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The Plant Growth Promoting Potential of Saline Tolerant Rhizobacteria under *In vitro* Saline Stressed Conditions

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Since the beginning of agricultural operations, soil salinity has been one of the most significant abiotic variables limiting agricultural production. Saline tolerant plant growth-promoting rhizobacteria (PGPR) enhance nutrient equitation and soil enzyme activities, which serve as indicators of soil biological health, and help to correct nutritional imbalances in plants. Potential bioinoculants for boosting crop yield in saline agriculture include saline tolerant plant growth-promoting rhizobacteria (PGPR). Therefore, in the present study the potential saline tolerant rhizobacteria isolated from saline patches of Karnataka were screened for Exopolysacchride production and Phosphate solubilization and phytohormone production potential under *in vitro* saline conditions. The results revealed that among the eight organisms were the highest EPS production, phosphate solubilization and phytohormone production recorded by *Bacillus subtilis* GAN-4 and *Staphylococcus cohnii* MAN-

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3 under both normal and saline stressed conditions compared to other organisms. Therefore, these strains show potential as plant growth-promoting rhizobacteria (PGPR) for mitigating salinity in saline agriculture.

Keywords: Exopolysacchride; saline tolerance; phosphate solubilization; phytohormones.

1. INTRODUCTION

"Climate change causes the sea level to increase, resulting in flooding and the incursion of saltwater into inland areas. According to reports, more than 50% of arable land will be in danger by 2050 as a result of soil salinization, which is brought on by incorrect irrigation methods, the overuse of chemical fertilizers, and an absence of adequate drainage systems" [1]. "Salinity negatively disrupts the physical and chemical properties of soil and has a greater impact on crop growth" [2]. "Beneficial microorganisms known as plant growth-promoting rhizobacteria (PGPR) may be crucial in reducing this scenario. As a beneficial substitute for inorganic fertilisers and pesticides, this group of rhizospheric bacteria effectively colonises plant roots and preserves soil fertility. PGPR has been shown to be beneficial at promoting crop growth in a variety of salt-stressed environments" [3]. "It has been reported that native strains of PGPR are more effective at boosting plant resistance to salinity stress than PGPR originating from the non-saline ecosystem" [4]. "The preliminary selection of locally-isolated salt-tolerant PGPR for salinity mitigation is crucial to ensure the effectiveness. These beneficial microbes possess several mechanisms for salt stress mitigation such as by retaining appropriate Na+/K+ ratio through secretion of extracellular polymeric substances called exopolysaccharide (EPS) that ensures their survivability under unfavourable soil conditions. Exopolysaccharides are also required for the generation of bacterial aggregation or flocculation yield, which is characterized by the selective adsorption of the polymeric segment and polymer bridging between cells" (Tenney and Stumm, 1958). Hence, the present study was conducted to determine the effect of salt-tolerant PGPR isolated from the saline patches of Karnataka.

2. MATERIALS AND METHODS

2.1 Saline Tolerant Isolates Source

This study utilized saline-tolerant strains isolated from the saline tracts of Karnataka, including *Staphylococcus gallinarum* GAN-1(OM491215), *Staphylococcus xylosus* GAN- 2(OM491216), *Bacillus subtilis* GAN-4(OM491217), *Staphylococcus simiae* GAN-6(OM491218), *Staphylococcus arlettae* GAN-7(OM491219), *Staphylococcus cohnii* MAN-3(OM491220), *Staphylococcus succinus* MAN-5(OM491221) and *Staphylococcus saprophyticus* BEL-2 (OM491222) [5].

2.2 Exopolysacchride Production

EPS helps to protect the bacteria from uncongenial conditions, thereby enabling their survival. The saline tolerant rhizobacterial isolates were tested for their ability to produce Exopolysacchride (EPS) in the absence and presence of 23% NaCl. The EPS was extracted from 3 days old isolates grown in Trypticase soya broth. Two ml of culture was centrifuged at 10,000 rpm for 10 min and 1 ml of supernatant was collected to which 2 ml of 90% ethanol was added and incubated at -20 ºC for 24 hours. The suspension was centrifuged at 8000 rpm for 15 minutes and the precipitate was dissolved in 2 ml of water, to this 200 µl of 5% phenol and 1 ml of 93% sulphuric acid was added and kept under room temperature for 10 minutes, the change of a yellow color was an indication for EPS production. The absorbance of the aliquot was recorded at 490 nm using a UV visible spectrophotometer (Thermo scientific, Biomate 3S, China). A calibration curve was prepared using standard stock solution of glucose at different concentrations. The EPS production was expressed as the concentration of reducing sugars [6].

2.2.1 Antioxidant activity of saline tolerant rhizobacteria

The antioxidant capacities of the bacterial extracts were determined based on the 2, 2'-azino-bis(3-ethyl-benzthiazolin-6-sulfonicacid) (ABTS) method according to Sun et al. [7] with few modifications. To form ABTS+, potassium persulfate (K2S2O8, Merck KGaA, Darmstadt, Germany) was added to ABTS, mixed and kept in dark conditions for 16 hrs at room temperature. Phosphate buffer (10 mM, pH 7.4

Table 1. HPLC conditions for quantification of phyotohomones

Properties	IAA	GA_3	ABA	SA
Stationary phase	C18 column	C18 column	C18 column	C18 column
Flow rate	1ml/min	0.8ml/min	0.8ml/min	1ml/min
Mobile phase	Methanol: water (80:20)	Methanol: water (70:30)	Acetonitrile:0.5%Acetic acid (80:20)	Acetonitrile:0.5%Acetic acid (90:10)
Wavelength	270 nm	208 nm	254 nm	302 nm
Column Temperature	30ºC	30ºC	30ºC	30ºC

Merck KGaA) was used to dilute $ABTS^+$ stock solution to a final absorbance of ca. 0.7 at 734 nm. Bacterial extracts or ascorbic acid (Sigma-Aldrich) which was used as standard (50 µL) were dissolved in 3 mL of diluted $ABTS^+$ solution. The scavenging activity of the bacterial extracts was assessed from the percentage of decolorization at 734 nm after 2 min of reaction at room temperature. The ABTS+ scavenging activity (%) was calculated using the equation as follows: $(OD_{734control}-OD_{734sample})/OD_{734control})*100$.

2.2.2 *In vitro* quantification of phosphate solubilization by the saline tolerant bacterial isolates

"The isolated bacterial culture was grown in nutrient broth and 1 ml of the actively grown culture was inoculated to the 100 ml of NBRIP medium with tri-calcium phosphate (without NaCl and with 23 % NaCl concentration) as a phosphate source and incubated at 28 °C for seven days. After seven days of incubation the pH of the medium were recorded and centrifuged at 10000 rpm for 5 minutes. To estimate the soluble phosphorus content in the medium 0.5 ml supernatant was taken and 1-2 drops of p-nitrophenol (0.25 %) was added as an indicator followed by addition of 5 N HCl drop wise to neutralize the colour. The above solution was diluted with 40 ml of double distilled water and 8 ml of ammonium paramolybdate-ascorbic acid reagent was added to the solution and incubated at room temperature for 20 minutes. The final volume of the solution was made up to 50 ml with double distilled water. The absorbance was read at 880nm by using a UV visible spectrophotometer (Thermo scientific, Biomate 3S, China)" [8,9].

2.3 Extraction and Estimation of IAA, GA_3 and ABA

2.3.1 Media conditions

a) Nutrient broth
b) Nutrient broth +Tryptophan
c) Nutrient broth + 23 % NaCl
d) Nutrient broth + 23 % NaCl +Tryptophan

2.3.2 Procedure for estimation of IAA, GA_3 ABA and SA

Twenty-four hours old grown culture was inoculated to the media described in section 3.9.5.1 and incubated at 37 °C for 7 days at dark condition. After seven days of incubation, it was centrifuged at 6000 rpm for 10 minutes. To the supernatant, 1N HCl was added and the pH was adjusted to 2.8. The total acidified supernatant taken in a 250 ml conical flask to which equal volume of diethyl ether was added and incubated in dark condition for 4 hrs. The samples were kept at 4 °C overnight in a separating funnel. Then organic phase discarded and the solvent phase collected. The upper layer allowed to evaporate and 2-3 ml of HPLC grade methanol was added and the IAA, GA_3, ABA and SA were quantified by a high-performance liquid chromatography (Shimdzu, Japan) by the conditions described.

2.4 Statistical Analysis

The experimental data was statistically analysed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool (www.icargoa.res.in/wasp2/index.php) and means were separated by Duncan Multiple Range Test (DMRT).

3. RESULTS AND DISCUSSION

3.1 Exopolysaccharides Production

The EPS produced by bacteria binds with excess Na+, enhances the uptake of water and helps stabilize the soil structure and aggregation under salinity stress, thereby aid during salinity stress [10]. The highest EPS was observed in *Bacillus subtilis* GAN-4 (64.72 µg ml^{-1}) followed by *Staphylococcus arlettae* GAN-7 (49.35 µg ml^{-1}) and *Staphylococcus cohnii* MAN-3 (45.09 µg ml^{-1}) in absence of NaCl. But in presence of 23%

NaCl the highest EPS production was recorded by *Bacillus subtilis* GAN-4 (66.39 µg ml^{-1}) followed by *Staphylococcus cohnii* MAN-3 (58.09 µg ml^{-1}) and *Staphylococcus succinus* MAN-5 (57.87 µg ml^{-1}) (Table 2).

EPS produced helps to protect the bacteria from uncongenial conditions, thereby enabling their survival. EPS producing saline tolerant bacteria could reduce the uptake of Na^+, Ca^+ and Mg^+ ions by binding with them in the soil and thereby reduces the plant's exposure to the ion under salinity stress conditions. In present study, *Bacillus subtilis* GAN-4 produced the highest levels of EPS as compared to other isolates under normal and salinity stress conditions. Similar results were reported by Mukherjee et al. (2019) who reported a halotolerant bacterium *Halomonas* sp. Exo1 was able to produce increased EPS at NaCl concentrations of upto 20%. Shultana et al., (2020) quantified the EPS production by saline tolerant bacterial isolates at different NaCl concentration. Results revealed that the highest EPS production was recorded by UPMRB9 (31.50 g L^{-1}) at 1.5M of NaCl concentration.

3.2 Antioxidant Activity

The antioxidant activity ($ABTS^+$ radical scavenging activity) of the saline tolerant rhizobacterial isolates was determined and the results revealed *Bacillus subtilis* GAN-4 had stronger antioxidant activity under both normal and saline stress conditions (60.48 and 69.12% respectively) when compared to other isolates (Fig. 1).

3.3 Phosphate Solubilization

"Phosphorus (P) is the second most vital macronutrient required by plants, next to nitrogen. Inorganic phosphorus readily gets transformed into less available forms by forming a complex with Al and Fe in acid soils or with Ca in calcareous soils. Some of the bacteria are known to improve the solubilization of fixed soil phosphorous and applied phosphates, resulting in higher yields even under stress conditions" [9]. The quantitative analysis of phosphate solubilization abilities of the saline tolerant rhizobacterial isolates was examined on NBIRP broth with Tri-calcium phosphate under normal and salinity stress conditions (Table 3). Results revealed that isolate *Staphylococcus cohnii* MAN-3 (33.55 µg ml^{-1}) and *Bacillus subtilis* GAN-4 (24.13 µg ml^{-1}) were able to solubilize higher phosphorous under saline stress condition. Similarly, under non-stress condition, these two isolates showed significantly higher phosphate solubilization abilities.

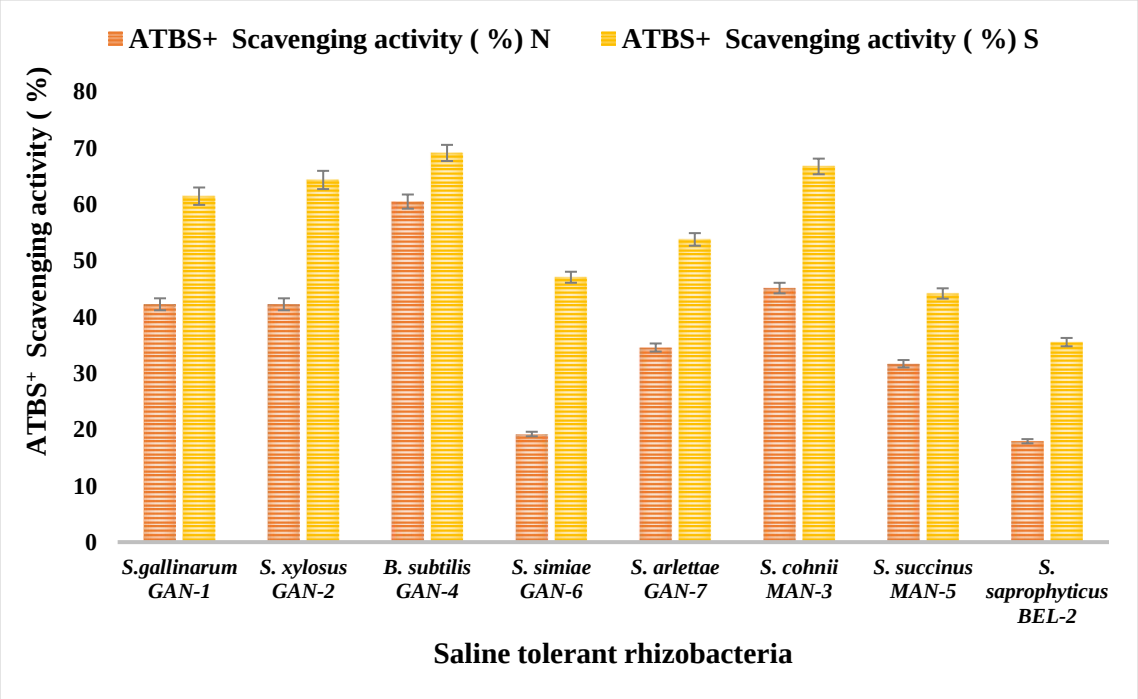

Fig. 1. Antioxidant activity ($ABTS^+$ scavenging activity (%) by the saline tolerant rhizobacteria under normal and salinity stress conditions

Table 2. Exopolysaccharide production of the saline tolerant rhizobacterial isolates under *In vitro* conditions

Saline Tolerant Rhizobacteria	EPS Production (µg ml^{-1})	
	N	S
S. gallinarum GAN-1	21.02^{e}	47.13^{d}
S. xylosus GAN-2	39.17^{d}	48.8^{d}
Bacillus subtilis GAN-4	64.72^{a}	66.39^{a}
S. simiae GAN-6	22.87^{e}	39.91^{e}
S. arlettae GAN-7	49.35^{b}	47.5^{d}
S. cohnii MAN-3	45.09^{c}	58.06^{b}
S. succinus MAN-5	38.8^{d}	57.87^{b}
S. saprophyticus BEL-2	24.3^{b}	10.02^{g}

Note: GAN- Gangavathi, MAN- Mandya and BEL-Bellary, N- Normal Condition, S- Saline Stress Condition Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

Table 3. Quantitative estimation of phosphate solubilization of the saline tolerant rhizobacterial isolates under *In-vitro* conditions

Saline Tolerant Rhizobacteria	Phosphate Solubilization (µg/ml)	
	N	S
S. gallinarum GAN-1	20.1^{d}	13.71^{f}
S. xylosus GAN-2	21.87^{c}	22.37^{c}
Bacillus subtilis GAN-4	26.49^{a}	24.13^{b}
S. simiae GAN-6	6.74^{f}	19.85^{d}
S. arlettae GAN-7	22.37^{c}	22.62^{c}
S. cohnii MAN-3	24.39^{b}	33.55^{a}
S. succinus MAN-5	14.39^{e}	17.16^{e}
S. saprophyticus BEL-2	24.3^{b}	10.02^{g}

Note: GAN- Gangavathi, MAN- Mandya and BEL-Bellary, N- Normal Condition, S- Saline Stress condition. Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

Bacteria capable of producing a halo/clear zone due to solubilization of inorganic phosphate in the surrounding medium were selected as potential phosphate solubilizers. Normal PGPR bacteria generally lose their plant growth promoting traits under saline conditions [10] hence, saline tolerant PGPR bacteria should be selected for employing to saline soils [11]. General mechanisms that are engaged by bacteria for solubilization of tri-calcium phosphate are lowering the pH of the media by secreting organic acids [12]. In this experiment, two saline tolerant rhizobacterial strains *Staphylococcus cohnii* MAN-3 and *Bacillus subtilis* GAN-4 showed higher in-vitro phosphate solubilizing efficiency compared to other strains, this might be due to secretion of organic acids by these strains. Similar results were obtained by Woranan et al. [13] who assessed the phosphate solubilization by saline tolerant bacteria PDMCd0501 which was recorded maximum phosphate solubilization (4.2) at 8 % NaCl concentration. Similarly, phosphate solubilizing bacterial isolates *Pantoea aallii* BD390, *Strenotrophomonas maltophilia* IAM 12423 and *Pseudomonas frederiksbergensis* DSM 13022 could form halozone in medium supplemented with 0.1 % TCP.

3.4 Phytohormone Production

The elite saline tolerant rhizobacterial isolates were examined for the production of IAA, GA_3, ABA and SA under stress and non-stress conditions, and the results are presented in Table 4 [14-16]. Among the eight isolates, *Bacillus subtilis* GAN-4 produced the highest IAA without tryptophan under both normal and salinity stress condition (2.33 and 5.22 mg^{-L} respectively) whereas *Staphylococcus cohnii* MAN-3 produced maximum IAA (11.14 and 38.08 mg L^{-1} respectively) with tryptophan under both normal and saline stress condition compared to other isolates. The results of GA_3 production recorded that isolate *Bacillus subtilis* GAN-4 (121.47 and 130.3 mg L^{-1} respectively) followed by *Staphylococcus cohnii* MAN-3 (117.52 and 126.53 mg L^{-1} respectively) under saline stressed and unstressed condition. The ABA and SA production were higher in *Bacillus subtilis* GAN-4 (4.39 and 3.39 mg L^{-1} respectively) when it was grown in stress conditions [17-19].

Table 4. Phytohormone (mg L^{-1}) production by saline tolerant rhizobacterial isolates under *In-vitro* conditions

Saline Tolerant Rhizobacteria	IAA				GA_3		ABA		SA	
	N		S		N	S	N	S	N	S
	- T	+T	-T	+T						
S. gallinarum GAN-1	1.47^{cd}	5.27^{e}	3.43^{c}	11.98^{d}	64.76^{c}	64.73^{d}	1.44^{cd}	2.04^{c}	1.21^{d}	2.53^{c}
S. xylosus GAN-2	1.79^{bc}	4.05^{f}	5.1^{a}	9.85^{e}	47.51^{f}	59.28^{e}	1.36^{cd}	2.38^{c}	2.02^{b}	1.95^{d}
Bacillus subtilis GAN-4	2.33^{a}	8.11^{b}	5.22^{a}	13.06^{c}	130.38^{a}	121.47^{a}	2.17^{a}	4.39^{a}	2.88^{a}	3.39^{b}
S. simiae GAN-6	2.00^{ab}	5.40^{e}	2.84^{d}	7.59^{f}	56.44^{e}	32.49^{g}	1.1^{d}	2.05^{c}	1.46^{cd}	2.56^{c}
S. arlettae GAN-7	1.18^{d}	5.21^{e}	4.10^{b}	10.14^{e}	37.59^{g}	35.7^{f}	1.45^{cd}	3.37^{b}	1.31^{cd}	2.05^{d}
S. cohnii MAN-3	2.15^{a}	11.14^{a}	5.17^{a}	38.08^{a}	126.53^{b}	117.52^{b}	1.85^{b}	4.65^{a}	2.14^{b}	3.94^{a}
S. succinus MAN-5	1.30^{d}	7.19^{c}	4.29^{b}	9.57^{e}	61.36^{d}	74.98^{c}	1.46^{c}	2.51^{c}	1.47^{cd}	2.63^{c}
S. saprophyticus BEL-2	1.19^{d}	6.08^{d}	2.41^{d}	16.24^{b}	65.94^{c}	35.95^{f}	1.13^{cd}	2.13^{c}	1.63^{c}	2.29^{cd}

Note: GAN- Gangavathi, MAN- Mandya and BEL-Bellary, N- Normal Condition, S- Saline Stress Condition, (-T)- without Tryptophan, (+T)- with Tryptophan, IAA- Indole Acetic Acid, GA_3- Gibberellin, ABA- Abscisic Acid, SA-Salicylic Acid. Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

"IAA is the most common type of auxin, it regulates various aspects of plant development and growth. Different levels of IAA production has been reported in bacteria" [20]. "A halotolerant bacterium, *Kocuria turfanensis* 2M4 was found to be dependent on L-tryptophan for producing IAA and could produce 38 µg ml^{-1} of IAA in presence of 600 µg ml^{-1} of tryptophan" [21]. Sarkar et al. [22] observed that "a halotolerant *Enterobacter* sp. produced higher IAA levels in the absence of NaCl. Which decreased with the increasing NaCl concentration. ABA is also synthesized by strains of ST-PGPR including *Proteus mirabilis*, *Bacillus megaterium*, *B. lichenifor mis*, *Pseudomonas fluorescens*, and *Achro mobacter xylosoxidans"* [23].

4. CONCLUSION

This study characterizes two locally-isolated PGPR strains, *B. subtilis* GAN-4 and *S. cohnii* MAN-3 which demonstrate saline tolerance and plant growth-promoting properties under saline conditions. Utilizing these microbial inoculants offers a cost-effective and environmentally friendly method to alleviate stress and enhance plant yields, thus improving the management of saline soils for better crop productivity. Consequently, the promising potentials of *B. subtilis* GAN-4 and *S. cohnii* MAN-3 make them suitable candidates for biofertilizer applications aimed at mitigating salinity in affected areas.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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