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Potential of *Bacillus amyloliquefaciens* for Biocontrol of Bacterial Wilt of Tomato Incited by *Ralstonia solanacearum*

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Abstract

Fifty seven rhizobacteria were isolated from rhizospheric soil of wilted tomato plants and among them two strains of rhizobacteria, having better antagonistic and plant growth promoting ability were characterized them as *Bacillus amyloliquefaciens* DSBA-11 and DSBA-12 based on morphological, biochemical, partial gene sequence analysis of 16S rRNA and fatty acid methyl ester analysis. Antagonistic activity of these strains DSBA-11, DSBA-12 was compared with other *Bacillus* species such as *B. subtilis* DTBS-5, *B. cereus* JHTBS-7, *B. pumilus* MTCC-7092 strains, against *Ralstonia solanacearum* race 1, bv 3, phylotype I, inciting bacterial wilt of tomato under*in vitro* conditions. *B. amyloliquefaciens* DSBA-11 showed maximum growth inhibition of *R. solanacearum* (4.91cm^2) followed by strains DSBA-12 (3.31cm^2) and *B. subtilis* (3.07 cm^2). Moreover, strains DSBA-11 was also have better phosphorus solubilizing ability (42.6 µg/ml) and indole acetic acid (95.4 µg/ml) production than other strains of *Bacillus* spp. *in vitro* conditions. Biocontrol efficacy and plant growth ability of these bacterial antagonists was tested against bacterial wilt of tomato cv. Pusa Ruby under glasshouse conditions. Minimum bacterial wilt disease incidenceincultivar Pusa Ruby (17.95%) was recorded in *B. amyloliquefaciens* DSBA-11followed by *B. amyloliquefaciens* DSBA-12 after 30 days of inoculation.The bio-control efficacy was higher in *B. amyloliquefaciens* DSBA -12 treated plants, followed by *B. pumilus* MTCC- 7092.

Keywords: *Bacillus;* Bacterial wilt; Fatty acid methyl ester; 16S rRNA; PGPR; Tomato

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi is a serious tomato disease (*Solanum lycopersicum. L.*) in tropical, subtropical and temperature areas of the world. The disease has been reported frommostly in coastal, hilly, as well as foothills areas, including Goa, Karnataka, Kerala, Maharashtra, Odisha, Jharkhand, West Bengal, Himachal Pradesh, Jammu & Kashmir, Uttarakhand and north-eastern states [1,2]. The disease causes very heavy losses, varying from 2 to 90% in different agro climatic conditions in India [3] particularly, during October-November in coastal areas [1] and August–October in northern and eastern parts of India [2]. The pathogen issurvived in soil for days to years [4]. In addition, very difficult to control the disease However, various strategies have been developed earlier to control bacterial wilt in tomato, including use of chemicals like bleaching powder and calcium chloride [5]. However, these chemicals applied in soil are not effective to control the disease. Thus, use of biological control of microbial antagonists is being emerged methodto manage bacterial wilt disease [6-8]. Besides disease suppression, the antagonists have some other advantages like not harmful to human beings, animals as well as environment, easy-to- apply by farmers and have ability to enhance plant growth and yield of the crops [9,10]. Several bacterial antagonists, such as *Pseudomonas fluorescens*, *P. putida*, *Bacillus* spp. and *Actinomyces* are used to control wilt disease in tomato. Among various bacterial antagonists reported, *Bacillus* spp. like *B. amyloliquefaciens, B. coagulans, B. cereus, B. licheniformis, B. pumilus, B. subtilis* and *B. vallismortis* have been used for effective control the disease in tomato [11-13]. The *Bacillus* spp. Have more advantages over other genera of bacterial antagonists, since they are resistant to desiccation and have better survivability at higher temperature due to endospore forming nature and also ability to promote plant growth [14,15]. Although, several bacterial antagonists are used to control the disease, but it is always a scope to search new potential strains of bacterial antagonists from rhizosphere of some plants. The characterization of new bacterial antagonists is done by morphological, biochemical, physiological [16] and by advanced methods such as fatty acid profiling [17] and DNA based techniques [18]. Fatty acid methyl esterase analysis has been used for characterization of bacteria [19]. The types of fatty acids present in a cell are determined by bacterial genotypes, and identify different species and strains of bacteria [20]. Sasser [21] developed commercial systems for streamline fatty acid extraction and detection procedures to the fatty acid profiling of agriculturally important [22]. A molecular marker based on 16S rRNA sequence analysis has been developed to differentiate *Bacillus* species [18,23].

The present investigations were undertaken to characterize potential bacterial biocontrol agents isolated from acidic rhizospheric soil of wilted tomato plantto control bacterial wilt disease and also ability to enhance promote plant growthunder glasshouse conditions.

Materials and Methods

Soil sample and isolation

Soil sample from wilted tomato rhizosphere were collected from Bhuwali and Nainital, in Uttarakhand state. Ten gram of soil (acidic

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soil, pH 6.5) was taken and mixed it well in a 100 ml of sterilized distilled water in a 250 ml of flask, then heated it for 15 min at 80°C. About 100 µl of diluted aliquot of soil suspension was inoculated on to TSA (Trypticase soy agar) medium and then incubated at 28 ± 1°C for 48 h. Suspected colonies of bacteria were selected and transferred in the slants and preserved them at –80°C.

Characterization of *Bacillus* spp.

Morphological and biochemical tests were done to characterize rhizobacteria using standard procedure [16]. For FAME analysis, strains of *B. amyloliquefaciens* DSBA-11 and DSBA-12 and other *Bacillus* spp.(*B. subtilis* DTBS-5, *B. cereus* JHTBS-7, *B. pumilus* MTCC-7092) showing antagonistic and plant growth promoting ability, were grown in TSA(trypticase soya agar) medium at 28 ± 1°C for 24 h. Liquid medium containing bacterial cells (1.0 L) was extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulphate, removed under reduced pressure at 45°C, and dried. Metabolite profiles of ethyl acetate extract were determined using gas chromatography mass spectrometry (Agilent) equipped with a DB-5 capillary column with size 30 m × 0.25 mm film thickness 0.25 µm). Chromatographic conditions were as follow: helium as carrier gas with 1 ml/min flow-rate (split mode, 1:20); injection volume 1 µl (10 mg extract/3 ml acetone). The column temperature was maintained at 60°C and then programmed at 3°C/min to 280°C, and kept for 5 minutes. The injector ion source and Mass spectrometric transfer line temperatures were kept at 250°C, 230°C and 280°C. The column was coupled directly to quadrupole mass spectrometer (EI mode, at 70 eV) with the mass range of 28-500 a.m.uat 1 scan/s. The compounds were identified individually by comparing their mass spectrum with the spectrum of compound available in NIST Mass Spectral Library and literature [24]. The FAME wasanalyzed by gas chromatographic (GC) using the Microbial Identification System (MIS, MIDI Inc., Newark, DE) software to identify the relative amount of fatty acids in bacteria and expressed as a percentages of whole cell fatty acid methyl ester as described by Whittaker et al. [17]. The fatty acid profiles generated were compared against an in-built Sherlock TSBA Library version 3.9 (MIDI Inc., DE and USA). A similarity index of >50% was considered to cluster of isolates at species level.

DNA extraction and 16S r RNA sequencing of *B. amyloliquefaciens*

The bacterial colonies were grown in Luria broth medium at 28 ± 1°C with 200 rpm for 48 h. The grown colonies of bacteria were harvested in the form of pellet after centrifugation. Total bacterial DNA was extracted by using CTAB methods [25]. Oligonucleotide primers UNI_OL5 and outer reverse UNI_OR based on 16 S rRNA were used to amplify from obtained template in a PCR reaction mixture and thermo cycling conditions as described by Sauer et al. [26]. Sequencing of purified PCR aliquots was done by Xcelris genomics, Ahmadabad (http://www.xcelrislabs.com), using both the primers. Nucleotide sequence similarities were determined using BLAST version 2.2.6 (NCBI databases; http://www.ncbi.nlm.nih.gov/). The Partial nucleotide sequences were aligned with the partial sequences of 16 rRNA gene sequences of other of *Bacillus* spp. taken from NCBI Gen Bank database. A phylogenetic tree was made using by neighbor-joining method of MEGA 5.0 software [27].

Antagonistic ability of *B. amyloliquefaciens* against *R. solanacearum in vitro*

Dual culture technique was used as described by Singh et al. [5] to study comparative antagonistic ability of *B. amyloliquefaciens* DSBA-11 and DSBA-12 isolated from rhizospheric soil of tomato along with other species of *Bacillus* such as *B. cereus* JHTBS-7, *B. subtilis* DTBS-5, obtained from Division of Plant Pathology,IARI, New Delhi and *B. pumilus* MTCC-7092, obtained from MTCC, Chandigarh against wilt pathogen *R. solanacearum* under *in vitro* conditions. The bacteria were grown in nutrient sucrose broth medium at 28 ± 1°C for 48 h. The population of bacteria was maintained to 0.1 OD at 600 nm by spectrophotometer (UV-VIS Spectrophotometer, Hitachi, U-2900). 100 µl cultures of bacteria were spread onto the Petri plates having nutrient sucrose agar medium to make a lawn of bacteria on the medium with three replications. Three wells of 5 mm diameter in each Petri plates were made with the help sterilized cork borer and poured 40 µl of 48 h old culture of *Bacillus* species including *B. amyloliquefaciens* strains DSBA-11 and DSBA-12 containing 0.1 OD bacterial population into each well. The inoculated plates were kept at 28 ± 1°C for 48 h to form of inhibition zone. The inhibition zone formed in diameter was converted into inhibition zone area by using formula πr^2.

Plant growth promoting parameters under *in vitro* condition

For plant growth-promoting parameters, *viz.* phosphate solubilization, siderophores production, indole 3-acetic acid (IAA) and ammonia production by *Bacillus* spp. were estimated under *in vitro* conditions. Phosphate solubilization was estimated quantitatively by using the method described by Mehta and Nautiyal, [28]. Indole -3-acetic acid was assayed by colorimetric method using ferric chloride acid [29]. Siderophores production was measured as described by Schwyn and Neilands [30]. The ammonia production by bacteria was inoculated in peptone water medium and incubated at it 30°C for 4 days. One ml of Nessler's reagent was added in to each tube and colour development as brown to yellow was recorded for positive to ammonia production of development of faint yellow colour indicating relatively less amount of ammonia production, while deep yellow to brown colour indicated the maximum production of ammonia.

Biocontrol of bacterial wilt and plant growth promotion

Twenty one days old tomato cv. Pusa Ruby seedlings were transplanted in 15 cm diameter pots having autoclaved soil mixture of peat moss, vermiculite and sand (2:1:1) at 25 -30°C. Bacterial colonies of pathogenic and antagonistic bacteria after 48 h harvested were scraped from the petri plates and mixed in 10 ml of sterile distilled water to maintain bacterial population 0.1 OD at 600 nm by using spectrophotometer. The 50 ml of *R. solanacearum* UTT-25 was inoculated at root zone of each plantafter4 days of transplanting. Subsequently 50 ml of antagonistic *B. amyloliquefaciens* DSBA-11 and DSBA-12, B. *cereus* JHTBS-7, *B. subtilis* DTBS-5,and*B.Pumilus*MTCC-7092were inoculated at root zone of each plant.The plants treated with pathogen (*R. solanacearum)* only and un-inoculated plants werealso maintained as positive and negative control. The observations were recorded at 5 days of intervals up to 30 days of transplanting. The wilt intensity percentage was recorded at initial stage and final stage (whole plant wilted). Disease rating was also recorded by using 1-5 scale andwilt intensity weredeterminate as described by Schaad et al. [16]. Biological control efficacy (BCE) of antagonistic bacteria was determined as described by Guo et al. [31]. To study plant growth-promoting ability of antagonistic bacteria in tomato, the same experiment was done. The whole plants with roots were uprooted from each treatment with 3 replicates. Root and shoot of each plant were cut from crown region for length (cm) measurement, whereas, fresh weight and dry weight (60^0C for 3 days) were taken after 30 days of inoculation.The growth promotion efficacy (GPE) of *Bacillus* spp. based on plant dry weight was calculated as described by Singh et al. [5].

Population of *R. solanacearum*

Ralstonia solanacearum in plant system was determined after 30 days of inoculation. Three asymptomatic plants of tomato from each treatment were randomly sampled and one g of root and shoot were crushed using 5 ml brine solution 0.85% and diluted serially. 100 µl of aliquot was inoculated and spread uniformly on the modified SMSA medium for growth of *R. solanacerarum* [32]. The inoculated Petri plates were incubated at 28 ± 1°C for 48 h. The *R. Solanacerarum* colonies were counted and colony forming unit (CFU) was calculated per g of plant fresh weight. The experiments conducted under same conditions in the glass house conditions repeating thrice and the data were pooled for statistical analysis.

Statistical analysis

The data was analyzed using Fisher's least significant differences (LSD) to determine the significant differences between treatments at P<0.05 level.

Results

Bacterial isolation, morphological and biochemical characterization

Based on colony characters, 57 different types of bacteria were isolated from the soil of tomato rhizospheric soil of tomato plant. Among them, 2 bacterial isolates DSBA-11 and DSBA-12 of *B. amyloliquefaciens* having highly antagonistic ability characterized by using morphological, biochemical, FAME analysis and 16s rRNA sequence analysis. The isolates were Gram positive, rod-shaped, cells in chains and motile, with peritrichous flagella, oval spores were central or paracentral in sporangia, and not swollen. Casein, gelatin and starch were degraded, whereas positive in oxidase and catalase tests showed positive reaction. Nitrate was reduced to nitrite by the bacteria.Both the isolatesof *B. amyloliquefaciens* did not utilize citrate as a main carbon source. They were differentiated from other *Bacillus* species. These isolates were grown at 10% NaCl and 3% H_2O_2 concentrations (data not presented).

Accordingly FAME analysis, variation in cellular fatty acid of two strains, DSBA-11 and DSBA-12 of *B. amyloliquefaciens* and other species *B. subtilis* DTBS-5, *B. cereus* JHTBS-7 and *B. pumilus* MTCC -7092 was observed. About 13 cellular fatty acids consisted with 12:0, 13:0 iso, 15:0iso, 15:anteiso, 16:0 iso, 16:0, 16:1 wllc, 17:1iso w10c, 17:0 anteiso, 18:1 w9c and 18:0 were found in all four species of *Bacillus*. However, variation in fatty acid profile in *Bacillus* species17: anteiso w9c was found only in B. *amyloliquefaciens* strains, which was distinguished from *B. cereus*, *B. subtilis*, and *B. pumilus*, variation in fatty acid profile within strainsof *B. amyloliquefaciens*was also observed like 10:0, 16:1 w7c alcohol sum in feature 3, 17:0, 18:0 iso, and summed feature 8, were unique in strain DSBA-11, whereas15:0 and 15:0 iso 3OH fatty acids were present only DSBA-12 but not in DSBA-11.

Molecular characterization of strains DSBA-11 and DSBA-12, of *B. amyloliquefaciens* based on partial 16S rRNA sequence analysis (≈ 709 bp) was done with homology of 99%. Nucleotide sequence data of these isolates were grouped along with the sequences of other *Bacillus* spp. obtained from NCBI database. Based on grouping of both the strains, DSBA-11 and DSBA-12, of *B. amyloliquefaciens* were phylogenetically affiliated to the genus *Bacillus*, and They were closely related to *B. amyloliquefaciens* by showing pair-wise sequence similarity>99% (Supplementary data provided). The nucleotide sequence of both the strains were submitted to Genebank NCBI and obtained accession number *B. amyloliquefaciens* DSBA-11(KF850150) and *B. amyloliquefaciens* DSBA-12 (KF850151) respectively.

Antagonistic and plant growth promoting ability *in vitro* conditions

Preliminary screening was done to test the antagonistic ability of 57 isolates, among them two strains, DSBA-11 and DSBA-12 of *B. amyloliquefaciens* along with three other species of *Bacillus* such as *B. subtilis* DTBS-5, *B.pumilus* MTCC- 7092 and *B. cereus* JHTBS-7 were tested for their comparative biocontrol efficiency against *R. solanacearum* UTT-25 under *invitro* conditions. Both the strains of *B. amyloliquefaciens* have better ability to inhibit the growth of *R. solanacearum* UTT-25 as compared to other speciesof *Bacillus* (Table 1). However, *B. amyloliquefaciens* DSBA-11 was found to be the best among other species of *Bacillus* and indicated maximum inhibited growth of *R. solanacearum* (4.91 cm^2). Based on *invitro* study, all strains of *Bacillus* spp. showed plant growth promotion expression attributes such as phosphorus solubilization, siderophores, IAA and ammonia production. However, strain DSBA-11 of *B. amyloliquefaciens* solubilized the maximum amount of phosphorus (42.6 µg/ml) and IAA production (95.4 µg/ml), whereas *B. subtilis* DTBS-5 has maximum ability to produce maximum siderophores (1.3 cm diameter) by making yellow zone on the medium and ammonia production as comparedto other species of *Bacillus* (Table 2).

Biocontrol of bacterial wilt disease and plant growth attributes

Bacillus amyloliquenfaciens DSBA-11 and DSBA-12, *B. cereus* JHTBS-7, *B. pumilus* MTCC-7092, and *B. subtilis* DTBS-5, were selected to test their comparative bio-efficacy to control wilt disease as well as promote growth of tomato cv. Pusa Ruby (susceptible cv.) under glass house conditions. The wilt disease was initiated only in *R. solanacearum* (UTT-25)infected plantsafter 6 days of inoculation, whereas in *Bacillus* treated plant delayed appearance of wilt disease 4–8 days. Minimum disease intensity 17.95% was recorded in *B. amyloliquefaciens* DSBA-11 followed by *B. amyloliquefaciens* DSBA-12 (20.81%) and *B. subtilis* DTBS-5 (21.63%) after 30 days. (Table 3) with maximum biocontrol efficacy of*B.amyloliquefaciens* DSBA-11 (68.19%) followed by *B. amyloliquefaciens* DSBA-12 and *B. subtilis* DTBS-5. However, no significant variation in reduction of wilt disease in tomato under glasshouse conditions between DSBA-11 and DSBA-12 was found (P>0.05) (Table 4). *R. solanacerarum* population was decreased by *Bacillus* treated plants, whereas untreated tomato plant remained high in shoot (5.85 log CFU/g of fresh weight) as well as root (7.85 log CFU/g of fresh weight) after 30 days (Table 3). The maximum reduction in population of *R. solanacerarum* in root and shoot of the plant was found in *B.amyloliquefaciens* DSBA-11treated plants. Maximum shoot length (39.50 cm) was recorded in *B. subtilis* DTBS-5 followed by *B.amyloliquefaciens* DSBA-11 (38.50 cm) and *B. amyloliquefaciens* DSBA-12 (38.40 cm), whereas root length was maximum in *B. amyloliquefaciens* DSBA-11, followed by *B. amyloliquefaciens* DSBA-12 without treated with *R. solanacerarum* after 30 days of inoculation (Table 4). Root dry weight (0.55 g) was recorded in *B. amyloliquefaciens*

Library	Sim index	Entry name
RTSBA6 6.20 - 1	0.675	*Bacillus amyloliquefaciens* (DSBA-11)
RTSBA6 6.20 – 2	0.568	*Bacillus amyloliquefaciens* (DSBA-12)
RTSBA6 6.21	0.617	*Bacillus subtilis* (DTBS-5)
RTSBA6 6.21	0.719	*Bacillus cereus* (JHTBS-7)
RTSBA6 6.21	0.535	*Bacillus pumilus* (MTCC-7092)

Table 1: Similarity index based on fatty acid profile of *Bacillus* spp.

***Bacillus* species**	**Inhibition zone (area in cm²)**	**PGPR activity of *Bacillus* spp.**			
		Phosphorous solubilized (µg/ml)	**IAA production (µg/ml)**	**Siderophores production [Dia. Of the orange yellow halo produced (cm)]**	**Ammonia production***
B. amayloliquefaciens DSBA-11	$4.11^{a} \pm 0.72$	$42.6^{a} \pm 1.9$	$95.4^{a} \pm 0.85$	$0.880^{b} \pm 0.16$	++
B. amayloliquefaciens DSBA-12	$3.31^{ab} \pm 0.55$	$36.6^{b} \pm 0.96$	$90.7^{b} \pm 1.58$	$0.893^{b} \pm 0.04$	++
B. subtilis DTBS-5	$3.07^{ab} \pm 0.15$	$30.0^{c} \pm 1.05$	$73.4^{bc} \pm 1.01$	$1.13^{a} \pm 0.03$	+++
B. cereus JHTBS-7	$2.52^{b} \pm 0.26$	$31.3^{c} \pm 1.85$	$68.0^{c} \pm 1.01$	$0.746^{bc} \pm 0.03$	++
B. pumulis MTCC-7092	$2.30^{c} \pm 0.1$	$24.6^{d} \pm 0.65$	$72.8^{bc} \pm 0.79$	$0.686^{c} \pm 0.02$	+

*+: less production, ++: Moderate production, +++: high production the values within a column with different letters are significantly different by using Fisher's LSD test (α=0.05). Data present means of the experiment within 3 replications each.

Table 2: Antagonistic and plant growth promoting activities of *Bacillus* species isolated from rhizosphere of wilted tomato plants *in vitro* conditions.

Treatment	**Disease incidence (%)**	**Biocontrol efficacy (%)**	**Population of *R. solanacearum* in tomato plant (Log value cfu/ml)**	
			Stem	**Root**
R. solanacerarum UTT-25	$56.43^{a} \pm 0.35$	-	$5.85^{a} \pm 0.03$	$7.45^{a} \pm 0.07$
B. amyloliquefaciens DSBA-12+*R. solanacerarum* UTT-25	$20.81^{d} \pm 1.7$	63.12	$4.74^{d} \pm 0.06$	$6.51^{c} \pm 0.04$
B. amyloliquefaciens DSBA -11+ *R. solanacerarum* UTT-25	$17.95^{d} \pm 3.2$	68.19	$4.98^{c} \pm 0.17$	$6.50^{c} \pm 0.06$
B.subtilis DTBS-5+ *R. solanacerarum* UTT-25	$21.63^{cd} \pm 2.5$	61.67	$5.02^{c} \pm 0.05$	$6.74^{b} \pm 0.04$
B.cereus JHTBS-7+ *R. solanacerarum* UTT-25	$28.38^{b} \pm 3.1$	49.71	$5.22^{b} \pm 0.02$	$6.79^{b} \pm 0.11$
B.pumulis MTCC-7092 + *R. solanacerarum* UTT-25	$25.58^{bc} \pm 1.8$	54.67	$5.12^{bc} \pm 0.15$	$6.58^{c} \pm 0.15$

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 within 3 replications each.

Table 3: Reduction of bacterial wilt disease intensity and population of *Ralstonia solanacearum* in tomato cv. Pusa Ruby plants by applying *Bacillus* spp. under greenhouse conditions.

Treatment	**Length tomato plant (cm)**		**Dry weight (g/ plant)**		**GPE (%) based on dry weight of root and shoot**
	Root	**Shoot**	**Root**	**Shoot**	
R. solanacerarum UTT-25	$3.50^{ef} \pm 0.4$	$25.87^{f} \pm 3.60$	$0.41^{abc} \pm 0.04$	$0.96^{b} \pm 0.36$	23.42
B. amyloliquefaciens DSBA-12 + *R. solanacearum* UTT-25	$4.23^{de} \pm 0.80$	$37.53^{ab} \pm 1.05$	$0.47^{ab} \pm 0.16$	$1.26^{b} \pm 0.26$	55.86
B. amyloliquefaciens DSBA-12	$6.30^{a} \pm 0.34$	$38.40^{ab} \pm 0.95$	$0.55^{a} \pm 0.08$	$1.84^{b} \pm 0.30$	115.31
B. amyloliquefaciens DSBA -11+ *R. solanacerarum* UTT-25	$6.00^{ab} \pm 0.26$	$35.23^{bc} \pm 1.80$	$0.30^{de} \pm 0.30$	$1.60^{b} \pm 0.35$	71.17
B. amyloliquefaciens DSBA -11	$6.67^{a} \pm 0.15$	$38.50^{ab} \pm 2.6$	$0.33^{de} \pm 0.05$	$1.85^{b} \pm 0.47$	96.39
B. subtilis DTBS-5+ *R. solanacerarum* UTT-25	$4.73^{cd} \pm 0.50$	$34.40^{cd} \pm 1.4$	$0.38^{abc} \pm 0.04$	$1.83^{b} \pm 0.37$	99.00
B. subtilis DTBS-5	$5.40^{bc} \pm 0.7$	$39.50^{a} \pm 1.83$	$0.31^{de} \pm 0.02$	$1.77^{b} \pm 0.28$	87.40
B. cereus JHTBS-7+ *R. solanacerarum* UTT-25	$2.93^{g} \pm 0.15$	$27.36^{ef} \pm 3.80$	$0.34^{abc} \pm 0.006$	$0.97^{b} \pm 0.05$	18.01
B. cereus JHTBS-7	$4.05^{de} \pm 0.23$	$33.17^{cd} \pm 2.05$	$0.213^{e} \pm 0.08$	$1.65^{b} \pm 0.75$	67.84
*B. pumulis*MTCC-7092**+***R. solanacerarum* UTT-25	$3.87^{e} \pm 0.251$	$34.67^{c} \pm 1.70$	$0.42^{abc} \pm 0.06$	$1.43^{b} \pm 0.10$	66.6
B. pumilus MTCC-7092	$5.13^{c} \pm 0.680$	$36.36^{abc} \pm 2.20$	$0.36^{abc} \pm 0.037$	$1.93^{b} \pm 0.76$	106.3
Control (Uninoculated)	$4.17^{de} \pm 0.404$	$30.60^{de} \pm 0.85$	$0.33^{de} \pm 0.08$	$0.78^{a} \pm 0.15$	-

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 within 3 replications each.

Table 4: Enhancement of biomass tomato plants treated with antagonistic *Bacillus* spp. under glass house conditions.

DSBA-12 treated plants and maximum shoot dryweight (1.85 g) in *B. amyloliquefaciens* DSBA-11 treated plants respectively. The growth-promoting efficacy was noticeable higher in *B. amyloliquefaciens* DSBA -12 treated plants followed by *B. pumilus* MTCC-7092. Although there was no significant variation in plant growth promotion activity was observed within the bioagents treated plants.

Discussion

Bacterial wilt of tomato is a very serious problem throughout the world and causes direct yield >90% and very widely based on the host cultivars, climate soil type, cropping pattern and strains of *R. solanacearum* [33]. Rhizospheric soil of tomato has a plenty of bacterial populations including antagonistic and plant growth stimulating bacteria. *Bacillus* species and *Bacillus* derived generadominate among bacterial populations isolated especially from the rhizospheric soil of wheat [34] and tomato [13] plants. Isolation of potential antagonistic bacteria from the soil is an important way to control plant disease successfully [35].To control this wilt disease through bacterial antagonists, we isolated 57 isolates of bacteria from wilted tomato rhizosphere and most of them are *Bacillus* spp. In this study,we isolated rhizobacteria from rhizosphere of wilted plant for this study as earlier reported by Huang et al. [36] that the isolates of bacteria from the rhizosphere of diseased plants performed better in reducing the intensity of the disease that those of the healthy plants. We targeted *Bacillus* spp. because of their ability to survive better in adverse conditions like high temperature resistance to desiccationas well as promoting plant growth [15]. In our case, we isolated bacteria from slight acidic soil and got maximum *Bacillus* spp. by treating the soil suspension at 80°C for 15 min to kill other rhizospheric bacteria, which was earlier reported by Edward et al. [37]. Ramesh and Phadke [38] isolated 109 strains of endophytic rhizobacteria from eggplants and screened for their antibacterial activity against *R. solanacearum* and effective isolates of *Pseudomonas* spp. and *Bacillus* spp.

All 57 bacterial isolates, isolated from the rhizospheric soil of tomato plants were screened for their antagonistic activity, which was found to be not <0.5 cm diameter of inhibition zone againsttest bacterial pathogens*R. solanacearumin vitro*. Tan et al. [13] reported that addition of $FeCl_3$ into the KB medium increased the antibacterial activity of *B. amyloliquefaciens* against *R. solanacearum* but in contrast, our study showed that both the strains of *B. amyloliquefaciens* DSBA-11 and DSBA-12 performed better antibacterial activity (Table 2) without addition of $FeCl_3$ as compared to strains CM-2 and T-5 of *B. amyloliquefaciens.* Most potential rhizospheric bacteria having best antagonistic ability as well as plant growth promoting abilities were characterized by using morphological, biochemical methods, FAME analysis and 16S rRNA sequence analysis. Partial 16S rRNA nucleotide sequences (709 bp) of these strains have shown 99-100% nucleotide sequence identity with *B. amyloliquefaciens* in the NCBI gene bank. (Data not provided). Based on the combined characters like phenotypic, physiological test, FAME analysis and 16S rRNA analysis (higher than the acceptable 97%, [39]) of both the strains DSBA-11 and DSBA-12, they belong to *B. amyloliquefaciens.*

In comparative study of antagonistic behavior of *Bacillus* spp. against *R. solanacearum* was done by using dual culture methods *in vitro*. Among four species of *Bacillus*,*B.amyloliquefaciens* formed maximum inhibition zone against *R. solanacearum*. Formation of inhibition zone by the bacteria is directly related to type of secondary metabolites produced by bacteria particularly in antibiosis, which acts against the target pathogens as described earlier [40,41]. Thescreening antagonistic bacteria like *B. subtilis* and *P. fluorescens* based on antibiotic production was done under *in vitro* assay. *Bacillus* spp.produce different group of secondary metabolites [42], which are suppressing the growth of bacterial pathogens. Besides antibiotics, strains DSBA-11 and DSBA-12 showed higher phosphorus solubilization and IAA production ability *in vitro* and similar results has also obtained in another strain FZB42 of *B. amyloliquefaciens,* which produces IAA to promote the plant growth [43], which is an important growth promoting hormones for the plant. However, siderophores and ammonia production was found higher in *B. subtilis* DTBS-5 along with all the species of *Bacillus*. The phosphorus was solubilized by all the species of *Bacillus* and availability of phosphorus is an important major nutrient element for plant. *In vitro* study, both the strains DSBA-11 and DSBA-12 produced siderophores, although it was slighter lower than the strains DTBS-5 of *B. subtilis,* which may contribute as iron chelating and produces soluble complexes which is taken by plant or it make insoluble to phyto pathogenic bacteria by binding the available form of iron in the soil [44].

In glass house study, both the strains of *B. amyloliquefaciens* DSBA-11 and DSBA-12 along with *B. cereus, B. subtilis* and *B. pumilus* significantly decreased the bacterial wilt disease incidence in tomato and enhance the plant growth. Maximum biocontrol efficacy was found in the treatments of DSBA-11 and DSBA-12of *B.amyloliquefaciens* in accordance with the earlier reports that *Bacillus* spp. reduces the bacterial wilt incidence in tomato [10] and potato [40]. Moreover population of bacterial pathogen reduced in the plants treated with bioagents may be due to production of antibiotics by antagonistic bacteria [45] in rhizosphere of tomato plants which suppressed the population of pathogenic bacteria. *B. subtilis* and *B. amyloliquefaciens* were found most effective to control various plant diseases [13,39,46]. In our case, *B. amyloliquefaciens* strainswere isolated from wilted tomato rhizospheric soil having acidic in nature. Several strains have been reported for production of PGPR attributes *in vitro* and also significantly promote the plant growth. [43,47]. In present study, all species of *Bacillus* have growth promoting ability; however, the variation was found in different plant growth promoting parameters.

Conclusion

Rhizosphere of tomato crop is a good source of potential antagonistic bacteria. Among *Bacillus* species, both the strains, DSBA-11 and DSBA-12 of *B. amyloliquefaciens* isolated from rhizosphere of wilted tomato pose excellent antagonistic ability to reducebacterial wilt disease incidence in tomato and suppress the *R. solanacearum* population and improved overall growth of tomato plants.

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