Journal of Academia and Industrial Research (JAIR)
Volume 4, Issue 3 August 2015

ISSN: 2278-5213

RESEARCH ARTICLE

Selection of Potential Isolates of Plant Growth Promoting Rhizobacteria (PGPR) in Conferring Salt Tolerance under *In Vitro*

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Abstract

Salinity is a major environmental stress which has been studied extensively and has its impact on agriculture in the past, present and future. The global importance of salt affected soils can be explained by their wide distribution on all continents covering about 10% of the total land surface. At present, there are nearly 954 million ha of saline soils on earth's surface. It is extremely difficult to quantify the social and economic costs of salt prone land and water resources. Plant Growth Promoting Rhizobacteria (PGPR) is a group of bacteria that actively colonize plant roots and increase plant growth and yield. They play an important role within the interaction between soil and plant. As plants grow on marginal soils such as saline soils, the importance of the rhizobacteria increases as they mobilize nutrients and provide tolerance ability to the plants. In the present study, an attempt was made and isolated 51 PGPR isolates (Phosphate Solubilizing Bacteria–18; *Azotobacter* spp.-16 and *Azospirillum* spp.-17) from different salt affected areas in Tamil Nadu and Puducherry. All the isolates were screened for their efficacy against different sodium salts namely sodium chloride, sodium citrate and sodium sulphate. It was observed that the absorbance of the culture broth grown in salt stress to sodium chloride decreased with increasing concentration of the salt. Similarly decrease in growth pattern in terms of the absorbance was also observed in salt stress to sodium citrate and sodium sulphate.

Keywords: Plant growth promoting rhizobacteria, *Azotobacter, Azospirillum*, phosphobacterium, saline soil.

Introduction

High salt concentrations in the root zone soil limit the productivity of nearly 8.11 million ha otherwise less productive land in India. Plant species vary in how well they tolerate salt affected soils. Some plants will tolerate high levels of salinity while others can tolerate little or no salinity. The relative growth of plants in the presence of salinity is termed their salt tolerance. Since soil salinity is a major abiotic stress in plant agriculture worldwide. It has led to research into salt tolerance with the aim of improving crop plants. However, salt tolerance might have much wider implications because transgenic salt-tolerant plants often also tolerate other stresses including chilling, freezing, heat and drought (Naseri and Reycroft, 2002). Salinity refers to the dissolved concentration of major inorganic ions (Na, Ca, Mg, K, HCO_3, SO_4 and Cl). Total salt concentration is expressed either in terms of the sum of either the cations or anions in mM/L or sum of cations plus anions in mg/L. For analytical reason practical index of salinity is calculated using Electrical Conductivity (EC). It is expressed in units of ds/m. An approximate relation between EC and salinity concentration is 1 ds/m=10 mmol/L=700 mg/L. All soil contains some water soluble salts. Plants absorb essential plant nutrients in the form of soluble salts, but the excessive accumulation of soluble salts called soil salinity, suppresses plant growth.

Saline or salt affected soils are common in arid and semi-arid regions. Salts in the soil occur as ions (electrically charged form of atoms or compounds). Ions contribute to the soil salinity include Cl^-, SO_4^{+2}, HCO^{-3}, Na^+, Ca^{2+}, Mg^{2+} and rarely NO_3^- or K^+. The salts of these ions occur in highly variable concentration and proportions. They may be indigenous, but more commonly they are brought in to an area in the irrigation water or in waters draining from adjacent areas. Natural drainage is often so poorly developed in arid regions that salts collect inland basins rather than being discharged to sea. Salinity in urban areas often results from the combination of irrigation and groundwater processes. Irrigation is also now common in cities (Rhoades *et al*., 1994). As soil salinity increases, salt effects can result in degradation of soils and vegetation (Blaylock, 1994). Nutrient deficiencies and excess salinity can inhibit plant growth. Plants are stressed in three ways by salinity: (1) low water potential in the root medium leads to water deficits in plants, (2) the toxic effects of ions, mainly Na and Cl) and (3) nutrient imbalance caused by depression in uptake or transport (Leon, 1975). Saline soils contain soluble salts in quantities that affect plant growth adversely, the lower limit for a saline soil being set conventionally at an electrical conductivity of 4 mmol/cm in the soil saturation extract.

Actually, sensitive plants are affected at half this salinity and highly tolerant ones at about twice this salinity. Sodic soils contain excess exchangeable sodium with by definition 15% or more of the cation exchange sites of the soil occupied by Na^+. Plants sensitive to sodium are however affected at lower exchangeable sodium percentages sodic soils may be either non saline or saline. There are extensive areas of salt affected soils on all the continents but their extent and distribution has not been studied in detail. In some countries even the existence of these soils was discovered only through a survey or the pressing demand for agricultural utilization of a region. A first attempt to compile information on the extent of salt affected soils on a worldwide basis was made by Massoud *et al.* (1988) based on the FAO, UNESCO Soil Map of the World. Any attempt to increase food production in coming years must pay adequate attention to the improvement of existing salt affected soils with little or no production and to prevent further deterioration of productive soils through these degradation processes. In India, salt affected areas were recorded as 23796 ha in which 23222 were saline and 574 were sodic soil (Massoud *et al.,* 1988).

Plant Growth Promoting Rhizobacteria (PGPR) is a group of bacteria that actively colonize plant roots and increase plant growth and yield. The mechanisms by which PGPR promote plant growth, include the ability to produce phytohormones, asymbiotic N_2 fixation-against phytopathogenic microorganisms by production of siderophores, the synthesis of antibiotics, enzymes and/or fungicidal compounds and also solubilization of mineral phosphates and other nutrients (Gholami *et al*., 2009). Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as Plant Growth Promoting Rhizobacteria (PGPR). PGPR are associated with plant roots and augment plant productivity and immunity; however, recent work by several groups shows that PGPR also elicit so-called 'induced systemic tolerance' to salt and drought (Yang *et al.,* 2009). Enormous numbers of microbial populations and species in the soil, especially in the rhizosphere, intensive and extensive interactions have been established between soil microorganisms and various other soil organisms, including plant roots, and plant growth promotion by rhizosphere microorganisms is well established (Bashan,1998). Rhizobacteria play an important role within the interaction between soil and plant. As plants grow on marginal soils such as saline soils, the importance of the rhizobacteria increases as they mobilize nutrients and provide tolerance ability to the plants. Research on the rhizosphere beneficial microflora in saline environment finds its direct application in the exploitation of those novel beneficial microbes for phytoremediation of saline soils (Paul and Sudha, 2008; Upadhyay *et al*., 2009). There are different ways to reclaim and rehabilitate salt affected areas.

To overcome salt stress problems, it is possible to select salt tolerant plants, use biological processes such as mycorrhizal interactions or beneficial microbial interactions or desalinate soil by leaching excessive salts (Munns, 2005). The desalination of soils is not economically viable for sustainable agriculture. Salt tolerant beneficial microbes from different sources i.e., saline soils, rhizosphere, rhizoplane and phylloplane of different plants growing in saline areas have been isolated (Yasmin and Hasnian, 1997). Recently, Mohan and Sangeetha Meon (2015) isolated and identified different isolates of PGPR from the salt affected areas in Tamil Nadu and Puducherry. These microbes were able to resist high levels of sodium chloride stress. The bacteria from different sources promote seedling growth under saline conditions (Siddique, 1997). The present study was undertaken to screen and select the potential isolates of PGPR microbes in conferring salt tolerance under *in vitro* condition.

Materials and methods

Collection of soil samples: Rhizosphere soil samples were collected under the root zone of different plants in selected salt affected study sites including coastal areas of Tamil Nadu and Puducherry in zip lock poly bags, sealed tightly and immediately transported to laboratory. The samples were kept in refrigerator at 4ºC until further use.

Isolation and identification of PGPR from saline soil samples: Serial dilution and plating techniques as described by (Parkinson *et al*., 1971; Subba Rao, 1993) were adopted for enumerating the status of Plant Growth Promoting Rhizobacteria (PGPR). Among different PGPRs, *Azotobacter* colonies were selected based on the appearance of mucoid, transparent, gummy colonies; *Azospirillum* colonies appeared as scarlet pink, round colonies and Phosphate Solubilizing Bacteria (PSB) colonies were identified based on the halo zone formed around the colonies. Population density of these PGPR organisms was also determined for each sample as CFU/g (Colony forming units/gm of soil (Rodriguez-Caceras, 1982; Subba Rao, 1993). All the isolates of PGPR viz., *Azotobacter, Azospirillum* and PSB were maintained in nutrient agar slants at 4ºC for further studies. All the PGPR isolates were identified up to species level based on the following growth characteristics, staining reactions and biochemical tests (Martin *et al*., 2006).

Maintenance of pure cultures of different PGPR: The colonies of *Azotobacter* sp. was observed as mucoid colonies on Jensen's agar medium plates were picked up and sub-cultured on to fresh medium plates. These were maintained in nutrient agar slants at 4ºC. The colonies of *Azospirillum* sp. appearing as scarlet pink colonies in Rojo-Congo agar medium were picked up and subcultured to fresh medium plates and maintained in nutrient agar slants at 4ºC.

Table 1. PGPR isolates obtained from saline samples of different salt affected sites in Tamil Nadu and Puducherry.

S. No.	Soil sample locations	PGPR			Total
		PSB	*Azotobacter* sp.	*Azospirillum* sp.	
1.	Saline soil paddy field, Semmaru 1, Sathanur (Villupuram)	1	1	1	3
2.	Saline soil outside paddy field, Semmaru 2, Sathanur (Villupuram)	1	1	1	3
3.	Saline soil with *Prosopsis juliflora* Semmaru-3, Sathanur (Villupuram)	2	1	1	4
4.	Inside *Casuarina equisetifolia* plantation, Kalapet-1 (Puducherry)	1	1	1	3
5.	Outside *Casuarina equisetifolia* plantation, Kalapet-2 (Puducherry)	1	1	1	3
6.	Coastal soil, Kalapet-3 (Puducherry)	Nil	1	1	2
7.	*Casuairna equisetifolia* Plantation, Sonangkuppam-1 (Cuddalore)	1	1	1	3
8.	Salt affected soil, Marakanam	2	1	1	4
9.	Outside *Casuairna equisetifolia* Plantation Sonangkuppam-2(Cuddalore)	3	1	1	5
10.	*Casuairna equisetifolia* Plantation, Singarathopu-1 (Cuddalore)	1	1	1	3
11.	Outside *Casuairna equisetifolia* Plantation, Singarathopu-2 (Cuddalore)	1	1	1	3
12.	Saline soil Eucalyptus trees, Kattugudalur-1 (Acchirupakkam)	Nil	1	1	2
13.	Saline soil (Neem and Phoenix trees), Kattugudalur-2 (Acchirupakkam)	1	1	1	3
14.	Coastal soil, Singarathopu-3 (Cuddalore)	1	1	1	3
15.	*Casuarina equistifolia* plantation, Moorthy palayam-1 (Karur)	1	1	1	3
16.	Outside *Casuarina equisetifolia* plantation, Moorthy palayam-2 (Karur)	Nil	1	1	2
17.	Saline soil, Ammapatty (Karur)	1	Nil	1	2

The colonies of PSB forming clear halo zone in the Pikovskaya's agar medium plates were sub-cultured in fresh medium plates and they were maintained in nutrient agar slants at 4ºC.

Salinity tolerance studies of PGPR isolates

Effect of various concentrations of sodium chloride (NaCl): PGPR isolates were tested for their tolerance to various concentration of NaCl. Selective nutrient broth tubes supplemented with various concentration (0, 5, 10, 15, 20, 25 and 30 mM) of sodium chloride was prepared and 0.2 mL of culture broth was added. The tubes were incubated at 28±2ºC for 2-4 d and the absorbance was measured using UV spectrophotometer (Labtronics LT 29) at 600 nm. Uninoculated nutrient broth for each concentration was maintained as negative control.

Effect of various concentrations of sodium sulphate: PGPR isolates were tested for their tolerance to various concentration of Na_2SO_4. Selective nutrient broth tubes for each type of PGPR supplemented with various concentration (0, 5, 10, 15, 20, 25 and 30 mM) of sodium sulphate was prepared and 0.2 mL of culture broth was added. The tubes were incubated at 28±2ºC for 2-4 d and the absorbance was measured using UV spectrophotometer (Labtronics LT 29) at 600 nm. Uninoculated nutrient broth for each concentration was maintained as negative control.

Effect of various concentration of sodium citrate: PGPR isolates were tested for their tolerance to various concentration of sodium citrate. Selective nutrient broth tubes for each type of PGPR supplemented with various concentration (0, 5, 10, 15, 20, 25 and 30 mM) of sodium citrate was prepared isolates and 0.2 mL of culture broth was added. The tubes were incubated at 28±2ºC for 2-4 d and the absorbance was measured using UV spectrophotometer (Labtronics LT 29) at 600 nm. Uninoculated nutrient broth for each concentration was maintained as negative control.

Results

Isolation of PGPR from saline soil samples: Collected soil samples from salt affected areas in Tamil Nadu and Puducherry were processed and serial dilutions were performed. Pour plate technique were used to isolate PGPR from different soil samples. Three types of PGPRs were isolated based on specific colonies formed in the respective selective media. These colonies were further purified and maintained in Nutrient slants. *Azotobacter* colonies appeared as mucoid, watery, transparent and gummy colonies. *Azospirillum* colonies appeared as scarlet pink round colonies. Formation of halo zone was observed around the colonies of phosphate solublizing bacteria. A total of 51 PGPR isolates were isolated viz., 18 PSB isolates, 17 *Azospirillum* species and 16 *Azotobacter* species were recorded (Table 1).

Saline tolerance studies of PGPR isolates under *in vitro:* All the PGPR isolates were screened for their efficacy on salt tolerant by using different concentrations of sodium chloride, sodium citrate and sodium sulphate salts under *in vitro* condition.

Effect of different concentrations of sodium chloride salt on growth of Azotobacter isolates: Total of 16 different isolates of *Azotobacter* was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium chloride salt and the data is presented in Fig. 1.

Fig. 1. Effect of various concentrations of sodium chloride on the growth of *Azotobacter* isolates.

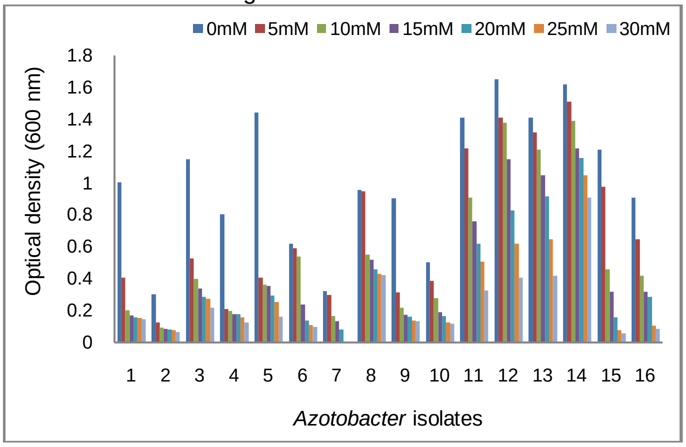


Fig. 2. Effect of various concentrations of sodium chloride on the growth of *Azospirillium* isolates.

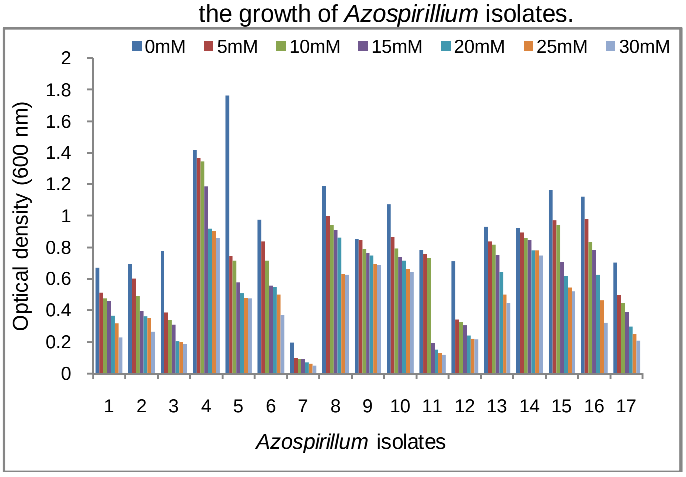
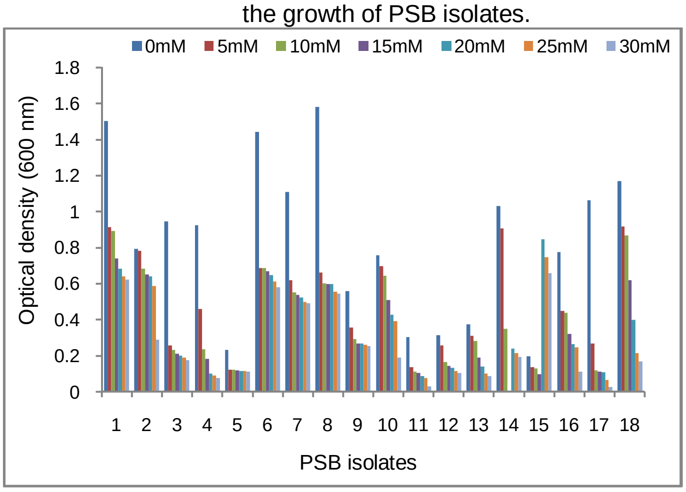


Fig. 3. Effect of various concentrations of sodium chloride on the growth of PSB isolates.



It was observed that the absorbance of the culture broth grown in different concentrations of sodium chloride decreased with increasing concentration of the salt. Among 16 different isolates, the isolate Azoto 16 revealed maximum growth up to 30 mM concentration and this is followed by isolate Azoto 11, Azoto 15 and Azoto 12.

Effect of different concentrations of sodium chloride (NaCl) salt on growth of Azospirillum isolates: Total of 17 different isolates of *Azospirillum* was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium chloride and the data is presented in Fig. 2. It was observed that the absorbance of the culture broth of *Azospirillum* isolates grown in different concentrations of sodium chloride decreased with increasing concentration of the salt. Among 17 different isolates, the isolate Azosp 4 revealed maximum growth up to 30 mM concentration and this is followed by the isolates Azosp14, Azosp 9, Azosp 10 and Azosp 15.

Effect of different concentrations of sodium chloride salt on growth of PSB isolates: Total of 18 different isolates of PSB was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium chloride and the data is presented in Fig. 3. It was observed that the absorbance of the culture broth of PSB isolates grown in different concentrations of sodium chloride decreased with increasing concentration of the salt. Among 18 different isolates, the isolate Piko 12:1 revealed maximum growth up to 30 mM concentration and this is followed by the isolates Piko 1:1, Piko 7:1, Piko 2:1 and Piko 8:1.

Effect of different concentrations of sodium citrate salt on growth of Azotobacter isolates: Total of 16 different isolates of *Azotobacter* was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium citrate and the data is presented in Fig. 4. It was observed that the absorbance of the culture broth grown in different concentrations of sodium citrate decreased with increasing concentration of the salt. Among 16 different isolates, the isolate Azoto 8 revealed maximum growth up to 30 mM concentration and this is followed by isolate Azoto 11, Azoto 15 and Azoto 14.

Effect of different concentrations of sodium citrate salt on growth of Azospirillum isolates: Total of 17 different isolates of *Azospirillum* was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium citrate and the data is presented in Fig. 5. It was observed that the absorbance of the culture broth of *Azospirillum* isolates grown in different concentrations of sodium citrate decreased with increasing concentration of the salt. Among 17 different isolates, the isolate Azosp 8 revealed maximum growth up to 30 mM concentration and this is followed by the isolates Azosp 1, Azosp 3, Azosp 2 and Azosp 4.

Effect of different concentrations of sodium citrate salt on growth of PSB isolates: Total of 18 different isolates of PSB was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium citrate and the data is presented in Fig. 6.

Fig. 4. Effect of various concentrations of sodium citrate on the growth of *Azotobacter* isolates.

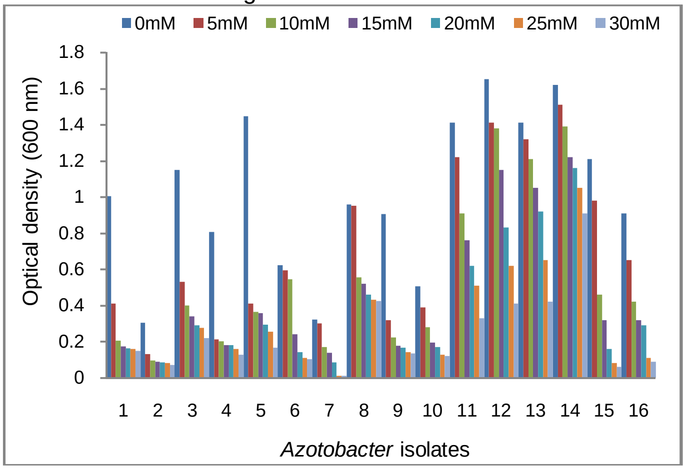


Fig. 5. Effect of various concentrations of sodium citrate on the growth of *Azospirillium* isolates.

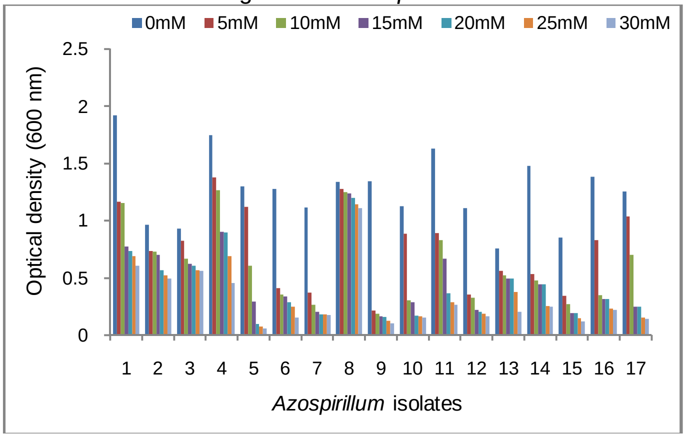
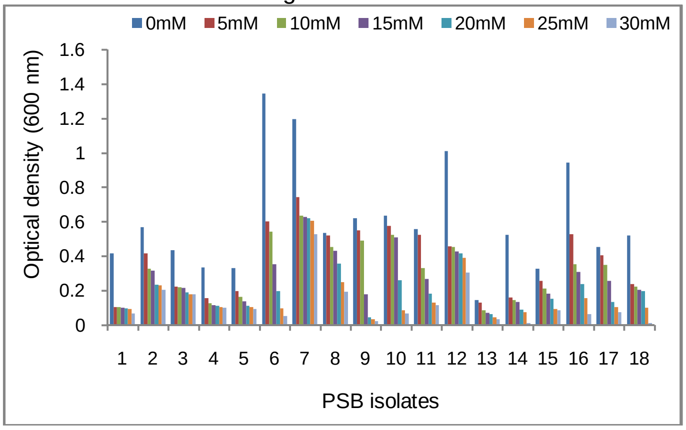


Fig. 6. Effect of various concentrations of sodium citrate on the growth of PSB isolates.



It was observed that the absorbance of the culture broth of PSB isolates grown in different concentrations of sodium citrate decreased with increasing concentration of the salt. Among 18 different isolates, the isolate Piko 6:1 revealed maximum growth up to 30 mM concentration and this is followed by the isolates Piko 9:2 and Piko 2:1.

Effect of different concentrations of sodium sulphate salt on growth of Azotobacter isolates: Total of 16 different isolates of *Azotobacter* was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium sulphate and the data is presented in Fig. 7. It was observed that the absorbance of the culture broth grown in different concentrations of sodium sulphate decreased with increasing concentration of the salt. Among 16 different isolates, the isolate Azoto 4 revealed maximum growth up to 30 mM concentration and this is followed by isolate Azoto 14, Azoto 15 and Azoto 10.

Effect of different concentrations of sodium sulphate salt on growth of Azospirillum isolates: Total of 17 different isolates of *Azospirillum* was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium sulphate and the data is presented in Fig. 8. It was observed that the absorbance of the culture broth of *Azospirillum* isolates grown in different concentrations of sodium sulphate decreased with increasing concentration of the salt. Among 17 different isolates, the isolate Azosp 7 revealed maximum growth up to 30 mM concentration and this is followed by the isolates Azosp 10, Azosp 9, Azosp 17 and Azosp 5.

Effect of different concentrations of sodium sulphate salt on growth of PSB isolates: Total of 18 different isolates of PSB was screened to determine the efficacy of the best isolates under various concentrations (0 mM to 30 mM) of sodium sulphate and the data is presented in Fig. 9. It was observed that the absorbance of the culture broth of PSB isolates grown in different concentrations of sodium sulphate decreased with increasing concentration of the salt. Among 18 different isolates, the isolate Piko 14:1 revealed maximum growth up to 30 mM concentration and this is followed by the isolates Piko 1:1, Piko 3:1, Piko 8:1 and Piko 13:1.

Identification potential isolates of PGPR: PGPR isolates most tolerant up to 30 mM sodium chloride showing higher absorbance were identified up to genus and species level tentatively by their growth characteristics, staining reactions and biochemical tests. The *Azotobacter* isolates were identified as *Azotobacter chroococcum* and *Azotobacter* species. The *Azospirillum* isolates were identified as *Azospirillum amazonense* and *Azospirillum lipoferum*. The PSB isolates belong to the genus *Bacillus* was identified as *B. subtilis* and *B. megaterium* (Table 2).

Effect of various concentrations of sodium chloride on growth of PSB, Azotobacter and Azospirillum isolates: Based on the salt tolerance experiments by using various concentrations (0 mM–30 mM) of sodium chloride, sodium citrate and sodium sulphate salts, some of the isolates of *Azotobacter*, *Azospirillum* and PSB revealed better salt tolerant ability.

Fig. 7. Effect of various concentrations of sodium sulphate on the growth of *Azotobacter* isolates.

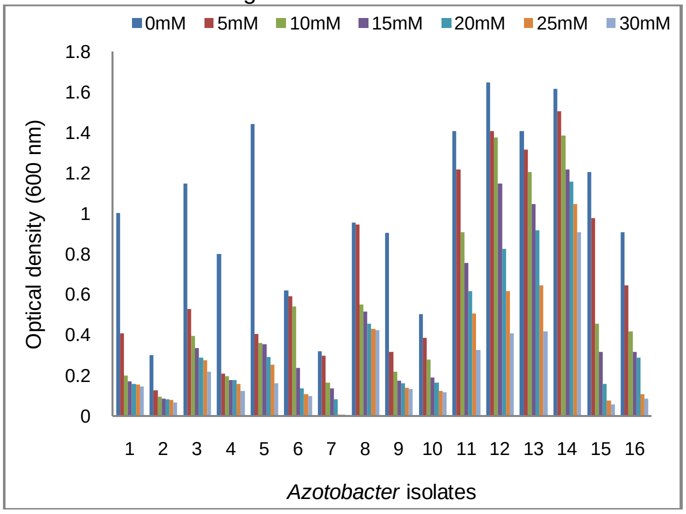


Fig. 8. Effect of various concentrations of sodium sulphate on the growth of *Azospirillium* isolates.

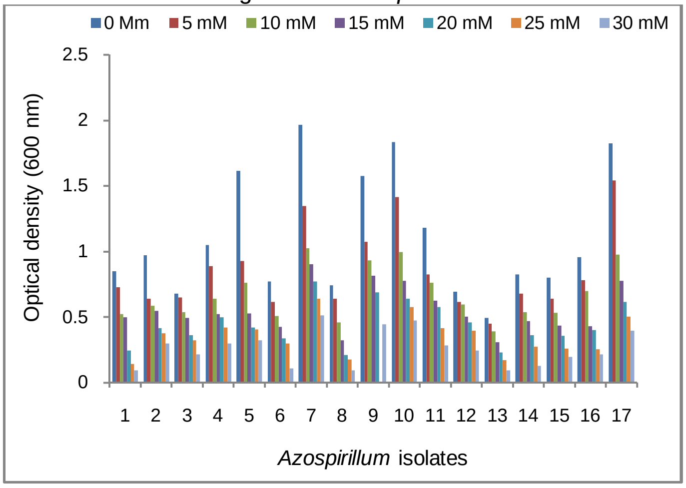


Fig. 9. Effect of various concentrations of sodium sulphate on the growth of PSB isolates.

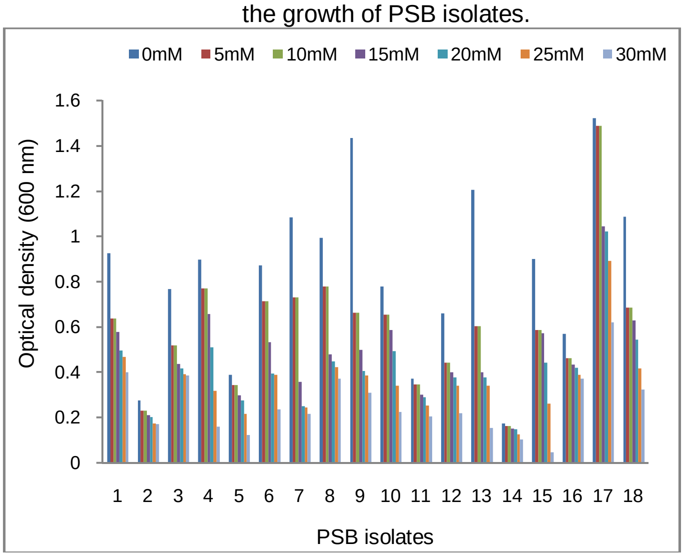


Table 2. List of identified PGPR isolates as salt tolerant.

Isolate code	Sample No.	Identified species
Azotobacter 1	11	*Azotobacter chroococum*
Azotobacter 2	16	*Azotobacter beijernickii*
Azospirillum 1	4	*Azospirillum amazonase*
Azospirillum 2	14	*Azospirillum lipoferum*
PSB 1	1	*Bacillus subtilis*
PSB 2	15	*Bacillus megaterium*

Hence, those selected PGPR organisms were further tested for their salt tolerance efficacy against higher concentrations (250 mM to 4000 mM) of all the three sodium salts (sodium chloride, sodium citrate and sodium sulphate) under *in vitro*. Data on effect of different concentrations of sodium chloride on growth of selected PGPR isolates is presented in Table 3. It was found that among isolates of *Azotobacter, A. chroococcum* had maximum growth up to 2000 mM concentration of sodium chloride salt. Whereas isolate of *A. beijernickia* had better growth up to 1000 mM concentration afterwards the growth was decreased drastically. Among the isolates of *Azospirillum, A. lipoferum* had maximum growth up to 4000 mM concentration, whereas the isolate *A. amazonense* had growth up to 1000 mM concentration. Among the isolates of PSB, *Bacillus megaterium* had better growth up to 3000 mM concentration as compared to *Bacillus subtilis* which grows up to 1000 mM concentration of sodium chloride salt under *in vitro*.

Effect of various concentrations of sodium citrate on growth of PSB, Azotobacter and Azospirillum isolates: Data on effect of different concentrations of sodium citrate on growth of selected PGPR isolates is presented in Table 4. It was found that among different isolates of *Azotobacter, A. chroococcum* had maximum growth up to 2000 mM concentration of sodium citrate salt. Whereas, isolate of *A. beijernickia* had better growth up to 1000 mM concentration afterwards the growth was decreased drastically. Among the isolates of *Azospirillum, A. lipoferum* had maximum growth up to 3000 mM concentration, whereas the isolate *A. amazonense* had growth up to 1000 mM concentration. Among the isolates of PSB, *Bacillus megaterium* had better growth up to 3000 mM concentration as compared to *Bacillus subtilis* which grows up to 1000 mM concentration of sodium chloride salt under *in vitro*.

Effect of various concentrations of sodium sulphate on growth of PSB, Azotobacter and Azospirillum isolates: Data on effect of different concentrations of sodium sulphate on growth of selected PGPR isolates is presented in Table 5. It was found that among different isolates of *Azotobacter, A. chroococcum* had maximum growth up to 3000 mM concentration of sodium sulphate salt. Whereas isolate of *A. beijernickia* had better growth up to 1000 mM concentration afterwards the growth was decreased drastically.

Table 3. Effect of different concentrations of sodium chloride on the growth of different PGPR isolates under *in vitro*.

S.No.	PGPR isolates	Concentration of NaCl (mM)							
		Control 0 mM	250 mM	500 mM	750 mM	1000 mM	2000 mM	3000 mM	4000 mM
1.	*Azotobacter chroococcum*	+++	+++	+++	+++	+++	+++	+	+
2.	*Azotobacter beijernickia*	+++	+++	+++	+++	+++	+	+	-
3.	*Azospirillum amazonense*	+++	+++	+++	+++	+++	+	-	-
4.	*Azospirillum lipoferum*	+++	+++	+++	+++	+++	+++	+++	+++
5.	*Bacillus megaterium*	+++	+++	+++	+++	+++	+++	+++	++
6.	*Bacillus subtilis*	+++	+++	+++	+++	+++	+	-	-

+++ = 50-100%, ++ = 10-50%, + = 1-10%, - = Absence of growth.

Table 4. Effect of different concentrations of sodium sulphate on the growth of different PGPR isolates under *in vitro*.

S.No.	PGPR isolates	Concentration of NaCl (mM)							
		Control 0 mM	250 mM	500 mM	750 mM	1000 mM	2000 mM	3000 mM	4000 mM
1.	*Azotobacter chroococcum*	+++	+++	+++	+++	+++	+++	+	+
2.	*Azotobacter beijernickia*	+++	+++	+++	+++	+++	+	-	-
3.	*Azospirillum amazonense*	+++	+++	+++	+++	+++	+	-	-
4.	*Azospirillum lipoferum*	+++	+++	+++	+++	+++	+++	+++	++
5.	*Bacillus megaterium*	+++	+++	+++	+++	+++	+++	++	+
6.	*Bacillus subtilis*	+++	+++	+++	+++	+++	+	+	-

Table 5. Effect of different concentrations of sodium citrate on the growth of different PGPR isolates under *in vitro*.

S.No.	PGPR isolates	Concentration of NaCl (mM)							
		Control 0 mM	250 mM	500 mM	750 mM	1000 mM	2000 mM	3000 mM	4000 mM
1.	*Azotobacter chroococcum*	+++	+++	+++	+++	+++	+++	+++	+
2.	*Azotobacter beijernickia*	+++	+++	+++	+++	+++	+	+	-
3.	*Azospirillum amazonense*	+++	+++	+++	+++	+++	+	-	-
4.	*Azospirillum lipoferum*	+++	+++	+++	+++	+++	+++	+++	+++
5.	*Bacillus megaterium*	+++	+++	+++	+++	+++	+++	+++	+
6.	*Bacillus subtilis*	+++	+++	+++	+++	+++	++	+	-

Among the isolates of *Azospirillum, A. lipoferum* had maximum growth up to 4000 mM concentration, whereas the isolate *A. amazonense* had growth up to 1000 mM concentration. Among the isolates of PSB, *Bacillus megaterium* had better growth up to 3000 mM concentration as compared to *Bacillus subtilis* which grows up to 1000 mM concentration of sodium sulphate salt under *in vitro*. Growth of *Azotobacter chroococcum, Azospirillum lipoferum* and *Bacillus megaterium* isolates showed a decline with increasing sodium salts up to 4000 mM; however, all these isolates reached an EC_{50} point (effective concentration inhibiting growth by 50%) below 3000 mM sodium salts. Hence, these three PGPR organisms have been selected and short listed to conduct nursery experiment to determine the efficacy of these organisms on growth enhancement of different clonal plants of *C. equisetifolia* in saline soil in nursery.

Discussion

Saline tolerance studies of PGPR isolates: In the present study, all the PGPR isolates were screened to select the best salt tolerant isolates by using varying concentrations of sodium salts viz., sodium chloride, sodium citrate and sodium sulphate under *in vitro* condition.

It was observed that the absorbance of the culture broth grown in salt stress to sodium chloride decreased with increasing concentration of the salt. Similarly, decrease in growth pattern in terms of the absorbance was also observed in salt stress to sodium citrate and sodium sulphate. Based on this, some of the PGPR isolates were selected and further screened their salt tolerance ability up to 4000 mM concentrations. Growth of *Azotobacter chroococcum, Azospirillum lipoferum* and *Bacillus megaterium* isolates showed a decline with increasing sodium salts up to 4000 mM; however, all these isolates reached an EC_{50} point (effective concentration inhibiting growth by 50%) below 3000 mM sodium salts. Hence, these three PGPR organisms have been selected and short listed to conduct nursery experiment to determine the efficacy of these organisms on growth enhancement of different clonal plants of *C. equisetifolia* in saline soil in nursery. Similar kind of studies has been carried out by many earlier researchers in India and other parts of the world. Upadhay *et al.* (2009) reported that rhizobacterial isolates from wheat rhizosphere were tolerant to 8% NaCl. In our study, the PGPR isolates were moderately tolerant up to 30 mM salt.

The salt tolerance of PGPR might be due to osmo tolerance mechanism where de-novo synthesis of osmolites and over production of salt stress proteins effectively nullifies the detrimental effects of high osomolarity (Paul and Sudha, 2008). The role of *Azotobacter chroococcum* in producing plant growth promoting substance and nitrogen fixation might be due to its role in (1) decreasing the absorption of sodium and (2) increasing both Nitrogen and Magnesium concentration. Tripathi *et al.* (1998) stated that production of the phytohormone indole acetic acid was not affected by salinity as strongly as nitrogenase activity. Lower levels of salinity, in fact stimulated indole acetic acid production, but at above 100 mM NaCl, IAA production was inhibited. At low levels of salinity, increase in IAA production may cause proliferation of roots and possibly aid in the uptake of proline or betaines released by the plant roots. This might be one of the strategies for the better survival of both plants and the associated *Azospirillum*.

Mayak *et al.* (2004) studied few mechanisms which unequivocally demonstrated in explaining the increased resistance to environmental stresses of plants treated with PGPRs. These include reduction of stress ethylene production via the action of ACC deaminase and increased expression of the ERD15 gene that was previously shown to be responsive to drought stress. Nevertheless, PGPRs found in association with plants grown under chronically stressful conditions, including high salinity, may have adapted to the stress conditions, and could provide a significant benefit to the plants. Glassker (1996) reported that the inorganic and organic compounds play an important role to maintain internal osmolarity of bacteria. The bacteria employ the adaptive strategy of accumulating a broad spectrum of osmotically active solutes by modulating their biosynthesis, catabolism, uptake of efflux. The ability of cells to grow under conditions of elevated osmolarity is determined both by the salt tolerance of their enzymes and by their capacity to accumulate compatible solutes in place of salts. Hartmann and Zimmer (1994) studied that *A. amazonense* DSM2787 acetylene reduction was very sensitive to the addition of salt showing 90% inhibition with only 40 mM NaCl but in *A. halopraferens* 24% nitrogenese activity of unstressed cells could still be 250 mM NaCl. Gaur (2004) reported *P. pulifora* isolates from the root free soil, rhizosphere and rhizoplane of growing in alkaline soils in 85% morphotypes solublized phosphate in agar. The incidence of PSB was highest in rhizoplane followed by rhizosphere and root free soil. Phosphate solublizing ability of strain NBR14 was higher than control in the presence of salt (NaCl, Ca_2Cl_2 and KCl) at 30^0C and further increased at 37^0C. Nautiyal (2000) has reported that the strain NBR12601 isolated from the rhizosphere of chickpea and alkaline soils could solublize phosphorus in presence of 10% salt, pH 12 at 45^0C suggesting that extensive diversity in appropriate habitats may lead to recovery of effective bacteria.

Several reports suggests that osmotolerance in *Azospirillum* is strain specific and depends on the extent of salinity of the habitat from where the strain is isolated. Tripathi *et al.* (1998) studied that species of *Azospirillum* are widely distributed in salt affected soils in Pakistan, India, and Brazil. Occurrence of these species of *Azospirillum* i.e., *A. brasilense, A. lipoferum, A. halopreferens* in the rhizosphere and rhizoplane of Kallar grass grown on pioneer in high salinity soils indicated that these isolates should be salt tolerant. A variety of *Azospirillum* strains isolated from other habitats and thus showed adaptation to their saline environments. Tilak *et al.* (2005), studied Phosphate solublizing microorganisms which can grow in media containing Tricalcium ion and Aluminium phosphate hydroxyapatate, bone meal, rock phosphate and similar in soluble phosphate compounds as a sole phosphate source. Such microbes are not only in quantities in excess of their own requirements. The most efficient phosphate solubilizing microorganisms belongs to genera *Bacillus* and *Pseudomonas* amongst bacteria and *Aspergillus* and *Pencillium* in fungi.

Conclusion
The findings of the present project study are much useful to the State Forest Departments (SFDs), Forest Development Corporations (FDCs), Tree growers, Farmers and NGOs for utilizing these potential beneficial microbes particularly PGPR isolated from different salt affected areas as bio-inoculants for application in nurseries for production of quality and healthy seedlings which can be successfully used for development of tree cover in saline and salt affected areas and other unproductive areas of the country.

Acknowledgements
Authors are highly grateful to the Director, IFGTB, Coimbatore, for providing all the facilities and encouragement.

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