Use of hairy roots extracts for 2,4-DCP removal and toxicity evaluation by Lactuca sativa *test*

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RESEARCH ARTICLE

Use of hairy roots extracts for 2,4-DCP removal and toxicity evaluation by *Lactuca sativa* test

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Abstract 2,4-Dichlorophenol (2,4-DCP) is widely distributed in wastewaters discharged from several industries, and it is considered as a priority pollutant due to its high toxicity. In this study, the use of different peroxidase extracts for 2,4-DCP removal from aqueous solutions was investigated. Tobacco hairy roots (HRs), wild-type (WT), and double-transgenic (DT) for tomato basic peroxidases (TPX1 and TPX2) were used to obtain different peroxidase extracts: total peroxidases (TPx), soluble peroxidases (SPx), and peroxidases ionically bound to the cell wall (IBPx). All extracts derived from DT HRs exhibited higher peroxidase activity than those obtained from WT HRs. TPx and IBPx DT extracts showed the highest catalytic efficiency values. The optimal conditions for 2,4-DCP oxidation were pH 6.5, H_2O_2 0.5 mM, and 200 U mL^{-1} of enzyme, for all extracts analyzed. Although both TPx extracts were able to oxidize different 2,4-DCP concentrations, the removal efficiency was higher for TPx DT. Polyethylene glycol addition slightly improved 2,4-DCP removal efficiency, and it showed some protective effect on TPx WT after 2,4-DCP oxidation. In addition, using *Lactuca sativa* test, a reduction of the toxicity of post removal solutions was observed, for both TPx extracts. The results demonstrate that TPx extracts from both tobacco HRs appear to be promising candidate for future applications in removing 2,4-DCP from wastewaters. This is particularly true considering that these peroxidase sources are associated with low costs and are readily available. However, TPx DT has increased peroxidase activity, catalytic efficiency, and higher removal efficiency than TPx WT, probably due to the expression of TPX1 and TPX2 isoenzymes.

Keywords Removal · 2,4-DCP · Hairy roots · Polyethylene glycol · *L. sativa* test

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Abbreviations

2,4-DCP	2,4-Dichlorophenol
HRs	Hairy roots
WT	Wild-type
DT	Double-transgenic
Px	Peroxidases
TPx	Total peroxidases
SPx	Soluble peroxidases
IBPx	Ionically bound to the cell wall peroxidases
PEG	Polyethylene glycol
PRS	Post-removal solutions

Introduction

Phenolic compounds are one of the most important groups of environmental pollutants. The world produces over 100,000 t of these compounds every year, and many of them, such as 2, 4-dichlorophenol (2,4-DCP), are listed as hazardous compounds in the Agency of Toxic Substances and Disease

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Registry. 2,4-DCP is highly toxic because it is easily absorbed by skin, causing poisoning (resulting in hepatic and renal failure, pulmonary edema, hemolytic anemia), or teratogenic and carcinogenic effects (Brandon and Leybor 2001; Petroutsos et al. 2008). 2,4-DCP and other chlorophenolics are widely used in agriculture (pesticides, insecticides, antiseptics), and they are also used by various industries like textiles, paper, wood. In many cases, wastewaters derived from these industries are dumped illegally into the environment without prior treatment; as a consequence, high concentrations of these compounds are found in lakes and rivers resulting in a potential severe impact to the respective ecosystems and other natural resources, since these pollutants can enter in food chains through agricultural products or drinking water involving potential risks to human and animal health (Paisio et al. 2009). Despite the fact that 2,4-DCP guide levels for drinking water and surface waters were established by Regulatory Organizations, higher concentrations than those suggested are frequently found in contaminated environments. In fact, reported levels of chlorophenols were found to be up to 50 times greater than allowable limits (Angelini et al. 2011). Although different methodologies involving physicochemical methods for 2,4-DCP removal have been developed, most of them have disadvantages, i.e., high cost, formation of toxic by-products, and/or low efficiency (Eapen et al. 2007; Busca et al. 2008).

Biological remediation technologies, such as bioremediation, phytoremediation, rhizoremediation, as well as the application of enzymes derived from microorganisms and plants, have been successfully used in order to remove environmental and industrial contaminants. Moreover, biotechnology can increase the capacity and efficiency of these systems through the incorporation of foreign enzymes into microorganisms or plants. For instance, plants have been genetically modified to overcome the inherent limitations of plant detoxification capabilities (Van Aken 2008; Van Aken and Doty 2009). However, the introduction of transgenic organisms in the environment is not totally accepted by society, and there are ethic barrier concerns preventing the release of such organisms in to the environment (Abhilash et al. 2009). Nevertheless, the use of enzymes derived from transgenic organisms would circumvent this problem for application in bioremediation processes, since the use of such isolated components does not involve the introduction of transgenic organisms into the environment. In addition, the use of enzymes for remediation processes is characterized by high efficiency and selectivity and is significantly more environmentally friendly compared with the use of a whole organism (Sutherland et al. 2004). Particularly, peroxidases (Px) have the capability to oxidize several phenolic compounds and have been used in a wide spectrum of remedial applications in a number of industrial processes in order to preserve water quality (Ashraf and Husain 2010). In this sense, horseradish peroxidase (HRP) is the most studied enzyme for decontamination processes (Diao et al. 2010). However, Px isoenzymes present disadvantages, such as the possibility of their inactivation. In phenol removal processes, peroxidases can be inactivated by three possible mechanisms: (a) adsorption of polymerized phenols on peroxidases resulting in hindering the access of a substrate to the enzyme active site, (b) irreversible reactions between the enzyme and phenyl or phenoxy radicals that occur by one-electron oxidation of phenolic substrates during the catalytic cycle, and (c) suicide-peroxide inactivation which is a significant and dominant type of inactivation in diluted phenol solutions (up to about 0.2 mM) (Nazari et al. 2007). Thus, in order to prevent or minimize the inactivation of these enzymes during the reaction, some researchers have suggested the use of additives, including high-molecular-weight polymers, such as polyethylene glycol (PEG) (Bratkosvkaja et al. 2004; González et al. 2008).

In previous works, Talano et al. (2012) described that double-transgenic tobacco plants (DT), which express basic Px genes from tomato (*tpx1* and *tpx2*), were able to remove 2, 4-DCP more efficiently than wild-type (WT) tobacco plants. In addition, we established that tobacco hairy roots (HRs) represented a good alternative compared with the use of whole plant for remediation processes, including phenol and 2,4-DCP removal (Sosa Alderete et al. 2009; Talano et al. 2010), as well as sources of Px isoenzymes. Furthermore, it was observed that double-transgenic tobacco HRs (DT) were more efficient than wild-type HRs for phenol removal. Thus, transgenic HRs could represent an interesting alternative to improve the efficiency of remediation processes. Therefore, the use of Px extracts derived from WT and DT tobacco HRs could be suitable for bioremediation, specifically for the treatment of water polluted with 2,4-DCP. To our knowledge, this is the first report describing the use of different extracts derived from WT and transgenic tobacco HRs for 2,4-DCP oxidation.

For this reason, the aims of the present work were to: (a) analyze 2,4-DCP removal capability and kinetic properties of different Px extracts obtained from WT and DT tobacco HRs, (b) evaluate the optimal conditions for 2,4-DCP oxidation, and (c) evaluate toxicities of post removal solutions obtained after treatment with WT and DT extracts.

Materials and methods

Plant material

HRs cultures derived from WT and DT tobacco plants (*Nicotiana tabacum* cv. Wisconsin), which express tomato Px genes (*tpx1* and *tpx2*) were used. Both HRs were obtained through *Agrobacterium rhizogenes* LBA 9402 infection as described by Sosa-Alderete et al. (2009). Plants were sub-

cultured every 30 days in Murashige Skoog liquid medium (Murashige and Skoog 1962), enriched with vitamins. They were incubated at 25±2 °C in darkness using an orbital shaker at 100 rpm (Agostini et al. 2003).

Total and differential enzyme extraction and peroxidase activity determination

Px extraction from WT and DT HRs was performed according to the methodology described by Sosa Alderete et al. (2009). To obtain total Px extracts (TPx), HRs were homogenized with 50 mM sodium acetate/acetic acid buffer pH 5, containing 1 M KCl, using a ratio 1:3 (tissue/buffer). These homogenates were centrifuged at 5,000 rpm for 5 min, and the supernatants were denominated TPx WT and TPx DT in accordance with their origin. The TPx DT extract contains both Px isoenzymes (TPX1 and TPX2). In order to carry out the differential enzyme extraction, it was necessary to extract soluble Px (SPx) and Px ionically bound to cell wall (IBPx). The SPx fraction from DT HRs only contained TPX2 Px isoenzyme, whereas the IBPx fraction contains TPX1 Px isoenzyme. To obtain SPx and IBPx, 400 mg of tissue were homogenized with 50 mM sodium acetate/acetic acid buffer pH 5 in a ratio 1:3 (tissue/buffer) without the addition of 1 M KCl. The homogenate was stirred for 2 h at 4 °C and then was centrifuged at 5,000 rpm for 20 min. The supernatants contained SPx. The pellet was washed with the same buffer until no Px activity was detected, and then it was resuspended in 1.2 mL of the same buffer, supplemented with 1 M KCl and maintained for 2 h at 4 °C. The extracts were centrifuged at 5,000 rpm for 5 min, and the supernatants were considered as source of IBPx.

Px activity was determined spectrophotometrically using *o*-dianisidine and H_2O_2 as substrates as was previously described by González et al. (2006). One unit of enzyme (U) was defined as the amount of enzyme, which generated 1 mmol of product in 1 min under pre-established conditions (0.63 mM *o*-dianisidine, 0.5 mM H_2O_2, and 100 mM sodium acetate/acetic acid buffer pH 5.3 in 1 mL reaction mixture). Activity was measured following the appearance of *o*-dianisidine oxidation product (ε_{460nm}, 11.3 mM^{-1} cm^{-1}) due to the increase in A_{460nm} at 37 °C.

For the detection of Px activity after 2,4-DCP removal, an aliquot of the post-removal solution was taken, and the activity was determined as described above. The results were expressed as percent with 100 % of enzyme activity representing the enzymatic concentration used at the beginning of the experiment.

Kinetic parameters determination

Kinetic properties of different HRs extracts (TPx, SPx, and IBPx) obtained from WT and DT HRs were studied by a spectrophotometric method using 2,4-DCP as substrate (ε_{510} =6,710 M^{-1} cm^{-1}) (Metelitza et al. 1991). Px concentrations necessary to estimate the catalytic efficiency was determined using a millimolar absorption coefficient (ε_{403}= 90 mM^{-1} cm^{-1}) (Agostini et al. 2002).

The assays were carried out with 2,4-DCP, H_2O_2, and 4-aminoantipyrine (4-AAP), which generate a colored product, a red quinoneimine. The reaction was followed over 1 min by monitoring A_{510nm}. The reaction mixture (1 mL) contained 2.4×10^{-3} M 4-AAP, 2,4-DCP in a concentration range of 0.1 to 10 mM, and H_2O_2 in a concentration range of 1×10^{-3} to 1 mM. To establish the optimal pH, a buffer solution composed by 0.2 M acetic acid, 0.2 M boric acid, 0.2 M phosphoric acid, and 1 M NaOH was used to provide a pH range between 4.0 and 8.0. The reaction rate was expressed as units per milliliter. The assays were conducted in triplicate, and the results are the mean of three independent experiments.

2,4-DCP removal

The removal reactions were performed using 10 mg L^{-1} 2,4-DCP, 0.2 mM H_2O_2, and 200 UmL^{-1} of different extracts (TPx, SPx, and IBPx) from both HRs. The reaction mixture was incubated for 1 h at 25 °C in an orbital shaker at 100 rpm. In order to establish the optimal concentration for the removal of this contaminant, solutions ranging between 10 and 100 mg L^{-1} of 2,4-DCP were used. The H_2O_2 concentration varied between 0.2 and 1 mM and the Px activity between 25 and 600 U mL^{-1}. To analyze the effect of PEG in the removal process, the reactions were carried out following the same protocols described above with the difference that 100 mg L^{-1} of PEG-3350 or PEG-6000 were added to the reaction medium. Several control assays were carried out to evaluate whether other mechanisms could be involved in 2,4-DCP removal. To analyze the possible 2,4-DCP loss by evaporation or autooxidation, a solution of this contaminant was incubated at the conditions previously described but without enzymatic extract. To evaluate the eventual 2,4-DCP/H_2O_2 interaction, a control incubating only both compounds was performed. Results are the average of at least three determinations, corresponding to three independent experiments.

Residual 2,4-DCP determination

After 1 h of reaction, the remaining 2,4-DCP was determined through a spectrophotometric assay (Klibanov and Morris 1980). A 5-mL aliquot of the solution after removal reacted with 0.025 mL of 6.0 M ammonium hydroxide, 0.025 mL of 4-aminoantipyrine (2 % *w/v* aqueous solution), and 0.05 mL of potassium ferricyanide (8 % *w/v*). After several minutes, the colored compound formed was extracted with 2.5 mL of chloroform. Then, the absorbance was determined at 510 nm and converted to 2,4-DCP concentrations using a calibration

curve obtained with known concentrations of the contaminant. The curve was proportional to the concentration of 2,4-DCP in the range of 0–10^{-4} M. Results were expressed as removal efficiency, which was defined as the percentage of the pollutant removed from solution under these experimental conditions.

Acute toxicity of post-removal solutions using *Lactuca sativa* seeds

For this assay, 20 lettuce seeds were placed on filter paper discs in a Petri dish, under aseptic conditions. Then they were wetted with 5 mL of the solution to be tested and left in the dark at 25±2 °C for 5 days. This test was selected because it was proposed for use in toxicity studies by the USEPA (http://nepis.epa.gov/) and the US Food and Drug Administration (http://www.fda.gov).

2,4-DCP solutions between 0 and 100 mg L^{-1}, H_2O_2 (0.5 mM), and post-removal solutions (PRS) from TPx WT and TPx DT originally containing 2,4-DCP 25 mg L^{-1} were tested for their potential toxicity. Distilled water was used as negative control. Three replicates were performed for each solution, and results are the mean of three independent experiments. At termination of the test, percentages of relative seed germination and early growth parameters such as hypocotyl and root length were measured.

Statistical analysis

In all experiments, three replicate experiments were performed for each sample. Results are presented as the mean and the standard error. The homogeneity of Variance analysis and Normality were verified trough Levenes's and Shapiro Wilk test, previously to analysis of variance ($p<0.05$). The post hoc Tukey's test was applied to determine significant differences between treatments. Results were considered statistically significant when $p<0.05$. For that, the STATISTICA 6.0 (Statsoft, Inc., Tulsa, OK, USA) software was used.

To calculate effective concentration (EC_{50}) values and corresponding 95 % confidence intervals depending on the raw data distribution for assessing the quality of the treatment applied, PROBIT program version 1.4 (http://nepis.epa.gov/) was employed according to the American Society for Testing and Materials.

Results and discussion

Peroxidase activity determination

Different Px extracts (TPx, SPx, and IBPx) from WT and DT HRs were obtained by simple extraction methods. All extracts showed high Px activity (Table 1). However, as was expected, peroxidase activity in TPx extracts from both cultures was significantly higher than in SPx and IBPx extracts, since TPx extracts contain all Px isoenzymes included SPx and IBPx. Moreover, the enzymatic activity of TPx from DT HRs was twice than that of TPx from WT HRs. Similarly, significantly higher Px activities were detected in SPx and IBPx from DT HRs, compared with the same extracts derived from WT HRs. The highest Px activity detected in TPx extracts could be associated with the presence of all Px isoenzymes present in tobacco HRs. Regarding the high Px activity detected in DT extracts, this is probably due to the expression of basic tomato Px (TPX1 and TPX2) in tobacco DT HRs, since SPx extract contains TPX2 Px isoenzyme, whereas the IBPx extract contains TPX1 Px isoenzyme, as was previously described by our group (Sosa Alderete et al. 2009). In this study, the authors described several Px isoenzymes with pI values between 9.6 and 3.6 detected by isoelectric focusing in TPx extracts from both HRs (WT and DT). In addition, the SPx extract from DT HRs showed a main basic band (pI higher than 9.6), which corresponds to TPX2 isoenzyme, whereas a basic Px band of pI 9.6 was detected in IBPx extract from DT HRs, corresponding to TPX1 isoenzyme (Sosa Alderete et al. 2009).

Table 1 Px activity of different extracts obtained from WT and DT tobacco HRs

	Peroxidase activity (10^{-3} U mL^{-1})
TPx WT	255±19 (a)
TPx DT	521±37 (b)
SPx WT	90±10 (c)
SPx DT	136±13 (d)
IBPx WT	62±9 (e)
IBPx DT	97±10 (c)

Data are expressed as mean value ± SE. Different letters indicate significant differences between the activities obtained from different Px source (Tukey's test, $p<0.05$)

Determination of kinetic parameters of different enzymatic fractions

Kinetic studies were performed with different extracts using 2, 4-DCP and 4-aminoantipyrine as substrates in order to determine the apparent kinetic constants for their oxidation at room temperature. Since pH and substrate concentration are important parameters to improve the efficiency of a biological process, the optimal pH and H_2O_2 concentration were determined for different extracts. All Px extracts oxidized 2,4-DCP in a pH range between 5 and 8 with the highest activity being obtained between pH 6 and 7 for all extracts analyzed (Table 2, Electronic supplementary material Figure 1). Based on these results, was selected pH 6.5 for all subsequent experiments. Similar results were described for other Px isoenzymes which

Table 2 Optimal pH and apparent kinetic parameters (K_m and catalytic efficiency) of different extracts obtained from WT and DT HRs, using 2, 4-DCP and H_2O_2 as substrates

	Optimal pH	K_m (mM)	Catalytic efficiency (L (mmoles.s)$^{-1}$)
TPx WT	6–7	4.7	349
TPx DT	6–7	2.3	590
SPx WT	6	5.2	28
SPx DT	7	6.2	79
IBPx WT	6–7	7.3	181
IBPx DT	6–7	3.0	370

showed adequate activity over a wide pH range. For instance, *Coprinus cinerereus* Px, HRP, soybean Px, as well as *Brassica napus* and *Solanum lycopersicum* Px showed high enzymatic activities between pH 4 and 9 (Masuda et al. 2001; Bódalo et al. 2006; Franzen et al. 2007; González et al. 2008). However, the use of pH close to neutrality is recommended for the treatment of phenolic compounds (Belcarz et al. 2008; Coniglio et al. 2008).

The effect of different H_2O_2 concentrations (0.2 to 1.0 mM) was evaluated, and typical hyperbolic curves were obtained. The highest activity values were obtained when using 0.5 mM H_2O_2 with the different extracts, except for SPx DT extracts. In this particular case, the highest activity was obtained with 0.2 mM H_2O_2. In general, Px activity of all extracts did not change or was only slightly inhibited at concentrations greater than 1 mM H_2O_2 (Electronic supplementary material Figure 2). It is known that Px isoenzymes from different plant species may show different requirements for H_2O_2 to oxidize diverse

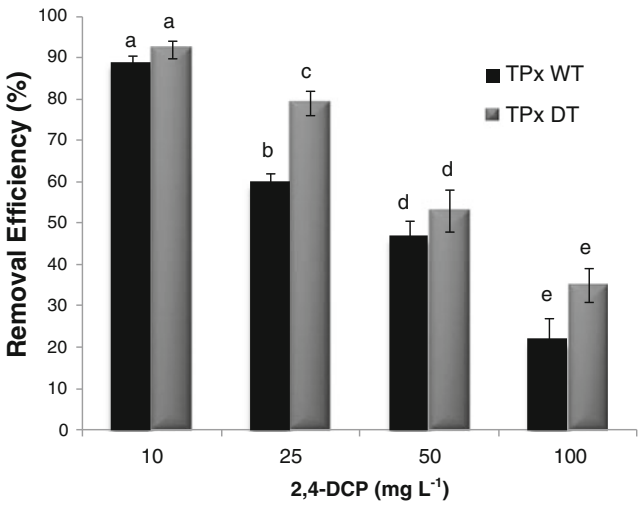

Fig. 1 Removal efficiency of different 2,4-DCP concentrations using TPx WT and TPx DT. Different *letters* indicate significant differences between all analyzed treatments. Removal efficiencies were compared between the different 2,4-DCP concentrations and between the Px sources used (Tukey's test, $p < 0.05$)

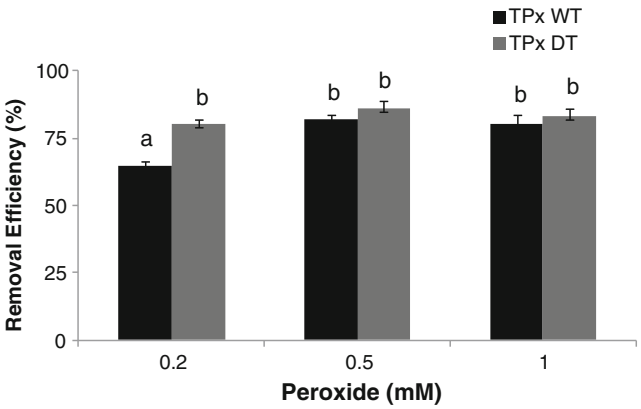

Fig. 2 Removal efficiency of 2,4-DCP (25 mg L^{-1}) using both TPx extracts and varying H_2O_2 concentrations. **a**, **b** indicate significant differences between treatments. Removal efficiencies were compared between the different H_2O_2 concentrations and between the Px sources used (Tukey's test $p < 0.05$)

phenolic compounds (Santos de Araujo et al. 2004; Kim and Lee 2005; Serrano-Martínez et al. 2008). Moreover, H_2O_2 concentrations required by one Px isoenzyme can be different to oxidize different substrates (Santos de Araujo et al. 2004).

To obtain apparent kinetic parameters for Px from different extracts, reactions were performed varying only 2,4-DCP concentration between 1 and 12 mM (Electronic supplementary material Figures 3 and 4), and by fixing pH and H_2O_2 to 6.5 and 0.5 mM, respectively, which were previously established as optimal conditions. The reactions followed the expected Michaelis Menten kinetics and the apparent K_m and Vmax constants were estimated to calculate the catalytic efficiency (Table 2). The latter is considered a good parameter

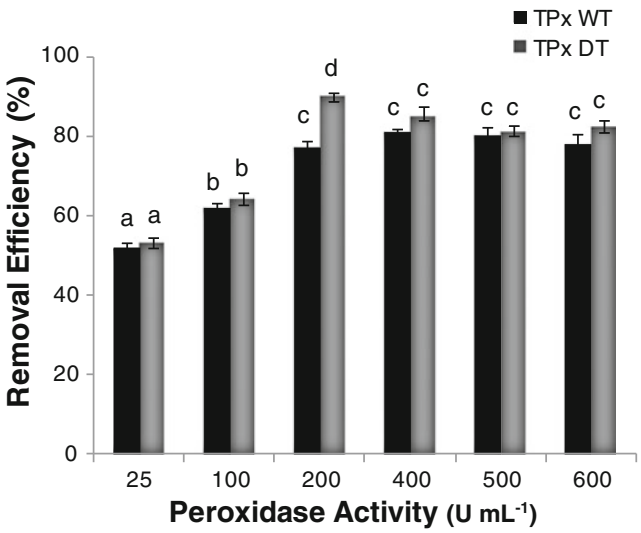

Fig. 3 Removal efficiency of 25 mg L^{-1} 2,4-DCP using both TPx extracts, 0.5 mM peroxide, and varying enzyme concentrations (25–600 U mL^{-1}). Different *letters* indicate significant differences between treatments. Removal efficiencies were compared between the different enzyme concentrations and between the Px sources used (Tukey's test, $p < 0.05$)

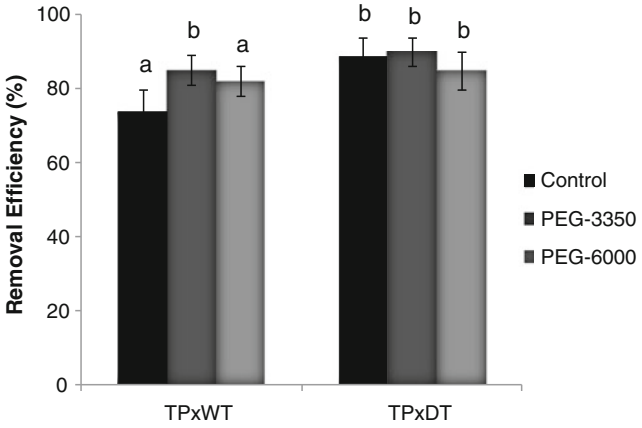

Fig. 4 Removal efficiency of 25 mg L^{-1} 2,4-DCP, H_2O_2 0.5 mM using both TPx extracts in presence of PEG of different molecular weights. **a**, **b** indicate significant differences between treatments (Tukey's test, $p<0.05$)

to compare the efficiency of different enzyme extracts, and it was defined as the ratio K_{cat}/K_m (Eisenthal et al. 2007; Fox and Clay 2008). TPx and IBPx extracts (from DT HRs) showed the lowest K_m values, and therefore these extracts had higher affinity for 2,4-DCP (Table 2). Regarding catalytic efficiencies, the highest value was observed for TPx DT, while TPx WT and IBPx DT extracts showed also high catalytic efficiency, and similar values between them (Table 2). In general, the catalytic efficiency of all extracts (TPx, SPx and IBPx) derived from DT HRs was higher than their respective extracts derived from WT HRs. These results could be due to the presence of one or two basic Px isoenzyme from tomato plants, in extracts derived from DT HRs.

It should be note that IBPx fractions showed higher catalytic efficiency than SPx fractions from both HRs. From these results, we suggest that the group of the basic Px isoenzymes present in IBPx extracts (including TPX1) could be responsible for 2,4-DCP removal, while the participation of SPx extracts (including TPX2) in the oxidation of 2,4-DCP would be minimal. According to previous studies carried out in our laboratory, basic Px isoenzymes would be more involved in phenol and 2,4-DCP removal than acidic Px in tomato HRs (Wevar Oller et al. 2005; González et al. 2008; Sosa Alderete et al. 2009). However, the presence of basic Px from tomato (TPX1 or TPX2) in the different DT extracts could have an important role in enhancing the oxidation of 2,4-DCP. These results demonstrate that TPX1 and TPX2 isoenzymes could have an important role in 2,4-DCP oxidation by enhancing it.

Removal of 2,4-DCP

In order to select the more suitable extract to remove 2,4-DCP, TPx, SPx, and IBPx extracts from both HRs, cultures were used for degradation studies. Removal reactions were carried out with the same enzyme units by the different fractions to evaluate the relative contribution of each isoenzyme in the process. The removal efficiencies were similar for all analyzed fractions, reaching values around 88 % (Electronic supplementary material Figure 5). From these results, both TPx extracts from WT and DT HRs were selected for all subsequent studies because TPx extracts are readily available, are easier and more economic to obtain than other extracts, and they have high Px activity and catalytic efficiency. Removal efficiency of different 2,4-DCP concentrations was evaluated with these extracts (Fig. 1). The removal efficiencies were concentration-dependent, since they decreased when the concentration of the contaminant increased, independently of the TPx used. Maximum removal efficiencies of TPx WT and TPx DT (89 and 92 % respectively) were achieved with the lowest concentration of pollutant analyzed (10 mg L^{-1}). When 2,4-DCP concentrations were increased from 10 to 100 mg L^{-1}, removal efficiencies significantly decreased to 25 and 35 % when TPx WT and TPx DT were used, respectively. It is important to note that, for all concentrations assayed, removal efficiencies reached with TPx DT were always higher than those obtained with TPx WT. However, this difference was only statistically significant when 25 mg L^{-1} 2,4-DCP was used ($p<0.05$). For this reason, this concentration was selected by further experiments.

Removal efficiencies can be enhanced by modifications of the reaction conditions. In this sense, H_2O_2 is a key reagent in Px reaction mechanism, and it is necessary to optimize its concentration to enhance catalytic efficiency. Low concentrations of H_2O_2 could limit the reaction rate by a low availability of substrate, whereas a H_2O_2 excess could cause suicide inactivation of the enzyme (Wagner and Nicell 2002; Nazari et al. 2007). Therefore, different H_2O_2 concentrations (0.2–1 mM) were assayed to remove 25 mg L^{-1} of 2,4-DCP using TPx WT and TPx DT (Fig. 2).

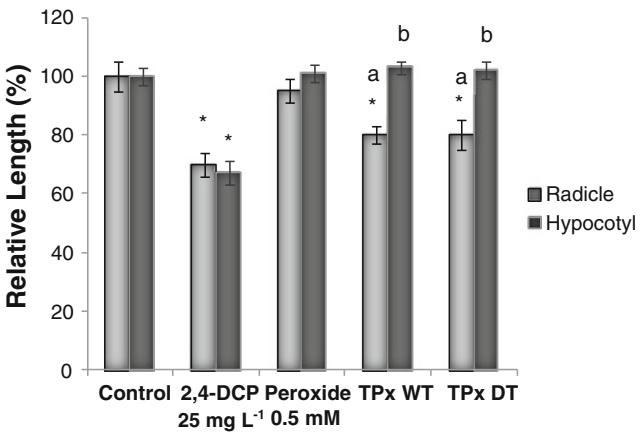

Fig. 5 Relative root and hypocotyl length of seeds germinated in presence of 25 mg L^{-1} 2,4-DCP, 0.5 mM H_2O_2, and PRS derived from treatments with TPx WT and TPx DT. Distilled water was used as control. *Asterisk* indicates significant differences with the control, (**a** and **b**) indicate significant differences respect to 25 mg L^{-1} 2,4-DCP ($p<0.05$)

A significant increase of 15 % in the removal efficiency was observed with increasing H_2O_2 concentration from 0.2 to 0.5 mM only when TPx WT was used. In contrast, removal efficiencies were not affected by changes in H_2O_2 concentrations in solutions treated with TPx DT. This would indicate that 0.2 mM H_2O_2 would be limiting for 2,4-DCP removal using TPx WT. Based on these results, 0.5 mM of H_2O_2 was selected as the optimal concentration for both TPx extracts, although 0.2 mM H_2O_2 could be also used to remove 2,4-DCP efficiently, when TPx DT is used.

When investigating the effect of enzyme concentration (from 25 to 600 U mL^{-1}) on removal efficiency, removal efficiency was dependent on enzyme concentration until 200 U mL^{-1} (Fig. 3). At greater concentrations, the removal efficiencies remained relatively constant. The removal efficiencies were similar regardless of whether TPx WT or TPx DT were used, showing only significantly differences for 200 U mL^{-1}. Based on these results, and to minimize the cost of the process, 200 U mL^{-1} were selected as the optimal enzyme concentration.

Effect of PEG on the removal efficiency and peroxidase activity

Several authors have reported that the use of some additives, like PEG, can improve the efficiency of phenolic compound removal (Cheng et al. 2006; González et al. 2008). In this sense, PEGs of different molecular weights (PEG-3350 and PEG-6000) were used in the reaction mixture. PEG addition improved 2,4-DCP removal efficiency when TPx WT extract was used, but significative differences were only observed with PEG-3350 addition (Fig. 4). This additive did not affect the removal efficiency when TPx DT was used. González et al. (2008) demonstrated that the use of PEG-3350 did not modify removal efficiency of 2,4-DCP and phenol when TPx extract from tomato HRs was used. On the contrary, when these authors used Px ionically bound to cell wall (IBPx) and a basic Px (BP) from tomato HRs for phenol removal, an increase on removal efficiency was observed. These results show that the effect of PEG is variable depending on the pollutant and the different isoenzyme composition of each extract used (González et al. 2008).

Generally, the effect of PEG was related to its known protective effect on Px activity. Thus, after 2,4-DCP removal with or without the addition of PEG-3350, remaining Px activity was measured in the reaction medium. The results showed that PEG-3350 addition in the reaction medium permited to conserve 40 % of the initial Px activity when TPx WT was used; however, only 15 % of activity was registered when TPx DT was used. These results demonstrated that PEG could protect Px activity. On the contrary, González et al. (2008) observed that in general Px activities of all extracts analyzed from tomato HRs after 2,4-DCP and phenol removal were not affected by the addition of PEG. Exceptions to these results were Px activity of IBPx and BP extracts after phenol removal.

Other authors have been able to increase the removal efficiency of different pollutants, by the addition of protective agents, such as kitosan, detergents, or amino acids to the reaction media, avoiding the enzyme inactivation (Quintanilla-Guerrero et al. 2008). In this context, Al-Ansari et al. (2010) demonstrated that the used of PEG showed no improvement on phenol removal efficiency when SBP was used as a Px source, whereas anionic surfactants like dodecyl sulfate showed a significant improvement.

The search of new insights in order to enhance the removal process as well as the protection of involved enzymes would reduce costs and improve the process on an industrial scale.

Toxicity of post-removal solutions using *L. sativa* test

In order to determine PRS toxicity, *L. sativa* test was performed. The toxicity of several 2,4-DCP concentrations and 0.5 mM H_2O_2 solution was also analyzed. Germination of seeds was inhibited by 100 mg/L of 2,4-DCP. The EC_{50} based on germination test was 51 mg L^{-1}, whereas the germination was not affected by lower 2,4-DCP concentrations. Because H_2O_2 is used in the removal process, toxicity of this solution was also evaluated on *L. sativa* seeds. Germination percentage did not show significant differences compared with the control (distilled water), indicating that this concentration was not toxic to *L. sativa* seeds. Similarly, the germination was not affected when PRS from TPx WT and TPx DT were analyzed (data no graphed). For this reason, other parameters including root and hypocotyl length were also evaluated. When the effect of 2,4-DCP on seed growth was analyzed, the results showed that concentrations equal to or greater than 10 mg L^{-1} root and hypocotyl length were significantly affected compared with the controls. In a similar way, 25 mg L^{-1} produced a significant diminution of approximately 35 % of both parameters. The EC_{50} values based on root and hypocotyl length were 33.5 and 26 mg L^{-1}, respectively. In contrast, 0.5 mM H_2O_2 did not affect root and hypocotyl length (Fig. 5). Regarding the effects produced by PRS from TPx WT and TPx DT on seed, hypocotyl length was not affected, and a significant reduction of 20 % in root length was observed compared with control for both PRS. However, both parameters showed an increase compared with those obtained for 25 mg L^{-1} 2,4-DCP. The root length significant increased by 12 %; however, hypocotyl length reached similar values to the control after treatment with both TPx HRs. No differences between PRS obtained from both TPx extracts were observed. These results indicated that toxicity of PRS was lower than 25 mg L^{-1} 2,4-DCP without treatment, which demonstrated that the proposed remediation process could be a promising system, since not only reduced 2,4-DCP concentration but also a significant toxicity reduction in PRS compared with 25 mg L^{-1} 2,4-DCP was observed.

Conclusions

Different enzyme extracts obtained from WT and DT tobacco HRs showed high Px activities with TPx extracts showing the highest activity. Moreover, all extracts derived from DT HRs exhibited significantly higher activities than those obtained from WT HRs. The optimal conditions to perform kinetic studies were established, namely pH 6.5, 0.5 mM H_2O_2 and 200 UmL^{-1} of enzyme. The highest catalytic efficiency was observed for TPx from DT, with TPx WT and IBPx DT extracts also showing high catalytic efficiencies. 2,4-DCP removal studies showed that TPx extracts from both HRs cultures were able to oxidize different concentrations of the contaminant. The results demonstrated that under optimal conditions Px extracts obtained from DT HRs were in general more efficient to remove 2,4-DCP than those obtained from WT HRs. This could be due to the presence of TPX1 and TPX2 isoenzymes in DT HRs, which could be responsible for the higher enzymatic activities and greater catalytic and removal efficiencies observed when these extracts were used. PEG addition slightly improved 2,4-DCP removal efficiency and retained more Px activity after 2,4-DCP oxidation when TPx WT extract was used. On the other hand, both PRS showed a reduction in their toxicities compared with 2,4-DCP solution without treatment in an acute toxicity test with *L. sativa* L seeds. Based on these results, TPx extracts from both tobacco HRs cultures appear to be promising in view of future applications to remove aqueous 2,4-DCPdue to their low cost and ready availability. More research on the scaling up of the process should be conducted for future commercial applications.

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References

Abhilash PC, Jamil S, Shing N (2009) Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. Biotechnol Adv 27:474–488

Agostini E, Hernández-Ruiz J, Arnao MB, Milrad SR, Tigier HA, Acosta M (2002) A peroxidase isoenzyme secreted by turnip (*Brassica napus*) hairy-root cultures by hydrogen peroxide and application in diagnostic kits. Biotechnol Appl Biochem 35:1–7

Agostini E, Coniglio MS, Milrad SR, Tigier HA, Giulietti AM (2003) Phytoremediation of 2,4-dichlorophenol by *Brassica napus* hairy root cultures. Biotechnol Appl Biochem 37:139–144

Al-Ansari MM, Modaressi K, Taylor KE, Bewtra JK, Biswas N (2010) Soybean peroxidase-catalyzed oxidative polymerization of phenols in coal-tar wastewater: comparison of additives. Environ Eng Sci 27:967–975

Angelini VA, Orejas J, Medina MI, Agostini E (2011) Scale up of 2,4-dichlorophenol removal from aqueous solutions using *Brassica napus* hairy roots. J Hazard Mater 185:269–274

Ashraf H, Husain Q (2010) Studies on bitter gourd peroxidase catalyzed removal of p-bromophenol from wastewater. Desalination 262:267–272

Belcarz A, Ginalska G, Kowalewska B, Kulesza P (2008) Spring cabbage peroxidases—potential tool in biocatalysis and bioelectrocatalysis. Phytochem 69:627–636

Bódalo A, Gómez JL, Gómez E, Hidalgo AM, Gómez M, Yelo AM (2006) Removal of 4-chlorophenol by soybean peroxidase and hydrogen peroxide in a discontinuous tank reactor. Desalination 195:51–59

Brandon C, Leybor G (2001) The environmental contamination in Argentine. http://www.santacruz.gov.ar/recursos/educacion/opiaus01/contamin.htm

Bratkosvkaja I, Vidziunaite R, Kulys J (2004) Oxidation of phenolics compounds by peroxidase in the presence of soluble polymers. Biochem (Moscow) 69:985–992

Busca G, Berardinelli S, Resini C, Arrighi L (2008) Technologies from removal of phenol from fluid streams: a short review of recent developments. J Hazard Mater 160:265–288

Cheng J, Mings S, Zuo P (2006) Horseradish peroxidase immovilized on aluminium-pillared interlayered clay for the catalytic oxidation of phenolic wastewater. Water Res 40:283–290

Coniglio MS, Busto VD, González PS, Medina MI, Milrad S, Agostini E (2008) Application of *Brassica napus* hairy root cultures for phenol removal from aqueous solutions. Chemosphere 72:1035–1042

Diao M, Oue'draog N, Baba-Moussa L, Savadogo P, N'Guessan A, Bassole' I, Dicko M (2010) Biodepollution of wastewater containing phenolic compounds from leather industry by plant peroxidases. Biodegradation 22:389–396

Eapen S, Singh S, D'Souza SF (2007) Advances in development of transgenic plants for remediation of xenobiotic pollutants. Biotechnol Adv 25:442–451

Eisenthal R, Danson MJ, Hough DW (2007) Catalytic efficiency and K_{cat}/K_m: a useful comparator? Trends Biotechnol 25:247–249

Fox RJ, Clay MD (2008) Catalytic effectiveness, a measure of enzyme proficiency for industrial applications. Trends Biotechnol 27:137–140

Franzen S, Gilvey LB, Belyea JL (2007) The pH dependence of the activity of dehaloperoxidase from *Amphitrite ornata*. Biochem Biophys Acta 1774:121–130

González PS, Capozucca CE, Tigier HA, Milrad SR, Agostini E (2006) Phytoremediation of phenol from wastewater, by peroxidases of tomato hairy root cultures. Enzym Microb Technol 39:647–653

González PS, Agostini E, Milrad SR (2008) Comparison of the removal of 2,4-dichlorophenol and phenol from polluted water, by peroxidases from tomato hairy roots, and protective effect of polyethylene glycol. Chesmosphere 70:982–989

Kim SJ, Lee DJ (2005) Purification and characterization of a cationic peroxidase C_s in *Raphanus sativus*. J Plant Physiol 162:609–617

Klibanov AM, Morris ED (1980) Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. Enyzm Microb Technol 3:119–122

Masuda M, Sakurai A, Sakakibara M (2001) Effect of enzyme impurities on phenol removal by the method of polymerization and precipitation catalyzed by *Coprinus cinereus* peroxidase. Appl Microbiol Biotechnol 4:494–499

Metelitza DI, Litvinchuk AV, Savenkova MI (1991) Peroxidase-catalyzed co-oxidation of halogen-substituted phenols and 4-aminoantipyrine. J Mol Catal 67:401–411

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Plant Physiol 15:473–479

Nazari K, Esmaeili N, Mahmoudi A, Rahimi H, Moosavi-Movahedi AA (2007) Peroxidative phenol removal from a aqueous solutions using activated peroxidase biocatalyst. Enzym Microb Technol 41:226–233

Paisio C, Agostini E, González P, Bertuzzi M (2009) Lethal and teratogenic effects of phenol on *Bufo arenarum* embryos. J Hazard Mater 167:64–68

Petroutsos D, Katapodis P, Samiotaki M, Panayotou G, Ketos D (2008) Detoxification of 2,4-dichlorophenol by de marine microalgae *Tetraselmis marina*. Phytochemistry 69:707–771

Quintanilla-Guerrero F, Duarte-Vázquez MA, García-Almendarez BE, Tinoco R, Vazquez-Duhalt R, Regalado C (2008) Polyethylene glycol improves phenol removal by immobilized turnip peroxidase. Bioresour Technol 99:8605–8611

Santos de Araujo B, Oliviera JO, Machado SS, Pletsch M (2004) Comparative studies of the peroxidases from hairy roots of *Daucus carota, Ipomoea batatas* and *Solanum aviculare*. Plant Sci 167: 1151–1157

Serrano-Martínez A, Fortea MI, del Amor FM, Núñez-Delicado E (2008) Kinetic characterization and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase. Food Chem 107:193–199

Sosa Alderete LG, Talano MA, Ibáñez SG, Purro S, Agostini E, Milrad SR, Medina MI (2009) Establishment of transgenic tobacco hairy roots expressing basic peroxidases and its application for phenol removal. J Biotechnol 139:273–279

Sutherland TD, Horne I, Russell RJ, Oakeshott JG (2004) Enzymatic bioremediation: from enzyme discovery to applications. Clin Exp Pharmacol Physiol 31:817–821

Talano MA, Frontera S, González PS, Medina MI, Agostini E (2010) Removal of 2,4-diclorophenol from aqueous solutions using tobacco hairy root cultures. J Hazard Mater 176:784–791

Talano MA, Busso D, Paisio C, González PS, Purro S, Medina MI, Agostini E (2012) Phytoremediation of 2,4-dichlorophenol using wild type and transgenic tobacco plants. Environ Sci Pollut Res 19: 2202–2211

Van Aken B (2008) Transgenic plants for phytoremediation: helping nature to clean up environmental pollution. Trends Biotechnol 26:225–227

Van Aken B, Doty SL (2009) Transgenic plants and associated bacteria for phytoremediation of chlorinated compounds. Biotechnol Genet Eng Rev 26:43–64

Wagner M, Nicell JA (2002) Detoxification of phenolic solutions with horseradish peroxidase and hydrogen peroxide. Water Res 36:4041–4052

Wevar Oller AL, Agostini E, Talano M, Capozucca C, Milrad SR, Tigier HA, Medina MI (2005) Overexpression of a basic peroxidase in transgenic tomato (*Lycopersicon esculentum* Mill. Cv. Pera) hairy roots increase phytoremediation of phenol. Plant Sci 169:1102–1111