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Mycoremediation of Organophosphorous Insecticide Chlorpyrifos by Fungal Soil Isolates

Sherif, H. Abd-Alrahman[1,2] and Ashraf A. Mostafa[3]

[1]Biochemistry Department, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia.
[2]Pesticides Residues and Environmental Pollution Department, Central Agricultural Pesticide Lab.,Agricultural Research Center, Giza 12618, Egypt.
[3]Botany and Microbiology Department, Collage of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia.

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Chlorpyrifos is organophosphorus pesticide widely used in cultivated fields of Egypt. Chlorpyrifos resides are persisted in environment for unpredictable time and threaten public health. Adequate measures to remove chlorpyrifos contamination are essential to minimize environmental pollution and reduce public health hazards. The vital role of soil fungi in degradation of organic matter prompted us to attempt biodegradation of chlorpyrifos using these fungi. This study aims to isolate biodegradable soil fungi capable of metabolizing chlorpyrifos. 5 of 13 fungal strains isolated form chlorpyrifos contaminated soils were able to degrade chlorpyrifos and the results were confirmed by broth assay. Based on their chlorpyrifos tolerance, five fungal strains identified as *Trichoderma viride, T. harzianum, Aspergillus niger, A. oryzae* and *Penicillium citrinum* were detected and confirmed as chlorpyrifos biodegradable fungal strains. *T. viride* was the most effective biodegradable fungi recording a highest dissipation of chlorpyrifos 70.61% while *A. niger, T. harzianum* followed by *A. oryzae* were moderate in their biodegradable activities reducing chlorpyrifos to 63.96, 60.31 and 50.79% respectively. On contrast, *P citrinum* was showed the lowest degradation rate 25.93%, after 15 days of incubation. Results indicated that these tolerant fungi are promising for potentially effective and environmentally safe of chlorpyrifos biodegradation

Key words: Organophosphorus pesticides, Chlorpyrifos, Fungi, Biodegradation.

The extensive use of pesticides in agriculture has led to their widespread release into environment. Environmental contamination with pesticide residues increasing the accumulation of these pesticides in environment resources and different food chain[1]. Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is one of the most extensively applied organophosphorus pesticides. Chlorpyrifos is a non-systemic insecticide, which is effective against a wide range of insect pests of economically important crops[2]. However, its residues persist in the environment for unpredictable period of time[3,4], and may enter vegetables cultivated on the polluted sites and pose a great threat to the human health[5,6]. Usually, it affects the nervous system of the target insects by inhibiting the activity of acetylcholinesterase by phosphorylation, both at the synapse of neurons and in the plasma[7]. As a result, acetylcholine is accumulated at the neuro-synapse which causes the death of the target insect. Use of organochlorine pesticides heptachlor and lindane have been discontinued due to their extended persistence and possible toxicity toward non-target organisms, and have been substituted by relatively more efficient and less persistent organophosphorous (OP) compounds[8]. The

* To whom all correspondence should be addressed.
E-mail: drsherif_hussein@yahoo.com

traditional physico-chemical treatments for the remediation of pesticide from polluted sites are costly, labor demanding and also disturbing to the environment[9]. Therefore, there is a need of an eco-friendly and efficient technology for remediation of soil contaminated with such hazardous pesticides.

The remediation of chlorpyrifos-contaminated sites to mitigate the hazardous effects of such toxic chemicals is required. A number of methods, including chemical treatment, volatilization, photodecomposition and incineration, can be applied for the detoxification of chlorpyrifos[10-12]. However, most of them are not applicable for diffused contamination at low concentration because they are expensive, inefficient and not always environmental friendly. Biotic degradation is one of the most viable options for the remediation of chlorpyrifos in soil and water. Several researchers have focused on the microbial degradation which has been reported as a primary mechanism of pesticide dissipation from the soil and water environment[13]. In some early studies, chlorpyrifos was reported to be resistant to biodegradation due to accumulation of the antimicrobial degradation products in soil[14, 15]. Later, several studies have revealed that many microorganisms are capable of degrading chlorpyrifos efficiently[16, 17].

MATERIALS AND METHODS

Chemicals

Chlorpyrifos formulation (Dursban 20 EC) used for fortification was obtained from Dow Agro Sciences Ltd., Indiana, USA. The analytical standard of chlorpyrifos (purity 97.5 %) was purchased from Sigma-Aldrich Laborchemikalien GmbH, UK(Fig. 1). A stock solution of chlorpyrifos at a concentration of 10 mg ml^{-1} was freshly prepared in Acetone. All other chemicals used were of analytical grade.

Collection of soil samples

Sixteen soil samples were collected from contaminated fields with chlorpyrifos in Qaliobia governorate- Egypt at the depth of 10 cm from the ground surface layer. Fifty grams were collected for each soil sample in plastic bags and transported at once in cold storage containers to laboratory for further investigation. The soil samples were spiked with chlorpyrifos solution at concentration of 10 mg l^{-1} after adding water to keep the soil moisture at 50 %. Soil samples were incubated at 30 °C ±2 for ten days. After the incubation the mass soil was a little air-dried, thoroughly mixed and then sieved through 0.20 mm mesh sieve for chemical analysis and fungal isolation experiments respectively.

Determination of chlorpyrifos residues in soil

To detect chlorpyrifos residues in soil, 5 g of the contaminated soil in clean 250 ml bottle was extracted with 50 ml of n-hexane: acetone (1:1 v/v) mixture with shaking for 9 hours. Samples were filtrated using Whatman paper No. 1. The organic layer was collected and water was removed by sodium sulphate anhydrous column. Samples were evaporated to get 0.5 ml volume, cleaned-up using SPE cartridge (C18, 200 mg, 3ml) and evaporated again under nitrogen to dryness then stored in the freezer at -20 °C till the chromatographic analysis.

Isolation of Biodegradable fungi

For fungal isolation, serial dilution method described by Hanlin and Ulloa 1979[18], was used. Five grams of chlorpyrifos contaminated soil was added to 45ml of sterile distilled water and shaked for 5 minutes to get stock solution of concentration 10^{-1}. One ml of the stock solution was pipetted into 9 ml of sterile distilled water to make serial dilution of 10^{-2}. Similar method was carried out to give final concentrations of 10^{-3},10^{-4} and 10^{-5}. One ml of dilutions: 10^{-3}, 10^{-4} and 10^{-5} for each soil sample was distributed on the surface of sterile petri-dishes containing Potato dextrose agar (PDA) supplemented with rose Bengal (1/15000) to reduce spread of the fast growing fungi and 0.5 g l^{-1} of each of penicillin G and streptomycin to eliminate bacterial growth. Plates were incubated at 25 °C for 8 days during which cultures were daily examined macroscopically for purification. Subculture was repeated several times to obtain pure culture which were preserved on potato dextrose agar slant till identified.

Identification of isolated fungi.

The isolated fungi were identified using different keys of identification. *Aspergillus* species were identified according to Raper and Fennell 1975 [19], *Fusarium* species were identified according to Nelson *et al.*, 1983[20] while other terrestrial fungi were identified according to Funder 1961[21], Ellis 1971[22] and Barnett and Hunter 1972[23].

Confirmation of chlorpyrifos fungal tolerance

Thirteen dominant fungal strains isolated form chlorpyrifos contaminated soils (Table, 1) were confirmed for their abilities to grow in the presence of chlorpyrifos. Sterile flasks containing 50 ml of potato dextrose broth supplemented with 10 mg/ml of chlorpyrifos were inoculated with 1 ml of fungal spore suspension (10^8 spores ml^{-1}) of the isolated fungal strains. Three replicates were performed for each fungal treatment and the control set was kept without chlorpyrifos. The Inoculated flasks were incubated at (25 °C ±2) for 7days and the growth of fungal strains used for standing the toxicity of chlorpyrifos pesticide was measured and recorded. Five fungal strains confirmed their pesticides-catabolising ability. Mycelial growth of *Aspergillus niger*, *A.oryzae*, *Penicillum citrinum*, *Trichoderma harzianum* and *T. viride* were similar to their control growth and were detected as the most effective chlorpyrifos tolerant fungal strains

Mycodegradation of chlorpyrifos with fungal isolates.

A series of 250 ml Erlenmeyer flasks containing 100 of sterile Burkes mineral broth medium of composition (in gl^{-1}); K_2HPO_4, 0.2 gm; KH_2PO_4, 0.8gm; $MgSO_4$, 0.2gm; $CaCl_2$, 0.1gm; NH_4SO_4, 0.1gm; yeast extract, 0.2gm; glucose, 10gm and Distilled water to make 1 litter at pH 7.2 (23) were amended with 10 ppm of chlorpyrifos. Flasks were inoculated with 1ml of fungal spore suspension (10^8spores ml^{-1}) of the tolerant fungal strains.

Three replicates were performed for each fungal treatment and positive control set was kept without chlorpyrifos while a separate set of un-inoculated flasks was maintained as negative control flasks to assess abiotic losses. Flasks were keptat 27 ± 2°C for a period of 15 days without shaking. Aliquots(5 ml each) of the mineral medium were withdrawn by micropipette after 0, 3, 6, 9, 12 and 15 days of incubation and subjected to chromatographic analysis. After incubation, content of each flask was filtered (Whatman No.1) and biomass of the filtered mycelium was determined after drying at 70°C for 4 days till their weights remains constant.

Chromatographic analysis

Agilent 6890 (USA) gas chromatography-coupled with nitrogen phosphorus detector (GC-NPD) was used. Separation was performed using capillary column HP-5 (30m × 0.25mm × 0.25µm). Nitrogen was used as the carrier gas at a flow rate 2ml/min. The following temperature program was employed:initial temperature of 180 °C held for 1 min; increased at 25 °C min^{-1} to 220 °C, held for 2 minutes; yet another increase at 3 °C min^{-1} to reach 245 °C. The injector temperature was 220 °C and the injection volume was 1 µl for all standard and samples. Data analysis was performed using Chemistation software. Calibration curves were generated by plotting peak area versus concentration. Standard calibration curves were presented excellent linearity with regression coefficient r > 0.995 with good separation and repeatability for the chlorpyrifos pesticides. The calibration curve and recovery validation study were all repeated three times (n = 3).

Statistical analysis

All measurements were repeated three times for each treatment and the data were reported as mean ± SE (Standard error). The data were also statistically evaluated by one-way analysis of variance (ANOVA) and the differences among means were carried out by using the least significant differences at $p \leq 0.05$. All statistical analyses were done using theStatistical Package for social sciences (SPSS, 16.0) program.

RESULTS AND DISCUSSION

Detection of chlorpyrifos residues in soil.

The soil samples collected from sixteen provinces of Qaliobia governorate were analyzed to detect their chlorpyrifos contamination. The residues of herbicide were evaluated by GC and tabulated in Table 1.

It was proved that chlorpyrifos was detected in 13 soil samples and concentration of the herbicide was varied among the governorate. Soil of Toukh, Banha, Ash Shimut and Qaha were the highly chlorpyrifos contaminated soil recording (0.31, 0.26, 0.18 and 0.17) µg/g of soil while chlorpyrifos was not detected in Nawa, IkyadDijwi and Al-Qulzum respectively.

Isolation of chlorpyrifos tolerant fungi

Isolation of tolerant fungi

Results in Table 2 showed the dominant fungal strains isolated form chlorpyrifos contaminated soils. Mycelial growth of the isolated

fungi reflected tolerance variation of chlorpyrifos as most of them were inhibited with different levels except five fungal strains. These fungal strains can stand the toxicity of chlorpyrifos pesticide at 10 ppm and their mycelial growth was not affected. Mycelial growth of *Aspergillus niger*, *A. oryzae*, *Penicillum citrinum*, *Trichoderma harzianum* and *T. viride* were similar to their control growth and were chosen as the most effective chlorpyrifos tolerant fungal strains

Biodegradation of chlorpyrifos in liquid medium

In the present study, chlorpyrifos was used at concentration of 10 ppm (Initial concentration) without inhibitory effects to tolerant fungi (Table, 1). In normal agriculture concentration chlorpyrifos did not exhibit measurable effect on fungal soil populations[24]. The culture of *Aspergillus niger* could tolerate 400 ppm of technical grade of endosulfan[25]. On the other hand,

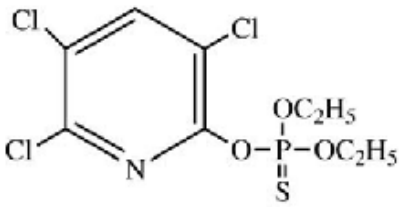

Fig 1. Chemical structure of chlorpyrifos

Table 1. Chlorpyrifos contamination of soil samples collected from different locations in Qaliobia governorate- Egypt

Serial	Locations	Chlorpyrifos residues in soil (µg/g)
1	Shalaqan	0.07
2	Nawa	0.13
3	Tanan	ND
4	Qaha	0.17
5	Kafr El-Shorafa	0.11
6	Alikhsas	0.08
7	Sintiris	0.01
8	Kafr Ad Dayer	0.02
9	Namul	0.13
10	IkyadDijwi	ND
11	Al-Qulzum	ND
12	Toukh	0.31
13	Biltan	ND
14	IzbatAkif	0.05
15	Ash shimut	0.18
16	Banha	0.26
Mean		0.13

complete degradation of DDT at concentrations up to 15 ppm in flasks, with shaking, had been achieved but inhibitory effects were observed at 50 ppm[26].

Data in Table 3, illustrated that the recovered amount of chlorpyrifos significantly declined from the initial concentration with increasing the incubation period in the media amended with all the tolerant fungal strains, while media without any amendment (i.e., un-inoculated control) showed less dissipation of chlorpyrifos. About 4.43–14.4% of chlorpyrifos degradation was observed after 3 days of incubation which increased to 9.8–28.45%, 15.18–42.51%, 20.56–56.56% and 25.93–70.61% after 6, 9, 12 and 15 days of incubation with the biodegradable fungi respectively. *Trichoderma viride* was the most effective biodegradable fungi recording a highest dissipation of chlorpyrifos (70.61%) while *Penicillium citrinum* was the lowest degradable one recording (25.93%) dissipation after 15 days of incubation respectively. On the other hand, *Aspergillus niger*, T. *harzianum* followed by *A. oryzae* were moderate in their biodegradable activities reducing the chlorpyrifos to 63.96, 60.31, and 50.79% after 15 days of incubation respectively.

Also, the recovered amount of chlorpyrifos significantly declined from the initial concentration with increasing the incubation period in the media amended with the biodegradable fungi, while media without any amendment (i.e., un-inoculated control) showed less dissipation of chlorpyrifos (Table 3). There were considerable variations between the herbicide tolerant fungi with respect to their abilities to degrade chlorpyrifos during the periods of 3–15 days of incubation. At the end of experiment the maximum removal of chlorpyrifos was observed with *T.viride* (70.61%) followed by *A. niger*(63.96%).

However, the half-life values $t_{1/2}$ values for chlorpyrifos were 10.82, 13.34 and 13.36 days in culture amended with *T. viride*, *T. harzianum* and *A. niger* while they were 18.25 and 38.59 days in media amended with *A. oryzae* and *P. citrinum* respectively.

These findings are in accordance with many investigators who reported that the kinetics of pesticides degradation in soil is commonly

Table 2. Screening of tolerant fungi isolated from chlorpyrifos contaminated soils

Fungal isolates	Mycelial dry weight (mg/50ml)	
	Control (PDB without cholorpyifos)	Treatment (PDB + 10 ppm cholorpyifos)
Alternaria alternate	438 ± 1.1	195 ± 0.6
A. flavus	311 ± 0.9	136 ± 1.7
A. niger	397 ± 1.3	401 ± 0.9
A. oryzae	341 ± 0.7	339 ± 1.3
A. terreus	378 ± 0.5	89 ± 1.1
F. oxysporium	359 ± 0.6	78 ± 1.9
F. solani	253 ± 0.9	104 ± 1.5
Mucorracemosus	309 ± 1.7	48 ± 0.9
Penicillium citrinum	289 ± 2.2	293 ± 2.3
P. ultimum	171 ± 1.5	65 ± 0.8
Rhizopus nigricans	195 ± 0.8	71 ± 0.3
Trichoderma harzianum	247 ± 0.7	243 ± 1.1
T. viride	295 ± 0.9	289 ± 1.3

Table 3. Biodegradation kinetics of chlorpyrifos in liquid medium with tolerant fungal isolates.

Time (days)	Control	*Trichoderma harzianum* Loss %	*T. viride* Loss %	*Penicillium-citrinum* Loss%	*Aspergillus niger* Loss %	*Aspergillus oryzae* Loss %
0	0.00	0.00	0.00	0.00	0.00	0.00
3	4.50	12.71	14.40	4.43	10.26	7.81
6	6.68	24.61	28.45	9.80	23.68	18.56
9	14.37	36.51	42.51	15.18	37.11	29.30
12	21.03	48.41	56.56	20.56	50.53	40.05
15	24.54	60.31	70.61	25.93	63.96	50.79
$T_{1/2}$	41.96	13.34	10.82	38.59	13.36	18.25

biphasic with a very rapid degradation rate at the beginning followed by a very slow prolonged dissipation[27, 28]. The relative importance of the phases depends on the availability of the pollutants, hydrophobicity and affinity for organic matter.

Chlorpyrifos was found to be more easily degradable than DDT and other organochlorine insecticides[29] showing the lowest accumulation hazard. Indeed, the trichloromethyl group of chlorpyrifos is extraordinary susceptible to carbon–carbon bond cleavage to form 4,4-0-dichlorobenzophenone[29-31]. The biodegradation of chlorpyrifos yielded nearly equal chloride (3.3lg from 20lg) than in the case of methoxychlor (2lg from 20lg) when calculated on total substituted chloride[29]. Also, the pathway between chlorpyrifos and 4,4-0-dichlorobenzophenone in fungus *Phanerochaete chrysosporium* might proceed in a manner similar to that observed in biodegradable fungi, in which trichloromethyl carbon undergoes successive reductive dechlorinations followed by oxidation to form the carboxylic acid which then undergoes decarboxylation to formdichlorobenzophenone[30]. In our experiment dichlorobenzophenone could not be detected using the HPLC during fungal biodegradation of chlorpyrifos. Also, the absence of chlorpyrifos metabolites may suggest that these tolerant fungal strains may degrade chlorpyrifos by another pathway different from previous reports[29-31]. The lack of chlorpyrifos metabolites may support its complete degradation. Complete disappearance of endosulfan was seen onday 12 of incubation with

A. niger with evolution of CO_2 and change in pH of medium to acid side indicating microbial transformation of endosulfan[25], while removal of chlorpyrifos by *T. viride*, *T. harzianum* and *A. niger* was lower than that of endosulfan[26]. In the present study, chlorpyrifos could biodegraded by *T. viride*, *T. harzianum* and *A. niger* while it was found to bioaccumulate in the *P. citrinum*[24] resulting in enhanced persistence of chlorpyrifos. In general organochlorine pesticides possess halogen electron with drawing groups which make these compounds resist aerobic degradation[32]. However, in some cases the degradation of other organochlorine pesticides such as Lindane has also been successfully conducted under aerobic conditions by white-rot fungi[33].

CONCLUSION

Degradation of chlorpyrifos proceeded rapidly in pure media amended with *T. viride*, *T. harzianum* and *A. niger* with $t_{1/2}$values of 10.82, 13.34 and 13.36 days compared with 41.96 days in case of control medium. For the remaining isolates like *A. oryzae* and *P. citrinum*, the $t_{1/2}$ values were 18.25 and 38.59 days respectively. Variations in rate of chlorpyrifos degradation by biodegradable fungi may be attributed to the adaptation of the tested microorganisms to degrade chlorpyrifos and to highly activity of their enzymatic systems. By the end of the experiment, *T.viride* degraded roughly 70.6% of chlorpyrifos, while *A. niger*, *T. harzianum* and *A. oryzae* degraded 63.96 – 50.79%. On contrary, *P. citrinum* was less degrader of DCF, with 25.93% of the compound degraded. During the incubation of chlorpyrifos with these microorganisms, no known intermediate or dead-end product could be detected using GC-NPD. These results demonstrate that tolerant fungal strains can reduce the persistence of chlorpyrifos in liquid media culture. The results have implications for the development of a bioremediation strategy by mixing fungal-inoculated substrates with the contaminated soil.

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