Plant Cell Rep (2009) 28:1725–1735
DOI 10.1007/s00299-009-0772-5

ORIGINAL PAPER

Metabolism of sulphonated anthraquinones in rhubarb, maize and celery: the role of cytochromes P450 and peroxidases

Valérie Page · Jean-Paul Schwitzguébel

Received: 7 July 2009 / Accepted: 6 September 2009 / Published online: 19 September 2009

Abstract Sulphonated anthraquinones are precursors of many synthetic dyes and pigments, recalcitrant to biodegradation, and thus contaminating many industrial effluents and rivers. In the development of a phytotreatment to remove sulphonated aromatic compounds, rhubarb (*Rheum rhaponticum*), a plant producing natural anthraquinones, as well as maize (*Zea mays*) and celery (*Apium graveolens*), plants not producing anthraquinones, were tested for their ability to metabolise these xenobiotics. Plants were cultivated under hydroponic conditions, with or without sulphonated anthraquinones, and were harvested at different times. Either microsomal or cytosolic fractions were prepared. The monooxygenase activity of cytochromes P450 towards several sulphonated anthraquinones was tested using a new method based on the fluorimetric detection of oxygen consumed during cytochromes P450-catalysed reactions. The activity of cytosolic peroxidases was measured by spectrophotometry, using guaiacol as a substrate. Results indicated that the activity of cytochromes P450 and peroxidases significantly increased in rhubarb plants cultivated in the presence of sulphonated anthraquinones. A higher activity of cytochromes P450 was also detected in maize and celery exposed to the pollutants. In these two plants, a peroxidase activity was also detected, but without a clear difference between the control plants and the plants exposed to the organic contaminants. This research demonstrated the existence in rhubarb, maize and celery of biochemical mechanisms involved in the metabolism and detoxification of sulphonated anthraquinones. Taken together, results confirmed that rhubarb might be the most appropriate plant for the phytotreatment of these organic pollutants.

Keywords *Apium graveolens* · Cytochromes P450 monooxygenases · Peroxidases · Phytoremediation · *Rheum rhaponticum* · Sulphonated anthraquinones · *Zea mays*

Introduction

Synthetic sulphonated anthraquinones are very important starting material to produce a large palette of dyes, and this family of xenobiotics has a potential and actual impact on the environment, especially rivers. Because these chemicals contain at least one sulphonic group and often also varying substitutions such as nitro groups, microorganisms have a limited ability to degrade them, and conventional wastewater treatment plants or biofilters are usually inefficient in managing the effluents of dye and textile industries. In this context, the development of alternative biological treatments to efficiently eliminate sulphonated aromatic compounds is needed (Schwitzguébel et al. 2002). More precisely, selected plant species are able to remove many pollutants from wastewater, by the use of constructed wetlands (Biddlestone et al. 1991; Davies and Cottingham 1994; Haberl et al. 2003; Davies et al. 2005; Bulc and Ojstrsek 2008), or hydroponic type

Communicated by J. Zou.

V. Page · J.-P. Schwitzguébel (✉)
Laboratory for Environmental Biotechnology (LBE),
Swiss Federal Institute of Technology Lausanne (EPFL),
Station 6, 1015 Lausanne, Switzerland
e-mail: jean-paul.schwitzguebel@epfl.ch

Present Address:
V. Page
Institute of Plant Sciences, University of Berne,
Altenbergrain 21, 3013 Bern, Switzerland

treatment systems (Furukawa and Fujita 1993; Dumont et al. 1999; Monnet et al. 2002). Both options offer a potentially low cost, low maintenance biological method for wastewater treatment. Most of the systems currently in use have been designed to treat domestic wastewater, but have a great potential to treat industrial effluents containing recalcitrant organics such as priority pollutants and dyes.

Anthraquinones naturally occur in several plant genera such as *Rheum*, *Rumex, Cinchona, Galium, Morinda* and *Rubia* (Van der Plas et al. 1998; Matsuda et al. 2001; Han et al. 2002). Furthermore, the biosynthetic pathways of natural anthraquinones, often glycosylated, have been recently unravelled and several enzymes involved in the process are characterised (Khouri and Ibrahim 1987; Van der Plas et al. 1998; Matsuda et al. 2001; Han et al. 2002).

It has been therefore assumed that the hardy and perennial rhubarb (*Rheum rabarbarum* or *R. rhaponticum*) might possess enzymes able of transforming sulphonated anthraquinones and could be harnessed to treat wastewater from the dye and detergent industries. As a first step, it has been shown that cells isolated from rhubarb and grown in bioreactors in the presence of different sulphonated aromatic compounds are able to accumulate and transform most of them (Duc et al. 1999; Schwitzguébel et al. 2002; Schwitzguébel and Vanek 2003).

The next step has been to check if whole plants of rhubarb cultivated under hydroponic conditions are also able to efficiently remove sulphonated anthraquinones from spiked water. Not only rhubarb, but also other plants producing anthraquinones, such as *Rumex hydrolapatum* and *R. acetosa*, have been tested. As a comparison, plants not producing anthraquinones, such as maize (*Zea mays*), rape (*Brassica napus*) and celery (*Apium graveolens*), have also been grown in the presence of different sulphonated anthraquinones. It has been shown that the most efficient plant to remove these xenobiotic compounds is rhubarb, especially the Valentine variety (Aubert 2003; Aubert and Schwitzguébel 2002, 2004).

However, the disappearance of a pollutant from the medium does not automatically mean that it is accumulated and degraded by the plant itself. The next step has thus been to investigate any possible adsorption, uptake, metabolism and degradation by the plant and its different organs. As measured by capillary electrophoresis, several sulphonated anthraquinones and different metabolites, not yet characterised, have been found in leaves of rhubarb and *R. hydrolapatum*, suggesting that these plant species are able to accumulate and metabolise these compounds (Aubert and Schwitzguébel 2002; Aubert 2003; Schwitzguébel et al. 2008).

Even if the precise biochemical mechanisms involved in the detoxification of sulphonated anthraquinones are not yet understood, they probably have cross talks with secondary metabolism, redox processes and plant energy metabolism. Enzymes involved in the early stages of the detoxification process, such as glutathione-*S*-transferases, cytochrome P450 monooxygenases or peroxidases are often linked to the plant redox biochemistry (Coleman et al. 1997; Gordeziani et al. 1999; Stiborová et al. 2000; Singer et al. 2003; Brankova et al. 2007; Jouili et al. 2008). The presence of xenobiotic compounds and their detoxification in plant cells could thus have significant effects on the redox balance and energy metabolism of the different plant organs. It is thus of utmost importance to avoid overloading of a plant with too high concentrations of xenobiotics requiring detoxification, both for phytoremediation efficiency and plant wellness.

It has recently been shown that microsomal cytochrome P450 monooxygenases isolated from plants producing natural anthraquinones are able to accept as substrates different sulphonated anthraquinones and that cytosolic peroxidases react to the presence of these xenobiotics (Page and Schwitzguébel 2009), whereas glutathione-*S*-transferase is apparently not involved in their detoxification (Aubert 2003; Schwitzguébel et al. 2008).

In the present study, the possible role of cytochrome P450 monooxygenases and peroxidases in the metabolism of sulphonated anthraquinones was investigated in two plant species, not producing natural anthraquinones, celery (*A. graveolens*), a dicotyledon, and maize (*Z. mays*), a monocotyledon, and compared to rhubarb. Microsomal and cytosolic fractions were thus isolated from plantlets cultivated under hydroponic conditions with or without sulphonated anthraquinones. Using a recent method based on the fluorimetric detection of oxygen consumed during cytochromes P450 catalysed reaction (Olry et al. 2007), it was possible to test directly the enzyme activity towards the different xenobiotics under investigation, whereas peroxidase was measured by spectrophotometry with guaiacol as a substrate.

Materials and methods

Plant material and cultivation

Seeds of *Rheum rhaponticum* L. (rhubarb) variety Victoria were provided by Graines Baumaux, Nancy, France. Seeds of *Apium graveolens* (celery) variety Secalinum were provided by Sativa Rheinau AG, Rheinau, Switzerland. Seeds of *Zea mays* (maize) variety LG 2185 were provided by Semences UFA, Switzerland. Seeds of rhubarb and maize were soaked in lukewarm water for 15 min before

being germinated on wet paper in plastic dish in darkness at a temperature of 16°C during the night and 22°C during the day. Seeds of celery were germinated without being soaked in water. After 1 week of germination, seedlings were grown hydroponically in brown glass culture pots containing 100 ml water. Luwasa (30110, Interhydro AG, Allmendingen, Switzerland) was supplied as plant nutrient. Five seedlings of rhubarb or celery and only one seedling of maize were placed in each pot and cultivated for 17 days (maize), 21 days (rhubarb) or 39 days (celery). Photoperiod was 14 h day and 10 h night and temperatures were the same as for germination.

Plant exposure to sulphonated anthraquinones

Plants of rhubarb and maize were exposed for 4 weeks to sulphonated anthraquinones, while plants of celery were exposed for 5 weeks. The five sulphonated anthraquinones were added together to the culture medium at a concentration of 0.2 mM each. This model effluent corresponded to the concentrations previously used in cell cultures (Schwitzguébel and Vanek 2003) and plant cultures (Aubert and Schwitzguébel 2004). The same quantity of rhubarb, maize and celery plants were grown at the same time with water and Luwasa only and used as control.

Anthraquinone-1-sulphonic acid (AQ-1-S), potassium salt, was obtained from Ciba-Geigy (Monthey, Switzerland) (Fig. 1); anthraquinone-2-sulphonic acid, sodium salt (AQ-2-S), anthraquinone-1,5-disulphonic acid (AQ-1,5-SS) and anthraquinone-2,6-disulphonic acid (AQ-2,6-SS), disodium salts, from Sigma-Aldrich (Buchs, Switzerland); and anthraquinone-1,8-disulphonic acid, disodium salt (AQ-1, 8-SS) from Huabei Foreign Trading Textile Corporation (Huabei, China).

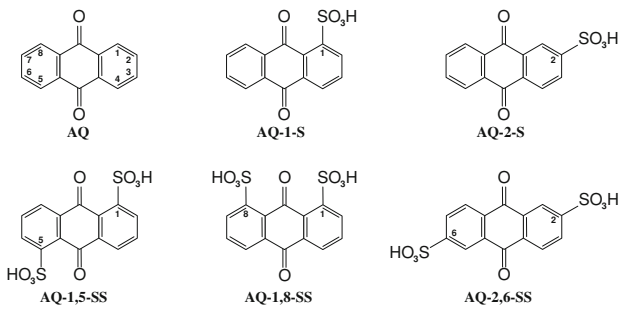

Fig. 1 Chemical structure of anthraquinone and sulphonated derivatives. The IUPAC names of sulphonated anthraquinones are: 9,10-dioxo-9,10-dihydro-1-anthracenesulphonic acid (AQ-1-S); 9,10-dioxo-9,10-dihydro-2-anthracenesulphonic acid (AQ-2-S); 9,10-dioxo-9,10-dihydro-1,5-anthracenedisulphonic acid (AQ-1,5-SS); 9,10-dioxo-9,10-dihydro-1,8-anthracenedisulphonic acid (AQ-1,8-SS); 9,10-dioxo-9,10-dihydro-2,6-anthracenedisulphonic acid (AQ-2,6-SS)

Design of the experiment

The experimentation started with the exposure of plants to the mixture of sulphonated anthraquinones. At the same time, samples from control plants were collected for extraction of cytosolic peroxidases or microsomal cytochromes P450. For each extraction, the plants of three pots were collected for the peroxidase assay and the plants of five (rhubarb and celery) and four (maize) pots were collected for the microsomal preparation. Afterwards, samples from control and exposed plants were collected once a week, during 4 weeks (rhubarb and maize) or 5 weeks (celery). Rhubarb plants were collected for the cytochromes P450 extraction after 1, 3 and 4 weeks of exposure to the sulphonated anthraquinones. To have enough plant material, roots and shoot (control or exposed) were pooled for the extraction of cytosolic peroxidases or microsomal cytochromes P450. At the end of the experimentation, roots and shoot of the plants exposed or not to sulphonated anthraquinones were collected separately for the cytosolic and the microsomal fraction extractions and for the enzymatic assays.

Microsomes extraction and assay of cytochromes P450 activity

For the extraction of microsomes, plants were washed with deionised water, dried with paper and the fresh weight was measured. The plant material was ground in a blender (Waring Laboratory Blender), 2 × 30 s, maximum speed, in the extraction buffer: 100 mM Na phosphate buffer pH 7.4, 250 mM saccharose, 1 mM EDTA (ethylenediaminetetraacetic acid), 40 mM ascorbic acid (vitamin C), 1 mM PMSF (phenylmethylsulphonyl fluoride), 10 mM β-mercaptoethanol. The ratio of the volume of the extraction buffer to the mass of plant material was 10–1. The obtained homogenate was filtered on Miracloth (Calbiochem) and centrifuged at 10,000×g for 15 min at 4°C. Supernatant was collected, filtered on Miracloth and ultracentrifuged at 100,000×g for 1 h at 4°C. Pellets of microsomes were collected and homogenised in 100 mM Na phosphate buffer pH 7.4 in a Potter. The procedure was carried out at 4°C. Concentration of proteins in the microsomal extract was measured by Lowry assay (Bio-Rad *DC* Protein Assay). Microsomes were stored at −20°C.

The fluorimetric assay for cytochromes P450 activity was adapted from Olry et al. (2007). Standard BD Oxygen Biosensor System 96-well plates with an oxygen-sensitive fluorophore incorporated in a permeable matrix at the bottom of each well were used for the assay. Oxygen consumption due to substrate oxygenation was measured at 27°C in a fluorimeter (FLEXstation, Molecular Devices,

USA). The excitation wavelength was set at 480 nm and the emitted light was collected at 620 nm. All experiments were carried out in 20 mM Na phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM NADPH, 3 mM glucose-6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase (all purchased from Sigma-Aldrich), 0.1 mM substrate (one of the five sulphonated anthraquinones) and 50 µl microsomes. The reaction medium was preheated for 1 h at 27°C in the fluorimeter before the initiation of the reaction by adding the microsomes preheated at 30°C. Fluorescence was recorded every 30 s for 2 h. Controls were done by omitting either NADPH or the substrate (one of the five sulphonated anthraquinones) or both of them (see Page and Schwitzguébel 2009, Fig. 2). Calculation of oxygen consumption converted from fluorescence changes during the assay, to evaluate the enzymatic activity towards the different substrates, was made according to Olry et al. (2007).

Extraction and assay of cytosolic peroxidases activity

Plants were washed with deionised water, dried with paper and the fresh weight was measured. The plant material was ground in a blender (Waring Laboratory Blender), 2 × 30 s, maximum speed, in the extraction buffer: 0.1 M sodium tetraborate adjusted to pH 8.0 with Tris–HCl (1 M), 2 µM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT (1,4-dithio-DL-threitol), 1 mM PMSF, 0.1% DMSO (dimethyl sulphoxide), 10 mg ml^{-1} PVP (polyvinylpyrrolidone). The ratio of the volume of the extraction buffer to the mass of plant material was 5–1. The homogenate obtained was filtered on Miracloth and centrifuged at 39,000×*g* for 30 min at 4°C. Supernatant was then collected for enzyme activity measurements. Concentration of proteins in the enzyme extract was measured by Lowry assay (Bio-Rad *DC* Protein Assay). Peroxidase activity was followed by spectrophotometry at 470 nm by the guaiacol oxidation rate in 1 ml reaction mixture containing 790 µl 0.1 M phosphate buffer pH 7.4, 5 µl guaiacol, 5 µl H_2O_2 (30%) and 200 µl of enzyme extract. Peroxidase activity was measured at room temperature.

Statistical analysis

The amount of microsomes extracted was large enough to do one measurement of the cytochromes P450 activity for each sulphonated anthraquinone, but repetitions of the measurement with the same extract were not possible. In order to know if the method for the measurement of cytochromes P450 activity can be repeated with the same results, a large extraction was made with higher amount of plant material, for the control and exposed plants. Fluorimetric analysis of the cytochromes P450 activity was then repeated three times. Standard deviations were calculated from these three replicates and were always smaller than 10% of the average of the three replicates. These repetitions of the measurement were made for rhubarb (Fig. 2), maize and celery (results not shown). The standard deviations in the case of maize and celery were also smaller than 10% of the average of the three replicates.

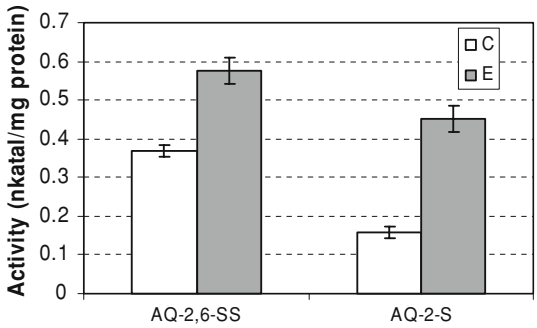

Fig. 2 Specific activity of cytochromes P450 in rhubarb. Average and standard deviations of three replicates are shown for cytochromes P450 activity (nanokatal per milligram of protein) in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*) when *AQ-2,6-SS* and *AQ-2-S* were used as substrate. The standard deviations were always smaller than 10% of the average of the three replicates

Results

Effect of sulphonated anthraquinones on plants

To know the possible effect of sulphonated anthraquinones on plants, the visual aspect of rhubarb, maize and celery was observed, and the fresh weight was measured at each collecting time. Plants of rhubarb and maize exposed to the mixture of xenobiotics looked very similar to the control plants (Fig. 3). This was not the case for celery, since the exposed plants showed yellow and dry leaves. Furthermore, the celery exposed to sulphonated anthraquinones showed a lower fresh weight than the control plants (Fig. 4). The difference between the fresh weight of exposed and control plants already appeared after 2 weeks of exposure to the contaminants. The plants of rhubarb and maize were growing normally despite the presence of sulphonated anthraquinones.

Cytochrome P450 activities

Cytochrome P450 monooxygenases are involved in the detoxification processes of many organic contaminants in plants (Khatisashvili et al. 1997; Werck-Reichhart et al. 2000; Morant et al. 2003; Isin and Guengerich 2007).

Fig. 3 Plants of rhubarb, celery and maize grown hydroponically. **a** Rhubarb, **b** celery and **c** maize plants grown hydroponically with (*right*) or without (*left*) sulphonated anthraquinones. **c** Two plants grown in the presence of AQs (*right*) and two control plants (*left*)

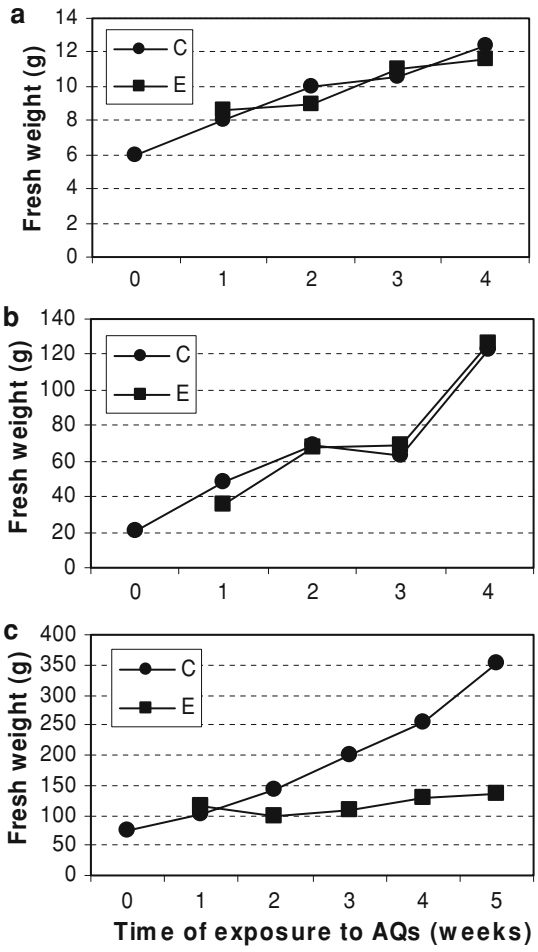

Fig. 4 Fresh weight of the plants. Comparison of the fresh weight (in grams) of plants grown in the presence of sulphonated anthraquinones (*E*) and control plants (*C*) for rhubarb (**a**), maize (**b**) and celery (**c**)

Plants exposed or not to the mixture of sulphonated anthraquinones were analysed at different times after the exposure to the contaminants in order to know if these enzymes were involved in the detoxification of the five sulphonated anthraquinones. A significant activity of cytochromes P450 was detected in the rhubarb plant with the different anthraquinones used as substrate (Fig. 5). Higher activity was detected in the plants exposed to the contaminants than in control plants. When AQ-2,6-SS, AQ-2-S and AQ-1-S were used as substrates, a higher activity was detected in the plants 1 week after the exposure to the xenobiotics, indicating an induction of these enzymes when plants were grown in the presence of the five sulphonated anthraquinones (Fig. 5a–c, week 1). Activity was not found at each collecting time when AQ-1,5-SS and AQ-1,8-SS were used as substrates. At the end of the experimentation, after 5 weeks of exposure, the roots and the shoot of the rhubarb were analysed separately. Activity was detected in the roots and in the shoot of control and exposed plants when AQ-2,6-SS, AQ-2-S and AQ-1-S were used as substrates, while activity was detected only in roots of exposed plants when AQ-1,5-SS was used as a substrate (Table 1). No activity was detected in the roots and in the shoot of control and exposed plants of rhubarb when AQ-1,8-SS was used as a substrate. This new method for the measurement of cytochromes P450 monooxygenases activity using sulphonated anthraquinones as substrates clearly showed that these xenobiotic compounds were accepted as substrates by the enzyme.

To compare the detoxification mechanisms of sulphonated anthraquinones in a plant producing natural anthraquinones (rhubarb) with plants that do not produce natural

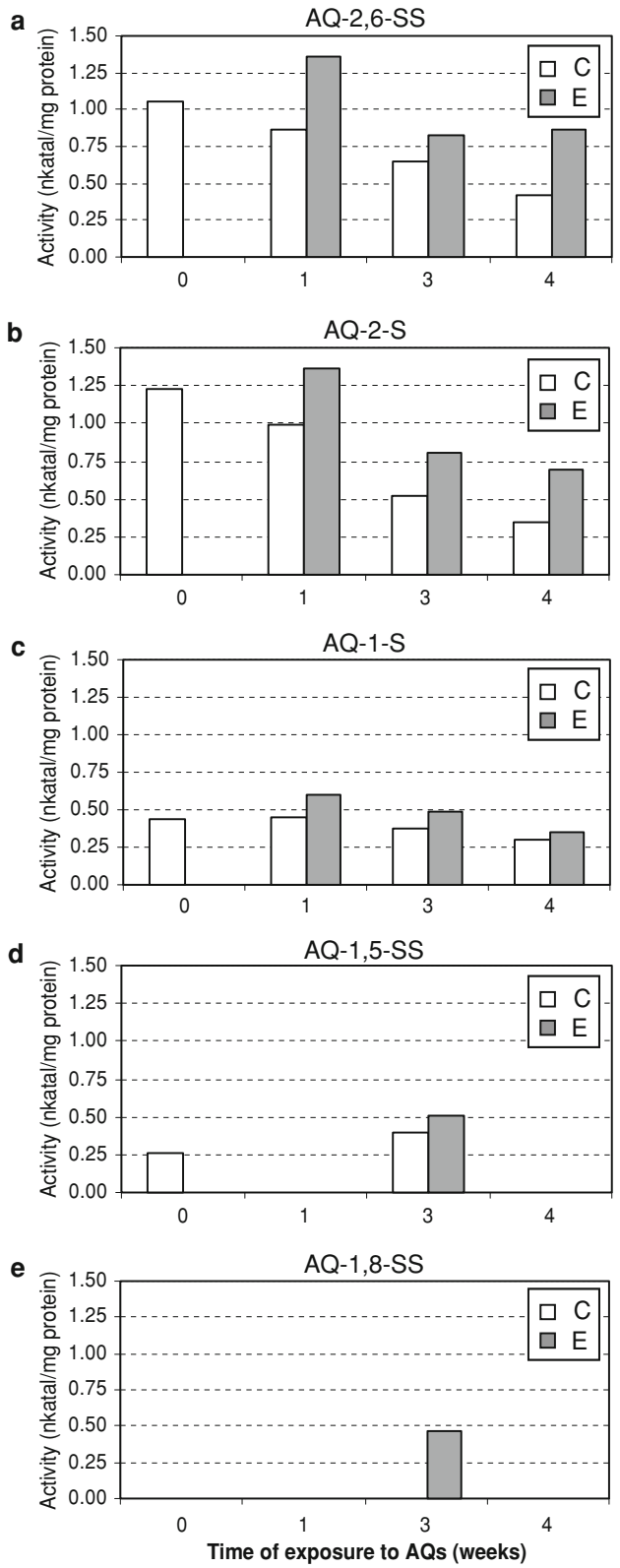

Fig. 5 Specific activity of cytochromes P450 in rhubarb. Comparison of the activity in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*) when AQ-2,6-SS (**a**), AQ-2-S (**b**), AQ-1-S (**c**), AQ-1,5-SS (**d**) and AQ-1,8-SS (**e**) were used as substrates. Activity is shown in nanokatal per milligram of protein

anthraquinones, cytochromes P450 activity was also analysed in maize and celery. An activity of cytochromes P450 towards all sulphonated anthraquinones was found in maize (Fig. 6). For most of the collecting times, a higher activity was detected in the exposed plants. In contrast to rhubarb, the activity after 1 week of exposure to the contaminants was not clearly higher than the activity detected at weeks 2 and 3 (Fig. 6a, b). At the end of the experiment, after 4 weeks, the activity of cytochromes P450 was detected in the shoot and in the roots of control and exposed maize plants, with the exception of control and exposed roots when AQ-1-S, AQ-1,5-SS and AQ-1,8-SS were used as substrates (Table 1). An activity towards all sulphonated anthraquinones was also found in celery plants, but no clear induction of the activity occurred after the first week of exposure to the pollutants (Fig. 7). The activity of cytochromes P450 when AQ-2,6-SS, AQ-2-S and AQ-1-S were used as substrates increased with the time of exposure to reach a higher level after 3 weeks and then it decreased. In contrary to maize, but similar to rhubarb, activity was not found at each collecting time when AQ-1,5-SS and AQ-1,8-SS were used as substrates. After 5 weeks of exposure to the xenobiotic compounds (end of the experiment), no activity was found in the shoot of control and exposed plants and in the roots of control plants when AQ-1,5-SS and AQ-1,8-SS were used as substrates, as well as in the roots of control plant with AQ-1-S (Table 1).

Cytosolic peroxidase activities

Beside several functions, peroxidases are multifunctional enzymes, also involved in a wide variety of detoxification mechanisms (Bhunia et al. 2001; Arrieta-Baez et al. 2002; Strycharz and Shetty 2002; Passardi et al. 2005; Karim and Husain 2009). To know if peroxidases were involved in the metabolism of sulphonated anthraquinones, samples of rhubarb, maize and celery were collected from the same experimentation as for cytochromes P450 and analysed for peroxidase activity. With guaiacol as a substrate, a significant activity was measured in rhubarb, maize and celery (Fig. 8). A higher activity was found in the exposed plants of rhubarb, while no significant difference was observed in the maize and celery plants growing with or without sulphonated anthraquinones. Peroxidase activity increased with the time of exposure to the xenobiotic compounds in rhubarb to reach the highest level at the end of the experiment (4 weeks). At that time, for exposed rhubarb plants, roots showed significantly higher activity than leaves. Even if there were no significant differences between control and exposed celery, activity increased also with the time of exposure in this plant (Fig. 8c). Maize roots, control and exposed, clearly showed a higher peroxidase activity than leaves (Fig. 8b).

Table 1 Specific activity of cytochromes P450 in shoot and roots of rhubarb, maize and celery plants exposed (E) or not (C) to sulphonated anthraquinones

Plants	Plant parts	Treatments	Cytochromes P450 activity (nkatal/mg protein)				
			AQ-1-S	AQ-2-S	AQ-1,5-SS	AQ-1,8-SS	AQ-2,6-SS
Rhubarb	Shoot	C	0.31	0.61	0.00	0.00	0.64
		E	0.26	0.47	0.00	0.00	0.53
	Roots	C	0.42	0.45	0.00	0.00	0.64
		E	0.52	0.45	0.48	0.00	0.49
Maize	Shoot	C	0.94	2.31	0.41	0.57	2.04
		E	0.72	1.93	0.21	0.41	1.67
	Roots	C	0.00	1.70	0.00	0.00	2.00
		E	0.00	0.83	0.00	0.00	0.83
Celery	Shoot	C	0.26	1.39	0.00	0.00	1.28
		E	0.35	0.53	0.00	0.00	0.65
	Roots	C	0.00	0.20	0.00	0.00	0.20
		E	0.48	0.52	0.35	0.51	0.62

Standard deviations were smaller than 10% (see Fig. 2 and statistical analysis)

Discussion

In the present study, rhubarb, maize and celery were grown with or without sulphonated anthraquinones to study their capacity for growing in the presence of these pollutants and their ability to metabolise these organic contaminants which are recalcitrant to biodegradation and not eliminated in normal wastewater treatments. The visual aspect of the plants and the fresh weight after 4 or 5 weeks of exposure to the xenobiotic contaminants showed that only celery suffered from the presence of sulphonated anthraquinones (see Figs. 3, 4). Celery was used with success in phytoremediation, for instance, in the treatment of industrial effluents (Dumont et al. 1999) and the remediation of polycyclic aromatic hydrocarbons contaminated soils (Yi and Crowley 2007). However, the fact that the celery exposed to the pollutants presented yellow leaves and was smaller than the control plants indicated that this plant might not be well adapted for the phytoremediation of sulphonated anthraquinones. It is important in phytoremediation that the plants tolerate the pollutant to be treated.

Cytochromes P450 have a large range of physiological functions in the plants, such as biosynthesis of hormones, lipids and secondary metabolites (Bolwell et al. 1994). The majority of these enzymes catalyse reactions consuming one molecule of oxygen and one molecule of NADPH (Olry et al. 2007). In the present work, a fast, reliable and simple method to detect the activity of cytochromes P450 was adapted from Olry et al. (2007). Moreover, this fluorimetric assay is a direct method to test potential substrates for a large variety of cytochromes P450 monooxygenases.

Chiapella et al. (1995) have shown that plant microsomes can contain other enzymes than cytochromes P450. Peroxidases for instance are also present in relatively high levels in this plant subcellular system. Microsomes were tested for peroxidase activity, using guaiacol as a substrate, but no activity of these enzymes was detected (data not shown). Furthermore, the fluorimetric reaction was negligible when NADH was used instead of NADPH (data not shown), but NADH has been reported to be less efficient as cofactor than NADPH (Stiborová et al. 2000). No reaction was observed when the NADPH regenerative system was omitted (data not shown). All these features and the fact that microsomes, NADPH and the sulphonated anthraquinones were all needed for the enzymatic reaction consuming oxygen, provided conclusive evidence for the involvement of cytochromes P450 in the oxidative metabolism of sulphonated anthraquinones.

A significant activity of cytochromes P450 was found in the roots and in the shoot of rhubarb, maize and celery plants when AQ-2-S and AQ-2,6-SS were used as substrates (Figs. 5, 6, 7; Table 1). These results suggest that the detoxification mechanisms of these two sulphonated anthraquinones took place in the roots and in the shoot of the plants studied. Furthermore, these results might also indicate that both xenobiotic compounds were transported from the roots to the shoot. Aubert and Schwitzguébel (2002) have shown that some sulphonated anthraquinones are found in the leaves of rhubarb and common sorrel after an exposure of 6 weeks to these compounds. When AQ-1,5-SS and AQ-1,8-SS were used as substrates, an activity of cytochromes P450 was detected only in the exposed roots of rhubarb (AQ-1,5-SS) and celery (both AQs). These results suggested that detoxification mechanisms of these two pollutants took place only in the roots or that these two organic compounds were not transported from the roots to

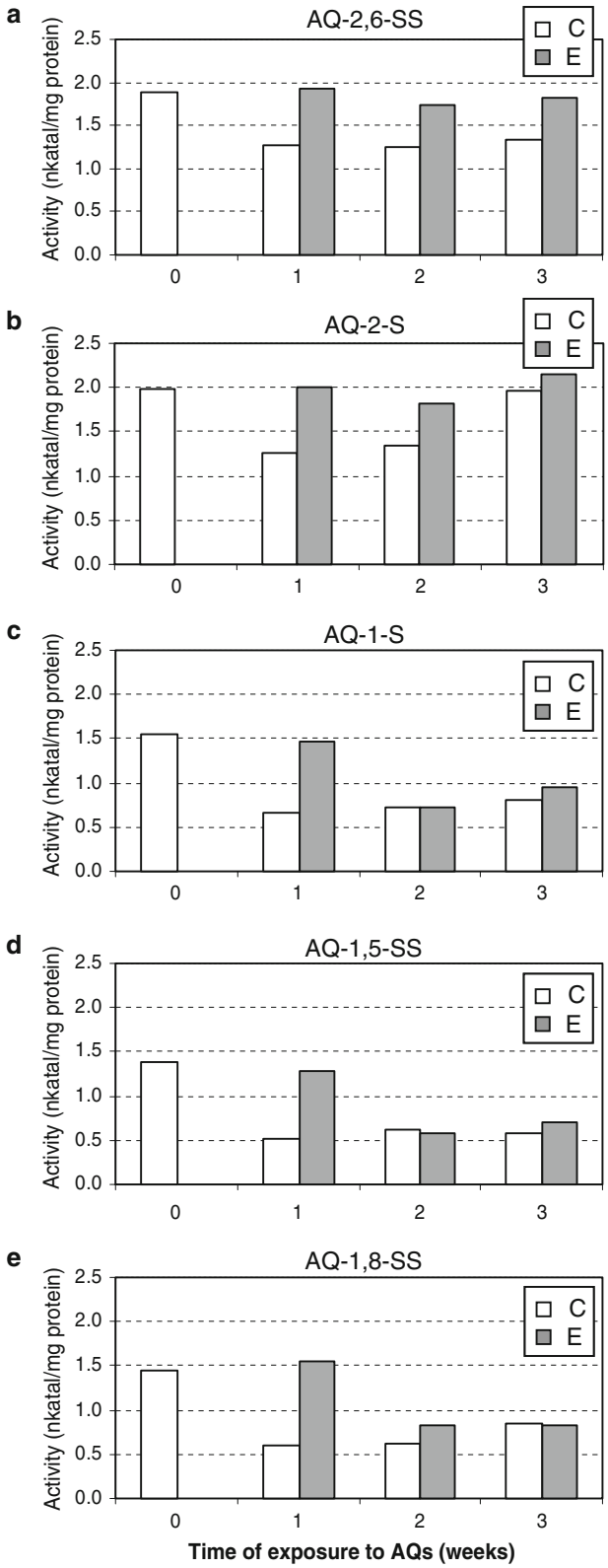

Fig. 6 Specific activity of cytochromes P450 in maize. Comparison of the activity in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*) when AQ-2,6-SS (**a**), AQ-2-S (**b**), AQ-1-S (**c**), AQ-1,5-SS (**d**) and AQ-1,8-SS (**e**) were used as substrates. Activity is shown in nanokatal per milligram of protein

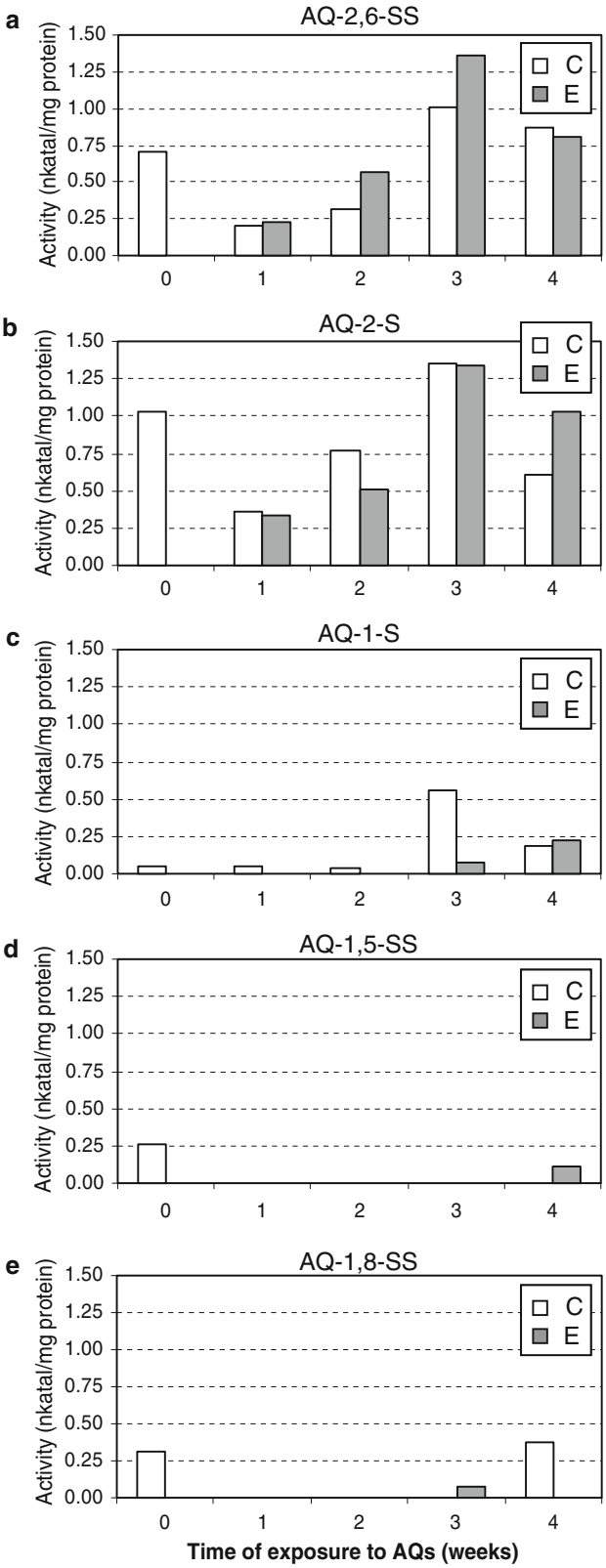

Fig. 7 Specific activity of cytochromes P450 in celery. Comparison of the activity in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*) when AQ-2,6-SS (**a**), AQ-2-S (**b**), AQ-1-S (**c**), AQ-1,5-SS (**d**) and AQ-1,8-SS (**e**) were used as substrates. Activity is shown in nanokatal per milligram of protein

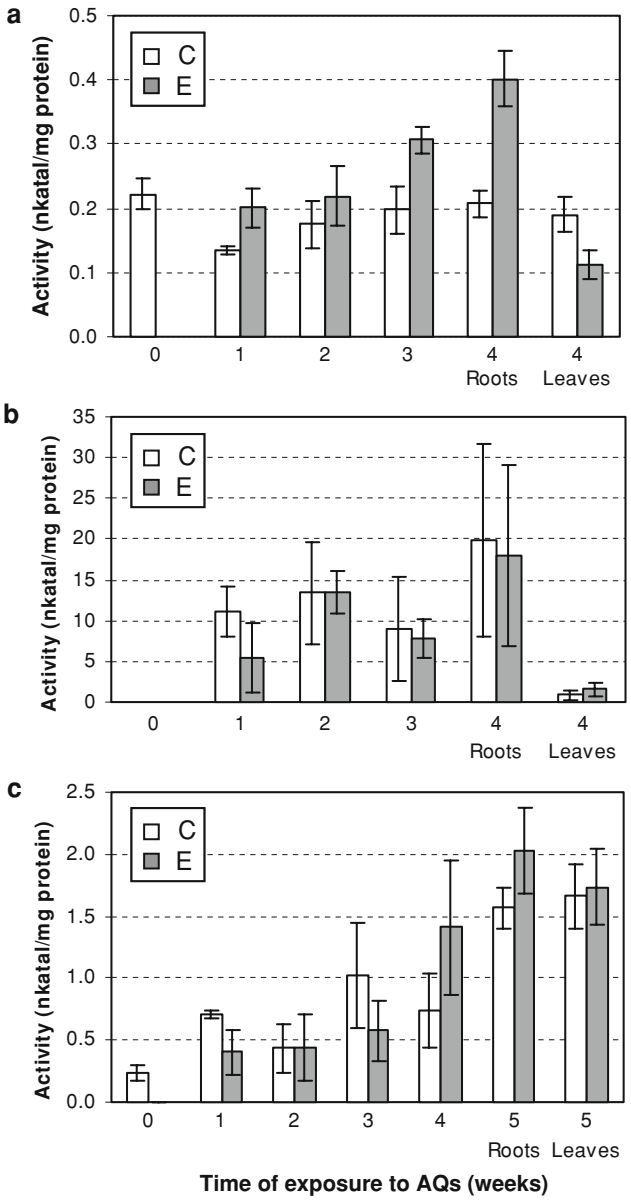

Fig. 8 Specific activity of peroxidases in rhubarb (**a**), maize (**b**) and celery (**c**). Average and standard deviations of six replicates are shown for peroxidases activity (nanokatal per milligram of protein) in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*)

the shoot. Sulphonated anthraquinones may also be transferred to the shoot after transformation as shown by Aubert (2003) and Schwitzguébel et al. (2009). Actually, new metabolites are found in leaf extracts from rhubarb plants cultivated in the presence of the sulphonated anthraquinones. On the other hand, Schwitzguébel et al. (2008) have shown that sulphonated anthraquinones and metabolites derived from them are present in leaves of rhubarb, maize and common sorrel after the direct injection of the pollutants in cut stems of these plants. The metabolites formed are different for the three plants tested and also different from those obtained in whole plants. These results indicate that transformations of the sulphonated anthraquinones can occur in the roots and in the shoot and that transformations occurring in the roots are different from those occurring in the shoot. All these features and the fact that cytochromes P450 were able to accept as substrates the five sulphonated anthraquinones suggest that these enzymes were involved in the metabolism of these pollutants.

Activity of cytochromes P450 was not found at each collecting time for rhubarb and celery plants when AQ-1,5-SS and AQ-1,8-SS were used as substrates (Figs. 5, 7), while an activity was found at each collecting time in maize plants (Fig. 6). Comparable results were already obtained with adult rhubarb plants (no activity at each collecting time) and common sorrel plants (activity at each collecting time) (Page and Schwitzguébel 2009). The fact that some plants are (rhubarb and common sorrel) or not (maize and celery) natural anthraquinones producing plants may not explain this difference, as well as the fact that the rhubarb, the celery and the common sorrel are dicotyledons while maize is a monocotyledon plant. A result found in this study, similar for the three plants tested and also in the adult plants of rhubarb and common sorrel (Page and Schwitzguébel 2009) is that the activity was smaller when AQ-1,5-SS and AQ-1,8-SS were used as substrates than with AQ-2-S or AQ-2,6-SS. The activity found with AQ-1-S was always in the middle. Maybe the affinity of cytochromes P450 for AQ-1,5-SS and AQ-1,8-SS was lower, due to the position of the sulphonated group in the molecule. The relatively high activity of cytochromes P450 found in all plants when AQ-2-S was used as substrate and the results of Schwitzguébel and Vanek (2003) showing that rhubarb cells cultivated in the presence of AQ-2-S release an increasing amount of sulphate suggest that the cytochromes P450 might be responsible for the desulphonation of AQ-2-S.

The activity of peroxidases was found in all plants studied (Fig. 8). A higher activity was measured in the exposed plants of rhubarb and this activity increased with the time of exposure to the sulphonated anthraquinones. Moreover, exposed roots of rhubarb showed four times higher activity than the shoot. These results suggest the involvement of peroxidases in the detoxification of sulphonated anthraquinones and this process took place mainly in the roots, the plant part directly in contact with the pollutants. In celery, the peroxidases activity increased also with the time of exposure, but in contrast to rhubarb, no significant difference between control and exposed plants was found. Whereas peroxidases are involved in mechanism of stress response and in the degradation of some xenobiotic compounds (Bhunia et al. 2001; Carias et al. 2008), they also have several functions in relation to the

growth of the cell (Passardi et al. 2005). The peroxidases activity found in the celery might be due to these functions rather than detoxification mechanism.

Arrieta-Baez et al. (2002) have shown that peroxidases isolated from horseradish and from *Senna angustifolia* can transform natural hydroxy-9,10-anthraquinone. Nevertheless, the ability of peroxidases to accept sulphonated anthraquinones remains unknown. Also, the method used in the present work did not allow to know if the peroxidases might accept sulphonated anthraquinones as a substrate and thus to prove the involvement of this family of enzymes in the detoxification mechanism of these compounds. But the promising results found with rhubarb, a plant producing natural anthraquinones, seemed to indicate a possible function of peroxidases in the detoxification of these aromatic compounds. Further studies should be performed to find the possible involvement of peroxidases in the metabolism of sulphonated anthraquinones by anthraquinone-producing plants.

The results obtained in the present work confirmed that rhubarb has a strong potential for the remediation of sulphonated anthraquinones from contaminated industrial effluents. In this plant, cytochromes P450 and peroxidases seemed to be involved in the metabolism of these xenobiotic compounds. Further investigation should be performed with this plant to find the next steps of this detoxification pathway. Glycosyltransferases might be enzymes of interest. Actually, natural anthraquinones are mostly present as glycosides in plants (Khouri and Ibrahim 1987; Van der Plas et al. 1998; Matsuda et al. 2001). Moreover, after hydroxylation of an organic pollutant by cytochromes P450, glycosylation is usually the next step of detoxification pathway (Coleman et al. 1997; Pflugmacher and Sandermann 1998). Anthraquinone-producing plants could possibly glycosylate synthetic anthraquinones. The next step should thus be to test this hypothesis by further investigation on glycosyltransferases.

Acknowledgments This work was supported by the Swiss Secretariat for Education and Research (Project SER C05.0027), in the framework of COST Action 859. We are grateful to Alexandre Olry for the advices for the adaptation of the method. We gratefully acknowledge Anita Maric, Corinne Weis, Raphaël Meylan and Blaise Gafsou for their technical assistance.

References

Arrieta-Baez D, Roman R, Vazquez-Duhalt R, Jiménez-Estrada M (2002) Peroxidase-mediated transformation of hydroxy-9,10-anthraquinones. Phytochemistry 60:567–572

Aubert S (2003) Accumulation and transformation of sulphonated anthraquinones by higher plants: a first step towards the phytotreatment of wastewater from dye and textile industry, PhD thesis Nr 2809, EPFL, 153 p

Aubert S, Schwitzguébel JP (2002) Capillary electrophoretic separation of sulphonated anthraquinones in a variety of matrices. Chromatographia 56:693–697

Aubert S, Schwitzguébel JP (2004) Screening of plant species for the phytotreatment of wastewater containing sulphonated anthraquinones. Water Res 38:3569–3575

Bhunia A, Durani S, Wangikar PP (2001) Horseradish peroxidase catalyzed degradation of industrially important dyes. Biotechnol Bioeng 72:562–567

Biddlestone AJ, Gray KR, Thurairajan K (1991) A botanical approach to the treatment of wastewaters. J Biotechnol 17:209–220

Bolwell GP, Bozak K, Zimmerlin A (1994) Plant cytochrome P450. Phytochemistry 37:1491–1506

Brankova L, Ivanov S, Alexieva V (2007) The induction of microsomal NADPH:cytochrome P450 and NADH:cytochrome b5 reductases by long-term salt treatment of cotton (*Gossypium hirsutum* L.) and bean (*Phaseolus vulgaris* L.) plants. Plant Physiol Biochem 45:691–695

Bulc TG, Ojstrsek A (2008) The use of constructed wetland for dye-rich textile wastewater treatment. J Hazard Mat 155:76–82

Carias CC, Novais JM, Martins-Dias S (2008) Are *Phragmites australis* enzymes involved in the degradation of the textile azo dye acid orange 7? Bioresource Technol 99:243–251

Chiapella C, Ysern P, Riera J, Llagostera M (1995) A plant metabolic activation system from *Persea americana* with cytochrome P450-dependent and peroxidase activities. Mutation Res 329:11–18

Coleman JOD, Blake-Kalff MMA, Davies TGE (1997) Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. Trend Plant Sci 2:144–151

Davies TH, Cottingham PD (1994) The use of constructed wetlands for treating industrial effluent (textile dyes). Water Sci Technol 29:227–232

Davies LC, Carias CC, Novais JM, Martins-Dias S (2005) Phytoremediation of textile effluents containing azo dye by using *Phragmites australis* in a vertical flow intermittent feeding constructed wetland. Ecol Eng 25:594–605

Duc R, Vanek T, Soudek P, Schwitzguébel JP (1999) Accumulation and transformation of sulfonated aromatic compounds by rhubarb cells (*Rheum palmatum*). Int J Phytorem 1:255–271

Dumont P, Phillipon P, Xanthoulis D (1999) Du célery pour épurer les eaux. Biofutur 188:40–43

Furukawa K, Fujita M (1993) Advanced treatment and food production by hydroponic type wastewater treatment plant. Water Sci Technol 28:219–228

Gordeziani M, Khatisashvili G, Ananiashvili T, Varazashvili T, Kurashvili M, Kvesitadze G, Tkhelidze P (1999) Energetic significance of plant monooxygenase individual components participating in xenobiotics degradation. Int Biodeterior Biodegrad 44:49–54

Haberl R, Grego S, Langergraber G, Kadlec RH, Cicalini AR, Martins Dias S, Novais JM, Aubert S, Gerth A, Thomas H, Hebner A (2003) Constructed wetlands for the treatment of organic pollutants. J Soil Sediments 3:109–124

Han YS, van der Heijden R, Lefeber AWM, Erkelens C, Verpoorte R (2002) Biosynthesis of anthraquinones in cell cultures of *Cinchona* "Robusta" proceeds via the methylerythritol 4-phosphate pathway. Phytochemistry 59:45–55

Isin EM, Guengerich FP (2007) Complex reactions catalyzed by cytochrome P450 enzymes. Biochim Biophys Acta 1770: 314–329

Jouili H, Bouazizi H, Rossignol M, Borderies G, Jamet E, El Ferjani E (2008) Partial purification and characterization of a copper-induced anionic peroxidase of sunflower roots. Plant Physiol Biochem 46:760–767

Karim Z, Husain Q (2009) Guaiacol-mediated oxidative degradation and polymerization of bisphenol A catalyzed by bitter gourd

(*Momordica charantia*) peroxidase. J Mol Catal B Enzym 59:185–189

Khatisashvili G, Gordeziani M, Kvesitadze G, Korte F (1997) Plant monooxygenases: participation in xenobiotic oxidation. Ecotoxicol Environ Safe 36:118–122

Khouri HE, Ibrahim RK (1987) Purification and some properties of five anthraquinone-specific glucosyltransferases from *Cinchona succirubra* cell suspension culture. Phytochemistry 26:2531–2535

Matsuda H, Morikawa T, Toguchida I, Park JY, Harima S, Yoshikawa M (2001) Antioxidant constituents from rhubarb: structural requirements of stilbenes for the activity and structures of two new anthraquinone glucosides. Bioorg Medicinal Chem 9:41–50

Monnet F, Vaillant N, Hitmi A, Vernay P, Coudret A, Sallanon H (2002) Treatment of domestic wastewater using the nutrient film technique (NFT) to produce horticultural roses. Water Res 36:3489–3496

Morant M, Bak S, Møller BL, Werck-Reichhart D (2003) Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Curr Opin Biotechnol 14:151–162

Olry A, Schneider-Belhaddad F, Heintz D, Werck-Reichhart D (2007) A medium-throughput screening assay to determine catalytic activities of oxygen-consuming enzymes: a new tool for functional characterization of cytochrome P450 and other oxygenases. Plant J 51:331–340

Page V, Schwitzguébel JP (2009) The role of cytochromes P450 and peroxidases in the detoxification of sulphonated anthraquinones by rhubarb and common sorrel plants cultivated under hydroponic conditions. Environ Sci Pollut Res. doi:10.1007/s11356-009-0197-2 (in press)

Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. Plant Cell Rep 24:255–265

Pflugmacher S, Sandermann H (1998) Taxonomic distribution of plant glucosyltransferases acting on xenobiotics. Phytochemistry 49:507–511

Schwitzguébel JP, Vanek T (2003) Some fundamental advances for xenobiotic chemicals. In: McCutcheon SC, Schnoor JL (eds) Phytoremediation: transformation and control of contaminants. Wiley, Hoboken, pp 123–157

Schwitzguébel JP, Aubert S, Grosse W, Laturnus F (2002) Sulphonated aromatic pollutants—limits of microbial degradability and potential of phytoremediation. Environ Sci Pollut Res 9:62–72

Schwitzguébel JP, Braillard S, Page V, Aubert S (2008) Accumulation and transformation of sulfonated aromatic compounds by higher plants—toward the phytotreatment of wastewater from dye and textile industries. In: Khan NA, Singh S, Umar S (eds) Sulfur assimilation and abiotic stress in plants. Springer, Berlin, pp 335–353

Schwitzguébel JP, Page V, Martins Dias S, Davies L, Vasilyeva G, Strijakova E (2009) Using plants to remove foreign compounds from contaminated water and soil. In: Schröder P, Collins C (eds) Organic xenobiotics and plants: from mode of action to ecophysiology. Springer, Berlin (in press)

Singer AC, Crowley DE, Thompson IP (2003) Secondary plant metabolites in phytoremediation and biotransformation. Trends Biotechnol 21:123–130

Stiborová M, Schmeiser HH, Frei E (2000) Oxidation of xenobiotics by plant microsomes, a reconstituted cytochrome P450 system and peroxidase: a comparative study. Phytochemistry 54:353–362

Strycharz S, Shetty K (2002) Peroxidase activity and phenolic content in elite clonal lines of *Mentha pulegium* in response to polymeric dye R-478 and *Agrobacterium rhizogenes*. Process Biochem 37:805–812

Van der Plas LHW, Hagendoorn MJM, Jamar DCL (1998) Anthraquinone glycosylation and hydrolysis in *Morinda citrifolia* cell suspensions: regulation and function. J Plant Physiol 152:235–241

Werck-Reichhart D, Hehn A, Didierjean L (2000) Cytochromes P450 for engineering herbicide tolerance. Trends Plant Sci 5:116–123

Yi H, Crowley DE (2007) Biostimulation of PAH degradation with plants containing high concentrations of linoleic acid. Environ Sci Technol 41:4382–4388