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Research Article

CULTURABLE ENDOPHYTIC BACTERIA FROM HALOTOLERANT *Salvadora persica* L.: SCREENING, ISOLATION AND PLANT GROWTH PROMOTING TRAITS

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Abstract- Twenty three endophytic bacterial isolates were isolated from the roots of *Salvadora persica* L. were characterized on the basis of various criteria such as morphology and biochemical characteristics, out of which six potential endophytes were selected based on their PGPR activity and identified by 16S rRNA gene sequence analysis as *Citrobacter sp.* A6 (KY587407), *Pantoea agglomerans* A10 (KY587408), *Pseudomonas oryzihabitans* A16 (KY963571), *Serratia marcescens* A20 (KY963572) *Enterobacter aerogenes* A23 (KY963573) and *Bacillus sp*. A26 (KY963574). Only *Citrobacter sp.* A6 (KY587407), *Pantoea agglomerans* A10 (KY587408) were able to produce IAA. Siderophore production was observed in *only Enterobacter aerogenes* A23. All isolates solubilized tricalcium phosphate except *Bacillus sp*. A26 and ACC deaminase production were observed in *Citrobacter sp.* A6 and *Bacillus sp*. A26. All isolates could withstand higher salt level (7 % NaCl) whereas *Serratia marcescens* A20 tolerated up to 5 % of NaCl.

Key words- *Salvadora persica*, Halophyte, Endophyte, Plant growth promotion

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Introduction

Salvadora persica L. of the family *Salvadoraceae* is an evergreen shrub, 4-6 m tall with a short trunk; also called as meswak, has white bark and smooth green leaves. It is one of the most commonly used medicinal plants for oral hygiene among global Muslim community [1]. Noumi and co-worker [2] reviewed the history, use of Meswak as an oral hygienic tool and the biological effects of its extract. *Salvadora* is the habitant of varieties of location ranging from mangroves, in saline land, swamps, thorn shrubs, deserts and flooded plains to near riverbanks where ground water level is high indicating their tolerance to a wide range of water, soil pH and salinity [3]. The *Salvadora* species have a number of proven pharmacological importance [4-7]. However, their medicinal values have been explored [8] but the efficiency of associative bacteria for plant growth promotion is not fully understood. Various bacteria collectively referred to as Plant Growth Promoting Rhizobacteria (PGPRs) colonize the rhizosphere of many plant species and some of which invades inner tissues without causing any symptoms [9], termed as endophytes, have beneficial effects on host plant growth, which is manifested physiologically as increased plant growth and reduced susceptibility to diseases [10]. These bacteria exhibit properties of phosphorus solubilization and siderophore production, which allow host plants to efficiently uptake phosphorus- and iron-derived nutrients from the soil, respectively. In addition, certain endophytes may interfere with the biosynthesis of phytohormones, especially auxin and ethylene. IAA produced by endophytes increases total pool of hormone along with plant IAA [11].The direct association effect of bacterial indole acetic acid (IAA) production and modified root architecture of the host was clearly demonstrated between *A. baselines* and wheat [12]. Also, many microbes excrete 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, which is encoded by the conserved *acdS* gene of endophytes and degrade the ethylene precursor ACC to ammonia and α-ketobutyrate, thereby reducing the level of ethylene which normally inhibits plant growth. Some of the other characteristics like phosphate solubilization [13] and N_2 fixation [14] have been extensively studied for plant growth promotion. As mentioned, the modes of action of PGPR related factors are thus diverse and complex, but the mechanistic basis is still largely unknown, especially at the molecular level. The underground *Salvadora persica* roots favour growth of various microbial communities or endophytes, which modulates plant growth through the synthesis of biochemical and secondary metabolites to adjust plant against salt stress. The present investigation was undertaken to study the endophytic bacterial community of the *Salvadora persica* roots and their response to the salinity stress and plant growth promoting activities.

Materials and Methods

Isolation of endophytes

For isolation of endophytic bacterial isolates, roots of *S. persica* L. were collected from the coastal area of Dandi, South 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 were 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 1500 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). Theamplicons 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 was retrieved by Nucleotide Basic Local Alignment Search Tool (BLAST N) program available at the National Center 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 100–400 µg $ml^{-1}$of L-tryptophan and harvested through centrifugation (8000 rpm, 10 min). Supernatant (2 ml) was mixed with 2 drops of orthophospheric acid and 4 ml of the Salkowski reagent (50 ml, 35 % of perchloric acid, 1 ml 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 tricalcium phosphate on agar plate and incubated at 28 ± 2 °C for 2–3 days [18]. Development of clear halo zone around the strains exhibited their positive phosphate solubilization activity.

Siderophore production

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

ACC deaminase production

The isolates were point inoculated on DF salt minimal medium containing ACC as sole nitrogen source [20]. 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

To check salt tolerance efficiency endophytes were streaked on LB media containing different concentration of NaCl (4-9%) and incubated at 28 ± 2 °C to check the salt tolerance of the isolates [21].

Results

A total of 23 different bacterial clones were isolated from the sliced *Salvadora persica* roots while no bacteria were observed near the surface sterilized root samples. Out of 21 clones 6 clones were identified based on their good PGPR activities by16S rRNA gene sequence as *Citrobacter sp.* A6 (KY587407), *Pantoea agglomerans* A10 (KY587408), *Pseudomonas oryzihabitans* A16 (KY963571), *Serratia marcescens* A20 (KY963572) *Enterobacter aerogenes* A23 (KY963573) and *Bacillus sp.* A26 (KY963574) which are belonged to γ Proteobacteria (*Pantoea, Serratia, Enterobacter*, *Citrobacter*) and Firmicutes (*Bacillus*) [Table-1] and [Fig-1].

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	*Salvadora persica*	A6	KY587407	*Citrobacter sp. BAN69*	99%	0
2	*Salvadora persica*	A10	KY587408	*Pantoea agglomerans*	92%	0
3	*Salvadora persica*	A16	KY963571	*Pseudomonas oryzihabitans strain 7*	98%	0
4	*Salvadora persica*	A20	KY963572	*Serratia marcescens strain JW-QS2*	100%	0
5	*Salvadora persica*	A23	KY963573	*Enterobacter aerogenes 8a*	99%	0
6	*Salvadora persica*	A26	KY963574	*Bacillus sp. NCCP-975*	99%	0

PGP traits

Only *Citrobacter sp.* A6 (KY587407) and *Pantoea agglomerans* A10 (KY587408) endophytic bacterial strains produced IAA on supplementation of 400 µg ml^{-1} L-tryptophan [Fig-2]. Siderophore production was observed in only *Enterobacter aerogenes* A23. All isolates solubilized phosphate except *Bacillus sp.* A26 and ACC Deaminase production were observed in *Citrobacter sp.* A6 and *Bacillus sp.* A26. All isolates could withstand higher salt level (7 % NaCl) whereas *Serratia marcescens* A20 tolerated up to 5 % of NaCl [Table-2].

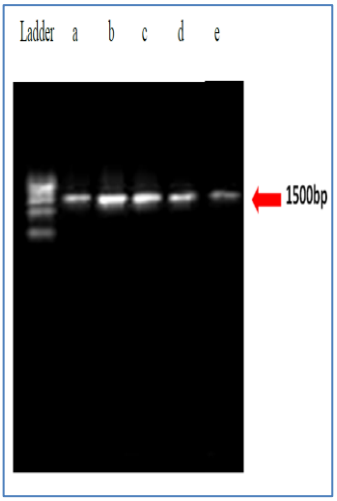

Fig-1 IAA production assay of bacterial endophyte.a) A6, b) A10, c) A16, d) A20 and e) A23.

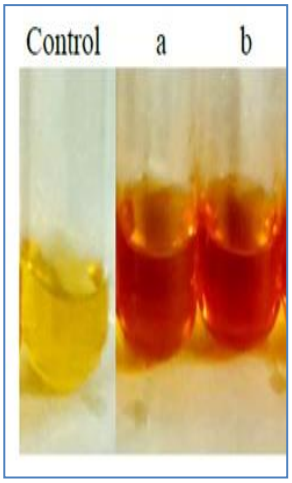

Fig-2 IAA production assay of bacterial endophytes. a) A6, b) A10.

Discussion

The *S. persica* roots were colonized by diverse endophytic bacteria. 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* [22], *Pantoea agglomerans* from citrus [23], *Serratia nematodiphila* from *Solanum nigrum* [24], *Enterobacter sp.* from *Curcuma longa* L [25]. Many endophytic microorganisms have the potential to produce IAA. This may be a reason for the increased growth promotion of some plants when the plant is colonized with endophytes as bacterial IAA increases total pool of IAA along with plants IAA [26]. IAA is the most common plant hormone, which stimulate the growth and reproduction in plants [27] and is also involved in cell enlargement and division, tissue differentiation, physiological processes [28].

Table-2 *Biochemical and plant growth promoting activity (intensity wise) of endophytes*

Sr. no.	Isolates	1.1M	Amylase Production	Cellulase Production	Protease Production	Siderophore Production	ACC Deaminase Production	Phosphatase Production	IAA Production
1	A4	+				+		++	
2	A5	+				+		+++	
3	A6	+	+	++	++		+		+++
4	A10	+		+++				+++	+++
5	A11	+						+++	
6	A14				+	++		++	
7	A16	+						+	+++
8	A18	+			++	++		++	
9	A19							+++	
10	A20	+		+	+			+	+
11	A21	+		+					+
12	A23	+				+++		+++	
13	A24					+	+	+++	+++
14	A26	+	+	+++	++		+		
15	A28	+				++		+++	
16	A29	+					+	++	+++
17	A32	+						+++	
18	A33	+						+	
19	A35			++				++	
20	A37	+		++					+
21	A39		+	+++					+
22	A41	+					+		+++
23	A42							+++	+++

For the microbial synthesis of IAA in tryptophan dependent route, tryptophan is used as the precursor. The amount of IAA produced by bacteria play important role in plant–microbe interaction [29]. The modulation of plant growths takes place by optimal IAA concentration range. In the study [30] it is found that inoculation of IAA producing bacteria *Pseudomonas thivervalensis* at the amount 10^5 CFU ml^{-1} in Arabidopsis resulting reproducible morphological changes but the amount of 10^6 CFU ml^{-1} inoculants inhibit the plant growth. The ability of the production of IAA by different species of *Pseudomonas* was reported by many authors [31]. During the plant growth promotion trait analysis, only *Citrobacter sp.* A6 (KY587407) and *Pantoea agglomerans* A10 (KY587408) strains produced significant amount of IAA. The extent of production was found maximum in case of *Citrobacter sp.* A6 in the presence of tryptophan. IAA produced by bacteria increases pool of total IAA along with plant IAA and affects plants by diverse ways from pathogenesis to phyto-stimulation. 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. Different species of *Bacillus* have been reported to have the ability to produce of siderophores [32,33]. Many reports reveal the ability of both gram negative bacterial isolates (*Pseudomonas* sp.) and bacterial genera of *Bacillus* and *Rhodococcus* that belongs to the gram positive group with the capability to produce siderophores [34]. In this study siderophore production was observed in *Enterobacter aerogenes* A23. Plant growth promoting bacteria solubilize insoluble phosphates to make them available to enhance crop productivity. When applied to seed, plant surfaces or soil, PSM colonizes the interior of the plant (endophytes) and facilitate growth by providing phosphate to growing plants [35]. All isolates except *Bacillus sp.* A26 solubilized phosphate. 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 [36-39]. 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 [40]. The endophytic bacterial strains of *Momordica charentia* showed tolerance to 4–10 % NaCl [41]. ACC, 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 [42], and hence, it strongly alleviates the stress induced by ethylene-mediated impact on plants by lowering the ethylene levels in plants [43]. This decrease in the levels of ACC and ethylene may prevent the ethylene-mediated plant growth inhibition. Endophytic microbes with these capabilities residing inside the host plants can benefit the host by reducing the stress and increasing the plant growth [44]. Alizadeh [45] has explained the application of the ACC deaminase which has been synthesised by different genera of *Pseudomonas* in increasing the senescence of the plants. The bacteria utilize the NH_3 so evolved from ACC as a source of N and thereby restrict the accumulation of ethylene within the plant, which otherwise inhibits plant growth [46]. ACC deaminase production was reported by *Citrobacter sp.* A6 and *Bacillus sp*. A26.

Conclusion:

The diverse endophytic bacterial strains (A6, A10, A16, A20, A23 and A26) were isolated from the root of *S. persica*. They harbour PGP traits of variable degrees to establish symbiotic relationship with the host. Out of six, two strains produced IAA; five solubilized phosphate, two produced ACC deaminase, one produced siderophore and five tolerated high salt (7 % NaCl) concentration during salinity tolerance. Further, these isolates need to be analyzed for beneficial effects on plants. Also, change in metabolites of treated plants need to be compared with control plants which might reveal exact mechanism of plant growth promotion.

Application of research: Identification of Plant Growth Promoting Bacterial endophytes help to investigate the possible association of these bacteria with crops and can combat adverse biotic and abiotic stresses of environment.

Research Category: Microbiology, Plant- Microbe interaction

Abbreviations:

PGP: Plant Growth promotion
ACC: 1- Amino Cyclopropane 1- Carboxylate
IAA: Indole Acetic Acid
NaCl: Sodium Chloride
LB: Luria Bertani
NaOCl: Sodium Hypochlorite

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