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Biodegradation of β-Cyfluthrin by Fungi

NIRMALI SAIKIA AND MADHUBAN GOPAL*

Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi-110012, India

Five fungal species, namely, *Trichoderma viride* strain 5−2, *T. viride* strain 2211, *Aspergillus niger*, *A. terricola*, and *Phanerochaete chrysoporium* were screened for degradation study of β-cyfluthrin. Each fungal species was allowed to grow in Czapek dox medium containing β-cyfluthrin (5 mg/mL) as the major carbon source of the medium. The highest degradation of β-cyfluthrin was observed by *T. viride* 5−2 ($T_{1/2}$ = 7.07 days), followed by *T.viride* 2211 ($T_{1/2}$ = 10.66 days). The degradation of β-cyfluthrin followed first-order kinetics with a fast degradation rate during first 7 days of growth of the fungi. In the case of *T. viride* strain 5−2, five degradation products were isolated after 20 days of growth of the fungi, out of which three products were identified as α-cyano-4-fluorobenzyl-3- (2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate, α-cyano-4-fluoro-3-phenoxy benzyl alcohol, and 3(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanoic acid.

KEYWORDS: **β-Cyfluthrin; biodegradation; *Trichoderma***

INTRODUCTION

With the decline in the usage of organochlorine pesticides, the use of synthetic pyrethroid has increased with time and they represent the most popular synthetic insecticide. The consumption of synthetic pyrethroids in India increased by 42% during the last five years. Generally, synthetic pyrethroids are recommended against Lepidopteran pests affecting Solanaceous crops (*1*). Various studies have demonstrated that synthetic pyrethroids are more efficient in controlling insect pest menace (*2*) and have a more favorable persistence profile than conventional insecticides (*3*). β-Cyfluthrin (α-cyano-4-fluoro-3-phenoxybenzyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo-propanecarboxylate) is a synthetic pyrethroid consisting of four active stereoisomers of the parent compound cyfluthrin (*4*). This photostable synthetic pyrethroid has an ester and ether linkage in addition to a dichlorovinyl group attached to a cyclopropane moiety (**Figure 1**). A special feature of this compound is the presence of a fluorine atom that enables it to overcome resistance developed in insect by the use of similar xenobiotics. β-Cyfluthrin insecticide has been tested against various pests of okra (*5*), cotton (*6*), and mustard (*7*). Persistence of β-cyfluthrin on various crops has been studied, and the pesticide, though effective for controlling the insect pest, leaves toxic residues on vegetables that are harvested almost daily, some of which are even consumed raw (*1*, *5*, *7*). The presence of toxic residues over maximum residue limit (MRL) of these insecticides in fruits, vegetables, and green leaves are of concern to the human health due to the hazardous nature of pesticide residues. It therefore becomes imperative that each pesticidal schedule on edible crops be investigated for quantification of amount of residue, and if residues are found above its MRL, efforts are required for decontamination of such contaminated food commodities.

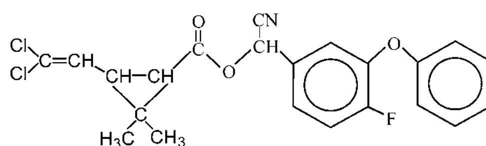

Figure 1. β-cyfluthrin (α-cyano-4-fluro-3-phenoxybenzyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo-propanecarboxylate).

Simple culinary processes were tested for this purpose that were found to be quite efficient in reducing the pesticide residues from 0 and 1 day samples of fruits. The limewater soaking was found to be better for decontamination of eggplant fruits than was simple washing with tap water (*1*). However, these treatments were not very effective at the later stage, as β-cyfluthrin, though not a systemic insecticide, penetrated slightly in the skin of eggplant fruit. Therefore, an efficient method for detoxification of this compound is needed. Preliminary studies with known microorganisms, namely *Bacillus subtilis*, *Bacillus polymyxa*, *Klebsiella plenticola* and *Proteus vulgaris* have shown maximum detoxification of this compound up to 37.0% (*8*). Therefore, selection of a suitable microorganism, for improving level of detoxification of this pesticide, is required.

Five fungi (*Trichoderma viride* 5−2, *T. viride* 2211, *Aspergillus niger*, *A. terricola*, and *Phanerochaete chrysoporium*) known to have the ability to degrade xenobiotics were taken for exploratory studies with different types of pesticides (*9*−*11*). After *Trichoderma viride* was found to be able to detoxify/break the compound fastest, it was taken for further studies (i.e., isolation and identification of the products) formed after degradation of β-cyfluthrin by spectroscopy.

* To whom correspondence should be addressed. Tel.: 91-11-25848706 (Office); 91-11-22013551 (Res.). FAX: 91-11-25733062; 91-11-25843272. E-mail: madhubangopal@hotmail.com.

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MATERIALS AND METHODS

Chemicals. (*a*) *Reagents.* Acetone and hexane were obtained from S.D.Fine-Chem Ltd., Mumbai, India. The solvents were distilled prior use.

(*b*) *Standard.* β-Cyfluthrin standard (97.8%) was procured gratis from Bayer India Ltd. The stock solution of β-cyfluthrin standard was prepared in hexane at 1 mg/mL and stored at 4° C. Working standard solutions (0.1−1.0 μg/mL) were prepared by appropriate dilutions.

(*c*) *Media*. Czapek dox medium was prepared by dissolving 2.5 g of $NaNO_3$, 0.5 g of KCl, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $FeCl_3$ in 1 L of distilled water (pH 6.5). It was used for the degradation studies of β-cyfluthrin, and the compound (5 μg/mL) was added as the major source of carbon in acetone. Liquid media without β-cyfluthrin were sterilized by autoclaving at 120 °C for 20 min. β-Cyfluthrin was sterilized by membrane filtration (Pore size, 0.22 μM., Millex GP, Millipore, Bedford, Mass.) and added to the medium aseptically.

Inoculums. The five strains of fungi, namely *Trichoderma viride* 5−2, *T. viride* 2211, *Aspergillus niger*, *A. terricola*, and *Phanerochaete chrysoporium* were obtained from Type Culture Lab, IARI, New Delhi.

Recovery Study. β-Cyfluthrin is highly soluble in dichloromethane and toluene (200 g/L; 20 °C) (*12*). Because this compound can be analyzed by GLC, therefore toluene and another nonpolar solvent hexane were tried as partitioning liquid for extraction of β-cyfluthrin from Czapek dox medium. The media (10 mL in each conical flask) was taken in a 50-mL conical flask and then fortified with 500 μL of 100 μg/mL stock solution in three replications. Two conical flasks, containing 50 mL of media, were taken as blank (without β-cyfluthrin). The whole content of a conical flask was transferred to a 125-mL separatory funnel, and 50-mL saturated saline solution was added to it. This was subsequently partitioned with 30 mL of hexane. The partitioning was repeated twice with the same solvent (2 × 15 mL). The β-cyfluthrin-containing organic phase was dried by passing through anhydrous sodium sulfate.

Inoculation and Extraction. β-Cyfluthrin was added to each conical flask, separately, at the rate of 5 μg/mL of medium. Extraction for day 0 was done 1 h after inoculation, following the same procedure as given under recovery study. Other conical flasks were kept in a BOD incubator at 28 ± 1 °C for subsequent extraction on 2, 4, 6, 8, 10, 15, 20, and 30 days after inoculation.

Gas Chromatograph. (*a*) *GC Apparatus.* The quantitative estimation of β-cyfluthrin present in each sample was carried out by using a Hewlett-Packard gas liquid chromatography instrument (Model 5890, series II) equipped with a Ni^{63} electron capture detector (ECD) and an autoinjector system connected to a Hewlett-Packard Computer (Model Vectra 05/165). The stationary phase was a Megabore column packed with OV-1 (10-m × 0.54-mm i.d.)

(*b*) *GC Operating Parameters.* ChemStation (Hewlett-Packard) was used for analysis of β-cyfluthrin. The injection port temparature was maintained at 280 °C. The column and detector temperature were 250 and 300 °C. The injection volume was 3 μL.

Quantification. The concentration of β-cyfluthrin was calculated on the basis of a peak area from calibration curve. Standard solutions of β-cyfluthrin of concentrations 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 $\mu g\ mL^{-1}$ were injected in GLC and a calibration curve was constructed by plotting peak area versus concentration. Each sample was injected twice, and detector response was measured in terms of peak area. From the values of peak area, the best concentration was chosen for sample dilution.

TLC Analysis. Cultures grown in Czapek Dox medium containing β-cyfluthrin was extracted with hexane. The extracts were dried over anhydrous sodium sulfate. A crude sample thus obtained was analyzed by thin-layer chromatography on silica gel G plate using acetone/hexane (1:4 v/v) as the developing solvent. The plates were visualized under UV lamp and R_f value of each compound was recorded. Purification of metabolites was done by preparative TLC method using the glass plates of size 20- × 20-cm coated with silica gel G (1000-μm layer) and using the same developing solvent.

^{1}HNMR. ^{1}HNMR of β-cyfluthrin and its metabolites were obtained in a Varian Model EM-368L, 60 MHz instrument using a continuous wave mode. The samples were dissolved in deuterated chloroform and TMS was used as reference standard.

FD-MS. FD-MS of β-cyfluthrin and its metabolites were carried out in a Micromass Autospecs Ultima E Field-Desorption Mass Spectrometer under a calibration range of 50−500 daltons.

IR. Infrared spectra were recorded on a Nicolet Fourier Transform Infrared spectrophotometer (Model, Impact 400).

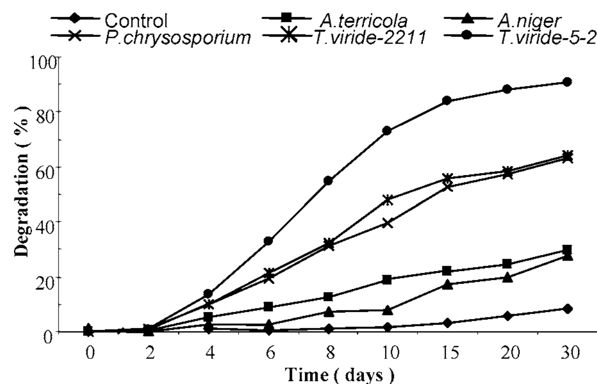

Figure 2. Curves for degradation of β-cyfluthrin by fungi.

Table 1. Correlation Coefficients, Regression Equations, and Half-Lives of β-Cyfluthrin in Various Fungal Cultures

fungal species	time period (days)	corr. coeff. (*r*)	degrdn const. (kf)	half-life in days ($T_{1/2}$)
control	0–30	−0.972	0.0023	216.52
T. viride (5–2)	0–10	−0.935	0.098	7.07
	15–30	−0.933	0.049	14.14
T. viride (2211)	0–10	−0.957	0.065	10.66
	15–30	−0.998	0.015	46.20
Phanerochaete chrysosporium	0–30	−0.962	0.037	18.73
Aspergillus terricola	0–8	−0.982	0.018	38.50
	10–30	−0.999	0.009	77.07
A. niger	0–30	−0.988	0.012	57.75

RESULTS AND DISCUSSION

β-Cyfluthrin Extraction. The recovery of β-cyfluthrin from Czapek dox medium was carried out using hexane as partitioning solvent. The efficiency of the method was satisfactory as more than 85% recoveries of the compound was obtained. There was no correction factor.

Fungal metabolites of β-cyfluthrin. Biodegradation of β-cyfluthrin by the various fungal species with the elapse of time is presented in **Figure 2**. The result shows that about 80−83% degradation was observed with in 10 days of growth with *Trichoderma viride* strain 5−2. Maximum degradation was observed during the first 7 days of growth of the fungus. The degradation constants (k_f), correlation coefficients (*r*) and half-lives of β-cyfluthrin in various fungal cultures are given in **Table 1**.

Structure Elucidation. Metabolites produced by the fungal degradation of β-cyfluthrin were separated by thin-layer chromatography. The R_f values of three major metabolites were 0.79, 0.46, and 0.11, whereas the R_f value of the parent compound was 0.51. The NMR data presented in **Table 2** about the insecticide and three degradation products separated by preparative TLC could be utilized for the identification of these products. Isolating the individual compound by preparative TLC in 30−40 mg and also subjecting the crude to FD-MS (**Table 3**) confirmed the identity of left over parent compound and produced products. The structures of three major products are presented in **Figure 3**.

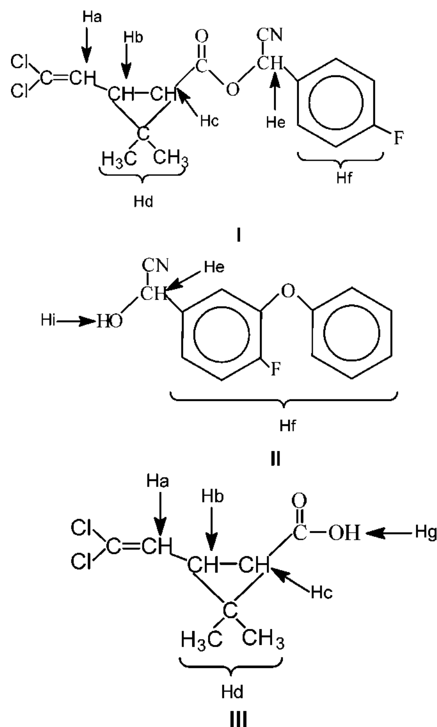

Figure 3. Degradation products of β-cyfluthrin. **(I)** α-cyano-4-fluorobenzyl-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate. **(II)** α-cyano-4-fluoro-3-phenoxy benzyl alcohol. **(III)** 3(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanoic acid.

Table 2. ^{1}HNMR of β-Cyfluthrin and Its Degradation Products

	δ-value			
type of proton	β-cyfluthrin	product I	product II	product III
H_a	5.5 (1H)	5.5 (1H)		5.5 (1H)
H_b	1.6 (1H)	1.6 (1H)		1.6 (1H)
H_c	2.2 (1H)	2.1 (1H)		2.1 (1H)
H_d	1.2 (6H)	1.1 (6H)		1.1 (6H)
H_e	6.3 (1H)	6.3 (1H)		
H_f	6.8–7.4 (8H)	6.8–7.2 (4H)	6.7–7.3 (8H)	
H_g				10.0 (1H)
H_h			9.2–9.8 (1H)	

Table 3. FD-MS of β-Cyfluthrin and Its Degradation Products

compounds	molecular mass	molecular formula
β-cyfluthrin	433	$C_{22}H_{18}O_3NCl_2F$
product I	341	$C_{16}H_{14}O_2NCl_2F$
product II	243	$C_{14}H_{10}O_2NF$
product III	208	$C_8H_{10}O_2Cl_2$

Cleavage of ether linkage resulted in the product **I** (MW 341), which was most nonpolar and showed an R_f of 0.79. In NMR spectra of this compound, four aromatic protons (d 6.8−7.2) were present, suggesting a loss of the phenyl ring from the parent compound (MW 433). The H_a proton was deshielded to d 5.5 due to the magnetic anisotropy of vinylic group. Six protons from the two methyl groups gave a singlet at d 1.1 as H_d. Two different singlets at d 1.6 and d 2.1, respectively, were observed due to H_b and H_c protons present in cyclopropane ring. The H_e proton gave a singlet at d 6.3 due to its direct bonding to electronegative oxygen and cyano group. Two additional spots below that of β-cyfluthrin (R_f 0.51) were of product **II** (R_f 0.46) and **III** (0.11). The formation of cyanohydrin (M. W. 243) and propanoic acid derivative (M. W. 208) proved the cleavage of ester linkage by the fungus. It suggests that the fungus contains enzymes having esterase activity. The presence of a product having molecular weight (243) in FDMS is more significant, as ether bonds are not easily cleaved even with chemical agents. Though less common such cleavage of ether (C−O−C) bond by microorganism is documented (*13*, *14*). This is an interesting finding, as both ester and ether bonds have been cleaved by the action of the fungal enzymes.

The NMR of the most polar product showed the presence of a proton at d 10.0, which could be exchanged by D_2O, proving that the product is an acid. Presence of C═O was confirmed by IR, which showed a band at 1696.79 cm^{-1} and broad OH stretch at 2500−3500 cm^{-1}. This proved the cleavage of ester bond present in β-cyfluthrin. The NMR of product **II** is relatively simple, as it exhibited eight protons in the aromatic region (d 6.7−7.3) as well as a proton deshielded due to its attachment to electronegative oxygen atom and inductive effect of adjacent cyano group. This product is not otherwise formed on carrying out the alkaline hydrolysis of the synthetic pyrethroid (unpublished work). The formation of cyanohydrin (M. W. 243) and propanoic acid derivative (M. W. 208) conclusively proved the cleavage of ester bond by the fungus-resulting detoxification of the insecticide. The Farca's acid (Product **III**) is already documented as non-toxic (*15*).

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