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Evaluation of Phosphate Solubilizing *Pseudomonas* Strains and their Mutants in Relation to Mustard (*Brassica compestris*)

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Phosphorous is involved in many essential biochemical processes like cell division, photosynthesis, sugar break down, transfer of energy and nutrient uptake. Two phosphate solubilizing *pseudomonas* strains 25MRP and 33MRP were taken in this study. Tn-5 derived mutants of 25MRP and 33MRP were obtained with altered phosphate solubilizing ability by using *E.coli* strain S17-1. Highest phosphate solubilization and maximum drop in pH were found with 33MRP-314 mutant. Under pot house conditions maximum plant dry biomass was observed with 33MRP-233 with URP (3.43g/pl) followed by 25MRP-61 with URP (3.32 g/pl) at 60 DAS. The phosphate uptake ranged between (11-298%), and maximum phosphorous uptake observed in 25MRP-233 with URP (0.726 mg/pl) followed by 25MRP-61 with URP (0.712 mg/pl). Better performance with low phosphate solubilizing mutants such as 25 MRP-179 and 33MRP-188 in relation to plant dry weight indicated that some other metabolites may also be involved in plant growth promotion.

Key words: Phosphate solubilization, Seed bacterization, Mustard, Mutation.

Phosphorous is the second most critical nutrient next to nitrogen in enhancing plant growth and development. It is known to be important constituent of nucleic acid, phospholipids, phytin, coenzymes, phosphorylated sugar and nucleotides. Majority of the Indian soils contain insufficient amount of available phosphorous to support plant growth (Kanwar *et al*., 1982). Phosphorous is involved in many essential biochemical processes like cell division, photosynthesis, sugar break down, transfer of energy and nutrient uptake (Sanyal and Dedatta 1991; Kapoor 1995). The majority of applied phosphorus is rapidly fixed in soils that are poorly available to plant roots (Yadav and Dadarwal 1997). Inorganic phosphates in acidic soils are associated with iron and aluminium compounds where as calcium phosphates is predominant form of inorganic phosphates in neutral or calcareous soils (Mclaughlin *et al*., 1988; Hao *et al*., 2002). The unbalanced use of chemical fertilizers is responsible of reduction in soil fertility and environmental degradation (Gyaneshwar *et al*., 2002). Phosphorus biofertilizers in the form of microorganisms can help in availability of accumulated phosphates for plant growth (Goldstein 1986). In addition, the microorganisms involved in phosphate solubilization as well as better scavenging of soluble phosphate can enhance plant growth by increasing the efficiency of biological nitrogen

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fixation, enhancing the availability of other trace elements and by production of plant growth promoting substances (Gyaneshwar *et al*., 2002). A range of bacteria, fungi and actinomycetes are known to solubilize phosphorus and enumerated from different sources such as soil (Roychoudhury and Kaushik 1989), compost [Thakkar *et al*., 1993], rhizosphere of various plants (Kundu *et al*., 2002). However, bacterial phosphate solubilization and their subsequent effects on plant growth have arriving little attention. Therefore, the present study was undertaken with objectives to evaluate the potential of *Pseudomonas* strains/mutants in plant growth promotion in relation to mustard.

MATERIALS AND METHODS

Pseudomonas strains and chemicals

Two phosphate solubilizing *Pseudomonas* strains 25MRP and 33MRP were taken in this study. All chemicals and media components used were of AR grade from Hi-Media, Glaxo, E.Merk or Sigma chemicals company, U.S.A.

Intrinsic antibiotic resistance pattern of Pseudomonas strains 25MRP and 33MRP

Intrinsic antibiotic resistance patterns of 25MRP and 33MRP strains were determined on Luria-Bertani (LB) plates containing the different concentrations of antibiotics. Filter sterilized stock solutions of antibiotics (ampicilin, kanamycin, nalidixic acid, neomycin, rifampicin, spectinomycin trimethoprim, and tetracycline) were added in LB molten agar at 50 ºC. The antibiotics concentration were (50 µg/ml) except nalidixic acid and tetracycline, which was (20 µg/ml). The medium was gently shaken after adding the desired concentration of antibiotic and plated out. After solidification of the medium, the 25MRP and 33MRP strains were spot inoculated and incubated at 28 ºC for 2-3 days. Controls without antibiotics were also kept for comparison of growth.

Transposon Tn-5 mutagensis in *Pseudomonas* strains 25MRP and 33MRP

The strains 25MRP and 33MRP were maintained on LB agar medium. *E. coli* strain S17-1 containing suicidal plasmid Psup2021 (having transposon Tn-5) was maintained on LB agar medium containing kanamycin (50 µg/ml). The *E.coli* strain (donor) and 25MRP and 33MRP (recipient) were mixed and grown on LB plates at 28 ºC overnight. The overnight growth after conjugation was inoculated into LB broth and incubated at 28 ºC h for 24 h. The serial dilutions of this LB broth were prepared upto 10^{-6} and plated on LB plates containing Kanamycin (50 µg/ml) and tetracycline (20 µg/ml). These plates were incubated at 28 ºC and mutant colonies (Kan^R + Tc^R) were selected and transferred to Pikovskaya medium (PVK) slants for further studies.

Phosphate solubilization by *Pseudomonas* strains/mutants under liquid culture conditions

Phosphate solubilization in liquid culture conditions was assessed by using Pikovskaya broth containing tricalcium phosphate (TCP) under stationary conditions. The Pikovskaya medium (Pikovskaya 1948) with the following composition (g/1) glucose, 10.0; $(NH_4)_2SO_4$ 0.2; NaCl, 0.2; $MgCl_2$, 0.1; KCl, 0.2; yeast extract, 0.5; $FeSO_4$, 0.02; $MgSO_4$, 0.02; tricalcium phosphate, 2.5; pH 7.0 and agar agar, 20.0 (whenever solid medium was used). Fifty ml PVK medium was taken into 150 ml Erlenmeyer flasks and inoculation was done with 1 ml suspension of 25MRP and 33MRP and their mutants containing approximately 10^7 cells/ml, uninoculated flasks were taken as control. The flasks were incubated at 30 ^{0}C for 4 days and the contents were centrifuged at 10,000 rpm for 10 min. The supernatant was analyzed for changes in pH and water soluble phosphorous content. The pH of supernatant was recorded and phosphorous content was estimated according to method (John 1970).

Evaluation of *Pseudomonas* strains/mutants for biomass and P uptake on mustard under pot culture conditions

Strains 25MRP and 33MRP and their mutants were evaluated under pot house conditions for their effect on biomass production and phosphate uptake with and without rock phosphate at different days after sowing (30, 45 & 60 DAS). Five Kg of loamy sand soil having pH, 8.21 and total phosphate, 8.16 kg/ha was filled in earthen pots. The inorganic fertilizers 60 Kg/ha (326 mg urea/5 kg soil) and 30 Kg URP/ha (250 mg URP/5 kg soil) were mixed in upper 5 cm of soil. Seed inoculation with PSB/mutants were done by dipping the mustard seeds in suspension containing approximately 10^7 cells/ml. Five seeds per pot were sown, after 8 days thinning was done and only three plants were left in each pot. Three

replications each of the following treatments were taken (a) control; (b) URP (30kg/ha); (c) 25MRP; (d) 25MRP+URP; (e) 25MRP-179; (f) 25MRP-179+URP; (g) 25MRP-61; (h) 25MRP-61+URP; (i) 25MRP-233; (j) 25MRP-233+URP (k) 33MRP; (l) 33MRP+URP; (m) 33MRP-188; (n) 33MRP-188+URP; (o) 33MRP-273; (p) 33MRP-273+URP; (q) 33MRP-314; (r) 33MRP-314+URP. Mustard plants were uprooted at 30, 45 and 60 DAS, dried in oven at 60ºC to a constant biomass weight. Total phosphorous in plant samples was determined by Vanadomolybdophosphoric yellow color method (John *et al*., 1970).

RESULTS AND DISCUSSION

Antibiotic resistant pattern of *Pseudomonas* strains 25MRP and 33MRP

Two phosphate solubilizing *Pseudomonas* strains 25MRP and 33MRP were taken in this study and screened for the phosphate solubilization. The phosphate solubilized by 25MRP and 33MRP was (260.8 µg/ml and 220.5 µg/ml) respectively. The antibiotic resistant pattern of 25MRP and 33MRP was determined on the basis of their growth on antibiotics containing plates. When growth was presents on antibiotic containing plate its means culture was resistant (+) towards the antibiotic. If growth was not present means culture was sensitive (-) towards the antibiotic as shown in (Table 1). The culture 25MRP was resistant towards antibiotics (Nm, Amp, Spc) and the culture 33MRP was resistant towards antibiotics (Amp, Tc, Tmp, Spc).

Table 1. Antibiotic resistance pattern of *Pseudomonas* strains 25MRP and 33MRP

Antibiotic	25MRP	33MRP
km	-	-
Nm	+	-
Tmp	-	+
Nal	-	-
Rif	-	-
Tc	-	+
Amp	+	+
Spc	+	+

Table 2. Effect of *Pseudomonas* strains/mutants on mustard biomass (g/pl) and phosphate uptake (mg/pl) under pot house conditions at different DAS

Treatment	Biomass (g/pl)			Phosphate uptake (mg/pl)		
	30 DAS	45 DAS	60 DAS	30 DAS	45 DAS	60 DAS
Control	0.20	0.45	0.91	0.055	0.132	0.182
URP(30kg/ha)	0.20	0.45	1.12	0.063	0.142	0.271
25MRP	0.23	0.57	1.66	0.098	0.195	0.321
25MRP+URP	0.49	1.69	3.16	0.215	0.753	0.699
25MRP-179	0.21	0.53	1.61	0.099	0.187	0.311
25MRP-179+URP	0.46	1.62	3.12	0.194	0.728	0.687
25MRP-61	0.24	0.58	1.59	0.097	0.199	0.325
25MRP-61+URP	0.58	1.96	3.32	0.235	0.783	0.712
25MRP-233	0.20	0.51	1.57	0.092	0.197	0.327
25MRP-233+URP	0.62	2.11	3.43	0.243	0.777	0.726
33MRP	0.23	0.56	1.63	0.096	0.199	0.215
33MRP+URP	0.35	1.16	2.54	0.143	0.420	0.423
33MRP-188	0.20	0.52	1.62	0.091	0.198	0.203
33MRP-188+URP	0.32	1.11	2.47	0.129	0.412	0.417
33MRP-45	0.22	0.57	1.68	0.089	0.193	0.221
33MRP-45+URP	0.43	1.37	2.88	0.237	0.494	0.442
33MRP-213	0.21	0.52	1.69	0.096	0.199	0.227
33MRP-213+URP	0.47	1.43	2.97	0.252	0.51	0.457

Tn-5 mutagenesis in *Pseudomonas* strains 25MRP and 33MRP and estimation of phosphate solubilization

Tn-5 mutagenesis of 25MRP and 33MRP were carried out by using *E. coli* strain S17-1. A total of 350 transconjugants from 25MRP and 425 transconjugants from 33MRP were obtained, screened for variation in phosphate solubilization and change in pH of medium, as a result of which four high phosphate solubilizing and two low phosphate solubilizing mutant were obtained. Two mutants 25MRP-61 (312.7 µg/ml) and 25MRP-233 (332.8 µg/ml) showed more phosphate solubilization, whereas the mutant 25MRP-179 (197.7 µg/ml) showed less phosphate solubilzation as compared to wild type strain 25MRP (260.8 µg/ml). Two mutants 33MRP-273 (292.3 µg/ml) and 33MRP-314 (334 µg/ml) showed more phosphate solubilization and one mutant 33MRP-188 (190.6 µg/ml) showed less phosphate solubilization as compared to wild type strain 33MRP (220.5 µg/ml) as shown in (Fig.1A). The strain 25MRP and 33MRP decreased the pH of the medium (3.5, 4.0) respectively. Maximum drop in pH (3.3) was found with 33MRP-314 mutant as shown in (Fig.1B). The decrease in pH of the medium was due to the release of organic acid by PSB (Singh *et al*., 1982; Tripura *et al*., 2007; Mishra 1985). Soil microbes play an important role in mobilizing phosphorous for the use of plants by bringing about changes in pH of the soils microenvironment by producing organic acids and chelating substances, which lead to solubilization of insoluble phosphates. However, the quality and quantity of organic acid is fully dependent on type of phosphate solubilizing organisims and will determine extant of phosphate solubilization (Krishnaraj *et al*., 1999).

Evaluation of *Pseudomonas* strains/mutants for biomass production in mustard under pot house conditions

Seed inoculations of phosphate solubilizing microorganisms have shown positive effect on crop yield (Gupta 2004; Panda *et al*., 2004). Under pot house conditions increase in plant dry biomass was recorded by the application of *Pseudomonas* strains/mutants with URP, as compared to *Pseudomonas* strains/mutants without URP. The plant biomass of mustard with URP at 30, 45 and 60 DAS did not increase as compared to control as shown in (Table 2). At 30 DAS, minimum response (0.20 g/pl) to plant biomass over control was noticed with URP and maximum response (0.62 g/pl) by 25MRP-233 with URP. At 45 DAS, highest plant dry biomass was observed by 25MRP-233 with URP (2.11 g/pl) followed by 25MRP-61 with URP (1.96 g/pl). At 60 DAS, maximum plant biomass weight was found in 25MRP-233 with URP (3.43 g/pl) followed by 25MRP-61 with URP (3.32 g/pl).

Evaluation of *Pseudomonas* strains/mutants for phosphate uptake in mustard under pot house conditions

Under pot house conditions increase in phosphorous uptake was recorded by the

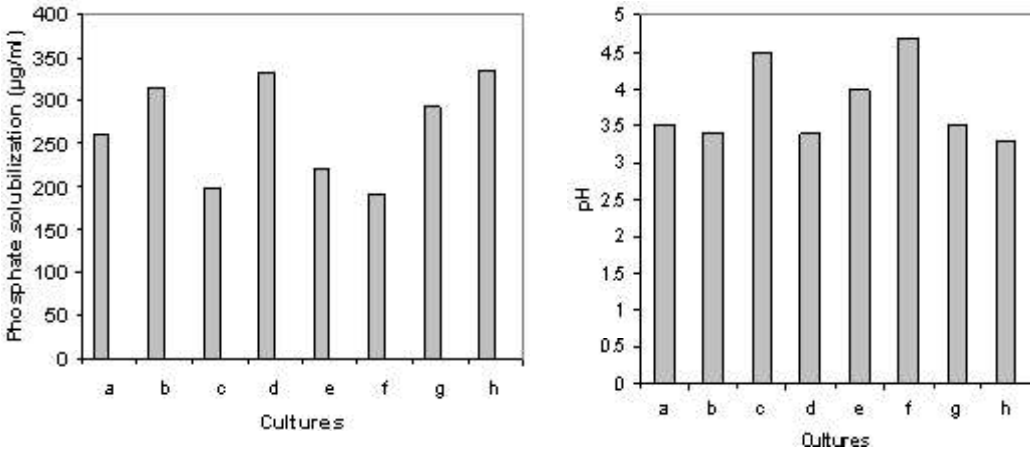

Fig. 1. Phosphate solubilization (A) and pH Change (B) by *Pseudomonas* strains/mutants in pikovskaya broth containing tricalcium phosphate (TCP). (a) 25MRP; (b) 25MRP-61; (c) 65MRP-179; (d) 25MRP-233; (e) 33MRP; (f); 33MRP-188; (g) 33MRP-273; (h) 33MRP-314

application of *Pseudomonas* strains/mutants with URP, as compared to *Pseudomonas* strains/ mutants without URP. The phosphate uptake of mustard with URP at 30, 45 and 60 DAS did not increase as compared to control as shown in (Table 2). It was observed that phosphate uptake was more when *Pseudomonas* strains /mutants were inoculated with URP as compared to without URP. At 30 DAS, in comparison to control (0.055 mg/pl), there was (0.063 mg/pl, 14%) increase with URP. At 30 DAS, maximum phosphate uptake (0.243 mg/pl) was observed, when 25MRP-233 inoculated with URP, which was 341% increase over uninoculated control. At 45 DAS, the maximum phosphate uptake observed in 25MRP-61 with URP (0.783 mg/pl) followed by 25MRP-233 with URP (0.777 mg/pl) and 25MRP with URP (0.753 mg/pl). At 60 DAS, maximum phosphorous uptake observed in 25MRP-233 with URP (0.726 mg/pl) which was (298%) followed by 25MRP-61 with URP (0.712 mg/pl) (291%). These results signify, that microbial phosphate solubilization play an important role in plant growth. Beside the supply of two major nutrients (N and P), phosphate solubilizing bacteria inoculation might also produce growth promoting substances (Liba *et al*., 2006; Rajkumar *et al*., 2006; Jat and Ahlawat 2004). The various mechanisms involved in phosphate solubilization are production of organic acid, chelating substances, mineral acids, proton extrusion, humic substances, siderophores, CO_2 release and hydrogen sulphide production (Kucey *et al*., 1989;). Better performance with low phosphate solubilizing mutants such as 25MRP-15 and 33MRP-45 in relation to plant dry weight was found; this indicates that some other metabolites could be involved in plant growth promotion. These studies suggest that phosphate solubilizing ability in *Pseudomonas* strains 25MRP and 33MRP/mutants is one of the best beneficial traits for screening and selection of plant growth promoting rhizobacteria (PGPR) strains from the rhizosphere of plants. Manipulation of such rhizobactria in relation to plant growth promotion and phosphate solubilization is a promising area for development and use PGPR strains as biofertilizer for sustainable crop production systems under field conditions.

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