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Technical Note

Phenol tolerance, changes of antioxidative enzymes and cellular damage in transgenic tobacco hairy roots colonized by arbuscular mycorrhizal fungi

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ABSTRACT

Phytoremediation has been recognized as a cheap and eco-friendly technology which could be used for the remediation of organic pollutants, such as phenolic compounds. Besides, the extent to which plants react to environmental pollution might depend on rhizosphere processes such as mycorrhizal symbiosis. In the present work, phenol tolerance of transgenic tobacco hairy roots (HR), namely TPX1, colonized with an arbuscular mycorrhizal fungus (AMF) was studied. However, the question is whether AMF symbiosis can moderate adverse effects of phenol to the plant tissues. Thus, the antioxidative response as well as parameters of oxidative damage, like malondialdehyde (MDA) content, were determined. Antioxidative enzymes such as peroxidase, superoxide dismutase, ascorbate peroxidase were higher in TPX1 HR colonized with AMF, compared to wild type HR colonized by AMF, in the presence of increasing concentrations of the pollutant. Besides, MDA levels remained unaltered in TPX1 HR associated with AMF treated with the xenobiotic. These results, suggested that this culture could tolerate phenol and moreover, it has an efficient protective mechanism against phenol-induced oxidative damage, which is of great importance in the selection of species with remediation capacities. Thus, transgenic HR colonized with AMF could be considered as an interesting model system to study different processes which play a key role in the phytoremediation of organic pollutants.

1. Introduction

Environmental pollution is a global problem that affects both the developing and developed countries (Suza et al., 2008). Common organic contaminants, such as herbicides, explosives and petrochemicals, are mainly produced by human activities and they contaminate air, soil and water. In particular, phenol is considered a priority pollutant since it is harmful to organisms at low concentrations and it has been classified as a hazardous pollutant because of its potential harm to human health (Agostini et al., 2010). Therefore, stringent effluent discharge is imposed. In this sense, the US EPA has established a limit concentration of 1 $\mu g\ L^{-1}$ for phenolic compounds for drinking water, while in our country, Argentina, the law 24051 of hazardous residues established guide levels for drinking water of 2 $\mu g\ L^{-1}$ (Coniglio et al., 2008). In recent years, as an alternative strategy to deal with environmental pollution, the interest in the study of plants with biodegradation capacity has increased. Although, phytoremediation is an useful technology, an important problem in this process is that high pollutant concentrations tend to inhibit plant growth, in part due to oxidative stress (Gerhardt et al., 2009), which results from the disruption of cellular homeostasis of reactive oxygen species (ROS) production (Hernández et al., 2010). To prevent ROS accumulation, plants have the ability to scavenge/detoxify ROS by producing different enzymatic antioxidants, including, superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (Px), catalase (CAT) and glutathione reductase (GR) (Ashraf, 2009). Plants under stress are well stocked with an array of protective and repair systems that minimize the occurrence of oxidative damage (Khalvati et al., 2010). However, although plants possess natural protection systems against different kind of stress, their interaction with soil microorganisms can alleviate symptoms (Marulanda et al., 2007). Thus, combining the advantages of microbe plant symbiosis within plant rizhosphere, results in an effective cleanup technology (Kamaludeen and Ramasamy, 2008). Besides, this microbe-assisted phytoremediation appears to be particularly effective for the removal and/or degradation of organic pollutants from contaminated soil (Gerhardt et al., 2009). Among rhizospheric organisms, arbuscular mycorrhizal fungus (AMF) form a ubiquitous symbiotic relationship with over 90% of land plants (Huang et al., 2007) and constitute an important functional component of the soil–plant system occurring in almost all habitats and climates (Leyval et al., 2002). Since one of the limitations for phytoremediation is the contact between roots and the contaminants, as well as the root growth rate, AMF should be beneficial because they increase the volume of soil explored by roots. They also improve plant

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growth, and contribute to alleviate toxicity of pollutants such as heavy metals (Leyval et al., 2002). Thus, plants growing in a polluted habitat with a mycorrhizal association may be often more tolerant to chemical stress than those growing without the symbiotic fungi (Gaspar et al., 2002). Several studies toward phytoremediation of pollutants mediated by AMF are mainly focused in the remediation of heavy metals and As (Liu et al., 2005; Janoušková et al., 2007; Lingua et al., 2008). These studies suggested that plants are protected against those compounds by AMF. However, few reports deal with the effects of organic compounds, such as phenolics on the symbiosis between plants and AMF.

Another useful strategy for enhancing effectiveness of phytoremediation is to express or overexpress in transgenic plants, the genes involved in metabolism, uptake, or transport of specific pollutants (Abhilash et al., 2009). Among the enzymes involved in phenol removal, plant Pxs have been reported as those implicated in this process (Coniglio et al., 2008; Sosa Alderete et al., 2009). In our laboratory, a basic Px (TPX1) from tomato was successfully expressed in tobacco hairy roots (HR), resulting in higher total Px activity and in higher phenol removal efficiency in the transgenic culture, compared to wild type (WT) HR (Sosa Alderete et al., 2009). However, it is not known if the interaction of this transgenic HR with rhizospheric microorganisms, such as AMF, can alleviate adverse effects produced by phenol and/or induce the antioxidant defense against oxidative stress. Therefore, we used WT and transgenic tobacco HR for TPX1, to evaluate AMF-induced changes in antioxidative enzymes and cellular damage under different phenol concentrations.

2. Materials and methods

2.1. Biological material

The experiments were carried out with HR cultures of *Nicotiana tabacum* (Sosa Alderete et al., 2009). WT and transgenic cultures expressing TPX1 from tomato were selected for the experiments described below. They were subcultured every 30–35 d in Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) enriched with vitamins. The AMF used was *Glomus intraradices* strain GA5, which was kindly provided by Dr. Alicia Godeas (FCEFQyN-UBA). AMF was routinely cultured in association with WT and TPX1 tobacco HR in a modified minimum medium (MM) (Bécard and Fortin, 1988) with 1% sucrose and solidified with 0.4% (w/v) gellan gel (Phytagel).

2.2. Culture of mycorrhizal tobacco roots in the presence of phenol

Phenol tolerance of tobacco HR was assayed *in vitro*, in order to avoid contamination. The experiment consisted of a randomized block design with three factors: the expression of a basic tomato peroxidase, the mycorrhizal symbiosis (with or without AMF) and phenol contamination. To prepare phenol enriched medium, a stock phenol solution (1000 mg L^{-1}) was filter sterilized and individually added to three different flasks containing autoclaved MM to provide final concentrations of 5, 10 and 25 mg L^{-1}. Then, the medium was placed on Petri dishes. MM without phenol was used as control. Phenol used in all experiments was purchased from Merck. Solutions were prepared with deionized water and all substances used were of Analytical Reagent Grade.

Cultures were started from standardized roots inoculums of 2-month-old monoaxenic cultures of tobacco HR colonized or not by *G. intraradices* strain GA5, which were placed in the middle of eight replicate Petri dishes per each phenol treatment. Cultures were kept in the dark in a growth chamber at 25 ± 2 °C for 5 months and routinely observed. The roots were collected from the medium by solubilizing the solidified media 30 min under agitation in 20 mL of 10 mM sodium citrate buffer pH 6 (Verdin et al., 2006).

After the roots were harvested, the root biomass, in each of the replicates per treatment, was divided into two sub-samples to determine: (1) root colonization and (2) enzyme activities and MDA content. The first root sub-samples from each replicate were cleared in KOH and stained with 0.05% (v/v) trypan blue in lactophenol as described by Phillips and Hayman (1970) and microscopically examined for AMF colonization using a gridline intercept method (Giovanetti and Mosse, 1980). The second root sub-samples were collected and immediately frozen in liquid nitrogen. Frozen tissues were ground in liquid nitrogen with mortar and pestle and stored at −80 °C until extraction for the determination of antioxidative enzymes (Px, SOD and APX) and MDA content, as it is described below.

2.3. Determination of enzyme activities: Px, SOD and APX

Frozen samples (0.1 g of pulverized tissues) were extracted with 50 mM acetic/sodium acetate buffer, pH 5.0 containing 1 M KCl. The homogenate was centrifuged at 10 000 rpm for 10 min at 4 °C. The supernatant was considered as total protein extract. Total Px (EC 1.11.1.7) activity was determined spectrophotometrically using *o*-dianisidine and H_2O_2 as substrates (Sosa Alderete et al., 2009). One unit of enzyme (U) was defined as the amount of enzyme, which generated 1 μmol of product in 1 min in the conditions described. Px isoforms patterns from the homogenates were analyzed by cationic (Reisfeld et al., 1962) and anionic (Davis, 1964) electrophoresis on 7.5% polyacrylamide gels using a BioRad Mini-protean III system following the manufacturer's instructions. Px activity in the gels was detected with 0.5 M acetic/sodium acetate buffer pH 4.6 containing 3.8 mM benzidine and 21 mM H_2O_2 as substrates (Sosa Alderete et al., 2009) and images were analyzed by measuring the band intensities by Scion Image program (Scion Corporation).

SOD (EC 1.15.1.1) activity was assayed by using the photochemical nitrobluetetrazolium (NBT) method (Beauchamp and Fridovich, 1973). One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50%.

Total APX activity (EC 1.11.1.6) was measured spectrophotometrically by monitoring the decline in A_{290} while ascorbate (ε = 2.8 mM^{-1} cm^{-1}) was oxidized, using the method of Hossain and Asada (1984).

2.4. Lipid peroxidation determination

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid-reactive substances (TBARs), as described by Heath and Packer (1968). MDA (ε = 155 mM^{-1} cm^{-1}) content was determined spectophotometrically at A_{532} and corrected for non-specific turbidity at A_{600}.

2.5. Statistical analysis

Results are presented with the mean and the standard error (SE). Statistical analysis was carried out using Duncan's multiple range tests ($P < 0.05$) by using software STATISTICA (version 6.0).

3. Results

3.1. Phenol tolerance of AMF

Microscopic observations of stained AMF showed the presence of spores, vesicles, and intraradical hyphae in WT and TPX1 HR

after 2 months of growth in MM medium. Regarding colonization percentages, we did not observe any difference between WT and TPX1 HR (data not shown). Thus, the expression of the tomato TPX1 in tobacco HR did not produce any effect either in the interaction with AMF or in the growth and development of the symbiosis.

To evaluate whether TPX1/GA5 HR had any advantage regarding phenol tolerance over WT/GA5 HR, the cultures were grown with different phenol concentrations. As shown in Fig. 1, cultures grown in the presence of 5 mg L^{-1} of the pollutant showed an increase in the colonization percentages, compared with their respective controls (HR in association with the AMF grown without the pollutant). However, only TPX1/GA5 showed a greater colonization percentage in the presence of 10 mg L^{-1} of the xenobiotic. Moreover, when both cultures (WT/GA5 and TPX1/GA5) were exposed to higher phenol concentrations (25 mg L^{-1}) a greater reduction in the colonization percentages was observed. Although, runner hyphae producing lateral simple branched structures was observed.

3.2. Effect of phenol on the antioxidative enzymes

Pxs are ubiquitous enzymes implicated in a broad range of physiological processes. They are also related to the degradation of toxic molecules, such as phenolic compounds. Generally, TPX1 HR showed high Px activity, compared with WT HR (Fig. 2a). Similarly, TPX1/GA5 HR showed higher Px activity than WT/GA5 HR, under all the concentrations of the xenobiotic tested. Moreover, it is important to note the lower activity obtained for these cultures, compared to WT and TPX1 HR uninoculated with AMF. Hence, the presence of the AMF exerts a decrease in Px activity. Since the enzymes activities were determined at the end of the experiment, we were not able to observe other changes that may have happened at an early developmental stage.

The isoenzyme analysis of Pxs by electrophoresis revealed at least three bands (Rf 0.06, 0.38 and 0.43) in the cationic gel and two bands (Rf 0.20 and 0.23) in the anionic gel. No differences in the number of bands were observed in Px isoform patterns (Fig. 2b and c), among different treatments with phenol. Besides, all basic isoenzymes were slightly affected by the symbiosis with the AMF and, thus, lower band intensity in both tobacco HR (WT and TPX1) colonized with AMF was observed, compared with uninoculated cultures.

SOD activity is considered of great importance in the detoxification process and related to numerous stress situations. As it is shown in Fig. 3a, WT, TPX1 and WT/GA5 did not show significantly different values in the activity of this enzyme after 5 months of growth with different phenol concentrations. On the other hand, SOD activity surprisingly increased in TPX1/GA5 HR, with values significantly higher than the rest of the cultures.

Regarding APX, an enzyme which catalyses the reductions of H_2O_2 using ascorbic acid as a cofactor, transgenic HR (TPX1 or TPX1/GA5) showed higher APX activity than WT HR (Fig. 3b). In addition, this activity was not affected by the treatment with phenol, since each culture did not show significantly differences compared with their respective control (untreated culture).

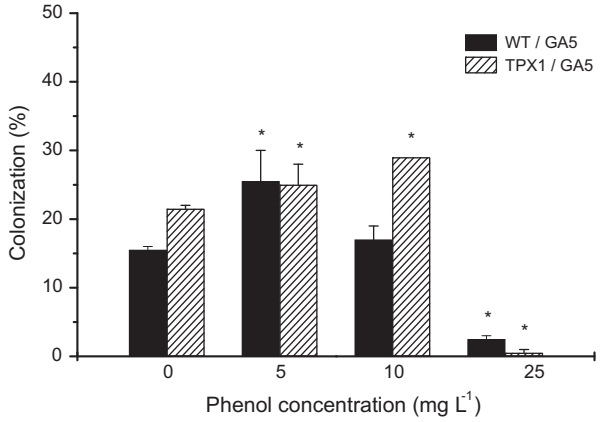

Fig. 1. Colonization percentages of WT and TPX1 HR after 5 months of growth with different phenol concentrations (5, 10 and 25 mg L^{-1}). Data are presented as means ± SE of the mean. The bars marked with asterisks are significantly different respect to WT/GA5 HR according to Duncan's test ($P < 0.05$).

3.3. Effects of phenol on lipid peroxidation

The damage produced by phenol to cellular membranes due to lipid peroxidation was estimated by determination of TBAR. Fig. 3c shows MDA concentrations of WT HR and TPX1 HR inoculated or uninoculated with *G. intraradices* strain GA5.

TPX1 HR and TPX1/GA5 seem to be less affected by phenol treatment, because lower levels of cellular damage were observed compare with WT HR and WT/GA5. The exception was TPX1 treated with 10 mg L^{-1} of the pollutant. Moreover, transgenic HR cultures in association with *G. intraradices* strain GA5 and treated with phenol, showed lower levels of MDA respect to TPX1 HR. This may mainly be attributed to the beneficial contribution of AMF in host roots under stress conditions.

4. Discussion

Since roots are the primary site of contact between plant tissues and contaminants in the soil or water, they provide a key assessment of the phytoremediation potential of a particular plant species (Suza et al., 2008). As an organized tissue, roots are closer in structure and function to the organs of whole plants, thereby offering a greater degree of authenticity with regard to their biological behavior and properties (Doran, 2009). Furthermore, HR cultures due to their short growing period, stable yield and conditions that eliminate the effects of microbes, appeared to be a suitable model system to study the activity of central detoxification enzymes in the cultivation medium (Nepovím et al., 2004; Doran, 2009). *In vitro* culture is an artificial system, but it may be a valuable tool to study fundamental and practical aspects of AMF symbiosis (Diop, 2003). In addition, the establishment of *in vitro* root-organ cultures has greatly influenced our understanding of the AMF symbiosis (Fortin et al., 2002). Although it is well known that mycorrhizal symbiosis causes important changes in plant metabolism, there is little information about the response of plants in mycorrhizal symbiosis towards stress conditions (Khalvati et al., 2010), such as those produced by organic pollutant, like phenol. In the present study, WT and TPX1 HR showed a significant decrease in the colonization percentages only with the highest concentration of the pollutant tested (25 mg L^{-1}). However, runner hyphae producing lateral simple branched structures, was observed with this phenol concentration, indicating that AMF could also grow in these conditions. Moreover, microscopic observations of fungus structures in stained WT and TPX1 HR grown in a matrix polluted with phenol indicated the ability of *G. intraradices* strain GA5 to tolerate this contaminant. Furthermore, the determination of AMF development in roots could be a sensitive indicator of changes in soil pollutant toxicity (Gaspar et al., 2002). To our knowledge, this is the first report describing the ability of this mycorrhizal species to tolerate phenol and to establish symbiosis in these adverse conditions.

It is well known that ROS are produced under normal plant growth conditions and their concentration remain low, whereas, most environmental stresses induce enhanced production of ROS

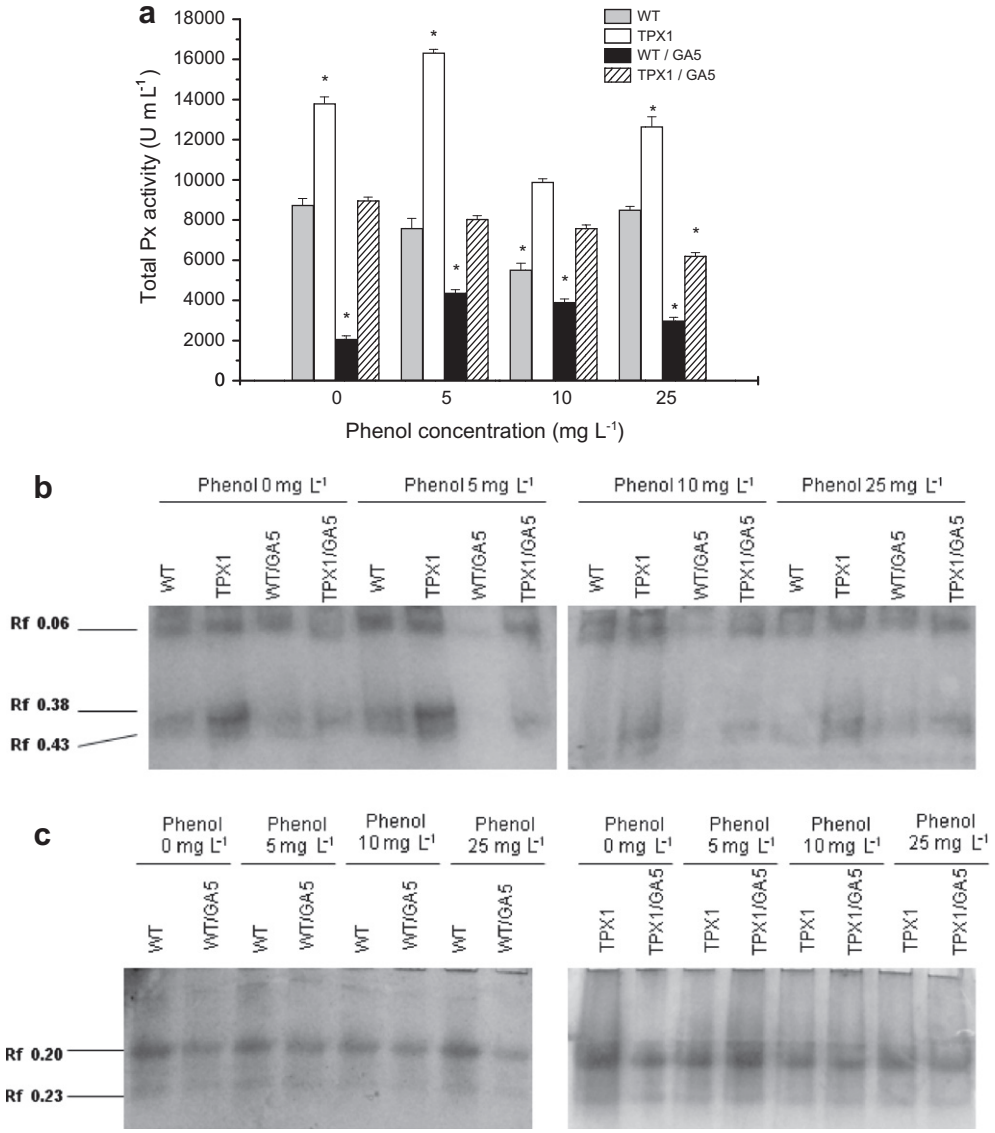

Fig. 2. (a) Total Px activity of WT and TPX1 HR noncolonized and colonized with *G. intraradices* strain GA5 after treatment with different phenol concentrations (0, 5, 10 and 25 mg L^{-1}). Data are presented as means ± SE of the mean. The bars marked with asterisks are significantly different respect to WT HR according to Duncan's test ($P < 0.05$). (b) Basic and (c) acidic Px isoforms of HR extracts.

(Ashraf, 2009). In addition, ROS accumulation induces oxidative damage of membrane lipids, nucleic acids and proteins. Therefore, a tight control of the steady-state concentration of ROS seems to be necessary to avoid oxidative damage at subcellular levels (Hernández et al., 2010). Moreover, the induction of ROS-scavenging enzymes, such as SOD, POD, APX and CAT is the most common mechanism for detoxyfing ROS synthesized during stress responses (He et al., 2007).

As it was already mentioned, Pxs can be involved in the interconversion of ROS as well as in the removal of phenolic compounds. In the present work, transgenic HR, (TPX1 and TPX1/GA5) showed higher Px activity, compared with WT HR, which is in agreement with previous results obtained with this HR culture (Sosa Alderete et al., 2009). On the other hand, it is important to note the lower activity showed by the HR colonized with the AMF. Fries et al. (1996) studying the symbioses of *G. intraradices* with corn (*Zea mays* L.) roots, observed an increase in Px activity during the first and second week of growth which could be related to the defense response of the host plant. However, this activity was suppressed after this time. Probably, the decrease in total Px activity could play an important role in the establishment of the symbiosis. In this sense, it is well known that an increase in the activity of the cell wall-bound anionic Pxs will increase the cross links between cell wall polysaccharides, as well as hydroxyproline-rich glycoproteins, thus making the cell wall more rigid and more resistant to rapid fungal spread (Fries et al., 1996).

Px isoenzyme patterns of different plant species could change in the presence of organic pollutants (Nepovím et al., 2004; Coniglio et al., 2008). In our study, Px zymograms of HR cultures treated with 10 and 25 mg L^{-1} of phenol, showed a decrease in the intensity of bands corresponding to basic isoforms, compared to the intensity of bands obtained for untreated HR cultures (controls). These results suggest that basic isoforms could be more related to phenol degradation thus, these isoforms could be inactivated during this process. This inactivation could be attributed to irreversible reactions between Px and phenyl or phenoxy radicals formed during oxidation of phenolic substrates, but also due to the absorption of the final polymeric product on Px, making difficult the access of the substrate to the enzyme's active site (Nazari et al., 2007; Coniglio et al., 2008). This kind of Px inactivation was

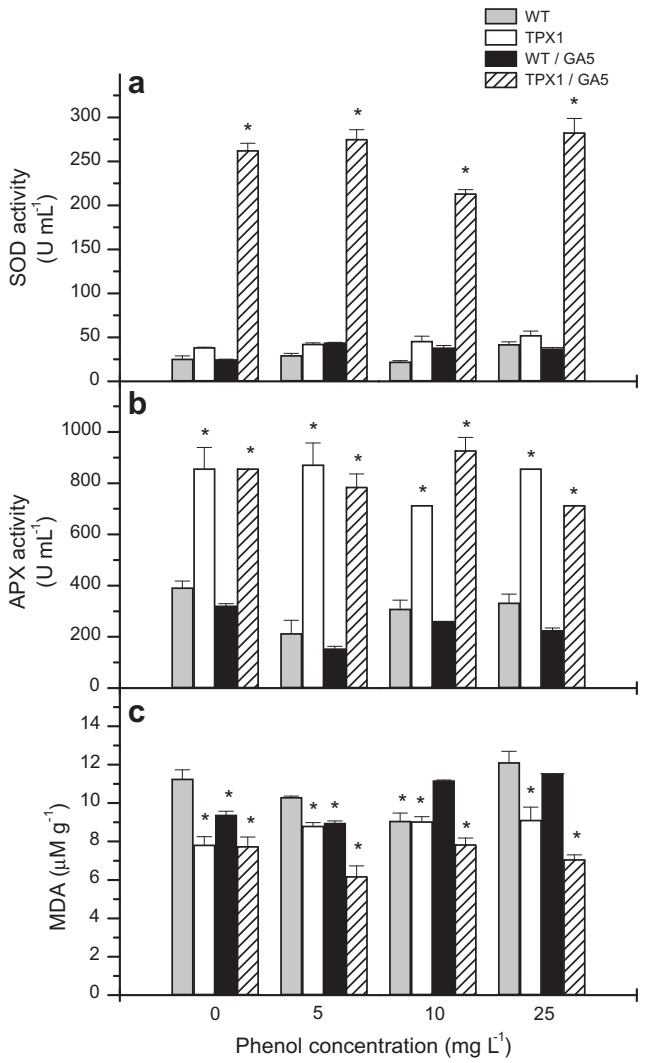

Fig. 3. (a) SOD activity, (b) APX activity, (c) MDA levels of WT and TPX1 HR noncolonized and colonized with *G. intraradices* strain GA5 grown with different phenol concentrations (5, 10 and 25 mg L^{-1}). Data are presented as means ± SE of the mean. The bars marked with asterisks are significantly different respect to WT HR according to Duncan's test ($P < 0.05$).

also observed in horseradish HR treated with heavy metals and nitroaromatic compounds (Nepovím et al., 2004).

It is important to note, that the results obtained in the present study agree with those previously obtained in our laboratory with tomato HR overexpressing TPX1 and treated with phenol (Wevar Oller et al., 2005). Authors observed a decreased or inhibition in the activity of basic and neutral Px isoforms. Moreover, these changes in Px patterns after the treatment with the pollutant, suggested an important role of these isoforms in the phytotransformation process. Similarly, González et al. (2006) proposed that a group of tomato basic Px isoforms was more related to phenol remediation.

SOD is an enzyme associated with stress situations that plays a vital role in detoxification processes by catalyzing the conversion of free $O_2^{\cdot-}$ to O_2 and H_2O_2 (He et al., 2007). In the present study, TPX1/GA5 showed higher SOD activity than the other colonized or uncolonized HR. Similar results were observed regarding APX activity, which was higher in transgenic HR compared to WT HR, probably indicating the enhanced ability of the antioxidative enzymes to scavenge/detoxify ROS in transgenic plant tissues. This increase in the activities of antioxidative enzymes (Px, SOD and APX) of transgenic HR agrees with results obtained with different transgenic plant species (Ashraf, 2009). For instance, in an attempt to produce transgenic *Arabidopsis* overexpressing Mn-SOD, Wang et al. (2004) observed that Mn-SOD activity was over two-folds in transgenic plants than in WT plants. The transgenic *Arabidopsis* showed higher tolerance to salt as compared to the non-engineered plants. In addition, further analyses revealed that despite the enhanced activities of Mn-SOD, the activities of other antioxidative enzymes such as Cu/Zn-SOD, Fe-SOD, CAT and Px of transgenic plants were markedly higher than those of WT plants. Also, the levels of MDA were lower in the transgenic plants than those of WT under salt treatment (Ashraf, 2009). This result clearly emphasizes the importance of a balanced interaction of protective enzymes and other metabolites and that manipulation of a single antioxidant gene could have a great extent to overall stress tolerance.

As it was previously mentioned, to estimate oxidative stress, the MDA content had been widely used as an indicator of lipid peroxidation and, thereby, of oxidative damage in pollutant exposed plant tissues (Mediouni et al., 2009). Besides, the plant capability to activate the defense system against oxidative destruction may be a key link in the mechanism of plant tolerance of unfavorable conditions (Lomonte et al., 2010). Interestingly, in the present study, the lowest damage in cell membranes was observed in transgenic HR cultures colonized by AMF. Thus, our results could mean that the intensity in the response of the antioxidant system of this symbiotic culture could avoid the deleterious effects produced by ROS accumulation in the presence of phenol. These findings, also demonstrate that mycorrhization had beneficial effects, which can alleviate the adverse effects of phenol on plant tissues.

5. Conclusion

Since phytoremediation is a multicomponent process, it is evident that an appropriate selection of plants and microorganisms together with the investigation of both enzymology and gene technology offer many advantages to improve phenol phytoremediation. Thus, based on the presented results, we concluded that tobacco HR (WT/GA5 and TPX1/GA5) could tolerate up to 25 mg L^{-1} of phenol. Besides, transgenic HR (TPX1 and TPX1/GA5), generally showed higher activity of antioxidative enzymes (Px, SOD and APX). It is well known that the antioxidant systems of plants act as important tolerance mechanisms by protecting cells against damage caused by toxic oxygen species such as superoxide radicals ($O_2^{\cdot-}$), H_2O_2, and $^{\cdot}OH$, being all of them generated under environmental as well as xenobiotic stresses. In addition, transgenic HR associated with *G. intraradices*, showed lower oxidative damage in the presence of the pollutant, compared with TPX1 HR and with WT HR colonized or uncolonized with the AMF. The results obtained in this study, could be considered as an interesting and successful evidence of combining different strategies, such as the use of transgenic plants associated with rhizospheric microorganisms, to improve the phytoremediation process.

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