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Induction, protein composition and DNA ploidy level of embryogenic calli of silver fir and its hybrids

A. GAJDOŠOVÁ, B. VOOKOVÁ, A. KORMUŤÁK, G. LIBIAKOVÁ and J. DOLEŽEL*

Institute of Plant Genetics, Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, 950 07 Nitra, Slovakia
*Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Sokolovská 6, 77200 Olomouc, Czech Republic**

Abstract

Somatic embryogenesis was initiated from immature zygotic embryos of intraspecific cross of *Abies alba* and interspecific combination of *Abies alba* × *Abies nordmanniana*. The most responsive explants for induction of embryogenic calli were zygotic embryos in precotyledonary stage of development. Biochemically the compared embryogenic lines were uniform irrespective of their morphology or embryogenic potential. The flow cytometric analysis of single embryogenic lines suggests that no changes in ploidy levels occur during induction and culture of embryogenic callus in *Abies alba* what confirms the convenience of this system for propagation of valuable seed material.

Key words: *Abies alba*, *Abies nordmanniana*, flow cytometry, somatic embryogenesis

Introduction

Increased sensitivity of silver fir to ambient air pollution is commonly recognized among coniferous woody species. One of the possibilities of extending the genepool of this species and subsequent increasing of its adaptability to environmental factors is intra- and interspecific hybridization (Kormuťák 1986). Unfortunately, the seeds obtained by artificial pollination are costly and their number is often limited.

That is why we have used the method of somatic embryogenesis to obtain embryogenic-suspensor masses in silver fir and its hybrids that are capable to produce large number of somatic embryos. In the genus *Abies* the induction of somatic embryogenesis has been achieved in both the immature (Schuller *et al.* 1989 - *A. alba*, Norgaard and Krogstrup 1991 - *A. nordmanniana*) and mature zygotic embryos (Gebhardt *et al.* 1988, Hristoforoglu *et al.* 1992 - *A. alba*).

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This paper describes the initiation of embryogenic-suspensor masses (ESM) and the formation of the somatic embryos from immature zygotic embryos from a controlled intraspecific cross-pollination of *A. alba* as well as from the interspecific cross-pollination of *A. alba* × *A. nordmanniana*. Total protein analysis was performed in individual embryogenic lines with the aim to characterize them biochemically. Flow cytometry of nuclear DNA content was employed to assess the stability of DNA ploidy levels during *in vitro* culture.

Materials and methods

Somatic embryogenesis was initiated from immature zygotic embryos of intraspecific cross of *Abies alba* (Mill.) and interspecific combination *A. alba* × *A. nordmanniana*. The cones were collected at regular intervals during the period of June - August in 1991 and 1992. Immature seeds were isolated from the cones, sterilized first in 80 % ethanol for 30 s, than in 0.1 % $HgCl_2$ for 15 min and washed in sterile water. Excised megagametophytes containing immature embryos were placed directly on the medium. One hundred to 312 megagametophytes of *A. alba* and 94 to 210 *A. alba* × *A. nordmanniana* were taken in each treatment. In total 286 *A. alba* and 641 *A. alba* × *A. nordmanniana* explants were used.

For the induction of somatic embryogenesis the following media were tested:

- SH medium with 1 mg dm^{-3} BAP - Schuller *et al.* (1989)
- DCR medium with 0.5 mg dm^{-3} BAP and 2 mg dm^{-3} 2,4-D - Klimaszewska (1989), Salajová and Salaj (1992)
- DCR medium with 1 mg dm^{-3} BAP
- 1/2 MS medium with 2 mg dm^{-3} BAP - Norgaard and Krogstrup (1991)

After that, all cultures were maintained on SH medium with 0.5 - 1 mg dm^{-3} BAP, containing 500 mg dm^{-3} L-glutamine and 1000 mg dm^{-3} casein hydrolysate. During the induction and proliferation period, the cultures were kept in the dark at 25 ± 2 °C and subcultured to the fresh medium every two weeks.

Two types of embryogenic lines with different morphology and stage of somatic embryo development were chosen for total protein and flow-cytometry analysis: *1.* White homogeneous calli without precocious germinating embryos (lines No. 2 - *A. alba* and No. 63 - *A. alba* × *A. nordmanniana*), *2.* White transparent calli with long suspensor cells and plenty of precocious germinating embryos (lines No. 1, 7, 8, 9, 12, 16 - *A. alba* and No. 41, 42, 45 - *A. alba* × *A. nordmanniana*).

Total protein analysis: Callus tissue (1 g) was homogenized in 2 cm^3 of 125 mM TRIS-HCl, pH 6.8; consisting of 22.5 % (v/v) 2-mercaptoethanol, 22.5 % (v/v) glycerol and 9 % (v/v) sodium dodecyl sulphate. After homogenization in *Potter-Elhevjem* homogenizer, the slurry was boiled in water bath (3 min) and centrifuged at 15 000 *g* (10 min). The supernatant obtained in this way was processed by the SDS-electrophoresis using 5 % stacking and 9 % separating polyacrylamide gels, respectively.

Flow cytometric analysis: Flow cytometric analysis of nuclear DNA content was performed on isolated nuclei. *Abies alba* protoplats were isolated from actively

growing embryogenic cultures cultivated on SH medium with 0.5 mg dm^{-3} BAP. The enzyme solution consisted of 0.2 % bovine serum albumine, 1.5 % cellulase, 0.3 % macerozyme, 0.1 % driselase, 0.3 % pectinase, 0.5 M sucrose, 5 mM MES/KOH and 5 mM $CaCl_2$, pH 5.8 (Libiaková and Dedičová 1995). The embryogenic calli were incubated in enzyme solution for 18 - 20 h at 25 °C in the dark. After purification on a sucrose-glucose gradient, the protoplasts were washed three times in WS solution (Medgyesy *et al.* 1980).

Settled protoplasts were resuspended in LB2 buffer (Doležel *et al.* 1989), gently shaken and left for 10 min on the ice. The suspension containing nuclei and cell fragments was passed through a 50 mm nylon filter. The nuclei in the filtrate were stained by propidium iodide at the final concentration 50 mg cm^{-3} in the presence of 50 mg cm^{-3} RNase. After 15 min, the suspension was filtered through a 15 mm nylon filter and analysed within 1 h with a *Leitz MPV-Compact Flow Cytometer* (*Ernst Leitz Wetzlar GmbH*, Wetzlar, Germany).

The instrument was calibrated using nuclei isolated from *A. alba* embryos so that the peak corresponding to G1 nuclei (2C DNA content) was positioned at channel 64. The nuclei were isolated from mature zygotic embryos by chopping with a scalpel in LB01 lysis buffer (Doležel *et al.* 1989) and stained by propidium iodide as described above. The histograms were collected over 512 channels and analysed with *FLOWSTAR* software (Doležel 1989). In each sample, 5 000 - 20 000 nuclei were analysed and the percentage of nuclei with different DNA levels was calculated.

Results and discussion

Induction of embryogenic cell lines: The most responsive explants for induction of embryogenic suspensor masses (ESM) were collected from seeds on July 23 in 1991 when the embryos were in precotyledonary stage of development. In 1992 induction of ESM confirmed repetivity of this process. ESM were induced on all tested media. While Schuller *et al.* (1989) and Norgaard and Krogstrup (1991) observed initiation of ESM on media with cytokinin as a sole plant growth regulator, in our experiments ESM was initiated on DCR medium where auxin 2,4-D was added to cytokinin BAP, as well (*A. alba* × *A. alba* - 5 %). The most effective was SH medium where we obtained the highest induction in *A. alba* × *A. alba* - 24 % and in *A. alba* × *A. nordmanniana* - 37.9 % (Fig. 1). In comparison with literature data we can see that induction potential in intraspecific hybrid was the same as in *A. alba* from open-pollinated (Schuller *et al.* 1989). Potential of induction in interspecific hybrid *A. alba* × *A. nordmanniana* was higher (38 %) than induction in *A. alba* (24 %) and *A. nordmanniana* (25 - 33 %) - Norgaard and Krogstrup (1991), respectively.

Excised megagametophytes cultured on media began to enlarge and produced translucent to white coloured embryogenic suspensor masses (ESM) (Fig. 2) after 4 weeks of culture. ESM consisted of single elongated highly vacuolated cells, clumps of small and densely cytoplasmic cells and somatic embryos at early stages of development (Fig. 3). Prematurely germinating somatic embryos occurred frequently in ESM during the cultivation on proliferation medium. Single lines of ESM were

distinguished by different vitality and proliferation rate. Proliferation rate was influenced by casein hydrolysate and glutamine presence in medium.

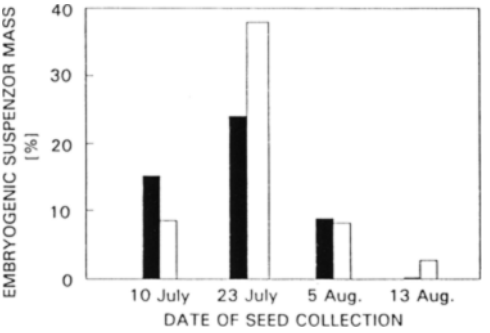
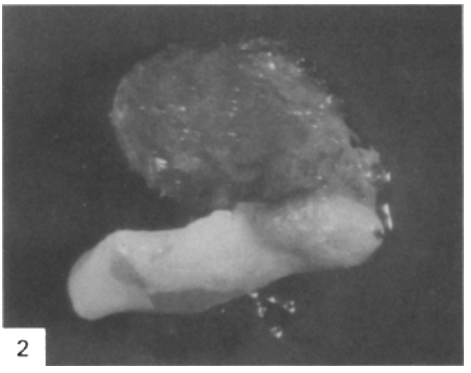


Fig. 1. Initiation frequency of embryogenic suspensor mass from immature silver fir hybrid seeds during 1991 year. Full columns: *A. alba* × *A. alba*, hatched columns: *A. alba* × *A. nordmanniana*.



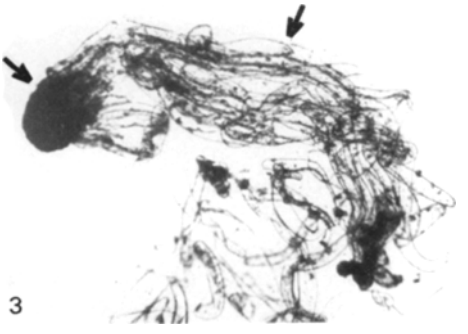


Fig. 2. Translucent and mucilaginous embryogenic suspensor mass extruded from micropile end of the megagametophyte of *Abies alba*.

Fig. 3. ESM of interspecific hybrid *A. alba* × *A. nordmanniana* consisting of elongated highly vacuolated cells and densely cytoplasmic embryogenic cells (arrows).

Total protein analysis: Biochemically the compared embryogenic lines were uniform irrespective of their morphology or embryogenic potential. There can be distinguished as many as 40 bands in the protein patterns of both *A. alba* and *A. alba* × *A. nordmanniana* calli with the three major fractions positioned at Rm 0.26, 0.62 and 0.80, respectively (Figs. 4, 5). The minor components in the lower parts of zymograms deviate seemingly in lines No. 1 and 7 of *A. alba* not being so distinct as in the remaining samples. The diffuse nature of these is probably due smaller amount of proteins applied in the corresponding samples and does not reflect the variation between the individual lines.

The lack of correlation between the morphological types of ESM and the protein profiles of individual callus lines indicates the abscence of association between the corresponding traits at both the morphological and biochemical levels. This finding

contradicts the postulated higher extent of variation of the biochemical traits in regenerated tissues and plants as compared with the variability of morphological characteristics (Noh and Minocha 1990). Also, the biochemical traits in spite of

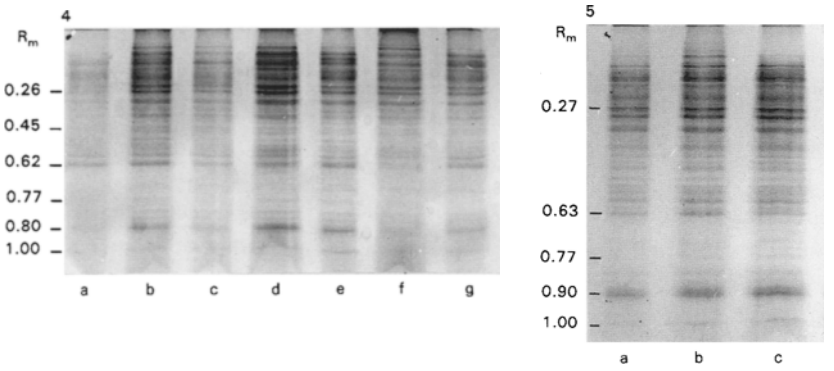


Fig. 4. SDS protein profiles in embryogenic callus lines No. 1 (a), 2 (b), 7 (c), 8 (d), 9 (e), 12 (f), and 16 (g) of *A. alba*.

Fig. 5. SDS protein profiles in embryogenic callus lines No. 41 (a), 42 (b) and 45 (c) of *A. alba* × *A. nordmanniana* combination.

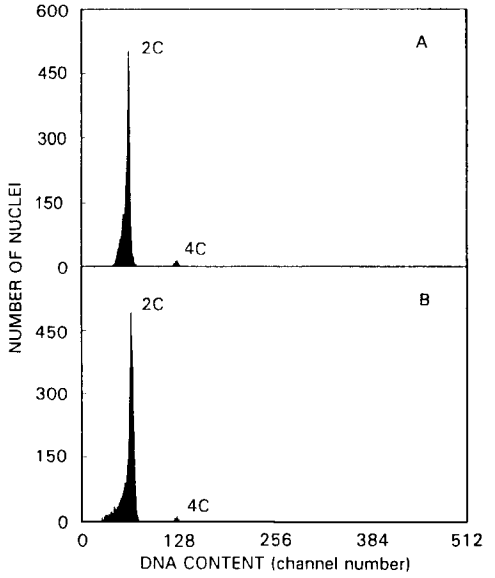


Fig. 6 A, B. Histograms of relative nuclear DNA content (A) of nuclei isolated from mature zygotic embryos of *A. alba* and (B) nuclei isolated from embryogenic callus line No. 7. In both cases, the most of the nuclei have 2C DNA content corresponding to diploid nuclei in G1 phase of the cell cycle.

being offered as an alternative tool to the study of the extent of somaclonal variation have preferably been used in discrimination between the embryogenic and non-

embryogenic calli in a variety of plant species (Kochba *et al.* 1977, Wann *et al.* 1987, Fransz 1988, Joersbo *et al.* 1989, Rao *et al.* 1990). There is only one report referring about the differences in the extracellular protein profiles in morphologically differentiated embryogenic cell lines of *Picea abies*. The observed differences in 8 protein fractions which have been excreted by the A and B-cell lines have, however, been of physiological nature arising probably as a result of their treatment with the different plant growth regulators (Mo *et al.* 1992). Fransz (1988) has also mentioned in this connection of the differentiated isozyme patterns of glutamate dehydrogenase in compact and friable embryogenic calli of maize but the enclosed zymogram had not proved these differences unequivocally. It seems therefore more probable that individual lines of an embryogenic callus showing the same potential for somatic embryo formation do not exhibit the profound differences in metabolic activities which determine their embryogenic abilities. With a special reference to the embryogenic callus lines of *A. alba* and *A. alba* × *A. nordmanniana* this statement is validated by the identical profiles of their soluble proteins.

Flow cytometric analysis: The present study confirmed the potential of flow cytometry for the analysis of DNA ploidy levels in cultured cells (Doležel 1991). While it was possible to prepare suspensions of intact nuclei by chopping mature *A. alba* embryos, attempts to use the same procedure to isolate nuclei from embryogenic calli were unsuccessful. The yield of nuclei was low and the quality of histograms was unacceptable due to high background levels (data not shown). Thus the nuclei were obtained from embryogenic calli *via* protoplast isolation and lysis. The quality of histograms obtained after the analysis of nuclei isolated this way was fully comparable to those obtained from embryos.

The histograms obtained after the analysis of nuclear DNA content in mature *A. alba* embryos contained a single dominant peak corresponding to nuclei in G_1 phase of the cell cycle with 2C DNA content (Fig. 6A). The remaining nuclei were localized in G_2 phase with 4C DNA content. The distributions of nuclear DNA content obtained after the analysis of nuclei isolated from embryogenic calli of *A. alba* were similar to those of mature embryos. Most of the nuclei (range 91.2 - 96.5 %) had 2C DNA content (peak at channel 64) which corresponds to G_1 phase of the cell cycle (Fig. 6B). Very few nuclei were found having 4C DNA content (range 3.1 - 7.6 %) and practically no nuclei were detected with DNA content higher than 4C. The differences in DNA content distributions obtained in individual embryogenic lines were negligible.

These finding suggest that no changes in ploidy levels occur during induction and culture of embryogenic callus in *A. alba*. This is in agreement with other reports on chromosome number stability during *in vitro* culture of this species (Schuller *et al.* 1989; Libiaková *et al.* 1995). Considering the fact that similar stability was noted also in tissue cultures of other conifers (Hakman *et al.* 1986, Mo *et al.* 1989, Wyman *et al.* 1992) it seems highly probable that the stability of ploidy level under *in vitro* conditions is a more general phenomenon in these plants.

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