Conservation of Agricultural Soil Using Entomopathogenic Fungi: An Agent With Insecticides Degradation Potential

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Abstract. A major current focus in agricultural soil conservation is to ensure a pest control program is sustainable, and therefore, entomopathogenic fungi have been considered and extensively studied as biopesticides. However, the ecological role of entomopathogenic fungi in degrading insecticides in soil is not well understood. In this study, the potential of entomopathogenic fungi Metarhizium anisopliae (Met.) in degrading two common agricultural insecticides, chlorpyrifos and cypermethrin was investigated by introducing M. anisopliae into autoclaved soils artificially contaminated with 500 ppm of chlorpyrifos, and cypermethrin. The concentration of chlorpyrifos and cypermethrin were determined after 21 days using UPLC/PDA detector. The residues, rate, and percentage of degradation between insecticides treated and control soil were compared using an independent t-test (SPSS 20.0). The degradation of both insecticides in Met. treated soil (>80%) was significantly higher than control soil (47-61%). The residues for chlorpyrifos and cypermethrin residue in Met. treated soils were 19.39±0.10 ppm and 19.68±0.36 ppm, respectively, significantly lower than control (residues of chlorpyrifos-262.6±7.6 ppm and cypermethrin-194.4±4.3 ppm, at p<0.05). The results suggested M. anisopliae may play a role in the bioremediation of soil.

1. Introduction
Insecticides contribute significantly to the success of modern farming and food production. Although biological control approaches have been commercially applied for some insect species [1], we still rely heavily on insecticides to limit insect damage in agriculture [2]. Insecticides such as chlorpyrifos and cypermethrin were commonly used in crops like corn, pepper, and potatoes, due to its capable effect in controlling soil-dwelling pest. Organophosphorus pesticides such as chlorpyrifos were once regarded as the most widely used insecticides and occupied an estimated 34% of worldwide insecticide sales [3]. However, the insecticides were highly persistent in the ecosystem. This therefore introduces possible incorporation into our food chain, thus affecting ecosystems including human beings [4], as stated by Singh and Walker [5] who suggested that these compounds possess high toxicity to mammals.

Extensive application of the insecticides led to soil contamination and therefore lowered soil fertility. The problem is accumulating due to deficiency of an applicable solution in the form of methods that are effective and ecologically friendly in the removal of toxic pollutants [6]. In general, physical and chemical clean-up technologies are relatively expensive, less sustainable and not appropriate. Boopathy [7] applied bioremediation on insecticides contaminated soil by using microorganisms, and the result was promising; but when bacteria was applied in degrading chlorpyrifos, its antibacterial metabolite
3,5,6-trichloro-2-pyridinol (TCP) prevented the proliferation of degrading bacteria [8], subsequently persisting in the environment.

The impediment of bioremediation on chlorpyrifos contaminated soil can be resolved by using fungi, as Chen et al. [9] demonstrated from the biodegradation of chlorpyrifos and TCP by fungus Cladosporium cladosporioides Hu-0. Abd El-Ghany and Masmali [10] showed a type of entomopathogenic fungus Metarhizium anisopliae (Met.) was able to biodegrade more than 90% of malathion. Ong et al [11] investigated the interaction between M. anisopliae and chlorpyrifos in the control of house fly, and found the residues of chlorpyrifos were significantly lower compared to the control in the potato-dextrose agar (PDA) culture media after 14 days. Therefore, this facilitated our interests on the possibility of M. anisopliae in biodegradation of chlorpyrifos in the common substrate-soil. In this study, we aimed to investigate the degradability of M. anisopliae on the chlorpyrifos and cypermethrin in soil by analysing the residues after certain incubation duration.

2. Materials and methods

2.1 Fungal Culture

Metarhizium anisopliae (Met.) Sorokin strain was isolated from the spores that form on the Oryctes rhinoceros (L.) beetle. The isolate was batch cultured on 10 Petri dishes containing potato-dextrose agar (PDA) at 27°C for 30 days. The conidial suspension prepared in the testing was harvested from young colonies from the surface of these colonies by using a sterilized L-rod and transferred aseptically to a tube containing a mixture of 0.1% (v/v) Tween 80 and autoclaved distilled water. The stock suspension was standardized at a concentration of 3.5 x 10^8 conidia ml⁻¹ using a Neubauer haemocytometer.

2.2 Identification of fungus

Fungus conidia morphology was observed using compound microscope (Leica® DM500) with Köhler illumination. Samples were prepared as proposed by Humber [12], where the conidia were taken by an insect pin with assistance of a stereo microscope (Olympus SZ Stereo Microscope). The morphology was identified according to the Key to Fungal Entomopathogens [12], in which the Metarhizium was confirmed with its formation of short to long chains of conidia, with rounded to broadly conical apices, branched, densely intertwined conidiophores that forming a compact hymenium, and the conidia borne in parallel chains and green in mass (figure 1).

![Figure 1. Conidia of Metarhizium.](image)
2.3 Degradability test on soil

Soil samples were collected from the field with no history of insecticides application at Penang Bayan Lepas, Malaysia from a depth of 10-20 cm. Soil samples were sieved through a 90-mesh sieve to remove plant debris and stones. These soil samples were autoclaved and stored at 4°C. Ten grams of sterile soil were introduced in Petri discs, with the variants of three controls; 1) soil, 2) soil + insecticides, 3) soil + fungal inoculums, and the treatment; soil + insecticides + fungal inoculums in five replicates. Each of the insecticides was added at 500 ppm, and inoculations were done with two mL of fungal spore suspension (3.5 x 10^8 conidia ml^-1). The Petri discs were incubated at 30°C for 21 days then proceed for insecticidal residue analysis.

2.4 Insecticidal residue detection

A standard curve was initially generated to determine the particular retention time for each tested insecticide by preparing serial dilutions of five standards ranging in concentration from 50 to 500 ppm by weighing analytical grade chlorpyrifos (99.7%) and cypermethrin (94.3%) (Sigma-Aldrich, Malaysia) in a 25-ml volumetric flask. Acetonitrile (HPLC grade, Fisher Chemical) was used as the solvent. Modified solvent direct-immersion extraction- SDIE [13] was used to extract insecticide from the soil. Two grams of homogenized soil for treatments and controls were prior extracted using acetone, water, and an insecticide-specific solvent (chlorpyrifos-acetone and cypermethrin-hexane) in a 1:1:2 ratio with rotary shaking and immersed for 24 h. The upper solvent layer of the sample later was subjected to centrifugation using an Eppendorf Centrifuge 5427R (Eppendorf Asia Pacific Sdn. Bhd, Selangor, Malaysia) at 2,000 rpm for 5 min. The supernatant was cleaned-up using solid-phase extraction (SPE) C-18 (Supelclean ENVI-18 SPE wt. 500 mg, volume 6 ml) cartridge (EPA 1996). The filtrate was concentrated until dry at 55-65°C in a ventilated oven. The dried residues were reconstituted in 1 ml of acetonitrile in 2-ml amber glass vials for UPLC analysis.

The residues were analysed using the ACQUITY UPLC WATER system (Waters Analytical Instruments Sdn. Bhd., Petaling Jaya, Malaysia), consisting of a PU-1580 pump coupled to an HG-1580-31 mixer and a photodiode array (PDA) detector with programmable excitation and emission wavelengths. Separation was achieved using an ACQUITY UPLC BEH C18 Column (1.7 mm by 2.1mm by 100 mm). The PDA detector was set at the excitation wavelengths of 220 nm for chlorpyrifos and 225 nm for cypermethrin, with the initial mobile phase of 10:90 (v/v) for cypermethrin and methanol/acetonitrile at 70:30 (v/v) for chlorpyrifos. The quantitative measurement of the insecticide residue followed the CDFA standards [14].

3. Results and discussion

Extensive use of chlorpyrifos and cypermethrin may cause various environmental consequences due to the natural degradation, particularly soil fertility. We proposed the application of biodegradation on both chlorpyrifos and cypermethrin in agricultural soils that were also demonstrated by Abd El-Ghany and Masmali [10] that using M. anisopliae in reducing organophosphates in the soil. The principle to support the investigation of using fungi on chlorpyrifos and cypermethrin was due to the characteristics of the fungus that did not have sensitive targets for the insecticidal action mode of chlorpyrifos and cypermethrin [15].

The characteristic of fungus was identified as described in the section of 2.2 “Identification of fungus”. The insecticides’ residue for the controls (soil, soil + insecticides, soil + M. anisopliae) were less than 0.01 ppm, and the chlorpyrifos and cypermethrin’s residue for the plates of soil + insecticides + M. anisopliae were significantly lower compared to the plate containing the soil with the two insecticides, respectively (P < 0.05, table 1). The result of this study was comparable to Ong et al. [11], in which the M. anisopliae + ChCy (a commercial insecticide that was having both chlorpyrifos and cypermethrin) showed significantly lower residues than the control. Our results agreed well with the study of Abd El-Ghanay and Masmali [10] that showed M. anisopliae was able to biodegrade more than 90% of malathion, which had the same category and action mode as chlorpyrifos. Siewiera et al.
used *Metarhizium robertsii* (genus *Metarhizium*) to enhance the tributyltin (TBT) degradation by estradiol (E2), in which the presence of *M. robertsii* significantly reduced the amount of tributyltin.

**Table 1.** Insecticide residues (ppm, means ± S.E) of the treatment soil after 21 days.

| Residue (ppm, means ± S.E) | Chlorpyrifos | Cypermethrin |
|-----------------------------|--------------|--------------|
| Soil                        | Nil          | Nil          |
| Soil + *M. anisopliae*      | Nil          | Nil          |
| Soil + chlorpyrifos         | 262.6±7.60a  | Nil          |
| Soil + chlorpyrifos + *M. anisopliae* | 19.39±0.10b  | Nil          |
| Soil + cypermethrin         | Nil          | 194.4±4.30a  |
| Soil + cypermethrin + *M. anisopliae* | Nil          | 19.68±0.36b  |

*Means ± S.E followed by same letter indicates not significantly different at P = 0.05 within row, post hoc test, LSD
*Nil indicated that the residue is less than 0.01 ppm; S.E standard error

Chlorpyrifos is suggested as a high persistency pollutant in the environment due to its antibacterial metabolite 3,5,6-trichloro-2-pyridinol (TCP) [17]; however, Chen et al. [9] has shown the possibility of using the fungus *Cladosporium cladosporioides* Hu-01 to degrade chlorpyrifos and hydrolyzed its antibacterial metabolites 3,5,6-trichloro-2-pyridinol (TCP). Similarly, a study by Fang et al. [18] showed that application of the fungus *Verticillium sp.* from soil successfully degraded chlorpyrifos, Mukherjeea and Gopala [19] also demonstrated that two soil fungi *Aspergillus niger* and *Trichoderma viride* are able to degrade chlorpyrifos. The fungus may be an excellent alternative for bacteria in bioremediation of chlorpyrifos contaminated soil. Bioremediation on insecticides contaminated soil was achieved, as demonstrated by Chalamala et al. [20] who used *Aspergillus niger*, as an alternative to other conventional technique for the degradation of malathion contaminated residue soils in which *Aspergillus sp.* showed tolerance limit of 800 mg of malathion and degraded 300 mg within 24 h of incubation. The fungus may synthesize phosphotriesterases (PTE), the main class of enzymes in the hydrolysis of organophosphate insecticide such as chlorpyrifos. Various PTEs have been identified such as organophosphate hydrolase (OPH), methyl parathion hydrolase (MPH), organophosphorus acid anhydrolase (OPAA), disopropylfluorophosphatase (DFP), and paraoxonase 1 (PON1), carboxylesterases from fungus [21]. In conclusion, future study could be broadened in the molecular extraction and catalytic enzymes from fungus on insecticides such as chlorpyrifos and cypermethrin, and also other organic insecticides.

4. References

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