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Selected pesticides inhibit phosphate solubilizing activity of *Gluconacetobacter* sp. and *Burkholderia plantarii*

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Native Phosphate solubilizing bacteria (PSB) possessing the ability to solubilize insoluble inorganic phosphates were isolated from rhizosphere soils of crop plants. Eighty one potential PSBs thus obtained were quantitatively screened for phosphate solubilization. The amount of P solubilized for different bacteria varied between 11.38-72.97 mg/100 mL. Of these, two bacteria (PSB 12 and 73) found to be efficient phosphate solubilizers. These organisms were characterized on the basis of morphological, cultural and biochemical characteristics as *Gluconacetobacter* sp. and *Burkholderia plantarii*. The isolates were further tested for their tolerance to pesticides, Ekalux, Tagban, Sevin, Metacid and Hinosan. The two organisms showed remarkable difference in pesticide tolerance. Their minimum inhibitory concentration (MIC) values range between 48-3125 µg/mL. Quantitative estimation of soluble P in pesticide containing Pikovaskaya's broth indicates that pesticides have an inhibitory effect on phosphate solubilizing activity.

Key words : Pesticides, P solubilization, Rhizosphere.

INTRODUCTION

In modern agriculture, pesticides are frequently applied to the crop fields to increase crop production. Besides combating pests, a significant amount of the pesticides eventually reaches the soil in the form of "insecticidal fall out" and is accumulated in top soil (0-10 cm) where maximum microbiological activities occur (Alexander, 1978). There are some pesticides, which exert adverse effect on proliferation of microorganisms and their associated transformations in soil (Martinez-Toledo *et al*., 1992). Moreover, the insecticidal effects on soil microorganisms and their associated transformations of nutrients are very specific since individual members within a group vary in toxicity (Simon and Fournier, 1979). The pesticides have direct effect on soil microbiological aspects by causing changes in the populations of *Azotobacter, Rhizobium*, cellulolytic and phosphate dissolving microorganisms (Kalam *et al.*, 2004). Among which phosphate solubilizing bacteria (PSB) solubilize insoluble phosphate and make it available to plant growth, development and reproduction (Anu and Kundu, 2005). These organisms are extremely important in maintaining P nutrition to plants since soluble forms of P fertilizers applied to soil are easily precipitated as insoluble non available forms. This often leads to an excess application of P fertilizer to crop land without being utilized by the plants (Kim *et al*., 1998). This unmanaged excess is both an environmental and economic problem. Hence an attempt has been made to isolate efficient phosphate solubilizing bacteria from rhizosphere soils and *in vitro* studies were made to find out the effects of selected pesticides on phosphate solubilizing activity.

MATERIALS AND METHODS

Isolation of phosphate dissolving bacteria :
Isolation of phosphate dissolving bacteria were carried out from rhizosphere soils of different crop plants. Pikovaskaya's medium was used for the isolation, cultivation and maintenance of phosphate solubilizing bacteria (Gaur, 1990).

Screening of phosphate solubilizing bacteria :
One hundred mL of Pikovaskaya's liquid medium containing tricalcium phosphate (TCP, 5g/L) as sole P source was dispensed in 250 mL Erlenmeyer flasks. The flasks were inoculated with 0.5 mL of 24 h active culture suspension of each culture. Uninoculated medium served as control in each case. Each experiment was done in triplicate set. All the flasks were maintained at 30^0C for 14 days with intermittent shaking twice a day. The soluble P_2O_5 in the supernatant solution was determined by vanadomolybdophosphoric yellow colour method, in nitric acid system (Subba Rao, 1982). The final pH of the culture medium was recorded using digital pH meter (Spectronic

20-D). All bacterial cultures were grown in Pikovaskaya's broth for 24-48 h, adjusted to equal OD (0.6) and a loopful of active culture was spot inoculated on to soil extract apatite agar medium precipitated with dicalcium phosphate. Plates were incubated for 4 d at 30 ± 2^{0}C. The size of each colony as well as that of clear zone were determined with a zone reader (Cimtex, Bombay). Phosphate solubilization efficiency (PSE) was measured as Z-C/C x 100, where Z is solubilization zone and C is diameter of bacterial colony (Srivastav *et al.,* 2004). Similarly solubilization index (SI) was the ratio of solubilization zone to growth zone (Nasreen *et al*., 2005)

Identification of selected bacteria :
The selected strains were subjected to cultural, morphological, biochemical and physiological characterization as mentioned in Bergey's manual of determinative bacteriology (Holt *et al*., 1994). The two isolates were also subjected to Biolog Identification system (Biolog Inc., Hayward, California). A standard phosphate solubilizing bacteria (*Pseudomonas striata*) obtained from Division of Microbiology, Indian Agriculture Institute (IARI), New Delhi was used as a reference strain.

Effect of pesticides on phosphate solubilizing activity:
Tolerances of bacteria to pesticides (Ekalux, Tagban, Sevin, Metacid and Hinosan) were studied by the method described by Gupta *et al*., (1994). All the pesticides used were of commercial grade and obtained from Unique Farm Pvt. Ltd., India. Concentrations of the pesticides were calculated on the basis of the percentage of the active ingredients in the commercial formulations. Stock solutions of these pesticides were prepared in an appropriate solvent and then supplemented in respective liquid media in varying concentrations from 3 to 12500 µg/mL. Tubes were then inoculated with 100 µL of the test culture (exponentially grown) and incubated at 28-30^{0}C for 48-78 h. The growth was determined turbidometrically at the end of incubation by measuring the OD at 600 nm in a Spectronic-20 spectrophotometer using plane nutrient broth as reference blank. A control experiment was also run simultaneously with standard pesticide dilutions. The lowest concentration of the pesticide which inhibited the growth of the organism was considered as minimum inhibitory concentration (MIC). Similarly minimum bactericidal concentration (MBC) was determined by subculturing a loopful of all the pesticide dilutions on to nutrient agar plates and incubated at 30^{0}C for 2 d. The lowest concentration of the pesticide, which killed the organism was denoted as MBC. The effects of pesticides on phosphate solubilizing isolates were studied by incorporating pesticides (5 mL quantity, concentration just below MIC) to Pikovaskaya's broth. The flasks were inoculated with 100 µL of inoculum in triplicate and incubate at 30^{0}C for 14 days. The soluble P_2O_5 in the culture medium was estimated and compared it with the control (without pesticide).

Results and Discussion

In the present investigation 81 potential phosphate solubilizing bacteria were isolated from rhizosphere of crop plants. Their extent of P solubilization ranged between 11.38 – 72.97 mg/100ml of P_20_5 in liquid medium. Out of this, 17 bacterial isolates showed promising results and reevaluated for their phosphate solubilization potential (Table1). Analysis of solubilization index and efficiency values indicated that PSB 13 and 67 were better solubilizers. But the formation of halo zone is not the only criteria of the ability of an organism to solubilize P. There was no correlation between P solubilization efficiency on solid and liquid medium as noticed earlier (Srivastav *et al*., 2004 and Kundu *et al*., 2002). Therefore, PSB 12 and 73 were selected as efficient strains on the basis of their potential phosphate solubilization in liquid medium. All the isolates decreased the pH of the medium. However, it could not be correlated with quantity of P solubilized. This may be attributed to many other factors responsible for P solubilization (Krishna *et al*., 1999 and Gaur *et al.*, 1973). Among the 17 isolates, two strains (PSB 40 and 56) were unable to produce solubiliztion zone in solid medium, but they could weakly solubilize TCP in liquid medium. Sangeeta Mehta and Chandra Shekhar Nautiyal (2001) also had reported isolates that did not show any clear zone on agar plates solubilized insoluble inorganic phosphates in liquid medium.

The two isolates were subjected to biochemical characterization as per Bergey's manual of determinative bacteriology (Table 2). Both the strains were Gram negative, motile, nonsporulating, oxidase negative rods. PSB 73 found to possess diffusible brownish pigment. The two strains were able to utilize glucose, mannitol, mannose and arabinose as their C source. But assimilation of inositol and galactose was only detected in PSB 73. Out of 95 C-source used in Biolog identification system, PSB 12 was able to utilize 19 substrates while PSB 73 assimilated 40 substrates as their sole source of carbon. On the basis of above cultural, morphological, biochemical, physiological characteristics the PSB 12 and PSB 73 were identified as *Gluconacetobacter* sp., *Burkholderia plantarii*, respectively. The identification of isolates were confirmed by MTCC. The genus *Burkholderia* have

Table 1 : Screening of phosphate solubilizing bacteria.

Phosphate solubilizing bacteria	pH	amount of P_2O_5 solubilized (mg/100 mL)**	solubilization zone diameter (mm)	solubilization efficiency (SE)
PSB 12	3.8	72.97	35	483
PSB 13	3.3	50.92	27	575
PSB 20	4.5	43.25	28	460
PSB 21	5.3	49.52	22	450
PSB 22	3.6	49.24	19	216
PSB 23	3.9	40.24	17	143
PSB 30	3.7	45.12	15	200
PSB 31	4.7	22.19	20	150
PSB 38	4.0	42.13	16	100
PSB 40	5.9	23.95	*	*
PSB 47	4.2	32.93	12	100
PSB 55	4.2	37.48	18	125
PSB 56	5.0	11.38	*	*
PSB 58	3.6	59.08	18	260
PSB 66	3.3	55.71	20	300
PSB 67	3.8	63.84	19	533
PSB 73	3.3	68.80	21	320
IARI standard	4.8	50.12	15	200
Control	6.5	1.55	-	-

* indicates no zone formation

** all the values are average of 3 replicates

nitrogen fixing ability, secretes phytohormones, ACC deaminase, solubilizes phosphates and is antagonistic to phytopathogens (Piyush *et al.*, 2005). Similarly Loganathan and Sudha Nair (2003) reported a novel, salt tolerant, N_2-fixing and phosphate solubilizing *Gluconacetobacter* sp. isolated from wild rice, substantiating our findings.

The isolates, *Gluconacetobacter* sp. and *Burkholderia plantarii* were tested for their tolerance to commonly available pesticides like Ekalux, Tagban, Sevin, Metacid and Hinosan. The *Gluconacetobacter* strain tolerated concentrations up to 390 µg of Ekalux/mL, 781 µg Sevin/mL, 48 µg Metacid/mL, and 3125 µg of Hinosan/mL In this case MIC value of Tagban found to be above the tolerance range (Table 3). However *Burkholderia plantarii* was able to tolerate the concentration 195 µg/mL for the three pesticides (Ekalux, Tagban and Hinosan) tested (Table 4). Our findings are almost similar to those reported by Ferrer *et al.* (1986) who reported that 100, 200,300.400 and 500 µg/mL of 2,3,6 TBA (2,3,6 trichlorobenzoic acid) caused 28.0, 47.8, 54.5, 61.2 and 95.6% inhibition, respectively after 24 h of incubation.

Tu (1994) studied the effect of nine pesticides on soil bacteria at concentrations of 10 µg/mL of soil for herbicides and 100 µg/mL for fumigants. He observed that most of the pesticide treatments affected nitrification of ammonium during a two week incubation. However, no inhibition was observed after three weeks incubation in all treatments. This suggests that nitrifying bacteria recover and nitrification proceeds in normal fashion. He also concluded that metabolites of pesticides such as 2, 4-D dichloropropene may also be a substrate for certain microorganisms. Different authors have reported both innocuous and inhibitory effects of certain pesticides on soil bacteria, depending on the concentrations used (Rajagopal *et al*., 1984).

The above mentioned isolates were further tested for their phosphate solubilizing potential in pesticide incorporated PV broth. All the pesticide added treatments showed a significant reduction in the amount of P_20_5 solubilized when compared to control. The only exception was in case of *Burkholderia plantarii* + Eka., where

Table 2 : Phenotypic and physiologicalproperties of selected strains.

Isolates	PSB 12	PSB 73
Configuration	circular	circular
Margin	entire	lobate
Elevation	dome	low convex
Surfac	smooth & shiny	pale brownish
Pigment	-	pale brown
Gram's reaction	negative	negative
Cell shape	rods	rods
Arrangements	singles & pairs	singles & pairs
Spore(s)	negative	negative
Motility	positive	positive
Growth at		
25^0C	positive	positive
30^0C	positive	positive
37^0C	positive	positive
2% NaCl	no growth	no growth
4 % NaCl	no growth	no growth
5 % NaCl	no growth	no growth
pH 6	positive	positive
pH 7	positive	positive
pH 8	no growth	no growth
Growth on MacConkey's agar	no growth	no growth
Indole test	negative	negative
Methyl red test	negative	negative
Voges Proskauer test	negative	negative
Nitrate reduction	negative	positive
Citrate utilization	no growth	no growth
Casein hydrolysis	negative	positive
Catalase test	negative	positive
Ornitine decarboxylase	positive	positive
Oxidase test	negative	negative
C-source utilization		
Glucose	positive	positive
Lactose	negative	negative
Sucrose	negative	negative
Mannitol	positive	positive
Galactose	negative	positive
Mannose	positive	positive
Inositol	negative	positive
Arabinose	positive	positive
Identification	*Gluconacetobacter* sp.	*Burkholderia plantarii*

Table 3 : Pesticide tolerance of *Gluconacetobacter* sp.

Tolerance range (µg/ml)	Pesticides				
	Ekalux	Tagban©	Sevin	Metacid	Hinosan
3	+	+	+	+	+
6	+	+	+	+	+
12	+	+	+	+	+
24	+	+	+	+	+
48	+	+	+	– *	+
97	+	+	+	– #	+
195	+	+	+	–	+
390	– *	+	+	–	+
781	–	+	– *	–	+
1563	– #	+	– #	–	+
3125	–	+	–	–	– *
6250	–	+	–	–	– #
12500	–	+	–	–	–

* MIC, # MBC, + indicate growth, – indicate no growth, © value above the range

Table 4 : Pesticide tolerance of *Burkholderia plantarii*..

Tolerance range (µg/ml)	Pesticides				
	Ekalux	Tagban	Sevin	Metacid	Hinosan
3	+	+	+	+	+
6	+	+	+	+	+
12	+	+	+	+	+
24	+	+	+	+	+
48	+	+	+	+	+
97	+	+	+	– *	+
195	– *	– *	+	–	– *
390	–	– #	+	– #	–
781	– #	–	– *	–	– #
1563	–	–	– #	–	–
3125	–	–	–	–	–
6250	–	–	–	–	–
12500	–	–	–	–	–

* MIC, # MBC, + indicate growth, – indicate no growth

we got almost similar response of corresponding control (Table 5). Maximum phosphate solubilization inhibition was detected in two treatments (*Gluconacetobacter* sp. + Meta. and *Burkholderia plantarii* + Sev.) and the least affected treatment was *Bukholderia plantarii* + Tag. Many authors (Kalam *et al*., 2004; Nasreen *et al*., 2005 and Shahin and Malik 2003) reported the inhibitory role played by pesticides on phosphate solubilizing activity. According to their observations, pesticides not only inhibit phosphate solubilizing bacteria but also interfere with other soil microbes and microbiological processes like soil respiration, nitrification and inhibit normal activities in soil. On the contrary, two reports (Das and Mukherjee, 1998 and Gaur, 1990) indicated the proliferation of phosphate

Table 5 : Phosphate solubilization in pesticide containing media.

Treatments	pH	Amount of P_2O_5 solubilized (mg/100 mL)*
Gluconacetobacter sp.	3.7	71.06±0.83
Burkholderia plantarii	3.2	67.65±0.67
Gluconacetobacter sp.+ Eka	4.6	47.81±0.37
Burkholderia plantarii + Eka	3.5	67.33±0.46
Gluconacetobacter sp.+ Tag	5.3	27.65±0.93
Burkholderia plantarii + Tag	4.2	53.33±0.57
Gluconacetobacter sp.+ Sev	5.2	49.34±0.37
Burkholderia plantarii + Sev	5.2	20.85±0.48
Gluconacetobacter sp.+ Meta	3.9	24.71±0.72
Burkholderia plantarii + Meta	4.0	32.39±0.54
Gluconacetobacter sp.+ Hino	4.1	32.90±0.40
Burkholderia plantarii + Hino	5.1	26.37±0.53

* mean of three replica, Eka; Ekalux, Tag; Tagban, Sev; Sevin, Meta; Metacid, Hino; Hinosan

solubilizing microorganism due to application of pesticides in soil. According to their findings phosphate solubilizers were able to use the insecticides and/or their degraded products for their growth and metabolism. In view of sterling role played by phosphate solubilizing bacteria on soil fertility, more cautious efforts should be made to find out new genera and species of PSB from our soil. A thorough knowledge about the impact of environmental hazards on these organisms is extremely important to retain the viability and vitality of our native agricultural soils.

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