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Response of microbial community to a new fungicide fluopyram in the silty-loam agricultural soil

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ABSTRACT

The impacts of fluopyram on a soil microbial community were studied at three application rates: at the recommended field rate (T1, 0.5 mg/kg soil), three-fold recommended field rate (T3, 1.5 mg/kg soil) and ten-fold recommended field rate (T10, 5 mg/kg soil). Soil samples were taken after 7, 15, 30, 45, 60 and 90 days of application to determine the fluopyram residue and microbial properties (i.e., basal respiration, substrate-induced respiration, microbial biomass carbon, microbial community function and structure). The half-lives of the fluopyram at levels of 0.5, 1.5 and 5 mg/kg in soil were calculated to be 64.2, 81.5 and 93.6 days, respectively. The results demonstrated that fluopyram treatment (T1, T3 and T10) decreased microbial biomass *C* but increased the basal respiration, substrate-induced respiration, and ecophysiological indices (qCO_2). Average well color development (AWCD) represents the oxidative capacity of soil microorganisms cultivated in the BIOLOG micro-plates and usually indicates the overall microbial metabolic capacity. The BIOLOG results revealed that the AWCD in the soil treated with 1.5 and 5 mg/kg fluopyram (T3 and T10) was significantly lower than that of the control during the incubation period. A similar variation in the diversity indices (Simpson index and McIntosh index) was observed. Phospholipid fatty acid (PLFA) analysis revealed that the addition of fluopyram decreased the total amount of PLFAs, bacterial biomass (both Gram-positive (GP) bacteria and Gram-negative (GN)), fungal biomass, the ratios of the GN/GP and fungi/bacteria at all incubation times. Principal component analyses (PCA) suggested that the addition of fluopyram shifted the soil microbial community structure and function. Hence, fluopyram has a harmful effect on overall soil microbial activity, and changed soil microbial community structure and function.

1. Introduction

Soil microorganisms play a unique role in maintaining soil productivity in soil ecosystems (Min Liao et al., 2007), and many environmental variables, including pesticides, can affect soil microbial communities. The effect of fungicides on soil microorganisms, typically broad-spectrum killing and inhibiting antimicrobials, has attracted more studies than other pesticides because of their toxic potential to non-target soil fungi as well as soil bacteria (Wu et al., 2012).

Fluopyram, *N*-[2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl]-α,α,α-trifluoro-*ortho*-toluamide, is a new pyridinylethylbenzamide fungicide introduced by Bayer Crop Science in 2010. Fluopyram is highly effective in controlling a variety of pathogens (*Sclerotinia spp, Monilia spp*) for more than 70 crops, including vines and table grapes, pome and stone fruits, vegetables and field crops (Labourdette et al., 2010), mainly through inhibiting succinate dehydrogenase and the fungal respiratory chain complex, when applied either alone or in combination with other fungicides even at low rates. Fluopyram has been registered in many countries and also in China which is applied to control powdery mildew.

It is reasonable to assume that fluopyram will affect soil health and productivity. However, to the authors' best of knowledge, information on fluopyram's impacts on soil microorganisms is limited, if available at all. In addition, only a few studies involving residual fluopyram analysis (Guan et al., 2012), microbiological activities in the presence of fluopyram (Veloukas and Karaoglanidis, 2012), and reaction mechanisms (Labourdette et al., 2010) have been reported.

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The aims of this work were to examine the following: (1) the persistence of fluopyram in soil, (2) the impacts of fluopyram on soil microbial activity, which is characterized by basal respiration and substrate-induced respiration, and (3) the impacts of fluopyram on soil microbial biomass (microbial biomass carbon and total PLFA), soil microbial community structure and function. These findings are useful to understand the interactions between fungicides and microbes in agricultural soils.

2. Materials and methods

2.1. Reagents and solvents

Fluopyram (purity, 99.4 percent), was purchased from the Fluka Co. Mixtures of fatty acid methyl esters were purchased from Supelco Co. Methyl nonadecanoate was purchased from Accustandard Co. The chemical reagents used in the experiments were all analytical grade.

2.2. Soil collection and handling

The soil was collected from a field of Shangzhuang Farm in Beijing, China. The soil had not been subjected to conventional-tillage and not treated with pesticide in the previous 2 years. Soil (top 0–15 cm) was collected with a stainless steel soil tube drill with a diameter of 3 cm. The soil samples were taken to the laboratory in coolers, then mixed, sieved with 2 mm mesh to remove the plant tissue, and subjected to physicochemical characterization according to Sparks et al. (1996). The soil samples were adjusted to a soil moisture of 40 percent water-holding capacity (WHC) and then pre-incubated for 10 days with distilled water in the dark at 25 °C and 50 percent humidity before use. The soil had pH 7.8 (soil:H_2O 1:2.5), organic carbon 17.5 g/kg determined by dichromate digestion method (Kalembasa and Jenkinson, 1973), and soil mechanical composition (silt 61.3 percent, sand 35.6 percent, clay 3.1 percent) was measured by hydrometer method (Bouyoucos, 1962), and $NH4^+$ −N 9.1 mg/kg, NO_3^- −N 7.9 mg/kg determined by Yan et al. (2013). Rapid available potassium 239.0 mg/kg was determined by extraction method with ammonium acetate (Pratt, 1965). Available phosphorus 11.3 mg/kg was determined photometrically as a blue phosphate molybdic acid complex (Olsen and Sommers, 1982).

Incubation experiments were conducted with fluopyram in 500 mL brown-glass pots covered with sterile cotton plugs. To avoid the potential effects of solvents upon the microbiological activity of the soils, the following methods were adopted. A portion of the soil (20 g) was first spiked with 0.1 mL of stock solution in acetone and stirred for 5 min. The spiked soils were allowed to air-dry for 10 min, and then the remaining soil (100 g) was added and mixed thoroughly for another 5 min, yielding the final concentrations of 0.5, 1.5 and 5 mg fluopyram/kg soil (based on dry weight) (T1, T3 and T10, respectively). T1, T3 and T10 correspond to the maximum recommended field dosage (75 g a.i./ha), three-fold recommended field dosage and ten-fold recommended field dosage, respectively. An equal volume of acetone (0.1 mL) was added to fluopyram-free controls (CK). The soil moisture content was 24.8 percent, adjusted to 60 percent water holding capacity (WHC). Each experiment was conducted in triplicate. The pots were incubated in the dark at 25 °C and 50 percent humidity for 90 days. Throughout the incubation period, distilled water was added to the soil to compensate for any water loss that exceeded 5 percent of the initial amount added. The pots were removed from the environmental chamber at random after different incubation time intervals (7, 15, 30, 45, 60 or 90 days). The soils were then analyzed for the soil microbial activity, microbial biomass, community structure and function, as well as the concentration of fluopyram.

2.3. Determination and analysis of fluopyram

The fluopyram was extracted from the soil using the QuEChERS method as described by Guan et al. (2012) with some modifications and detected by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Briefly, a 10.0 g sample of soil was extracted for 2 h in 5 mL of water and 20 mL of acetonitrile. After centrifugation, 1.5 mL of the upper layer was transferred into a 2.0 mL dispersive-SPE tube containing 50 mg of PSA and 150 mg of $MgSO_4$. Then, the tubes were vortexed for 1 min and centrifuged for 5 min at a RCF of 2077 g. The resulting supernatants were filtered through 0.22 μm nylon syringe filters for UPLC–MS/MS analysis.

Chromatographic separation of fluopyram was performed on a Waters ACQUITY ultra-performance liquid chromatography system. The mobile phase consisting of methanol (solvent A) and water (solvent B) was pumped at a flow rate of 0.3 mL/min. The gradient elution program was as follows: 0–1 min, 10–90 percent A and 1–1.5 min, 90–10 percent A and then held at 10 percent A for 3.5 min. The temperature in the sample manager was set at 5 °C, and the column oven temperature was maintained at 45 °C. The sample volume injected was 5 μL.

A triple-quadrupole mass spectrometer (TQD, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source was used for fluopyram detection. MS/MS detection was performed in positive ionization mode, and the monitoring conditions were optimized. Multi-reaction monitoring mode (MRM) was selected as the scan mode. In addition, 397.3 (m/z) was selected as the precursor ion, and its quantitative and qualitative product ions were 173 (m/z) and 208 (m/z), when the collision energies were 30 and 25 V, respectively. Under the described conditions, the retention time of fluopyram was approximately 1.7 min.

2.4. Respiratory activity

Soil respiration rate was measured based on Muñoz-Leoz et al. (2011) with minor modifications. For basal respiration, 20 g of fresh soil was transferred to a flask along with a vial containing 0.2 mol/L NaOH (10 mL) to trap the released CO_2. As a blank, one flask did not contain soil. The soil samples and the blank were all incubated at 25 °C for 24 h. Then, NaOH solution was precipitated with 3 mol/L $BaCl_2$ and back-titrated with 0.1 mol/L HCl using a Brand Titrette® (Germany). Substrate-induced respiration was determined by adding 10 g of glucose/kg soil (based on dry weight) to soil samples and then measuring CO_2 evolution 6 h later.

2.5. Determination of microbial biomass carbon (MBC)

Microbial biomass carbon (an indicator of the overall size of the soil microbial community) was determined by the chloroform fumigation-extraction with minor modification (Wang et al., 2007; Zhang et al., 2010). The soil sample was divided into two portions, each containing 20.0 g of soil (based on dry weight). One portion was fumigated with ethanol-free chloroform for 24 h, whereas the other was left untreated. Both the fumigated and unfumigated soil samples were extracted using 40 mL of 0.5 mol/L K_2SO_4 for 1 h on a shaker. Then 10 mL of filtrate was mixed with 5 mL of 0.2 mol/L $K_2Cr_2O_7$ and 5 mL H_2SO_4. The mixture was boiled for 10 min at 170–180 °C before cooling and then titrated using Fe_2SO_4 (0.05 mol/L) and $C_{12}H_8N_2$ as an indicator. The MBC was calculated as MBC=2.64 × (C extracted from fumigated soil−C extracted from unfumigated soil).

2.6. Community-level substrate utilization analysis

BIOLOG ECO plates (MicroPlate., BIOLOG Inc., Hayward, USA) were used to study the substrate utilization pattern of soil microbial communities as described by Girvan et al. (2003). Each 96-well plate consists of three replicates, each one comprising 31 sole carbon sources and a blank. Briefly, soil was extracted by sterile saline solution (0.85 percent, m/v). The supernatant was serially diluted to the 10^{-3} dilution, and 150 μL of the suspension was added to each well of a BIOLOG ECO plates. The microplates were then incubated at 25 ± 2 °C in the dark. Color development in the plates was measured every 24 h at 590 nm for 7 days using a BIO-TEK Elx808 automated microplate reader (Biolog, Hayward, CA, USA).

Average well color development (AWCD) represents the oxidative capacity of soil microorganisms cultivated in the BIOLOG micro-plates and usually indicates the overall microbial metabolic capacity (Garland and Mill, 1991). The diversity indices (Shannon, Simpson, and McIntosh) are used to assess soil microbial functional diversity (Gomez et al., 2006). The Simpson index is weighted toward the abundances of the most common species, and the Shannon index indicates the richness of soil microorganisms (Magurran, 1988), whereas the McIntosh index indicates the evenness or homogeneity of soil microorganisms (Atlas, 1984). The AWCD, Shannon index, Simpson index and McIntosh index were determined by calculating the mean of every well's absorbance value after of 96 h incubation, which corresponded to the time of maximal microbial growth in the BIOLOG ECO plates.

$$\mathrm{AWCD} = \sum OD_i/31 \quad (1)$$

where OD_i is the optical density value from each well after subtracting the value of the blank (water).

$$\text{Shannon index}: H' = -\sum P_i \times \ln(P_i) \quad (2)$$

$$\text{Simpson index}: D = \sum(n_i(n_i-1)/(N(N-1) \quad (3)$$

$$\text{McIntoshindex}: U = \sqrt{\sum(n_i^2)} \quad (4)$$

where p_i is the ratio of microbial activity on each substrate (OD_i) to the sum of the microbial activities on all substrates $\sum OD_i$. n_i refers to absorbance value; N is the total absorbance values of all wells, and the Simpson index is expressed as the reciprocal ($1/D$).

2.7. Analysis of soil the microbial community structure by PLFA

The performed PLFA analysis was based on previous reports of Bossio et al. (1998) and Zhang et al. (2010). Briefly, a 5.0 g-sample of freeze-dried soil samples