5 at 540 nm) in MEA before plating. The fungal bit was placed in the middle of the plates and incubated at 24±1°C for seven days. In control, only nutrient broth was added in place of the bacterial cell suspension (three replicate plates per each biocontrol agent–pathogen). The inhibitory effect of cell pellet was determined using dilution technique (Warnock 1989). Per cent growth inhibition was calculated according to Vincent 1947 using the equation:

$$I = \frac{C - T}{C} \times 100$$

Where, I = per cent growth inhibition; C = growth in control; T = growth in treatment.

Phenotypic and metabolic characterization

Morphological and biochemical tests were performed by using standard protocol (Indole test, Methyl red test, Citrate utilization, Starch hydrolysis, Casein hydrolysis, Gelatin

hydrolysis, Hydrogen supplied production, Catalase test, Voges Proskauer test, Urea hydrolysis, Fermentation of carbohydrates).

Molecular taxonomic characterization

Bacillus isolate was grown overnight in nutrient broth at 37°C. Bacterial cells were harvested by centrifugation at 5,000×g for 5 min and DNA was isolated from these bacterial cells by using Real genomic DNA extraction kit as per manufacturers instruction. The isolated DNA was finally suspended in 100 µl of elution buffer and quantified on 1% agarose gel**.** The total genomic DNA was kept at -20°C before use (Sambrook and Russel 2001).

16S rRNA region was amplified using the *Bacillus* genus-specific 16S rRNA gene PCR primers. The forward primer Bs-for 25 mer (5´-GCAAG TCGA GCGG ACA GA T GGG AGC-3´) and reverse primer Bs-rev 25 mer (5´-AACTCTCGTGGTGTGACGGGCGGTG-3´) were used to amplify the total genomic DNA. The PCR reaction was carried out in 20 µl reaction containing ~50µg of template DNA, 20 pmoles of each primer, 0.2 mM dNTPs and 1 U Taq polymerase (MP Biomedicals) in 1x PCR buffer. Reaction were cycled 35 times as 94ºC for 30 s, 58ºC for 30 s, 72ºC for 1 min 30 s followed by final extension at 72ºC for 10 min. The PCR products were analyzed on 1% agarose gel along with 100 bp ladder as marker in 1x TAE buffer, run at 100V for 1 hr. Gels were stained with ethidium bromide and photographed. Amplified PCR products were eluted from the gel using real genomic (Hi Yield ™ Gel/PCR DNA Extraction Kit), eluted fragment was then sequenced using PCR primers.

Sequence analysis

The sequence was aligned with corresponding sequences of 16S rRNA from the database using BLAST (Altschul *et al.*, 1997). Multiple alignments were generated by the MULTALIN program (Corpet 1988). Phylogenetic tree was constructed with the help of ClustalW (Higgins *et al.*, 1994). Tree was viewed with the help of TreeView (Page 1996).

Statistical Analysis

All the experiments were conducted in triplicates along with equal number of appropriate controls. Data were statistically analyzed by analysis of various techniques (one way classification) following Gomez and Gomez (1976).

Results and Discussion

Isolation and selection of PGPR

A total of hundred bacterial strains were isolated by serial dilution method for multiple plant growth promoting activities. In the present study (Fig.1) the eleven bacterial isolates showed maximum variation in their ability for phosphate solubilization, indole-3-acetic acid and siderophore production were selected for further studies

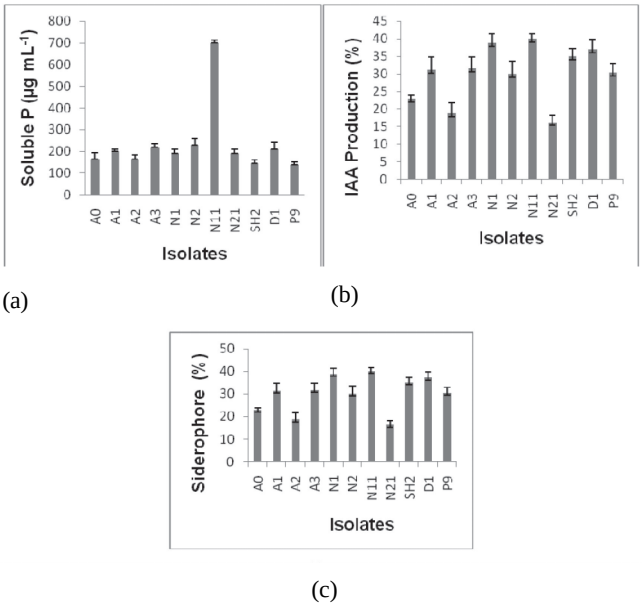

Fig. 1: Phosphate solubilization (a), IAA production (b) and Siderophore production (c) observed in the bacterial strains obtained from tomato plants. Each value represents the mean of three replicates and the error bars represent the standard deviations of the average

(Lee *et al.*, 2005; Ashrafuzzaman *et al.*, 2009).

P-solubilization and IAA production

It was observed that maximum P-solubilization (700µg/ml) occurred up to 72 h of incubation. P-solubilization was accompanied by a decrease in pH of the culture filtrate from 7.2 initially to 5.07. The decrease in pH indicates the production of organic acids considered responsible for P-solubilization (Daimon *et al.*, 2006). Maximum growth ($39.67x10^7$ cfu/ml) coincides with the maximum amount of P-solubilization and is in agreement with the earlier reports on P-solubilization (Trivedi and Tongmin, 2008). These phosphate solubilizing microorganisms (PSM) render

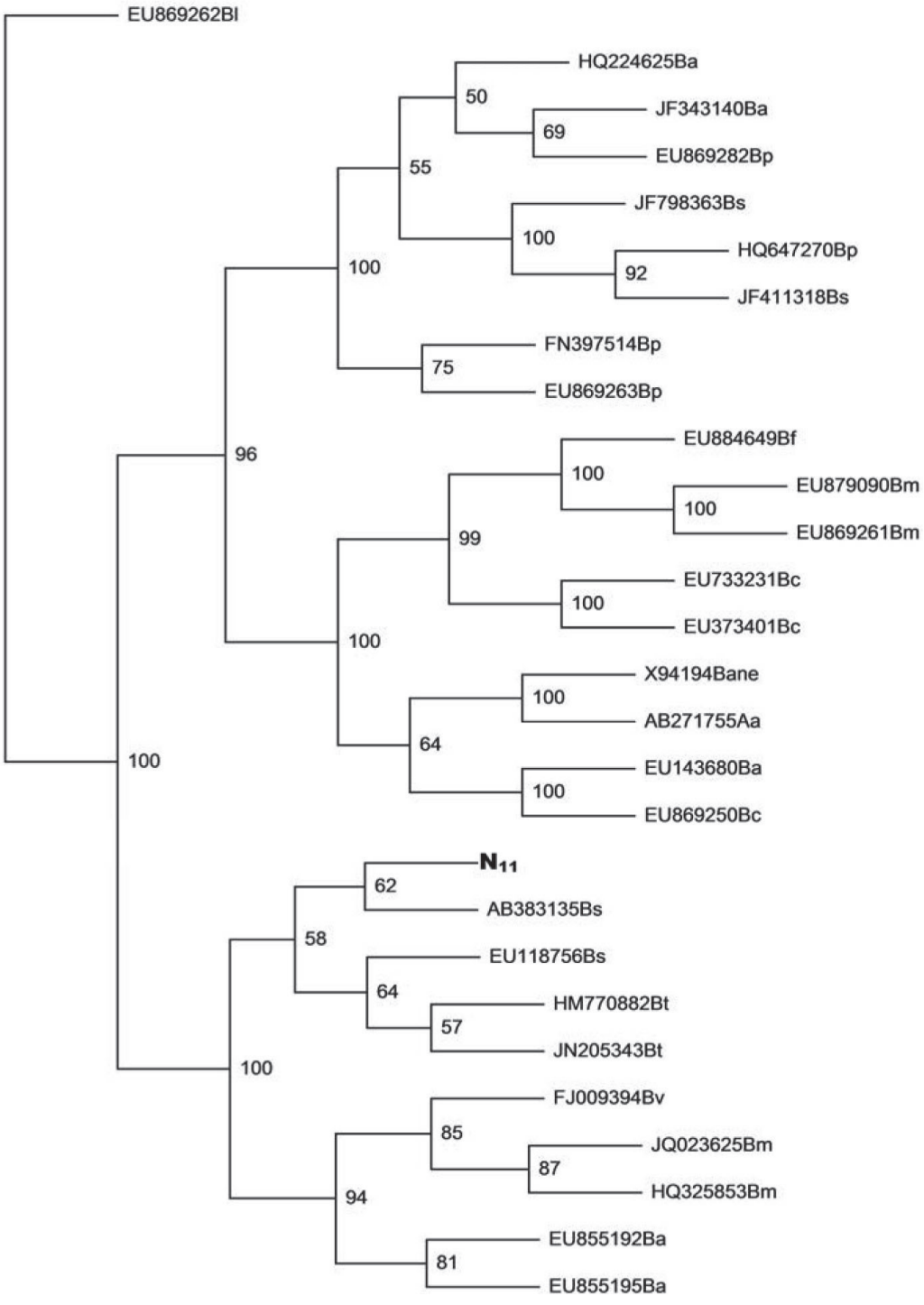

Fig. 2: Neighbor-joining tree based on 16Sr RNA gene sequences showing the phylogenetic relationship of isolate *Bacillus subtilis* CKT1 (N_{11}) to related strains. Numbers at nodes indicate the level of bootstrap support based on data for 1,000 replicates; inferred values greater than 50 % only are presented. Scale bar Ten substitutions per nucleotide position.

insoluble phosphate into available forms by the process of acidification, chelation, and exchange reaction.

Besides having P-solubilization activity, isolate N_{11} produced a significantly higher concentration of IAA (30.27 µg/ml) after 72 hour of incubation and might account for a more extensive root system in treated seedlings. Thus, the substantial production of IAA and P-solubilization by N_{11} clearly suggests its inherent plant growth promoting potential. This is in accordance with earlier studies demonstrating the plant growth promoting activities of such PGPRs which has been recently reviewed by Vassilev *et al.*, (2006) and Mehta *et al.*, (2010). Auxins are quantitatively the most abundant phytohormones secreted by the PGPRs and it is generally agreed that auxin production rather than phosphate solubilization and siderophore production is major factor responsible for the stimulation of root growth. IAA production by microbes promotes the root growth by directly stimulating plant cell elongation or cell division (Khalid *et al.*, 2004; Glick 1995).

Siderophore production

In the present study all the bacterial isolates except A_2 and N_{21} exhibited siderophore production on CAS medium. Bacterial isolate N_{11} produced (40.00 percent siderophore unit) at 72 h of incubation which was statistically at par with amount of siderophore produced by isolate N_1 (38.75 percent siderophore unit). Mechanism of plant growth promotion not only involves direct promotion but also by indirect mechanisms such as siderophore production and suppression of deleterious phytopathogenic microorganisms (Shirkot and Sharma, 2005; Kloepper *et al.*, 2007). The present studies showed the potential of eleven isolates for the simultaneous synthesis and release of pathogens suppressing metabolites, mainly siderophores and indole acetic acid. Secondary metabolites produced by certain species and strains of *Bacillus* are known for their antifungal potential against phytopathogenic fungi (Kumar *et al.*, 2012).

Production of Hydrogen Cyanide (HCN)

Of eleven strains, only N_{11} showed the production of HCN as indicated by the discoloration of the filter paper from orange to brown when incubated with picric acid (0.5%) and sodium carbonate (2%). This gains significance as HCN production release by rhizospheric microbes has been proposed as a possible line of defence against soil borne plant pathogens (Selvakumar *et al.*, 2008).

***In vitro* compatibility assessment**

Dual culture compatibility assessment

Compatibility of individual isolates in mixture among themselves is a major consolidate in the use of multiple isolate mixture of PGPR. However, multiple isolate mixtures of PGPR, each with specific capability and functions, may have certain advantages allowing the combination of various traits of plant growth promotion. All eleven bacterial isolates were screened for their antagonistic and synergistic activities amongst themselves. The results (Table 1) revealed that isolate D_1 and P_9 showed synergism with rest of the bacterial isolates, Isolate N_{11} showed synergism with six isolates (A_3, N_1, N_{21}, SH_2, D_1 and P_9) and antagonism against four bacterial isolates (A_o, A_1, A_2, N_2). Isolate N_2 showed synergism only with isolate N_{11} and antagonism with the rest. The information generated will help in formulation of PGPRs in field studies.

In vitro evaluation of the antagonistic activity of isolated

Table 1: Dual culture compatibility assessment amongst eleven bacterial isolates of tomato seedlings

Isolates used for over layering	Synergism with bacterial isolates	Antagonism with bacterial isolates
Ao	A_1, A_2, N_1, N_2, N_{11}, N_{21}, SH_2, D_1	A_3, P_9
A_1	A_2, N_1, N_2, N_{11}, N_{21}, SH_2, D_1, P_9	A_0, A_3
A_2	A_0, A_1, A_3, N_1, N_2, N_{11}, N_{21}, SH_2	P_9
A_3	N_{11}, SH_2	A_0, A_1, A_2, A_3, N_1, N_{21}, SH_2, D_1, P_9
N_1	N_2, N_{11}	A_0, A_1, A_2, A_3, N_{21}, SH_2, D_1, P_9
N_2	N_{11}	A_0, A_1, A_2, A_3, N_1, N_{21}, SH_2, D_1, P_9
N_{11}	A_3, N_1, N_{21}, SH_2, D_1, P_9	A_0, A_1, A_2, N_2
N_{21}	A_2, A_3, N_1, N_{11}, SH_2, D_1, P_9	A_0, A_1, N_2
SH_2	A_1, A_2, A_3, N_2, N_{11}, N_{21}	A_0, N_1,D_1,P_9
D_1	A_0, A_1, A_2, A_3, N_1, N_2, N_{11}, N_{21}, SH_2, P_9	-
P_9	A_0, A_1, A_2, A_3, N_1, N_2, N_{11}, N_{21}, SH_2, D_1	-

bacteria against soil-borne fungi. Various mechanisms account for the ability of biocontrol PGPR to control plant pathogens, including competition for iron and other nutrients, production of antifungal metabolites such as antibiotics. For effective biocontrol, these antimicrobial metabolites must be delivered at a right time and site by an efficient root colonization process. All the isolates used in the present study are efficient root colonizer as is evident by the large populations (based on morphotype) both in rhizosphere soil and endorhizobacterial counts. The antifungal activities of individual isolates were compared using dual culture method as well as by using dilution technique.

Antagonistic activity by using dual plate method

The individual isolates arrested mycelia growth six days after incubation and no overgrowth was observed even after eight days of fungal growth. N_{11} bacterial isolate showed comparatively maximum growth of inhibition against *F. oxysporum* (42.57 %), *R. solani* (47.30 %) and *S. sclerotiorum* (48.71 %) which was found to be significantly higher as compared to the growth inhibition exhibited by other isolates (Table 2). However isolate A_0 and A_3 showed no antagonistic activity against all the three fungal pathogens. Isolate A_2 was not effective against *F. oxysporum.* Antagonistic effects of the PGPR's may have occurred through the action of metabolites fungicidal to fungal pathogens. Siderophore producing and iron-chelating isolates of *Pseudomonas fluorescens* isolates are reported to antagonize *F. oxysporum* (Gamliel and Katan 1993). In a previous study, *Bacillus megaterium, Bacillus subtilis and Pseudomoas fluorescens* also suppressed the rhizosphere population of *F. oxysporum* (Sultana *et al*., 2004; Kumar *et al*., 2012).

Table 2: Antifungal activity of bacterial isolates against *F. oxysporum, S. sclerotiorum, R. solani* using agar streak method

Isolates	Growth inhibition (%)*		
	F. oxysporum	*S. sclerotiorum*	*R. solani*
A_0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
A_1	37.84 (37.96)	39.74 (39.08)	36.49 (37.11)
A_2	0.00 (0.00)	30.77 (33.69)	35.13 (36.34)
A_3	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
N_1	33.11 (35.12)	42.31 (40.57)	38.51 (38.36)
N_2	27.03 (31.32)	35.90 (36.81)	32.43 (34.72)
N_{11}	42.57 (40.72)	48.71 (44.26)	47.30 (43.45)
N_{21}	33.78 (35.54)	0.00 (0.00)	35.81 (36.76)
SH_2	33.78 (35.54)	30.77 (33.68)	37.16 (37.56)
D_1	33.78 (35.51)	39.74 (39.08)	41.22 (39.94)
P_9	29.05 (32.61)	32.05 (34.48)	35.81 (36.76)
$CD_{0.05}$	1.85	1.07	2.26

Figures in parentheses are arc sine transformed values

Antifungal antibiotic activity by using agar diffusion assay

The results (Table 3) revealed that on an average the per cent growth inhibition of the fungus increased significantly with increase in bacterial cell density from 75.00 to 90.70 in case of *Fusarium oxysporum*, 72.09 to 83.14 in case of *Rhizoctonia solani* and 67.44 to 90.12 in case of *Sclerotinia sclerotiorum.*

The interaction study between cell density and per cent growth inhibition revealed that N_{11} showed maximum per cent growth inhibition against *F. oxysporum* (90.70%), *R. solani* (83.14) and *S. sclerotiorum* (90.12) at 1.50 O.D which was statistically higher as compared to other isolates.

Results from the present study (Table 3) clearly establish the antifungal activity of N_{11} against fungal pathogens i.e. *F. oxysporum, S. sclerotiorum* and *R. solani*. These results are in agreement with earlier report that *Bacillus* sp. produced antifungal substances with activity against a number of mycelia fungi (Sultana *et al*., 2004; Cazorla *et al.,* 2007). We suppose that this effect is caused by different antifungal metabolites including siderophores, organic acid, IAA and antifungal antibiotics in the cell pellets (Sadfi *et al.,* 2002).

Table 3: Effect of cell density of *B. subtilis* CKT1 (N_{11}) on the growth inhibition of *F. oxysporum, R. solani* and *S. sclerotiorum* in mixed culture on malt extract medium

Cell density (O.D at 540 nm)	% Growth inhibition at different cell density (O.D)			
	F. oxysporum	*R. solani*	*S. sclerotiorum*	Mean
0.25	75.00 (60.03)	72.09 (58.11)	67.44 (55.21)	71.51
0.50	81.98 (64.88)	74.42 (59.62)	69.77 (56.65)	75.39
1.0	83.72 (66.22)	79.07 (62.78)	73.26 (58.86)	78.68
1.50	90.70 (72.25)	83.14 (65.75)	90.12 (71.68)	87.99
Mean	82.85	77.18	75.15	

$CD_{0.05}$
Fungus (T) = 0.48
Cell density (I)= 0.56
T × I = 0.97

Figures in parentheses are arc sine transformed values

Interesting observation made during the *in vitro* antifungal antibiotic production studies was the induction of antibiotic production only in the presence of fungal pathogen and

lack antifungal activity in culture filtrate which was the *novelty* of the isolates. The literature indicates (Sadfi et al. 2002) that antibiotic production in such cases is induced by the presence of fungal host.

Phenotypic and metabolic characteristics of *Bacillus subtilis* CKT1

On the basis of the results obtained during preliminary characterization and *in vitro* screening against soil borne phytopatogenic fungi, the isolate N_{11} was selected for further identification.

The strain CKT1 was preliminary characterized by morphological, physiological and biochemical characteristics (Table 4). The pure colonies of the isolate on nutrient agar medium after 48 hr of incubation were cream coloured, irregular having rough surface, raised elevation and erose margin. The isolated colonies on the Azotobacter medium after 72 hr of incubation were large sized with an irregular configuration. The isolate produced a yellowish orange siderophore in the standard CAS agar medium. The isolate produced yellowish halozone on the PVK medium. The morphological characteristics of the strain are as follows: the cells were gram positive, spore forming, motile rods, occurring in chains. The spores were oval and subterminal.

The physiological and biochemical characteristics of the isolated strain CKT1 are given in Table 4. The isolate is an aerobic, catalase producing strain. The isolate tested positive for gelatine liquefaction, Voges proskauer test and urea hydrolysis. However, the isolate showed negative reaction for production of hydrogen sulphide, casein hydrolysis, starch hydrolysis, citrate utilization, methyl red test and indole test. In addition to this, acid was produced from carbohydrates such as glucose, maltose, sucrose and fructose. The organism grew in the temperature range of 25 to 50° C and over a wide range of pH 5 to 10.

Table 4: Differential phenotypic and metabolic characteristics of the *Bacillus subtilis* CKT1

Characteristics	*Bacillus subtilis* CKT1
Colony morphology	Irregular/cream coloured/raised/erose
Gram character	Gram+
Sporulatiing	+
Motile	+
Catalase	+
Gelatin liquefaction	+
Starch hydrolysis	-
Hydrogen sulphide	-
Voges Proskauer	+
Urea hydrolysis	+
Casein hydrolysis	-
Citrate utilization	-
Methyl red test	-
Indole test	-
Utilization of :	
Dextrose	+
Maltose	+
Sucrose	+
Fructose	+
Galactose	+

Phylogenetic analysis of *Bacillus subtilis* CKT1 by 16S rRNA analysis

Designed primers were used successfully for amplification of 16S rRNA from *Bacillus* sp. (N_{11}). Amplicon of expected size, i.e. ~1375 bp was obtained. The PCR product was eluted from gel, and sequenced using PCR primers. Molecular characterization based on 16S rRNA homology of partial sequence (1262bp) with the sequences available in NCBI database website http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.,* 1997) confirmed the earlier reported presumptive identification (Kloepper *et al.,* 1999) as *B. subtilis*. Sequence was analyzed with corresponding sequences of 19 different *Bacillus* isolates reported from different parts of the world. *B.* isolate (N_{11}) showed 99% homology with *B. subtilis* (AB383135, from Viet Nam), *B. subtilis* (EU118756, from China), *B. vallismortis* (FJ009394, from China) and with *B. amyloliquefaciens* (EU855195, from China).

To trace out the evolutionary patterns of the test isolate and to find out relationship of the same with other selected sequences at NCBI, phylogenetic tree was also constructed using Neighour- Joining (J) method of mathematical averages (UPGMA) among 16S rRNA sequence of CKT1 and corresponding sequence of 19 different *Bacillus* isolates. Strain CKT1 was united with quite high statistical

support by the bootstrap estimates for 1,000 replications. Phylogenetic tree (Fig. 2) also verified CKT1 as *B. subtilis* as the strain CKT1 clustered closely with *B. subtilis* with boot strap value (62 %).The 16S rRNA sequence of the strain has been deposited in the GenBank database under accession number GQ379201.

Based on above morphological, biochemical and molecular characterization, the strain CKT1 was identified as *B. subtilis* CKT1.

Conclusion

This study elucidates the multifarious role of bacterial isolates with broad spectrum antifungal activity and plant growth promoting potentials. The present study, therefore, suggests the use of single strain of PGPR with multiple plant growth promoting activities as biofertilizers is an efficient approach to replace chemical fertilizers and pesticides for sustainable tomato cultivation as they induced simultaneous antifungal production and other plant growth promoting activities. Further investigations, including efficiency test under field conditions, are needed to clarify the role of PGPR as biofertilizers that exert beneficial effects on plant growth and development.

Acknowledgement

Thanks are due to ICAR (All India Network Project on Soil-Biodiversity and Biofertilizer), New Delhi, India for their generous financial support.

References

Altschul, S.F., Thomas, L.M., Alejandro, A.S., Jinghui, Z., Zheng, Z., Webb, M. and David, J.L. 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Arshad, M. and Frankenberger, W.T. 1998. Plant growth-regulating substances in the rhizosphere: microbial production and functions. *Advances in Agronomy* **62**: 45-151.

Ashrafuzzaman, M., Hossen F. Akhtar., Ismail M. Razi., Hoque Md, Anamul., Islam L, Zahurul., Shahidullah, S.M., and Meon, Sariah. 2009. Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. *African Journal of Biotechnology* **8**(7): 1247-1252.

Cazorla, F.M., Romero, D., Garcia, A.P., Lugtenberg, B.J.J., Vincente, A. and Bloemberg, G. 2007. Isolation and characterization of antagonistic *B. subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *Journal of Applied Microbiology* 1-10.

Corpet, F. 1988. Multiple sequence alignment with hierarchial clustering. *Nucleic Acids Research* **16**: 10881-10890.

Daimon, H., Nobuta, K., Ohe, M., Harada, J. and Nakayama, Y. 2006. Tricalcium phosphate solubilization by root nodule bacteria of *Sesbania cannabina* and *Crotalaria juncea*. *Plant Production Science* **9**: 388-389.

De Freitas, J.R., Banerjee, M.R. and Germida, J.J. 1997. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake to canola (*Brassica napus* L.). *Biology and Fertility of Soils* **24**: 358-364.

Frankenberger, W.T., and Arshad, M. 1995. Phytohormones in Soils: Microbial Production and Function. Marcel Dekker, New York, Pp 520.

Gamliel, A. and Katan, J. 1993. Suppression of major and minor pathogens by fluorescent pseudomonads in solarized and nonsolarized soil. *Phytopathology* **83**: 68-75.

Glick, B.R. 1995. The enhancement of plant growth of free living bacteria. *Canadian Journal of Microbiology* **41**: 109-117.

Glick, B.R., Panrose, D.M. and Li, J. 1998. A model for the lowering of plant ethylene concentration by plant growth promoting rhizobacterium *Pseudomonas putida* GR 12-2. *Soil Biology and Biochemistry* **29**: 1233-1239.

Gomez, K.A. and Gomez, A.A. 1976. Statistical procedures for agricultural research, 2nd edn. JohnWiley and Sons, Singapore.

Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Khalid, A., Arshad, M. and Zahir, Z.A. 2004. Screening of plant growth promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology* **96**: 473.

Kloepper, J.W., Estrad, G. and Mclnroy, J.A. 2007. Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Canadian Journal of Microbiology* **53**: 159-167.

Kloepper, J.W., Leong, J., Terntze, M. and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**: 885-886.

Kloepper, J.W., Rodriguez, R., Zehnder, G.W., Murphy, J.F., Sikora, E. and Fernadez, C. 1999. Plant root bacterial interactions in biological control of soil borne diseases and potential extension to systemic and foliar diseases. *Australian Plant Pathology* **28**: 21-26.

Kumar, P., Dubey, R.C. and Maheshwari, D.K. 2012. Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiological Research* **167**: 493-499.

Kumar, S.R., Ayyadurai, N., Pandiaraja, P., Reddy, V.A., Venkateswarlu, Y., Prakash, O. and Sakthivel, N. 2005. Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits. *Journal of Applied Microbiology* **98**: 145–154.

Lee, K.D., Bai, Y., Smith, D. and Han H.S.S. 2005. Isolation of plant-growth-promoting endophytic bacteria from bean nodules. *Research Journal of Agricultural and Biological Science* **1**: 232-236.

Mehta, P., Chauhan, A., Mahajan, R., Mahajan, P.K. and Shirkot, C.K. 2010. Strain of *Bacillus circulans* isolated from apple rhizosphere showing plant growth promoting potential.

Current Science **98**: 538-542.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applied Bioscience* **12**: 357-358.

Raaska, L., Viikari, L. and Mattila-Sandholm, T. 1993. Detection of siderophores in growing cultures of *Pseudomonas* spp. *Journal of Industrial Microbiology* **11**: 181-186.

Sadfi, N., Cherif, M., Hajlaovi, M.R., Boudabbous, A. and Belanger, R. 2002. Isolation and partial purification of antifungal

