Ecotoxicology and Environmental Safety 93 (2013) 87–92

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Microbial degradation of fipronil by *Bacillus thuringiensis*

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ARTICLE INFO

Article history:
Received 16 December 2012
Received in revised form
30 March 2013
Accepted 1 April 2013
Available online 23 April 2013

Keywords:
Fipronil
Metabolites
Bacillus
Soil
Biodegradation

ABSTRACT

Fipronil, a phenyl pyrazole insecticide has been found to be effective for the control of various insect pests. Due to its higher persistence in soil bioremediation is a promising approach to degrade the pesticide from soil. Isolation and identification of soil microbes was conducted for bioremediation of fipronil contaminated soils. Soil samples collected from different sugarcane growing fields in Gurdaspur district with extensive use of pesticide history served as a source of pesticide degrading microbes. The microbe cultures were grown in Luria broth and maintained at 28 °C. After that Dorn's broth enrichment culture supplemented with fipronil was used and *Bacillus thuringiensis* were isolated. Clay loam soil samples were fortified with fipronil @ 0.50, 0.75, 1.00, 1.25 and 1.50 mg kg^{-1} along with 45×10^7 microbe cells. Each treatment was replicated thrice and from each fortified (insecticide +microbes) sample, 50 g soil sample was taken at 7, 14, 28, 35, 42, 49 and 56 days after initiation of this experiment. Residues were not detected after 28, 35, 35, 35 and 42 days in soil samples after fortification with fipronil @ 0.50, 0.75, 1.00, 1.25 and 1.50 mg kg^{-1}. Among metabolites, sulfide was found to be the main metabolite followed by sulfone and amide. Desulfinyl metabolite was not produced in any of the sample. Total fipronil residues were not found to follow the first order kinetics.

1. Introduction

Fipronil,5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile, is a phenyl pyrazole insecticide first synthesized by Rhône Poulenc Ag Company (now Bayer Crop Science) in 1987, introduced for use in 1993, and registered in the U.S. in 1996 (Tingle et al., 2003; Tomlin, 2000; Ware, 2000). Fipronil formulations in form of solid, liquid and granular products influence the environmental fate (White, 1998). Fipronil degrades to its major metabolites by reduction to sulfide MB45950 (Ramesh and Balsubramanian, 1999), oxidation to sulfone MB46136 (Bobe et al., 1998a), hydrolysis to amide RPA200766 (Bobe et al., 1998b; Ngim and Crosby, 2001) and photolysis to desulfinyl MB46513 (Hainzl and Casida, 1996; Fig. 1). The desulfinyl photodegrade is extremely stable and actually more toxic than the parent compound (USEPA, 1996). The metabolite MB 46513 is about 10 times more acutely toxic to mammals than fipronil itself. The metabolite MB 46136 is highly toxic to birds, and the metabolites MB 46136 and MB 45950 are more toxic to freshwater invertebrates than fipronil (Pesticide Action Network-UK, 2000). The half-lives of fipronil have been observed to be as long as 342 days in loam soil (Tingle et al., 2003) and 126 days in sandy loam soil (Rhône-Poulenc Ag Company, 1998). Similarly, $t_{½}$ of fipronil in sandy loam was reported to be 122 days with fipronil-amide and fipronil-sulfone accounting for 27–38% and 14–24% of the total byproducts, respectively (USEPA, 1996).

Microbial degradation is an important mechanism controlling the fate of pesticides in soils and is generally considered to be desirable both from an environmental as well agricultural perspective (Parkin et al., 1991). Biodegradation of pesticides in soil is controlled by the number and activity of microbes in the soil and environmental conditions (Shelton and Parkin, 1991). The widespread use of increasing number of pesticides in agriculture has acquired great importance earlier due to their pest control activities and now due to their deleterious impact on the human health and environment. Degradation studies in soils are essential for evaluation of persistence of pesticides and their breakdown products. Data on the rate of pesticide degradation are extremely important as they permit prediction of the potential risk associated with exposure. Information on the nature and amounts of pharmacological active metabolites of fipronil in soil is necessary to know the fate of pesticide. Microbial degradation of fipronil has been studied by different scientists (Ying and Kookana, 2002; Zhu et al., 2004; Masutti and Mermut, 2007; Lin et al., 2008; Tan et al., 2008; Kumar et al., 2012). To date, only one bacteria *Paracoccus* sp. (Kumar et al., 2012) has been reported to degrade fipronil in soil. Therefore, the present studies were undertaken to isolate and identify microbes for bioremediation of fipronil contaminated soils. Soil samples

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http://dx.doi.org/10.1016/j.ecoenv.2013.04.001

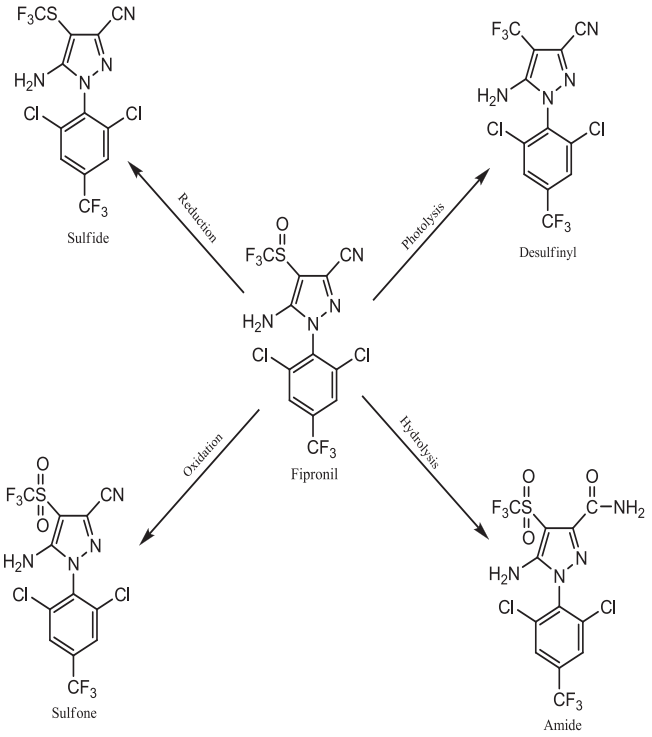

Fig. 1. Metabolites or degradation products of fipronil.

collected from different sugarcane fields with known history of extensive pesticide usage and located in Gurdaspur district served as source of pesticide degrading microbes.

2. Materials and methods

2.1. Chemicals and media

Fipronil formulation (Jump 80 WG) used for fortification was obtained from M/s Bayer CropScience India Ltd., Mumbai, India. The technical grade analytical standards of fipronil MB-46030 (purity 97.5 per cent), sulfone MB-46136 (purity 99.7 per cent), sulfide MB-45950 (purity 98.8 per cent), desulfinyl MB-46513 (purity 97.8 per cent) and amide RPA-20076 (99.8 per cent) were also supplied by M/s Bayer CropScience India Ltd., Mumbai, India. Analysis of acetone extract of the formulation showed only fipronil, and none of its metabolic products and interfering peaks under the retention time of the compound being estimated. Moreover, the concentration of fipronil was found to be accurate with respect to its purity as claimed by the manufacturers. All other solvents and reagents used in this study were of analytical reagent grade.

Two different bacteriological media were used for isolation of fipronil degrading bacteria and their subsequent molecular identification. Dorn's broth media was used for isolation of degrading bacterial species. The composition of Dorn's broth was—$Na_2HPO_4 . 12H_2O$ 3.0 g, KH_2PO_4 1.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4 . 7H_2O$ 10.0 g, $CaCl_2 \cdot 2H_2O$ 2.0 g, $MnSO_4 \cdot H_2O$ 3.0 g, $FeSO_4 . 7H_2O$ 0.2 g, ammonium ferric citrate 0.01 g, yeast extract 0.1 g, distilled water 1.0 L, pH 7.0. Luria broth was used for growth and maintenance of isolated bacteria. The composition of Luria broth was—trypton 20.0 g, yeast-extract 1.5 g, NaCl 1.5 g, distilled water 1.0 L, pH 7.0. When needed Luria broth (LB) was supplemented with bacteriological agar @ 1.6% before autoclaving to make solidified Luria agar medium. The pH of the each medium was adjusted and all the media were sterilized by autoclaving (121 °C, 15 psi of steam, 20 min) before use. Calculated amounts of fipronil as sole carbon were added to each lot of respective pre-sterilized medium before use.

2.2. Enrichment of fipronil degrading microbial species

Soil samples (one kg each) were collected from different sugarcane fields in Gurdaspur district and pooled by thorough mixing. 200 g of pooled soil sample was suspended in one litre of sterilized distilled water with the help of a hand held blender and suspension was filtered by sieving through two layers of muslin cloth. The microbial biomass in the filtrate was recovered in pellet by centrifugation at 10,000 rpm for 10 min and resuspended in 0.5 ml of sterile distilled water. For selective growth of only fipronil degrading microbes, the mixed microbial suspension was grown in Dorn's broth containing fipronil as sole source of carbon. For this purpose, 50 ml of Dorn's broth supplemented with fipronil (50 μg ml^{-1}) was inoculated with 0.1 ml of microbial suspension and allowed to grow at 28 °C on an orbital shaker (120 rpm). After 96 h of growth, the procedure was repeated once by subculturing of 0.1 ml of fresh growth into 50 ml of fresh Dorn's broth containing fipronil (50 μg ml^{-1}) and allowing it to grow as above for 96 h.

2.3. Isolation and purification of fipronil degrading bacterial species

Bacterial cultures were isolated by surface plating of the enriched culture broth (100 μl) on the solidified Dorn's medium supplemented with fipronil, in 90 mm Petri dishes followed by incubation at 28 °C. After seven days, when individual colonies appeared on the surface of the medium, individual colonies were picked up with a sterile needle loop and streaked on the same medium. The growing individual colonies from the streak plate were ultimately maintained by growth in slant tubes prepared with the fipronil supplemented Dorn's medium as well as on LB agar medium and maintained at 4 °C.

2.4. Screening of bacterial isolates for growth and fipronil degradation in liquid medium

For studies on relative growth of different bacterial isolates and fipronil degradation capacities, individual bacterial isolates were allowed to grow in liquid Dorn's medium supplemented with fipronil at 28 ± 1 °C on shaker at 150 rpm before estimation of the bacterial growth and analysis of fipronil degradation. For this purpose, 50 ml of Dorn's broth in a 250 ml Erlenmeyer flask was supplemented with fipronil (50 μg ml^{-1}) and inoculated with one ml of overnight grown culture of the respective bacterial isolate in 5 ml of Luria broth. The inoculated medium was incubated at 28 °C on an orbital shaker (120 rpm) for 14 days.

Estimation of bacterial growth was measured as increase in optical density (OD_{600}). For this purpose, bacterial cell culture (5 ml) drawn at regular intervals was centrifuged (600 rpm, 5 min). The collected cell mass in the pellet was suspended in 5 ml of distilled water and its optical density was measured in a spectrophotometer using water as control blank.

2.5. Quantification of fipronil degradation

Five ml samples of culture broths were drawn at specific intervals. The sample was transferred into 1 l separatory funnel along with rinsings of acetone. The sample in the separatory funnel was diluted with 600 ml brine solution (almost saturated sodium chloride solution), and partitioned the contents two times into 100 mL dichloromethane and two times into 75 ml hexane. Combined both the dichloromethane and hexane layers after passing through anhydrous sodium sulfate and treated with 500 mg activated charcoal powder for about 2–3 h at room temperature. The clear extract obtained was filtered through Whatman filter paper No.1 and concentrated to near dryness using rotary vacuum evaporator at < 40 °C. The final volume was made upto 5 ml using acetone.

2.6. Identification of selected bacterial isolates

Taxonomic identification of bacterial isolates showing potential for fipronil metabolization was based upon broad morphological characteristics (shape and arrangement of bacterial cells, grams reaction) and 16s ribosomal RNA (16s rRNA) nucleotide sequence homology with GenBank database of NCBI (National Center for Biotechnology Information) as described below:

Bacterial DNA isolation: A loopful of bacterial mass taken from an isolated clone on LB-agar plate was inoculated into 3 ml of Luria Broth (LB) and allowed to grow for 48 h at 28 ± 1 °C on an orbital shaker (150 rpm). The bacterial cell mass was harvested in pellet by centrifugation at 10,000 rpm for 1 min. The cell mass was lyzed by suspending in 10 mM Tris EDTA buffer (TE) containing 20 μg ml^{-1} lysozyme, followed by incubation at RT for 10 min. The cellular proteins in the lysate were removed by three extractions with phenol:chloroform (1:1 v/v) followed by chloroform:isoamyl alcohol (25:1 v/v). The total DNA in the aqueous phase was precipitated with equal volume of isopropanol in the presence of 0.25 M sodium acetate, pH 5.2. The bacterial DNA was collected in pellet by centrifugation at 10,000 rpm, for 5 min. The DNA pellet was washed once with 70% ethanol and allowed to dry at RT. The Dried DNA pellet was dissolved in 100 μl of TE buffer and stored at −20 °C until used. The quality of DNA isolated from bacteria was determined by horizontal agarose 0.7 per cent containing ethidium bromide @1 μg per ml gel electrophoresis in 1 × TAE (Tris Acetate EDTA) buffer at 75 V for 1 h. The DNA bands were visualized and photographed under a UV transilluminator in 'UltraCam Gel documentation system'.

2.7. PCR amplification of 16s rRNA gene region

Specific amplification of 16s rRNA was preformed using 16s rRNA Gold primers set (name of primer Forward: QUGP-F4-CCGCCTGGGGAGTACG and Name of primer Reverse: QUGP-Rn2- TGACGGGCGGTGTGTACAAG) as per Barghouthi (2011). The primers were custom synthesized through facility of Integrated DNA Technologies,