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Azoxystrobin Causes Oxidative Stress and DNA Damage in the Aquatic Macrophyte *Myriophyllum quitense*

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Abstract Among the search for new types of pesticides, the fungicide azoxystrobin (AZX) was the first patent of the strobilurin compounds, entering in the market in 1996. Its use worldwide is growing, mainly linked to soybean production, although its effects in non-target organisms are almost unknown. The goal of the present work was to evaluate effects of short-term AZX exposure to the aquatic macrophyte *Myriophyllum quitense*, focusing on oxidative stress parameters and DNA fragmentation. Significant inhibition of the antioxidant enzyme systems were observed at 50 μg/L AZX for catalase and peroxidase ($p < 0.05$). Lipid and DNA damage were significant at 50 and 100 μg/L AZX. These biomarkers were sensitive to AZX and can be used in a battery to evaluate the occurrence of AZX in freshwater ecosystems.

Keywords Strobilurin fungicide · Antioxidant enzymes · Lipid peroxidation · Comet assay

The impact of strobilurin fungicides on agriculture is reflected by the widespread use of azoxystrobin (AZX), a chemical which has been approved for use on more than 80 different crops representing over 400 crop/disease systems (Bartlett et al. 2001). Fungicide use on crops in the US was estimated to be 52,000 metric tons per year in 2002, and at that time, less than 1 % of US soybean acres were treated with a fungicide (Battaglin et al. 2011). By 2009, 14 fungicides were registered for treatment of soybean rust because cultural practices have little or no effect on that disease (Battaglin et al. 2011). Currently fungicides are the only option for managing the fungus until disease-resistant soybean varieties are developed (Deb et al. 2010). AZX is expected to be mobile in the environment because it is highly water soluble, slow to degrade in soil and water, and unlikely to volatilize (Battaglin et al. 2011). AZX bioconcentration factors, bioaccumulation potential or biomagnification values have been scarce in the literature (Rodrigues et al. 2013). Currently, its impact on non-target species is not fully known (Deb et al. 2010) and its use has created concern because the mode of action is not specific to just fungi. AZX concentrations in run-off waters range from 1 to 30 μg/L (Berenzen et al. 2005; Deb et al. 2010; Battaglin et al. 2011), levels considered toxic to calanoid copepods (Gustafsson et al. 2010) and fish like the Atlantic salmon (Olsvik et al. 2010). In relation to genotoxicity, AZX showed to be clastogenic in invertebrates (Han et al. 2014) as well as in vertebrates (Bony et al. 2008). Most of the reports available in the literature show effects of AZX in aquatic vertebrates and invertebrates, but the information on aquatic macrophytes effects are scarce.

The aquatic macrophyte *Myriophyllum aquaticum* has been used for testing in sediment toxicology (Maltby et al. 2010), and its relative sensitivity and recovery potential when exposed to some pesticides like the herbicide atrazine (Teodorovic et al. 2012) have been examined. *M. quitense,* which is a representative species from freshwater ecosystems in North, Central and South America (Orchard 1981), has been used for biomonitoring water quality in Argentina (Nimptsch et al. 2005) as well as for assessing the effects of endosulfan (Menone et al. 2008). Currently, the primary parameters employed for toxicity testing in plants are

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growth rate and biomass measurements. However, because toxicity in plants is first manifested at the biochemical level before whole organism effects are evident, biochemical effect parameters are typically more sensitive and can be early indicators of xenobiotic stress (Ferrat et al. 2003). Brain and Cedergreen (2008) reviewed the groups of contaminants that have been evaluated by a given biomarker. Fungicides are only linked to stress proteins, denoting the lack of studies carried out for their potential biomarker use. Taking into account the current use of AZX and the lack of information on its effects in aquatic plant species, the goal of the present work was to evaluate possible effects of short-term AZX exposure in the aquatic macrophyte *M. quitense*, focusing on antioxidant enzyme activities, lipid peroxidation, and genotoxicity to determine if this fungicide induces oxidative damage.

Materials and Methods

Myriophyllum quitense was obtained from Estación Experimental Agropecuaria Balcarce (INTA), Argentina. Species identification was done according to Orchard (1981). Plants were acclimated prior to the experiments for 15 days in 30 L tanks containing Hoagland's medium (pH = 5). Supplementary light was provided by fluorescence lamps with an irradiance of 100–120 μE m^{-2} s^{-1} at a light/dark cycle of 12:12 h. Room temperature was maintained at 20–22°C. Approximately 10 g fresh weight of *M. quitense* was exposed to concentrations of 0, 0.1, 1, 10, 50 or 100 μg/L AZX [(methyl (E)-2-2-(6-(2-cyanophenoxy) pyrimidine-4-yloxy) phenyl-3-methoxyacrylate)] (CAS No. 131860-33-8) (Fluka, Germany) in a volume of 350 mL each, for 24 h, under constant light and temperature conditions. AZX was dissolved in dimethyl sulfoxide (DMSO, Mallinckrodt) in a stock solution of 2,000 mg/L. Afterwards each exposure concentration was prepared by diluting the appropriate amount of AZX in Hoagland medium to a final volume of 350 mL. In the negative control treatment (Co-) AZX was omitted from the medium, but pure DMSO was added. DMSO in water solutions never exceeded 0.005 %. One positive mutagenic control (CO^{+}) consisting of 7.14 g/L of hydrogen peroxide was also included. After exposures, plants were rinsed with bidistilled water to remove AZX adsorbed on the surface and immediately frozen using liquid N_2, before being stored at −80°C until processing.

Preparation of soluble enzymes was performed according to Pflugmacher (2004). Three grams of leaf material were used in five independent preparations. Plants were ground with mortar and pestle under liquid nitrogen before 10 mL of sodium-phosphate buffer (0.1 M, pH 6.5), containing 20 % glycerol, 14 mM of dithioerythritol (DTE) and 1 mM of ethylenediamine tetraacetic acid (EDTA) were added. Cell debris was removed by centrifuging at 10,000*g* for 10 min. Protein determination was done according to Bradford (1976) using bovine serum albumin as the standard. Enzyme activity was calculated in nanokatals per milligram of protein from percent substrate conversion. Measurement of the peroxidase (POD) activity using guajacol as the substrate was performed as described by Drotar et al. (1985). Determination of glutathione S-transferase (GST) activity with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) was done according to Habig et al. (1974), and catalase (CAT) activity was assayed according to Claiborne (1985). Lipid peroxidation was measured in terms of tiobarbituric acid reactive species (TBARS) concentration according to Shi et al. (2006). A leaf sample of 0.3 g was homogenized in 3 mL of 0.1 % (w/v) trichloroacetic acid (TCA), centrifuged at 10,000*g* for 10 min, and 3 mL of 20 % TCA containing 0.5 % (w/v) thiobarbituric acid (TBA) was added to 1 mL of supernatant. The mixture was heated at 95°C for 30 min, placed into an ice-bath and centrifuged at 10,000*g* for 10 min, and the absorbance of the supernatant was read at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the TBARS concentration was calculated by its extinction coefficient of 155 mM/cm.

The alkaline protocol of the plant Comet (Single Cell Gel Electrophoresis) Assay was calibrated for detecting DNA damage in *M. quitense*. Leaves were cut, fragmented to small pieces with scissors and homogenized in an Eppendorf tube containing phosphate-buffered saline (PBS), 0.5 M EDTA, 10 % DMSO and 1 % Triton. The homogenate was filtered through a layer of cotton tissue and centrifuged at 130*g* for 5 min. The pellet was re-suspended in a buffer containing 1 mM $MgCl_2$, 1× PBS buffer, and S-buffer (1 M sorbitol, 25 mM phosphate buffer, pH 6.5 and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Because the photosynthetic active plant tissues have higher level of pigments and secondary metabolites which deteriorate the isolated plant nuclear fraction, a sucrose cushion was used in order to isolate the nuclear fraction intact (Peycheva et al. 2011). Therefore, centrifugation at 2,000*g* for 10 min was accomplished through a sucrose cushion containing 320 mM sucrose in 1× PBS and 1 mM $CaCl_2$. Afterward, the nuclear pellet was re-suspended in S-buffer. Slides were prepared with a layer of agarose normal melting point (NMPA) 1 %, an aliquot of the extract was mixed with agarose low melting point (LMPA) 1 %, and finally a layer of 1 % LMP agarose was added. These preparations were subjected to alkaline electrophoresis, the slides were submerged during 10 min in a denaturing solution (1 mM Na_2EDTA and 300 mM NaOH), and after that, electrophoresis was run in the same solution at 0.72 V/cm for 30 min. Neutralization of the

gels in buffer Tris (400 mM) pH 7.5 was performed. Gels were silver stained according to Nadin et al. (2001) for optic microscope observation using a microscope OLYMPUS CX31. The quantification of the level of DNA damage was made by measuring the relative length of the tail of the comet. Six independent preparations were prepared for each treatment, and 50 nucleoids were analyzed for each slide. Each nucleoid was classified into five classes according to tail size (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (Damage Index, DI) for each slide. The DI was calculated as follows: $DI = n_1 + 2n_2 + 3n_3 + 4n_4$; where n_1, n_2, n_3 and n_4 are the number of cells in class 1, 2, 3 and 4 of damage respectively (Poletta et al. 2009).

Since the studied variables failed both normal distribution and variance homogeneity, data sets were described using the median as the measure of central tendency. The Kruskal–Wallis non-parametric test was applied and, a posteriori, differences among treatments were tested by the Dunn test (Zar 1999). Statistical analyses were determined at a 0.05 % significance level.

Results and Discussion

A general inhibition response of the antioxidant enzyme systems was observed, although it was only significant at 50 µg/L AZX for CAT and POD ($p < 0.05$) (Fig. 1a, b). The activity of GST did not show changes in comparison to controls ($p > 0.05$) (Fig. 1c). In addition, lipid peroxidation-measured as TBARS content-increased at 50 and 100 µg/L AZX, being threefold-higher in comparison to controls ($p < 0.05$) (Fig. 1d). Oxidative stress due to AZX has been previously reported for terrestrial agronomic plants like spring barley and wheat in which changes in CAT and POD were observed (Wu and von Tiedeman 2002; Zhang et al. 2010). The inhibition of CAT detected in *M. quitense* has been previously reported to be caused by other insecticides, and could be due to: (a) the inactivation of the molecules of CAT by reactive oxygen species (ROS) (Feieraband and Engel 1986); (b) a change in assembly of enzyme subunits (Hertwig et al. 1992); or (c) a degradation of CAT by peroxisomal proteases (Sandalio et al. 2001). AZX mode of action/mechanism of toxicity is known to be the specific inhibition of mitochondrial respiration by blocking electron transfer between cytochrome b and cytochrome c1. This occurs at the ubiquinol oxidizing site (Q_0) of the mentioned complex, resulting in cellular oxidative stress triggered by electrons escaping from the mitochondrial respiratory chain (Bartlett et al. 2002). Therefore, the effect on enzyme activities observed here would be interpreted as being caused by increased levels of ROS able to inactivate CAT molecules. Together with CAT, POD belongs to the enzymatic defending system controlling membrane lipid peroxidation, but due to a threshold of enzyme activity, the protective function of them is limited (Ding et al. 2007). Afterwards, inhibition of these enzymes can cause a delay in the removal of H_2O_2, and an enhancement in lipid peroxidation under AZX exposure would be expected, as it was observed in *M. quitense*. On the other hand, in plants, AZX is extensively metabolized via biotic and abiotic reactions to 17 identified metabolites. The most abundant metabolite is cyanophenoxypyrimidinol, which is finally converted to a N-glucoside conjugate (Balba 2007). Therefore, GST activity would not be expected to catalyze conjugation of AZX with glutathione, but the current study worked under the hypothesis that GST could have an antioxidant role, because some plant-GSTs functions as glutathione peroxidases to detoxify lipid peroxides directly (Marrs 1996; Cummins et al. 1999). Under the conditions of this work, GST activity from *M. quitense* was not affected by AZX.

DNA damage, measured as DNA fragmentation, was significantly higher in the positive control with respect to the negative control, showing the sensitivity of *M. quitense* (Fig. 2). Increased DNA damage was observed at 50 and 100 µg/L AZX, being threefold-higher in comparison to the negative controls ($p < 0.05$) (Figs. 2, 3). The occurrence of mutagenic/genotoxic compounds in aquatic ecosystems was first perceived in the 1970s, leading to one of the first toxicity test as developed by Ames (Ames et al. 1975). Since that time, several tests have been developed for evaluating DNA alterations in aquatic organisms, which include the comet assay (Singh et al. 1988), applied to ecotoxicology about 15 years ago. In the plant kingdom the first reports were done by Koppen and Verschaeve (1996); Gichner (1997). Several authors stated that genotoxicity induced by environmental pollutants may involve the interaction of them with DNA, either directly or indirectly via the induction of oxidative stress (Gichner et al. 2008; Patnaik et al. 2011). For example, Gichner (2003) reported genomic damage induced by mutagenic compounds, measured by the comet assay, in catalase-deficient tobacco plants. Interestingly, results obtained in the present work show the coincidence in AZX concentrations (50 and 100 µg/L) at which oxidative stress and DNA damage occurred, suggesting the linkage between both processes. Lastly the resulting DNA-lesions that vary from innocuous molecular changes to highly mutagenic or genotoxic alterations in the genome lead to genomic instability or genotoxic stress which may result in genetic disease, cancer, senescence, aging and change in gene expression (Patnaik et al. 2011). Increased genomic instability has been suggested to play important roles in decreased fitness of plant and animal populations (Jha 2008).

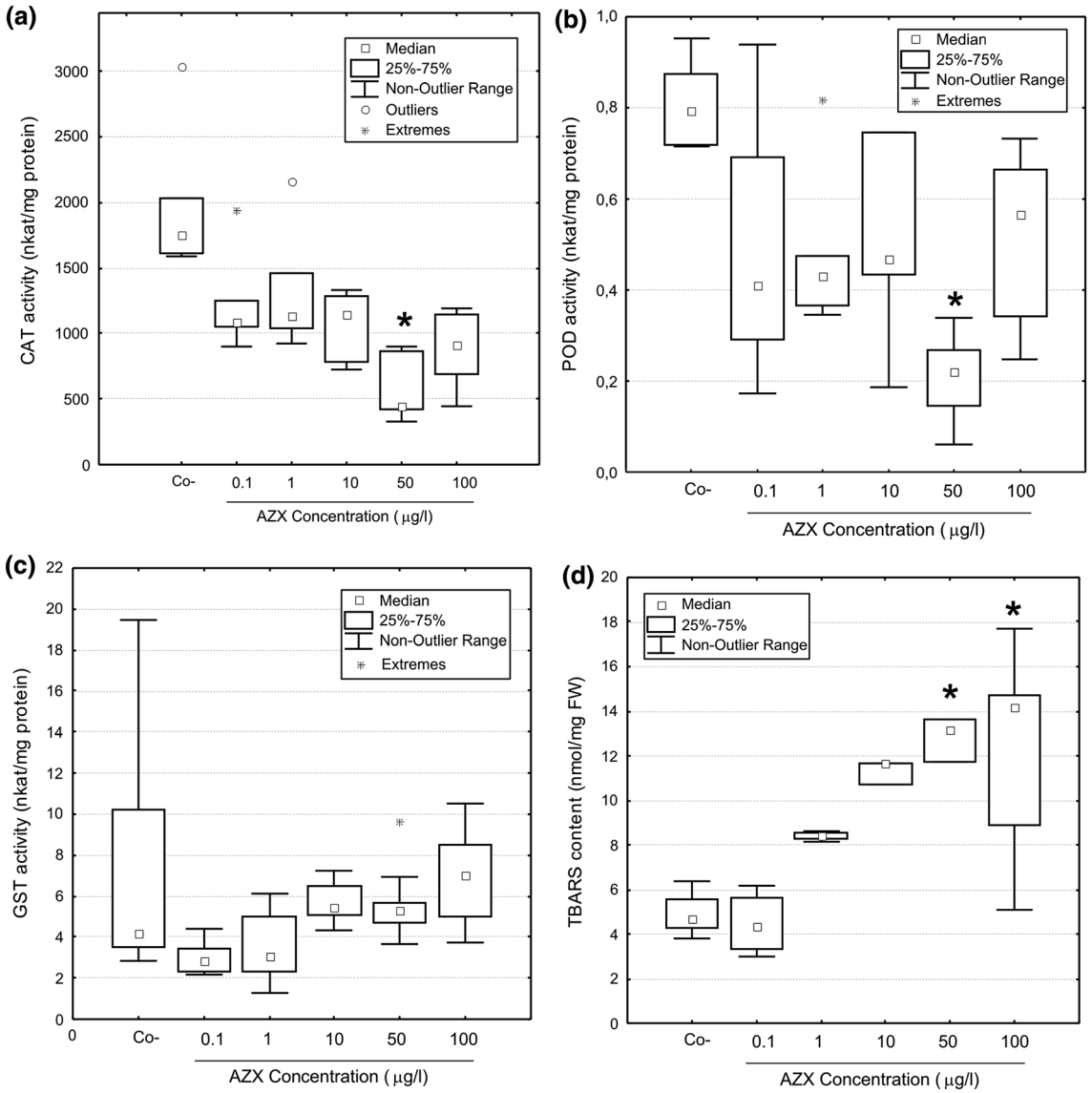

Fig. 1 Oxidative stress biomarkers in *Myriophyllum quitense* exposed to azoxystrobin (µg/L). **a** Catalase (CAT), **b** guaiacol peroxidase (POD), **c** glutathione-S-transferase (GST), **d** lipid peroxidation (TBARS content). *Significantly different from the control (Kruskall–Wallis with post hoc Dunn's test) ($p < 0.05$)

AZX is considered stable to hydrolysis in the pH range of 4–9, appearing to degrade at a slightly faster rate at alkaline pH; and is degraded only slowly by photolysis (Komárek et al. 2010; Rodrigues et al. 2013). At 20°C, the solubility of AZX in water is 6.7 mg/L (pH 5.2 and 7.0) and it has a low vapor pressure of 1.1×10^{-7} mPa (Rodrigues et al. 2013). AZX dissition rates from water with half-life values of 18 days and 15–25 days were also determined by Zafar et al. (2012); Gustafsson et al. (2010), respectively. Analytical measurements of AZX were not carried out in the aqueous media of exposure used but the design of the present study was made taking into account the physicochemical characteristics of AZX detailed above. According to that, static conditions were appropriated in a 24 h-bioassay; and also similar measured- and nominal-concentrations of the fungicide would be expected. Effects observed should be attributable to a range of concentrations close to 50–100 µg/L AZX, the nominal concentrations at which oxidative stress and genotoxicity were observed. Few studies have investigated the occurrence of AZX in surface waters, being one of the causes that the analytical methodology for its determination is from 2007 on (Rodrigues et al. 2013). Therefore, analytical development for measuring realistic concentrations of AZX in the natural water samples deserves more attention. In addition, because few data on AZX toxicity to different aquatic

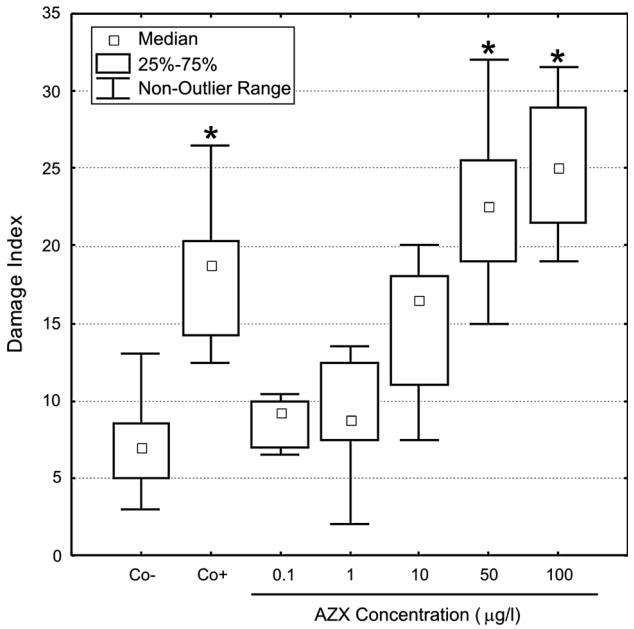

Fig. 2 Damage index calculated from the comet assay in *Myriophyllum quitense* exposed to azoxystrobin (µg/L) *Significantly different from the negative control (Kruskall–Wallis with post hoc Dunn's test) ($p < 0.05$)

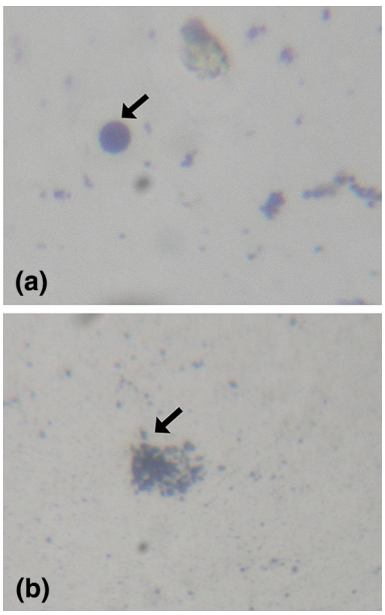

Fig. 3 Undamaged nucleoid (class 0) **a** (amplification ×160) and damaged nucleoid (class 2) **b** (amplification ×160) in *Myriophyllum quitense* exposed to azoxystrobin

organisms is available, further studies are necessary to know effects of AZX in aquatic non-target organisms such as *M. quitense*.

In conclusion, results of the present study demonstrate that cells of the aquatic macrophyte *M. quitense* were significantly sensitive to short-term exposure to AZX. In *M. quitense*, AZX decreased the activity of key enzymes of the antioxidant system of cells such as CAT and POD and caused lipid oxidative damage, indicating the effect of oxidative stress. DNA damage was observed constituting the first report of clastogenicity of AZX in plants. Therefore, all these parameters may be sensitive biomarkers of AZX in aquatic ecosystems and can be used in a larger assessment to know its effects at environmentally relevant concentrations.

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