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Phosphate solubilization by *Bacillus sphaericus* and *Burkholderia cepacia* in presence of pesticides

Vimal Ramani[1*] and H.H. Patel[2]

[1]Department of Microbiology and Biotechnology, Smt. U. B. Bhagat Science Mahila College, Smt. S. H. G. Saikshanik Sankul, Chakkargadh Road, Amreli -365601. Gujarat. India.
[2]Department of Life sciences, Bhavnagar University, Bhavnagar-364001 Gujarat, India.

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Screening of phosphate solubilizing bacteria with genetic potential for increased tolerance to high salt, high pH and high temperature could enhance production of food and forage in semi-arid regions. Emphasizing particularly on this hypothesis 165 phosphate solubilizing bacteria were isolated. Among these, two culture *Bacillus sphaericus* and *Burkholderia cepacia* were selected on the basis of salt tolerance property and Phosphate Solubilizing activity with different forms of phosphates. Both the bacterial culture showed significant result with pot culture experiments in green house conditions and field trail experiments. In the present investigation both these culture were assessed for the effect of six different pesticides, to confirm its successful realistic application as microbial inoculants in actual farm conditions. Both cultures showed more phosphate solubilizing activity with phosphate containing pesticides, while surprisingly B. *cepacia* showed more good phosphate solubilizing activity with all different pesticides, may be due to its well documented extraordinary versatile metabolic activity. This advance would prove them a better candidate as microbial inoculants.

Keywords: *Bacillus sphaericus, Burkholderia cepacia* and Phosphate Solubilization

Introduction

In agriculture the use of pesticides became very common as plant protection measures and during storage of food-grains. Pesticides include insecticides, herbicides, fungicides, nematocides and rodenticides. Pedticides come under a broad range of chemical types such as chlorinated hydrocarbons, organic phosphorous compounds, carbmates, organic sulphur compounds, heterocyclic molecules, phenols, quaternary ammonium compounds, halogenated and nitro-aromatic or aliphatic substances and a number of inorganic substances.

* Corresponding author: Vimal Ramani; e.mail: vimalramani@rediffmail.com

Organophosphorous and carbamate insecticides use has been increasing whereas the use of chlorinated insecticides has decreased as these are very stable and persist in the environment for a long time. Although, scientists continue to identify less persistent agro-chemicals, microbiologists are concerned regarding their use, because some of these chemicals may adversely affect non-target soil microflora and fauna. The direct effect of pesticides on soil microbiological aspects, which in turn, affect the plant growth are (i) change in the population of *Azotobacter, Rhizobium,* cellulolytic microorganisms and phosphate dissolving microorganisms which determine soil fertility, (ii) change in the number of nodules and lateral roots on roots of nodulating legumes and effect on microrrhizal symbioses in plants, (iii) change in the quantitative aspects of several microorganisms in soil which disturb the microbiological equilibrium, (iv) alteration in the nitrogen balance in soil by changes in the growth and activity of nitrifying bacteria;*Nitrosommonas* and *Nitrobacter* and (v) interference with ammonification in soil. Organic pesticides applied to soil, may undergo degradation by microorganisms and resulting in the formation of new compounds, which may prove to be far more deleterious to plants than the parent molecules. Occasionally, the applied pesticides may undergo detoxification or inactivation by microorganisms as in the conversion of the herbicide dalapon to pyruvic acid by *Arthrobacter* spp. Phosphate solubilizing microorganisms (PSM), which are useful soil inhabiting microflora and of importance to soil fertility may be affected by some insecticides and herbicides. Sivasithamparam (1970) examined the effect of chloropyrifos on phosphorus dissolving microflora and found slight inhibition of anaerobic PSB in a submerged clay loam. Tulabaev (1972) reported that triflusalin had no effect on phosphorous mineralization but simazine and 2,4-Dichlorophenoxyacetic acid reduced the growth of phosphate solubilizing bacteria. Lewis *et al.,* (1978) applied alchlor in loamy sand and silt clay loam and found that solubilization of tricalcium phosphate (TCP) in soil was not affected. Kazuhito *et al.,* (2002, 2001) also reported the role of *Bradirhizobium–Agromona–Nitorbacter–Afipia* (BANA) cluster of alpha proteobacteria on basis of 2,4-Dichlorophenoxyacetic acid degradation. In the present investigation, an attempt has been made to examine effect of different pesticides (normally used for different crops of Saurashtra region) on phosphate solubilizing (PS) activity of *Bacillus sphaericus* and *Burkholderia cepacia*.

Materials and methods

Organisms: *B. sphaericus* and *B. cepacia* were maintained on Pikovskaya's agar slant [glucose, 10 g; tricalcium phosphate (TCP), 5g; ammonium sulphate, 0.5g; sodium chloride, 0.2g; potassium chloride, 0.2g;

magnesium sulphate, 0.1g; yeast extract, 0.5g; manganese sulphate, trace; ferrous sulphate, trace; agar, 15g were added to 1 L of distilled water; the pH was adjusted to 7.0 ± 0.2 before sterilization. After sterilization 50µg/ml cyclohexamide was added asceptically to the cooled (60ºC) medium to inhibit the growth of fungi.

Culture media

Nutrient broth medium was used for preparation of bacterial inoculum, while Pikovskaya's broth medium was used for evaluating phosphate solubilizing activity. The required number of flasks containing 100 ml Pikovskaya's broth were sterilized. To each flask the pesticides (monochrotophos, carbandazim, phoret, endosulphan, chlorpyrifos, in the quantity of "recommended dose (RD)" as mentioned on the commercial packing and double the quantity of "recommended dose" (double dose) were added aseptically to separate flasks. The flasks containing Pikovskaya's broth without added pesticides were kept as control. Preparation of an inoculum: A flask containing 100ml Nutrient broth was inoculated with culture from the Pikovskaya,s agar slant and incubated at 30° ± 0.2° C for 48 h. The cells were separated from the broth by centrifugation at 10,000 rpm for 20 min., washed twice with sterile distilled water and resuspended in sterile distilled water such that suspension contained 6.65 x 10^7 cells/ml (1.0 O.D. at 660 nm) This suspension was used as an inoculum. All the steps were carried out aseptic condition.

Inoculation and growth condition

Two sets, each of ten flasks containing 100ml modified Pikovskaya's broth (with added pesticides), were inoculated aseptically with 1ml inoculum in each flask. All the inoculated flasks were incubated at 28° ± 0.2° C for 21 d for phosphate solubilization under static condition and shaken at 12 h intervals. An uninoculated flask was incubated as control. The experiment was carried out in triplicate.

Phosphorus estimation and pH measurement

At periodic intervals, 10ml medium was withdrawn from each flask aseptically and centrifuged at 10,000 r.p.m. for 20 minutes. Supernatant was analysed for water soluble - P content by chlorostannous reduced molybdophosphoric acid blue method. (Jackson, 1973) The pH of the medium was determined by 'Elico' pH meter.

Results

The effect of various pesticides on TCP solubilization by *B. sphaericus* and *B. cepacia* maximum PS activity occurred on different days with various pesticides was shown in table 1 and 2.

Table 1. Effect of pesticides on TCP solubilization by *B. sphaericus*.

Treatments	Days of Incubation													
	3		6		9		12		15		18		21	
	P_2O_5*	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH
B. sphaericus	103.70	3.7	176.49	3.3	96.34	3.9	142.02	3.6	138.98	3.5	181.94	3.2	166.07	3.3
B + Mono X	22.50	6.4	20.40	6.8	15.77	6.9	17.05	6.8	43.82	5.4	39.65	5.1	28.39	5.3
B + Mono 2X	31.99	5.6	41.13	5.7	35.68	5.8	40.33	5.6	34.72	5.6	44.33	5.3	30.25	5.4
B + Endo X	33.08	6.6	16.89	6.7	19.62	6.5	11.28	6.7	40.78	5.9	21.86	6.5	20.37	5.6
B + Endo 2X	36.48	6.2	21.45	6.3	29.59	6.3	20.61	5.4	19.98	5.4	36.16	5.9	25.88	5.9
B + Chlo X	15.80	5.3	37.75	4.5	45.85	5.0	34.72	5.2	19.01	6.2	182.39	6.3	79.35	7.8
B + Chlo 2X	27.82	5.3	27.82	4.9	60.04	5.4	47.40	6.0	186.38	6.1	35.13	5.6	30.58	5.6
B + Car X	7.98	7.8	12.79	6.3	14.39	7.5	6.37	6.0	36.15	6.4	33.05	6.1	28.36	5.8
B + Car 2X	9.97	7.8	17.88	8.0	14.61	6.1	11.73	7.9	11.73	6.2	23.85	6.3	20.54	5.8
B + Pho X	14.39	6.4	17.76	7.3	15.19	6.3	27.21	7.4	25.45	6.2	35.13	6.3	31.65	6.4
B + Pho 2X	11.88	6.4	14.45	6.5	13.97	6.8	12.85	6.0	24.17	6.8	13.97	6.5	23.18	6.5

*P solubilized as net mg % P_2O_5 after deducting respective control.
Mono = Monochrotophos , Endo = Endosulphan, Chlo = Chlorpyriphos Car = Carbendazim, Pho= Phoret,
X = Recoomended dose, 2X = Double dose, B= *Bacillus sphaericus*

Table 2. Effect of pesticides on TCP solubilization by *B. cepacia*.

Treatments	Days of Incubation													
	3		6		9		12		15		18		21	
	P_2O_5*	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH
B. Cepacia	115.73	4.0	152.28	3.2	101.63	3.6	136.57	3.8	69.73	4.7	71.81	4.5	93.29	4.2
B + Mono X	4.88	4.8	86.94	4.6	39.33	4.9	70.27	4.5	160.38	4.0	7316	5.8	65.12	5.6
B + Mono 2X	48.66	5.6	44.17	5.8	35.52	5.9	54.92	5.6	170.38	5.2	82.97	5.6	120.47	5.8
B + Endo X	44.30	4.6	77.16	4.3	83.58	5.0	95.32	4.8	158.99	5.8	71.71	5.8	42.03	6.0
B + Endo 2X	47.38	4.6	42.73	4.6	43.05	4.5	74.95	5.5	154.68	5.8	86.65	5.8	82.36	6.0
B + Chlo X	73.86	4.5	67.61	4.6	89.02	5.1	92.58	4.7	183.39	6.7	178.36	6.3	60.23	6.4
B + Chlo 2X	44.91	4.8	114.96	4.0	120.38	4.8	152.41	5.7	95.86	5.9	189.38	4.8	114.36	5.0
B + Car X	57.35	4.0	69.53	3.8	80.75	4.8	123.71	5.4	130.35	6.3	152.39	5.9	70.39	6.0
B + Car 2X	51.26	4.1	65.37	4.0	47.09	4.8	101.27	5.6	120.33	6.5	153.38	6.4	140.21	6.5
B + Pho X	44.65	4.9	72.55	4.3	62.13	4.0	79.22	3.9	152.39	5.9	152.48	6.3	40.78	6.4
B + Pho 2X	37.44	5.1	83.93	4.4	41.13	5.4	102.57	4.9	152.30	6.5	129.57	5.3	75.68	5.5

*P solubilized as net mg % P_2O_5 after deducting respective control.
Mono = Monochrotophos , Endo = Endosulphan, Chlo = Chlorpyriphos Car = Carbendazim, Pho = Phoret,
X = Recoomended dose, 2X = Double dose, B = *Burkholderia cepacia*

Maximum TCP solubilization (mg% P_2O_5) by *B. sphaericus* in the presence of various pesticides and in control can be arranged in the following decreasing order as chlorpyriphos 2x (186.38) > chlorpyriphos x (182.39) > control (181.94) > monochrotophos2x (43.82) > monochrotophos x (44.33) > endosuphan x (40.78) > endosuphan 2x (36.16) > carbandszim x (36.15) >phoret x (35.13) > phoret 2x (24.17) > carbandazim 2x (23.85).

Among five pesticides, maximum PS activity was observed with chlorpyriphos with double dose (186.39 mg % P_2O_5) and single dose (182.39 mg % P_2O_5) on 18th and 15th d, respectively, which surpassed the PS activity of control also. Lowest maximum PS activity (23.85 mg % P_2O_5) was observed in presence of double dose of carbandazim on 18th d. Except carbandazim and phoret, differences between PS activity in presence of single and double doses were not much.

Between the two P containing pesticides (monochrotophos and cholpyriphos), cholrpyriphos supported four times higher PS activity than that of monochrotophos and was slightly less than that of control. Other pesticides in presence of single and double doses reduced the PS activity of the organism. In case of *B. cepacia*, surprisingly all pesticides, showed more PS activity compared to control. The maximum phosphate solubilization i.e.189.38 mg% P_2O_5 and 183.39 mg% P_2O_5 was observed with double dose and single dose of chlorpyriphos, respectively. Maximum TCP solubilization (mg% P_2O_5) by *B. cepacia* in the presence of various pesticides and in control can be arranged along with maximum TCP solubilization in the following decreasing order as chlorpyriphos 2x (189.38) > chlorpyriphos x (183.39) >monochrotophos 2x (170.38) > monochrotophos x (160.38) >endosuphan x (158.99) > endosulphan 2x (154.68) > carbandazim 2x (153.38) > carbandazim x (152.39) > phoret x (152.39) > phoret 2x (152.30) > control (152.28).

Among P containing pesticides, cholrpyriphos showed better PS activity than monochrotophos and than that in control [Figure 1(A)]. With all the pesticides the PS activity of the organism was not found to be affected as in the case of *B. sphaericus*. Maximum phosphate solubilization by *B. cepacia* in presence of all pesticide occurred on either 15th day or 18th day except the maximum PS activity with control was observed on 6th day. It is evident [Figure 1(B)] that maximum PS activity of *B. cepacia* was more in presence of P containing pesticides i.e. cholrpyriphos and monochrotophos than that of rest of pesticides and control also. Some organophosphate pesticides such as diazion, chlorpyriphos and gursathion are also reported susceptible to microbial hydrolysis and may serve as carbon source for growth of *Flavobacterium* sp. *Pseudomonas* sp. and *Arthrobacter* sp.

Fig. 1 (A) Effect of pesticides on TCP solubilization by B. sphaericus.

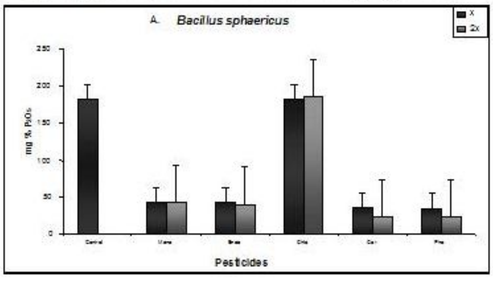
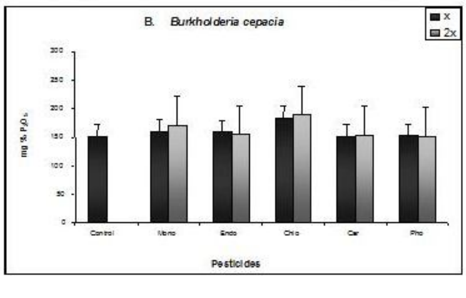


Fig. 2 (B) Effect of pesticides on TCP solubilization by B. cepacia



The drift in the pH of the medium was towards acidic side with phosphate solubilization in all experiments. The correlation coefficient between phosphate solubilization and ph of the medium was 0.04 and 0.33 for *B. sphaericus* and *B. cepacia*, respectively, which also indicate drop in pH of the medium usually accompanied by P solubilization. This may indicate that the possible mechanism of phosphate solubilization is organic acid production.

Discussion

The results are in contrast with the results of Dave (1999) with *Pseudomonas fluorescens* and Samaha (1998) with *Debaryomyces hansenni* and *Aspergillus aculeatus*. They reported negative effect of different pesticides tested on TCP solublization by concerned organisms. However, Sangodkar et al. (1998) and MacLoughlin *et al.,* (1992) reported extraordinary metabolic versatility of *B. cepacia* and its ability to degrade chlorinated aromatic substances, which are toxic compounds in complex pesticides and herbicides, some with carcinogenic potentials also. *B. cepacia* utilized these compounds as carbon and energy sources. The other important toxic compound degraded by *B. cepacia* is 2,4,5 - chlorophenoxy acetic acid (2,4,5-T), a potent herbicide that is not easily biodegradable and persists for long periods in an environment. Our results are supported by these observations.

Ghisalba *et al.,* 1987; Siddaramppa *et al.,* 1973. observed that the *Streptomyces pilosus* was capable of utilizing several organophosphorus pesticides. Noted the enhancement of PS activity with chlorpyriphos and quinalphose by *A. aculeatus*. Our results are in affirmation with these observations.

Dave and Patel, 2000; Narsian and Patel, 2000; Samaha, 1998; Gaur and Sachar, 1980; Chhonkar and Subba Rao, 1967; Sperber, 1957 have also reported release of phosphorus with a fall in the pH of the medium. Finally, we can conclude that the phosphate solubilization by *B. cepacia* was enhanced in presence of different pesticides, so it can be exploited as a good candidate for microbial inoculant.

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