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ScienceDirect
Phytochemistry 69 (2008) 1150–1157
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Endosulfan induces oxidative stress and changes on detoxication enzymes in the aquatic macrophyte *Myriophyllum quitense*

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Received 19 April 2007; received in revised form 15 November 2007
Available online 7 January 2008

Abstract

Endosulfan (**1**) is a chlorinated insecticide still in use in both developed and emerging countries. Although its toxicity on animals has been studied in the last years, scarce information is available on its effects on plants. In this study, we exposed the aquatic macrophyte *Myriophyllum quitense* to environmentally relevant concentrations of endosulfan (μg/L) (**1**) for a short time, simulating exposures that might occur after either accidental spills or toxic run-off from agricultural areas. The main goal was to evaluate changes in both detoxication and antioxidant enzymatic systems of this plant upon exposure to endosulfan (**1**). Thus, we measured the activities of catalase (CAT), soluble and membrane associated glutathione-S-transferases (s- and m-GSTs) and glutathione reductase (GR), as well as the hydrogen peroxide (H_2O_2) content. Results showed that endosulfan (**1**) exerts oxidative stress on *M. quitense*, which was evidenced by the increase of CAT activity and the H_2O_2 content in exposed plants. At 5 μg/L endosulfan (**1**), we found a generalized induction of activities of tested enzymes, indicating that this xenobiotic activates the protection system of this plant, increasing its capacity to scavenge reactive oxygen species. On the other hand, we did not find significant changes at 0.02 μg/L endosulfan (**1**), which is the maximal concentration allowed for freshwater. We conclude that runoff events, which can produce significant amounts of endosulfan (**1**) in aquatic environments during short time, can result in oxidative stress on *M. quitense*, and probably on similar macrophytes.

Keywords: *Myriophyllum quitense*; *Haloragaceae*; Catalase; Glutathione-S-transferases; Hydrogen peroxide; Insecticide endosulfan

1. Introduction

Endosulfan (**1**) is a chlorinated insecticide belonging to the cyclodiene subgroup, which has been widely used due to a decline in the application of other organochlorine insecticides such as endrin and DDT (Dubois et al., 1996) (see Fig. 1). It is still extensively used in Europe, USA (Vorkamp et al., 2004) and other parts of the world like Argentina (Jergentz et al., 2004). Its presence in surface water is a concern because of its potential adverse impact on humans and aquatic life. According to the USA environmental protection agency (EPA), endosulfan (**1**) has adverse impact on the health of aquatic organisms at concentrations above 0.22 μg/L (acute) and 0.05 μg/L (chronic) (Mersie et al., 2003).

It has been reported that the run-off from fields treated with this insecticide can contain high concentrations of

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doi:10.1016/j.phytochem.2007.11.016

endosulfan (**1**), ranging from 1 to over 100 μg/L, with an average concentration of 13.4 μg/L (Antonious and Byers, 1997; Mersie et al., 2003). Values from other places of important application such as Australia range from 2.5 to 45 μg/L (Kennedy et al., 2001; Leonard et al., 2001) and in the Ganga River (India) they can reach 66.5 μg/L (Selvakumar et al., 2005).

Although there is scarce data available on the concentration of endosulfan (**1**) from natural aquatic ecosystems in Argentina, its presence has been reported in suspended particles and run-off sediments (Jergentz et al., 2004). Additionally, the presence of endosulfan sulfate (**2**) (a metabolite of this insecticide) has been reported in biota and bottom sediment by our group (Menone et al., 2000, 2001).

Dubois et al. (1996) discovered selectively increased levels of CYP3A-family transcripts in rat hepatic, as well as in Hep G2 human hepatoma, cells after exposure to endosulfan (**1**). Yadwad (1989) proved the induction of glutathione-S-transferase (GST) activity by sublethal dose of this insecticide in crabs. Additionally, Kalender et al. (2004) demonstrated enhanced activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in rats treated with endosulfan (**1**). Ramachandran et al. (1984) have reported the effects of endosulfan (**1**) on photosynthesis and respiration in marine macrophytes. Moreover, DeLorenzo et al., 2002 reported EC_{50} 427.8 μg/L in the freshwater green alga *Pseudokirchneriella subcapitatum* and 24 h immobilization EC50 of 366.3 μg/L; 5 h EC_{50} for filtration and ingestion 165.6 and 166.44 μg/L, respectively in *Daphnia magna.* On the other hand, LC_{50} 96 h = 1.5 μg/L have been determined for rainbow trout (Ferrando et al., 1991 in Dorval et al., 2003).

There is a surprising similarity to the metabolic pathways of xenobiotic metabolism in mammalian liver. This has led to the "green liver" concept of plant xenobiotic metabolism (Sandermann, 1994). Plant metabolic sequences of biotransformation is mostly done by cytochrome P-450 monooxygenases (phase I), followed by conjugation (e.g. through glutathione-S-transferases and glucosyltransferases) (phase II), and internal compartmentation reactions, occurring mostly in cell wall fractions or in the vacuole (phase III) (Sandermann, 1994). Recently, Fatima and Ahmad (2006) demonstrated enhanced activity of ethoxyresorufin-O-deethylase (EROD) (a phase I enzyme) in the terrestrial plant species *Allium cepa* exposed to endosulfan (**1**). However, to the extent of our knowledge, the effects of endosulfan (**1**) on antioxidant enzyme system as well as changes in the activities of phase II-detoxication in plants have not been characterized.

Basic cellular metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical reactive species (reactive oxygen species; ROS), including superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) (Valavanidis et al., 2006). The imbalance between the generation and the neutralization of ROS by antioxidant mechanisms within an organism is called oxidative stress (Davies, 1995). Although a re-evaluation of this concept takes place nowadays, and it could be more useful to describe it as "oxidative signalling" (Foyer and Noctor, 2005), the fact that high concentrations of ROS trigger genetically programmed cell suicide events, and the discovery of the importance of free radical damage in the mechanisms of toxicity of many environmental pollutants (xenobiotics), lead to an increased study and application of biomarkers of oxidative stress in living organisms.

Antioxidant enzymatic activities (e.g. catalase) in plants have been considered as relevant endpoints in different stress situations, because this enzymatic system neutralizes and scavenges ROS, protecting cellular membranes and organelles from oxidative damage. However, to date, there is no report of ROS production upon exposure of plants to endosulfan (**1**).

Glutathione-S-transferases (GST) constitute a complex family of proteins, grouped into six classes in plants, with a large range of functions (Foyer and Noctor, 2005). They play roles in both normal cellular metabolism and in the detoxification of a wide variety of xenobiotic compounds. GST has been intensively studied with regard to the detoxication of herbicides in plants. GST conjugate reduced glutathione (GSH) to electrophilic xenobiotics as well as endogenously produced electrophiles such as membrane lipid peroxides. Some GST functions as glutathione peroxidases to detoxify such products directly (Marrs, 1996; Cummins et al., 1999).

The aquatic macrophyte genus *Myriophyllum* has a cosmopolitan distribution. Nimptsch and Pflugmacher (2005) highlighted the need to understand biotransformation of xenobiotics as well as the oxidative stress metabolism of different species of this genus, due to its potential use as biosensor. It is also worthy to remark the importance of using early warning systems (biomarkers) when monitoring aquatic ecosystems.

In this study we exposed the aquatic macrophyte *M. quitense* to environmentally relevant concentrations of endosulfan (**1**) for a short period of time. Thus, we looked to simulate exposures that might occur after accidental spills or run-off events from agricultural areas. Our main goal was to evaluate the induction of oxidative stress by this insecticide in *M. quitense*. We studied changes in the activity of several enzymes related to antioxidant response, phase-II detoxication, and H_2O_2 content. Because of their importance in oxidative stress responses and phase-II detoxication process, we focused on the evaluation of changes in the activities of membrane associated and soluble glutathione-S-transferases (m- and s-GSTs), glutathione reductase (GR) and catalase (CAT).

2. Results

DMSO did not affect the activity of CAT, s-GST, m-GST, GR or H_2O_2 content, since no differences between the DMSO-control and the H-control were detected (data not shown).

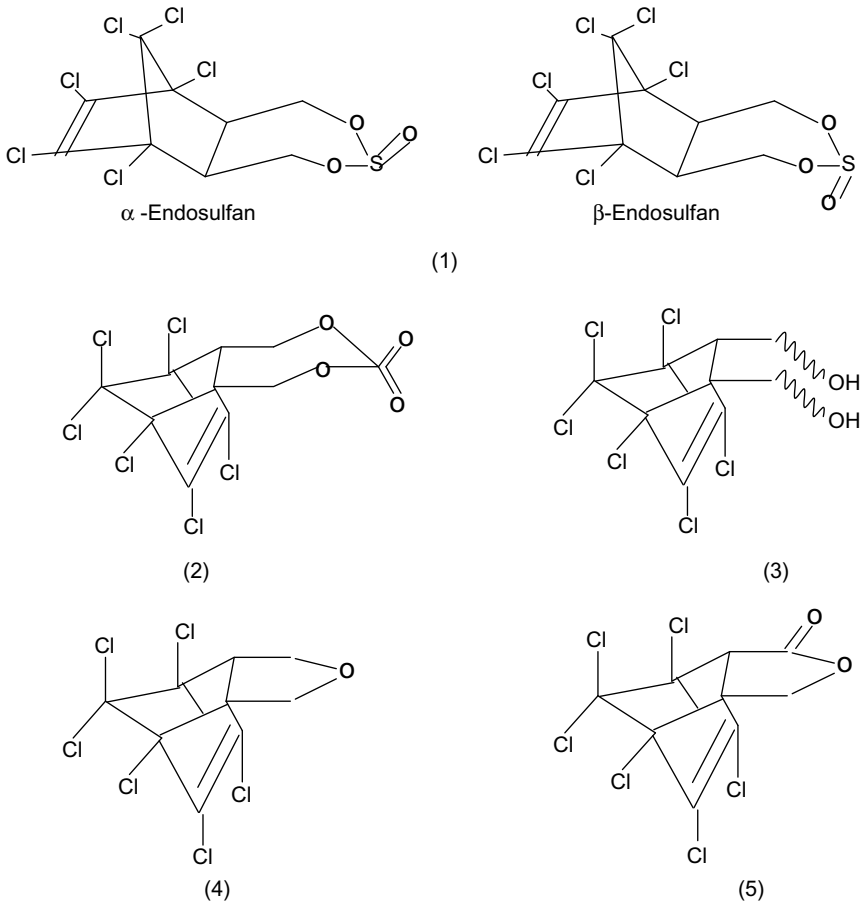

Fig. 1. Chemical structure of α- and β-endosulfan isomers (1), endosulfan sulfate (2), endosulfan diol (3), endosulfan ether (4) and endosulfan lactone (5).

At 5 μg/L endosulfan (**1**), a general and significant induction of activity of the enzymes tested was found, while at 0.02 μg/L activities of all enzymes were not different from control plants (Figs. 2–4).

The concentration-response relationship plot corresponding to CAT is shown in Fig. 2, where it is evident of a significant ($p < 0.05$) increase of the enzymatic activity in plants exposed to 0.5 and 5 μg/L endosulfan (**1**) (2-fold and 2.9-fold, respectively).

The concentration-response relationships for m- and s-GST are shown in Figs. 3A and B. The activities displayed a trend to become more intense as endosulfan (**1**) concentration was increased. Significant ($p < 0.05$) increase of activity (2.5-fold for m-GST and 2.2 fold for s-GST) was observed at 0.5 μg/L endosulfan (**1**). Similarly, the activities showed a significant ($p < 0.05$) augment (5.8 fold for m-GST and 4-fold for s-GST) at 5 μg/L endosulfan (**1**) (Fig. 3A and 3B).

The concentration-response relationship of GR showed an elevation of the activity (3.2-fold) in plants exposed to 5 μg/L endosulfan (**1**) (Fig. 4).

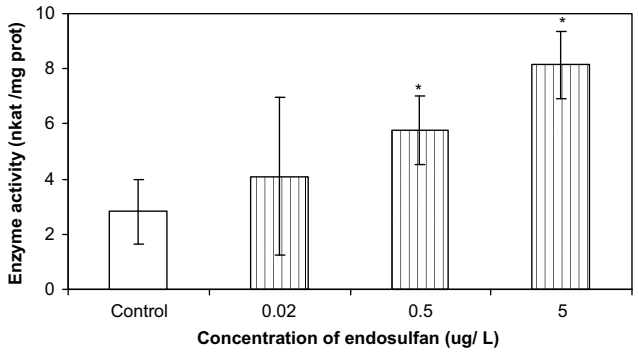

Fig. 2. Concentration-response relationship of catalase activity in *Myriophyllum quitense* after exposure to endosulfan (1). Data are mean ± standard deviation. *: Significantly different to control ($p < 0.05$) $n = 9$.

2.1. H_2O_2 content

The estimation of hydrogen peroxide brought values ranging from about 8.0 to 14.9 μmol g/fr.wt. The concentration-response relationship for H_2O_2 is shown in Fig. 5. At 5 μg/L endosulfan (**1**), the H_2O_2 content was significantly higher than controls ($p < 0.05$), while at 0.02 and 0.05 μg/L no changes were detected.

3. Discussion

Simply determining the pollutant contents of organisms does not necessarily provide information on the toxicological significance of the body burden, or of the many factors

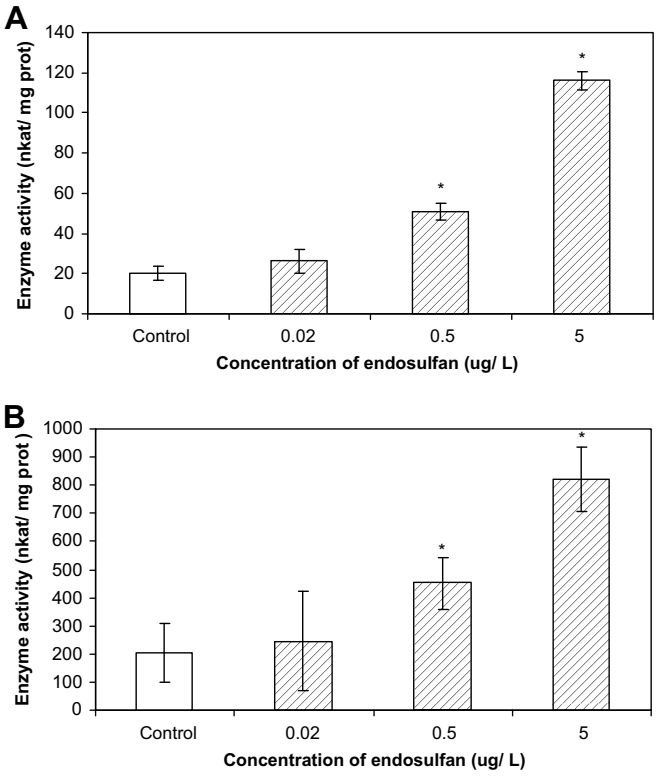

Fig. 3. Concentration-response relationship of the glutathione-S-transferase activity in (A) membrane associated fraction, (B) soluble fraction in *Myriophyllum quitense* after exposure to endosulfan (1). Data are mean ± standard deviation. *: Significantly different to control ($p < 0.05$) $n = 9$.

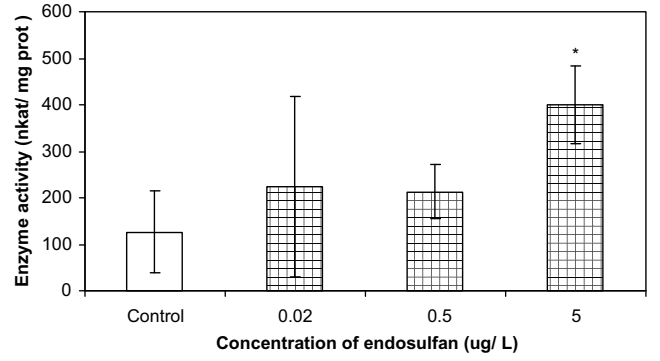

Fig. 4. Concentration-response relationship of the glutathione reductase activity in *Myriophyllum quitense* after exposure to endosulfan (1). Data are mean ± standard deviation. *: Significantly different to control ($p < 0.05$) $n = 9$.

that can influence contaminant accumulation. An alternative and potentially more useful approach is the use of indexes of sublethal stress, or biomarkers (Padinha et al., 2000). Nimptsch and Pflugmacher (2005) investigated induction of GST activity in five different species of *Myriophyllum* and recommended the use of this endemic plant species for active monitoring, due to the invasive character of the non-native species of this macrophyte genus. GST in *M. quitense* has been demonstrated to be a powerful tool

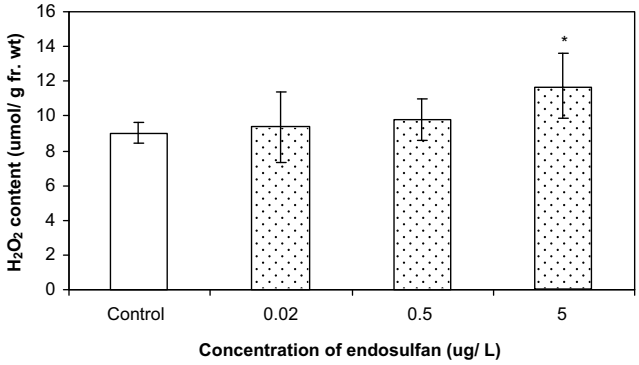

Fig. 5. Concentration response relationship of H_2O_2 content in *Myriophyllum quitense* after exposure to endosulfan (1). Data are mean ± standard deviation. *: Significantly different to control ($p < 0.05$) $n = 12$.

for the identification of aquatic sites with different pollution levels (Nimptsch et al., 2005). Biomarkers should react to environmentally realistic concentrations, showing a good dose-response to different levels of pollution (Au, 2004). On the other hand, a battery of biomarkers could be necessary to demonstrate evidence for different processes and damage induced by diverse pollutants. During this work, we thus evaluated the induction of CAT, m- and s-GST, GR and H_2O_2 levels as representative of biomarkers signalling both oxidative stress and detoxication activity upon acute exposure to endosulfan (**1**).

CAT is a hematin-containing enzyme that facilitates the removal of hydrogen peroxide (H_2O_2), which is metabolised to molecular oxygen (O_2) and water. Unlike some peroxidases that can reduce various lipid peroxides as well as H_2O_2, CAT can reduce only H_2O_2 (van der Oost et al., 2003). It has been demonstrated that CAT is a part of the antioxidant defence system in mussels exposed to lindane (Khessiba et al., 2005). The induction of CAT upon exposure to 0.5 and 5 μg/L endosulfan (**1**) for 24 h, together with the enhanced levels of H_2O_2 at 5 μg/L, shows that endosulfan (**1**) might, directly or indirectly, generate an excess of ROS (e.g. H_2O_2) in *M. quitense* cells. These can not be efficiently removed by CAT, thereby leading to increased H_2O_2 levels. At the organismic level, fundamental lesions associated with ROS include oxidation of membrane lipids, proteins, nucleic acids and altered cellular redox status. These perturbations are believed to underlie specific tissue injuries associated with redox active contaminants. Numerous studies concerning the activities of antioxidant enzymes have suggested adaptative responses of antioxidant system components in aquatic organisms exposed in vivo to either conditions or xenobiotics likely to enhance oxyradical fluxes (Rand, 1995). On the other hand, in cells under a hyperoxidative condition, the enzymes could be inactived (due to its threshold in the capacity of detoxication) leading to damage and lastly to cell death. Although it is difficult at present to demonstrate clearly that changes in the proteins are linked to either overt disease or reproductive problems in aquatic species

(Connell, 1997), if deleterious effects persist and take place in many organisms, a demoecological effect (e.g. decline of populations) could happens.

Although GST activity could be induced by different xenobiotics, its efficiency as a biomarker of organochlorine pesticides and PCBs in molluscs (Fitzpatrick et al., 1997; Hoarau et al., 2001; Blanchette and Singh, 2002) and in the aquatic macrophyte *Ceratophyllum demersum* (Menone and Pflugmacher, 2005), remains to be demonstrated. According to our present results, GST can act by protecting *M. quitense* against damage induced by endosulfan (**1**) through different mechanisms: (a) by playing a role in oxidative stress tolerance (e.g. GST could be induced to detoxify peroxides); (b) by catalysing the conjugation of some phase-I metabolites of endosulfan (**1**) with GSH, since the biotransformation of endosulfan (1) produces the corresponding sulfate (2), diol (3), ether (4), and lactone (5) (Arrebola et al., 2001; Kim et al., 2001).

GR plays a role in the antioxidant defence processes, by reducing GSH to GSSG with consumption of NADPH, thus maintaining a high intracellular GSH/GSSG ratio. Thus, the enhanced activity of GR observed during acute stress (24 h exposure) could reflect the need to restore the oxidative balance between GSH and GSSG, which is altered due to GST detoxication and ROS scavenge, both processes being induced upon exposure to endosulfan (**1**).

The fate of endosulfan (**1**) in an aquatic system will thus be largely dependent on how it is introduced. Pollution with pesticides normally arises from diffuse sources such as runoff and overspray. Although endosulfan (**1**) is persistent in soils, aquatic organisms are typically exposed to short-term peak concentrations rather than chronic levels (Gormley and Teather, 2003). A one-off input from overspray or storms can have severe short-term impact, while constant input from runoff or stream flow may not produce noticeable effects at any particular time but may chronically deteriorate the system (Peterson and Batley, 1993).

We did not observe any significant response of the defensive system of *M. quitense* when this macrophyte was exposed to 0.02 μg/L endosulfan (**1**), which is the maximal concentration allowed for freshwater, in according to Argentinean law as well as other international laws (e.g. Canadian guidelines, Bisson and Hontela, 2002). On the other hand, when *M. quitense* was exposed to 0.5 and 5 μg/L, effects were evident and significant after 24 h exposure. Thus, our present results demonstrate that this aquatic macrophyte is affected at environmentally relevant concentrations of endosulfan (**1**), which can be reached during either accidental spills or by runoff from fields that have been recently treated with this insecticide. At the organismic level, fundamental lesions associated with ROS include oxidation of membrane lipids, proteins, nucleic acids and altered cellular redox status. These perturbations are believed to underlie specific tissue injuries associated with redox-active contaminants. Numerous studies concerning the activities of antioxidant enzymes have suggested adaptative responses of antioxidant system components in aquatic organisms exposed in vivo to either conditions or xenobiotics likely to enhance oxyradical fluxes (Rand, 1995). On the other hand, in cells under a hyperoxidative condition, the enzymes could be inactive (due to its threshold in the capacity of detoxication) leading to damage and lastly to cell death. Although it is difficult at present to demonstrate clearly that changes in the proteins are linked to overt disease or reproductive problems in aquatic species (Connell, 1997), if deleterious effects persist and take place in many organisms, a demoecological effect (e.g. decline of populations) could happen.

3.1. Concluding remarks

The induction of CAT, GSTs and GR demonstrates that the exposure of *M. quitense* to endosulfan (**1**) activates its enzymatic protection system, increasing its capacity to scavenge reactive oxygen species (ROS). Furthermore, our present results show that endosulfan (**1**) exerts oxidative stress in the aquatic macrophyte *M. quitense,* which is mainly represented by the induction of the antioxidant CAT activity and the significant increase of H_2O_2 observed at 5 μg/L. To the extent of our knowledge, this is the first report of oxidative stress in plants induced by endosulfan (**1**).

Our results also extend the current knowledge by identifying the participation of CAT, GST and GR as defence mechanisms against endosulfan (**1**) in the aquatic macrophyte *M. quitense*. Our present results indicate that the tested enzymes have the potential to act as biomarkers for water quality biomonitoring, using the genus *Myriophyllum* as a good bioindicator. However, further studies are required to improve the knowledge on the usefulness of phase II and antioxidant enzymes as biomarkers in aquatic plants considering effects of natural factors (e.g. temperature) that could influence enzymatic responses.

4. Experimental

4.1. Plant material

M. quitense was collected in La Brava pond (37° 53′ South, 57° 59′ West), Buenos Aires Province-Argentina, and cultivated prior to the experiments for two months in 100 L tanks using Hoagland's medium. Identification of plant species was done according to Orchard (1981). Healthy plants with approximately the same biomass were selected and used for all the experiments, including controls.

4.2. Plant exposure

Experiments were carried out under a light/ dark photoperiod of 14/10 h, keeping the temperature at 20–22 °C.

Relevant environmental concentrations (μg/L) were tested throughout the study, considering levels detected in surface waters after spraying, as well as the maximal allowed quantities (MAQ) for the protection of aquatic life in freshwater (Argentine Dangerous Wastes Law NO. 24051/93) (Rovedatti et al., 2001).

Considering that commercial formulations of endosulfan (**1**) consist in a mixture of α- and β-endosulfan (70:30), we used this mixture throughout the experiment. Thus, we prepared two stock solutions by diluting α- endosulfan (Riedel-de Haën) (0.04 g in 25 mL in dimethyl sulfoxide-DMSO, Mallinckrodt) and β-endosulfan (0.04 g in 50 mL in dimethyl sulfoxide-DMSO, Mallinckrodt). Afterwards, we prepared the appropriate exposure medium by diluting the appropriate amount of both α- and β-endosulfan (70:30) (**1**) in Hoagland medium to a final volume of 300 mL.

Separate pools of *M. quitense* (ca. 6 g fresh weight each) were exposed to three different concentrations of endosulfan (**1**): 0.02, 0.05 and 5 μg/L. Exposures were carried out in triplicate during 24 h under the light and temperature conditions described above. Two different controls were assayed. The first contained DMSO added to Hoagland medium (DMSO-control). The second contained only Hoagland medium without DMSO and endosulfan (**1**) (H-control).

After exposure, the plants were rinsed with ultrapure water (Milli-Q, Millipore) to remove the endosulfan (**1**) adsorbed on the surface, immediately frozen using liq. N_2, and stored at −80 °C until extraction or analysis.

4.3. Enzyme extraction

The extraction of enzymes was done according to the method described by Pflugmacher and Steinberg (1997) with minor modifications. Briefly, we used five grams of plant material, arising from independent exposures. Frozen plants were ground to a fine powder with a mortar and pestle under liquid N_2, followed by the addition of 10 mL sodium-phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol, 14 mM DTE, and 1 mM EDTA. Cell debris was removed by centrifugation at 10,000*g* for 10 min. The supernatant was centrifuged at 40,000*g* for 60 min to get the membrane associated fraction, which was re-suspended in sodium phosphate buffer (20 mM, pH 7.0 supplemented with 20% glycerol and 1.4 mM DTE), and homogenized in a glass potter. Selective protein precipitation was achieved by adding solid ammonium sulphate to the supernatant until reach 35% saturation. After centrifugation at 20,000*g* for 20 min, the pellet was discarded and additional ammonium sulphate was added to the supernatant until reach 80% saturation. After centrifugation at 30,000*g* for 30 min, the pellet (containing soluble enzymes) was re-suspended in sodium-phosphate buffer (20 mM, pH 7.0), desalted by gel filtration on NAP-10 columns (GE-Healthcare), concentrated to 1.5 mL, immediately frozen using liquid N_2, and stored at −80 °C until measurement.

4.4. Measurement of enzymatic activities

Enzymatic activities were determined by spectrophotometry. The activities of membrane associated and soluble glutathione-S-transferase (m-GST and s-GST) were determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR) was assayed in the soluble fraction according to Tanaka et al. (1994). The enzymatic activities were calculated in terms of the protein content for each sample (Bradford, 1976), using bovine serum albumin as standard. Enzymatic activities are reported in nanokatals per milligram of protein (nkat/mg prot), where 1 kat is the conversion of 1 mol of substrate per second. Each enzymatic assay was carried out by triplicate.

4.5. Assay of H_2O_2

The cell content of H_2O_2 was measured by spectrophotometry according to Jana and Choudhuri (1981). Briefly, four independent preparations (0.5 g plant material each) were used. Plant tissue was homogenized in 3 mL 50 mM sodium phosphate buffer (pH 6.5) and centrifuged at 10,000*g* and 4 °C for 2 min. Afterwards, 250 μL supernatant were mixed with 750 μL 0.1% titanium sulfate dissolved in a H_2SO_4 solution (20% v/v), measuring the absorbance at 410 nm. The amount of H_2O_2 in plant tissues was determined using a calibration plot, constructed from solutions containing known amount of H_2O_2, and its concentration is expressed as μg H_2O_2 per g plant (fresh weight – fr. wt). Each preparation was assayed by triplicate.

4.6. Statistics

All values are expressed as mean ± standard deviation. One-way ANOVA models were fitted for each measured parameter (enzymatic activities and content of H_2O_2) to estimate effects of exposures on the macrophyte. We verified that DMSO and H controls did not present significant differences. Thus, we report controls as the mean ± standard deviation of both controls for each measured parameter. Due to the presence of outliers within the data set, we used weighted ANOVA models to fit the contribution of individual cases to the outcome of an analysis, weighting those cases in proportion to the values of the selected variable. We used planned contrasts to assess significant difference between control groups and concentrations.

Acknowledgements

We would like to thank Dr. E. Camadro (Lab. Genetics-Faculty of Agrochemistry UNMdP- EEA Balcarce-INTA) for enabling plant exposures in her laboratory, to Lic. D. Pérez for her assistance during experiments and to Mr. J. Espinillo for his help during field collection of plants. This

work was supported by grants: FONCYT-PICTR/2003-327 and CONICET PIP 6497.

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