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Removal of 2,4-diclorophenol from aqueous solutions using tobacco hairy root cultures

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ABSTRACT

2,4-Dichlorophenol (2,4-DCP) is harmful for aquatic life and human health, so many attempts have focused on removing it through innocuous technologies. Hairy roots (HR) represent an interesting plant system to study the process and to remove efficiently this compound. In the present work, tobacco HR clones were obtained and one of them was selected for 2,4-DCP phytoremediation assays. These cultures removed 2,4-DCP in short time and with high efficiency (98%, 88% and 83%) for solutions initially containing 250, 500 and 1000 mg/L, respectively. Removal process was mainly associated with peroxidase activity. The highest efficiency for 2,4-DCP (500 mg/L) removal was reached at 60 min and using 10 mM H_2O_2. Moreover, HR could be re-used, almost for three consecutive cycles. The diminution of pH and the increase of chloride ions in post-removal solutions suggested that 2,4-DCP dehalogenation was mediated by peroxidases. Moreover, changes in deposition pattern of lignin in HR exposed to 2,4-DCP suggested that cell walls of xylem and phloem elements would be the site of deposition of some products formed and they would be a lignin-type polymer. These findings contribute to understand 2,4-DCP removal process with tobacco HR and it might have implications in the use of this system for decontamination of polluted waters.

1. Introduction

Human activities generate large amounts of chlorinated phenolic chemicals that are often introduced into soil and water environment during pesticide and insecticide application, industrial releases and accidental spills. Also they are used as antiseptics, disinfectants and wood preservatives. These phenolic compounds are listed as priority environmental pollutants by the US EPA because of their high toxicity, carcinogenicity and persistence [1]. Their toxicity increases with the degree of chlorination of the phenol ring.

In fact, chlorophenols toxicity has been proven both *in vitro* and *in vivo*. In particular, 2,4-DCP has been reported to cause lethargy, tremors and convulsions in mice [2], while workers who manufacture pesticides or were exposed to chlorophenols developed acne and mild liver injuries [1]. Further, various cases of death have also been reported in workers who were sprayed with 2,4-DCP containing steam [3].

The World Health Organization (WHO) established a maximum level for chlorophenols in drinking water of 0.1 μg/L [4], while in Argentina, the law 24051 established a guide level of 0,3 μg/L for 2,4-DCP for human consumption water [5].

In spite of these restrictions, a great amount of 2,4-DCP containing wastewater produced through anthropogenic activities, is drained into rivers and marine environment every year [6]. Moreover, many industries are responsible of contaminating the main rivers of our country, because they discharge many toxic residues such as methoxiphenolchlorine, di and trichloro phenols. These toxic elements are found at concentrations 140 times higher than those accepted by the law of hazardous wastes [7]. So, 2,4-DCP is frequently found in higher concentrations than those established by regulatory organisms which could modify the biota of the environment and produce several toxic effects in most of the living organisms.

Physical, chemical and biological methods, including incineration, carbon adsorption, chemical or enzymatic oxidation, solvent extraction, microbial degradation, and phytoremediation have been proposed for removal or degradation of several chlorophenols from wastewaters [8]. The use of plants to absorb, stabilize and degrade contaminants is gaining acceptance as a more cost-effective alternative to other cleanup approaches. In particular, hairy root (HR) cultures have potential to become a powerful tool for studying the phytoremediation capacity of plants and they have been used as good alternatives for efficient phenol and 2,4-DCP removal treatment [9,10]. These cultures produce peroxidase isoenzymes, which may be useful to remove phenol compounds

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from aqueous solutions. These enzymes catalyze the oxidation of aromatic compounds using hydrogen peroxide as substrate with the consequent formation of highly reactive radical species. Once formed, these unstable radicals react with another to yield phenolic polymers. These polymeric products have limited water solubility and tend to precipitate [11]. So, insolubilization of halogenated phenols is considered interesting since it allows their removal, reduces their bioavailability and thus their negative environmental impact [12].

Therefore, the specific objectives of this research were to obtain and to study tobacco HR as a potential system to efficiently remove 2,4-DCP as well as to elucidate the metabolic transformation of this contaminant and the localization of at least some of the formed product.

2. Materials and methods

2.1. Plant material

Hairy root (HR) cultures derived from tobacco (*Nicotiana tabacum* cv. W*isconsin*) sterile explants were obtained through *Agrobacterium rhizogenes* LBA 9402 infection (Fig. 1) as it was described by Sosa Alderete et al. [13]. They were grown in Murashige Skoog (MS) medium [14] with 3% (w/v) sucrose and enriched with vitamins [15] with agitation at 100 rpm in dark at $25 \pm 2\,^{\circ}C$. They were subcultured every three weeks into fresh MS medium. Only one clone of these hairy root cultures was selected for further studies based in its high growth, morphological stability, the presence of *rol C* gene (Fig. 1C), a marker of the effective transformation event [16], and high peroxidase activity (data not shown), properties which were opportunely studied in our laboratory with this objective. Twenty eight-day-old hairy roots were used as plant material for 2,4-DCP removal assays and further studies.

2.2. Analysis of tobacco HR growth

The evolution of tobacco hairy root growth was followed during 35 d. Inoculum of 0.2 ± 0.05 g of hairy root tissues were incubated in 50 mL of MS liquid medium and harvested each 7 d (7, 14, 21, 28 and 35 d). The HR growth was determined by measurements of fresh weight and expressed as g/L. Growth index (GI = final fresh weight/initial fresh weight) was also calculated.

2.3. Peroxidase activity determination

Total peroxidase activity was determined in HR tissues during growth. For this purpose, 0.2 g of root tissues 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 5000 *g* for 10 min and crude extracts obtained were considered as total peroxidases (TPx). Peroxidase activity was determined using 0.63 mM *o*-dianisidine and 0.5 mM H_2O_2 as substrates in 1 mL reaction mixture, as previously described [17]. The activity was measured following the increase in $A_{460\,nm}$ at 35 °C due to the appearance of the coloured reaction product ($\varepsilon_{460\,nm}$: $11.3\,mM^{-1}\,cm^{-1}$). Spectrophotometric assays were carried out with a Beckman 640 spectrophotometer. One unit of enzyme (IU) was defined as the amount of enzyme, which generated 1 μmol of product in 1 min in the previously described conditions.

2.4. Study of 2,4-DCP removal process

2.4.1. Effect of 2,4-DCP concentration on removal efficiency

Root inoculums of 0.4 g from 28 day-old cultures were incubated with 10 mL of solutions containing different 2,4-DCP concentrations: 250, 500 and 1000 mg/L. The required volume of a H_2O_2 stock solution was added to reach the desirable concentration (5, 10 and 20 mM for each 2,4-DCP concentration, respectively). These 2,4-DCP/H_2O_2 ratios were selected based on previous studies performed in our laboratory with other plant species.

Different controls were performed to estimate enzymatic or non-enzymatic mechanisms of 2,4-DCP removal. A 2,4-DCP solution (500 mg/L) without H_2O_2 was incubated under described conditions to evaluate auto-oxidation and/or evaporation, while a solution of 2,4-DCP (500 mg/L) with the addition of H_2O_2 (10 mM) was analysed to evaluate the possible reaction between both substrates. When 0.4 g of HR were incubated with 2,4-DCP solution without H_2O_2 we analysed the oxidation of this compound by peroxidases with endogenous H_2O_2 as well as the participation of other phenol oxidases like laccases and/or tyrosinases in the process. With previous treatment of root tissues with catalase (0.1 mg/L) (an enzyme which converts H_2O_2 to O_2) during 15 min, we evaluated

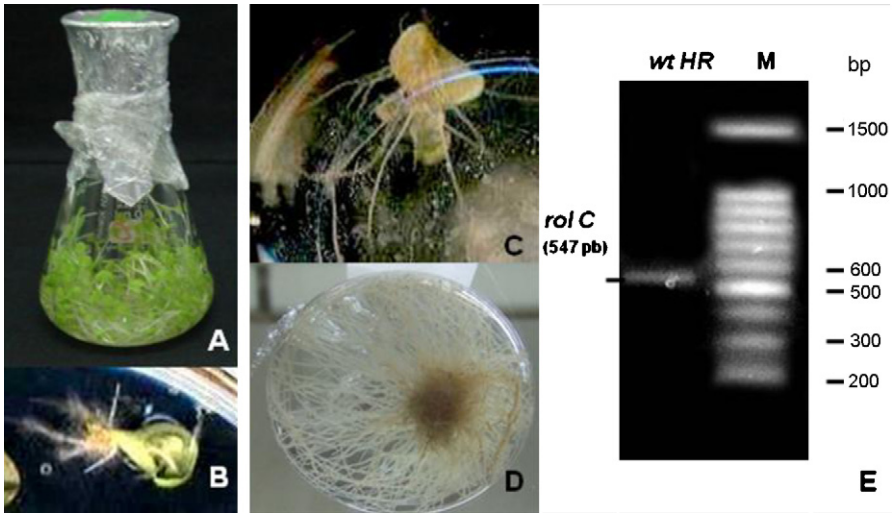

Fig. 1. (A) Sterile tobacco plants grown *in vitro* and used as explants source for *A. rhizogenes* infection. (B) A transformed leaf explant after 10 days from inoculation with bacteria grown in MS solid medium with ampicilin. (C) First passage to MS liquid medium from a transformed HR clone. (D) Tobacco HR culture after 15 days in MS liquid medium. (E) PCR analysis of a *rol C* segment of a tobacco HR clone used for further studies. M, 100 bp DNA ladder molecular weight marker (Promega).

the contribution of endogenous H_2O_2 on 2,4-DCP removal. Other controls were those with autoclaved HR (20 min, 121 °C, 1 atm), with and without H_2O_2, to estimate physical mechanisms of 2,4-DCP elimination.

Removal assays were carried out in darkness at 25 ± 2 °C with agitation (100 rpm), during 1 h for all the studies described below. Residual 2,4-DCP was determined in all removal assays as is described later.

2.4.2. Optimal conditions determination

From the different 2,4-DCP concentrations, previously analysed, we selected 500 mg/L for further studies. The minimum time required to reach the highest removal efficiency, as well as, the optimal H_2O_2 concentration were analysed. The 2,4-DCP removal efficiency was checked through determination of residual 2,4-DCP at 30 min, 1 h and 2 h with different H_2O_2 concentrations such as 1, 10, 20 and 30 mM.

2.4.3. Re-use of HR for consecutive removal assays

An inoculum of 0.4 g of fresh HR was incubated with 500 mg/L 2,4-DCP solution and 10 mM H_2O_2 for 1 h. Then, the post-removal solution was kept for residual 2,4-DCP quantification and replaced by a new 2,4-DCP/H_2O_2 mixture to repeat this procedure every 1 h, for four cycles consecutively. Peroxidase activity of HR was determined before removal and after the end of the fifth cycle.

2.4.4. Study of 2,4-DCP transformation

We determined Cl^- ions concentration from reaction mixture containing 2,4-DCP, HR and H_2O_2 and from the following controls: HR plus H_2O, HR plus H_2O_2 and HR plus 2,4-DCP. These samples were incubated during 24 and 72 h using 500 mg/L 2,4-DCP and 10 mM H_2O_2. The quantification of Cl^- ions was made using a commercial kit RANDOX Laboratories Ltd. from Wiener laboratory (Rosario, Santa Fé, Argentina). The determination is based on the reaction of Cl^- ions with a Hg-TPTZ (mercury-2,4,6-tri-(-2-piridyl)-1,3,5-triazine) complex. This reaction produces TPTZ that reacts with Fe(II) to form a blue Fe–TPTZ complex which is measured at 603 nm after 10 min of incubation. The pH value was measured, in the mentioned removal assays, using a digital *Fisher Scientific Accumet Basic AB15* pHmeter.

2.4.5. Evaluation of HR cell wall structure after 2,4-DCP removal

In order to evaluate *in situ* modifications of HR cell wall, we stained HR fragments using phloroglucinol (1% w/v)–HCl technique [18]. This staining allows us to evidence aldehyde groups of lignin and suberin [19]. After removal assays with 500 mg/L and 10 mM H_2O_2 during 24 and 72 h, 5 cm HR samples were analysed and compared with the following controls: HR/H_2O and HR/2,4-DCP. After the staining, root segments were observed with a Carl Zeiss Axiophot microscope and microphotographs were taken with a digital camera.

2.5. Residual 2,4-DCP determination

Residual 2,4-DCP was quantified through a colorimetric assay [20] as follow. Aliquots of 5 mL of each sample were mixed with: 25 μL of 4-aminoantypirine (2% w/v), 25 μL of 6 M NH_4OH and 50 μL of potassium ferricyanide (8% w/v). After 5 min, 2.5 mL of chloroform was added to recover the remaining coloured 2,4-DCP and the absorbance was determined at 510 nm. The results were expressed as removal efficiency, which was defined as the percentage of the pollutant removed from solution under pre-established experimental conditions.

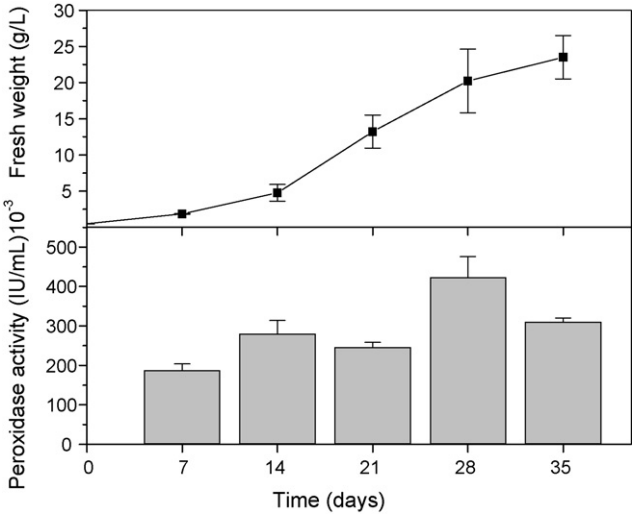

Fig. 2. (A) Evolution of tobacco HR growth during 35 days. (B) Peroxidase activity during the time of culture of tobacco HR.

2.6. Statistical analysis

In all experiments three replicates were performed for each sample. Results are presented with the mean and the standard error. Statistical analysis was carried out using Duncan' multiple range tests ($P < 0.05$) by using the STATISTICA (version 6.0) software.

3. Results and discussion

3.1. Growth performance and peroxidase activity determination of tobacco HR cultures

Since HR obtained by each transformed plant cell is expected to be genetically different due to the specific insertion of T-DNA [21] each HR culture can behave differently in growth as well as metabolite production. Therefore, the evaluation of different HR properties such as biomass yield and peroxidase production represents important aspects to be considered in 2,4-DCP phytoremediation process. So, in order to determine the required time to reach the maximum growth of HR, we analysed the evolution of growth for 35 d. As it can be seen in Fig. 2A these HR showed a characteristic sigmoidal growth curve with a lag phase during the first 7 d and followed by an increase of growth until the 28th d. Then, HR reached a stationary phase of growth with a final fresh weight of 23 g/L and a growth index of 4.6. Similar growth performance has been shown for tomato HR [17], *Panax ginseng* [22] and *Beta vulgaris* L. HR cultures [23]. The biomass accumulation of HR cultures studied in the present work was similar to that obtained for almost red beet HR clones grown in the same culture medium except one of them, which only showed a final fresh weight of 2.0 g [23]. Since peroxidases have been associated with phenolic compounds degradation [15,24] is useful to establish the levels of these enzymes during growth in order to select the better time to collect the roots for phytoremediation assays. So, peroxidase activity of these HR cultures was determined during 35 d. Peroxidase activity was variable during the time of growth showing a continues increasing until 28th d and declining further at the exponential phase of growth (Fig. 2B). High levels of peroxidase activity, coincident with the exponential phase of growth, were also observed for *Armoracia lapathifolia* HR [25] and *Beta vulgaris* HR [23].

We considered that HR cultures of approximately 28 d of growth could be used for further phytoremediation studies since biomass

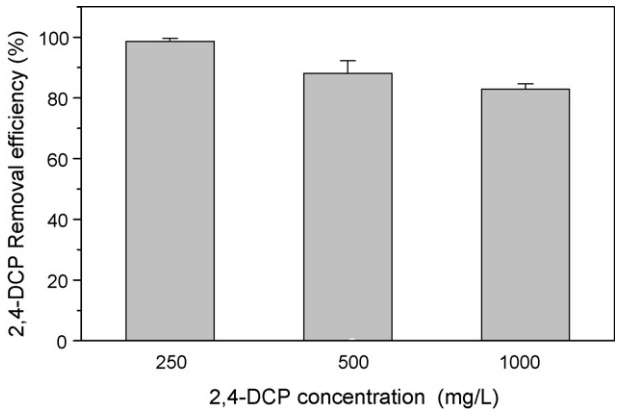

Fig. 3. Removal efficiencies of different 2,4-DCP concentrations (250, 500 and 1000 mg/L) by tobacco HR.

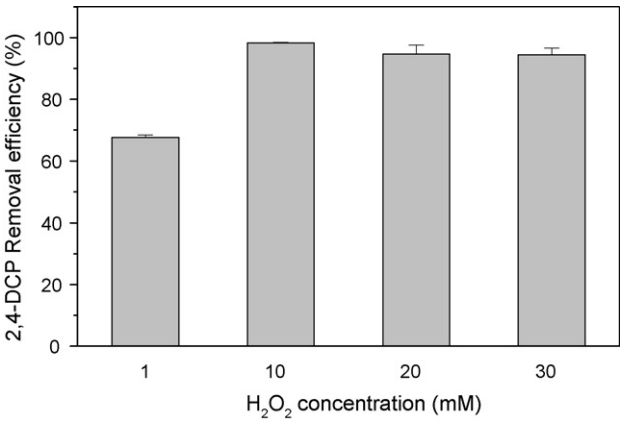

Fig. 4. Comparison of removal efficiencies of tobacco HR incubated with 2,4-DCP (500 mg/L) and different H_2O_2 concentrations during 1 h treatments.

as well as peroxidase activity were both high at this time of growth.

3.2. Study of 2,4-DCP removal process

3.2.1. Effect of 2,4-DCP concentration on removal efficiency

Some reports have shown that HR cultures of different plant species constitute appropriate systems to remove phenol and their derivatives [9,10,15,24,26]. In particular, the efficiency of tobacco HR to remove 100 mg/L of phenol has been studied comparing *wild type* cultures and other ones which expressed foreign basic peroxidases (*tpx1* and/or *tpx2*). These transgenic HR showed higher efficiency in the removal process [13]. Nevertheless, there are no reports if HR cultures of tobacco are useful and efficient for the removal of the halogenated derivative, 2,4-DCP. So, we studied the efficiency of this culture to remove 2,4-DCP solutions with different concentrations (250, 500 and 1000 mg/L), which correspond with those frequently found in waste waters (10–1000 mg/L) [27]. As it is shown in Fig. 3, the removal efficiencies were high under selected conditions (0.4 g HR inoculums, 5, 10 or 20 mM H_2O_2, 25 °C, 1 h) for all 2,4-DCP concentrations assayed and they were 98%, 88% and 83% for solutions initially containing 250, 500 and 1000 mg/L, respectively. So, even though we increased 2,4-DCP concentration until 1000 mg/L the removal efficiency reached for this assays was high. These results showed the high capability of tobacco HR to remove this halogenated compound with high efficiency and in a short time. Similar removal efficiencies for 2,4-DCP were observed by Agostini et al. [15] using *Brassica napus* HR. On the other hand, another halogenated phenol 2,6-DCP (50 mg/L) was also removed efficiently by *Daucus carota, Ipomoea batatas* and *Solanum aviculare* HR with efficiencies of 83%, 57.7% and 73.1%, respectively, although these assays were carried out during 72 h and without exogenous H_2O_2 [24]. In contrast, other plant system such as willow trees were not a good option for 2,4-DCP phytoremediation since the toxicity to these trees was high and the removal efficiency was limited even for low concentrations of this phenolic compound (5 and 150 mg/L for hydroponic solution and between 9.1 and 44.4 mg/L in sand) [28].

It should be noted that many papers reported the use of purified enzymes (peroxidases, laccases) for 2,4-DCP degradation. However, large amounts of enzyme are required as a consequence of enzyme inactivation, thus limiting its use [29]. In this way, HR could be useful tools in phytoremediation process not only for the high efficiency shown for the removal of phenolic compounds but also because the root *per se* acts as matrix for enzyme immobilization and it has a protective effect. In this context, it is necessary to study different plant systems because removal efficiencies are variable depending not only on the different toxicity of xenobiotics as well as on the removal capabilities of different HR or plant species.

In order to analyse enzymatic or non-enzymatic mechanisms involved in 2,4-DCP removal process, different kinds of controls were performed. So, 2,4-DCP removal cannot be attributed to chemical effect or interaction between H_2O_2 and 2,4-DCP since there was not removal when only H_2O_2 was added to 2,4-DCP solution. In addition, only 18% of removal was detected if the roots were incubated with 2,4-DCP without H_2O_2, which reflected the oxidation of 2,4-DCP by endogenous H_2O_2 and/or the involvement of other phenoloxidases non-H_2O_2 dependent [30]. Through the use of autoclaved tobacco HR and the consequent inactivation of biologic components, which could be involved in the removal process, we detected a small percentage of 2,4-DCP removal (23%) with or without H_2O_2. This removal could be associated to the adsorption of the pollutant on root surfaces, as was described by Dec and Bollag [30]. When HR were previously treated with catalase and without exogenous H_2O_2 the same removal values could be seen so, the contribution of endogenous H_2O_2 would be depreciable. Thus, as it could be checked by these different controls, the transformation of 2,4-DCP could be mainly associated with peroxidases participation since when we incubated HR with 2,4-DCP and H_2O_2 the removal was high, 88%.

These control assays and the previous results show the important requirement of H_2O_2 to gain efficiency. The H_2O_2 addition reduces removal time, besides it shows the specific involvement of peroxidases in the process, since it is an essential substrate for these enzyme activity [24,31].

3.2.2. Optimal conditions for removal process

Is frequent the study and looking for optimal conditions (inoculum size, time, substrate concentrations and substrate ratio) for a determined phytoremediation process [9]. Hence, we studied two important parameters to be considered in the removal of 500 mg/L 2,4-DCP, such as H_2O_2 concentrations and time required to reach high removal efficiency. From a biotechnological point of view, the requirement of one essential substrate as H_2O_2, not only influences removal efficiency but also, the final cost of the process mainly at large-scale so, it is an important aspect to be studied.

The removal of 500 mg/L 2,4-DCP with tobacco HR and 10 mM H_2O_2 was followed for 30, 60 and 120 min. We observed high removal efficiency (84.4%) at 30 min, although the removal efficiency increased approximately 5% with longer time, reaching the highest efficiency at 60 min (data not shown).

It is important to point out that 10 mM was optimal to reach maximum removal efficiency, while 20 and 30 mM produced a slow diminution in the removal process (Fig. 4). As it was shown, low

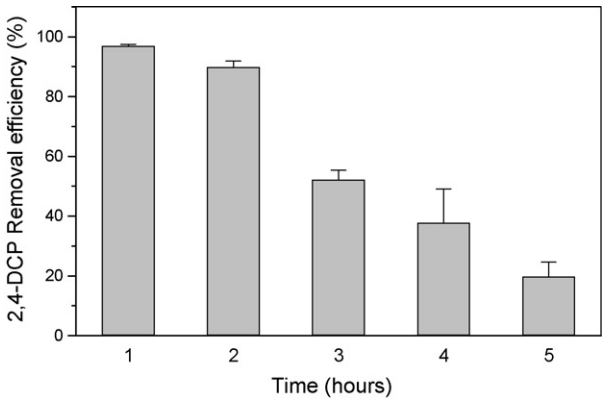

Fig. 5. Re-use of HR tissues for consecutive cycles of 2,4-DCP (500 mg/L) removal.

H_2O_2 concentration could be insufficient to give adequate quantity of substrate for peroxidases; however, high H_2O_2 concentration could produce suicide inactivation of peroxidases with the consequent formation of inactive intermediates [32] and could result in lower removal efficiency. On the other hand, 5 mM of H_2O_2 was needed to reach maximum phenol removal using tomato HR [9] while Park et al. [33] found that 20 mM H_2O_2 was needed, for an efficient removal of 2,4-DCP (500 mg/Kg) with *Capsella bursa-pastoris* L. roots extracts. Therefore, H_2O_2 requirement for different removal process depends on the nature and concentration of the contaminant as well as the plant species used.

3.2.3. Re-use of HR for consecutive removal assays

In order to evaluate the possibility of re-using tobacco HR in a continuous process, consecutive cycles of removal were performed (Fig. 5). The highest important removal values were obtained for the first and second hour. For the following cycles the removal efficiencies were low until to reach just a 20% in the fifth cycle. This behaviour was probably associated with the inactivation of peroxidases, which are considered the main enzymes involved in the removal process, since peroxidase activity diminished approximately 80% after the first cycle. As it was postulated by several authors enzymatic inactivation could be due to different mechanisms: irreversible interaction of free radicals with the active site of the enzyme [20], final product (polymer) adsorption to cell wall [30] and diverse reactions produced by H_2O_2 and/or other intermediates of the catalytic cycle [32].

Similar results, with high removal efficiencies of 2,4-DCP during 1 h consecutive cycles, were obtained using *B. napus* HR, although for low concentrations (100 mg/L) [15]. Another study of re-use of tissue for a phenolic compound like phenol (200 mg/L) was carried out by Singh et al. [26] with *Brassica juncea* HR but without H_2O_2 addition. In this work, the removal percentages were high during four cycles; however, each cycle takes 72 h of incubation. These results showed the importance of exogenous H_2O_2 addition to develop a fast and efficient removal process because of the scarce H_2O_2 content or production in tobacco HR.

3.2.4. Study of 2,4-DCP transformation

The first evidence of 2,4-DCP transformation was the change in pH after 24 and 72 h treatments in post-removal solutions (Fig. 6A). The pH of control mixtures (HR/H_2O_2 and HR/H_2O) showed near neutral values (6.75–6.9), while in the control assay HR/2,4-DCP, pH was 5.5, a little minor than those previously mentioned controls due to the acidic nature of 2,4-DCP solution (pH 5). However, a notable diminution in the pH of removal solution with HR plus 2,4-DCP and H_2O_2 was observed, reaching values of approximately 3.75 at 24 h and 72 h. This fact could be associated with the enzymatic

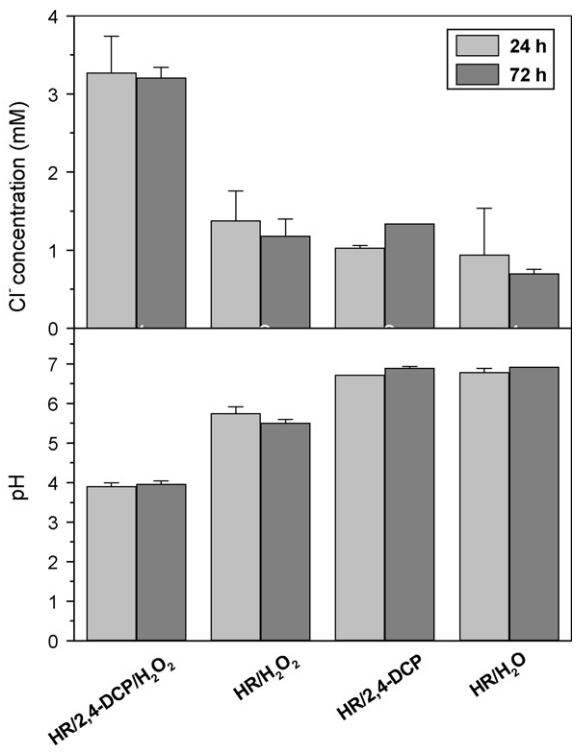

Fig. 6. (A) pH values of post-removal reaction mixtures of different assays after 24 and 72 h using 2,4-DCP (500 mg/L). (B) Chloride ions concentration in the medium of different removal assays and their respective controls after 24 and 72 h.

transformation of 2,4-DCP, which could indicate the involvement of peroxidase enzymes in 2,4-DCP transformation and the consequent chloride ions liberation from this halogenated compound.

On the other hand, while in the reaction medium of control assays, the Cl^- concentration varied from 0.7 to 1.4 mM, in the removal assay with HR plus 2,4-DCP and H_2O_2 the Cl^- ions increased until 3.27 mM at 24 h and 72 h of treatment (Fig. 6B). The detection of an increase of Cl^- ions in the removal solution, suggested that, as a consequence of 2,4-DCP transformation, the formation of HCl could produced a change in pH.

In this context we can suggest that during the transformation of this halogenated compound the loss of Cl^- involves the participation of peroxidases, since in control assays no increase in Cl^- concentrations was observed. As it was demonstrated by Dec et al. [34], the basic peroxidase isoenzyme of horseradish (HRP) produces different free radicals from 2,4-DCP which are then coupled under different combinations. This coupling produces several kinds of dimmers with specific combinations and the consequent loss of one or two Cl^- atoms, only if the free electron is placed just in the aromatic carbon with chlorine atom [35]. So, the chloride substituent release was the result of an oxidative coupling reaction [36] and it constitutes an initial step leading to further decomposition of organic compounds. Dehalogenation of chlorinated phenols was also shown using fungi peroxidases [35] or other oxido-reductases [37]. So, with these findings in mind we could establish that dehalogenation process was underwent during 2,4-DCP removal assays using plant tissues with high peroxidase activity like tobacco HR. Peroxidases participate in phenolic substrate oxidation with the consequent unstable free radical or quinones generation which form spontaneous dimmers first, then trimmers, tetramers and eventually polymers later [8,36]. These polymers are not soluble in water and they precipitate in water phase, so it is considered useful and interesting for the removal of toxic compounds from the environment [36].

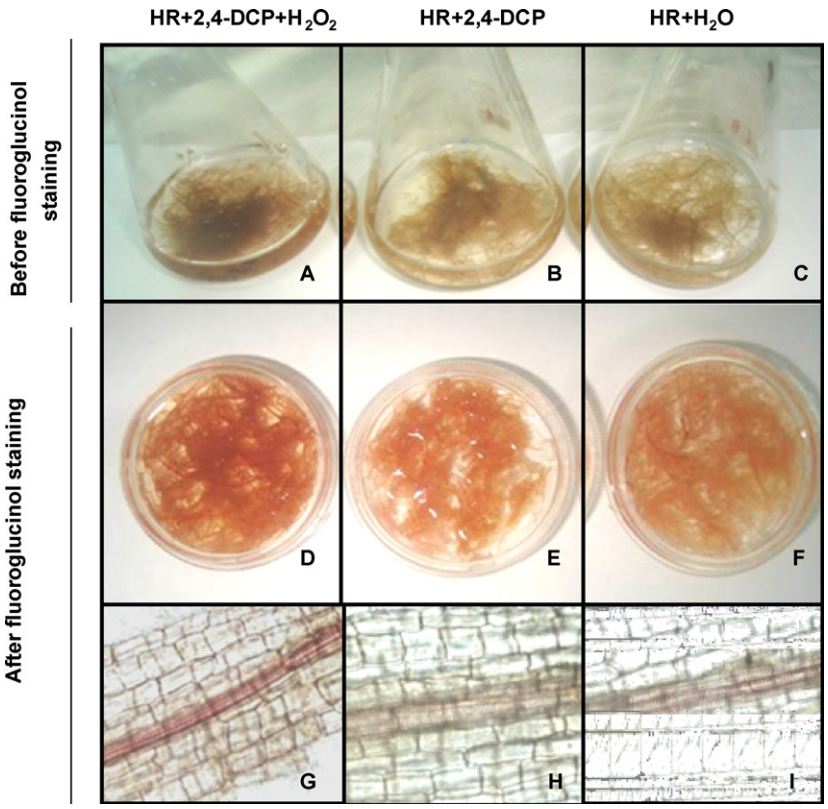

Fig. 7. Macroscopic and microscopic photographs from HR after 2,4-DCP removal during 24 h and HR of control assays, before (A, B, C) and after phloroglucinol staining (D, E, F, G, H, I).

3.2.5. Evaluation of HR cell wall structure after 2,4-DCP removal

We evaluated *in situ* modifications of tobacco HR produced by their exposition to 2,4-DCP solutions. So, we used an histochemical technique in order to find possible changes produced in the roots during 2,4-DCP removal.

In Fig. 7, roots of different 24 h assays before (Fig 7A–C) and after (Fig 7D–F) the phloroglucinol–HCl staining are shown. After 24 h of 2,4-DCP removal assays (Fig. 7A) we could see a browning of HR surface which could indicate through an indirect manner, the possible adsorption of some products on root surface. This surface browning was also reported by Wevar Oller et al. [38] during phenol removal by tomato HR. This phenomenon was probably due to the adsorption of products derived from 2,4-DCP transformation in root surface, since a common feature of the metabolism of phenolic compounds in soil and plants is the formation of inextricable residues.

Moreover, the red colour which is characteristic of phloroglucinol–HCl staining, appeared immediately and it was more intense in HR after 2,4-DCP removal assays with the addition of H_2O_2. However, some red colour was also appreciable in HR of control assay (HR/2,4-DCP, HR/H_2O) (Fig. 7E and F) which could be related with polymer formation through the participation of other phenol oxidases, as well as, the own and natural content of lignin and suberin of roots.

Microscope observations of longitudinal slices of roots were done to visualize possible changes in root cells (Fig. 7G, H and I). Those HR slices from HR/2,4-DCP/H_2O_2 treatment showed higher red colour intensity in comparison with HR of control treatments. These stained lignin and/or suberin like deposits were associated with cell walls of xylem and phloem elements in the vascular cylinder. Naturally, lignin is present in xylem vessels but frequently the deposition pattern in roots changes in response to salinity, heavy metals and nutrient stresses, as a strategy to limit the incorporation of harmful molecules [39] (and references there in). As it was shown in Talano et al. [40] the use of phloroglucinol–HCl technique have allowed demonstrating changes in lignosuberization pattern of tomato HR in response to NaCl treatment. The exposition of HR to high 2,4-DCP concentrations could be considered as an stressful condition for this plant tissue. In addition, this phenolic compound is oxidized by peroxidases and other oxidases (probably laccases) first, and some of its products could be then polymerized forming lignin/suberin as it could be seen in this work through the changes observed in lignosuberization pattern. So, the change in the deposition pattern of lignin in cell walls, in response to 2,4-DCP treatment, is at least an approximation about the *in situ* localization and nature of some of the formed products. Similarly, Sorroche [41] have shown that p-coumaric, cafeic and ferulic acids, natural phenolic compounds from plant cells, were metabolized by *B. napus* HR and transformed in lignin/suberin polymers which were deposited preferentially in xylem cell walls. The chemical similarity between 2,4-DCP and the natural phenolic compounds (monolignols) from plant tissues, which are the source for synthesis of lignin/suberin, suggest that the structural changes observed in xylem cell walls would be related with the removal of 2,4-DCP from aqueous solution. Many attempts have been made to elucidate the final fate of 2,4-DCP and/or the final products of its metabolism. Ensley et al. [42] identified metabolites less toxic for plants such as β-glycosides and phenyl-glycosides as intermediate products of phenol and 2,4-DCP transformation by *Lemna gibba in vitro* cultures. On the other hand, Santos de Araujo et al. [24], using radioactive C^{14}2,4-DCP, suggested that 2,4-DCP is incorporated by HR of different plant species, then dehalogenated and finally transformed in high polar conjugated molecules possibly glycosides. These steps have been pointed out in the "*green-liver*" model (transformation, conjugation and elimination or storage), which is based on metabolic processes by which plants can detoxify or store organic compounds that

enter tissues [43]. In this context, peroxidases have been postulated as an important enzyme family with significant role in the transformation step of the *green-liver* model. After the conjugation step, with malonic acid, D-glucose, glutation, cysteine, as the constituents more frequently found, water soluble compounds and with reduced toxicity are obtained [44]. Glycosidation, a specific type of conjugation frequently associated with chlorinated phenols transformation, catalysed by o-glycosiltransferase enzymes results in soluble glycosides that can be stored in vacuoles and/or in the apoplast or incorporated through covalent union into lignin from cell walls, a phenomenon that is called sequestration or compartmentalization. However, many times the study of localization of the product has failed because the bound residues formed from phenol compounds transformation are not extractable substances and they are strongly associated with lignin, which is not available for further analysis [44]. So, here, we present an evidence about the nature and localization of some of the products of 2,4-DCP transformation in tobacco HR.

4. Conclusion

This study has shown the great efficiency of tobacco HR to remove high concentrations of 2,4-DCP and it has contributed to show the lignin-type nature of some products formed from 2,4-DCP transformation, as well as, its compartmentalization in HR cell walls. However, as it was reported by many authors, a complicated mixture of intermediate products would be formed (dimmers, trimmers and oligomers) from 2,4-DCP transformation. For these reason, toxicity tests are likely to be needed to confirm the detoxifying effect of chemical oxidation of chlorophenols. Now, studies related with the analysis of post-removal solution toxicity are undergoing in our laboratory, in order to consider the future application of this technology for wastewater treatments.

2,4-DCP removal process with this plant system would be possible in the future since recent progress are being developed in the construction of new types of bioreactors adapted to root growth. However, also, scaling up HR to industrial levels constitutes a great challenge at the moment. Therefore, in the near future the HR technology might be used more commonly in biotechnological process such as phytoremediation [45]. In this context, the high efficiency shown by tobacco HR for the removal of 2,4-DCP at high concentrations, even higher than those frequently found in sites of discharge of many industries, would make this system a useful strategy for phytoremediation of 2,4-DCP. In this context, the interest of private companies, such as German Company ROOTec in the development of large-scale HR cultures, would probably give an advance in the use of HR to clean up industrial effluents.

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References

[1] http://www.atsdr.cdc.gov/cercla/05list.html, ATSDR, Agency for Toxic Substances and Disease Registry, 2005.
[2] J.F. Borzelleca, J.R. Hayes, L.W. Condie, J.L. Egle, Acute and subchronic toxicity of 2,4-dichlorophenol in CD-1 mice, Toxicol. Sci. 5 (1985) 478–486.
[3] http://www.epa.gov/opptintr/24dcp.htm, EPA/OSHA, Advisory on 2,4-DCP. Chemical advisory and notice of potential risk:skin exposure to 2,4-DCP can cause rapid death, 2000.
[4] World Health Organization (WHO), Chlorophenols in drinking water. 2003, Ginebra.(Suiza).
[5] http://www.aguamarket.com.
[6] D. Petroutsos, P. Katapodis, M. Samiotaki, G. Panayotou, D. Kekos, Detoxification of 2,4-dichlorophenol by the marine microalga *Tetraselmis marina*, Phytochem 69 (2008) 707–714.
[7] http://www.cecopal.org/derecho/DNacional/DN-831-93.htm, Ley Nacional Argentina No. 24051-Decreto 831/93. Reglamentación de Residuos Peligrosos.
[8] E. Laurenti, E. Ghibaudi, S. Ardissone, R.P. Ferrari, Oxidation of 2,4-dichlorophenol catalysed by horseradish peroxidase: characterization of the reaction mechanism by UV–visible spectroscopy and mass spectrometry, Inorg. Biochem. 96 (2003) 171–176.
[9] P.S. González, C. Capozucca, H. Tigier, S. Milrad, E. Agostini, Phytoremediation of phenol from wastewater by peroxidases of tomato hairy root cultures, Enzyme Microb. Technol. 39 (2006) 647–653.
[10] M.S. Coniglio, V.D. Busto, P.S. González, M.I. Medina, S. Milrad, E. Agostini, Application of *Brassica napus* hairy root cultures for phenol removal from aqueous solutions, Chemosphere 72 (2008) 1035–1042.
[11] G. Zhang, J.A. Nicell, Treatment of aqueous pentachlorophenol by horseradish peroxidase and hydrogen peroxide, Water Res. 34 (2000) 1629–1637.
[12] A. Longoria, R. Tinoco, R. Vázquez-Duhalt, Chloroperoxidases-mediated transformation of highly halogenated monoaromatic compounds, Chemosphere 72 (2008) 485–490.
[13] L.G. Sosa Alderete, M.A. Talano, S.G. Ibáñez, S. Purro, E. Agostini, S.R. Milrad, M.I. Medina, Establishment of transgenic tobacco hairy roots expressing basic peroxidases and its application for phenol removal, J. Biotechnol. 139 (2009) 273–279.
[14] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Plant Physiol. 15 (1962) 473–479.
[15] E. Agostini, M.S. Coniglio, S. Milrad, H. Tigier, A. Giulietti, Phytoremediation of 2,4-dichlorophenol by *Brassica napus* hairy roots cultures, Biotechnol. Appl. Biochem. 37 (2003) 139–144.
[16] V. Veena, C.G. Taylor, *Agrobacterium rhizogenes*: recent developments and promising applications, In Vitro Cell. Dev. Biol. Plant 43 (2007) 383–403.
[17] M.A. Talano, E. Agostini, M.I. Medina, S. Milrad de Forchetti, H.A. Tigier, Tomato *(Lycopersicon esculentum* cv. Pera) hairy root cultures: characterization and changes in peroxidase activity under NaCl treatment, In vitro Cell. Dev. Biol. Plant 39 (2003) 354–359.
[18] A. D'ambrogio de Argüeso, Manual de técnicas en Histología Vegetal. Ed Hemisferio Sur S.A., Buenos Aires, Argentina 1986, pp. 83–90.
[19] R.P. Baayen, G.B. Oullette, D. Rioux, Compartmentalization of decay in carnations resistant to *Fusarium oxysporium* f sp. Dianthi, Phytopatology 86 (1996) 1018–1031.
[20] A.M. Klibanov, B.N. Alberti, E.D. Morris, L.M. Felshin, Enzymatic removal of toxic phenol and anilines from waste waters, Appl. Biochem. 2 (1980) 414–421.
[21] P.M. Doran, Properties and applications of hairy root cultures, in: K.M. Oksman-Caldenty, W.H. Brarz (Eds.), Plant Biotechnology and Transgenic Plants, Marcel Dekker Inc, New York, 2002, pp. 143–162.
[22] G. Sivakumar, K.W. Yu, E.J. Hahn, K.Y. Paek, Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors, Curr. Sci. 89 (2005) 641–649.
[23] R. Thimmaraju, L. Venkatachalam, N. Bhagyalakshmi, Morphometric and biochemical characterization of red beet (*Beta vulgaris* L.) hairy roots obtained after single and double transformations, Plant Cell Rep. 27 (2008) 1039–1052.
[24] B. Santos de Araujo, J. Dec, J.M. Bollag, M. Pletsch, Uptake and transformation of phenol and chlorophenols by hairy root cultures of *Daucus carota, Ipomoea batatas* and *Solanum aviculare*, Chemosphere 63 (2006) 642–651.
[25] C.G. Flocco, M.A. Alvarez, A.M. Giulietti, Peroxides production *in vitro* by *Armoracia lapathifolia* (horseradish)-transformed root cultures: effect of elicitation on level and profile of isoenzymes, Biotechnol. Appl. Biochem. 28 (1998) 33–38.
[26] S. Singh, J.S. Melo, S. Eapen, S.F. D'Souza, Phenol removal using *Brassica juncea* hairy roots: role of inherent peroxidase and H_2O_2, J. Biotechnol. 123 (2006) 43–49.
[27] I. Buchanan, J.A. Nicell, Model development for horseradish peroxidase-catalyzed removal of aqueous phenol, Biotechnol. Bioeng. 54 (1997) 251–261.
[28] A.S. Ucisik, S. Trapp, K.O. Kusk, Uptake, accumulation, phytotoxicity, and removal of 2,4-dichlorophenol in willow trees, Environ. Toxicol. Chem. 26 (2007) 1165–1171.
[29] M. Hamid, K.-U. Rehman, Potential applications of peroxidases, Food Chem. 115 (2009) 1177–1186.
[30] J. Dec, J.M. Bollag, Dehalogenation of chlorinated phenols during oxidative coupling, Environ. Sci. Technol. 28 (1994) 484–490.
[31] M. Wagner, J.A. Nicell, Detoxification of phenolic solutions with horseradish peroxidase and hydrogen peroxide, Water Res. 36 (2002) 4041–4052.
[32] A.N.P. Hinner, J. Hernández-Ruiz, M.B. Arnao, F. García Canovas, M.A. Acosta, Comparative study of the purity, enzyme activity and inactivation by hydrogen peroxide of commercially available horseradish peroxidase isoenzyme A and C, Biotechnol. Bioeng. 50 (1996) 655–662.
[33] J.W. Park, B.K. Park, J.E. Kim, Remediation of soil contaminated with 2,4-dichlorophenol by treatment of minced Sherpherd's purse roots, Environ. Contam. Toxicol. 50 (2006) 191–195.
[34] J. Dec, K. Haider, J.M. Bollag, Decarboxylation and demethylation of naturally occurring phenols during coupling reactions and polymerization, Soil Sci. 166 (2001) 660–671.
[35] J.W. Park, J. Dec, J.E. Kim, J.M. Bollag, Transformation of chlorinated phenols and anilines in the presence of humic acid, J. Environ. Qual. 29 (2000) 214–220.

[36] J. Dec, K. Haider, J.M. Bollag, Release of substituens from phenolic compounds during oxidative coupling reactions, Chemosphere 52 (2003) 549–556.
[37] R.D. Minard, S. Minard, S.Y. Liu, J.M. Bollag, Oligomers and quinones from 2,4-dichlorophenol, J. Agric. Food Chem. 29 (1991) 250–253.
[38] A. Wevar Oller, E. Agostini, M. Talano, C. Capozucca, S. Milrad, H.A. Tigier, M.I. Medina, Overexpression of a basic peroxidase in transgenic tomato (*Lycopersicon esculentum* Mill. cv. Pera) hairy roots increases phytoremediation of phenol, Plant Sci. 169 (2005) 1102–1111.
[39] E. Hose, D.T. Clarkson, E. Steudle, L. Schreiber, W. Hartung, The exodermis: a variable apoplastic barrier, J. Exp. Bot. 52 (2001) 2245–2264.
[40] M.A. Talano, E. Agostini, M.I. Medina, H. Reinoso, M.C. Tordable, H.A. Tigier, S. Milrad de Forchetti, Changes in lignosuberization of cell walls of tomato hairy roots produced by salt treatment: the relationship with the release of a basic peroxidase, J. Plant Physiol. 163 (2006) 740–749.
[41] F.G. Sorroche, Grade thesis: Biotransformation of phenylpropanoid compounds through the application of *Brassica napus* hairy root cultures, 2006.
[42] H.E. Ensley, H.A. Sharma, J.T. Barber, M.A. Polito, Metabolism of chlorinated phenol by Lemma gibba, in: E.L. Kruger, T.A. Anderson, J.R. Coats (Eds.), Phytoremediation of Soil and Water Contaminants, American Chemical Society, Washington, 1997, pp. 238–253.
[43] H.J. Sandermann, Higher plant metabolism of xenobiotics: the "green-liver" concept, Pharmacogenetics 4 (1994) 225–241.
[44] J.G. Burken, Uptake and metabolism of organic compounds: green-liver model, in: S.C. McCutcheon, J.L. Schnoor (Eds.), Phytoremediation: Transformation and Control of Contaminants, Wiley, New York, 2003, pp. 59–84.
[45] W. Suza, R.S. Harris, A. Lorence, Hairy roots: from high-value metabolite production to phytoremediation, Electron. J. Integr. Biosci. 3 (2009) 57–65.