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Phosphorus uptake and growth promotion of chickpea by co-inoculation of mineral phosphate solubilising bacteria and a mixed rhizobial culture

M. Gull[A], *F. Y. Hafeez*[A,C], *M. Saleem*[B] *and K. A. Malik*[A]

[A]National Institute for Biotechnology and Genetic Engineering, PO Box 577, Jhang Road, Faisalabad, Pakistan.
[B]Department of Botany, University of Agriculture, Faisalabad, Pakistan.
[C]Author for correspondence: e-mail: fauzia@nibge.org

Abstract. Isolation of phosphate solubilising bacterial strains was carried out from rhizosphere, roots and nodules of chickpea, to study the viability for solubilisation of tri-calcium phosphate and the effect on growth of chickpea plants. The potential of isolated bacterial strains to solubilise phosphate was qualitatively evaluated by the measurement of a clear zone around the colonies. The diameter of this zone ranged from 21 to 83 mm. Phosphate solubilisation, by phosphate solubilising bacterial isolates, was quantified by spectrophotometry and was found to range from 65 to 130.5 μg/mL. The drop in pH ranged from 5.6 to 3.6. The plant growth, shoot phosphorus and nitrogen concentrations, nodulation efficiency and nitrogenase activity were significantly enhanced, showing the positive effect of phosphate solubilising bacteria inoculation. Phosphate solubilising bacterial strains CPS-2, CPS-3 and Ca-18 had the maximum positive effect on shoot length, shoot dry weight and nodulation of chickpea plants. Treatments inoculated with non-phosphate solubilising bacterial strains IFA_1 and IFA_2 showed the minimum values in all the parameters.

Introduction

Phosphorus (P) is a limiting factor for plant growth as, compared with other major nutrients, it is the least mobile and available to plants in most soil conditions. Indeed, it is estimated that Pakistani soils contain sufficient total P for crops (400–3000 μg/mL), but usually a very low amount (3–10 μg/mL) of P is available for the plants and most (>90%) of these soils are P-deficient (Zia 1990; Ahmad *et al.* 1996).

The poor mobility of soil inorganic P is due to the large reactivity of phosphate ions with numerous soil constituents and to the consequent strong retention of most of soil P. In soils with high pH (>7.5), P is present mostly in the insoluble form of calcium phosphate (Brady 1990). In acidic soils, complex iron and aluminium phosphates predominate. Only ~10–20% of applied P was taken up by the crop and the remainder goes to the building up of soil reserves (Orvis and Hellums 1993).

As a component of the plant nutritional system, phosphate biofertilisers help in increasing the availability of P (which is already present in the soil in sparingly insoluble form) and can enable the farmer to get greater benefits from his investment in fertiliser P (Gerke 1992). Mycorrhizosphere interactions, between phosphate solubilising bacteria (PSB) and fungal plant associates, contribute to the biochemical P cycling, thus promoting a sustainable nutrient supply to plants (Vazquez *et al.* 2000).

Several soil bacteria (particularly belonging to the genera *Pseudomonas* and *Bacillus*) and fungi (belonging to the genera *Penicillium* and *Aspergillus*) possess the ability to bring insoluble phosphate into soluble form, by secreting organic acids (Chunningham and Kuiack 1992; Nahas 1996). These acids decrease the pH, resulting in effective solubilisation and utilisation of phosphate (Whitelaw 1999).

In the literature, many studies report the beneficial effect of PSB inoculation on plant growth (Mikanova *et al.* 1997; Pozan *et al.* 1997). These describe its influence on several vital physiological processes, such as utilisation of sugar and starch, photosynthesis, transfer of energy (Liu *et al.* 1992; Goldstein *et al.* 1993), ion uptake and total P content (Reyes *et al.* 1999; Fenice *et al.* 2000). Improved P uptake after PSB inoculation has also been observed in barley, maize, mungbean, lentil, chickpea and other crops (Subba Rao 1993; Belimov *et al.* 1999; Narula *et al.* 2000; Peix *et al.* 2001; Vazquez *et al.* 2000; Ramirez *et al.* 2001). It is, however, necessary to improve the PSB biofertiliser performance and survival under field conditions.

With this in view, the present study was conducted to isolate and characterise PSB, quantify the available P solubilised by these PSB strains and to see the effect of different bacterial strains on nodulation, nitrogen (N) fixation, P uptake and growth of chickpea plants at different levels of N and P.

Materials and methods

Isolation and characterisation of phosphate solubilising bacteria

PSB were isolated from rhizosphere, roots and nodules of chickpea plants, obtained from NIBGE/NIAB fields, by dilution-plate method (Brielry *et al.* 1928), using Pikovskaya's medium (Pikovskaya 1948).

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Table 1. Identification and characterisation of phosphate solubilising bacterial strains

Bacterial strains	Host	Source	Gram staining	Zone diameter[A] (mm)	Indole acetic acid test	Acetylene reduction assay
CPS-1	Chickpea	Rhizosphere	—	26.7	—	—
CPS-2	Chickpea	Rhizosphere	—	25.1	—	—
CPS-3	Chickpea	Rhizosphere	—	83.7	—	+
LPS-3	Lentil	Nodules	—	41.0	+	—
WPS-5	Wheat	Rhizosphere	+	47.1	—	—
Ca-18	Chickpea	Nodules	—	56.3	+	—
AP	Soil	Soil	—	21.0	+	+
IFA_1	Maize	Roots	—	—	+	+
IFA_2	Maize	Roots	—	—	+	+

[A]Values are the means of 3 readings.

Transparent zones surrounding bacterial colonies indicated the extent of P solubilisation. Three PSB isolates (CPS-1, CPS-2 and CPS-3), from the rhizosphere of chickpea plants, were isolated and maintained on Pikovskaya agar slants. Another 6 strains (LPS-3, WPS-5, Ca-18, AP, IFA_1, IFA_2) were obtained from the Biofertilizer Resource Center (BIRCEN), NIBGE culture collection. All 9 strains were characterised for further studies (Table 1) by Gram staining, cell morphology and motility (Vincent 1970), indole acetic acid production (Gordon and Weber 1953) and acetylene reduction assay (Hardy *et al.* 1973).

Quantification of available phosphorus solubilised by PSB strains

The phospho-molybdate blue colour method (Murphy and Riley 1962) was used for this study. Fresh bacterial cultures were grown in Pikovskaya broth (100 mL), in a 250 mL volumetric flask on a bench top rotary shaker, for 12 days at 200 rpm and 24°C. The suspensions were centrifuged (8149 × *g* for 15 min). The supernatants were decanted and filtered, then the pH of each sample was measured. Available P was determined by spectrophotometry at 882 nm using a standard phosphate solution.

Effect of PSB strains on growth of chickpea

Chickpea seeds cv. CM-98, obtained from the Mutation Breeding Division at NIAB, were surface sterilised with 0.1% $HgCl_2$ and were germinated on 1% agar plates. Healthy, uniform seedlings (5–7 mm long) were transplanted to plastic pots filled with sterile vermiculite at the rate of 3 seedlings/pot. A completely randomised design experiment was performed with 13 treatments (see Table 3) and 3 replicates.

Treatment T_1 was the control and was treated with a N-free Hoagland solution (Arnon and Hoagland 1940) with insoluble phosphate $Ca_3(PO_4)_2$ and a mix inoculum of rhizobial culture. Treatments T_2, T_3 and T_4 were not inoculated with PSB strains or mix culture of rhizobia and supplied Hoagland solution with N. T_2 was supplemented with soluble phosphate KH_2PO_4, T_3 was not supplemented with phosphorous and T_4 was supplemented with insoluble phosphate $Ca_3(PO_4)_2$. Treatments T_5–T_{11} were inoculated with a single PSB strain and mix rhizobial culture with N-free Hoagland solution. Treatments T_{12} and T_{13} were supplemented with N-free Hoagland solution with single, non-PSB strain culture and mix culture of rhizobial inoculum. Single strain PSB inoculum was prepared by incubating bacterial culture in Pikovskaya broth medium, at 30°C in a shaking incubater for 24 h. Inoculation of PSB strains was performed with a suspension of 1×10^9 CFU/mL, applying 0.5 mL per seed according to treatments (see Table 3). BioPower mix culture (a commercial product of NIBGE, PAEC, Pakistan) of chickpea-compatible mesorhizobial strains Ca-34, Ca-31, Ca-25 were co-inoculated at the rate of 1×10^9 cells/mL and these strains were observed as non PSB. Plants were watered when required. In all treatments 1/4 strength Hoagland solution was used.

Different growth parameters (plant height, shoot weight, nodulation and nitrogenase activity of nodules) (Hafeez *et al.* 1998) were recorded 6 weeks after transplantation. Shoot N was measured by the Kjeldhal method (Yoshida *et al.* 1976), P by vanadomolybdate phosphoric acid yellow colour method (Yoshida *et al.* 1976) and ion (Na^+, K^+, Cl^-) uptake was measured by flame photometry. The results were compared statistically by Duncan's multiple range test.

Results and discussion

Isolation and characterisation of PSB

Phosphate solubilising bacterial strains were isolated from rhizosphere, roots and nodules of chickpea plants. A total of 83, 73 and 53 bacterial colonies/mL were recorded from rhizosphere, root and nodule samples, respectively. Only 3 isolates obtained from chickpea rhizosphere (CPS-1, CPS-2, CPS-3) were able to solubilise phosphate, accounting for 4% of the bacteria. No PSB strains were isolated from root and nodule samples of chickpea plants. The characterisation of 9 PSB isolates (CPS-1, CPS-2, CPS-3,

Table 2. Available phosphorus, solubilised by bacterial strains isolated from different sources, and final pH of cultures

Bacterial strains isolated from different sources
Cultures grown for 12 days in insoluble P medium
Values are means of 2 readings

Bacterial strains	O.D.	Available P (μg/mL)	Percentage increase over control	pH
Control	1.28	36.1		6.8
CPS-1	2.14	77.4	114.0	4.3
CPS-2	2.98	84.1	132.4	3.6
CPS-3	3.66	103.3	186.1	4.3
LPS-3	3.63	100.3	177.8	5.4
WPS-5	4.62	130.5	261.4	5.6
Ca-18	3.32	95.4	164.2	3.9
AP	2.46	65.3	80.9	5.7
IFA_1	1.30	36.7	1.7	6.5
IFA_2	1.32	37.2	3.0	6.5

Table 3. Effect of co-inoculation of phosphate solubilising bacteria (PSB) and mesorhizobial strains on nodulation, nitrogen fixation and growth of chickpea plants

Values are the means of 3 readings
Means followed by the same letter are not significantly different at $P = 0.05$
treatments T_2, T_3, T_4 were uninoculated; treatments T_5 to T_{11} were co-inoculated with PSB and rhizobial strains; treatments T_{12} and T_{13} were co-inoculated with non PSB and rhizobial strains; treatments T_1 and T_5–T_{13} were provided with 1/4 strength N-free Hoagland solution with insoluble phosphate (P^+ins) — 50 mg/L $Ca_3(PO_4)_2$
P^+s, soluble phosphate 2 mL/L KH_2PO_4; N^+, nitrogen was applied as 1 mol/L $Ca(NO_3)_2$ and 1 mol/L KNO_3 at the rate of 10 mL/L

Treatment	Nodules/plant	Nodule dry weight (mg)	Acetylene reduction assay[A]	Shoot length (cm)	Shoot dry weight (mg)
Control					
T_1 (P^+ins + N^-)	11.7h	3.8i	109j	21.4f	371g
T_2 (P^+s + N^+)	0	0	0	33.4a	558a
T_3 (P^+s + N^+)	0	0	0	25.8d	471d
T_4 (P^+ins + N^+)	0	0	0	24.5e	451de
PSB					
T_5 (CPS-1)	17.3b	5.5cd	164bc	28.3c	478c
T_6 (CPS-2)	21.2a	6.7a	177a	30.7ab	532b
T_7 (CPS-3)	16.0cd	5.8b	153e	25.8d	433e
T_8 (LPs-3)	14.1f	4.8f	140g	26.1cd	439e
T_9 (WPs-5)	15.5e	5.1e	147f	23.6e	398f
T_{10} (Ca-18)	16.9cd	5.7d	162cd	25.4d	457de
T_{11} (AP)	14.7ef	4.4fg	128h	23.4e	410f
Non-PSB					
T_{12} IFA_1	11.9gh	3.9h	109	20.7f	356g
T_{13} IFA_2	12.2g	4.0h	109	20.7f	351g

[A]Measured as nmole of C_2H_4 produced/h/g nodule dry weight.

LPS-3, WPS-5, Ca-18, AP, IFA_1, IFA_2) indicated that all of them were rod shaped, motile, fast growers and varied in indole acetic acid production and acetylene reduction assay activity. All the isolates were Gram negative, except WPS-5. Qualitative evaluation of these PSB isolates showed that transparent zone diameter ranged from 21 to 83.7 mm (Table 1). Earlier studies have shown that bacterial strains of *Pseudomonas, Bacillus* and *Flavobacterium* are very active

Table 4. Effect of co-inoculation of phosphate solubilising bacteria (PSB) and mesorhizobial strains on nutrient uptake of chickpea plants

Values are the means of 3 readings
Means followed by the same letter are not significantly different at $P = 0.05$
For treatments see Table 3

Treatments	Total N (mg/g)	Total P (μg/g)	Na^+ (μg/g)	Ca^{2+} (μg/g)	K^+ (μg/g)
Control					
T_1 P^+ins + N^-	7.6h	3.8i	10.1g	9.6h	23.3g
T_2 P^+s + N^+	11.5b	6.1c	20.4a	15.5a	38.8a
T_3 P^+s + N^+	8.9f	4.0g	11.6f	10.7f	25.2f
T_4 P^+ins + N^+	8.7f	3.9h	11.6f	11.0f	24.5f
PSB					
T_5 CPS-1	9.7d	5.8d	12.7e	13.1bc	30.5d
T_6 CPS-2	13.3a	6.5a	15.0bc	13.8b	35.0b
T_7 CPS-3	11.6b	6.1c	12.9de	13.0c	30.5d
T_8 LPS-3	10.1c	5.5f	13.2d	12.2e	30.4d
T_9 WPS-5	9.8d	5.9d	16.0b	12.3e	33.4c
T_{10} Ca-18	10.4c	6.3b	13.6c	12.7d	34.2bc
T_{11} AP	9.1e	5.6ef	11.8f	11.7ef	29.5e
Non-PSB					
T_{12} IFA_1	7.7g	3.9h	8.5h	9.9g	23.4g
T_{13} IFA_2	7.7g	3.9h	8.6h	9.8g	23.4g

in phosphate solubilisation by production of organic acids (Chunningham and Kuiack 1992; Nahas 1996; Baryosef *et al.* 1999).

Quantification of dissolved phosphorus

Spectrophotometric quantification indicated that dissolved P solubilised by PSB strains ranged from 65 to 130.5 μg/mL and drop in pH ranged from 5.6 to 3.6 (Table 2). Numerous studies have shown a fall in pH, by production of organic acids when PSB strains are grown in liquid cultures (Asea *et al.* 1988; Halder *et al.* 1990; Rodriguez and Fraga 1999).

The greatest amount of phosphate (130.5 μg/mL) was solubilised by strain WPS-5, followed by strain CPS-3 (103.3 μg/mL). The lowest amount of phosphate (65.3 μg/mL) was solubilised by strain AP.

Effect of PSB on growth of chickpea

The effect of these PSB strains on chickpea plants was determined by a pot trial. Although all the PSB strains were useful in enhancing the plant growth by inoculation, CPS-2 was the most effective strain in improving plant height and shoot dry weight, as well as the number, dry weight and nitrogenase activity of nodules of chickpea plants. This enhancement represented 43, 43, 82, 76 and 63% over the control, respectively (Table 3). This strain was followed by PSB strains CPS-1 and Ca-18, which significantly increased plant height, shoot dry weight, nodulation and nitrogenase activity. Plants inoculated with non-PSB strains IFA_1 and IFA_2 showed significantly lower values in all the parameters. Earlier studies expressed that several PSB strains played an effective role in P uptake and growth promotion of plants by solubilisation of inorganic phosphate (Tomar *et al.* 1997; Thakkar *et al.* 1993; Belimov *et al.* 1999; Desouza *et al.* 2000). PSB strains are known to improve vital physiological processes (Liu *et al.* 1992; Goldstein *et al.* 1993; Reyes *et al.* 1999), by mineralising organic P compounds and by converting inorganic phosphate into a more available form (Gaur 1990; Marschner 1995). Uninoculated plants showed the negative impact of P and N deficiency on growth.

It is further observed that PSB strains (CPS-1, CPS-2, CPS-3 and Ca-18) isolated from chickpea, showed more effectiveness for growth promotion of experimental chickpea plants. The PSB strains LPS-3 and WPS-5, which solubilised more P, showed less impact on growth of chickpea plants. This effect may be due to the isolation from other crops; that is lentil and wheat and may have less affinity for chickpea roots.

Similarly, PSB inoculation positively affected the P uptake and, consequently, increased the shoot P content significantly with respect to uninoculated control plants. Maximum P contents of 6.5 and 6.3 μg/mL were recorded in plants inoculated with strains CPS-2 and Ca-18, respectively. PSB inoculation caused more P translocation from root to shoot. This led to a better P-accumulating mechanism which, in turn, enhanced vegetative and reproductive growth and nutrient uptake (Chabot *et al.* 1996; Narula *et al.* 2000; Peix *et al.* 2001). Many researchers have reported an increase in P uptake and seed yield, due to PSB inoculation of wheat, barley, mungbean, chickpea and maize genotypes (Singh and Kapoor 1999; Ramirez *et al.* 2001). Other PSB strains also improved N and P content, but to a lesser extent.

Our physiological studies, of nitrogenase activity of nodules and total N contents, revealed an increasing trend of these nodule parameters of plants by PSB inoculation. It is further evidenced that chickpea plants exhibited a greater increase in total N contents by inoculation of chickpea isolates CPS-1, CPS-2, CPS-3 and Ca-18 (Table 4)

The enhanced P uptake caused by PSB inoculation allowed the plants to utilise P from fixed resources. This demonstrates the need to improve and modify PSB performance, survival under field conditions and selection of PSB strains that can produce growth-promoting hormones, along with P solubilising organic acids. These are new areas to be explored. Conclusively, there is a great scope for improving P deficiency in crop plants if the criteria of PSB inoculation are incorporated in high yielding germplasm, through conventional breeding and biotechnological techniques.

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