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BRIEF COMMUNICATION

Optimization of Algerian fir somatic embryos maturation

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Abstract

The effect of maturation pretreatment on development and growth of *Abies numidica* De Lann. somatic embryos was studied. The most beneficial was pre-culturing on Schenk and Hildebrandt medium without growth regulator for 2 weeks. Dry mass accumulation of emblings was lower than that of seedlings after 50 d of culturing. Contents of microelements in seedlings were higher than in emblings, but macroelements contents were higher in emblings. Contents of chlorophyll *a* and chlorophyll *b* in cotyledons were higher in seedlings than in emblings while no qualitative differences were detected between the protein profiles of seedlings and emblings.

Additional key words: *Abies numidica,* chlorophyll, germination, macroelements, preculturing, proteins.

Somatic embryogenesis is a multi-step regeneration process starting with formation of embryogenic tissue, followed by somatic embryo development, maturation, desiccation, germination and plantlet regeneration. Although great progress has been made in improving the protokol used, it has been revealed that some treatments, coinciding with increased yield of somatic embryos in conifers can influence embryo quality and impairing germination (Von Arnold *et al.* 2002, Von Aderkas *et al.* 2007). In *Abies*, the most difficult parts seems to be the embryo conversion and germination phase (Salajová and Salaj 2003/04, Krajňáková *et al.* 2008). In order to stimulate development and germination of *Abies numidica* somatic embryos, the effect of maturation pretreatment was studied. Emblings, regenerants obtained from somatic embryos under studied conditions were compared with seedlings. Accumulation of dry mass, element concentration, storage proteins and chlorophyll content were investigated.

Embryogenic tissue of *Abies numidica* de Lann. was induced from immature embryos of seeds colected from Arboretum Mlyňany according to Vooková and Kormuťák (2002). To stimulate development and growth of *Abies numidica* somatic embryos, the effect of maturation pretreatments was studied as follows. Embryogenic tissue was transferred from Schenk and Hildebrandt (1972; SH) proliferation medium with 1 mg dm^{-3} benzylaminopurine (BAP) and 20 g dm^{-3} sucrose to the same SH medium lacking growth regulator. Cultures were maintained on this medium for 1 - 2 weeks. Embryogenic tissues proliferated on SH media with or without BAP were transferred on maturation medium. The pieces of embryogenic tissue (*ca*. 350 mg) were cultured in 60 mm plastic Petri dishes on maturation medium in the dark at 21 to 23 °C. Maturation of somatic embryos was achieved on modified (Murashige and Skoog 1962; MS) medium supplemented with 40 g dm^{-3} maltose, 100 g dm^{-3} polyethylene glycol (PEG) 4000 and 10 mg dm^{-3} abscisic acid (ABA; Vooková and Kormuťák 2002). Each Petri dish was replicated ten times. The whole experiment was repeated twice. Mature somatic embryos were subjected to a partial desiccation under high humidity during three weeks at 24 ºC in the dark (Vooková *et al*. 1997/98). Embryos were allowed to germinate on ½ SH medium with 10 g dm^{-3} sucrose, 10 g dm^{-3} charcoal, 3 g dm^{-3} *Phytagel* either without myo-inositol (MI), or with 100 mg dm^{-3} MI, or with MI + 0.05 mg dm^{-3} indole-3-butyric acid (IBA). Six replications of ten embryos were

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Abbreviations: ABA - abscisic acid; BAP - benzylaminopurine; IBA - indole-3-butyric acid; MI - myo-inositol; MS - Murashige and Skoog (1962) medium; PEG - polyethylene glycol; SH - Schenk and Hildebrandt (1972) medium; SDS - sodium dodecyl sulphate.
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cultivated in Ehrlenmayer flasks with 50 cm^3 media per treatment. Germination percentages were evaluated after 40 d of cultivation. Zygotic embryos and somatic embryos after partial desiccation were cultured on half strengh SH germination medium with MI during 50 d.

Comparisson of dry mass accumulation of seedling and emblings (regenerants from somatic embryos) was made after 14, 20, 30, 40 and 50 d of cultivation on ½ SH medium with 100 mg dm^{-3} MI and 0.05 mg dm^{-3} IBA under temperature of 21 - 23 °C and 16-h photo-period with irradiance of 110 μmol m^{-2} s^{-1}. Dry mass was determined by drying fresh tissue at 85 ºC for 48 h. Dry samples (0.5 g) were digested by 5 cm^3 of 65 % HNO_3 in the *Plasmatronic Uniclever* microwave oven. After filtration, a sample solution was made up to a volume of 50 cm^3. Contents of Ca, Mg, K, Na, Cu, Zn, Mn and Fe were determined by atomic absorption spectrometry (*GBC Avanta*, Dandenong, Australia). and P by Inductively Coupled Plasma atomic-emission spectro-metry (*ICP 3000*, *Leco*, St. Joseph, MI, USA). .Three replicates of each samples were prepared and each sample was measured 3 times. Accuracy of analytical results was controlled through the Spruce needle powder, B 101 (Chemmea, Bratislava, Slovakia). Element concentration populations were normally distributed (Kolmorogov-Smirnov test), which was supported with almost equal median values and relatively low values of kurtosis and skewness. Due to the very high difference between the lowest and highest contents recorded at individual elements a semi-logarithmic scale was used. *F*-test was used to compare two variances of seedlings and emblings standard deviations and Student's *t*- test to assess the difference between seedlings and emblings element concentration means.

The amount of material used in protein analysis was 0.9 g for seedlings and 1.2 g for emblings. The material was homogenized in a mortar using liquid N_2 and 30 mM TRIS-HCl buffer, pH 6.8 with admixture of 1.5 % sodium dodecyl sulphate (SDS), 5.3 % glycerol and 11.2 % mercaptoethanol. The crude homogenate was heated for 8 min followed by centrifugation at 20 000 *g* and at 10 °C for 20 min. The obtained supernatant was analyzed by SDS polyacrylamide electrophoresis using 10 % stacking and 12 % separating gels (Laemmli 1970). A standardized amount of 40 mg proteins of each sample was loaded onto the gel. The molecular masses of the proteins were determined relative to the broad range protein marker (*New England Biolalbs*, Ipswich, USA). The gels were stained with Coomasie Brilliant Blue R-250.

The sampling for quantitative analysis of chlorophylls was performed from cotyledon material of 20-d-old seedlings and emblings. The samples were homogenized to powder, extracted using 80 % acetone solution and chlorophyll *a* and *b* contents were determined spectrophotometrically (*Jenway UV/vis 6405*, Essex, UK). The contents of chlorophylls were calculated according to Lichtenthaler (1987). Each determination was repeated two times ($n = 6$).

Statistical evaluation of the data from somatic embryo maturation and germination, accumulation of dry mass and chlorophyll analysis was carried out by Student's *t*-test.

Embryogenic tissue of *A. numidica* proliferated on SH medium with BAP and BAP free medium without any visual changes during one or two weeks. Maturation pretreatment of this tissue on medium without BAP showed a positive effect on maturation of somatic embryos (Table 1). The duration of maturation process was from 6 to 8 weeks. Eight weeks maturation without pretreatment resulted in 31.45 % yield of qood-quality somatic embryos. One week pretreatment increased total yield of embryos to 36.09 % but yield of good-quality somatic embryos was nearly the same as without pretreatment (32.43 %). The most beneficial was somatic embryo pre-culturing on SH medium without growth regulator during two weeks. The duration of maturation was shorter. After 6 weeks of maturation the first cotyledonary embryos were observed. Though number of all matured embryos per g of embryogenic tissue decreased (20.27 %), the yield of embryos without abnormality increased to 56.71 %, which is very significant difference between control and 7-d pretreat-ment. After partial desiccation, mature embryos germinated on three different media with charcoal (Table 1). Germination of embryos was affected by maturation pretreatment in dependence on type of germination medium. Significant difference was found between germination percentage on medium with MI and medium without MI, where embryos were obtained without prematuration treatment (control). Also addition of IBA to medium with MI affected positively germination. Difference in germination percentage on medium without MI and medium with MI and IBA was not significant. But 14-d maturation pretreatment attenuated differences in germination media effect.

Table 1. Effect of maturation pretreatment (see text) on yield of mature somatic embryo (means ± SE, $n = 20$) and germination of somatic embryos cultured on ½ SH medium without myo-inositol (MI), with 100 mg dm^{-3} MI, and with MI and IBA (0.05 mg dm^{-3}). Means ± SE, $n = 6$; Values followed by the same letters are not significantly different ($P \leq 0.05$). EMS - embryonic suspensor mass.

Pretreatment [d]	Number of embryos [g^{-1}(ESM)]	Germination [%] 1/2 SH	1/2 SH + MI	1/2 SH + MI + IBA
0	26.51 ± 4.09ab	77.61 ± 10.31a	47.47 ± 5.64b	55.38 ± 12.43b
7	36.09 ± 2.97a	60.01 ± 3.33b	30.00 ± 4.26c	76.65 ± 3.34a
14	20.27 ± 3.54b	83.30 ± 3.31a	82.86 ± 4.05a	81.87 ± 4.99a

Accumulation of dry mass in seedlings and emblings during 50 d of culture was investigated. Emblings contained nearly the same percentage of dry mass (10.51 %) as seedlings (11.17 %) after 14-d cultivation. Over next days culture dry mass percentage of emblings increased from 11.48 to 13.44 %, while from 18.88 to 22.20 % in seedlings.

The content of macro- and microelements differed between seedlings and emblings (Fig. 1). Except for P and S, these differences were statistically significant ($P < 0.05$). While higher micronutrients contents were found in seedlings, C and N contents (nt shown) were higher in emblings. The highest value of K [54.43 mg(K) g^{-1}(d.m.)] was found in emblings, which was nearly 2-fold higher compared to that in seedlings [30.23 mg(K) g^{-1}(d.m.)]. The highest nearly 3-fold discrepancy was recorded at iron [99.19 *versus* 37.45 mg(Fe) g^{-1}(d.m.)].

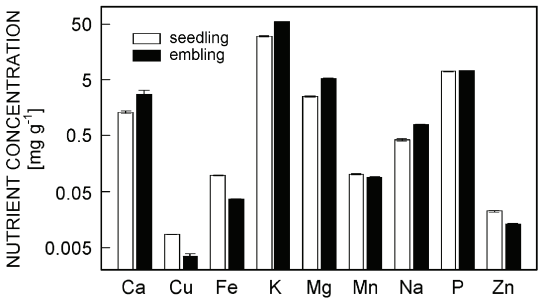

Fig. 1 Concentrations of essential elements in seedlings and emblings (semilogarithmic scale).

No qualitative differences were detected between the protein profiles of seedlings and emblings. In both cases the electroforetic patterns consisted of 11 protein bands of comparable molecular mass with major proteins of 55.6 and 26.6 kDa. The 43 kDa protein, which is considered to be the major storage protein in *Abies* (Jensen and Lixue 1991), was less abundant than 26 kDa protein, which is another storage protein specific for *Abies*.

Chlorophyll contents in cotyledons of seedlings were higher ($P \leq 0.05$) than in emblings and the chlorophyll *a*/*b* ratio in both seedling and emblings was approximately 2/1. This ratio was in line with the relationships usualy found in higher plants (*e.g.* Šalgovičová and Kmeť 2004). Seedlings and emblings contained 0.616 and 0.270 mg g^{-1}(d.m.) of chlorophyll *a*, and 0.288 and 0.103 mg g^{-1}(d.m.) of chlorophyll *b*, respectively.

Results of this study clearly show on posibilities of promoting more effective plant regeneration of *A. numidica* by modification of condition for development of somatic embryos. Prematuration of somatic embryos in medium lacking growth regulators inhibites proliferation and stimulates somatic embryo formation and early development (Von Arnold *et al.* 2002). Also positive effect of MI omission in germination medium on yield of regenerants was observed. These results are in line with the observation of our previous work (Vooková *et al*. 2001). Also in other plant species, it is possible to influence embryo quality by modification of some treatments (Hong *et al.* 2008, Hu *et al.* 2008*)* and obtain efficient regenerant system (Wang *et al.* 2008). Number of embryos exhibiting morphological abnormalities was decreased and emblings were morphologically similar to zygotic seedling. However, dry mass was mainly accumulated in seedlings after 21 - 50 d of culturing. In present experiment no differences were found in protein composition of seedlings and emblings. With respect to these characteristics seedlings and emblings share the same quality. It seems that protein composition is not changed during *in vitro* conditions. Only a small number of needle proteins of Norway spruce showed early responses to the experimental treatment (Blödner *et al.* 2007). But contents of chlorophylls were lower in emblings than in seedlings. Some connection between chlorophyll and protein content in plant is known and the developmental stage of photosynthetic apparatus is important for relation of these components. It was confirmed that all components of photosystem 2 were determined in very early stage of needle development (Fulgosi *et al.* 2005). Probably, emblings manifested adaptation which allowed them to grow under *in vitro* conditions all the time. Under field conditions, shade needles of Norway spruce showed chlorophyll content 59 % lower than sun needles (Bertamini *et al*. 2006). Similarly, a reduction of chlorophyll content under shade was also observed in *A. alba* seedlings (Robakowski *et al.* 2004).

The analysis of the elements content of the needles is a common method both for assesment of nutrition status, and for the early diagnosis of some environmental disturbances of forest trees (Schleppi *et al.* 2000). Contents of microelements were higher in seedlings, contents of Ca, Mg, Na and K were elevated in emblings. Ca and Mg contents of embling were twice higher compared to those of seedlings. Unlike seedlings, development of emblings occured in condition *in vitro*. From this point of view many differences in these tissues can be expected. Growth conditions influenced dry mass and increase a K/N ratio in needle of phytotron-grown seedlings of Aleppo pine (Fernández *et al.* 2003). In seedlings, elevated concentrations of Cu, Zn, Fe and Mn were found as compared to those in emblings. Higher contents of these micronutrients can be explained by antagonistic relationships of some microelements to Ca and Mg (Szymura 2003).

Elevated macroelement contents in emblings and higher chlorophyll and dry mass content in seedlings indicate that the comparison of photosynthesis, water relationship, enzyme activity of emblings and seedlings will be required. In the next also monitoring the genetic fidelity of embryogenic tissues (Yang *et al.* 2008) cultured for a many years would be suitable.

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