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Evaluating subchronic toxicity of fluoxastrobin using earthworms (*Eisenia fetida*)

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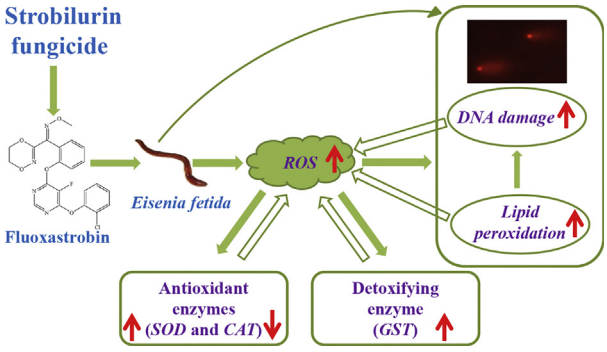
HIGHLIGHTS

- The subchronic toxicity of fluoxastrobin to earthworms was investigated.
- Fluoxastrobin can cause oxidative stress and oxidative damage in earthworms.
- The comet assay was the most sensitive of the biomarkers used in the present study.

GRAPHICAL ABSTRACT



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ABSTRACT

Potential toxicity to soil organisms by fluoxastrobin, a new strobilurin-type fungicide has drawn increasing attention. Thus, the present study investigated the subchronic toxicity induced by exposure to several concentrations (0, 0.1, 1.0, and 2.5 mg kg^{-1}) of fluoxastrobin to earthworms on days 7, 14, 21, and 28. Biochemical indicators (e.g., reactive oxygen species (ROS) content, activities of antioxidase and detoxifying enzymes (superoxide dismutase, catalase, and glutathione S-transferase), lipid peroxidation (malonaldehyde) and degree of DNA damage) were measured. No earthworm deaths were observed during the entire experimental period. For ROS and malonaldehyde, the bioassay values of the three doses reached a maximum on day 21 and then decreased. For superoxide dismutase and glutathione S-transferase, the values increased with the exposure doses of 0.1 and 1.0 mg kg^{-1} and then decreased. In contrast, the values for catalase were lower on days 7, 14, and 28 and greater on day 21 compared to those of the controls. In addition, the comet assay was more sensitive than other biomarkers, and the degree of DNA damage was dose and time -dependent.

1. Introduction

Fluoxastrobin is primarily applied to various green vegetables, cereal crops, coffee, potatoes, and other crops. Previous studies found that fluoxastrobin inhibits mitochondrial respiration of the target fungus by transferring electrons between cytochromes b and C_1 (Yin et al., 2003; Zhang et al., 2018b). Fig. 1 is the structure of fluoxastrobin. Because fluoxastrobin is a new type of strobilurin fungicide related to azoxystrobin and pyraclostrobin, there is potential for its broad application. Fluoxastrobin may lodge in the soil due to its high solubility in water of 2.27 (pH = 9), 2.29 (pH = 7), and 2.43 mg L^{-1} (pH = 4) at 20 °C (Yin et al., 2003).

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Zhang (2015) stated that the degradation half-life of fluoxastrobin in soil was ranging from 16 to 119 d. Zhang et al. (2018b) stated that fluoxastrobin had high toxicity to *Danio rerio* and could induce oxidative stress and DNA damage in zebrafish livers. They also stated that fluoxastrobin was stable in the experimental period.

Han et al. (2014) studied the oxidative stress induced by azoxystrobin, another strobilurin fungicide, on earthworms (*Eisenia fetida*) selected as a biological indicator by the Organization for Economic Cooperation and Development (OECD 222, 2004) and International Organization for Standardization (ISO, 1993). The effects on this species have been assessed in numerous studies (Gu et al., 2017; Li et al., 2016; Y.H. Wang et al., 2015). Han et al. (2014) concluded that azoxystrobin led to oxidative stress and DNA damage in earthworms. Fluoxastrobin, a new strobilurin fungicide, has a mode of action similar to azoxystrobin (Yin et al., 2003). This raises the question of whether the toxicity of fluoxastrobin is similar to that of azoxystrobin in soil due to their similar chemical structures.

Zhang (2015) stated that the LC_{50} value of earthworm after exposure to fluoxastrobin for 14 d was >1000 mg kg^{-1} soil. According to Zhang et al. (1986), fluoxastrobin is low toxic to earthworms in the short term. Here comes the question: what are the toxic effects of low doses of fluoxastrobin for long exposure? However, there is little information on the subchronic toxicity of fluoxastrobin on *Eisenia fetida*. Furthermore, there was little study focus on the residue of fluoxastrobin. However, there were some studies focuses on the residue of pyraclostrobin. Pyraclostrobin, also strobilurin fungicide, has the similar chemical structure with fluoxastrobin. The initial residue in soil after exposure to pyraclostrobin (56 to 187.5 g ha^{-1}) was ranging from 0.01 to 0.72 mg kg^{-1} soil (Li et al., 2010). Thus, we set the doses of 0.1, 1.0, and 2.5 mg kg^{-1} in the present to evaluate the subchronic toxicity of fluoxastrobin to *Eisenia fetida* before it is widely applied.

Specifically, the present study used static tests to evaluate the subchronic toxicity of fluoxastrobin to reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), malonaldehyde (MDA), and the degree of DNA damage (comet assay) and identified the most sensitive indicator for evaluating the toxic effects of fluoxastrobin on the soil ecosystem. In addition, the present study provides a theoretical scientific basis for assessing the environmental safety of fluoxastrobin.

2. Materials and methods

2.1. Chemicals

Fluoxastrobin (CAS Nos. 361377-29-9) of 99.3% purity, with a molecular weight of 458.83, was provided by Dr. Ehrenstorfer (GmbH, Augsburg, Germany). The acetone used to dissolve the fluoxastrobin was chromatographically pure. All the analytically pure chemicals and reagents were purchased from the Solarbio Science &Technology Company (Beijing, China), which were listed in Table S1.

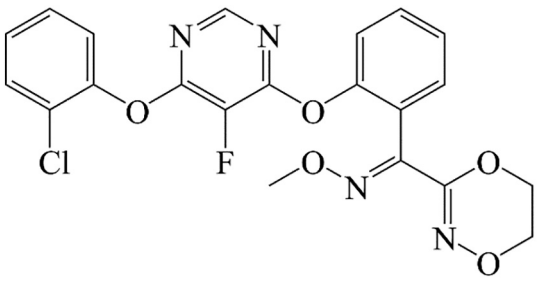

Fig. 1. The structural formula of fluoxastrobin.

2.2. Soil and earthworms

The composition of the artificial soil substrate used in the present study was as follows: 20% kaolin, 70% silica sand, and 10% turf soil (ISO, 1993; OECD 207, 1984). The soil pH was adjusted to 5.5–6.5 using calcium carbonate. According to OECD 207 (1984), the moisture content in the artificial soil was adjusted to 35% of dry soil. Each beaker used in the experimental period was sealed with plastic wrap to retain moisture. Furthermore, gravimetric method was used to check the moisture every day.

Eisenia fetida was recommended as the species of earthworm for measurements of chemical toxicity (ISO, 1993; OECD 207, 1984). These earthworms were purchased from Shandong Agricultural University and cultured with a mixture of cattle feces and sphagnum at 20 ± 2 °C for 14 days before the subchronic toxicity test. Healthy mature adults, which had well-developed clitella and weights ranging from 300 to 600 mg, were randomly selected and precultured for 24 h in the aforementioned artificial soil in an incubator (RXZ-500B-LED, Ningbo Jiangnan Instrument Factory, China) before exposure to fluoxastrobin. We also performed the sensitivity test of earthworms (*Eisenia fetida*) to chloroacetamide, which can prove the results of the toxicological tests in the present study were accurate. The specific methods and results were listed in the section "Supplementary Text" in the file "Supplementary Material".

2.3. The toxicological test design of fluoxastrobin for earthworms

The toxicological testing was performed following the procedure for the use of animals in toxicology (Zhang et al., 2018a). The exposure doses in the present study were 0.1, 1.0, and 2.5 mg fluoxastrobin per kg in the artificial soil described above in 1-L beakers, into which fifteen earthworms were transferred. Next, the beakers were cultivated in the incubator with a photoperiod of 12 h light:12 h dark at 20 ± 2 °C for 28 days. The controls and exposure treatment groups of each indicator were analyzed in triplicate.

2.4. Enzyme extraction for measurement of protein contents, enzyme activities and lipid peroxidation

One of the three earthworms from each beaker, which had been subjected to preculture to void the gut contents, was rinsed using DI water and cleaned using clean filter paper for enzyme extraction (Liu et al., 2016). The earthworms exposed to the same concentration were homogenized with 0.05 mol L^{-1} solution of phosphate buffered saline (PBS, pH = 7.8) at 4 °C. Afterward, the homogenate was centrifuged (Centrifuge 5810R, Eppendorf AG, Germany) at 4 °C at 12,857*g* for 10 min. The supernatant was transferred to a 10-mL centrifuge tube for measurement of protein content, enzyme activities and lipid peroxidation.

2.5. Measurement of protein content

The measurements of the protein content were conducted at 595 nm using an ultraviolet-visible spectrophotometer (UV spectrophotometer, UV-2550, Shimadzu, Japan) and were compared to a standard curve prepared using bovine serum albumin (Bradford, 1976).

The measurement of SOD activity was conducted using the reported method (Giannopolitis and Ries, 1977). The SOD reaction liquid consisted of a mixture of DI water (5 mL), 0.05 mol L^{-1} SOD-PBS (30 mL, pH = 7.8), 130 mmol L^{-1} solution of methionine (6 mL), 750 μmol L^{-1} solution of nitroblue tetrazolium (NBT, 6 mL), 100 μmol L^{-1} solution of disodium ethylenediamine tetraacetate (6 mL), and 20 μmol L^{-1} solution of riboflavin (6 mL). The treatment tubes were exposed to 4000 lx for 30 min, and one control tube was placed in the dark. Next, the absorbance was determined at 560 nm with the dark treatment tube serving as the negative control. The SOD activity is