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Acute and subchronic toxicity of pyraclostrobin in zebrafish (*Danio rerio*)

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HIGHLIGHTS

- Acute and subchronic toxic effects of pyraclostrobin on zebrafish were investigated.
- Pyraclostrobin can cause oxidative stress and oxidative damage in zebrafish.
- The comet assay was the most sensitive of all biomarkers used in the present study.
- The doses of pyraclostrobin are at a relatively stable level in the present study.

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ABSTRACT

The aim of the present study was to assess the toxic effects of pyraclostrobin on DNA damage and antioxidant enzymatic activities in the zebrafish (*Danio rerio*) liver. Based on the 96-h median lethal concentration (96 h LC_{50}, 0.056 mg/L) of this chemical, fish were exposed to three doses (0.001, 0.01, and 0.02 mg/L) and sampled on days 7, 14, 21 and 28 after the initiation of a subchronic toxicity test. The levels of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione S-transferase (GST), reactive oxygen species (ROS) and DNA damage were determined. The amount of pyraclostrobin residue in the water was also measured. The concentrations in the three treatment groups varied no more than 5% during the exposure periods, indicating that pyraclostrobin is relatively stable during this time in an aquatic environment. ROS and MDA levels significantly changed in a dose dependent manner during the experiment. Enzymatic activities were inhibited to a certain extent. DNA damage was significantly enhanced. These results collectively indicate that pyraclostrobin induces oxidative stress and DNA damage in zebrafish.

1. Introduction

Strobilurin fungicides use the natural antibiotic strobilurin A as a lead compound (Mick et al., 2002). Pyraclostrobin is used around the world and has been recently demonstrated to have broad applications. However, people have often used pesticides unscientifically, resulting in a low utilization rate during the application process. This causes most of the pesticide residues to be located in the soil or water ecosystem (Zhang et al., 2017b), which can decrease its ecological and environmental safety. The contamination of aquatic ecosystems by these fungicides is therefore an environmental concern.

Pyraclostrobin transfers electrons between cytochrome b and c_1 to inhibit mitochondrial respiration in the target fungus (Mick et al., 2002). Pyraclostrobin, or methyl N-[2-[[1-(4-chlorophenyl)-1*H*-pyrazol-3-yl]oxymethyl]phenyl]−N-methoxycarbamate, can be applied to crops including cereal, cucumber, peanuts, and banana (Joshi et al., 2014).

Numerous studies have focused on pyraclostrobin (Brühl et al., 2013; Fulcher et al., 2014; Raina-Fulton, 2015), but few have evaluated the sub-lethal effects of this fungicide on aquatic organisms. Pesticide residues that reach the aquatic environment may be

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harmful to both aquatic organisms and human health. Hence, zebrafish are used in biology toxicological tests to evaluate the ecological toxicity of chemical pollutants in an aquatic environment (Spence et al., 2008).

Zebrafish (*Danio rerio*) are a model organism (Organization for Economic Cooperation and Development (OECD)), that is widely used in laboratory tests (Altenhofen et al., 2017; Chen et al., 2017; Liu et al., 2017; OECD, 1992; Yang et al., 2017; Yan et al., 2015; Zhang et al., 2017a, 2017b). The aim of the present study was to use acute and subchronic toxicity tests to evaluate the potential toxicity of pyraclostrobin by determining the levels of ROS, MDA, antioxidant enzymes (i.e., SOD and CAT), GST, and DNA damage in zebrafish. Under physiological condition, reactive oxygen species (ROS) are continuously produced by O_2 metabolism and commonly associated with cellular injuries. Antioxidant enzymes eliminate free radicals to slow oxidative damage. The MDA contents were measured to evaluate the extent of lipid peroxidation.

2. Materials and methods

2.1. Chemicals and reagents

The pyraclostrobin (99.0%, CAS No. 175013-18-0) used in the present study was obtained from Dr. Ehrenstorfer GmbH (Augsburg Germany) and dissolved in chromatographically pure acetone to make stock solutions. The molecular structural formula of pyraclostrobin is shown in Fig. 1. All other analytical grade reagents were obtained from Sigma Chemical (St. Louis, Missouri, USA) and Solarbio Science &Technology Company (Beijing, China).

2.2. Fish domestication and experimental design

In the present study, we followed principles for the use of animals in toxicology that were in line with the Guiding Principles adopted by the Society of Toxicology in 1989 (Van, 2002). Adult zebrafish with a mean weight and length of 0.14 ± 0.01 g and 2.50 ± 0.02 cm, respectively, were acquired from the Kaixin Tropical Fish Aquarium (Taian, China). Fish of different genders were uniformly mixed and randomly selected for measurement. Six hundred fish were purchased and divided equally into four tanks containing 20 L each. They were then acclimated for half of one month before the formal toxicological tests were performed. A 12-h light:12-h dark cycle was implemented, and the animals were housed in dechlorinated tap water that was aerated for 7 day at an oxygen saturation greater than 70%, a temperature of 26 ± 1 °C and a pH ranging from 7.4 to 8.1. The fish used in the acute and subchronic tests were domesticated under the aforementioned conditions for two weeks to acclimate them to the test conditions. The fish were fed bait each day at regular intervals until 24 h before the acute and subchronic tests were performed. Half of the water was replaced at the time every 2 days, and feces, redundant bait and dead fish were extracted using the siphon method to avoid interference. The acute toxicity test is a static test that was performed to acquire the 96 h LC_{50} of pyraclostrobin. The concentrations that led to acute toxicity were 0, 0.001, 0.01, 0.05, 0.06, 0.07, 0.08 and 0.1 mg/L. Each sample consisted of ten randomly selected fish and 1.5 L of exposed solution. Based on Passino and Smith (1987), the resulting 96 h LC_{50} was used to evaluate the acute toxicity (mg/L) of the pesticides in zebrafish as follows: less than 1, highly toxic; 1–10, moderately toxic; 10–100, slightly toxic; 100–1,000, practically harmless; and greater than 1,000, relatively harmless. The subchronic toxicity test for pyraclostrobin was performed a control group and three groups exposed to different levels of pyraclostrobin (i.e., 0.001, 0.01 and 0.02 mg/L). One hundred and twenty fish were randomly selected and assigned to a vessel containing 20 L of water at one of the three concentrations. The subchronic toxicity test is a semistatic test, and half of the exposed solution was replaced at the same time every 2 days to maintain the concentration of pyraclostrobin throughout the subchronic toxicity experiment. The fish were sampled in triplicate to analyze the levels of ROS, SOD, CAT, GST, MDA, and DNA damage on days 7, 14, 21 and 28. The control was set up using 1 mL of acetone dissolved in the same source of dechlorinated tap water to prevent interference from the solvent. Three replicates were performed for each trial in both the acute and the subchronic toxicity tests.

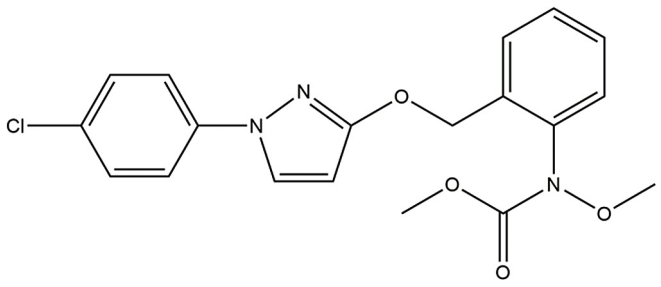

Fig. 1. The molecular structural formula of pyraclostrobin.

2.3. Determination of pyraclostrobin concentrations

The concentration of pyraclostrobin was determined using the methods described in Wang et al. (2011). In the present study, we also used gas chromatography (GC; Agilent 7890B; Agilent Technologies Inc., USA) to accurately measure pyraclostrobin concentrations in the tested water during the experimental period. These tests were performed in triplicates to ensure the reliability of the results. First, 100 mL of pyraclostrobin-exposed solution, 20 mL of 10% NaCl, 50 mL of acetone and 40 mL of dichloromethane were added to a separatory funnel. The separatory funnel was then vigorously shaken for 2 min. After the mixture was incubated for 30 min, it was demixed. Second, the dichloromethane under layer was collected by passing the columella of anhydrous sodium sulfate into a 250 mL Florence flask. The superstratum remained in the separatory funnel, and 30 mL of dichloromethane was added to re-extract it, and the extracted solutions were collected into the same Florence flask. Third, the mixed solutions in the Florence flask were evaporated at 40 °C using rotary evaporators (RE-52, Shanghai Yarong Biochemistry Inc., China). Normal hexane was added to rinse the Florence flask. Fourth, the solutions were transferred to a graduated test tube and blow-dried using nitrogen evaporators (N-EVAP, N-EVAP-112, Organomation Associates, Inc., USA). The solutions were then diluted in normal hexane to a volume of 1 mL. The temperatures of the inject port and electron-capture detector (ECD) were 280 °C and 300 °C, respectively. The initial column oven temperature was 160 °C, and this temperature was maintained for 1 min. In the present study, we used temperature programming. Specifically, the temperature was increased at a rate of 20 °C per min until it reached 260 °C. It was then maintained for 10 min. The samples were separated using a HP-5 column (19091J-413, 30 m × 320 μm × 0.25 μm) and measured at constant-voltage. Nitrogen was used at a flow rate of 1.6 mL/min for the mobile phase. The inject volume and flow rate of the make-up gas were 1 μL and 30 mL/min, respectively. In theory, the level of pyraclostrobin was expected to be 80%–120%, if not (or even > 120% or < 80%), the actual values that were determined were used to analyze the toxicity of pyraclostrobin in zebrafish (OECD 203, 1992).