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Full Length Research Paper

Performance studies of free-living tomato (*Lycopersicon exculentum* L.) rhizospheric *Bacillus* for their multiple plant growth promoting activity

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The present investigation is based on the study of the diversity of *Bacillus* spp. isolated from tomato rhizosphere and their evaluation as plant growth promoter. A total of twenty eight rhizospheric gram positive rod-shaped bacteria (DPNSB-1 to DPNSB-28) were isolated on nutrient agar and confirmed as *Bacillus* spp. on the basis of phenotypic characterization. Carbon source utilization pattern based on BIOLOG and 16S rDNA-RFLP analysis of these isolates using three tetra cutter restriction enzymes (*Alu*I, *Hae*III and *Msp*I) was employed for diversity studies. All the 28 strains deliberated with multiple plant growth promotion (PGP) attributes such as production of Indole acetic acid (IAA), siderophore, ammonia, HCN and phosphate solubilization. The diversity pattern followed amongst isolates was based on carbon utilization profiling, which revealed 9 distinct clusters ranging from 10 to 50%, whereas RFLP based resulted into pattern 5 determined distinct clusters with a range of 10 to 70%. However, all the isolates were endowed with variable range of PGP activities. Among them, DPNSB-2, DPNSB-11 and DPNSB-28 produced significant levels of IAA production 503.65, 687.94 and 845.28 µg mg^{-1} respectively. While 19 (67%) isolates were capable of solubilizing phosphate, 13 (46%) isolates produced siderophore, 21 (75%) isolates produced Ammonia and 20 (71%) were produced HCN. The positive isolates appeared attractive for exploring their plant growth- promoting activity and may be useful for control of *Fusarium* wilt in tomato field.

Key words: *Bacillus*, carbon source profiling, ARDRA, plant growth promoting activity.

INTRODUCTION

Tomato (*Lycopersicon exculentum* L.) ranks third in priority after Potato and Onion in India but ranks second after potato in the world under vegetable crop system. Its cultivation has increased from 596, 000 ha (year 2007) to 634, 000 ha (year 2010), whereas its production is increased from 10055 to 12433 thousand tons. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici,* is a major disease of tomato in both green house and field condition, cause loss up to 10 to 50% of yield (Kallo, 1991; Mao et al., 1998).

Modern agriculture is heavily dependent on the application of chemical pesticides for disease control. Due to the concerns regarding both human health and environmental protection, viable alternatives to these chemicals are being sought (Franks et al., 2006). The

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interest in the use of biological approaches to replace hazardous pesticides in fertilizing soils or improve plant resistance against phytopathogens is steadily gaining worldwide acceptance. In this regard, the use of plant growth promoting rhizobacteria (PGPR) has depicted potential in developing sustainable agricultural systems for crop production and protection (Govindasamy et al., 2011, Erturk et al., 2010). The study of root-associated bacteria and their potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants but also for many biotechnological applications. *Bacillus* spp may protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as, by induction of host resistance. it depends on a wide variety of traits, such as the production of structurally diverse antibiotics (Liu et al., 2006), production of iron chelators and exo-enzymes such as proteases, lipases, chitinases and β 1,3-glucanases (Hayat et al., 2010, Beneduzi et al., 2008, Swain and Ray, 2006, Deepa et al., 2010), formation of viable spores (Cenci et al., 2006), promotion of plant growth (Ryu et al., 2004), production of bacterial phytohormones and/or the solubilization of mineral phosphates (Calvo et al., 2010, Viruel et al., 2011) and an ubiquitous presence in soil (Gajbhiye et al., 2010).

Considering the multiple applications of *Bacillus* strains, it is essential to develop efficient strains in field conditions. The research on the concept of *Bacillus* diversity studies from rhizospheric regions of different crop system of India has been reported recently (Kumar et al., 2011, Gopalakrishnan et al., 2011, Gajbhiye et al., 2010, Deepa et al., 2010). Moreover, the characterization on diversity studies and plant growth promoting activities in an area is essential for the efficient management of diseases and crop productivity. As such, keeping in view the aforementioned constrains, the present study was designed to screen certain tomato rhizospheric *Bacillus* isolates for their multiple plant growth promoting activities, along with the profiling of carbon source utilization. ARDRA analysis was also carried out to access diversity for recognition of the isolates. The screened isolates will help to control the wilt consequence in tomato crop which is caused by *Fusarium oxysporum* f.sp. *lycopersici* organically.

MATERIALS AND METHODS

Bacterial isolates and phenotypic characterization

In total, twenty eight Bacillus isolates were isolated from 16 tomato rhizospheric soil samples collected from four different agricultural sites of Indo-Gangetic plain regions including; 1). Farm of Indian Institute of Vegetable Research –Varanasi, Uttar Pradesh (82.52° E longitude and 25.10° N latitude) 2) Farm of Indian Institute of Horticultural Research – Bangalore, Karnataka (78°E longitude and 13.58° N latitude), 3) Farm of Indian Agricultural Research Institute, New Delhi (77°E longitudes and 28.38°N latitude) and 4) Farm of Andhra Pradesh Horticultural University, Tadepalligudem, West Godavari District, Andhra Pradesh (16°E longitudes and 81.30°N latitude), during 2009. The intact root systems were collected and transported to the laboratory immediately by using cold box. Loosely adhering soils were shaken and detached from the roots and discarded. These root portions with just a layer of closely adhering rhizoshpere soil was used. Heat treatment method as described by Walker et al. (1998) was used for isolation, 1 g of soil sample was suspended in 9 ml sterile distilled water and was incubated in water bath at 80°C for 10 min. 100 µl of the suspension was spread on the nutrient agar medium plates enriched with 1% dextrose. The plates were incubated at 28 to 30°C for 24 to 40 h. Cream whitish colored colonies were purified by culturing on nutrient agar (NA) and maintained in 20% glycerol at -80°C. Gram staining and motility test were performed for preliminary identification of the strains (Allegrucci and Sauer, 2007). In order to determine the phenotypic nature, morphological characterization was done on the basis of colony color, size, shape and margin (Cappucino and Sherman, 2002). Biochemical tests include indole formation, nitrate reduction, citrate utilization, casein and starch hydrolysis, catalase, oxidase production and gas production from glucose were carried out as per the methods of Bergey's Manual of Determinative Bacteriology (1994). Results of these tests were scored either positive or negative. All the subsequent experiments were conducted after maintaining fresh culture.

Variability on carbon sources utilization

The ability to use different carbon sources was tested using BIOLOG Phenotype Micro-Array™ plates GP2 (Biolog, Inc., Hayward, California, USA). These plates comprise of 95 different sole carbon substrates, primarily carbohydrates, but they also contain other carbons such as amino and carboxylic acids. Tetrazolium redox dyes are used to calorimetrically indicate the utilization of the carbon sources or resistance to inhibitory chemicals. Fresh cultures were raised on Biolog Universal (BU) Growth medium and 24 h old cultures were used to inoculate into inoculation fluid by using sterile cotton swab. Turbidity of the inoculants was adjusted to 85% for Inoculation Fluid A (IFA) and 65% for Inoculation Fluid B (IFB) by using turbidity meter. The microbial suspensions of 150 µl were inoculated into each well of microplate using 8-channel repeating pipette. Plates were incubated at 30°C and observed for color development at incubation of 48 h. Color development was recorded using a micro plate reader (Model EL311, BioTek Instruments, USA) with 590 nm wavelength. On the basis of data derived from the carbon source utilization profiles, a matrix with binary code composing positive (1) and negative (0) values was made.

ARDRA analysis

The genotypes of representative isolates were determined using ARDRA. DNA was directly extracted from bacterial cultures by a direct lysis method (Wilson, 1992). The extracted DNA was dissolved in 20 µl TE buffer and used as the template for PCR reaction. 16S r DNA gene was amplified followed by primers 65F1 (50-GTGCTACAATGGACAGAACAA-30) and reverse primer 265R1 (50-GTGAGATGTTGGGTTAAGTC-30) (Vardhan et al., 2011). The reaction mixture contained 1.5 µl of 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]; 0.9 µl of $MgCl_2$ (25 mM); 6.24 µl of sterile distilled water; 1.5 µl of deoxynucleoside triphosphate (2 mM each); 0.75 µl of primer solution (10 µM each); 0.15 µl Taq polymerase (5U/µl) and 3.96 µl of template. Cycling conditions were as follows: 95°C for 5 min, then 30 cycles for 1 min at 95°C, 55°C for 1 min and 72°C for 1 min, followed by a final extension step of

72°C for 5 min. Amplified 16S r DNA product (560 bp) were restricted digested for 3 h at 37°C in 25 µl reaction mixture containing 8 µl (300 ng) of PCR product, 2.5 µl of 10X PCR buffer and 10 U of either the restriction enzymes namely; *Alu*I, *Hae*III or *Mbo*I. Restriction digestion was analyzed by agarose gel (1.5%) electrophoresis containing 0.5 µg ml^{-1} ethidium bromide (Sambrook et al., 1989). A 100 bp ladder was used as molecular size marker. The analysis was done at least twice with each enzyme. The whole restriction profile information obtained was used to discriminate each isolate. On the basis of data derived from the restriction profiles, a matrix with binary code was composed, in which "1" represented presence value and "0" represented absence value.

Screening of plant growth promoting (PGP) activities

IAA production was determined by method of Glickman and Dessaux (1995). Briefly, test bacterial culture was inoculated in the nutrient broth with L-tryptophan (50 µg/mL), incubated at 28 ± 2°C for 5 days. Cultures were centrifuged at 2,600 g for 30 min. Two ml of the supernatant was mixed with two drops of orthophosphoric acid and 4 mL of Salkowaskis reagent (50 mL, 35% perchloric acid; 1 mL 0.5 $FeCl_3$). Development of red color indicated IAA production; OD was read at 530 nm using spectrophotometer. The level of IAA produced was estimated by standard IAA graph and expressed as lg/mL (Gordon and Paleg, 1957). Production of siderophores determined on Chrom azurol S agar medium as described by Alexander and Zubere (1991). Development of yellow-orange halo around the growth was considered as positive for siderophore production. Detection of phosphate solubilization ability on Pikovskaya's agar (Pikovskaya, 1948), inoculated colonies were incubated for 72 h at 28 ± 2°C, presence of clear zone around the colonies were considered as positive. Freshly grown cultures were inoculated in 10 ml peptone water (10%) in each tube and incubated for 72h at 28 ± 2°C. Nessler's reagent was added in each tube. Development of brown to yellow color was confirmed as the positive test for the production of ammonia (Cappuccino and Sherman, 1992). HCN was estimated qualitatively by sulfocyanate colorimetric method (Lorck, 1948), development of reddish brown color on the filter paper indicated positive.

Data analysis

The different isolates of *Bacillus* were compared on the basis of the obtained values (1 and 0) from positions of unequivocally scorable RFLP bands and carbon utilization patterns were transformed into a binary character matrix (1 for the presence and 0 for the absence of a band at a particular position). The pairwise distance matrices were compiled by the NTSYSpc- 2.02e software (Rohlf, 1993) using the Jaccard coefficient of similarity (Jaccard, 1912). Phylogenetic tree was created by the unweighted pair-group method arithmetic (UPGMA) average cluster analysis (Sneath and Sokal, 1973; Dias, 1988).

RESULTS

Phenotypic characterization

Among the twenty eight isolates, 7 (DPNSB 1 to DPNSB 7) were obtained from IIVR, 8 (DPNSB 8 to DPNSB 16) from IIHR farm, 5 (DPNSB 17 to DPNSB 21) from IARI farm and 8 (DPNSB 22 to DPNSB 28) from APHU farm respectively (Data not shown). All the isolates were confirmed as *Bacillus* based on microscopic and biochemical tests such as indole formation, nitrate reduction, citrate utilization, casein and starch hydrolysis, catalase, oxidase production and gas production from glucose (Table 1). Morphological/Microscopic observation showed that most of the strains were rod shaped, motile and endospore forming. All the isolates were positive in citrate utilization, starch hydrolysis and casein hydrolysis tests. However, in other biochemical tests, the strains showed a variation, for example, out of 28 isolates, 10 isolates (DPNSB-1, 3, 4, 6, 10, 15, 17, 18, 20 and 24) positive in indole reduction assay; 22 isolates (DPNSB-1, 3, 4, 5, 6, 7, 8, 10, 11, 13, 14, 15, 17, 18, 19, 20, 21, 22, 24, 25, 27 and 28) positive in nitrate reduction assay; 24 isolates (DPNSB-1, 2, 3, 4, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 23, 24, 25, 26, 27 and 28) positive in catalase test; 22 isolates (DPNSB-1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, 18, 19, 20, 21, 22, 23, 25, 26 and 28) positive in oxidase production and 18 isolates (DPNSB-1, 2, 3, 4, 6, 9, 10, 12, 13, 15, 16, 17, 18, 20, 23, 24, 26 and 27) showed gas production in the presence of glucose as a substrate respectively (Table 1).

Carbon source utilization

Differentiation of 28 strains was done using Biolog. The test yielded a characteristic pattern of substrate utilization of each isolates; Figure 1 shows a similarity index less than 0.2. Based on the Biolog GP2 microplate results, the present study showed that the assay can be readily used to search for the specific carbon sources preferentially used by each strain. However, few C sources were unavailable to all strains. Table 2 list the carbon substrates utilized by strains. There were large differences in the C utilizations among the strains. All *Bacillus* strains were commonly utilized following carbon sources namely, Dextrin, Tween-80, N-Acetyl-D-glucosamine, N-Acetyl-β-D-Mannosamine, L-Arabinose, D-Arabitol, Arbutin, D-Cellobiose, D-Fructose, L-Fucose, D-Galactose, Gentiobiose, D-Gluconic acid, α-D-Glucose, m-Inositol, α-D-Lactose, Maltose, Maltotriose, D-Mannitol, D-Mannose, α-Methyl-D-galactoside, β-Methyl-D-glucoside, Palatinose, D-Psicose, L-Rhamnose, D-Ribose, Salicin, D-Sorbitol, Sucrose, D-Tagatose, Turanose, Xylitol, D-Xylose, Acetic acid, β-Hydroxybutyric acid, γ-Hydroxybutyric acid, p-Hydroxy- Phenylacetic acid, α- Ketoglutaric acid, α-Ketovaleric acid, L-Lactic acid, D-Malic acid, Pyruvatic acid Methyl Ester, Succinic acid Mono-methyl Ester, Propionic acid, Pyruvic acid, Succinic acid, D-Alanine, L-Alanine, L-Asparagine, L-Glutamic acid, Glycyl- L-Glutamic acid, Putrescine, but exhibited varying degree of utilization towards other carbon sources such as α-Cyclodextrin, β-Cyclodextrin, Glycogen, Inulin, Mannan, Tween 40, Amygdalin, D-Galacturonic acid, Lactulose, D-Melezitose, D-Melibiose, β-Methyl-Dgalactoside, 3-Methyl Glucose, α-Methyl-D glucoside, α-Methyl-Dmannoside, D-Raffinose, Sedoheptulosan, Stachyose, D-Trehalose, α-

Table 1. Biochemical characterization of rhizosphere associated *Bacillus* spp. Where BCC1 = Indole reduction assay, BCC2 = Nitrate reduction assay, BCC3 = Citrate utilization assay, BCC4 = Starch hydrolysis assay, BCC5 = Casein hydrolysis assay, BCC6 = Oxidase production assay, BCC7 = Catalase production assay and BCC8 = Gas production assay from glucose as a substrate.

Isolate code	Biochemical characterization							
	BCC1	BCC2	BCC3	BCC4	BCC5	BCC6	BCC7	BCC8
DPNSB-1	+	+	+	+	+	+	+	+
DPNSB-2	-	-	+	+	+	+	+	+
DPNSB-3	+	+	+	+	+	-	+	+
DPNSB-4	+	+	+	+	+	+	+	+
DPNSB-5	-	+	+	+	+	+	-	-
DPNSB-6	+	+	+	+	+	+	+	+
DPNSB-7	-	+	+	+	+	+	+	-
DPNSB-8	-	+	+	+	+	+	-	-
DPNSB-9	-	-	+	+	+	+	+	+
DPNSB-10	+	+	+	+	+	-	+	+
DPNSB-11	-	+	+	+	+	+	+	-
DPNSB-12	-	-	+	+	+	+	+	+
DPNSB-13	-	+	+	+	+	-	+	+
DPNSB-14	-	+	+	+	+	+	+	-
DPNSB-15	+	+	+	+	+	+	+	+
DPNSB-16	-	-	+	+	+	+	+	+
DPNSB-17	+	+	+	+	+	-	+	+
DPNSB-18	+	+	+	+	+	+	+	+
DPNSB-19	-	+	+	+	+	+	-	-
DPNSB-20	+	+	+	+	+	+	+	+
DPNSB-21	-	+	+	+	+	+	+	-
DPNSB-22	-	+	+	+	+	+	-	-
DPNSB-23	-	-	+	+	+	+	+	+
DPNSB-24	+	+	+	+	+	-	+	+
DPNSB-25	-	+	+	+	+	+	+	-
DPNSB-26	-	-	+	+	+	+	+	+
DPNSB-27	-	+	+	+	+	-	+	+
DPNSB-28	-	+	+	+	+	+	+	-

Hydroxybutyric acid, Lactamide, D-Lactic acid Methyl Ester, L-Malic acid, Succinamic acid, N-Acetyl-LGlutamic acid, L-Alaninamide, L-Alanyl-Glycine, L-Pyroglutamic acid, L-Serine, 2,3-Butanediol, Glycerol, Adenosine, 2'-Deoxy Adenosine, Inosine, Thymidine, Uridine, Adenosine-5'- Monophosphate, Thymidine-5'-Monophosphate, Uridine-5'- Monophosphate, D-Fructose-6- Phosphate, α-D-Glucose- 1-Phosphate, D-Glucose- 6-Phosphate and D-L-α-Glycerol Phosphate (Table 2). Numerical analysis of phenotypic characteristics revealed a high degree of polymorphism. All *Bacillus* strains were grouped into two major phenons at 80 % similarity co-efficient level (Figure 1). Statistical analyses employing Jaccard coefficients were used to group the isolates, reflecting the relatedness of their individual carbon substrate utilization patterns (Figure 1). The similarity coefficient range among bacterial strains was 0.50 to 1.00. All the 28 strains were grouped into 10 groups based on their carbon utilization pattern. Group no. 5 contained more strains (16 no's) compared to other groups (Figure 1) and isolates of IARI and APHU farm were congregated on this large group. The remaining was grouped individually.

16S RNA amplification and ARDRA analysis

Strains of putative rhizospheric bacilli belonging to different region were distinguished by RFLP-PCR of the 16S r DNA gene. The restriction digestion patterns of the isolates were analysed using selected enzymes (*Alu*I, *Hae*III and *Mbo*I), patterns of three cutter enzymes were monomorphic across the isolates (Figure 2). All clusters, showed digestion yielding monomorphic and polymorphic binding patterns respectively. Although, amplified fragments corresponding to 16S r DNA gene sequences

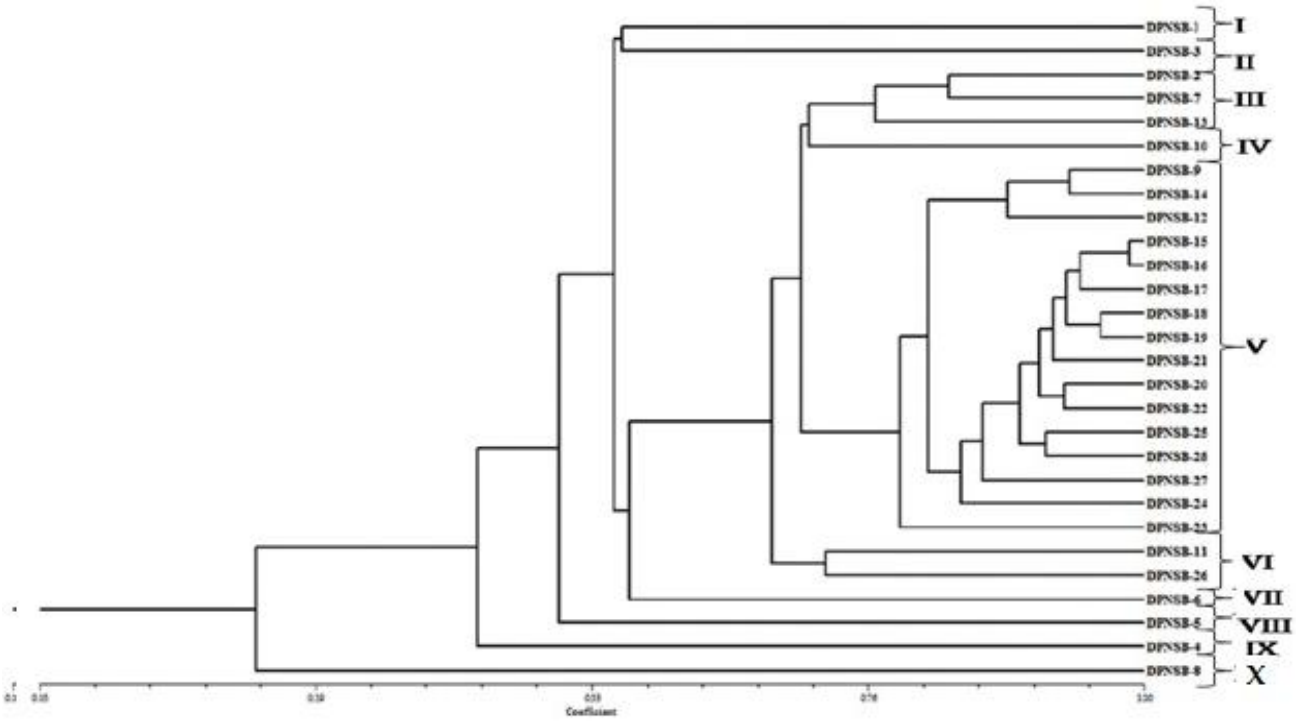

Figure 1. Dendrogram showing the clustering of the type strains of 28 bacillus isolates on the basis of 95 carbon sources utilization pattern studied by the BIOLOG system. The simple matching (SSM) coefficient and unweighted-pair-group method with average (UPGMA) clustering were used.

Table 2. Physiological characteristics with scores of 0.5 or above strains. Where, 1 = DPNSB-1, 2 = DPNSB-2, 3 = DPNSB-3, 4 = DPNSB-4, 5 = DPNSB-5, 6 = DPNSB-6, 7 = DPNSB-7, 8 = DPNSB-8, 9 = DPNSB-9, 10 = DPNSB-10, 11 = DPNSB-11, 12 = DPNSB-12, 13 = DPNSB-13, 14 = DPNSB-14, 15 = DPNSB-15, 16 = DPNSB-16, 17 = DPNSB-17, 18 = DPNSB-18, 19 = DPNSB-19, 20 = DPNSB-20, 21 = DPNSB-21, 22 = DPNSB-22, 23 = DPNSB-23, 24 = DPNSB-24, 25 = DPNSB-25, 26 = DPNSB-26, 27 = DPNSB-27 and 28 = DPNSB-28.

Carbon sources used	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Cyclodextrin	-	-	-	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
β-Cyclodextrin	-	+	+	-	-	+	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Dextrin	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	+	+	+	+
Inulin	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+
Mannan	-	+	-	-	-	+	+	-	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-
Tween 40	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Tween 80	-	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-Dglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-β-Dmannosamine	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
L-Arabinose	-	+	-	-	-	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabitol	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Fucose	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	-	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galacturonic acid	+	+	-	-	+	-	+	-	+	-	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	-	-	+
Gentiobiose	+	+	-	-	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-Gluconic acid	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-Glucose	-	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. Contd.

m-Inositol	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-Lactose	-	+	-	-	-	-	+	-	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+
Lactulose	-	+	-	+	-	-	+	+	+	-	-	+	-	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+
Maltose	-	+	-	-	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltotriose	+	+	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	-	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Melezitose	+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	-	-	-	-	+	-	-	+	+	-	+	-	-
D-Melibiose	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-
α-Methyl-Dgalactoside	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
β-Methyl-Dgalactoside	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-
3-Methyl Glucose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-
α-Methyl-D glucoside	-	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-
β-Methyl-Dglucoside	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
α-Methyl-Dmannoside	-	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-
Palatinose	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
D-Psicose	-	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-Raffinose	-	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+
L-Rhamnose	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Ribose	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	+	-	-	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Sedoheptulosan	-	+	-	+	-	+	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
D-Sorbitol	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Stachyose	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	-	+	-	-	+	-	+	-	-	-	+	+	+
Sucrose	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Tagatose	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	-	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Turanose	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylitol	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Hydroxybutyric acid	+	+	+	-	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
β-Hydroxybutyric acid	+	+	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
γ-Hydroxybutyric acid	+	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p-Hydroxy- phenylacetic acid	-	+	-	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α- Ketoglutaric acid	-	-	+	-	+	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Ketovaleric acid	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactamide	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
D-Lactic acid methyl ester	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	+	-	+	-	-
L-Lactic acid	-	+	-	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Malic acid	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Malic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Pyruvatic acid methyl ester	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinic acid mono-methyl ester	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionic acid	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyruvic acid	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinamic acid	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+
Succinic acid	-	+	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-lglutamic acid	-	+	-	-	-	+	+	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	-	-
L-Alaninamide	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
L-Lactic acid	-	+	-	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Malic acid	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. Contd.

D-Alanine	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Alanine	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Alanyl-Glycine	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
L-Asparagine	-	+	-	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Glutamic acid	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycyl- LGlutamic acid	-	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Pyroglutamic acid	+	+	-	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
L-Serine	+	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Putrescine	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2,3-Butanediol	-	+	+	+	-	+	+	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Glycerol	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Adenosine	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
2'-Deoxy adenosine	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Inosine	-	-	+	+	-	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Thymidine	+	-	-	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Uridine	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Adenosine-5'- Monophosphate	+	-	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Thymidine-5'- Monophosphate	-	-	+	+	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Uridine-5'- Monophosphate	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
D-Fructose-6- Phosphate	+	-	+	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
α-D-Glucose- 1-Phosphate	+	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
D-Glucose- 6-Phosphate	-	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
D-L-α-Glycerol Phosphate	-	+	-	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+

did not contain the full 16S r DNA gene, the determined nucleotide sequences of the 1300 bp of each isolate analyzed enabled the discrimination between several bacilli isolates. To assess the diversity of bacterial isolates from the 28 isolates based on 16D r DNA gene amplification followed by RFLP analysis isolates presented identical band. NT-Sys analysis was used to investigate relationships between *Bacillus* isolates. At the similarity value of 73%, the isolates were divided into a total of five groups (I to V). In Figure 2, Group I has 8 (DPNSB-1, 5, 6, 2, 3, 4, 7 and 16) isolates included exclusively strains from IIVR (7 isolates) and IIHR (1 isolate), While Group II included predominantly 11 (DPNSB-8, 9, 13, 15, 19, 21, 12, 14, 22, 24 and 25) isolates from IIHR (6 isolates), IARI (2 isolates) and 3 isolates of APHU respectively. Group III have one isolate (DPNSB 20) and Groups IV and V each contained 4 isolates. APHU isolates of DPNSB – 23, 26, 28 and 27 farmed Group IV. 3 isolates (DPNSB- 10, 11 and 16) from IIHR and 1 isolate (DPNSB 17) from IARI were combindly made Group V.

PGP traits of the isolates

Firstly, ability to produce indole-3-acetic acid (IAA) in the presence and absence of L-tryptophan as precursor was tested. All those isolates showed IAA production in culture supplemented with tryptophan in the range of 42.46 to 845.28 µg ml^{-1} (Table 3). Substantial production of IAA was produced from the isolates of DPNSB-2, DPNSB-11 and DPNSB-28 respectively (Figure 3). The P-solubilizing ability of the isolates was evidently visible on some plates of Pikovskaya agar. Only 9 of the 28 isolates were able to solubilize phosphate. Of the 28 isolates, only 13 were able to produce siderophores. Positive numbers were observed more for the production of HCN (20) and NH_3 (21) respectively (Figure 4). Significantly among the strains, 1 isolate (DPNSB 2) of IIVR, 2 isolates (DPNSB 10 and 15) of IIHR and 1 isolate (DPNSB 18) of IARI were expressed costiveness in all PGP traits (Table 3).

DISCUSSION

The tomato root-soil obtained from different agro climatic zones of India restrains diverse community of microbes which play a central role in gross production and nutrient cycling. In this study, we analyzed the genotypic and functional (C utilization) diversity of *Bacillus* and its possible consequences in terms of plant growth promoting activity. Plant rhizosphere is known to be preferred to ecological niche for various types of soil micro-organisms due to rich nutrient availability. The beneficial effects of rhizobacteria on plant health management have been demonstrated for several host pathogen

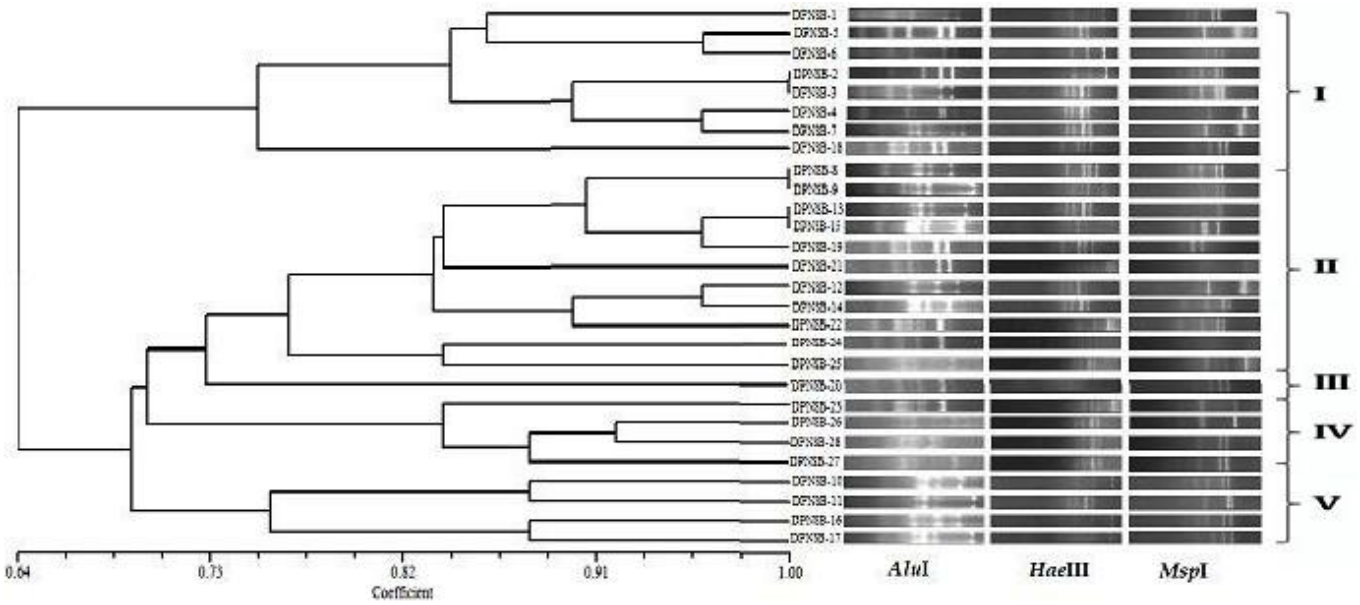

Figure 2. Dendrogram based on UPGMA cluster analysis with the NTSYS-PC program using the 16S r DNA-RFLP data obtained from 28 representative isolates. The profile obtained with restriction endonucleases viz., *AluI, HaeIII* and *MboI* are shown.

systems (Rosales et al., 1986; Weller and Cook, 1986; Sakthivel et al., 1986). In this explorative study, we used a cultivation-based approach to characterize the *in vitro* studies amongst dominant culturable bacteria in sixteen soil samples from different geographic locations (4 no's) in India. On the basis of cultural, morphological and biochemical characteristics, a total of 28 isolates were characterized as *Bacillus* spp. While multiple species of bacilli was detected in the soils and rhizosphere (Garbeva et al., 2003; Ding et al., 2005; Nishijima et al., 2005). Comparatively limited research has been carried out to indicate the most commonly isolated species. However, Beneduzi et al. (2008) isolated nitrogen fixing *Bacillus* (*Paenibacillus borealis* and *Paenibacillus graminis*) from seven distinct wheat production zone of the Brazil. A substantial number of *Bacillus* species from both environments, rhizosphere soil, was detected in this work with most of the isolates belonging to the *Bacillus*. According to Rosch et al. (2002) bacteria belonging to these groups are widely distributed in the soil. All the strains studied were Gram positive rods, motile and spore forming.

Most of the *Bacillus* strains were capable of using numerous carbon sources. Fifty one compounds of the Biolog GP were metabolized by these strains commonly and forty five compounds were partially utilized (Table 2). Patterns of carbon substrate utilization have been reported for *Halomonas* species (Valderrama et al., 1991, Litzner et al., 2006). Results based on turbidity data set, quantitatively reflecting C-substrate utilization and spore production, were used for cluster analysis. We also found some differences in the nutritional test carried out using classical and automated methods. These discrepancies in the results were probably due to their different operational modes: increased growth by assimilation of carbon sources (classical method) or oxidation of substrates via an electron transport chain (BIOLOG method) (Ruger and Krambeck, 1994). Cluster analysis separated the strains into ten distinct clusters. Among these, five clusters were strongly separated from the other nine clusters with a combination of 16 isolates, namely DPNSB- 9, 14, 12, 15, 16, 17, 18, 19, 21, 20, 22, 25, 28, 27, 24 and 22, most of which are strains obtained from APHU and IARI with a few from IIHR. Other isolates were farmed in to groups with combination and individually.

For the diversity analysis, ARDRA analysis was done. Lawongsa et al. (2008) studied the culturable pseudomonad community associated with the roots of field grown rice and maize rhizosphere using ARDRA analysis. In this study, a combination of three tetra cutter restriction endonuclease namely; *Alu*I, *Msp*I and *Hae*III were used for ARDRA analysis as comparable to the earlier reports by Upadhyay et al. (2009) and Saikia et al. (2011). For supporting of current studies, recent studies dealt with the genetic diversity of strains of *Bacillus* spp. (Deepa et al., 2010, Kumar et al., 2011, Gajbhiye et al., 2010, Vardhan et al., 2011, Ouoba et al., 2004, Xu and Cote, 2003). It has been observed that five clusters were obtained among the 28 isolates. A significant variation was obtained in genetic diversity analysis amongst isolates. Bakker et al. (2002) further controlled agents on the entire bacterial community in the rhizosphere of wheat Many studies have been undertaken to understand the

Table 3. Isolates and their characteristics on PGPR activity, Where + = Positive and - = Negative.

Isolate code	Plant growth promoting traits				
	IAA (µg mg^{-1} protein)	HCN	NH_3	Siderophore	P-solubilisation
DPNSB-1	98.99	+	-	-	-
DPNSB-2	687.94	+	+	+	+
DPNSB-3	42.46	+	+	-	-
DPNSB-4	63.04	-	+	-	+
DPNSB-5	272.49	+	+	+	-
DPNSB-6	91.53	+	+	+	-
DPNSB-7	392.4	+	-	+	+
DPNSB-8	97.1	-	+	-	-
DPNSB-9	99.56	+	+	+	-
DPNSB-10	79.63	+	+	+	+
DPNSB-11	845.28	+	-	+	+
DPNSB-12	60.21	-	+	-	-
DPNSB-13	72.55	+	+	-	-
DPNSB-14	83.29	+	+	-	-
DPNSB-15	94.1	+	+	+	+
DPNSB-16	132.35	-	+	-	-
DPNSB-17	89.65	+	+	-	-
DPNSB-18	127.25	+	+	+	+
DPNSB-19	258.23	-	-	+	-
DPNSB-20	60.21	+	-	-	-
DPNSB-21	304.1	+	+	-	+
DPNSB-22	83.29	-	-	-	-
DPNSB-23	109.84	-	+	-	-
DPNSB-24	115.25	+	+	-	-
DPNSB-25	88.97	+	+	-	-
DPNSB-26	65.65	-	+	+	+
DPNSB-27	76.76	+	+	+	-
DPNSB-28	503.65	+	-	+	-

nature and properties of these unique microbes which harbour potential plant growth-promoting traits with increasing awareness about the chemical-fertilizers based on agricultural practices (Ahmed, 1995), it is important to search for region-specific microbial strains which can be used as a potential plant growth promoter to achieve the desired product. In the present study, PGP traits for the isolates were further investigated under *in-vitro* condition. IAA production was quantified as ranging from 50 to 850 µg mg^{-1}. Similar reports for the IAA production have been reported by Kumar et al. (2011). This phenomenon can be attributed to the ability of the isolate to produce IAA, as IAA positively influences root growth and development, thereby, enhancing nutrient uptake (Khalid et al., 2004). For the siderophore production, it was observed that most of the *Bacillus* strains were positive for siderophore production and fifteen isolates were given positive results and 13 isolates were shown as negative for siderophore production. The role of siderophore production for the plant growth promotion was described by Buyer and Leong (1986). Siderophores produced by a number of *Pseudomonas* spp. are attracted for their possible roles in the biological control of the number of plant pathogens (Budzikiewicz, 1988). In terms of HCN production among the 28 isolate tested, 20 isolates were shown positive for HCN production and 8 isolates exhibited negative results. It was also reported that the production of HCN may play a critical role in PGP activities and proposed as a defense regulator against phytopathogens. As reported earlier by Forchetti et al. (2007), most organic phosphorous compound is carried out by phosphate enzymes. Among the 28 isolated tested for Phosphate solubiliztaion, 19 isolates were tested positive and 9 isolates were tested negative in this study. Examples of studied associations include *Azotobacter chroococcum* and wheat, *Bacillus circulans* and wheat, *Pseudomonas chlororaphis* and *P. putida* and soybean (Vessey, 2003).

The present study was successful in studying the effective characters on isolates of bacteria from tomato

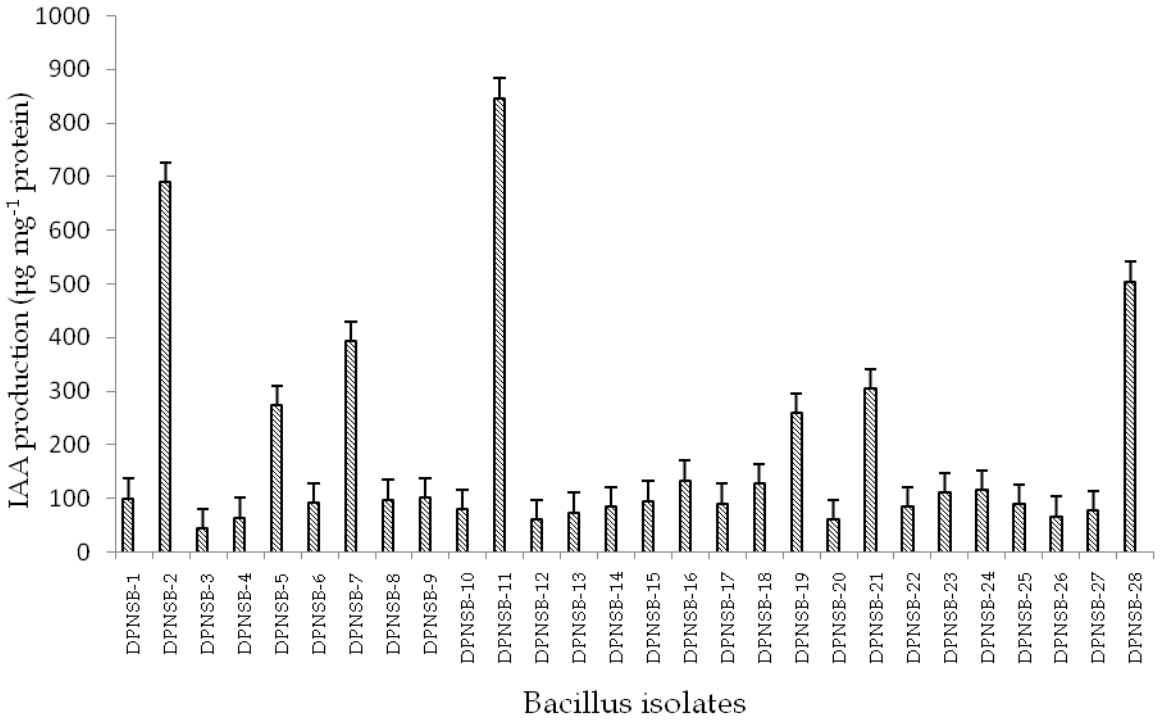

Figure 3. IAA production of *Bacillus* isolates.

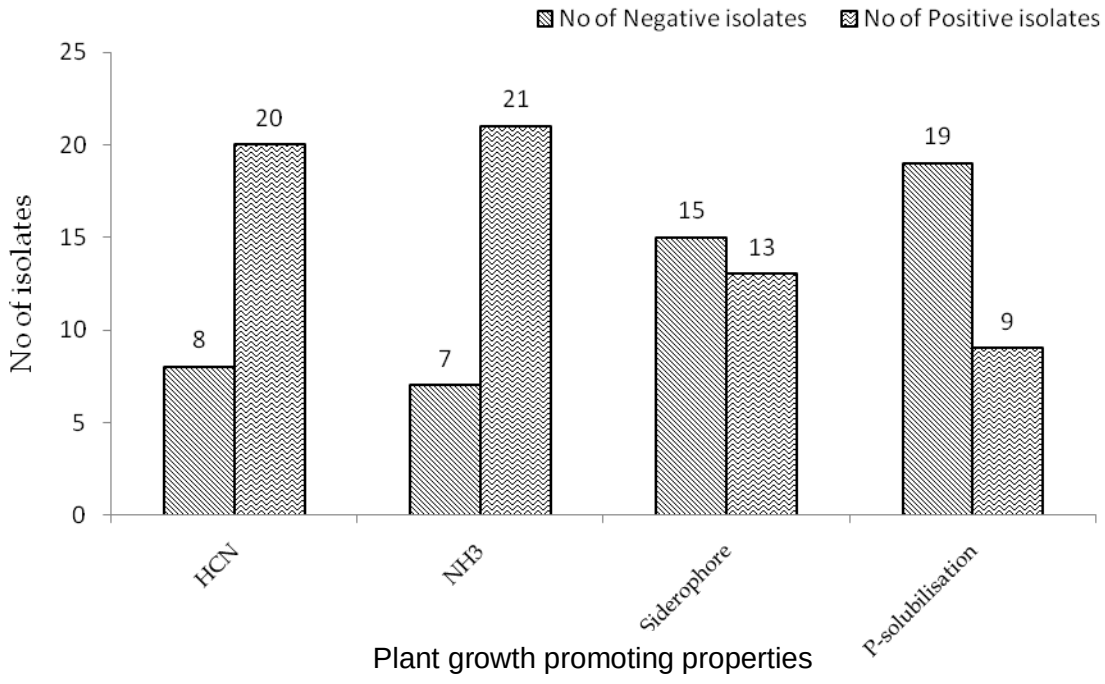

Figure 4. PGP characteristic of the isolates.

rhizosphere that can be a useful component of integrated disease management. The multipositiveness contained strains like DPNSB-2, 10, 15 and 18 as well as the highly IAA produced isolates like DPNSB- 2, 11 and 28 could be used as plant growth promoters for the control of wilt of tomato. In the absence of high level of genetic resistance

in high-yielding varieties, these bio-agents could be effective in controlling wilt diseases and related loss in tomato. Overall the findings of this study suggest that; i) *Bacillus* isolated from rhizospheric soil of tomato have shown more than one mechanism may be involved in the suppression of the pathogen and plant growth promoting activity; ii) high diversity was observed among the isolates. Further studies are in progress to; i) evaluate the interaction of these PGPR with other plants and to identify other native strains isolated from tomato rhizosphere in this study; ii) needed to determine the effectiveness of these isolates under different field conditions and to understand the nature of interaction with the pathogen and the host plant. However, it is becoming important to map these *Bacillus* populations in order to select “consortia” of bacteria for biological control from plant.

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