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Phenolic accumulation and peroxidase activity in *in vitro* selected alfalfa callus cultures resistant to filtrate of *Fusarium* spp.

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Abstract

Changes in the phenylalanine ammonia-lyase (PAL) activity, accumulation of phenolic acids and ionically-bound peroxidase activity in the *in vitro* selected embryogenic and nonembryogenic *Medicago sativa* callus cultures resistant to the filtrate of *Fusarium* spp. were found. The PAL activity in both *in vitro* selected cultures during a 4-week cultivation on a medium with phytotoxins was higher than in the control calli grown on a medium without toxin. The filtrate from *Fusarium* spp. evoked an increase in the contents of all determined phenolic acids in the selected calli. They occurred predominantly bound as esters. The most pronounced portions in the elevated acids level were of ester-bound *p*-hydroxybenzoic, vanillic, p-coumaric and ferulic acids. The ionic cell wall-bound peroxidase activity in both selected calli cultivated on a medium with a filtrate was twice as high as the activity determined in the control cultures. The activity of soluble peroxidase was not influenced by challenge with a filtrate. No significant differences were found between the *in vitro* selected embryogenic and nonembryogenic alfalfa callus cultures in the response to the phytotoxic filtrate.

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Abbreviations: EC - embryogenic callus culture, NEC - nonembryogenic callus culture, PAL - L-phenylalanine ammonia-lyase, AA - anisic acid, CA - cinnamic acid, *p*CA - *p*-coumaric acid, FA - ferulic acid, *p*HBA - *p*-hydroxybenzoic acid, SaA - salicylic acid, SiA - sinapic acid, VA - vanillic acid, PhA - phenolic acids, HPLC - High-Performance Liquid Chromatography, FM - fresh mass.

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Introduction

The complex response of the plant to various stresses includes changes in phenylpropanoid metabolism and an increase in the peroxidase activity. The commonly reported induction of L-phenylalanine ammonia-lyase activity following a fungal infection indicates the important role of this phenolic biosynthesis regulating the enzyme in an invaded tissue (Pascholati *et al.* 1985, Farmer 1985, Jorrin and Dixon 1990). The function of an increased accumulation of phenolic substances accompanying the response of different plant tissues to bacterial and fungal pathogens is linked to both direct antifungal effects (phytoalexins) and formation of a more effective mechanical barrier against the penetrating pathogen. A rapid accumulation of isoflavonoid phytoalexins in the area of attempted penetration is a major defence response of leguminous plants to a fungal infection (Dixon 1986). The response to a fungal elicitor also comprises an increase in the amount of the phenolic acids ester-bound to a cell wall (Bolwell *et al.* 1985) that, together with the induced lignification, may effectively localize the pathogen (Latunde-Dada and Lucas 1986).

A brown discolouration of the infection sites, usually followed by the cell death in a "hypersensitive response", represents another very obvious response of the pathogen infected plants. The brown pigments melanins originate from the oxidative polymerization of phenolic compounds (Mayer and Harel 1979). The formation of these small necrotic local lesions is accompanied by a distinctly increased phenolic biosynthesis (Legrand *et al.* 1976) and increased peroxidase activity, the highest increase occurring at the lesion margin (Van Loon and Ceelen 1971).

Another very important role of peroxidase during pathogenesis is its participation in the formation of a mechanical barrier that may effectively localize the pathogen. The peroxidases present in the intercellular spaces and ionically-bound to cell walls are involved in the polymerization of phenylpropanoid lignin precursors (Harkin and Obst 1973) and oxidize ester-bound phenolic acids (mostly ferulic esters) that are to a higher extent incorporated in the cell wall material of the challenged plant cell. The peroxidase catalysed oxidative coupling of phenolic esters enables a cross-link of polysaccharide and glycoprotein molecules (to which the phenolic acids are bound), thus stabilizing the cell wall (Fry 1986, Gaspar *et al.* 1986). The peroxidase activity and resistance do not seem to be directly related; however, peroxidase may well affect the extent of the resistance expressed (Van Loon 1986).

Phenylpropanoids are known to be also involved in the processes of differentiation and organogenesis (Beaudoin-Eagan and Thorpe 1984). Although both the enhanced shikimic pathway enzymes and their intermediates and the increased phenolic compounds level in various organogenesis-active systems were reported (Mato *et al.* 1988), the role of phenolics still remains unclear.

In this paper we report the changes in phenylpropanoid metabolism and peroxidase activity observed in the *in vitro* selected alfalfa callus cultures resistant to the filtrate of *Fusarium* spp. Both embryogenic and nonembryogenic alfalfa calli (differing in phenolic acid levels) appear to be suitable systems for studying the response of tissues with different potentials for phenolic biosynthesis to the fungal irritant.

Materials and methods

Plant material: A highly embryogenic and nonembryogenic callus cultures of alfalfa (*Medicago sativa* L.) were derived from petiole or leaf explants of a genotype G_{13} sensitive to *Fusarium oxysporum* as described by Binarová and Novák (1985). Toxin less sensitive cell lines were selected *in vitro* on culture filtrates of *F. oxysporum* and *F. solani*. The toxicity of various filtrates was determined by a laboratory method (Nedělník 1988). Growth inhibiting concentrations of filtrates were used for selection of G_{13} in cell suspension culture (Binarová *et al.* 1990). The embryogenic and nonembryogenic calli grown on the basic medium of Blaydes (1966) complemented by 5 μM 2,4D and 1 μM kinetin and cultivated at 25 °C under 10/14 light/dark cycles, were used as controls. The calli treated with phytotoxin were grown on a medium containing 15 % toxic filtrate of *F. solani*, isolate 147. The cultures were subcultivated after a 4-week cultivation on control and 15 % toxic filtrate containing media and 4-week old calli were analysed.

Peroxidase extraction and assay: The calli cells (0.5 g) were resuspended into 100 mM $CaCl_2$ solution, shaken for 5 min and then the suspension was vacuum filtrated. The extracellular wall bound peroxidase activity was determined in the filtrate after a dialysis against distilled water. A soluble fraction of peroxidase was obtained from the cells after calcium chloride extraction. The cells were washed with deionized water for several times, ground in N_2 liquid and homogenized in a 100 mM phosphate buffer (pH 6.5) with insoluble polyvinylpyrrolidone 1 g g^{-1}(tissue). The homogenate was centrifuged at 4 °C for 20 min at 14 000 *g*. The peroxidase activity was determined in crude extract, and the assay was performed at 25 °C in 2 cm^3 of 50 mM acetate buffer (pH 5.5) containing 13 mM guaiacol and 5 mM H_2O_2. The increase in absorbance was recorded at 470 nm with *Specol 20*.

PAL extraction and assay: PAL was extracted and its specific activity determined by the method described by Meravý (1979). The amount of the enzyme catalyzing the formation of 1 230 μmol of cinnamic acid within 1 h at 40 °C was defined as one enzyme unit.

Determination of total phenolic substances: Total phenolics were estimated by Folin-Ciocalteau reagent (Bray and Thorpe 1954) using gallic acid as a standard.

Determination of phenolic acids: Phenolic acids were extracted as described earlier (Cvikrová *et al.* 1988). Briefly: Free (F_1) and ester-bound (F_2) phenolic acids (released after an alkaline hydrolysis) were obtained from the methanol extract of the tissue grinded in fluid nitrogen. The third fraction (F_3) was obtained after the alkaline hydrolysis of the residual tissue after methanol extraction. Phenolic acids were analysed by HPLC using *Pye Unicam PU 4002-Video Liquid Chromatograph* on a column (250 × 4.6 mm) prepacked with *Spherisorb 5 ODS*.

Elution: at a flow rate of 0.5 cm^3 min^{-1} linear gradient elution within 70 min from 10 to 35 % B, then for 15 min from 35 to 50 % B, for 5 min 50 to 100 % B, 5 min 100 % B and 5 min from 100-10 % B, solvent A = 5 mM citric acid + 5 mM sodium

dihydrogen orthophosphate + 0.3 mM caprylic acid (adjusted to pH 1.5 by sodium hydroxide); solvent B = 80 % methanol; column eluate was monitored at 260 and 300 nm using *Multichannel detector PU 4021*; temperature 40 °C.

Statistical evaluation of results: The presented values of HPLC analyses of phenolic acids are the means of three determinations with the deviations not exceeding 10 to 15 % in one experiment. The pattern of changes was similar in both independent experiments.

Results and discussion

The cultivation of EC and NEC filtrate sensitive controls on a medium with a toxic filtrate mostly resulted in a brown discolouration of the cultures, and after a short time of cultivatings it was followed by the cell death. The brown melanins are most probably the products of the oxidation of the phenolic substances which were synthesized and accumulated in a defence response of the cells to the filtrate (Mayer and Harel 1979).

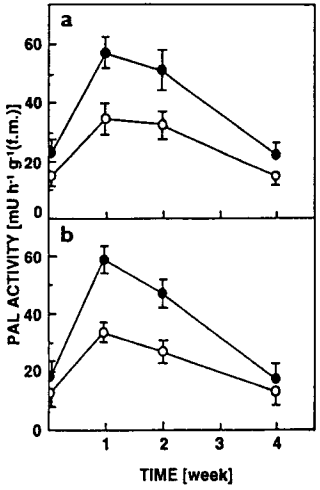

Fig. 1. Activity of phenylalanine ammonia-lyase in embryogenic (*A*) and nonembryogenic (*B*) alfalfa calli during four week cultivation. *full symbols* - *in vitro* selected resistant calli grown on a medium with a filtrate of *Fusarium* spp., *empty symbols* - control calli grown on a medium without a filtrate of *Fusarium* spp.

The development of certain degree of resistance of the *in vitro* selected alfalfa EC and NEC to the phytotoxins of *Fusarium* spp. is connected with the changes in the biosynthesis of phenolic substances and their accumulation and with the alterations in the peroxidase activity.

The PAL activity in the selected EC and NEC during a 4-week cultivation on a medium with phytotoxins was higher than in the control calli grown on a medium

without phytotoxins. The pattern of changes in the enzyme activity during the cultivation period was similar in both the control and filtrate treated cultures (Fig. 1) and it resembled the course of the PAL activity seen in the callus culture of the *Nicotiana tabacum* (Zádor *et al.* 1985). The enzyme activity culminated in the first week after inoculation, and the maximum PAL activity in the selected cultures was approximately twice as high as in the control calli. PAL catalyzes the first step in the biosynthesis of a whole range of phenylpropanoid secondary compounds which are synthesized in the response of both the intact tissue and cell cultures to the fungal infection or treatment with the elicitors present in the culture filtrates (Ebel 1986, Dixon 1986). The rate of increase in the PAL activity was also correlated with the resistance of alfalfa callus lines to the infection with *Verticillium albo-atrum* (Latunde-Dada *et al.* 1987).

Table 1. Total contents of phenolic substances in 4-week *in vitro* selected toxin resistant alfalfa callus cultures and in controls [μg g^{-1} (FM)]. The values are average ± S.D. of three experiments with two replicates in each experiment.

EC control	EC filtrate	NEC control	NEC filtrate
43.4 ± 3.4	71.6 ± 6.4	27.2 ± 2.1	53.2 ± 3.2

The stimulated phenolic biosynthesis was accompanied by an increased level of phenolic substances in both *in vitro* selected cultures (determined by Folin-Ciocalteau reagent, Table 1). The total contents of phenolic substances were markedly elevated in both EC and NEC (about 65 % and 95 %, respectively). The transitory accumulation of soluble phenols in the tomato plants inoculated with *Fusarium oxysporum* was already reported (Ferraris *et al.* 1987); however, without an evidence of its direct participation in the resistance mechanism. In contrast, cercosporin, a toxin isolated from fungus *Cercospora canescens*, had a reverse effect on the polyphenol metabolism in the mungbean, *i.e.* it resulted in a decrease in total phenols, orthodihydroxyphenols and flavonols, thus inhibiting the plant defence response (Bajaj *et al.* 1985). HPLC analysis of phenolic acids divided into three

Table 2. Content of phenolic acids in the *in vitro* selected toxin resistant alfalfa callus cultures cultivated on a medium with a filtrate and in the controls after a four week cultivation. The values represent the sum of all three determined fractions.

	Phenolic acids [μg g^{-1} (FM)]								
	*p*HBA	VA	SaA	AA	*p*CA	FA	SiA	CA	Σ PhA
EC cont.	2.782	1.260	-	0.219	1.505	3.376	0.521	0.323	9.989
EC filt.	6.008	2.546	0.419	0.450	3.206	6.683	0.868	0.479	20.659
NEC cont.	2.766	0.225	-	-	0.711	2.465	0.635	0.025	6.827
NEC filt.	5.831	0.524	0.363	-	1.664	6.292	1.259	0.097	16.030

fractions (free, soluble ester-bound and ester-bound to cells walls) confirmed our results obtained with Folin-Ciocalteau reagent (Table 2, Fig. 2). The phenolic accumulation in NEC was more intense but because of a lower basal level it did not reach the value of Ec. The filtrate obtained from *Fusarium* spp. evoked an increase in the contents of all the determined phenolic acids in selected NEC and EC. The acids occured predominantly bound as esters and their portion in the elevated acid level was pronounced. The esters of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids rose by 230 %, 160 %, 115 % and 90 %, respectively, in selected EC, *vs.* 115 %, 280 %, 160 % and 170 %, respectively, in NEC (Fig. 2).

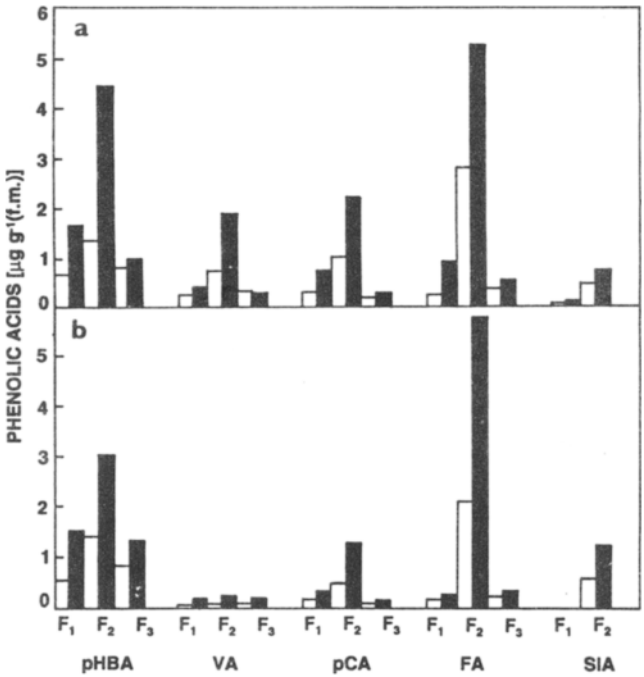

Fig. 2. Fraction contents of phenolic acids extracted from 4-week *in vitro* selected EC (*A*) and NEC (*B*) alfalfa callus cultures grown on a medium with a filtrate of *Fusarium* spp. and from controls. F_1 - free phenolic acids, F_2 - ester-bound phenolic acids, F_3 - phenolic acids bound to cell structures (*full columns* - *in vitro* selected calli, *empty columns* - control calli).

The appearence of salicylic acid in both selected cultures may correspond with its role in the defence mechanism. The manifestation of the alfalfa callus response to the filtrate irritant appears to be similar to that one of the cultured carrot cells treated with the mycelial walls of *Chaetonium globosum*. After stimulated PAL activity the increased levels of *p*-hydroxybenzoic, caffeic and ferulic acids were determined and were considered to be an early stage in the lignification of cell walls (Kurosaki *et al.*

1986). The infection with *Fusarium oxysporum* induced the formation of fungistatic phytoalexins in both the susceptible and resistant carnations. However, susceptible plants accumulated more of some lower phenolics instead of phytoalexins (Niemann and Baayen 1988).

It is speculated that a rapid increase in the peroxidase activity is part of the plants defence response to pathogens. The extracellular peroxidases, which include the enzymes ionic-bound to cell walls, are known to be involved in the polymerization of lignin precursors and in the cross-linking of polysaccharides in the walls of elicited cells. The stimulation of phenylpropanoid biosynthesis and the accumulation of phenolics coincide with the increase in the peroxidase activity (Van Loon 1986).

Table 3. Peroxidase activity in the *in vitro* selected toxin resistant alfalfa callus cultures and in the controls. The values are average ± S.D. of four different experiments with three replicates in each experiment. IP - ionically bound peroxidase, SP - soluble peroxidase

	Peroxidase activity [ΔA_{470} g^{-1}(FM) min^{-1}]	
	IP	SP
EC control	1436 ± 44	3985 ± 119
EC filtrate	3018 ± 105	3840 ± 122
NEC control	1368 ± 38	6195 ± 179
NEC filtrate	3597 ± 125	5067 ± 152

The induction of ionic cell wall-bound peroxidase activity occurred in our *in vitro* selected NEC and EC cultures cultivated on a medium with a filtrate. The enzymatic activities in both selected cultures were twice as high as the activities determined in the control calli; only insignificant differences were found between EC and NEC (Table 3). The activity of soluble peroxidase was not influenced by a challenge with the filtrate and the different values in EC and NEC may be due to differences in the metabolisms of these cultures. It was shown earlier that extracellular peroxidases are more sensitive to oxidant stress than those extracted from cells (Castillo *et al.* 1984). Besides the role of peroxidases in the stabilizing of cell walls (lignification and oxidative coupling of phenolic esters), the products of phenolic oxidation are toxic and may inhibit the extracellular enzymes of pathogens (Mayer 1987) or kill the plant tissue (lethal "browning") and so confine the pathogen to the site of infection (Van Loon 1986).

One of the causes of the death of sensitive control cultures during the cultivation on a medium with a filtrate may be a high accumulation of phenolic substances and their quick oxidation in the defence response. In the *in vitro* selected cultures we also found a raised synthesis and accumulation of phenolics and an increased ionically-bound peroxidase activity (characteristics of defence response), but the cells seem to be capable of withstanding the elevated level of phenolics.

In conclusion, we can summarize that the alterations in the metabolism of phenolics and the ionically-bound peroxidase activity are part of the metabolic changes contributing to the development of a certain degree of resistance of *in vitro* selected alfalfa cultures.

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