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Genetic diversity of iturin producing strains of *Bacillus* species antagonistic to *Ralstonia solanacerarum* causing bacterial wilt disease in tomato

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***Bacillus* spp. is a potential bacterial antagonist to manage bacterial wilt disease of tomato incited by *Ralstonia solanacearum,* which is one of the most threatening diseases of tomato in India. Genetic diversity of *Bacillus* strains and their potentiality to control bacterial wilt of tomato isolated from rhizospheric soil and endophytic tomato plants from different agro-climatic regions of India were studied. Rhizospheric soil and plants of tomato were pasteurized at 80°C for 15 min before dilution and then inoculated onto the Petri plates containing tryptic soy agar medium and incubated at 28± 2°C. Out of 250 isolates of *Bacillus* species, 47 strains showed antagonistic ability against *R. solanacearum.* Maximum growth of *R. solanacearum* was inhibited by strain DTBS-5 to form inhibition zone of 5.5 cm^2 *in vitro* and lowest wilt incidence of 14.3 and 7.6 % in Pusa Ruby and Arka Abha cultivars under glass house conditions, respectively. Plants treated with strain JTBS-9 had maximum fresh weight of 42.0 and 49.0 g and dry weight 6.1 and 6.6 g in tomato cultivars Pusa Ruby and Arka Abha after 45 days of transplanting, respectively. Out of 47 strains, 11 strains of *Bacillus* spp. were detected as iturin antibiotic producing strains by using iturin gene based marker. Genetic variability was found in *Bacillus* spp., which was made 5 clusters at 50% similarity coefficient. However, iturin producing and iturin non-producing strains as well as rhizospheric and endophytic *Bacillus* spp. could not be distinguished by using 16S rRNA sequence analysis and genetic fingerprinting.**

Key words: BOX-PCR, *Bacillus* spp., iturin, *Ralstonia solanacearum*, phylogenetic analysis, tomato.

INTRODUCTION

Bacterial wilt of tomato caused by *Ralstonia solanacearum* is one of the most devastating and wide spread diseases of crops worldwide (Hayward, 1991). In India, *R. solanacearum* race 1 biovar 3 is dominated mostly in coastal and hilly and foot hill area including Goa, Karnataka, Kerala, Maharashtra, Orissa, Jharkhand, West Bengal and state of North eastern hills, like Himachal Pradesh, Jammu and Kashmir and Uttarakhand (Devi and Menon, 1980). This disease causes very heavy loss varying from 2 - 90% in different climates and seasons in India (Mishra et al., 1995).

Management of this disease is very difficult due to soil borne nature of the pathogen, its wide host range of 200 species and 50 families including tomato, potato, egg

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plant, pepper, ground nut, tobacco, weeds and also roots of non host plants (Hayward, 1991; Poussier et al., 1999). There are no such chemicals available for effective management of this disease. Moreover, it causes soil and water pollution due to pesticide residues, which affects human health as well as development of resistant mutant by pathogen against pesticides. Hence, non-chemical methods including cultural methods, resistant cultivars and biocontrol with antagonistic bacterial agents have been made successfully to manage bacterial diseases of plants (Marten et al., 2000; Almoneafy et al., 2012). However, resistant cultivar is not completely effective due to lack of stability or durability (Boucher et al., 1992).

In biocontrol method, various fungal and bacterial antagonists were used to control plant diseases successfully. Among these bioagents, *Bacillus* species, a endospore forming, Gram positive bacterium is now a good candidate as a biocontrol agent and plant growth promoting bacteria (Chung et al., 2008). *Bacillus* spp. are able to colonize rhizosphere of plants and endophytic to form biofilm, which improve their ability to act as a bioagents against plant pathogens (Timmusk et al., 2005). Biological control of *R. solanacearum* through the antagonistic bacteria was done, which reduced the incidence of bacterial wilt disease (Almoneafy et al., 2012; Singh et al., 2012). The bacterium is found quite effective to suppress the bacterial pathogen (Rajendran et al., 2012; Singh et al., 2012) due to well developed secretary system and produces structurally diverse secondary metabolites with a wide spectrum of antibiotic activity. The *Bacillus subtilis* group was mostly able to synthesize surfactin and arthrobacin (Wulff et al., 2002). Chung et al. (2008) screened *B. subtilis* through PCR based on gene involved in biosynthesis of 11 antibiotics produced by various isolates of *B. subtilis.* Beside antagonistic ability, this bacterium can either directly or indirectly facilitate rooting and growth of plants by producing plant growth promoting substances like indole acetic acid, phosphate solubilization, ammonia and siderophore(Chaiharn et al., 2009).

Characterization of bacteria by using classical methods as morphological, biochemical, physiological is very laborious, time consuming. Hence, various advanced DNA based techniques are used to distinguish bacteria at species and sub species level. However, a molecular marker based on 16S rRNA sequence was developed to differentiate *Bacillus* species (Wattiau et al., 2001). Genetic diversity analysis of bacteria is studied by using BOX-PCR (Martin et al., 1992) and sequence analysis of 16S rRNA (Marten et al., 2000). These techniques have been successfully applied to *Bacillus* species for investigations of the relationships among isolates of *Bacillus* (Almoneafy et al., 2012). A little information is available on distinguishing antagonistic and plant growth promoting strains of *Bacillus* species by using genomic fingerprinting.

Thus, the present study was done to study genetic diversity of iturin producing strains of *Bacillus* species collected from different agro climatic conditions having antagonistic ability to suppress *R. solanacearum* and determine plant growth promoting attributes *in-vitro and vivo*.

MATERIALS AND METHODS

Sample collection

Rhizospheric soil and tomato plants were collected from 2009 - 2011 from different agro-climatic regions of India *viz.* Delhi, Jammu and Kashmir, Jharkhand, Himachal Pradesh, Manipur, Meghalaya, Uttarakhand and Uttar Pradesh states (Table 1). Physiochemical parameters of soil were analyzed and most of soils have electrical conductivity (EC) > 8.5, pH ranging from 6.0 - 8.5, sandy - loam to laterite soil (red soil). The plants were carefully removed from the soil and the whole plants with adherent soil were kept in plastic bags for isolation of bacteria.

Isolation of *Bacillus* from tomato plant and rhizosphere soil

For isolation of bacteria from rhizosphere soil, roots of tomato plants were shaken to remove excess soil and 10 g of rhizospheric soil from each sample was added to 90 ml of sterile distilled water and shaken for 30 min on a rotary shaker at 150 rpm. Soil samples were pasteurized at 80°C for 15 min for specific isolation of *Bacillus* spp. and then aliquots were further diluted up to 10^{-5}. The 100 µl aliquot of 10^{-3} and 10^{-5} dilutions were placed on to a tryptic soy agar (TSA) medium (Singh et al., 2012). The Petri plates were incubated at 28±1°C for 48 h.

For endophytic isolation of *Bacillus* spp., individual tomato plants were isolated as described by Zinniel et al. (2002) with slight modification as pasteurization at 80°C for 15 min. The single colony of bacterium was picked up and cultures were maintained on the TSA slants and stored at 4°C for further use.

Morphological, physiological and biochemical characters

Morphological and biochemical characterization of 44 isolates of *Bacillus* spp. was done based on the Gram reaction, KOH test, shape, motility, endospore formation and growth at different temperatures and NaCl concentrations. Biochemical characters like starch hydrolysis, utilization of citrate, indole production and H_2S production were done by using standard procedure (Schaad et al., 2001). Prototype strains used in taxonomic comparison were obtained from Institute of Microbial Technology, Chandigarh, India and Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India.

Antagonistic properties against *R. solanacearum*

Dual culture method was used for the screening of antagonistic properties of bacteria against *R. solanacearum* strain UTT -25 of *R. solanacerarum* was isolated from wilted tomato plant from District Nainital, Uttarakhand on TTC medium in 2009 as per standard procedure (Schaad et al., 2001). The *R. solanacearum* was grown in CPG broth medium for 48 h at 28 ±1°C and maintained the population of bacteria 10^8 cfu/ml. 100 µl culture of *R. solanacearum* was spread onto the Petri plates containing CPG medium to make a lawn of bacteria. Then 0.5 cm diameter of three wells in each Petri plate was made with sterilized cork borer. 40 µl of 48 h old

Table 1. Evaluation of antagonistic and plant growth promoting *Bacillus* species isolated from rhizospheric and endophytic of tomato from different agro-climatic conditions of India.

S/N	Strain of *Bacillus* spp.	Source	Location of collection	Area of inhibition zone against *R. solanacearum* (cm^2)	16SrRNA amplification	Iturin production	Phosphorus solubilization (µg/ ml)	IAA production (µg/ ml)	Siderophore production (µg/ ml)
1	MTBS-1	Rhizosphere	Nambol, Bishnupur, Manipur	1.2^{efgh}	+	-	34.3^{pq}	74.8^{cd}	0.63^{ijkl}
2	MTBS-2	,,	Nambol, Bishnupur, Manipur	1.1^{efgh}	+	-	30.3^{rs}	74.8^{cd}	0.67^{hijkl}
3	MTBE-1	Endophytic	Nambol, Bishnupur, Manipur	1.2^{efgh}	+	-	$44.7^{ijklmno}$	55.5^{cd}	0.90^{def}
4	MTBE-2	,,	Nambol, Bishnupur, Manipur	1.3^{defgh}	+	-	48.3^{ghi}	48.5^{cd}	0.73^{fghijk}
5	MTBS-3	Rhizosphere	Heikujm, Imphal west, Manipur	1.3^{defgh}	+	-	34.0^{pq}	48.5^{cd}	0.87^{defg}
6	MTBS-4	,,	Heikujm, Imphal west, Manipur	1.6^{defgh}	+	-	26.0^{tu}	55.46^{cd}	0.83^{defgh}
7	MTBE-3	Endophytic	Heikujm, Imphal west, Manipur	1.6^{defgh}	+	-	24.0^{u}	48.5^{cd}	1.0^{bcd}
8	MTBE-4	,,	Heikujm, Imphal west, Manipur	1.6^{defgh}	+	-	53.7^{f}	55.5^{cd}	0.77^{efghij}
9	MTBS-5	Rhizosphere	Wabagai, Thoubal, Manipur	0.8^{fgh}	+	-	66.7^{bc}	41.0^{d}	0.80^{efghi}
10	MTBS-6	,,	Wabagai, Thoubal, Manipur	1.3^{defgh}	+	-	66.7^{bc}	$41.^{d}$	0.70^{ghijkl}
11	MTBE-5	Endophytic	Wabagai, Thoubal, Manipur	1.0^{efgh}	+	-	42.3^{mno}	55.5^{cd}	0.57^{kl}
12	MTBE-6	,,	Wabagai, Thoubal, Manipur	1.6^{defgh}	+	-	41.3^{o}	41.0^{d}	0.93^{cde}
13	UTTBS-1	Rhizosphere	Khandi,Nainital, Uttarakhand	1.4^{defgh}	+	+	65.0^{cd}	55.5^{cd}	0.77^{efghij}
14	JHTBS-2	,,	Pithoria, Ranchi, Jharkhand	1.0^{efgh}	+	+	42.0^{no}	254.3^{bc}	0.87^{defg}
15	UTTBS-3	,,	Tihari, Nainital, Uttarakhand	1.3^{defgh}	+	+	42.3^{mno}	90.0^{bcd}	1.30^{a}
16	HPTBS-4	,,	Nagav, Solan, Himachal Pradesh	1.0^{efgh}	+	-	69.0b	55.5^{cd}	1.17^{ab}
17	JHTBS-5	,,	Pithoria, Ranchi, Jharkhand	1.5^{defgh}	+	+	60.7e	254.3^{bcd}	1.10^{bc}
18	JHTBS-6	,,	B A U, Ranchi, Jharkhand	1.2^{defgh}	+	-	46.3^{hijkl}	48.5^{cd}	1.13^{ab}
19	JHTBS-7	,,	B A U, Ranchi, Jharkhand	1.9^{def}	+	-	47.0^{ghij}	55.5^{cd}	0.87^{defg}
20	JHTBS-8	,,	ICAR Research Complex for Eastern Region, Palandu, Ranchi, Jharkhand	1.8^{defg}	+	+	41.3^{o}	55.5^{cd}	0.73^{fghijk}
21	JHTBS-9	,,	ICAR Research Complex for Eastern Region, Palandu, Ranchi, Jharkhand	1.9^{cde}	+	-	41.0^{o}	48.5^{cd}	0.57^{kl}
22	DTBS-4	Rhizosphere	Vegetable Farm, IARI, New Delhi	1.3^{defgh}	+	+	36.7^{p}	55.5^{d}	0.57^{kl}
23	DTBS-5	,,	Vegetable Farm, IARI, New Delhi	5.5^{a}	+	_	31.0^{qr}	48.5^{cd}	0.77^{efghij}
24	DTBS-6	,,	Vegetable Farm, IARI, New Delhi	1.9^{cde}	+	-	29.0^{rst}	48.5^{cd}	0.90^{def}
25	DTBE- 8	Endophytic	Vegetable Farm, IARI, New Delhi	1.8^{def}	+	-	32.0^{qr}	55.5^{cd}	0.63^{ijkl}
26	DTBE-9	,,	Vegetable Farm, IARI, New Delhi	1.5^{defgh}	+	-	36.7^{p}	90.1^{bcd}	1.30^{a}
27	JTBS-9	Rhizosphere	KVK, Kathua, Jammu & Kashmir	1.7^{defg}	+	-	72.7^{a}	55.5^{cd}	0.90^{def}
28	JTBE-14	Endophytic	KVK, Kathua, Jammu & Kashmir	2.1^{cde}	+	-	24.0^{u}	55.5^{cd}	0.77^{efghij}
29	JTBS-17	Rhizosphere	Basht, Udham Nagar, Jammu & Kashmir	1.5^{defgh}	+	-	24.7^{u}	48.5^{cd}	0.73^{fghijk}

Table 1. Contd

30	JTBE-20	Endophytic	Basht, Udham Nagar, Jammu & Kashmir	2.1^{cde}	+	-	31.0^{qr}	141.4^{a}	0.60^{jkl}
31	JTBS-21	Rhizosphere	S. K. University of Agriculture and Technology, Jammu, Jammu & Kashmir	1.5^{defgh}	+	-	29.3^{rst}	48.5^{cd}	0.73^{fghijk}
32	JTBE-23	Endophytic	S. K. University of Agriculture and Technology, Jammu, Jammu & Kashmir	1.0^{efgh}	+	-	$45.3^{hijklmn}$	48.5^{cd}	0.63^{ijkl}
33	UTBS-24	Rhizosphere	Naini, Allahabad, Uttar Pradesh	2.1^{cde}	+	-	62.3^{de}	48.5^{cd}	0.83^{defgh}
34	UTBE-25	Endophytic	Naini, Allahabad, Uttar Pradesh	0.5^{h}	+		63.7^{cde}	55.5^{cd}	0.73^{fghijk}
35	UTBE-26	,,	Naini, Allahabad, Uttar Pradesh	2.4^{cd}	+	-	64.3^{cd}	48.5^{cd}	0.83^{defgh}
36	UTBS- 32	Rhizosphere	SardarBallabhabhaiAgricutural University, Modipuram, Mereet, Uttar Pradesh	1.1^{efgh}	+	-	46.7^{hijk}	55.5^{cd}	0.57^{kl}
37	UTBS-33	,,	SardarBallabhabhaiAgricutural University, Modipuram, Mereet, Uttar Pradesh	0.7^{gh}	+	-	46.0^{hijklm}	288.5^{b}	0.83^{defgh}
38	UTBE-29	Endophytic	SardarBallabhabhaiAgricutural University, Modipuram, Mereet, Uttar Pradesh	2.0^{cde}	+	-	42.7^{lmn}	48.5^{cd}	0.70^{ghijkl}
39	UTBS-30	Rhizosphere	N. D. University of Agriculture &Technology, Faizabad, Uttar Pradesh	1.9^{cdef}	+	-	$45.3^{hijklmn}$	48.5^{cd}	0.80^{efghi}
40	UTBE-30	Endophytic	N. D. University of Agriculture &Technology, Faizabad, Uttar Pradesh	2.9^{bc}	+	-	49.0^{gh}	41.0^{d}	0.60^{jkl}
41	UTBE-31	,,	N. D. University of Agriculture &Technology, Faizabad, Uttar Pradesh	1.7^{defg}	+	-	50.3^{g}	48.5^{cd}	0.73^{fghijk}
42	METBS-31	Rhizosphere	ICAR, Research Complex, for NEH Region, Shilong, Meghalaya	3.6^{b}	+	-	41.7^{no}	48.5^{cd}	0.77^{efghij}
43	METBS-32	,,	ICAR, Research Complex, for NEH Region, Shilong, Meghalaya	2.1^{cde}	+	+	41.3^{o}	40.5^{cd}	0.93^{cde}
44	METBE-35	Endophytic	ICAR, Research Complex, NEH Region, Shilong, Meghalaya	3.4^{b}	+	+	43.0^{klmno}	55.5^{cd}	0.90^{def}
45	*B. Subtilis*	MTCC -2387	Institute of Microbial Technology, Chandigarh	1.5^{defgh}	+	+	43.7^{jklmno}	48.5^{cd}	0.73^{fghijk}
46	*B. cereus*	MTCC -7278	Institute of Microbial Technology, Chandigarh	1.7^{defg}	+	+	46.6^{hijk}	55.5^{cd}	0.87^{defg}
47.	*B. licheniformis*	-	Division of Plant Pathology, IARI, New Delhi- 110012	1.6^{defgh}	+	+	$45.3^{hijklmn}$	48.5^{cd}	0.77^{efghij}

+ = Positive reaction; - = negative reaction; Means followed by the same letter within a column are not significantly different as determined by LSD test (α = 0.05). Data present means of the experiment with 3 replications each.

culture of 250 isolates of *Bacillus* spp. including *B. cereus, B. licheniformis* and *B. subtilis* grown in the TS broth containing 10^9 cfu/ ml were poured into each well separately. The plates were incubated at 28 ±1°C for 24 h and inhibition zone formed by these isolates of *Bacillus* was recorded. These isolates, which did not form >0.5 cm diameter of inhibition zone, were not included in this study. The value of inhibition zone was converted into area of inhibition zone using the formula:

Area of circle = πr^2

Plant growth promoting attributes *in vitro*

The strains of *Bacillus* spp. which showed antagonistic ability to form >0.5 cm diameter of inhibition zone against *R. solanacearum* were screened for the expression of plant growth promoting attributes. The plant growth promoting attributes *viz*., phosphate solubilization, siderophore production and indole acetic acid (IAA) production possessed by bacteria were measured. Quantitative estimation of phosphate solubilization by *Bacillus* spp. was done as described by Mehta and Nautiyal (2001). Indole acetic acid produced by isolates of *Bacillus* was assayed colorimetric using ferric chloride-perchloric acid reagent (Vikram et al., 2007) and total soluble protein estimation was done according to Lowry et al. (1951). Siderophore production by *B. subtilis* isolates was measured using method described by Schwyn and Neilands (1987).

Assessment of antagonistic and plant growth promoting ability of *Bacillus* under glasshouse condition

Disease assessment

Among 47 isolates, 4 of *Bacillus* sp. *viz.,* DTBS-4, DTBS-5, JTBS-9 and METBS-31 were taken based on antagonistic and plant growth promoting attributes *in vitro* to evaluate their performance under glass house conditions in 2010-11. Aqueous suspensions (10^9 cfu/ml) of these strains *Bacillus* were prepared from 24 h old pure culture grown on TSA medium. Pots were filled with one kilogram of sterilized soil mixture containing vermiculite scaled, peat soil and sand in the ratio of 2:1:1 for raising the seedlings of tomato cultivars viz. Pusa Ruby (susceptible to bacterial wilt) and Arka Abha (resistant to bacterial wilt). 100 ml/per pot of 24 h old culture of each isolates of *Bacillus* was thoroughly mixed with the soil in the pot. After half an hour, 100 ml of 48 h old culture of *R. solanacearum* grown on CPG medium containing 2.5 x 10^9cfu/ml was mixed with the soil in each pot and five replications for each treatment was maintained. A set of treatment without adding antagonistic bacteria was maintained as control. Subsequently, 25 days old, five seedlings of tomato cv. Pusa Ruby and Arka Abha was transplanted in each pot separately. Wilt incidence was observed by counting the proportion of wilted plants in total plants per pot using 0-5 scale (Winstead and Kelman, 1952) and calculated wilt incidence/wilt intensity as mentioned by Schaad et al. (2001). Biological control efficacy (BCE) was calculated as described by Guo et al. (2004).

Assessment of plant growth parameters

The same procedure was followed as mentioned in disease assessment except pathogen *R. solanacearum,* which was not inoculated in the soil. After 45 days of inoculation, the effect of tested antagonist on growth was measured in terms of plant height (cm), fresh and dry weight (g) of root and shoot were assayed. Plants were measured for their height and uprooted whole plant with root from the pot. Root and shoot were cut from crown region, and fresh weight of root and shoot were recorded. The plant parts were dried at 60°C for three days and dry weight of root and shoot was recorded. The comparative growth promotion efficacy (GPE) by the strains of *Bacillus* was calculated as described by Singh et al. (2012):

$$GPE = [(G_T-G_C)/G_C] \times 100$$

Where, GPE is growth promotion efficacy, G_T is growth promotion by the treatment group and G_C is growth by the control.

Molecular characterization and genetic diversity

Extraction of genomic DNA

Total DNA of bacteria was extracted as described previously by using CTAB method (Murray and Thompson, 1980). The purified DNA was used for molecular characterization, detection of iturin production and 16s rRNA sequence and fingerprinting of *Bacillus* species.

Detection of iturin D producing strains of Bacillus spp.

A set of primer (ITU-F: 5'- ATTGACGGAGGACGCGCCTAT-3' and ITU- R: 5'- CCGAGGA TCGCTACATCAAT-3') was developed from iturin D gene of *B. subtilis* with predicted product size of 617 bp. For validation of primer, 47 strains of *Bacillus* spp. including *B. cereus, B. licheniformis* and *B. subtilis* were performed in gradient thermal cycler (BIO RAD C100™Thermal cycler). The amplifications were carried out in a final volume of 20 µl of PCR master mix containing 4 µl of PCR buffer, 0.5 µl of dNTPs (Promega), 0.5 µl of each primer, 2.0 µl $MgCl_2$, 0.25 µl of Taq polymerase and 100 ng of DNA template. In each PCR experiment, a control without DNA template was used as negative control. The PCR reactions were run with initial denaturation of 5 min at 94°C, 30 cycles each consisting of 94°C for 30 s, 62°C for 45 s and 72°C for 1 min and final extension 72°C for 5 min. A 15 µl aliquot of each amplified PCR product was separated on a 1.0% agarose gel in 0.5% TBE buffer, gel electrophoresis unit was run on 70 V for 1.0 h and stained with ethidium bromide and visualized under gel documentation (BIORAD, GEL DOC™ XR+ with image Lab™software).

PCR amplification of 16S rRNA and sequencing

For identification of 44 strains of *Bacillus* spp. (Table 1), a PCR technique was used as described by Wattiau et al. (2001). For sequence analysis of 21 strains of *Bacillus* spp. (Table 1), another set of universal primer (UNI_OL5 :5`-GTGTAGCGGTGAAATGCG-3`) UNI_OR (5` ACGGGCGGTGTGTACAA-3`) based on 16s rRNA for bacteria was used as described by Sauer et al. (2005). 15 µl of PCR product were analyzed in 1.5% agrose gel at 80 V for 1.0 h, and the gel was viewed under gel documentation system (BioRad,GEL DOC™ XR+ with image Lab™software) and photographed. Sequencing of 21 strains of *Bacillus* species belonging to different agro-climatic region was performed using ABI3730XL sequencer and reactions were analyzed on a capillary sequencer. 16S rRNA sequences of all the strains were compared with available database with high sequence matching >95% using GenBank BLAST N to determine approximate (≈700 bp) phylogenetic affiliation. The phylogenetic relationships were inferred by MEGA4 (Tamura et al., 2004, 2007). The sequence data were submitted in NCBI and accession no. of each strain of *Bacillus* spp. is given in bracket as follows, DTBS-4 (JQ688021), DTBS-5 (JQ688022), DTBS-6 (JQ688023), HPTBS-4 (JQ688024), HPTBS-5 (JQ688025), JHTBS-2 (JQ688026), JHTBS-6 (JQ688027), JHTBS-

Table 2. Effect of antagonistic isolate of *Bacillus* sp. on wilt incidence on susceptible and resistant cultivars of tomato and biocontrol efficacy cultivars under glasshouse condition.

Isolate of *Bacillus* sp.	Pusa Rubi(susceptible to bacterial wilt)		Arkha Abha(resistant to bacterial wilt)	
	Wilt incidence (%)	Biocontrol efficacy (%)	Wilt incidence (%)	Biocontrol efficacy (%)
HTBS-4	21.2^{c}	66.7	11.5^{c}	47.7
DTBS-5	14.3^{d}	77.6	7.6^{d}	65.9
JTBS-9	27.3^{bc}	57.1	15.7^{b}	19.5
METBS-31	29.4^{b}	53.9	19.9^{b}	23.2
Control	63.3^{a}	-	22.0^{a}	-

Means followed by the same letter within a column are not significantly different as determined by LSD test (α = 0.05). Data present means of the experiment with 5 replications each.

7 (JQ688028), JHTBS-8 (JQ688029) JHTBS-9 (JQ68830), JHTBS-21 (JQ68831), JTBE-23 (JQ68832), JTBS-9 (JQ68833), JTBS-17 (JQ68834), METBE-35 (JQ68835), METBS-32 (JQ68836), UTTBS-1 (JQ68838) and UTTBS-3 (JQ68839).

Genotypic diversity

The genomic DNA (50 ng) from 47 strains of *Bacillus* spp. including *B. cereus, B. subtilis* and *B. licheniformis* used to perform template in 25 µl of reaction mixture for fingerprinting was carried out using BOX primer (BOXA1R 5'- CTACGGCAAGGCGACGCTGACG-3') described by Schaad et al. (2001). The PCR product of 15 µl were separated by electrophoresis on a 1.5% agarose gel in 0.5 x TAE buffer for 6.5 h at 100 V. Gel was stained with ethidium bromide and photographed on gel documentation system. For cluster analysis, the data were converted to a binary matrix, where the digit 1 or 0 represented the presence of or absence of DNA band in the gel. NTSYS (2.02e version) software was used to analyze the fingerprinting. The similarity coefficient of BOX-PCR fingerprint was calculated with Pearson coefficient. Cluster analysis of the similarity matrix was performed by unweighted pair group method using arithmetic averages (UPGMA) algorithm (Saitou and Nei, 1987).

Data analysis

The analysis of variance for antagonistic ability, PGP attributes was performed by using standard procedure (Gomez and Zomez, 1984). Mean comparisons were conducted using a least significant difference (LSD) test (P = 0.05).

RESULTS

Antagonistic property of *Bacillus* isolates

Out of 250 isolates of *Bacillus* screened during preliminary test, 44 isolates collected from different agro-climatic regions of India and *B. subtilis, B. cereus* obtained from IMT, Chandigarh and *B. licheniformis* from Division of Plant Pathology, IARI, New Delhi presented inhibition activity against *R. solanacearum*. Hence, these 44 bacterial isolates were considered as bacteria with antagonistic characteristics. Maximum growth of *R. solanacearum* was inhibited by strain DTBS-5, which formed area of inhibition zone of 5.5 cm^2 followed by METBS- 31 (3.6 cm^2) and METBE- 35 (3.4 cm^2) *in vitro.* Whereas the other strains of *Bacillus* species had a wide range of antagonistic activity to form inhibition zone ranging from 0.51 to 2.94 cm^2 in laboratory *in vitro* test (Table 1). Significant variation in formation of inhibition zone against *R. solanacearum* was recorded.

Antagonistic strains of *Bacillus* showed significant differences for their abilities to suppress tomato bacterial wilt. Out of 47 strains showing potential for antagonistic ability, 4 strains namely HTBS-4, DTBS-5, JTBS-9 and METBS-31 were chosen for antagonistic and plant growth promoting ability under greenhouse condition. Strain DTBS- 5 showed the lowest value of wilt incidence of 14.3 and 7.6% in Pusa Ruby and Arka Abha cultivars, which was the highest biocontrol efficacy (77.6 and 65.9%) against *R. solanacearum*, respectively (Table 2). While other strains HTBS-4, JTBS-9 and METBS-31 also reduced the disease incidence as significantly compared to control. However, Arka Abha showed resistance against the disease under glass house condition, and further reduced wilt incidence and improved further biocontrol efficacy of *Bacillus* strains.

Plant growth promoting attributes

Screening of soil and endophytic bacteria for plant growth promoting activities were studied. The strains having antagonistic property >0.5 mm diameter of inhibition zone was considered for plant growth promoting attributes. 44 isolates of *Bacillus* were tested for solubilizing phosphorous *in vitro* and all strains have ability to solubilize the phosphorus (Table 1). However, strain JTBS-9 solubilize the highest phosphorus (72.7 µg/ml) followed by HPTBS-4 (69.0 µg/ml) (Table 1). A significant variation in solubilizing phosphorous was recorded among the strains of *Bacillus* spp. The bacteria also had ability to produce siderophore, which is an iron chelating agent. Strains DTBE-9 and UTTBS-3 produced maximum (1.30 µg/ml) siderophore in the medium followed by HPTBS-4 (1.17 µg/ ml) *in vitro.* Besides these strains, other strains of *Bacillus* produced siderophore ranging from 0.57 to 1.13 µg/ml in the medium. Indole acetic acid production is one of the criteria for screening of PGP bacteria and all

Table 3. Effect of antagonistic isolate of *Bacillus* sp. on growth and biomass of susceptible and resistant cultivars of tomato cultivars under glasshouse condition.

Isolate of *Bacillus* sp.	PusaRubi							ArkaAbha						
	Shoot length (cm)	Fresh shoot wt. (g)	Dry shoot wt. (g)	Root length (cm)	Fresh Root wt. (g)	Dry root wt. (g)	GPE (%)	Shoot length (cm)	Fresh shoot wt. (g)	Dry shoot wt. (g)	Root length (cm)	Fresh root wt. (g)	Dry root wt. (g)	GPE (%)
HTBS-4	46.6^{a}	39.4^{a}	5.3^{ab}	6.9^{a}	5.2^{ab}	1.1^{a}	14.3	4.8^{ab}	43.1^{bc}	5.2^{ab}	10.1^{ab}	7.7^{a}	0.9^{a}	7.0
DTBS-5	41.0^{a}	36.7^{a}	5.0^{ab}	6.2^{a}	5.7^{ab}	1.2^{a}	10.7	3.5^{ab}	43.4^{abc}	5.6^{ab}	9.3^{b}	7.6^{a}	1.0^{a}	15.7
JTBS-9	47.9^{a}	42.0^{a}	6.1^{a}	6.9^{a}	6.3^{a}	1.3^{a}	32.1	56.0^{a}	49.0^{ab}	6.6^{a}	11.8^{a}	8.0^{a}	1.1^{a}	35.1
METBS-31	46.3^{a}	37.4^{a}	5.0^{ab}	5.9^{a}	5.5^{ab}	1.1^{a}	8.9	57.1^{a}	48.2^{a}	6.2^{ab}	9.7^{b}	7.9^{a}	0.9^{a}	21.1
Control	41.2^{a}	31.0^{b}	4.7^{b}	6.0^{a}	5.1^{b}	0.9^{a}	-	47.1^{b}	39.8^{c}	4.8^{b}	8.5^{b}	5.9^{b}	0.9^{a}	-

wt.: Weight; Means followed by the same letter within a column are not significantly different as determined by LSD test (α = 0.05). Data present means of the experiment with 5 replications each.

all tested strains of *Bacillus* spp. produced IAA ranging from 41.0 to 288.5 µg/ml. However, UTBS- 33 produced the highest amount of IAA 288.5 µg/ml followed by UTBS-33 (254.3 µg/ml) *in vitro*.

Out of 47 strains of *Bacillus* spp. 4 strains viz. HTBS-4, DTBS-5 JTBS-9 and METBS-31 were used to study the effect of these bioagents on plant height and biomass production under glass house conditions. Plants treated with strain JTBS-9 of *Bacillus* sp. had maximum fresh shoot weight of 42.0 and 49.0 g and dry weight of 6.1 and 6.6 g of Pusa Ruby and Arkha Abha after 45 days of transplanting, respectively, which had high significant differences among other bioagents HTBS-4, DTBS-5 and METBS-31 and untreated control (Table 3). Fresh weight and dry weight of root and shoot of the cultivars treated with the strain of *Bacillus* was significantly increased and found variable in treated and untreated plants.

Morever, plant growth efficiency was found higher in JTBS-9 treated Pusa Ruby and Arka Abha, 32. 1 and 35.1% based on dry weight of root and shoot of the plant. Although, the highest dry weight of root of both cultivars was found in treated with JTBS-9 but variation in dry weight of root treated with bioagents and untreated control was insignificant.

Characterization of bacteria

The cells of *Bacillus* isolates were rod shaped, some cells are in pair, Gram positive and endospore forming. The 250 strains of *Bacillus* spp. were analyzed based on morphological observations, physiological and biochemical characters, showing their similarities to *Bacillus* species like endospore formation of the cell, swelling of bacillary body and no growth in glucose broth under anaerobic condition.

The *Bacillus* hydrolyzed the starch and gelatin and make clear zone. They also showed oxidase test, growth in 7% NaCl, and citrate utilization tested positive. The *Bacillus* strains showed similar properties in utilization of carbon sources and biochemical reactions as reported earlier for *Bacillus* spp.

Molecular characterization of *Bacillus* spp.

Based on the large multiple alignments of *Bacillus* 16S rRNA sequences, the two PCR primers, *Bsub*-5F and Bsub-3R were chosen (Wattiau et al., 2001), which was specific to *B. subtilis* group as reported and predicted to specifically amplify at 595 bp DNA fragments of *B. subtilis.* All strains of *Bacillus* spp. were detected as positive with this test except *B. cereus*, which were very close from a taxonomic point of view (Table 1).

21 strains of *Bacillus* species were confirmed to belong to the *B. subtilis* group which included *B. licheniformis*, *B. subtilis*, *Bacillus pumilus*, *B. atrophaeus*, and *Bacillus amyloliquefaciens* by using 16S rRNA based primer. However, these strains were isolated from rhizospheric soil and endophytic of tomato plants from different agro-climatic regions belonging to *B. subtilis* group.

Iturin production

A primer was developed to screen iturin producing isolates of *Bacillus* spp. isolated from diverse climatic conditions across India. PCR amplification for iturin locus showed 617 bp amplification in 11 strains out of 47 strains of *Bacillus* spp. Including *B. subtilis*, *B. cereus* and *B. licheniformis* (Figure 1).

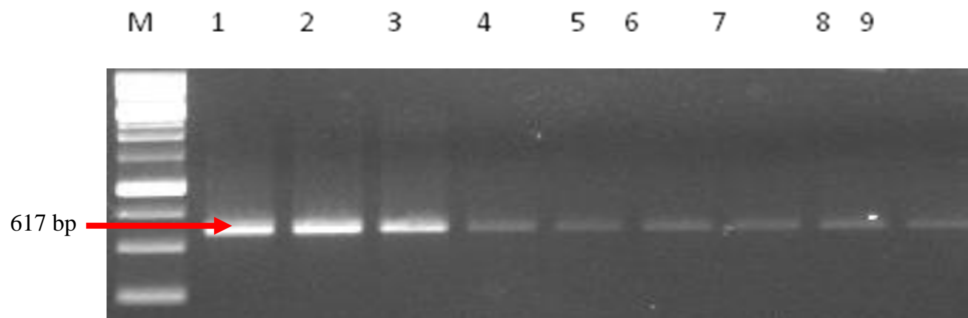

Figure 1. The amplified DNA fragments at 617 bp produced strains of *Bacillus* spp. by ITU primer isolated from rhizosphere and endophyte of tomato plants. Lane M: 1 Kb DNA Ladder, lanes 1: *Bacillus subtilis*, 2: UTTBS-1, 3: JHTBS-2, 4: UTTBS-3, 5: HPTBS-4, 6: JHTBS-5, 7: DTBS-4, 8: METBS-32, 9: METBE-35.

All the strains producing iturin were soil inhabitant under diverse climatic conditions belonging states Delhi, Uttarakhand, Himachal Pradesh, Jharkhand and Meghalaya of India.

Genomic fingerprinting

The fingerprinting patterns of 47 strains of *Bacillus* spp. isolated from rhizosphere and endophytic of tomato plants from different agro-climatic regions of India was generated by BOX- PCR considered as 5 - 15 fragments ranging in size of about from 200 to 4.5 kb (Figure 2b). The variations in number of fragments, their size among the strains of *Bacillus* spp. were observed. The highest 15 fragments were noted in strain MTBE-35. Computer assisted analysis of the BOX-PCR fingerprinting showed that all tested strains could be grouped into five distinct clusters at 50% similarity coefficient. Cluster 1 represented the 40 strains of *B. subtilis* and unidentified *Bacillus* spp., cluster 2 comprised three strains including *B. cereus,* JHTBS-6 andJ HTBS-8. Whereas strain DTBE-4 and UTBS-24 clustered in cluster 3. *Bacillus licheniformis* and JTBS-17 formed clusters 4 and 5, respectively. Cluster 1 further separated into 7 subgroups A, B, C, D, E, F and G at >75% similarity coefficient (Figure 2a) and subgroup A contained the highest 18 strains of *Bacillus* spp. irrespective of agro-climatic regions belonging to state Manipur, Uttar Pradesh, Delhi, Jammu and Kashmir followed by subgroup C comprising of 9 strains *viz*., JHTBS-5, JHTBS-7, METBS-32, UTBE-25, UTBE-29, UTBS-30, UTBE-31, METBS-31and UTBE-30. Moreover, sub group B, D, E, F and G comprised 4, 4, 2, 1 and 2 numbers of strains of *Bacillus* sp., respectively.

Genetic diversity of 21 strains of *Bacillus* spp. based on the partial 16S rRNA sequence analysis (≈ 700 bp) isolated from endophytic and rhizospheric of tomato plant were determined with homology of 95-100. These strains of data base were grouped along with sequences of other *Bacillus* spp. obtained from NCBI clustered. Based on the grouping, strain JHTBS- 8, JHTBS-9, JHTBS-2, JTBE-23 were very close to *B. subtilis*, strain JHTBS-1 close to *B. amyloliqueficience*, whereas JHTBS-6 and JTBS-9 were very close to *B. cereus* K17 and HTBS-4 close to *Bacillus* sp. YXA. However, the remaining 15 isolates made separate 3 groups (Figure 3). The grouping of the strains was not based on climatic conditions and also not disguised by their nature of inhabitants.

DISCUSSION

Bacterial wilt of tomato is a serious disease in India and severely infected field as high as 95% disease incidence was caused by *R. solanacearum* (Mishra et al., 1995; Singh et al., 2012). Managing the bacterial disease by biocontrol agents has become increasingly important. Among various bioagents, rhizobacteria and endophytic bacteria have an impressive effect which protect the soil borne bacterial pathogens as well as improve plant growth (Glick et al., 2007; Almoneafy et al., 2012; Singh et al., 2012).

In our study, rhizobacteria and endophytes belonging to genus *Bacillus* were isolated from tomato plants from different agro-climatic conditions to find the potential strains representing high bacterial wilt control and also to offer several plant growth promoting attributes such as phosphorus solubilizing, siderophore production and indole acetic acid production to improve health and growth of tomato plant. *In vitro* study, 250 isolates of *Bacillus* spp. were tested and those that formed >0.5 cm diameter of inhibition zone were considered for antagonistic ability, in which 47 strains represented remarkable antagonistic activity against *R. solanacearum*. The strains DTBS-5, METBS- 31, METBE- 35, UTBE- 26 and UTBE-30 have potential for highest inhibition effect against bacterial pathogen. These results confirm the previous studies reporting the antagonizing activity of *Bacillus* species against *R. solanacerarum* (Li et al., 2008; Almoneafy et al., 2012;

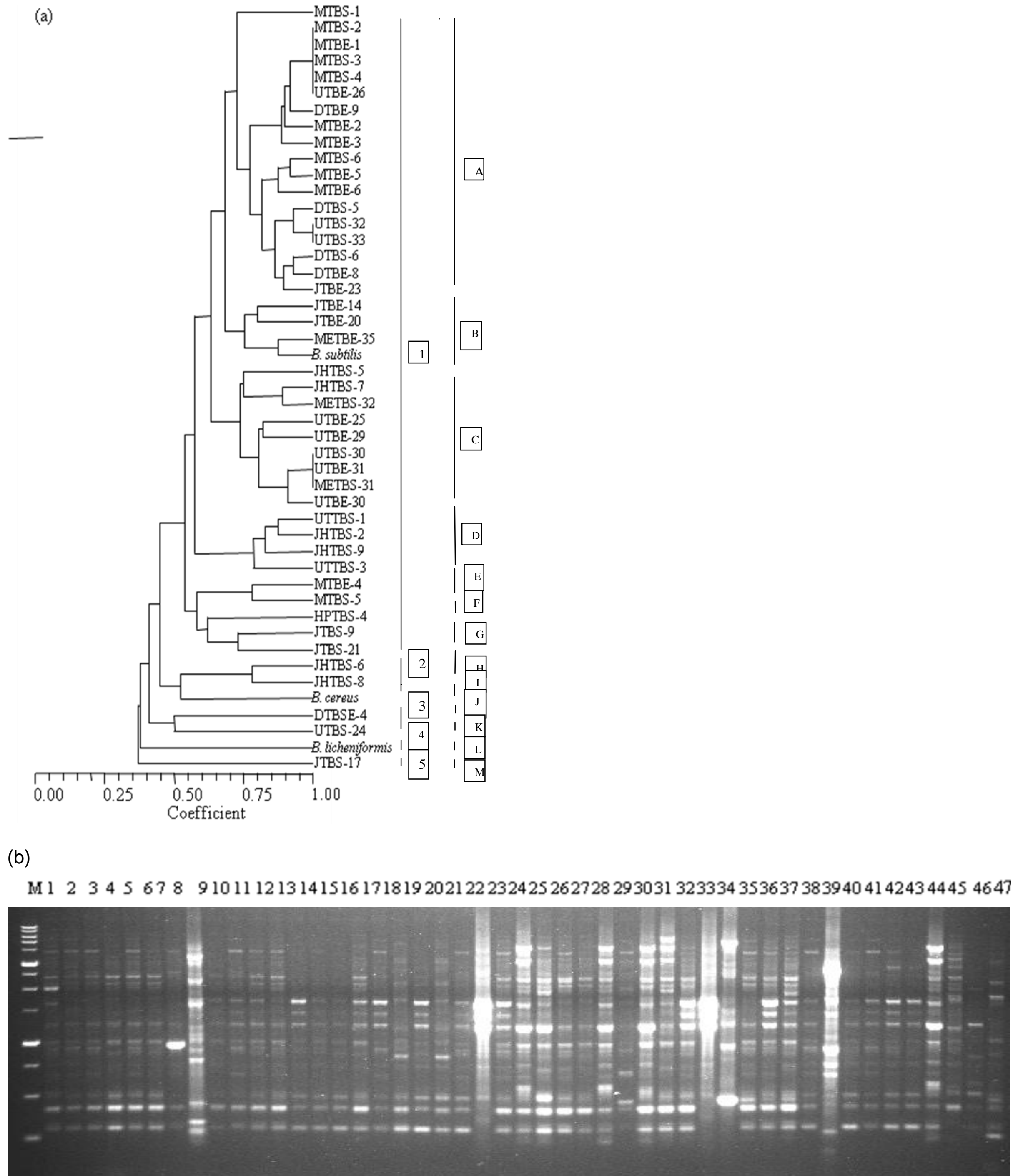

Figure 2. (a) Cluster analysis of genetic diversity of 47 strains of *Bacillus* spp. reaction by using BOX-PCR. The phylogenic tree was constructed by the UPGMA method for the similarity matrix generated using Pearson's correlation coefficient applied to the whole patterns. The minimum similarity coefficient of all isolates was used to define distinct groups, which are labeled numerically. Distinct subgroup of genomic variability of Bacillus spp. isolates are labeled alphabetically A - M. (b)The amplified DNA fragments produced by BOX primer for strains of *Bacillus* species. Lane name with respective strains. Lane M: 1 Kb DNA ladder, Lanes 1 - 12: Strains from Manipur (MTBS-1, 2: MTBS-2, 3: MTBE-1,4: MTBE-2, 5: MTBS-3, 6: MTBS-4, 7: MTBE-3, 8: MTBE-4, 9: MTBS-5, 10: MT*BS*- 6,11: MT*BE-5,* 11: MTBS-6, 12: MTBE-5), lanes 13 and 15: strains from Uttarakhand (UTTBS-1 and UTTBS-3) lanes 14, 17 - 21: strains from Jharkhand (JHTBS-2, JHTBS-5, JHTBS-6, JHTBS-7, JHTBS-8 and JHTBS-9), lane 16: strain from Himachal Pradesh (HPTBS-4), lanes 22 - 26: strains from Delhi (DTBSE-4, DTBS-5, DTBS-6, DTBE-8, DTBE-9), lanes 27 - 32): strains from Jammu and Kashmir (JTBS-9, JTBE-14, JTBS-17, JTBE-20, JTBS-21 and JTBE-23), lanes 33- 41: strains from Uttar Pradesh (UTBS-24, UTBE-25, UTBE-26, UTBS-32, UTBS-33, UTBE-29, UTBS-30, UTBE-30, UTBE-31, lanes 42- 44: strains from Meghalaya (METBS-31, 43: METBS-32, 44: METBE-35), 45: *B. subtilis*, 46: *B. licheniformis*, 47: *B. cereus.*

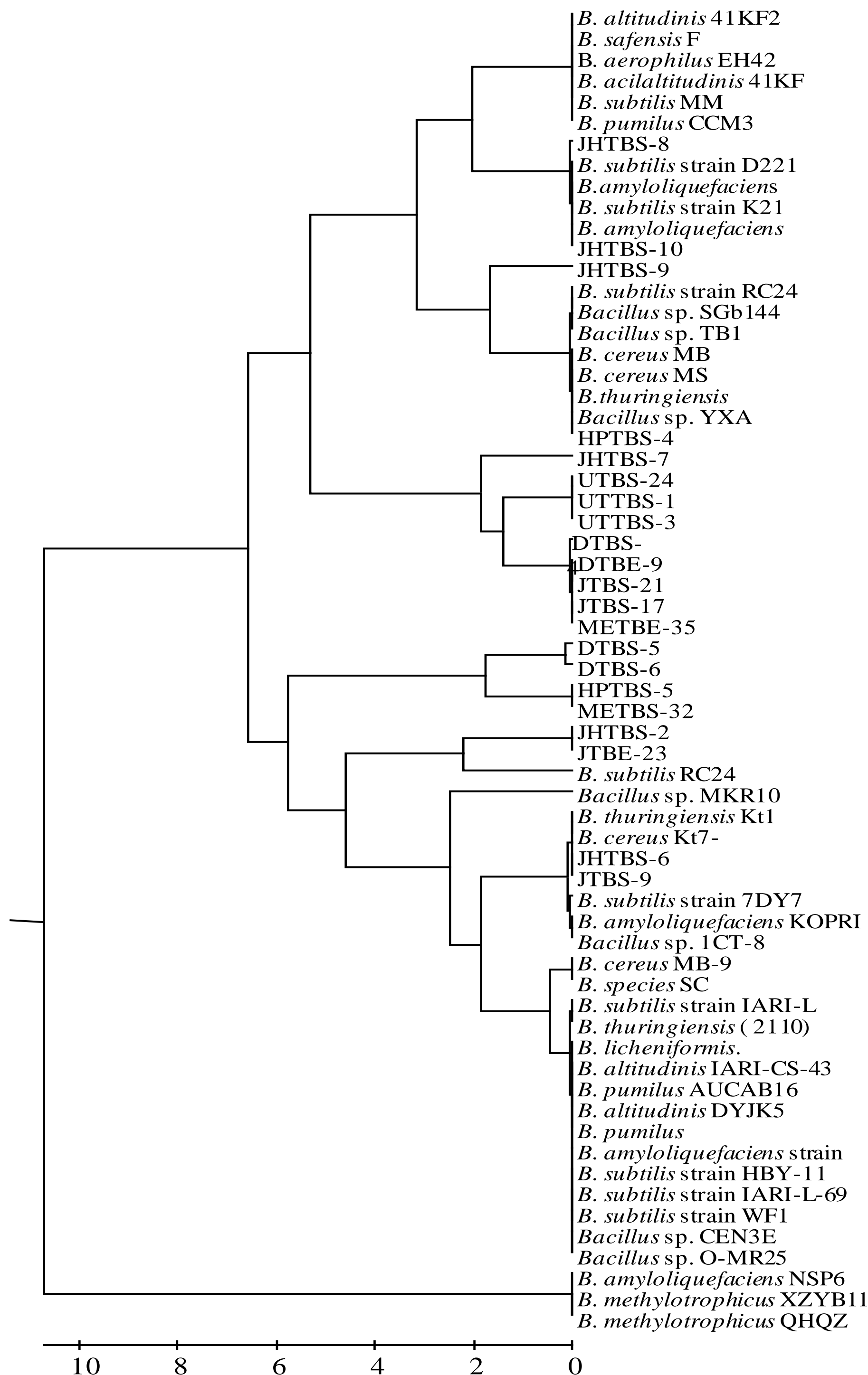

Figure 3. Phylogenetic analysis based on 16S r RNA sequences of 21 isolates of *Bacillus* spp. showing the position with respect to related species available from NCBI database. The evolutionary history was inferred using the UPGMA method. There was a total of 650 positions in the final dataset and phylogenetic analyses were conducted in MEGA4.

Singh et al., 2012). Growth inhibition zones on the agar media may be formed due to chemicals like antibiotic substances with broad spectrum of activity or more specific bacteriocin (Gross and Vidaver, 1990) or production of siderophore or both of them produced by bacteria (Adesina et al., 2007; Lemessa and Zeller, 2007).

In this study, CPG medium was used for *in vitro* screening assay, because the medium was suitable for *R. solanacearum* growth and agrees with other earlier studies, which reported that the type of culture medium strongly affects antagonistic activity mediating the production of chemical substances responsible for inhibition (Montesinos et al., 1996). *Bacillus* species are known as very efficient producers of antibiotic molecules of three main family, surfactins, iturins and fengycins. *B. subtilis* has the potential to produce > 24 structurally diverse antimicrobial compounds (Stein, 2005), which have been reported for their inhibitory activity against plant pathogens.

Variability in production of PGP attributes were found in *Bacillus* spp., which are influenced by various factors such as nutritional richness, physiological and growing status of the bacterium (Upadhyay et al., 2009). Strains JTBS-9, HPTBS-4, MTBS-5 and MTBS-6 have demonstrated the high ability to solubilize inorganic phosphate *in vitro*. *Bacillus* spp. is capable of increasing availability of phosphorus in soil. Phosphorus solubilizing bacteria has also been isolated from soil (Mehta and Nautiyal, 2001) and endophyte (Rajendran et al., 2012), which supported our findings. Siderophore production is another plant growth promoting attributes, which is produced by rhizobacteria and endophytes (Beneduzi et al., 2008). Strains UTTBS-3, HPTBS-4 and JHTBS-5 produce >1.0 µg/ml siderophores *in vitro*. In our study, all the strains of *Bacillus* spp. produced IAA, which range from 41.0 - 288.5 µg/ml. However, strains UTBS-33, JHTBS-5 and JTBE-20 have high potential to produce IAA, which help in promoting plant growth (Loper and Schroth, 1986). It has been observed that strain like DTBS-5 has the highest antagonistic ability to suppress the bacterial wilt incidence but has lower IAA production. It might be due to inactivation of gene responsible for IAA biosynthesis which causes reduction in IAA concentration, resulting in low plant growth promotion activity (Idris et al., 2007). The overall result showed that strains isolate HPTBS-4, JHTBS-5, UTTBS-3 and UTBS-33 have better PGP attributes, which may be good candidates for PGP trait. Moreover, the strains UTTBS-3 and JHTBS-5 produce iturin antibiotic, which was confirmed through molecular techniques.

Molecular characterization the 47 strains of *Bacillus* spp. was performed for further confirmation. The primer based on 16s rRNA gene specific to *B. subtilis* group was applied for characterization of isolate in which *B. licheniformis*, *B. subtilis*, *B. pumilus*, *B. atrophaeus*, and *B. amyloliquefaciens* are included (Wattiau et al., 2001). All the strains used in this study were amplified except *B. cereus*. It indicates that these strains isolated from rhizospheric soil and endophyte of tomato plants from different agro-climatic regions belong to *B. subtilis* group as earlier reported by Wattiauet al. (2001). But in contrast to the earlier study, we found that the strains JHTBS-6 and JTBS-8 are grouped with *B. cereus* in BOX- PCR and and JHTBS-6 in 16S rRNA sequence analysis which are amplified.

The dendrogram (Figure 2a) was generated by use of the Jacord coefficient to analyze BOX-PCR data. Our result discriminated *B. cereus*, *B. subtilis* and *B. licheniformis* along with other strains of *Bacillus* spp. In contrast to our result, it did not reliably discriminate between *B. cereus* and *B. thuringiensis* (Kim et al., 2002). Another genetic diversity study of *Geobacillus* and *Bacillus* strains isolated from a volcanic region compared different molecular techniques such as *rpo*B sequence analysis, repetitive extragenic palindromic-PCR (REP-PCR) and BOX-PCR (22). Upadhyay et al. (2009) reported that *Bacillus* and *Bacillus* derived genera dominated in rhizoplane of soil of wheat and found a lot of genetic diversity in *Bacillus*. Genetic variation in strains of *Bacillus* spp. may be due to mutation and other genetic changes like recombination. Though BOX-PCR allowed grouping of strains but it was not possible to clearly distinguish the iturin antibiotic producing strains from other non-iturin producing strains. In our case, since we took only those strains of *Bacillus* having antagonistic ability and plant growth promoting attributes, hence they could not be differentiated genetically. Moreover, the results of BOX-PCR did not generate any molecular marker that was associated with antagonistic and plant growth activity.

Conclusion

The strains of *Bacillus* spp. isolated from rhizospheric soil and endophyte of tomato posses both tracks, that is, antagonistic and PGP attributes and few strains are able to produce iturin antibiotics. However, non iturin producing strains have better antagonistic property, which indicates that they produced some other antibiotics. *B. subtilis* strains were discriminated by using 16S rRNA primer and BOX-PCR. The BOX-PCR genomic fingerprinting could be used as highly discriminatory technique to determine the genetic relatedness and diversity among the strains of *Bacillus* species.

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