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MULTI-TRAIT PLANT GROWTH PROMOTING BACTERIA FROM TOMATO RHIZOSPHERE AND EVALUATION OF THEIR POTENTIAL AS BIOINOCULANTS

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ABSTRACT

Present study was aimed to assess plant growth promoting activity of rhizobacteria isolated from mid hill region of Himachal Pradesh (India). One hundred rhizobacteria were isolated and after replica plating on 3 media *viz.*, chrome-azurol-S (CAS), Pikovskaya's and N-free Jensen's medium, 26 isolates were further characterized on the basis of functional plant growth promoting traits *viz.*, phosphorus solubilization, indole-3-acetic acid (IAA) production, siderophore production, growth on N-free medium, lytic enzymes production as well as antifungal activity against *Alternaria solani*, *Fusarium oxysporum* f. sp. *lycopersici*, *Pythium aphanidermatum*, *Phytophthora* spp. and *Sclerotium rolfsii*. Strain S_{25} showed significantly higher phosphate solubilization (345 µg mL^{-1}) and IAA production (95 µg mL^{-1}). The highest siderophore production (209% SU) was observed in isolate S_{14}. Of the 26 isolates, 18 were nitrogen fixers while 23 and 20 isolates showed chitinase and protease activity, respectively. On the basis of *in vitro* screening, 10 most efficient isolates were screened for growth promotion on tomato under net house conditions. Strain S_{25} exhibited highest seed germination (78%), root length (16.9 cm), root dry weight (1.32 g $plant^{-1}$), shoot length (31.5 cm) and shoot dry weight (6.3 g $plant^{-1}$). Random amplified polymorphic DNA (RAPD) fingerprinting was performed to ascertain the genetic variability among the ten most efficient PGPR. Overall strain S_{25}, identified as *Bacillus subtilis* on the basis of biochemical and molecular characterization, proved most efficient. This strain could be used as an PGPR inoculant having multiple PGP-traits for plant growth promotion under mid hilly conditions.

Key words: Growth promotion, IAA, PGPR, phosphate solubilization, siderophore production, tomato

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the important and widely consumed vegetable crops belonging to the family *Solanaceae*. India is a principal tomato growing country and ranks 2nd in production after China (FAO, 2011). Tomato is the main cash crop grown in mid hills of Himachal Pradesh (HP) during summer season (Thakur *et al.*, 2010). The area under tomato cultivation in HP is 8,035 ha with production of 3,50,950 metric tonnes (Anonymous, 2010). Most of this tomato (>75%) is produced in two districts *viz.*, Solan and Sirmaur, lying in mid hill regions of the state.

Tomato has rich phytochemical contents so its consumption is beneficial to human health. Lycopene, one of the nature's most powerful antioxidants found in tomato, is implicated in the prevention of various diseases including prostate cancer, coronary heart diseases and cataract (Ranieri *et al*., 2004).

With increasing concern for food and environmental quality, the use of indigenous soil bacteria for reducing chemical inputs in agriculture appears potentially important. Numerous soil bacteria which flourish in plant rhizosphere stimulate plant growth by a plethora of mechanisms and collectively known as plant growth promoting rhizobacteria (PGPR). PGPR have been applied to various crops to enhance growth, seed emergence and crop yield (Hameeda *et al*., 2008). The mechanisms by which PGPR promote plant growth and yield are not yet fully understood. The direct mechanisms include atmospheric N_2 fixation, phosphate solubilization, siderophore production and secretion of plant growth hormones *i.e.*, indole-3-acetic acid (IAA), cytokinins, ethylene and gibberellic acid (Glick, 2012). The indirect mechanisms involve biological control of phyto-pathogens/deleterious microbes through antibiotic production, lytic enzymes, siderophore and HCN secretion or through competition. These mechanisms remarkably improve plant health and promote growth in terms of increase in seedling emergence, vigor index and crop yield (Gholami *et al*., 2009).

Recently, there has been a resurgence of interest in PGPR application due to their plant growth promoting (PGP) traits and their effectiveness as biocontrol agents against many crop diseases (Thakuria *et al*., 2004). Hill and mountain agro-ecosystem is characterized by difficult terrain, inadequate infrastructure, fragile ecosystem and societies entrenched in severe top soil erosion and low input application. Hence by default, hill agriculture largely remains a low external input based production system. The use of biofertilizers can efficiently solve the problem by improving crop production and ensuring environment safety. There is no commercial biofertilizer formulation available for tomato grown in hill and mountain agro-ecosystem. In view of this, the rhizosphere of tomato was investigated to explore potential PGPR from two sites of Solan and Sirmaur districts lying under mid hill regions of Himachal Pradesh and to study their inoculation effect on tomato growth promotion.

MATERIALS AND METHODS

Isolation and screening of rhizobacteria

Rhizosphere soil and root samples were collected from one month old tomato seedlings in April, 2011. Sampling was done from two sites Solan and Rajgarh lying in the mid hills of HP (India). Ten samples were collected randomly from each site for the isolation of bacteria. Bacteria were isolated by serial dilution using standard spread plate technique. Total bacterial population was determined using nutrient agar (NA) medium. Endorhizobacteria were isolated using surface sterilization, dilution plating assay (Mehta *et al*., 2013). After incubation at 35±2°C for 24 h, distinct morphotypes, differentiated by their colony morphology, pigmentation and growth rate, were selected. The bacterial isolates screened from sites Solan and Rajgarh were designated with letter 'S' and 'R', respectively. A total of 100 rhizobacterial isolates were subjected to replica plating so as to screen them for multiple PGP-traits *viz.*, CAS medium for siderophore production (Schwyn and Neilands, 1987), N-free Jensen's medium for nitrogen fixation and Pikovskaya's medium for phosphate solubilizing ability (Pikovskaya, 1948). After replica plating, the most predominant isolated colonies simultaneously growing on all the media (i.e. nutrient agar, Pikovskaya's, Jensen's and CAS) were characterized for additional traits that are commonly associated with plant growth promotion. The growth of rhizobacteria on N-free medium was observed by testing their ability to grow on N-free medium (Baldani and Dobereiner, 1980).

Quantitative estimation of IAA

IAA production by bacterial isolates was determined in Luria-Bertani (LB) broth colorimetrically. Bacterial cultures were grown in 25 mL LB broth (amended with 5 mM L-tryptophan, 0.065% sodium dodecyl sulphate and 1% glycerol) for 72 h at 37°C under shake conditions. Two mililitre of Salkowsky's reagent (2 mL 0.5 M $FeCl_3$ + 98 mL 35% $HClO_4$) was added to 3 mL culture supernatant and the mixture incubated at room temperature for 25 min. Absorbance was measured spectrophotometrically at 535 nm for the development of pink colour (Gorden and Paleg, 1957).

Tricalcium phosphate solubilization in liquid medium

An aliquot (0.1-1.0 mL) from culture supernatant was made to final volume of 5 mL with distilled water and then 5 mL ammonium molybdate was added. The mixture was thoroughly shaken and the contents of flasks diluted to 20 mL. To this, 1.0 mL chlorostannous acid was added and final volume made to 25 mL. The blue coloured intensity was measured after 10 min. at 660 nm (Bray and Kurtz, 1945).

Quantitative estimation of siderophore

For determination of siderophore, 0.5 mL cell free supernatant was mixed with 0.5 mL chrome-azurol-S (CAS) assay solution along with 10 µL shuttle solution (0.2 M 5-sulfosalicyclic acid). After 10 min. the absorbance was noted at 630 nm for change in colour from blue to pink. A reference was prepared using exactly the same components, except the cell free extract of culture supernatant (Schwyn and Neilands, 1987).
The siderophore units (SU) were calculated using the equation:

$$SU\ (\%) = \frac{Ar - As}{Ar} \times 100$$

Where, Ar is absorbance of reference at 630 nm and As the absorbance of test sample at 630 nm.

Production of cell wall degrading enzymes

The ability to produce chitinase was detected by formation of clear halos surrounding bacterial colonies on minimal agar plates amended with 0.3% colloidal chitin (Robert and Selitrennikoff, 1988). Similarly, protease activity was detected by measuring clear halo zones around bacterial colonies on skim milk agar plates (Fleming *et al*., 1975).

Broad spectrum antifungal activity

Broad spectrum antifungal activity was assessed by dual culture technique against *Alternaria solani*, *Fusarium oxysporum* f. sp. *lycopersici*, *Sclerotium rolfsii*, *Phytophthora* spp. and *Pythium aphanidermatum*. The fungal pathogens were procured from the Department of Plant Pathology, YSPUHF, Nauni, Solan (India). Bacterial isolates were cross-streaked on one edge of petri-plates containing potato dextrose agar (PDA). A mycelial disc of 5 day old test pathogen was placed on one side of the streak and growth inhibition calculated after 5 days incubation at 28±2°C (Vincent, 1947). The percent growth inhibition (GI %) was calculated according to formula:

$$GI\ (\%) = \frac{C - T}{C} \times 100$$

Where, C = Growth of the pathogen in control; T = Growth of pathogen in treatment

Growth promotion effect on tomato seedlings under net house conditions

Ten most efficient PGPR isolates displaying PGP-traits and antifungal activity were selected for growth promotion study on tomato under net house conditions. The experiment was conducted in April-May, 2012. First, the bacterial isolates were inoculated into nutrient broth and incubated at 35±2°C for 48 h on a rotary shaker at 120 rpm followed by centrifugation at 3000 g for 10 min. The pellets were washed with sterile distilled water; and the concentration of cells adjusted to 1×10^9 cfu mL^{-1} by dilution to give bacterial suspensions with optical density of 1.0 at 600 nm using

spectrophotometer and the suspensions were used as inoculum (Mortensen, 1997). The soil, sand and farm yard manure were mixed in 2:1:1 ratio to make potting mixture and sterilized by 3 successive autoclave cycles of 1 h each at 121°C. Five kg potting mixture was filled in pots. Tomato seeds cv. Solan Vajr were surface sterilized with 2% sodium hypochlorite for 2 min. and soaked in 10 mL bacterial suspension (10^9 cfu mL^{-1}) amended with 0.2% carboxymethyl cellulose to facilitate the adherence of bacteria to seeds and then incubated at 35±2°C for 6 h. Ten treated seeds were sown in each pot containing potting mixture. After 5 days of seedling emergence thinning was done and 3 plants/ pot maintained. Suitable control (without culture) was also maintained. The experiments were conducted in a completely randomized design with each treatment replicated three times. The average temperature in net house during experimentation period was 33±3°C. Plant growth parameters such as root-shoot length and dry weight were measured after 60 days growth. The data on plant growth was subjected to the analysis of variance (one way classification) using SPSS software version 2.0. Comparisons of means were performed by the Fisher's projected LSD test at $P_{0.05}$.

Random amplified polymorphic DNA analysis (RAPD)

Genetic diversity amongst the ten most efficient bacterial strains was analyzed using RAPD-PCR analysis. Three random decamer oligonucleotide primers namely RBa-1 (5′-AAAACCGGGC-3′), RBa-2 (5′-ACAGGGCTCT-3′) and RBa-3 (5′-ACAGGGGTGT-3′) were screened initially for RAPD analysis. All PCR-RAPD reactions were repeated three times, and fingerprints compared. Only those RAPD bands that appeared consistently were evaluated. The genomic fingerprints obtained were compared for similarity by visual inspection of band patterns. Similarity matrices were calculated with Dice coefficient (Kumar *et al*., 2002). Cluster analysis of similarity matrices was performed by unweighted pair group method with arithmetic mean (UPGMA) and computer-assisted analysis was performed with NTSYS-pc2 program for Windows (Saito and Nei, 1987).

Morpho-biochemical characterization of selected PGPR

Morpho-biochemical characterization of most efficient PGPR was done based on its colony morphology, microscopic observation and biochemical tests. The biochemical characterization of the isolate was performed by using KB009 Hi carbohydrate™ kit and KB013 HiBacillus™ Identification Kit (Himedia Laboratories Pvt. Ltd., Mumbai, India) following the manufacture's instruction. The tentative identification of culture was made following the *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 1994).

Identification of bacterial isolate by 16S rDNA sequence analysis

The 16S rDNA of selected bacterial strain was amplified using genus specific primers (forward 5'GCAAGTCGAGCGGACAGATGGGAGC3' and reverse 5'AACTCTCGTGGTGTGACGGGC GGTG3'). DNA was isolated from selected bacterial isolate by using total DNA isolation kit (RBCs Real Genomics, Taiwan) as per manufacturer's instructions. PCR reaction was carried out in a 20 µL reaction volume containing 50 ng of template DNA, 20 pmoles of each primers, 0.2 mM dNTPs and 1U Taq polymerase in 1x PCR buffer. PCR carried out involved 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min. 30 s followed by final extension at 72°C for 10 min. The PCR product was analyzed on a 1% agarose gel in 1 x TAE buffer, run at 100 V for 1 h. The band of 1375 bp was excised from the gel and purified using gel extraction kit (RBCs Real Genomics, Taiwan). The purified fragment was sequenced by commercial sequencing facility (Xcleris Lab., Ahemadabad, India) using both forward and reverse primers. Both forward and reverse sequences were corrected and finally based on the overlapping regions complete 16S rRNA gene sequence of selected strain was obtained. The 16S rDNA sequence of this strain was compared with 20 other 16S rDNA sequences of genus *Bacillus* retrieved from the EMBL database and aligned by ClustalW (Thompsons *et al*., 1994). The phylogenetic dendrogram was generated by the

Neighbour-joining method (Saito and Nei, 1987), using the MEGA 6 software. The sequence was submitted to NCBI GenBank database and accession number was assigned.

RESULTS AND DISCUSSION

Isolation and screening of rhizobacteria

Of the 100 rhizobacterial isolates obtained from soil and tomato root samples and after preliminary replica plating for one step screening of traits like P-solubilization, siderophore production and nitrogen fixation, 26 bacterial isolates were selected on the basis of simultaneously displaying triple PGP-traits of P-solubilization, siderophore production and nitrogen fixation for further screening. The majority of isolates were Gram positive and rod shaped, except S_{18} and R_1 which were cocobacillus (Table 1). Our results are in agreement with Aranda *et al*. (2011) and Joshi *et al*. (2011) who found Gram positive bacteria as predominant bacteria in rhizosphere and root-associated microbial communities in many plant species.

Detection of PGP-traits

In present study the selected 26 PGPR isolates exhibited IAA production in the range of 15 to 95 µg mL^{-1} (Table 1). Isolate S_{25} produced maximum IAA which resulted in significantly higher root length and dry weight. IAA production by microbes promotes root growth by stimulating plant cell elongation or cell division (Khalid *et al.,* 2004). PGPR promote plant growth through production of plant growth regulators or phytohormones (Glick, 2012). Auxins stimulate root development and improve water and nutrient absorption from soil (Fatima *et al.*, 2009; Ahmad and Khan, 2011).

The phosphate solubilizing ability of microbes is considered to be one of the most important traits associated with plant nutrition (Rodriguez *et al*., 2006) as it renders insoluble phosphate available to plants through acidification, chelation and/or exchange reaction (Mehta *et al*., 2014). In present study, bacterial isolates effectively solubilized insoluble tri-calcium phosphate in Pikovskaya's liquid medium and highest P-solubilization (345 µg mL^{-1}) was noted in isolate S_{25}. The substantial P-solubilization potential of isolate S_{25} suggests its inherent plant growth promoting potential. This is in accordance with earlier studies wherein plant growth promoting potential of phosphate solubilising bacteria has been reported (Mehta *et al.,* 2013; Kumar *et al.*, 2015).

All the isolates exhibited variable siderophore production in CAS liquid medium, the highest production (209.0% SU) was in isolate S_{14} which was at par with isolates S_5 (184.4% SU) and S_{13} (168.0% SU). The ability to produce siderophore and metabolites contributed to antibiosis has been the focus of many studies on PGPR (Sayyed *et al*., 2005; Maksimov *et al*., 2011).

Twenty three isolates (88.4%) exhibited chitinase activity with significantly highest activity (6.6 enzyme index (E.I) in isolate S_{27}. Protease activity was present in 20 isolates (76.9%) and highest activity was observed in isolate S_8 (2.56 E.I) which was at par with 10 isolates (S_1, S_3, S_6, S_{15}, S_{18}, S_{19}, S_{20}, S_{22}, S_{25} and S_{27}). The mechanism of plant growth promotion not only involves direct promotion but also indirect mechanisms like siderophore production and suppression of deleterious phytopathogenic microorganisms (Kloepper *et al.*, 2007).

Broad spectrum antifungal activity

A high number of PGPR showed *in vitro* antibiosis thereby suggesting their biocontrol potential against various fungal pathogens (Table 2). Twenty isolates (76.9%) showed inhibition against *Alternaria solani*, 6 isolates (23.1%) against *Fusarium oxysporum* f. sp. *lycopersici*, 14 isolates (53.8%) against *Sclerotium rolfsii* and *Phytophthora* spp. and 9 isolates (34.6%) against *Pythium aphanidermatum*. The antagonistic activity may be attributed to the production of secondary metabolites like antibiotics (Joshi and Gardener, 2006; Cazorla *et al*., 2007). Study revealed that the

Table 1: Plant growth promoting traits of rhizobacterial isolates from tomato

Isolates	Morphology	IAA (µg mL^{-1})	P-Solubilized (µg mL^{-1})	Siderophore production (%SU)	Growth on N-free medium	Chitinase [b](E.I)	Protease (E.I)
S_1	G (+) Rods	33.0	95.0	53.28 (1.72)	ND	ND	2.00
S_2	G (+) Rods	28.0	95.0	32.79 (1.50)	+	2.53	1.50
S_3	G (+) Rods	33.0	95.0	143.44 (2.15)	+	2.42	2.19
S_5	G (+) Rods	31.0	115.0	184.43 (2.26)	ND	2.25	1.42
S_6	G (+) Rods	42.0	95.0	118.85 (2.07)	+	2.88	1.69
S_7	G (+) Rods	15.0	110.0	110.66 (1.71)	+	2.62	1.52
S_8	G (+) Rods	45.0	85.0	73.77 (1.86)	ND	3.50	2.56
S_9	G (+) Rods	20.0	95.0	122.95 (2.09)	+	3.00	ND
S_{11}	G (+) Rods	20.0	70.0	24.59 (1.39)	+	4.40	ND
S_{12}	G (+) Rods	43.0	80.0	110.66 (2.04)	+	1.61	ND
S_{13}	G (+) Rods	55.0	95.0	168.03 (2.22)	+	2.55	1.47
S_{14}	G (+) Rods	61.0	80.0	209.02 (2.32)	+	3.33	1.44
S_{15}	G (+) Rods	65.0	90.0	40.98 (1.61)	+	2.25	1.57
S_{16}	G (+) Rods	51.0	90.0	106.56 (2.02)	+	1.90	1.33
S_{17}	G (+) Rods	55.0	100.0	65.57 (1.81)	ND	3.88	1.35
S_{18}	G(+) Cocobacillus	47.0	80.0	69.67 (1.84)	+	2.09	1.66
S_{19}	G (+) Rods	36.0	95.0	129.63 (2.11)	ND	ND	2.04
S_{20}	G (+) Rods	20.0	90.0	73.77 (1.86)	ND	4.20	1.56
S_{21}	G (+) Rods	38.0	125.0	139.34 (2.14)	+	2.09	1.50
S_{22}	G (+) Rods	32.0	90.0	4.10 (0.61)	+	4.20	1.75
S_{24}	G (+) Rods	38.0	180.0	73.77 (1.86)	ND	1.85	ND
S_{25}	G (+) Rods	95.0	345.0	110.66 (2.04)	+	2.66	1.80
S_{27}	G (+) Rods	42.0	95.0	90.16 (1.95)	+	6.60	2.00
R_1	G (+) cocobacillus	23.0	100.0	3.27 (0.50)	ND	ND	ND
R_2	G (+) Rods	40.0	95.0	12.30 (1.08)	+	2.80	1.26
R_3	G (+) Rods	63.0	95.0	65.57 (1.81)	+	1.50	ND
$LSD_{0.05}$	-	1.17	6.78	0.133	-	1.10	1.03

Figures in parentheses are log transformed values; ND = Not detected;

Enzyme index (E.I) = $\frac{A}{B}$ where, A= diameter (colony + halo zone); B = diameter colony

growth inhibition of fungal pathogens by bacterial strains ranged from 15.5 to 77.5%. Seventeen (65.4%) rhizobacterial isolates inhibited at least two fungal pathogens tested. The results are in agreement with Cazorla *et al*. (2007) who reported that *Bacillus* strain UCR 236 and *Bacillus* spp. produced antifungal substances with activity against a number of mycelial fungi.

Growth promotion of tomato under net house conditions

Ten isolates with maximum PGP-traits and higher *in vitro* antibiosis were evaluated for growth promotion in tomato under net house conditions. Significant increase in seed germination and other plant growth parameters was observed in treated plants over uninoculated control (Table 3). The increase in seed germination and plant growth parameters over uninoculated control is depicted in Fig. 1. Isolate S_{25} showed highest increase (20%) in seed germination over uninoculated control followed by 15.4% increase due to isolate S_{21}. Strain S_{25} also showed highest increase in root length (81.7%) over control followed by strain S_{21} (68.8%). Isolates S_{17}, S_{16} and S_8 showed significant increase in root length (65.6, 62.4 and 58.1%, respectively). Highest increase in root dry weight was observed in strain S_{25} (65%) followed by strain S_{21} (61.3%). Similarly, PGPR isolates showed a remarkable increase in shoot length and shoot dry weight over uninoculated control. Highest increase in shoot length (53.7%) and shoot dry weight (80.0%) also corresponded to the isolate S_{25} followed by S_{21} with 52.68% increase in shoot length and 71.43% increase in shoot dry weight. The increase in length and dry weights of root and shoots may be attributed to the concurrent improvement in phosphorus solubilization, IAA production and nitrogen fixing ability of

Table 2: Broad spectrum antifungal activity of PGPR isolates from tomato against different fungal pathogens

Isolates	Antifungal activity (G.I %)				
	Alternaria solani	*Fusarium oxysporum* f. sp. *lycopersici*	*Sclerotium rolfsii*	*Phytophthora* spp.	*Pythium aphanidermatum*
S_1	53.00	53.33	40.00	46.66	42.22
S_2	75.55	ND	43.33	ND	ND
S_3	62.22	48.88	47.77	46.66	ND
S_5	46.66	ND	ND	ND	ND
S_6	55.55	ND	60.00	46.66	ND
S_7	63.33	ND	46.66	51.11	46.66
S_8	54.44	ND	50.00	41.11	ND
S_9	68.88	ND	ND	42.22	46.66
S_{11}	ND	ND	ND	48.88	ND
S_{12}	15.55	ND	ND	ND	ND
S_{13}	54.44	ND	45.55	ND	ND
S_{14}	60.00	45.55	ND	45.55	52.22
S_{15}	62.22	ND	54.44	44.44	45.55
S_{16}	58.88	ND	46.66	ND	ND
S_{17}	ND	ND	ND	ND	ND
S_{18}	74.22	ND	38.88	40.00	46.66
S_{19}	62.22	ND	ND	44.44	ND
S_{20}	ND	42.22	ND	ND	ND
S_{21}	61.11	60.00	40.00	51.11	42.22
S_{22}	64.44	ND	42.22	42.22	46.66
S_{24}	ND	ND	ND	ND	ND
S_{25}	57.77	54.76	44.44	ND	ND
S_{27}	54.44	ND	ND	ND	ND
R_1	ND	ND	ND	ND	ND
R_2	58.88	ND	46.66	46.66	47.77
R_3	ND	ND	ND	ND	ND
$LSD_{0.05}$	5.20	2.44	4.61	2.80	2.53

ND = Not detected

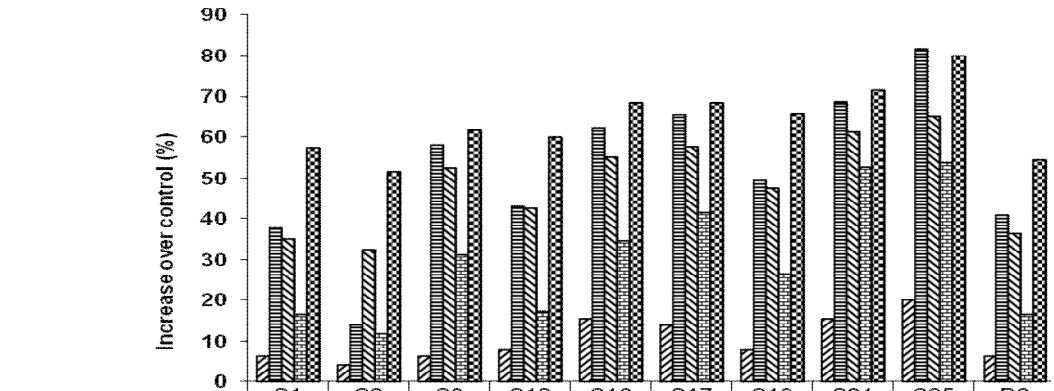
these strains, especially isolate S_{25}. The findings are supported by Ibiene *et al*. (2012) who found that most of the PGPR isolates were instrumental in enhancing shoot length, root length and stem width in tomato seedlings. The increase in growth and biomass yield by PGPR have previously been reported for other crops (Shirkot and Sharma, 2005; Fatima *et al*., 2009).

Table 3: Growth promotion effect of selected PGPR isolates on tomato seedlings under net house condition

Isolate	Germination (%)	Root length (cm)	Root dry weight (g plant^{-1})	Shoot length (cm)	Shoot dry weight (g plant^{-1})
Control	65.00	9.30	0.78	20.50	3.50
S_1	69.00	12.80	1.08	23.90	5.30
S_2	68.00	10.60	1.06	22.90	5.30
S_8	69.00	14.70	1.22	26.90	5.80
S_{12}	70.00	13.30	1.14	24.00	5.60
S_{16}	75.00	15.10	1.24	27.60	5.90
S_{17}	74.00	15.40	1.26	29.00	5.90
S_{19}	70.00	13.90	1.18	25.90	5.80
S_{21}	75.00	15.70	1.29	31.30	6.00
S_{25}	78.00	16.90	1.32	31.50	6.30
R_2	69.00	13.10	1.09	23.90	5.40
$LSD_{0.05}$	1.70	1.05	0.53	1.19	0.60

Genomic fingerprinting using RAPD analysis

Among three primers (RBa-1, RBa-2 and RBa-3) used in the present study for RAPD analysis, primer RBa-2 was found more stable and reproducible and gave more number of distinct polymorphic bands in comparison to the other two RAPD primers. Hence, RBa-2 amplified products were used to construct the dendrogram and to study the genetic relatedness of PGPR strains.



	S1	S2	S8	S12	S16	S17	S19	S21	S25	R2
Germination (%)	6.15	4.12	6.15	7.69	15.39	13.85	7.69	15.39	20	6.15
Root length (%)	37.63	13.98	58.07	43.01	62.37	65.59	49.46	68.82	81.72	40.86
Root dry weight (%)	35	32.5	52.5	42.5	55	57.5	47.5	61.25	65	36.25
Shoot length (%)	16.59	11.7	31.22	17.07	34.63	41.46	26.34	52.68	53.66	16.59
Shoot dry weight (%)	57.43	51.43	61.71	60	68.57	68.57	65.71	71.43	80	54.29

Fig. 1: Increase in root/shoot length and root/shoot dry weight of tomato seedlings over untreated control under net house conditions

RAPD of isolates revealed DNA bands ranging from 100 to 1100 bp (Fig. 2). Variations were clearly observed in PCR products. Dendrogram analysis of RBa-2 amplified products could clearly differentiate among the isolates (Fig. 3). Nine out of 10 isolates were grouped in one main clusture,

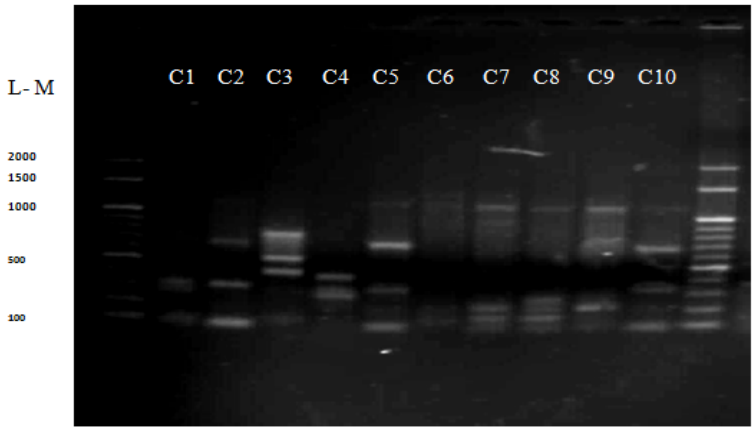
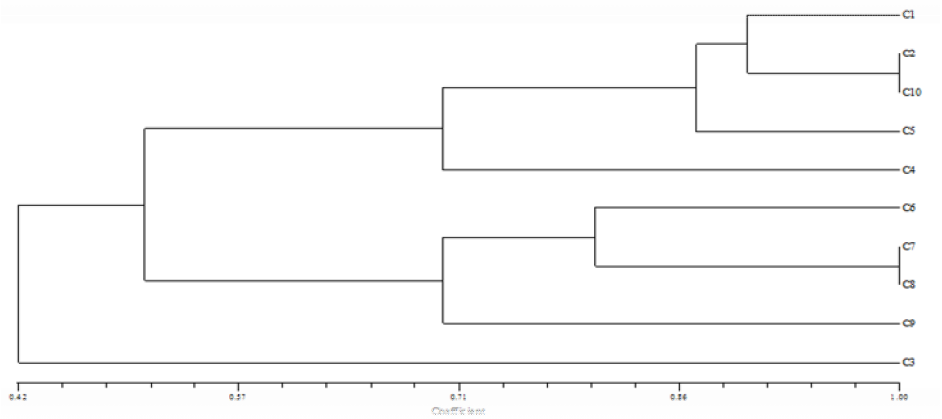


Fig. 2: Random amplified polymorphic DNA pattern of selected 10 PGPR using RBa-2 primer Lane M, DNA marker; Lane C1, S1; Lane C2, S25; Lane C3, S19; Lane C4, S21; Lane C5, S17; Lane C6, S12; Lane C7, R2; Lane C8, S2; Lane C9, S16 and Lane C10, S8



C1,S1; C2, S25; C3,S19; C4, S21; C5, S17; C6, S12; C7, R2; C8, S2; C9, S16; C10, S8

Fig. 3: Dendrogram showing the genetic relationship amongst selected 10 PGPR isolates based on RAPD analysis

separating isolate S_{19} from the main clusture. The main clusture was subdivided into 2 sub-clustures, upper subclusture consisting of 5 isolates (S_1, S_{25}, S_8, S_{17} and S_{21}) and lower subclusture consisting of 4 isolates (S_{12}, E_1, S_2 and S_{16}). Isolate S_{25}, S_8 and isolate E_1 shared 100% homology with S_2 in upper and lower subclustures. The level of 100% polymorphism detected indicated that the primer RBa-2 could be employed in future RAPD based genetic diversity studies on Gram positive rod shaped rhizobacteria. The polymorphism in the amplification products may be due to the changes in sequence of primer binding site (*e.g.* point mutations) or from the changes which alter the size or prevent the successful amplification of target DNA (*e.g.* insertion, deletions, inversions) as suggested earlier (Rani *et al*., 1995). The utility of RAPD markers for the analysis of genetic diversity among bacterial isolates, fungi and plants have previously been reported (Girgis *et al*., 2008; Chakraborty *et al*., 2010).

Phenotypic and metabolic characterization of selected isolate

The isolate S_{25} with maximum number of PGP-traits was characterized by morpho-biochemical characteristics. The isolated colonies on nutrient agar were creamish and round with radiating edge and the cells were Gram positive, rod shaped and arranged singly. The biochemical characterization revealed the isolate to be positive for catalase test, citrate utilization, arginine dihydrolase and esculin hydrolysis and could utilize xylose, fructose, dextrose, sucrose, mannose, inulin, glycerol, salicin, cellobiose, sucrose, glycerol, salicin and mannitol as sole carbon source but was negative for malonate, Voges Proskauer, ONPG, nitrate reduction, indole production and hydrogen sulphide production. The strain could not utilize trehalose, erythritol, melibose, rhamnose, melezilose, α-methyl-D-mannoside, sodium glucanate, α-methyl-D-glucoside, D-arabinose, sorbose, dulcitol, adoitol, inositol, lactose, sorbitol, arabitol, xylito, galactose and raffinose. The starin was tentatively identified as *Bacillus* sp. as per the criteria of *Bergey's Manual of Systemic Bacteriology* (Holt *et al*., 1994).

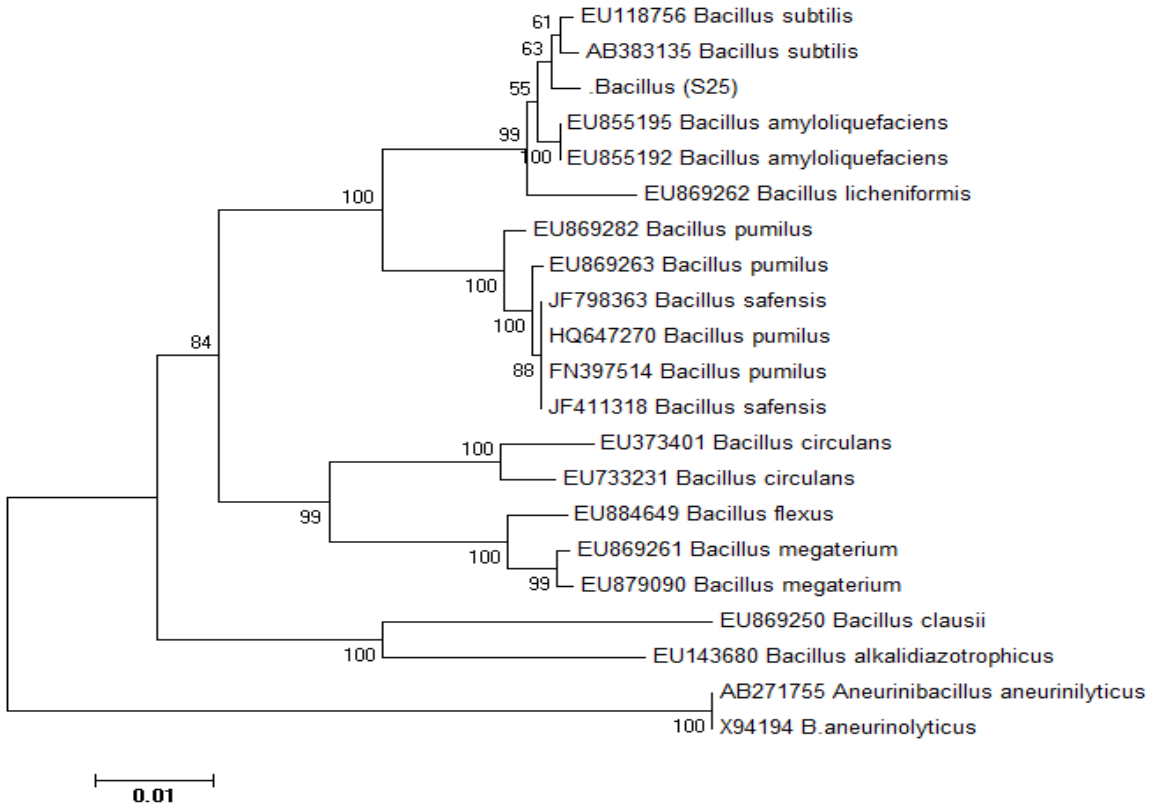


Fig. 4: Neighbour-joining phylogenetic dendrogram based on 16S rRNA sequences showing relationships between strain S_{25} and related organisms. Scale bar represents 0.01 inferred substitutions per nucleotide position

Phylogenetic analysis of selected isolate by 16S rDNA sequence analysis

The 16S rDNA fragment was successfully amplified using designed primers. A fragment of 1375 bp was obtained and sequenced. Both forward and reverse sequences obtained were corrected, and finally based on the overlapping regions a complete sequence of 1251 bp was obtained. Sequence analysis revealed that isolate S_{25} had 99% homology with *B. subtilis* (AB383135). Phylogenetic tree (Fig. 4) verified the identity of strain S_{25} as *B. subtilis* as it was closely clustered with *B. subtilis* (AB383135), an isolate from Vietnam available in NCBI, with high boot strap value (63%). The sequence of strain S_{25} was deposited in NCBI Genbank under Accession No. JX993903 and strain designated as *Bacillus subtilis* strain CKTR. *Bacilli* are well known for their aggressive root colonization, disease suppression by inhibition of pathogens and stimulation of plant growth by possessing multiple plant growth promoting traits (Mohamed and Gomaa, 2012; Walia *et al*., 2013).

Conclusion: The isolate S_{25} was found most efficient PGPR for tomato under net house studies and was identified as *Bacillus subtilis* strain CKTR on the basis of biochemical and molecular characterization. The strain can be used as inoculant biofertilizer and biofungicide for sustainable tomato cultivation in hilly and mountain areas. Further investigations including efficiency evaluation under field conditions are desired to establish their effectiveness as biofertilizers under natural conditions.

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