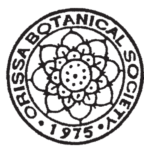

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The growth and biochemical responses of *Aspergillus niger* to treatment of malathion

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ABSTRACT

Organophosphates are widely used pesticide in various agricultural fields causing soil pollution and has health hazards. The persistence nature of these pesticides in environment and its toxic effect on biodiversity make it necessary for rapid removal from the environment. A study was undertaken to determine the effect of organophosphate malathion on growth and biochemical activities of *Aspergillus niger* with an aim to examine the potential of the fungus for decontamination of malathion from soil. The fungus was treated for five days at an initial inoculums of 10^3 spores/ml on exposure to graded concentrations of malathion (10-1000 mM). The species was affected differentially by malathion, with concentrations up to 50 mM causing growth acceleration and toxicity observed thereafter. Quite a significant growth of organism was obtained at 1000 μM on prolongation indicating the high tolerant nature of the fungus towards pesticide. As expected there was biochemical alteration of cellular metabolism resulting in the reduction of cellular protein and carbohydrate and concentration dependent changes of carbohydrate enzymes (cellulase, invertase and amylase) and dehydrogenase. There was also major change in stress enzymes like phosphatases and esterases, both the enzymes being more active as a response to insecticide treatment. Enhanced activities of these enzymes was considered to be the basis for tolerance of the fungus to high concentrations of malathion.

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1. Introduction

One of the most widely used agricultural pesticide groups are the organophosphates (OP), which are being exclusively used worldwide for control of the sulking and chewing pests in the orders diptera, lepidoptera, hemiptera, and coleoptera, due to their low effective concentrations. One such OP insecticide is malathion, S-(1,2-dicarbethoxyethyl)-O,O-dimethyldithiophosphate, which is being widely used since 1950 as a non-systemic, wide spectrum, phosphorodithioate organophosphorus compound (Goda *et al.,* 2010; Xie *et al.,* 2013). The pesticide is used in over 100 food crops in agriculture sector and against domestic pests like mosquitoes, head lice, animal parasite, etc. worldwide because of its quick knock down action against target pests by inhibition of acetylcholine esterase and comparatively low persistence in the environment (Barlas, 1996; Singh *et al.,*2012). However, prolonged use of the chemical has caused residual toxicity in crop fields affecting the soil microbiota (Megharaj *et al.*, 1987; 1994; Jena *et al.,* 2012). Residual toxicity of OP insecticides including malathion at field concentrations, has been reported in crop fields and in aquatic conditions leading to the decrease of productivity of the agricultural sectors and freshwater ecosystems (Van Donke *et al*., 1992; Megharaj *et al*., 1994; Mohapatra and Schiewer, 1996; Pandey and Gopal, 2012).

Any pesticide when applied not only affects the target organism but the main sufferers are the non-targeted soil microorganisms which get adversely affected by the application of the compounds. Pesticides get incorporated in soil directly, during plant treatment, and indirectly, via residues of plant or water and animal origin (Johnen, 1977; Anderegg and Madisen, 1983). A study on the effect of Triazophos-AN shows a great reduction in the population of soil bacteria up to 81%. There is also reduction in the population of the soil fungi by field application of

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insecticides but bacteria are more sensitive to the pesticide as compared to fungi (Kalyani *et al*., 2015). However, similarly profenofos, diazinon and malathion caused adverse impact on soil bacteria, nitrogen fixing bacteria and actinomycetes and effects are similar to that of the soil fungi (El-Ghany and Masmali, 2016). The application of organophosphate herbicides affects the size and composition of the soil organisms (Milosevia and Govedarica 2002; Ayansina *et al*., 2003). Liu *et al.* (2001) observed that dimethoate is inhibitory to the growth and biochemical activities of *A. niger* but the fungus also metabolically degrade the insecticides dimethoate and chlorpyriphos. There is no proper study of the effects of malathion on growth of *A. niger* in general and on enzymatic activities in particular. This study deals with the effect of malathion on growth and cellular enyme activities of *A. niger.*

2. Material and methods

2.1 The experimental conditions

The fungal strain *Aspergillus niger* was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. The pesticide used in the study was commercial grade malathion (Kalyani Industries Ltd., Mumbai). All the chemicals used for the sample analysis were of Hi media. The selected fungus was batch-cultured in 250ml Erlenmeyer flasks, each containing 100ml of liquid CzepakDox culture medium and with an initial inoculum of 10^3 spores/ml. Aqueous solution of malathion, after extraction of the active ingredient in acetone, was added to the medium to achieve the desired concentrations (10 - 1000 µM). The stock and experimental cultures were grown in an incubator shaker (NBiotech, Korea) at 26 ± 1°C in dark under continuous shaking (120 rpm).

2.2 Analytical procedures

Samples were taken at twenty four hours intervals up to five days for the estimation of the growth (fresh and dry weight). However, for protein, carbohydrate, and for other enzyme assays, samples were taken after 5 days of incubation. The mycelium was harvested using Whatman filter paper and the fresh weight was taken after one hr of shade drying. The samples were then kept in hot air oven at 80°C for 24 hours to obtain the dry weight. Total cellular protein content was determined by the methodof Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. The total carbohydrate content was quantified by method of Roe (1955), using glucose as standard. The activities of carbohydrate enzymes (invertase, amylase and cellulase) were measured after incubating the mycelia, macerated in Sorensen's buffer for 24 hour in the respective substrate (1% starch for amylase, 1% carboxymethyl cellulose for cellulase and 1% sucrose for invertase). The amount of glucose produced was measured using 3,5 – dinitrosalicilic acid (DSA) and the standard was prepared using glucose (Chhotaray *et al.*, 2014). Dehydogenase activity was measured following the method of Casida *et al.* (1964), using triphenylformazan as standard. Phosphatase activity was determined spectrofluorimetrically by the breakdown of 4-Methylumbelliferyl phosphate to methyl umbelliferone using two buffers- citrate buffer for acidic phospatase and Tris HCL buffer for alkaline phosphatase. Quantification of phosphatase activity was done by using the linear regression equation of standard curve of methyl-umbeliferone (Chandrakala, 2016). Esterase activity was determined by breakdown of florescein-diaceate into florescein in Tris HCL buffer at pH 7. Quantification of esterase activity was made using the linear regression equation of standard curve of fluoriscein disodium salt (Chandrakala, 2016). For all biochemical and enzyme assays, a mass of 0.05g of fresh mycelium (after 1 hr of shade drying) was taken and macerated in the respective buffers.

2.3 Statistical analysis

The treatments were made in triplicates and the experiments were done twice. The data presented as figures and tables are the means of the replicates pooled together from two experiments. Comparisons among the concentrations as well as among days of treatment were made using least significant difference test (LSD). The trend of change was made by regression analysis among the replicates of the treatments. As and when needed the regression coefficients have been given in the text. The data analysis was done in Excelstat following the standard statistical procedures (Gomej and Gomej, 1984).

3. Results and discussion

From the pattern of change in the hyphal biomass during the period of observation showed that both fresh weight and dry weight of *A. niger* increased with increase in the days of incubation irrespective of the applied concentration of the malathion indicating linear growth increment of the fungus (Table 1). However, significant concentration dependent change (enhancement/reduction) in the growth could be seen on any day of the observation. The insecticide, up to concentration of 50µM, caused increase in growth of the fungus resulting in increase in biomass as well as cellular protein and carbohydrate content. The enhancement of growth was found to be high in the beginning as compared to prolongation for five days. The fresh weight after one day of treatment was 2.85 times higher at 50µM concentration as compared to control. Corresponding increase in the dry weight was also observed

at this concentration. On the other hand, after five days of incubation the growth enhancement at 50µM malathion was 35% higher than that observed in control (Table 1). The protein and carbohydrate content at this concentration were also higher than of control but such difference was found insignificant (LSD = 1.837% and 1.002% for protein and carbohydrate, respectively). However, with further increase in the concentration of the insecticide, protein and carbohydrate content significantly decreased but reduction of carbohydrate content was only significant at 1000µM (Fig 1).

The activity of invertase was found to be the highest followed by amylase and cellulase in the fungus. The activity of these enzymes were high in culture treated with the insecticide up to 50µM but further increase of concentration caused a continuous concentration dependent decrease of the enzyme activity (Fig 2). As compared to control, the highest rate of reduction in activity (at 1000µM compared to control) was 29% for cellulase followed by amylase (45%) and invertase (80%). However, at 50µM of the insecticide the enhancement of the enzyme activity was highest for cellulase (105%) followed by amylase (26%) and invertase (23%). The difference in the enhancement rate of invertase and amylase was found insignificant (t=0.48; n=12). This indicated that at non-toxic concentrations of the insecticide, the activities of the carbohydrate enzymes and effectively enhanced to encourage the growth of the fungus.

The activities of phosphatases and esterases were measured in order to estimate the insecticide metabolizing efficiency of the fungus. As expected, alkaline and acidic phosphatase activity increased significantly at each of the applied concentrations of the insecticide up to 50µM (LSD = 1.36 and 1.85 for alkaline and acid phosphatase, respectively). The alkaline phosphatase activity drastically decreased at 100µM concentration but further increase of the insecticide concentration did not cause significant change in the enzyme activity (Fig 3). The activity at these concentrations (100-1000µM) also did not significantly differ from that of untreated control. The acid phosphatase on the other hand continuously decreased with increase in malathion concentration beyond 50µM but at 100µM the activity was significantly higher than of untreated control. At other higher concentration the acid phosphatase activity was insignificantly different from the untreated control.

Similarly esterase activity increased continuously up to 50µM concentration of the insecticide but showed a concentration dependent decrease thereafter (Fig. 3). The activity enhancement at 50µM concentration was about 2.7 times of the untreated control. At other higher concentration also there was significantly high activity of the esterase, when compared to the untreated control (LSD = 2.113).The activity of the dehydrogenase was estimated as a measure of metabolic efficiency of the fungus. Dehydrogenase activity showed enhancement up to 50µM of the insecticide and reduction thereafter (Fig. 4). The rate of enhancement were found insignificant (LSD = 0.039) but reduction beyond 50µM insecticide concentration was found significant.

Table 1

The fresh weight and dry weight (g/100 ml) after different days of treatment with different concentrations of malathion

Conc. (mM)	Fresh wt (g/100 ml) Days after treatment					Dry wt (g/100 ml) Days after treatment				
	1	2	3	4	5	1	2	3	4	5
0	0.024 ±0.002	0.391 ±0.022	1.743 ±0.078	2.663 ±0.139	2.887 ±0.149	0.002 ±0.001	0.057 ±0.003	0.233 ±0.012	0.341 ±0.014	0.384 ±0.021
10	0.053 ±0.003	0.392 ±0.015	1.798 ±0.094	2.679 ±0.148	3.145 ±0.166	0.004 ±0.001	0.062 ±0.003	0.245 ±0.011	0.359 ±0.019	0.424 ±0.022
50	0.061 ±0.003	0.581 ±0.035	1.913 ±0.086	2.757 ±0.153	3.875 ±0.191	0.006 ±0.001	0.066 ±0.003	0.273 ±0.014	0.368 ±0.018	0.508 ±0.021
100	0.023 ±0.001	0.355 ±0.017	1.577 ±0.082	2.318 ±0.126	2.685 ±0.142	0.003 ±0.001	0.051 ±0.002	0.208 ±0.011	0.344 ±0.013	0.359 ±0.015
500	0.009 ±0.001	0.183 ±0.009	0.932 ±0.054	2.117 ±0.101	2.258 ±0.113	0.001 ±0.001	0.021 ±0.001	0.143 ±0.009	0.222 ±0.015	0.282 ±0.016
1000	0.004 ±0.001	0.011 ±0.001	0.448 ±0.026	0.475 ±0.029	1.073 ±0.055	0.001 ±0.001	0.002 ±0.001	0.071 ±0.003	0.065 ±0.003	0.133 ±0.007

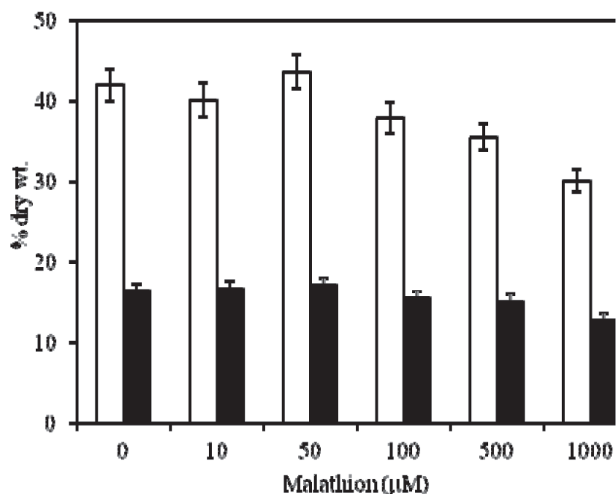

Fig. 1. The percentage of protein (empty columns) and carbohydrate (solid columns) contents of *A. niger* in response to 5 days of treatment with graded concentrations of malathion.

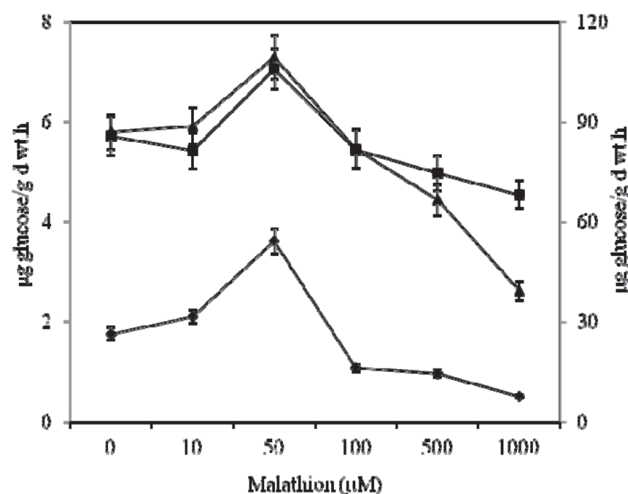

Fig. 2. The cellulase (diamond), amylase (triangle) and invertase (square) activities of *A. niger* in response to 5 days of treatment with different concentrations of malathion. The activity of invertase has been presented in secondary Y axis.

The fungus was found to have significantly high tolerance to malathion, being encouraged to grow up to 50 mM of the insecticide (Table 1). Enhanced growth and biochemical performances (Fig. 1 and 2) indicated that the fungus probably metabolised the insecticide and used it as a carbon source. The day dependent increase in growth and metabolic activities in the treated cultures supports this assumption. There are a number of cyanobacteria, algae, bacteria and fungi, which are known to metabolically degrade insecticides and other organochemicals causing decrease in their environmental toxicity (Ratna Kumari *et al.,* 2012; Ibrahim *et al.,* 2014; Kadhim *et al.,* 2015; Azmy *et al.,* 2015; Khan *et al.,* 2016; Nadalian *et al.,* 2016). Many fungal species belonging to phycomycetes, ascomycetes and white-rot fungi are found to be very useful for biodegradation of pesticides (Liu *et al.,* 2001; Jauregui *et al.,* 2003, Mohapatra *et al.,* 2018). Fungal species like *Aspergillus glaucus* (Anderegg and Madisen, 1983), *A. flavus* and *A. sydowii*

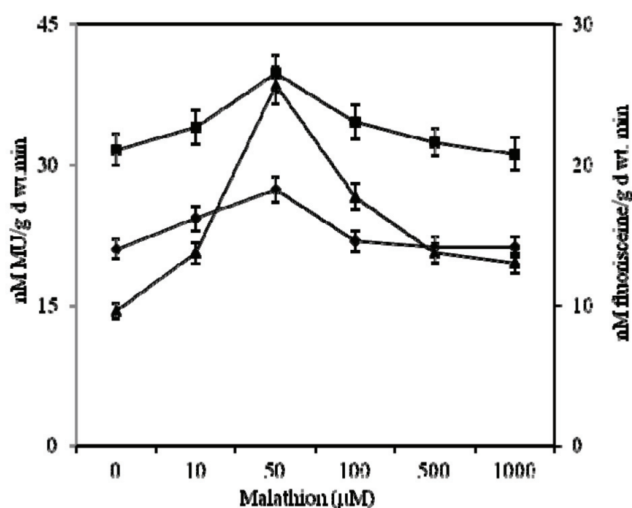

Fig. 3. The alkaline (diamond) and acid (triangle) phosphatase and esterase (square) activities of *A. niger* in response to 5 days of treatment with graded concentrations of malathion. The activity of estarase has been presented in secondary Y axis.

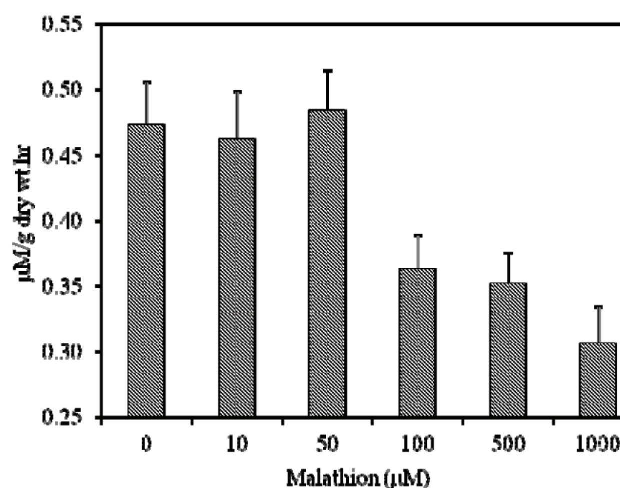

Fig. 4. The dehydrogenase activity of *A. niger* in response to 5 days of treatment with malathion.

(Hasan, 1999), *A. niger* (Liu *et al*., 2001) and *Trichoderma viride* (Matsumura and Boush, 1966) are known to degrade the pesticides under ambient environmental set up, even when exposed concentrations are much higher than of the residual level. Ramadevi *et al.,* (2012) reported that *Aspergillus niger* isolated from soil samples from malathion contaminated cotton cultivated field soils of Guntur district showed degradation ability of the chemical under laboratory conditions.

Significantly high level of esterases and phosphatases was noticed in the cultures treated with 50 mM of malathion. Liu *et al.* (2001) have reported that *A. niger* expressed a special enzymatic protein, comparable to bacterial phosphotriesterase on prolonged exposure to the OP insecticide dimethoate. They also observed the degradation of chlorpyrifos by the dimethoate tolerant *A. niger* strain indicating the broad substrate range of the induced enzyme. The high esterase activity in the present case proved that in *A. niger*, malathion treatment enhanced esterase level but the type of esterase so formed needs to be characterized. Further it was also observed that malathion treatment enhanced phosphatase activity which has not been reported earlier with OP treatment in fungal systems.

The result showed that *A. niger* has quite a high degree of tolerance to malathion showing improved growth and metabolic performance under treated condition up to 50 mM of dimethoate. This indicates that the fungus has the potential to degrade the organophosphate pesticide malathion under in vitro condition and can be a very good organism for the degradation of this compound. Moreover the organism also showed potential to grow in high concentration, though slowly, and also showed good sign to have the ability to survive in natural condition.

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