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Research Article

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Phosphate Solubilizing Rhizobia Promote the Growth of Chickpea under Buffering Conditions

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ABSTRACT

In the present study rhizobial isolates from root nodules of various legumes were screened for phosphate (P) solubilizing ability in both non-buffered and buffered media. Thirty five percent isolates showed P. solubilizing ability in buffering conditions. There was reduction in pH of media with increase in P. concentration up to 15 days. Isolate RASH6 selected on the bases of highest P. solubilizing ability in buffering condition also showed IAA, siderophore, ammonia and HCN production. HPLC analysis showed production of succinic and gluconic acids in the non-buffered culture medium.In buffered media citric, succinic and gluconic acids were produced in higher concentrations in comparison to non-buffered media, due to enhanced requirement of organic acids for reduction of pH in buffered condition. Pot study showed that treatment with RASH6 had a significant effect on chickpea growth in soil amended with tricalcium phosphate in comparison to without any amendment, indicating the role of P. solubilization ability of rhizobia in plant growth enhancement. In treatment of soil amended with soluble phosphate indicates that as soluble phosphate is already present in the soil, phosphate solubilization mechanism may not be involved however other plant growth mechanism may be responsible for plant growth promotion. There was enhancement in nodulation and nitrogenase on RASH6 treatment in soil amended with phosphates indicated that improvement in P nutrition of the plant was responsible for increased nodulation and N_2-fixation. Rhizobial isolates having N_2 fixing as well as high P solubilizing capability can be of great value for sustainable yield enhancement.

Key words: *Buffering condition, chickpea, organic acids, phosphate solubilizing microorganisms, rhizobia.*

INTRODUCTION

Phosphorus (P) after nitrogen is the most important nutrient limiting agricultural production[1,2]. Soils are often abundant in insoluble P, either in organic or inorganic forms, but deficient in soluble phosphates essential for growth of most plants and microorganisms. Soluble forms of phosphate fertilizers are widely applied to agricultural soils in order to circumvent P-deficiency but 75 to 90% of added P is rapidly precipitated as insoluble forms and becomes unavailable to plants[3]. Converting insoluble phosphates (both organic and inorganic) to a form available for plants is a necessary goal to achieve sustainable agricultural production. Several reports show the ability of diverse bacteria to solubilize inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate[4]. Among the bacterial genera reported to express phosphate solubilization are *Pseudomonas, Bacillus,* rhizobia*, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium* and *Erwinia*[5].

Phosphate solubilization ability of microorganisms in soil may be different from that found under laboratory conditions[6]. In soil pH buffering capacity is governed mostly by protonation/deprotonation of acidic group on organic matter, oxides and hydroxides, dissolution/precipitation of carbonates etc[7]. The buffering capacity of soils can limit solubilization of soil phosphates by microorganisms as it has been shown that solubilization of calcium phosphate (Ca-P) complexes are mediated mainly by lowering the pH of the medium[8]. According to Gyaneshwar *et al.*[9], it is common to obtain phosphate solubilizing microorganisms (PSM) under laboratory conditions, while field performances by the PSM are highly variable. No increase in crop yield or P uptake was found in 70% of field experiments. To find a highly efficient PSM that performs well under laboratory conditions and in soil remains a challenge.
Rhizobia are well known for biological nitrogen fixation, an important source of nitrogen, and various legume crops could fix as much as 200 to 300 kg of nitrogen per hectare or about 70 million metric tons of nitrogen per year[10,11]. It has been reported that certain strains of *Rhizobium* can also solubilize both organic and inorganic phosphates[12,13]. But studies on phosphate solubilizing ability of rhizobia are very limited[14,15,16]. The main advantage of using rhizobia as a PSM will be their beneficial nutritional effect resulting both from phosphate mobilization and nitrogen fixation[17]. The aim of this work is (i) to screen rhizobial isolates with phosphate solubilizing ability even under buffering conditions and (ii) check the plant growth promoting ability of selected isolates taking chickpea (*Cicer arietinum*) as test crop.

MATERIALS AND METHODS

Isolation of phosphate solubilizing rhizobia

Root nodules from leguminous plants were collected from Kanpur (UP, India) and adjoining areas. Rhizobia were isolated on congo red yeast extract mannitol (YEM) agar medium according to Somasegran and Hoben[18]. Prolific colonies developed after 48 h of incubation at 28°C were picked and re-streaked on fresh prepared plates to obtain pure cultures. The purified rhizobial isolates were stored at -20°C in 25% glycerol and checked for production of nodules in their respective host plants. Briefly surface sterilized seeds were inoculated by immersion for 15 min in a RASH6 culture in YEM broth (48 h) and then potted in sterilized soil for 20 days.

Phosphate solubilization ability

The phosphate solubilizing ability of the isolates was tested on Pikovskaya's agar medium containing (per liter): 0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$,0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4.7H_2O$ and 15 g agar. After 7 days of incubation at 28°C, isolates that induced clear zone around the colonies were considered as positive[19]. The solubilization index (SI) of isolates was calculated as follows:

$$SI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

The effect of buffering condition on phosphate solubilizing activity of rhizobial isolates was checked on RP minimal agar medium containing 100 mM glucose, 25 µM $MgSO_4$, 10 mM NH_4Cl and the following micronutrients per liter: 3.5 mg $FeSO_4.7H_2O$, 0.16 mg $ZnSO_4.7H_2O$, 0.08 mg $CuSO_4.5H_2O$, 0.5 mg H_3BO_3, 0.03 mg $CaCl_2.H_2O$, 0.4 mg $MnSO_4.H_2O$ and 15 g agar. The phosphate source was 5 g of tricalcium phosphate. The pH of the medium was adjusted to 7.0. Sterilized tricalcium phosphate was added to media before pouring. For buffering of media 100 mM tris HCl, pH 7.0 was used. Methyl red indicator dye was used at 0.01% in plates. The effect of buffering on phosphate solubilization was recorded by measuring the diameter of red zone around the colony after different time intervals up to 7 days and SI calculated.

Quantitative estimation of phosphorus contents

The phosphorus solubilization potential of rhizobial isolates was tested under both non-buffering and buffering conditions in the Pikovskaya's broth and RP minimal broth amended with known amount of

tricalcium phosphate as a substrate, respectively. Uninoculated broth was used as control. The flasks were incubated at 28°C for 30 days and centrifuge at 21129 x g. Available phosphorus was determined in supernatant following the procedure of American Public Health Association[20]. The pH of the culture medium was recorded.

Other plant growth promotory characteristics

Rhizobial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in tubes and incubated at 28°C. After 72 h Nessler's reagent (0.5 ml) was added in each tube and observed for the change in color from yellow to brown. Siderophore production was determined in CAS agar medium according to the Schwyn and Neilands[21]. Color change of the medium from blue to orange was taken as indication of siderophore production by rhizobial isolates. For siderophore estimation cell free-supernatant of RASH6 was subjected to detection and estimation of siderophore by CAS shuttle assay[22]. The production of IAA by rhizobial isolates was determined according to the method of Bric *et al.*[23]. The log phase culture of rhizobial isolates was inoculated in YEM broth supplemented with tryptophan (500 µg/ ml) and IAA concentration determined using Salkowski's reagent. The concentration of IAA was evaluated by comparison with standard curve. Production of HCN was observed on YEM agar medium containing glycine (4.5 g/l) according to Baker and Schippers[24]. A change in color of filter paper from yellow to reddish brown was recorded as an index of cyanogenic activity.

Organic acid production

For the analysis of organic acids produced by selected isolate RASH6, non-buffered and buffered broths (7 days after inoculation) were filtrated through a 0.22 ml filter (Millipore, TBP) and injected (20µl) in HPLC (Perkin Elmer) C-18 column. The mobile phase was 0.1% phosphoric acid at a flow rate of 1 ml/min; the column temperature was 55°C. UV absorption was measured at 210 nm. Organic acid in purified solutions were determined by comparing the retention time and peak areas of chromatograms with the standards of citric, gluconic, succinic, lactic, fumaric, oxalic, malic and tartaric acids[25].

Acid production *in vitro* (tube)conditions

Chickpea (*Cicer arietimum*) seeds were surface sterilized with 2 % sodium hypochlorite for 5 min followed by three washes with sterile distilled water (DW). Seeds were germinated onto water-agar plates (1% agar) at 25°C. The seedlings were inoculated by immersion for 15 min in a RASH6 culture in YEM broth (48 h) and placed into tubes containing non buffered and buffered (using 100 mM Tris HCl) semi solid agarized Fähraeus medium with 0.01 % methyl red as acid-base indicator[26]. Uninoculated seedlings in both non-buffered and buffered media were taken as negative controls.

***In vitro* nodulation and plant growth promotion**

The PGP activity of selected rhizobial isolate RASH6 was detected taking chickpeaas a test crop. Seeds were surface sterilized with 70 % ethyl alcohol for 2 min followed by 2% sodium hypochlorite (10 min) and germinated in tubes (2 seed/tube) 1/3 filled with plant growth media[27] (PGM)and PGM modified by replacing K_2HPO_4 and KH_2PO_4 with tricalcium phosphate. Buffering condition was maintained using 100 mM Tris HCl. The experiment was conducted using non-modified and modified PGM in following sets: (i) seeds (control) (ii) seeds + 100 mM Tris HCl (iii) Seeds + RASH6 (iv) Seeds + RASH6 + 100 mM Tris HCl.

Seeds were bacterized by dipping in cell suspension of RASH6 (OD_{610} = 0.1) for 10 min and dried overnight under aseptic condition. After 10 days of growth root length and shoot length were measured[28]. The experiment was conducted in triplicate.

***In vivo* plant growth promotion**

Chick pea plants were raised from surface-sterilized seeds in plastic pots filled with steam-sterilized local soil (500 g; P = 0.0908 %, pH = 8.4). The experiment was carried out in six different treatments: (i) soil (control) (ii) soil + 0.2% tricalcium phosphate (w/w) (iii) soil + 0.1% K_2HPO_4 (w/w) + 0.1% KH_2PO_4 (w/w) (iv) soil + RASH6 (v) soil + 0.2% tricalcium phosphate (w/w) + RASH6 (vi) soil + 0.1% K_2HPO_4 (w/w) + 0.1% KH_2PO_4 (w/w) + RASH6. After emergence of seedlings, plants were thinned to five plants

per pot. At harvest (30 days), root length, shoot length, nodule number per plant, fresh weight and dry weight were determined[29]. Symbiotic nitrogenase activity was measured in situ as C_2H_4 evolution according to Minchin *et al.*[30].

RESULT AND DISCUSSION

In the present study, we screened rhizobial isolates from root nodules of chickpea, pigeon pea, sweet pea, masoor and lobia. All the isolates showed nodule formation in their respective host plants.Out of 100 rhizobial isolates 70% showed phosphate solubilizing index in a range of 2.2 to 4.1 on Pikovskaya's agar media. Isolate RASH6 showed maximum solubilization index. (Fig. 1a). The study reports that in buffering condition only 35 % showed phosphate solubilizing ability. Under buffering condition maximum phosphate solubilization index (3.8) was showed by RASH6. (Fig. 1b). In buffered medium phosphate solubilizing ability of rhizobial isolates got reduced. There are several reports of reduced or loss in P-solubilizing ability of the bacteria under buffered media conditions[6,31]. Maximum phosphate was solubilized by isolate RASH6 in both non-buffered (Pikovskaya's broth) and buffered (RP minimal broth) condition. Isolate RASH6 showed 275 µg/ ml phosphate solubilization and lowered the pH to 3.4 on twentieth day in Pikovskaya's broth (Fig. 2a). However, in buffering condition RASH6 showed phosphate solubilization of 202.5 µg/ml with reduction of pH to 3.9 (Fig. 2b) on fifttenth day in RP minimal medium. After 15 days no further increase in phosphate solubilization ability and reduction in pH were observed (Fig. 2). The study showed reduction in pH of media with increase in amount of phosphate solubilized by rhizobial isolates. Walpola and Yoon[32] demonstrated that symbiotic nitrogenous rhizobia, which fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plants, have also shown phosphate solubilization activity. The PSMs are known to solubilize Ca-P complexes mainly by lowering the pH of the media by secreting organic acids[33,34]. Phosphate solubilizing bacteria produced more available P by the production of organic acids which act like chelates and solubilized insoluble phosphorus[35]. Phosphorus solubilization ability of PSB has direct correlation with pH of the medium[36]. The decrease in phosphate solubilizing ability after a certain period of incubation in both Pikovskaya's media and in RP media may be due to production of certain toxic metabolites during late log or decline phase or due to autolysis of cells as suggested by Trivedi and Sa[29].

The selected rhizobial RASH6 isolate also had the ability of IAA, siderophore, ammonia and HCN production. Isolate RASH6 produced 98.80 µg/ ml of IAA and 27.6% of siderophore units after 48 h of growth. PSB can exert a direct effect on plant growth through various mechanisms such as phosphate solubilization, production of phytohormones, biological nitrogen fixation, and increased iron nutrition through iron-chelating siderophores and volatile compounds that affect the plant signaling pathways[37]. Most root promoting bacteria synthesize IAA causing rapid establishment of roots advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their capacity for survival[27,28]. Microbial siderophores are well known for their property to promote plant growth by supplying iron[28]. Siderophore production also is one of the mechanisms involved in the solubilization of iron-bound phosphorus by the microorganisms[25]. Ammonia produced by bacteria is taken up by plants as a source of nitrogen for their growth[39]. Earlier studies reported the role of HCN in disease inhibition in vitro conditions[40].

HPLC analysis showed two peaks in case of non-buffered media, identified as succinic (305.14 µg/ ml) and gluconic (16.86 µg/ ml) acids by comparing the retention time with those of authentic standards. In case of buffered media peak for citric acid (368.54 µg/ ml) was also found in addition to succinic (453.27 µg/ml) and gluconic acids (24.20 µg/ml). The concentrations of succinic and gluconic acids were higher in buffered over non-buffered media, with the highest increment observed for succinic acid (Fig. 3). HPLC analysis showed the production of organic acids in media containing glucose. Hwangbo*et al.*[41] reported production of organic acids during the metabolism of glucose, a mechanism responsible for the dissolution of inorganic insoluble phosphate. The amount of succinic and gluconic acids produced in buffered media was higher than that in non-buffered media may be due to enhanced requirement of

organic acids for reduction of pH in buffered condition. Only in buffering condition RASH6 showed production of citric acid. Gyaneshwar *et al.*[6] showed that oxalic and citric acid are most effective to solubilize soil phosphates.

In vitro acid production analysis showed that tubes containing seedlings developed from seeds inoculated with RASH6 developed a significant area of acidification in both un-buffered and buffered condition (Fig. 4). This acidification can be ascribed to the production of organic acid/s. This assumption is made on the basis that no acidification was observed around seedlings from non-inoculated seeds. Crespo *et al.*[42] showed an intense acidification in the root environment of tomato and wheat seedlings inoculated with a *G. diazotrophicus*.

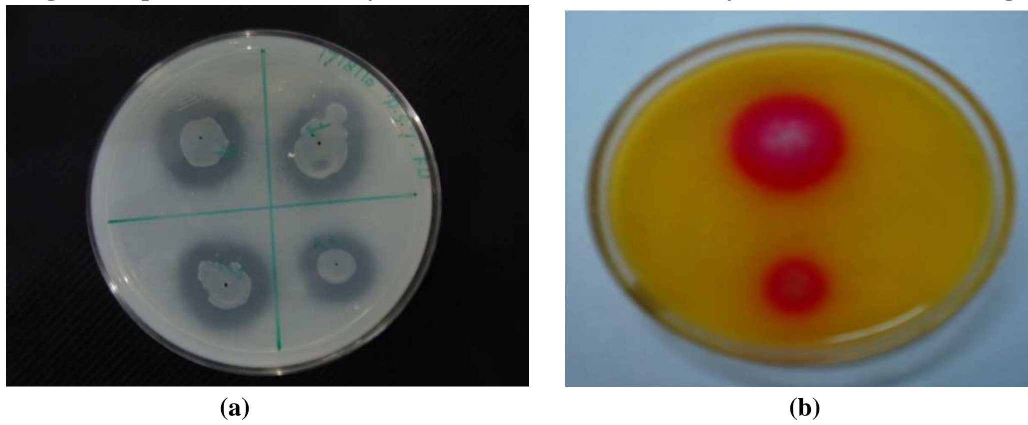
Treatment with phosphate solubilizing rhizobial strain RASH6 showed 20.10 % and 16.54 % enhancement in root and shoot length over control in non-buffering condition. However, in buffering condition there was 16.31 % and 12.27 % enhancement of root and shoot length respectively. In both *in vitro* and *in vivo* studies, RASH6 inoculation had a positive effect on all the chickpea growth parameters. Best growth of chickpea plants was observed when soluble phosphate was added to soil both with and without RASH6 treatment. Nodules developed only in cases of RASH6 treatment. In case of soil amended with tricalcium phosphate and soluble phosphates there was 38.10 % and 52.38% enhancement of nitrogenase activity over only soil control. There was 66.67 % enhancement in dry weight by RASH6 over control in absence of any phosphate amendment. Addition of tricalcium phosphate RASH6 caused 131.82 % enhancement in dry weight in comparison to control (without RASH6 treatment in soil amended with tricalcium phosphate). Soil amended with soluble phosphate and RASH6 showed 152 % increase in dry weight over control (Table 1). In case of the soil amended with tricalcium phosphate, treatment with RASH6 had a significant effect on plant growth. This shows the role of rhizobia in solubilization of insoluble forms of phosphate in actual soil conditions. It is a well-established fact that improved phosphorous nutrition influences overall plant growth and root development[43]. The enhancement in nitrogenase activity of RASH6 in soil amended with phosphates indicates an improvement in P nutrition of the plant resulting from the solubilization of phosphates by inoculated rhizobia. The enhancement in nitrogenase activity correlated with increase in nodulation and N_2-fixation. It is well-known that nodulation and N_2-fixation processes are P-dependent[44]. RASH6 showed enhanced PGP activity in soil amended with soluble phosphate amended soil over tricalcium amnded soil, indicates that as phosphate is already available to plant, the P-solubilization mechanism of plant growth promotion was not effective in this condition, and other PGP mechanisms are involved. This is a known fact that most of the phosphorous occur in insoluble form as calcium phosphates in alkaline soils[37]. Soil with Ca–P as a major phosphorous source also has high buffering capacity[45]. The present study reported rhizobial isolate with strong Ca-P solubilizing activity even in buffering condition. The plant growth promotory bacteria having a number of beneficial mechanisms for plant health improvement is of immense importance to improve plant health. It is a well known fact that phosphorus gives strength and maturity to the crop[46]. Therefore, finding agricultural inoculants with high phosphate solubilizing capability in buffered soil condition and nitrogen fixing ability would be of immense interest for sustainable improvement of plant health.

Table 1: Effect of various treatments on growth of chickpea plant

Treatment	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)	Nodule No./ Plant	Symbiotic Nitrogenase Activity (μmol C_2H_4/plant/h)
Seed (Control)	$07.17^a \pm 0.01$	$12.10^a \pm 0.02$	$0.53^a \pm 0.02$	$0.18^a \pm 0.01$	0.0^a	0.00^a
Seed + RASH6	$10.12^c \pm 0.01$	$18.58^d \pm 0.01$	$1.50^b \pm 0.01$	$0.34^b \pm 0.02$	$7.0^b \pm 0.02$	$0.21^b \pm 0.01$
Seed + RP	$07.80^a \pm 0.02$	$14.70^b \pm 0.01$	$0.65^a \pm 0.02$	$0.22^a \pm 0.01$	0.0^a	0.00^a
Seed + RP + RASH6	$11.18^d \pm 0.01$	$20.64^e \pm 0.01$	$1.73^b \pm 0.01$	$0.51^c \pm 0.02$	$8.5^c \pm 0.01$	$0.29^c \pm 0.01$
Seed + SP	$08.40^b \pm 0.02$	$16.30^c \pm 0.01$	$0.84^a \pm 0.01$	$0.25^a \pm 0.01$	0.0^a	0.00^a
Seed + SP + RASH6	$13.00^e \pm 0.01$	$19.72^e \pm 0.02$	$2.14^c \pm 0.02$	$0.63^d \pm 0.01$	$9.5^d \pm 0.01$	$0.32^d \pm 0.02$

Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range test. RP = Tricalcium phosphate, SP = $K_2HPO_4 + KH_2PO_4$

Fig.1: Phosphate solubilization by rhizobial isolates in (a) Pikovskaya's and (b) RP minimal agar media

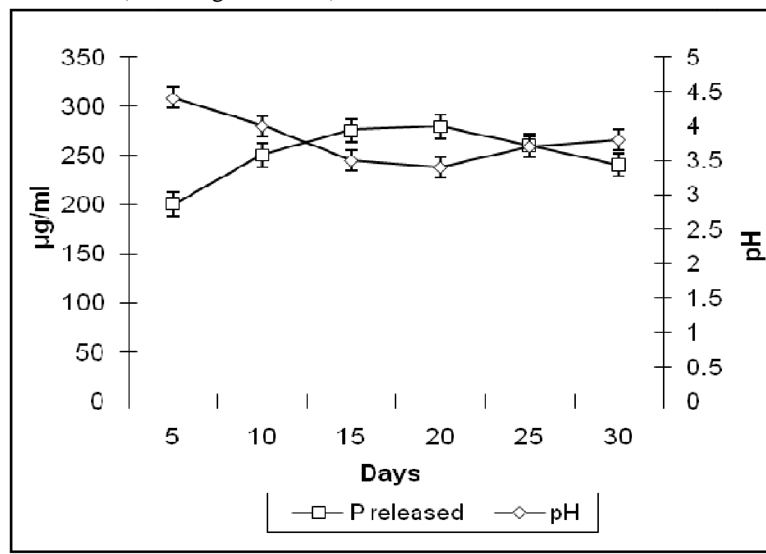


(a) **(b)**

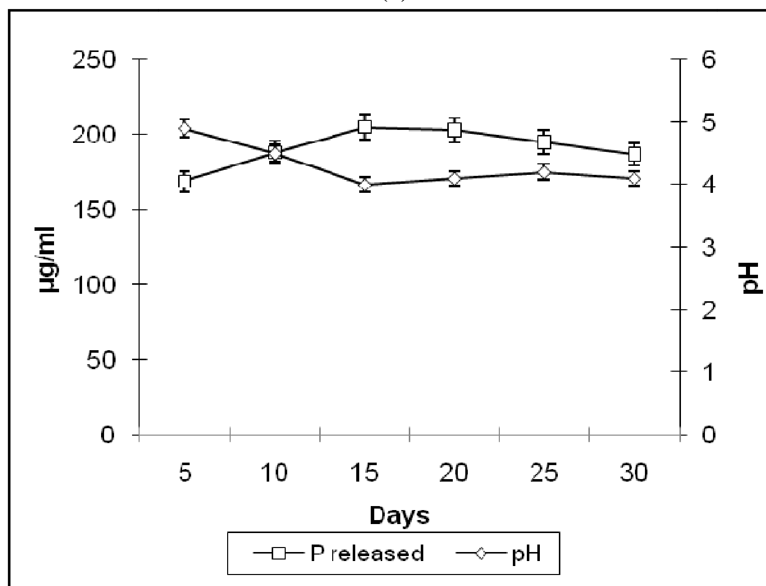
Fig.2: Changes of pH and phosphate solubilized in media containing tricalcium phosphate during 30 days of incubation

(a) Pikovskaya's broth (non-buffering condition)

(b) RP minimal broth (buffering condition)

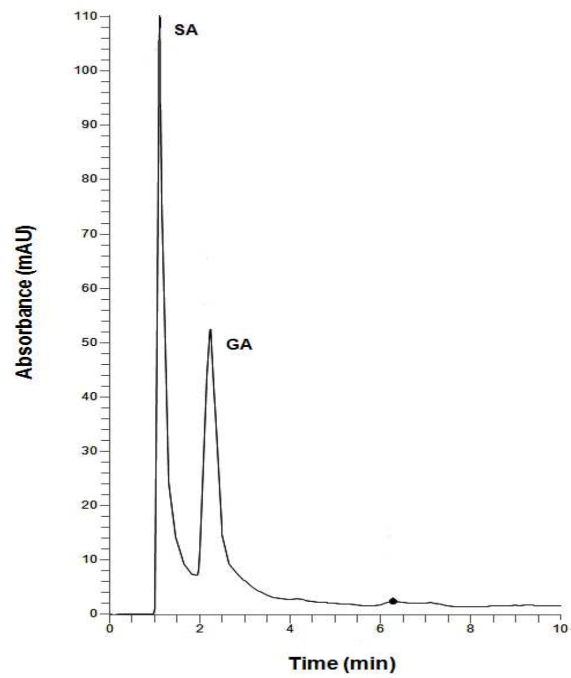


(a)

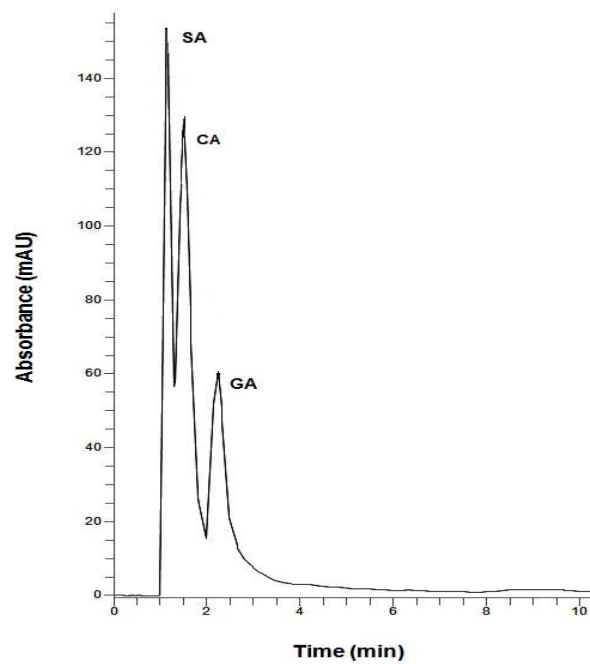


(b)

Fig.3: HPLC chromatograms of culture supernatant of rhizobial isolate RASH6 in (a) non-buffered and (b) buffered broth. SA = Succinic acid, GA = Gluconic acid, CA = Citric acid



(a)



(b)

Fig.4: Acidification in the root environment of chickpea seedlings developed from non-inoculated seeds (control) and RASH6 inoculated seeds

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