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ORIGINAL ARTICLE

Screening, isolation and characterization of culturable stress-tolerant bacterial endophytes associated with *Salicornia brachiata* and their effect on wheat (*Triticum aestivum* L.) and maize (*Zea mays*) growth

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Abstract

Globally more than 5.2 billion hectares of farming fields are damaged through erosion, salinity and soil deterioration. Many salt stress tolerant bacteria have plant growth promoting (PGP) characteristics that can be used to overcome environmental stresses. Isolation and screening of salt-tolerant endophytes from *Salicornia brachiata* were achieved through surface sterilization of leaves followed by cultivation on 4% NaCl amended media. Performance of isolates towards indole-3-acetic acid (IAA) production, phosphate solubilization, ACC deaminase activity, ammonia production, siderophore production and stress tolerance were determined. On the basis of the highest plant growth promoting activity, SbCT4 and SbCT7 isolates were tested for plant growth promotion with wheat and maize crops. In the present study, a total of 12 morphologically distinct salt-tolerant endophytic bacteria was cultured. Out of 12 isolates, 42% of salt-tolerant endophytes showed phosphate solubilization, 67% IAA production, 33% ACC-deaminase activity, 92% siderophore production, 41.6% ammonia production and 66% HCN production. A dendrogram, generated on the basis of stress tolerance, showed two clusters, each including five isolates. The bacterial isolates SbCT4 and SbCT7 showed the highest stress tolerance, and stood separately as an independent branch. Bacterial isolates increased wheat shoot and root dry weights by 60–82% and 50–100%, respectively. Similarly, improved results were obtained with maize shoot (27–150%) and root (80–126%) dry weights. For the first time from this plant the bacterial isolates were identified as *Paenibacillus polymyxa* SbCT4 and *Bacillus subtilis* SbCT7 based on phenotypic features and 16S rRNA gene sequencing. *Paenibacillus polymyxa* SbCT4 and *B. subtilis* SbCT7 significantly improved plant growth compared to non-inoculated trials.

Keywords: endophyte, MEGA X, plant growth promotion, *Salicornia brachiata*, salt stress

Introduction

As per report published by a world economic forum, the global food security challenge is straightforward: by 2050, the world must feed 9 billion people and the demand for food will be 60% greater than it is today (FAO 2018). Securing food availability for the world population is one of the major challenges for existing agricultural systems (Zaheer *et al.* 2016). All living forms on earth are completely dependent on plants for their various major needs i.e. oxygen and staple foods (Abhilash *et al.* 2016). More than 90% of global nutrition requirements are met by 12 crop varieties and 14 animal varieties (Wani *et al.* 2007; Zaheer *et al.* 2016; Wintermans *et al.* 2016). Of these 12 major plant species, crops such as wheat, rice and maize, are major energy providing crops for more than 50% of the world population (Oerke 2006). In the natural habitat, plants share a huge amount of space and nutrients with many naturally available microorganisms. The nutrient rich

root neighbouring habitat is highly promotive for initiating positive mutualistic interactions between plants and microbes (Zhu *et al.* 2015).

Salt stress is one of the major agricultural problems reducing crop yield in arid and semi-arid regions of the world. In the natural system, abiotic stresses (i.e. drought and salinity) limit and work as a major constraint for the growth, yield and quality of crops (Ul Hassan *et al.* 2016). Plants have well established mechanisms to cope with such stress. One of the mechanisms adopted by plants is the accumulation of compatible solute 'osmolytes' which provide protection to plant cell organelles under stress conditions. However, at elevated salt concentrations these mechanisms are not sufficient to tolerate consistent salt stress. Under such conditions the role of plant associated bacteria such as plant growth promoting (PGP) bacteria in alleviating salt stress has been documented through enhanced plant growth and modulation of the concentration of different osmolytes and antioxidant enzymes (Zhao et *al.* 2016).

The usage of microbial inoculants to improve effective adaptation and survival of crops may promote sustainable crop yields and restoration of soil fertility and composition. This would be a positive approach that can help in stress management of crops (Shrivastava and Kumar 2015; Panwar *et al.* 2016). These microorganisms have various biochemical pathways, e.g. deaminase activity, nodule formation, siderophore production, organic acid production and other physiological activities that help plants to tolerate stress (Zhu *et al.* 2015; Xun *et al.* 2015). Many researchers have reported that the efficacy of PGPRs become ineffective with sudden changes in environmental conditions and fail to respond in the field due to stress conditions. Therefore, selection for suitable stress resistance has become an essential parameter during the screening of microbial isolates suitable for the production of microbial inoculants. Salt stress in fields has resulted in a significant decline due to the production of salt-susceptible as well as, salt stress tolerant plants (Shrivastava and Kumar 2015). It was noted (Ullah and Bano 2015) that the majority of crops have low salt stress tolerance e.g. salinity tolerance for wheat is up to 6 dSm^{-1}, whereas for maize it is ~2 dSm^{-1}. Earlier research (Zhu *et al.* 2015; Ul Hassan *et al.* 2016; Singh *et al.* 2014) demonstrated the beneficial roles of microorganisms in alleviating salinity stress in crops and product yield.

Bacteria that may improve plant development are named PGP bacteria. These bacteria may be free-living in nature or in symbiotic associations with plants, or as endophytic bacteria in host tissues (Glick 2012). PGP bacteria may promote plant growth through direct or indirect mechanisms. A direct mechanism of plant growth includes the production of phytohormones, siderophore, HCN, ACC deaminase, N fixation, ammonia production, phosphate solubilization, decomposition of organic materials for smooth absorption, etc. Indirectly, these bacteria decrease the effect of phytopathogenic microorganisms by increasing the immunity of the host plant to resist phytopathogens (Lim and Kim 2013).

In the present investigation, screening, characterization and PGP activities of salinity stress tolerant bacteria isolated from *Salicornia brachiata* were investigated with two worldwide important staple crops, wheat and maize.

Materials and Methods

Isolation of bacterial endophytes

The present study was conducted with endophytic culturable bacteria, isolated from *S. brachiata* growing in Pudupalayam, Cuddalore, Tamilnadu, India located between the latitude 11°44'37.7"N and longitude 79°46'17.3"E. Leaf samples were collected from healthy plants, placed in sterilized ziploc bags and brought to the Microbiology Laboratory, Bhojia Institute of Life Sciences, Baddi, India. The samples were washed with distilled water three times to remove adhering soil and other chemicals. Surface sterilization of leaves was carried out by dipping in 75% ethanol for 30 sec followed by 0.2% $HgCl_2$ for 3 min (Bertani *et al.* 2016; Karnwal 2009). Then the surface sterilized leaves were surface washed with sterilized distilled water five times to remove any residue of surface sterilizing chemicals and aseptically shredded into 0.5 cm^2 leaf pieces, placed on modified nutrient agar medium amended with 4% NaCl (Karnwal 2018). These plates were incubated for up to 72 h at 28 ± 1°C in an unlighted area (Karnwal 2018). Plates were inspected every 12 h for any bacterial growth. Depending on macroscopic and microscopic characteristics and appearance individual bacterial colonies developing around the inoculated leaf parts were chosen. Pure culture was established and then subsequently preserved in 30% glycerol for further studies (Karnwal 2017). All chemicals used in the present experimental studies were of an analytical grade and procured from Himedia Laboratories, Mumbai, India.

Screening for plant growth promoting traits

Solubilization of phosphate

Phosphate solubilization assay was conducted by applying spot inoculation of each bacterial isolate on modified Pikovskaya's agar (yeast extract 0.5 gm · l^{-1}, dextrose 10 gm · l^{-1}, calcium phosphate 5 gm · l^{-1}, ammonium sulphate 0.5 gm · l^{-1}, potassium chloride 0.2 gm · l^{-1}, magnesium sulphate 0.1 gm · l^{-1}, manganese sulphate 0.0001 gm · l^{-1}, ferrous sulphate

0.0001 gm · l^{-1} and agar 15 gm · l^{-1}) (Himedia). Results were considered in the expression of solubilization index (SI) (Karnwal 2017). The plates were incubated in a BOD incubator for 48 h at 28 ± 1°C. A clear halo zone near the bacterial growth was assumed as +ve results for phosphate solubilization and measured by applying the subsequent equation of Edi-Premono *et al.* (1996).

$$\text{Phosphate solubilization index (SI)} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

The qualitative examination of inorganic phosphate, the liquefaction capability of the bacterial isolates was assessed with Pikovskaya's broth *in vitro* by calculating existing liquid phosphate in 0.5% tri-calcium phosphate (TCP) amended media. The broth was inoculated in triplicate with isolated salt tolerant endophytic bacteria. All flasks were incubated at 28 ± 1°C in a rotary shaker incubator for 120 h at 180 rpm and then centrifuged for 10 min at 11,180 g. The available phosphate in the culture medium was measured, followed by the phosphomolybdate method (Shyla *et al.* 2011). During the experiment the pH of the broth was also measured using a systronics-304 pH meter at regular intervals (Ambardar and Vakhlu 2013).

Indole-3-acetic acid-like auxin production

Indole-3-acetic (IAA) production was measured on DF medium enriched with 0.1% L-tryptophan with Van Urk Salkowski reagent using Salkowski's method (Ehmann 1977). The bacterial isolates 100 µl were incubated in DF (Dworkin and Foster medium) medium amended with L-tryptophan for 48 h at 28 ± 1°C. The culture broth was centrifuged at 11,963 g in a cooling centrifuge and 1 ml of supernatant with 2 ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$) was incubated in a test tube. This mixture was left undisturbed in the dark at room temperature for 30 min. The optical density (OD) was recorded at 530 nm and the amount of IAA-like auxins was documented against the non-inoculated control.

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

ACC (1-aminocyclopropane-1-carboxylate) deaminase activity for salt-tolerant isolates was analyzed on ACC supplemented Dworkin and Foster salts minimal medium (Dworkin and Foster 1958) as the sole nitrogen source. The DF agar plates were inoculated with the bacterial cultures and incubated at 28 ± 1°C. The plates were observed for bacterial growth as a positive result after 48 h.

Siderophore production

One liter of chrome-azurol S (CAS) blue agar medium was prepared using 60.5 mg CAS dissolved in 50 ml distilled water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3$ $6H_2O$, 10 mM HCI). Stirring constantly, this solution was slowly added to 72.9 mg H.D.T.M.A. (hexa decyl tri methyl-ammonium bromide) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved for 20 min. A mixture of 750 ml water, 15 g agar, 30.24 g Pipes and 12 g of a 50% (w/w) NaOH solution to raise the 6.8 pH was also autoclaved and used for confirmation of siderophore production potential of bacteria isolates as described by Schwyn and Neilands (1987). The bacterial culture after 24 h incubation was inoculated on CAS agar and incubated for 48–72 h at 28 ± 1°C. A change of CAS agar medium from blue to orange or with a yellow halo around bacterial growth confirmed siderophore production.

Ammonia production

The potential of bacterial isolates for ammonia production was investigated by inoculation of bacterial isolates on peptone medium. Bacterial inoculated medium was incubated for 48 h at 28 ± 1°C. The incubated medium was amended with Nessler's reagent (0.5 ml) at a 2 : 1 ratio. A change from brown to yellow was recorded as a positive result for ammonia production. The uninoculated medium was used as a reference.

Hydrogen cyanide (HCN) determination

HCN product capability of bacterial isolates was tested as described by Bakker and Schippers (1987) with few modifications. Nutrient agar medium supplemented with 4.4 g glycine · l^{-1} was streaked with bacterial isolates. Sterile Whatman 1 filter paper was placed on the upper lid of a Petri plate saturated with picric acid solution (2.5 g of $(O_2N)_3C_6H_2OH$; 12.5 g of Na_2CO_3, 1,000 ml of distilled H_2O). Parafilm sealed Petri plates were incubated at 28 ± 1°C for the development of light/moderate/strong brown color as a positive HCN production result.

Stress tolerance screening

Salt stress tolerance potential of bacterial isolates was investigated on nutrient agar medium (NAM) supplemented with different concentrations of NaCl, i.e. 5.0% (0.86 M), 7% (1.2 M), 8.5% (1.46 M), 10.0% (1.71 M), and 12% (2.054 M). These plates were streaked with bacterial culture and incubated in a BOD incubator for 48 h at 28 ± 1°C. Bacterial isolates that grew ≥5.0% NaCl were documented as salt stress resistant isolates and were examined *in vitro* for temperature stress (25–40°C), pH stress (4–8) and PGP attributes.

The results of the growth of bacterial strains were scored as binary numbers, 1 representing growth and 0 indicating no growth. The data were subjected to cluster analysis using PAST 3.22 software.

Effect on plant growth

The effect of halophilic isolates SbCT4 and SbCT7 on plant growth was analyzed with wheat (*T. aestivum)* and maize (*Zea mays*) seeds. Crop seeds were obtained from IARI, Pusa, Delhi, India. Seeds were washed with sterilized distilled water three times and after that surface sterilized with 3% sodium hypochlorite (NaOCl) for 5 min. Seeds were washed five times with sterilized distilled water for removing any traces of NaOCl. Thereafter, the seeds were dipped and left for 20 min with bacterial culture in a conical flask having 48-h-old bacterial growth with 9 log10 CFU · ml^{-1}. Bacterial inoculated seeds were air dried in a laminar air flow for 60 min. Air dried bacterial coated seeds were planted in earthen pots (five seeds per pot, diameter and height of pot were 18 inches). For every treatment three replicates were prepared. The trials for both crops were designed in the following manner:

- Control_W: unsterilized garden soil and wheat seeds dipped in 10 ml of non++-inoculated nutrient broth medium
- PGP_W1: unsterilized garden soil and wheat seeds coated with bacterial strain SbCT4
- PGP_W2: unsterilized garden soil and wheat seeds coated with bacterial strain SbCT7
- Control_M: unsterilized garden soil and maize seeds dipped in 10 ml of non-inoculated nutrient broth medium
- PGP_M1: unsterilized garden soil and maize seeds coated with bacterial strain SbCT4
- PGP_M2: unsterilized garden soil and maize seeds coated with bacterial strain SbCT7.

A randomized block design was used for the layout of the pots in a natural environment and growth parameter data i.e. shoot and root lengths and total dry weight were recorded after 60 days.

Characterization and identification of the bacterial isolate

Microscopic, biochemical (starch hydrolysis, H_2S production, citrate utilization, oxidation reaction, casein hydrolysis, 3-ketolactose production, urease production, catalase test, lipolysis activity, pigment production, lipolysis activity and gelatin liquefaction) and molecular methods were applied for the identification of isolates. Biochemical identification of SbCT4 and SbCT7 was carried out as described in Bergey's manual of determinative bacteriology (Holt *et al.* 1994). For phenotypic characterization Gram's staining, motility testing, and endospore staining were performed (Krieg and Holt 1984).

Phylogenetic analysis and 16S ribosomal RNA sequencing were carried out for both bacterial isolates (Karnwal 2017). 16s rRNA was isolated by utilizing the Qiagen DNeasy Plant Mini Kit. Gene amplification, thermocycling conditions, and sequence analysis have been described earlier in detail (Gulati *et al.* 2008). MUSCLE algorithm was used for multiple sequence alignment (MSA) with sequences collected from BLAST results and MEGA X software (Kimura 2-parameter substitution model and neighbor-joining statistical method) was applied for computing the evolutionary distance of the stress tolerant strains for phylogenetic analysis.

Trials' design and statistical analysis

All trials were carried out by applying a randomized block design during the experiments. Unless stated otherwise, all values are the standard means of three replicates. Data on plant growth promotion was analyzed by analysis of variance (ANOVA). The mean of the treatments was compared by Fisher's significant difference (LSD) test at p values of 0.05.

Results and Discussion

Isolation of bacterial endophytes

In a natural environment plants have to tackle various biotic and abiotic stresses (Abhilash *et al.* 2016). Abiotic stresses are classified as the inanimate components associated with the environment whose impact is experienced by the living section of nature (Ahmad *et al.* 2011; Liu *et al.* 2014). Nutrients, salt concentration, water availability, temperature change and pH are the main abiotic factors that directly influence plant growth in the agricultural field. Many researchers (Gulati *et al.* 2008; Karnwal 2009) observed and revealed the use of beneficial microbes in agriculture to deal with increased salt tolerance in plants and utilized as substitute methodology to use salt susceptible fields in farming. Plant growth promoting rhizobacteria (PGPR) are bacteria that reside under the influence of plant roots and effectively participate in plant growth development and abiotic stress tolerance (Karnwal 2017). These microbes enhance soil-water-plant relationships, manipulate phytohormonal signaling and trigger several other mechanisms that work in an integrated fashion to enhance salt and drought stress tolerance in plants (Shrivastava and Kumar 2015).

In the present study, a total of 12 salt tolerant endophytic bacterial isolates were successfully screened in pure form from leaf samples of *S. brachiata* on 4%

NaCl concentration amended nutrient agar media and designated as SbCT1 to SbCT12. Colony characters of 12 isolates we are shown in Table 1. It was observed that all 12 isolates had colonies from irregular to circular. SbCT6, SbCT9, SbCT12 colonies were raised; SbCT3, SbCT4, SbCT7, SbCT8, SbCT10, SbCT 11 were convex and SbCT1, SbCT2 were flat. The colony margin, color and Gram staining results significantly varied between all bacterial isolates as shown in Table 1. It was observed that the variety and type of endophytic microbes depended on natural host plants. Many workers (Chung *et al.* 2015; do Amaral *et al.* 2016) reported less variable diversity of endophytic bacteria in plants (4 log 10 to 8 log 10 CFU · g^{-1} of plant tissue) compared to rhizospheric bacteria (6 log 10 to 9 log 10 CFU · g^{-1} of soil) of a host crop. In recent years, various scientific studies have reported numerous pathways related to endophytes that are used for enhancing plant development under varied abiotic stresses. These include phytohormone production, ACC deaminase activity, HCN production, organic acid production for solubilization of minerals, antagonistic activity, etc. (Ul Hassan *et al.* 2016; Ullah and Bano 2015).

These 12 bacterial isolates were further accessed for their PGP traits i.e. IAA production, phosphate liquefaction, siderophore production, ACC deaminase activity and HCN production.

Phosphorus is a macronutrient that is required by all living organisms (Xun *et al.* 2015). However, plants require much less of this particular macronutrient than animals although a critically low availability could lead to deficiencies and have an adverse impact on plant growth (Karnwal 2017). Plant requirements of phosphorus range from 25 µmol · l^{-1} to 30 µmol · l^{-1} for optimum growth but the actual amount of phosphorus available in most soil types ranges from only 1 µmol · l^{-1} to 1.7 µmol · l^{-1} (Melo *et al.* 2016). In soil the maximum quantity of phosphorus exists in solid or powder form but cannot be directly utilized by the plant. Research workers (Zaheer *et al.* 2016; Zhu *et al.* 2015) have documented the use of soil residing bacteria for liquefaction of mineral phosphates into a plant utilizable form. Phosphate solubilizing assay results of the present study showed that only 5 isolates have transparent areas surrounding bacterial growth with diameters ranging from 2.0 to 18.5 mm and phosphate solubilization indices ranging from 6.25 to 13.73 for phosphate solubilizing isolates (Table 2). The phosphate solubilizing efficiency of all 12 isolates in 0.5% tri-calcium phosphate supplemented Pikovskaya's broth suggested that bacterial isolates successfully liquefied mineral phosphate in the inoculated broth (Table 2). Phosphate solubilizing bacteria (PSB) SbCT4 and SbCT7 were estimated with 214.59 µg · ml^{-1} and 316.72 µg · ml^{-1} soluble phosphate in the broth after 48 h and 78 h incubation, respectively. Phosphate solubilizing efficiency results of other strains were reported in Table 2. Inorganic phosphate solubilization by microbes is of economic importance in crop nourishment. Research workers (Das *et al.* 2014) have documented the application of numerous soil bacteria genera, i.e. *Achromobacter, Pseudomonas, Flavobacterium, Enterobacter, Serratia, Bacillus, Mycobacterium, Erwinia, Agrobacterium* and *Escherichia* as phosphate solubilizers.

Table 1. Micro and macroscopic characteristics of bacterial isolates

Bacterial isolates	Shape	Color	Margin	Elevation	Gram stain
SbCT1	rod	yellow	entire	flat	+
SbCT2	rod	creamy	irregular	flat	+
SbCT3	rod	yellow	entire	convex	+
SbCT4	cocci	yellow	entire	convex	+
SbCT5	cocci	whitish	entire	flat	–
SbCT6	rod	creamy	entire	raised	+
SbCT7	cocci	whitish	entire	convex	+
SbCT8	rod	whitish	unbonate	convex	–
SbCT9	cocci	light orange	entire	raised	+
SbCT10	cocci	whitish	irregular	convex	+
SbCT11	rod	pale yellow	unbonate	convex	+
SbCT12	rod	pale yellow	regular	raised	–

Production of IAA is one of the most important traits of a wide variety of soil microorganisms used for plant growth promotion (Gulati *et al.* 2009). IAA is a plant development hormone associated with rhizome propagation, plant cell proliferation and cell duplication so that IAA biosynthesis activity of microbes is essential for plant development (Das *et al.* 2014). In all reported auxins, IAA is the most important growth enhancer for plant root system development. Karnwal (2009) reported the significance of rhizo-competent stress resistant microbes with diverse functions of IAA intended for the elimination of salt anxiety in crops. In the present study, all 12 isolates were analyzed for IAA production in the availability of L-tryptophan. A total of 8 bacterial isolates showed positive attributes towards IAA production ranging from 0.08 μg · ml^{-1} to 17.1 μg · ml^{-1} as shown in Table 3. Isolates SbCT4 and SbCT7 produced the maximum levels of IAA in broth, 17.1 μg · ml^{-1} and 14.6 μg · ml^{-1}, respectively. Strains SbCT2, SbCT3, SbCT8 and SbCT11 did not produce any amounts of IAA and were reported as negative for IAA production. The biosynthesis of iron scavenger siderophores is another valuable process of PGPRs (do Amaral *et al.* 2016) consisting of a dual impact on promoting plant development by raising the supply of beneficial nutrients for the plant (Banik *et al.*

Table 2. Screening of bacterial isolates for phosphate solubilization

Bacterial isolates	Phosphate solubilization efficiency		Phosphate solubilization index	Phosphate solubilization [μg · ml^{-1}]
	colony diameter [mm]	halo zone diameter [mm]		
SbCT1	0.4 ± 0.002	2.1 ± 0.002	6.25 ± 0.018	103.83 ± 0.04
SbCT2	1 ± 0.01	0	1.00 ± 0.01	0
SbCT3	2 ± 0.02	18.5 ± 0.14	10.25 ± 0.04	176.21 ± 0.01
SbCT4	1.1 ± 0.001	14 ± 0.08	13.73 ± 0.06	214.59 ± 0.15
SbCT5	0.2 ± 0.02	0	1.00 ± 0.02	0
SbCT6	1 ± 0.04	0	1.00 ± 0.04	0
SbCT7	1.3 ± 0.02	15 ± 0.08	12.54 ± 0.06	316.72 ± 0.08
SbCT8	1.8 ± 0.03	0	1.00 ± 0.03	0
SbCT9	0.7 ± 0.06	0	1.00 ± 0.06	0
SbCT10	0.2 ± 0.001	0	1.00 ± 0.001	15 ± 0.01
SbCT11	0.2 ± 0.003	2 ± 0.01	11.00 ± 0.02	158.1 ± 0.11
SbCT12	1.6 ± 0.01	0	1.00 ± 0.01	0

Values are the means of three replicates ± SE mean

Table 3. Screening profile of bacterial isolates for various PGP traits

Bacterial isolates	IAA production [μg · ml^{-1}]	HCN production	Ammonia production	Siderophore production zone [mm]	ACC deaminase activity
SbCT1	0.08 ± .001	+	–	3.2 ± .01	–
SbCT2	–	+	++	2 ± .07	–
SbCT3	–	–	–	20.2 ± .12	–
SbCT4	17.1 ± .02	++	+++	22.7 ± .06	+++
SbCT5	1.24 ± .008	–	+++	11.7 ± .02	–
SbCT6	8.6 ± .01	++	+	17 ± .07	+++
SbCT7	14.6 ± .04	+++	+++	26.9 ± .15	++
SbCT8	–	–	–	3.8 ± .1	–
SbCT9	10.27 ± .02	++	–	7.1 ± .02	–
SbCT10	4.53 ± .01	+++	–	9.2 ± .06	++
SbCT11	–	–	–	–	–
SbCT12	1.7 ± .03	+	–	3.6 ± .01	–

Values are the means of three replicates ± SE mean; (+++) – luxuriant growth/strong activity, (++) – good growth/moderate activity, (+) – poor growth/weak activity, (–) – no growth/no activity

2016). PGPRs in soil are able to produce ACC deaminase and can promote plant growth as well as protect plants against abiotic (drought, salt, flooding, inorganic and organic contaminants) (Gulati *et al.* 2008) and biotic stress (bacterial and fungal pathogens) (Karnwal 2017). In our study, 8 isolates showed positive results for HCN production, 05 ammonia production, 11 siderophore production, and 04 ACC deaminase activity as shown in Table 3. These secondary metabolites instantly impact shoot and root development and seed growth of various agricultural crops.

On the basis of all PGP trait results, the two best stress tolerant endophytic isolates (SbCT4 and SbCT7) were selected for further study.

Screening for stress tolerance

During the process of evolution, microbes established a variety of variable mechanisms to live with stress in the environment (Lim and Kim 2013; Panwar *et al.* 2016). Numerous bacterial genera, such as: *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium* are competent to withstand abiotic stresses by synthesizing a significant volume of exopolysaccharides (EPS) (Vurukonda *et al.* 2016). In recent years, it has been revealed that bacteria belonging to different genera, such as: *Rhizobium*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Achromobacter*, *Methylobacterium*, and *Variovorax*, can provide tolerance to host plants against different abiotic stresses (Yaish *et al.* 2015; Zhao *et al.* 2016). These types of microbes can remove environmental anxiety in farming and will be useful for decreasing soil salinity. In the present study, bacterial isolates SbCT4 and SbCT7 were tested for stress tolerance against varying levels of salinity, pH and temperature. Salinity studies have revealed that both isolates tolerate higher NaCl concentrations up to 10% in agar medium. Both isolates showed good growth on 5.0% (0.86 M), 7% (1.2 M) and 8.5% (1.46 M) salt concentrations. SbCT4 was able to tolerate 10.0% NaCl whereas on a 12% concentration no growth was observed. In the same manner, the growth of SbCT7 was observed up to 8.5% (1.46 M) of NaCl and no growth was observed above this NaCl concentration. Variable growth was observed with different pH values. The best supportive pH for SbCT7 growth was 6, 7 and 8 whereas SbCT4 was able to grow on 5, 6 and 7, however on pH 8 no growth of SbCT4 was observed. A temperature stress study revealed that both isolates exhibited growth at different temperatures ranging from 25 to 40°C.

A dendrogram created by using UPGMA cluster analysis (similarity index: euclidean) for stress tolerance at different levels of salinity, temperatures, and pH for bacterial isolates showed two clusters. Cluster 1 included 5 isolates (SbCT2, SbCT5, SbCT8, SbCT10, SbCT11) and cluster II included 5 isolates (SbCT1, SbCT3, SbCT6, SbCT9, SbCT12) as shown in Figure 1. The bacterial isolates SbCT4 and SbCT7, which showed the highest stress tolerance, stood separately as an independent branch.

Effect on plant growth promotion

The potential use of PGPRs in the alleviation of salt stress in plants has been reported by many researchers in various plants, including licorice, and chili pepper (Singh *et al.* 2014; Zhu *et al.* 2015). In a recent study, the inoculation effect of PGPRs at early development stages was reported and resulted in better plant development and biomass production through direct effects

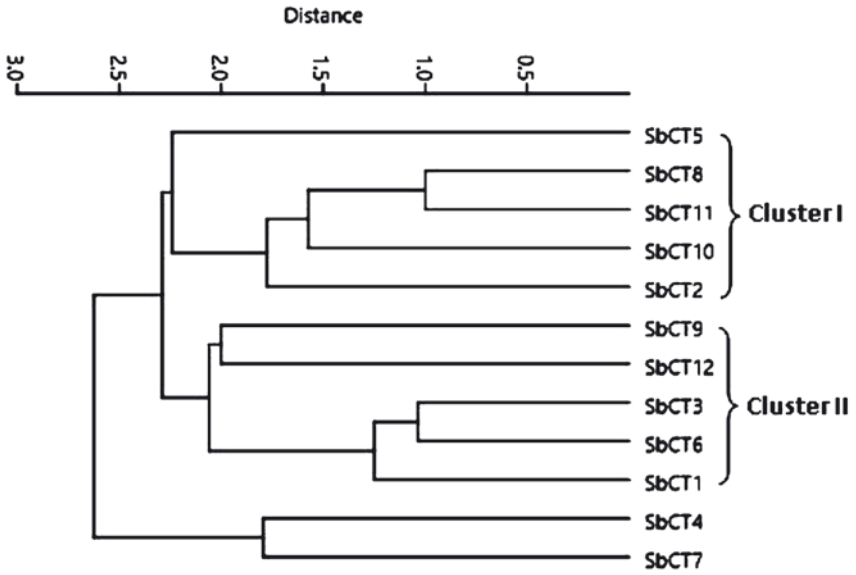

Fig. 1. Dendrogram based on UPGMA cluster analysis (similarity index: euclidean) by using PAST software 3.22 for salt tolerant isolates of *Salicornia Brachiata* using growth pattern data for different stress levels (salinity, temperature and pH stress)

Table 4. Effect of stress-tolerant bacterial endophytes on growth promotion of wheat and maize

Bacterial isolates	Wheat				Maize			
	shoot length cm]	root length [cm]	shoot dry weight [g]	root dry weight [g]	shoot length [cm]	root length [cm]	shoot dry weight [g]	root dry weight [g]
SbCT4	28.33 b	17.23 b	0.09 b	0.08 b	42.30 c	20.40 b	0.59 c	0.45 c
SbCT7	34.65 c	20.67 c	0.12 c	0.09 b	33.00 b	15.13 a	0.47 b	0.23 b
Control	21.46 a	13.20 a	0.06 a	0.05 a	27.10 a	14.13 a	0.26 a	0.18 a
LSD value at 5%	2.29	2.91	0.025	0.024	5.33	2.24	0.078	0.057

Values are the means of three replicates with five plants each ± SE_{mean}. Values with different letters in each column differ significantly from one another at $p \leq 0.05$

on root and shoot development (Mahmood *et al.* 2017). In the present study, 60 days after sowing, all plants were harvested for analysis and the average of three replicates for each isolate was used for statistical analysis. Results of the pot study showed that both isolates significantly ($p \leq 0.05$) increased the growth of wheat and maize compared to the control (Table 4). Salt stress negatively impacts plant development and decreases product formation (Panwar *et al.* 2016). Salt tolerant and root-colonizing bacteria have the potential to tolerate abiotic stresses and adaptively grow in stressful environments that directly help the plant to bear and resist salt stress (Vaishnav *et al.* 2016). In an earlier study, Egamberdieva and Kucharova (2009) reported the effect of salt tolerant *P. extremorientalis* strain on root colonization and plant development. Dixit *et al.* (2018) documented that salt tolerant bacteria was the most efficient during crop development of tomato in non-saline and saline soil. Our results also support other studies since the shoot and root lengths were the longest with SbCT7 inoculated wheat treatment while SbCT4 plant growth promotion results were also significantly better than uninoculated treatments for wheat. Similar improvement has been mentioned by inoculation of PGPB during wheat farming. Almaghrabi *et al.* (2013), reported that the application of salt stress tolerant *P. putida* and *P. fluorescens* increased plant growth parameters. Earlier reports (Almaghrabi *et al.* 2013; Shrivastava and Kumar 2015) demonstrated the effect of improved root growth by bacterial inoculants which facilitated plant development and increased the overall access of plant roots to soil minerals, i.e. nitrogen (N), phosphorus (P) and potassium (K). It was noted that in the case of maize seeds, root length was higher with SbCT4 than with SbCT7 and uninoculated pots. Root dry weight was significantly ($p \leq 0.05$) higher in SbCT7 and SbCT4 treatments than uninoculated treatments for both crops. Shoot dry weight was significantly ($p \leq 0.05$) increased in SbCT4 for maize and SbCT7 for wheat (Table 4).

Characterization and identification of the bacterial isolate

Earlier reports documented that Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes are the primary abundant populations belong to plant endophytic bacteria participate in plant growth promotion (Banik *et al.* 2016). The most commonly found genera of bacterial endophytes are: *Microbacterium*, *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Bacillus*, *Pantoea* and *Micrococcus* (Almaghrabi *et al.* 2013; Chung *et al.* 2015). Microscopic, biochemical and molecular methods were used for the characterization and identification of both bacterial isolates. Microscopic results revealed that bacterial isolates SbCT4 and SbCT7 were rod shaped bacilli, Gram +ve, and endospore former.

The 16S rRNA gene sequencing method was adopted for the molecular characterization of bacterial isolates. SbCT4 (NCBI Accession Number: MK680506) 16s rRNA gene sequence showed 97% identity with *Paenibacillus polymyxa* strain DSM 36 while SbCT7 (NCBI Accession Number: MK680507) 16S rRNA gene sequence showed 98% identity with *Bacillus subtilis* strain NRRL B-4219, *B. subtilis* strain JCM 1465, *B. subtilis* strain NBRC 13719 and *B. subtilis* strain DSM 10, as shown in Figure 2. The phylogenetic tree for both isolates was constructed by using MEGA X software as shown in Figure 2.

Conclusions

This study has confirmed that the growth of both crops (wheat and maize) in the presence of inoculated salinity stress tolerant bacteria endophytes was improved. Inoculation of isolates showed an effective increase in shoot and root lengths, as well as shoot and root dry weights. These improvements in plant growth traits were related with phyto hormone production (auxins-IAA), phosphate liquefaction, siderophore production,

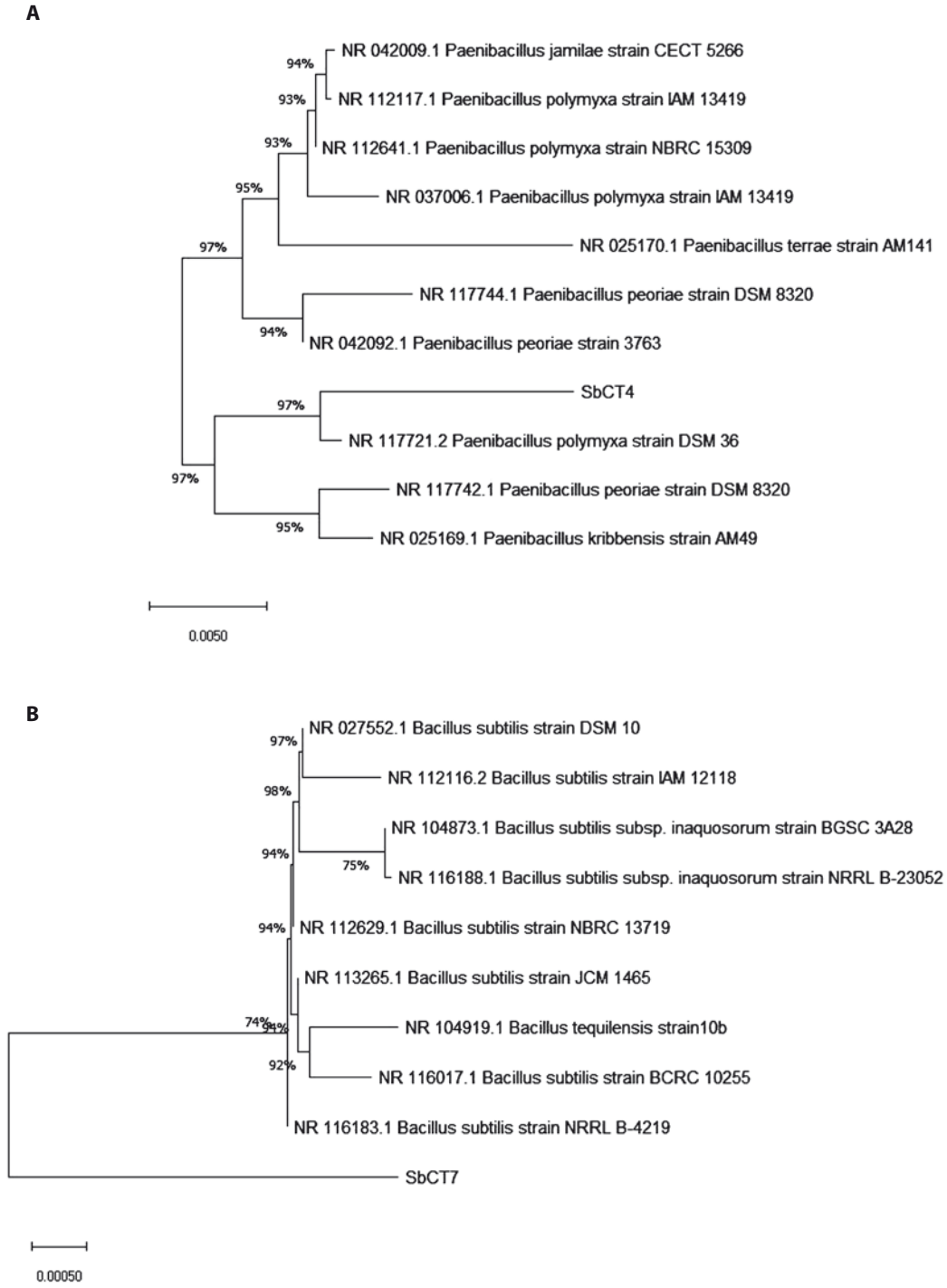

Fig. 2. Phylogenetic tree of bacterial isolates based on 16S rRNA sequence similarity by using MEGA X software: A – BLAST similarity search results and phylogenetic tree for isolate SbCT4, B – phylogenetic tree for isolate SbCT7

ACC deaminase activity, HCN production, and ammonia production by bacteria. This suggests that the use of salinity stress tolerant bacteria was helpful and beneficial in enhancing the growth of wheat and maize.

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