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# Peroxidase activities and contents of phenolic acids in embryogenic and nonembryogenic alfalfa cell suspension cultures

M. HRUBCOVÁ, M. CVIKROVÁ and J. EDER

*Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Ke dvoru 15, 166 30 Praha 6, Czech Republic*

## Abstract

Contents of phenolic acids, peroxidase activities and growth curves showed significant differences in embryogenic (EC) and nonembryogenic (NEC) suspension cultures of *Medicago sativa* L. NEC gave a typical growth curve while in EC the distinct phases were absent. The total content of phenolic acids was higher in NEC (related to EC), changed during the growth cycle and most of the acids occurred in ester-bound methanol soluble form. The level of phenolic acids in EC was significantly lower and did not change during 12-d cultivation. The major fraction was formed by phenolic acids ester-bound to the cell wall. The cytoplasmic peroxidase activity in NEC increased continuously during the growth and reached the maximum value at the end of exponential phase. In EC the extremely low cytoplasmic peroxidase activity did not change during cultivation. Ionically bound peroxidases in NEC represented 14 to 30 % of the total extracted activity in dependence on the growth phase while in EC formed about 50 % of the total activity and did not change during studied period. A possible participation of ionic peroxidase in the incorporation of phenolics into the cell wall is discussed.

## Introduction

Oxidation of phenolic compounds, the natural occurring substrates in the plant cell, is one of the functions of peroxidases. In addition to these phenol oxidising reactions, peroxidases catalyse several physiologically relevant oxidase reactions as indole-3-acetic acid oxidation (Ros Barceló *et al.* 1989). The phenolic cofactors act as

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*Abbreviations*: EC - embryogenic suspension culture; NEC - nonembryogenic suspension culture; BL - medium of Blaydes; 2,4-D - 2,4-dichlorophenoxyacetic acid; PO - peroxidase activity; FM - fresh mass.

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electron donors to allow recycling $Fe^{3+}$-form of peroxidase in the catabolism of IAA (Pedreño *et al.* 1990). Monophenols generally increase the rate of IAA degradation and the peroxidase catalysed degradation of IAA results in the formation of indole-3-methanol in the presence of phenolic compounds (Grambow and Langenbeck-Schwich 1983). So, peroxidase activity as a whole is involved in the regulation of growth processes partly through the control of the endogenous auxin level and partly through the participation in the processes resulting in the growth inhibition via regulation of cell wall rigidification and lignification (Gaspar *et al.* 1985). Recently Zheng and Huystee (1992) demonstrated the inverse relationship between peroxidase and elongation of segments from peanut hypocotyls and concluded that peroxidases, especially cationic peroxidases, directly regulate cell elongation through catalysing the crosslinks of wall phenolic compounds. It was shown that several different morphogenetic processes in *Cichorium intybus* are associated with changes in peroxidase activities (Bouazza *et al.* 1993). The level and accessibility of phenolic substrate is an important factor for the two peroxidase catalysed reactions. In our previous work we showed that qualitative and quantitative differences in the pool of phenolic acids occurred in alfalfa embryogenic and nonembryogenic cultures (Cvikrová *et al.* 1991). The aim of this work was to investigate the relationship between peroxidase activities and phenolic acid contents in these cultures.

## Material and methods

**Plant material:** Alfalfa (*Medicago sativa* L.) embryogenic culture (EC) and nonembryogenic culture (NEC) were derived from leaf explants and cultivated as described by Binarová and Doležel (1988). Starting from friable callus cells of embryogenic culture were inoculated into liquid BL hormone-free medium (Blaydes 1966), cells of nonembryogenic callus into liquid BL-medium supplemented with 5 μM 2,4-D and 1 μM kinetin. The cell suspensions were grown on a rotary shaker under a 10/14 h photoperiod regime at 26 °C and subcultured at 10-d intervals. Nonembryogenic culture consisted of single cells and small cellular clumps; the embryogenic culture contained besides single cells and the small clusters of non differentiated cells also the earliest developmental stages of somatic embryos. Somatic embryos were removed from EC before analyses.

**Peroxidase extraction and assay:** Cells of NEC and EC were frozen in liquid nitrogen, homogenised with 10 mM phosphate buffer (pH 6.0) and centrifuged (4 °C, 14 000 *g*, 20 min). Soluble peroxidase (PO) activity was determined in the crude extract. The pellet was purified by washing with phosphate buffer and distilled water. Peroxidases ionically bound to cell walls were extracted from the purified pellet with 100 mM NaCl solution and de-salted by dialysis. Peroxidase activity was determined in 3 $cm^3$ of reaction mixture containing 13 mM guaiacol, 5 mM $H_2O_2$ and 50 mM Na phosphate buffer (pH 6.5). Oxidation of guaiacol was followed by the increase of absorbance at 470 nm.

**Determination of phenolic acids:** Phenolic acids were analysed in NEC and EC durind 12-d culture. Phenolic acids were extracted as described earlier (Cvikrová *et al.* 1988). Soluble ester-bound phenolic acids ($F_1$) were obtained after alkaline hydrolysis from a methanolic extract of tissue ground in liquid nitrogen. The fraction of cell wall bound phenolics ($F_2$) was obtained after alkaline hydrolysis of the residual tissue after methanol extraction. Phenolic acids were analysed by means of HPLC using a *Pye Unicam PU 4002-Video Liquid Chromatograph* with a *Spherisorb 5 ODS* column (Cvikrová *et al.* 1991).

**Statistical evaluation of results:** Absolute values of peroxidase activities and the contents of phenolic acids depended largely on the physiological state and embryogenic capacity of subcultivated cultures. Therefore the results of one representative experiment are given. The pattern of changes was uniform in all three experiments.

## Results

**Growth of cultures:** Growth of NEC and EC was recorded on a fresh mass basis (Fig. 1). The NEC gave a typical growth curve with lag, exponential, linear and stationary phases. In contrast, the EC did not follow the typical growth curve. The increase in fresh mass of EC was mostly caused by the growth of developing embryos in the culture.

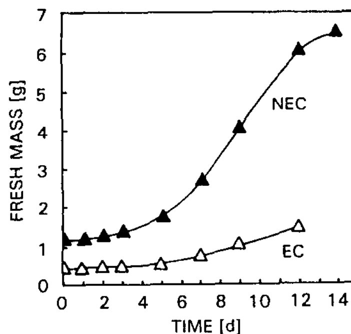


Fig. 1. Growth curves, expressed by the relative fresh mass increase from nonembryogenic (NEC) and embryogenic (EC) alfalfa cell suspension cultures.

**Peroxidase activities in NEC and EC:** Changes in the soluble PO activity in NEC growing on the medium supplemented with 2,4-D and kinetin were during the studied growth period much more pronounced than those observed in EC growing on hormone free medium (Fig. 2). Two peaks of soluble PO activity were observed in NEC cells after their transfer into the fresh medium (Fig. 2A). The first increase occurred at the end of the lag-phase and this phenomenon could be associated with dilution of culture after inoculation (Fig. 3A,B,C). The second transient increase at the end of exponential phase ($7^{th}$ day) was followed by a decrease in the linear phase

of growth. In EC the soluble PO activity was 3 - 5 times lower (Fig. 2B) than the activity found in NEC and no significant changes were observed during the studied period.

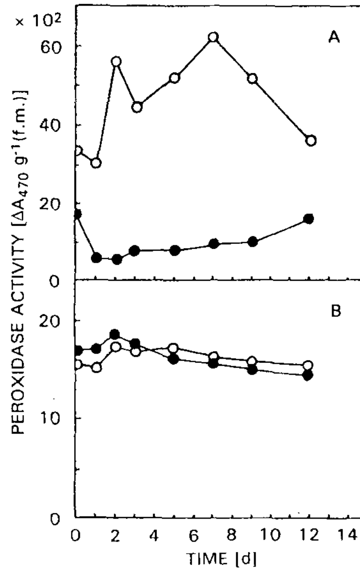


Fig. 2. Soluble (*open circles*) and ionically bound (*closed circles*) peroxidases extracted from nonembryogenic (A) and embryogenic (B) alfalfa cell suspension during the growth of cultures.

The ionically bound PO activity in NEC (Fig. 2A) was low till the end of exponential phase and the proportion of ionically bound PO activity represented approximately 14 % of the total extracted activity (Table 1). The increase in this proportion to 30 % ($12^{th}$ day of culture) coincided with a decrease of the rate of NEC growth. In EC was the activity of ionically bound PO relatively constant during the studied period (Fig. 2B). Although EC showed a low total extracted PO activity (related to NEC), ionically bound PO formed approximately 50 % of the total activity (Table 1).

Table 1. Proportion of ionically bound peroxidase activity in the total extractable peroxidase activity.

| | Ionically bound PO [%] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 d | 2 d | 3 d | 5 d | 7 d | 9 d | 12 d |
| NEC | 15.9 | 8.9 | 14.7 | 13.3 | 13.8 | 16.3 | 30.9 |
| EC | 52.8 | 51.4 | 52.0 | 48.0 | 47.7 | 48.1 | 49.7 |

**Contents of phenolic acids in NEC and EC:** Contents of methanol soluble ester-bound ($F_1$) and ester-bound to the cell wall ($F_2$) phenolic acids found in NEC changed during the suspension culture. Most of the phenolic acids occurred in methanol soluble form and this fraction underwent pronounced changes during the culture

period. The decline of the level of soluble esters in the transition to exponential phase was caused predominantly by the decrease of *p*-hydroxybenzoic, ferulic and *p*-coumaric acid contents. The contents of soluble esters rose again in the late linear phase (Table 2) and the increase was due predominantly to ferulic and *p*-coumaric acids. The contents of phenolic acids esterically bound to the cell wall ($F_2$) did not change significantly during the culture and the alterations were similar to those observed in soluble esters content. Compared with soluble esters their level was much lower (Table 2).

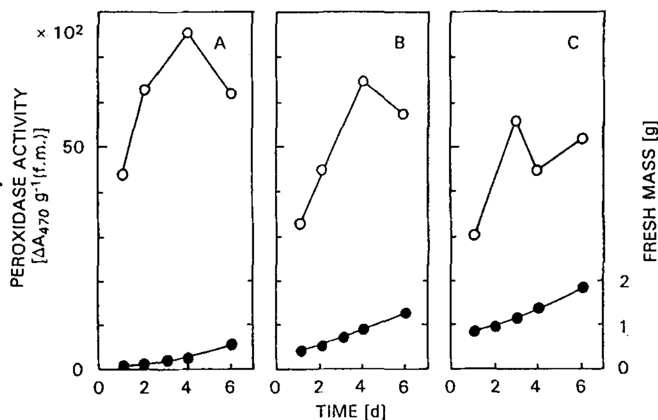


Fig. 3. The effect of inoculum dilution on peroxidase activity (*open circles*) in the lag-phase. Fresh mass of inoculum: A - 0.15 g, B - 0.4 g, C - 0.9 g.

Table 2. Contents of phenolic acids in alfalfa nonembryogenic suspension culture.

| Time [h] | Growth phase | Fraction of PhA | Phenolic acids [μg g-1(f.m.)] pHBA | VA | SaA | AA | pCA | FA | CA | SiA | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | inoculation | $F_1$ | 0.79 | 0.08 | - | 0.02 | 0.67 | 5.99 | 0.20 | - | 7.75 |
| | | $F_2$ | 0.35 | 0.05 | - | 0.04 | 0.36 | 0.29 | 0.02 | - | 1.11 |
| 12 | lag-phase | $F_1$ | 4.11 | 0.44 | 0.24 | 0.61 | 1.69 | 7.76 | 0.20 | 0.30 | 15.35 |
| | | $F_2$ | 1.02 | 0.29 | 0.14 | 0.24 | 0.95 | 0.41 | 0.04 | 0.02 | 3.11 |
| 72 | transition to | $F_1$ | 1.93 | 0.05 | - | 0.16 | 0.25 | 0.26 | 0.16 | - | 2.81 |
| | exp. phase | $F_2$ | 1.12 | 0.09 | - | 0.09 | 0.11 | 0.08 | 0.02 | - | 1.51 |
| 288 | late linear | $F_1$ | 0.79 | 0.14 | 0.08 | 0.02 | 0.87 | 7.42 | 0.07 | - | 9.39 |
| | phase | $F_2$ | 0.43 | 0.08 | - | 0.12 | 0.23 | 0.32 | - | - | 1.18 |

$F_1$ - methanol soluble phenolic acids; $F_2$ - ester-bound cell wall phenolic acids; pHBA - *p*-hydroxybenzoic acid; VA - vanillic acid; SaA - salicylic acid; AA - anisic acid; pCA - *p*-coumaric acid; FA - ferulic acid; CA - cinnamic acid; SiA - sinapic acid;

In contrast to NEC, in EC the contents of phenolic acids ester bound to the cell wall ($F_2$) was higher than the content of soluble form ($F_1$). The contents of phenolic acids incorporated in the cell wall of EC was three times higher than the $F_2$ contents found in NEC (with the exception of 12 h, lag-phase). The level of both, soluble and bound forms, did not change significantly during the culture. The highest

concentrations in EC showed *p*-hydroxybenzoic and vanillic acids, while the levels of p-coumaric and ferulic acids were many times lower (Table 3).

Table 3. Contents of phenolic acids in alfalfa embryogenic suspension culture (embryos removed). Since the embryogenic culture does not follow the typical growth curve the growth phases are not indicated.

| Time [h] | Fraction of PhA | Phenolic acids [μg g$^{-1}$(f.m.)] pHBA | VA | SaA | AA | pCA | FA | CA | Total |
|---|---|---|---|---|---|---|---|---|---|
| 0 | $F_1$ | 0.79 | 0.22 | 0.07 | 0.12 | 0.14 | 0.74 | 0.08 | 2.16 |
| | $F_2$ | 3.02 | 0.46 | 0.16 | 0.23 | 0.29 | 0.19 | 0.01 | 4.36 |
| 12 | $F_1$ | 1.28 | 0.23 | 0.05 | 0.14 | 0.16 | 0.25 | 0.10 | 2.21 |
| | $F_2$ | 2.52 | 0.41 | 0.15 | 0.37 | 0.15 | 0.20 | 0.04 | 3.84 |
| 72 | $F_1$ | 1.02 | 0.15 | 0.08 | 0.11 | 0.15 | 0.11 | 0.07 | 1.69 |
| | $F_2$ | 2.69 | 0.51 | 0.14 | 0.60 | 0.31 | 0.21 | - | 4.46 |
| 288 | $F_1$ | 0.46 | 0.21 | 0.11 | 0.12 | 0.15 | 0.76 | 0.08 | 1.89 |
| | $F_2$ | 3.02. | 0.46 | 0.18 | 0.34 | 0.34 | 0.23 | - | 4.57 |

Abbreviations see Table 2.

## Discussion

In our experiment nonembryogenic culture (NEC) exhibited a conspicuous accumulation of soluble esterically bound phenolic acids in the early lag-phase. The increased level of phenolic substances as a consequence of induction of phenylalanine ammonia-lyase in stress response to inoculation was described in alfalfa cultures (Cvikrová *et al.* 1991). The increase in the contents of phenolic substances in the lag-phase coincided with the first peak of soluble PO activity which absolute value (similarly as the level of phenolic esters, data not shown) depended on the size of inoculum and the physiological state of culture. In contrast with the first peak the second maximum in soluble PO activity in NEC occurred at the end of exponential phase when the level of phenolic esters was rather low. In this growth phase the activity of soluble PO may participate in the catabolism of auxin and the cofactors of IAA oxidation may origin from the decrease in the contents of *p*-hydroxybenzoic, *p*-coumaric and first ferulic acid soluble esters. Monophenols are known to serve as cofactors of IAA oxidation by peroxidases (Grambow 1986, Pedreño *et al.* 1990). Relation between soluble PO level, IAA metabolism and plant cell growth potential was described in the hypocotyl of legumes. Our results are in agreement with the observations of Ferrer *et al.* (1991) who detected higher soluble PO activity in the fast growing cells than in older, slowly growing cells. In embryogenic culture the activity of soluble PO was many times lower (related to NEC) and remained relatively constant during the studied period. This is in good agreement with its assumed role in utilisation of phenolics, as the level of soluble phenolic esters ($F_2$) in EC was low and did not change significantly during the culture.

The slight increase in ionically bound PO activity in the late linear phase of growth of NEC correlated with the increase of both soluble and cell wall-bound phenolic esters (mainly ferulic acid esters). In this growth phase activity of ionic-bound PO formed 30 % of total PO activity while representing only 14 % by the end of the exponential phase. The increase of PO bound form was associated with the decrease in the rate of NEC growth. Although EC showed a low total PO activity (related to NEC), ionic-bound PO activity formed 50 % of the total activity. This relatively large proportion of ionically bound peroxidases may be associated with the high level of phenolic esters incorporated into the cell wall of EC (high contents of *p*-hydroxybenzoic, vanillic and anisic acids).

The importance of peroxidases in process of somatic embryogenesis probably consists in their participation in the restriction of cell wall expansion in the early stages of embryo formation (Van Engelen and De Vries 1992). It has been shown that the specific cationic isoperoxidase is involved in the control of somatic embryogenesis in carrot cell culture (Cordewener *et al.* 1991). We suppose that phenolic acids esterically bound to the cell walls may become cross-linked after oxidation by ionically peroxidase and so influence the expandability of alfalfa embryogenic cells.

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