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Passive defense in somatic embryos of *Abies alba* from two cell lines tested with *Phaeolus schweinitzii*

Abstract: Differential inhibition in growth of fungus *Phaeolus schweinitzii* inoculum was tested with somatic embryos of two cell lines of silver fir (*Abies alba* Mill.) by the method of dual cultures with pre-cultivation of somatic embryos and subsequent removal. It was confirmed that inhibitory substances from pre-cultivated somatic embryos L27 spreading to agar cultivation medium by simple diffusion cause greater inhibition of tester mycelial growth during subsequent co-cultivation compared to somatic embryos L122. Characteristics of embryogenic cell lines L27 and L122, from which somatic embryos were formed, are provided. Increased levels of peroxidase activity characterized the cell line L27. Natural non-induced constitutive defense of plant somatic embryos established by quantitatively measured restriction of growth in tester *Phaeolus schweinitzii* was discussed.

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Introduction

Defense is a common strategy in plants which, being immobile, have difficulty in avoiding their enemies. Plants contain an extremely rich and diverse array of chemicals. Many of these chemicals were originally thought to be secondary by-products of cellular mechanism, hence their common name secondary plant metabolites (Berryman 1988). There are two basic approaches to defense: passive or constitutive defense, which is analogous to fortification, and dynamic or induced defense, which is more similar to counter attack (Cowling and Horsfall 1980).

The report of using embryogenic tissue lines in studying host-parasite interaction between Scots pine and *Gremmeniella abietina* was presented by Terho et al. (2000). The role of *Pinus nigra* lectin (PNL) during initial stages of the host-parasite interaction was described by Nahálková et al. (2001).

In our laboratory, defense reactions of conifers, such as *Pinus nigra* megagametophyte in dual cultures with the basidiomycete *Phaeolus schweinitzii* were studied. Strong defense reaction of the megagametophyte against the fungus was observed in pre-cultivated cultures. After megagametophyte removal, the inhibition of mycelial growth by diffused

substances (DS) was stronger than in the case of simultaneous inoculation of megagametophytes and the fungus. These results indicate that substances diffused into the agar medium are responsible for defense reactions *in vitro* (Hřib et al. 1996). Differential mycelium growth of the fungus *Phaeolus schweinitzii* was observed in the presence of *Pinus nigra* embryogenic and non-embryogenic calli. We have evidence that defense system in European black pine is probably formed at the very early stage of somatic embryo development (Hřib et al. 1995). Defence reactions of embryonal suspensor mass, precotyledonary, cotyledonary and desicated cotyledonary somatic embryos of Algerian fir (*Abies numidica*) were tested by dual cultures with fungus *Phaeolus schweinitzii* (Vooková et al. 2006).

SDS analysis showed differences in the accumulation of low molecular weight storage proteins in hybrid fir somatic embryos (Salaj et al. 2004). Storage proteins can play an important role in the defense of embryos (Vooková et al. 2006).

Objective of this study is to verify presence and quantify magnitude of a non-induced passive defense in somatic embryos of two silver fir cell lines producing substances which diffuse in medium during pre-cultivation with tester fungus *Phaeolus schweinitzii* in dual cultures. Detection of non-induced passive defense of *Abies alba* against fungal tester *Phaeolus schweinitzii* can provide helpful information unavailable in previous reports.

For better understanding the differences in defense reactions of somatic embryos L27 and L122, guaiacol peroxidase activity analysis and total protein concentration were determined.

Material and methods

Plant material

The embryogenic cell lines L27 and L122 were initiated from different immature zygotic embryos of silver fir (*Abies alba Mill.*). Immature cones were collected on July 26th from open-pollinated family D5 tree in Dobroč primeval. Induction of embryogenic tissue on SH medium (Schenk and Hildebrandt 1972) supplemented with 5 μM 6-benzylaminopurine (BAP), and maturation of somatic embryos on modified MS medium (Murashige and Skoog 1962) supplemented with 40 g/l maltose, 100 g/l polyethylene glycol-4000 and 40 μM abscisic acid (($\pm$) cis-trans, ABA) was published by Vooková and Kormuťák (2002). Mature somatic embryos were isolated from embryogenic tissue and placed in Petri dish (Ø 60 mm) and subjected to high relative humidity treatment. Petri dish was opened and placed on moist filter paper in Petri dish (Ø 90 mm), which was sealed with parafilm. The embryos in Petri dish were culti-

vated in the dark at 22–23°C during three weeks. In this way, desiccated cotyledonary somatic embryos obtained from cell lines L27 and L122 were used in the experiment.

Fungal tester

The fungus *Phaeolus schweinitzii* (Fr.) Pat., isolate No. 151 was used as a model tester. This strain was obtained from the Collection of Wood Destroying Fungi, J. E. Purkyně University (now Masaryk University). This culture was grown at 23±1°C in the dark on 3% malt extract and 2% Difco Bacto Agar.

Dual culture

Tests were carried out on agar MS medium without growth regulators, supplemented with 20 g/l sucrose and 10 g/l Difco Bacto Agar in Petri dishes (Ø 90 mm). The somatic embryos L27 and L122 were placed at the margin of the dishes and were cultured 14 days in the dark on the MS medium and then removed. In day 14, fungal inoculums 10×10 mm were taken from the base culture on malt agar and were inserted in the Petri dishes about 2.5 cm from the embryo imprint. Position of the somatic embryos was marked on the bottom of Petri dish by Centropen®. In the first variant, only one somatic embryo was placed. In the second variant, three somatic embryos were used. The controls contained only the fungus inoculums. The cultures were maintained at 23 ± 1°C in the dark. Experimental treatments included two factors: line of somatic embryos and number of embryos. Factor of embryo line had two levels: L27 or L122. Factor number of embryos had two levels: single embryo (E1) or three embryos (E3). Control dishes were not treated with embryos to display uninhibited growth of fungal mycelium. Dishes treated with embryo imprint were anticipated to show growth of mycelium affected by differential constraints due to line of treatment somatic embryos and number of treatment embryos. The measurements of fungal mycelium size were taken in 2-day intervals in the direction towards the embryos and in the opposite direction, starting with day zero and ending with day 10-post inoculation. Mycelium size in control dishes was measured on four peripheral spots corresponding to two perpendicular diameter axes. The measurements were taken using a mm rule in counter light from the bottom of the dish. For every combination of experimental treatments and control, four replications of Petri dish were established.

Peroxidase analysis of embryogenic tissue

Peroxidase activity was determined spectrophotometrically (Ultra Spec UV 2100, GE Healthcare) using guaiacol as substrate at 436 nm with molecular absorption coefficient 25 000 l/mol/cm (Bergmeyer

1974). The reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol and 0.15 mM hydrogen peroxide was temperated at 25°C. The enzyme reaction was started by adding 0.1 ml of the supernatant from embryogenic cell line L27 or line L122.

Protein determination of embryogenic tissue

For protein extraction, 0.5 g of embryogenic tissue of silver fir (*Abies alba*) cell lines (L27, L122) was used. After homogenization in Potter-Elvehjem homogenizator, the mixture was centrifuged at 22,000 g for 5 min. The resulting supernatant was used for the analysis. In the protein assay bicinchoninic acid was used (Smith et al. 1985). Proteins were quantified with bovine serum albumin using standard by QuantiPro BCA Assay Kit (Sigma, USA). The protein concentration was measured in three replicates and represented by mean ± standard deviation.

Statistical analysis

Early review of mycelium growth data from control and experimental cultivations pointed to a sigmoid growth process, represented by three-parametric logistic equation assumed by Koch (1975) for modeling mycelial growth

$$Y = \frac{\alpha}{1 + e^{((\beta_0 - X)/\beta_1)}} + \varepsilon$$

In this equation, Y denotes the size of mycelium [mm], α is the coefficient of upper asymptote of the logistic curve, β_0 is the coefficient representing time required to reach inflection point placed approximately at $\alpha/2$, β_1 was the scale parameter indicating time required for mycelium to grow from half to $1/(1+e^{-1}) \approx 3/4$ of the asymptotic height, X was number of cultivation days from inoculation to the mycelium size measurement and ε represented random disturbances. Interpretation of the logistic curve parameters is illustrated in Fig. 1 (Pinheiro and Bates 2000).

Estimated parameters of the logistic model were anticipated to be positive. Model coefficients, especially α and to a smaller extent β_0 and β_1 are assumed to reflect apparent inhibitory effects of diffused substances (DS) from pre-cultured somatic embryos on treated mycelium growth. Several generalized fixed and mixed models were, as a result, constructed to quantify extent of the growth inhibition in response to experimental treatment or control. A general logistic model was constructed early for the combined data. In models with experimental treatments, the parameters were allowed to fluctuate. Competing nested models were compared by generalized likelihood ratio χ^2 test to determine simultaneous signifi-
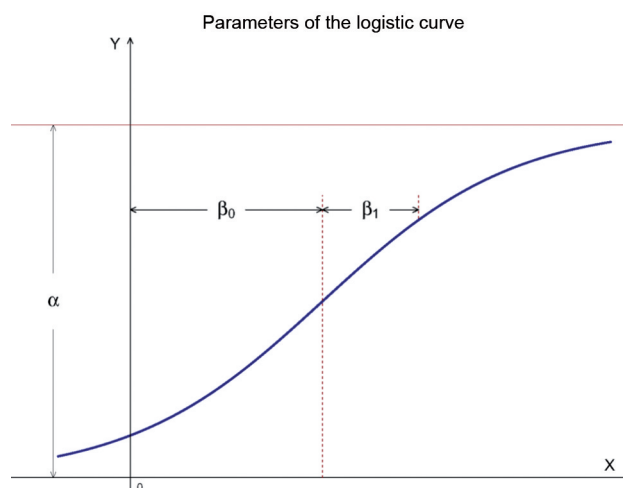

Fig. 1. Interpretation of the logistic curve parameters

cance of the added effects. Adding random effects of Petri dishes on the logistic parameters was aimed at validating random variation associated with the dishes apart from the error term. Solutions to the coefficients α, β_0 and β_1 were obtained by iterative algorithm.

Estimated parameterizations applied by statistical software due to identifiability, were linearly transformed to facilitate interpretations and allow comparisons of treatment effects. Linear combinations of the coefficients were estimated by $\beta^* = C'\beta$, where C is a matrix storing coefficients of linear parametric combinations and β is vector of the untransformed coefficients derived by software. Covariance matrix of the linear combinations was obtained by applying $Var(\beta^*) = C'Var(\beta)C$, where $Var(\beta)$ is the covariance matrix of the primary coefficients. Statistical significance of the estimates and parametric combinations was verified by t-test.

Properties of the transformed growth curves were evaluated by finding root of the equation received by taking second derivative of the model with respect to cultivation time and setting equal to zero. Maximum gradient at the point of inflection was computed by maximizing the model first derivative with respect to cultivation time. Statistical analyses and production of plots were accomplished with R software (www.r-project.org), principally the built-in *nlme* library (version 3.1-104) and *lattice* library (version 0.20-6).

Results

Embryogenic cell lines L27 and L122 differed during proliferation process. Embryogenic tissue of line L27 showed faster browning during sub-cultivation relative to embryogenic tissue line L122 (Fig. 2). These cell lines differed also in number of mature somatic embryos obtained by maturation process in 2008.
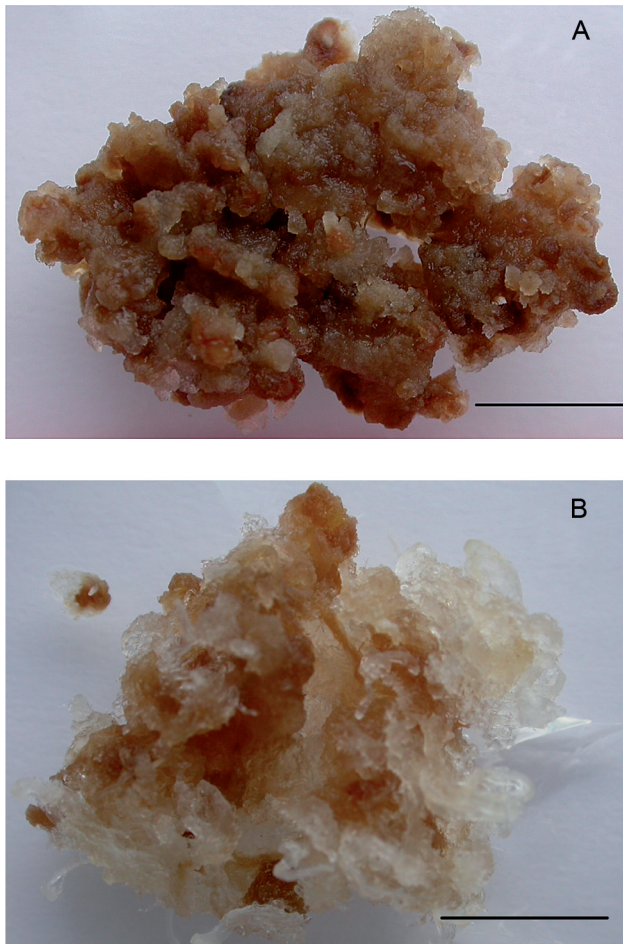
Fig. 2. Embryogenic tissues line L27(A) and line L122(B) of silver fir (*Abies alba*) after culturing for 27 days. Scale is 5 mm

Totally 74.87 ± 13.98 somatic cotyledonary embryos were obtained from 1g of L27 embryogenic tissue. This number included 37.76 ± 7.20 quality somatic embryos with fully developed radicula and at least 4 cotyledons. Cell line L122 was more productive; totally 95.86 ± 4.46 somatic embryos and 62.59 ± 8.75 quality embryos were obtained from g of
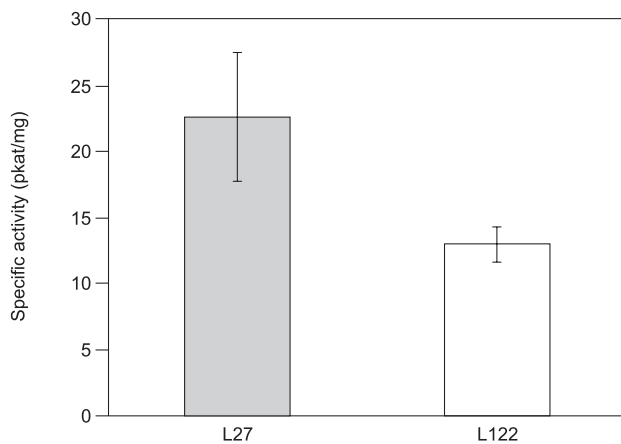

Fig. 3. Peroxidase activity of silver fir (*Abies alba*) embryogenic tissues represented by mean ± standard deviation from three replicates
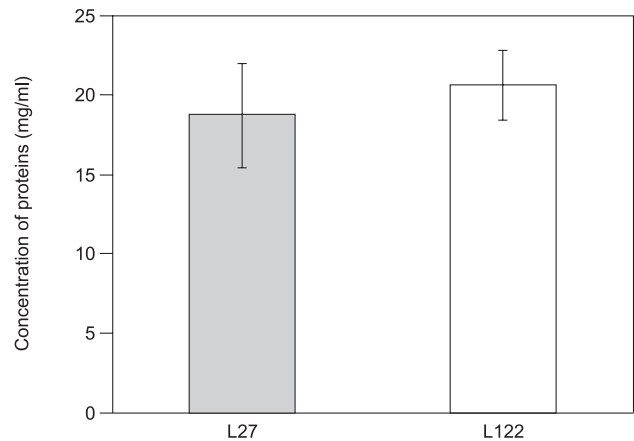

Fig 4. Concentration of proteins in embryogenic tissues of silver fir (*Abies alba*) represented by mean ± standard deviation from three replicates

embryogenic tissue. Subsequently, specific peroxidase activity in embryogenic tissue of *Abies alba* was measured. The specific peroxidase activity 22.70 ± 4.90 pkat/mg of protein was found higher in cell line L27 relative to line L122 with 13.00 ± 1.30 pkat/mg of protein (Fig. 3).

Somatic embryogenic tissue extracts from cell lines L27 and L122 were prepared in protein amounts 18.64 ± 3.24 mg/ml and 20.59 ± 2.20 mg/ml, respectively (Fig. 4).

The coefficients of logistic model obtained from the combined data, which disregard the experimental variants, line of somatic embryos and amount of DS corresponding to the number of embryos, were significantly different from zero ($P < 0.01$, Table 1). Residual standard deviation from the combined model was $\sigma_E = 2.20$. In the final nonlinear mixed-effects model with experimental treatment grouping, the estimated error standard deviation was smaller ($\sigma_E = 0.81$) compared to the combined model. The mixed model, in overall, had better fit.

In accordance with previous anticipation, the experimental treatments caused inhibition to speed of growth in Petri dishes treated with DS from different number of somatic embryos of *A. alba* in comparison to the untreated control variants. Linearly reparametrized coefficients for the experimental treatment combinations of embryo cell lines, DS from different

Table 1. Estimated logistic coefficients, residual standard deviation and associated 95% confidence limits for the general fixed-effects model

Parameter	Estimate	2.5%	97.5%
α	18.78	16.26	21.29
β_0	6.36	5.66	7.07
β_1	1.84	1.42	2.26
σ_E	2.20	1.95	2.52

f(x) at point of inflection = 9.39 mm; maximum gradient = 2.55 mm/day

Table 2. Estimated parameters of logistic curve, response at inflection point and maximum gradient at inflection point for experimental treatment fixed-effects combinations from the treatment effects model

Combination	α	β_0	β_1	f(x) at point of inflection [mm]	Maximum gradient of growth at point of inflection [mm/day]
L27:E1	15.71**	6.35**	1.83**	7.86	2.15
L27:E3	15.21**	6.35**	1.83**	7.61	2.08
L122:E1	19.64**	6.35**	1.83**	9.82	2.69
L122:E3	17.29**	6.35**	1.83**	8.65	2.37
Control	25.70**	6.35**	1.83**	12.85	3.52

Notation: **estimated coefficients significant at $\alpha = 0.01$

number of somatic embryos or untreated control and respective standard errors were deduced from the estimated model. Linearly reparametrized coefficients were statistically different from zero ($P < 0.01$, Table 2). Among logistic model coefficients, only α coefficients, representing the upper asymptotic height, showed oscillations among experimental variants. Relative to the control dishes, the presence of DS from embryos significantly reduced the height of the logistic asymptote. Parameters β_0 and β_1, on the contrary, appeared unaffected by the presence or absence of DS from plant somatic embryos during mycelium cultivation. The extent of mycelium growth suppression caused by experimental variants is depicted in Figure 4. Relative to experimental variants, control Petri dishes displayed unimpaired growth with inflection point at larger mycelium size and larger growth gradient at the inflection point.

There is indication, that presence of the DS from somatic embryos relative to control, embryo line of *Abies alba* (L27 or L122) and DS from one or three embryos slowed down the speed of mycelium growth. Impact of the experimental variants of embryo line, DS from number of somatic embryos on logistic coefficients of the upper asymptote α is presented in Table 3. Magnitude of the general inhibitory effects by DS
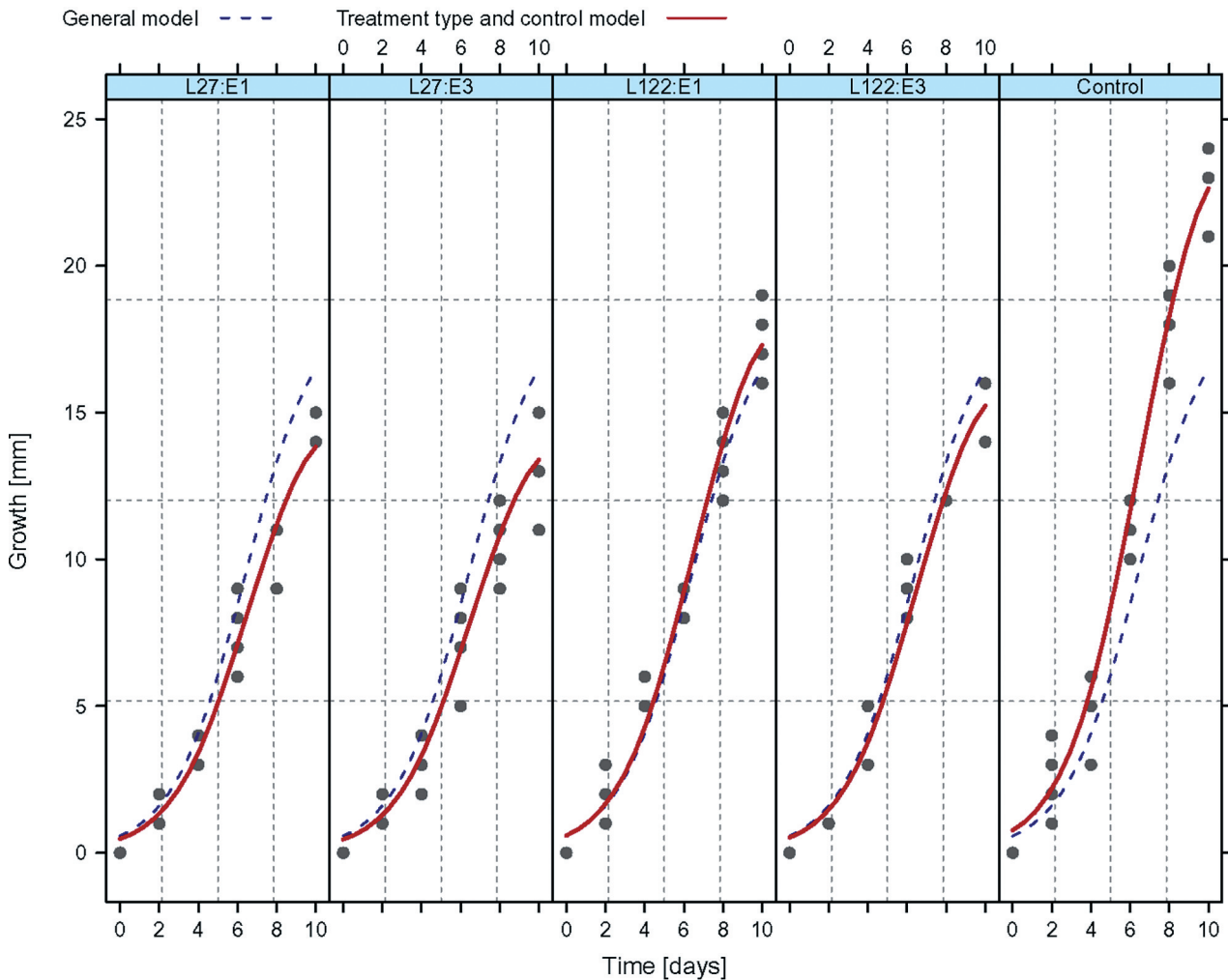

Fig. 5. Estimated logistic curves for combinations of embryo line of *A. alba* (L27 or L122) or control and number of somatic embryos (single or three inserted embryos) in the treatment or control model and the unstructured general model. Points designate actual measurements

from added somatic embryos on mycelial growth is shown in the form of tests of mean effects. It can be concluded that somatic embryos L27 provide stronger inhibitory effect on mycelium growth compared to somatic embryos L122. Also, presence of DS from three embryos in general causes a larger inhibition of mycelium growth, relative to presence of DS from just one embryo. Statistically significant averaged effects of the DS from embryo treatment, line of embryo and

Table 3. Tests of main effects for the experimental variants performed for parameter α of the logistic curves from the treatment effects model

Factor	Main effects	Estimated parametric difference in α coefficients [mm]
No inhibition/ Inhibition	Control – L27	10.24**
	Control – L122	7.24**
	Control – E1	8.03**
	Control – E3	9.45**
Number of embryos	E1 – E3	1.43*
Line of embryo	L122 – L27	3.01**

Notation: parametric difference significant at $^{..}\alpha = 0.01$ $^{.}\alpha = 0.05$

Table 4. Tests of simple effects for the experimental variants holding the other factor fixed, for parameter α of the logistic curves from the treatment effects model

Simple effects	Estimated parametric difference in α coefficients [mm]
Control – L122E1	6.06**
Control – L122E3	8.41**
Control – L27E1	9.99**
Control – L27E3	10.49**
L122E1 – L122E3	2.35*
L122E1 – L27E1	3.93**
L122E1 – L27E3	4.43**
L122E3 – L27E1	1.58[ns]
L122E3 – L27E3	2.08*
L27E1 – L27E3	0.50[ns]

Notation: difference significant at $^{..}\alpha = 0.01$, $^{.}\alpha = 0.05$ ^{ns} not significant

Table 5. Estimated random effects associated with Petri dish and random error from the treatment effects model

Source of variation	Random effect parameter	Estimate	2.5%	97.5%
Petri dish	σ_P	1.10	0.71	1.68
Random error	σ_E	0.81	0.71	0.93

number of embryos, on β_0 and β_1 coefficients of the logistic curve, however, were not confirmed.

Experimental treatment with DS from three somatic embryos produced a small differential inhibitory response in mycelial growth relative to treatment with DS from only single embryo depending on the embryo line. The mycelium inhibitory reaction to presence of DS from somatic embryos L122 is by 4.43 mm larger due to presence of DS from one somatic embryo relative to three embryos of L27 ($p < 0.01$). Statistical tests of the simple effects are presented in Table 4. Estimated random effects associated with Petri dish and random errors are shown in Table 5. Inclusion of random variation associated with Petri dish in the model was justified due to significant impact of stochastic effects of the Petri dish on the observed results of the experiment ($p < 0.01$). It can be hypothesized that variation among fungal inocula used in this *in-vitro* experiment played a significant role together with other sources of variation.

Discussion

The inhibition of mycelial growth of fungus *P. schweinitzii* on diffused substances (DS) in agar MS medium from pre-cultured somatic embryos L27 was stronger compared to somatic embryos of line L122. It is evident, that embryos L27 show more intense passive defense and / or non-induced natural defense.

Numerous results from past dual culture experiments confirmed the idea, that tester *P. schweinitzii* shows inhibition of mycelial growth on substances causing growth correlative inhibitions of tissues and organs in plant. Outline of theory describing constitutive defense system in plants on the basis of integrity was presented by Hřib (1985). Among these substances determining growth correlative inhibitions and their network in plant organism, it is primarily auxin (IAA) at higher concentrations (Hřib 1985; Hřib et al. 1999) similar to cytokinin (Hřib et al. 1993), which may influence the formation of phenolic compounds (Schnablová et al. 2006). The mechanism of growth correlative inhibitions is formed also by growth inhibitors, such as ethylene, abscisic acid and others.

It must be emphasized that observed phenolic compounds play a significant role in the defense reactions of many plants against pathogenic fungi, mainly in induced defense. This is well-documented evidence concerning quantitative changes in these compounds caused by a pathogen (Farkas and Kiraly 1962, Cruickshank and Perrin 1964), as well as changes in oxidative-reductive activity of the tissue. Increase of the oxidative activity leads to accumulation of quinones and quinone-like derivatives, which have higher fungi toxic activity.

Peroxidase and polyphenol oxidase are directly involved in the defense reactions of resistant plants against fungi (Rubin and Artsikhovskaya 1964, Lehrer 1969) and others. Also, in our experiments, increased activity of peroxidase, a manifestation of browning after 27 days in embryogenic line L27 was observed, compared to line L122. Second part of the study confirmed higher passive defense of somatic embryos of line L27 compared to line L122 tested with *P. schweinitzii* due to substances produced by the embryos and diffused in the medium.

The peroxidase and polyphenol oxidase activities and their relationship to browning in such cultures were compared with pine embryogenic and non-embryogenic cultures (Laukkanen et al. 1999). Visible browning was a typical feature of callus cultures derived from shoots tips of mature Scots pine (*Pinus sylvestris L.*). Accumulation of phenolic substances was shown by histochemical staining. In the experiment, peroxidase activity increased rapidly soon after the start of the culture period, and especially between the second and third weeks of culture. They conclude that browning is associated with cell changes. Callus cultures from shoot tips were characterized by rapid browning and inability to regenerate (Laukkanen et al. 2000).

An interesting experiment was carried out by Dowd and Norton (1995). Callus of the fungus and insects resistant corn inbred, Mp313E of differing ages and degrees of brownness was tested for effect on neonate larvae of the corn earworm, *Helicoverpa zea*. Older, browner, 8 weeks old callus caused significantly higher mortality of *H. zea* after two days compared to larvae fed 5 weeks old callus (brown or not) or pinto bean diet. Larvae fed on the 5 weeks old calli were significantly smaller after nine days compared to those fed on nonbrown callus of the same age. Corn allelochemicals associated with cell walls, were oxidized, i.e. produced a brown product, up to 10-fold more rapidly by peroxidase preparations of the brown callus compared to nonbrown callus. This information suggests the peroxidases are contributing to the browning through oxidation of phenolic acids and thereby enhancing resistance to insects.

Generally, enzymatic browning occurs mainly due to the oxidation of natural phenolic compounds to quinones, which in turn are polymerized to brown or dark pigments (Valentines et al. 2005). These pigments are mostly melanin-based. Melanin is commonly thought of as the agent that protects numerous life forms (Pugh et al. 2005), although melanin has been identified previously within plants (Kamei et al. 1997). The structural characterization of melanin polymers has been notoriously difficult due to their general insolubility of most solvents and because the subunits are linked by non-hydrolysable bonds.

It has been suggested that there is an association between peroxidase and the metabolism of aromatic compounds, plant hormones and lignification (Butt 1980). Kansagra et al. (2008) concluded that anionic peroxidase plays an important role in browning of the tissue.

In vivo, auxin oxidase is a complex consisting of peroxidase and number of effectors which regulate its activity. These effects involve mineral substances, phenols, cumarins, IAA protectors and organic acids. Auxin protectors occur especially in juvenile tissues. These phenolic compounds are also produced in wounded tissues (Šebánek et al. 1991).

Production of indole-3-acetic acid (IAA) and melanin by root-nodule bacteria *Rhizobium*-like strains described by Pongsilp and Nuntagij (2009). It is interesting that authors observed melanin production and 21.92 ± 1.67 μg IAA equivalent per ml culture in strain DASA 57003 (positive strain) and only 12.88 ± 2.45 μg IAA equivalent per ml culture in DASA 57053 (negative strain) where no melanin production was observed.

In future, it is our intention to perform testing of passive defense in regenerants from lines L27 and L122 by the method of dual cultures with pre-cultivation of emblings and subsequent removal with tester *P. schweinitzii*.

Acknowledgements

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