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The role of cytochromes P450 and peroxidases in the detoxification of sulphonated anthraquinones by rhubarb and common sorrel plants cultivated under hydroponic conditions

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Abstract
Background, aim and scope Sulphonated anthraquinones are precursors of many synthetic dyes and pigments, recalcitrant to biodegradation and thus not eliminated by classical wastewater treatments. In the development of a phytotreatment to remove sulphonated aromatic compounds from dye and textile industrial effluents, it has been shown that rhubarb (*Rheum rabarbarum*) and common sorrel (*Rumex acetosa*) are the most efficient plants. Both species, producing natural anthraquinones, not only accumulate, but also transform these xenobiotic chemicals. 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. The aim of the present study was to investigate the possible roles of cytochrome P450 monooxygenases and peroxidases in the detoxification of several sulphonated anthraquinones.
Materials and methods Both plant species were cultivated in a greenhouse under hydroponic conditions, with or without sulphonated anthraquinones. Plants were harvested at different times and either microsomal or cytosolic fractions were prepared. The monooxygenase activity of cytochromes P450 toward 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 A significant activity of cytochromes P450 was detected in rhubarb leaves, while no (rhizome) or low (petioles and roots) activity was found in other parts of the plants. An induction of this enzyme was observed at the beginning of the exposition to sulphonated anthraquinones. The results also indicated that cytochromes P450 were able to accept as substrate the five sulphonated anthraquinones, with a higher activity toward AQ-2,6-SS (0.706 nkat/mg protein) and AQ-2-S (0.720 nkat/mg protein). An activity of the cytochromes P450 was also found in the leaves of common sorrel (1.212 nkat/mg protein (AQ-2,6-SS)), but no induction of the activity occurred after the exposition to the pollutant. The activity of peroxidases increased when rhubarb was cultivated in the presence of the five sulphonated anthraquinones (0.857 nkat/mg protein). Peroxidase activity was also detected in the leaves of the common sorrel (0.055 nkat/mg protein), but in this plant, no significant difference was found between plants cultivated with and without sulphonated anthraquinones.
Discussion Results indicated that the activity of cytochromes P450 and peroxidases increased in rhubarb in the presence of sulphonated anthraquinones and were involved in their detoxification mechanisms.
Conclusions These results suggest the existence in rhubarb and common sorrel of specific mechanisms involved in the metabolism of sulphonated anthraquinones. Further investigation should be performed to find the next steps of this detoxification pathway.
Recommendations and perspectives Besides these promising results for the phytotreatment of sulphonated anthraquinones, it will be of high interest to develop and test, at

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small scale, an experimental wastewater treatment system to determine its efficiency. On the other hand, these results reinforce the idea that natural biodiversity should be better studied to use the most appropriate species for the phytotreatment of a specific pollutant.

Keywords Common sorrel · Cytochromes P450 monooxygenases · Detoxification pathway · Dye industry · Hydroponic systems · Peroxidases · Phytoremediation · *Rheum rabarbarum* · Rhubarb · *Rumex acetosa* · Sulphonated anthraquinones · Textile industry · Xenobiotic chemicals

1 Background, aim and scope

Every year, thousands of different synthetic molecules of dyes and pigments are produced worldwide for a total of around one million tons. A significant amount of these chemicals are discharged into the environment, mainly via industrial effluents (Willetts and Ashbolt 2000). Synthetic sulphonated anthraquinones (Fig. 1) are the parent compounds for a large palette of dyes and an important starting material in their production. Wastewater from the dye and textile industries, as well as leachates from landfills, are thus often contaminated with sulphonated aromatics, giving to these chemicals an actual impact on the environment, especially fresh water (Riediker et al. 2000; Schwitzguébel et al. 2002). The pollution of many rivers by sulphur-organic xenobiotics is largely due to this class of chemicals and many of them have acute and/or chronic effects on aquatic organisms (Greim et al. 1994).

Removal of sulphonated aromatic compounds is thus a major challenge for dye and textile industries, not only because of the colour of these chemicals, but also due to their recalcitrance and toxicity (Schwitzguébel et al. 2002; Dos Santos et al. 2005; Liu and Sun 2009). During the last decade, several physical techniques have been tested such as adsorption, precipitation, ion exchange or filtration (Nigam et al. 2000; Robinson et al. 2001). Chemical treatment possibilities based on electrochemistry, oxidative processes or enzymatic degradation, have also been investigated. However, these physical-chemical treatments have major disadvantages, including high cost, low efficiency and inapplicability to a wide variety of dyes, or the formation of by-products, creating waste disposal problems (Vandevivere et al. 1998; Robinson et al. 2001).

Dyes and pigments are intentionally designed to be hard-wearing under typical usage conditions, and this property makes treatment difficult. Because they usually contain at least one sulphonic group and often also varying substitutions such as nitro groups, these chemicals are not uniformly susceptible to biodegradation (McMullan et al. 2001).

Bacterial degradation of dyes and by-products often requires unusual catabolic activities rarely found in a single species (Cook et al. 1999). An important step appears to be catalysed by dioxygenases adding oxygen across the double bond bearing the sulphonate group, leading to its elimination (McMullan et al. 2001). Unfortunately, a rather limited substrate range has been observed for bacterial isolates containing these enzymes and the accumulation of dead-end products often occurs (Schwitzguébel et al. 2002). Furthermore, toxic effects of several anthraquinone dyes on aerobic bacteria or on methanogens have been reported (Dos Santos et al. 2005; Liu and Sun 2009). The decolourisation of synthetic dyes including azo and anthraquinone derivatives has also been examined in white-rot

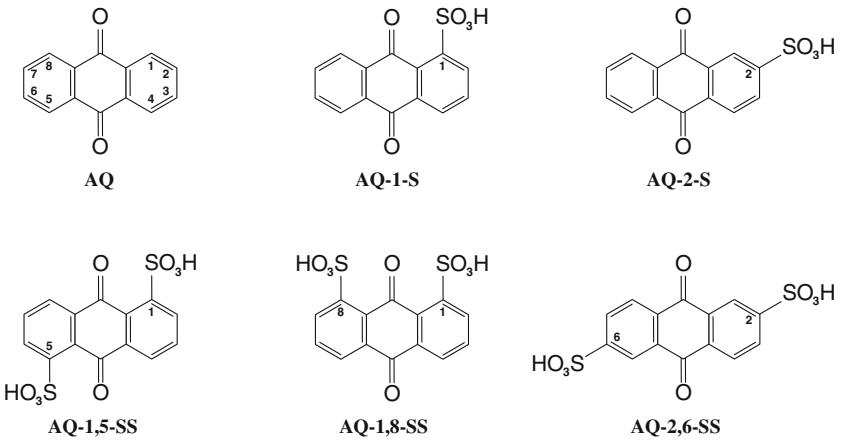

Fig. 1 Chemical structure of anthraquinone and sulphonated derivatives. The IUPAC names of sulphonated anthraquinones are: 9,10-dioxo-9,10-dihydro-1-anthracenesulphonic acid (*AQ-1-S*); 9,10-dioxo-9,10-dihydro-2-anthracenesulphonic acid (*AQ-2-S*); 9,10-dioxo-9,10-dihydro-1,5-anthracenedisulphonic acid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

fungal cultures, known to produce powerful laccases and other peroxidases (Claus et al. 2002; Nyanhongo et al. 2002). However, it appears that high concentrations, depending on the individual dye structure and on the fungal species, cause slower decolourisation (Wesenberg et al. 2003). The limited ability of micro-organisms to degrade sulphonoaromatic compounds, and thus to cope with various mixtures of these xenobiotics, limits the efficiency and, therefore, the use of conventional wastewater treatment plants. In this context, the development of alternative biological treatments to eliminate these pollutants from industrial effluents is a requirement (Robinson et al. 2001). More precisely, constructed wetlands or hydroponic systems based on selected plant species are able to remove a wide range of xenobiotics from industrial effluents (Biddlestone et al. 1991; Furukawa and Fujita 1993; Davies and Cottingham 1994; Haberl et al. 2003; Davies et al. 2005; Bulc and Ojstrsek 2008; Carias et al. 2008). They can offer a low cost, low-maintenance green approach to treat wastewater.

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). Higher plants also produce a large number of secondary metabolites, which often play a major role in the interactions of the plant with its environment or serve as signal molecules (Singer et al. 2003). Plant metabolism is extremely diverse and can be exploited to treat recalcitrant pollutants, not degradable by bacteria or fungi, and 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).

Anthraquinones (AQ) are an important group of plant secondary metabolites occurring in several genera like *Rheum*, *Cinchona*, *Morinda*, *Rumex* and *Rubia* (Van der Plas et al. 1998; Han et al. 2002). Even if the precise functions of anthraquinones in plants remain largely unknown, they have been reported to have antioxidant, antimicrobial, antifungal, antiviral, hypotensive, analgesic, laxative, antimalarial and anti-tumour activities (Demirezer et al. 2001; Matsuda et al. 2001). On the other hand, some peroxidases are able to catalyse the formation of dimeric AQ from hydroxy-9,10-anthraquinones (Arrieta-Baez et al. 2002). Furthermore, most of the natural AQs are glycosylated, whereas glycosyltransferases are enzymes known to be involved in the conjugation of many xenobiotic compounds (Khouri and Ibrahim 1987; Pflugmacher and Sandermann 1998; Jones and Vogt 2001; Brazier et al. 2002).

Plants producing natural anthraquinones might thus possess enzymes able to accept sulphonated anthraquinones as substrates. To test this hypothesis, cells have been isolated from rhubarb (*Rheum rabarbarum*) and cultivated in shake flasks and bioreactors in the presence of up to 700–800 mg/l anthraquinones—a usual concentration in primary effluents from dye production lines—with sulphonated groups in different positions (see Fig. 1). The most significant results indicate that: AQ-1-S totally disappears from the medium, phytotransformation occurs, but not desulphonation; AQ-2-S is partially but rapidly taken up by rhubarb cells, metabolised and desulphonated, as indicated by the stoichiometric release of sulphate into the medium; AQ-1,5-SS and AQ-1,8-SS rapidly disappear from the medium, but no intermediates are released (Schwitzguébel et al. 2002; Schwitzguébel and Vanek 2003). The ability of rhubarb cells to accumulate, transform and/or degrade other sulphonated aromatic compounds, all being by-products and pollutants present in effluents from a dye-producing company has also been investigated: 2-hydroxy-4-sulpho-1-naphthalenediazonium is completely removed and transformed; 2-hydroxy-4-sulpho-6-nitro-1-naphthalenediazonium is efficiently accumulated and desulphonated; 7-nitro-1,3-naphthalenedisulphonic acid, 7-amino-1,3-naphthalenedisulphonic acid and 2-chloro-5-nitro benzene sulphonic acid are taken up and transformed by rhubarb cells (Duc et al. 1999).

Since rhubarb is a hardy and perennial plant, it might be a promising candidate in developing new biological processes to decontaminate effluents containing sulphonated aromatic compounds. It has been the aim of a further investigation to test if whole rhubarb, sorrel and other plants grown under hydroponic conditions can cope with sulphonated anthraquinones. Results have shown that mono- as well as disulphonated anthraquinones are efficiently taken up by rhubarb, especially the Valentine variety, and other anthraquinone-producing species, and translocated to the shoot. The presence of putative metabolites, not yet identified, in leaves has also been observed, indicating the transformation of several sulphonated anthraquinones (Aubert and Schwitzguébel 2002, 2004; Haberl et al. 2003; Schwitzguébel et al. 2009). The metabolites found in the different plant species tested are not similar among them, indicating that the transformation of these xenobiotics is dependent on plant species.

Many of the enzymes involved in the early stages of the detoxification process are closely associated with the redox biochemistry of the cell. The activity of enzymes such as glutathione transferases, peroxidases and cytochrome P450 monooxygenases have implications with respect to the maintenance of redox homeostasis (Gordeziani et al. 1999; Stiborová et al. 2000; Werck-Reichhart et al. 2000).

Whereas plant "peroxidases have more functions than a Swiss army knife" (Passardi et al. 2005), cytochrome P450 monooxygenases represent a multigenic family of enzymes involved in the detoxification process of many xenobiotic compounds (Khatisashvili et al. 1997; Werck-Reichhart et al. 2000; Morant et al. 2003; Isin and Guengerich 2007; Olry et al. 2007). They catalyse complex reactions, NAD(P)H reducing the iron in the heme centre to the ferrous form, which then binds O_2. The oxygen molecule is cleaved: one atom is inserted into the xenobiotic molecule, whereas the second is released as water. Electrons from NAD(P)H are transferred to P450 via flavoproteins called cytochrome P450 reductases. Both plant P450 and their reductases are usually bound via their N-terminus to the cytoplasmic surface of the endoplasmic reticulum. The hydroxylation of an aromatic ring can generate a phenolic intermediate that may have the potential to dissipate proton gradients and uncouple oxidative phosphorylation. In addition to activating xenobiotics, cytochromes P450 play an important role in the normal secondary metabolism of plants, which produce compounds involved in cell signalling and defence mechanisms. Overloading a plant with high concentrations of xenobiotics requiring oxidation by cytochromes P450 may thus compete with the normal functions of these enzymes. There is evidence that the presence of xenobiotics can induce the synthesis of numerous cytochromes P450. An overall increase in the activity of the mixed functional oxidases may impose a major demand on both the oxygen and the NADPH pool within the cell. This could have significant effects on the redox balance of the cell, thus compromising the primary metabolic processes in the plant, making it more sensitive to environmental stresses (Gordeziani et al. 1999).

Enzymatic studies were performed to determine if sulphonated anthraquinones might be transformed by enzymes of the classical detoxification pathways in plants. Preliminary results obtained with glutathione *S*-transferases have shown that this class of enzymes is probably not significantly involved in the metabolism of these xenobiotic compounds (Aubert 2003). In the present study, the possible role of cytochrome P450 monooxygenases and peroxidases in the metabolism of synthetic sulphonated anthraquinones was investigated. For such a purpose, microsomal and cytosolic fractions were isolated from plant species producing natural anthraquinones, namely rhubarb (*R. rabarbarum*) and common sorrel (*Rumex acetosa*), cultivated under hydroponic conditions in the presence or absence of 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.

2 Materials and methods

2.1 Plant material and cultivation

Plants of *R. rabarbarum* (rhubarb), Valentine variety, and *R. acetosa* (common sorrel) were provided by Schilliger, Gland, Switzerland. Rhubarb and common sorrel were grown in a greenhouse under hydroponic conditions in polypropylene tanks (40×30×32 cm deep) for rhubarb and (40×30×17 cm deep) for common sorrel. Each tank contained one plant and was filled with 12 L (rhubarb) or 8 L (common sorrel) of water and Luwasa (30110, Interhydro AG, Allmendingen, Switzerland) was supplied as plant nutrients. Oxygenation of the liquid medium was performed by means of aquarium pumps. Plants were maintained in the tank through polypropylene colander. Roots were half immersed into water to avoid any lack of oxygenation and were protected from light by black plastic bags covering the tanks. Tanks were refilled twice a week. The temperature was kept between 20 and 25°C, with an illumination of 300 W/m^2 for 15 h per day. Rhubarb and common sorrel plants were cultivated under hydroponic conditions for several months before the exposure to sulphonated anthraquinones.

2.2 Plants exposure to sulphonated anthraquinones

Three plants of rhubarb were exposed for 30 weeks to sulphonated anthraquinones, while four plants of common sorrel were exposed for 5 weeks. The five sulphonated anthraquinones were added together to the culture media 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). Three rhubarb plants and four common sorrel 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; see 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).

2.3 Design of the experiments

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 and microsomal cyto-

chromes P450. For each extraction, 10 g of leaves were collected from the four control plants of the common sorrel. For rhubarb, one leaf was collected from each of the three control plants for each extraction. Afterwards, samples from control and exposed plants were collected once a week, during 5 (common sorrel) or 30 (rhubarb) weeks. To have enough plant material, the three leaves of rhubarbs (control or exposed) were pooled for the cytosolic peroxidases or the cytochromes P450 extractions. At the end of the experimentation, leaves, petioles, rhizomes and roots of rhubarbs exposed or not to sulphonated anthraquinones were collected for the cytosolic and the microsomal fraction extractions and for the enzymatic assays.

2.4 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 to 1. The homogenate obtained 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 a 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, 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 (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).

2.5 Extraction and assay of cytosolic peroxidases activity

The plants were washed with deionised water, dried with paper and the fresh weight was measured. The plant

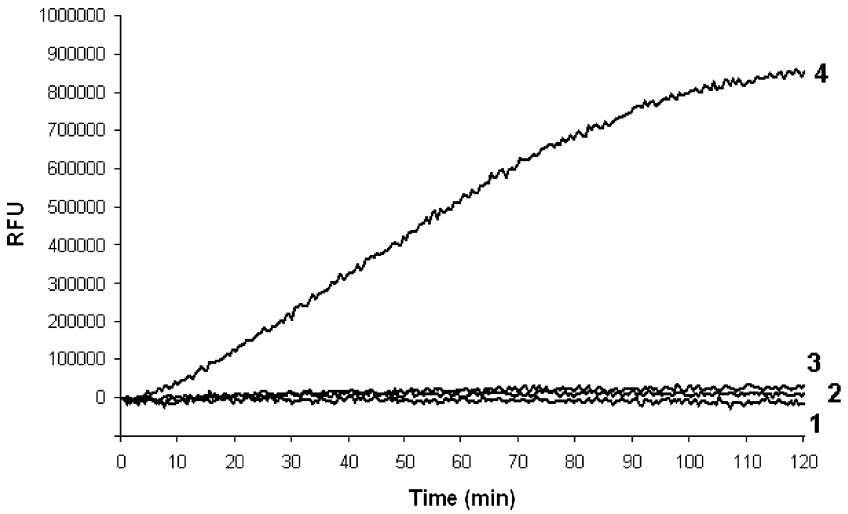

Fig. 2 Evolution of the normalised relative fluorescence (*RFU*, relative fluorescence units) detected with preparation of microsomes of rhubarb cultivated in the presence of the five sulphonated anthraquinones. *Line 1* microsomes alone, *line 2* microsomes with only AQ-2,6-SS, *line 3* microsomes with only NADPH, *line 4* microsomes with AQ-2,6-SS and NADPH. The essay contained 50 µL microsomes, 0.1 mM NADPH, 3 mM glucose-6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase, 0.1 mM substrate AQ-2,6-SS

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 on the mass of plant material was 5 to 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 a 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.

2.6 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 six leaves of rhubarb, for the control and exposed plants, at two different days of sampling. 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 at different times of exposure for rhubarb (Fig. 3) and common sorrel (results not shown). The standard deviations in the case of common sorrel were also smaller than 10% of the average of the three replicates.

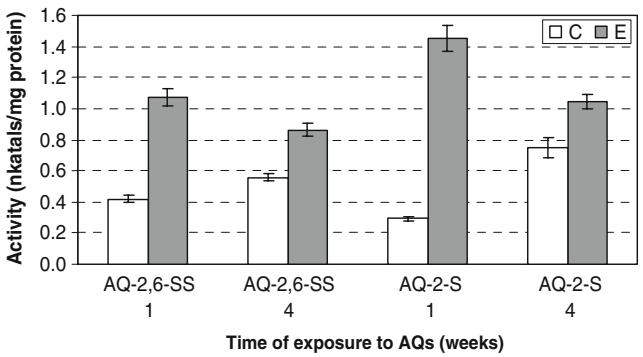

Fig. 3 Specific activity of cytochromes P450 in rhubarb leaves. Average and standard deviations of three replicates are shown for cytochromes P450 activity (nanokatal per milligramme of protein) in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*). The standard deviations were always smaller than 10% of the average of the three replicates

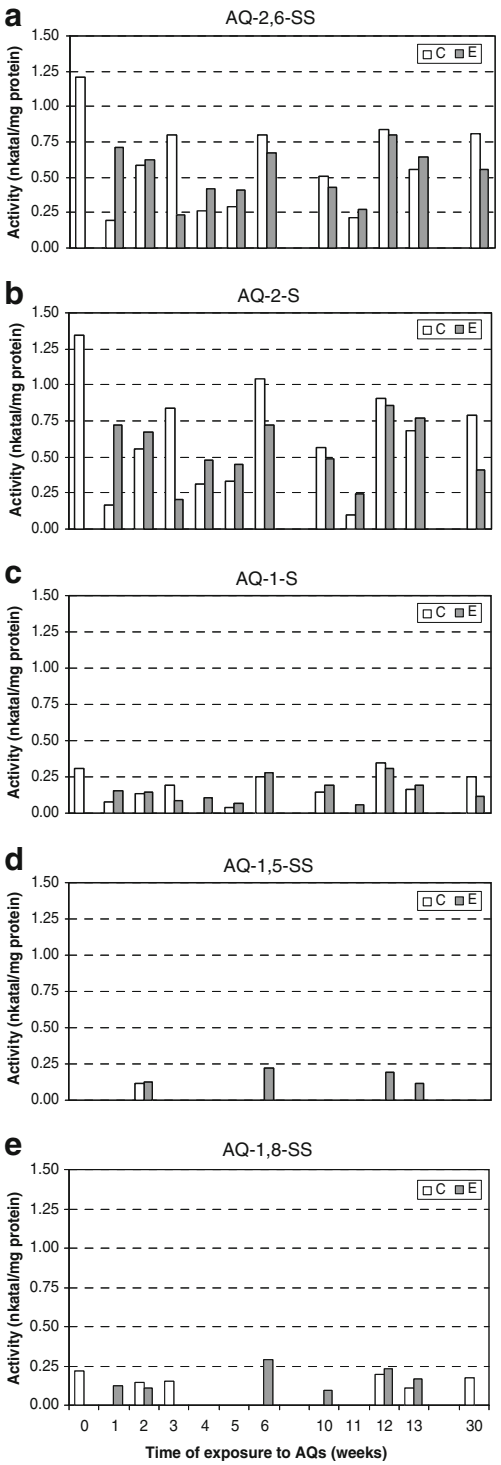

Fig. 4 Specific activity of cytochromes P450 in rhubarb leaves. Comparison of the activity in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*) when **a** AQ-2,6-SS; **b** AQ-2-S; **c** AQ-1-S; **d** AQ-1,5-SS and **e** AQ-1,8-SS were used as substrate. Activity is shown in nanokatal per milligramme of protein

3 Results

3.1 Cytochrome P450 activities

Cytochrome P450 monooxygenases are responsible for the detoxification of many organic contaminants in plants (Khatisashvili et al. 1997; Werck-Reichhart et al. 2000; Morant et al. 2003). To know if these enzymes are involved in the detoxification process of sulphonated anthraquinones in rhubarb and common sorrel, plants exposed or not to the mixture of the five sulphonated anthraquinones, were analysed at different times after the exposure to the contaminants. A significant activity of cytochromes P450 was detected in the leaves of rhubarb with the different sulphonated anthraquinones as substrates (Fig. 4). The higher activity detected in rhubarb leaves after 1 week of exposure to the pollutants indicated an induction of these enzymes when plants were grown in the presence of the five xenobiotic compounds (see Fig. 4a and b, week 1). The plants were exposed to the five sulphonated anthraquinones for a long period to mimic the continuous exposure to pollutants of plants growing in a constructed wetland or in a rhizofiltration process for wastewater treatment. Activity of cytochromes P450 was detected during the whole period of exposure to sulphonated anthraquinones. At the end of the experimentation, after 31 weeks, the different parts of the rhubarbs (leaves, petioles, rhizome and roots) were analysed separately. No activity of cytochromes P450 was detected in the rhizome, while low activity was measured in the roots and in the petioles (Table 1). The highest activity was found in the leaves when AQ-2,6-SS and AQ-2-S were used as substrates (see Fig. 4 and Table 1). Activity of cytochromes P450 was also observed when AQ-1-S, AQ-1,5-SS and AQ-1,8-SS were used as substrates, but at a lower level and not at each collecting time, especially when AQ-1,5-SS and AQ-1,8-SS were used as substrates. This new method for the measurement of the cytochromes P450 activity using sulphonated anthraquinones as substrates clearly showed that these pollutants were accepted as substrates by the enzyme.

To compare the detoxification mechanisms of sulphonated anthraquinones in two plants producing natural anthraquinones, cytochromes P450 activity was also analysed in the common sorrel. An activity toward all sulphonated anthraquinones was also found in the leaves of this plant, but no clear induction of the activity occurred after the exposure to the pollutants (Fig. 5). The activity in the control plants and the plants exposed to the five sulphonated anthraquinones were similar. In contrast to rhubarb, the activity of cytochromes P450 was found for each collecting time. These results indicated that common sorrel used the cytochromes P450 activity already present in the plants for the metabolism of these pollutants. As already shown for rhubarb, the activity was higher when AQ-2,6-SS and AQ-2-S were used as substrates.

3.2 Cytosolic peroxidases activity

The activity of peroxidases often increases in response to stress and one of their principal functions is the protection of the cell against hydrogen peroxide. Besides this function, peroxidases are also involved in a wide variety of detoxification processes (Bhunia et al. 2001; Arrieta-Baez et al. 2002; Passardi et al. 2005). To know if peroxidases are involved in the metabolism of sulphonated anthraquinones, some samples of rhubarb and common sorrel 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 the leaves of rhubarb (Fig. 6). For most of the collecting

Table 1 Specific activities of peroxidases and cytochromes P450 in the different parts of rhubarb plants exposed (E) or not (C) to sulphonated anthraquinones

Plant parts	Treatment	Peroxidases activity (nkat/mg protein)	Cytochromes P450 activity (nkat/mg protein)				
			AQ-1-S	AQ-2-S	AQ-1,5-SS	AQ-1,8-SS	AQ-2,6-SS
Leaves	C	0.42±0.09	0.18	0.77	0.00	0.17	0.73
	E	0.47±0.09	0.20	0.72	0.11	0.14	0.65
Petioles	C	0.49±0.16	0.09	0.38	0.00	0.00	0.35
	E	0.15±0.06	0.00	0.13	0.00	0.00	0.20
Rhizome	C	0.17±0.04	0.00	0.00	0.00	0.00	0.00
	E	0.01±0.00	0.00	0.00	0.00	0.00	0.00
Roots	C	0.19±0.04	0.00	0.00	0.00	0.00	0.00
	E	0.02±0.00	0.05	0.00	0.00	0.00	0.11

Average and standard deviations of six replicates are shown for peroxidases. For the cytochromes P450 activity, the standard deviations were smaller than 10% (see Fig. 3 and Section 2.6)

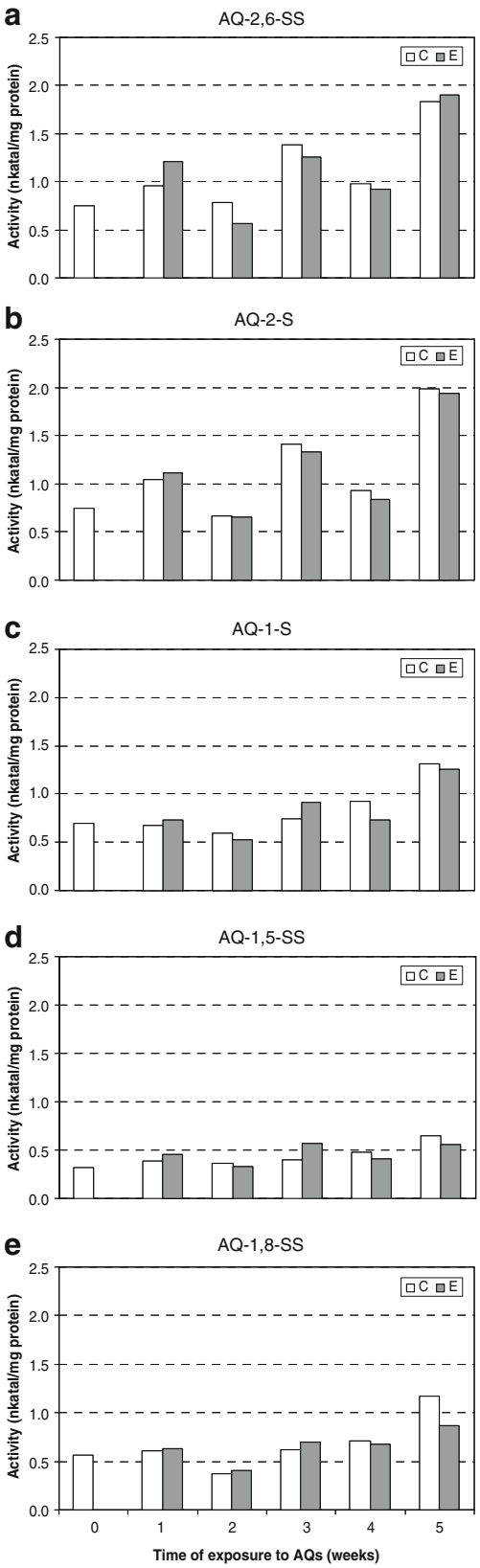

Fig. 5 Specific activity of cytochromes P450 in common sorrel leaves. Comparison of the activity in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*) when **a** AQ-2,6-SS; **b** AQ-2-S; **c** AQ-1-S; **d** AQ-1,5-SS and **e** AQ-1,8-SS were used as substrate. Activity is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

times, a higher activity was found in the rhubarb plants growing in the presence of the five sulphonated anthraquinones. Peroxidases were active during the whole period of exposure to pollutants. At the end of the experimentation, after 31 weeks of exposure to the five sulphonated anthraquinones, the different parts of the rhubarb were also analysed separately for peroxidases activity. An activity of these enzymes was found in all parts of rhubarb plants, with a higher level in the leaves and in the petioles (see Table 1). The activity of peroxidases was also measured in the leaves of common sorrel, but no significant difference was observed in the plants growing with or without sulphonated anthraquinones (Fig. 7).

4 Discussion

It cannot be excluded that some other enzymes than cytochromes P450 were present in the microsomal fraction. Plant microsomes contain not only cytochromes P450 as do microsomes of animals, peroxidases for example are also present in relatively high levels in this plant subcellular system (Chiapella et al. 1995). However, the fact that microsomes, fragments from the membrane of the endoplasmic reticulum that contain cytochromes P450, NADPH and the sulphonated anthraquinones were all needed for the enzymatic reaction consuming oxygen (see Fig. 2), provided conclusive evidence for the involvement of cytochromes P450 in the oxidative metabolism of sulphonated anthraquinones. Furthermore, no reaction was observed when the

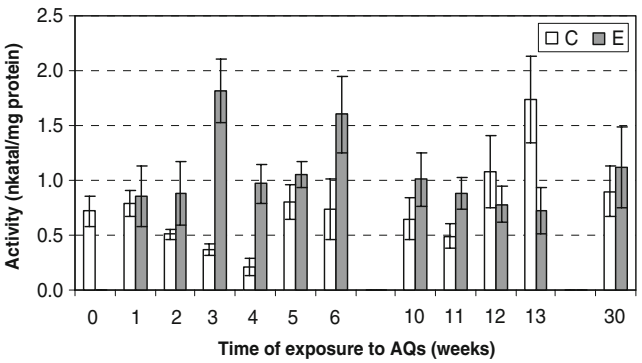

Fig. 6 Specific activity of peroxidases in rhubarb leaves. Average and standard deviations of six replicates are shown for peroxidases activity (nanokatal per milligramme of protein) in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*)

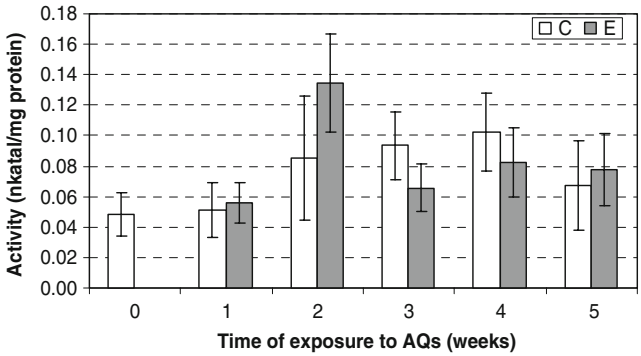

Fig. 7 Specific activity of peroxidases in common sorrel leaves. Average and standard deviations of six replicates are shown for peroxidases activity (nanokatal per milligramme of protein) in control plants (*C*) and in plants exposed to sulphonated anthraquinones (*E*)

NADPH regenerative system was omitted (data not shown). The reaction was also 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).

The comparison of the activity of cytochromes P450 in different parts of the rhubarb plant and the fact that the activity was higher in leaves than in petiole, roots or rhizome (see Table 1) suggests that the five sulphonated anthraquinones were taken up from the hydroponic medium and then translocated from the roots to the leaves where they were transformed by these enzymes. As measured by capillary electrophoresis, several sulphonated anthraquinones have been found in the leaves of rhubarb and common sorrel after an exposure of 6 weeks to these compounds (Aubert and Schwitzguébel 2002). Furthermore, new metabolites, not yet identified, are found in leaf extracts from rhubarb plants cultivated in the presence of these xenobiotics (Aubert 2003; Schwitzguébel et al. 2009), suggesting that at least some of them are transformed by the plant. 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 detoxification pathway of these xenobiotics.

A significant activity of cytochromes P450 was observed in rhubarb leaves, while no (rhizome) or low (petioles and roots) activity was found in other parts of the plants (see Table 1). These results suggest that the detoxification mechanisms took place mainly in the leaves of rhubarb. On the other hand, the direct injection of sulphonated anthraquinones in cut stems of rhubarb and common sorrel has been done (Schwitzguébel et al. 2008). Parent compounds and metabolites derived from them have been found in leaves of both species. The metabolites formed are different for both plant species, but are also different from those obtained in whole plants. This indicates that a transformation of the sulphonated anthraquinones can also occur in roots and rhizome and that this transformation is different from the transformation occurring in the leaves.

Growing hydroponically adult terrestrial plants such as rhubarb and common sorrel is possible, even if difficult (Aubert and Schwitzguébel 2004). Moreover, collecting every week leaves from rhubarb, a plant that grows slowly, is a challenge. It was sometimes not possible to collect leaves of the same age or stage of development and consequently with the same level of enzymes activity. Cytochromes P450 have several physiological functions in the plants, such as biosynthesis of hormones, lipids and secondary metabolites (Bolwell et al. 1994). It has been shown that the content of cytochromes P450 in microsomal fraction of root of etiolated maize seedlings changes with growth (Khatisashvili et al. 1997). All these facts might explain some variability of the results obtained for the measurement of the cytochrome P450 activities (see Fig. 4), over the long time of cultivation.

Peroxidases activity was found in the leaves of both plants studied, as well as in the petioles, the roots and the rhizome of rhubarb (see Table 1). The activity of peroxidases increased when rhubarb was cultivated in the presence of sulphonated anthraquinones (see Fig. 6), but in common sorrel, no significant difference was found between plants cultivated with and without the xenobiotics (see Fig. 7). The peroxidases activity present in all plant parts may be explained by the fact that there is always a basal level of peroxidases activity in plants, probably to perform housekeeping functions, such as growth by elongation and lignification (Passardi et al. 2005). In contrast, an induction of peroxidases activity was found in rhubarb leaves cultivated in the presence of sulphonated anthraquinones, except for weeks 12 and 13 of sampling (see Fig. 6). At this time, the control leaves collected were rapidly growing and the high activity of peroxidases found in these leaves might be due to this phenomenon rather than detoxification functions (Passardi et al. 2005).

Due to the method used in this work, it was not possible to know if peroxidases might accept sulphonated anthraquinones as a substrate and, consequently, if peroxidases might be directly involved in the detoxification mechanism of these compounds. On the other hand, peroxidases isolated from horseradish and from *Senna angustifolia* can transform natural hydroxyl-9,10-anthraquinone (Arrieta-Baez et al. 2002); but their ability to accept sulphonated anthraquinones remains, however, unknown. Further studies should be performed to find the possible involvement of peroxidases in the metabolism of sulphonated anthraquinones by anthraquinone-producing plants.

The uptake of xenobiotics by plants is an important step for any phytotreatment. The ability of rhubarb and common sorrel to remove sulphonated anthraquinones from synthetic wastewater has been previously studied. Both species have

a significant effect on the removal of the five sulphonated anthraquinones and rhubarb, especially the Valentine variety, gives the highest remediation level (Aubert and Schwitzguébel 2004). Due to this fact, the activity of cytochromes P450 and peroxidases and the fact that rhubarb is able to grow for a long period in the presence of sulphonated anthraquinones without showing any symptoms of toxicity (data not shown), this plant appears to have the strongest potential for the remediation of industrial effluents containing sulphonated aromatic compounds.

The fluorimetric assay adapted here for plant microsomes from Olry et al. (2007) was a fast, reliable and simple means to detect cytochromes P450 activity. This procedure was suitable not only for the detection, but also for the quantitative evaluation of enzyme activity. Due to the fact that the majority of the cytochromes P450 catalysed reactions consume one molecule of oxygen and one molecule of NADPH, the assay used in the present work was a direct method to test potential candidate substrates for a large variety of cytochrome P450 monooxygenases.

5 Conclusions

The results obtained in the present study show that the five sulphonated anthraquinones were accepted as substrate by the cytochromes P450 of rhubarb and common sorrel. In rhubarb, an induction of these enzymes was observed at the beginning of the exposition to these xenobiotics. An activity of the cytochromes P450 was also found in the leaves of common sorrel, but no induction of the activity occurred after the exposition to the pollutant. In both plants, the activity was higher in the presence of AQ-2,6-SS and AQ-2-S. The activity of peroxidases increased when rhubarb was cultivated in the presence of sulphonated anthraquinones. Peroxidases activity was also detected in the leaves of common sorrel, but in this plant no significant difference was found between plants cultivated with and without sulphonated anthraquinones. These results suggest the existence in rhubarb of specific mechanisms involved in the metabolism of sulphonated anthraquinones. Further investigation should be performed with this plant to find the next steps of this detoxification pathway.

Experiments with rhubarb cells cultivated in bioreactor in the presence of sulphonated anthraquinones have shown a significant desulphonation occurring during the phytotransformation of AQ-2-S as indicated by the increasing release of sulphate (Schwitzguébel and Vanek 2003). The activity of cytochromes P450 observed in the present work when AQ-2-S was used as a substrate was relatively high in rhubarb and common sorrel. The cytochromes P450 monooxygenases might thus be responsible for the desulphonation of AQ-2-S.

5.1 Recommendations and perspectives

After hydroxylation of a xenobiotic by cytochromes P450, the next step of detoxification pathway is usually glycosylation (Coleman et al. 1997). Moreover, in plants, the natural anthraquinones are mostly present as glycosides (Khouri and Ibrahim 1987; Van der Plas et al. 1998; Matsuda et al. 2001). Anthraquinone-producing plants could possibly glycosylate synthetic anthraquinones. Further investigation on glycosyltransferases activity should be performed to test this hypothesis.

Rhubarb, a plant producing natural anthraquinones, appears to have a strong potential for the remediation of sulphonated anthraquinones from contaminated sites and industrial wastewater. These results reinforce the idea that natural biodiversity should be better studied to use the most appropriate species for the phytotreatment of a specific pollutant. Screening of plants that produce natural chemicals whose structures are similar to the xenobiotic compounds should be the first step of any phytoremediation process. On the other hand, on the basis of these promising results, it will be of high interest to develop and test an experimental wastewater treatment system at pilot scale. It is important to know the ability of the plants to tolerate the pollutants and to determine the maximal possible amount of pollutants that can be detoxified by the plants without damage. Experimental phytoremediation system with real effluent should give useful information on the real detoxification capacity of the plants and allow determining the appropriate design and size of the future constructed wetland or hydroponic system to clean up the contaminated wastewater.

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