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Optimalization of the peroxidase production by tissue cultures of horseradish *in vitro*

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Abstract

Tissue cultures of *Armoracia rusticana* L., both transformed with *Agrobacterium rhizogenes* and nontransformed, were screened for peroxidase activity. Most of the derived and tested strains exhibited 20 times higher activity [from 99 to 723 U g^{-1}(d.m.)] than the root of the intact plant [(30 U g^{-1} (d.m.)]. The highest peroxidase activity was found in tumour culture growing on the medium without growth regulators. The influence of the addition of sugars and heavy metal ions in the medium on peroxidase production was tested. Increase in peroxidase activity was observed after cultivation of horseradish culture with cadmium, cobalt, nickel or lead ions.

Additional key words: *Agrobacterium rhizogenes*, *Armoracia rusticana*, cadmium, calcium, cobalt, iron, lead, nickel, sugars.

Introduction

Peroxidases (E.C. 1.11.1.7.) are very abundant in plant tissues. In cell walls peroxidases occur either in soluble form or linked by ionic or covalent bonds to other components of cell walls (Ros Barceló *et al*. 1988, Veitch 2004). Peroxidase may play an important role in cell wall repair by forming an impermeable barrier to water at the site of injury *via* aliphatic and aromatic compounds (Espelie *et al*. 1986). Peroxidases catalyse cross-linking of various macromolecules, such as those involved in lignin biosynthesis (Goldberg *et al*. 1991), hemicellulose and ferulic acid (Fry 1983), cross-linking of proteins by *iso*-dityrosine (Fry 1982) or *via* lysine residues as determined by oxidation (Grambow and Langenbeck-Schwich 1983), indolylacetic acid oxidation (Moerschbacher 1992) and response to pathogens (Cassab and Varner 1988) Due to the high diversity of substrates and to the existence of many different isoenzymes (Shannon 1968), the precise physiological role of peroxidases has not yet been elucidated. Nevertheless, due to their versatile character and ability to form coloured products, peroxidases are widely used in numerous genetic, physiological and pathological studies (Dutta Gupta and Datta 2003/4, Skorzynska-Polit *et al.* 2003/4, Chouliaras *et al.* 2004, Molassiotis *et al.* 2004).

The largest amount peroxidase is used in human medicine in clinical biochemical tests. Horseradish (*Armoracia* sp.) roots represent a reliable source of commercially produced peroxidase. The isolation of enzymes from field-grown plants is made difficult by the fact that the plants do not produce seeds and must therefore be propagated vegetatively. In addition, a viral disease has recently started spreading. For these reasons there is considerable interest in investigating the possibilities of exploiting plant cultures *in vitro* as an alternative source of these enzymes (Toivonen 1993).

The aim of this study was the selection of a strain of horseradish culture with high peroxidase activity and induction of hyperproduction by altering the composition of the media and cultivation conditions.

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Abbreviations: BAP - 6-benzylaminopurine; CC - callus culture of transformed horseradish cultivated on agar medium with 0.5 μM 2,4-D; 2,4-D - 2,4-dichlorophenoxyacetic acid; HRC - root culture of transformed horseradish cultivated on agar medium without hormones; IAA - indole-3-acetic acid; MS medium - Murashige and Skoog (1962) basal medium; NAA - naphthaleneacetic acid; RC - root culture of transformed horseradish cultivated on agar medium with 3 μM NAA.

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Materials and methods

Plant transformation and regeneration: Seeds of *Armoracia rusticana* L. were surface sterilized in 5 % (m/v) sodium hypochlorite for 2 min, soaked in sterile water three times for 10 min and germinated on hormone-free Murashige and Skoog (MS) medium at 27 °C, 16-h photoperiod (irradiance of 115 μmol m^{-2} s^{-1}). After emergence of the second pair of leaves the nodal cuttings were transferred to fresh medium, and subcultured at intervals of four weeks. Callus was cultured on hormone-free MS medium at the same temperature and photoperiod.

"Hairy root" cultures were obtained by inoculation of the callus of cuttings with a suspension of *Agrobacterium rhizogenes* A4 at 10^7 cm^{-3}. "Hairy root" cultures were incubated on MS medium with 0.6 μM of naphtalene-acetic acid (NAA) and 4.44 μM of 6-benzylaminopurine (BAP) and 500 mg dm^{-3} ticarciline. Ticarciline was used for three subculture cycles (2 weeks per cycle) to prevent the growth of bacteria. In the second and third culture cycle MS medium was either supplemented with 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 3 μM NAA, or used hormone-free. For "hairy root" cultures rapid growth was characteristic and they did not exhibit strong geotropism.

Four weeks old horseradish callus or tumour tissues grown *in vitro* on MS medium supplemented with 0.5 μM 2,4-D or 3 μM NAA or without growth regulators were used as the source for peroxidase extraction.

Confirmation of transformation: DNA was isolated from 2 g of frozen 3-week-old roots (-80 °C). The roots were homogenized in liquid nitrogen and extracted in 5 cm^3 of 1 % solution of cetyltrimethylammonium bromide (CTAB) in 1 M Tris-HCl buffer supplemented with 1 M NaCl, 5 mM EDTA, and 0.01 % mercapto-ethanol (pH 7.5) for 1 h at 60 °C. Cold isopropanol (-20 °C) was added to the cold extract (5 min, ice) and incubated at 4 °C for 30 min. DNA was collected using a glass rod and washed by 75 % ethanol overnight. Primers designed for amplification of genes *rol B* and *rol C* were proposed and synthesised according to the known nucleotide sequence of TL-DNA of *A. rhizogenes* A4 (Slightom *et al*. 1986). The primers for *rol B* (fw GCACTTTCTGCATCTTCTCG, rev CCTGCATTTC CAGAAACGAT) of size 206 bp and rol C (fw GCACTCCTCA CCAACCTTCC, rev ATGCCTCACC AACTCACCA) of size 257 bp were used.

Polymerase chain reaction (PCR) was done for 35 cycles with the following steps: 94 °C 1 min; 50 °C 1 min; 72 °C 1 min; 4. 92 °C 30 s; 50 °C 1 min; 72 °C 1 min. The amplified oligonucleotides were separated by electrophoresis in agarose gel (2 %) supplemented by ethidium bromide (20 mg dm^{-3}) under UV radiation (Nepovim *et al*. 2004).

Influence of sugars on peroxidase production: Two different strains of horseradish culture were used for the experiment. The MS medium supplemented by agar (8 g dm^{-3}) was modified by 30 g dm^{-3} of one of the sugars (saccharose, galactose, ribose, lactose, glucose, fructose, sodium-galactosaethiolate or sodium-glucosaethiolate). The cultures were harvested after 26 d, frozen at -80 °C, extracted and peroxidase activity was measured.

Influence of iron and calcium ions on peroxidase production: "Hairy root" culture of horseradish was used. MS medium had a 0.1, 0.2, 0.3 or 0.4 mM concentration of Ca^{2+} or 2.3, 4.6, 6.9 or 9.2 mM concentration of Fe^{3+}. The culture was cultivated on this medium for 14 d, the samples were then frozen at -80 °C, extracted and peroxidase activity was measured.

Influence of heavy metals on peroxidase production: "Hairy root" culture was used and MS medium was supplemented by 0.25 mM Cd, Co, Cu, Ni, Pb, U or Zn. After 14 d peroxidase activity was measured .

Protein extraction: Callus or tumour tissue was homogenised with mortar and pestle in cold 0.1 M phosphate buffer [pH 7.2; 3 cm^3 of extraction buffer per 1 g(f.m.)]. The homogenate was centrifuged at 2 000 *g* at 4 °C for 45 min. The supernatant was stored at -18 °C. Samples were diluted in a ratio of 1:9 with 0.1 M phosphate buffer (pH 7.2) before peroxidase activity estimation.

Peroxidase assay: The reaction mixture used to test peroxidase activity contained guaiacol (18 mM, 0.1 cm^3), phosphate buffer (0.1 M, pH = 7.2, 1.7 cm^3) and the diluted supernatant (0.1 cm^3) in a total volume of 2 cm^3. The enzyme activity was measured spectrophotometrically (at 436 nm) using a UV-VIS spectrophotometer (*UV mini 1240*, *Shimadzu*, Kyoto, Japan). A coefficient of absorbance of 25.5 mM^{-1} cm^{-1} for H_2O_2 was used and one unit of the enzyme was defined as the amount capable of catalyzing the degradation of 1 μmol of H_2O_2 per 1 min under the standard assay conditions. Peroxidase activity was expressed in U g^{-1}(d.m.)

Growth value calculation: Growth value (GV) was calculated during the experiments. GV = [m(x)-m(0)]/ m(0), where m(0) is f.m. of "hairy root" culture at the start of experiment and m(x) is f.m. of "hairy root" culture after x days (in our case x = 2, 4, 8, 16 and 32) (Soudek *et al*. 2004).

Results and discussion

Ten different tumour cultures were derived from sterile horseradish plantlets (Table 1). The peroxidase activity of individual cultures, as well as that of natural root was estimated. Natural roots exhibited an activity of 30 U g^{-1}(d.m.). Transformed cultures showed approximately 20 times higher activity [*e.g.* RC 3 - dark 723 U g^{-1}(d.m.), HRC 9 - dark 574 U g^{-1}(d.m.), CC 7 - light 552 U g^{-1}(d.m.)]. Differences between cultures cultivated under different light conditions were generally not significant. The type of horseradish culture and type of phytohormone in MS medium were also without significant influence on peroxidase production. The peroxidase activity in transformed cultures of *Armoracia rusticana* was about 10 times higher (if we consider 90 % of water in fresh material) than in transformed *Armoracia lapathifolia* [651 U g^{-1}(f.m.)] (Flocco *et al.* 1998). Tissue cultures exhibiting activity higher than 400 U g^{-1}(d.m.) were selected for optimization of peroxidase production.

Table 1. Effects of genotype, medium composition and cultivation conditions on peroxidase activity [U g^{-1}(d.m.)] of *Armoracia rusticana* L. cultures. Means ± SD, n = 5). CC - callus culture of transformed horseradish cultivated on agar medium with 0.5 μM 2,4-D; RC - root culture of transformed horseradish cultivated on agar medium with 3 μM NAA; HRC - root culture of transformed horseradish cultivated on agar medium without hormones; light - 16-h photoperiod. Peroxidase activity in natural horseradish roots was 30 ± 4 U g^{-1}(d.m.).

Genotypes	CC		RC		HRC	
	light	dark	light	dark	light	dark
1	-	-	308 ± 14	-	-	535 ± 21
2	347 ± 7	319 ± 10	546 ± 13	253 ± 6	304 ± 12	270 ± 11
3	407 ± 20	331 ± 15	312 ± 15	723 ± 21	222 ± 10	440 ± 12
4	295 ± 13	417 ± 18	188 ± 9	249 ± 5	202 ± 9	280 ± 14
5	249 ± 10	218 ± 10	452 ± 12	315 ± 10	323 ± 11	354 ± 12
6	508 ± 24	399 ± 15	302 ± 11	231 ± 5	238 ± 9	446 ± 13
7	552 ± 26	427 ± 21	351 ± 15	153 ± 5	99 ± 4	180 ± 9
8	415 ± 18	441 ± 20	288 ± 14	197 ± 8	219 ± 10	220 ± 11
9	-	-	329 ± 14	-	-	574 ± 21
10	516 ± 23	282 ± 14	247 ± 12	255 ± 12	179 ± 5	334 ± 10

A sugar, generally saccharose, is an indispensable ingredient of all culture media, as the photosynthetic ability of the cultures tissues is limited because of low irradiance and limited gas exchange (Kozai 1991); it is also required as an osmotic agent (Thorpe 1985). In the paper of Jain and Babbar (2003/4) various sugars were tested for the best caulogenic response of the black plum (*Syzygium cuminii*). They found the best caulogenic response to saccharose. The number of shoots and shoot length was decreased in the presence of other sugars in comparison with saccharose. In our experiments the effect of the sugar composition of the cultivation medium on peroxidase activity was tested using galactose, ribose, lactose, glucose, fructose, saccharose or thio derivatives of galactose and glucose. Cultures cultivated on media with galactose or fructose showed similar activity as those cultivated on saccharose (Table 2). The sugar modified with sulphur did not affect the peroxidase activity positively. Low activity was determined in cultures cultivated on media that contained sodium-glucosaethiolate or sodium-galactosaethiolate [in three of four examples below 90 U g^{-1}(d.m.)]. Cultures growing on the media with ribose, lactose or glucose also had low peroxidase activity (in comparison with control culture grown on saccharose).

The changes of peroxidase activity during the growth cycle were studied in detail in tissue cultures RC 5 - light

Table 2. Comparison of peroxidase activity [U g^{-1}(d.m.)] of cultures RC 2 - light and HRC 9 - dark on the medium with different sugars. (RC - root culture of transformed horseradish cultivated on agar medium with 3 μM NAA; HRC - root culture of transformed horseradish cultivated on agar medium without hormones; light - 16-h photoperiod). Means ± SD, n = 5.

Sugar	RC 2 - light	HRC 9 - dark
Saccharose	128 ± 5	185 ± 9
Ribose	90 ± 3	94 ± 4
Lactose	68 ± 3	132 ± 5
Fructose	128 ± 6	159 ± 5
Galactose	134 ± 7	160 ± 8
Sodium-galactosaethiolate	119 ± 5	86 ± 3
Glucose	89 ± 3	127 ± 5
Sodium-glucosaethiolate	64 ± 2	80 ± 4

cultivated on fructose, galactose or saccharose (Fig. 1*A*,*B*,*C*). A quick and high increase of peroxidase activity was found in the culture cultivated with fructose (Fig. 1*A*). The maximum was measured after 12 d of cultivation [150 U g^{-1}(d.m.)]. Sugars have been recognized as important signal molecules that affect a variety of physiological responses and in particular regulate genes involved in photosynthesis, sink metabolism, and defense response (Koch 1996, Smeekens 1998, Roitsch 1999, Sheen *et al*. 1999). We presumed that the increase of peroxidase activity at the beginning of cultivation could be due to fructose regulation of genes of stress response. The biomass production increased very slowly and after 14 d the culture passed into a lag phase; at the same time peroxidase activity decreased to the initial level. The biomass growth on fructose containing medium was 8 times lower than that when saccharose was used.

The peroxidase production by the culture cultivated with galactose remained at the initial level during the first 9 d of cultivation (Fig. 1*B*). A high increase of peroxidase production [260 U g^{-1}(d.m.)] was observed after 12 d of growth and afterwards it decreased slowly to the level of 120 U g^{-1}(d.m.) (4 times higher in comparison with the

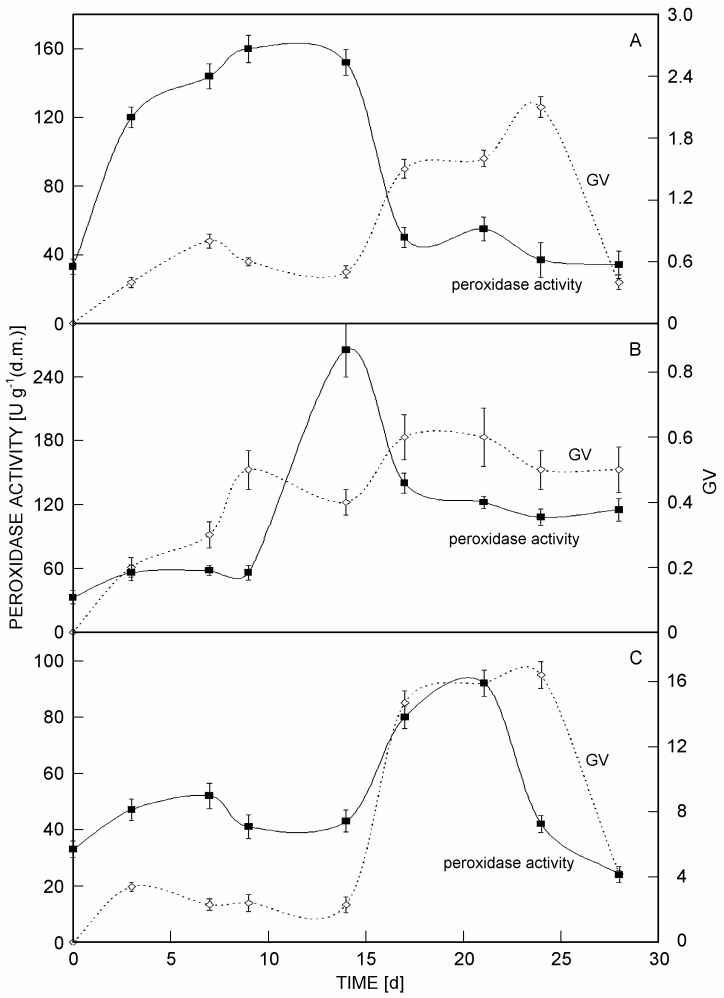

Fig. 1. Biomass production (GV) and peroxidase activity in culture HRC 3 - dark cultivated on liquid medium without hormones with fructose (*A*), galactose (*B*) or saccharose (*C*). Means ± SD, $n = 5$.

Table 3. The effect of Ca^{2+} and Fe^{3+} ionts on the peroxidase activity [U g^{-1}(d.m.)] in culture HRC 3 - dark (liquid MS medium). Means ± SD, $n = 5$.

Fe^{3+} [mM]				Ca^{2+} [mM]			
0.1 (control)	0.2	0.3	0.4	2.3 (control)	4.6	6.9	9.2
206 ± 9	185 ± 7	103 ± 5	39 ± 1	206 ± 9	152 ± 8	134 ± 4	122 ± 6

initial peroxidase activity). We suppose that galactose does not have a regulatory function like fructose. Plants are able to use galactose as a carbon source, but they must change the metabolic pathway and galactose utilization is not efficient. In this case the growth is very slow and biomass production is low (32 times lower than that when saccharose was used). At the beginning of cultivation, the culture used the remainder of saccharose deposited in cells. When saccharose was exhausted, the culture started to be under nutrient stress and peroxidase activity quickly increased.

At the very beginning of the cultivation of culture with saccharose the peroxidase activity started increasing considerably with the first small maximum on day 6 (Fig. 1*C*). This was followed by a decrease to almost the initial value. During the exponential phase of biomass growth the peroxidase activity increased again. In the late stationary phase the activity decreased. The fast increase of activity immediately after inoculation might have been caused by adaptation processes in the culture together with a defensive reaction towards negative environmental effects.

The effect of the concentration of Ca^{2+} and Fe^{3+} ions, which are cofactors of peroxidase, in the cultivation medium on the production of peroxidase activity was tested. The normal concentration in the MS medium (0.1 mM Fe^{3+} and 2.3 mM Ca^{2+}) was used as a control. At four times higher concentration of Fe^{3+}, a quarter of peroxidase activity of that found in the control was observed. At a four times higher concentration of Ca^{2+} we also obtained lower peroxidase activity than at the control concentration (Table 3). Our assumption that peroxidase activity would increase with growing ion concentration was not confirmed.

Production of peroxidase in a response to stress evoked by the presence of heavy metals has also been investigated. Generally, heavy metals are able to induce the formation of hazardous free radicals (Baccouch *et al*. 1998, Richards *et al*. 1998), which are detoxified by several antioxidative systems, in which enzymes such as peroxidase, catalase or superoxide dismutase participate. Mazhoudi *et al*. (1997) reported that a higher concentration of Cu induced an increase of peroxidase activity in seedlings of tomato plants. Chen *et al*. (2002) also published an increase of peroxidase in *Raphanus sativus* which was cultivated with copper ions. Flocco *et al.* (1998) tested the influence of abiotic elicitors (Ag^{+} and Cu^{2+}) on peroxidase activity in transformed roots of *Armoracia rusticana*. With $CuSO_4$ (3 or 5 mM) a significant decrease in peroxidase activity occurred after 48 h. In our experiments copper ions (0.25 mM) did not induce changes in peroxidase activity during the 14 d of cultivation. We found a significant increase of peroxidase activity in cultures of horseradish cultivated on media with cadmium, cobalt, nickel or lead ions and partially also in the presence of zinc ions (Table 4). However, a considerable decrease of peroxidase activity was determined in a culture of horse-radish cultivated with uranyl ions. The highest peroxidase activity [330 and 194 U g^{-1}(d.m.)] after 16 d of cultivation was recorded in the presence of nickel and cobalt ions, respectively. The presence of these metals evidently induced free radicals in cultures of horseradish, which were detoxified by increasing peroxidase production. In comparison with tomato or *Raphanus sativus*, horseradish is obviously more resistant to the incidence of copper ions.

Table 4. Peroxidase activity [U g^{-1}(d.m.)] in hairy root culture (HRC 3 - dark) on liquid medium with different heavy metals in concentration 0.25 mM. Means ± SD, n = 5.

	0 d	2 d	4 d	8 d	16 d
Cd	-	84 ± 4	123 ± 5	151 ± 6	85 ± 4
Co	-	162 ± 6	95 ± 4	113 ± 5	194 ± 7
Cu	-	53 ± 2	42 ± 2	66 ± 3	32 ± 1
Ni	-	48 ± 2	124 ± 2	74 ± 3	330 ± 10
Pb	-	86 ± 3	86 ± 3	64 ± 2	99 ± 4
U	-	9 ± 1	9 ± 0	10 ± 3	12 ± 1
Zn	-	134 ± 6	73 ± 3	98 ± 4	52 ± 2
control	33 ± 2	53 ± 2	72 ± 3	30 ± 1	23 ± 1

Conclusion: Production of peroxidase in most of the derived and tested strains of *in vitro* culture of horseradish is possible as 20 times higher than peroxidase activity in natural horseradish roots was found. Changes of the sugar component in the cultivation medium did not have a positive effect on peroxidase production. Cultures cultivated on the medium with galactose, fructose and sucrose had similar peroxidase activity at the end of the cultivation cycle and an increase of production during cultivation could not be important for potential commercial production. Cultures with other tested sugar components exhibited even lower peroxidase production. The assumption that peroxidase production would increase after cultivation on the medium with higher concentrations of Ca^{2+} and Fe^{3+} was not confirmed. Induction of peroxidase activity was observed after cultivation of horseradish culture with cadmium, cobalt, nickel or lead ions. Generally, the highest peroxidase activity was detected in root culture of transformed horseradish cultivated on agar medium without hormones in the dark. This level can be sufficient for real biotechnology production of horseradish peroxidase.

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