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Degradation of chemically related organochlorine pesticides (γ - hexachlorocyclohexane and vinclozolin) in rice soil, pre-exposed to each other

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A B S T R A C T

Accelerated degradation of soil-applied pesticides, upon their repeated application, is the result of proliferation of microorganism degrading candidate pesticide and can undermine the efficacy of the pesticide under consideration. In the present study, experiments were conducted both in green house and in laboratory conditions to examine the development of enhanced degradation of vinclozolin in mineral salts medium inoculated with soil suspension from unplanted and planted flooded alluvial soils untreated or pre-treated with commercial HCH and vice versa. Results demonstrated that 15 days after fourth application, approximately 97% of vinclozolin was degraded in the suspension from planted pots. The development of enhanced biodegradation of γ-HCH was examined in a mineral salts medium inoculated with soil suspensions from unplanted or planted flooded alluvial soils untreated or pre-treated with commercial vinclozolin. Fifteen days after third application, only a trace of γ-HCH was recovered from the soil suspension from planted pots. The rice plants played a definite and important role in influencing the development of enhanced degradation of both the pesticides.

1. Introduction

Organochlorine pesticides like DDT, HCH, aldrin, vinclozolin, heptachlor and endosulphan, extensively used for control of agricultural pests and vector born diseases, are of much concern because of their prolonged persistence, lipophilic nature and tendency to accumulate in animal and plant tissues. The use of these pesticides have been banned or restricted in several countries including India. However, neither the ban nor the restricted use has reduced the levels of residues of these compounds in the soil environment (Simonich *et al*., 1995; Cerkvenik *et al*., 2000; Noren *et al*., 2000; Akbar *et al*., 2003). This is especially true for hexachlorocyclohexane (HCH), a highly recalcitrant pesticide, extesively used for control of agricultural pests over the past five decades (Deo and Karanth, 1994), and also for vonclozolin (Vanni *et al*., 2000; Flynn *et al*., 2001; Sandra *et al*., 2001).

The spontaneous or induced microbial degradation is one of the possibilities for decontamination of these highly persistent organochlorine residues. Unfortunately, spontaneous microbial degradation proceeds rather slowly (Johri *et al*.,1996; Lal and Saxena, 1982). Thus addition of naturally occuring microbes to contaminated soils as an alternate strategy, can be assisted to a great extent by exploring the potentials of such isolates.

Manonmani *et al*. (2000) reported that amongst the auxillary carbon-sources, ethanol, benzoate and glucose (at higher concentrations) retarded γ-HCH degradation whereas addition of cellulose, saw dust and low concentration of glucose (< 200 mg/ml) and acetone enhanced the rate of degradation. Enhanced microbial degradation occurred readily in a soil treated with analytical grade carbofuran, chlorpropham, cloethocarb diazinon and fensulfothion (Chapman and Harris, 1990).

The dicarboxymide fungicide vinclozolin [(RS)-(3.5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] is used to control disease caused by *Botrytis* sp., *Alternaria*

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sp., *Sclerotinia* sp. and *Monilia* sp. of vegetables and other field crops (Spencer, 1982). The fungicide undergoes hydrolysis and produces several degradation products including 3,5-dichloroaniline (Szeto *et al*., 1989a; Golovleva *et al*., 1991). Golovleva *et al*. (1991) showed that microbial strains utilizing vinclozolin as the sole source of carbon and energy belonged to genera *Pseudomonas* and *Bacillus*. Frederick *et al*. (1994) reported the degradation of vinclozolin in soil, thatch and grass clippings. Pothuluri *et al*. (2000) reported the biotransformation of vinclozolin by the fungus *Cunninghamella elegans*. They reported 93% of transformation of vinclozolin to four major metabolites after 96h incubation.

About 50% of the fungicide is reported to be degraded in aerobic soils within 23 days and the degradation is rather slow in acidic soil (Walker *et al*., 1986). Many a reports regarding the enhanced degradation of fungicide vinclozolin (Walker *et al*., 1986, 1987; Milhomme, 1989; Cork and Krueger, 1991; Golovleva *et al*., 1991; Vega-Palas *et al*., 1992; Cain *et al*., 1996; Mercadear *et al*., 1998) showed the involvement of microorganisms in its degradation. Pirsisi *et al*. (1986) reported the isolation and identification of the major breakdown products of chlozonilate vinclozolin. Cain *et al*. (1996) showed the rapid onset of the accelerated degradation of dicarboximide fungicides including viclozolin in a U.K. soil; with its long history of agrochemical exclusions. Szeto *et al*. (1989b) analysed the kinetics of the hydrolysis of the Dicarboximide fungicide vinclozolin. This report presents the efficiency of degradation of γ-HCH and vinclozolin in rice soil on pre-exposure to each other.

2. Materials and Methods

2.1. Soil

A deltaic alluvial soil from the experimental farm of Central Rice Research Institute, Cuttack, Orissa, was used for the present study. The soil was air-dried in shade and after breaking the clods, sieved through a < 2mm mesh and stored in polyethylene bags at room temperature. The physico-chemical properties of the soil (Table 1) were determined by the following methods (Jackson, 1973). Soil pH was measured by 1:1.25 soils to water ratio using a digital pH meter with Calomel glass electrode assembly. Organic carbon content of the soils was determined and the organic matter was calculated by multiplying the organic carbon values with 1.72. Total nitrogen content of the soils was estimated by Kjeldahl method. The cation exchange capacity (CEC) of soils was determined by 1 N ammonium acetate (pH 7.0) by summation of exchangeable Na, K, Ca, Mg and H. Physical analysis for clay, silt and sand fractions was measured by employing the Bouyoucos hydrometer method (Black, 1965).

2.2. Pesticides

Technical formulation of hexachlorocyclohexane (HCH) (99.1% purity) used in this study were obtained from M/s Lachat chemicals, Mequon, Wisconsin, U.S.A. Commercial formulation of HCH containing 50% active ingredient (γ-HCH) was obtained from M/s Das Enterprise, Calcutta, India. Both commercial formulation of the fungicide vinclozolin (Ronilan 50% w.p.) and analytical grade vinclozolin (99% a.i.) were obtained from M/s BASF, Germany.

2.3. Greenhouse and laboratory experiment

Earthenware pots (25.5 x 9.5 cm dia.) were separately filled with 5 kg of alluvial soils. 30-d old rice seedlings (*cv.* IR-72) were transplanted with 4 hills of one plant each per pot. Phosphorus as single super phosphate (SSP) and potassium as muriate of potash (MOP) at 20 mg/kg soil each were applied to soils of all the pots as basal dressing. Nitrogen (60 mg N/kg soil) was applied as urea to all the pots in three splits with 50% at basal and 25% each at maximum tillering and panicle initiation stages of the crop. There were three uniform replicated pots of each soil for each treatment (HCH-treated or untreated) and amended with commercial formulation of HCH or vinclozolin at 10 mg/g.

First and second application of HCH was made at 10 d and 15 d, respectively after flooding. Fifteen days after each application (before the next application was made) of HCH, duplicate 1g surface soil (1-2 cm) samples collected from HCH treated and untreated pots, were separately shaken with 10ml sterile distilled water in pre-sterilized test tubes (200 x 25 mm) to prepare soil suspensions for use as inoculum for the γ-HCH degradation studies.

Table 1
Physico-chemical characteristics of the soil used in the study

Soil type	Taxonomic group	pH	Organic matter (g/kg)	Total Nitrogen (g/kg)	SO_4 (mg/g)	EC (dS/m)	CEC (cMoles/ 100g soil)	Soil Separates (g/kg)		
								clay	silt	sand
Alluvial	Aeric endoaquept	6.16	8.2	0.9	10.2	0.51	95.5	25.9	21.6	52.5

2.3.1.1. Laboratory methods

A mineral salts (MS) medium [$(NH_4)_2HPO_4$, 0.5 g; $MgSO_4.7H_2O$, 2 g; $FeSO_4.7H_2O$, 0.001 g; K_2HPO_4, 0.1 g; $Ca(NO_3)_2$, 0.01 g and distilled water 1 litre, pH 7.0] was shaken with analytical grade of γ-HCH for 48 hr on a mechanical stirrer and subsequently sterilized by filtration through a Millipore® filter (0.3 mm). 10ml portions of this medium were aseptically dispensed in sterile 100ml Erlenmeyer flasks and later inoculated with 1ml of soil suspension (flooded) from untreated or HCH-treated pots. The un-inoculated medium served as control. The samples were incubated under intermittent shaking (for 4 hr after every 4 hr interval) to provide aerobic conditions. At periodic intervals, 1 ml portion of the inoculated and un-inoculated medium were withdrawn aseptically from duplicate flasks and shaken with 2-4 ml of hexane for 2-3 min for extraction of HCH residues. HCH residues in hexane fraction were analyzed by GLC (Varian, 3400) equipped with a ^{63}Ni detector and a metal column (2m length, 1/8" OD). Column, injector and detector were maintained at 220, 240 and 240^{0}C, respectively with a flow rate of the carrier gas (95% argon in 5% methane) at 20 ml/min.

Accelerated degradation of vinclozolin was studied under greenhouse conditions as described by Bharati *et al*. (1998). The first and second application of vinclozolin was made to the soil in the pots at 10 d and 25 d, respectively after moistening/flooding (unplanted pots) or transplantation (planted pots). Subsequent applications were made at 40 d (50-d after moistening/flooding) and 55 days (65-d after moistening/flooding). At regular intervals, soil samples from pots treated as above were collected as described by Panda *et al.* (1988) and tested for vinclozolin degradation.

2.4. Degradation of γ-HCH or vinclozolin through cross-inoculation

Accelerated degradation of γ-HCH or vinclozolin was measured in soils enriched with commercial formulation of HCH or vinclozolin respectively at concentration mentioned above. Soils were pre-enriched with either HCH or vinclozolin in unplanted or planted pots as described. Flask containing MS medium supplemented with γ-HCH/vinclozolin and inoculated with soil suspension pre-enriched with γ-HCH/ vinclozolin served as positive control for γ-HCH/ vinclozolin degradation.

3. Results and discussion

In most of the experimental studies on the development of xenobiotic-degrading microbial enrichment cultures, the target ecosystem is exposed to repeated application of the candidate insecticide. However, enhanced degradation of many of the pesticides has often been reported wherein the enrichment was developed through the exposure of chemically related pesticide (Racke and Coats, 1990). Both γ-HCH and vinclozolin are being organochlorine compounds, it was thought worthwhile whether any enrichment of vinclozolin-degrading microorganisms developed upon the exposure to γ-HCH or vice versa.

3.1. Degradation of vinclozolin in mineral salts medium inoculated with suspension of soil pretreated with commercial HCH

Accelerated degradation of soil-applied pesticides, upon their repeated application, is the result of proliferation of microorganism degrading candidate pesticide and can undermine the efficacy of the pesticide under consideration (Sethunathan, 1971; Felsot, 1989; Racke, 1990). From the earlier reports (Bhuyan *et al*., 1992, 1993; Sahoo *et al*., 1990) and from the results of present study, it has been established that γ-HCH is degraded faster upon its repeated application to the flooded soil planted to rice. Besides, accelerated degradation of vinclozolin occurred upon repeated application of the fungicide especially under flooded condition. In the present experiment attempts were made to examine the development of enhanced degradation of vinclozolin in mineral salts medium inoculated with soil suspension from unplanted and planted flooded alluvial soils untreated or pre-treated with commercial HCH. The results showed no accelerated degradation after first application of HCH and approximately 60% of vinclozolin was degraded in planted flooded soil after second application. After third application of HCH, vinclozolin degradation was comparatively faster, especially in flooded soils planted to rice. 15 days after fourth application, approximately 97% of vinclozolin was degraded in the suspension from planted pots (Table 2). During the same period, there was some loss of vinclozolin from un-inoculated control and from the medium inoculated with suspensions from untreated soils, possibly due to volatilization/chemical degradation.

3.2. Degradation of γ-HCH in mineral salts medium inoculated with suspension of soil pre-treated with vinclozolin

In a follow up experiment, the development of enhanced biodegradation of γ-HCH was examined in a mineral salts medium inoculated with soil suspensions from unplanted or planted flooded alluvial soils untreated or pre-treated with commercial vinclozolin. In this case, no accelerated degradation of γ-HCH was observed after first and even second application of fungicide to the soil. Comparatively, degradation of γ-HCH was faster after third application of vinclozolin to the soil. Fifteen days after third application, only a trace of γ-HCH was recovered from the soil suspension from planted pots. Enhanced degradation of γ-HCH became more pronounced after fourth application. Five days after fourth application, 91% of γ-HCH was degraded and the entire amount of γ-HCH was degraded within 10 days (Table 3).

Interestingly, soil planted to rice and maintained under flooded condition exhibited clear-cut enhancement of γ-HCH degradation in the present experiment. It was observed that rice plants play a definite and important role in influencing the development of enhanced degradation of γ-HCH. It is known that flooded soil planted to rice is characterised by an interactive aerobic-anaerobic interface that is dynamic in nature. Such aerobic-anaerobic interface seems to trigger the development of HCH degrading factor in low land rice field upon repeated addition of HCH (Bhuyan *et al*., 1990;1992). As flooded soil planted to rice is microbially more active (Yoshida, 1975), high microbial activity in the flooded soil planted to rice may enhance γ-HCH and/or vinclozolin degradation in these conditions.

Barraga´n-Huerta *et al.* (2007) reported the biodegradation of organochlorine pesticides by bacteria grown in micro-niches of the porous structure of green bean coffee and concluded that defective green bean coffee can be used as both a nutrient source and a support for organochlorine pesticide degrading bacteria in liquid media. Baczynski *et al.* (2010) reported that anaerobic biodegradation of organochlorine pesticides in contaminated soil is significantly influence by the temperature and availability of the pesticide in the soil.

Many reports indicate the inhibitory effect of organochlorine pesticides on their own degradation and their toxic effects on the soil and the microbes responsible for their degradation (Ghadiri *et* al., 1995; Nawab *et al*., 2003; Reddy *et al*., 2012). It has been reported that certain pesticides have inhibitory effects on bacterial growth (Nawab *et al*., 2003). Report suggest that application of endosulfan to a clay soil aged with aldrin, dieldrin, endrin and chlordane significantly reduced the rates of degradation of both aldrin and dieldrin, indicating possible toxic effect of this pesticide on the micro-organisms responsible for the degradation of

Table 2
Degradation of vinclozolin (µg/ ml) in a mineral salts medium inoculated with suspension from unplanted or planted flooded alluvial soil untreated or pretreated with commercial HCH

Incubation (days)	Vinclozolin (µg/ml) recovered soil pretreated with HCH						
	Uninoculated	2nd application		3rd application		4th application	
		Unplanted	Planted	Unplanted	Planted	Unplanted	Planted
0	9.2 ± 0.2	6.6 ± 0.3	5.7 ± 0.5	6.5 ± 0.1	5.4 ± 0.2	6.3 ± 0.7	5.2 ± 0
5	7.4 ± 0.6	5.0 ± 0	3.9 ± 0.3	6.5 ± 0.1	3.5 ± 0.5	2.9 ± 0.2	1.7 ± 0.1
10	6.5 ± 0.1	3.4 ± 0.2	2.7 ± 0.6	3.0 ± 0	1.9 ± 0.1	1.3 ± 0	0.9 ± 0.1
15	5.8 ± 0.5	3.4 ± 0.2	0.9 ± 0.1	1.5 ± 0.5	0.6 ± 0	0.9 ± 0.1	0.2 ± 0

Note: vinclozolin added @ 10 µg/ml mineral salts medium

Table 3
Degradation of γ-HCH in a mineral salts medium inoculated with suspension from unplanted or planted flooded alluvial soil untreated or pretreated with commercial Vinclozolin

IIncubation (days)	HCH (µg/ml) recovered from soil pretreated with Vinclozolin						
	Uninoculated	2nd application		3rd application		4th application	
		Unplanted	Planted	Unplanted		Unplanted	Planted
0	9.7 ± 0.1	6.4 ± 0.2	5.2 ± 0.5	5.7 ± 0.1	4.6 ± 0.5	5.3 ± 0.1	3.8 ± 0.1
5	6.9 ± 0.6	4.5 ± 0	3.7 ± 0.6	3.8 ± 0.2	2.5 ± 0.5	1.7 ± 0.1	0.6 ± 0.1
10	6.0 ± 0	3.6 ± 0	2.1 ± 0.1	1.8 ± 0.2	0.7 ± 0.1	0.8 ± 0	0
15	5.3 ± 0.1	2.1 ± 0.5	0.8 ± 0.1	1.0 ± 0	trace	0.5 ± 0.1	0

Note: vinclozolin added @ 10 µg/ml mineral salts medium the aged organochlorine pesticides already in the soil (Ghadiri *et al*., 1995). Extensive applications of persistent organochlorine pesticides like endosulfan on cotton led to the contamination of soil and water environments at several sites in India (Reddy *et al*., 2012).

Increasing pesticide usage in agriculture adds to the rise in concern for the environmental contamination (Zhu *et al*., 2004). Pesticides reaching the soil may affect non-target soil microorganisms, thereby disturbing pesticide degradation processes (Pal *et al*., 2006). Adverse effect of pesticidal chemicals on soil microorganisms (Araújo *et al*, 2003), may affect soil fertility (Schuster and Schröder, 1990) becomes a foreign chemicals major issue. Soil microorganisms show an early warning about soil disturbances by foreign chemicals than any other parameters. But the fate and behaviour of these chemicals in soil ecosystem is very important since they are degraded by various factors and have the potential to be in the soil, water etc. So it is indispensable to monitor the persistence, degradation of pesticides in soil and is also necessary to study the effect of pesticide on the soil quality or soil health by in depth studies on soil microbial activity.

Development of enrichment cultures through the exposure of chemically related pesticides have been reported earlier in case of organophosphorous (Sethunathan, 1973) and carbamate pesticides (Racke and Coats, 1990).

The present study indicates that HCH and Vinclozolin, being included in the same organochlorine group, on their repeated applications to the same soil, encouraged an increase in the number of microorganisms capable of degrading the chemical and resulted in more rapid degradation of the chemical compared to untreated soil supporting the assumption that repeated applications of a pesticide may result in an increase in the enzyme activity, but not community size, specifically toward the degradation of the pesticide. Similar mechanism may be in operation resulting into development of organochlorine-degrading enrichment cultures.

It is known that micro-organisms represent the richest repertoire of molecular and biological diversities in nature as they comprise the most diverse forms of life. They are nature's original recyclers, converting toxic organic compounds to harmless end products, offten CO_2 & H_2O. Ever since, it was discovered that microbes have the ability to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, particualrly of contaminated areas in search for organism that can degrade a wide range of pollutants. Microbial diversity offers an immense scope of environment friendly options for minerlization of contaminants or their transformation into less harmful hazardous compound. In this context, the development of a broad spectrum of the organochlorine-degrading enrichment cultures as observed and discussed in the present study may have an influencial importance in the decontamination process of the agricultural fields/ lands having the previous history of pesticidal practice and can also be used in the bioremediation programme of xenobiotics in the contaminated sites.

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