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RESEARCH ARTICLE

Enzymatic basis for fungicide removal by *Elodea canadensis*

Rachel Dosnon-Olette · Peter Schröder · Bernadett Bartha · Aziz Aziz · Michel Couderchet · Philippe Eullaffroy

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Abstract
Purpose Plants can absorb a diversity of natural and man-made toxic compounds for which they have developed diverse detoxification mechanisms. Plants are able to metabolize and detoxify a wide array of xenobiotics by oxidation, sugar conjugation, glutathione conjugation, and more complex reactions. In this study, detoxification mechanisms of dimethomorph, a fungicide currently found in aquatic media were investigated in *Elodea canadensis*.
Methods Cytochrome P450 (P450) activity was measured by an oxygen biosensor system, glucosyltransferases (GTs) by HPLC, glutathione S-transferases (GSTs), and ascorbate peroxidase (APOX) were assayed spectrophotometrically.
Results Incubation of *Elodea* with dimethomorph induced an increase of the P450 activity. GST activity was not stimulated by dimethomorph suggesting that GST does not participate in dimethomorph detoxification. In plants exposed to dimethomorph, comparable responses were observed for GST and APOX activities showing that the GST was more likely to play a role in response to oxidative stress. Preincubation with dimethomorph induced a high activity of O- and N-GT, it is therefore likely that both enzymes participate in the phase II (conjugation) of dimethomorph detoxification process.
Conclusions For the first time in aquatic plants, P450 activity was shown to be induced by a fungicide suggesting a role in the metabolization of dimethomorph. Moreover, our finding is the first evidence of dimethomorph and isoproturon activation of cytochrome P450 multienzyme family in an aquatic plant, i.e., *Elodea* (isoproturon was taken here as a reference molecule). The detoxification of dimetomorph seems to proceed via hydroxylation, and subsequent glucosylation, and might yield soluble as well as cell wall bound residues.

Keywords Cytochrome P450 · Detoxification · Glucosyltransferase · Glutathione S-transferase · Pesticide · Phytoremediation

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R. Dosnon-Olette (✉) · A. Aziz · M. Couderchet · P. Eullaffroy
Laboratoire Plantes, Pesticides et Développement Durable (PPDD), URVVC-SE EA 2069,
Université de Reims Champagne-Ardenne,
BP 1039, 51687 Reims Cedex 2, France
e-mail: rachel.olette@gmail.com

P. Schröder · B. Bartha
Abteilung Mikroben-Pflanzen Interaktionen,
Helmholtz-Zentrum München,
85758 Neuherberg, Germany

1 Introduction

In intensively cultivated areas, agriculture and viticulture are significant sources of surface water contamination by pesticides via spray drift and/or runoff (Groenendijk et al. 1994; Kloeppel et al. 1997; Shultz 2001). When entering the aquatic environment, chemicals may affect directly or indirectly human and ecosystem health by inducing a significant threat to aquatic environments and drinking water resources (Dabrowski and Schulz 2003; Moore et al. 2007). Restoration of the pesticide-contaminated soil or groundwater could be improved by chemical methods, but these are prohibitively expensive or even impracticable, and the resulting products may not always be safer than the parent compounds (Chaudhry et al. 2002). To clean up the contaminated water and yet reduce the impact of pesticides on the environment, considerable interest has been focused on biological innovative technologies. In recent years, the use of plants to remediate polluted soils, air, and water (so-

called phytoremediation) has gained popularity as a cost effective, environmentally friendly, and efficient in situ technology for a variety of pollutants (Cunningham et al. 1995; He et al. 2005; Pilon-Smits 2005; Dhir et al. 2009) and, among them, many pesticides (Gao et al. 2000; Schröder and Collins 2002; De Carvalho et al. 2007; Dosnon-Olette et al. 2009; Moore et al. 2009).

Because plants are static and live in a competitive and sometimes hostile environment, they have evolved mechanisms that protect them from environmental abiotic stress, including the detoxification of xenobiotic compounds (Sandermann 2004). Plant metabolism is extremely diverse and can be exploited to treat recalcitrant pollutants, not degradable by bacteria or fungi. Plants may therefore be considered as "green livers", acting as an important global sink for environmental pollutants, in many cases detoxifying them (Sandermann 2004). Since there is often an analogy of structure between xenobiotics and plant secondary metabolites, it is likely that the metabolism of xenobiotics uses at least partially secondary metabolic pathways (Singer et al. 2003). It is known that plants often metabolize xenobiotic pollutants by three sequential steps (Sandermann 1992, 1994; Coleman et al. 1997): (1) Phase I involves conversion/activation (oxidation, reduction, and hydrolysis) of lipophilic xenobiotic compounds (Komives and Gullner 2005); this transformation is mostly catalyzed by cytochrome P450 monooxygenases (P450, Pflugmacher et al. 1999). (2) Phase II involves conjugation of xenobiotic metabolites of phase I to endogenous hydrophilic molecules such as sugars, amino acids, and glutathione (GSH) (Coleman et al. 1997; Dietz and Schnoor 2001); this conjugation is mostly catalyzed by glucosyltransferases (GTs) and glutathione S-transferases (GSTs) (Pflugmacher et al. 1999; Loutre et al. 2003). (3) Phase III is a storage/excretion phase, in which modified xenobiotics are compartmentalized in vacuoles or getting bound to cell wall components such as lignin or hemicellulose (Coleman et al. 1997; Dietz and Schnoor 2001; Eapen et al. 2007).

Presently, little information is available on the mechanisms that may be involved in the detoxification of fungicides in aquatic plants. Here we investigate some mechanisms that could participate in the detoxification of dimethomorph (DMM, Fig. 1) in *Elodea canadensis*, an aquatic macrophyte efficient in the removal of phytosanitary products (Gao et al. 2000; Dosnon-Olette et al. 2009). The possible role of cytochrome P450 monooxygenases, glucosyltransferases, and glutathione S-transferases in the metabolism of dimethomorph was investigated. GSTs are also known to be involved in antioxidative stress defense, with glutathione peroxidizing activity or a general induction in the presence of reactive oxygen species. To underpin the lacking role of GSTs in dimethomorph metabolism, but rather in oxidative stress response, another oxidative stress enzyme was assayed, the ascorbate peroxidase (APOX).

Using a recent method based on the fluorimetric detection of oxygen consumed during cytochromes P450 catalyzed reaction (Olry et al. 2007; Page and Schwitzguébel 2009), it was possible to test directly the enzyme activity towards the fungicide under investigation. To compare the P450 activity, another pesticide, isoproturon (IPU), was taken as a reference molecule. Isoproturon was chosen as a positive control, since P450 participates in isoproturon detoxification (De Prado et al. 1997) and it had previously been used successfully as substrate for P450 (Siminszky 2006).

2 Materials and methods

2.1 Plant material and cultivation

Cultures of *E. canadensis* were supplied from a specialized provider (Animalis, Cormontreuil, France). Plants were maintained in large polyvinyl chloride aquaria containing growth medium (Chollet 1993) at pH 6.5±0.5. All aquaria were maintained at 23±2°C under continuous light (65 μmol m^{-2} s^{-1}photosynthetic active radiation) provided by cool white fluorescent lamps (Sylvania Gro Lux F36W).

2.2 Measurement of cytochrome P450 activity

Samples (20 g) from plants incubated 24 h either with DMM (30 μM) or IPU (1 μM) or without pesticide were frozen and homogenized under liquid nitrogen with mortar and pestle to a fine powder, and extracted at 4°C in ten volumes (*w*/*v*) of 0.1 M potassium phosphate buffer pH 7.4, containing 10 mM 1,4-dithioerythritol (DTE), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, and 40 mM ascorbic acid (adapted from Page and Schwitzguébel 2009). Then, the crude extract was filtered with

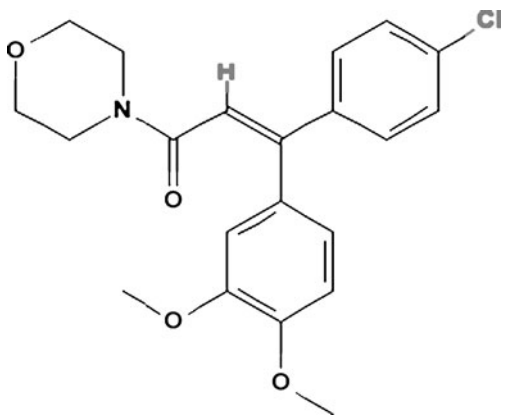

Fig. 1 Chemical structure of *E*-dimethomorph

Miracloth (50 μm) and centrifuged at 10,000×*g* for 15 min (4°C). The supernatant was filtered again and ultra-centrifuged at 100,000×*g* for 1 h (4°C). The pellets were resuspended in 1 mL of 0.1 M potassium phosphate buffer pH 7.4 and 20% glycerol and stored at −20°C.

For the quantification of P450 (E.C. 1.14.-) activity, we used standard BD™ Oxygen Biosensor system (BD Biosciences) according to the method described by Page and Schwitzguébel (2009). Briefly, the reaction was started by adding dimethomorph (25, 50, 75, and 100 μM) or isoproturon (50 and 100 μM) as substrate. A control was run without substrate. Calculation of oxygen consumption converted from fluorescence changes during the assay, to evaluate the enzymatic activity towards the different substrates, was recorded according to Olry et al. (2007).

Isoproturon was taken as a reference molecule in these experiments, since it had previously been used successfully as substrate for P450 (Siminszky 2006).

2.3 Measurement of glucosyltransferase activity

For the GT (E.C. 2.4.1.-) experiments, 10 g of plants were taken after incubation with or without dimethomorph at 1.55 μM during 24 h. The extraction procedure is adapted from Loutre et al. (2003) and the determination of GT activity from Brazier et al. (2002). Frozen plants were homogenized under liquid nitrogen with mortar and pestle to a fine powder and extracted at 4°C in ten volumes (*w*/*v*) of 0.1 M potassium phosphate buffer pH 6.5, containing 10 mM DTE, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF, and 1% polyvinylpyrrolidone (PVP, K30). After 30 min, the crude extract was centrifuged at 20,000×*g* and 4°C for 30 min. Proteins in the supernatant were precipitated by stepwise addition of solid ammonium sulfate to 35% and in a second step to 75% saturation. After each step, the extracts were centrifuged at 18,500×*g* and 4°C for 30 min and the final pellets were suspended in 2.3 mL of 200 mM Tris/HCl buffer pH 7.3, containing 1 mM DTE, and 2 mM $MgCl_2$. The extracts were desalted and further purified by passing through gel-filtration columms (PD 10; Pharmacia, Freiburg, Germany). Samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. GT activity was assayed as the conjugation of activated glucose to a substrate. Two substrates known for conjugation with O-glucosyltransferase were tested: 2,4,5-trichlorophenol (2,4,5-TCP; Merck, Darmstadt, Germany) and quercetin (Merck, Darmstadt, Germany), as well as a substrate of the N-glucosyltransferase 3,4-dichloroaniline (DCA; Merck, Darmstadt, Germany), and the pesticide of interest, dimethomorph (certified standard; Sigma, Saint-Quentin-Fallavier, France). The reaction mixture consisted of a Tris/HCl buffer (50 μL, 0.2 M, pH 7.3), the substrate (10 μL, 2 mM), uridine 5′-diphosphoglucose (20 μL, 20 mM), 4-nitrophenyl β-glucoside (10 μL, 25 mM), salicin (10 μL, 25 mM), and the enzyme extract (100 μL). After different incubation times, the reaction was stopped with phosphoric acid (85%, 10 μl), diluted 1:4 (*v*:*v*) with ultrapure water, centrifuged (10,000×*g*, 1 min), and the supernatant injected into the HPLC system (Varian Prostar 210, C18 Hypersil-ODS: 5 μm, 250×4.6 mm). Elution was carried out at 1 mL min^{-1} with a gradient of A (H_2O and 0.1% of trifluoroacetic acid, TFA) and B (acetonitrile and 0.1% of TFA), B increased from 8% to 100% in 16 min and remained stable at 100% during 3 min. Peaks were detected with a diode array detector (Varian, 335 detector). Identification of the compounds was confirmed by UV spectrum and concentration was determined at 205 nm for 2,4,5-TCP, 370 nm for quercetin, and 255 nm for DCA, by comparison with standard curves obtained with compounds certified standard.

2.4 Measurement of glutathione S-transferase and ascorbate peroxidase activities

For GST (E.C. 2.5.1.18) and APOX (E.C. 1.11.1.11) assays, 5 g of plants were taken after 24- or 96-h incubation with or without dimethomorph at 1.55 μM. The extraction procedure is according to Schröder et al. (2005). Frozen plants were homogenized under liquid nitrogen with mortar and pestle to a fine powder and extracted at 4°C in ten volumes (*w*/*v*) of 0.1 M Tris/HCl buffer pH 7.8, containing 5 mM DTE, 5 mM EDTA, 1% Nonidet, and 1% PVP. After 30 min, the crude extract was centrifuged at 20,000×*g* for 30 min (4°C). Proteins in the supernatant were precipitated by stepwise addition of solid ammonium sulfate to 40% and in a second step to 80% saturation. After each step, the extracts were centrifuged at 18,500×*g* for 30 min (4°C) and the pellets were resuspended in 2.3 mL of 25 mM Tris/HCL buffer pH 7.8. This step was followed by desalting with Sephadex PD-10 desalting columns (Pharmacia, Freiburg, Germany). Samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

GST activity was evaluated as the increase of absorbance at 345 nm due to the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST, following the method of Schröder et al. (2002). The reaction mixture contained Tris/HCl buffer (0.1 M, pH 6.4), CDNB (1.0 mM), GSH (1.1 mM), and enzyme extract (10 μL in a final volume of 200 μL of the mixture). Activity was measured spectrophotometrically by following the increase of the absorbance for 5 min at 25°C due to the conjugation of the GSH with the substrate CDNB. The amount of conjugated substrate was determined using the extinction coefficient ε (conjugate GSH/CDNB=9.6 mM^{-1} cm^{-1}).

APOX activity was assessed according to Vanacker et al. (1998) as the decline of absorbance at 290 nm due to the

decrease in ascorbate concentration and activity was calculated using the extinction coefficient ε (2.8 mM^{-1} cm^{-1} for ascorbate). The reaction mixture contained Tris/HCl buffer (50 mM, pH 7.0), Na-ascorbate (645 μM), and H_2O_2 (0.1%). The enzymatic reaction was started by addition of the enzyme extract (10 μL in a final volume of 200 μL of the mixture) and the absorbance decrease was recorded for 5 min at 25°C.

After each extraction the protein content was determined according to Bradford (1976) using bovine serum albumin for calibration.

2.5 Statistical analysis

In this study, all statistical analyses were performed with SigmaStat 3.5 (Systat Software Inc, San Jose, CA, USA). Significant differences between controls and contaminated samples were determined by the one-way ANOVA test and p values <0.05 were considered significant.

3 Results and discussion

3.1 Cytochrome P450 activity

Direct addition of dimethomorph (25–100 μM) or isoproturon (50–100 μM) in the reaction medium as substrates (without preincubation of *Elodea*) resulted in an increase of cytochrome P450 activity in a concentration-dependent manner (Fig. 2a). Both 50 and 100 μM isoproturon led to a significant increase in P450 activity compared to the control ($p<0.05$). These results are in accordance with the fact that isoproturon is taken as substrate by P450 (Siminszky 2006). Dimethomorph at 100 μM yielded a significant P450 activity (i.e., oxygen consumption) compared to the control, indicating that the fungicide was recognized as substrate by the P450.

A 24-h preincubation of plants with 30 μM dimethomorph (Fig. 2b) resulted in elevated P450 activity when substrates were added to enzymatic extract. P450 activity was significantly higher ($p<0.05$) with dimethomorph (50, 75, and 100 μM) or isoproturon (50 and 100 μM) as substrates than in control. In the presence of dimethomorph (50–100 μM) as substrate, a significantly higher P450 activity was noticed due to preincubation with dimethomorph compared to results without preincubation (Fig. 2a). The higher activity detected in *Elodea* after 24 h of exposure to dimethomorph indicates an induction of these enzymes when plants were grown in the presence of this fungicide. This fact was not observed in the presence of isoproturon as substrate, with or without preincubation with dimethomorph, no significant change in P450 activity was noticed (Fig. 2a, b).

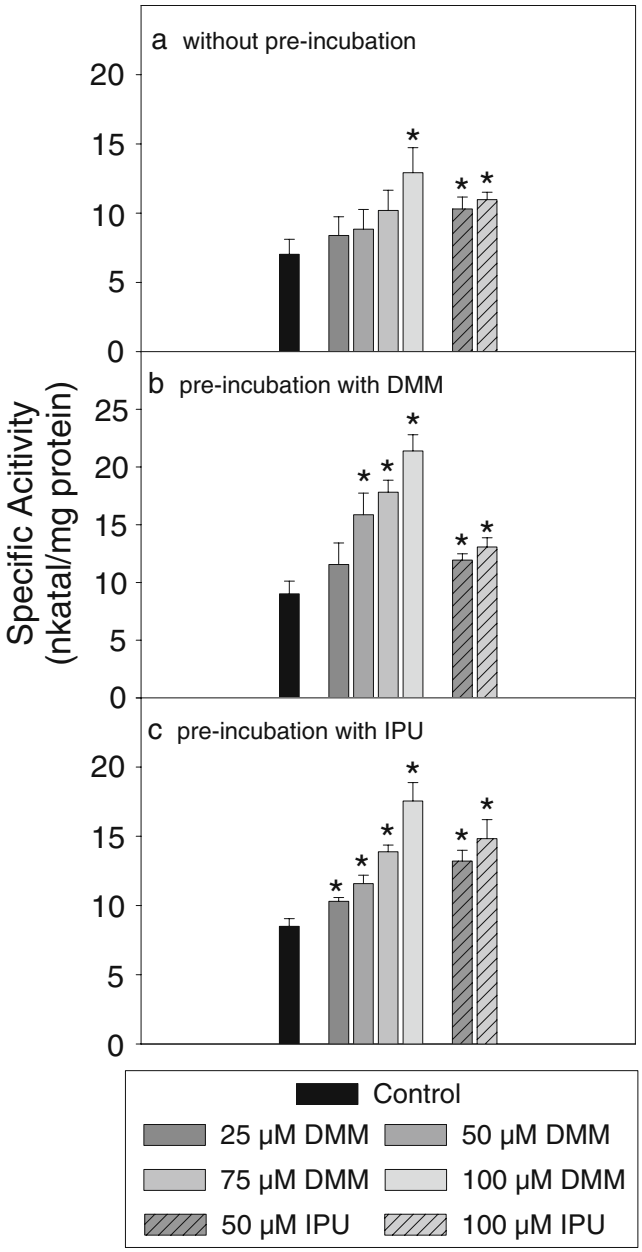

Fig. 2 Specific activity of cytochromes P450 in *E. canadensis*. Comparison of the activity in plants **a** without preincubation, **b** after 24-h preincubation with DMM (30 μM), and **c** after 24-h preincubation with IPU (1 μM); without addition of substrate (control) in the reaction mixture or with DMM (25, 50, 75, and 100 μM) or IPU (50 and 100 μM) as substrates. Activity is shown in nanokatals per milligram of protein. *Asterisk* indicates a significant difference with the control (one-way ANOVA, $p<0.05$)

Without preincubation, dimethomorph as substrate was recognized by P450, but this enzyme had low affinity for this substrate in the reaction medium. This affinity was higher when plants were preincubated with dimethomorph. However, P450 affinity for isoproturon as substrate was not changed by dimethomorph preincubation. Hence, the preincubation might affect a different P450 isoform.

As expected preincubation of *Elodea* with 1 μM isoproturon (Fig. 2c) resulted in a subsequent increase of P450 activity, in the presence of dimethomorph or isoproturon in the reaction medium as substrates, in a concentration-dependent manner with both pesticides. However, the activity observed in control or with dimethomorph as substrate was not significantly different from that observed in *Elodea* without preincubation (Fig. 2a) or with dimethomorph incubation (Fig. 2b). However, significant increase of P450 activity was noticed with isoproturon as substrate, compared to results obtained without preincubation and with dimethomorph preincubation.

Here, preincubation with dimethomorph led to an activation of P450 activity with dimethomorph as substrate in the reaction medium compared to the activity without preincubation. No change was observed in this case with isoproturon as substrate. However, the same trend was observed with isoproturon preincubation; addition of isoproturon as substrate in the reaction mixture yielded a higher P450 activity in plants preincubated with isoproturon compared to the activity in plants without preincubation. And, no change was observed in presence of dimethomorph as substrate. To resume preincubation with dimethomorph induced a higher P450 affinity for dimethomorph as substrate, and preincubation with isoproturon induced a higher P450 affinity for isoproturon as substrate. It seems here that each pesticide activated a specific P450. This was in agreement with other work showing the existence of a multitude of P450 involved in the detoxification process in plants (Werck-Reichhart and Feyereisen 2000). In any case, P450 was likely to participate in the phase I (activation/conversion) of dimethomorph detoxification processes.

3.2 Glucosyltransferase activity

Different substrates were tested (Table 1) to characterize O-GT and N-GT activity in response to dimethomorph incubation: 2,4,5-TCP and quercetin as substrates for the O-GT (Brazier et al. 2003), 3,4-DCA for the N-GT (Lao et al. 2003) and dimethomorph.

The presence of enzyme extract from *Elodea* led to a significant decrease in substrates concentrations compared to the control (without enzyme extract). Moreover, except for dimethomorph, we observed a significant decrease of substrate concentration when *Elodea* was preincubated with dimethomorph (24 h, 1.55 μM) than in the presence of enzyme extract from *Elodea* without preincubation. In fact, the decrease of these substrates was due to GT activity, which catalyzed the glucoside products' formation. Therefore, the amount of glucoside product formed was significantly higher in the presence of enzyme extract after dimethomorph preincubation of *Elodea* than without preincubation (Fig. 3). However, with dimethomorph as substrate, the amount of substrate and glucoside product was not significantly changed in the presence of enzyme extract from *Elodea* with or without preincubation. From these results, it appeared that these enzymes had little affinity for dimethomorph; this might be explained by the nature of dimethomorph molecule. Dimethomorph added as substrate in the reaction mixture come from initial chemical form (certified standard) and was not undergone a previous activation (i.e., hydroxylation) by plants P450, first step of the detoxification mechanism.

In the presence of 2,4,5-TCP, a higher GT activity was noticed, while this activity was lower in the presence of quercetin or DCA as substrates. Here, O-GTs seemed to have higher affinity to 2,4,5-TCP than to quercetin as substrate. This result was in agreement with those of Brazier et al. (2003) which suggested that plants could recruit distinct O-GTs to detoxify xenobiotics, rather than utilize the same enzymes in each case. And, this has resulted from the great diversification of the O-GTs in plants both in terms of their gene sequences and functions in natural product metabolism (Vogt and Jones 2000; Ross et al. 2001).

Preincubation with dimethomorph induced a high activity of O- and N-GT. It is therefore likely that both enzymes

Table 1 Concentrations of substrates and glucoside products resulting from GT activity in the presence of different substrates: dimethomorph, 2,4,5-TCP, quercetin and DCA, with enzyme extract from *Elodea* incubated 24 h with or without DMM (1.55 μM) and without enzyme extract as control

	Control		*Elodea* without DMM		*Elodea* with DMM	
	Substrate (μM)	Glucoside product (μM substrate eq mg^{-1} protein)	Substrate (μM)	Glucoside product (μM substrate eq mg^{-1} protein)	Substrate (μM)	Glucoside product (μM substrate eq mg^{-1} protein)
DMM	98.5	0	94.8[a]	7.2[a]	93.9[a]	8.4[a]
2,4,5-TCP	102.5	0	94.9[a,b]	72.5[a,b]	98.3[a,b]	161.0[a,b]
Quercetin	99.4	0	90.9[a,b]	0.6[b]	80.8[a,b]	6.9[a,b]
DCA	100.4	0	95.5[a,b]	26.3[a,b]	85.5[a,b]	35.4[a,b]

[a] Mean value is significantly different from control (without enzyme extract)

[b] Significant difference between *Elodea* without DMM and *Elodea* with DMM ($p<0.05$)

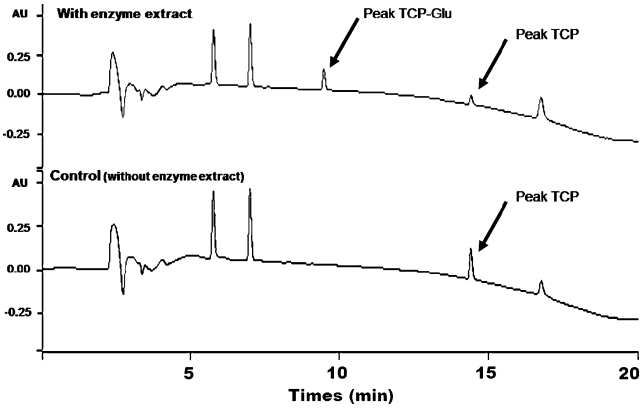

Fig. 3 Example of HPLC elution profile obtained during the measurement of GTs activities. Comparison of the activity in presence or not (control) of enzyme extract from *E. canadensis* in the reaction mixture, after 1 h of incubation with TCP as substrate

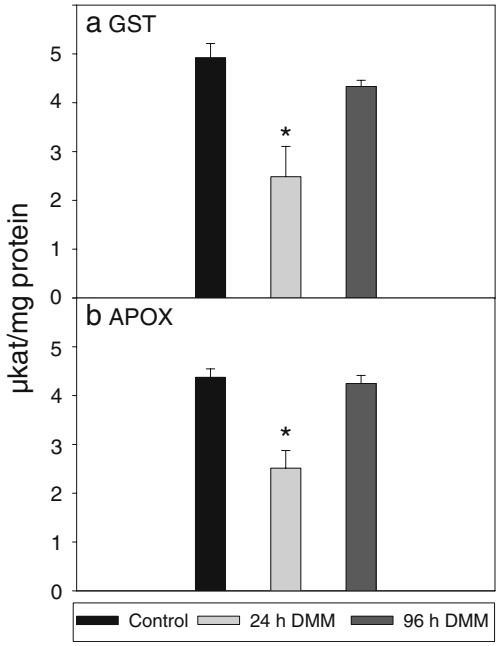

Fig. 4 Specific activity of **a** GST and **b** APOX in *E. canadensis*. Comparison of the activity in plants without preincubation (control), or after 24- or 96-h preincubation with DMM (1.55 µM). Activity is shown in microkatals per milligram of protein. *Asterisk* indicates a significant difference with the control (one-way ANOVA, $p<0.05$)

participate in the phase II (conjugation) of dimethomorph detoxification process.

3.3 Glutathione S-transferase and ascorbate peroxidase activities

Glutathione S-transferases are known to be involved in the phase II of pesticide detoxification process and are also known to be involved in antioxidative stress defense. In this study, GST activity was not stimulated in *Elodea* plants after incubation with DMM (Fig. 4a).

The effect of preincubation (24 or 96 h) with 1.55 µM DMM on *Elodea* GST activity was similar to that of APOX (Fig. 4b), another enzyme involved in the oxidative stress response. These results suggest that in this case, GST was more likely to play a role in response to oxidative stress (if any occurs) rather than participate directly in the dimethomorph detoxification process by conjugation.

4 Conclusions

Incubation of *Elodea* with dimethomorph induced an elevated enzymatic activity in those enzymes that are involved in its detoxification (i.e., P450 and GTs). Previous results have shown that this plant has high potential in phytoremediation of this fungicide (Dosnon-Olette et al. 2009). More studies have to be done to understand if a correlation does exist between plant enzymatic activity level and the removal rate of the fungicide. If this correlation is confirmed, such information may prove beneficial to facilitate phytoremediation management and this future improvement. Therefore, phytodegradation of organic pollutants may be optimized by selecting or engineering plant species with higher activities of the enzymes thought to be involved and rate limiting (Pilon-Smits 2005).

Concerning the enzyme catalyzing phase I reactions of the detoxification process, the incubation of *Elodea* with dimethomorph induced an increase of the P450 activity. Hence, it is very likely that the cytochrome P450 system plays a role in the metabolism of dimethomorph. It seems that dimethomorph and isoproturon are not detoxified via the same enzyme of the P450 family. Our finding is the first evidence of the hydroxylation of dimethomorph and isoproturon by members of cytochrome P450 multienzyme family in an aquatic plant, i.e., *Elodea*.

Experiments on glucosyltransferases, enzymes catalyzing phase II reactions in the metabolism of xenobiotics, indicate that incubation with dimethomorph induced a higher glucosylation with either O- and N-GT. This is also consequent in light of the previous activation (i.e., hydroxylation) of the DMM molecule by P450.

The glutathione S-transferase is known as an enzyme involved in phase II reactions of detoxification. However, its activity was not stimulated by dimethomorph suggesting that GST did not participate in dimethomorph detoxification. In plants exposed to dimethomorph, comparable responses were observed for GST and APOX activities showing that the GST was more likely to play a role in response to oxidative stress.

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