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Effect of Non-Organophosphate Pesticides on Phosphatase Activity of a Soil Fungus *Aspergillus awamori* Kc316117 Isolated from a Moist Deciduous Forest in Odisha, India

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Abstract: This investigation was undertaken to study how microbial activities have been minimized by applying heavy amount of pesticides in agricultural fields. Four non-organo phosphate pesticides (Cypermethrin, Cartap hydrochloride, Acetamiprid, Carbendazim) were considered in this investigation to study the effect on phosphatase activity of a soil fungus, isolated from rhizospheric soil of Similipal Biosphere Reserve. The isolate showed better enzymatic activity in presence of Acetamiprid in the medium, in comparison to the other selected pesticides. During the investigation, it was also noted that Cypermethrin, and Cartap hydrochloride highly repressed the enzymatic activity and growth of the isolate. Hence, the study suggests how use of different pesticides decrease the mineralizing capacity of microorganisms (fungi).

Key words: *Aspergillus awamori*, phosphatase activity, pesticides.

Introduction

In recent years, demand for agricultural products have been increasing rapidly due to population explosion. This necessitates the farmers to use synthetic fertilizers and chemical pesticides to increase production [1,2]. Most of the chemical pesticides and fertilizers are recalcitrant and have serious negative impact to the living organisms including soil microorganisms and ultimately, which in turn influence plant growth. Some of the most important effects caused by pesticides is the decrease in soil fertility and crop productivity ceaseless changes in the soil microflora. While, now a days, different types of organophosphate and non organophosphate pesticides used frequently to eradicate different infection. Therefore, many beneficial soil microorganisms are either killed or their beneficial effects are retarded [3-7]. In this perspective, use of microbial inoculants (biofertilizers) specially Phosphate Solubilizing Microorganisms (PSM) in agriculture signifies an environment friendly alternative for future applications in sustainable agriculture [8-9]. However, the greatest challenge of microbial inoculant is their survival and performance under field conditions where farmers often apply pesticides for crop protection. Since most of the pesticides have detrimental impact on soil biota and biological functioning it is necessary to screen and select efficient phosphate-solubilizers that could overcome such consequences.

The objective of the present investigation was to study the effect of selective non-organophosphate pesticides on phosphate solubilising activity of *Aspergillus awamori* in order to exploit its potential for use as a microbial inoculants.

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Materials and methods

Isolation and identification of source organism

The fungus (*Aspergillus awamori*) used in the present study was isolated from soil samples collected from Similipal biosphere reserve by standard culturable method [10,11]. The isolate was screened for phosphatase activity on Pikovskaya's agar medium [11]. It was one of the several isolates that showed considerable phosphatase activity and was selected for further study. The organism was identified by morphological traits and microscopic observation referring standard identification manual [12-13].

Effect of pesticides on phosphatase activity

Four different pesticides **(**Table 1) were selected and their effect on phosphatase activity of the isolate was evaluated *in-vitro*. The isolate was grown in Pikovskaya's broth medium supplemented with various concentrations of pesticides separately and incubated at 29^0C. The enzyme activity and soluble phosphate content were assayed at two days intervals from 6- 16 days.

Assay for phosphatase activity of the isolate

The isolate (S3-4) was cultured in Pikovskaya's broth, at 28±2°C, for 6 days. After the incubation period, the culture was filtered out by Whatman filter paper. The filtrate was centrifuged at 1000 rpm for 10 min and the supernatant was used for phosphatase assay (as crude extract), spectrophotometrically using p-nitrophenyl phosphate (p-NPP) as substrate. Phosphatase activity was transformed to absolute units using a standard curve based on increasing concentrations of p-nitrophenol. The presence of acid phosphatase and alkaline phosphatase were determined. The activity was expressed in n mol p-NP released/ml/min [11] . The p-nitrophenol was used to make a standard curve for determination of phosphatase activity.

Estimation of available phosphate

An attempt was made to measure the available phosphate in the culture medium following ascorbic acid method [14]. Briefly, after the incubation period (as mentioned previously), the culture broth was centrifuged (150 rpm). The supernatant was taken for estimation of soluble phosphate. 50.0 mL supernatant was taken to which 0.05 mL (1 drop) phenolphthalein indicator was added. On development of a red colour 5*N* H_2SO_4 solution was added drop wise to just discharge the colour. 8.0 mL combined reagent [50 mL of 5N *Sulfuric acid,* 5mL *Potassium antimonyl tartrate solution* (1.3715 g K(SbO)C4H_4O_6/2H2O in 400 mL distilled water in a 500-mL volumetric flask and dilute), 15 mL of Ammonium molybdate solution (Dissolve 20 g (NH4) 6Mo_7O_{24}4H_2O in 500 mL distilled water.) and 30 mL of 0.1M Ascorbic acid, 0.1M was added to the sample. After, 10-30 min of incubation, the absorbance was measured at 880 nm, using reagent blank as the reference solution.

Results

The fungus, *Aspergillus awamori* was isolated from Similipal Biosphere Reserve showing phosphate solubilising activity and sulphur oxidation

Table 1. List of pesticides used in the experiment

No.	Commercial name	Pesticides	Chemical formula	Concentration % in the solution	Recommended concentration use in agricultural sectors (µl per 100 ml and %)
1	Stark-10	Cypermethrin	($C_{22}H_{19}C_{12}NO_3$)	10	20 (0.02 %)
2	Cartop	Cartap hydrochloride	$C_7H_{15}N_3O_2S_2$.HCl	50	100 (0.1 %)
3	Nagcarizim	Carbendazim	$C_9H_9N_3O_2$	50	100 (0.1 %)
4	Rider	Acetamiprid	$C_{10}H_{11}ClN_4$	20	40 (0.8 %)

characters. The isolate was identified by both morphological and molecular analysis. The molecular identification was carried out by sequence analysis of 18S rDNA study and the isolate was observed to have closest homology (100 %) with *Aspergillus awamori* . The sequence data also was deposited in NCBI with accession no. S3-4KC316117. Assay for phosphatase activity was carried out at different time intervals (6 -16 days, at 2 days intervals) at different concentration of pesticides. The highest activity was found on 6 days of incubation under control conditions (Table 2). Therefore, all experiments further were carried on 6 days of incubation.

The result indicated that Cartap hydrochloride inhibited the growth and activity of the fungus at concentration of 0.0125 % in the medium up to 6 days. Surprisingly, it was observed that after 6 days of incubation, the fungus could be able to establish its mycelial growth up to 12 days in that concentration. But, higher concentration of Cartap hydrochloride (0 .025 % and 0.05 %) inhibited the growth of fungus between 6-10 days, thereafter, mycelial growth was observed. The soluble phosphate in the medium was determined after 16 days of incubation and found to be 397.18 µg/mL and 390.45 µg/mL and 186.42/mL at the concentration of 0.0125 %, 0.025 % and 0.05 % respectively. However, the growth of the isolate was totally inhibited at 0.1 % concentration of Cartap hydrochloride (Fig. 1 A) in the medium without any phosphatase activity.

The isolate was found to be highly sensitive to pesticide, Carbendazim. It was observed that Carbendazim inhibited the growth of the isolate even at the concentration of 0.0125 % in the medium, upto 14 days. However, very less growth was observed on 16 days of incubation, and only with acid phosphatase activity. The activity was very less and found to be 5.11n mol p-NP /ml/ 20min. The soluble phosphate in the medium was observed to be 104.7 µg/ml (Fig. 1B).

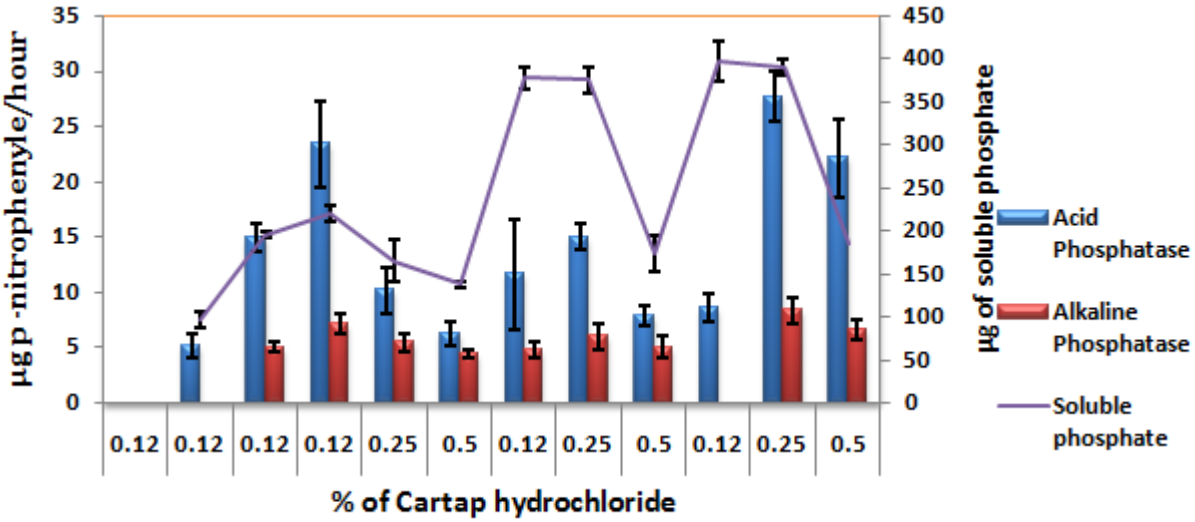
Acetamiprid is wildly used in agricultural sector. Therefore, the effect of Acetamiprid on enzyme production was also studied during the investigation. It was observed that the isolate retained its activity at concentrations of 0.01 %, 0.02 % and 0.03 % of Acetamiprid and acid phosphatase enzymes was found to be 22.14 n mol n-PN/ml/20 min, 17.54 n mol n-PN/ml/20 min and 11.68 n mol n-PN/ml/20 min respectively on 8 days of incubation. The production of alkaline phosphatase was found to be less in comparison to acid phosphatase. The alkaline phosphatase was observed to be 8 n mol n-PN/ml/20 min, 6 n mol n-PN/ml/20 min, 5.1 n mol n-PN/ml/20 min at the concentrations 0.01 %, 0.02 % and 0.03 % respectively upto 8 days of incubation. Surprisingly, it was observed that 0.4 % of Acetamiprid in the medium inhibited the fungal growth upto 14 days and thereafter, growth of the fungus was re-established in the medium. The soluble phosphate in the medium was estimated to be 1033 µg/ml, 436.04 µg/ml, 373.4 µg/ml and 298.7 µg/ml at the concentration of 0.01 %, 0.02 %, 0.03 % and 0.04 % of pesticide, respectively at 16 days of incubation (Fig. 1C).

Among the pesticides, Cypermethrin at the concentration of 0.005 % and 0.01 % partially inhibited both growth and enzyme activity of the isolate. Pesticide concentration higher than 0.01 % totally inhibited the growth of the isolate upto 6 days. However, the isolate showed very less enzymatic activity with slow growth after 14 days

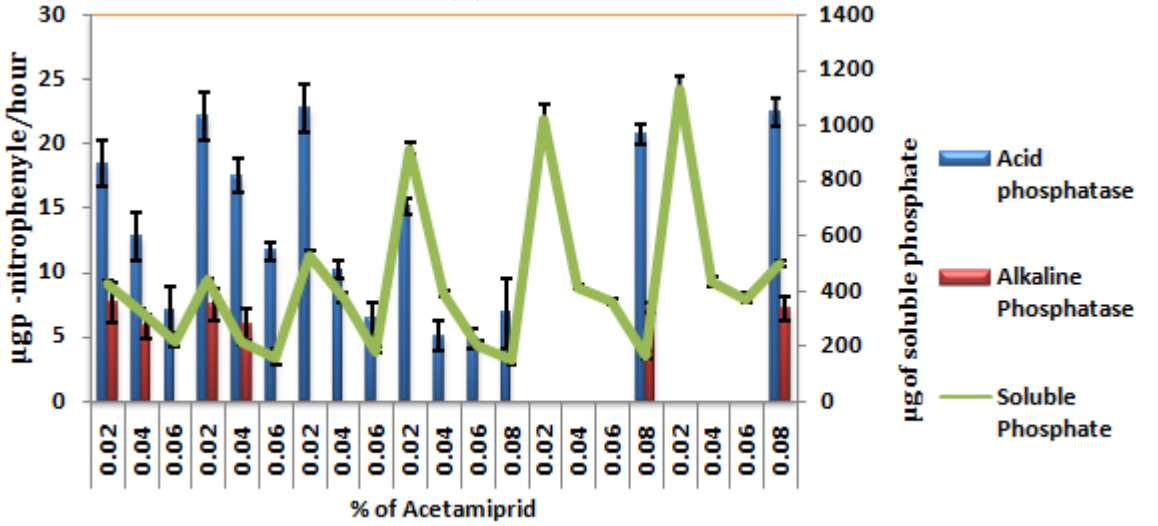
Table 2. Phosphatase activity of the isolate *Aspergillus awamori*

Activity	6 days	8 days	10 days	12 days	14 days	16 days
Acid Phosphatase	29±2.526	21.95±1	6.153±.106	4.153±0.106	4.15±1.52	3.5±1
Alkaline Phosphatase	15.68±0.58	10.89±0.53	6±1.158	3±1.15	00	00
Soluble phosphate	1228.1±26.2	1402±45	1446±65	1466±60	1470.1±28	1470±45
Dry weight*	0.88±.04	0.71±.07	0.7±.11	0.64±.11	0.6±.04	0.6±.05

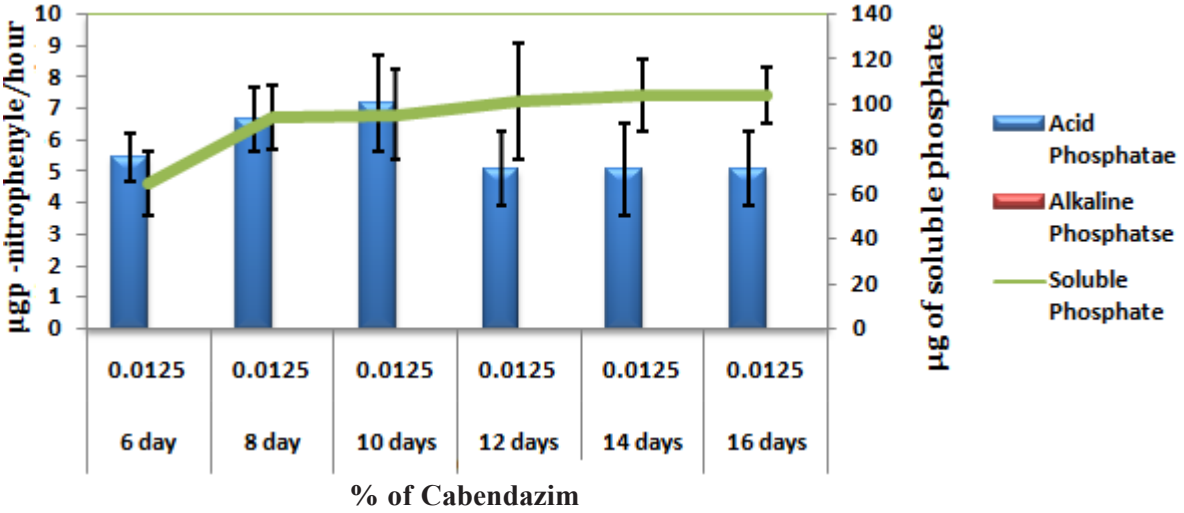
*Dry mass of the isolate was determined by simple oven drying at 80°C for 20 hrs of the filtrate cell mass


µg p -nitrophenyle/hour
µg of soluble phosphate
Acid Phosphatase
Alkaline Phosphatase
Soluble phosphate
0.12 0.12 0.12 0.12 0.25 0.5 0.12 0.25 0.5 0.12 0.25 0.5
% of Cartap hydrochloride

(A)


µgp -nitrophenyle/hour
µg of soluble phosphate
Acid phosphatase
Alkaline Phosphatase
Soluble Phosphate
0.02 0.04 0.06 0.02 0.04 0.06 0.02 0.04 0.06 0.02 0.04 0.06 0.08 0.02 0.04 0.06 0.08 0.02 0.04 0.06 0.08
% of Acetamiprid

(B)


µgp -nitrophenyle/hour
µg of soluble phosphate
Acid Phosphatae
Alkaline Phosphatse
Soluble Phosphate
0.0125 0.0125 0.0125 0.0125 0.0125 0.0125
6 day 8 day 10 days 12 days 14 days 16 days
% of Cabendazim

(C)

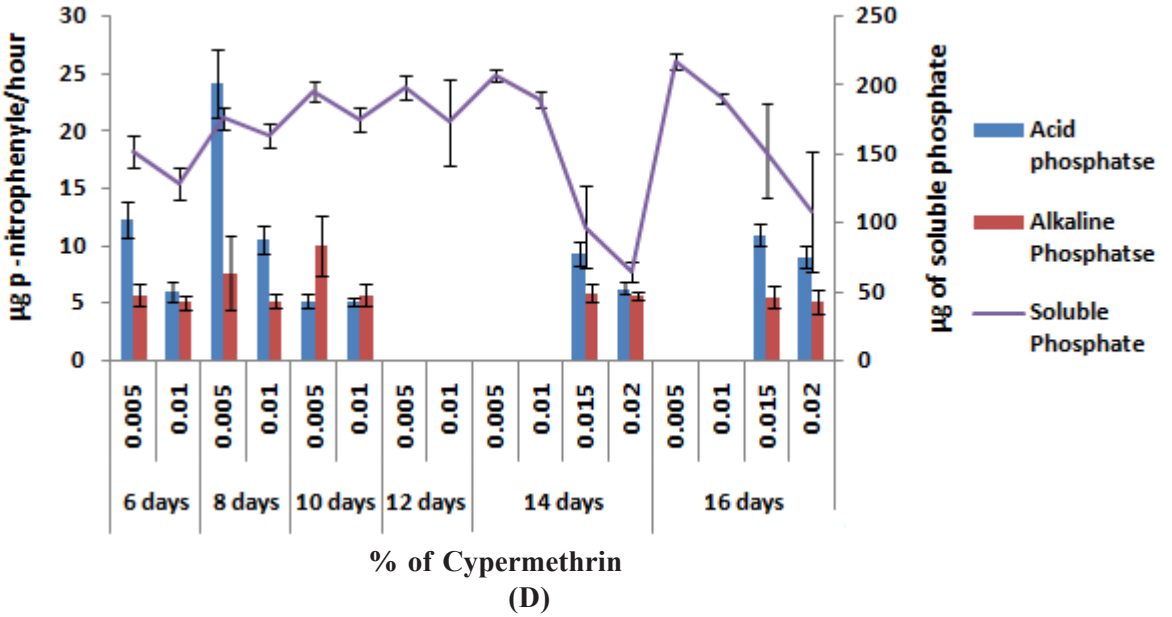


Fig. 1. Effect of pesticides on phosphatase activity of soil fungus (*A. awamori*) : A) Cartap HCL (B) Acetamiprid (C) Carbendazim (D) Cypermethrin

of incubation in the medium at concentrations of 0.015 % and 0.02 %. The final production of soluble phosphate at 16 days of incubation was estimated to be 218.07 µg/ml, 191.53 µg/ml, 152.93 µg/ml and 109.03 µg/ml against the concentration of 0.005 %, 0.01 %, 0.015 % and 0.02 % respectively (Fig. 1D).

Discussion

In the present study a soil fungus identified as *Aspergillus awamori* isolated from Similipal Biosphere Reserve in Odisha, India was shown to produce phophatase enzyme against different concentrations of commercially used non organophosphate pesticides in agriculture. Cartap hydrochloride is a pesticide commonly used by farmers and applied at a concentration of 0.1% or 100 µl/1L water. It is used to control chewing and sucking pests that results into insect paralysis [15]. This chemical is known to be highly stable and very toxic to living organisms, causing long-term adverse effects to the environment [16]. Although many soil microorganisms are not able to tolerate high concentration of pesticides like cartap HCl, there are reports that native microorganisms from soil and sediment are capable of degrading pesticides [15]. It was observed that our isolate was inhibited by cartap HCL upto 6 days; thereafter it re-established in the medium and showed phosphatase activity. Therefore, it can be inferred that the fungus is with an established mechanism to tolerate Cartap HCl and could produce phosphatase enzyme in presence of it. Similarly, many workers have reported the degradation of pesticides by several soil inhabiting microorganisms [17] in which nitrifying organisms were affected by high concentrations of Cartap HCI whereas, phosphatase activity was decreased when the soil was treated with 1,000 ppm Cartap HCI initially but then recovered after few days.

Carbendazim is used to control a broad range of fungal diseases on several crops, fruits, vegetables and ornamentals by interfering with spindle formation during mitosis [18-19]. In this investigation, it was found that *Aspergillus awamori* growth and phosphatase enzyme production were minimized at 0.0125 %. Therefore, use of this species could be limiting in the field accumulated with carbendazim previously.

It is also reported that Acetamiprid moderately inhibit the enzyme production of microorganisms. Punitha *et al.*[20] reported the decrease of both alkaline and acid phosphpatase production by soil microorganisms with increased amount of pesticides application in the field. Again our observations corroborate with other researches that this

pesticide was no longer effective on enzyme production by fungal isolates [21]. The production of phosphatase enzyme by microorganism was inhibited by high concentration of Acetamiprid [7]. Initial reduction in the phosphatase activity was found as observed in this investigation may be due to the inhibitory action of acetamiprid on the isolate. But later, the pesticides may be degraded or which alter the membrane permeability of the microorganisms releasing Phosphatase enzymes [21]. Yao *et al*.[7] reported that acetamiprid had a strong negative influence on soil respiration and phosphatase activity.

Cypermethrin ($C_{22}H_{19}C_{l2}NO_3$) is a synthetic pyrethroid used as an insecticide. Cypermethrin pesticide affects both microbial growth and enzyme action. Goswami *et al*.[22] reported that the microbial growth was highly influenced by presence of cypermethrin. In accordance with this, during this investigation, it was clearly observed that microbial growth was retarded even in the low concentration of cypermethrin in the medium. Cypermethrin is stable for long period in the soil and affect the soil biota [23]. They, also reported that cypermethrin drastically decreased the production of soil phosphates. Our findings also touch all the above reports that microbial mass and production of phosphatase enzymes were decreased in presence of cypermethrin.

Conclusion

In conclusion it can be told that pesticides affect both soil biota and microbial activity, hence, use of biofertilizers, require a prior examination of soil for presence of pesticides. In this regard our isolate *A. awamori* showed better phosphatase activity in presence of acetamiprid, Cartap HCL is suggestive of its use in agriculture soil, contaminated with these pesticides with necessary scientific investigations.

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