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Evaluation of the toxic response induced by azoxystrobin in the non-target green alga *Chlorella pyrenoidosa*☆

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ABSTRACT

The top-selling strobilurin, azoxystrobin (AZ), is a broad-spectrum fungicide that protects against many kinds of pathogenic fungi by preventing their ATP production. The extensive use of AZ can have negative consequences on non-target species and its effects and toxic mechanisms on algae are still poorly understood. In this work, *Chlorella pyrenoidosa* that had been grown in BG-11 medium was exposed to AZ (0.5–10 mg L^{-1}) for 10 d. The physiological and molecular responses of the algae to AZ treatment, including photosynthetic efficiency, lipid peroxidation level, antioxidant enzyme activities, as well as transcriptome-based analysis of gene expression, were examined to investigate the potential toxic mechanism. Results shows that the photosynthetic pigment (per cell) increased slightly after AZ treatments, indicating that the photosystem of *C. pyrenoidosa* may have been strengthened. Glutathione and ascorbate contents were increased, and antioxidant enzyme activities were induced to relieve oxidative damage (e.g., from lipid peroxidation) in algae after AZ treatment. Transcriptome-based analysis of gene expression combined with physiological verification suggested that the 5 mg L^{-1} AZ treatment did not inhibit ATP generation in *C. pyrenoidosa*, but did significantly alter amino acid metabolism, especially in aspartate- and glutamine-related reactions. Moreover, perturbation of ascorbate synthesis, fat acid metabolism, and RNA translation was also observed, suggesting that AZ inhibits algal cell growth through multiple pathways. The identification of AZ-responsive genes in the eukaryotic alga *C. pyrenoidosa* provides new insight into AZ stress responses in a non-target organism.

The main finding of the work

Azoxystrobin (AZ) treatment did not inhibit ATP generation in *C. pyrenoidosa*. It did, however, significantly alter amino acid metabolism, which is considered to be a key AZ target in this algal species.

1. Introduction

Strobilurins are potent antifungal agents that were first reported in 1977 and were structurally modified to produce strobilurin-type fungicides (Anke et al., 1977; Bartlett et al., 2002). In 2015, strobilurins collectively accounted for 27% of the fungicide sales worldwide (Casida and Durkin, 2017). Azoxystrobin (AZ), is the top-selling strobilurin (with $1.2 billion in global sales reported for 2014) and is a broad-spectrum fungicide that protects against ascomycetes, deuteromycetes, basidomycetes, and oomycetes (Casida and Durkin, 2017). Azoxystrobin inhibits fungal growth by blocking electron transfer at the cytochrome *bc*1 complex between cytochrome *b* and cytochrome *c*1, ultimately preventing ATP generation, mitochondrial respiration, and energy production (Balba, 2007; Bartlett et al., 2002).

The extensive use of AZ-containing formulations, e.g., Amistar®, Quadris®, Heritage®, and Priori®, would inevitably result in remnants of the fungicide remaining in the air, soil, or water after field application, and may contribute to water pollution through spray drifts, surface runoff and leaching processes (Liu et al., 2015; Rodrigues et al., 2013). In aquatic environments, pesticides can easily dissolve in the water, bind to sediments or suspended solids

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(Lv et al., 2017), and then get transferred to various organisms and their tissues through bioaccumulation processes, resulting in negative consequences for non-target species (Rodrigues et al., 2013). The residual quantities of AZ in various freshwater environments (i.e., streams, ponds, groundwater, and lakes distributing in Denmark, Germany, France, and other countries) were found to range from 0.01 to 29.70 μg L^{-1} (Jorgensen et al., 2012; Liess and von der Ohe, 2005; Rodrigues et al., 2013). Although AZ was reported to have low toxicity in mammals, birds, and bees, it was found to be harmful to some freshwater species, having effects such as growth suppression in *Daphnia magna* and algae (230–320 μg L^{-1}), expression inhibition of growth- and energy-related genes in grass carp (50–250 μg L^{-1}), and even reproductive toxicity in adult zebrafish (20–200 μg L^{-1}) (Bartlett et al., 2002; Cao et al., 2016; Liu et al., 2013; Ochoa-Acuna et al., 2009).

Algae play important roles in the primary production of aquatic ecosystems. Different algae have significantly different tolerances to AZ. For example, the median inhibitory concentration (IC_{50}) of AZ for the unicellular green alga *Pseudokirchneriella subcapitata* is 230 μg L^{-1} (Ochoa-Acuna et al., 2009), but the EC_{50} value of AZ for *Anabaena flosaquae* was as high as 120–140 mg L^{-1} (US-EPA, 2012). Liu et al. (2015) reported that AZ could induce severe oxidative stress and affect energy-photosynthesis-related mRNA expression in *Chlorella vulgaris*. Although these studies show that AZ can display inhibitory effects on algal growth, data on its toxicology is still limited and the toxic mechanism of AZ on algae is unknown.

In this study, we used the microalgal species *Chlorella pyrenoidosa* and *Microcystis aeruginosa* as representatives of eukaryotic and prokaryotic algae, respectively. In a preliminary experiment, it was found that 0.5–10 mg L^{-1} AZ could inhibit the growth of *C. pyrenoidosa* but had no negative effects on *M. aeruginosa*; therefore, we chose *C. pyrenoidosa* as the study species to investigate the potential toxic targets of AZ. We examined the effects of AZ treatment on *C. pyrenoidosa* growth, subcellular structure, lipid peroxidation level, antioxidant contents, antioxidant enzyme activities, photosynthetic efficiency variation (photosynthetic pigment contents and F_v/F_m value), and used a transcriptome-based analysis of gene expression to reveal the mechanism underlying the physiological changes induced by AZ treatment in eukaryotic algal cells. The present study aimed to provide insights into the potential toxic mechanisms of AZ on eukaryotic algae.

2. Material and methods

2.1. Microalgae and growth conditions

The green alga *C. pyrenoidosa* (FACHB-9) and the freshwater cyanobacterium *M. aeruginosa* (FACHB-905) were provided by the Institute of Hydrobiology at the Chinese Academy of Sciences (Hubei, China). The tested species were maintained as stationary cultures in 250 mL Erlenmeyer glass flasks containing 150 mL of BG-11 medium (initial pH = 7.1) at 22 °C (±0.5 °C) under cool-white fluorescent light (≈46 μE m^{-2} s^{-1}) with a photoperiod of 12:12 h light: dark cycle. The BG-11 medium and all other materials used in the maintaining and testing of the algal cultures were autoclaved at 121 °C for 25 min. Azoxystrobin was purchased from Aladdin (Shanghai, China) and stock solutions (50 g L^{-1}) were diluted in acetone. The final concentration of acetone was 0.01% (v/v) in treatment and control groups, and this amount of acetone was assumed to have negligible influence on algal growth (Tang et al., 1997).

2.2. Measurement of algal cell yield after AZ treatment

In our pre-test, the initial AZ concentrations below 0.5 mg L^{-1} did not have an inhibition effect on *C. pyrenoidosa* or *M. aeruginosa*, however, concentrations greater than 10 mg L^{-1} were seen to restrain the growth of *C. pyrenoidosa.* Based on these findings, we chose to use the initial test AZ concentrations: 0.5, 1, 2.5, 5, and 10 mg L^{-1}. The cell density of the culture was measured every 24 h until the tenth day, using a spectrophotometer at an optical density of 680 nm (OD_{680}). The following regression equation was used to estimate cell density: y, in $\times 10^5$ cells·mL^{-1}, the OD_{680} was (x): $y = 186.512x - 1.263$ ($R^2 = 0.9734$). A standard curve that expressed cell density as a function of OD_{680} was generated using a standardized diatom culture and a hemocytometer. The initial cell density of each experiment was standardized at approximately 4.3×10^4 cells·mL^{-1}. All cultures were manually agitated three times a day. Data are representative of four replicates performed for each bioassay.

2.3. Cellular and subcellular structure observation

Algal cells in the control and 5 mg L^{-1} AZ treatment groups were harvest after 96 h, and then fixed with 2.5% glutaraldehyde at 4 °C for 12 h. The fixed cells were washed and dehydrated following the method described in Song et al. (2017). The dehydrated samples were then embedded in epoxy resin. Ultrathin sections were obtained using a ultramicrotome and then observed in a Hitachi Model H-7650 transmission electron microscope (TEM) (Tokyo, Japan). The dehydrated samples were coated with gold-palladium in a Hitachi Model E–1010 ion sputter for 4–5 min and observed in a Hitachi Model E–1000 scanning electron microscope (SEM) (Tokyo, Japan).

2.4. Analysis of photosynthetic pigment and chlorophyll fluorescence

Samples of *C. pyrenoidosa* that had been treated with 0, 2.5, and 5 mg L^{-1} AZ for 96 h were collected to measure chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), and the total chlorophyll (total-Chl) content with *N*,*N*-Dimethylformamide (DMF), and measured as described by Inskeep and Bloom (1985). The photosystem II (PSII) activity of algal cultures was measured with a MINI-PAM-II system (Heinz Walz GmbH, 910090 Effeltrich, Germany) equipped with actinic light. All the samples were dark treated for 15 min before measurements were taken. The maximum quantum yield of PSII was evaluated using the following: $F_v/F_m = F_m - F_0/F_m$, where F_0 and F_m represent the minimal fluorescence and the maximum fluorescence signal, respectively.

2.5. Protein content, malondialdehyde, and enzyme activity assays

After 96 h treatment with 0, 2.5, and 5 mg L^{-1} AZ, 40 mL of the algal cultures were collected and centrifuged at 7000 × g for 10 min at 4 °C. The samples were homogenized on ice with 1 mL cold PBS buffer. The homogenate was further centrifuged at 4000 × g at 4 °C for 15 min to obtain the supernatant used in the following assays: the protein content, glutathione (GSH) content, malondialdehyde (MDA) content, antioxidant enzyme superoxide dismutase (SOD) activity, and peroxidase (POD) activity. Results were determined using commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The supernatant was processed according to the instruments of the MDA assay kit. The absorbance of red thiobarbituric acid-MDA complex was measured at 532 nm and the MDA content (nmol/10^8 cells) was calculated according to detailed instructions of the