Establishment of *lacZ* marked strain of phosphate solubilizing bacterium in the rhizosphere and its effect on plant growth in mungbean

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Abstract The establishment of *lacZ* marked strain of P-solubilizing bacterium *Pseudomonas* in the rhizosphere of mungbean (*Vigna radiata*) under pothouse conditions was studied. The *lacZ* marker was transferred to *Pseudomonas* P-36 on LB medium using donor strain of *E. coli*. The *lacZ* marked strain formed blue colonies on selective media and could be identified from soil on the basis of this character. The *lacZ* marked strain was able to survive in rhizosphere of mungbean under pothouse conditions and maintained a population of about 10^4 g⁻¹ of rhizosphere soils up to 60 days study period. Positive effect of inoculation with P-solubilizing bacterium on dry matter yield, P and N-uptake was observed using rock phosphate and single super phosphate as P sources with and without farmyard amendment.

Keywords Phosphate solubilization · *lacZ* marked strain · Mungbean · Rock phosphate

Introduction

Phosphorus is one of the major plant nutrients limiting plant growth. Soluble P applied in the form of phosphatic fertilizers gets converted into insoluble Al, Fe and Ca phosphates depending upon soil pH [1]. Soil microbes play a significant role in mobilizing P for the use of plants by bringing about changes in pH of soil microenvironment by production of organic acids and chelating substances which leads to solubilization of insoluble phosphates [2, 3]. So, there is a possibility of using phosphate-solubilizing bacteria for increasing P release from insoluble P sources such as rock phosphate in neutral and alkaline soils.

One of the major problems in inoculation studies with P-solubilizing bacteria is the survival and establishment in bulk soil and rhizosphere. Such studies are often limited by lack of suitable method to detect inoculated bacteria in soil [4]. The introduction of reporter gene(s) that can be easily identified on chromogenic substrates is a rapid technique for strain identification. Stable genetic markers such as *lacZ*, *gusA*, *luxA* and *gfp* have been introduced in many agriculturally important bacteria such as *Rhizobium*, *Azotobacter*, *Pseudomonas* and *Azospirillum* [5, 8]. These markers provide simple and direct method for strain identification in soil. The present study reports the construction and establishment of *lacZ* marked strain of phosphate-solubilizing bacterium (PSB) in crop rhizosphere and response of inoculation on the growth and N and P-uptake of mungbean.

Materials and methods

Construction of *lacZ* marked strains of PSB: *Pseudomonas* sp strain P-36 was tagged with *lacZ* gene using suicidal

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plasmid pSUP101 as described earlier [6]. The exconjugants were screened on LB (Luria Bertini) plates containing nalidixic acid (Nx) ($25 \mu\text{g ml}^{-1}$), and kanamycin (Km) ($50 \mu\text{g ml}^{-1}$). Exconjugants obtained were purified on $\text{Km}_{50}\text{Nx}_{25}$ plates containing X-Gal (5-Bromo-4-chloro-3-indoyl- β -D-galactopyranoside) 20 mg l^{-1} . Transconjugants containing lacZ gene in right orientation formed blue colonies on X-Gal plates. The P solubilization efficiency of parent and marked strain was compared on the Pikovskaya's medium containing tricalcium phosphate (TCP) [10]. The colony and zone diameter of different isolates was measured to calculate solubilization efficiency [22]. The β -galactosidase activity of marked strain was determined in LB broth by measuring the ability to hydrolyze o-nitrophenyl-D-thiogalactoside as described by Miller [29].

Pot culture experiment: A phosphorus deficient loamy sand soil was used to study establishment of lacZ marked strain with mungbean as a test crop. The soil was air dried and passed through a 2 mm sieve. The physico-chemical properties of soil are shown in Table 1. Seven kg of soil was filled in earthen pots. Mussoorie rock phosphate (MRP) was added to supply $60 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$. The MRP used in present course of investigation was obtained from M/S Pyrites, Phosphates and Chemicals Ltd., Dehradun, which is a carbonate apatite mineral of sedimentary origin containing about 67% apatite. The single super phosphate was added to supply 30 and $60 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$. The farmyard manure (FYM) was added @ 10 t ha^{-1} . These additives were mixed in upper 5 cm of soil. Seeds of mungbean (*Vigna radiata* cv Asha) were inoculated with 1 ml liquid inoculant of *Rhizobium* sp. (*Vigna*) containing $10^8 \text{ cells ml}^{-1}$. Inoculation with lacZ marked strain of PSB was carried out by dipping the seeds in suspension of 2-day old culture containing about $10^7 \text{ cells ml}^{-1}$. Five plants per pot were sown with the treatments as per Table 2 with six replications. Rhizospheric soil samples were collected after uprooting the plants and

analyzed for the population of marked strains at 30 and 60 days as described earlier [6]. Plants were harvested at 60 days, washed with distilled water and dried on oven at 80°C to a constant weight and analyzed for dry matter yield, total N and total P content [11, 12]. The P and N uptake was calculated by multiplying plant dry weight with P and N concentration respectively.

Results and discussion

Random Tn5 mutagenesis was used to introduce lacZ marker in *Pseudomonas* sp. P-36. The transconjugant P-36 produced blue colonies on X-Gal plates containing $\text{Km}_{50}\text{Nx}_{25}$. Insertion of lacZ gene was confirmed by estimating the β -galactosidase activity of parent as well as its exconjugant. Transconjugant P-36 showed β -galactosidase activity of 50 Millers units, while no activity was detected in the parent. The P-solubilization of parent as well as its transconjugant was examined on solid and in liquid Pikovskaya's medium using TCP as P-source. Transconjugant was as good as its parent as regards the P-solubilizing efficiency (data not shown), suggesting that introduction of lacZ did not affect P solubilizing efficiency. No other PSB was able to grow on TCP plates containing $\text{Km}_{50}\text{Nx}_{25}$, indicating that lacZ can be used as effective marker for PSB. No blue colonies were formed on LB plates containing $\text{Km}_{50}\text{Nx}_{25}$ plus X-gal when soil dilutions from controls (without inoculation of lacZ marked *Pseudomonas*) were plated indicating that the enumeration method specifically identified the lacZ marked strain.

The PSB population in the rhizosphere soil of mungbean is shown in Table 2. The total population of PSB was up to 10^4 per gram of rhizosphere soil at 30 days and increased at 60 day sampling. In general the PSB population was more in inoculated treatments with different doses of SSP and MRP or FYM over their respective uninoculated controls. The count of blue colonies was less as compared to total PSB and ranged between 2.6 to 8.0 and 4.4 to $9.4 \times 10^4 \text{ g}^{-1}$ soil at 30 and 60 days respectively. The parent failed to grow on LB medium containing antibiotics ($\text{Km}_{50}\text{Nx}_{25}$) and did not form blue colonies on LB medium containing X-gal. We developed marked strain of phosphate solubilizing bacterium that incorporated lacZ from *E. coli* through conjugation.

Exconjugants were obtained with conjugation frequency of 2.7×10^{-4} Simon [25] observed transposition frequency between 10^{-3} to 10^{-6} using pSUP and pUT vectors in different Gram-negative bacteria. The transposition frequency varies with recipient strain and growth conditions. The introduction of foreign gene(s) that can be easily detected on

Table 1 Physiochemical properties of the soil used in the experiment

Texture	Loamy sand
Mechanical analysis (%)	
Sand	90.4
Silt	4.0
Clay	5.6
Cation exchange Capacity (meq 100^{-1}g)	4.9
Electrical conductivity (dS m^{-1})	0.15
pH	7.7
Organic carbon (%)	0.12
Total N (%)	0.04
NaHCO_3 -soluble P (mg kg^{-1})	2.5

a chromogenic substrate has been widely used to identify strains of *Pseudomonas*, *Erwinia*, *Rhizobium*, and *Bradyrhizobium* in natural environments [6, 7, 14, 15, 18, 23, 26]. Earlier Hofte et al. [17] have constructed a *lacZ* marked strain of *P. aeruginosa* MPB1 and found that β -galactosidase activity was expressed constitutively under culture conditions used. The *P. aeruginosa* MPB1 strain was recovered with an efficiency of about 100 per cent from loamy sand soil on medium containing X-Gal. In the present study also the marked strain exhibited β -galactosidase activity which was expressed constitutively in Pikovasakaya (1948) medium containing glucose as the carbon source. The *Pseudomonas* sp P-36 showed comparable P solubilization activity indicating that this strain can be used for ecological studies in soil as no gene essential for phosphate solubilization is affected. Since the use of antibiotic resistance genes for tagging bacteria for ecological studies is undesirable

from environmental point of view, the use of genetic markers such as *lacZ* is very useful.

There was no response of rock phosphate application on dry matter production as well as P uptake in uninoculated treatments compared to control without MRP (Table 3). There was positive effect of inoculation of PSB on dry matter production without rock phosphate application in conjunction with FYM but the results were statistically nonsignificant. However, application of PSB along with rock phosphate either in absence or presence of FYM led to significant increase in dry matter production and N and P-uptake. There was 10 per cent increase in dry matter production, 16 per cent increase in P-uptake, 45 per cent increase in N-uptake in soil treated with MRP+FYM+PSB over its control without inoculation. The SSP application increased plant growth to a large extent over unfertilized control because of P being in soluble form in this fertilizer.

Table 2 Population of *lacZ* marked PSB in rhizosphere soil of mungbean under pothouse conditions

Treatment	Uninoculated				Inoculated			
	Total PSB Count ($\times 10^4$ g $^{-1}$ soil)		Count of marked strain ($\times 10^4$ g $^{-1}$ soil) on $Km_{50}Nx_{25}$ plates with X-gal		Total PSB Count ($\times 10^4$ g $^{-1}$ soil)		Count of marked strain ($\times 10^4$ g $^{-1}$ soil) on $Km_{50}Nx_{25}$ plates with X-gal	
	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
Soil	8.2	10.1	-	-	6.9	10.1	4.5	6.5
Soil+MRP(60 kg P $_2$ O $_5$ ha $^{-1}$)	6.6	9.2	-	-	15.3	18.5	5.8	6.4
Soil + FYM	9.5	12.7	-	-	17.2	20.3	6.6	8.5
Soil + MRP (60 kg P $_2$ O $_5$ ha $^{-1}$) + FYM	6.1	14.4	-	-	11.1	14.3	8.0	9.2
Soil + SSP (30 kg P $_2$ O $_5$ ha $^{-1}$)	8.7	24.3	-	-	10.6	18.2	6.7	9.4
Soil + SSP (30 kg P $_2$ O $_5$ ha $^{-1}$) + FYM	17.1	20.0	-	-	5.5	8.2	2.9	4.4
Soil + SSP (60 kg P $_2$ O $_5$ ha $^{-1}$)	12.0	16.3	-	-	8.4	14.4	3.8	6.3
Soil + SSP (60 kg P $_2$ O $_5$ ha $^{-1}$) + FYM	6.5	14.2	-	-	6.1	18.3	2.6	8.9

Table 3 Effect of *lacZ* marked PSB inoculation on dry matter production total-P and total-N uptake of mungbean under pothouse conditions after 60 days

Treatments	Uninoculated			Inoculated		
	Dry matter (g pot $^{-1}$)	P-uptake (mg pot $^{-1}$)	Nuptake (mg pot $^{-1}$)	Dry matter (g pot $^{-1}$)	Puptake (mg pot $^{-1}$)	Nuptake (mg pot $^{-1}$)
Soil	2.05	2.90	4.61	2.12	3.05	5.57
Soil+MRP(60 kg P $_2$ O $_5$ ha $^{-1}$)	2.08	3.07	5.82	2.29	3.44	10.77
Soil M + FY	2.12	3.08	6.12	2.23	3.31	7.20
Soil + MRP (60 kg P $_2$ O $_5$ ha $^{-1}$) M + 2.17 FY	2.17	3.09	6.88	2.41	3.67	12.52
Soil + SSP (30 kg P $_2$ O $_5$ ha $^{-1}$)	3.60	9.06	17.90	3.87	11.78	18.93
Soil + SSP (30 kg P $_2$ O $_5$ ha $^{-1}$) + FYM	3.74	10.06	18.33	4.16	12.72	20.82
Soil + SSP (60 kg P $_2$ O $_5$ ha $^{-1}$)	4.31	12.95	18.13	4.70	14.81	23.61
Soil + SSP (60 kg P $_2$ O $_5$ ha $^{-1}$) + FYM	4.51	13.80	21.89	4.88	16.68	25.13
LSD (P=0.05)	0.06	0.21	0.32	0.10	0.27	0.41

The effect of inoculation in presence of SSP and FYM was also significant.

The field and pot trials of PSB inoculation with or without phosphatic fertilizers, and rock phosphates have shown increase in P and N-uptake and dry matter production [16, 20, 21]. The increased P availability has a positive effect on nitrogen fixation in legumes, with the effect N uptake is also increased by application of phosphate sources and PSB [24, 28]. The application of rock phosphate directly to soil as a source of P is recommended practice for acidic soils [19], but their application in neutral to alkaline soils is not beneficial because of their low solubility in such soils. Many workers have investigated usefulness of using rock phosphates in combination with P-solubilizing microorganisms so as to increase P-availability in neutral and alkaline soils [13, 21, 22]. Increase in yield of various crops by inoculation with P-solubilizing organisms has been reported [22, 24, 27, 28]. Generally, the positive response due to inoculation is associated with high organic matter content and low available P status of soil. Presence of FYM was helpful in increasing of availability by PSB. During this study also the positive effect of inoculation was improve by FYM amendment.

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