Journal of Academia and Industrial Research (JAIR)
Volume 5, Issue 12 May 2017

ISSN: 2278-5213

RESEARCH ARTICLE

Screening of Salt Tolerant and Growth Promotion Efficacy of Phosphate Solubilizing Bacteria

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Abstract

An attempt was made for screening of salt tolerant against sodium chloride (NaCl) salt and growth promotion efficacy of eight different phosphate solubilizing bacterial (PSB) isolates under *in vitro* conditions. An efficient isolate which exhibited maximum phosphate solubilization, salt tolerant, Indole-3-Acetic Acid (IAA) production and root colonization potential in maximum concentration of NaCl (7%) was identified as *Bacillus cereus* based on 16S rRNA sequencing. It was recorded that highest phosphate solubilization in 7% NaCl concentration (70% and 374 µg/mL) in agar plate and broth assay respectively. IAA production was observed to be decreased when salt concentration increases, the isolate showed significant amount of IAA in normal culture media without amendment of NaCl (74.94 µg/mL) and at 7% gradual decrease of IAA production was observed (30 µg/mL). Maximum colonization of isolate in the roots of *Zea mays* was also observed in the study.

Keywords: *Bacillus cereus*, salt tolerant, phosphate solubilization, salinity, *Zea mays*.

Introduction

More than 6% of the world's total land area is salt-affected; most of this salt-affected land has arisen from natural causes and the accumulation of salts over long periods of time in arid and semiarid zones (Rengasamy, 2002). Salinity affects almost all aspects of plant development including, germination, vegetative growth and reproductive development. Soil salinity imposes ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants and thus, limits water uptake from soil. Improving soil fertility is one of the most common tactics to increase agricultural production. Maintaining high levels of available nitrogen (N) and phosphorus (P), the two most limiting nutrients in the soils, remains a major challenge to ecologists and land managers. Phosphorus is one of the most essential macronutrients and its deficiency in the soils is limiting the crop yields (Hameeda *et al.,* 2008). However, many soils in the world are P-deficient and thus, cannot sustain the good crop yield (Hinsinger, 2001). Although P is abundant in soil in both inorganic and organic forms, but mostly they are found as insoluble mineral complexes, some of them appearing after frequent application of chemical fertilizers, but a large proportion of available phosphate are rapidly transformed into its poorly soluble forms before plant absorbing it (Sulbaran *et al*., 2009). The rhizosphere phosphate utilizing bacteria could be a promising source for plant growth promoting agent in agriculture (Chaiharn *et al*., 2008). The use of phosphate solubilizing bacteria as bio-inoculants can increase the P uptake by the plants (Chen *et al.,* 2006). Husen (2003) and many others reported the inoculation of PSB to solubilize phosphate and thus helps in the growth of plants in normal soil. But the reports on salt tolerant PSB are found to be very scanty, so the present study was taken up to isolate, screen and identify the potential salt tolerant P-solubilizing rhizobacteria.

Materials and methods

Collection of soil samples: Rhizosphere soil samples were collected at a depth of 3 to 5 cm under the root zone of different plants grown in selected salt affected study sites at Pugalur, Karur district, Tamil Nadu 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 Phosphate Solubilizing Bacteria (PSB) from saline soil samples: Serial dilution and plating techniques as described by Parkinson *et al.* (1971) and Subba Rao (1993) were adopted for enumerating the status of P-solubilizing bacteria (PSB). The PSB colonies were identified based on the halo zone formed around the colonies. Population density of these organisms was also determined for each sample as Colony forming units/gm (CFU/g) of soil (Rodriguez-Caceras, 1982; Subba Rao, 1993). All the isolates of PSB were maintained in nutrient agar slants at 4ºC for further studies. All the PSB isolates were identified up to species level based on the following growth characteristics, staining reactions and biochemical tests (Martin *et al*., 2006).

Qualitative estimation of P-solubilization under various concentrations of NaCl salt: Pikovskaya's agar medium supplemented with different concentrations of NaCl (0%, 5%, 6% and 7%) was used to screen the P-solubilization efficacy of different isolates and it was incubated at 28°C for 5 d. Presence of growth indicates the salt tolerance level of microbes. Zone of phosphate solubilization around the colonies was measured and Solubilization Efficiency (SE) was calculated using the following formula:

$$\text{Solubilization Efficiency (SE)} = \frac{\text{Colony diameter}}{\text{Zone diameter}} \times 100$$

Quantitative estimation of P-solubilization under various NaCl concentrations: Bacterial cultures were inoculated into 25 mL of Pikovskaya's broth medium amended with various concentrations of NaCl (0%, 3%, 5% and 7%). Cultures were kept for incubation on rotary shaker at 150 rpm for 28°C or 5 d. After incubation, the culture broth was centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected and filtered using Whatman's No. 1 filter paper and supernatants were used for estimation of phosphorus. According to Peterson (1978), P estimation was carried out. A sample volume of 0.5 mL was mixed with 0.9 mL of 5% (w/v) sodium dodecyl sulfate (SDS) (Sigma-Aldrich) solution. This was followed by addition of 1 mL of 1.25% (w/v) ammonium molybdate (Merck) solution in 2M HCl (Hi-Media). Thereafter, 0.1 mL of 1 g/L ascorbic acid (Fluka) solution was added and mixed well. Incubating the mixture at room temperature for 30 minutes and Absorbance was read at 700 nm.

Effect of various NaCl concentrations on Indole-3-Acetic Acid (IAA) production: Based on Vishal and Punkaj (2013), IAA production was evaluated. All the isolates were grown in Luria Bertain (LB) media supplemented with 50 mg/L of L-Tryptophan and incubated in dark along with various NaCl concentrations (0%, 3%, 5%, and 7%) on orbital shaker at 200 rpm for 72 h. The broth cultures were centrifuged at 5000 rpm for 15 minutes. About 1 mL supernatant was mixed with 2 mL of Salkowski reagent (Sarwer and Kremer, 1995), the tubes were kept in dark at room temperature for 30 minutes. The development of pink colour was observed and it was read at 530 nm using UV-Vis Spectrophotometer (HITACHI, U-2000). Concentration of IAA (µg/mL) in the sample was determined using standard graph of IAA.

Gnotobiotic root colonization assay: Gnotobiotic root colonization assay was used as a method of evaluating the potential of individual rhizobacterial strains to colonize the roots of maize (*Zea mays*) seedlings. Steam sterilized sand was filled in glass tubes and used for the test. Sterilized Hoagland's nutrient solution was added into glass tubes containing sand and stored at room temperature for 48 h to equilibrate. Pre-germinated seeds of maize were inoculated into 24 h culture broth of respective isolate for normal condition and various concentrations of salt was imposed by adding different concentrations of NaCl in broth culture, allow it to stand for 30 minutes for better adherences of bacterial isolates on seed. For each tube, two seeds were inoculated using forceps. Mixture of sterilized broth and sugar solution (sucrose) was used to treat control seeds. The glass tubes were kept in controlled climate for 7 d at 28°C. After 7 d of germination, the roots were cut off and dipped in phosphatic buffer. The roots were crushed and shaken vigorously with sterile water and bacterial isolates were isolated by using serial dilution plate technique (Wollum II, 1982) and 1 mL was plated on petri plates containing Pikovskaya's agar medium, incubated at 28°C for 48 h and the number of colonies was counted. The experiment was replicated and the number of bacterial colony forming units (CFU/g of root tip) was calculated (Simons *et al*., 1996). Parametric statistics of ANOVA analysis was carried out to estimate the effect of rhizobacterial isolate inoculation on plant growth and biomass.

Molecular characterization of bacterial isolate using 16S rRNA method: Isolation of genomic DNA from the best salt tolerant antagonistic bacterial isolate and PCR amplification of 16S rRNA sequences were performed following Cho *et al.* (2006). Sequence data was aligned and analyzed for identifying the sample. The program MUSCLE 3.7 was used for multiple alignments of sequences. The 16S rRNA sequence was blast using NCBI blast similarity search tool (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were deposited in Gene Bank (http://www.ncbi.nlm.nih.gov/genbank). The construction of phylogentic tree was done by neighbour joining tree method.

Statistical analysis: All data were subjected to analysis of variance and the significant differences among the means were compared by Duncan's Multiple Range Test (DMRT) at P=0.05 level using SPSS (Version 10.0, SPSS Inc.).

Results and discussion

Isolation of PSB from saline soil samples: Collected soil samples from salt affected areas at Pugalur, Karur district, Tamil Nadu were processed and serial dilutions and pour plant technique were performed. Total of 8 isolates of PSB were obtained. Based on different morphological characteristics and the zone of clearance, the colonies were pure cultured and they were coded as S-3, S-6, S-11, S-12, S-21, S-25, S-26 and S-27. Sleator and Hill (2001) reported that the salt tolerant bacteria adapted to adverse environments of high osmolarity by the accretion of a constrained range of low molar mass molecules, termed compatible solutes owing to their compatibility with cellular processes at high internal concentrations. It was found that all the PSB isolates showed P-solubilization up to 7% NaCl concentration and further increase in NaCl concentration have no halo

zone of clearance but colony growth was observed. The isolate S-27 exhibited maximum solubilization efficacy (95% in 0% and 70% in 7% NaCl) followed by S-25 (91% in 0% and 65% in NaCl), when compared with other isolates. In one of the studies, Sharan *et al*. (2008) have reported that the P-solubilization efficacy of PSB isolates revealed up to 5% concentration. Upadhyay *et al*. (2009) isolated 10 bacterial isolates and they found that maximum P solubilization efficacy up to 8% concentration and they concluded that the bacterial adaptation to the high saline cultivated soil depends on their genetic diversity.

In this study, all the isolates were subjected to quantitative P-solubilization with different NaCl concentrations and it was recorded that P-solubilization was decreased when salt concentration was increased. The PSB isolate S-27 exhibited maximum P-solubilization (324.41 µg/mL) from Pikovaskya's broth without NaCl and at 7% NCl amended broth, the P-solubilization was found to be 218.64 µg/mL in decreasing way (Fig. 1). Kumar *et al.* (2010) found increased P-solubilization with an increase in NaCl concentration. Therefore, it is clear that the microbes isolated from salt affected soils are capable to tolerate the salt stress. In the present study, even in high salt concentration, all the PSB isolates confirmed noticeable levels of P-solubilization. It was also observed that the PSB isolate S-3 has produced IAA in higher level (79.96 µg/mL) in normal broth medium without NaCl, however in 7% NaCl concentration only trace amount (0.07 µg/mL) of IAA production was observed. In case of the PSB isolate S-27 at 0% NaCl concentration, the IAA production was 74.94 µg/mL and in 7% salt concentration the IAA production was 30 µg/mL (Fig. 2). Similar to this study, Upadhyay *et al*. (2009) have reported that the level of IAA production was decreased in all the PSB isolates with increase in NaCl concentrations.

Growth characteristics such as shoot and root lengths, fresh and dry weights of shoot and root of maize plants were recorded after 7 d of the seedlings inoculated with PSB isolates and without application of PSB isolates were kept as control. It was found that 6 PSB isolates showed the ability to promote plant growth in *Zea mays* seedlings and has the ability to colonize in the roots. The PSB isolates S-27 followed by S-25 revealed maximum plant growth in normal soil when compared with other PSB isolates. The shoot and root biomass was also found to be significant in PSB isolate S-27 (Fig. 3). In case of salinity condition, only the PSB isolate S-27 had the ability to promote plant growth and increase shoot and root biomass of maize plants in significant ratio. The PSB isolates S-3, S-11, S-12 and S-26 showed significant growth and shoot and root biomass production of *Zea mays* in normal condition but it failed to support the seed germination in high saline condition.

Fig. 1. Quantitative estimation of P-solubilization under different salt concentrations.

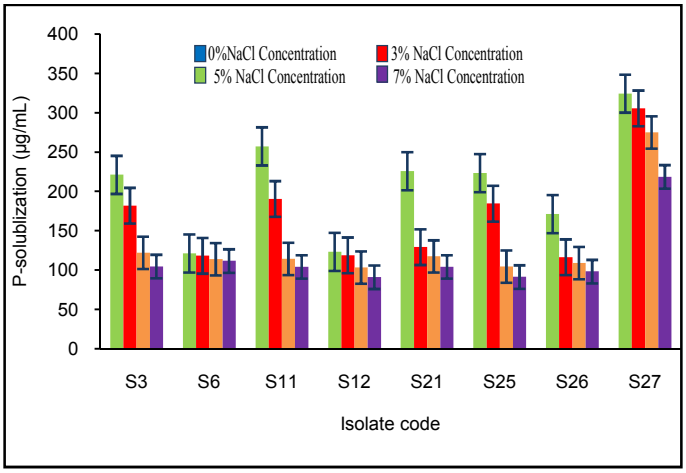

Fig. 2. Effect of IAA production in different concentration of NaCl.

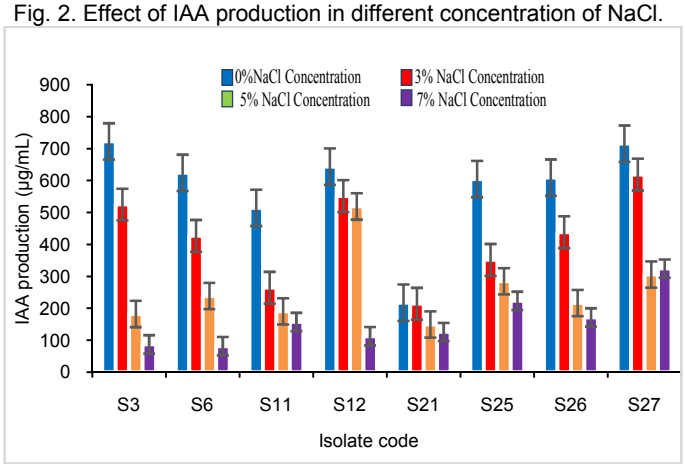

Fig. 3. Effect of Phosphate solubilizing microbes on the growth and biomass data of *Zea mays* in normal condition.

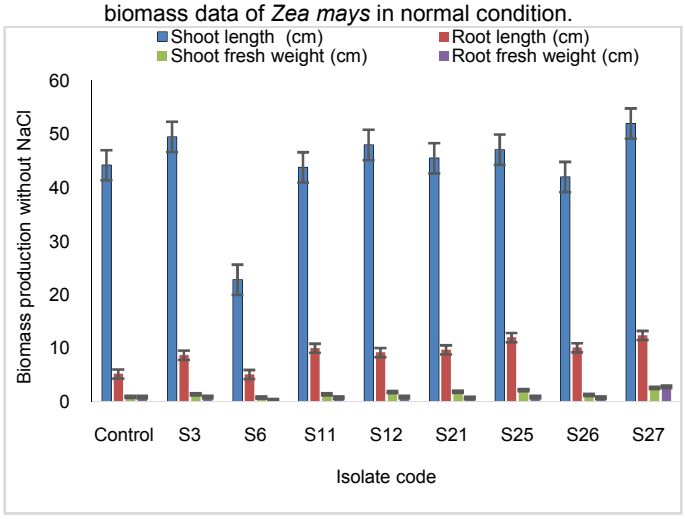

Table 1. Assessment of colony forming units in per gram of root samples from gnotobiotic root colonization assay.

PSB inoculants	Normal condition (without NaCl) CFU/g root*	Saline condition (7% NaCl) CFU/g root*
Control	2×10^5	No growth
S-3	10×10^5	No growth
S-6	12×10^5	No growth
S-11	10×10^5	3×10^5
S-12	8×10^5	No growth
S-21	11×10^5	7×10^5
S-25	12×10^5	17×10^5
S-26	4×10^5	No growth
S-27	26×10^5	20×10^5

*Mean value of three replicates.

Garcia and Hernandez (1996) reported that salinity negatively affects biological activity by high osmotic stress which causes toxic effect on microbial growth excepting tolerant halophytic bacteria. Nautiyal *et al*. (2000) also reported that bacterial strains with their genetic potential for increased tolerance to high salt and high temperature can enhance crop production in semi-arid and arid regions of the world. In root colonization assay, the ability of PSB inoculant to colonize the roots was assessed and the result clearly support the fact that greater the bacterial colonization in root helps the plant to thrive in saline condition. So it reveals that the PSB isolate S-27 has capacity to colonize the roots even in saline condition and support the plants to grow (Table 1). The findings of the study are in accordance with the findings made other researchers Csonka (1989) and Sleator and Hill (2001). They explained that the ability of prokaryotic cells to adapt in extreme environment, by evolving number of osmoadaptive strategies.

Mayak *et al*. (2004) reported that the salt tolerant root colonizing bacteria have succeeded to survive adverse environmental factors could greatly help in harnessing them for their beneficial properties in such environments in which other microorganisms hardly survive. Lugtenberg *et al*. (2001) said that these microbes may be able to increase plant growth, increase the rate of seed germination, improve seedling emergence and responses to external stress factors and also protect plants from disease. The best PSB isolate S-27 was selected for 16S rRNA identification and it shows maximum similarity with *Bacillus cereus.* The salt tolerance of *Bacillus* sp. was reported by many earlier workers. The P-solubilizing *Bacillus* sp. was found to be predominant in the study of Deepika *et al*. (2013). Upadhyay *et al*. (2009) reported that P-solubilizing bacteria from the genus *Bacillus* have evolved highly sophisticated regulatory networks for protection against sudden unfavourable environmental changes, including nutrient starvation, changes in temperature and humidity, oxidative stress, sudden elevation in medium salinity. So it may be the reason for the occurrence of *Bacillus cereus* in adverse saline conditions.

Conclusion

In the present study, all the 8 PSB isolates have an ability to solubilize P even in high concentrations. Based on IAA production, seed germination, root and shoot dry weight and root colonization assay of maize plants under saline condition, the efficient PSB isolate was selected and identified as *Bacillus cereus*. Soil fertility management by using microbial fertilizers is one of the basic components of sustainable agriculture production. Hence, proper formulation of the saline tolerant P-solubilizing bacterial bio-inoculants is very much essential for saline soil and other problematic areas in the country.

Acknowledgements

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

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