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Peroxidase production in *armoracia sp.* Transformed hairy roots

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Abstract

Peroxidase (E.C. 1.11.1.7) has been widely used as a component of reagents for chemical diagnosis and various laboratory experiments because a wide range of chemicals can be modified by the catalytic activity of this enzyme. Several novel applications of this enzyme have been suggested: treatment of wastewater containing phenolics, the synthesis of various aromatic chemicals etc. Horseradish (Armoracia sp.) roots contain peroxidase currently used for commercial applications. However there is interest in an alternative production system and the transformed tissues consists one of them.

In the present study it is shown that Armoracia sp. hairy root cultures obtained after transformation with an Agrobacterium rhizogenes strain harboring a cloning vector derived both from Ri plasmid and pBin 19 produce larger amounts of peroxidases compared with normal tissues. An enhancement of peroxidases production and secretion was detected when roots were grown on kanamycin containing medium. The effect of plant extracts on phenol compounds degradation was also tested.

Keywords: hairy roots, horseradish (Armoracia sp.), peroxidase, phenol degradation

Introduction

Plant-derived chemicals are valuable sources for a variety of pharmaceuticals, flavors, dyes, oils and resins [14]. Plant peroxidases (E.C. 1.11.1.7) as multifunctional enzymes have been associated with several physiological processes (including regulation of growth and cell proliferation, auxin metabolism, lignification) and are closely linked to plant defense responses[7]. Moreover, these enzymes have a key role in different stress-related processes such as browning; wound healing and disease resistance [7].

These enzymes are extensively used as reagents for laboratory tests. Several novel applications including the removal of peroxides from industrial wastes and the treatment of wastewater containing phenols and aromatic amines brought plant peroxidases again in the attention of scientists. Horseradish (*Armoracia sp.*) roots represent the traditional source or commercial production of peroxidases. Considering the fact that traditional methods are affected by climatic changes and cultivation limits (horseradish does not produce seeds and being propagated vegetatively some virus diseases have been recently identified [13], there is a big interest in exploiting the *in vitro* system of cell, tissue and organ cultures as an alternative for the improvement of enzyme biosynthesis[10]. Moreover, in vitro transformation of different kind of plant explants with cloning vectors derived from Ri plasmid of *Agrobacterium rhizogenes* could offer a possibility of improving the enzyme production. It was shown that organ cultures, including root cultures tend to show a superior genetic and biochemical stability over time [8].

In this context our experiments aimed to obtain *in vitro* cultures of transformed horseradish tissues trough infection with an *A.rhizogenes* recombinant strain in order to induce hairy roots and to test the biodegradation capacities of a root extract on a phenolic solution.

Materials and methods

1. **Plant material**. Roots of *Armoracia sp.* were forced in laboratory conditions and the resulted shoots served as explant sources for establishing axenic cultures further used for transformation experiments.
2. **Bacterial strains.** The strain *Agrobacterium rhizogenes* K599 (Rfm^r, His^-) harboring a recombinant cloning vector derived from Ri plasmid (Km^R, *gus*) was provided by the Institute of Biology from Bucharest. The strain was maintained on APM agar medium containing kanamycin (20 μg/ml). For transformation experiments, *A.rhizogenes* K599 strain was initially cultivated for 48 hours at 30ºC in APM liquid medium [4] and then transferred on solid medium, in Petri plates. After 48 h of cultivation at 30ºC, cells were suspended in sterile distillated water. The bacterial suspension was used for transformation.
3. **Plant transformation protocol.** Pieces of approx. 1 cm^2 detached from leaves of *Armoracia sp.* plantlets grown in aseptic conditions were incubated for 5 to 30 minutes in the bacterial suspension, and then placed on Petri plates between two pieces of sterile wet filter paper. The Petri plates were maintained at room temperature for 48 h in dark conditions. The tissue fragments were transferred onto solid MS hormone free medium, supplemented with cefotaxim (500μg/ml) for removing bacteria. After three subcultures on culture medium with progressively reduced concentrations of cefotaxime (400 μg/ml; 300 μg/ml; 200μg/ml), the tissue fragments were placed onto kanamycin containing MS medium, in three variants, with different concentrations (25μg/ml, 75μg/ml and 100μg/ml) in order to select the transformed cells. The morphogenetic cultures were maintained under a photoperiod of 16/8 light/dark cycles and a temperature of 25ºC (±2ºC). Hairy roots and shoots were regenerated on all variants of MS medium containing Km and they were tested for the ability of peroxidase production and phenol degradation.
4. **Peroxidase extraction**. Approximately 1g of transformed plant tissues (hairy roots, leaves and stems) was mashed into a jar with quartz sand and suspended in 10ml of sterile water. The suspension was centrifuged 5 minutes at 5000 rpm and the supernatant was collected. Peroxidase activity was determined with benzidine reagent using hydrogen peroxide as substrate, according to Brad method [6].
5. **Phenol degradation** with plant extract. Samples of 0,5 ml plant extract were treated with 1 ml of solution 1 (0,5ml of 3% phenol, 7,2ml distilled water, 2ml of 1% H_2O_2) and 0,5ml Folin Ciocâlteu reagent. After filtration, 0,5 ml filtrate was mixed with 4,5ml of 20% sodium carbonate. The absorbance at 660nm was determined with Helios gamma UV-VIS spectrophotometer. Phenol degradation was estimated by quantification of the residual phenol not oxidized through peroxidase activity.

Results and discussion

1. Selection of transformed horseradish roots and shoots

Hairy roots can be obtained by genetic transformation of wounded plant tissue by a pathogenic soil bacterium, *Agrobacterium rhizogenes*. The expression of hairy root disease phenotype is determined by a stable integration and expression of part of Ri plasmid (T-DNA) in the genome of the infected plant. The transferred genes are modifying the production and sensitivity to auxin in the plant cells, resulting in the proliferation of fast growing adventitious roots at wound site [12,13].

Using explants from "in vitro" plants grown in aseptic conditions permitted to eliminate the sterilizing procedure that could lead to tissue degradation. In order to remove *A.rhizogenes* K599 used for transformation, repeated subcultures of transformed tissues on cefotaxim containing MS medium were performed. This antibiotic removes all contaminant bacterial cells after three weekly passages.

Short time after infection (three weeks) with *A.rhizogenes* K599, callus, root and shoot primordial appeared at the infection sites and developed abundantly on MS hormone-free medium (figure 1).

Figure 1. Shoots and hairy roots of *Armoracia sp.* transformed with *A.rhizogenes* Ar40.

The best results were obtained when the explants were incubated for 30 minutes with bacterial suspension, followed by the co-cultivation for 48 h.

Fragments of the morphogenetic cultures with roots and shoots were excised and transferred on solid MS hormone free medium supplemented with different concentrations of kanamycin. The rapid growth of hairy roots at the edges of the plant tissue cultivated on antibiotic containing medium (in all variants of concentration) confirms the stable integration and expression of T-DNA from recombinant Ri plasmid into the plant genome.

As it is known, kanamycin is a selective agent for detection the transformation events in plants and could induce a specific biochemical answer in plant tissues. Different authors observed that peroxidases production is stimulated in unusual culture conditions as well as when the plants are infected by pathogens. Despite all these observations, the molecular explanation of the stimulation of POD biosynthesis remains unclear. In this stage of our researchs we are not able to give another explanation to the stimulation of POD increased level than that of the stimulation of plant defense mechanisms.

Control horseradish shoots obtained from axenic material in the absence of *A.rhizogenes* were subjected to the same culture conditions (solid MS hormone-free medium).

When they were subcultured on kanamycin containing medium, untransformed shoots turned brown and died (figure 2).

Figure 2. The aspect of untransformed shoots cultivated on kanamycin containing medium.

The morphological aspect of the transformed shoots was similar to the control variant but their growth in the presence of the antibiotic is the proof of the expression of *nptII* gene in these tissues.

2. Production of peroxidases in plant tissues

Transformed material selected after subcultivation in the presence or in the absence of kanamycin was used for protein extraction and total peroxidases (POD) production was assessed.

In the absence of kanamycin, the level of peroxidase was reduced, both in roots and shoots, but it was slightly increased in transformed plant tissues (control 1) comparing to untransformed material (control 2)(table 1). In this order, peroxidase activity grew with approx. 58,6% in transformed roots and with 26,6% in shoots comparing with control material (untransformed plant tissue).

Table 1. Peroxidase activity in normal and transformed roots and shoots.

Experimental variant	Peroxidase activity (μM/ml/min)		Kanamycin concentration in culture medium (μg/ml)
	Hairy-roots	Shoots	
V1	12	3,14	25
V2	22,4	18,5	75
V3	48	33,6	100
Control 1	2,38	1,9	0
Control 2 (untransformed plant tissue)	1,5	1,3	0

These observations are different from data presented in literature [1,7] where it was demonstrated that transformation with *A.rhizogenes* LBA 9402 did not affect the POD level and isoenzyme profile of *Armoracia lapatipholia*.

Employing genetic hormonal, nutritional and other environmental strategies can enhance growth and production of secondary metabolites and their related enzymes. Of these,

elicitation seems an appropriate technique to improve production rates. The various agents termed elicitors include molecule of microbial origins (biotic elicitors) and abiotic elicitors such as heavy-metal salts and detergents. These substances trigger different plant defense reactions, such as the production of enzymes such as chitinases and peroxidases that are involved in many defense mechanisms like lignification or phenol oxidation [11]

In our experiments, the presence of kanamycin in culture medium induced a spectacular increase of peroxidase activity both in adventitious roots as well as in transformed shoots. Significant variations in enzymatic activity were encountered when different concentrations of antibiotic were used (figure 3).

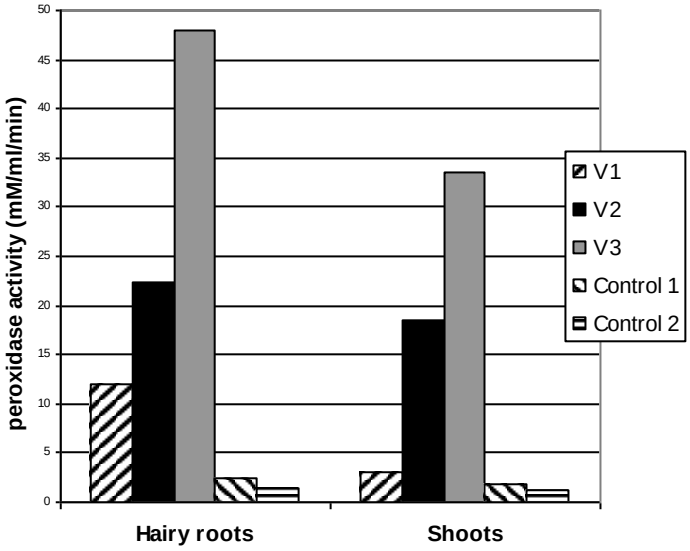

Figure 3. Influence of kanamycin concentration on peroxidase activity in transformed roots and shoots of *Armoracia sp.*

The best results were obtained when transformed plant material was cultivated in the presence of 100 μg/ml kanamycin (V3): 32 times higher activity in hairy roots and 25,8 times higher in transformed shoots comparing to untransformed (control 2) similar material (roots and shoots). If these activities are compared with those of transformed shoots and roots cultivated in the absence of antibiotic (control 1), the level of peroxidase activity was 20,1 times higher in hairy roots and 17,7 times higher in shoots.

An increased level of peroxidase activity as well the diversity of isoenzymes was observed previously in *Nicotiana tabacum* transformed with *A.tumefaciens* [2,3]. These data suggested that the transformation events and the cultivation of transformed cells on selective medium containing kanamycin could have the capacity to elicit POD activity.

It is interesting that the level of peroxidase produced by transformed tissues, especially by hairy root, remained relatively constant during 10 months of subcultivations (plant tissues were subcultured at two weeks intervals). This is a proof that the regenerated organs are transformed and are genetic and productive stable.

3. Phenol removal with plant extracts

It is well known that oxidoreductive enzymes are capable of oxidizing phenols and aromatic amines to free radicals or to quinones and benzoquinone imines (Dec and Bollag, 1994). Klibanov et al (1983) proposed that an oxidative coupling reaction mediated by horseradish peroxidase be exploited for the removal of phenols, anilines and other aromatic

compounds from aqueous solutions. In our study we investigated the ability of hairy roots extracts to remove the phenol from an aqueous solution (with 3% phenol). As source of peroxidase we used proteins extracted from normal roots and from hairy roots grown on MS hormone free solid medium, supplemented with 100µg/ml kanamycin. The obtained results showed a significant decrease of phenol content in samples treated with horseradish peroxidases, comparing with control (fig. 4).

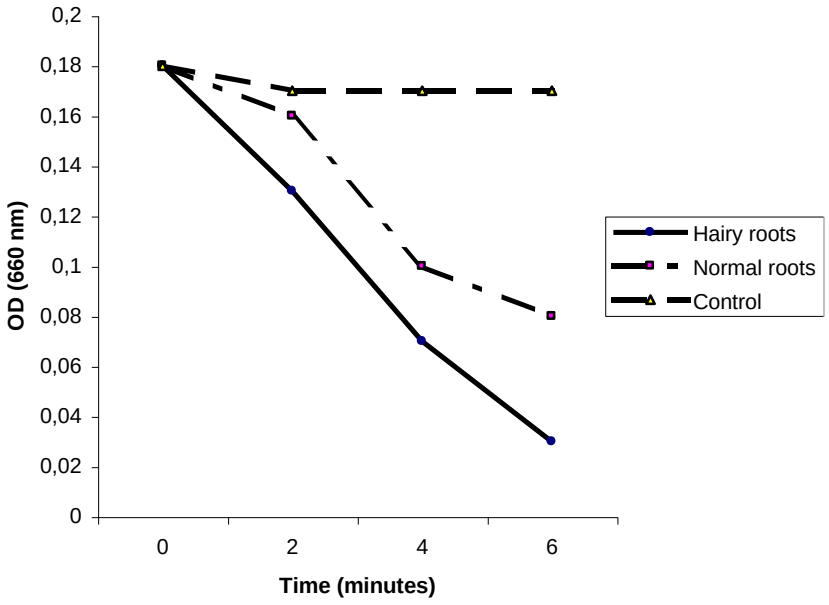

Figure 4. Effect of the activity of horseradish from normal and hairy roots on removal of phenol from an aqueous solution

It is also clear that transformed roots had the ability to remove phenol in about 25% less timed than normal tissues.

These results could be a good argument for the application of plant technology to wastewater treatment; the used plant material from various treatments may then be disposed of by composting, plowing into soil or combustion. The major reason that enzymatic treatment has not been applied on an industrial scale is the huge volume of polluted environments demanding bioremediation and the cost of this treatment. In this situation, the use of minced plant materials that contain oxidoreductases may represent a good alternative [5,9].

Conclusions

The results obtained in our experiments allow for the following conclusions:

1. Hairy roots and transformed shoots were obtained when *Agrobacterium rhizogenes* K599 was used for infection of horseradish explants.
2. The transformed nature of roots and shoots was assessed both by phenotypic observations and by selection on MS hormone free medium supplemented with kanamycin.
3. In the absence of kanamycin, the level of peroxidase was reduced, both in roots and shoots, but it was slightly increased in transformed plant tissues (control 1) comparing to untransformed material (control 2). In transformed roots and shoots,

the peroxidase activity increased by approx. 58,6% and 26,6% respectively, as compared to the control material (untransformed plant tissue).

4. The presence of kanamycin in the culture medium induced a significant increase of peroxidase activity both in adventitious roots as well as in transformed shoots: between 8-32 times higher activity in roots and 2,4-25,8 times higher in shoots, comparing with untransformed similar plant material.
5. The protein extracts, both from transformed and untransformed roots were able to remove the phenol from an aqueous solution (containing 3% phenol).

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