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Isolation and Characterization of Saline Tolerant Rhizobacteria from Saline Tracts of Karnataka

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Abstract

The present study aims to isolate and characterize the saline tolerant rhizobacteria from rhizosphere soil samples collected from saline tracts of Karnataka *viz.,* Gangavathi, Bellary and Mandya. Totallyeighty five isolates were isolated by using TSA medium. These isolates were screened for different salt concentrations upto 23 per cent of NaCl. Among the isolates, only eight isolates were able to tolerate 23 per cent of salt concentration compare to other isolates and these isolates characterized morphologically and biochemically. These isolates were further screened for their plant growth promotion potential under *in-vitro* condition. The results showed that among the isolates, GAN-4 and MAN-3 exhibited positive response to all the *in-vitro* PGPR characteristics studied. Similar results were recorded for root and shoot length of tomato where isolates GAN-4 and MAN-3 showed significantly increased root and shoot growth.

Keywords : Rhizobacteria, Salinity, Saline tolerance

In recent times, environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides and soil pH are major limiting factors in crop production because they affect almost all plant functions. Climate change leads to the rise of seawater level, which causes flood and triggers the intrusion of saltwater into the inland areas. It is reported that more than 50 per cent of arable land will be threatened by 2050 due to the effect of soil salinization which is the consequence of climate change, improper irrigation practices, excess application of chemical fertilizers, and lack of proper drainage systems (Chandrasekaran, *et al.,* 2014). Inappropriate management of soil and application of impermissible levels of fertilizers ruptures the overriding connection between the soil and microbes (Yalavarthi *et al*., 2020). This is currently evidenced in the degraded soils (*i.e.,* saline soils of India) which are caused by modern agricultural practices. Salinity destructively interrupts the physical and chemical properties of soil as well as affects crop growth to a higher extent (Singh, 2016). To mitigate this situation, beneficial microorganisms known as plant growth-promoting rhizobacteria (PGPR) could play an important role. This group of rhizospheric bacteria could effectively colonize plant roots and maintain soil fertility by offering a favourable alternative to inorganic fertilizers and pesticides (Majeed *et al.,* 2015). The effectiveness of PGPR to increase the growth of various crops under salt stress conditions have been reported previously (Cardinale *et al.,* 2015; Soldan *et al.,* 2019). The preliminary selection of locally-isolated salt-tolerant PGPR for salinity mitigation is crucial to ensure the effectiveness and it has been reported that the indigenous strains are more efficient in boosting plant resistance to salinity stress compared to PGPR organisms originated from the non-saline ecosystem (Etesami and Beattie, 2017). The term 'induced systemic tolerance' (IST) has been proposed for PGPR induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress. In comparison to reports of inducing systemic resistance in plants by PGPR, fewer reports have been published on PGPR as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess. The PGPR are the cheap and easily available sources for the mitigation of diferent biotic and abiotic stresses. PGPR usually improve plant growth promotion by triggering plant growth hormones, antioxidant system, produce siderophore and enhances nutritional capacity of the plants (Kumar and Verma,

2017). Bio-inoculants are the promising and updated technology in spite of many advantages not only over agrochemicals but also carrier based biofertilizer several reasons major being the viability of organism (Gurumurthy and Shivaprakash, 2017). Addition of beneficial microorganisms in the form of biofertilizers to soil will help replenish soil health (Sneha and Brahmaprakash, 2017). Therefore the present study is focused to survey soil habitat in the saline soils and isolate rhizobacteria from saline environment, to characterize and screen the isolates for PGPR and salinity tolerance.

Material and Methods

Isolation and Characterization of the Saline Tolerant Rhizobacterial Isolates from the Saline Soils of Karanataka

Collection of Soil Sample

Survey has been conducted to collect soil samples from saline soils of karanataka, (Gangavathi, Mandya and Bellary). Soil samples were collected from crop fields of maize, rice, sorghum and tomato. From each crop field five soil samples at a depth of 10 to 15 cm were taken randomly at a distance of about 1 m and about five hundred gram of soils was placed in plastic bags and transported to the laboratory. A total of 10 soil samples were collected from each location.

Isolation of Saline Tolerantrhizo Bacteria from Soil

The soil sample collected from different locations were further used to isolate salt tolerant rhizo bacteria on NaCl amended Trypticase soya agar medium by serially dilution method and incubated at 30 °C for 48 hrs. Purified by four way streak plate method and preserved on agar slants at 4 °C for further study. The isolates obtained has been examined for their salt tolerant efficacy as per the standard protocols.

Screening of Saline Tolerant Rizobactreial Isolates at different Salt Concentrations

The isolates were further examined for salt tolerance capacity on NaCl 5, 10, 15, 20 and 23 per cent amended Trypticase soya agar by streak plate method and selected elite isolates for further study.

Morphological Studies of Saline Tolerant Rhizobacterial Isolates

All the bacterial isolates were subjected to study their morphological characters based on the colony characters, Gram reaction, cell shape as per the standard procedures given by (Barthalomew and Mittewer, 1950).

Biochemical Characterization

The biochemical characterization of the isolates were essentially carried out as per the procedures outlined by (Cappuccino and Sherman, 1992). The tests conducted are detailed below.

Hydrogen Sulphide Production

Sulfide indole motility (SIM) agar stabs were inoculated with the bacterial isolates and incubated at 30 °C for 48 hrs. Black coloration along the line of stab inoculation indicated H_2S production.

Catalase Test (Blezevic and Ederer, 1975)

The nutrient agar slants were inoculated with test organisms and incubated at 30 °C for 24 hours. After incubation the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for production of gas bubbles. The occurrence of gas bubbles was scored positive for catalase activity.

Starch Hydrolysis (Eckford, 1927)

The ability of the isolates to hydrolyse starch was examined by the petri plates containing two per cent starch agar. Inoculated with test cultures and incubated at 30 °C for three days. After incubation the plates were flooded with Lugols iodine solution and allowed to stand for 15 - 20 minutes. The clear halo zone around the colony was considered as positive for the test.

Screening of Saline Tolerant Rhizobactreial Isolates for Plant Growth Promoting Activity under Normal and Stress Condition

Ammonia Production (Cappuccino and Sherman, 1992)

Actively grown 48 hrs old cultures of 100µl were added to 10 ml of the peptone water with and without NaCl (23%) separately into individual tubes. Inoculated tubes were kept for 72 hrs of incubation. After 72 hrs of incubation, 0.5 ml of Nessler´s reagent was added and observed for colour development. Indication of brown to yellow colour indicates the positive test for ammonia.

Zinc Solubilization

Bacterial isolates were inoculated to check the zinc solubilizing capacity in Bunt and Rovira medium with and without 23 per cent NaCl concentration for maintaining stress and controlled conditions, respectively, containing 0.1 per cent zinc oxide as insoluble source (Saravanan *et al.*, 2004) for zinc solubilization. The growth of bacterial isolates was assayed by spotting 10 µl of cultures on media, incubated at 30 °C for 48 h. The colonies exhibiting clear zones around them were considered as positive result for zinc solubilization potential.

Siderophore Production

CAS agar was used to estimate the production of siderophore. One single colony of culture was spot inoculated on the CAS agar plates with and without NaCl and incubated at 30 °C for 3 - 4 days. Orange halo zone around the colony is indicated as positive and no zone as negative. Nutrient agar was prepared with and without NaCl and autoclaved it separately and the 20 ml CAS dye (Chromoazural) was prepared (Schwyn and Neilands, 1987).

Preparation of Chrome Azurol S (CAS) Solution

Dehydrated chrome azurol S (CAS) solution was prepared by dissolving 60.5 mg dehydrated chrome azurol S in 50 ml double distilled water and further mixing with 10 ml of iron solution (1 mM $FeCl_3.6H_2O$ in 10 mMHCl). This was then slowly added to 40 ml aqueous solution containing 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) by continuous stirring and the final solution was autoclaved.

Phosphate Solubilization

Phosphate solubilizing ability of each saline tolerant rhizobacterial isolate was analysed on Pikovskya's (PVK) (Pikovskaya, 1948) media plates with and without 23 per cent NaCl concentration for maintaining stress and controlled conditions, respectively. PVK containing fivegram of Tri-calcium phosphate (TCP) as sole phosphorus source. The growth of bacterial isolates was analysed by spotting 10 µl of cultures on media, incubated at 30 °C for 7 days. The ability of the bacteria to solubilize insoluble phosphorus and form clear halozones around them were considered as positive result for phosphorus solubilization potential.

Effect of Saline Tolerant Rhizobacterial Isolateson Tomato Seedlings under *In-Vitro* Condition

Tomato seeds were surface sterilized with 70 per cent ethanol and imbibed in 24 h old saline tolerant bacterial suspension for 4 hrs. Then the seeds were placed in germination paper (Mia and Shamsuddin, 2009) supplemented with different NaCl solutions (0 mM, 50 mM, 100 mM and 150mM) and incubated for 7 days at 30 ± 2 ºC to assess the shoot and root length of tomato.

Statistical Analysis

The data obtained from pot experiment 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).

Results and Discussion

A transect survey was carried out in saline tracts of karnataka, India. The locations surveyed included Gangavathi, Mandya and Bellary. These locations were observed to have extended patches of saline soils with pH ranging from 7.2 to 8.5 and EC were >4

ds/ m. Totally eighty five isolates were isolated from saline soils.These isolates were purified by four way streak plate method and preserved on agar slants at 4 °C for further study. The isolates obtained has been examined for their salt tolerant efficacy as per the standard protocols.

Screening of Isolates for different Salt Concentration

All the isolates were examined at different salt concentration (15, 20 and 23 %). Among the isolates only eight isolates were able to grow at 23 per cent NaCl concentration compare to other isolates. Selected isolates were further used to characterize morphologically and biochemically and evaluate their plant growth promotion activity under *in-vitro* condition.

Among the eight isolates five isolates were with whitish creamy color colonies (GAN-1, 2, 6 and MAN-5, BEL-2) and others were whitish yellow colonies (GAN-4,7 and BEL-2) (Table 1). The isolates were of irregular, spherical, cocci, round and rod shaped. All the isolates were showed positive for gram reaction. Results have indicated that all the isolates were unable to assimilate starch, negative for H_2 production and while all the eight were able to utilize citrate and were positive for indole production and catalase activity (Table 2).

TABLE 1

Morphological characteristics of saline tolerant rhizobacterial isolates

Isolates	Cell shape	Colony characteristics	Gram reaction
GAN-1	Rod	White, creamy	+
GAN-2	Cocci	White, creamy	+
GAN-4	Cocci	White, creamy yellowish	+
GAN-6	Cocci	White, slimy, tiny	+
GAN-7	Cocci	White yellowish, tiny	+
MAN-3	Cocci	White yellowish	+
MAN-5	Cocci	White, transparent	+
BEL-2	Cocci	Whitish, tiny	+

TABLE 2

Biochemical characteristics of saline tolerant rhizobacterial isolates

Isolates	Starch hydrolysis	H_2s production	Indole production	Citrate utilization	Catalase activity
Control	-	-	-	-	-
GAN-1	-	-	+	+	+
GAN-2	-	-	+	+	+
GAN-4	-	-	+	+	+
GAN-6	-	-	+	+	+
GAN-7	-	-	+	+	+
MAN-3	-	-	+	+	+
MAN-5	-	-	+	+	+
BEL-2	-	-	+	+	+

Note : GAN- Gangavathi, MAN- Mandya and BEL-2, (+) Positive, (-) Negative

Eight isolates were further screened for PGPR traits (Table 3) like ammonia, siderophore production, phosphate, Zinc solubilization ability. Among the isolate GAN-4 and MAN-3 isolate able to produce ammonia, siderophore compare to other isolates under normal and stress condition. Similarly, the isolates GAN-4 and MAN-3 had extensive zone formation for Phosphorus and Zinc solubilization followed by BEL-2 and GAN-6 compare to other isolates. Among the eight

TABLE 3

Plant growth promotion activities of the saline tolerant rhizobacterialisolates under *in-vitro* conditions

Isolates	Ammonia production		Zinc Solubilization		Siderophore production		P Solubilization	
	N	S	N	S	N	S	N	S
Control	-	-	-	-	-	-		
GAN-1	++	++	++	++	++	++	++	++
GAN-2	++	++	++	++	++	++	++	++
GAN-4	+++	+++	+++	++	+++	++	+++	++
GAN-6	++	++	++	++	++	++	++	++
GAN-7	+	+	+	+	+	+	+	+
MAN-3	+	+++	+	+++	+	+++	+	+++
MAN-5	+	+++	+	+++	+	+++	+	+++
BEL-2	+++	++	+++	++	+++	++	+++	++

Note: GAN- Gangavathi, MAN- Mandya and BEL-2, (-) - negative, (+)- good, (++)- very good, (+++) – excellent, N- normal condition, S-stress condtion

isolates screened, two (GAN-4 and MAN-3) of them exhibited positive response to all the *in-vitro* PGPR characteristics studied.

In the study, eighty five isolates from natural selection in the rhizopshere of crops grown in saline soils were isolated and sorted into eight different pure colonies. The results were in accordance with (Tank and Saraf, 2010) where majority of the bacterial isolates were identified as *Bacillus* spp. based on biochemical and morphological observations. PGP activity of the bacteria present in the rhizosphere is found to exert beneficial effects on plant growth mechanism. Several mechanisms such as production of ammonia, siderophore, activation of phosphate and zinc solubilization are believed to be involved in plant growth promotion.

The pre-germinated seeds of tomato was treated with eight saline tolerant rhizobacterial isolates subjected to different NaCl concentration using germination paper towel method. The result showed that, among the isolates used, two isolates GAN-4 (12.70, 6.58, 5.6, and 2.54 cm at 0, 50, 100 and 100 Mm NaCl concentration, respectively) and MAN-3 (10.50, 5.32, 6.1 and 2,4 cm at 0, 50, 100 and 100 Mm NaCl concentration, respectively) showed significantly increased shoot growth compared to other isolates, similarly, GAN-4 (10, 7.06, 6.48 and 3.7 cm at 0, 50, 100 and 100 Mm NaCl concentration, respectively) and MAN-3 (8.76, 6.3, 5.98 and 3.4 cm at 0, 50, 100 and 100 Mm NaCl concentration, respectively) showed significantly increased root growth, indicating their superiority inimparting salinity stress tolerance to tomato (Table-4). Further, the isolates possessplant growth promoting activity as the inoculated plants increased the root and shoot lengthover untreated plants.The growth promotion due to bacteria inoculation is attributed to phytoharmones production (choi *et al.*, 2016) and the salt stress tolerance is due to production of anti-oxidants like superoxide dismutase (SOD) and Peroxidase (POD) by bacteria in inoculated plants.

The study reported the characterization of eight locally-isolated PGPR strains which have shown saline tolerance and plant growth promoting characteristics under saline conditions. The plant

TABLE 4

Influence of saline tolerant rhizobacteria on shoot and root length of tomato after 7 days of incubation

Treatments	0mM NaCl		50mM NaCl		100mM NaCl		150mM NaCl	
	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)
Control	7.80 ^{e}	5.9 ^{e}	3.48 ^{d}	4.28 ^{c}	3.7 ^{c}	3.7 ^{e}	1.24 ^{d}	2.58 ^{c}
GAN-1	8.78 ^{cde}	7.1 ^{de}	3.9 ^{d}	5.62 ^{b}	5.9 ^{a}	5.6 ^{bc}	2.26 ^{abc}	3.16 ^{abc}
GAN-2	8.70 ^{cde}	6.58 ^{e}	4 ^{d}	6 ^{b}	4.9 ^{b}	4.9 ^{cd}	2.32 ^{abc}	3.58 ^{a}
GAN-4	12.70 ^{a}	10.00 ^{a}	6.58 ^{a}	7.06 ^{a}	5.6 ^{a}	6.48 ^{a}	2.54 ^{a}	3.7 ^{a}
GAN-6	9.60 ^{bc}	8.4 ^{bc}	4.3 ^{cd}	5.8 ^{b}	5.8 ^{a}	5.36 ^{bc}	1.94 ^{bc}	3.12 ^{abc}
GAN-7	8.20 ^{de}	8.06 ^{bcd}	4.44 ^{bcd}	5.52 ^{b}	4.9 ^{b}	4.9 ^{cd}	2.16 ^{abc}	3 ^{abc}
MAN-3	10.50 ^{b}	8.76 ^{b}	5.32 ^{ab}	6.3 ^{ab}	6.1 ^{a}	5.98 ^{ab}	2.4 ^{ab}	3.4 ^{ab}
MAN-5	8.8 ^{cde}	7.2 ^{cde}	5 ^{abc}	5.74 ^{b}	4.5 ^{bc}	4.5 ^{de}	1.82 ^{c}	2.84 ^{bc}
BEL-2	9.1 ^{cd}	6.4 ^{e}	4.3 ^{cd}	6 ^{b}	4.2 ^{bc}	4.2 ^{de}	1.92 ^{bc}	2.66 ^{c}
CD (P<0.05)	1.87	2.46	1.27	1.30	0.92	1.04	0.38	0.70

Note: Means with same superscript in a column do not differ significantly as per Duncan multiple Range Test (DMRT).
Values represented are mean ±SE (n=5)

inoculation test revealed that these strains are capable of increasing the root and shoot length under different saline conditions.

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