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POTENTIAL OF NATIVE *TRICHODERMA HARZIANUM* TO TOLERATE AND REMEDIATE OF ORGANOPHOSPHATE PESTICIDES USED IN AGRICULTURE FIELDS

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ABSTRACT

Pesticide toxicity has become a major threat to human health and ecosystem, due to heavy and repeated inputs in agriculture fields and persistent nature of this chemical compound in the agriculture fields. Monocrotophos and dimethoate belongs to organophosphate group of pesticides are known to have a significant adverse impact on human health directly affect CNS. Thus, there is a need to metabolize these compounds in the environment and removal of the residual amount of them. With this aim we have studied the tolerance and break down of environment through rhizoremediation methods. Tolerance of native isolate *Trichoderma harzianum* towards monocrotophos and dimethoate enrichment was studied through assays (plate assay/ liquid culture) at concentrations ranging from 0-2000 mg/l. At 1500 mg/l concentration of dimethoate pesticide we observed isolates exhibiting 100% growth and afterwards the growth was reduced to 89% at 2000 mg/l. With monocrotophos the isolate showed constant growth till 1250 mg/l and afterwards it was reduced to 37% at 2000 mg/l concentration on solid agar plate. However, in liquid media isolate with dimethoate showed maximum growth only till 500 mg/ml concentration an initial increase in growth (dry weight) attaining and subsequently reduction upto 54% at 2000 mg/l concentration however there was a negative growth impact with increasing pesticides concentration varied with the type of pesticides, with monocrotophos there was a progressive growth reduction which reached to 31% at 2000 mg/l concentration. Additionally, *Trichoderma harzianum* was observed to prefer monocrotophos and dimethoate as nitrogen source in minimal condition. Isolate *Trichoderma harzianum* recorded production of degradative enzyme of acid phosphate enzyme activity was 0.65 U/min/ml in culture treated dimethoate and alkaline phosphitase activity was 0.55 U/min/ml by culture treated with monocrotophos. Esterase activity was shown 14 U/min/ml in culture treated with monocrotophos. The native *Trichoderma harzianum* was not only able to endure higher pesticides concentration but can also offer a safe natural way for the removal of pesticides residue.

KEY WORDS : Monocrotophos, Dimethoate, *Trichoderma harzianum*, Bioremediation, OPP.

INTRODUCTION

Organophosphate (OP) based insecticides are most prevalent and unrelenting environmental pollutants that are in use in agriculture since decades. Monocrotophos Dimethyl (E)-1-methyl-2-(methylcarbamoyl)vinyl phosphate) and dimethoate (O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] dithiophosphateare insecticides belonging to the aromatic group of OP with toxic effect on the environment as well as on humans (Anonymous, 2001). Like other organophosphates, dimethoate and Monocrotophos also act as an acetylcholinesterase inhibit or disabling cholinesterase, an enzyme essential for central nervous system function. In agriculture dimethoate is applied on different crop varieties like bajra, maize, sorghum, castor, mustard, safflower, bhindi, brinjal, cabbage and cauliflower, chillies, onion, potato, tomato, apple, apricot, banana, citrus, fig, mango and rose etc, where as

monocrotophos is applied on cotton, paddy, maize, black gram, green gram, pea, red gram, sugarcane, citrus, mango, coconut, coffee and cardamom (Mücke, 1994; Singh *et al.,* 2006).

Many microorganisms have the ability to remove organophosphate pesticides from the environment completly. *Pseudomonas aeruginosa, Clavibacter michiganense, Arthrobacter atrocyaneus, Agrobacterium radiobacter, Bacillus megaterium* and *Pseudomonas mendocina* (Ramanathan and Lalithakumari, 1999; Omar *et al*., 1993 and Singh *et al.,* 2006) have been studied for the degradation of OP in solutions and soils. Fungal degradation of OP pesticides was only studied by few species. like white-rot fungi and *Aspergillus* sp. (Horne *et al*., 2002, Omar *et al*., 1993). Therefore, there is need of degradation of these pesticides by native agriculture isolates which also provide plant protection and growth promotion attributes in the agriculture system. *Trichoderma harzianum* is known for its Biocontrol activity in the agriculture fields and showed resistance to many toxic compounds (Bhadbhade *et al*., 2002a, Horne *et al*., 2002). In the previous study we have studied the growth promotion; biocontrol and tolerance towards malathion pesticides by *Trichoderma harzianum* the data were already published. In today's agriculture there is requirement to have native microorganisms which can act on these pesticides so that toxicity associative by the accumulation of pesticides at different level in the food chain (ecosystem) can be avoided. In the present study we have designed to screen the tolerance and breakdown of monocrotophos and dimethoate pesticide by *Trichoderma harzianum*.

MATERIALS AND METHODS

Pesticides: Monocrotophos and dimethoate pesticides were used in this study. A stock solution of 1µl per litter was prepared in water. Working solutions of 10, 50, 100, 150, 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 were prepared by appropriate dilution of stock. All the other chemicals used in this study were of analytical grade and were obtained from Sigma, Himedia and CDH India.

Fungal isolates and inoculums preparation: The isolation and characterization of *Trichoderma harzianum* was already reported and published in the previous paper. For the revival of the culture MDA medium (malt dextrose agar-dextrose: 10 g/l, malt extract: 20 g/l, peptone: 2 g/l, yeast extract: 2 g/l, agar 1.5% after addiction of all the component except agar the pH was adjusted to 7 and after pH agar 1.5%) were used. After autoclaving were checked for sterility and then the culture was inoculated and incubated for 5 days at 30 °C in the incubator (Patel *et al.,* 2013). After incubation, 5 mm disc was cut from growing mycelium of *T. harzianum* colony and was used as fresh inoculum for every experiment.

Tolerance tomonocrotophos and dimethoate in solid media: Monocrotophos and dimethoate were tested for its effect on growth and proliferation on *Trichoderma harzianum*. From the stock, the pesticides were added to MDA media with the concentration ranging from 0-2000 ppm with the interval of 250 and the plates were prepared in triplicate. Fresh culture was inoculated on pesticide amended plates and the radial growth was recorded after 5 days of incubation (Singh *et al*., 2014).

Tolerance to monocrotophos and dimethoate in liquid media: In 250 ml of flasks 50 ml of MDB media were prepared with the concentration ranging from 100-2000 ppm. A control was prepared with MDB media without pesticide. Fresh culture was inoculated into the broth and incubated for 5 days at 150 rpm on 30 °C. fungal growth was observed in dry weight (Singh *et al*., 2014).

Substrate exclusion studied

Supplement pesticides in place of carbon source by *Trichoderma harzianum:* The fungal isolates were grown on MDA after incubation the culture was inoculated into flask containing 50 ml of minimal media prepared and the pesticides concentration ranges from 250-2000 ppm with the interval of 250 ppm. In the MSM media, pesticides serve as sole carbon and flask with 1% glucose as carbon source served as one control (C1) and another test tube without glucose and without pesticide, taken as second control (C2). Flasks were then incubated on a incubator shaker at 30 ppm, 150 rpm. Fungal growth with pesticides as sole carbon source was evaluated by dry weight. Similar protocol was followed for nitrogen utilization and carbon/ nitrogen utilization on minimal media (Wang *et al.,* 2012).

Studies of enzyme involved in pesticides metabolism

***Trichoderma harzianum acid and alkaline phosphates enzyme production*:** Fungal strain was inoculated into MDA plates and after incubation fresh culture was inoculated into 50 ml of sterilized

Pikovskaya's broth in 250 ml flasks for each strain. The flasks were incubated at 30 ºC for 7 days. The acid and alkaline phosphatase activity of each strain was estimated on 7th day. 1 ml of the culture was taken and centrifuged at 10,000 rpm for 10 min and the supernatant was used. To estimate acid and alkaline phosphatase activity aliquots of 150 µl of the supernatant of the respective strains were taken. Aliquots of each sample were added to 0.48 ml universal buffer 0.1 M, pH 6.5 or pH 11 for acid and alkaline phosphatase activity respectively. Later 0.12 ml of 0.05 M p-nitro phenyl phosphate (PNPP) solution was added to each sample followed by 1h incubation at 37 ºC. The yellow color was measured at 405 nm (Prasanna *et al.*, 2011).

Esterase production by *Trichoderma harzianum:* Fungal isolates grown in the media for 5 days after incubation, cells were harvested and washed with citrate buffer pH 8 followed by sonication and recovered by centrifugation at 6000 rpm for 40 mins. 100 µl of Supernatant as enzyme is added to assay mixture containing 100 µl 0.3 mM α-napthyl acetate and 4.8 ml 40 mM phosphate buffer (pH 6.8). Incubate the assay mixture for 20 mins in dark, to it than add 1ml of staining solutions (1% fast blue B salt in 40 mM phosphate buffer) and incubate at 20 ºC for 30 mins and the OD were taken at 590 nm (Robles-Mendoza *et al.*, 2011).

RESULTS

Tolerance to monocrotophos and dimethoate in solid media: Our native isolates *Trichoderma harzianum* was able to withstand with both the pesticides upto 2000 mg per litter concentration. In plate assay the media was supplemented with pesticides with the corresponded concentration and fresh culture was inoculated after incubation the plates were observed for the radial growth of the isolates. For monocrotophos, the *Trichoderma harzianum* showed sporulation and full growth till 1250 mg/l and at 1500 mg/l of pesticides concentration the growth was affected and so was sporulation. Afterwards the growth of the isolate was decreased as the concentration of pesticides increases. For dimethoate, the *Trichoderma harzianum* showed speculation and full growth till 1500 mg/l and at 1750 mg/l of pesticides concentration the growth was affected and sporulation also. Afterwards the growth of the isolate was affected at the 2000 mg/l as compared to control results was tabulated in Table 1 (Figure 1 and 2).

Tolerance to monocrotophos and dimethoate in liquid media

Tolerance studies were also attempted in liquid media the growth of the fungus by observed as dry weight after the incubation period. For monocrotophos, in liquid media the growth of the fungus is decreased as the concentration of pesticides increases at 10 µl concentration the dry weight was 677 mg comparable to control 680 mg dry weight, and after wards the growth was subsequently decrease with each pesticides increment. At 50, 100, 250 and 500 mg/l concentration the growth as dry were recorded as 573, 520, 504 and 429 mg. At 750 mg/L pesticides

Table 1. Radial growth of *Trichoderma harzianum* with monocrotophos and dimethoate

Pesticide concentration	Monocrotophos		Dimethoate	
	Growth in diameter(cm)	Mycial morphology	Growth in diameter(cm)	Mycial morphology
Control	8.2	full growth and sporulation	8.2	full growth and sporulation
10 ppm	8.2	full growth and sporulation	8.2	full growth and sporulation
50 ppm	8.2	full growth and sporulation	8.2	full growth and sporulation
100 ppm	8.2	full growth and sporulation	8.2	full growth and sporulation
250 ppm	8.1	full growth and sporulation	8.2	full growth and sporulation
500 ppm	8.1	full growth and sporulation	8.2	full growth and sporulation
750 ppm	8.1	full growth and sporulation	8.2	full growth and sporulation
1000 ppm	8.1	full growth and sporulation	8.2	full growth and sporulation
1250 ppm	8.1	full growth and sporulation	8.2	full growth and sporulation
1500 ppm	7.9	growth and sporulation	8.2	full growth and sporulation
1750 ppm	3.8	growth	7.5±0.2	full growth and sporulation (change in colour light white)
2000 ppm	3.1	growth	7.3 ±0.05	full growth and sporulation (change in colour light white)

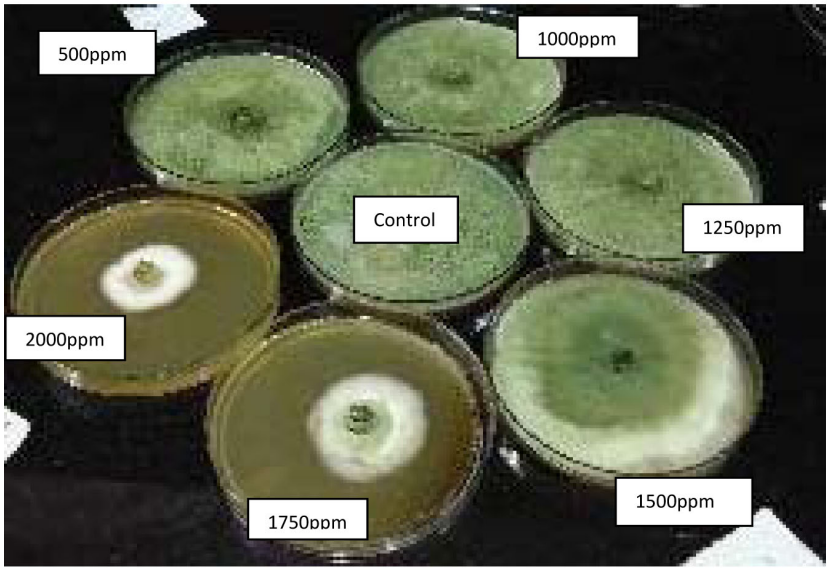

Fig. 1. *Trichoderma harzianum* growth studies with monocrotophos

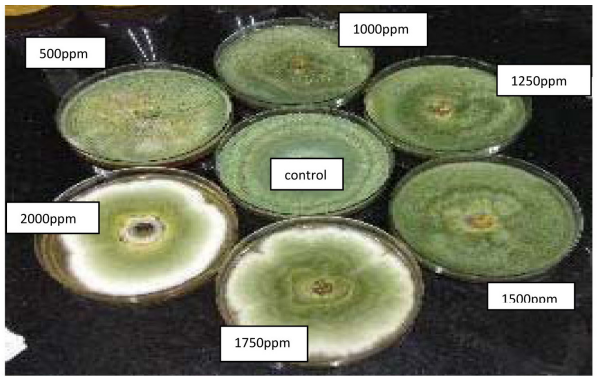

Fig. 2. *Trichoderma harzianum* growth studies with dimethoate

concentration the dry weight was at 50% decrement i.e 354 mg and from 1000-2000 mg/l concentration 321 to 217 mg of dry weight was recorded (Fig. 3).

For dimethoate, in liquid media the growth of the fungus is increased as the concentration of pesticides

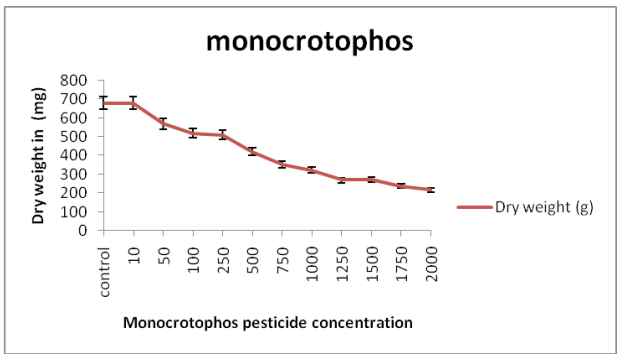

Fig. 3. Monocrotophos tolerance with *Trichoderma harzianum*

increase, at 500 µl concentration the growth was increasedup to 1400 mg as compared to control 700g and the dry weight was decreased as the concentration of pesticides increases but still the growth of the fungus was higher as compared to control. After 1500 µl the growth was decreased as compared to control.

Supplement pesticides in place of nitrogen source by *Trichoderma harzianum* : In pesticides supplementation studies minimal salt medium were used to check whether the fungus was taking the pesticides as carbon source or as nitrogen source. Nitrogen exclusion studies revile that in comparison to the control dry weight of fungi was decreased from 130 g to 48 g with the pesticides in minimal salt media. Medium contained 250 mg/l concentration of monocrotophos showed increment in the growth as the dry weight of the fungus increases by 133 g which indicate that the fungus was using this pesticide as nitrogen source. Further, at 500 mg/l concentration the growth is decreased as compared to control, i.e 127g and afterwards the dry weight was decreased with each increasing pesticides concentration the dry weight was affected (Fig. 3). For dimethoate the fungus was also able to utilize the pesticides as nitrogen source as compared to control the growth was affected in the form of dry weight was decreased as the pesticide's concentration increases (Fig. 5).

Pesticides as carbon source: *Trichoderma harzianum* was able to withstand in the absence of carbon source and it was using the pesticides as carbon

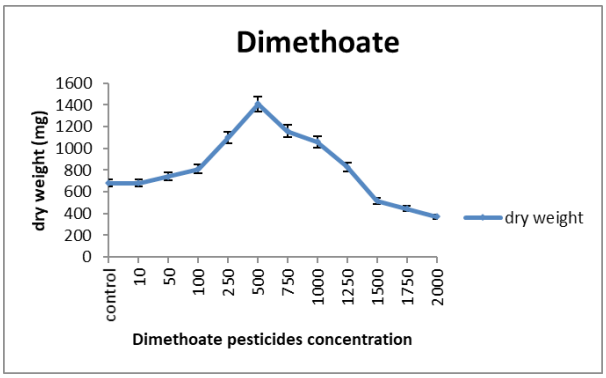

Fig. 4. Dimethoate tolerance with *Trichoderma harzianum*

source (Fig. 6). In the supplementation studies when we have excluded both the carbon and nitrogen source the growth was higher as compared to control but in carbon exclusion studies the growth were constantly decreasing as the pesticide's concentration increased (Fig. 7).

Enzymes involved in the metabolism of the pesticides:A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric. Phosphatase enzymes are also used by soil microorganisms to access organically bound phosphate nutrients.Acid and alkaline phospahtase enzymes were checked without any pesticides and induce pesticides stress condition with 1000 mg/L concentration for both monocrotophos and dimethoate. Higher production was recorded with dimethoate pesticdies followed by non-inducefungus and lower activity were recorded with monocrotophostreated sample. Whereas alkaline phosphatase activity was 0.55 U/min/ml higher in monocrotophos treated sample followed by dimethate 0.41 U/min/ml at 1000 mg/L concentration 0.3 U/min/ml (Fig. 8 and 9).

Esterase is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water. The enzyme was studied for both the

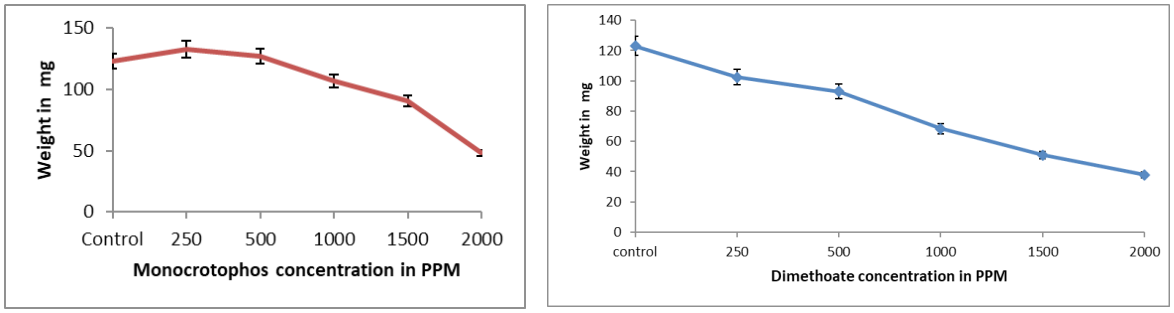

Fig. 5. Nitrogen exclusion studies in MSM with monocrotophos and dimethoate pesticides

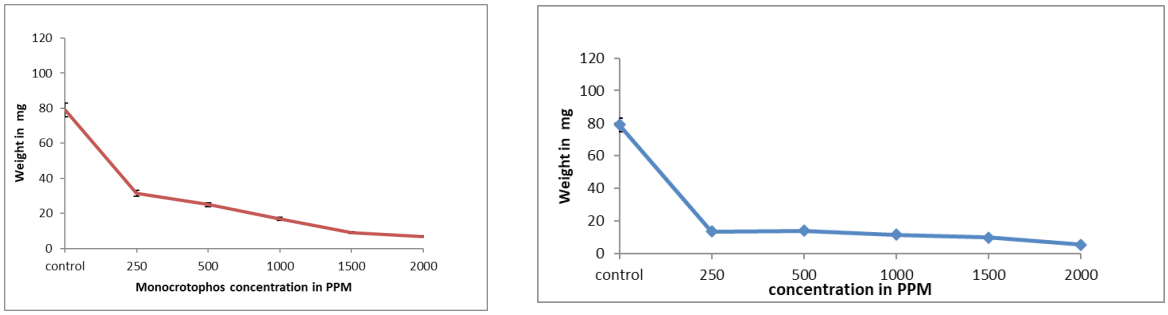

Fig. 6. Carbon exclusion studies in MSM with monocrotophos and dimethoate pesticides

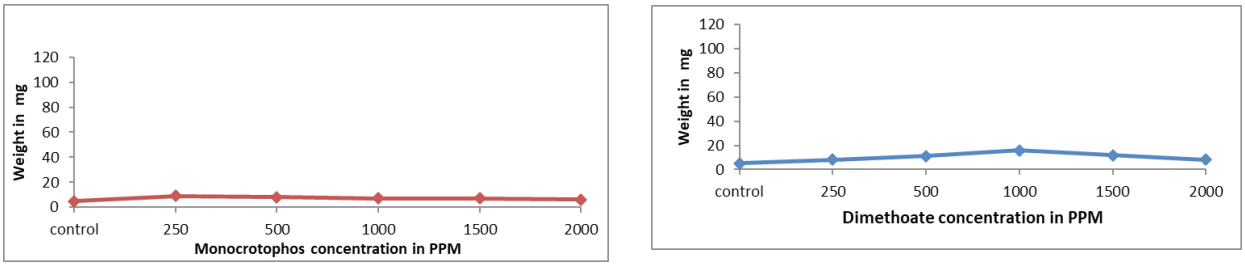

Fig. 7. Nitrogen and carbon exclusion studies in MSM withmonocrotophosand dimethoate pesticides

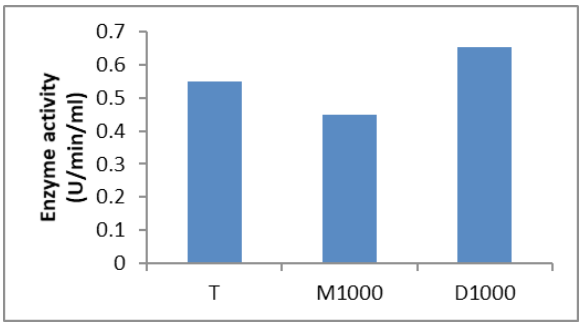

Fig. 8. Acid phosphatase enzyme production

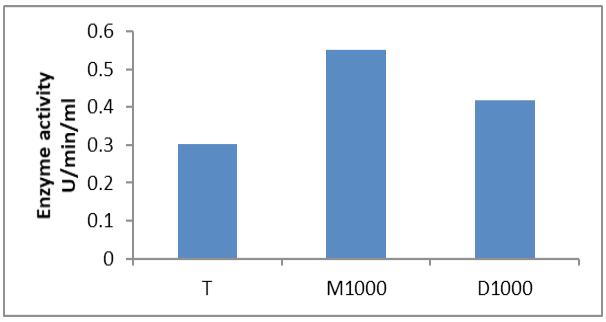

Fig. 9. Alkaline phosphatase enzyme production

pesticides metabolism. Esterase production was higher with induce condition. For monocrotophos the enzyme activity was 14U/min/ml with 100 mg/l concentration of pesticides followed by 8.9 U/min/ml 50 mg/l concentration of pesticides and in dimethoate the production was 10 U/min/ml at 100 mg/l concentration as compared to non-induce *trichoderma harzianum* and 4 U/min/ml shown at 50 mg/l concentration of dimethoate which was lower than the non induce pesticides control (Fig. 10**)**

DISCUSSION

Every year in the agriculture field farmers used different pesticides to control pest and protect crop cultivation. The pesticides exposure begins with

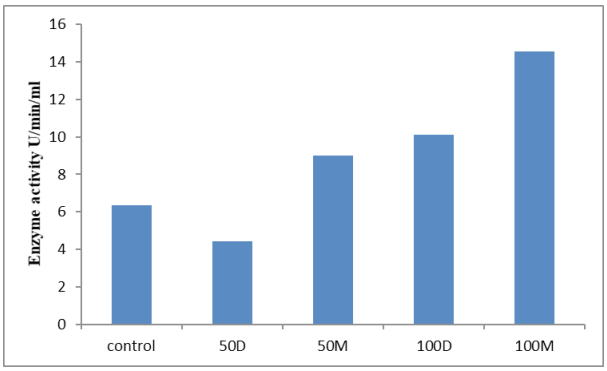

Fig. 10. Esterase enzyme production

farmers to the microorganism in the applied agriculture land. In India, a study reviled that in Punjab farmers experience health problem like occurrence of kidney failure, still birth, infertility, etc due to implication of pesticides (Abhilash and Singh, 2009: Rani *et al.,* 2020). In general, it has been observed that organophosphorus pesticides are responsible for death in more than 70% cases and intentional poisonings make up a large proportion of the poisonings by pesticides of high toxicity in certain developing countries (Abhilash and Singh, 2009). Most of the microorganism dies due to its toxic effect but some of them were able to survive caused they were able to use the pesticides as source of nutrient. Several studies were conducted on the breakdown of the pesticides compound and reduce the toxicity of parental pesticides. Chlorpyrifos, endosulfan, parathion, coumaphos, ethoprop and atarzin are the pesticides which were well studied for the complete removal of it by the application of bacteria and fungus (Chandini *et al.,* 2014). In our studies we have checked the broad spectrum of *Trichoderma harzianum* towards monocrotophos and dimethoate. In the enrichment method with monocrotophos at 10, 50, 100, 250 and 500 mg/l there was 10%, 73%, 66%, 65% and 50% decrement as compared to control which indicate that up to 500 mg/l concentration was 50% survival rate of the isolates. And afterwards at 750, 1000, 1250, 1500, 1750 and 2000 mg/l isolates showed the 42%, 37%, 29%, 29%, 24% and 21% inhibitory effect on the isolate's growth. *Jain et al.,* 2012 reported *Aspergillus niger* growth were affected and it was found that under optimized condition pH-8, Temperature 25-30°C and monocrotophos concentration 150 mg l^{-1}. Were ableto degrade 90% monocrotophos in 10 days. Further MCP degradation studies were supported by microbes such as *Bacillus*, *Pseudomonas*, *Aspergillus*, *Anabaena* and *Nostoc* at 25–37 °C and pH 5.5–8.5 were able to utilize monocrotophos compound as nutrient source for their growth and were able to tolerate 500–1200 mg/m concentration of pesticides. Thus, resulting its complete or partial degradation into nontoxic from of compound like dimethyl phosphate, phosphoric acid, valeric or acetic acid (Kaur and Goyal, 2019).

In Dimethoate *Trichoderma harzianum* studies showed opposite growth pattern as compared to monocrotophos. In the initial concentration the growth was increasing as shown in Fig 4 from 0-1250 mg/l highest growth were recorded at 500 mg/l concentration, i.e 100%. But at 1500-2000 mg/l

onwards concentration the growth was reduced as compared to control i.e., 24%, 35% and 45%. In the pesticides supplementation studies *Trichoderma harzianum* in minimal salt media with pesticides showed that the isolate was able to use the pesticides as nitrogen source of nutrient for both the pesticides. Algae was also able to break xenobiotic compounds, for monocrotophos two different algae, *Aulosira fertilissima* ARM 68 and *Nostoc muscorum* ARM 221, were shown the metabolism of it and it was using the pesticides as source of phosphorus (13). Another studies by Singh. and Singh (2003) also showed breakdown of monocrotophos by *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* ssp. insidiosum SBL 11 where these bacteria utilizing the pesticides as phosphorus source but not as a carbon source. Dash, and Osborne, (2020) showed degradation of monocrotophos by 95% treated with strain VITNNDJ5 for 5 days. Degradation of pesticides by microbes showed that they were able to complete mineralize the compound and utilize the pesticides as carbon or nitrogen or phosphorous (Singh and Walker, 2006). Deshpande *et al.,* 2001 showed degradation of dimethoate by *Bacillus licheniformis* and *Pseudomonas aeruginosa*

Microbes were able to produce certain enzyme or metabolite which has ability to break the pesticides into other from. The enzyme studies also shown that the alkaline phosphates enzyme production was 0.55U/min/ml higher with monocrotophos treated sample followed by 0.41U/min/ml activity in dimethoate treated sample as compared with non induce pesticides (0.31U/min/ml) where as for acid phosphatise dimethoate treated sample shown 0.65U/min/ml activity higher than control 0.54U/min/ml and with dimethoate treated sample the enzyme activity was low as compared to non induce pesticides condition 0.41U/min/ml. Studies on organophosphate degradation suggested that hydrolysis, oxidation, microbial metabolism and other biological process are the process through which different breakdown pathways occur (Singh, and Walker, 2006). Phosphatase, esterase, hydrolase and oxygenase are the key enzyme which involve principally in the breakdown of P-O, P-S linkages in OPP and the enzymatic metabolism with 12 commonly known used OPP shown faster results as compared to chemical process (Hassal, 1990).

Arthrobacter atrocyaneus MCM B-425 and *Bacillus megaterium* MCM B-423, were isolated from soil exposed to MCP and able to degrade MCP by 93% and 83% respectively. The bacterial isolate was able to break MCP into carbon dioxide, ammonia and phosphates through formation of one unknown compound – Metabolite I, valeric or acetic acid and methylamine (Bhadbhade *et al.,* 2002) Two of the potential microbial isolates, *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* subsp were able to degrade monocrotophos as phosphorous source by 98.9 and 86.9% and the enzyme responsible for breakdown of MCP was phosphotriesterase (Subhas and Singh, 2003). Esterase production was shown by both the treated sample. Studies of monocrotophos metabolism revile that, methylamine was the end product which was produced by esterase enzyme as it has ability to break amides bond (Bhadbhade *et al.,* 2002). In our studies we have studied all the metabolic or breakdown aspects as suggested by different reported literature. As *Trichoderma harzianum* spp. is known for Biocontrol abilities against different disease this study reviled a new aspect of agriculture pesticides residual removal without damaging the native micro flora.

CONCLUSION

In the environment there are enormous no of pollutants present which affecting the natural micro and macro flora as well as human and polluting the soil, water and air.

Many microbes have an ability to break the compound into nontoxic form further which can be utilize by others microorganism. In our study we have found that *Trichoderma harzianum* spp. was also able to metabolize the compound and protect it from spoiling into water and soil. The study suggests the futuristic aspect of metabolism and there is a need to explore more and more microbes which reduce the toxic compound from the environments and make the place contaminant free.

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