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In vitro screening of native phosphate solubilizing bacteria for P solubilization and herbicides tolerance

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ABSTRACT

The phosphate (P) solubilization efficiency, glyphosate and oxyfluorfen tolerance of fifteen phosphate solubilizing bacterial (PSB) isolates collected from the rhizosphere of native plant species grown in red laterite soil of Purulia District in West Bengal were tested in vitro. Based on P solubilization efficiency as judged by the zone of P solubilization on solid and percent P solubilization in liquid Pikaovskaya's media, six PSB isolates were screened as high, five as medium and four as low P solubilizers. In spite of inhibition in growth and P solubilization of majority of the isolates at recommended and double of the recommended doses of glyphosate, WPB-2, PSB-3 and PSB-26 registered high degree of P-solubilization. On the other hand growth of most of the PSB isolates was not suppressed even in double the concentration of oxyfluorfen but P solubilization of majority of the isolates except WPB - 2 and PSB – 26 was affected greatly. Based on lesser degree in inhibition of P solubilization in presence of herbicides, WPB - 2 and PSB – 26 were assumed to be promising glyphosate and oxyfluorfen tolerant P bioinoculants and could be exploited for P nutrition in these two herbicides applied situation.

Key words: Glyphosate, oxyfluorfen, phosphate solubilizing bacteria, tolerance

Phosphorus is an essential and major plant nutrient. Its availability in soil is low due poor mobility [0.006 – 0.8 cm in four days (Patel, 2002)]. High fixation (50% of applied chemical P goes to insoluble pool through precipitation with calcium in saline alkaline soil and with Fe and Al in acidic soil) and immobilization make the efficiency regime of applied P fertilizer throughout the world around 10 – 20% (Khan *et al.*, 2009).

Several heterotrophic and chemoautotrophic bacteria have the capacity to release P from such fixed and insoluble mineral and organic phosphate sources, commonly known as phosphate solubilizing bacteria (PSB), mainly by the production of various organic acids but sometimes by the production of CO_2, H_2S, chelating agents, humic acid, proton extrusion and siderophores (Lal, 2002; Ivanova *et al.*, 2006). PSB are known to improve plant growth and health, enhance hormonal level and produce various secondary metabolites detrimental to plant pathogen (Dey *et al.*, 2004). The population, growth, survival activities and P solubilization efficiency of PSB are greatly influenced by soil physical, chemical and biological stresses. PSB species/ strains that are tolerant and adapted to such stresses of any particular agro-ecological situation would be of paramount importance.

Various agrochemicals e.g. herbicides when applied intensively and erratically on herbicide resistant non transgenic and transgenic crops to control the noxious weeds leads to their accumulation in soil to a dangerous level that affects growth, survival, efficiency and quality of beneficial microbial communities of soil (Srinivas *et al.*, 2008; Pereira *et al.*, 2008; Ahemad *et al.*, 2009). The naturally abundant plant growth promoting rhizobacteria are also metabolically inactivated through the uptake of herbicides applied in excess to the soil (Barriuso *et al.*, 2010). In contrast, a few micro-organisms found to be tolerant or resistant towards specific herbicides. It is of great concern that how to reduce the effect of herbicides on the beneficial micro-organisms and at the same time it is of great interest to screen out micro-organisms which are tolerant/ resistant to herbicides.

Root and soil samples of some native plant species for the experiment were collected from red lateritic soil having the history of deficiency in major plant nutrients specially P due to high P fixation, acidity, aridity, low soil depth with honey comb structures at sub surface, soil degradation, erosion and also having the history of occasional application of pesticides including herbicides. The reasons behind the selection of native plant species for sampling were that in spite of such stresses, some native plant species did not show any P deficiency, grew well and remained more healthy than cultivated crops. It was assumed that those native plant species might be harbouring some beneficial micro-organisms including PSB. Keeping these background and assumptions in mind, present investigation has been designed to identify efficient PSB native to red laterite soil and to judge their level of tolerance/growth and P solubilization in presence of two commonly used herbicides *viz.*, glyphosate and oxyfluorfen *in vitro*.

MATERIALS AND METHODS

Isolation, purification and maintenance of phosphate solubilizing bacteria

Root samples of twelve native plant species [Bamboo (*Bambusa vulgaris* Schrad. ex J.C. Wendl.),

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Ber (*Zizyphus jujube* Lamk.), Andropogon (*Andropogon aciculatum* Retz.), Goat weed (*Ageratum conyzoides* L.), Wild sage (*Lantana camara* L.), Pigeon pea {*Cajanus cajan* (L.) Millsp}, Neem (*Azadirechta indica* L.), Guava (*Psidium guajava* L.), Eucalyptus (*Eucalyptus grandis* Hill ex Maiden), Arjun {*Terminalia arjuna* (Roxb.) W & A.}, Citrus {*Citrus lemon* (L.) Burm. F.}, Palas {*Beautea frondosa* (Roxb.) Sans}] grown at Anara ($23^0$29'N $86^0$33'E), Ladhurka ($23^0$21'N $86^0$32'E) and Jaichandi ($23^0$33'N $86^0$40'E) locations under stress prone red laterite soil (sand – 52.2%, silt – 20.0%, clay – 25.8, water holding capacity – 31.3%, organic carbon – 0.18%, pH – 5.5, total nitrogen – 0.025%, available P – 5.1ppm, available K – 98 ppm) of Purulia District in West Bengal were collected, cleaned, crushed, diluted following 10 fold dilution series, plated with Pikovskaya's agar medium taking 1 ml from each dilutions of 10^{-4} to 10^{-7} in each Petri plate and incubated at 30° ± 1°C for 72 hours. The isolates showing a discrete clear halo zone around bacterial colony were considered as phosphate solubilizing bacteria and were picked up, purified and maintained on Pikovskaya's slant. A total of 30 PSB isolates were obtained primarily from the root samples collected from three different locations. However, only 15 PSB isolates showing greater than 5 mm clear halo as zone of P solubilization in the Pikovskaya's agar medium under repeated sub-culturing were considered for experimental use and the rests were discarded.

Determination of P solubilization ability

Phosphate - solubilization ability of each PSB isolate was assessed by measuring the zone of P solubilization on the Pikovskaya's agar medium as well as by estimating percent P solubilization in Pikovskaya's broth medium. For determining zone of P solubilization, ten fold dilution series from10^{-5} to 10^{-7} were prepared taking 1ml from each of 24 hours active growth PSB culture. Three Petri plates for each dilution were inoculated with 1 ml of each PSB culture followed by pouring and mixing with melted Pikovskaya's agar medium. After solidification plates were incubated at 30° ± 1°C for 96 hours. The diameter of cleared zone considered as zone of P solubilization was measured from PSB colonies remained discrete and scattered (15 – 20 colonies on 9.0 cm diameter Petri plate) sufficiently.

Table 1: Categorization of PSB isolates based on phosphate solubilization capacity in solid and liquid Pikovskaya's medium

PSB isolates	Diameter (mm) clear zone of P solubilization on solid medium	£Categorization of PSB isolates based clear zone of P solubilization	Percent P solubilization in liquid medium	€Categorization of PSB isolates based on percent P solubilization	Categorization of PSB isolates based on clear zone of P and percent P solubilizations
WPB-1	5.3 h	L	15.3 (23.0) h	M	**L**
WPB-2	13.0 a	H	32.6 (34.8) ab	H	H
WPB-3	13.0 a	H	31.4 (34.1) b	H	H
WPB-4	12.0 ab	H	29.4 (32.8) c	H	H
WPB-5	13.0 a	H	32.9 (35.0) a	H	H
WPB-7	12.0 ab	M	28.1 (32.0) cd	H	M
PSB-3	8.0 f	M	18.8 (25.7) f	M	M
PSB-8	5.0 h	L	6.0 (13.8) k	L	L
PSB-9	10.0 de	H	24.8 (29.9) e	H	H
PSB-15	8.3 f	M	15.0 (22.8) h	M	M
PSB-23	11.3 be	H	27.3 (31.5) d	H	H
PSB-26	10.7 cd	H	16.4 (23.9) g	M	M
PSB-27	9.0 ef	M	17.5 (24.7) g	M	M
PSB-38	5.0 h	L	7.1 (15.5) h	L	L
PSB-41	6.7 g	L	13.4 (21.50) i	L	L
SEm(±)	**0.4**	-	**0.3**	-	-
LSD(0.05)	**1.0****		**0.9****		

*Note: Figures in parentheses are arch-sine transformed values; ** Significance at 1 % level, Figures followed by same letter are not significantly different*

£ >10 mm zone of P solubilization=High (H)
£ >8 mm but <10 mm zone of P solubilization= Medium (M)
£ <8 mm zone of P solubilization=Low (L)

€ >24 % of P solubilization=High (H)
€ >15 % but <24 % of P solubilization = Medium (M)
€ <15 % of P solubilization= Low (L)

Table 2: Growth and P-solubilization of PSB-isolates on two concentrations of glyphosate

PSB isolates	Growth in glyphosate containing media at (mg/ 100ml medium)			P solubilization in glyphosate containing media at (mg/ 100ml medium)		
	0.0 (Control)	410	820	0.0 (Control)	410	820
WPB - 1	++++	+	+	††	††	††
WPB - 2	++++	++	+	††††	†††	†
WPB - 3	++++	+	+	†††	†	†
WPB - 4	++++	+	+	†††	††	†
WPB - 5	++++	+	+	††††	††	†
WPB - 7	++++	++	++	††	††	†
PSB - 3	+++++	+	+	††††	†††	†††
PSB - 8	+++++	+	+	††	††	†
PSB - 9	+++++	++	+	††	††	††
PSB - 15	++	+	+	†††	-	-
PSB - 23	+++++	++	++	†††	††	††
PSB - 26	+++	+	+	††††	††††	†††
PSB - 27	+++	+	+	†††	††	††
PSB - 38	+++++	++	+	††	††	††
PSB - 41	+++++	+	+	††	††	††

Note: - = No growth, - = No solubilization, + = Negligible growth, † = Negligible solubilization, ++= Low growth †† = Low solubilization, +++ = Medium growth, ††† = Medium solubilization, ++++ = Good growth †††† = High solubilization, +++++ = High growth

Table 3: Growth and P-solubilization of PSB-isolates on two concentrations of Oxyflourfen

PSB isolates	Growth in oxyfluorfen containing media at (mg ml^{-100} medium)			P solubilization in oxyfluorfen containing media at (mg ml^{-100} medium)		
	0.0 (Control)	235	470	0.0 (Control)	235	470
WPB - 1	++++	++++	++++	††	†	†
WPB - 2	++++	++++	++++	††††	††	†
WPB - 3	++++	++	++	†††	†	-
WPB - 4	++++	++++	+++++	†††	†	-
WPB - 5	++++	++++	+++++	††††	†	-
WPB - 7	++++	++	++++	††	-	-
PSB - 3	+++++	++++	++++	††††	†	†
PSB - 8	+++++	++++	++++	††	†	†
PSB - 9	+++++	++++	++++	††	†	†
PSB - 15	++	++	+++	†††	††	†
PSB - 23	+++++	++++	++++	†††	†	-
PSB - 26	+++	++++	+++	††††	††	††
PSB - 27	+++	+++++	++++	†††	†	†
PSB - 38	+++++	+++	++	††	†	†
PSB - 41	+++++	++++	+++	††	†	†

Note: - = No growth, - = No solubilization, + = Negligible growth, † = Negligible solubilization, ++= Low growth †† = Low solubilization, +++ = Medium growth, ††† = Medium solubilization, ++++ = Good growth †††† = High solubilization, +++++ = High growth

For testing percent P solubilization of different PSB isolates, 125 mg of tri-calcium phosphate equivalent to 25 mg phosphate was added to 25 ml of Pikovskaya's broth medium and inoculated separately in triplicate each with 1 ml of 24 hours active growth PSB cultures and incubated at 30° ± 1°C in shaker incubator for 120 hours. Thereafter, the culture broths were centrifuged at 10,000 rpm for 10 min. The supernatant was taken out and analyzed for soluble P-content using the method as described by Jackson (1973).

Pikovskaya's medium supplemented with carrot decoction (100 g/ 1000 ml medium) was mixed with recommended (410 mg of Glyphosate and 235mg of Goal per 100 ml of water) and doubled of the recommended doses of two commonly used herbicides *viz.* Glyphosate and Oxyfluorfen . Twenty millilitre of mixed medium was poured in each sterilized Petri plate

in triplicates along with suitable control without any herbicide. Single long straight streak per plate was made at the centre of Petri plate from the suspension prepared 24 hours actively grown culture of each PSB isolate. Plates were then incubated at 30° ± 1°C for 120 hours and growth and P solubilization of PSB isolates were qualitatively assessed.

RESULTS AND DISCUSSION

In vitro growth and P solubilization response of PSB isolates to glyphosate and oxyfluorfen

Fifteen PSB isolates were grown both on Pikovskaya's agar and broth media to assess their capacity in forming zone of P solubilization and percent P solubilization respectively. The results indicated that PSB isolates differed with respect to P solubilization as measured by the diameter of clear halo zone of P solubilization (ranged from 5-13 mm) and in percent P solubilization (6-32.9 %) (Table -1). On the basis of diameter measurement of the cleared zone of P solubilization, all the 15 PSB isolates were grouped in three categories - high, medium and low P- solubilizers and accordingly, seven PSB isolates were considered as high, four isolates as medium and four isolates as low P solubilizers.

Attempt was also made to categorize the PSB isolates on the basis of percent P solubilization on Pikovskaya's broth medium. Seven PSB isolates having >24 % of P solubilization were categorized as high, five isolates having >15 % and 24 % P solubilization as medium and three isolates having 15% P solubilization as low P solubilizers.

Considering both the parameters together, six isolates *viz.* WPB-2, WPB-3, WPB-4, WPB-5, PSB-9 and PSB-23 were identified to have high, five isolates *viz.* WPB – 7, PSB-3, PSB-15, PSB – 26 and PSB-27 to have medium and four isolates *viz.*WPB – 1, PSB-8, PSB-38 and PSB-41 to have low P solubilization efficiency. Only a few exceptions, the isolates showing high zone of P solubilization on Pikovskaya's solid medium also exhibited high percent of P solubilization in Pikovskaya's liquid medium and were considered as efficient P solubilizing isolates. A positive correlation (r =51* at 13 degrees of freedom) was also found to exist between these two parameters.

P- solubilization ability of micro-organisms is considered to be one of the most important trait associated with plant phosphorus nutrition (Chen *et al.*, 2006). To judge the P solubilization efficiency of 15 PSB isolates, we considered the measurement of clear zone of P solubilization and the estimation of percent P solubilization as dependable criteria. Similar emphasis on these two parameters was given by Souchie *et al.* (2007) at the time of isolation and initial screening of phosphate solubilizing microorganisms. It was evident from results of the present investigation that PSB isolates differed in their capacity to form the clear zone of P solubilization as well as on the efficiency in percent P solubilization. On the basis of differential performance in P solubilization, six PSB isolates were categorized as high, five as medium and four as low P solubilizers. The findings of the experiment corroborate with results obtained by Kundu *et al.* (2009) wherein they also classified 193 PSB isolates but into five categories based on P- solubilization efficiency and P – solubilization (as $\mu g\ ml^{-1}$) potential.

Studies on growth of 15 PSB isolates in Pikovskaya's agar medium containing glyphosate herbicide at two different concentrations revealed that the growth of all isolates was demonstrably inhibited in presence of glyphosate (Table 2). Except 3 isolates, the growth inhibition of other isolates remained unchanged with increase in glyphosate concentrations in the medium. P-solubilization abilities of majority of the PSB isolates were strongly inhibited as compared to control. In some of the isolates *viz.* WPB-2, WPB-4, WPB-5, WPB-7, PSB-8 and PSB-26, P-solubilization ability exhibited decreasing trend with increasing concentration of glyphosate whereas in others it remained unaltered. Interestingly, PSB-15 did not show any solubilization. On the contrary, in spite of their growth inhibition, WPB-2, PSB-3 and PSB-26 registered high degree of P-solubilization. Similar variation in inhibition of growth and P solubilization trait of PSB by the application of herbicide glyphosate at and higher the recommended doses was also recorded earlier (Encheva and Rankov, 1990; Santos *et al.*, 2004, 2006; Weaver *et al.*, 2007; Ahemad and Khan, 2010). However, the findings of the present experiment contradict with the observations made earlier by Sandhu *et al.* (1990) wherein they reported that the application of glyphosate enhanced bacterial propagules. In separate experiment Stratton and Steward (1992) observed that glyphosate application had no significant effect on number of bacteria, fungi and actinomycetes.

In oxyflorfen containing Pikovskaya's agar medium, the growth of seven out of fifteen PSB isolates was either marginally or strongly inhibited, two isolates was not affected, two isolates was stimulated and rest four isolates was increased marginally at higher concentration of oxyfluorfen but P solubilization in majority of isolates was affected greatly (Table - 3). Substantial amount of P was solubilized by WPB-2, PSB-15 and PSB-26 at lower concentration though it was decreased at higher concentration except PSB-26. Five out of 15 PSB isolates did not show any P solubilization at higher concentration of oxyfluorfen. But, the PSB isolate, WPB-7 did not exhibit any P solubilization on both the concentration of oxyfluorfen. It is evident from the results of experiment that the growth response of PSB isolates may be stimulatory, neutral and inhibitory. Inhibitory effect of oxyfluorfen to some PSB isolates as was observed here corroborate with the findings of Selvamani and Sankaran (1993) wherein they noted significant reduction in the population of bacteria and actinomycetes by oxyfluorfen along with two other herbicides *viz.* pendamethalin and fluchloralin. Mondal *et al.* (1987) made the same comment on the

initial depression of bacterial population by oxyfluorfen herbicide in direct seeded rice during *kharif* season. The stimulatory effect of oxyfluorfen to some PSB isolates was in tune of the results obtained by Das and Debnath (2006). They observed that oxyfluorfen was the most stimulatory followed by fluchloralin and oxadiazon for the population of phosphate solubilizing micro-organisms in the rhizosphere.

It is apparent from the results that out of fifteen PSB isolate *in vitro* tested six isolates were identified as high, five as medium and four as low P solubilizers. In terms of growth and P solubilization, WPB – 2 among high and PSB – 26 among medium P solubilizers have emerged as tolerant to both glyphosate and oxyflorfen which could be exploited as bio-inoculants for plant phosphorus nutrition.

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