



Technical Note

Bioremediation of fipronil by a *Bacillus firmus* isolate from soilKousik Mandal^{a,*}, Balwinder Singh^a, Monu Jariyal^b, V.K. Gupta^c^a Pesticide Residue Analysis Laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana 141004, Punjab, India^b Department of Microbiology, Punjab Agricultural University, Ludhiana 141004, Punjab, India^c Insect Molecular Biology Laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana 141004, Punjab, India

HIGHLIGHTS

- *Bacillus firmus* proved its potential in efficient metabolism of fipronil.
- Fipronil were not detected after 42 d in soil when fortified @ 1.50 mg kg⁻¹.
- Fipronil sulfide was found to be the main metabolite followed by sulfone and amide.
- Total fipronil residues were not found to follow the first order kinetics.

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ABSTRACT

Persistence of fipronil, a new molecule in extensive use against various insect pests is causing serious problems to the environment. *Bacillus firmus* was isolated by selective enrichment from soil samples collected from sugar fields with known history of pesticide usage and evaluated for metabolism of fipronil in clay loam soil. Soil samples in 50 g aliquotes were fortified with fipronil @ 0.50–1.50 mg kg⁻¹ and inoculated with *B. firmus* cells (45×10^7 CFU mL⁻¹) and incubated at 25 °C. Each sample in triplicates was drawn periodically up to 56 d and residual fipronil contents analyzed by gas liquid chromatograph. Fipronil residues were not detected after 35 d at lower doses of fipronil (@ 0.50, 0.75 and 1.00 mg kg⁻¹). However, at higher doses (@ 1.25 and 1.50 mg kg⁻¹) than this total metabolism of fipronil could be observed after 35 and 42 d, respectively. Thus whereas, *B. firmus* proved its potential in efficient metabolism of fipronil, the period required for the same was dose dependent. Amongst various metabolites of fipronil degradation, fipronil sulfide was found to be the main metabolite followed by fipronil sulfone and fipronil amide. Though, desulfinyl metabolite earlier reported as one of the main metabolite of fipronil degradation, the existence of the same was not detected in any of the treatment.

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1. Introduction

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 the deleterious impact of their residues on the human health and environment. In nature, slow activity of natural microflora for degradative elimination of these toxic chemicals is proving insufficient to provide needed cure of heavily contaminated pesticide rich environment. Microbial degradation is an important mechanism controlling the fate of pesticides in soils and is generally considered to be desirable both from an environmental perspective as well as an agricultural point of view (Parkin et al., 1991). Studies on microbial degradation are useful in the development of strategies for the detoxification of insecticides by selective species of various microorganisms (Hayatsu et al.,

2000). Biodegradation of pesticides is controlled by bioavailability of the pesticide to a pesticide-degrading microorganism and its activity (Anhalt et al., 2007). In this respect, data on the rate of pesticide degradation are extremely important as they permit prediction of the potential risk associated with their exposure.

Fipronil, a phenyl pyrazole insecticide is labeled for use in large number of crops and is effective against a wide range of insect pests. Through contact and ingestion activity of insect, it controls a broad spectrum of insects such as rice stem borer, leaf folder, cockroaches, mosquitoes, locust, ticks and fleas at both their larval and adult stages (Chanton et al., 2001; Aajoud et al., 2003). Fipronil degrades to its major metabolites by reduction to sulfide, oxidation to sulfone, hydrolysis to amide and photolysis to desulfinyl (Fig. 1). The half-life of fipronil in soil varies greatly, ranging from 3 d to 7 months (Bobe et al., 1998; Tingle et al., 2003). Due to its higher persistence of residue in soil, bioremediation is a promising approach to degrade the pesticide. Microbial degradation of fipronil was studied by different scientists (Ying and Kookana, 2002;

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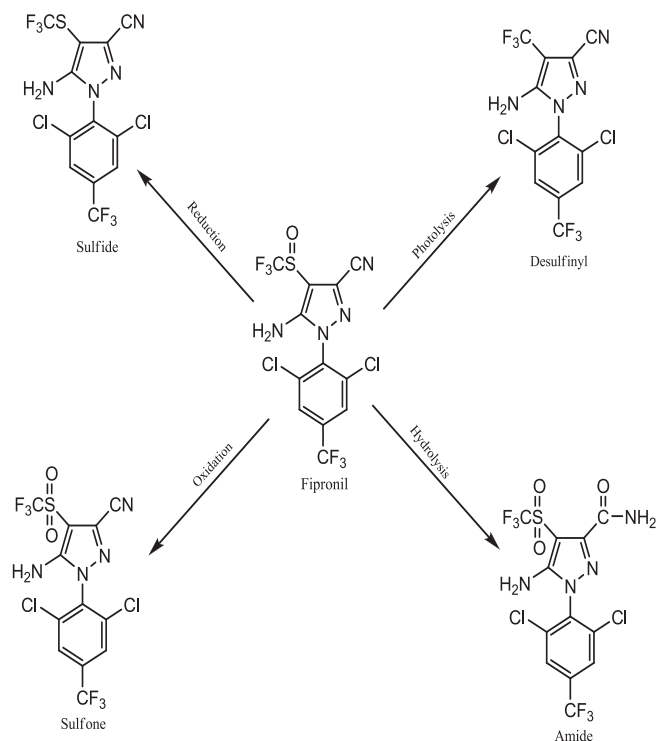


Fig. 1. Metabolites or degradation products of fipronil.

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. has been reported to degrade fipronil in soil (Kumar et al., 2012). Since less information on microbial degradation of fipronil in soil is available, the present studies reports on isolation and identification of specific soil microbes and their evaluation for bioremediation of fipronil contaminated soils.

2. Materials and methods

2.1. Chemicals and media

The technical grade analytical standards of fipronil MB-46030 (purity 97.5%), sulfone MB-46136 (purity 99.7%), sulfide MB-45950 (purity 98.8%), desulfinyl MB-46513 (purity 97.8%) and amide RPA-20076 (99.8%) were supplied by M/s Bayer CropScience India, Mumbai, India. Fipronil (Jump 80 WG) formulation used for fortification was also obtained from M/s Bayer CropScience India, Mumbai, India. Analysis of acetone extract of the formulation showed only fipronil, and none of its metabolic products and no interfering peak was observed 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. Semi-synthetic Dorn's broth media was used for isolation of fipronil degrading bacterial species and was consisted (g L^{-1}) of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$: 3.0, KH_2PO_4 : 1.0, $(\text{NH}_4)_2\text{SO}_4$: 1.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 10.0, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 2.0, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 3.0, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.2, ammonium ferric citrate-0.01 and yeast extract 0.1 in distilled water and pH adjusted to 7.0. Other natural medium Luria broth (LB) used for growth and maintenance of isolated bacteria was consisted (g L^{-1}) of Tryptone – 20.0, Yeast-extract – 1.5, NaCl – 1.5 in distilled water and pH adjusted to 7.0. When needed these broths

were supplemented with bacteriological agar @ 1.6% before autoclaving to make solidified agar media. All media in appropriate aliquots were steam sterilized (15 kPa of steam, 20 min) before use. Calculated amounts of fipronil as sole source carbon were added to each lot of pre-sterilized Dorn's medium before use.

2.2. Enrichment of fipronil degrading microbial species

Soil samples collected from different sugarcane fields with known history of extensive pesticide usage and located in Gurdaspur district (Punjab, India) served as source of pesticide degrading microbes. Representative soil samples (1 kg each) were collected from four different fields were mixed thoroughly mixed. Pooled soil sample (200 g) was suspended in 1 L of sterilized distilled water with the help of a hand held blender and suspension filtered by sieving through two layers of muslin cloth. The microbial biomass in the filtrate was recovered in pellet by centrifugation at 10000 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 50 mL of Dorn's broth containing fipronil ($50 \mu\text{g mL}^{-1}$) as sole source of carbon and allowed to grow at 28°C on an orbital shaker (120 rpm). After 96 h of growth, the procedure was repeated once by sub culturing of 0.1 mL of the mixed culture into 50 mL of fresh Dorn's broth containing fipronil ($50 \mu\text{g mL}^{-1}$) and allowing it to grow as above for 96 h to form enriched culture.

2.3. Isolation and purification of fipronil degrading bacterial species

Enriched culture (100 μL) was surface plated on the solidified Dorn's medium supplemented with fipronil, in 90 mm Petri dishes followed by incubation at 28°C . After 7 d, when well isolated bacterial colonies appeared, individual colonies were picked up with a sterile needle loop and streak purified 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 and allowed to grow at $28 \pm 1^\circ\text{C}$ 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 ($50 \mu\text{g mL}^{-1}$) and inoculated with 1 mL of overnight grown culture of the respective bacterial isolate in 5 mL of LB. The inoculated medium was incubated at 28°C on an orbital shaker (120 rpm). 5 mL Samples of culture broths were drawn at specific intervals. The samples were extracted and cleaned up by following a standardized analytical methodology.

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 (6000 rpm, 5 min). The collected cell mass in the pellet was suspended in 5 mL of distilled water and its Optical density measured in a spectrophotometer at 600 nm using water as control blank.

2.5. Identification of selected bacterial isolates

Taxonomic identification of bacterial isolates showing potential for fipronil metabolization was based upon (i) broad morphological

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