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In situ Bioremediation of Chlorpyrifos in Cotton Fields: Possible Role of Plant-Microbe Interaction

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Indiscriminate use of oganophosphate pesticides has made their mitigation from soil essential. Among the pesticides, chlorpyrifos is a widely used broad-spectrum organophosphate effective in controlling a variety of insects. In the present study, *Pseudomonas fluorescence and Pseudomonas aeruginosa* were inoculated in the fields with cotton plants to determine the role of the plant-microbe interaction in the degradation of chlorpyrifos in the contaminated plots. After 25 days of inoculation, *P. fluorescence* and *P. aeruginosa* degraded 78 and 85% of chlorpyrifos in plots without cotton plants whereas 99% degradation of chlorpyrifos was observed in soil, where cotton plants were inoculated with either *Pseudomonas fluorescence* or *Pseudomonas aeruginosa* as compared to un-inoculated control soil. REP-PCR showed an increase in population of *P. fluorescence* from 1.52 X 10^7 at the time of inoculation to 2.0 X 10^7 in plots without crops whereas 3.1 X 10^7 in plots with cotton plants on day 25. Similar trend was shown by the *P. aeruginosa*. Product formation indicated the appearance of 3, 5, 6-trichloro-2-pyridinol (TCP), the major metabolite of chlorpyrifos degradation, in the plots inoculated with *P. aeruginosa*, which disappeared to negligible amounts.

Key words: Plant-microbe interaction, chlorpyrifos degradation, REP-PCR, *P. aeruginosa* and TCP.

Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate) is an organophosphorus insecticide that is widely used for pest control in agriculture and, to a lesser degree, for indoor use and soil applications to control termites[1] . Chlorpyrifos is one of the most widely used pesticides in the US and accounts for 11% of total pesticide use[2]. Its environmental fate has been studied extensively, and the reported half-life in soil varies from 10 to 120 days[3], with 3, 5, 6-trichloro-2-pyridinol (TCP) as the major degradation product. This large variation in half-life has been attributed to variation in factors such as pH, temperature, moisture content, organic carbon content, and pesticide formulation[4]. The persistence of chlorpyrifos leads to bio-

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accumulation in the ecosystem Including blue-green algae[5,6,7], aquatic plants, and goldfish[8]. On the other hand, plants growing in soils that have been contaminated by pesticides may be able to biodegrade the pesticide residues by means of their rhizosphere bacterial influence. In our previous study, we have isolated bacterial cultures which were able to degrade chlorpyrifos both in basal medium and in soil[1, 9]. The main objective of this study was to investigate the degradation of chlorpyrifos by the selected bacteria when introduced in the cotton fields and the survival of the bacteria with REP-PCR technique.

MATERIAL AND METHODS

Bacterial cultures *Pseudomonas fluorescence and Pseudomonas aeruginosa* capable of degrading chlorpyrifos were isolated from a pesticide contaminated soil[1]. Chlorpyrifos was purchased from Rallis India (20% active component). All other chemicals and solvents were of analytical grade.

In-situ degradation of chlorpyrifos in plots

1.3-acre land was divided into small plots of 9m X 6m. These plots were sprayed with chlorpyrifos to a concentration of 50mg/Kg. Soil moisture was maintained at 30% approximately, throughout the experiments. The plots were tilled, racked and maintained properly for a few days before sowing. Randomized block design was used for this study. Bacterial isolates *Pseudomonas fluorescence and Pseudomonas aeruginosa* were grown on a mass scale, pelleted, re-suspended in sterile distilled water. American cottonseeds (Mahyco seeds, Maharashtra) were inoculated with *Pseudomonas fluorescence and Pseudomonas aeruginosa* in jaggery for adhesion and sowed in plots at a depth of 2.5cm and with a distance of 0.5m by length and 1m by width between each row. There were three treatments: plots contaminated with chlorpyrifos were sown with cotton plants and inoculated with *Pseudomonas fluorescence and Pseudomonas aeruginosa; Pseudomonas fluorescence and Pseudomonas aeruginosa* were inoculated in plots without cotton plants and plots were kept without both cotton plants and bacterial cultures, which served as control.

Cultures were enumerated on LA + Kan (50 µg/ml) plates, so that approx. 2×10^8 cells/ gm soil was inoculated into these plots. Samples of soil and plants were withdrawn at the time of sowing and after regular time intervals from each plot. Soil samples were analyzed for residual chlorpyrifos and population survival was estimated by antibiotic plating.

Extraction of chlorpyrifos absorbed by plants

Plant samples were air-dried, then crushed in pestle mortal. The powdered plant samples (10gm) were left overnight in acetone (100ml), re-suspended in 5% (w/v) brine solution (500ml) so as to dilute aqueous phase and partitioning was done using 75ml dichloromethane and 75ml hexane in a separating funnel (1000ml). This extraction process was repeated twice as described above. Organic phase was evaporated to 5ml and mixed with silica gel (20gms), activated charcoal (5gms) and anhydrous sodium sulfate (20gms) in a beaker and wet packing of this mix was done in a column with dichloromethane (DCM).and drop-wise elution was again carried out, followed by elution with DCM/Hexane (1:1). The samples were dried by rotary flash evaporation, dissolved in 5ml acetone and samples were analyzed by GC as described elsewhere[1]. A column check with standard chlorpyrifos (50µg) was also done to know the efficiency of extraction of chlorpyrifos by this method.

Extraction of chlorpyrifos residues from soil

Chlorpyrifos was extracted from sample soil using Hexane-Acetone (9:1). For extraction of residual chlorpyrifos, the soil sample was distributed into conical flasks (5gm in each) and 10ml solvent was added. The flasks were incubated at 37°C, 130 rpm in a rotary shaker for two hours; the organic phase was decanted into a glass crucible using pasteur pipettes (2ml). The sample was re-extracted with additional solvent (total volume 25ml), the solvent was evaporated, using rotary flash evaporation, under an exhaust fume chamber. Samples were re-dissolved in acetone (1ml) and quantified by GC as done earlier.

Extraction of Genomic DNA for molecular fingerprinting

Genomic DNA of the isolates from soil were extracted by suspending bacterial colonies in sterile distilled water (100 µl) followed by PCR

Pseudomonas aeruginosa was always better in the fields with cotton plants as compared to the plots without cotton plants, which is in agreement with the enhanced degradation of chlorpyrifos in soils with cotton plants due to the plant-microbe interaction. No residual chlorpyrifos was found in any of the sampled plants. No chlorpyrifos was detected on the 60th day soil samples from plots with crops inoculated with the cultures.

Metabolite analysis during *in situ* degradation of chlorpyrifos in plots with and without cotton plants inoculated with

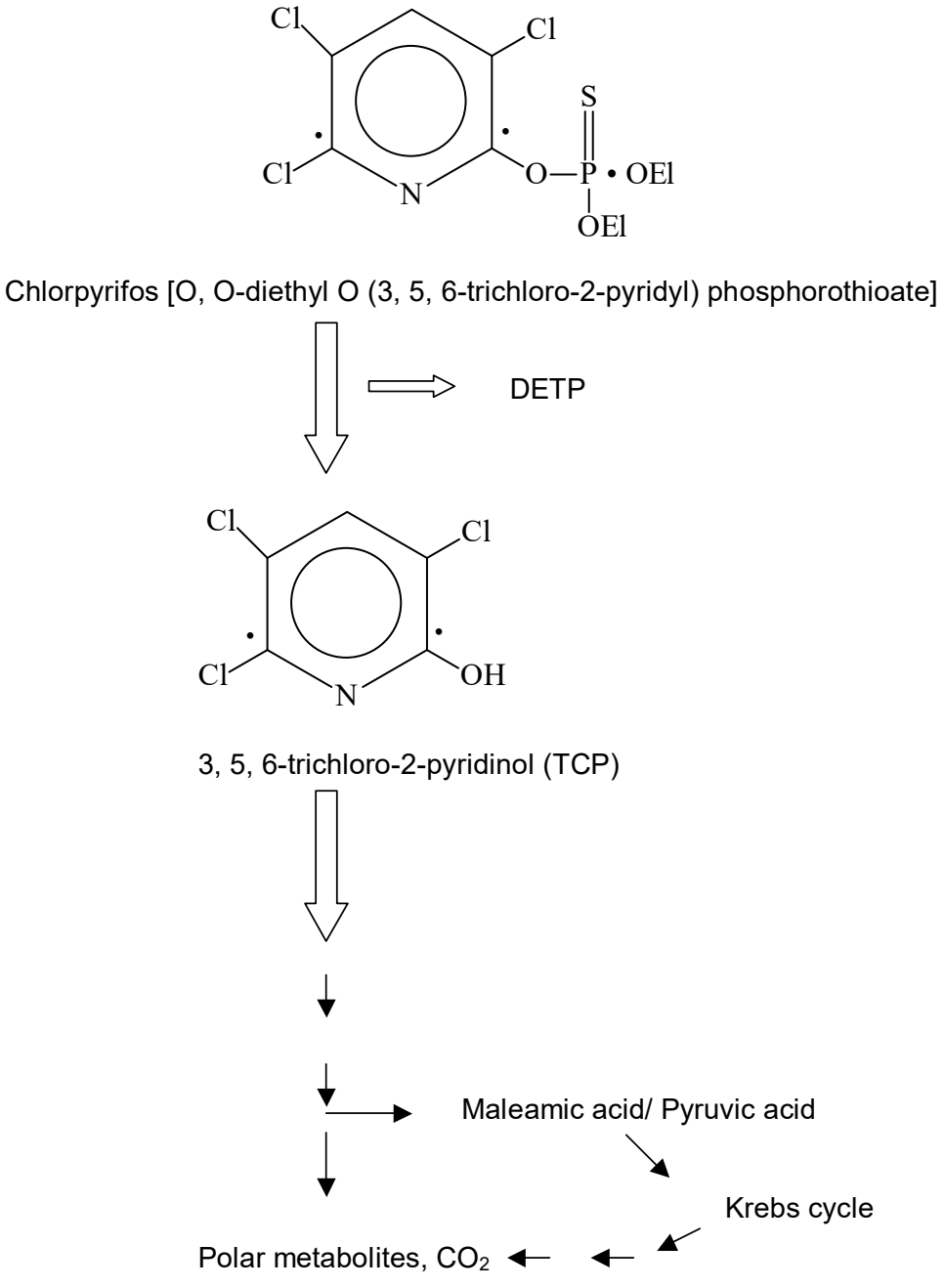

Fig. 3. Proposed metabolic pathway of chlorpyrifos degradation by *Pseudomonas aeruginosa*

Table 1. Survival of *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* based on REP-PCR in Plots

Genomic DNA of *Pseudomonas fluorescence* was extracted along with genomic DNA of bacterial strains isolated during different time intervals of *In situ* degradation of chlorpyrifos in plots and amplified by touch down PCR with REP primers and the PCR products (10 µl) were analyzed on agarose gel.

	P. fluorescence		***P. aeruginosa***	
Days	Without Cotton plants	With Cotton plants	Without Cotton plants	With Cotton plants
0	1.5×10^7	1.6×10^7	1.6×10^7	1.6×10^7
10	2.8×10^7	3.0×10^7	3.1×10^7	3.4×10^7
20	1.5×10^7	2.6×10^7	3.3×10^7	4.6×10^7
30	2.0×10^7	2.1×10^7	2.6×10^7	3.6×10^7
45	1.7×10^6	2.2×10^6	7.7×10^6	8.0×10^6
60	2.8×10^5	4.5×10^6	6.8×10^5	2.0×10^6

Pseudomonas aeruginosa indicated the formation of TCP (2.5mg/L) after 25 days which disappeared to negligible amounts at the end of the study. However, TCP was not detected in the plots inoculated with *Pseudomonas fluorescence*. Based on the soil studies and the whole cell uptake studies and literature review, a metabolic pathway of chlorpyrifos degradation by *Pseudomonas aeruginosa* has been proposed (Fig 3). The parent compound chlorpyrifos was degraded to TCP which is further mineralized to polar products which were not recovered in the present study.

DISCUSSION

Many researchers have studied the role of inoculated microorganisms into soil in the degradation of chlorpyrifos by fumigating the soil[12-15]. The main objective of *in situ* bioremediation trial in plots with plants was to study the effect of plant-microbe interaction on enhanced degradation of chlorpyrifos. Cotton plants were chosen because of predominant usage of chlorpyrifos on cotton fields in major agricultural areas around Punjab where the studies were carried out. In plants there have been reports of delayed seedling emergence, fruit deformities and abnormal cell division due to prolonged exposure to chlorpyrifos[16]. However none of these effects were visually observed in our studies. In fact, all the chlorpyrifos sprayed fields showed better growth than the one without chlorpyrifos. When the crops were analyzed for chlorpyrifos, no chlorpyrifos was found in any of the samples at any stage indicating that no uptake of chlorpyrifos by the plant occurred at any stage. Yang *et al*, 2005 treated cabbage growing soils with their strain DSP3 which then showed enhanced degradation of chlorpyrifos, though they did not study whether there was any uptake of chlorpyrifos by the cabbage plants. Studies on the absorption, translocation and metabolism of ^{14}C and ^{36}Cl labeled chlorpyrifos in plants had shown that for all practical purposes uptake of the chemical or its degradation products into plants does not occur, either through the foliage or through the roots and nutrient culture experiments have shown uptake of chlorpyrifos by plant roots to be essentially nil[18,19]. Many researchers have reported disappearance of chlorpyrifos in soils with Bermuda grass and maize plants[20, 21]. There has been no evidence of residue build up on plants from repeated application of chlorpyrifos, which is all in total agreement with our studies.

DNA fingerprints based on REP-PCR of isolates were successfully developed to study the population dynamics of the introduced population for the degradation of chlorpyrifos. For this DNA of the native isolates and the isolates from soil were subjected to REP-PCR and the original DNA fingerprints of the isolates were compared with the fingerprints of microorganisms isolated from the chlorpyrifos contaminated soil to score the survival of the population. Molecular fingerprinting profiles indicated that the degradation of chlorpyrifos was in concurrence with the proliferation of the introduced cultures. Contrary to the Racke *et al*, 1990 report that there was no evidence for proliferation of

microorganisms in soils or in pure cultures of bacteria using chlorpyrifos[22], our isolates showed an increase in population in the initial days after inoculation concomitant with chlorpyrifos degradation. The extent of proliferation of our population is similar to that of the bacterial isolates in the studies of Mallick *et al*, 1999 but their studies were carried out in liquid media. Proliferation also occurred for the chlorpyrifos degrading organisms in the UK soils[14]. Even though only 10^6 cells/ gm soil was required for degradation of chlorpyrifos in the studies by Singh *et al* (2004), these were studied in fumigated soil under laboratory conditions with only 35mg/kg chlorpyrifos, while our experiments were conducted under natural environmental conditions with 50 mg/Kg chlorpyrifos and the inoculated microorganisms had to compete with inherent microorganisms of soil and hence, the difference in inoculum needed for effective degradation of chlorpyrifos. Even in the studies by Yang, 2005, addition of 10^8 cells/ gm soil *Alcaligenes faecalis* was needed for effective bioremediation of 100 mg/kg chlorpyrifos contaminated soil.

CONCLUSION

Degradation of chlorpyrifos was more in the cotton plots which were inoculated with *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* thus showing the important role of plant-microbial interaction in the dissipation of the pesticide chlorpyrifos. Biodegradation of chlorpyrifos by the introduced population of *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* was concomitant with the proliferation of these isolates in the cotton fields.

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