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Single and mixture toxicity of strobilurin and SDHI fungicides to *Xenopus tropicalis* embryos

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ABSTRACT

The decline in amphibian populations is a critical threat to global biodiversity, and pesticide pollution is considered as one of the major factors. Although effects of single pesticides on amphibians have been documented, toxicological interactions prevailing in mixtures of pesticides have not been well elucidated. Strobilurin and succinate dehydrogenase inhibitor (SDHI) fungicides are new types of commonly used pesticides. In this study, effects of three strobilurins (pyraclostrobin, trifloxystrobin and azoxystrobin), two SDHIs (isopyrazam and bixafen), and their mixtures on *X. tropicalis* embryos were fully investigated. Results showed that exposure to individual fungicides induced lethal and teratogenetic effects; and malformed embryos displayed similar phenotypes including microcephaly, hypopigmentation, somite segmentation and narrow fin. Exposure to two strobilurins or two SDHIs at equitoxic concentrations caused additive or synergetic effects at environmentally relevant concentrations. TU for mixtures of isopyrazam and bixafen was 0.53 and 0.30 for lethal and teratogenic toxicity, respectively. Finally, binary mixtures of strobilurins and SDHIs also exhibited additive or synergetic effects on amphibian embryos. Overall, these results reveal that the mixtures of multiple fungicides caused a higher incidence of lethality and teratogenicity of amphibian embryos, compared to a single fungicide at the corresponding doses. Our findings provide important data about the ecotoxicology of agricultural fungicides on non-target organisms, which is useful for guiding management practices for pesticides.

1. Introduction

Large declines in amphibian populations have been reported since the 1990s (Houlahan et al., 2000; Brühl et al., 2013). 32% of approximately 6000 amphibian species are threatened with extinction, and at least 43% are experiencing serious declines in the world (Stuart et al., 2004). Another report showed that as many as 40% of amphibian species face imminent extinction (Wake, 2012). Amphibians are disappearing at a rate faster than any other vertebrate animal. Pressures faced by amphibian populations are mostly derived from anthropogenic influences including habitat change or loss, climate change, introductions of foreign species, pollution and diseases (Hof et al., 2011). Additionally, the chytrid fungus is capable of causing sporadic death in some amphibian populations (Hayes et al., 2010). Except for land use change, chemical pollution is probably the single most important cause of amphibian decline (Sodhi et al., 2008; Wagner et al., 2013).

A wide range of agricultural chemicals negatively affect amphibians both in the field and in laboratory-based studies (Egea-Serrano et al., 2012), in part because they are sensitive to both aquatic and terrestrial contaminants (Quaranta et al., 2009). Agricultural fungicides are widely used globally and the application of fungicides, especially new fungicides, such as strobilurins and succinate dehydrogenase inhibitor (SDHI) fungicides, has dramatically increased over the past decade (Lucintel, 2016), including in the US and Europe. Strobilurin fungicides are part of the larger group of QoI inhibitors, which act to inhibit the mitochondrial respiratory chain at the level of Complex III. After several years of development, strobilurin fungicides have become the largest fungicide category by market value globally, whose market value accounts for about 23–25% of the global fungicides total (Reportlinker, 2016). As inhibitors of succinate dehydrogenase (i.e. complex II in the mitochondrial respiration chain), SDHI fungicides are rapidly achieving market share in many crops and new SDHIs are currently in development (Lucintel, 2016). These new fungicides are frequently detected out in the environment. For example, the fungicides pyraclostrobin,

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trifloxystrobin and azoxystrobin, were detected at maximal concentrations of 0.10, 0.73 and 0.03 μg/L, respectively, in surface water from natural rivers in Australia (Wightwick et al., 2012). Azoxystrobin was detected with a maximum concentration of 1.13 μg/L in 45% of 103 samples from streams in the USA (Battaglin et al., 2011). However, potential adverse effects of these new fungicides on non-target organisms like amphibians have not been well studied (Hooser et al., 2012; Cui et al., 2017), but the potential toxicity of fungicides has received increasing attention (Belden et al., 2010; Di Renzo et al., 2010; Wightwick et al., 2012; Li et al., 2016).

Amphibian embryos or tadpoles may be particularly susceptible to agricultural fungicides. Reproduction and early development in amphibians coincides seasonally with agricultural fungicide application in crops such as wheat and soy-bean (Belden et al., 2010; McMullen et al., 2012). A previous study showed that embryonic and larval development of *Oryzias latipes* was affected by 0.1 mg/L trifloxystrobin (Zhu et al., 2015b). Belden et al. (2010) reported that pyraclostrobin caused acute toxicity to Bufo at levels of 15 mg/L to 150 mg/L. 1.7 mg/L pyraclostrobin could cause chronic effects on *Bufo cognatus* tadpoles (Hartman et al., 2014). Our recent study showed strong lethality and teratogenicity of ten commonly used pesticides, including strobilurins, on *Xenopus tropicalis* embryos (Li et al., 2016). Because many agricultural fungicides exist as mixtures, further investigation is needed to explore whether these agricultural fungicides produce joint toxicity, and or have synergistic effects. For example, pyraclostrobin, trifloxystrobin and azoxystrobin were simultaneously detected in rivers (Battaglin et al., 2011; Wightwick et al., 2012). Additionally, the occurrences of multiple pesticides were seemingly related to the malformation in amphibians (Mann et al., 2009). However, toxicological interactions prevailing in fungicide mixtures have been scarcely studied in amphibian animals (Relyea, 2009; Battaglin et al., 2011; Wightwick et al., 2012).

The West African clawed frog *X. tropicalis* has a number of advantages as an amphibian embryological system including small body, short generation time and abundant eggs (Schmitt et al., 2014). To date, *X. tropicalis* has been extensively used as an amphibian model for chemical screening and toxicity assays (Hoke and Ankley, 2005; Hu et al., 2015; Li et al., 2016). In this study, pyraclostrobin, trifloxystrobin and azoxystrobin were selected as representatives of strobilurin fungicides; isopyrazam and bixafen selected as SDHI fungicides. The objective of this study was to evaluate and compare potential adverse effects of either individual or binary combinations of fungicides on amphibian embryos using *X. tropicalis*. We examined lethal and teratogenetic effects for single or joint exposures to strobilurins, SDHIs and their mixtures.

2. Materials and methods

2.1. Chemicals

Pyraclostrobin (PY), trifloxystrobin (TR), azoxystrobin (AZ), isopyrazam (IS) and bixafen (BI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Structural formulas of these fungicides are shown in Fig. 1. Dimethyl sulphoxide (DMSO), 3-amino-benzoic acid ethyl ester (MS-222) and other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Xenopus tropicalis

The husbandry of *X. tropicalis* adults and breeding were performed as described previously (Li et al., 2016), and conducted in accordance with protocols approved by Science and Technology Commission of Shanghai Municipality. The experimental procedures were consistent with national guidelines for the protection of human subjects and animal welfare. Adult *X. tropicalis* were purchased from Nasco (Fort Atkinson, WI, USA) and were maintained in aquaria with dechlorinated tap-water at a 26 ± 0.5 °C, alternating 12 h light/dark cycles and fed a semi synthetic diet (Zhejiang, China). Breeding was induced by subcutaneous injection of human chorionic gonadotrophin (hCG) (Zhejiang, China). Six pairs of mature frogs were used for breeding, and healthy embryos from the paired frogs were collected and used for experiments.

2.3. Single fungicide exposures

Exposure experiments were conducted using the standard protocol of the frog embryo teratogenesis assay-*Xenopus* (FETAX) (ASTM, 1998; Fort et al., 2000). Fungicide solutions were dissolved in DMSO (< 0.1%) and prepared just prior to the exposure. The nominal concentration of DMSO was 0.05% in all solutions. Based on preliminary experiments, five appropriate exposure concentrations were formulated for each fungicide as follows: 0.5–6 μg/L pyraclostrobin, 5–40 μg/L trifloxystrobin, 10–200 μg/L azoxystrobin, 0.1–2.0 mg/L isopyrazam, and 0.1–2.0 mg/L bixafen (Table S1 of Supplementary material). As described previously, exposure duration was adjusted to 48 h (Hu et al., 2015; Li et al., 2016). Briefly, embryos from paired frogs were collected before exposure experiments. Twenty embryos with jelly coats at stage 10 were put into each glass Petri dish (10 cm diameter) with FETAX medium control, DMSO solution control or test fungicide solutions, with a medium renewal after 24 h. Four replicates were performed and any dead embryos were removed at 12 h intervals.

2.4. Measurement of actual concentrations

Actual concentrations of exposed fungicides were measured at the beginning of the treatment using high-performance liquid chromatography (Agilent 1260 fitted with a photodiode array detector, Palo Alto, CA, USA), with a ZORBAX Eclipse XDB-C18 reverse phase column. Three replicate samples were assayed in each concentration group. The mobile phase was a mixture of acetonitrile and Millipore water with 0.01 M formic acid (75:25, v/v). The flow rate was set at 1.0 ml/min. The optimum wavelengths were 254 nm (PY), 250 nm (TR), 254 nm (AZ), 254 nm (IS) and 250 nm (BI), respectively.

2.5. Observations and measurements of embryos

The embryos were observed under a Carl Zeiss Discovery V8 Stereovmicroscope (MicroImaging GmbH, Göttingen, Germany) and images were acquired using an AxioCam digital camera. Developmental stages were determined following Nieuwkop and Faber (1956). The whole body length was measured in five tadpoles of each replicate dish using computer-assisted image analysis (iSeeV3.873). According to the protocol described previously (Hu et al., 2015; Li et al., 2016), the phenotypes of malformations were distinguished in all surviving embryos. The main observed phenotypes included abnormal eyes, enlarged proctodaeum, bent axis, narrow fins, and skin hypopigmentation. The percentages of survival and malformation respectively were calculated in each concentration group.

2.6. Joint exposure and toxicity assessments

Joint binary exposures were prepared as equitoxic mixtures (Table 1), using concentrations of each fungicide that produced a similar toxic effect individually, i.e., the LC50 values which produce a 50% lethality rate (Fulladosa et al., 2005). Five concentration grades (1–5 with increasing concentrations) of equitoxic binary mixtures were designed with intervals equally spaced on a log scale (OECD/OCDE, 2015), and are shown in Table 1. The median lethal concentration (LC50) and the median teratogenic concentration (TC50) were selected as indicators of lethal and teratogenic toxicity for fungicides, respectively.

The joint effects were presented as the sum of toxic units (TU) as