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ORIGINAL ARTICLE

Stress-tolerant antagonistic plant growth-promoting rhizobacteria from *Zea mays*

Amandeep Kaur, Soibam Richan Devi, Pratibha Vyas*

Microbiology Department, School of Bioengineering and Biosciences, Lovely Professional University, India

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*Corresponding address:
vyasp2000@yahoo.co.in

Abstract

Biological control of plant diseases is strongly emerging as an effective alternative to the use of chemical pesticides and fungicides. Stress tolerance is an important attribute in the selection of bacteria for the development of microbial inoculants. Fourteen salt-tolerant bacteria showing different morphological features isolated from the rhizosphere of maize were evaluated for different plant growth-promoting activities. All isolates showed auxin production ranging from 5 to 24 μg · ml^{-1} after 48 h incubation in tryptophan supplemented media. Phosphate solubilization ranged from 15 to 419 μg · ml^{-1}. 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was shown by 6 isolates, ammonia production by 9 isolates, siderophore production by 8 isolates while HCN production by 4 isolates. Four bacterial isolates with all plant growth-promoting properties also showed strong antagonistic activities against *Fusarium oxysporum, F. verticillioides, Curvularia lunata* and *Alternaria alternata* and abiotic stress tolerance against salinity, temperature, pH and calcium salts. Two selected bacterial isolates significantly enhanced the growth of pea and maize test plants under greenhouse conditions. The bacterial isolate M1B2, which showed the highest growth promotion of test plants, was identified as *Bacillus* sp. based on phenotypic and 16S rDNA gene sequencing. The results indicated that *Bacillus* sp. M1B2 is a potential candidate for the development of microbial inoculants in stressful environments.

Key words: antagonism, *Bacillus*, maize, phosphate solubilization, plant growth promotion, stress tolerance

Introduction

Pathogenic microorganisms affect plant health and are a major threat to crop production. Large-scale use of chemicals for controlling plant pathogens has led to the disturbance of ecological balance due to the development of pesticide resistance in plant pathogens (Compant *et al*. 2010; Krauss *et al*. 2010; Dalal and Kulkarni 2013). In response to continued use of pesticides, the interest in finding eco-friendly and safe alternatives for promoting agricultural productivity has increased. Biological control of plant diseases is strongly emerging as an effective alternative to the use of chemical pesticides (Błaszczyk *et al*. 2014; Arzanlou *et al*. 2016). Many plant growth-promoting rhizobacteria (PGPR) residing in the rhizosphere of plants have been reported to enhance plant growth and inhibit plant pathogens by various mechanisms including nitrogen fixation, phosphate solubilization, phytohormone production, 1-amino-cyclopropane-1-carboxylate (ACC) deaminase activity, siderophore production, ammonia production and hydrogen cyanide (HCN) production (Rajendran *et al*. 2007; Gulati *et al*. 2009; Vyas and Gulati 2009; Kaur *et al*. 2017). These rhizobacteria also help the plants to tolerate abiotic stresses like alkalinity, salinity and nutrient deficiencies (Glick 2014).

Fusarium species are well known human, animal and plant pathogens. *Fusarium oxysporum* and

Curvularia lunata cause diseases in a wide range of plant species belonging to different families (Lal *et al.* 2013). Similarly, *Alternaria alternata* with a wide host range cause leaf spots and blights in many plants while *F. verticillioides* is an economically important fungal pathogen reported to cause stalk rot, ear rot and kernel rot of maize (Murillo-Williams and Munkvold 2008; Ghosh *et al.* 2016). In addition to phytopathogens, different biotic and abiotic factors also affect the performance of microorganisms and plant growth (Vyas *et al.* 2010; Das *et al.* 2015). Abiotic stress includes temperature, pH, moisture status, salinity and salts present in soils. Many PGPR which show good results *in vitro* fail to give the same results in the field, when applied as microbial inoculants due to the stress imposed by the sudden change in the environment (Praveen Kumar *et al.* 2014). Therefore, screening for stress tolerance is an important parameter in the selection of bacterial strains for the development of microbial inoculants. Many bacteria have adapted themselves to temperature fluctuations, pH and osmolarity while living in stressful environments. Limited reports are available on the selection of bacterial strains based on stress tolerance for the development of microbial inoculants (Rangarajan *et al.* 2002; Sharan *et al.* 2008; Vyas *et al.* 2009; Vyas *et al.* 2010). One approach is to select stress tolerant bacteria with multiple plant growth-promoting (PGP) and biocontrol properties.

The present study was designed to isolate stress-tolerant rhizobacteria from maize growing in Dharamshala, Himachal Pradesh, India and to screen them for antagonism and PGP activities as a first step in the development of microbial inoculants.

Materials and Methods

Soil sampling and isolation of salt-tolerant rhizobacteria

Samples were collected from the rhizosphere of maize (*Zea mays*) growing in Dharamshala, Himachal Pradesh, India located between the latitude 32°13'8.5584" N and longitude 76°19'24.2472" E. From each plant, a sample was collected by digging the soil at two spots around each plant to a depth of 10–12 cm. Four composite soil samples, prepared by mixing four soil samples collected from two different plants, were employed for the isolation of salt-tolerant bacteria. Salt-tolerant rhizobacteria were isolated by spreading serial soil dilutions on nutrient agarplates with 0.42 M NaCl (2.5% NaCl) and incubating the plates at 28°C for 48 h. Morphologically distinct bacterial colonies were picked and streaked on nutrient agar plates for purification and stocked in 30% glycerol (w/v) for further studies.

Screening for plant growth-promoting activities

Phosphate solubilization

Phosphate solubilization by salt-tolerant bacterial isolates was detected qualitatively on modified Pikovskaya (PVK) agar (Gupta *et al.* 1994). The zone of phosphate solubilization around the bacterial colonies was measured on the 5th day of incubation at 28°C.

For quantitative estimation, 50 ml NBRIP (National Botanical Research Institute Phosphate) broth was inoculated with 500 µl of the bacterial cultures ($\sim 3 \times 10^9$ CFU · ml^{-1}). The flasks were incubated in a refrigerated incubator shaker at 180 rpm at 28°C. The uninoculated sterilized NBRIP medium served as control. The liberated P was estimated by the yellow colour method on the 5th day of incubation as described earlier (Nautiyal 1999; Gulati *et al.* 2008). The values of phosphorus liberated were expressed as microgram per ml over uninoculated control.

IAA-like auxin production

Indole-3-acetic acid (IAA) production was quantified in nutrient broth with 0.1% DL-typtophan using Salkowski reagent (Loper and Schroth 1986). In brief, 100 µl of 24 h old bacterial cultures were inoculated in the medium and incubated for 48 h in dark at 28°C. Thereafter, the cultures were centrifuged at 10,000 rpm and 4 ml Salkowski reagent was added to 1 ml culture filtrate. After 10 min, the absorbance of the resultant pink colour was measured at 535 nm and the values of IAA-like auxins were expressed as microgram per ml over uninoculated control.

1-aminocyclopropane-1-carboxylate (ACC) deaminase activity

The salt-tolerant bacterial isolates were screened for ACC deaminase activity on Dworkin and Foster (DF) salts minimal medium with ACC as the sole nitrogen source (Jacobson *et al.* 1994). The DF agar plates were streaked with bacterial cultures and incubated at 28°C. The plates were observed for the appearance of growth after 48 h.

Siderophore production

Screening of the bacterial isolates for siderophore production was carried out on Chrome Azurol sulphonate (CAS) agar plates qualitatively (Schwyn and Neilands 1987). The CAS agar plates were spot inoculated with the bacterial cultures and incubated at 28°C. The diameter of an orange halo was measured on the 5th day of incubation.

Ammonia production

Ammonia production was detected by adding Nessler's reagent to bacterial isolates grown in peptone broth for

24 h. The change in colour from faint yellow to dark brown indicated the production of ammonia (Cappuccino and Sherman 1992).

Hydrogen cyanide (HCN) production

HCN production was detected using the Castric method (1975). Briefly, the bacterial isolates were streaked on nutrient agar amended with glycine. Filter paper discs soaked in 2% Na_2CO_3 prepared in 0.5% picric acid solution were kept inside the lid of the Petri plates and incubated for 3 days at 28°C. Colour change of filter paper discs from orange to brown indicated HCN production.

Antagonism against fungal plant pathogens

The bacterial isolates were evaluated for antagonism against fungal phytopathogens *F. oxysporum* strain 1008 (MTCC 284), *F. verticillioides* strain 1 (MTCC 3322), *C. lunata* strain 716 (MTCC 283) and *A. alternata* strain 6663 (MTCC 1362) on yeast extract medium by well diffusion assay. The bacterial cultures were grown in nutrient broth for 48 h at 28°C and centrifuged at 10,000 rpm. The fungal cultures were grown in potato dextrose agar (PDA) for 7 days and a spore suspension was prepared by homogenizing the fungal cultures and suspending in sterile normal saline. One ml of fungal suspension was spread plated onto yeast extract agar plates in triplicate. A well was bored in the center of the plates with a sterile cork-borer and 50 µl bacterial supernatant was added to each well. For comparison, 50 µl of sterile distilled water was added to control plates. The zone of inhibition was measured after incubating the plates at 28°C for 7 days.

Stress tolerance screening

Stress tolerance screening was carried out by growing the bacterial isolates at different stress levels of temperature, pH, salinity and calcium salts. Temperature effect was studied by streaking the bacterial isolates on nutrient agar plates and incubating at 10, 30 and 40°C for 48 h and observed for growth. Similarly, for pH stress, nutrient agar with pH 5, 7, and 9 was prepared using citrate-phosphate buffer and the bacterial isolates were grown at 28°C. Likewise, for salinity stress nutrient agar medium with 0.86 and 1.2 M NaCl (5% and 7% NaCl) was prepared and the bacterial isolates were grown on the medium at 28°C. The bacterial isolates were also screened for tolerance against calcium salts by growing on nutrient agar plates with 0.18 and 0.36 M $CaSO_4$ (2.5 and 5% $CaSO_4$), 0.22 and 0.45 M $CaCl_2$ (2.5 and 5% $CaCl_2$), and 0.25 and 0.5 M $CaCO_3$ (2.5 and 5% $CaCO_3$).

Effect on plant growth promotion

Two selected bacterial isolates M1B2 and M3B4 were evaluated for growth promotion of pea (*Pisum sativum* var. Palam Priya) and maize (*Zea mays* var. Parbhat) as described earlier (Vyas and Kaur 2017; Kaur *et al.* 2017). Briefly, maize and pea seeds were sterilized by dipping for 3 min in 20% sodium hypochlorite and washed thrice with sterile distilled water. Thereafter, the surface sterilized seeds were dipped for 30 min in 48 h old bacterial cultures (OD adjusted to 1.0 ~10^9 CFU · ml^{-1}). The initial count of bacteria per seed was determined by serially diluting a single bacterized seed in normal saline up to 10^{-6}. From each dilution, 100 µl was spread plated onto nutrient agar plates and the plates were incubated for 24–48 h at 28°C. Two seeds were sown in each 10-cm diameter pots containing unsterilized garden soil. Seeds dipped in sterilized nutrient broth and sown in pots served as uninoculated controls. The experiment consisted of three treatments with four replicates each. The pots were kept under natural conditions in randomized block design and data were collected after 45 days on shoot length, root length, and total dry weight. For dry weight calculation, the plants were dried at 70°C for 3 days in an oven till constant weight was obtained.

Identification of the bacterial isolate

The bacterial isolate M1B2 was partially characterized and identified on the basis of phenotypic characters and 16S rDNA gene sequencing. For phenotypic characterization, Gram's staining, motility, endospore staining, methyl red Voges Prauskauer, catalase, citrate, oxidase and urease tests were performed following standard methods (Krieg and Holt 1984).

16S rDNA gene sequence analysis was carried out as described earlier (Gulati *et al.* 2008). DNA was isolated using the Qiagen DNeasy Plant Mini Kit. Gene amplification, thermocycling conditions, and sequence analysis has been described earlier in detail (Gulati *et al.* 2008). The sequences were aligned with ClustalW and MEGA software package version 7 using Kimura's two-parameter model to calculate the evolutionary distance of M1B2 and its related taxa. The sequence was submitted to NCBI GenBank with accession number MG711590.

Experimental design and statistical analysis

Randomized block design was implemented for carrying out the experiments. Unless stated otherwise, all values are the means of three replicates. Data on plant growth promotion were analyzed by analysis of variance (ANOVA) using XLSTAT 2016. The means of the treatments were compared by Fisher's significant difference (LSD) test at p values of 0.05.

Results and Discussion

Isolation of stress tolerant rhizobacteria

Looking into the large-scale use of chemical pesticides and fertilizers, there is a need for safe alternatives to

be developed for sustainable agriculture. Plant growth-promoting rhizobacteria are important components of sustainable agriculture as they are cost-effective, safe and are able to enhance plant growth. However, different biotic and abiotic factors affect the performance of these PGPR (Vyas *et al*. 2010; Das *et al*. 2015). Abiotic stresses include temperature, pH, moisture status, salinity and salts present in soils. Importantly, even though many plant growth-promoting rhizobacteria show good results *in vitro*, they fail to give the same results in the field, when applied as microbial inoculants. One main reason for their failure is the stress imposed on them by the sudden changes in the environment (Praveen Kumar *et al*. 2014). Screening for stress tolerance is an important parameter in the selection of bacterial strains for the development of microbial inoculants. Therefore, with the objective of selecting stress-tolerant PGP and antagonistic bacteria, 14 morphologically distinct salt-tolerant bacteria were isolated by spread plating the serially diluted rhizosphere samples on nutrient agar plates with 0.42 M NaCl. Stress-tolerant bacteria have also been isolated earlier from various sources, however, limited reports are available on the stress-tolerant rhizobacteria isolated from *Z. mays* (Nadeem *et al.* 2007).

The salt-tolerant bacteria were further assessed for PGP activities including phosphate solubilization, auxin production, ACC deaminase activity, ammonia production and HCN production.

Plant growth promoting activities

Of 14 salt-tolerant bacterial isolates, phosphate solubilization zones varying from 3 to 18 mm were shown by 12 isolates on modified Pikovskaya agar while two bacterial isolates did not show any zone of phosphate solubilization. However, all isolates exhibited phosphate solubilization ranging from 15 to 419 $\mu g \cdot ml^{-1}$ over uninoculated control at 28°C on the 5th day of incubation in liquid medium (Table 1). No correlation was observed between the zone of phosphate solubilization and solubilization in liquid culture since M1B2, showing the highest solubilization in liquid medium, did not produce the largest solubilization zone on agar plates. The bacterial isolates differed in their ability to solubilize phosphate with the highest solubilization shown by M1B2 and the lowest by M4B8. The results are in accordance with an earlier report where *Pseudomonas* strains showed differences in the solubilization of various phosphate substrates (Gulati *et al*. 2008). Recently, *Bacillus pumilus* and *B. subtilis* isolated from maize rhizosphere have been reported for phosphate solubilization (Kuan *et al.* 2016; Karnwal 2017). The application of these strains could prove to be highly beneficial in calcareous soils where phosphorus deficiency is attributed to the binding of phosphate with calcium.

Another important mechanism for plant growth promotion is the production of phytohormones

Table 1. Exhibition of plant growth-promoting activities by salt-tolerant rhizobacteria from maize (*Zea mays*)

Isolate	TCP solubilization [$\mu g \cdot ml^{-1}$]	Auxin production [$\mu g \cdot ml^{-1}$]	Ammonia production	Siderophore production zone [mm]	ACC deaminase activity	HCN production
M1B1	151.3 ±2.33	23.0 ± 1.52	+++	8.0 ± 0.57	–	–
M1B2	430.7 ± 2.18	21.3 ± 1.76	++	16.0 ± 1.52	++	+++
M1B5	26.3 ± 1.76	18.7 ± 1.45	–	–	–	–
M2B3	278.0 ± 1.15	20.3 ± 0.88	+	–	+	–
M2B5	419.3 ± 1.45	11.0 ± 0.81	++	12.3 ± 1.20	+	++
M2B6	97.3 ± 1.20	5.0 ± 1.15	+	–	–	–
M3B1	114.0 ± 2.51	24.3 ± 0.88	++	9.0 ± 0.57	++	++
M3B2	85.7 ± 1.45	21.0±1.52	–	–	–	–
M3B4	332.3 ± 1.85	22.0 ± 1.73	+	11.3 ± 0.88	+	+++
M3B6	151.0 ± 1.00	19.3 ± 1.45	++	5.0 ± 0.58	–	–
M4B2	125.7 ± 2.02	13.7 ± 1.33	–	–	–	–
M4B5	26.3 ± 1.33	8.3 ± 0.66	–	7.0 ± 1.54	–	–
M4B7	132.0 ± 2.64	17.3 ± 1.20	+	5.3 ± 0.88	+	–
M4B8	15.3 ±1.76	6.7 ± 0.66	–	–	–	–

TCP – tricalcium phosphate; ACC – 1-aminocyclopropane-1-carboxylate; HCN – hydrogen cyanide
Values are the means of three replicates ± SE mean. "+++" – strong activity/luxuriant growth, "++" – moderate activity/good growth, "+" – weak activity/poor growth, "–" – no growth/no activity

by PGPR strains. In the present study, all 14 salt--tolerant bacteria showed the production of IAA--like auxins in tryptophan-amended media after 48 h incubation as detected using Salkowski reagent. However, the isolates varied in their ability to produce auxins ranging from 5 to 24 μg · ml^{-1} with the highest production shown by M3B1 while the lowest was by M2B6 (Table 1). Indole-3-acetic acid (IAA) production has been reported for many PGPR in the presence of tryptophan (Tsavkelova *et al*. 2007; Vyas *et al*. 2010; Vyas and Kaur 2017). Plant rhizosphere contains tryptophan from root exudates, which is used by bacteria for the production of auxins (Stachecki *et al*. 2004).

In addition to auxin production and phosphate solubilization, siderophore production and hydrogen cyanide production are important attributes of the microorganisms that influence plant growth by suppressing fungal pathogens. In the present study, four isolates showed hydrogen cyanide production ranging from weak to strong as indicated by the colour change of filter paper discs from yellow to orange/brown (Table 1). Siderophore production zones ranged from 5 to 16 mm after 5 days incubation on CAS agar plates by the bacterial isolates.

Several plant growth-promoting bacteria produce the enzyme ACC deaminase which breaks the plant ethylene precursor ACC into ammonia and α-ketobutyrate (Glick 2014). ACC-deaminase producing bacteria helps to promote root elongation and plant growth by hydrolyzing ACC from germinating seeds and increasing the active rhizosphere zone. Many workers have used the ability of bacteria to utilize ACC as the sole nitrogen source while screening ACC-deaminase producing bacteria (Dey *et al*. 2004; Glick 2014). In the present study, six isolates exhibited growth on DF salts minimal medium with ACC as the sole nitrogen source (Table 1). These bacteria are known to protect plants against drought, flooding, salts, heavy metals as well as from bacterial and fungal pathogens (Glick 2014; Orhan 2016; Vurukonda *et al*. 2016; Li and Jiang 2017). Therefore, bacteria producing ACC deaminase are important components of agriculture in stressful environments. Ammonia production is also an important trait as it is used by plants for their growth as a source of nitrogen (Ahmad *et al*. 2008). Herein, ammonia production was shown by nine isolates (Table 1). Plant growth-promoting bacteria from maize rhizosphere have been reported (Lawongsa *et al*. 2008; Karnwal 2017).

Screening for antagonism

Plant pathogens cause huge damage to crop plants worldwide. Large-scale use of chemicals for controlling phytopathogens has led to the buildup of pesticide resistance among pathogens (Krauss *et al*. 2010; Dalal and Kulkarni 2013). Biological control of plant diseases is an effective alternative to the use of chemical pesticides and fungicides. Four bacterial isolates showing all PGP activities were tested for antagonism against four wide host range phytopathogenic fungi including *F. oxysporum, F. verticillioides, C. lunata* and *A. alternata* by well diffusion assay. The highest antagonistic activity was shown by M1B2 against all test pathogens while the lowest was by M3B1 (Table 2). The antagonistic activity can be correlated with the production of HCN as all isolates showed cyanide production ranging from weak to strong. Hydrogen cyjanide (HCN) is one of the most important compounds inhibiting the growth of fungal pathogens, among the volatile compounds produced by bacteria (Reetha *et al*. 2014). The bacterial isolates showing antagonism also produced ammonia, siderophores and auxins, which have been implicated in inhibiting the growth of fungal pathogens (Tables 1–2). Recently, strains of *B. amyloliquefaciens*, *B. subtilis*, and *Paenibacillus polymyxa* have been shown to show antagonistic activity against *F. graminearum* (Zalila-Kolsi *et al*. 2016). Likewise, *Bacillus*, *Pseudomonas* and *Paenibacillus* isolated from maize rhizosphere have been reported to inhibit the common maize pathogen *F. verticillioides* (Figueroa--López *et al*. 2016).

Table 2. Antagonistic activity of salt-tolerant rhizobacteria from maize (*Zea mays*) against fungal pathogens by well diffusion assay

Isolate	Zone of inhibition [mm]			
	Fusarium oxysporum (MTCC 284)	*Fusarium verticillioides* (MTCC 3322)	*Curvularia lunata* (MTCC 283)	*Alternaria alternata* (MTCC 1362)
M1B2	19.3 ± 1.76	14.3 ± 1.20	12.0 ± 1.73	13.0 ± 1.52
M2B5	11.3 ± 1.45	9.0 ± 0.58	ND	5.7 ± 0.88
M3B1	ND	ND	8.0 ± 1.00	ND
M3B4	16 ± 1.52	10.3 ± 0.88	6 ± 0.58	7 ± 0.58

Values are the mean of three replicates ± SE mean. ND – not detected

Screening for stress tolerance

Screening for stress tolerance is an important parameter while selecting bacterial strains for developing biofertilizers as the performance of PGPR is constrained by the stress generated by environmental factors including temperature, desiccation, pH, alkalinity/acidity and salinity in the soil (Rangarajan *et al*. 2002; Vyas *et al*. 2010; Das *et al*. 2015). Therefore, the selection of stress-tolerant bacterial strains is essential for consistency in field performance for application as microbial inoculants. Four salt-tolerant bacteria exhibiting broad-spectrum antagonism and PGP activities were tested for tolerance against different levels of pH, temperature, salinity and calcium salts (Tables 3–4). The bacterial isolates exhibited differences in tolerating stress conditions (Tables 3–4). All bacterial isolates showed growth on agar medium with 0.86 M NaCl (5% NaCl); 0.18 and 0.36 M $CaSO_4$ (2.5 and 5% $CaSO_4$) and 0.25 M $CaCO_3$ (2.5% $CaCO_3$) (Table 3). None of the isolates showed growth on 0.45 M $CaCl_2$ (5% $CaCl_2$). The bacterial isolate M3B1 showed the lowest salt tolerance as it could not grow in the presence of $CaCl_2$ and 1.2 M NaCl. The highest salt tolerance was shown by M1B2 followed by M3B4 (Table 3). Among different levels of pH tested, all isolates showed growth on pH 7, and three on pH 9. The isolates M1B2 and M3B4 could grow on agar medium of pH 5. Similarly, all isolates exhibited growth at 30°C, three at 10°C and two at 40°C. Similar results were observed earlier while screening stress-tolerant *Pseudomonas* strains from Lahaul and Spiti valley in Himachal Pradesh, India (Vyas *et al*. 2009). Many reports are available on screening rhizobacteria for PGP properties, however, limited

Table 3. Salinity and calcium salts-tolerance of rhizobacteria from maize (*Zea mays*) on nutrient agar

Isolate	NaCl [M]		$CaSO_4$ [M]		$CaCl_2$ [M]		$CaCO_3$ [M]	
	0.86	1.2	0.18	0.36	0.22	0.45	0.25	0.5
M1B2	+++	+	+++	+++	+++	–	+++	+
M2B5	++	+	+++	++	+	–	++	–
M3B1	++	–	++	+	–	–	++	+
M3B4	+	+	+++	++	++	–	++	+

"+++" – good growth, "++" – medium growth, "–" – no growth

Table 4. pH and temperature tolerance of salt-tolerant rhizobacteria from maize (*Zea mays*)

Isolate	pH			Temperature [°C]		
	5	7	9	10	30	40
M1B2	++	+++	++	++	+++	++
M2B5	–	+++	+	–	+++	+
M3B1	–	+++	–	–	+++	–
M3B4	+	+++	+	++	+++	+

"+++" – good growth, "++" – medium growth, "–" – no growth

Table 5. Effect of salt-tolerant bacterial isolates from maize rhizosphere on growth promotion of pea (*Pisum sativum*) and maize (*Zea mays*) under greenhouse conditions

Isolate	*Pisum sativum* var. Palamp Priya			*Zea mays* var. Parbhat		
	root length [cm]	shoot length [cm]	dry weight [g]	root length [cm]	shoot length [cm]	dry weight [g]
Uninoculated control	10.5 a	17.1 a	0.18 a	16.5 a	31.5 a	0.19 a
M1B2	14.8 b (40.9)	24.5 b (43.3)	0.26 b (44.4)	21.5 b (30.3)	41.0 b (30.1)	0.28 b (47.4)
M3B4	12.5 c (19.5)	20.5 c (19.9)	0.21 c (16.7)	19.5 c (18.2)	38.2 c (21.3)	0.24 b (26.3)
Fisher's LSD at 5%	1.6	1.7	0.03	1.5	1.9	0.04

Values are the mean of four replicates with two plants each. Values in parentheses indicate % increase over uninoculated control. Values with different letters in each column differ significantly from one another at $p \leq 0.05$

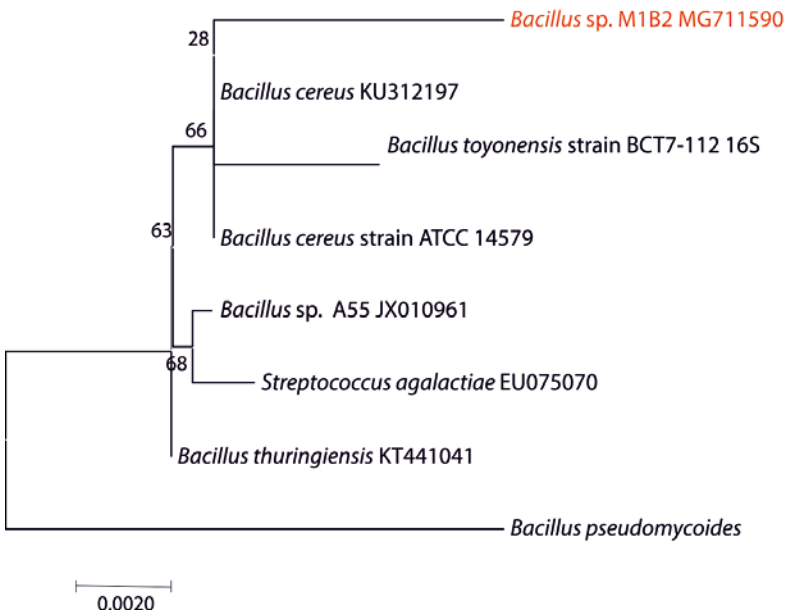

Fig. 1. Phylogenetic tree constructed based on 16S rRNA gene sequences of M1B2 and its nearest neighbours using neighbour-joining method

reports have shown the selection of bacteria based on stress tolerance along with other activities for the development of microbial inoculants (Vyas *et al.* 2009; Orhan 2016; Panwar *et al.* 2016; Molina-Romero *et al.* 2017). Selection of bacteria based on stress tolerance is important as bacteria screened *in vitro* fail to give the same response in the field due to the stress imposed by the environmental conditions (Vyas *et al.* 2009).

Plant growth promotion

Based on initial studies on PGP activities, antagonism and stress tolerance, bacterial isolates M1B2 and M3B4 were tested in pots for growth promotion of pea and maize. The initial count of M1B2 and M3B4 per seed was 5.1×10^5 and 3.2×10^5 colony forming units, respectively. These two bacterial isolates significantly enhanced the growth of both pea and maize (Table 5). However, of the two isolates, M1B2 showed significantly higher growth promotion than M3B4, except for the dry weight of maize where both treatments were statistically at par with one another. Earlier, many PGP bacterial species belonging to *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Rahnella* have also been reported to enhance growth promotion in various plants (Gulati *et al.* 2009; Vyas *et al.* 2009; Vyas *et al.* 2010; Oteino *et al.* 2015; Kaur *et al.* 2017; Tahir *et al.* 2017; Vyas and Kaur 2017). A *Bacillus pumilus* strain isolated from maize rhizosphere has shown growth-promoting effects on maize plants in a controlled environment (Kuan *et al.* 2016). *Bacillus subtilis,* able to produce indole-3-acetic acid, enhanced plant growth by increasing the number of root hairs (Araujo 2008).

Characterization and identification of the bacterial isolate M1B2

The selected bacterial isolate M1B2, based on phenotypic features, biochemical tests and 16S rDNA gene sequencing, was characterized. M1B2 was Gram-positive, motile, rod shaped, arranged in chains; positive for endospore, catalase and citrate utilization. The isolate tested negative for oxidase, methyl red, Voges Proskauer, indole and urease. Based on the results, the bacterial isolate M1B2 was tentatively identified belonging to the genus *Bacillus*.

To confirm the identity of the bacterial isolate M1B2, 16S rDNA gene sequencing was carried out since the 16S rDNA gene is the most commonly used marker for deducing the phylogenetic relationship between bacteria due to its universal distribution, highly conserved nature and its rate of evolution (Wang *et al.* 2007). 16S rDNA gene analysis of 1499-bp sequence of M1B2 showed 98.7% similarity with *B. cereus* ATCC 14579$^{(T)}$ (Fig. 1). However, the phylogenetic tree constructed on the basis of 16S rDNA gene sequences of M1B2 and its nearest neighbours showed four distinct groups. The first group consisted of M1B2 along with two *B. cereus* strains and one *B. toyonensis* strain whereby, M1B2 and *B. toyonensis* formed separate branches (Fig. 1). The bacterial isolate M1B2 was identified as *Bacillus* sp. Plant growth promoting and antagonistic *Bacillus* spp. have earlier been reported for their stress tolerance against salinity, temperature and desiccation (Praveen Kumar *et al.* 2014).

Conclusions

In the present study, the *Bacillus* sp. M1B2 was selected as a potential candidate to be used as a microbial inoculant in a stressful environment as it showed stress tolerance towards all tested stress levels and also exhibited antagonisms and multiple PGP activities.

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