

# Tolerance and metabolism of phenol and chloroderivatives by hairy root cultures of *Daucus carota* L.

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**“Capsule”:** *A root culture assay allows screening for tolerance and assesses the role of roots in metabolism.*

## Abstract

Hairy root cultures are shown to be suitable experimental systems to screen higher plants for tolerance to various inorganic and organic pollutants, and for determining the role of the root matrix in the uptake and further metabolism of contaminants. A number of clones were obtained by infection of carrot tissues with *Agrobacterium rhizogenes* and two (the fastest and the slowest growing root clones) were chosen for further experimentation. Both clones showed a similar degree of tolerance towards phenol and its chlorinated derivatives, i.e. the growth of root biomass was maintained in concentrations of phenol equivalent to 1000  $\mu\text{mol/l}$ , whilst the chlorophenols were tolerated only at concentrations 20 times lower (50  $\mu\text{mol/l}$ ). Transformed carrot roots were able to remove more than 90% of the exogenous phenolic compounds from the culture medium within 120 h after treatment. Metabolism of these compounds occurred in the root tissue and was accompanied by an increase in peroxidase activity. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Phenol; Chlorophenols; Hairy roots; *Daucus carota*; Phytoremediation

## 1. Introduction

Phenol and its chlorinated derivatives are used in the formulation of insecticides, herbicides and fungicides and can be found as toxic pollutants in industrial wastes. The use of these substances imposes severe risks to human health (Ensley et al., 1997) and is, therefore, a matter of public concern. In order to treat land contaminated by these compounds, chemical and biotechnological methods, such as solvent extraction and enzyme systems, are available. Whilst both approaches are very efficient, they suffer from the disadvantage of being expensive and give rise to toxic by-products (Klibanov et al., 1983; Maloney et al., 1986). In recent years, intact plants have been used for the remediation of areas contaminated by a variety of pollutants, including organic compounds (Cunningham et al., 1996). However, in order fully to exploit phytoremediation technology, there is a need for basic understanding of many aspects of xenobiotic metabolism, together with the screening of plants suitable for the remediation of specific compounds.

In this context, some valuable work has been performed regarding the degradation of nitroaromatic explosives by *Myriophyllum aquaticum* (Bhadra et al., 1999), polychlorinated biphenyls by *Armoracia rusticana*, *Atropa belladonna*, *Solanum aviculare* and *S. nigrum* (Mackova et al., 1997; Kucerova et al., 1999) and phenanthrene by *Helianthus annuus* and *Pisum sativum* (Liste and Alexander, 1999, 2000). These experiments show that the degradation of xenobiotics may take place either in the plant body, through its own enzymatic machinery, or in the rhizosphere where the enzymes produced by associated microorganisms play a major role in conjunction with the enzymes released by the roots.

In order to determine the levels of phenol and chlorophenols that could be tolerated by the roots of a model plant, we have used hairy root cultures which have previously proven to be excellent systems for investigations on the accumulation, biosynthesis and regulation of bioactive secondary metabolites (Caetano et al., 1999; Argôlo et al., 2000). The application of hairy roots to phytoremediation has been suggested by various authors (Metzger et al., 1992; Pletsch et al., 1999), mainly because of their biochemical resemblance to the roots of the plant from which they have derived. Furthermore, growth and maintenance of such cultures is a cheap and straightforward

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procedure which allows studies to be carried out without the interference of the microbiota.

The aims of the present work were: (1) to determine whether hairy roots of carrots (which are easily induced and normally contain high levels of peroxidase) actively take up, store and/or further metabolize phenol (P), 2-chlorophenol (CP), 2,6-dichlorophenol (DCP) and 2,4,6-trichlorophenol (TCP); (2) to study the effects of treatment of two individual hairy root clones with phenols; (3) to establish if there was a relationship between the dynamics of removal/metabolism of phenols by the plant roots and in situ peroxidase activity.

## 2. Materials and methods

### 2.1. Materials

Phenol, 2-chlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol and type I peroxidase (EC 1.11.1.7, 40 U/mg of solid) were purchased from Sigma (St. Louis, MO, USA). Chromatographic solvents (HPLC grade) were purchased from Grupo Química (Rio de Janeiro, Brazil). *Agrobacterium rhizogenes* LBA9402 was a gift from Dr. M.J.C. Rhodes (Institute of Food Research, Norwich, UK).

### 2.2. Induction and maintenance of hairy root cultures

*Agrobacterium rhizogenes* was inoculated into 5 ml of yeast mannitol broth medium (0.5 g/l  $K_2HPO_4$ , 0.2 g/l  $MgSO_4 \cdot 7H_2O$ , 0.1 g/l NaCl, 10 g/l mannitol, 0.4 g/l yeast extract, pH 7.0) and incubated for 48 h at  $25 \pm 1^\circ C$ , under constant agitation at 120 rpm. The suspension was centrifuged (4000 g; 5 min), the supernatant was discarded, the pellet was washed twice with sterile distilled water and finally resuspended in 50  $\mu$ l of sterile water. Roots of *Daucus carota* L. were washed thoroughly with tap water, scraped and sterilized with hypochlorite solution (1% of active chlorine). Carrot discs (3–5 mm thickness) were cut into sections (1  $cm^2$ ), each one containing the pericycle, and placed onto Petri dishes containing agar (1%). An aliquot of the bacterial suspension was spread over the surface of each fragment after which the plates were sealed and incubated in the dark at  $25 \pm 1^\circ C$ . After approximately 12 days, individual root tips (clones) were placed onto half strength Gamborg B5 medium (Gamborg et al., 1968), supplemented with sucrose (30 g/l), ampicillin (500 mg/l) and agar (10 g/l). Subcultures were carried out at 20 day intervals. The ampicillin was omitted from the growth medium following three subcultures.

### 2.3. PCR analysis

Proof of transformation, as well as confirmation that the roots were not contaminated with *Agrobacterium*,

was achieved by PCR following a strategy previously described (Argôlo et al., 2000), using primers for the amplification of segments of  $T_L$ -DNA and *vir* D1. PCR products were analyzed by electrophoresis on agarose gel (20 g/l) in TBE buffer (Sambrook et al., 1989) containing 2  $\mu$ l of ethidium bromide solution (500  $\mu$ g/ml).

### 2.4. Selection of carrot root clones

Thirty independent clones were chosen randomly and their growth indices were determined by culturing an inoculum of each clone onto the maintenance medium and determining the wet and dry weight after 30 days (stationary phase). The growth index (GI) was calculated using the formula  $GI = W_f/W_i$ , where  $W_f$  is the fresh weight of the culture after 30 days and  $W_i$  is the fresh weight of the inoculum (50 mg). The doubling time ( $T_D$ ) of each clonal culture was calculated using the formula  $T_D = \ln 2(t_1 - t_0)/\ln(GI)$ , where  $t_1 - t_0$  corresponds to the period between inoculation and the stationary phase. The clones which demonstrated the smallest and the largest GI and  $T_D$  values were selected for further experiments.

### 2.5. Tolerance towards phenols

Tolerance was defined in this experiment as the highest concentration of toxic substance which would allow normal culture growth, i.e. when the GI of the treated culture was not significantly ( $P = 0.05$ ) reduced compared with that of the control culture.

In order to determine the degree of tolerance of the cultures towards phenol and chlorophenols, the selected clones were inoculated individually (50 mg each) onto the maintenance medium which had been supplemented separately with aliquots of aqueous stock solutions of P, CP, DCP and TCP to give final concentrations of 50, 500, 1000, 2000 and 5000  $\mu$ mol/l. The cultures were incubated in the dark at  $25 \pm 1^\circ C$ . The GI of the treated cultures was determined after 30 days as described above and compared with that of non-treated control cultures.

### 2.6. Removal of phenols from the culture medium and their accumulation in roots

The concentration of phenols in the culture medium, as well as in the root tissue, of each selected clone was determined in liquid culture. Inoculation of roots (50 mg) was carried out in the maintenance medium (100 ml) contained in conical flasks (250 ml). The flasks were kept under constant agitation at 100 rpm under the conditions described earlier. Phenols were added separately to the medium 21 days after inoculation (middle exponential phase of growth). The initial concentrations were 1000  $\mu$ mol/l of P, and 50  $\mu$ mol/l of CP, DCP, and

TCP. Samples of the culture medium and root tissue were taken 24, 120 and 240 h after the treatment. The controls used were untreated root cultures, root cultures inactivated by heat (autoclave for 1 h) and the culture medium itself (without roots) to which were added P (1000  $\mu\text{mol/l}$ ) and TCP (50  $\mu\text{mol/l}$ ).

### 2.7. Extraction of phenols and HPLC analysis

The extraction and the quantitative analyses of phenols in the medium and in the roots were performed according to a modification of the method described by Frébortová (1995). Culture medium (10 ml) was extracted with chloroform (3 $\times$ 2 ml) and the bulked extract was re-extracted with NaOH 0.1 mol/l (3 $\times$ 2ml). The aqueous extract was kept in a water bath (70°C) until any residual chloroform had evaporated. The pH of the solution was adjusted to 5–6 with concentrated HCl. SepPak C18 mini-columns (Waters Corporation, Milford, USA), previously equilibrated with methanol and distilled water (2 $\times$ 2 ml each), were used to concentrate the phenols, which were desorbed from the columns with methanol (1.5 ml). Root samples (100 mg) were soaked in chloroform (6 ml) for 24 h, after which time they were macerated using a mortar and pestle. The mixture was centrifuged (7000 g; 10 min) and the supernatant was treated in the same manner as the culture medium. All extracts were filtered through 0.22  $\mu\text{m}$  filters (Sigma) and aliquots (25  $\mu\text{l}$ ) were analyzed by HPLC. The efficiency of the extraction method was 85% from liquid medium and roots.

The liquid chromatographic system used was a Waters isocratic pump model 515, attached to a Rheodyne injector, a model 486 UV/VIS detector and a model 746 integrator. The Spherisorb ODS2 column (250 $\times$ 4.6 mm i.d.; particle size 5  $\mu\text{m}$ ) and appropriate pre-column (50 $\times$ 4.6 mm i.d.) were purchased from Phase Separations Ltd (Deeside, UK). The mobile phase was a mixture of phosphoric acid (7 mmol/l) and acetonitrile in the proportion 70:30 (P and CP) and 54:46 (DCP and TCP). The flow rates were 1.2 ml/min (P), 1.5 ml/min (CP) and 1 ml/min (DCP and TCP). Detection was carried out at 225 nm. A calibration curve was constructed using the integrator values obtained from the quantification of standard solutions.

### 2.8. Peroxidase activity

Peroxidase activity was determined in the medium and root tissue using the guaiacol method described by Kim and Yoo (1996). The activity was measured before the addition of phenols to the culture medium and at 24, 120 and 240 h after addition. The controls were cultures of roots treated with sterilized distilled water. The calibration curve was constructed using peroxidase solutions in the concentration range of 0.08, 0.16, 0.24, 0.32

and 0.4 U/ml. The spectrophotometer used was a Perkin-Elmer UV/VIS Lambda 2 (Veberlingen, Germany)

## 3. Results

All experiments were carried out using two hairy root clones of *D. carota* (B6 and B28) which were selected from 30 different clones in respect of their significantly distinct ( $P < 0.001$ ) growth characteristics: clone B6 showed one of the largest GI values ( $76.56 \pm 6.99$ ;  $T_D = 4.79$  days) whilst clone B28 showed the smallest GI ( $6.29 \pm 2.23$ ;  $T_D = 11.3$  days).

Both root clones tolerated a maximum concentration of phenol equivalent to 1000  $\mu\text{mol/l}$ , whilst the chlorophenol derivatives (CP, DCP and TCP) were tolerated only in concentrations 20 times lower (50  $\mu\text{mol/l}$ ). Above these limits, the growth of biomass was significantly reduced ( $P < 0.001$ ) (Fig. 1). Concentrations

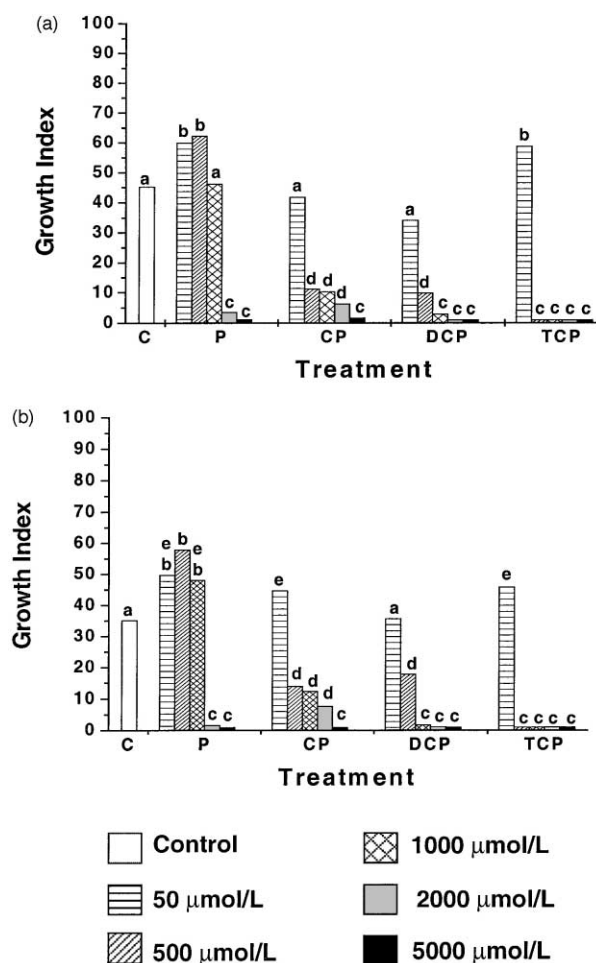


Fig. 1. Relative tolerance of hairy root clones B6 (a) and B28 (b) towards phenol and chlorophenols. Bars containing similar lower case letters indicate that the GI values are not significantly different ( $P < 0.05$ ).

below tolerance limits allowed the cultures either to grow normally or to grow significantly faster ( $P=0.01$ ) than the controls.

Liquid cultures of clones B6 and B28 were used to study their potential for the removal of phenols from the culture medium and the possible accumulation or metabolism of these toxic substances. In order to demonstrate that the disappearance of the additives was due to the uptake and intensive metabolism (inside or outside of the root matrix) and not because of evaporation, root cultures of clone B6 were fed separately with P and TCP using as controls culture medium without roots and root cultures which had been heat inactivated. Fig. 2 shows the rate of loss of P and TCP during a period of 120 h. It is clearly demonstrated that the concentration of P and TCP in the medium diminished by only a small amount when the additives were supplied to the culture medium containing no roots (6.1 and 10%

loss of P and TCP, respectively) and somewhat more when added to heat-inactivated root cultures (17.6 and 28% loss of P and TCP, respectively). On the other hand, most of the additives (more than 90%) had disappeared from the medium within this time in the presence of active root cultures. The B6 root cultures were able to uptake 46% of P from the medium within 24 h, although the uptake of TCP was somewhat slower (only 24% of the initial concentration). Seventy-two hours after inoculation, the concentration of phenols remaining in the medium had further decreased, i.e. 58% of P and 44% of TCP had been removed. At 120 h, most of the initial concentration of P (98.5%) and TCP (91.4%) had disappeared from the medium.

As shown in Figs. 3 and 4, the disappearance of phenols from the medium was accompanied by an enhancement in peroxidase activity in the roots of up to 2.6-fold. This increased activity, compared with the control cultures, was observed within 24 h after treatment and was maintained during a further 240 h. Preliminary experiments (data not shown) indicated that phenol and its chlorinated derivatives enhanced peroxidase activity in carrot roots when present at levels greater than half of the maximum tolerated, i.e. concentrations of P at 500  $\mu\text{mol/l}$  or above, and of CP, DCP and TCP at 25  $\mu\text{mol/l}$  or above augmented peroxidase activity. Very low peroxidase activity (below the limits of the calibration curve) was detected in the culture medium after addition of phenols.

Although more than 90% of the exogenous phenols had been removed from the medium within a period of 120 h, the levels of accumulation of the additives in the root tissue were considerably lower, as demonstrated in Table 1. Moreover, the levels of phenols accumulated by the metabolically active roots decreased during further incubation, such that after 240 h the concentration of P, CP, DCP and TCP found in the roots did not exceed 25% of the initial amount added to the cultures.

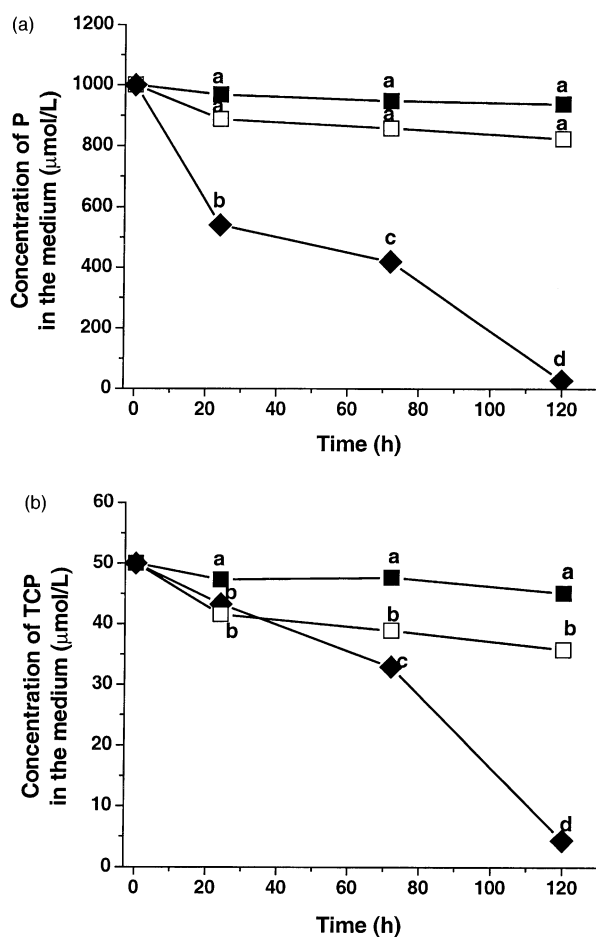


Fig. 2. Removal of phenol (a) and 2,4,6-trichlorophenol (b) from the culture medium by the B6 cultures during a period of 120 h and comparison with appropriate controls: (◆) metabolically active roots, (□) heat inactivated roots, (■) culture medium without roots. Symbols containing similar lower case letters indicate that the concentration values are not significantly different ( $P < 0.05$ ).

#### 4. Discussion

B6 and B28 root clones showed similar tolerance towards phenol and chlorophenols. A considerable decrease in growth was observed for both clones, independent of their growth characteristics, when the roots were submitted to concentrations above the tolerated levels. Within the limits of tolerance (up to 1000  $\mu\text{mol/l}$ ), growth was either comparable with control cultures or significantly increased ( $P < 0.001$ ) suggesting a growth regulator effect of the phenols. The chlorinated derivatives were more toxic to the transformed carrot roots than was phenol itself, and this toxicity appeared to increase with the number of chlorine substitutions in the aromatic ring.

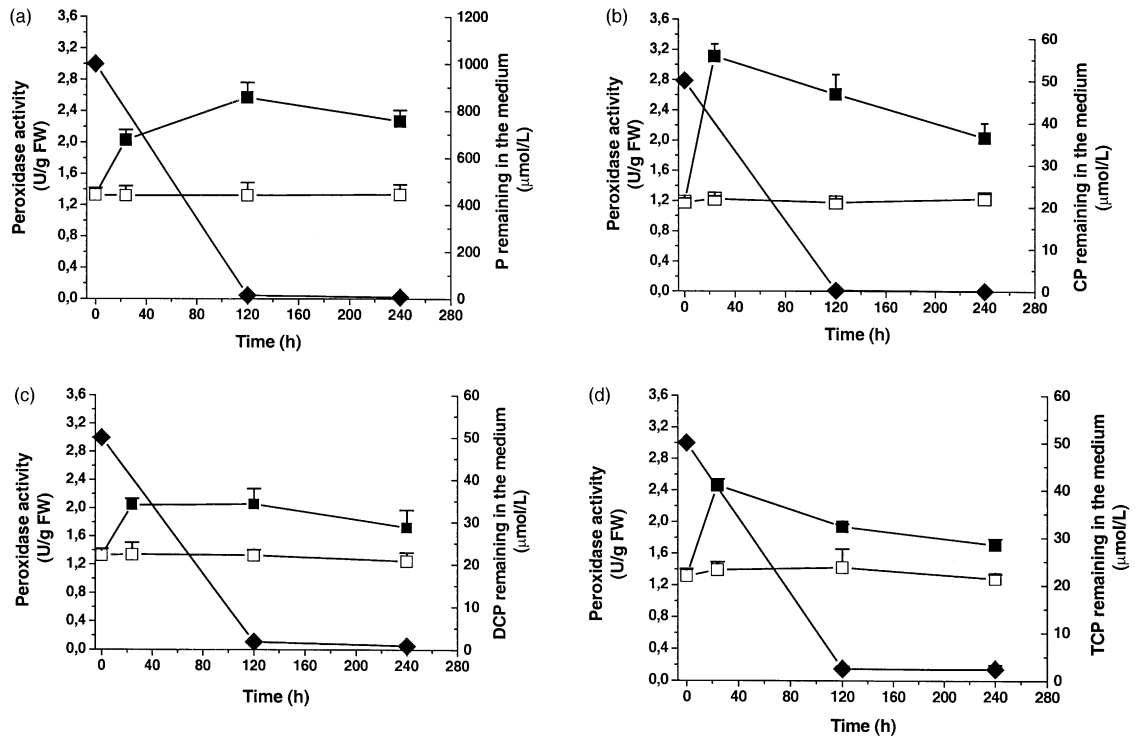


Fig. 3. Removal of phenol (a), 2-chlorophenol (b), 2,6-dichlorophenol (c) and 2,4,6-trichlorophenol (d) from the culture medium by clone B6 during a period of 240 h and concomitant peroxidase activity in the roots: (◆) concentration of phenolic compounds in the medium, (□) peroxidase activity in the control cultures, (■) peroxidase activity in the treated cultures.

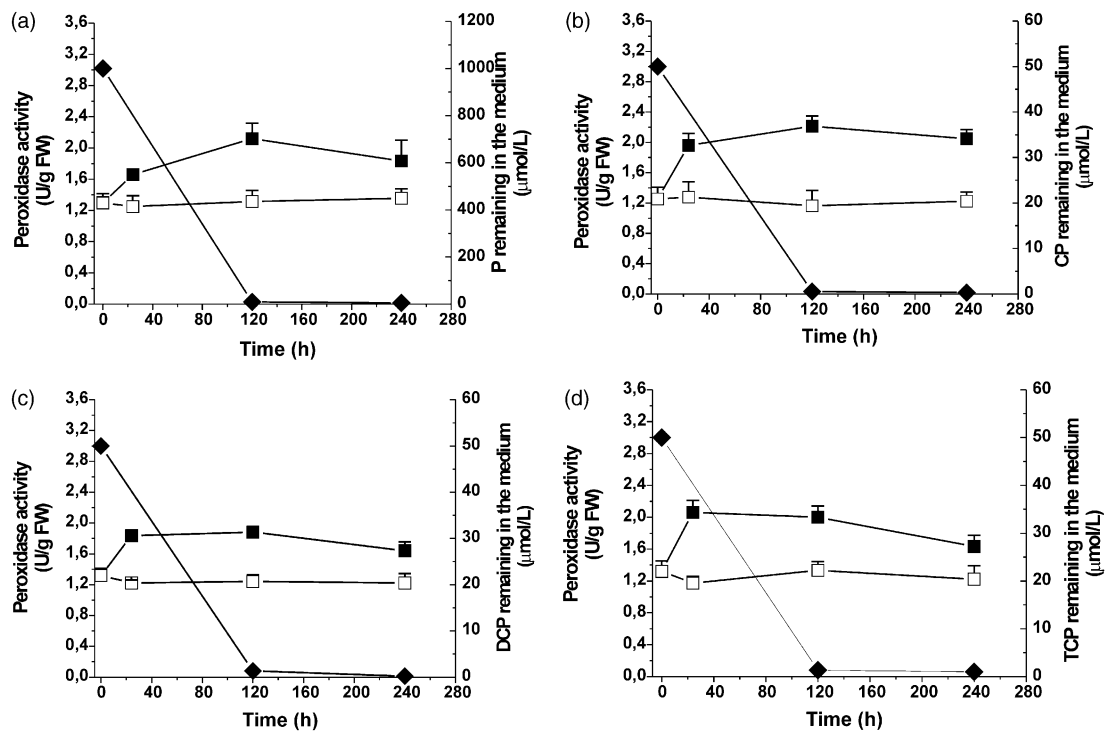


Fig. 4. Removal of phenol (a), 2-chlorophenol (b), 2,6-dichlorophenol (c) and 2,4,6-trichlorophenol (d) from the culture medium by clone B28 during a period of 240 h and concomitant peroxidase activity in the roots: (◆) concentration of phenolic compounds in the medium, (□) peroxidase activity in the control cultures, (■) peroxidase activity in the treated cultures.

As demonstrated by the experiment in which the medium alone and inactivated roots were used as controls (Fig. 2), absorption and metabolism by the roots were the main factors which accounted for the disappearance of phenols, although evaporation and unspecific adhesion to the plant tissues also contributed slightly. The suggestion that phenols were converted into other substances within the root tissue and not in the culture medium was supported by two observations: (1) insoluble polymers were not detected in the medium; (2) there was no darkening of the medium which would indicate the presence of oxidation products. Previously, Dec and Bollag (1994) showed that phenols can passively adhere to pieces of horseradish roots.

The capacity of transformed carrot roots to take up phenol and chlorophenols from the medium and further transform them was also independent of their growth characteristics. This is important because different species and varieties of plants can clearly be tested without concern for the selection of specific hairy root clones, which would invalidate the application of this system in phytoremediation proposed mainly because of its simplicity. In contrast, the selection of root clones has been shown to be very important in the case of accumulation of natural products (Argôlo et al., 2000).

Results indicate that phenol was metabolised more rapidly than the polychlorinated derivatives, which were accumulated longer within the root tissue (Table 1). The slow rate of conversion of chlorinated phenols, particularly TCP, might explain a higher toxicity compared with phenol. Furthermore, the breakdown of the phenol ring is perhaps more efficiently attained than the elimination of chlorine ions. The intracellular release of free chlorine ions and the lack of mechanisms for their prompt removal might be another explanation for the toxicity of TCP.

Table 1  
Accumulation of phenols in the root tissue of B6 and B28 cultures after 120 and 240 h

Clones	Treatment <sup>a</sup>	Phenols in the total root mass (% of the initial concentration)	
		120 h	240 h
B6	P	5.7	2.7
	CP	19.0	3.2
	DCP	14.9	6.7
	TCP	53.3	24.8
B28	P	11.0	0.8
	CP	9.3	4.4
	DCP	59.8	18.8
	TCP	62.7	0.8

<sup>a</sup> Initial concentration in the medium: *p* = 1000 µmol/l; CP, DCP, TCP = 50 µmol/l.

In the present work, it was shown that peroxidases are associated with the metabolism of phenols within carrot roots and not in the culture medium. As the phenols were depleted from the medium and from the root tissue after 240 h, peroxidase activity tended to return to the levels found in unperturbed cultures. Kim and Yoo (1996) reported that peroxidase activity in carrot hairy roots increased after the addition of pyrogallol, 2-aminophenol, *p*-cresol, catechol, 2,4-dichlorophenol and guaiacol. The authors observed that the surface of the roots changed to a reddish colour when guaiacol was added in consequence of its transformation to tetraguaiacol. They suggested that there was a large quantity of peroxidases outside the root cells.

The involvement of peroxidases in the oxidative polymerization of phenols, as well as in the formation of dimers was demonstrated previously by a number of authors (Maloney et al., 1986; Yu et al., 1994; Roper et al., 1996). However, the application of pure enzyme preparations for the treatment of water has been questioned by Maloney et al. (1986) on the basis that some reaction products, such as dioxins and furans, are also toxic. Therefore, the use of plant pieces and whole plants rich in peroxidases were investigated as an alternative to pure enzymes. The former methodology was proven to be very efficient in the removal of 2,4-DCP from industrial wastes in the case of potato and horseradish fragments (Dec and Bollag, 1994). The application of whole plants rich in peroxidases for the treatment of soil and water contaminated by phenol was recommended by Adler et al. (1994), after the authors had shown that peroxidases on the surface of tomato and hyacinth roots were able to convert guaiacol to tetraguaiacol.

The research focus until now has been on peroxidases, but other catabolic enzymes such as laccases, tyrosinases and phenoloxidases are most likely involved in the process of transformation of phenols in the roots. Furthermore, the roles of the various classes of peroxidases need clarification. The correlation between laccases and the metabolism of phenols in carrot roots is being investigated in our laboratory at present.

The application of whole plants to the decontamination of soil or water must take into account the levels of xenobiotics present in a particular location. The information concerning the levels of phenols in industrial wastes is very scarce, but according to Maloney et al. (1986), the concentration of CP normally found in industrial sludge is approximately 100 mg/l (or 770 µmol/l) which would be lethal to carrot roots. Of course, for application purposes, it is the bioavailable fraction of the hazardous substance which must be taken into consideration and not the absolute level. In the soil, the available fraction which can be absorbed by plants is usually much smaller than the total fraction of the pollutant, thus carrots possibly would tolerate higher levels of phenols than those tested.

## 5. Conclusions

The main conclusions derived from this work are: (a) hairy root cultures could supply valuable information about the metabolism of organic pollutants in plants. This model system can be used to predict if a plant in the field is able to tolerate specific compounds and also what the maximum level of tolerance would be. Furthermore, it is possible to predict if a plant can either accumulate the compounds of interest or further transform them into innocuous products. Depending on the answers to these questions, it would be possible to decide the fate of the plant material, for example, if it is suitable as animal fodder (when the plant used has a nutritional value) or if it needs further chemical processing; (b) carrot hairy roots are able to metabolise exogenous phenol and chlorophenols and the reduction of these compounds within the root tissue is associated with the increased production of peroxidase enzymes.

The model system presented here seems to be a good screening method for selecting plants which may be suitable for use in phytoremediation, and could be extended to other plant species. Ornamental plants particularly seem to be desirable targets for remediation studies because they can be used with advantage in the landscaping of a degraded area. Hairy root cultures of edible, medicinal and ornamental plants are being studied in our laboratory with respect to their potential uses in the remediation of phenol and chlorophenols.

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