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Physiological and Structural Aspects of In Vitro Somatic Embryogenesis in *Abies alba* Mill

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Abstract: Initiation of somatic embryogenesis from immature zygotic embryos, long-term maintenance of embryogenic tissue in vitro or by cryopreservation, as well as maturation, of somatic embryos of *Abies alba* Mill. are reported in this study. For the initiation of embryogenic tissues, a DCR medium containing different types of cytokinins (1 mg·L^{-1}) were tested. During three consecutive years, 61 cell lines were initiated out of 1308 explants, with initiation frequencies ranging between 0.83 and 13.33%. The type of cytokinin had no profound effect on the initiation frequency within one given year. Microscopic observations revealed presence of bipolar somatic embryos in all initiated embryogenic tissues. Besides the typical bipolar somatic embryos, huge polyembryonal complexes, as well as "twin" embryos, were observed. Maturation of somatic embryos occurred on a DCR medium supplemented by abscisic acid (10 mg·L^{-1}), polyethylene glycol (PEG-4000, 7.5%) and 3% maltose. The maturation capacity was cell-line dependent. All of the four tested cell lines produced cotyledonary somatic embryos, though at different quantities, of 16 to 252 per g of fresh weight. After germination, seedlings developed, but their further growth soon stopped after the formation of a resting bud. Altogether, seven cell lines were cryopreserved, using the slow-freezing technique. After rewarming, all tested cell lines showed regrowth rates between 81.8 and 100%.

Keywords: cryopreservation; cytokinins; cytology; silver fir

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1. Introduction

Somatic embryogenesis is the process of in vitro formation of embryos from somatic plants cells without fusion of gametes. Somatic embryogenesis from immature zygotic embryos in the conifer Norway spruce was first reported in 1985 [1,2]. Since then, considerable successes have been achieved, and efforts have been focused on the embryogenic tissue initiation, somatic embryo maturation and somatic seedlings regeneration in many conifers [3,4]. This process provides an experimental tool for the study of the early development of conifer trees, and this knowledge can be applied to zygotic embryos, since somatic embryos strongly resemble their zygotic counterparts in seeds [5]. Somatic embryogenesis in conifers also represents an efficient plant regeneration system important for genetic transformation studies and cryopreservation. In addition, production of embryos from somatic cells can be used for mass propagation of conifers, while the process is amenable to automation [6–8].

The members of the genus *Abies* represent valuable and economically important trees grown in various parts of the world. The micropropagation of *Abies* species by adventitious bud/shoot initiation

and subsequent rooting resulted in limited success. Adventitious buds were initiated in Fraser fir [9], silver fir [10] or in hybrid firs [11], but the rooting of elongated shoots was poor. The application of somatic embryogenesis led to new opportunties for micropropagtion of *Abies* species, with a focus on the initiation of embryogenic tissues from immature or mature zygotic embryos. In *Abies nordmanniana* [12], *A. lasiocarpa* [13], *A. cephalonica* [14] or some *Abies* hybrids [15–18], embryogenic tissue was initiated from immature zygotic embryos. Other members of the genus, such as *A. balsamea*, *A. fraseri* and *A. procera*, proved to be more recalcitrant toward somatic embryogenesis [19]. Likewise, Rajbhandari and Stomp [20] reported only limited success with *A. fraseri* embryogenic tissue initiation and maintenance. Using a new medium containing Al salts, abscisic acid, brassinolide, paclobutrazol and sodium thiosulfate for the initiation of somatic embryogenesis in Fraser fir, Pullman et al. [21] succeeded in obtaining relatively high initiation frequencies (6–62%, depending on the genotype), and 50% of initiations were captured on a maintenance medium. Cotyledonary somatic embryos capable of germination and somatic seedlings regeneration were obtained. The maturation of somatic embryos was genotype dependent and influenced by the salt composition in the medium. During maintenance, the maturation capacity of cell lines decreased. To resolve this problem, cryopreservation was applied.

Further experimentations focused on broadening the "initiation window" to mature zygotic embryos in *A. nordmanniana* [19,22], *A. balsamea* [23] and hybrid firs [24].

A first study on somatic embryogenesis in *A. alba* was reported by Erdelsky and Barancok [25]. They observed the initiation of rapidly proliferating white tissues; however, the embryogenic nature of the tissue was neither tested nor recognized. Gebhardt et al. [26] reported somatic embryogenesis from immature zygotic embryos of open pollinated seeds of *A. alba*. Similarly, in *A. alba*, the megagametophytes containing immature zygotic embryos produced tissue in which early stage somatic embryos have been identified. Some of these structures developed and formed cotyledons, while the differentiation of roots was suppressed [27]. Mature zygotic embryos of *A. alba* cultured on media with 2.2 μM BA and 2.3 μM kinetin (KIN) formed embryogenic suspensor masses at a 40% initiation frequency [28]. Initiation was induced only by cytokinins, but for further proliferation, 0.2 μM 2,4–D was also included. Zoglauer and Rheuter [10] described the early stages of *A. alba* somatic embryo differentiation at the structural level. The somatic embryos arose as single structures on explants, and their origin was traced to epidermal and subepidermal cells. Periclinal cell divisions resulted in formation of nodular structures isolated from surrounding tissues, indicating their autonomy. These structures have been recognized as early stage somatic embryos. Despite the many reports on the successful initiation and long-term maintenance on solid media or by cryopreservation, somatic embryo maturation, as well as somatic seedling regeneration, in *A. alba* remains problematic [29].

In this paper, we report the intiation of embryogenic tissues from immature zygotic embryos of *A. alba* Mill., using a DCR medium. The effect of five different cytokinins on the initiation was tested, and the maintenance of initiated tissue on solid media or by cryopreservation is reported. Cultivation of four different cell lines on a maturation medium resulted in the development of somatic embryos capable of germination and somatic seedling regeneration.

2. Materials and Methods

2.1. Plant Material and Culture Conditions

Green cones were collected from open pollinated trees of *Abies alba* Mill. growing in natural stands (Slovakia). The cones were harvested from different (3–5) trees in the second half of July as follows: July 24 in 2013, July 19 in 2017 and July 27 in 2019. Immature seeds, containing embryos at their early cotyledonary developmental stage, were extracted from the cones, and their surface was sterilized by immersion in 10% H_2O_2, for ten min, followed by four rinses in sterile distilled water. The megagametophytes were aseptically excised from seeds and placed horizontally on the culture medium.

For culture of explants, a DCR medium [30] containing enzymatic caseinhydrolysate (1000 mg·L^{-1}), glutamine (500 mg·L^{-1}), myoinositol (200 mg·L^{-1}), glycine (2 mg·L^{-1}) and the vitamins thiamine (1 mg·L^{-1}), nicotinic acid (0.5 mg·L^{-1}), pyridoxine (0.5 mg·L^{-1}) and 2% sucrose was used. The medium was further supplemented with cytokinins N6-benzyladenine (BA), zeatin (ZEA), thidiazuron (TDZ), kinetin (KIN) or 2-isopentenyladenine (2iP) at a concentration of 1 mg·L^{-1}. The medium was solidified with gelrite 0.3% (Duchefa).

The megagametophytes were placed on the surface of a medium in plastic Petri plates (diameter 6 cm). In each dish, 6 explants were cultured, in the dark, at 23 °C. Following initiation, the embryogenic tissue was maintained by transfers to new media at two-to-three-week intervals. For the initiation experiments, a total of 1308 explants were used. During the first 6 months, the cultures were maintained on the same initiation medium. Thereafter, they were transferred to a DCR medium supplemented with 1 mg·L^{-1} BA. Initiation frequencies (IF) were calculated as the number of explants that produced embryogenic tissues relative to the total number of cultured explants.

2.2. Microscopic Observations

The structure of early somatic embryos in tissue was followed by using squash preparations. Small pieces of tissues were placed on glass slide, stained with two drops of 2% acetocarmine for two to three min, squashed and covered by a cover glass. The preparations were examined under a light microscope Axioplan 2 (Carl Zeiss Microscopy) equipped with a camera system (Sony DXC-5500).

First observations were executed when the initiated tissue was already intensively proliferating and later at two-to-three-month intervals, regularly, during the maintenance. For each observation, at least three samples were used per cell line.

2.3. Fresh and Dry Mass Accumulation

At day 8 after the last subculture, 0.5 g of tissue was transferred to a DCR proliferation medium. The fresh and dry mass were evaluated at days 7, 14 and 21 after transfer. The tissues were taken from the proliferation medium, and their fresh mass was recorded. The samples were then dried in an oven, for 3 h, at 80 °C, following 2 h at 105 °C, and the dry mass was recorded. Then, the samples were dried at 105 °C, for 2 h, and dry mass again recorded. The two measurements were compared, and the second measurement was considered in case it did not differ from the first one by more than 1 mg. Nine cell lines were included in the experiments that were repeated twice, with 5 samples for each cell line. The obtained data were analyzed by using a t-test.

2.4. Somatic Embryo Maturation and Germination

For the maturation experiments four cell lines were selected (A01, A30, A31 and A32). On day 12 after the last subculture, 1 g of vigorously growing tissue was resuspended in a liquid medium containig half the strength of DCR macro- and microelements, and an aliquot of 100 mg of fresh mass was then pipetted and spread onto the stacked filter paper discs, for the liquid to be absorbed. The upper filter paper disc was placed on the maturation medium that consisted of DCR macro-and microelements, 10 mg·L^{-1} abscisic acid (ABA) and supplemented with 7.5% polyethylene glycol (PEG-4000) and 3% maltose and solidified with gelrite (0.3%). The maturation of cell lines was also tested on a medium without PEG-4000 of the same composition as indicated above. The cultures were kept in a culture room, in darkness, at 23 °C. After eight weeks of cultivation, the developing somatic embryos were transferred to a medium devoid of ABA and PEG-4000 (post-maturation treatment, two to three weeks). The developing somatic embryos were categorized according to von Arnold and Hakman [31] as Stage 2 (precotyledonary somatic embryos), Stage 3 (cotyledonary somatic embryos) and Stage 4 somatic seedlings. The number of developing somatic embryos (Stages 2 and 3) was calculated on 1 g of fresh mass. The number of precotyledonary embryo was calculated after five weeks of cultivation on a maturation medium. The number of cotyledonary embryos was evaluated after eight weeks, on a maturation medium. The data were analyzed by using a t-test.

The maturation experiments were repeated three times, with each four to five Petri dishes. After post-maturation treatment, the somatic embryos on the filter paper discs were transferred to empty Petri dishes that were 6 cm in diameter and placed into Nalgene ™ container with sterile distilled water on the bottom, for two weeks, in darkness, at 23 °C. For germination, well-developed cotyledonary embryos that consisted of at least four cotyledons were selected. The germination was evaluated by the *t*-test. The germination medium contained DCR macro and microelements, 2% maltose and 1% activated charcoal and was solidified with 0.5% gelrite. During the first week of germination, Petri dishes with somatic embryos were kept in darkness; the second week, the somatic embryos were cultured in dimmed light, and, finally, when elongation of hypocotyl and cotyledons occurred, the somatic embryos, which were already small plantlets, were transferred to light. Lastly the small plantlets were transferred into Magenta baby-food jars on the same medium as for germination and cultured in a growth chamber room, at 23 °C, at a 16 h photoperiod, under cool white fluorescent tubes, at a photosynthetic photon flux density of 50 μmol^{-2} s^{-1}.

2.5. Cryopreservation

For cryopreservation, the two-step slow-freezing technique was applied as described elsewhere in detail [32,33]. Briefly explained, 1.5 g of vigorously growing tissue of the cell lines A01, A02, A07, A11, A13, A16 and A23 was precultured on a solid DCR proliferation medium containing 0.5 M sorbitol, for 24 h. Then, 3 g of tissue collected from two plates was resuspended in a liquid medium of the same composition as the preculture medium. Thereafter, a 10% DMSO (dimethylsulfoxid) solution dissolved in a proliferation medium was added, in three steps, on ice, to reach a final concentration of 5% DMSO. The suspension was pipetted to 1.8 ml cyrovials, and the cryovials were placed to Mr. Frosty (Nalgene™) container filled with isopropanol. The container with samples was put into the deep freezer, at −80 °C. The temperature was measured by placing a thermocouple in a representative cryovial, and when −40 °C was reached, the cryovials were plunged into liquid nitrogen (LN) and kept in this condition for one year. Then the vials were rewarmed in a 40 °C water bath, for the cells to be cultured on the proliferation medium. Samples designated as control (C) were preteated but not exposed to liquid nitrogen. Visual observations were executed at 3–5-day intervals, to monitor the tissue regrowth. The experiments were repeated three times; one repetition included five Petri dishes for each cell line, as control, as well as cryopreserved tissue.

3. Results

3.1. Initiation Experiment in 2013

The embryogenic tissue was initiated from female gametophytes containing immature zygotic embyos. Then, 12–15 days after placing immature zygotic embyos on initiation media, white embryogenic tissues were observed, protruding from explants (Figure 1a). Twenty-five out of the 312 cultured explants, i.e., 8.03%, produced embryogenic tissue. The frequency of embryogenic tissue formation was not profoundly affected by the type of cytokinin used (Table 1). ZEA was the most effective, with 13.33% initiation frequency. Treatments with BAP or KIN gave lower initiation frequencies, but the results with respect to the PGR treatment were statistically not significant ($p \leq 0.05$). Once a size of approximately 5 mm was reached, the embryogenic tissue was separated from the explant and subcultured as a cell line, derived from an individual zygotic embryo.

All of the established cell lines proliferated intensively over one year. Subsequently, four out of the 25 cell lines started to show signs of necrosis, and, in the second year of cultivation, some cell lines changed the mucilaginous consistency, resembling non-embryogenic callus tissue.

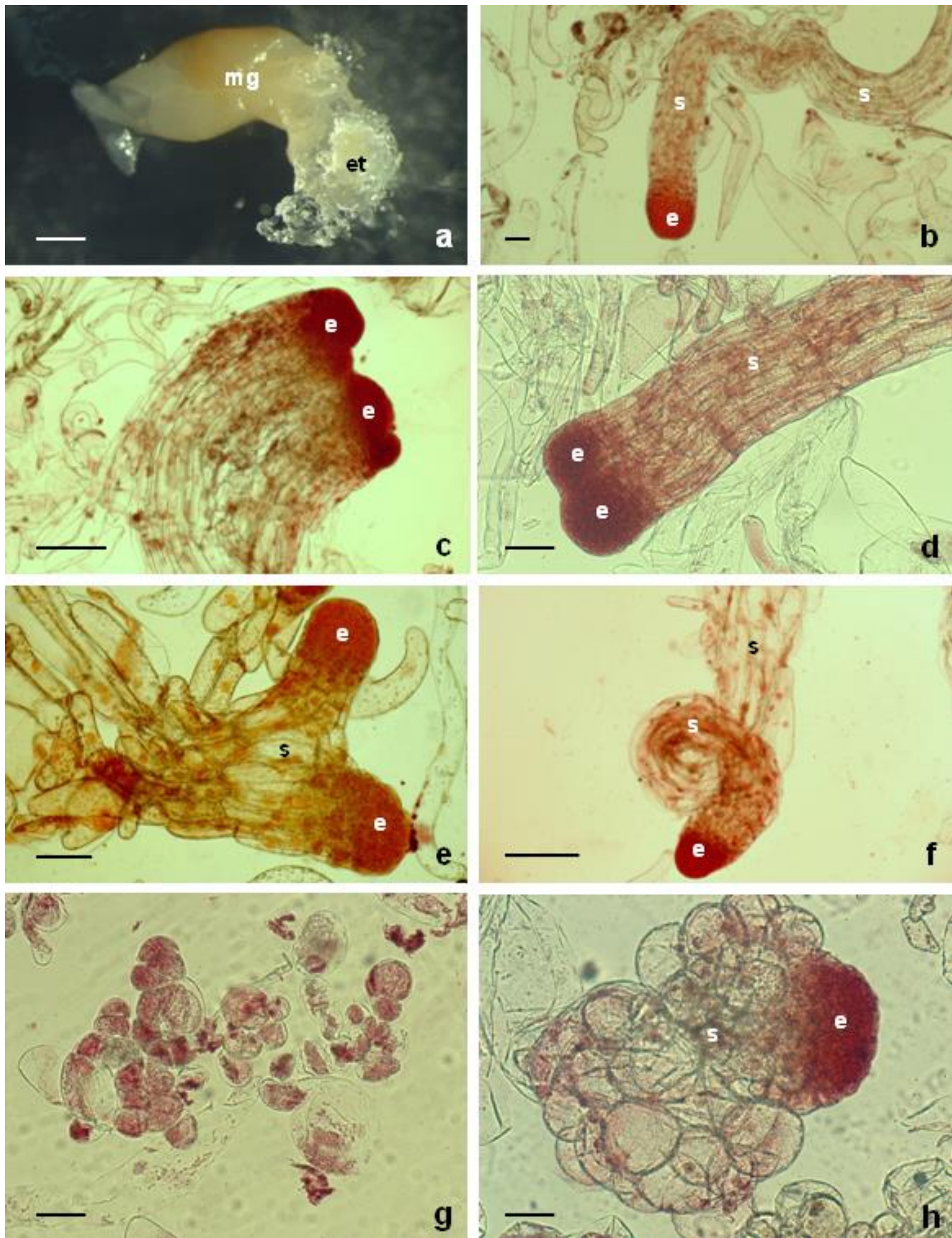

Figure 1. Initiation of somatic embryogenesis and structural characterization of embryogenic tissue in *Abies alba*: (**a**) extrusion of embryogenic tissue (et) from megagametophyte explant (mg), (**b**) bipolar somatic embryo with embryonal meristematic cells (e) and long vacuolized suspensor cells (s), (**c**) polyembryonal complex observable in embryogenic tissue, (**d**) twin somatic embryo, (**e**) somatic embryos with branched suspensor (s), (**f**) curling of suspensor (s) of a somatic embryo; (**g**) unorganized cell mass observed in tissue that lost the embryogenic character, (**h**) somatic embryo with changed micromorphology after five years of maintenance on a proliferation medium. Bars: a—1000 µm; (**b**,**d**,**e**,**g**,**h**) 200 µm; (**c**,**f**) 500 µm.

Table 1. Initiation of embryogenic tissues from immature zygotic embryos of *Abies alba* Mill., in three years, and the effect of different cytokinins (CKs) in the initiation medium.

	2013			2017			2019		
	Explant No.	**RE**	**IF (%) [1]**	**Explant No.**	**RE**	**IF (%) [1]**	**Explant No.**	**RE**	**IF (%) [1]**
CKs									
BA	60	4	6.66 (3.69)	114	1	0.88 (0.87)	72	6	8.33 (3.24)
ZEA	60	8	13.33 (4.84)	120	1	0.83 (0.32)	90	6	6.67 (3.17)
TDZ	60	3	5.0 (3.55)	120	2	1.67 (0.47)	78	7	8.97 (3.05)
KIN	66	7	10.6 (4.64)	114	2	1.75 (1.21)	78	6	7.69 (3.07)
2iP	66	3	4.54 (7.42)	120	2	1.67 (0.47)	90	3	3.33 (1.78)
Average			8.03 (1.66)			1.36 (0.21)			6.99 (0.99)

IF—initiation frequency. RE—responding explants. [1] Standard errors of mean are in parenthesis. BA—N6-benzyladenine; ZEA—zeatin; TDZ—thidiazuron; KIN—kinetin; 2iP—2-isopentenyladenine.

3.2. Initiation Experiment in 2017

The initiation frequencies were lower than in 2013, giving very low values, ranging from 0.83 to 1.75, averaging 1.36% (Table 1). Altogether, eight cell lines were initiated from a total 588 of explants, five died shortly after initiation and the remaining three cell lines were maintained on a proliferation medium (A30, A31 and A32). The effect of different types of cytokinins was again not apparent.

3.3. Initiation Experiment in 2019

In that year, the megagametophyte explants gave rise to embryogenic tissue at an overall rate of 6.99%. Altogether, 28 cell lines were obtained from 408 cultured explants (Table 1). After more than one year of maintenance, 18 cell lines were well proliferating. The extrusion of embryogenic tissue occurred within the first two to three weeks of explant culture. The ability to produce embryogenic tissue was not dependent on the type of cytokinin used.

3.4. Overall Assesment of Initiation

In the experiments performed over three consecutive years, 1308 explants were plated on a culture medium, and, altogether, 61 embryogenic cell lines were initiated. Our results suggest that the used cytokinins were equally efficient in their induction capacity.

3.5. Structural Organization of Embryogenic Tissue

The cellular composition of the different cell lines (tissue) was variable. Bipolar somatic embryos, composed of meristematic embryonal part ("head") subtended by a long suspensor, were observed in most of proliferating tissues (Figure 1b). Besides these structures, huge polyembryonal complexes (Figure 1c) or "twin" embryos (Figure 1d) could be observed. In these structures, two or several meristematic "heads" joined sharing a common suspensor. Occasionally the long suspensor branched or coiled (Figure 1e,f). Additionally, meristematic cell clumps interspersed with long vacuolized cells containing an apparent nucleus were frequently observed.

3.6. Changes in Structural Organization during Proliferation

Some cell lines lost embryogenic features in time, showing a consistence of non-embryogenic hard callusses. In these cell lines, the typical bipolarity of somatic embryos dissappeared, and round-shaped cells became apparent (Figure 1g). During the first five years of maintenance, cell line A01 was

characterized by the presence of bipolar somatic embryos. After this period, structural changes occurred. The meristematic "heads" were still present, but the long suspensor was replaced by small round-shaped cells (Figure 1h).

3.7. Maintenance of Embryogenic Tissue

The embryogenic tissues were maintained over a long period of time, by serial subcultures, at two-to-three-week intervals, on a fresh DCR proliferation medium supplemented with BA (1 mg·L^{-1}). No auxins were needed for tissue proliferation. On a maintenance medium, the embryogenic tissue remained at the proliferation stage. During the followed growth period of 21 days, fresh and dry mass accumulation gradually increased, but the tested cell lines differed (Figure 2). Cell line A01 showed the maximum fresh mass accumulation after three weeks of proliferation, approximately with a nine-fold increase. Some cell lines (A02 and A07) proliferated intensively, with a seven-fold increase in fresh mass accumulation, while others showed only a three-to-four-fold increase (A06, A10, A23, A30 and A31). The dry mass accumulation displayed a similar pattern and was positively correlated with the fresh mass accumulation (Figure 3).

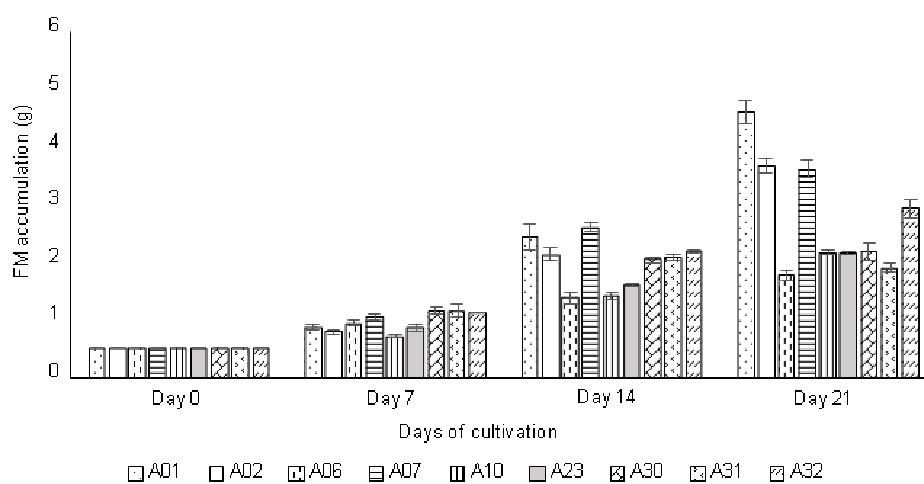

Figure 2. Fresh mass (FM) accumulation (g) in selected cell lines (A01...A32) during the growth cycle; at day 0, the fresh mass was 0.5 g for all cell lines (n = 10).

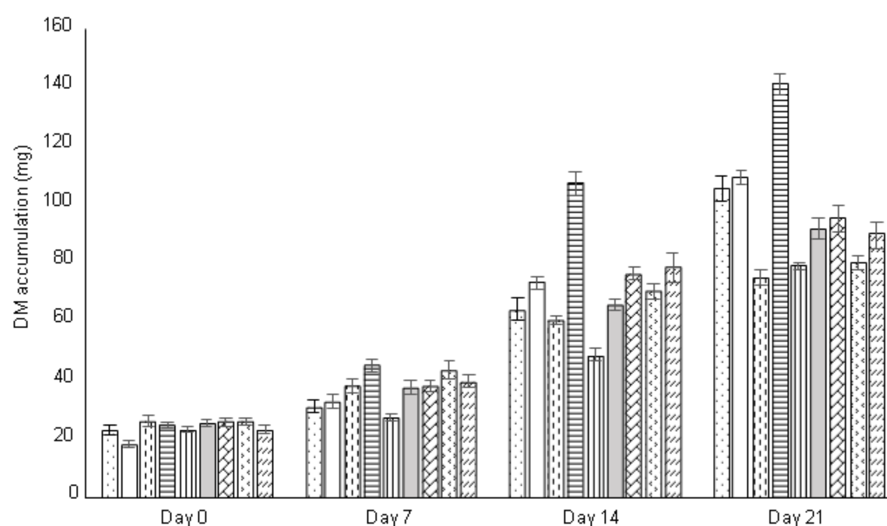

Figure 3. Dry mass (DM) accumulation (mg) in selected cell lines (A01...A32) during the growth cycle (n = 10).

Regular subcultures at two-to-three-week intervals were necessary to maintain vigorously growing tissues. Longer subculture intervals caused progressive tissue browning and necrotization. Occasionally white sectors with a proliferating capacity appeared on the surface of browning cultures. Isolation of such sectors from necrotic tissue and subsequent culture on a proliferation medium resulted in their further growth.

3.8. Somatic Embryo Maturation

During the maturation process, the somatic embryos of different cell lines followed similar patterns of development, despite their quantitative differences in somatic embryo production. After plating of the resuspended embryogenic tissue to a maturation medium, tissue regeneration started at days 4–5. After four to five weeks, precotyledonary somatic embryos became visible as structures anchored in embryogenic tissue by huge suspensors and distinguishable embryonic regions (Figure 4a). Following this period, gradually somatic embryos with emerging cotyledons developed (Figure 4b).

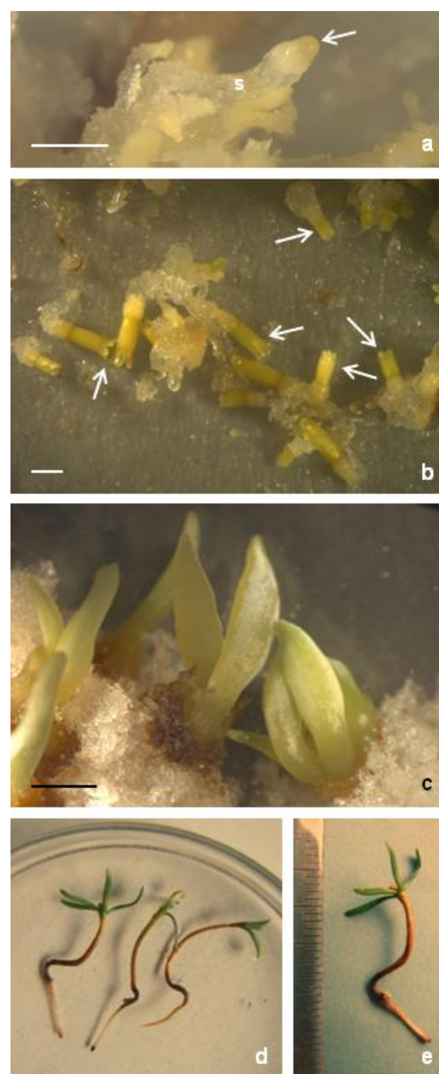

Figure 4. Somatic embryo maturation in *Abies alba*: (**a**) precotyledonary somatic embryo with apparent embryonal part (arrow) and suspensor (s) anchored in embryogenic tissue; (**b**) cotyledonary somatic embryos (arrows) developed on a maturation medium, approximately after eight weeks of cultivation; (**c**) abnormally developed structures with overproliferated tissue; (**d**,**e**) regenerated plantlets (somatic seedlings). Bars: (**a**,**b**) 000 µm; (**c**) 2000 µm.

3.9. Quantitative Assessment

At the end of the fifth week of maturation, numerous precotyledonary somatic embryos appeared on the surface of the callus. All of the tested cell lines produced precotyledonary somatic embryos (Stage 2), but significant differences were observed among cell lines (Table 2). Similarly, the cell lines differed in their ability to form fully developed cotyledonary somatic embryos. In the tested cell lines, many precotyledonary somatic embryos stopped their development without cotyledon differentiation. In the most productive cell line (A01), the development of precotyledonary somatic embryos to cotyledonary developmental stage reached 42.93%. A very weak development was achieved in cell line A30, with 6.55% conversion only. In this cell line, strong proliferation occurred, and the Stage 2 embryos were overgrown by proliferating tissue.

In all the tested cell lines, besides the well-developed somatic embryos, high numbers of abnormally developed structures, characterized by abnormal cotyledons were also present (Figure 4c). The aberrant strucures showed no further development. Moreover, the development of somatic embryos toward the cotyledonary stage (Stage 3) was unsynchronized. Somatic embryo maturation on a medium without PEG-4000 was not satisfactory, showing extreme proliferation of tissue in cell lines A01, A30 and A31, as well as somatic embryos degeneration, at the very early developmental stage, in cell line A32.

Table 2. Somatic embryo (se) maturation and germination in selected embryogenic cell lines of *Abies alba* Mill. (the number of somatic embryos was calculated per 1 g of fresh mass).

Cell Line	Precotyledonary Se [1]	Cotyledonary Se [1]	RP [2]	Germination (%)	SE [3]
A01	587 (45.67)	252 (23.30)	49	53 [a] (5.13)	93
A30	244 (48.17)	16 (5.36)			
A31	119 (36.21)	58 (10.22)	32	45 [a] (13.07)	70
A32	312 (33.54)	101 (23.21)	22	43 [a] (3.12)	51

[1] The standard errors of mean is in parenthesis. [2] Regenerated plantlets. [3] Number of somatic embryos tested for germination. [a] Statistically not significant.

3.10. Germination

The partially dessicated somatic embryos were placed horizontally on a germination medium and cultured in darkness for approximately 7 to 10 days. During this period, there was a slight elongation of hypocotyl and cotyledons. Further culture under dimmed light for another 7–10 days followed by culture on full light for another 1–2 weeks resulted in small plantlets with a 1.0–1.5 cm hypocotyl and elongated green cotyledons. The small plantlets were subsequently cultured in Magenta jars in light where they developed further. The lenght of root ranged between 3 mm and 1 cm (Figure 1d,e). Thereafter the somatic seedlings ceased to grow. The germination frequencies were similar in all tested cell lines (Table 2)

3.11. Cryopreservation

Cryopreservation of embryogenic tissues with the classical slow cooling method was successful and resulted in tissue regeneration of all tested cell lines, with frequencies between 81.1 and 100%. The differences observed among individual cell lines were statistically insignificant (Figure 5). Tissue regeneration started soon after thawing (four to seven days) and starting the third week of post-thaw culture; all regenerated tissues showed intensive proliferation.

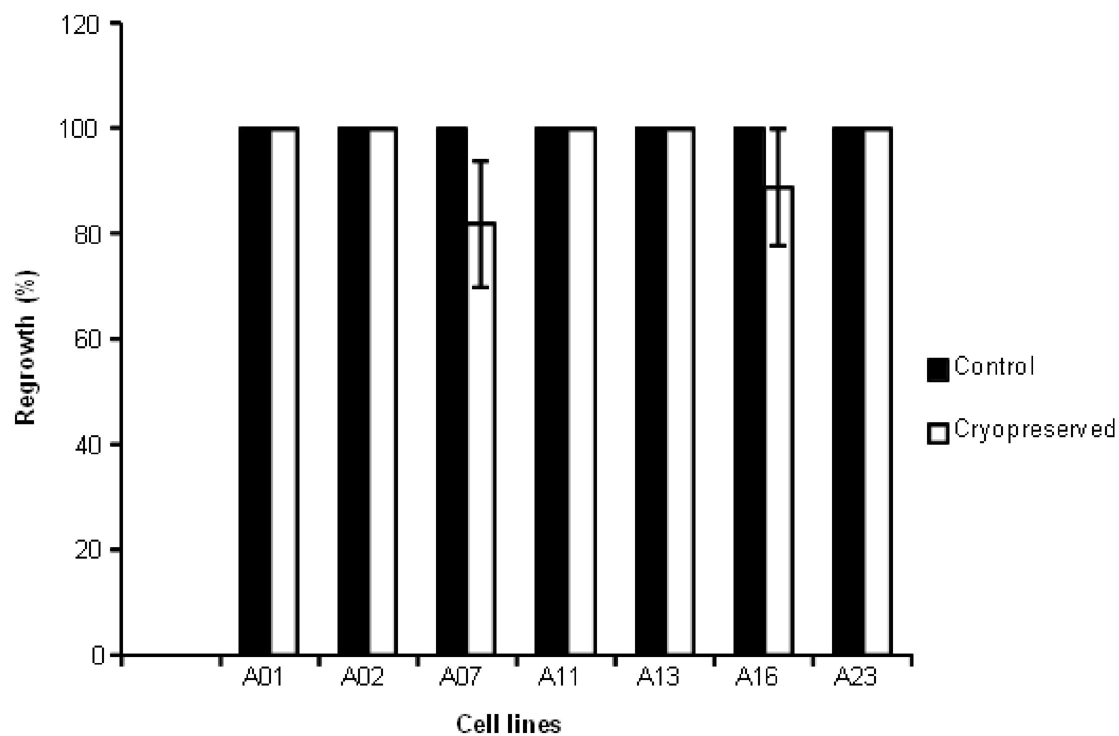

Figure 5. Regrowth (%) of different cell lines after cryopreservation (comparison to control tissue that was pretreated but not cryopreserved); three repetitions, each containing five Petri dishes; bars indicate standard errors.

4. Discussion

The successfull initiation of somatic embryogenesis in conifers depends on several factors, including the explant, exogenous plant growth regulators and basal medium composition [4,34]. In our experiments, the embryogenic tissue was initiated from immature zygotic embryos enclosed in the megagametophytes. Megagametophytes containing immature zygotic embryos have been used successfully as explants for the initiation of embryogenic tissues in *Abies* species or their hybrids [15,16,21,27,29,35,36], but the specific role of the megagametophyte in embryogenic tissue initiation has not been exactly determined. It is hypothesized that the megagametophyte provides nutrients and/or endogenous plant hormones that may be suboptimal in the culture medium [37], or can act as a mechanical barrier protecting the zygotic embryo during the cultivation [38].

In our experiments, the effect of different cytokinins on the initiation of somatic embryogenesis was tested over three consecutive years. Depending on the applied cytokinin and the year, initiation frequencies ranged between 0.83 and 13.33%. For the majority of conifer species, somatic embryogenesis initiation requires the presence of both auxins and cytokinins [4]. For species belonging to the genus *Abies,* cytokinin as a sole plant-growth regulator was sufficient for both the initiation of embryogenic cultures and the maintenance of initiated tissue [12,14,39,40]. The endogenous auxin production might be sufficient [11]. The role of exogenous auxin has been less extensively studied in *Abies*. Vondrakova et al. [41] showed a positive effect of exogenously applied 2,4–D on *Abies alba* somatic embryogenesis. The embryogenic tissues growing on a medium supplemented with 2,4–D yielded a higher number of mature somatic embryos, although, during the maturation period, no 2,4–D was needed. In *Abies* hybrids, embryogenic tissue initiation occurred also on a medium containing 2,4–D combined with BA, but during proliferation, non-embryogenic callus formation was observed [42]. Zancani et al. [43] evaluated the hormone-like effect of fulvic acid (FA) on somatic embryogenesis in *Abies cephalonica*. It stimulated the proliferation rate at the early growth stage.

In 2013, the initiation frequency reached 8.03% and is comparable to those obtained by Krajnakova et al. [29] for *Abies alba*: 5.8% in total, and 1.7 to 16.6%, depending on sampling date of

cones, but lower in comparison to the initiation frequencies of 22.5% obtained by Schuller et al. [27]. Such differences may originate from different experimental conditions applied in different laboratories.

In our experiments, somatic embryo maturation was achieved on a medium containing ABA (10 mg·L^{-1}) combined with PEG-4000 (7.5%) and 3% maltose. This medium was chosen based on our previous experiences with *Abies* hybrids [24,44]. The yield of cotyledonary embryo with developed cotyledons reached 16 to 252 per g of fresh mass inoculum, depending on the cell lines. Most studies on somatic embryo maturation in conifers reported the combination of the hormone ABA and high-molecular-weight osmoticum (PEG). Frequently observed responses to ABA are stimulation of storage reserves accumulation [31], reduction of tissue proliferation [45] and inhibition of cleavage and polyembryony [46]. Proper timing of exposure, as well as the concentration of ABA, significantly improved somatic embryo yield and their quality in loblolly pine. The mRNAs abundance in response to exogenous ABA could be used as a possible expression marker in somatic embryo maturation [47].

The addition of PEG as a non-plasmolysic osmoticum was an important factor in obtaining fully developed cotyledonary somatic embryos in *Abies alba*. PEG-4000 exerts non-osmotic moisture stress at the cellular level, preventing plasmolysis [48]. In white spruce, the addition of PEG-4000 stimulated storage lipid deposition and increased desiccation tolerance [49]. Storage proteins that were accumulated during maturation of white spruce somatic embryos resembled that of mature zygotic embryos [50]. The timing and concentration of PEG-4000 effect are also important. In *Abies nordmanniana*, Norgaard [51] reported the optimum PEG-4000 effect in the period of precotyledonary somatic embryo formation. The optimal concentration of PEG-4000 depends on the species, e.g., 7.5% for white spruce [50], 4% for the same species referred by Kong and Young [52] or 3.75% for *Picea abies* [53]. Tissue overproliferation during somatic embryo maturation in *Abies* species is a serious problem. In *Abies nordmannina*, PEG-4000 reduced tissue proliferation [51].

Besides well-developed cotyledonary somatic embryos, abnormal structures with fused cotyledons have also been frequently observed in our study. The abnormal somatic embryo development in conifers may be the result of a disturbed polar auxin transport [54]. The insufficiently developed somatic embryos of Norway spruce were eliminated by low doses of an anti-actin drug, latrunculin B, applied at the beginning of somatic embryo maturation [55].

In *Abies alba*, the germination was tested after a slight dessication of somatic embryos, and the germination percentages were lower compared to other *Abies* species. In *Abies nordmanniana*, different medium compositions for germination of somatic embryos were used [51], and the maximal percentages, around 60%, were obtained on a $\frac{1}{2}$ MS medium, as well as on a medium solidified with agar and supplemented with activated charcoal. The combined effect of sucrose and maltose resulted in 61% germination and also vigorous planlets development in *Abies alba* [28]. In both species mentioned, the somatic seedlings were transferred to soil. The growth of somatic seedlings obtained in *Abies alba* came to a halt seven to eight weeks after germination, probably due to dormant bud formation.

Zoglauer et al. [56] reported 80–90% conversion of somatic embryos of *A. nordmanniana* into somatic seedlings, independent of the genotype and with a 90% survival rate of somatic seedlings under appropriate conditions. Somatic seedlings often go into dormancy, but a cold treatment could break this for the somatic seedlings to start growing again [56].

Cryopreservation has frequently been applied for the long-term maintenance of conifer embryogenic tissues [57–59], including *Abies* species such as *A. nordmanniana* [60], *A. cephalonica* [22,61], *A. alba* [29] and *A. fraseri* [21], or *Abies* hybrids [32,33].

Storage in liquid nitrogen provides several advantages over the long-term in vitro culture on solid media or suspension cultures. Cryopreservation avoids the time-consuming frequent transfers to fresh media and may prevent the decline or complete loss of regeneration capacity of cell lines observed in conifers embryogenesis [62]. The conifer somatic embryos can be stored in liquid nitrogen in their very early developental stage, as bipolar structures, regenerated after storage and used for maturation. The slow-freezing technique was mostly used for conifer embryogenic tissue, including *Abies* species. Based on our previous experience with *Abies* hybrids [32,33], this method was also chosen for seven

cell lines of *Abies alba*. All tested cell lines recovered at high frequencies: 81.1 to 100% for individual cell lines. In *A. alba,* Krajňáková et al. [29] reported that four out of 12 cell lines recovered after cryostorage for six years. In our cryopreserved cell lines of *A. alba*, no significant differences in recovery frequencies among cell lines were recorded, although the cryotolerance in some conifer species was genotype dependent [56,58]. In control cell lines that were pretreated but not cryostored, the recovery frequencies reached 100%, indicating the cryoprotectant solutions had no harmful effect on recovery.

5. Conclusions

In the presented work, the successful initiation of the economically important conifer species, *Abies alba* Mill., is described. The embryogenic tissues were initiatied from immature zygotic embryos, and the effect of five types of cytokinins was evaluated, using a DCR medium, in three consecutive years. Althogether, 61 cell lines were obtained. The initiation frequencies ranged from 0.83 to 13.33%, although no statistically significant differences in the effect of the cytokinins in the given year were obtained. On maturation medium with ABA (10 mg·L^{-1}), as well as PEG-4000 (7.5%), cotyledonary somatic embryos developed, and their germination was terminated by somatic seedling regeneration.

Storage in liquid nitrogen for one year resulted in successful tissue regeneration in seven tested cell lines, reaching regrowth frequencies of 81.1 to 100%. This successful regeneration system for *Abies alba* via somatic embryogenesis needs further follow-up with the establishment and growth of somatic seedlings under natural conditions. We hypothize that this step could be improved by using a cold treatment, as demonstrated in other conifer species. Owing to the fact that the differerent embryogenic cell lines apparently differ in maturation capacity, a proteomic approach is forseen to study this phenomenon in more detail. The "ageing" effect, as shown in our study on the structural level, needs further physiological and biochemical investigations.

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