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Culturable endophytic bacteria from halotolerant *Salicornia brachata* L. : Isolation and plant growth promoting traits

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Abstract: Twenty two endophytic bacteria were isolated from the roots of *Salicornia brachiata* L. were characterized on the basis of morphology and biochemical characteristics, out of which five potential endophytes were selected based on their PGPR activity and identified by 16S rRNA gene sequence analysis as *Bacillus aereus* SA1 (KY953566), *Serratia nematodiphila* SG1 (KY953567), *Pantoea agglomerans* SG2 (KY953568), *Enterobacter sp.* SL (KY953569) and *Enterobacter sp*. SRh (KY953570). All the five selected endophytic bacterial strains produced IAA. Siderophore production was observed in *Serratia nematodiphila* SG1, *Pantoea agglomerans* SG2 and *Enterobacter sp.* SL. Only *Serratia nematodiphila* SG1 solubilised tricalcium phosphate and ACC Deaminase production were observed with *Bacillus aereus* SA1 and *Enterobacter sp.* SL. *Bacillus aereus* SA1, *Serratia nematodiphila* SG1 and *Enterobacter sp*. SRh withstand higher salt concentration of (8 % NaCl) whereas *Pantoea agglomerans* SG2 and *Enterobacter sp.* SL tolerated 7 % of NaCl. Endophyte *Pantoea agglomerans* SG2 was tolerant to 17 antibiotics whereas *Enterobacter sp.* SL tolerant to only 4 antibiotics.

Key words: *Salicornia*, Halophyte, Endophyte, Plant growth promotion

Introduction

Salicornia from family Amaranthaceae, widely known as pickle weed, [1] found at the edges of wetlands, marshes and alkaline flats [2]. *Salicornia* is a succulent, bushy plant with a high salt

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tolerance which is rich in vitamins A and C, calcium, iron, iodine, magnesium, sodium, and amino acids, low in calories and fat, contain no cholesterol and add fibre to the diet. However their medicinal values have been explored [3] but the efficiency of associative bacteria for plant growth promotion not fully understood.

Rhizosphere is the most prominent zone for the microbial interactions with plant roots. However, some of the bacterial species enter inside the plant tissue and resides as endophytes without causing any symptoms [4]. During colonization the endophytic bacteria resides in almost every internal part of plant [5,6]. Endophytes are superior in growth promotion over the rhizobacteria due to their adaptations against abiotic or biotic stresses [7]. Bacterial endophytes affect plant growth through direct mechanisms like, producing phytohormones IAA (Indole 3-Acetic Acid), gibberellins, cytokinins [8], phosphate solubilisation [9], N_2 fixation [10] or indirectly by production of antibiotics [11], siderophores [12] and lytic enzymes against the pathogens [13]. Common soil bacteria such as *Pseudomonas, Burkholderia* and *Bacillus* that produce diverse range of secondary metabolites, antibiotics and volatile organics invade plants and combat the deleterious effects of pathogens by mechanisms in line with the Plant Growth Promoting Rhizobacteria (PGPR) [14, 12] and counteract the adverse effects of salinity for sustainable agriculture.

The underground *Salicornia* roots favour growth of various microbial communities or endophyte, which modulates plant growth through the synthesis of biochemical and secondary metabolites to adjust plant against salt stress. The present study was undertaken to study the endophytic bacterial community of the *Salicornia* roots and their response to the salinity stress and plant growth promoting activities.

Materials and Methods

Isolation of endophytes

For isolation of endophytic bacteria, roots of *S. brachiata* L. were collected from the Danti farm, substation of Navsari Agricultural University, Gujarat, India. Fresh and healthy roots were washed to remove soil thoroughly under running tap water and dissected in small pieces followed by surface sterilization (70 % C_2H_5OH, 3 min,0.5 % NaOCl, 3 min and 70 % C_2H_5OH, 30 sec) and rinsed thrice with sterile distilled water [15]. Surface sterilization efficiency was checked by inoculating surface sterilized root samples on nutrient agar plate, prior to inoculation of endophytic bacteria. The surface sterilized roots were air dried, further sliced into thin sections and placed aseptically over LB agar plate and incubated at 30°C for 2–4 days in bacteriological incubator. The bacterial colonies surrounding root sections were picked and streaked on the fresh LB agar for the selection of single endophyte. Aseptic condition was maintained during whole isolation procedure.

Characterization of bacterial isolates

Endophytic bacterial isolates were characterized on the basis of biochemical characteristics by Bergey's manual of determinative bacteriology [16] and molecular phylogeny by 16S rRNA gene

sequencing. Genomic DNA was isolated using GeneiPure™ bacterial DNA purification kit (Bangaluru, India) following the manufacture's protocol. Universal eubacterial primers 27F- 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R- 5' CGGTTACCTTGTTACGACTT 3' were used to amplify approximately 1400 bp region of 16S rRNAgene using a thermal cycler (Eppendorf, Germany). Amplified products were resolved by agarose-gel electrophoresis (1.5%), and visualized using a gel documentation system (Bio Rad, USA). The amplicons were purified using GeneiPure™ quick PCR purification kit and quantified at 260 nm using a Nanodrop (Thermo scientific). The purified partial 16S rDNA amplicons were sequenced in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, CA, and USA).

Analysis of 16S rDNA sequences

The partial sequences of nucleotides were compared with available sequences from NCBI databases and sequences showing >98% similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLAST N) program available at the National Centre for Biotechnology Information(NCBI) BLAST server (www.ncbi.nlm.nih.gov/BLAST).

PGP traits analysis

Production of IAA

Bacteria were cultivated at 28 ± 2 °C for 48 h in LB broth supplemented with 400 µg ml^{-1} of L-tryptophan and harvested through centrifugation (8000 rpm, 10 min). Supernatant (2 ml) was mixed with 2 drops of ortho-phosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35 % of perchloric acid, 1 ml of 0.5 M $FeCl_3$ solution) [17]. Production of IAA was confirmed by the development of pink colour.

Phosphate solubilization

The bacterial strains were inoculated on the Pikovskaya medium containing tri-calcium phosphate on agar plate and incubated at 28 ± 2 °C for 2–3 days [18]. Development of clear halozone around the strains exhibited their positive phosphate solubilisation activity.

Siderophore production

The cultured bacterial strains were spotted on the Chrome azurol S agar plate [19]. Development of yellow orange hallow zone around the bacterial spot has been considered as positive indication for siderophore reproduction.

Antibiotic sensitivity test

The bacterial endophytes were inoculated in the Luria broth. The fresh culture was spreaded on Luria Agar plate and after drying discs containing antibiotics (Icosa universal 1A, Icosa disc I and II, HiMedia) were placed on the plates aseptically followed by incubation at 37 ± 2 °C for overnight [20]. After incubation, the zone of inhibition was observed.

ACC deaminase production

The isolates were point inoculated on DF salt minimal medium containing ACC (1-Aminocyclopropane 1- Carboxylate) as sole nitrogen source ([21]. Briefly, the composition of salt minimal media containing ACC as sole nitrogen source in g L^{-1} is as follows, KH_2PO_4, 1.36; Na_2HPO_4, 2.13; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.7; $FeSO_4.7H_2O$, 0.2; $CuSO_4.5H_2O$, 0.04; $MnSO_4.H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; H_3BO_3, 0.003; $CoCl_2.6H_2O$, 0.007; $Na_2MoO_4.2H_2O$, 0.004; Substrate ACC, 5 mM; Glucose, 1.0% dissolved in 1000 mL of distilled water. Growth on these plates shows positive result for ACC deaminase production.

Salt tolerance Test

To check salt tolerance efficiency, endophytes were streaked on LB media containing different concentration of NaCl (1-9%) and incubated at 28 ± 2 °C to check the salt tolerance of the isolates [22].

Results

A total of 21 different bacterial clones were isolated from the sliced *Salicornia brachiata* roots while no bacteria were observed near the surface sterilized root samples. Out of 21 clones 5 clones were identified based on their good PGPR activities by16S rRNA gene sequence as *Bacillus aereus* SA1 (KY953566), *Serratia nematodiphila* SG1 (KY953567), *Pantoeaagglomerans*SG2 (KY953568), *Enterobacter sp.*SL(KY953569) and *Enterobacter sp*. SRh (KY953570) which are belonged to γ Proteobacteria (*Pantoea, Serratia, Enterobacter*) and Firmicutes(*Bacillus*) (Table 1).

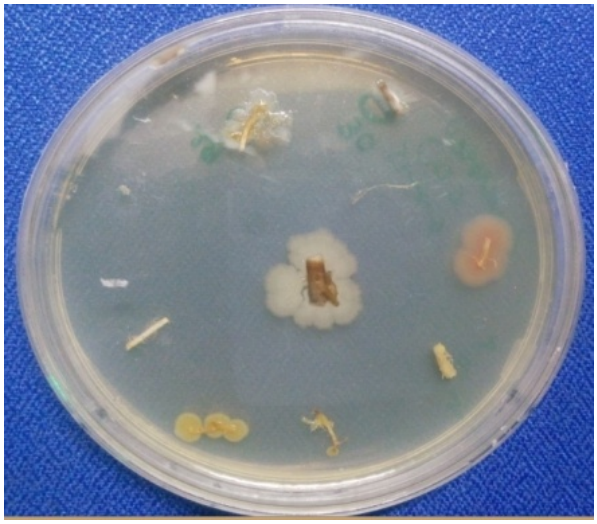

Fig. 1. Isolation of bacterial endophytes from surface sterilized root tissues.

Table 1 Closest relative of the isolated strains as revealed by 16S rRNA gene sequencing

Sr no.	Source	Strain	Accession no.	Nearest Phylogenetic Neighbour	Similarity (%)	E- value
1	*Salicornia brachiata*	SA1	KY953566	*Bacillus aerius strain* YM-14	98%	0
2	*Salicornia brachiata*	SG1	KY953567	*Serratia nematodiphila* strain PDRT	98%	0
3	*Salicornia brachiata*	SG2	KY953568	*Pantoea agglomerans* strain HPC126	100%	0
4	*Salicornia brachiata*	SL	KY953569	*Enterobacter sp.* GN21	99%	0
5	*Salicornia brachiata*	SRh	KY953570	*Enterobacter sp.* TF1-40	100%	0

PGP traits

All the five selected endophytic bacterial strains produced IAA with maximum by *Serratia nematodiphila* SG1 and minimum by *Enterobacter sp*. SRh on supplementation of 400 µg ml^{-1} L-tryptophan. Siderophore production was observed in *Serratia nematodiphila* SG1, *Pantoea agglomerans* SG2 and *Enterobacter sp.* SL. Only *Serratia nematodiphila* SG1 solubilised tri-calcium phosphate. ACC deaminase production was observed in *Bacillus aereus* SA1 and *Enterobacter sp.* SL. The endophytes strains showed different level of tolerance to the increasing salt concentration strain. *Bacillus aereus* SA1, *Serratia nematodiphila* SG1 and *Enterobacter sp*. SRh withstand higher salt level (8 % NaCl) whereas *Pantoea agglomerans* SG2 and *Enterobacter sp.* SL tolerated 7 % of NaCl (Table 2).

Table 2: Biochemical and plant growth promoting activity (intensity wise +++ is Highest, ++ is Intermediate and + is Lowest producing isolates) of selected bacterial endophytes.

Sr. no.	Isolate Code	Salt tolerance	Amylase production	cellulase production	Protease production	Siderophore production	ACC deaminase production	Phosphate solubilization	IAA production
1	SA1	+++	+++	+++			++		+
2	SA2								+
3	SB	++				+		+	+
4	SC		++	++		+		+	

5	**SD**					+			
6	**SF**								
7	**SG1**	+++	+++	+++		+		++	++
8	**SG2**					+			++
9	**SI**								
10	**SJ**	++	+++	+++					
11	**SL**					++	++		+
12	**SN**		+++	+++				+	
13	**SO1**								
14	**SP**		+++	+++					
15	**SQ**		+++	+++				+	
16	**SRh**	++	+++	+++					+
17	**SS**		+++	+++					
18	**ST**		+++	+++	+			+	
19	**SU**		+++	+++	+				
20	**Sd**		+++	+++		+	+	+	
21	**Sh**	+	+++		+				
22	**Se**							+	

Antibiotic sensitivity test

Antibiotic sensitivity pattern of the endophytic bacterial isolates were determined against thirty four different antibiotics by Icosa antibiotic disc diffusion method. Results shown in Table 3 depict that endophyte *Pantoea agglomerans* SG2 of *S. brachiata* was tolerant to 17 antibiotics whereas *Enterobacter sp.* SL tolerant to only 4 antibiotics.

Discussion

The *S. brachiata* roots were rich in endophytic bacterial diversity. These endophytic bacterial isolates belonged to four different genera *Bacillus, Pantoea, Serratia* and *Enterobacter.* These strains previously reported as endophytes in different plant species like *Bacillus aerius* from *Spharanthus indicus* [23], *Pantoea agglomerans* from citrus [24], *Serratia nematodiphila* from *Solanum nigrum* [25], *Enterobacter sp.* from *Curcuma longa* L. [26].

Table 3: Antibiotic resistance of selected isolates

Sr no.	Antibiotic name	Conc. (mg)	SA1	SG1	SG2	SL	SRh
1	Roxithromycin	30	+				
2	Clarithromycin	15		+	+		
3	Cefadroxil	30	+	+	+		+
4	sulbactrim	10			+	+	
5	Augmentin	30	+		+		+
6	Cephaloxin	32		+	+		
7	Clindamycin	2			+		
8	Erythromycin	15			+	+	
9	Vancomycin	30		+	+		
10	Oxacillin	1			+	+	
11	Linezolid	30	+		+		
12	Teicoplanin	10			+		
13	Methicillin	5			+		
14	Amoxyclave	30			+		+
15	Novobiocin	5		+	+	+	
16	Ampicillin	10	+		+		
17	Amoxycillin	10			+		+
18	Cefoxitin	30	+		+		+

IAA is the most common plant hormone, which stimulate the growth and reproduction in plants [27] and also involved in cell enlargement and division, tissue differentiation, physiological processes [28, 29]. IAA produced by bacteria increases pool of total IAA along with plant IAA and affects plants by diverse ways from pathogenesis to phytostimulation. The amount of IAA produced by bacteria play important role in plant–microbe interaction [30]. During the plant growth promotion trait analysis, all 5 endophytic strains produced significant amount of IAA. The extent of IAA production was found maximum in case of *Serratia nematodiphila* SG1 and minimum in *Enterobacter sp*. SRh in the presence of tryptophan. The modulation of plant growth takes place by optimal IAA concentration range. The study of Persello-Cartieaux [31], proved that inoculation of IAA producing bacteria *Pseudomonas thivervalensis* at the amount of 10^5 CFU ml^{-1} in *Arabidopsi*s resulting reproducible morphological changes but the amount of 10^6 CFU ml^{-1} inoculants inhibit the plant growth.

Siderophore production by the bacterial strain is one of the biocontrol mechanisms. The iron-chelation by bacteria makes them better competitors for the available iron and in this way, prevents growth of the pathogenic microorganisms. In this study siderophore production was observed in *Serratia nematodiphila* SG1, *Pantoea agglomerans* SG2 and *Enterobacter sp.* SL.

Plant growth promoting bacteria solubilizes insoluble phosphates to make them available to plants which enhances crop productivity. Viable and sufficient number of efficient phosphate solubilizing microorganisms (PSM) which are often called as "microphos" has provided some solution to the phosphorus insolubility problems [32]. When applied to seed, plant surfaces or soil, PSM colonizes the interior of the plant (endophytes) and facilitate growth by providing solubilized phosphorus to growing plants [33]. *Serratia nematodiphila* SG1 solubilised phosphate which strengthens the results as reported previously in *Bacillus sp.* and *P. putida* [34, 35].

The endophytic bacterial isolates reside and multiply in the plants where the environment contains relatively high ionic strength which successively tolerated both the biotic and abiotic factors. Previously many authors reported the endophytic strain which successively tolerated the high salt concentration [35, 36, 37]. In this study the endophytic isolates were able to grown differentially at different salt levels. In a previous study, *Pseudomonas sp.* tolerated up to 4 % NaCl, while *Bacillus sp.* 2 % NaCl [36]. The endophytic bacterial strains of *Momordic acharentia* showed tolerance to 4–10 % NaCl [38].

The ACC, which is the immediate precursor of C_2H_4 (ethylene), mainly exuded by plant and taken up by the bacteria and hydrolysed by ACC deaminase results in NH_3 and α-ketobutyrate formation [39], and hence, it strongly alleviates the stress induced by ethylene-mediated impact on plants by lowering the ethylene levels in plants [40]. The bacteria utilize the NH_3 evolved from ACC as a source of N and thereby restrict the accumulation of ethylene within the plant, which otherwise inhibits plant growth [41]. Here, ACC Deaminase production was observed in *Bacillus aereus* SA1 and *Enterobacter sp.* SL.

Many endophytic bacterial strains exhibited antibiotic properties that inhibit the growth of an antagonistic bacterium. The allele-chemicals including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes [42, 43] synthesized by PGPR and used in the management of plant diseases. *Pantoea agglomerans* SG2 was tolerant to 17 antibiotics. The antibiotic disc acts differentially on the growth of same bacterial strains of different isolation source.

Conclusion

The diverse endophytic bacterial strains (SA1, SG1, SG2, SL and SRh) are isolated from the root of *S. brachiata*. They harbour PGP traits of variable degrees to establish symbiotic relationship with the host. All five strains produced IAA; one solubilized phosphate; two produced ACC deaminase; one is tolerant to most antibiotics; three produced siderophore and tolerated high salt (7.5 % NaCl) concentration during salinity tolerance. The use of culture-dependent method has a certain practical significance of reflecting diversity and distribution of endophytic bacteria and their functional role in plant ecological adaptation especially with special habitats such as in salty soil of south Gujarat. Further, this isolation is also dependent on time, tissue and procedure used to isolate these bacteria. However, such isolation opens up new horizon for understanding plant-endophytes symbiotic relationship. Therefore, isolating the endophytes from root and providing

them to crops with perfect formulation might help plants to overcome deleterious effects of abiotic stresses at certain extent.

Author Contributions

Haidar Abbas and Ramesh Patel designed the experimental scheme. Haidar Abbas optimized the scheme and performed the research. Vipul Parekh analyzed the sequencing data. Ramesh Patel supervised the research.

Conflict of interest

The authors declare no conflict of interest.

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