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Effect of succinate on phosphate solubilization in nitrogen fixing bacteria harbouring chick pea and their effect on plant growth

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ABSTRACT

Diverse nitrogen fixing bacteria harbouring chick pea rhizosphere and root nodules were tested for multiple plant growth promoting traits like tricalcium phosphate (TCP) and rock phosphate (RP) solubilization, production of ammonia, indole 3-acetic acid, chitinase, phytase and alkaline phosphatase. Isolates belonged to diverse genus like *Enterobacter*, *Acinetobacter, Erwinia, Pseudomonas, Rhizobium, Sinorhizobium, Ensifer, Klebsiella,* etc. Most isolates solubilized TCP and RP along with the lowering of media pH, indicating acidification to be the chief mechanism behind this solubilization. However, lowering of media pH and P release decreased by 32–100% when media was supplemented with succinate, a major component of plant root exudates indicating succinate mediated repression of P solubilization. Maximum TCP and RP solubilization with P release of 850 µg/mL and 2088 µg/mL was obtained with lowering of media pH up to 2.8 and 3.3 for isolate E43 and PSB1 respectively. This pH drop changed to 4.4 and 4.8 with 80% and 87% decrease in P solubilization in the presence of succinate. Maximum 246 µg/mL indole 3-acetic acid production in Lh3, 44.8 U/mL chitinase activity in MB3, 11.3 U/mL phytase activity in I91 and 9.4 U/mL alkaline phosphatase activity in SM1 were also obtained. Most isolates showed multiple PGP traits which resulted in significant plant growth promotion of chick pea plants. Present study shows repression of P solubilization by succinate for various bacterial groups which might be one of the reasons why phosphate solubilizing bacteria which perform well *in vitro* often fail *in vivo.* Studying this repression mechanism might be critical in understanding the *in vivo* efficacy.

1. Introduction

Rhizosphere is a narrow region of soil in immediate proximity to the root system inhabited by a large group of beneficial bacteria called as plant growth promoting rhizobacteria (PGPR) which influence plant growth through diverse direct and indirect mechanisms. Direct mechanisms of plant growth promotion (PGP) encompass nitrogen (N_2) fixation, phosphate (P) solubilization, improving plant nutrient uptake, siderophore production, modulating levels of phytohormones like indole-3 acetic acid (IAA) whilst production of antibiotics, lytic enzymes, ability to survive competition and inducing systemic resistance in plants comprise the indirect mechanisms (Ahemad and Kibret, 2014). P is an important macronutrient which occurs in soil in organic and inorganic forms. However, most of the soil P is in a form unavailable for plant uptake, but can be converted to plant available forms by phosphate solubilizing bacteria (PSB) (Zaidi et al., 2009) like *Flavobacterium, Achromobacter, Agrobacterium, Aerobacter, Bacillus, Erwinia, Micrococcus, Pseudomonas, Burkholderia, Rhizobium*, etc (Rodriguez and Fraga, 1999). In addition to providing P to the plant, PSBs augment N_2 fixation, improves availability of trace elements and secrete chemicals which aid plant growth (Zaidi et al., 2009). Ability of bacteria to solubilize dicalcium phosphate (DCP), tricalcium phosphate (TCP), rock phosphate (RP) and hydroxyapatite have been studied (Shashidhar and Podile, 2010). Rock phosphates in the form of apatite, hydroxyapatite and oxyapatite along with mineral phosphate in association with metals form the biggest inorganic soil P reserve. This is followed by organic phosphates in the form of phyate which comprises 50% of soil organic P (McLaughlin et al., 1990). Major mechanism of inorganic P solubilization is organic acid production leading to acidification. Gluconic acid is an important organic acid produced by fluorescent *Pseudomonas* (Vyas and Gulati, 2009), P. *cepacia* (Goldstein et al., 1993), *E. herbicola* (Liu et al., 1992), etc. whereas 2-ketogluconic acid mediated P solubilization was found in *Pseudomonas* (Vyas and Gulati, 2009), *B. firmus* (Banik and Dey, 1982) and *R. leguminosarum* (Halder et al., 1990). Organic P is mineralized by acid phosphatases in *Serratia* sp. (Behera et al., 2017), *Pseudomonas* (Gugi et al., 1991), *Rhizobium* (Abd-Alla, 1994), *Enterobacter, Citrobacter, Serratia* and *Klebsiella* (Thaller et al., 1995), etc whereas phytase activity has been shown in *B. subtilis, P. putida,* etc

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(Richardson and Hadobas, 1997, Singh et al., 2014). Though P solubilization has been studied in wide range of bacteria, succinate mediated repression of P solubilization have been shown only in *P. aeruginosa* (Patel et al., 2011) and *K. pneumoniae* (Rajput et al., 2013). Present study is aimed at characterizing free living and symbiotic N_2 fixers for inorganic P solubilization and its succinate mediated repression which might be one of the reasons why these PSB which perform well *in vitro* often fail *in vivo*.

2. Materials and methods

2.1. Isolation and characterization of isolates

Composite soil samples collected from fields of chharodi, district Ahmedabad, Gujarat were clay loam soil having good water holding capacity and pH of 7.4. Healthy chick pea plants, 25–30 days after germination were uprooted and packed into sterile polythene covers. The plants were transported immediately to the laboratory where healthy root nodules and rhizospheric soil gently adhering to the root hairs were collected. Soil samples were serially diluted and plated onto Luria Bertani Agar and incubated at 30 °C for 24–48 h. Root nodules were surface sterilized with 0.1% $HgCl_2$, crushed in sterile distilled water, nodule sap was spread on Yeast extract mannitol agar with congo red and incubated at 28 °C for 48–72 h. Colonies with distinct morphology were plated onto Ashby's mannitol agar to check their ability to grow on N free media. N_2 fixers were selected and characterized biochemically according to Bergey's Manual of Systematic Bacteriology. All selected isolates were characterized for lactose fermentation, phenylalanine deaminase production, acetoin production, citrate utilization, ability to hydrolyse starch, etc. Molecular identification was carried out through 16S rDNA sequencing using universal primers F27 (5′-AGA GTT TGA TCA TGG CTC AG-3′) (Cheneby et al., 2000) and R1492 (5′-TAC GGT TAC CTT GTT ACG ACT T-3′) (Wang and Wang, 1996). Multiple sequence alignment was carried out with sequence data available in the GenBank using BLASTn algorithm (Altschul et al., 1997).

2.2. Characterization of isolates for P solubilization

To check the ability of isolates to solubilize TCP and RP, isolates were spot inoculated on Pikovskaya's agar (Pikovskaya, 1948) and 25 mM Tris buffered rock phosphate (TRP) agar with methyl red pH indicator (Gyaneshwar et al., 1998) respectively. 100 mM glucose was added as sole carbon source. Clear halo around the colonies in Pikovskaya's agar plate and red coloration around the colonies in TRP agar indicated TCP and RP solubilization respectively. P solubilization was quantitatively estimated in Pikovskaya's broth and 25 mM Tris buffered RP broth inoculated with test cultures and incubated at 30 °C for 4 days. Samples were withdrawn at 24 h intervals, centrifuged at 9000 rpm for 10 min and the cell free supernatant was used to determine the pH change, residual glucose concentration and soluble P using Ame's method (Ames, 1966).

2.3. HPLC analysis of organic acids produced

Isolates showing maximum TCP solubilization, E43 and RM1 were grown in TRP broth with 100 mM Glucose as sole carbon source. Cultures with minimum pH and maximum P release were selected for HPLC analysis. Cells were pelleted down at 9000 rpm for 10 min at 25° C, supernatant was filter sterilized using 0.22-μm Nylon filter and subjected to HPLC analysis in Perkin Elmer series 200 with Hypersil C18-column. pH of 25 mM KH_2PO_4 was set to 2.5 with orthophosphoric acid and was used as a mobile phase at a flow rate of 1 mL/min. Organic acids were detected using UV/VIS detector at 214 nm. Gluconate, malate, citrate, acetate and succinate were taken as standards. Retention time of the organic acid and area under curve were compared with that of standards to identify the type and concentration of the organic acid produced.

2.4. Determining succinate mediated repression of P solubilization

To check succinate mediated repression of TCP and RP solubilization, isolates were spot inoculated on Pikovskaya's agar (Pikovskaya, 1948) and 25 mM tris buffered RP agar (Gyaneshwar et al., 1998) with 50 mM glucose and 50 mM succinate. Repression of P solubilization was quantitatively estimated in Pikovskaya's broth and TRP broth with carbon sources as indicated above, incubated at 30 °C for 4 days. Samples were withdrawn at 24 h intervals, centrifuged at 9000 rpm for 10 min and the cell free supernatant was used to determine the pH decrease, residual glucose and soluble P using Ame's method (Ames, 1966).

2.5. Characterization of isolates for ammonia and IAA production

For checking ammonia production, all the isolates were grown in peptone water at 30 °C for 48–72 h and NH_3 production was tested using Nessler's reagent (Cappuccino and Sherman, 1992). For checking IAA production and related substances, isolates were inoculated in Tryptophan broth and incubated at 30 °C for 48 h. The concentration of IAA produced was determined spectrophotometrically at 530 nm using Salkowski's reagent (Gordon and Weber, 1951).

2.6. Characterization of isolates for enzyme production

2.6.1. Chitinase activity

Isolates were grown on Chitinase detection medium as described by Kim et al. (2003). Chitinase assay was performed with colloidal chitin as a substrate. Release of N-acetylglucosamine was estimated using Dinitrosalicylic acid (DNS) (Miller, 1959). One unit of chitinase activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of *N*-acetylglucosamine per mL per min.

2.6.2. Phytase activity

Phytase activity was determined in liquid phytate solubilization media (PSM) (Engelen et al., 1994). One unit of phytase activity was defined as the amount of enzyme that hydrolyzed 1 μM of Ca-phytate per ml per min.

2.6.3. Alkaline phosphatase activity

Alkaline phosphatase activity was checked as described (De Prada et al., 1996). One unit of phosphatase activity was defined as the amount of enzyme that hydrolyzed 1 μM pNPP per mL per min.

2.7. Pot experiments

Fertile field soil was collected and sterilized by repeated cycles of autoclaving. Soil used for plant growth experiments was clay and loamy with pH 7.5 and total P content of 0.10 g/L. All pots were filled with about 1 kg sterile soil and watered with 150 mL autoclaved water. Chick pea seeds were surface sterilized with 0.1% $HgCl_2$ and allowed to imbibe overnight. The seeds were bacterized overnight and then placed at a depth of 5 cm in plastic pots with sterilized soil and watered with sterile water. Pots were inoculated with the four isolates RS1, Pt1, E43 and PSB1 in all combinations which showed good P solubilization and other PGP traits. Inoculated pots were maintained under greenhouse conditions (10 h day and 14 h night cycle) at 25 °C. Uninoculated plants served as control. Plants were uprooted after 30 days of inoculation and were scored for multiple plant growth parameters.

2.8. Statistical analysis

All the experiments were performed in triplicates and result values