ISOLATION OF SALT TOLERANT BACTERIAL ENDOPHYTES FROM HALOPHYTIC GRASSES

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Abstract. The present study was aimed at isolating halotolerant endophytic bacteria from three halophytic grasses, namely *Sporobolus marginatus* A. Rich., *Urochondra setulosa* (Trin.) C.E. Hubb and *Leptochloa fusca* (L) Kutnth. Out of total 56 bacterial isolates, 24 were screened from *S. marginatus*, 15 from *U. setulosa,* and 17 from *L. fusca*. The identified bacterial endophytes belong to diverse bacterial genera, i.e., *Pseudomonas, Bacillus, Enterobacter, Microbacterium, Rhizobium, Chryseobacterium, Brevibacillus, Klebsiella, Proteus, Escherichia, and Agrobacterium.* Among these, *Pseudomonas*, *Bacillus,* and *Enterobacter* were more prominent in all three halophytic grasses. *S. marginatus* seems to be more enriched as compared to *U. setulosa* and *L. fusca* in terms of the number of isolates. Root and node seem to be the preferred explants. The isolates were further tested for their salt tolerance potential, as a result of which 24 isolates belonging to *Pseudomonas*, *Bacillus,* and *Enterobacter* genera demonstrated continual growth up to 10% salt concentrations (NaCl), suggesting their significant salt tolerating capability. Thus, the three halophytic grasses seem to be excellent reservoirs of halo-tolerant bacterial endophytes. *Pseudomonas*, *Bacillus,* and *Enterobacter* were more frequent as well as more competent genera in dealing with salinity stress and could be exploited for their plant growth-promoting potential in bioremediation of extreme saline soils and to enhance crop yield under salinity stress.

Key words: salinity; halophytic grasses; endophytes; sustainability; halotolerant.

INTRODUCTION

Salinity is a major threat to the agricultural world in various parts of the world. About 1,000 million hectares (M ha) of agricultural land throughout the world is affected by salt to varying degrees. In India, 6.74 million ha of land are affected by sodicity and salinity, which are considered to be increasing in the coming decades [21, 24]. Certain geogenic and anthropogenic factors led to deterioration of the soil quality and productivity, raising the issue of high salinity in the soil. This causes impairment in the water and osmotic potential in the plant, leading to interrupted plant growth via reduced water uptake. A large number of sodium ions interfere with native soil physiochemistry by affecting nutrient absorption and uptake mechanisms [9, 11, 17]. In addition, the presence of excess salt in water and soil leads to several morphological and physiological abnormalities in plants. These abnormalities have been due to osmotic stress and an ionic imbalance due to ionic stress [21, 23]. The toxic Na^+ and Cl^- ions interfere with the normal physiological processes that lead to diminished membrane stability, altered nutrient balance, altered levels of growth regulators, enzymatic inhibition, and metabolic dysfunction, including photosynthesis, which ultimately leads to plant death [13, 14, 25]. Halophytes can naturally reproduce and endure in environments with salt concentrations exceeding 200 mM of NaCl, i.e., 20 dS/m, constituting approximately 1% of the world's flora [7, 12]. The economic yield and biomass of halophytes are higher than those of non-halophytic plants in the same saline soil. So, these plants could serve as model systems to study adaptation mechanisms in saline conditions. All plants are colonized by a remarkable number of microorganisms that have profound effects on plant growth and development, seedling vigor, seed germination, nutrition, diseases, and productivity. In the presence of high salt concentrations and extreme pH ranges, the microbiota of halophytes should be adapted to the saline nature of the soil, contributing to the growth of the plants. Endophytic bacteria or fungi are endosymbiont microorganisms that reside inside the plant for its entire life cycle or part of it without causing harm to the plant. After forming mutualistic interactions with the host plant, they stimulate plant growth, health, and productivity due to their biocontrol and biofertilizing activities [16, 19]. Halophytes are able to adapt to harsh and extreme habitats because of their genetic makeup and association with the endophytic microbiome. This association enhances plant tolerance to saline habitats and promotes growth simultaneously. Today, the harmful effects of chemical usage in agriculture are not hidden in terms of the number of harmful diseases and severe allergies caused by polluted water, land, and air. Moreover, the constant use of specific, high-yielding genetically modified varieties has become one of the major reasons for the loss of approximately 75% of genetic diversity in crops [4]. Thus, there is an urgent need for eco-friendly farming approaches that forbid the use of synthetic fertilizers and chemical methods to treat saline soils and strongly favor crop-raising methods that ensure soil health, biological diversity, and sustainability. Moreover, no earlier reports were available on the isolation and screening of endophytes from these halophytic grasses, namely *S. marginatus, U. setulosa,* and *L. fusca.* Hence, the present study is the first attempt to screen salt-tolerant endophytes from these three halophytic grasses.

MATERIALS AND METHODS

Collection of raw materials and chemicals

The study was conducted on the three grass halophytes, namely *S. marginatus, U. setulosa,* and *L. fusca*. Seeds and root slips of *S. marginatus* and *U. setulosa* were collected from severe saline lands of the Kutch plains, Bhuj, Gujarat, India, and *L. fusca* (Karnal grass) was acquired from an extreme sodic region of Uttar Pradesh, India. These grasses were raised through root cuttings (as germination through seeds was very poor) during the month of April in pots packed with sandy loam soil under natural conditions. After getting established in pots, grasses were shifted to microplots of dimensions of 2.5m·1.5m·0.5m in the Division of Crop Improvement, ICAR-Central Soil Salinity Research Institute (CSSRI), Karnal (29°43'N, 76°58'E and 245 m above the mean level of sea), Haryana, India. The irrigation was provided with treatment of salinity i.e., EC_e: 30 dS·m^{-1}. To maintain the desired saline level in the pots, the screen house was covered with a good-quality polythene sheet to avoid rainwater. The sample explants (leaves, roots, and nodes) were collected in the months of August to September from all three grasses and packed in sealed plastic bags until reaching the laboratory, where further work on the isolation and characterization of bacterial endophytes was done. All the chemicals were of high purity analytical grade and purchased from Hi-Media Laboratories Pvt. Ltd.

Isolation of endophytic bacteria

The explants of all three grasses were thoroughly rinsed with running tap water for at least 20 minutes, followed by washing with mild detergent (Tween-20) and subsequently with double-distilled water before processing. The explants were then surface-sterilized to eliminate surface microbes by soaking in 70% ethanol for 60 seconds, followed by a sodium hypochlorite (1% available chlorine) solution for 90 seconds, and subsequently rinsing in sterile double-distilled water for 2-3 times under a laminar air flow cabinet. The small pieces (0.5–1.0 cm) of explants were placed on nutrient agar medium using the aseptic procedure. The media was supplemented with an antifungal agent, Amphotericin B, at a concentration of 10 mg/mL to suppress fungal growth. After the completion of the incubation period (37°C for 24-48 hours), morphologically distinct colonies were selected and streaked repeatedly to obtain pure isolates.

Morphological and phylogenetic analysis of endophytic bacteria

The morphological analysis was done via gram's staining. The phylogenetic and molecular characterization was carried out through outsourcing via commercial company (BIOKART INDIA Pvt. Ltd., Bengaluru) using 16S rDNA sequencing method by means of PCR (Polymerase chain reaction) via the following universal primers [6].

Table 1. Universal primers used for molecular characterization

S. No.	Forward primer sequence
1	16S- 27F: 5'-AGAGTTTGATCCTGGCTCAG-3'
2	16S-63F: 5'-CAGGCCTAACACATGGAAGTC-3'
3	16S-395: 5'-AGAGTTTGATCMTGGCTCAG-3'
S. No.	**Reverse primer sequence**
1	16S- 1492R: 5'- GGTTACCTTGTTACGACTT-3'
2	16S-1387R: 5'-GGCGGATGTGTACAAGGC-3'
3	16S-396: 5'TACGGYTACCTTGTTAACGACTT-3'

These universal primers were used for amplification of the 1500-bp region of the 16S rDNA gene of the isolate. The DNA sequence reaction was carried out by using a chemistry cycle sequencing kit, 'Big Dye Terminator version 3.1, via a sequencing machine, the ABI 3130xl Genetic Analyzer. The sequences obtained were compared with the sequences of the closest relatives in GenBank of the NCBI (National Center for Biotechnology Information) by way of BLAST. A distance matrix was generated using the Jukes-Cantor corrected distance model, in which alignment inserts were ignored, preferring only the alignment model positions. The phylogenetic tree was created using the weighted neighbour joining method (clustering-based method) with an alphabet size of 4 and a length of 1000 [3]. The estimation of sampling distribution was analyzed by boot strapping (the statistical method) by creating a pseudo-alignment for generating a distance matrix and a tree, repeating the process 100 times.

Halotolerant assay

All the isolates were screened at varying concentrations of sodium chloride (NaCl), ranging from 0.5 to 10% on nutrient agar medium providing incubation at 37°C for 48 hours.

RESULTS

In the present study, a total of 56 pure bacterial isolates were obtained from the surface-sterilized explants (root, node, and leaf) of three halophytic grasses established under saline conditions (EC_e: 30 dS m^{-1}) in which 24 were screened from *S. marginatus*, 15 from *U. setulosa* and 17 from *L. fusca*. The highest number of isolates were obtained from root explants of *S. marginatus* (KC1) as shown in Table 2. The effectiveness of the surface sterilization protocol was a crucial step for disinfecting explants by achieving total elimination of epiphytic microorganisms from sample explants. This step proved to be accurate in our study due to the absence of any growth on the control plate after culturing the aliquots of water from the last rinsing of the surface sterilization process, followed by incubation at 37°C for 24-48 hours.

Preliminary identification was done on the basis of gram staining in which 17 were found to be gram-positive and 39 were gram-negative. The identified bacterial endophytes were from diverse bacterial genera i.e., *Pseudomonas, Bacillus, Enterobacter, Microbacterium, Rhizobium, Chryseobacterium, Brevibacillus, Klebsiella, Proteus, Escherichia,* and

Table 2: Identified bacterial endophytes isolated from halophytic grasses - *S. marginatus* (KC1), *U. setulosa* (KC2) and *L. fusca* (KC3)**.**

S.No.	Halophytic grasses	Type of explant	No. of isolates	Culture code	Name of the organism identified	Closest Homologue
0	**1**	**2**	**3**	**4**	**5**	**6**
1	*S. marginatus* (KC1)	Leaf	3	KC1-d	*Bacillus* sp. EGY-SC*R3	*Bacillus cereus* strain EGI100
				KC1- k	*Pseudomonas* sp. strain C8	*Pseudomonas guguanensis* strain 4-n-1
				KC1-1-1L	*Bacillus* sp. Pc10	*Bacillus cereus* strain NC7
		Node	4	KC1-g	*Pseudomonas aeruginosa* strain CNEB5	*Pseudomonas aeruginosa* strain CNEB25
				KC1-1-3N	*Chryseobacterium indologenes* strain CIG 2219	*Chryseobacterium indologenes* SB1
				KC1-1-5N	*Bacillus* sp. strain J-ZH13	*Bacterium* FJAT-13834
				KC1-1-6N	*Agrobacterium* sp. RA65	*Rhizobium* sp. strain BD1
		Root	17	KC1-a	*Pseudomonas aeruginosa* strain RLimb	*Pseudomonas aeruginosa* strain PA75
				KC1-b	*Pseudomonas yangonensis* strain MY63	*Pseudomonas yangonensis* strain MY50
				KC1-c	*Pseudomonas* sp. strain SS46	*Pseudomonas* sp. strain ZZH-1
				KC1-e	*Pseudomonas aeruginosa* strain HB6(39)	*Pseudomonas aeruginosa* strain JAYT3
				KC1-f	*Pseudomonas monteilii* strain NBFPALD_RAS131	*Pseudomonas aestus* strain RKS80
				KC1- i	*Enterobacter hormaechei* subsp. xiangfangensis strain CDDS 11	*Enterobacter hormaechei* subsp. xiangfangensis strain HFB11
				KC1-j	*Pseudomonas hibiscicola* strain R4-721	*Pseudomonas hibiscicola* strain R4-790
				KC1-1-7R	*Pseudomonas* sp. strain H16S-48	*Pseudomonas* sp. strain H11S-28
				KC1-1-8R	*Bacillus altitudinis* strain 11-1-1	*Bacillus* sp. strain P13-3
				KC1-1-10R	*Pseudomonas* sp. strain MBL0322	*Pseudomonas* sp. strain PF37X
				KC1-1-12R	*Pseudomonas hydrolytica* strain DSWY01	*Pseudomonas alcaliphila* strain TXF8
				KC1-1-13R	*Klebsiella quasipneumoniae* strain MAHI 4	*Klebsiella pneumoniae* strain VRBG-48
				KC1-1-14R	*Pseudomonas aeruginosa* strain MLTBM2	*Pseudomonas aeruginosa* strain AB18
				KC1-1-16R	*Bacillus* sp. A6(2008)	*Bacterium* strain 27
				KC1-1-17R	*Pseudomonas aeruginosa* strain XSF-65	*Pseudomonas aeruginosa* strain XSF-39
				KC1-1-19R	*Enterobacter cloacae* strain R16	*Enterobacter cloacae* subsp. dissolvens strain L3
				KC1-1-20R	*Pseudomonas sp.* strain NT-4	*Pseudomonas oleovorans* strain CEMTC_4335
2	*U. setulosa* (KC2)	Leaf	4	KC2-g	*Pseudomonas aeruginosa* strain DM_U24H	*Pseudomonas aeruginosa* strain ERI036-MG5-IND
				KC2-i	*Enterobacter cloacae* strain MC4	*Enterobacter* sp. strain EF2
				KC2-2-1L	*Bacillus cereus* strain CUMB ASB-06	*Bacillus cereus* strain SL1
				KC2-2-2L	*Bacillus licheniformis* strain RC-2.	*Bacillus licheniformis* strain LET 603
		Node	6	KC2-a	*Pseudomonas aeruginosa* strain RLimb	*Pseudomonas aeruginosa* strain PA75
				KC2-b	*Pseudomonas oryzihabitans* strain TH10	*Pseudomonas psychrotolerans* strain JUQ310
				KC2-d	*Enterobacter hormaechei* strain KA3	*Enterobacter hormaechei* subsp. xiangfangensis strain RSM5
				KC2-h	*Pseudomonas guguanensis* strain 4-n-1	*Pseudomonas mendocina* strain 3-n-1
				KC2-2-3N	*Pseudomonas* sp. strain NT-4	*Pseudomonas mendocina* strain Y20
				KC2-2-4N	*Bacillus manliponensis* strain YEBN5.	*Bacillus manliponensis* strain KH4-3
		Root	5	KC2-f	*Escherichia coli* strain ABRL132	*Salmonella* sp. S13
				KC2-2-6R	*Pseudomonas* sp. strain P3	*Pseudomonas* sp. strain B-BETUL-M
				KC2-2-N6R	*Pseudomonas guguanensis* strain 4-n-1	*Pseudomonas mendocina* strain 3-n-1
				KC2-2-8R	*Bacillus cereus* strain BXC6	*Bacillus thuringiensis* strain SEM1H4
				KC2-2-10R	*Pseudomonas* sp. YAZ41	*Pseudomonas* sp. strain FB68

0	1	2	3	4	5	6
3	*L. fusca (KC3)*	Leaf	4	KC3-c	*Microbacterium* sp. strain rgb91	*Microbacterium* sp. strain TRB173
				KC3-k	*Bacillus* sp. (in: Bacteria) strain 4N	*Bacillus* sp. (in: Bacteria) strain 7LG
				KC3-l	*Enterobacter cloacae* complex sp. R_G8	*Enterobacter* sp. strain DeltaPSK
				KC3-3-6L	*Bacillus* sp. (in: Bacteria) strain 4N	*Bacillus* sp. (in: Bacteria) strain 7LG
		Node	9	KC3-a	*Escherichia coli* strain CFS3313	*Escherichia coli* strain EC1
				KC3-b	*Pseudomonas yangonensis* strain MY63	*Pseudomonas yangonensis* strain MY50
				KC3-e	*Enterobacter cloacae* strain GX1Z-1L	*Enterobacter cloacae* strain FDAARGOS 1431
				KC3-f	*Bacillus* sp. strain LS13	*Bacillus* sp. BAC S1
				KC3-h	*Pseudomonas guguanensis* strain 4-n-1	*Pseudomonas mendocina* strain 3-n-1
				KC3-i	*Brevibacillus laterosporus* strain DY23	*Brevibacillus laterosporus* strain 4SG
				KC3-3-1N	*Bacillus velezensis* strain EBS4	*Bacillus velezensis* strain JS12Q
				KC3-3-2N	*Enterobacter* sp. strain SP-203	*Enterobacter cloacae* subsp. dissolvens strain L3
				KC3-3-4N	*Pseudomonas* sp. XC1	*Halopseudomonas formosensis* strain CC-CY503
		Root	4	KC3-d	*Rhizobium* sp. strain BD1	*Rhizobium* pusense strain 76
				KC3-g	*Bacillus stratosphericus* strain PD6	*Bacillus pumilus* strain 15.2'
				KC3-j	*Bacillus mycoides* strain EB66	*Bacillus* sp. (in: Bacteria) strain SWP1
				KC3-3-5R	*Proteus faecis* strain TJ1636.	*Proteus vulgaris* strain P3M

Agrobacterium. Among them, species of the genera *Pseudomonas*, *Bacillus,* and *Enterobacter* were more abundant in all three halophytic grasses. *S. marginatus* (KC1) seems to be more enriched with endophytic bacteria as compared to *U. setulosa* (KC2) and *L. fusca* (KC3). The root seems to be the most preferred explant. In the cases of *U. setulosa* and *L. fusca,* the node explants were more prolific as compared to leaf and root explants. All of these bacterial species were further tested for their halotolerant potential at varying concentrations of NaCl (salt), ranging from 0.5 to 10%. All of them were able to grow up to 0.5–5% salt concentration range. After this range, 24 isolates (species of *Pseudomonas*, *Bacillus,* and *Enterobacter* genera) demonstrated continual growth up to 10% salt concentrations suggesting their significant salt-tolerating ability (Figure 1).

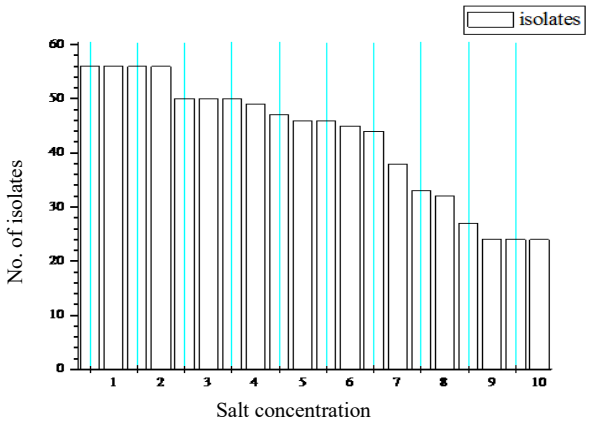

Figure 1. Effect of varying salt concentration (0.5-10%) on isolated bacterial isolates of the three halophytic grasses

DISCUSSION

In our study, a total of 56 pure bacterial isolates were screened from all three grass halophytes in which 24 were screened from *S. marginatus*, 15 from *U. setulosa,* and 17 from *L. fusca*. Albdaiwi et al. [1] isolated 74 bacterial isolates from durum wheat (*Triticum turgidum* subsp. durum) plants cultivated in saline environments in the Ghor region near the east of the Dead Sea, of which 38 were endophytes and 36 were rhizospheres. The root and node explants seems to be more abundant as compared to the leaf. According to some reports, the diverse endophytes may be located in different plant parts, i.e., root, stem, and leaf [8, 20]. The most preferred explant for endophytic localization is the root, followed by the stem and leaf. Root-associated bacteria may help the glycophytic plant survive in salt-affected soil and increase productivity by improving plant water relationships and mineral uptake [10]. The identified bacterial endophytic isolates were from diverse bacterial genera, i.e., *Pseudomonas, Bacillus, Enterobacter, Microbacterium, Rhizobium, Chryseobacterium, Brevibacillus*, *Klebsiella, Proteus, Escherichia,* and *Agrobacterium.* Many other studies also support the dominance of the genera *Bacillus*, *Enterobacter*, *Pseudomonas*, *Azotobacter*, *Arthrobacter*, *Streptomyces*, and *Isoptericola* in the plant microbiome, which successfully alleviate drought, salt, and heat stress in diverse crop plants [5, 15, 18]. The isolates were further tested for their salt tolerance potential. Out of total isolates, 24 were able to survive up to 10% salt concentrations. The plant growth promotion activity and the higher salinity tolerance capability of bacterial endophytes may be used as a significant alternative in agriculture in salt-stress-affected areas [2, 22]. Therefore, the screened halo-tolerant endophytic bacterial isolates can be explored successively for their biofertilizing and bioremedial potential of extreme saline soils and to enhance crop yield under salinity stress. Further, these isolates with high salt tolerance can collectively be employed for development of a microbial consortium which act as a natural and eco-friendly tonic/cushion and immunity booster for crops growing in extreme saline and acidic habitats.

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