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Toxic effects of three strobilurins (trifloxystrobin, azoxystrobin and kresoxim-methyl) on mRNA expression and antioxidant enzymes in grass carp (*Ctenopharyngodon idella*) juveniles

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ABSTRACT

The strobilurins are used widely in the world as effective fungicidal agents to control Asian soybean rust. In this study, the early life stage of grass carp (*Ctenopharyngodon idella*), which is one of the most important aquaculture species in China, was chosen to measure the acute toxicity of three common strobilurin-derived fungicides (trifloxystrobin (TFS), azoxystrobin (AZ) and kresoxim-methyl (KM)). As endpoints, normal developmental parameters (lethal concentration (LC_{50}) and average heart rate), expression of relative genes, and three antioxidant enzyme activities in the developing juveniles were recorded during a 48 h exposure. The results revealed that values of LC_{50} were TFS 0.051 (0.046–0.058) mg L^{-1}, AZ 0.549 (0.419–0.771) mg L^{-1} and KM 0.338 (0.284–0.407) mg L^{-1} for juveniles. For the potential toxicity mechanisms, these three fungicides increased catalase (CAT) and peroxidase (POD) activity and decreased superoxide dismutase (SOD) activity, significantly inhibited expressions of three growth-related genes (*IGF-1*, *IGF-2* and *GHR*) and two energy-related-genes (*CCK* and *PYY*), and caused pronounced up-regulation a stress-gene (*HSP70*). The present study demonstrated potential toxic effects of TFS, AZ and KM on the early development of *C. idella*. Overall, three strobilurins (TFS, AZ and KM) might cause serious damages to the aquatic species; therefore, their pollution supervision in water ecological environment should be strengthened.

1. Introduction

With the rapid development of modern agriculture, new pesticides have played an important role, acting at novel molecular targets to cope with pathogen resistance (Rodrigues et al., 2013). One group is strobilurin-derived fungicides that were developed from strobilurin A in *Strobilurus tenecellus*, manufactured and distributed for industrial–commercial use since the 1990s, which were considered an outstanding new fungicides used to gain popularity around the world against the pathogenic fungi (Bartlett et al., 2002; Sauter et al., 1999). The molecular target of strobilurins to fungus is the mitochondrial respiratory electron transfer chain, by blocking between cytochrome b and cytochrome c1 at the ubiquinol oxidizing site (Qo) of the complex III, and thus can cause losses of ATP synthesis to inhibit cellular respiration in eukaryotes (Bartlett et al., 2002; Hnatova et al., 2003; Balba, 2007). In addition, the inhibition can lead to electron escaping from the mitochondrial respiratory chain to induce cellular oxidative stress, which can be detoxified by mitochondrial manganese superoxide dismutase (MnSOD) (Kim et al., 2007). Therefore, strobilurins have a dual effect (lack of ATP and oxidative stress) on controlling fungi cell apoptosis.

Due to special physical and chemical properties, strobilurins has been used widely in the United States as an effective fungicidal agent applied on crops, with increased from less than 2% treated in 2004 and 2005 to 25–30% treated in 2009 (http://www.epa.gov/pesticides/regulating/headline-letter.pdf). Similarly, the use of strobilurins has been increased in Great Britain during the last decade (Hooser et al., 2012), because strobilurins were deemed the only effective means to combat the disease (Shaner et al., 2005). In the past decade, strobilurins was infrequently detected in aquatic habitats, and major investigators trended to explore environmental behavior, fate and occurrence of strobilurins in aquatic ecosystems, which lead to the absence of relevant environmental concentrations. Nowadays, even though lack of concrete data, some studies has showed that several different primary metabolites of some strobilurins are soluble in water and have a very high mobility/dissipation rates from soil/air to water (Belden et al., 2010; European Food Safety Authority (EFSA), 2010; Lefrancq et al., 2013; Jia et al., 2013), and eventually lead to significant transport, reaching downstream

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aquatic ecosystems. Widely application and high residue rates indicate that strobilurins may pose a major hazard for aquatic organisms exposed at relevant environmental concentrations during air spraying, or indirectly, leaching in soil, drain flow. Although strobilurins were considered to have low acute and chronic toxicity to humans, birds, mammals, and bees (Bartlett et al., 2002; European Food Safety Authority (EFSA), 2010), recent researches have clearly indicated that these fungicides were classified as acutely toxic to non-target fresh-water organisms. For instance, toxic effects of one strobilurin trifloxystrobin on *Bufo cognatus* tadpoles were obtained at 0.04 mg L^{-1} (Belden et al., 2010; Australian Pesticides and Veterinary Medicines Authority (APVMA), 2000) noted that the median lethal concentration (96-h LC_{50}) of trifloxystrobin for *Oncorhynchus mykiss* trout ranged from 15 to 0.078 mg L^{-1}, and for *Mysidopsis bahia* the median effective concentration EC_{50} ranged from 0.009 to 0.034 mg L^{-1}. Furthermore, several authors highlighted that another strobilurin azoxystrobin can cause acute toxicity to common frogs (*Rana temporaria*) at 0.5 μg mL^{-1} (Johansson et al., 2006), while pyraclostrobin to fresh-water mussels was at concentrations below 0.1 mg L^{-1} (Bringolf et al., 2007). Additionally, both European Pesticide Risk Assessment (EFSA) (2010) and United States Environmental Protection Agency (US EPA) (1997) concluded that AZ was considered as very toxic to aquatic organisms, including fresh water, estuarine/marine fish, and aquatic invertebrates, potentially to cause long-term adverse effects in the aquatic environment, and issued instructions for keeping it out of lakes, streams, ponds, tidal marshes, or estuaries. Even though a series of researches have assessed toxic and lethal doses of strobilurin fungicides in aquatic organisms, the limits still exist about other potential effects and action mechanisms. Thus, considerable attentions have been received about monitoring and researching the strobilurin fungicides pollution in water ecological environment.

Among the different aquatic organism toxicity evaluation experiment, fish acute toxicity tests play important roles in water environmental risk assessment and hazard classification, and fish early life stage (ELS) assays are particularly suitable for chemical testing because early developmental stages are particularly sensitive to chemicals, easy to produce and to look after and also small-sized (Zhu et al., 2013).

Information about action mechanism of strobilurins on non-target aquatic organisms is such scant that little is known about their potential acute toxicity, especially fish. In the present study, juveniles bioassay with grass carp (*Ctenopharyngodon idella*), one of the most important aquaculture species with a total production of 3.56 million tons in China (FBMA, 2008), was used to make a further exploration of potential acute toxicity of three commonly strobilurin-derived fungicides (trifloxystrobin (TFS), azoxystrobin (AZ) and kresoxim-methyl (KM)). We examined different critical monitor endpoints, such as drug lethal concentrations (LC_{50} and LC_{95}) and juveniles average heart rate, certain gene expression investigated (growth-related genes (*IGF-1*, *IGF-2*, and *GHR*), energy-related-genes (*CCK* and *PYY*) and environmental-stress-gene (*HSP70*)), as well as three common antioxidant enzyme activities (catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD)) measured in the present study. The intent of work presented herein was to provide the possible acute toxicity influence of TFS, AZ and KM on multi-level biomarker responses in the grass carp juveniles.

2. Material and methods

2.1. Test chemical and organisms

The strobilurins both AZ and KM with a purity of 98.5% were purchased from Zenith Chemicals Corp., LTD (Jiangsu, China), chemical name: methyl-(E)-2-{2[6-(2-cyanophenoxy)-pyrimidin-4-yloxy]-phenyl}-3-methoxyacrylate and (E)-α-(methoxyimino)-2-((2-methylphenoxy)-methyl)-benzeneacetic acid methyl ester (IUPAC), respectively.

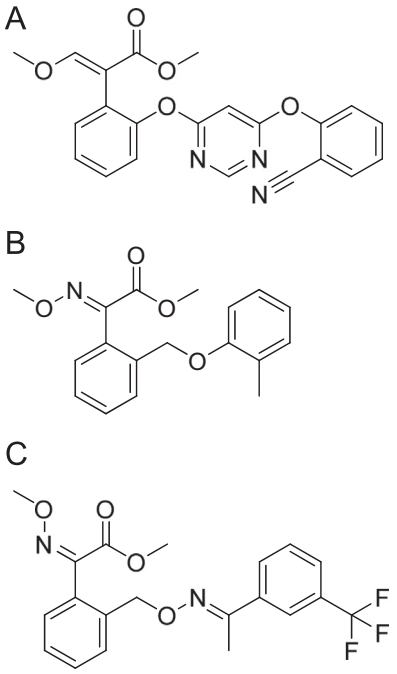

Fig. 1. Chemical structures of azoxystrobin (A), kresoxim-methyl (B) and trifloxystrobin (C).

The strobilurin TFS (purity 99%) was provided by Jintan Huashang Chemical Auxiliaries Corp., LTD (Jiangsu, China), chemical name: (E,E) methoxyimino-{2-[1-(3-trifluoromethyl-phenyl)-ethylideneamin-ooxymethyl]-phenyl}- acetic acid methyl ester (IUPAC). The chemical structure of these strobilurins was showed in Fig. 1. High performance liquid chromatography (HPLC) was used to confirm the purity of these fungicides. Stock solutions were prepared at a concentration of 20 mg mL^{-1} in dimethyl sulfoxide (DMSO) and stored in at 4 °C during the experiment, where the stock solutions were sonicated for approximately 10 min to ensure that the compounds were fully dissolved and mixed. The different nominal concentrations solutions were prepared by appropriate dilution of 20 mg mL^{-1} stock solutions.

Two thousand *C. idella* juveniles (hatching 4–5 days) were provided by Xinmin Aquatic Breeding Center (Weinan city, Shaanxi province, China), with the total length (cm) of 0.89 ± 0.10 (data are presented as mean values ± SD). The juveniles were reared in re-circulating aerated fresh water maintained at 25 ± 0.5 °C with a photoperiod of 16:8 h (light:dark). The juvenile cultural condition was per-formed according to established protocols (Zhu et al., 2011).

2.2. Larval acute toxicity assay

The effects of TFS, AZ, and KM on larval development of *C. idella* were investigated by exposing the hatched juveniles (hatching less than 10 d) to a range of concentrations (0.010, 0.016, 0.025, 0.040, 0.063, and 0.100 mg L^{-1} for TFS; 0.10, 0.16, 0.25, 0.40, 0.63, and 1.00 mg L^{-1} for AZ and KM) of these compounds, where the setting of concentration was based on the previous research in our laboratory. A DMSO-control (2.5‰, v/v, the highest percentage of DMSO in treatment groups) was served as the control and control survival rate was always above 95%. For the performance of the tests, every 25 juveniles were introduced into 6-well microtiter plates filled with 10 mL freshly prepared test solutions or controls per well and then incubated at 25 ± 0.5 °C with a photoperiod of 16:8 h light:dark for 48 h. The plates were covered with self-adhesive foil to avoid the possible effect of evaporation and the solution was removed and replaced with fresh solution every 6 h to ensure actual concentrations deviated little from initial target concentrations and dissolved oxygen concentrations. Three replicates were set for the tests. The survival juveniles were examined at regular intervals (every 3 h) for all treatment and control groups, where the optical microscopy (Olympus BX41) was used to define juveniles as no movement or response to gentle prodding. After the incubation period (48 h), average heart rate was also noted and described for five randomly selected survival juveniles from each replicate, which followed the video method (Lema et al., 2007), via a digital camera attached to a inverted microscope (Motic AE31, Hong Kong, China).

2.3. RNA extraction and reverse transcription (RT)

For the determination of gene expression by TFS, AZ, and KM treatments, juveniles were exposed to a range of concentrations (0.001, 0.005, and 0.025 mg L^{-1} for TFS; 0.01, 0.05, and 0.25 mg L^{-1} for AZ and KM) for 48 h. Three replicates were set for the tests, with 50 juveniles per replicate. These test compounds concentrations and