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Screening of PGPR from saline desert of Kutch: Growth promotion in *Arachis hypogea* by *Bacillus licheniformis* A2☆

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ABSTRACT

Rhizosphere of a halotolerant plant *Suaeda fruticosa* from saline desert of Little Rann of Kutch, Gujarat (India) was explored for isolation of PGPR form the rare ecological niche having4.33% salinity. Total 85 isolates from the rhizosphere belonging to different species were isolated. Out of 85 isolates, 23 could solubilize phosphate and 11 isolates produced IAA. Seven isolates showed both the traits of phosphate solubilization and IAA production. All isolates which showed either of IAA production or phosphate solubilization or both were further screened for other PGP traits like production of ammonia, siderophore, chitinase, HCN and assessment of their antifungal activity. Out of all the screened isolates, *Bacillus licheniformis* strain A2 showed most prominent PGP traits *in vitro* and it was tested *in vivo* for growth promotion of Groundnut (*Arachis hypogaea*) under saline soil condition. In presence of soil supplemented with 50 mM NaCl, *B. licheniformis* treated plants showed increase in fresh biomass, total length and root length by 28%, 24% and 17% and in absence of NaCl it was 43%, 31% and 39% respectively.

1. Introduction

Little desert of Kutch is a salt marsh with salinity up to 12,094 ppm (Gupta and Ansari 2012). It is located between 22°55″ to 24°35″ North latitudes and 70°30″ to 71°45″ East longitudes near the Great Rann of Kutch, Gujarat, which covers an area of 4953.7 Sq km (Ishnava et al. 2011). Majority of land in desert contains 60% of clay unlike other deserts, which contain high sand in soil (Gupta and Ansari 2012). This salt marsh has rich plant ecosystem containing 253 flowering plants, 23 shrubs, 18 climbers, 157 herbs, 18 tree species and 33 grass types. *Suaeda* spp., *Salvadora persica*, *Capparis decidua*, *Capparis deciduas*, *Calotropis procera*, *Tamarix* spp., *Aeluropus lagopoides*, etc. are few plant species, which dominate in this region (Ishnava et al. 2011). Little Rann of Kutch is nominated to be a "biosphere reserve" which is defined by the areas of terrestrial and coastal ecosystems internationally recognized within the framework of UNESCO's Man and Biosphere (MAB) program (UNESCO World Heritage Centre).

In spite of such ecosystem uniqueness possessed by Little Rann of Kutch, it is least explored for studying rhizobacterial diversity harbored in the rhizosphere of plants adapted to this ecosystem. Rhizobacteria is the group of bacteria residing in the rhizosphere of plant, and they are widely studied for plant growth promoting (PGP) traits they possess (Ahmad et al. 2008). Term PGPR was introduced in 1978 by Kloepper and colleagues (1978). Cultivable rhizobacterial isolates can be screened for obtaining potent PGPR by assessing their PGP traits using *in vitro* methods. Apotheosis for such a screening involves biochemical estimations for Phosphate solubilization, production for Ammonia, Indole 3 Acetic acid (IAA), Siderophore, Chitinase, Hydrocyanic Acid (HCN) (Park et al. 2005; Ahmad et al. 2008). Soluble Phosphate and Ammonia directly support plant growth as they act as macronutrient where as phytohormone IAA accelerates root growth (Bhattacharyya and Jha 2012). Bacterial Chitinase, Siderophores, HCN, etc. produced in the rhizosphere can indirectly support plant growth by suppressing hazardous effects of biotic stresses (Aeron et al. 2011; Ribeiro and Cardoso 2012).

Suaeda fruticosa is a unique plant adapted to grow luxuriously in the harsh ecosystem of Little Rann of Kutch, which can grow optimally at 300 mM NaCl concentration (Khan et al. 2000). So the study is designed to screen PGPR associated with *S. fruticosa* using an *in vitro* methods and the organism showing the maximum PGP traits *in vitro* was tested for *in vivo* pot study under saline conditions. *In vivo* pot experiments were carried out on Groundnut (*Arachis hypogaea*), as it is one of the important legume oil crop across the world. Groundnut is an annual herbaceous plant,

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shows rapid growth and it can easily tolerate soil salinity up to 100 mM NaCl concentration. However, salinity decreases germination, seedling growth and dry matter production of Groundnut (Singh et al. 2008). Therefore, groundnut was selected for *in vivo* pot trials to check the efficacy of plant growth promotion by screened PGPR under saline soil.

2. Materials and methods

2.1. Rhizospheric soil sampling and soil analysis

Rhizospheric soil of halotolerant plant *S. fruticosa* from a unique saline desert of Little Rann of Kutch, Gujarat, India (23°51′ N, 71°28′ E) was collected during the month of February with replicated sampling. 5 g rhizospheric soil from *S. fruticosa* was collected by uprooting the plant with the root system and the soil attached to the roots was scraped using forceps. Microbial flora of collected soil sample was analyzed within 4 days of sample collection. Further, soil sample was examined for its physical and chemical properties. For analysis of physical properties, Sieve analysis and Atterberg limit (Plastic limit, Liquid Limit and Plasticity Index) of soil was performed (Evett and Liu 2007). For chemical properties; total N_2, conductivity, TDS, pH, PO_4^{2-}, Na^+, K^+, Ca^{2+} and Mg^{2+} were estimated from the soil sample.

2.2. Isolation of rhizobacteria and informational diversity analysis

Soil sample was suspended in standard normal saline and vortexed for 20 min at room temperature. The suspension of soil in normal saline was serially diluted and its 0.1 ml was inoculated on various differential medium using spread plate technique for isolation of desired rhizobacteria. *Actinomyctes*, *Azotobacter*, *Pseudomonas*, *Rhizobia* and Fungi were isolated on various differentialmediums such as glucose aspargine agar (GAA), Ashby's mannitol agar (AMA), King's B (KB) agar, yeast extract mannitol agar (YEMA) and rose Bengal agar (RBA), respectively. Other non-specific bacteria were isolated on Nutrient Agar (NA) (Ilyas et al. 2008; Shamseldin et al. 2008). All the differential media were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India and additionally supplemented with 5% NaCl to isolate halotolerant microbes. Plates inoculated with suspensions were incubated at 27 ± 2 °C for 24 h for bacteria and 72 h for actinomycetes. Numbers of colonies obtained on all these agar plates were counted, and their colony characteristics were recorded. Names designated to the isolates were based on the medium from which they were isolated. From the data so obtained, microbial species diversity for cultivable microorganisms was computed. Species richness (Margalef's equation and Menhinick's equations), evenness (Pielou's equation) and diversity indices (Shannon-Weaver index and Simpson's index) along with their derivatives were calculated for cultivable rhizobacterial flora using the method adopted by Jha et al. (2010).

2.3. In vitro assessment of PGP traits of isolated Rhizospheric flora

2.3.1. Quantitative estimation of indole-3-acetic-acid (IAA) production

Spectrophotometric estimation of IAA was performed as per the method developed by Brick et al. (1991) with modifications adapted by Goswami et al. (2013). 72 h old Culture supernatant of the bacterial isolates grown in the respective medium supplemented with L-tryptophan (200 μg ml^{-1}) and 2% NaCl at 27 ± 2 °C was mixed with Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) in the ratio of 1:1. Development of pink color indicates production of IAA and its optical density was recorded at 530 nm. Concentration of IAA produced was estimated against standard curve of IAA (Hi-media) in the range of 10–100 μg ml^{-1}.

2.3.2. Qualitative and quantitative estimation of phosphate solubilization

Each rhizobacterial isolate was spot inoculated on Pikovskaya's agar plate amended with bromophenol blue to test phosphate solubilization ability (SubbaRao 1982). Phosphate solubilization index was calculated (dividing phosphate solubilization zone on Pikovskaya's agar by growth diameter of spot inoculant) after 5 days of incubation at 27 ± 2 °C. The method developed by Pikovskaya (1948) was used for quantitative estimation of tri-calcium phosphate solubilization by the isolate in the liquid Pikovskaya's medium and the pattern of decrease in the pH was also recorded. The concentration of the soluble phosphate was estimated from the supernatant by stannous chloride method (King 1932) after five and ten days of incubation. All the organisms that showed traits of either phosphate solubilization or IAA production were checked for other plant growth promoting traits as well as salt tolerance by growing in respective medium containing 1 M NaCl (Patel et al. 2011).

2.3.3. Ammonia production

Bacterial isolates were grown in peptone water broth for five days at 27 ± 2 °C.0.2 ml culture supernatant was mixed with 1 ml Nessler's reagent and volume of this mixture was made up to 8.5 ml by addition of ammonia free distilled water. Development of brown to yellow color is the indication of ammonia produced, and its optical density was measured at 450 nm using spectrophotometer (Cappucino and Sherman 1992). The concentration of ammonia was estimated using the standard curve of ammonium sulphate in the range of 0.1–1 μmol ml^{-1}.

2.3.4. Quantitative estimation siderophore estimation

Siderophore produced by various isolates were quantified using CAS-shuttle assay (Payne 1994). Cultures were grown in iron-free Fiss minimal medium. Sample was withdrawn and centrifuged at 2700 × *g* for 15 min. CAS assay solution was added to culture supernatant in equal proportion, mixed and allowed to stand for 20 min. Siderophore if present removes the iron from the dye complex, causing reduction in the intensity of blue color which was recorded at 630 nm. For the measurements, minimal medium was used as blank and % siderophore units were calculated by following formula – [(Ar–As)/Ar] × 100 = % siderophore units. Where, Ar = absorbance of reference (minimal media + CAS assay solution), As = absorbance of sample.

2.3.5. Qualitative screening of HCN and chitinase production

For the qualitative estimation of HCN production, Picrate assay described by Castric (1974) was used. Bacterial isolates were streaked on nutrient broth supplemented with 4.4 gm% glycine. A Whatman filter paper No. 1 (soaked in solution of 2% Na_2CO_3 in 0.5% picric acid) was placed in-between base and lid of the culture-plate. Plates were sealed with parafilm and incubated at 27 ± 2 °C for 96 h. Production of HCN was indicated by color change of filter paper from yellow to orange-brown. For qualitative estimation of chitinase, chitin agar plate amended with 2% phenol red was prepared and isolates were spot inoculated and incubated for 120 h at 27 ± 2 °C. Clear zone around the spot inoculants indicates the presence of chitinase (Wang et al. 2008).

2.3.6. Assessment of antifungal activity

The agar well diffusion method described by Mehmood et al. (1999) was used to assess antifungal activity. Antifungal activity of bacterial isolates was tested against *Fusarium oxysporum* f. sp. *Cubense* (KC351189), isolated previously by Thakker et al. (2011). A test fungus was grown on potato dextrose agar (PDA). The