Effect of sucrose concentration, charcoal, and indole-3-butyric acid on germination of *Abies numidica* somatic embryos

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Abstract

The dependency of radicle elongation in *Abies numidica* somatic embryos on germination media has been studied. No significant differences were detected between the Murashige and Skoog (MS) and Schenk and Hildebrandt (SH) medium. The addition of 10 g dm^{-3} activated charcoal or 0.05 mg dm^{-3} indole-3-butyric acid (IBA) into both media had positive influence on embryo germination. Difference between activated charcoal and IBA effects were significant. The high rooting percentage (85 %) was recorded on half SH medium with 10 g dm^{-3} sucrose and activated charcoal. After IBA addition rooting percentage was increased to 95 %. During 7 months 73 % of plantlets survived transfer to soil and in 54 % of plantlets shoot growth was observed.

Additional key words: Algerian fir, conifers, root formation.

Introduction

Somatic embryogenesis has became a convenient technique for multiplication of many conifers including genus *Abies*. The protocols elaborated for the different species and hybrids of *Abies* with regard to induction, proliferation, maintenance of embryogenic tissue and maturation of somatic embryos were relatively similar (Schuller and Reuther 1995, Guevin *et al.* 1994, Hristoforoglu *et al.* 1995, Nørgaard 1997, Gajdošová *et al.* 1995, Salajová *et al.* 1996, Rajbhandari and Stomp 1997, Guevin and Kirby 1997). Induction takes place with cytokinin as the sole plant growth regulator. Proliferation and maintenance of the cultures may take place with or without cytokinin. Casein hydrolysate and glutamine increase the proliferation. Maturation of somatic embryos is improved by abscisic acid (ABA) and sugars with their stimulating effect on formation of mature embryos. Maltose seems to be superior to lactose and sucrose (Hristoforoglu *et al.* 1995, Nørgaard 1997). The addition of polyethylene glycol-4000 (PEG-4000) to maturation medium has promotive effect and increases the yield of somatic embryos. Partial drying of matured somatic embryos is necessary for subsequent root development (Hristoforoglu *et al.* 1995, Nørgaard 1997, Vooková *et al.* 1997/98).

In 1998 the embryogenic tissue of *A. numidica* was initiated from immature zygotic embryos in our laboratory. We achieved somatic embryo maturation but with low ability of germination. Despite of many studies on induction, proliferation and maturation, there is a lack on information about factors affecting germination of *Abies sp.* somatic embryos. Media for germination are routinely used with sucrose in concentration 20 g dm^{-3} with (Vooková *et al.* 1997/98) or without activated charcoal (Salajová *et al.* 1996, Guevin and Kirby 1997). Sixty one percent of *A. alba* plantlets with a roots and primary needles developed on BM medium when a combination of 10 % sucrose and 20 % maltose was provided as a carbon source. Charcoal but not the gelling agent was important for radicle protrusion of *A. nordmanniana* (Hristoforoglu *et al.* 1995, Nørgaard 1997).

In present study the effect of sucrose concentration, charcoal and IBA on germination ability of *A. numidica* somatic embryo was defined.

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Abbreviations: ABA - abscisic acid; ac - activated charcoal; BA - 6-benzyladenine; CH - casein hydrolysate; ESM - embryogenic suspensor mass; GL - L-glutamine; IBA - indole-3-butyric acid; MI - myo-inositol; MS - Murashige and Skoog (1962) medium; PEG-4000 - polyethylene glycol-4000; SH - Schenk and Hildebrandt (1972) medium.

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Materials and methods

Embryogenic cell line of *Abies numidica* De Lann. was initiated from immature zygotic embryos in 1998 on SH medium (Schenk and Hildebrandt 1972) with 100 mg dm^{-3} myo-inositol (MI) and 1 mg dm^{-3} benzyladenine (BA). Proliferation of this embryogenic suspensor mass (ESM) was obtained on the same medium supplemented with 500 mg dm^{-3} L-glutamine (Gl) and 1000 mg dm^{-3} casein hydrolysate (CH). ESM was maintained on proliferation medium in the dark and subcultured in a three week interval.

Maturation medium contained half strength Murashige and Skoog (MS, 1962) macro- and micro-elements, and FeEDTA, and modified vitamins: 1 mg dm^{-3} nicotic acid, 1 mg dm^{-3} thiamine HCl, 1 mg dm^{-3} pyridoxin HCl, 2 mg dm^{-3} glycine, 100 mg dm^{-3} MI. The medium was supplemented with 40 g dm^{-3} maltose, 100 g dm^{-3} polyethylene glycol-4000 (PEG-4000), 10 mg dm^{-3} abscisic acid (ABA), CH and Gl in concentration of 500 mg dm^{-3}.

Prior to germination the isolated mature somatic embryos were undertaken to partial drying in the dark at 21 - 23 °C during 3 weeks as described previously for hybrids (Vooková *et al.* 1997/98). After partial desiccation, selected mature somatic embryos (at least with three cotyledons) were transferred to germination medium and cultured at 16-h photoperiod (irradiance of 110 µmol m^{-2} s^{-1}) and temperature of 21 - 23 °C. Embryos were selected in order to avoid premature germination of embryos with a lower number of cotyledons which readily germinated.

Five types of treatments were used for somatic embryo germination: *1*) modified MS medium containing half strength concentration of MS macroelements, original microelements, and FeEDTA, supplemented with 25 mg dm^{-3} thiamine HCl, or modified SH medium containing half strength SH medium salts and full SH vitamins; *2*) modified MS and SH media supplemented with 10 dm^{-3} activated charcoal (*Darco G 60, Serva*, Heidelberg, Germany); *3*) modified MS medium with or without activated charcoal supplemented with 0.05 mg dm^{-3} indole-3-butyric acid (IBA, *Sigma*, USA); all these media contained 20 g dm^{-3} sucrose; *4*) modified SH medium with sucrose in concentration 10, 20, 40 and 60 g dm^{-3}; *5*) modified SH medium with sucrose in concentration 10 g dm^{-3} supplemented with 0.05 mg dm^{-3} IBA.

The efficacy of 10 g dm^{-3} sucrose in germination medium was tested also for germination of somatic embryos *A. cilicica* (3 cell lines), *A. cilicica* × *A. nordmanniana* (3 cell lines) and *A. nordmanniana* × *A. weitchii* (6 cell lines). Induction of ESM and somatic embryo maturation was achieved on the same media as in case of *A. numidica*.

Induction, maturation and germination media were solidified with 3 g dm^{-3} *Phytagel* (*Sigma*). Growth regulators were coautoclaved with other ingredients of media. Plantlets with root and epicotyl were selected and than transplanted into pots containing autoclaved mixture of soil, peat and perlite (2:2:1).

Six replications of ten embryos were cultivated in Erlenmayer flask with 50 cm^3 media per treatment under irradiance of 110 µmol m^{-2} s^{-1} for 16-h photoperiod. Germination percentages were evaluated after 40 d of cultivation. The experiment was repeated two times. Statistical evaluation of the data was carried using Student's *t*-test.

Results and discussion

In this study we observed differences among germination media in terms of radicle elongation (Tables 1 and 2). After the partial drying treatment the root was observed between day 6 and 10 on germination medium. Only 9.82 % and 11.36 % of somatic embryos of *Abies numidica* germinate on modified MS and SH media, respectively. Less than 1 % of *A. fraseri* somatic embryos germinated on BLG medium (Guevin and Kirby 1997) and 12 % of *Abies* hybrid somatic embryos germinated on DCR basal medium (Salajová *et al.* 1996). Many of the

Table 1. The effect of medium, activated charcoal (ac, 10 g dm^{-3}) and IBA (0.05 mg dm^{-3}) on germination of *A. numidica* somatic embryos. Means ± SE, $n = 12$.

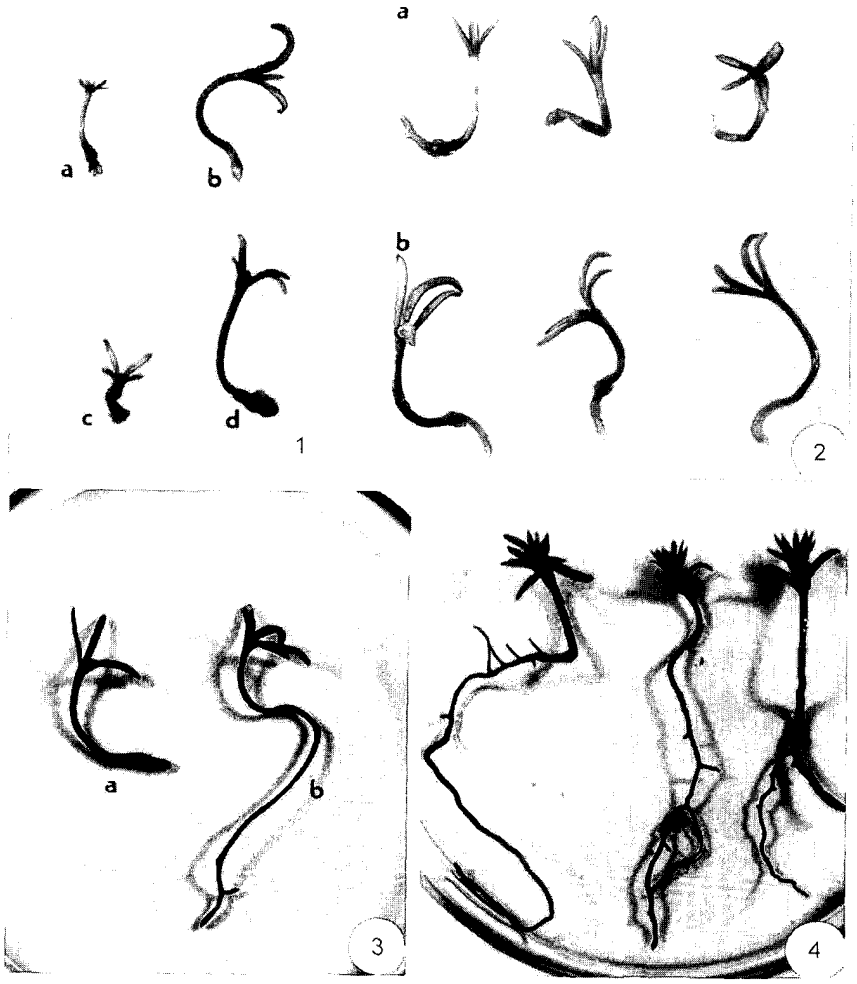
Treatment	Rooting [%]
MS	11.36 ± 3.97
SH	9.82 ± 2.41
MS + ac	44.36 ± 5.86
SH + ac	31.88 ± 6.84
MS + IBA	24.00 ± 5.51
MS + ac + IBA	80.00 ± 4.10

Table 2. Combined effect of sucrose and activated charcoal (10 g dm^{-3}) added to SH medium on germination of *A. numidica* somatic embryos. Means ± SE, $n = 12$.

Sucrose concentration [g dm^{-3}]	Rooting [%]
10	85.45 ± 4.11
20	30.14 ± 6.12
40	1.72 ± 1.42
60	1.50 ± 3.00

embryos formed small callus at the hypocotyl base which later necrotized. No significant differences were detected between these two basal media. The addition of 10 g dm^{-3} activated charcoal into both media had very significant influence ($P \leq 0.01$) on embryo germination. Also Nørgaard (1997) achieved the high germination percentage (60 %) of *A. nordmanniana* somatic embryos on medium with charcoal. The effect of 0.05 mg dm^{-3} IBA added to MS basal medium was significant compared with embryo germination on basal medium. Differences between charcoal and IBA effect were significant. The highest germination percentage (80 %) was achieved on medium including IBA and charcoal (Table 1). IBA has been used widely in rooting treatments in tissue cultured conifers at higher concentrations ranging from 1 - 10 mg dm^{-3}, including the pulse treatment with 20 - 127 mg dm^{-3} IBA (reviewed by Mohammed and Vidaver 1988). The beneficial effects of medium containing IBA during spruce somatic embryos maturation on subsequent root elongation and growth have been reported (Roberts *et al.* 1990, Flinn *et al.* 1991). Root elongation and further plant development of Norway spruce was supported by addition of 0.05 mg dm^{-3} IBA to conversion medium (Slabý and Havel 1999).

The embryos germinating on two different basal media without or with charcoal were different in size (Fig. 1). The eight percent of the plantlets exhibited primary needles on modified SH medium with charcoal. Based on this fact, sucrose addition was tested on SH medium with charcoal (Table 2, Fig. 2). The highest rooting percentage was recorded on medium with 10 g dm^{-3} sucrose. High rooting percentage was achieved



Figs. 1 - 3. *A. numidica* plantlets after 40 d germination of somatic embryos on different germination media.
Fig. 1. *a* - modified MS medium, *b* - modified SH medium, *c* - MS medium with activated charcoal, *d* - SH medium with activated charcoal.
Fig. 2. SH medium containing *a* - 20 g dm^{-3} sucrose + activated charcoal + IBA, *b* - 10 g dm^{-3} sucrose + activated charcoal.
Fig. 3. SH medium with activated charcoal supplemented with *a* - 10 g dm^{-3} sucrose, *b* - 10 g dm^{-3} sucrose + IBA.
Fig. 4. *A. numidica* regenerants with apical shoot after five month growth in the soil. Germination was achieved on SH medium with 10 g dm^{-3} sucrose.

also for other cell lines of *Abies* sp. and hybrids (Table 3). It seems that this medium is widely applicable for germination of other *Abies* sp. in spite of the importance of genotype. With increasing sucrose concentration the germination percentage was reduced. Root initiation is a energy requiring process. Following ^{14}C-sucrose uptake, it was determined that spruce germinants consumed only 25 % of the sucrose available in a 1 % (m/v) sucrose-containing medium (Carrier *et al.* 1997). *In vitro* studies with other woody species have shown that elevated sucrose concentration (40 g dm^{-3}) significantly increased stem lignification and acclimatization success (Mohammed and Vidaver 1988). Percentage of germinated embryos on medium with charcoal and 10 g dm^{-3} sucrose was nearly the same as on medium with combined effect of charcoal and IBA. However, it seems that IBA can compensate negative effect of higher sucrose concentration in the medium (20 g dm^{-3}).

Table 3. Germination of somatic embryos of other tested cell lines on SH germination medium with charcoal (10 g dm^{-3}) and sucrose (10 g dm^{-3}). Means ± SE, $n = 6$.

Species/hybribs	Cell line	Rooting [%]
A. cilicica	50	74.99 ± 6.81
	91	94.42 ± 2.71
	98	92.13 ± 3.95
A. cilicica ×	102	99.60 ± 1.03
A. nordmanniana	106	83.61 ± 11.4
	145	98.33 ± 1.18
A. nordmanniana ×	33	73.70 ± 2.21
A. weitchii	35	75.00 ± 2.31
	36	90.90 ± 4.13
	38	90.00 ± 3.11
	44	92.31 ± 1.14
	45	91.64 ± 1.60

At the end of the experiment all substances with beneficial effect on root development were used to optimise the germination medium. Number of developing roots was not significantly increased after IBA addition into SH medium with charcoal and 10 g dm^{-3} sucrose. In total, 95 ± 1.82 % of germinated embryos was achieved. Embryos germinated on this medium had longer radicles than embryos germinated on the same medium without IBA (Fig. 3). During cultivation in the soil no differences in growth were confirmed.

In the experiment we have succeeded in improving germination of *A. numidica* somatic embryos from 9.82 - 11.36 % (on basal media) to 95 %. Plantlets that had formed radicle were transferred to autoclaved soil/peat/perlite mixture. During 7 months 73 % of plantlets survived transfer to soil and 54 % of them exhibited shoot growth (Fig. 4).

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