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Phytoremediation of Phenol at pilot scale by tobacco hairy roots

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ABSTRACT

Phenol is a toxic compound commonly found in the wastewater released from several industries. In the present work, we optimized the use of tobacco hairy roots (HRs), wild type (WT) and double transgenic (DT) for two peroxidases, as an alternative strategy for the treatment of this pollutant. The optimal conditions for the removal process were evaluated using different phenol (100-800 mg L^{-1}) and H_2O_2 (2,5-10 mM) concentrations. Tobacco HRs were able to remove solutions with increasing phenol concentrations. Regarding the effect of exposition time, prolonged time of treatment improved phenol remediation when high pollutant concentrations were used. A decrease in basic peroxidase (Px) isoenzymes was detected after phenol treatment. Kinetic studies were performed using different Px extracts, such as soluble Px (SPx) and ionically bound Px (IBPx) from WT and DT HRs. SPx and IBPx extracts from DT HRs, showed high affinity towards phenol as substrate and higher catalytic efficiency than extracts derived from WT HRs. Moreover, IBPx extract from DT HRs, showed the highest catalytic efficiency, demonstrating that these HRs could provide an adequate matrix for the successful phenol removal.

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Keywords: Phytoremediation, organic pollutants, transgenic peroxidases, kinetic parameters.

1. Introduction

Phenol is one of the major organic compounds produced by a wide variety of industrial activities, such as, coal conversion processes, petroleum refineries, manufacturing of phenolic resins, herbicides, among others. Wastewater release from the above cited industries frequently contains phenols in concentrations ranging from 50-600 mg L^{-1} (Paisio et al. 2009) and even more. Although there are different conventional methods for phenol removal, they are not suitable for treating moderate to high concentrations of this xenobiotic (Diao et al. 2010). Thus, the development of more effective treatment processes is necessary for the decontamination of this pollutant from industrial waste streams and the environment (Diao et al. 2010). One of the alternatives is based on the application of oxidoreductive enzymes to catalyze the removal of this compound from wastewater (Gómez et al. 2008). Moreover, based on the ability of a natural enzyme such as peroxidase (Px), which polymerizes phenol in plants, an enzyme-based method for the treatment of water polluted with this xenobiotic was developed (Zuo et al. 2009). Peroxidases (EC 1.11.1.7) are the heme-containing versatile biological catalysts that have been used for wide spectrum applications in a number of industrial processes in order to preserve water quality (Ashraf and Husain 2010). Furthermore, horseradish Px is the most studied enzyme to use in decontamination processes (Diao et al. 2010) and it is the preferred plant Px in environmental and biotechnological applications (Ashraf and Husain 2010). However, due to its high cost and limited stability (Zuo et al. 2009), the development of low-cost sources of enzymes in quantities that are required at

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industrial scale is an important goal (Husain, 2010). In this sense, hairy roots (HRs) offer an attractive system for this purpose, because they produce large quantities of exudates which contain enzymes, like Px, and some metal chelating compounds that can detoxify or sequester harmful inorganic or organic pollutants, such as phenol (Angelini et al. 2011). Moreover, they have another attractive characteristic such as low maintenance requirement and fast grow in hormone-free medium, providing a greater surface area of contact between contaminant and tissue (Angelini et al. 2011).

In our laboratory, tobacco HRs were established from wild type (WT) and double transgenic (DT) plants (Sosa Alderete et al. 2009). Preliminary results showed the ability of tobacco HRs to remove phenol efficiently (Sosa Alderete et al. 2009). As an extension of this work, the aims of the present study were to: (i) determine the optimal conditions for phenol removal using tobacco HRs (WT and DT) exposed to different phenol and H_2O_2 concentrations; (ii) analyze the effect of different reaction time on treatment effectiveness; and (iii) study the kinetic parameters such as, Km and catalytic efficiency of two different Px isoenzyme extracts, soluble Px (SPx) and ionically bound Px (IBPx), derived from these HRs.

2. Material and Methods

2.1. Plant material

Tobacco (*Nicotiana tabacum*) DT HRs which expressed two basic Px genes (*tpx1* and *tpx2*) from tomato (*Solanum lycopersicon*) and WT HRs were used (Sosa Alderete et al. 2009). HRs were maintained for successive subcultures, each 25-30 d, on MS medium (Murashige and Skoog 1962) supplemented with vitamins, at 25 ± 2 ºC in darkness on an orbital shaker at 70 rpm.

2.2. Determination of the optimal conditions for phenol removal

The experiments were performed according to Agostini et al. (2003). A standard root inoculum (0.4 g), derived from HRs in stationary phase of growth (30 d of development), was incubated in 50 ml Erlenmeyer flasks containing a reaction mixture composed of 10 mL of an aqueous phenol solution and H_2O_2. The effects of different phenol (100, 200, 400, 600 and 800 mg L^{-1}) and H_2O_2 (2.5, 5, 7.5 and 10 mM) concentrations were investigated. After the reaction time (60 min) roots were taken and the residual phenol was determined according to the spectrophotometric method described by Kinsley and Nicell Kinsley and Nicell (2000). Results were expressed as residual phenol, which is defined as the ratio between the concentration of phenol removed and the initial concentration of phenol in the incubation medium (Coniglio et al. 2008).

To analyze the evolution of phenol removal along time, a HR inoculum (0.4 g) was exposed to two different phenol concentrations (100 and 600 mg L^{-1}) and 5 mM H_2O_2. Phenol removal efficiency was evaluated at different times of exposure (15, 30, 60, 150, 300 and 1440 min) according to the method described above (Kinsley and Nicell 2000).

2.3. Total and differential enzyme extraction

Extraction of total Px (TPx), soluble Px (SPx) and ionically bound to cell wall Px (IBPx) was carried out according to Sosa Alderete et al. 2009. The roots were homogenized with 50 mM sodium acetate/acetic acid buffer pH 5, containing 1 M KCl, using a ratio 1:3 (tissue/buffer).

This homogenate was centrifuged at 5000 g for 10 min and the supernatants contained TPx. In order to obtain SPx and IBPx extracts, 0.4 g of fresh weight (FW) root tissue was homogenized with 50 mM sodium acetate/acetic acid buffer pH 5 in a 1:3 ratio (tissue/buffer). The homogenate was centrifuged at 5000 g for 20 min. Supernatants contained SPx. The remaining pellet was washed with the same buffer until no Px activity was detected. Then, it was resuspended with 1.2 ml of the same buffer supplemented with 1M KCl at 4°C for 2 h with agitation. The extracts were centrifuged at 5000 g for 5 min. The supernatants were considered as IBPx extracts.

2.4. Analysis of peroxidase isoform profiles

Px isoenzymes from different extracts (TPx, SPx and IBPx) were analyzed by anionic (Davis, 1964) and cationic (Reisfeld et al. 1962) electrophoresis and isoelectric focusing (IEF) (in a pH range 3-10] in polyacrilamide gels using a Bio Rad Mini Protean III system. Samples were previously desalted through a Sephadex G-25 column. To determine pI values in the IEF analysis, a pI standard provided by Bio Rad (pI 4.45-9.6) was used. Gels were stained with benzidine and H_2O_2 as substrates to detect Px activity (de Forchetti and Tigier 1990).

2.5. Determination of kinetic parameters

Kinetic properties of SPx and IBPx isoforms, with phenol (millimollar absorption coefficient= 7870 M^{-1} cm^{-1}) as substrate were studied by a spectrophotometric method [12]. The catalytic efficiency was determined using a millimolar absorption coefficient (ε_{403} = 90 M^{-1} cm^{-1}) to estimate Px concentration (Coniglio et al. 2008). The assays were performed with phenol, 4-aminoantipyrine (4-AAP) and H_2O_2 as color-generating substrates. The formation of the reaction product, a red quinone, was followed by monitoring A_{510} at a final time of 1 min. The reaction mixture (1 mL) contained 2.4 M 4-AAP, phenol in the concentration range of 0-24 mM and H_2O_2 in the concentration range of 0-250 x 10^{-3} mM.

2.5. Statistical analysis

In all experiments three replicates were performed for each sample. Results are presented with the mean and standard error from three independent experiments. Statistical analysis was carried out using Duncan´s test ($p < 0.05$) with the software STATISTICA (version 6.0).

3. Results and discussion

3.1. Determination of the optimal conditions for phenol removal

In the present study, the efficiency for phenol removal of DT and WT HRs was evaluated using different phenol and H_2O_2 concentrations (Table 1), since several researches have reported the use of H_2O_2 as oxidant agent in *in vitro* remediation systems (Lin et al. 2004). Moreover, H_2O_2 is a co-substrate of Px, which catalyzes oxidation reactions of several organic substrates, like phenol. As it was expected, the efficiency of phenol removal decreased in both WT and DT HRs with increasing concentrations of the pollutant. When both HRs were treated with 200, 400, 600 and 800 mg L^{-1} of the pollutant and 5 mM H_2O_2, DT HRs showed significantly higher phenol removal efficiency than WT. However, efficiencies lower than 50% were observed in both HRs treated with the highest phenol concentration (800 mg L^{-1}) (Table 1). This low removal efficiency could be related with the phytotoxic effects of phenol, such as changes in the composition and structure of membranes

(Scragg 2006). Moreover, this pollutant and its chloral derivatives can potentially affect a wide range of hydrophobic domains in the cell (Biswas et al. 2010).

Table 1: Phenol removal efficiencies (%) of WT and DT HR cultures treated during 1 h to different phenol concentrations (100, 200, 400, 600 and 800 mg L^{-1}) and different H_2O_2 contents (2.5, 5, 7.5 and 10 mM)

	H_2O_2 concentrations (mM)							
Phenol (mg L^{-1})	2.5		5		7.5		10	
	WT HR	DT HR	WT HR	DT HR	WT HR	DT HR	WT HR	DT HR
100	70.4 ± 0.44	78.8 ± 0.95	72.3 ± 0.91	82.6 ± 0.16*	67.1 ± 1.21	71.9 ± 3.04	62.9 ± 0.63	56.7 ± 2.25
200	43.9 ± 0.14	61.8 ± 0.77	49.5 ± 0.14	68.4 ± 1.28*	46.3 ± 0.39	50.3 ± 3.48	46.4 ± 1.18	50.4 ± 1.64
400	41.6 ± 0.54	51.7 ± 1.44	46.2 ± 1.97	68.6 ± 0.53*	36.7 ± 0.12	63.7 ± 0.04	36.2 ± 2.07	64.5 ± 1.35
600	30.7 ± 0.11	42.2 ± 0.92	36.9 ± 1.79	52.1 ± 3.17*	33.7 ± 0.96	52.1 ± 3.42	34.2 ± 3.37	49.5 ± 3.25
800	25.9 ± 0.27	34.6 ± 1.24	30.7 ± 1.73	41.6 ± 0.85*	26.5 ± 0.32	36.8 ± 0.26	24.7 ± 0.81	23.2 ± 5.72

Footnote: Values are mean of three independent experiments and asterisk (*) represents significant differences according to Duncan´s test ($p < 0.05$).

Regarding H_2O_2, its concentration might be optimized for each system to determine the conditions under which maximal removal of phenol could be accomplished with minimum consumption of this reagent (Coniglio et al. 2008). As it is shown in Table 1, no significant differences in phenol removal efficiencies were obtained in the presence of higher H_2O_2 concentrations (7.5 and 10 mM). Besides, as high quantities of H_2O_2 were supplied, the removal efficiency decreased. Therefore, treatment with high H_2O_2 levels did not improve phenol removal efficiency of both HRs. Similarly, Bódalo et al. (2008), observed that when H_2O_2 concentration increased, conversion of 4-chlorophenol by horseradish and soybean Px increased until an optimum value was reached, after which no additional improvement in the conversion was found. In addition, Li et al. (2011) also observed an increase in pentachlorophenol (PCP) removal with increasing H_2O_2 concentration from 0 to 5 μM. However, PCP removal was not largely promoted with further increased concentration of H_2O_2 (Li et al. 2011). The results obtained by these authors and those from this work, could be related to a suicide inactivation of Px caused by a H_2O_2 excess. This type of inactivation is a characteristic aspect of the Px mechanism occurring at the level of the complex between compound I and H_2O_2, where a partition occurs leading to either enzyme turnover or inactivation (Sidrach et al. 2006). In addition, earlier studies pointed out that Px are susceptible to inactivation by H_2O_2 excess through the production of the catalytically inactive form called verdohaemoprotein (denoted as P-670) and a low-reactive intermediate called compound III [peroxy-iron (III) porphyrin free radical] (Nazari et al. 2007; Puiu et al. 2008). Unlike P-670, the formation of compound III does not represent terminal inactivation of the enzyme since it can spontaneously revert to the native state (E). However, its return to the catalytic cycle is slow and can represent a significant loss in Px activity (Hong-Mei and Nicell 2008). Thus, in order to minimize H_2O_2-induced inactivation, it will be necessary to maintain an adequate concentration of this reagent in the reaction mixture over time of treatment to ensure that all H_2O_2 is consumed by the end of the reaction. Based on the results obtained in this work, it can be concluded that the exogenous addition of 5 mM H_2O_2, is the optimum concentration that should be used to obtain the highest phenol removal efficiencies avoiding the inactivation of Px, in both HRs.

3.2. Analysis of phenol removal along different time of treatment

One of the main parameters that determine the cost of an enzymatic removal process is the optimal reaction time (González et al. 2006). Thus, in order to analyze the effect of exposition time on phenol removal efficiencies, HRs were treated with two concentrations of the pollutant (100 and 600 mg L^{-1}) and 5 mM H_2O_2 (Figure 1A). For this assay, the low pollutant concentration (100 mg L^{-1}) was used since HRs reached the highest removal efficiency and 600 mg L^{-1} was selected because the HRs were still able to remove about 40% of the pollutant.

DT HRs showed removal efficiencies significantly higher than WT HRs, either with 100 mg L^{-1} or 600 mg L^{-1} phenol after treatment times (15, 60, 150, 300 and 1440 min). The highest efficiencies were observed after 1440 min, for both HRs. Besides, prolonged exposition times were more significant on phenol removal process when both tobacco HRs were treated with the highest pollutant concentration. In this sense, the increase in phenol removal efficiency was about 20% respect to the increase reached (10%) at the lowest pollutant concentration. Since one important limitation in phytoremediation is the time required to achieve this process with the high pollutant concentrations detected in industrial effluents, it is noteworthy the fast reaction rate obtained with DT and WT HRs treated with 600 mg L^{-1} of phenol. Moreover, this is an interesting advantage of these *in vitro* cultures respect to other methods, which frequently required a long time of treatment. Our results also showed that longer times of treatment can improve the efficiency to remove high pollutant concentrations.

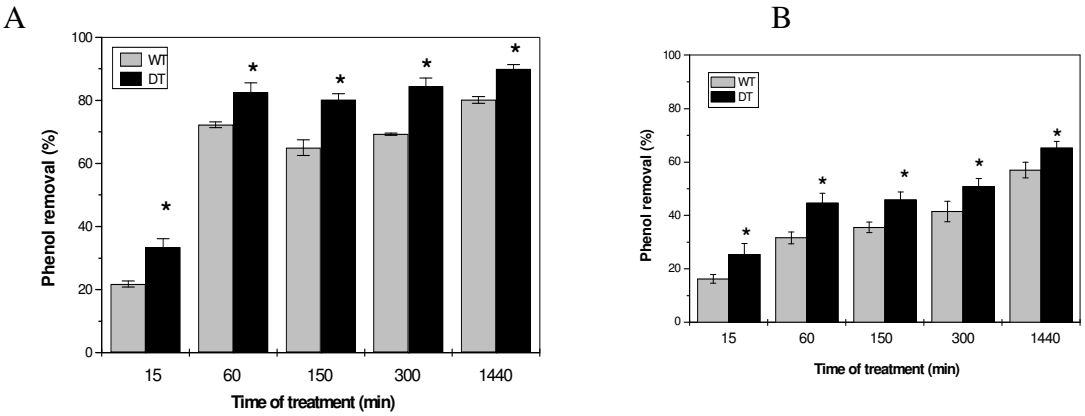

Figure 1: Phenol removal efficiencies (%) of WT and DT HR cultures treated with H_2O_2 5 mM and different phenol concentrations: (A) 100 mg L^{-1}, (B) 600 mg L^{-1}, during different times (15, 60, 150, 300 and 1440 min). Values are mean of three independent experiments and asterisk * represent significant differences according to Duncan´s test ($p < 0.05$)

Changes in acidic and basic Px isoforms, due to the treatment with the xenobiotic (100 mg L^{-1} and 600 mg L^{-1}) were analyzed and results are shown in Figure 2. Acidic Px isoforms of both HRs (WT and DT) did not show any differences after phenol treatment compared with the control (data not shown). On the other hand, TPX1 and TPX2 were detected as bands with Rf values of 0.56 and 0.86, respectively. Both Px and other basic Px with Rf between 0.56 and 0.86, showed a decrease in the intensity of their bands. It is important to note that WT HRs has a Px isoenzyme with the same electrophoretic mobility of TPX2 (Rf: 0.86). In addition, this decrease in the intensity of Px bands was more pronounced with increasing concentrations of the pollutant, suggesting that these basic Px isoforms may be implicated in phenol removal and, as the reaction took place, they were partially inactivated. It is well

known that Pxs are able to remove phenol through oxidative polymerization (Wright and Nicell 1999), leading to the formation of insoluble polymers which can be easily separated through filtration or sedimentation (Angelini et al. 2011). However, as it could be seen in the present study, these enzymes can lose their biological activity due to the inactivation or inhibitory effects during the reaction, such as: (a) irreversible reactions between Px and phenyl or phenoxy radicals formed by one-electron oxidation of phenolic substrates during the catalytic cycle; (b) adsorption of polymerized products on Px, resulting in steric hindrance of the substrate access to the enzyme's active site (Nakamoto and Machida 1992), (c) suicide inactivation due to excess of H_2O_2 or associated radical species and intermediates of the enzyme's catalytic cycle (Sidrach et al. 2006), which are able to irreversible inactivate through P-670 formation, as it was described above.

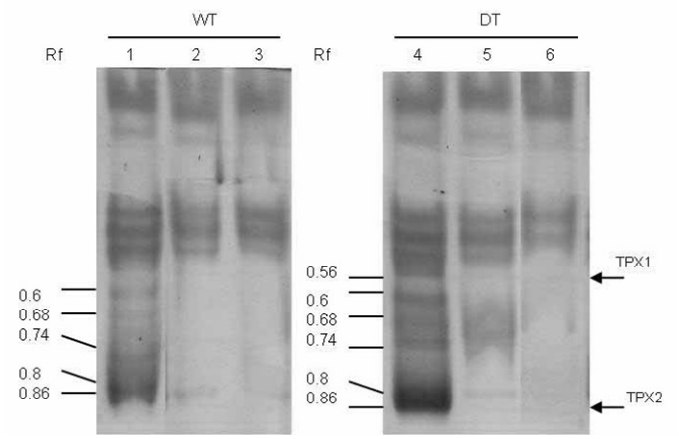

Figure 2: Basic Px isoform profiles from WT and DT tobacco HR treated with 100 mg L^{-1} and 600 mg L^{-1} of phenol and H_2O_2 5 mM for 24 h. Lines 1, 4: HR treated with water and used as controls. Lines 2, 5 and 3, 6 correspond to HR treated with 100 mg L^{-1} and 600 mg L^{-1} of phenol, respectively. **Rf**: relative mobility of Px isoforms

3.3. Analysis of the participation of different Px isoform in phenol removal

Simple extraction methods were used to separate different Px fractions. Since TPX1 is a protein ionically bound to cell wall, it is possible to obtain it in IBPx fraction, while TPX2 is obtained in the SPx fraction. As it is shown in Figure 3, several Px isoforms, with pI values from 9.6 to 3.6, were detected by IEF in TPx extracts from both HRs (WT and DT).

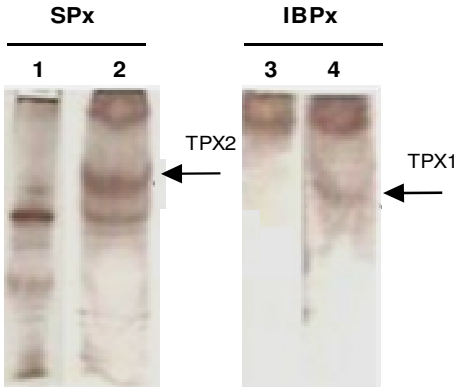

Figure 3. IEF in the pH range 3.0-10.0 of partially purified Px isoenzymes. Lines 1, 3: WT HR extracts, Lines 2, 4: DT HR extracts

In addition, the SPx extract from DT HRs showed a main basic band (pI higher than 9.6) which corresponds to TPX2 isoenzyme. On the other hand, a basic Px band of pI 9.6 was detected in IBPx extract from DT HRs, which corresponds to TPX1 isoenzyme. Then, kinetic studies were performed with different extracts (SPx and IBPx) from WT and DT HRs, in order to determine the apparent kinetic constants for phenol oxidation. The Km and catalytic efficiency are frequently used to evaluate the preference of the different enzyme extracts for the substrate, and represent the constant rate of the reaction to form the enzyme-substrate complex. SPx and IBPx extracts from WT and DT, showed high affinity towards phenol as substrate (Table 2). Besides, Px extracts from DT HRs showed higher catalytic efficiencies than WT HR extracts. Moreover, IBPx extract from DT HRs, which contained TPX1 isoenzyme, showed the highest value of catalytic efficiency.

Table 2: Kinetic parameters of different WT and DT HR extracts (SPx and IBPx) obtained with phenol as substrate

	Kinetic parameters	
	Km (mmol l^{-1}) efficiency	**Catalytic ($l\ mmol^{-1}\ seg^{-1}$)**
WT SPx	0.22	1.2
DT SPx	0.19	4.6
WT IBPx	0.36	1.5
DT IBPx	0.20	15.5

Previously, in our laboratory, a direct relationship between the over-expression of a basic ionically bound to cell wall Px isoenzyme from transgenic tomato HRs and phenol removal efficiency was established (Wevar Oller et al. 2005). Thus, the results obtained in this study with tobacco HRs could be considered as another interesting evidence of the active participation of TPX1 isoenzyme in phenol removal process. In addition, the present results demonstrated that TPX1 could act similarly in other plant species which normally do not express this isoenzyme, like tobacco. On the other hand, the high catalytic efficiency showed by SPx extract from DT HRs could indicate the participation of another basic Px such as TPX2 isoenzyme in phenol removal. These results are in agreement with those previously found by Sosa Alderete et al. (2009), who demonstrated that a single TPX2 transgenic tobacco HRs showed higher phenol removal than WT HRs. Therefore, the results obtained in the present study demonstrated that TPX2 isoenzyme could also have a role in phenol removal process.

As it is widely known, before one realizes the full potential of enzymes, a number of significant issues should be addressed, including the development of low-cost sources of enzymes in quantities that are required at industrial scale (Husain 2010). Since purification processes to obtain pure enzymes are expensive, the use of tobacco HRs could be considered as an alternative strategy for removal processes, taking into account that the main disadvantage of using pure enzymes is its inactivation during the reaction previously mentioned (Gómez et al. 2008). In this sense, as Px are trapped in root tissues, their catalytic activity could be better maintained than pure enzymes. Probably, the hydrophobic products precipitate and inactivate Px in aqueous phase reactions tending to adsorb and concentrate on

the root surface, thereby reducing this type of inactivation. Therefore, HRs could provide an adequate matrix at which phenol conversion can be achieved while simultaneously protecting Px from inactivation.

4. Conclusion

Tobacco HRs could be an interesting system to remove phenol and DT HRs, showed removal efficiencies significantly higher than WT HRs. Although phenol removal decreased with increasing pollutant concentrations, prolonged exposition times could improve these efficiencies. The ability of DT HRs to remove phenol more efficiently could be correlated with their higher Px activity due to the expression of TPX1 and TPX2 isoenzymes. Moreover, the adequate affinity towards phenol and the high catalytic efficiency showed by the basic Px isoform TPX1 could be considered as another evidence of its participation in phenol oxidation. However, TPX2, also exhibited a role in this process.

Finally, this first study on a pilot scale on the elimination of phenolic compounds by tobacco HRs Px may serve as a basis for an experimental application in industrial effluents. Thus, further studies on the scale-up of phenol removal by tobacco HRs could be very useful for the treatment of great amounts of effluents polluted with high phenol concentrations, and they are under study in our laboratory.

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