Testing of Algerian fir zygotic and somatic embryos on defence reactions in vitro

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ABSTRACT

Defence reactions of desiccated cotyledonary somatic embryos and mature zygotic embryos of *Abies numidica* were tested by dual cultures with tester, fungus *Phaeolus schweinitzii*. Both types of embryos expressed defence reactions. Mutual comparisons of zygotic and somatic embryos showed important differences between defence reactions against mycelial growth towards these embryos. Greater defence reactions were observed in zygotic embryos relative to defence found in somatic embryos.

Keywords: *Abies numidica*; mycelial growth; *Phaeolus*; dual cultures

Conifer somatic embryos, obtained from somatic embryogenesis, morphologically resemble zygotic embryos. Data of several authors indicate similar or identical storage proteins contents in somatic and zygotic embryos (Klimaszewska et al. 2004, Kormuťák and Vooková 2005). Marked differences were observed in carbohydrate spectra between developing seeds and maturing somatic embryos of *Picea abies* (Gösslová et al. 2001). The decrease in total carbohydrate as well as the accumulation of sucrose in later developing stages was a common features in both systems (Konrádová et al. 2002). The concentrations of total lipids exhibited marked variation during maturation of *P. abies* somatic embryos, indicating the importance of lipid reserves during embryo development (Svobodová et al. 1999, Grigová et al. 2007).

To study the quality of the produced somatic embryos under various treatments a progression in building up knowledge of biochemical events is logical. Consequently, production of viable artificial seeds can be achieved (Klimaszewska et al. 2004). From this point of view, it is also interesting to understand defence reactions of somatic embryos. Use of in vitro cultured embryos in breeding programmes and the incorporation of other tissue culture techniques, such as somatic embryogenesis in the identification of resistance mechanisms in tree species, would provide a rapid method of screening genotypes for susceptibility to pathogens and the subsequent rapid clonal propagation of resistant individuals (Daub 1986). The using embryogenic tissue cultures in studying host-parasite interaction between Scots pine (*Pinus sylvestris* L.) and *Gremmeniella abietina* reported Terho et al. (2000). In our laboratory, defence reactions of conifer zygotic embryos were studied in 1995 (Hřib et al. 1995a). This work described defence reactions of zygotic embryos and megagametophyte of *Pinus nigra* Arn. Basidiomycete *Phaeolus schweinitzii* was successfully used as tester for in vitro study of defence reactions. *P. nigra* seeds presented defence reaction when growth of mycelium was relatively intensively inhibited in presence of single or two embryos. From testing of embryogenic and non-embryogenic callus tissue of *P. nigra* and *Abies alba* resulted that the constitutive defence system of plant had already been formed in early developmental stage of embryogenesis (Hřib et al. 1995b). The same result was confirmed when

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defence reactions of developing somatic embryos of *A. numidica* were studied against the tester *Ph. schweinitzii* (Vooková et al. 2006). In continuation of these experiments, the present study of growth of *Ph. schweinitzii* mycelium in presence of *A. numidica* zygotic and somatic embryos was undertaken. The objective of the study was to describe and compare dynamics of defence reactions in somatic and zygotic embryos to a plant pathogenic fungus *Ph. schweinitzii* over time.

MATERIAL AND METHODS

Plant material. Zygotic and somatic embryos of *Abies numidica* De Lannoy ex Carrière were used as a plant material. Zygotic embryos were extirpated from seeds collected from open pollinated trees in Banská Štiavnica, Slovakia. They were sterilised in 70% (v/v) ethanol for 30 s followed by 15 min treatment in 0.1% (v/v) $HgCl_2$. Mature desiccated somatic embryos of *A. numidica* were obtained from embryogenic cell line L12 induced from immature zygotic embryo (Vooková et al. 2001). Selected mature somatic embryos with at least four cotyledons were used in the experiment.

Defence reactions. Defence reactions of mature desiccated cotyledonary somatic embryo and zygotic embryo of *A. numidica* (Figure 1) were tested by simple method of dual cultures. One somatic or one zygotic embryo were co-cultured with inoculum of basidiomycete *Ph. schweinitzii* (Fr.) Pat., which was used as a tester of defence reactions.

The tester, *Ph. schweinitzii*, isolate No. 151 was obtained from Collection of Wood Destroying Fungi, 1969, J.E. Purkyně University (now Masaryk University) in Brno, Czech Republic. The culture was grown at a temperature of 24 ± 1°C in the dark on 3% malt extract and 2% agar.

The growth responses of *Ph. schweinitzii* mycelium to the embryos were studied in Petri dishes (Ø 90 mm) on agar B-25 medium (Hřib and Rypáček 1981) without growth regulators (Table 1). One mature somatic or one zygotic embryo were placed on medium at the margin of the dish and cultured in the dark. Inoculation of mycelium was done after 6 days of cultivation. A 10 × 10 mm pieces of the fungus were taken from the margin of the basic culture on malt agar and placed at a distance of about 2.5 cm from the embryo. The control dishes were inoculated only with the fungus. The cultures were incubated at 24°C in the dark. Substrate mycelium of *Ph. schweinitzii* was very fine, especially hyphae growing at the margin of the fungal colonies. Therefore mycelium growth was measured by the ruler from below of the Petri dish against the light background in 2-day intervals. The fungal mycelium was measured in both directions, e.g. towards the embryo and in reverse direction from the embryo. Control dishes were measured on four peripheral spots corresponding to two perpendicular diameter axes. Each Petri dish was replicated four times. The whole experiment was repeated twice.

Statistical analyses. The objective of statistical analyses was to describe growth in fungal tester reflecting defence reactions between zygotic and somatic embryos and the control (without embryo) and also between the variants reflecting side of embryo placement (towards and opposite to embryo) as a function of time from zero to ten days post inoculation.

An early inspection of raw mycelium data suggested sigmoid growth process, described by three-parametric logistic formula, as assumed by Koch (1975) for mycelial growth. Interpretations of the logistic parameters are illustrated in Figure 2.

It is assumed that the experiment follows 2 × 3 factorial design with replications. It is hypothesized,

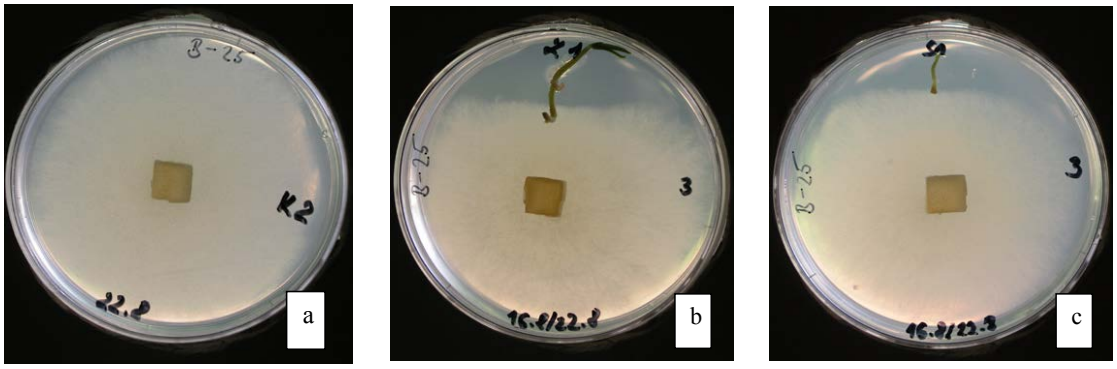

Figure 1. Control (a), zygotic (b) and somatic (c) embryos of *A. numidica* exhibited defence reactions as manifested by inhibition of mycelial growth of the tester *Ph. schweinitzii*

Table 1. Composition of B-25 medium used in dual culture of embryos with fungus inoculum

Inorganic nutrients (mg/L)	Organic supplements (mg/L)
NH_4NO_3 1 650.0	nicotinic acid 0.50
KNO_3 1 900.0	pyridoxine HCl 0.10
$CaCl_2 \cdot 2\ H_2O$ 440.0	thiamine HCl 0.10
$MgSO_4 \cdot 7\ H_2O$ 370.0	myo-Inositol 100.0
KH_2PO_4 170.0	asparagine 100.0
H_3BO_3 6.20	sucrose 30 000.0 (3%)
$MnSO_4 \cdot 4\ H_2O$ 16.90	bacto agar 10 000.0 (1%)
Zn $SO_4 \cdot 7\ H_2O$ 10.60	
KJ 0.83	pH 5.6
$Na_2MoO_4 \cdot 2\ H_2O$ 0.25	
$CuSO_4 \cdot 5\ H_2O$ 0.025	
$CoCl_2 \cdot 6\ H_2O$ 0.025	
Fe/as Fe EDTA/5.60*	

*5 mL/L of a stock solution containing 5.57 g $FeSO_4 \cdot 7\ H_2O$ and 7.45 g Na_2EDTA per litre of H_2O

that the model coefficients, primarily α, are likely to diverge, depending on all or some of the sources of variation. Several variants of fixed models were, for this reason, prepared to prove magnitude of defence reactions in mycelium in response to embryo type and control and sides of measurement (towards or opposite to embryo). A general logistic model was fitted early to the combined data. In more complex models, the coefficients were allowed to vary depending on embryo types and sides of measurement. Competing nested models were compared by likelihood ratio chi-square test to establish significance of the added terms. Estimates of the coefficients α, β_0 and β_1 were obtained by iterative Gauss-Newton algorithm. The process of iteration to convergence was traced.

Estimated coefficients in parametrizations applied by statistical software due to issues of identifiability, were linearly transformed to improve interpretations and enable intelligible comparisons of model effects. Linear combinations of the parameters were calculated from:

$$\beta^* = C'\beta$$

Where: C – a matrix storing coefficients of linear contrasts; β – vector of estimated non-transformed coefficients.

Transformed covariance matrix was obtained by applying:

$$Var(\beta^*) = C'Var(\beta)C$$

Where: Var(β) – covariance matrix of the primary coefficients. Statistical significance of the transformed coefficients was verified by *t*-test. Statistical analysis and construction of plots were carried out with R software (www.r-project.org) particularly the nlme library, version 3.1-95.

RESULTS AND DISCUSSION

Zygotic and somatic embryo of *A. numidica* exhibited defence reactions as manifested by inhibition of mycelial growth of the tester *Ph. schweinitzii* (Figure 1). Greater defence reactions of about 12% were observed in zygotic embryo relative to somatic embryo. The inhibition was contingent upon age post inoculation in all cultures. Early defence reactions were mainly evident in tester growth towards zygotic embryos, starting second day post inoculation of fungus. Coefficients of logistic growth curves in control (the only mycelial growth of tester) and experimental variants (zygotic or somatic embryo of *A. numidica*) were estimated by iterative procedure after reaching convergence with the criterion 10^{-6}. The coefficients of logistic regression obtained from the combined data, which disregard the experimental grouping (Table 2) were statistically significant from zero ($P < 0.01$). In the final model with experimental

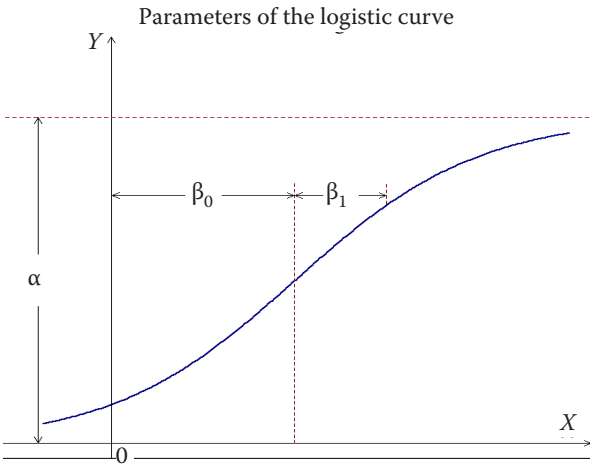

Figure 2. Interpretations of the parameters of the logistic curve:

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

Y was observed size of mycelium (mm), α was the coefficient of upper asymptote, β_0 was the coefficient representing time required to reach inflection point sited approximately at α/2, β_1 was the scale parameter indicating time required for mycelium to grow from half to of the asymptotic height, *X* was cultivation time of mycelium after inoculation in days and ε represented random disturbances

Table 2. Estimated coefficients and associated 95% confidence limits obtained from the general model of combined data

Parameter	Estimate	2.5%	97.5%
α	30.77	29.14	32.40
β_0	4.43	4.12	4.73
β_1	1.51	1.27	1.76
σ_e	3.57	3.20	4.04

$f(x)$ at point of inflection = 15.39 mm; maximum gradient = 5.08 mm/day

grouping, the residual standard deviation was smaller (σ = 1.47). In the experimental variants, speed of growth measured in the opposite direction from the embryo reached larger levels also in comparison to the control variants. Linearly reparametrized coefficients for zygotic and somatic embryo of *A. numidica* and control and sides level combinations and respective standard errors were deduced from the original model (Table 3).

There is evidence, that presence of the embryo relative to control, embryo type (zygotic and somatic embryo of *A. numidica*) and sides of the measurement (towards or opposite to embryo) influenced the mycelium growth. Impact of the averaged effects of sides (towards, opposite to embryo) on all logistic coefficients is illustrated in Table 4. Opposite variants had on average significantly higher upper asymptotes, required fewer days to reach the inflection point and needed fewer days to reach ¾ of the asymptote. Augmented speed of growth in the direction opposite to embryo was obvious mainly in variants with embryos.

Relative to control variants, the presence of embryo significantly reduced the height of the logistic asymptote, especially in the variant with zygotic embryo (reduction by almost 10 mm), while it only slightly increased β_0 parameter having impact on duration of the acceleration phase (Figure 3). Mean effects of the embryo placement on β_1 coefficient, however, were not substantiated. Significant differences in magnitude of growth curves (size of α parameter) were evident only between both embryo types. The embryo presence appears to inhibit the growth on the side away from the embryo, especially in the late stages of the growth experiment. The effects of embryo placement on mycelium growth are evident in the direction opposite to embryo, but to a lesser extent. Differences among the curves displayed in Figure 3 could be seen in part in Table 5.

Table 3. Estimated parameters** of logistic models and associated 95% confidence limits for combinations of control, somatic and zygotic embryos and sides of measurement

Combination	Parameter	Estimate	2.5%	97.5%	$f(x)$ at point of inflection (mm)	Maximum gradient at point of inflection (mm/day)
Control towards	α	35.84	33.84	37.83	17.94	5.70
	β_0	5.05	4.74	5.35		
	β_1	1.57	1.34	1.80		
Control opposite	α	37.50	35.58	39.43	18.64	5.77
	β_0	4.77	4.47	5.06		
	β_1	1.63	1.40	1.85		
Somatic towards	α	27.80	25.65	29.94	13.93	3.96
	β_0	4.84	4.39	5.30		
	β_1	1.75	1.42	2.09		
Somatic opposite	α	32.70	31.32	34.07	16.26	6.03
	β_0	4.01	3.77	4.26		
	β_1	1.36	1.15	1.56		
Zygotic towards	α	24.06	21.91	26.20	12.05	3.36
	β_0	4.74	4.21	5.28		
	β_1	1.79	1.40	2.19		
Zygotic opposite	α	29.34	28.17	30.52	14.79	6.18
	β_0	3.68	3.45	3.92		
	β_1	1.19	0.99	1.39		

**α = 0.01

Table 4. Tests of main effects for measurement of sides and experimental variants performed for parameters of logistic curves

Factor	Contrasts	Estimated differences between the coefficients of logistic curves		
		A	β_0	β_1
Sides	opposite – towards	3.95**	–0.72**	–0.32**
Experimental variants	control – zygotic	9.97**	0.69**	0.11^{ns}
	control – somatic	6.42**	0.48**	0.04^{ns}
	somatic – zygotic	3.55**	0.22^{ns}	0.06^{ns}

**α = 0.01; *α = 0.05, ^{ns}not significant

This observation could be explained by release of substances inhibiting mycelium growth by the embryo and successive spread by diffusion of the inhibitory substances (indole-3-acetic acid (IAA), abscisic acid (ABA) and others) through the cultivation medium. The key experiment (Hřib et al. 1999) confirmed that our fungus-tester is sensitive to auxin. Mycelium of *Ph. schweinitzii* responded from concentration 10^{-6} mol/L IAA in agar medium with inhibition of mycelial growth and at concentration of 10^{-3} mol/L IAA total growth inhibition was observed. In our previous work (Vooková et al. 2006) the defence reactions were found at a very early stage of *Abies numidica* somatic embryo development. Both embryogenic tissue and early somatic embryos inhibited mycelial growth of

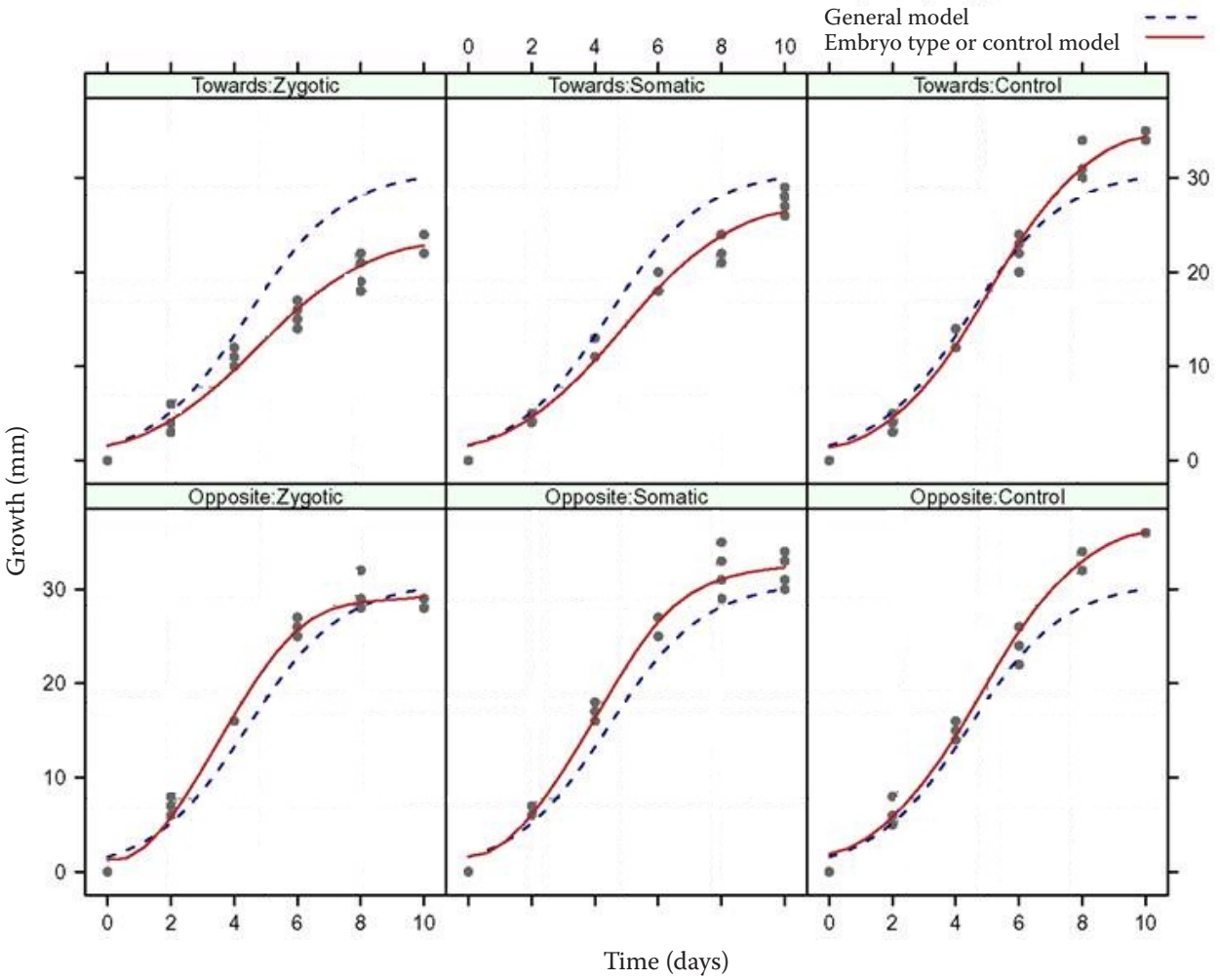

Figure 3. Comparisons of estimated logistic curves for combinations of embryo type of *A. numidica* or control and sides levels (towards or opposite to embryo) in the embryo type or control model and general model. Points designate actual measurements

Table 5. Tests of simple effects for experimental variants and measurement of side factors holding the other factor fixed, for parameters of the logistic curve

Simple effects	Estimated parametric difference		
	A	β_0	β_1
Control opposite – control towards	1.67^{ns}	-0.28^{ns}	0.05^{ns}
Somatic opposite – somatic towards	4.90^{**}	-0.83^{**}	-0.40^{*}
Zygotic opposite – zygotic towards	5.29^{**}	-1.06^{**}	-0.60^{**}
Control opposite – somatic opposite	4.81^{**}	0.75^{**}	0.27^{ns}
Control opposite – zygotic opposite	8.16^{**}	1.09^{**}	0.44^{**}
Somatic opposite – zygotic opposite	3.35^{**}	0.33^{ns}	0.17^{ns}
Control towards – somatic towards	8.04^{**}	0.20^{ns}	-0.18^{ns}
Control towards – zygotic towards	11.78^{**}	0.30^{ns}	-0.22^{ns}
Somatic towards – zygotic towards	3.74^{*}	0.10^{ns}	-0.04^{ns}

$^{**}\alpha = 0.01$; $^{*}\alpha = 0.05$; ^{ns}not significant

Ph. schweinitzii and the strongest defence shown by precotyledonary embryos. Therefore, it is interesting that endogenous levels of IAA was relatively high in embryogenic tissue, then decreased in the period of embryo development and increased again in the late maturation stage. This pattern was described during development of *Pinus sylvestris* zygotic embryos (Sanberg et al. 1987) and during early stages of somatic embryogenesis of *Picea abies* (Vágner et al. 1998). IAA concentrations rose over the course of development. Final concentration of IAA in somatic embryos was lover but comparable to that found in zygotic embryos of hybrid larch (von Aderkas et al. 2001). Peroxidase is considered to be the main enzyme responsible for the catabolism of the phytohormone IAA in higher plants (Campa 1991). Our previous studies aimed at biochemistry of embryogenesis somatic end zygotic embryos in silver fir confirmed that mature zygotic embryos have lower specific activity of peroxidase than mature somatic embryos (Kormuťák and Vooková 2005).

Woodward and Pearce (1988) studied the effect of variations in naphthalene acetic acid (NAA) and BAP concentrations on inhibition of *Ph. schweinitzii* mycelial growth. In general, increasing the NAA concentration increased the inhibition of fungal growth in presence of Sitka spruce callus. Later Rittich et al. (1992) found that phenylacetic (PAA), IAA, and indole-3-propionic acids (IPA) were fungicidally active.

In future experiments, we will try to confirm our idea about presence of IAA mechanism in defence of plant embryos by study of its basic isoperoxidases activity.

REFERENCES

Campa A. (1991): Biological role of plant peroxidases: known and potential function. In: Everse J., Everse K.E., Grisham M.B. (eds): Peroxidases in Chemistry and Biology. CRC Press, Boca Raton, Ann Arbor, Boston, 25–50.

Daub M.E. (1986): Tissue culture and the selection of resistance to pathogens. Annual Review of Phytopathology, *24*: 159–186.

Gösslová M., Svobodová H., Lipavská H., Albrechtová J., Vreugdenhil D. (2001): Comparing carbohydrate status during Norway spruce seed development and somatic embryo formation. In Vitro Cellular and Developmental Biology-Plant, *37*: 24–28.

Grigová M., Kubeš M., Drážná N., Řezanka T., Lipavská H. (2007): Storage lipid dynamics in somatic embryos of Norway spruce (*Picea abies*): histochemical and quantitative analyses. Tree Physiology, *27*: 1533–1540.

Hřib J., Rypáček V. (1981): A simple callus test to determine the aggressiveness of wood-destroying fungi. European Journal of Forest Pathology, *11*: 270–274.

Hřib J., Vooková B., Janisch R. (1995a): In vitro study of defense reactions of pine embryo and megagametophyte. Archives of Phytopathology and Plant Protection, *29*: 343–348.

Hřib J., Vooková, B., Fľak P. (1999): *In vitro* growth response of wood-rotting fungi to IAA. Archives of Phytopathology and Plant Protection, *32*: 503–520.

Hřib J., Vooková B., Salajová T., Bolvanský M., Fľak P. (1995b): Testing of embryogenic and non-embryogenic calli of European black pine (*Pinus nigra* Arn.) for defense reactions to the fungus *Phaeolus schweinitzii*. Biologia, *50*: 403–410.

Klimaszewska K., Morency F., Jones-Overton C., Cooke J. (2004): Accumulation pattern and identification of seed storage proteins in zygotic embryos of *Pinus strobus* and in somatic embryos from different maturation treatments. Physiologia Plantarum, *121*: 682–690.

Koch A.L. (1975): The kinetics of mycelial growth. Journal of General Microbiology, *89*: 209–216.

Konrádová H., Lipavská H., Albrechtová J., Vreugdenhil D. (2002): Sucrose metabolism during somatic and zygotic embryogeneses in Norway spruce: content of soluble saccharides and localisation of key enzyme activities. Journal of Plant Physiology, *159*: 387–396.

Kormuťák A., Vooková B. (2005): Storage proteins and peroxidase activity during zygotic and somatic embryogenesis of firs (*Abies* sp.). In: Mujib A., Šamaj J. (eds): Somatic Embryogenesis. Plant Cell Monography (2), Springer-Verlag, Berlin, Heidelberg, 1–14.

Rittich B., Pirochtová M., Hřib J., Jurtíková K., Doležal P. (1992): The antifungal activity of some aliphatic and aromatic acids. Collection of Czechoslovak Chemical Communications, *57*: 1134–1142.

Sandberg G., Ernstsen A., Hamnede M. (1987): Dynamics of indole-3-acetic acid and indole-3-ethanol during development and germination of *Pinus sylvestris* seeds. Physiologia Plantarum, *71*: 411–418.

Svobodová H., Albrechtová J., Kumštýřová L., Lipavská H., Vágner M., Vondráková Z. (1999): Somatic embryogenesis in Norway spruce: Anatomical study of embryo development and influence of polyethylene glycol on maturation process. Plant Physiology and Biochemistry, *37*: 209–221.

Terho M., Pappinen A., von Weissenberg K. (2000): Growth reactions of a *Gremmeniella abietina* isolate and Scots pine embryogenic tissue cultures differ in a host-parasite *in vitro* system. Forest Pathology, *30*: 285–295.

Vágner M., Vondráková Z., Strnadová Z., Eder J., Macháčková I. (1998): Endogenous levels of plant growth hormones during early stages of somatic embryogenesis of *Picea abies*. Advances in Horticultural Science, *12*: 11–18.

Von Aderkas P., Lelu M.-A., Label P. (2001): Plant growth regulator levels during maturation of larch somatic embryos. Plant Physiology and Biochemistry, *39*: 495–502.

Vooková B., Kormuťák A., Hřib J. (2001): Effect of myo-inositol on somatic embryogenesis of *Abies numidica*. Journal of Applied Botany, *75*: 46–49.

Vooková B., Hřib J., Kormuťák A., Adamec V. (2006): Defence reactions of developing somatic embryos of Algerian fir (*Abies numidica*). Forest Pathology, *36*: 215–224.

Woodward S., Pearce R.B. (1988): Responses of Sitka spruce callus to challenge with wood decay fungi. European Journal of Forest Pathology, *18*: 217–229.

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