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Biochemical differences between normal callus and embryogenic suspensor mass of silver fir

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Abstract

Normal callus and embryogenic suspensor mass (ESM) were induced from the same immature embryo of *Abies alba* Mill. These two tissues were found to differ in their isoenzyme patterns of peroxidase, glutamate dehydrogenase and non-specific esterase and in their requirement for myo-inositol in culture medium.

Additional key words: *Abies alba*, isoenzymes, tissue culture.

Introduction

Embryogenic suspensor mass (ESM) of wide range genera and conifer species was initiated on medium with auxin and cytokinin (for review see Attree and Fowke 1993). An interesting feature in genus *Abies* is that somatic embryogenesis can be expressed with cytokinin as the sole growth regulator (Schuller *et al.* 1989, Norgaard and Krogstrup 1991). In 1991 and 1992 the ESM of silver fir and some of its hybrids have also been induced in our laboratory (Gajdošová *et al.* 1995). Following initiation, the tissues were maintained throughout the period of four years on medium with cytokinin but without any supplement of auxin.

In the present work the differences between normal callus and ESM of *Abies alba* were investigated comparing isoenzyme patterns of three enzyme systems and requirement of the tissues for exogenous myo-inositol.

Materials and methods

Normal (non-embryogenic) callus and embryogenic suspensor mass (ESM) were induced from the same seed of *Abies alba* Mill. (collected in J. Kostolany on 5 August 1992). Immature embryo inside megagametophyte was used as a primary explant. The seeds were sterilized first in 70 % ethanol for 30 s, followed by their

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Abbreviations: BAP - benzylaminopurine; ESM - embryogenic suspensor mass; NAA - naphthaleneacetic acid.
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treatment with 0.1 % $HgCl_2$ for 15 min and rinsing in sterile water. Normal callus and ESM were induced on modified SH medium (Schenk and Hildebrandt 1972) solidified with 3 mg dm^{-3} phytagel. Salts and vitamins of SH medium were supplemented with 100 mg dm^{-3} myo-inositol, 500 mg dm^{-3} L-glutamine, 1000 mg dm^{-3} casein hydrolysate and 2 % saccharose. Normal callus line was initiated on this medium with 1 mg dm^{-3} 6-benzylaminopurine (BAP). After transferring of this callus together with the primary explant on the same medium which had been supplemented with 1 mg dm^{-3} α-naphthaleneacetic acid (NAA), the embryogenic suspensor mass was produced. Its proliferation has been achieved on the SH medium with 1 mg dm^{-3} BAP. For achievement of normal callus proliferation SH medium with 1 mg dm^{-3} BAP and 1 mg dm^{-3} NAA was suitable.

Myo-inositol, thiamine and pyridoxine requirement: Normal callus and ESM were cultured on proliferation medium with 100 mg dm^{-3} myo-inositol as well as on myo-inositol free medium. ESM was also cultured on proliferation medium where thiamine-HCl (5 mg dm^{-3}) or pyridoxine-HCl (0.5 mg dm^{-3}) was omitted. Medium was gelled with 3 mg dm^{-3} of phytagel. The cultures were kept in dim light at 25 ± 1 °C and subcultured every three weeks.

Isoenzyme analysis: The normal callus and ESM were characterized by analysis of the isoenzyme profiles of peroxidase, non-specific esterase and glutamate dehydrogenase. Tissue material was selected randomly from 2 - 3 pieces of calli of two plates of each tissue type investigated. Tissue (1 g) was homogenized in *Potter-Elvehjem* homogenizer using 1.5 cm^3 of 0.1 M TRIS-HCl buffer, pH 7.6, containing 10 mM mercaptoethanol, 1 mM Na_2EDTA and 0.5 % (v/v) Tween 80 (Pitel *et al.* 1992). The homogenates were centrifuged and supernatants used for enzyme analysis. All operations were carried out at 4 °C. Electrophoretic separation of isoenzymes was performed according to Frič (1971) applying the discs variant of polyacrylamide gel electrophoresis with 1.25 % stacking and 7.5 % separation gels and TRIS-glycine electrophoretic buffer, pH 8.3. The gels were run over 2 h under current of 3 mA per gel. The peroxidases were visualized by incubation of the gels in a solution of 0.1 M Na-acetate buffer, pH 4.5, with 10 mg of benzidine and 0.05 % H_2O_2. The total volume of mixture was 6 cm^3. The incubation mixture for non-specific esterases consisted of 0.1 M Na-phosphate buffer, pH 6.0, 3 mg of *Fast Blue B* and 3 mg of α-naphthylacetate in a total volume of 6 cm^3. The isoenzymes of glutamate dehydrogenase were detected in the enzyme assay solution containing 0.1 M Na-phosphate buffer, pH 7.4, 2 mg of NAD, 1 mg of phenazinemetasulphate, 1 mg tetrazolium nitroblue and 0.1 M neutralized L-glutamic acid, pH 7.5, in a total volume of 5 cm^3.

Results and discussion

Normal callus and embryogenic suspensor mass (ESM) were established from the same immature embryo but they differed mutually in their requirements for the

presence of growth regulators in culture medium. ESM proliferated on medium with BAP but without auxin. The callus culture and ESM showed differences also in their morphology and growth rate.

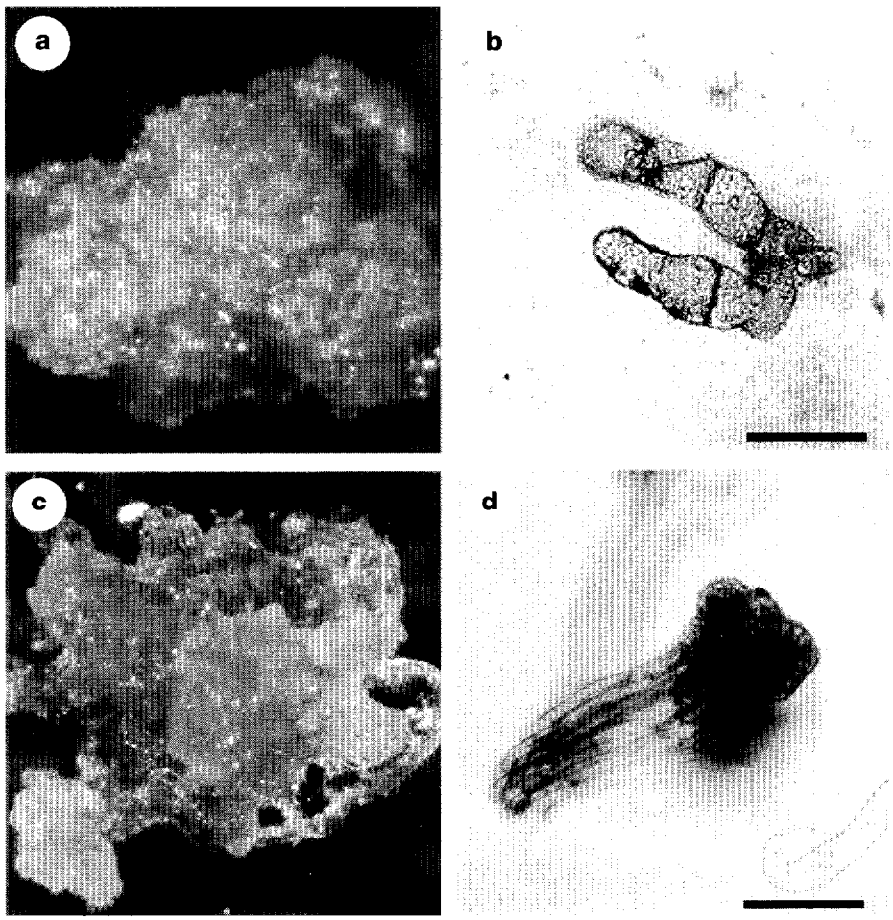

Fig. 1. Normal callus and embryogenic suspensor mass of silver fir (*Abies alba* Mill.): *a* - normal (non-embryogenic) callus, *b* - parenchymatic cells of normal callus, *c* - embryogenic suspensor mass (ESM), *d* - meristematic cells of somatic embryo in early globular stage of development and suspensor-like cells; *bar* = 100 μm.

Normal callus was yellow-green and compact. It consisted of parenchymatic cells and showed weak callus proliferation (Fig. 1*a,b*). ESM was translucent to white and predominantly mucilaginous in consistency. This tissue showed very intensive cell proliferation. As in other *Abies* species, the ESM consisted of somatic embryos at early globular stage, composed of clusters of small densely cytoplasmic cells interspersed with suspensor-like cells (Fig.1*c,d*). The habituated calli are usually non-

organogenic but in some cases they may share the organogenic or embryogenic potencial (Bisbis *et al.* 1993, Kochba and Spiegel-Roy 1977).

The ESM of *Abies alba* shows many similarities with habituated non-organogenic sugar beet callus (Gaspar *et al.* 1988, Bisbis *et al.* 1994). Sugar beet callus was composed of two parenchymatic cell types, without chlorophyll, containing more water and exhibiting a lower dry mass. The dry matter of *A. alba* ESM was also lower, ranging from 3.2 to 3.6 % , while the dry matter of normal callus was 10 %. Poorly differentiated cells of sugarbeet habituated callus exhibited incompletely structured plastids and mitochondria (Crèvecoeur *et al.* 1992). Isodiametric highly cytoplasmic cells of *A. alba* × *A. cephalonica* ESM were with prominent nuclei, abundant small vacuoles and proplastids (Salajová *et al.* 1996).

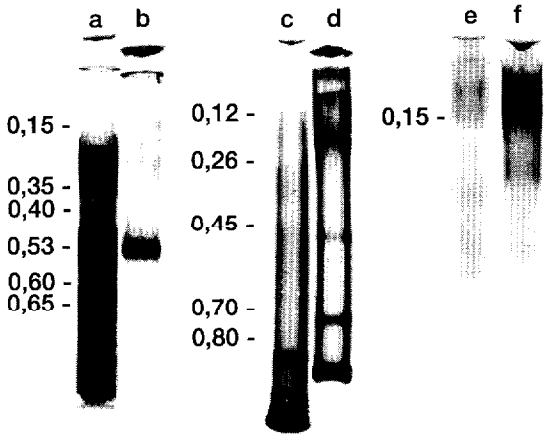

Fig. 2. Determination of isoenzymes of peroxidase, glutamate dehydrogenase and non-specific esterase in normal callus and ESM of silver fir (*Abies alba* Mill.) by polyacrylamide gel electrophoresis: *a,b* - peroxidase; *c,d* - non-specific esterase; *e,f* - glutamate dehydrogenase; *a,c,e* - normal callus; *b,d,f* - ESM.

Normal callus and ESM were differentiated with respect to all the three enzyme systems studied (Fig. 2). High peroxidase activity in normal callus contrasted with the reduced number of isoperoxidases in ESM represented by one major peroxidase band of a relative mobility Rm 0.53 (Fig. 2*a,b*). The reverse situation has been typical for the non-specific esterase with a higher number of isoenzymes registered in ESM. Conspicuous was in this connection the absence of isoenzyme of non-specific esterase in the normal callus at the positions corresponding to Rm 0.12 (Fig. 2*c,d*). Very interesting situation was observed with respect to the glutamate dehydrogenase (GDH). Only a smear of GDH activity found in normal callus (Fig. 2*e*), contrasted with a high GDH activity in the embryogenic cell line (Fig. 2*f*). Bouchet *et al.* (1978), Krsnik-Rasol and Jelaska (1991) have also shown that habituated calli exhibit lower peroxidase activity. Le Dily *et al.* (1993) are of the opinion that hormonal autonomy may be also the consequence of a high auxin level due to increase of synthesis and lack of degradation. A possible reduction in auxin degradation may be associated

with the deficiency in auxin degrading peroxidases. There is some evidence indicating that peroxidase-mediated control of IAA may be one of the major mechanisms controling auxin levels and thus the auxin action (Wareing and Phillips 1978).

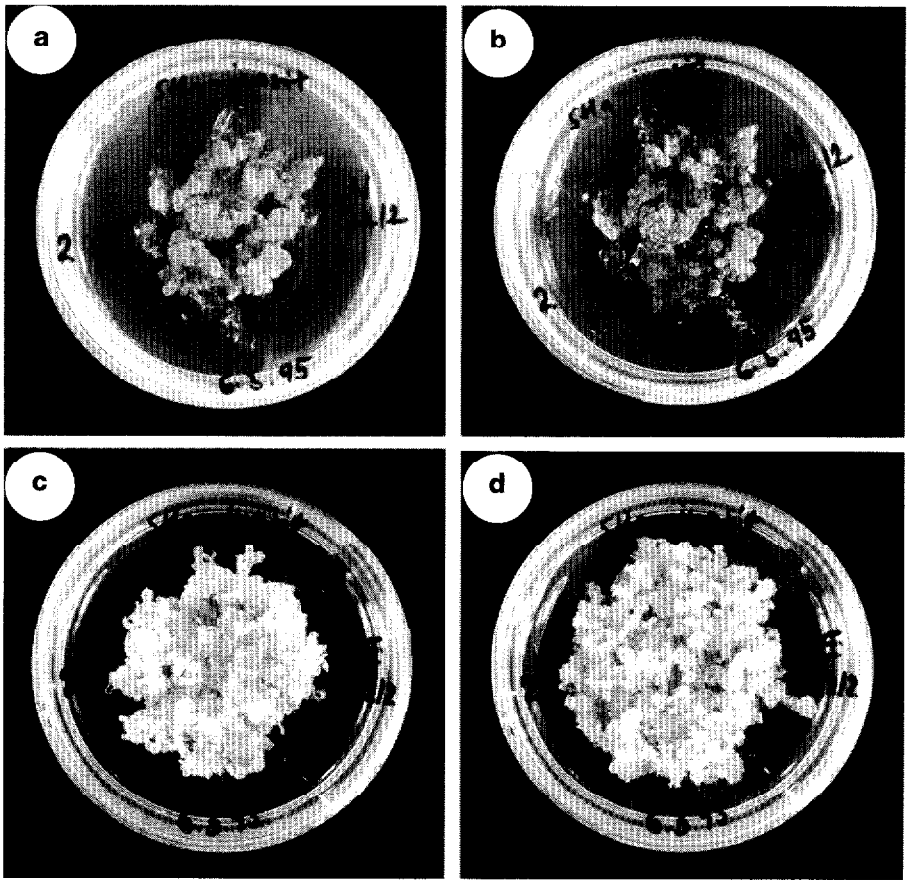

Fig. 3. Testing of myo-inositol requirement of normal callus and ESM of *Abies alba* Mill.: *a* - normal callus placed on SH medium without myo-inositol; *b* - normal callus showed necrosis symptoms after 3 weeks of cultivation on myo-inositol free medium; *c* - ESM placed on SH medium without myo-inositol; *d* - proliferation of ESM after 3 weeks of cultivation on medium where myo-inositol was omitted.

Increased activity of GDH in ESM of silver fir correlates with the finding by De Jong *et al.* (1967) who found the higher activity of GDH in young dividing and expanding cells as compared with the coresponding activity of stationary phase cells.

Another mechanism which could control the quantity of IAA present in tissue is the formation or dissolution of conjugates of IAA, such as indoleacetylarabinose, IAA-myo-inositol, and proteins. This mechanism is long overdue for investigation (Wareing and Phillips 1978). Myo-inositol is better known to function in synthesis of

cell phospholipids and cell wall pectins (Grey *et al.* 1987) and probably in osmoregulation of the plant cell (Zimmerman and Cobb 1989). Glycerol and cyclic myo-inositol are important components of cell lipids (Voet and Voet 1995). Myo-inositol played the role as a precursor of cell wall polysaccharides in suspension cultures of wild-carrot cells (Verma *et al.* 1979). Inositol-1,4,5-phosphate probably has a function of a secondary mediator inside plant cells.

In our experiment normal callus cultured on medium without myo-inositol showed necrosis symptoms and died after 3 - 4 weeks of cultivation (Fig. 3). ESM did not change appearance and proliferated on myo-inositol free medium during 14 months. In our previous work (Vooková and Hřib 1996) the necrosis of transformed tumour tissue and normal callus that were derived from seedling hypocotyl of *A. alba* and which were cultured on medium without myo-inositol had also been observed. The ESM of *A. alba* proliferated also on the medium free of thiamine and also when pyridoxine was omitted.

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