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Research article

# Antioxidant response of tobacco (*Nicotiana tabacum*) hairy roots after phenol treatment

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ABSTRACT

Phenol is released to the environment from a wide variety of industrial effluents and it causes severe problems to human health and ecosystem. In the present study, we determined that *Nicotiana tabacum* hairy roots (HRs) double transgenic (DT) for two peroxidase genes (*tpx1* and *tpx2*) showed higher phenol removal efficiency than wild type (WT) HRs after 120 h of phenol treatment at the expense of endogenous $H_2O_2$. Besides, to determine whether phenol could induce oxidative stress on tobacco HRs, we analyzed the antioxidant response, superoxide anion ($O_2^{\bullet -}$) localization and malondialdehyde (MDA) levels. Both HRs treated with phenol, showed significant increases in peroxidase (PX) activity mainly at the end of the assay (120 h) being PX activity from transgenic HRs 40% higher than that of WT HRs. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities showed significant increases from 24 to 120 h of phenol treatment. PX, SOD and APX isoforms were also analyzed and slight changes were observed only in PX patterns. Both HRs showed significant differences in total glutathione (TGSH) content during treatment, being higher in DT HRs than in WT HRs. At the end of the assay, a greater accumulation of $O_2^{\bullet -}$ in different root zones was observed in WT and DT HRs. Moreover, phenol was able to increase the MDA levels in WT HRs from 48 to 120 h of the treatment, but no significant changes were observed in DT HRs. Results suggest that under these experimental conditions, DT HRs would be more tolerant to phenol than WT HRs.



## 1. Introduction

Phenol is frequently found in the wastewater of different industries at concentrations between 100 and 1000 mg $l^{-1}$ depending on the industrial activities [1]. Due to its high toxicity and persistence in soil and water, it has been classified as a priority pollutant by the United States Environmental Protection Agency (USEPA). Although different methodologies have been developed to remove phenol, some of them have many disadvantages such as, high cost, production of toxic compounds and low efficiency [2]. Therefore, it is necessary to introduce new alternative methods such as, phytoremediation [3,4], which involves the use of plants, as well as *in vitro* cultures obtained from them, in order to remove or transform diverse environmental pollutants in less toxic compounds [5]. Different plant systems have been used in phytoremediation studies, among them hairy roots (HRs) which are obtained from *Agrobacterium rhizogenes* infection, followed by the genetic integration of one segment from its Ri plasmid to plant genome. In the last decade, HRs have become, an attractive model system mainly due to the maintenance of the same properties of the mother plant roots, particularly genetic and biochemical stability [6]. Therefore, HRs can be used in diverse studies, such as the identification of plant species with high ability to remove pollutants or to analyze in detail the different mechanisms involved in phytoremediation [7]. In addition, roots are the main organ which have contact with the environmental pollutants and they are also the sites where the first reactions against the pollutant take place, among them, production of bioactive molecules like enzymes, such as laccases and peroxidases (PX) [8,9].

Regarding phenol, little is known about its metabolism and effects on plant tissues as well as the aspects related to the stress produced by the pollutant, which could negatively affect the phytoremediation process [10]. In this context, Singh et al. [11] using *Brassica juncea* HRs observed that phenol treatment induced peroxidase activity and increased hydrogen peroxide ($H_2O_2$) content. As it is well known, one of the typical plant responses against stressors is the production of reactive oxygen species (ROS), like $H_2O_2$, superoxide anion ($O_2^{\bullet -}$), oxhydrile radical ($HO^{\bullet}$), and perhydroxile radical ($O_2H^{\bullet}$). ROS can modify the redox homeostasis

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and damage plant cells producing an oxidative stress and cell death [12]. Diverse environmental stressors, including biotic factors (pathogen bacterium, virus or fungi), abiotic factors (UV light, extreme temperatures, high salt contents, etc.) and several pollutants can alter the normal metabolism of the plant and induce oxidative stress, which occurs when the levels of ROS exceed the antioxidant defence system. ROS production is carried out by different organelles during the normal aerobic metabolism of the cell, among them, chloroplast, mithocondria and peroxisomes [12]. However, under stressful conditions, the quick increase of ROS production may be produced not only by the diverse organelles, but also by the activation of NADPH oxidase enzymes located in the plasma membrane [13]. To protect themselves against these toxic oxygen intermediates, plant cells and its organelles employ antioxidant defence systems, which involves enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include PX, SOD and APX whereas non-enzymatic antioxidants include glutathione (GSH), ascorbic acid, among others [12].

Several organic compounds are able to induce an antioxidant response in plants under oxidative stress. For instance, treatment with the herbicide Paraquat, cinamic acid and phenantrene increased the activity of antioxidant enzymes (PX, SOD, APX) and MDA contents in wheat plants [14], cucumber roots [15] and *Arabidopsis thaliana* plants [16], respectively. Previous results from our laboratory demonstrated that DT and WT HRs showed high ability to phenol removal when $H_2O_2$ was exogenously added [17]. However, according to current literature, little is known about phenol removal at expense of endogenous $H_2O_2$, the antioxidant response or oxidative stress that may occur in plant cells exposed to phenol and how it could affect the pollutant removal process. Therefore, the aims of the present work were to analyze: (i) phenol removal efficiencies; (ii) the response of antioxidant defence system; (iii) distribution and accumulation of $O_2^{\bullet -}$ and (iv) MDA content in DT and WT HRs after phenol treatment.

## 2. Materials and methods

### 2.1. Plant material

Tobacco (*Nicotiana tabacum*) DT HRs (clone 5) which expressed two basic peroxidase genes from tomato *(tpx1* and *tpx2*) and WT HRs were used [17]. For successive subcultures, the HR cultures were maintained for 25–30 d on Murashige and Skoog (MS) [18] medium supplemented with vitamins, at 25 ± 2 °C in the dark, on an orbital shaker at 70 rpm.

### 2.2. Phenol removal assay along different times of treatment

Inoculums of 0.4 g of HRs were placed in Erlenmeyer flasks containing 50 ml of MS medium and incubated at 25 ± 2 °C in the dark on an orbital shaker at 70 rpm for 15 d. After this time, the cultures were in the exponential phase of growth, which is considered as the optimal stage of growth for this assay. Then, a volume of phenol from a stock solution, previously sterilized by filtration using 0.22 μm Millipore filters was added to obtain a final concentration of 100 mg $l^{-1}$ of phenol in the growth medium of HR. Distilled and sterile water was added, as control. Then, aliquots of liquid medium were taken at different times: 1, 6, 24, 48 and 120 h for residual phenol analysis, which was determined according to the spectrophotometric method described by Kinsley and Nicell [19]. Results were expressed as percentage of the initial phenol concentration.

HRs harvested at each time were dried using filter papers, crushed into fine powder under liquid nitrogen, and stored at −80 °C for further studies, as described below.

### 2.3. Antioxidant enzymes

#### 2.3.1. Determination of PX activity

HRs were homogenized in 50 mM sodium acetate/acetic acid buffer, pH 5, supplemented with 1 M KCl, in the ratio 1:10 (tissue:buffer). Then, the homogenates were centrifuged at 10 000 rpm for 30 min at 4 °C. The supernatants were considered as enzyme extracts and used for PX activity assays. The PX activity was determined spectrophotometrically at 470 nm using *o*-dianisidine (*o*-D) 0.63 mM as substrate and 0.5 mM $H_2O_2$ as oxidant agent. One unit of enzyme was defined as the amount of enzyme that generated 1 μmol of product in 1 min under the conditions previously described [17].

#### 2.3.2. Determination of SOD activity

It was performed according the procedures described by Beauchamp and Fridovich [20] and Beyer and Fridovich [21], with some modifications. The root homogenate was prepared in the ratio 1:10 with 50 mM potassium phosphate buffer (pH 7.8), containing 5 mM EDTA and 2% Polyvinyl Pyrrolidone (PVP) (w/v) at 4 °C. Then, the homogenate was centrifuged at 15 000 rpm for 30 min. Supernatant was collected and considered as an enzyme extract for the subsequent analysis. The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.8), with 0.1 mM EDTA, 4 μM riboflavin, 13 mM methionine, 75 μM Nitroblue tetrazolium (NBT) and 20 μl of enzyme extract. The reaction was started by adding riboflavin. Absorbance at 560 nm was measured immediately, after 7 min illumination, with light intensity of 1 μmol $m^{-2}$ $s^{-1}$ at 25 °C. Control assays were performed without the enzyme extract. One unit of SOD was defined as the amount of enzyme required to inhibit the NBT photoreduction by 50%.

#### 2.3.3. Determination of APX activity

This assay was performed according to the method described by Shalata et al. [22] and Hossain and Asada [23] with slight modifications. Enzyme extracts were obtained in the same way as described above (section 2.3.2) for SOD activity determination. The enzyme activity was determined spectrophotometrically following the decrease in absorbance at 290 nm ($\varepsilon$ 290 = 2.8 $mM^{-1}$ $cm^{-1}$) due to ascorbate oxidation. The reaction mixture (1 ml) contained 0.4 mM ascorbate (ASA), 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM $H_2O_2$ and the enzyme extract. The reaction was started by adding $H_2O_2$ (0.1 mM). One unit of APX was defined as the amount of enzyme necessary to produce the disappearance of 1 μmol of ASA in 1 min at 25 °C.

### 2.4. Determination of total glutathione (TGSH) concentration

It was carried out according to the method previously described by Anderson [24], where simultaneous concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSH) were determined. HRs (0.2 g) were homogenized in 5% sulfosalycilic acid (1 ml). Then, the homogenate was centrifuged at 10 000 rpm for 30 min at 4 °C and the supernatant was collected for subsequent analysis. The reaction mixture contained 143 mM sodium phosphate buffer (pH 7.5), 6.3 mM EDTA, 0.248 mg $ml^{-1}$ NADPH freshly prepared, 6 mM DTNB [5,5′-Dithiobis (2-nitrobenzoic acid)]. This mixture was incubated at 30 °C for 10–12 min. The reaction was started by adding the supernatant previously obtained and enzyme GSSG reductase (Sigma). It was performed spectrophometrically, following the increase in absorbance at 412 nm. The amount of total glutathione was determined from a standard curve in which the GSH equivalents present (0.25, 0.5, 0.75, 1 and 1.25 μM) were plotted against the rate of change of absorbance at 412 nm.

### 2.5. Analysis of PX, SOD and APX isoform profiles

PX, SOD and APX were analyzed by anionic [25] and cationic [26] polyacrilamide gel electrophoresis using a Bio Rad Mini Protean III system. After completion of electrophoresis, the gels were stained to detect SOD activity following the method described by Rao et al. [27]. Visualization of Cu, Zn and Fe SOD was achieved by using specific inhibitors. Gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM KCN (for Cu/Zn-SOD) or 5 mM $H_2O_2$ (for Cu/Zn-SOD and Fe-SOD) during 30 min before staining for SOD activity [27]. APX and PX activities were detected following the procedures described by Rao et al. [27] and by de Forchetti and Tigier [28] respectively.

### 2.6. Analysis of the localization of superoxide anion

$O_2^{\bullet -}$ was detected according to the method previously described by Dunand et al. [29]. WT and DT HRs, after 1, 6, 24, 48 and 120 h of treatment with 100 mg $l^{-1}$ phenol and water, as control, were excised and immersed in 20 mM phosphate buffer (pH 6.1) containing 2 mM NBT for 10 min. The reaction was stopped by transferring the HRs to distilled water. In presence of the $O_2^{\bullet -}$, NBT is reduced producing dark blue formazan salts. Then the samples were fixed in the slides and examined under the light microscope at 100× (Axiolab-Zeiss). Each experiment was repeated at least three times with similar results. The Photoshop (Adobe Systems Inc., San Jose, CA, USA) histogram function was used to assess the mean staining intensity of the elongation zone (0, white; 255, black).

### 2.7. Determination of $H_2O_2$ concentration

The hydrogen peroxide content was determined according to the method described by Sergiev et al. [30]. Samples were homogenized with 1 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 rpm for 15 min. Then, a 0.5 ml aliquot of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7) and 1 ml 1 M KI. Samples were incubated at room temperature, for 15 min and the absorbance was measured at 390 nm. $H_2O_2$ content was calculated from a standard curve prepared from commercial $H_2O_2$ of known concentrations.

### 2.8. Determination of MDA concentration

MDA contents were determined according to the protocol described by Heath and Packer [31]. HRs were homogenized in 20% TCA in the ratio 1:10 (w/v). An equal volume of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA was added. Then, the homogenate was incubated at 95 °C for 25 min, and it was centrifuged at 3000 rpm for 10 min after it was cooled on ice for 2 min. The supernatant was collected, the absorbance at 532 nm was measured, and the amount of MDA was calculated ($\varepsilon\, 560_{nm} = 155\ mM^{-1}\ cm^{-1}$). The correction for unspecific turbidity was done by subtracting out the absorbance at 600 nm. The MDA concentration was expressed as μmol $g^{-1}$ fresh weight.

### 2.9. Statistical analysis

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

## 3. Results

### 3.1. Phenol removal along time of treatment

WT and DT HR, at exponential phase of growth (15 d), were exposed to 100 mg $l^{-1}$ of phenol solution and removal efficiencies were evaluated at different times: 1, 6, 24, 48 and 120 h (Fig. 1). Since $H_2O_2$ was not added in the reaction mixture, we assumed that phenol removal was achieved at expense of endogenous $H_2O_2$, which was detected in both HRs at levels of 4.5–5.5 μmol $g^{-1}$ FW. At the first hour of treatment with the pollutant, phenol removal efficiencies of both HRs did not show significant differences and the values were under 20%. However, from 6 h to 120 h of treatment, DT HRs were significantly more efficient than WT HRs. The higher efficiencies were obtained at the end of the treatment (120 h), with values of 70% and 40% for DT and WT HRs, respectively.

### 3.2. Analysis of the antioxidant responses of tobacco HR during phenol treatment

To analyze whether the treatment of the HRs with phenol could produce oxidative stress, we studied some indicators of this type of stress. In this context, we evaluated the antioxidant defence systems, such as enzymatic: PX, SOD and APX activities, as well as a non-enzymatic indicator, like GSHT concentration. Moreover, to determine whether phenol was able to induce the accumulation of ROS, like $O_2^{\bullet -}$ and to produce unspecific oxidation of membrane lipids, the distribution of $O_2^{\bullet -}$ and the MDA contents were determined in HRs.

#### 3.2.1. Evaluation of enzymatic defence system

In presence or absence of phenol, DT HRs showed higher total PX activity than WT ones. HRs treated with 100 mg $l^{-1}$ phenol solution for 48 h, did not show significant changes in PX activity (Fig. 2A and B). However, at the end of the treatment (120 h), both HRs, WT and DT, showed a significant increase in the PX activity compared to the controls treated with water. The increase of PX activity in DT HRs caused by phenol treatment was 40% higher than in WT HRs.

On the other hand, to study whether the pollutant could affect the activity of some PX isoenzymes, electrophoretic profiles in polyacrilamide gels were analyzed. Several PX isoenzymes could be distinguished in WT and DT HRs. In the cationic gels, from DT HRs,

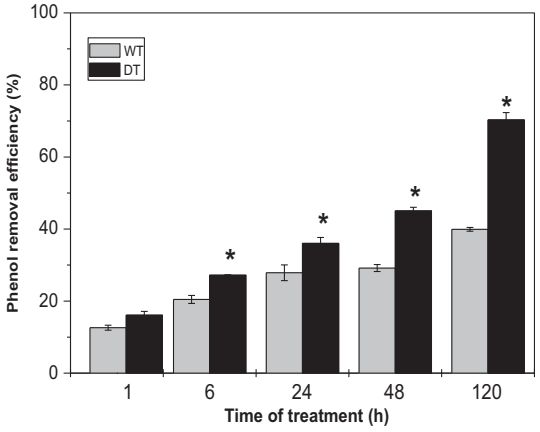


**Fig. 1.** Phenol removal efficiencies using WT and DT HRs at different times of treatment (1, 6, 24, 48 and 120 h) with phenol (100 mg $l^{-1}$). Values are the mean of three independent experiments. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$).

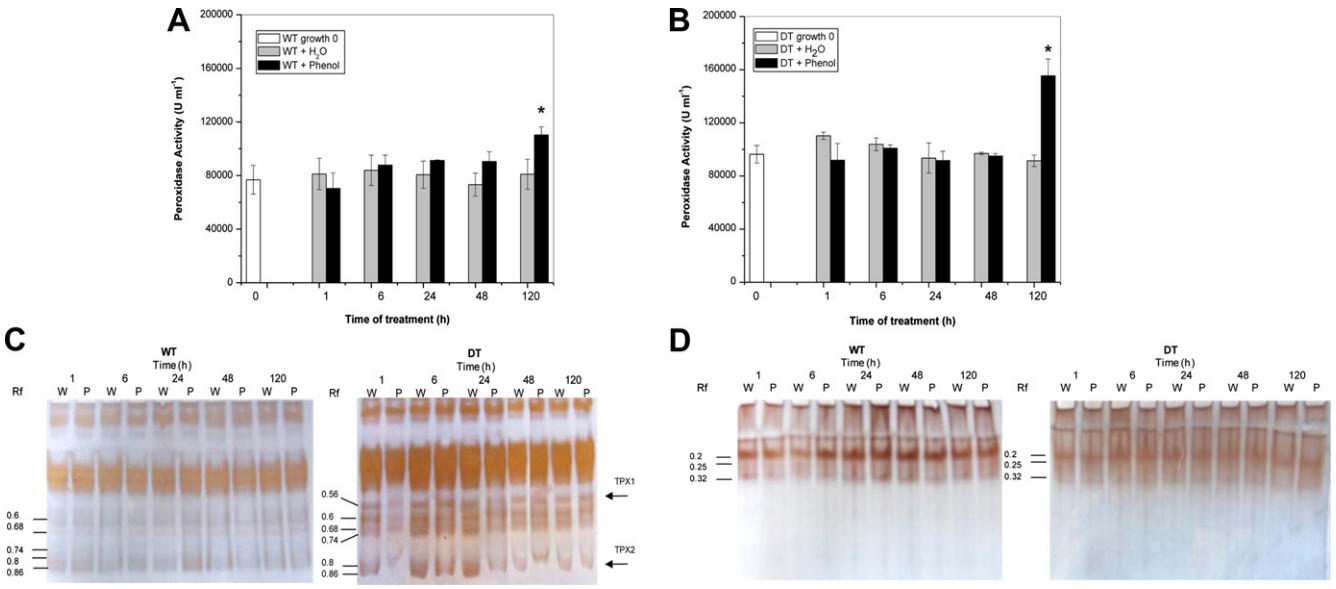


**Fig. 2.** Total PX activity (PX) (U ml$^{-1}$) from WT (A) and DT (B) HRs. Basic (C) and acidic (D) peroxidase isoform profiles from WT and DT HRs treated with phenol (P) (100 mg l$^{-1}$) and water (W) as control during different times (1, 6, 24, 48 and 120 h). Values are the mean of three independent experiments. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$). Time 0: represents a control of HRs after 15 d of growth in MS medium. Rf: relative movility of PX isoforms.

TPX1 and TPX2 isoenzymes could be observed as bands with Rf values of 0.56 and 0.86 respectively (Fig. 2C). After 1 h and 24 h of phenol treatment, DT HRs showed a decrease in the intensity of bands corresponding to basic PX isoforms (Rf: 0.56–0.86), among them TPX1 and TPX2. It is important to note that WT HRs has a PX isoenzyme with the same electrophoretic mobility of TPX2 (Fig. 2C). However, no changes were observed in basic PX isoform profiles of WT HRs after phenol treatment (Fig. 2C). Moreover, acidic PX isoform profiles were also analyzed, but no changes either in WT or in DT HRs were detected (Fig. 2D).

Regarding SOD activity, both HRs, showed similar values in presence or absence of phenol (Fig. 3A and B). Phenol treatment did not affect SOD activity after the first 6 h, compared to the control treated with water, in both HRs. However, from 24 h to the end of

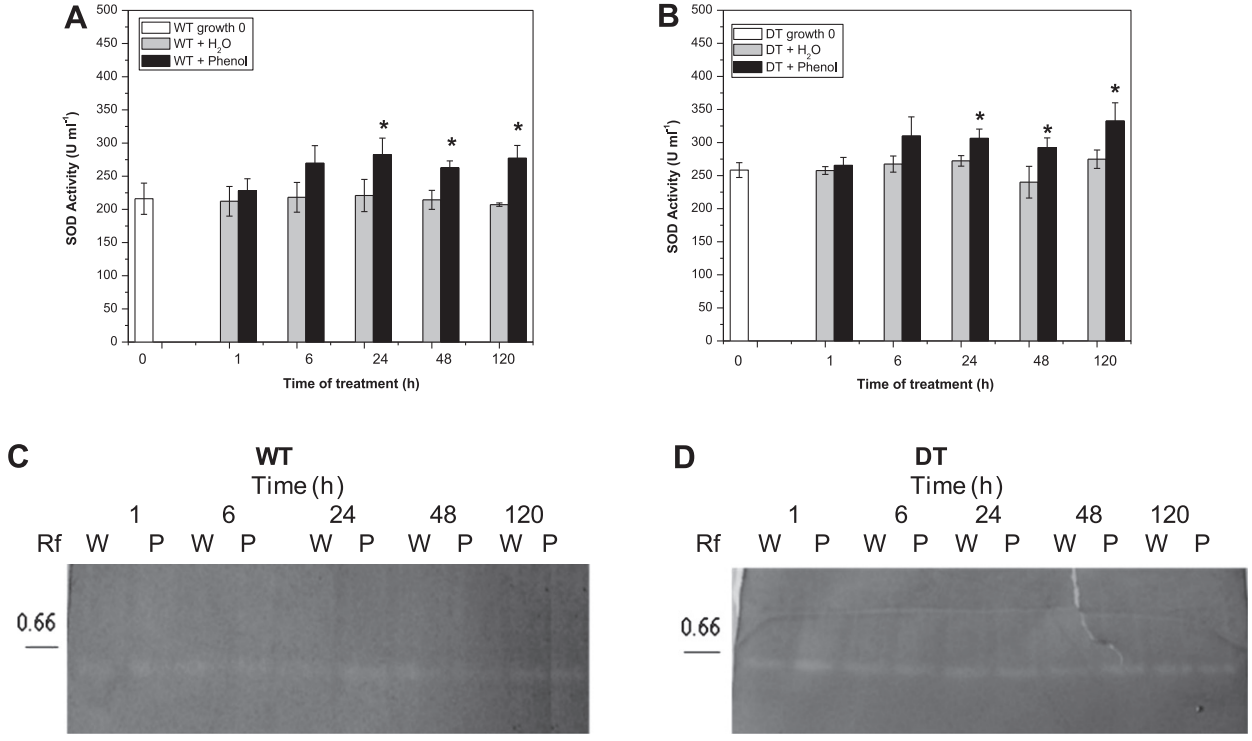


**Fig. 3.** SOD activity (U ml$^{-1}$) (A–B) and anionic SOD isoform profiles (C–D) from WT and DT HRs respectively, treated with phenol (P) (100 mg l$^{-1}$) and water (W) as control at different times (1, 6, 24, 48 and 120 h). Values are the mean of three independent experiments. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$). Time 0: represents a control of HRs after 15 d of growth in MS medium. Rf: relative movility of SOD isoforms.

the assay (120 h), WT and DT HRs showed an increase of 28 and 18% in SOD activity respectively, compared with their controls.

To investigate whether the changes in SOD activity could be due to variations in the isoform composition, the samples were subject to anionic and cationic gel electrophoresis. The SOD zymograms showed no bands corresponding to SOD basic isoforms. However, in the anionic polyacrilamide gel, one band corresponding to an acidic SOD isoform of Rf 0.66 was found in both HRs, which remained without changes during the phenol treatment (Fig. 3C and D). Incubation of gels in either 2 mM KCN or 5 mM $H_2O_2$ before staining for SOD activity revealed no activity in the gel suggesting that this isoform is a Cu/Zn-SOD (data not shown).

A behavior similar to that of SOD activity was observed in the APX activity of both HRs. At the first 6 h, no significant changes were found compared with the controls (Fig. 4A and B). However, in both HRs, the APX activity significantly increased after 24–120 h of treatment with phenol. However, the increase was only 11% higher than the controls.

Two isoforms exhibiting APX activity were detected for both HRs in the gels, one of them with high intensity corresponded to a basic APX isoform (Rf 0.33) (Fig. 4C), and the other with low intensity, corresponded to an acidic APX isoform (Rf 0.86) (Fig. 4D). The intensity of these APX bands was not affected by the presence of phenol.

### 3.2.2. Analysis of the non-enzymatic defence system

*3.2.2.1. Total Glutathione determination (TGSH).* WT HRs showed significant changes in the TGSH levels after phenol treatment. At the first hour of treatment with the pollutant, there was a slight decrease in the TGSH levels, whereas at 6 h there was no significant difference compared with the controls treated with water. However, after 24–120 h of treatment, the TGSH contents significantly increased, reaching the highest value of 23.6 μmol $ml^{-1}$ at the end of the assay (120 h), which was 27% higher than the control (Fig. 5A). DT HRs also showed some changes in the TGSH contents after phenol treatment. After the first 6 h of treatment, the TGSH levels significantly decreased compared with the control. In contrast, from 48 h to 120 h, DT HRs showed a response similar to that of WT HRs, where the TGSH contents significantly increased (27.78 μmol $ml^{-1}$). Thus, the TGSH levels were 37% higher than the control treated with water (Fig. 5B).

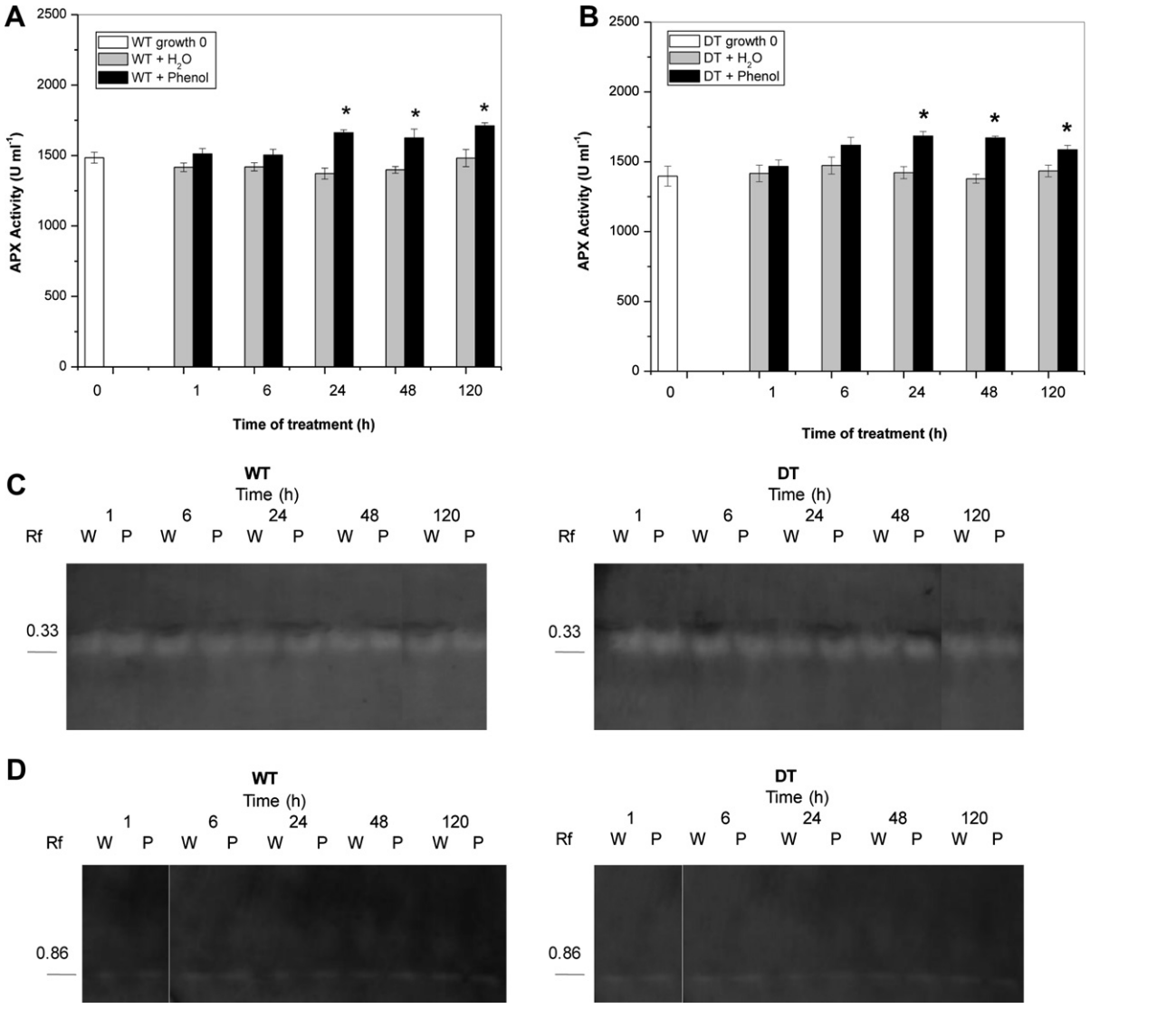


**Fig. 4.** APX activity (U $ml^{-1}$) (A–B) and cationic and anionic APX isoform profiles (C–D) from WT and DT HRs respectively, treated with phenol (P) (100 mg $l^{-1}$) and water (W) as control at different times (1, 6, 24, 48 and 120 h). Values are the mean of three independent experiments. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$). Time 0: represents a control of HR culture after 15 d of growth in MS medium. Rf: relative movility of APX isoforms.

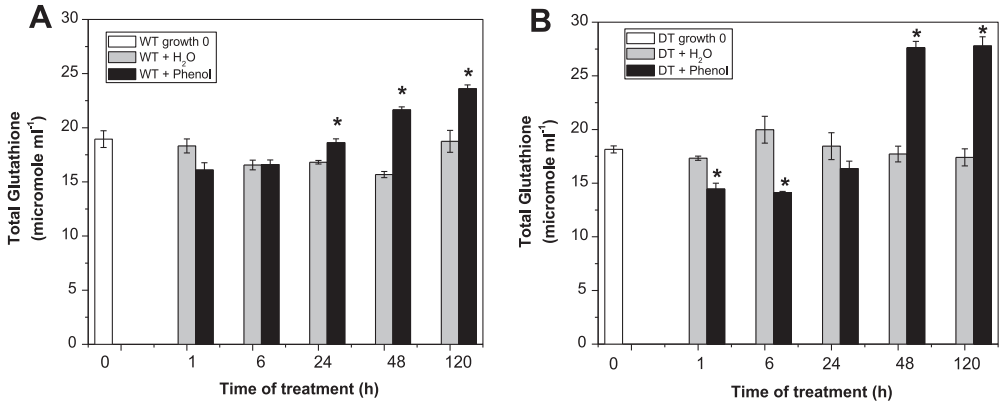


**Fig. 5.** Total Glutathione (TGSH) content of WT (A) and DT (B) HRs treated with phenol (100 mg $l^{-1}$) and water as control at different times (1, 6, 24, 48 and 120 h). Values are the mean of three independent experiments. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$). Time 0: represents a control of HRs after 15 d of growth in MS medium.

### 3.3. Localization of superoxide anion in tobacco HRs

One of the first responses of the plant under stressful conditions is the production of reactive oxygen species (ROS), like $O_2^{\bullet -}$. Therefore, to detect the localization and distribution of $O_2^{\bullet -}$ in HRs, we used NBT, a reagent which in presence of $O_2^{\bullet -}$ is reduced and precipitated as salts of blue-violet formazan [29]. In this assay, WT and DT HRs were used after treatment with 100 mg $l^{-1}$ of phenol for 1, 6, 24, 48 and 120 h. Significant differences between control and treated HRs were only detected after 120 h of incubation. In control conditions, the reaction products were detected mainly in the meristematic region and the staining intensity was similar (Table 1). However, both HRs, treated with the pollutant, showed higher accumulation of $O_2^{\bullet -}$ than the control. The largest deposit of NBT salts, which were also quantified as intensity levels by the method described by Dunand et al. [29], was mainly observed in the elongation zone, in root tip cells, and in cell wall of the differentiation region (Table 1 and Fig. 6).

### 3.4. Evaluation of the oxidative damage on tobacco HRs

In the lipid peroxidation analysis, the MDA contents obtained from WT HRs after the treatment with phenol were, on average, significantly higher (Fig. 7A) than those obtained from the DT HRs (Fig. 7B). The MDA levels of WT after the first 24 h of phenol treatment did not show significant differences compared with the control. However, the treatment of WT HRs with phenol from 48 to 120 h significantly increased the MDA contents. On the other hand, in DT HRs, no significant differences were found in MDA levels compared with the control. Moreover, these MDA levels were lower than those obtained in WT HRs. Therefore, this result could indicate that under these conditions, phenol would not produce oxidative damage on membrane lipids from DT HRs.

**Table 1**
Intensity of NBT staining (±SE) in tobacco HRs after 120 h of treatment with phenol (100 mg $l^{-1}$) and water as control.

| Tobacco HR | Staining intensity ± SE | |
|---|---|---|
| | Control | Phenol |
| WT | 143 ± 4.0[a] | 199 ± 1.7[b] |
| DT | 165 ± 3.3[a] | 207 ± 2.2[b] |

Numbers with the same superscript letters indicate non-significant differences according to Duncan's test ($P < 0.05$).

## 4. Discussion

HRs are convenient laboratory tools for phytoremediation studies and they offer important technical advantages compared with whole plant [6]. In addition, transgenic HRs are useful in biochemical and molecular studies and for screening of genetic transformants prior regeneration of whole plants with enhanced phytoremediation potential. In a previous work, we have established WT and DT HRs. The transgenic nature of DT HRs as well as TPX1 and TPX2 protein expression was confirmed [17]. In the present study, we have demonstrated that DT HRs were significantly more efficient to remove phenol than WT HRs at the expense of endogenous $H_2O_2$, whose levels were about 4.5–5.5 μmol $g^{-1}$ FW. The highest values of phenol removal efficiencies of DT and WT HRs were obtained at the end of assay, being 70 and 40%, respectively. Moreover, total PX activity also showed a significant increase at the end of the experiment, being DT HRs PX activity 40% higher than that of WT. This increase in the PX activity might be related to the high phenol removal efficiency obtained at the end of the treatment. Therefore, these results support the hypothesis that PX would be the main enzymatic group involved in phenol removal. It is important to note that transgenic HRs significantly increased the ability to remove the pollutant. Many authors have reported that PX activity may either increase or decrease after treatment with some organic pollutants, in several *in vitro* cultures from different plant species [32,33]. The results obtained in the present work were in agreement with those reported by Araujo et al. [34], who showed that disappearance of phenol from the reaction mixture was accompanied by an increase in the PX activity in *Daucus carota*, *Ipomoea batatas*, and *Solanum aviculare* HRs.

Despite the increase of PX activity in DT HRs after 120 h of phenol treatment, the analysis of PX isoform profiles did not show changes in the number of isoforms detected. However, in DT HRs, a decrease of the intensity of some basic PX bands, with Rf from 0.56 to 0.86, was observed at different times (1 and 24 h), indicating that the activity of these isoforms could be affected by phenol, at the early stages of treatment. Similar results were found in *in vitro* cultures from *Armoracia rusticana* and *Atropa bella-donna*, which showed the disappearance of some constitutive isoenzymes after treatment with polychlorinated biphenyls (Delor 103) [35]. It is well known that PX might lose activity during the catalytic cycle using phenolic substrates, probably due to different mechanisms such as irreversible reaction between enzyme and phenoxy radicals or by physical adsorption of phenol polymer affecting the active site of the enzyme [36]. This could explain why the above mentioned

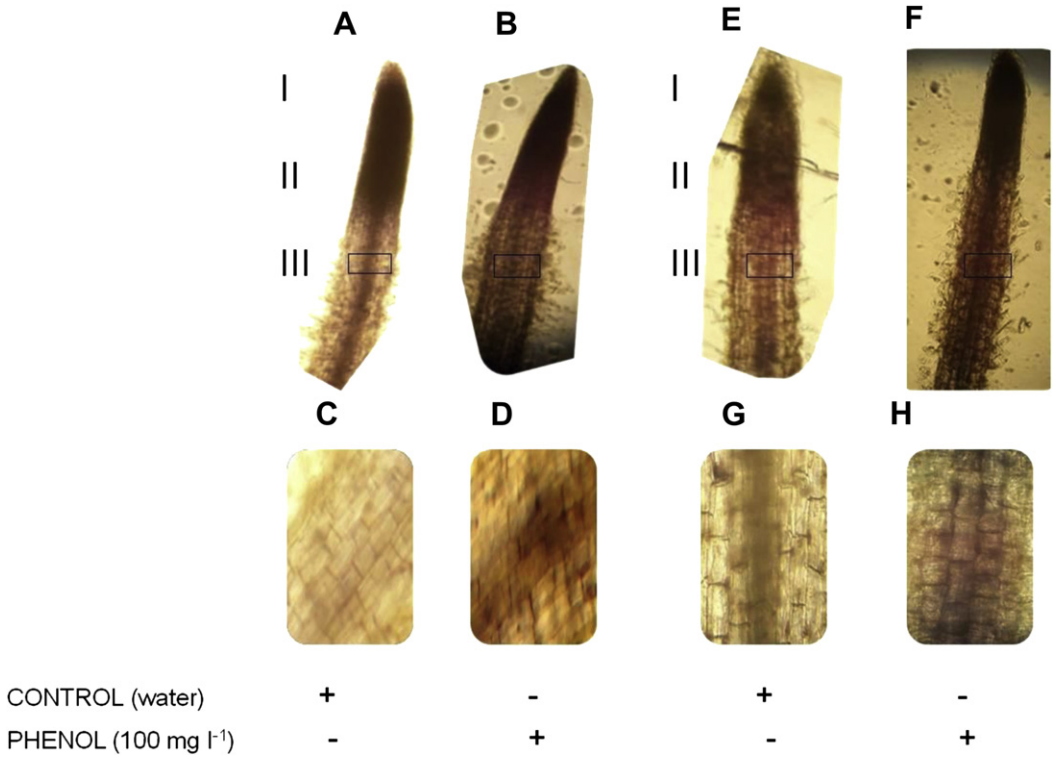


**Fig. 6.** Superoxide anion localization in tobacco HRs using NBT. WT (A, B) and DT (E, F) HRs at 120 h of treatment with phenol (100 mg $l^{-1}$) and water as control. C, D and G, H are magnifications of the boxed area in A, B and E, F, respectively. I, II and III correspond to meristematic, elongation and differentiation zones, respectively.

basic isoenzymes, including TPX1 and TPX2, showed a decrease in their bands intensity. However, after 24 h no changes were detected in PX profiles compared with the controls treated with water.

In the present study, other antioxidant enzymes such as Cu/Zn-SOD and APX also showed high activity after 24–120 h in both HRs. However, no changes in SOD and APX electrophoretic profiles were detected. Thus, the high activity detected in phenol treated roots would be due to the enhancement of the activities of existing antioxidant isoenzymes, because new isoforms were not observed. The enhancement of PX, SOD and APX activities under stressful conditions is a typical response in order to eliminate the excess of ROS during stress, as reported by several authors. For instance, different plant species subject to a wide range of abiotic stresses like heavy metals, sludge, methyl viologen (MV), salt, drought, among others, showed increase in antioxidant enzyme activities, such as SOD [37,38] and APX [39,40]. The higher activities of these antioxidant enzymes may prevent the oxidative damage in the root tissues.

On the other hand, TGSH contents showed changes after phenol treatment, decreasing at 1 h, followed by a significant increase from 48 h to 120 h. As it is well known, glutathione (GSH) is a very important antioxidant molecule responsible for maintaining the redox homeostasis inside the cells, since it controls the ROS levels through the cycles of glutathione-peroxidase and ascorbate-glutathione preventing the oxidation of proteins. In this sense, it was reported that plants with increased GSH contents showed higher tolerance to oxidative stress induced by heavy metals like Cd [41]. Metabolism of activated oxygen species has been related to the high redox state of ascorbate and glutathione. Moreover, a high redox state of the latter has been reported to induce Cu/Zn-SOD as was detected in the present work, which would have enhanced APX

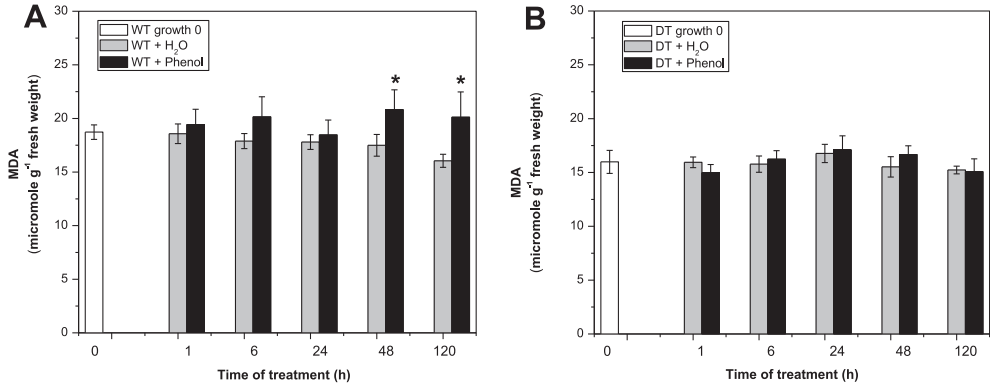


**Fig. 7.** MDA content (μmol $g^{-1}$ fresh weight) in WT (A) and DT (B) HRs, treated with 100 mg $l^{-1}$ phenol and water as control, at different times (1, 6, 24, 48 and 120 h). Values are the mean of three independent experiments. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$). Time 0: represents a control of HRs after 15 d of growth in MS medium.

because the mechanisms that control the expression of Cu/Zn SOD and APX are believed to be similar [27].

It has been described that under stressful situations, different sources of ROS production could be activated, like the NADPH oxidases localized in the plasma membrane, which would be responsible for increasing levels of $O_2^{\bullet -}$ at membranes [13]. In the present work, phenol treated HRs showed higher accumulation of $O_2^{\bullet -}$ in several parts of the root-elongation zone, root tip cells and cell wall of the differentiation region compared with controls, after 120 h of treatment. In the formation of oxygen derivatives, such as $O_2^{\bullet -}$, the transplasma membrane NADPH oxidase, in most cases, seems to be a key player. However, many aspects of ROS formation and mechanisms of action remain unclear. In this context, Dunand et al. [29] reported that *Arabidopsis* roots treated with diphenylene iodonium (DPI) (an specific inhibitor of the NADPH oxidases) reduced $O_2^{\bullet -}$ levels compared with the controls, and decreased root and root hair elongation, demonstrating the important participation of $O_2^{\bullet -}$ in these physiological processes.

Phenol induced oxidative damage on WT membrane lipids from 48 h to 120 h, as was shown by MDA levels. However, in DT HRs no significant differences were found in the MDA content compared with the control. Therefore, under this experimental condition, phenol could not produce oxidative damage on membrane lipids of DT HRs, which is in agreement with other results found in transgenic plants for different antioxidant enzymes. For instance, Mn-SOD transgenic plants were more tolerant to oxidative stress induced by MV and ozone, and showed less oxidative damage on the membrane lipids than WT plants [42]. Similarly, Guan et al. [37] reported that transgenic *B. juncea* plants for one catalase (CAT) gene had higher tolerance to Cd toxicity compared with WT plants. In addition, transgenic tobacco plants for an APX isoenzyme also showed high tolerance to MV and pathogen fungi treatment [43]. In this context, important evidences suggest that transgenic plants for two or more antioxidant genes could improve the survival under a wide variety of stressful conditions [39,40].

In conclusion, our results showed that phenol treatment after 120 h was able to induce the antioxidant response in both cultures, by the increase of antioxidant enzymatic (PX, SOD and APX) and non-enzymatic (TGSH) mechanisms. However, DT HRs had higher PX activity than WT HRs at the end of the assay. This would explain why DT HRs treated with phenol are able to remove the pollutant more efficiently. Furthermore, they showed increased TGSH content after 120 h of phenol treatment, demonstrating a more efficient antioxidant response than WT HRs. Therefore, this antioxidant response detected in DT HRs under phenol treatment, could be correlated with the absence of oxidative damage and also with the higher ability of these roots to tolerate and to remove phenol more efficiently.

Finally, the results obtained in this study may indicate that the use of trangenic HRs expressing foreing peroxidases would be an interesting tool to improve the ability for phenol removal, and also to enhance tolerance to the oxidative stress produced by the pollutant.

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