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EXPLORATION OF PLANT GROWTH-PROMOTING ENDOPHYTIC BACTERIA FROM THE CROPS GROWN IN SODIC SOILS

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Abstract

Isolation of the potential endophytic bacteria from plants that are grown in sodic soils, their diversity and screening their plant growth-promoting activities for testing their efficiency as bio inoculants for the problem soils were studied. Twelve different isolates were selected out of 35 endophytic bacterial isolates on the basis of their richness in the endophytic population of different crop plants and the nucleotide sequences of 16S rRNA genes were observed. The endophytic bacteria for their plant growth-promoting traits such as nitrogen fixation, IAA production, ACC deaminase production, phosphate solubilization, siderophore secretion and antifungal activity were assessed. The best performing strains were evaluated using principal component analysis (PCA). The phylogenetic tree from the endophytic bacterial isolates of different crops grown in sodic soil was categorized into three clusters *i.e. Firmicutes, β-Proteobacteria* and *γ-Proteobacteria.* The PCA results identified two potential endophytic strains *viz*., Ma11 (*Bacillus mojavensis*) and Ri 2 (*Pseudomonas fluorescens*) from sodic soil-grown crops. The present findings revealed that the isolates, Ma 11 and Ri 2 were capable of improving crop growth under sodicity. These multifaceted endophytic bacterial isolates would be developed as new inoculants which will be highly suitable for sodic soils.

Introduction

India comprises 6.73 Mha of salt-affected soils, of which 3.72 Mha is sodic soils mostly present in Indo-Gangetic plains (Singh *et al*. 2010). The reduction in the overall production of crops was recorded due to the steady formation of sodic soils in the northwest plains of the Indo-Gangetic basin and Yellow river basin of China (Gupta and Abrol 2000). Sodic soils have pH $>$ 8.5 and exchangeable sodium per cent $>$ 15, higher concentration of free carbonates and bicarbonates and hydraulic conductivity of sodic soil is low and exhibits high impedance to root growth (Qadir and Schubert 2002). These soil properties influence the rhizosphere populations, consequently affecting the yield of the crops (Tank and Saraf 2010). Endophytic bacteria colonize plants through roots and found in leaf, stem, fruits and seeds (Pirhadi *et al.* 2016, Dombrowski *et al.* 2017). Being in mutual interaction with the plant, endophytic bacteria can directly or indirectly promote the plant growth by nutrients mobilization, synthesis of plant growth hormones and also facilitate to combat with other pathogens and pests (Compant *et al.* 2010). The internal tissues of plants afford congenial environment for endophytes than the plant surfaces, where exposure to environmental conditions, such as temperature, osmotic pressure and UV radiation are major limiting factors for bacterial survival.

In India, the study area (Tamil Nadu state) alone consists of an area of about 4.7 lakh ha of sodic soils (Sureshkumar *et al*. 2015). The observation made in the study is that the crops cultivated in this soil comprises efficient plant growth-promoting endophytes which can be explored for the sustainable productivity of sodic soil agro-ecosystem. Hence, the aim of the

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present study was to isolate the potential endophytic bacteria from plants that are grown in sodic soils, study their diversity and screen their plant growth-promoting and biocontrol activities to examine and verify their potential as bio inoculants for the problem soils.

Materials and Methods

The healthy and disease symptom free crops *viz*., rice (*Oryza sativa*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), cotton (*Gossypium hirsutum*) and bhendi (*Abelmoschus esculentus*) that are grown in sodic soils of Tiruchirappalli, Thanjavur, Nagapattinam, Thiruvarur, Pudukkottai, Madurai and Sivaganga of Tamil Nadu, India were collected. The sodic soils are characterised with high pH (8.5) with low salinity (EC < 4) and high exchangeable sodium percentage (ESP) of >15%. Collected stems of the crops were washed in running tap water to remove soil particles and other impurities and differentiated by surface appearance to eliminate samples that exhibited superficial damage. The selected healthy stems from the fields were placed on ice and brought to the laboratory.

The stems were split into longitudinal sections and cut into 1-2 cm pieces with a sterile surgical blade and further they were kept in a beaker, soaked in distilled water and drained. Then, the stem bits were surface sterilized with sodium hypochlorite with 3% available chlorine followed by 70% ethanol. The 4-6 mm long stem bits were placed on TS agar media amended with benomyl (50 µg/ml) to arrest fungal growth. Plates were incubated in an incubator at 28ºC for 1-10 days to allow the growth of endophytic bacteria from the cut pieces (Araújo *et al*. 2002).

In other method, stem bits were homogenized in 5ml of sterile phosphate buffer saline (containing 8 g/l of NaCl, 0.2 g/l of KCl, 1.4 g/l of Na_2HPO_4 and 0.24 g/l of KH_2PO_4) by taking a blender and serial dilutions were plated onto TS agar. The plates were incubated at 28ºC for 1-10 days or until growth was noticed (Araújo *et al*. 2002). Both methods were conducted for isolation and the bacterial colonies from each sample were chosen on the basis uniqueness of the colony characteristics and purified.

The isolates of endophytic bacteria were analyzed through 16S rRNA gene sequencing. Total DNA of the endophytes was isolated using the standard protocol of CTAB method of Clark (2013) and dissolved in distilled water to a final concentration of 20 ng/µl and stored at 4ºC. Almost full-length of 16S rRNA gene was amplified from elite isolates by universal eubacterial primers, FD1 and RP2 (Weisburg *et al.* 1991). The gene amplification was done in the thermocycler (Eppendorf Master cycler, Germany) with a 25 µl reaction mixture containing 50ng of genomic DNA, 0.2 mM of each dNTP, 1µM of each primer, 2.5 mM of $MgCl_2$ and 2.5 µM of Taq DNA polymerase and the buffer provided with the enzyme (Thermo Scientific). The conditions of the polymerase chain reaction were initial denaturation performed at 95°C for 10 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final elongation at 72°C for 10 min. The amplified products were checked on a 1.5% agarose gel in 1X TBE buffer and documented in Alpha imager TM 1200 documentation and analysis system. The band of the required size was gel-purified using spin columns as mentioned in the manufacturer's instructions (Thermo Scientific) and cloned using PTZ57R/T vector supplied with TA cloning kit (Fermentas) before sequencing. Sequencing reactions were performed and phylogenetic tree was constructed.

Acetylene reduction assay (ARA) was used to analyze the nitrogenase activity of the endophytic isolate (Hardy *et al.* 1968). Nitrogenase activity was calculated as nmol of ethylene per tube per hr. Production of IAA by the endophytic isolates was estimated through the method suggested by Gordon and Paleg (1957). Twenty four hours old cultures of endophytic isolates were inoculated in the mid of Pikovaskaya agar plates supplemented with 0.5% tricalcium

phosphate, incubated at 28 ± 1ºC for 4 days. The phosphate solubilization (mm) zone developed around colonies was observed after 48 hr of inoculation (Pikovaskaya 1948).

1-Aminocyclopropane-1-carboxylate deaminase (ACCD) regulation of the bacterial endophytes was determined by the method previously explained by Shaharoona *et al.* (2006). The capacity of the endophytic bacteria for the synthesis of siderophore was experimented by the Chrome azurol S (CAS) method (Schwyn and Neilands 1987). Qualitative analysis of siderophore production by the endophytic cultures was done by spot inoculation on the plates containing CAS medium and kept at 28 ± 2ºC for 2-3 days. The presence of orange/reddish-brown color indicates positive for siderophore production.

Production of antimicrobial substances by the endophytic cultures was screened through cross-streak assay method (Williston *et al.* 1947) using four test organisms. The fungal pathogens *viz.*, *Rhizoctonia solani, Fusarium oxysporum,* and *Macrophomina phaseolina, Pythium* sp. were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. Inoculation of nutrient agar plates with bacterial endophytes as a single streak was done at the center of the Petri plate and incubated for 5 days at 30ºC. The grown test organisms were streaked at the right angle to the producer endophyte and observed for its growth/inhibition after 24 - 48 hrs of incubation at 30ºC. The length of the inhibition zone was measured to the nearest mm. Similarly the experiment was repeated thrice.

All the data were subjected to analysis of variance and Duncan's multiple range tests at 5% significance level using XLSTAT (version 2010.5.05) for Microsoft Excel- Windows 2007 to reveal the significant statistical difference among the treatments. Principal component analysis (PCA) was performed for the recorded variables of plant growth promoting traits to display the similarities and differences between isolates and to identify the best performing strains (Wold *et al.* 1987).

Results and Discussion

Several endophytic bacteria were isolated from crops cultivated in sodic soils of Tamil Nadu. Totally 35 endophytic bacteria were isolated, of which 6 bacterial endophytes were isolated from Rice,7 from Cotton,10 from Maize,6 from Sorghum, 3 from Pearl millet and 3 from Bhendi. The first two letters of the host plant name were labelled to the isolates from which they isolated. Twelve different isolates were chosen on the basis of their richness in the endophytic population of different crop plants and taken for the entire study (Table 1). The nucleotide sequences of 16S rRNA genes of 12 isolates were determined and aligned with those of reference strains in GenBank. As shown in Table 1, all isolates showed high similarities (≥ 98%) with their closest related species. The phylogenetic tree explains the relationships between the isolates and related reference species is depicted in Fig. 1. The phylogenetic tree could discriminate against the endophytic bacterial isolates of different crops and be arranged into three clusters: *Firmicutes, β -Proteobacteria* and γ *-Proteobacteria*.

The cluster *Firmicutes* consists of Gram-positive bacteria with low G + C content; *Bacillus* was the most dominant group among the isolates. Three members in the cluster γ*-Proteobacteria* belonging to the genera *Pseudomonas* and one isolate, *Alcaligens* from *β-Proteobacteria* were also identified. The plant-associated environment is a unique habitat which influence species composition of bacterial communities colonize plant tissues. Some of these factors are plant tissues (Mocali *et al.* 2003), plant species and soil type (Kuklinsky-Sobral *et al.* 2004). The problem (sodic soil) soil and the host plants might have influenced the composition of endophytic communities. The results proved the predominant population and wide distribution of the genus *Bacillus* in all the crop plants grown under sodic soil. Bacterial genera, *Bacillus* and *Pseudomonas*

are amenable for culturing in the laboratory and previous reports established them as frequently occurring endophytes (Seghers *et al.* 2004). Reports on the existence of bacteria belonging to *Bacillus* and *Pseudomonas* genera inside the different of parts of plants as endophytic bacteria already exist (Saidi *et al.* 2013, Etminani and Harighi 2018). Earlier studies also showed that *Bacillus* and *Pseudomonas* genera existed pre-dominantly in saline soils (Tank and Saraf 2010).

Table 1. Phylogenetic affiliation of different plant growth promoting endophytes isolated from different crop plants grown in the sodic soil.

Isolate	Host crop	16S rRNA gene homology	Similarity (%)
Co 2	Cotton	*Bacillus* sp.	99
Co6	Cotton	*Bacillus subtilis*	98
Co7	Cotton	*Pseudomonas* sp.	99
Ma9	Maize	*Alcaligenes* sp.	100
Ma11	Maize	*Bacillus mojavensis*	100
Ri2	Rice	*Pseudomonas fluorescens*	99
Ri5	Rice	*Bacillus* sp.	99
Ri6	Rice	*Bacillus* sp.	99
So2	Sorghum	*Pseudomonas* sp.	99
So4	Sorghum	*Bacillus* sp.	100
So5	Sorghum	*Bacillus* sp.	100
Bh1	Bhendi	*Bacillus marcorestinctum*	99

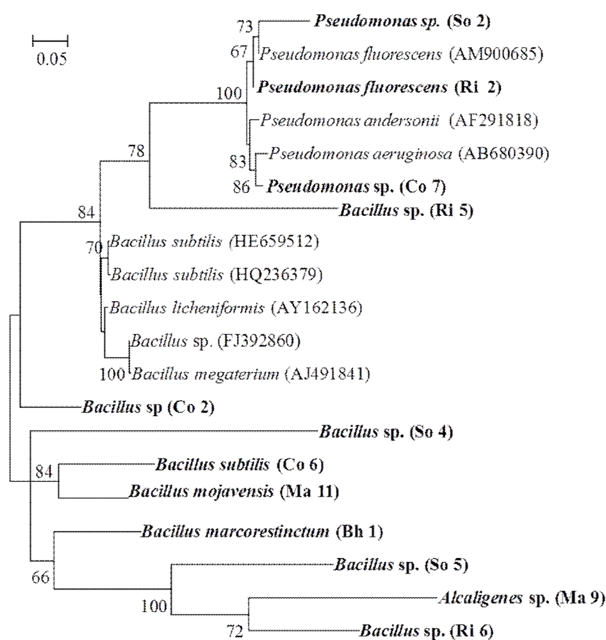

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence (1500 bp) from endophytic bacterial isolates using neighbor-joining method. The data of other 16S rRNA gene sequences of soil bacteria were from GenBank. The bacterial species, strain and GenBank accession number used in this analysis are given in the parenthesis. The boot-strap values of 500 and above are shown as per cent at the nodes.

The endophytic bacteria assimilate atmospheric nitrogen and convert it into ammonia, transferring this molecule to the plant metabolism (Gaiero *et al*. 2013). The nitrogen fixing ability of bacterial endophytes was assayed in the present study by acetylene reduction assay (Fig. 2). All the 12 isolates showed nitrogen-fixing activity, however, the isolate Ma11 (*Bacillus mojavensis*) showed higher acetylene reduction activity of 97.4 n moles C_2H_4 mg protein/hr followed by Ri 2 (*Pseudomonas fluorescens*) with 81.8 n moles C_2H_4 mg protein/hr. Similarly, Yan *et al*. (2018) reported the nitrogenase activities of five endophytic nitrogen-fixing isolates with acetylene reduction assay.

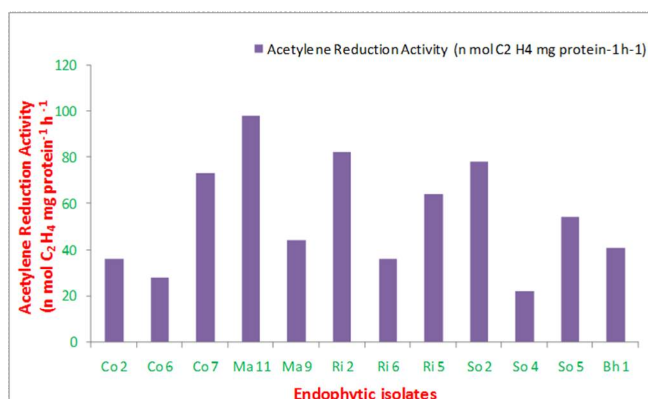

Fig. 2. Acetylene reduction activity of endophytic bacterial isolates from crops grown in sodic soils.

The well-known phytohormones that are produced by endophytic microbiota are IAA, which is synthesized via the indole-3-pyruvate (IPyA) pathway (Singh *et al*. 2017). The capacity to produce IAA is widespread among soil and plant-associated bacteria. In the present study, all the endophytic bacterial isolates produced considerable amounts of IAA in nutrient broth supplemented with tryptophan (Table 2). The isolate Ri 2 (*P. fluorescens*) produced a higher amount of IAA (7.12 µg/ml) subsequently by the isolate Ma 11(*B. mojavensis*- 5.52 µg/ml). Previous results showed that many endophytic bacteria including, *Pseudomonas*, *Serratia* and *Bacillus* are capable to synthesize IAA (Liu *et al*. 2010). Endophytic isolates, namely *Bacillus, Lysinibacillus, Pseudomonas, Psychrobacillus* and *Microbacterium* were reported to secrete IAA more efficiently (Yu *et al*. 2016).

All the selected endophytic bacterial isolates were scrutinized for their phosphate solubilizing functions by detecting extracellular solubilization of precipitated $Ca_3 (PO_4)_2$ by keeping glucose as the sole source of carbon. Except for two (Ri 5 and Bh1), all the other endophytic isolates showed notable phosphate solubilization activity (Table 2). On the basis of solubilization zone, the isolate Ma 11 (*B. mojavensis*) showed higher solubilization of mineral phosphate (0.58 mm) followed by Ri 2 (*P. fluorescens*) with a solubilization zone of 0.46 mm. Phosphate solubilization by *Bacillus* sp. isolated from the salt-stressed environment had been observed by earlier researchers (Son *et al*. 2006). Siderophore secreted by bacteria aid in promoting plant growth through enhanced direct iron availability to plants under iron-deficient conditions or by inhibiting the availability of iron to plant pathogens (Ahmad *et al*. 2008). Among the 12 isolates, 8 isolates (Co-2, Co-7, Co-6, Ma-11, Ri-2, Ri-6, So-4 and So-5) produced siderophore, as evidenced by the change of color in the CAS blue medium from bluish-green to orange (Table 2). *Bacillus* sp., *Stenotrophomonas* sp. and *Pseudomonas* sp. identified as endophytic bacterial isolates are efficient in siderophore production (Jasim *et al*. 2014).

Synthesis of 1-aminocyclopropane-1-carboxylate deaminase is one of the vital characteristics of plant growth-promoting microbes and endophytes. ACC deaminase cleaves ACC, the precursor of the hormone ethylene, to produce *α*-ketobutyrate and ammonia (Todorovic and Glick 2008). The isolate Ri 2 (*P. fluorescens*) showed higher deamination of ACC (405.2 nmol α-ketobutyrate mg/hr) subsequently by the isolate Ma 11(*B. mojavensis*) with a value of 387.8 nmol α-ketobutyrate mg/hr (Table 2). It has been noticed that ACC deaminase-producing bacteria improve plant growth under stress conditions. The present results are co-supported by previous reports of ACC deaminase production in many isolated strains of *Paenibacillus* and *Bacillus* (Xu *et al*. 2014, Gupta and Pandey 2019). ACC deaminase producing endophytes isolated from saline environments mitigated salinity stress in a various plants by reducing ethylene levels (Etesami and Beattie 2018). This trait of endophytic bacterial isolates can be attributed to significant growth enhancement of crop plants in the sodic soil stress environment.

Table 2. IAA production, Phosphate solubilization, ACC deaminase production and Siderophore production of endophytic bacterial isolates.

Endophytic bacterial isolate	IAA production (µg/ml)	Phosphate solubilization zone (mm)	ACC deaminase (nmol of α-ketobutyrate mg/hr)	Siderophore production
Co2	1.57	0.22	265.8	+
Co6	0.84	0.38	128.7	+
Co7	2.14	0.36	205.6	+
Ma9	3.46	0.24	106.2	-
Ma11	5.52	0.58	387.8	+
Ri2	7.12	0.46	405.2	+
Ri6	3.89	0.15	258.2	+
Ri5	2.04	0.0	56.5	-
So2	0.77	0.36	61.2	-
So4	1.28	0.28	194.2	+
So5	0.58	0.26	212.0	+
Bh1	2.14	0.0	58.4	-

+ presence; - absence.

It is apparent from Table 3 that only 50% of the selected endophytic isolates exhibited antifungal activity. The isolate Ma 11 (*B. mojavensis*) developed fungal resistance against three fungal pathogens *viz*., *M. phaseolina, F. oxysporum* and *Pythium* sp. Likewise, Ri 2 (*P. fluorescens*) isolate showed antifungal activity against *M. phaseolina, R. solani* and *Pythium* sp. The capacity of endophytic bacteria colonizing internal plant tissues to safeguard host plants from soil-borne pathogens was reviewed by Eljounaidi *et al.* (2016). Several reports examining the ability of *Bacillus* strains in suppressing plant pathogens exist (Ren *et al*. 2013). Egamberdieva *et al.* (2017) reported antagonistic activity of the endophytic isolate *B. subtilis* NUU4 against *Fusarium oxysporum, F. solani, F. culmorum, Alternaria alternata,* and *Botrytis cinerea* under salt stress condition. Similarly, Hernandez *et al.* (2018) studied the antifungal activity of certain endophytic bacteria *viz*., *Pantoea* sp., *Enterobacter* sp., *Citrobacter* sp. and *Paenibacillus* sp. and found that they exhibited resistance against *Fusarium oxysporum*.

The use of beneficial microbial inoculants for plants is a successful partial alternative for hazardous chemical fertilizers (Suman *et al*. 2020). To achieve this, several endophytic bacterial isolates were isolated from various agricultural crops grown in sodic soils. Endophytic organisms

were examined for their plant growth-promoting traits such as, N_2 fixation, phosphate solubilization, IAA production, ACC deaminase production, siderophore secretion and antifungal activity. In addition, all the observed PGP traits were assessed for relating them with the crop-wise endophytic isolates by principal component analysis. The observation plot showed the orthogonal positions of endophytic bacterial isolates (Fig. 3A) and loading plot showed their assessed PGP traits (Fig. 3B) explained by the first two components (PC1 and PC2) are presented as Fig. 3. The PC1 added 47.07% variability with PC2 added additional variability of 21.60% to a total cumulative variability of 68.67%. All the endophytic isolates were evenly and randomly arranged in the observation plot. This result revealed no relation between the PGP traits of the endophytes and the crop source being used for isolation (Fig. 3A).

Table 3. Screening of endophytic bacterial cultures for antimicrobial activity against plant pathogens.

Endophytic bacterial isolate	*In vitro* antagonistic assay			
	Macrophomina phaseolina	*Fusarium oxysporum*	*Rhizoctonia solani*	*Pythium* sp.
Co2	+	-	-	-
Co7	-	-	-	-
Co6	-	+	-	-
Ma9	-	-	-	-
Ma11	+	+	-	+
Ri2	+	-	+	+
Ri6	-	-	-	-
Ri-5	-	-	-	-
So2	-	-	-	-
So4	-	-	-	-
So5	-	+	-	-
Bh 1	-	-	+	-

+ presence and (-) absence of *in vitro* antagonistic activity.

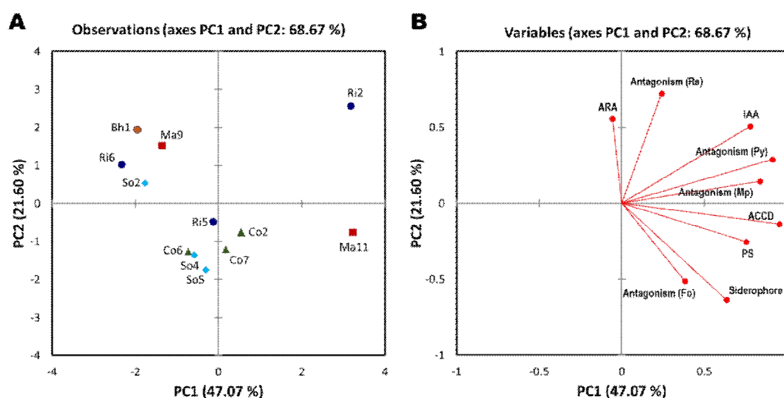

Fig. 3. Principal component analysis plots relating the endophytic bacterial isolates from crops grown under sodic soil and their PGP traits.(A) Scoring plot showing the positions of PGPR strains from different crops; (B) Loading plot showing orthogonal positions of assessed PGP traits. The % variance explained by each component (PC1 and PC2) is given in parentheses in axes. ARA - acetylene reduction assay; IAA - indole-3-acetic acid production; ACCD - 1-aminocyclopropane-1-carboxylate deaminase; Antagonism against Rs - *Rhizoctonia solani*; Py - *Pythium* sp., Mp - *Macrophomina phaseolina*; Fo - *Fusarium oxysporum*.

All the assessed variables taken for PCA were orthogonally organized in the PC1 and PC2 positive quadrant (left-hand top) and the PC1 positive and PC2 negative quadrant (left-hand bottom) of the plot. Corresponding to these two plots, the prospective endophytic isolates (Ma 11 and Ri 2) were positioned in the "high" (left-hand top) and "moderate" (left-hand bottom) quadrants of the PCA plot (Fig. 3B). The endophytic bacteria with less-PGP traits are negatively correlated with the variables and positioned in the "low" quadrant, which is negative for both PCs. All the observed variables had a significant contribution to either PC1 or PC2. The ACCD activity, IAA, P solubilization, siderophore, antagonism against *Macrophomina* and *Pythium* contributed 10 to 21% variability to PC1 and nitrogenase, IAA, siderophore and antagonism against *Fusarium* and *Rhizoctonia* contributed 13 to 20% contribution to PC2. The PCA results identified two potential endophytic strains *viz*., Ri 2 and Ma11 from sodic soil-grown crops.

The present findings demonstrated that the endophytic bacterial isolates Ma 11 (*Bacillus mojavensis*) and Ri 2 (*Pseudomonas fluorescens*) have positive response to the plant growth-promoting qualities proving their role in promoting the growth of crop plants under sodicity. These multifaceted endophytic bacterial isolates possessing plant growth-promoting and antagonistic potential can be developed as new inoculant suitable for sodic soils.

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