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The role of auxins in somatic embryogenesis of *Abies alba*

Research Article

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Abstract: The somatic embryogenesis of conifers is a process susceptible to exogenous phytohormonal treatments. We report the effects of the synthetic auxin 2,4–dichlorophenoxyacetic acid (2,4–D) and the auxin inhibitor *p*–chlorophenoxyisobutyric acid (PCIB) on the endogenous level of the auxin indole–3–acetic acid (IAA) and on the anatomical composition of early somatic embryos of *Abies alba* (European silver fir). The embryogenic suspensor mass (ESM) of *Abies alba* proliferated on a medium supplemented by 2,4–D as well as on an auxin-free medium. The endogenous level of IAA was significantly higher in the ESM cultivated on a medium supplemented by 2,4–D. The decrease in the endogenous level of IAA in the first week of maturation is one of the most important stimuli responsible for the subsequent development of embryos. However, suppression of IAA synthesis by an auxin inhibitor did not stimulate the development of embryos. The maturation of somatic embryos from the globular to the cotyledonary stage occurs when the concentration of endogenous auxin in the ESM (including the embryos) increases. Early somatic embryos proliferating on a medium supplemented by auxin had an increased probability of maturing successfully. Exogenous auxin treatment during maturation did not compensate for the auxin deficiency during proliferation.

Keywords: *Auxin inhibitor • Fir • Phytohormone • Somatic embryo*

Abbreviations

ABA – abscisic acid;
BAP – 6–benzylaminopurine, N^6–benzyladenine;
2,4–D – 2,4–dichlorphenoxyacetic acid;
ESM – embryogenic suspensor mass;
IAA – indole–3–acetic acid;
kin – kinetin;
PCIB – *p*–chlorophenoxyisobutyric acid;
PEG – polyethylene glycol 4000.

1. Introduction

Auxin is categorized as a morphogen and a phytohormone and it has long been postulated that polar auxin transport plays a central role in plant embryogenesis [1,2]. Changes in auxin fluxes have been linked with exogenous effects that induce a new distribution of auxin and a developmental response [3]. The formation of the apical meristem is regulated by auxin during early embryogenesis. The polar auxin efflux and the auxin response result in the apical–basal axis formation of the embryo [4-6], suggesting an analogous regulation of zygotic and somatic embryos.

The somatic embryogenesis of conifers is a process controlled by exogenous phytohormonal treatments [7,8]. The first stage, which is usually aided by cytokinins and auxins, is the induction of the embryogenic suspensor mass (ESM) from the mother explants (zygotic embryos). When cultivated on a proliferation medium supplemented with auxin and cytokinin, new embryogenic structures emerge while older embryogenic structures degrade. Further development of ESM into mature embryos can be induced by transfer onto a maturation medium lacking auxin and cytokinins, but supplemented with abscisic acid (ABA). Under maturation conditions, meristematic

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centres gradually increase in size. After five weeks of maturation, the largest embryos with distinct cotyledons can be desiccated in controlled conditions in high relative humidity for 3 weeks and germinated on pure phytohormone–free medium.

This scheme is optimal for the somatic embryogenesis of spruce [7,9]. However, when considering the potential for the application of the approach to other conifers the demand for plant growth regulators in the cultivation protocol can differ. The regulation of somatic embryogenesis of firs is even more complicated: investigations of somatic embryogenesis in species and hybrids such as *A. alba, A. alba x numidica, Abies balsamea, A. concolor, A. cephalonica, A. fraseri, A. lasiocarpa, A. nordmanniana* and *A. numidica* have been made [10-21]. Usually cytokinin(s) alone are able to trigger the process of induction of ESM in fir. Norgaard and Krogstrup [22], Schuller *et al*. [14], Guevin and Kirby [13] and Salajova *et al*. [12] described the inhibitory effect of auxin in the proliferation of early somatic embryos of *Abies nordmanniana*. In contrast, Tautorus *et al*. [23] and Guevin *et al*. [10] documented the positive effect of auxins during proliferation. ABA is only necessary in the maturation stage.

The phytohormone treatment of the ESM of conifers typically involves the synthetic auxin 2,4–D. The ESM may also synthesize a significant amount of auxin. Somatic embryos are not supplemented by exogenous auxin during the maturation period. However, Astarita *et al*. [24] have demonstrated that during the zygotic embryogenesis of *Araucaria* the presence of auxin is critical for the development of seeds, especially in the initial phase and during the growth of cotyledons.

In contrast to other conifers, the embryogenic cultures of firs require different saccharides in the medium for full development. Maltose is the most commonly used saccharide for maturation [23] while sucrose is fundamental for proliferation. Schuller and Reuther [25] and Schuller *et al*. [14] reported a positive effect of lactose, fructose, and galactose supplementation during the first days of maturation. The effects of auxin and saccharides appeared to be strongly contingent on the specific fir species and cell lines used.

The aim of our experiments was to assess the effect of exogenous synthetic auxin in cultivation media on the endogenous level of IAA during the development of somatic embryos of *Abies alba* (European silver fir). We tested the effects of treatment by exogenous auxin on the anatomical composition of early somatic embryos. We also tested the effects of the auxin inhibitor PCIB during the process of somatic embryogenesis in an attempt to improve the yield and quality of mature somatic embryos of silver fir.

2. Experimental Procedures

2.1 Plant material

The ESM of *Abies alba* Mill. was derived from immature zygotic embryos. The seeds were collected in southern Bohemia (Orlík region, Czech Republic) in June and July, 2000. The growth ability of embryogenic cell lines and their sensitivity to exogenous treatments varied. For the study, we selected cell line OR4, which exhibits rapid growth. The yield of embryos in this cell line is rather low and consequently the stimulatory and/or inhibitory effects of all treatments were detected easily in this embryogenic culture. The results were verified by applying the entire course of treatments to the genotypes OR7, OR5, JH151/1 and to 3 genotypes of *Abies cephalonica* (unpublished data).

2.2 Cultivation

2.2.1 Induction

The ESM was induced from immature zygotic embryos. Seeds isolated from strobilus were rinsed in ethanol and sterilized with sodium hypochlorite solution (20%) for 30 min. Whole embryos or their parts were placed into Magenta boxes (Sigma) on the induction medium (1/2 MS [26], 2 μM BAP, 2 μM kin, 3% sucrose, 0.4% gelrite). The boxes were placed in a cultivation room and kept in constant darkness at 25°C. The subcultivation interval was 3 weeks. During this time, a white ESM formed on the explants. From week four, the ESM was cultivated separately with the same period of planting.

2.2.2 Proliferation

In the first experimental treatment (A-) the ESM was cultivated on an auxin-free medium (1/2 MS [26], 2 μM BAP, 2 μM kin, 3% sucrose, 0.4% gelrite). The medium in the second treatment (A+) was supplemented by 2,4–D (1/2 MS, 2 μM BAP, 2 μM kin, 0.25 μM 2,4–D, 3% sucrose, 0.4% gelrite). Both variants were planted within a period of 14 days and cultivated in constant darkness at 25°C for a period of one year.

2.2.3 Maturation

ESMs from both conditions was transferred onto the basic maturation medium (1/2 MS, 20 μM ABA, 4% maltose, 3.75% PEG-4000, 0.4% gelrite). Nine ESM clusters, each consisting of approximately 0.1 g of fresh mass, were placed into separate Magenta boxes at the beginning of maturation. The subcultivation interval was 2 weeks. Samples from both experimental conditions were cultivated in constant darkness at 25°C. The yield of embryos was determined after 5 weeks of maturation.

The effect of auxin was tested in both variants. After proliferation, they were transferred onto the maturation medium containing auxin (basic maturation medium + 0.25 μM 2,4-D).

To test the effect of auxin inhibitor the A+ variant was transferred onto the maturation medium containing PCIB (2 μM, 10 μM and 20 μM).

2.3 An examination of anatomical structure

For each of the experiments an assessment was made of the anatomical structure of the ESM during proliferation. Changes induced by the transfer of ESM from a proliferation to a maturation medium were recorded after 1 and 3 weeks. We described the first changes in anatomy of early somatic embryos, polyembryonic complexes and suspensors.

The upper part of the ESM loaf was placed onto a microscopic slide and dyed with a drop of trypan blue. The cover glass was placed onto the ESM after 2 minutes and the dye was rinsed out with distilled water. The microscope preparations were examined using a Jenaval microscope (Carl Zeiss Jena).

Microscope images were recorded using a TV camera (Hitachi), and were analysed and stored using the computer image analysis system Lucia G, version 4.71 (Laboratory Imaging, Czech Republic).

2.4 Analysis of endogenous IAA

Endogenous IAA was determined in the ESM in proliferation just prior to transfer onto the maturation medium, and during the maturation process of both the A+ and the A- variants. The IAA concentrations were determined at weekly intervals.

Samples for chemical analysis were wiped carefully and frozen in liquid nitrogen. Methanol extracts of the frozen material were purified and endogenous IAA was determined using a HPLC equipped with a fluorimetric detector [27]. All of the experiments were repeated at least twice.

3. Results

3.1 Proliferation

The ESM cultivated on the (A-) medium was not supplemented by exogenous auxin. Nevertheless, it grew prolifically and the mass of early somatic embryos increased gradually. The ESM was observed to have a compact structure and a pale yellow colour.

The ESM in the A- variant consisted of simple, very small somatic embryos. The meristematic embryonal heads were small and linked with short suspensors. A large fraction of the suspensor cells had no connection with the suspensors and/or the meristems (Figure 1).

The ESM in the second variant (A+) was cultivated throughout the proliferation period on the medium supplemented by 2,4–D. Under these conditions the A+ variant grew faster than the A- variant. A structured, hairy surface of white ESM was characteristic for the A+ variant.

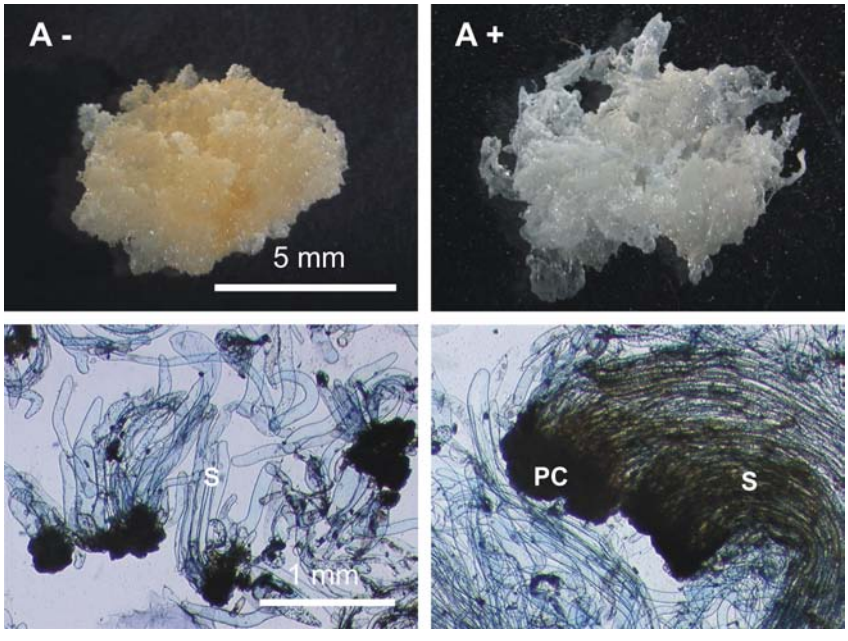

Figure 1. Embryogenic suspensor mass (ESM) during proliferation on a medium without 2,4–D (A-) (left) and on a medium with 0.25 μM 2,4–D (A+) (right): macroscopic view of ESM (upper panel) and the structure of ESM (lower panel); PC = polyembryonic centre, S = suspensor.

The ESM in the A+ variant was also composed of early somatic embryos and free suspensor cells. The early somatic embryos were larger than in the A- variant with large polyembryogenic complexes being formed. The meristematic heads were robust and linked with large suspensors. The suspensor cells were larger than seen under the A- conditions and the suspensors were longer and adopted a more organized structure (Figure 1).

3.2 The endogenous level of IAA during proliferation

Different levels of endogenous IAA were recorded in the proliferating ESM of A+ and A- variants. Endogenous IAA correlated with exogenously supplied 2,4–D. Very low IAA content (<2 ng/g of fresh mass) was found in the A- ESM. The A+ ESM was rich in IAA, which reached 25 ng/g of fresh mass (Figure 2 – week 0).

3.3 Maturation

At the start of maturation the ESM was supplemented with only a single phytohormone, ABA, and thus was considered to be free of auxins and cytokinins. Changes to the anatomical structure of the early somatic embryos were clearly visible after one to three weeks of maturation (Figure 3).

In the A- ESM the meristematic heads elongated after one week of maturation. The suspensor parts had a more structured organization compared to their state in the proliferation stage (Figure 1), being composed entirely of longer suspensor cells. However, the development of many young somatic embryos stagnated and these embryos did not form longer suspensors.

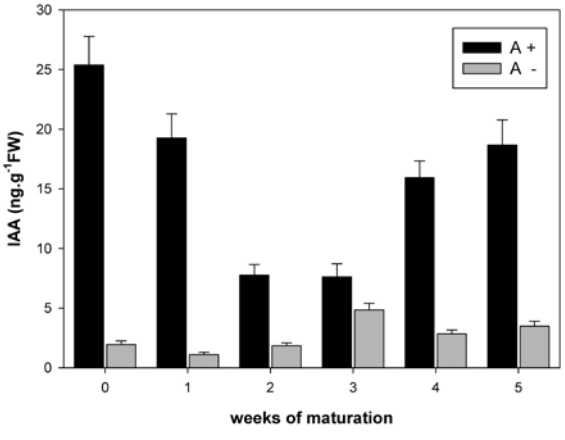

Figure 2. Endogenous level of IAA in ESMs during maturation. Before maturation, cultures were grown either on A- (no auxin, grey columns) or A+ (0.25 μM 2,4–D, black columns). Maturation medium was the same for both of the experimental variations. Week 0 = proliferation (beginning of maturation). Bars indicate standard errors.

After three weeks of maturation, the elongation of small meristematic heads could be discriminated in A- embryos (Figure 3). Suspensors were broken up and a small number of the suspensor cells remained in contact with the embryonal heads. The remainder of the suspensors contributed to the structure of the ESM. The somatic embryos were small and compact and very few of the embryos matured completely. In a large fraction of embryos, development was arrested and cells from the surface of the root pole of these embryos became detached during maturation. After this point the embryos disintegrated.

After one week of maturation, early somatic embryos of the A+ variant were composed of growing meristematic heads and large suspensors. The large suspensors were formed from organized long thin cells and single somatic embryos were separated from the polyembryogenic complexes at the beginning of maturation. All of the early A+ somatic embryos started development and were larger than for the A- variant. During three weeks of maturation the meristematic heads grew and elongated and were connected by long, compact, rope-like suspensors. These suspensors were typically frail and prone to damage. Large parts of suspensors lacking meristems were visible in the ESM of the A+ variant early in the maturation phase. During the following weeks, parts of suspensors disintegrated, and as found for the A- condition, a large number of embryos also disintegrated in this variant. The superficial cells of meristematic heads were released and the process resulted in the destruction of whole embryos within several weeks of maturation.

Morphological changes occurred in both variants within 4 weeks of maturation. The most fully developed embryos were located on the surface of the ESM clusters and were eventually released from the ESM. However, the majority of embryos remained connected with the ESM by the suspensors for the entire maturation period. Green cotyledons formed at the end of maturation. In both variants the somatic embryos matured within 7 weeks of cultivation on the maturation medium.

The final yield of somatic embryos was found to be low under both the A- and A+ conditions. Variant A- yielded, on average, 5.4 embryos in 1 g of ESM placed on maturation medium. In A+ the yield of somatic embryos was higher with 16.9 embryos in 1 g of ESM on average. In addition, the quality of somatic embryos differed between the two conditions. For A-, thick, short embryos with calluses formed on the root pole. In contrast, for A+ the number of short and thick embryos was low. The somatic embryos were often elongated with green cotyledons and the callus was absent.

3.4 The endogenous level of IAA during maturation

In the A- ESM, the content of IAA was extremely low during maturation with a maximal value of 5 ng/g of fresh mass after 3 weeks.

A drop in IAA content was found in the A+ ESM at the beginning of maturation (on the auxin-free maturation medium). During the 2nd and 3rd week of maturation the level of IAA was 7.5 ng/g of fresh mass. With advancing maturation the content of IAA gradually increased to 19 ng/g of fresh mass (Figure 2).

3.5 The effect of 2,4D during maturation

Additional small embryos with short suspensors were formed in the A- ESM treated with 2,4–D (Figure 4) in contrast to the characteristic small somatic embryos with long suspensors found in the A- ESM cultivated on the basic maturation medium (Figure 3). The number of free suspensor cells was higher and the suspensor tails were shorter. The embryos that maturated for three weeks on the medium supplemented by 2,4–D were smaller and their suspensors were poorly organized.

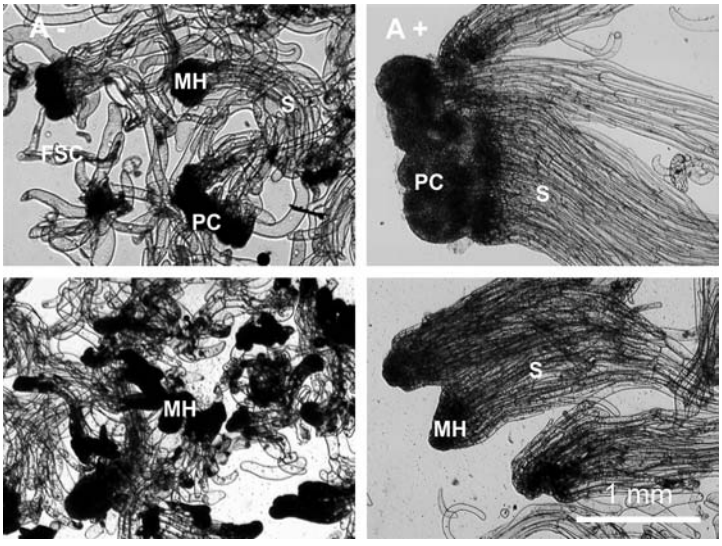

Figure 3. Somatic embryos on a basic maturation medium supplemented with 20 μM ABA after 1 week of maturation (upper panel) and after 3 weeks of maturation (lower panel). Before maturation, the cultures were grown either on A- (no auxin, left) or A+ (0.25 μM 2,4–D, right) proliferation medium. FSC = free suspensor cells, MH = meristematic head, PC = polyembryonic centre, S = suspensor.

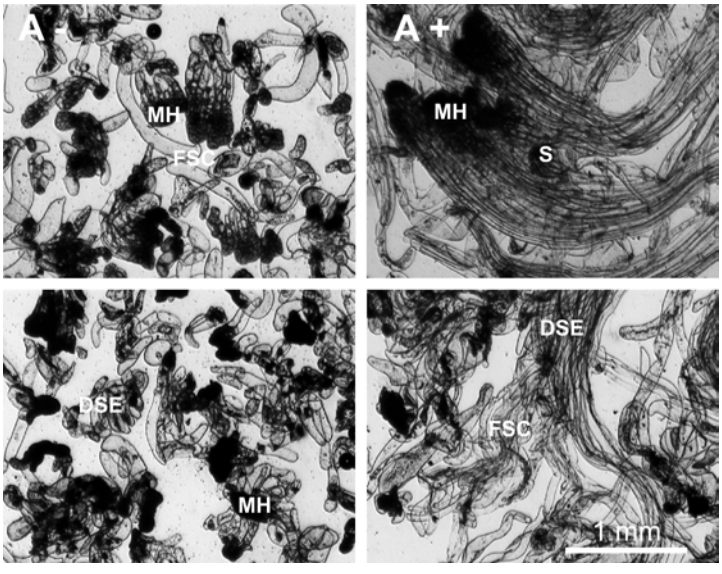

Figure 4. The effect of 0.25 μM 2,4–D on maturation of somatic embryos after 1 week (upper panel) and after 3 weeks of maturation. Before maturation, the cultures were grown either on an A- (no auxin, left) or A+ (0.25 μM 2,4–D, right) proliferation medium. DSE = degenerated somatic embryo, FSC = free suspensor cells, MH = meristematic head, S = suspensor.

The effect of 2,4–D was less visible in the first week of maturation in the A+ ESM. Large somatic embryos started their development on the basic maturation medium (Figure 3) and on the maturation medium supplemented by 2,4–D (Figure 4). The suspensors were poorly organized and the number of free suspensor cells was higher on the medium with 2,4–D. After three weeks, the ESM was composed of free meristematic cells, free suspensor cells, and strands of suspensors. All of the embryos decayed on the medium supplemented by 2,4–D, in contrast to the embryos on a basic maturation medium, which matured.

Somatic embryos of both the A+ and the A- variants failed to complete their development when matured on the medium supplemented by 2,4–D.

3.6 The effect of auxin inhibitor

The A+ ESM was cultivated on a maturation medium enriched by the auxin inhibitor PCIB at concentrations of 2 μM, 10 μM and 20 μM. The effect of the inhibitor strongly depended on the concentration used (Figure 5). The early somatic embryos developed more rapidly on the maturation medium with 2 μM PCIB (Figure 5) than on the basic maturation medium (Figure 3). Single embryos with large meristematic heads and well-organized suspensors were observed after 1 week of maturation with 2 μM PCIB. The ESM on a maturation medium supplemented by 10 μM PCIB generated well-organized embryos with compact suspensors together with small embryos with decaying small suspensors. In contrast, all of the somatic embryos disintegrated on the

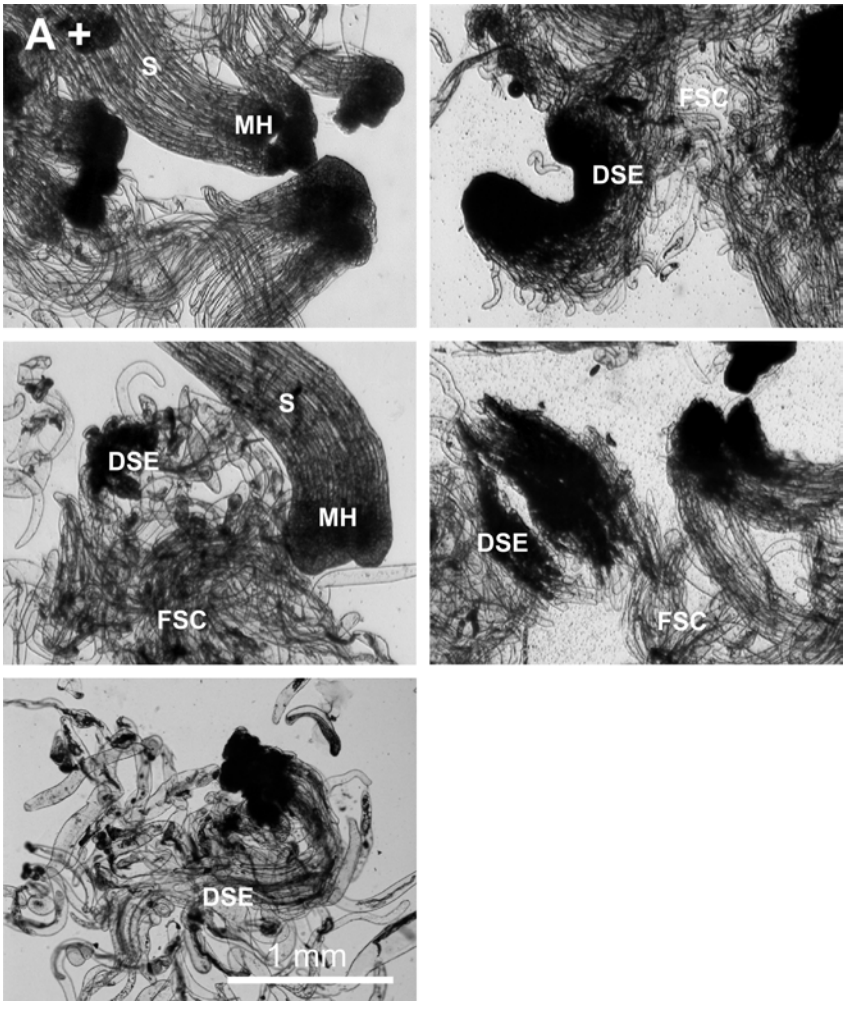

Figure 5. The effect of auxin inhibitor PCIB on maturation of somatic embryos (A+) after 1 week (left) and after 3 weeks of maturation with PCIB (right). The effects of 2 μM (upper), 10 μM (middle) and 20 μM (lower) concentrations of PCIB are compared. Before maturation, the cultures were grown on A+ (0.25 μM 2,4–D) proliferation medium. The concentration of 20 μM PCIB after 3 weeks of cultivation was found to be lethal. DSE = degenerated somatic embryo, FSC = free suspensor cells, MH = meristematic head, S = suspensor.

medium supplemented with 20 µM PCIB during the first week of cultivation. Under these conditions the ESM did not develop further and there was pronounced decay in all of the embryos 3 weeks later. A loss of polarity and an absence of mature embryos was noted in all of the media supplemented with PCIB; the presence of 10 µM PCIB in the medium resulted in the destruction of suspensors and more rapid progression of the meristematic heads (Figure 5). During the next few weeks of maturation all of the somatic embryos were destroyed. PCIB had similar destructive effects on the somatic embryos in other fir genotypes (unpublished results). The control variant, which matured on the basic maturation medium, produced the highest yield of embryos.

4. Discussion

In this study, we confirmed that the ESM of *Abies alba* can proliferate on an auxin-free medium, as suggested by Schuller and Reuther [25]. In addition we found that supplementation with exogenous auxin does not inhibit the growth of early somatic embryos of *Abies alba.*

The structure of the ESM at the start of maturation differs significantly between A- and A+. In addition, the endogenous level of IAA differs between the variants by one order of magnitude. Changes in the IAA level during the somatic embryogenesis of fir are similar to those found in larch by von Aderkas *et al.* [28] and to those in megagametophytes during the development of seeds of Douglas fir [29]. Vágner *et al*. [30] observed the same pattern of IAA changes in the embryogenic culture of Norway spruce grown in liquid medium. In subsequent experiments [31], the content of IAA was measured in isolated somatic embryos which matured on a solid medium where the quality of embryos was higher. The highest concentration of IAA was found after 3 - 4 weeks of cultivation. As the level of IAA in embryos grows, the concentration of IAA in the mixture of ESM and embryos is proportional to the ratio between the weight of embryos and the weight of the embryogenic rest mass. It indicates the number, the developmental stage and quality of embryos at the time of sampling. The increase in IAA level in the late stages of somatic embryogenesis has also been reported in developing zygotic embryos of conifers [32,33].

Auxin signalling is required for the correct division of cells and for the establishment of the fate of the suspensor cells [34]. The role of auxin as a patterning signal in embryos has been demonstrated in angiosperms as well as in gymnosperms. Its role in the organization of shoots and root meristems and generally in plant morphology was demonstrated by Berleth and Sachs [35]. The polar transport of auxin plays an important role in the early stages of the development of zygotic and somatic embryos. It determines the differentiation of vascular strands in the first step of the development of *Arabidopsis* zygotic embryos [36] and constitutes the polarity in the development of embryos. Auxin and its polar transport are believed to be mainly established by the PIN family of proteins in *Picea abies* (Norway spruce) somatic embryos [37]. The expression of the auxin efflux transporter PIN1 had been observed in differentiating procambium, running from the tips of cotyledons down throughout the somatic embryo axis and to the root apical meristem of *Picea abies* [38]. The centrality of polar auxin transport for the correct patterning of both apical and basal parts of spruce embryos throughout the whole developmental process has been demonstrated by Larsson *et al*. [39] using naphthylphthalamic acid (NPA) treatments. The establishment of an auxin transport system is a prerequisite for patterning events in the apical region of embryos at the beginning of the transition from the globular to the heart stage. This was shown by the effect of the inhibitor of auxin transport TIBA in *Brassica napus* zygotic embryos by Ramesar-Fortner and Yeung [40] and in spruce zygotic embryos by Ramarosandratana and van Staden [41]. Auxin is the most important phytohormone regulating the formation of lateral organs during the final stages of embryogenesis. The determination of the radial position and the size of lateral organs were demonstrated in tomato and *Arabidopsis* meristems by Reinhardt *et al*. [42].

The yield of somatic embryos was low in both treatments. Auxin (2,4–D) treatment during maturation triggered inhibition of the development of embryos as a result of accelerated destruction of suspensors and embryos. This effect was more pronounced in A+ where a higher level of IAA was seen. These results concur with the findings of Hristoforoglu *et al*. [43] who introduced an additional cultivation step between the proliferation and the maturation of somatic embryos of *Abies alba*. This prematuration step involves the cultivation of ESM on a phytohormone-free medium prior to maturation. The significance of this step was later confirmed by Bozhkov *et al*. [9] in the somatic embryogenesis of spruce, where it increased the number of immature embryos in the tissue. These embryos were able to continue their development when ABA was applied. Norgaard and Krogstrup [22] clearly demonstrated the inhibitory effect of auxin on the proliferation of *Abies nordmanniana*. In contrast, our results show no inhibitory effect of auxin on proliferation of early somatic embryos *of Abies alba* but do indicate a negative effect of auxin during maturation. We can therefore only speculate

about the optimal timing of auxin or antiauxin treatment during somatic embryogenesis [44].

It appears that the decrease in IAA level at the start of maturation may be the most important step for the subsequent development of embryos. The presence of auxin inhibitors may amplify this effect. We chose to use *p*–chlorophenoxyisobutyric acid (PCIB), a putative antiauxin. PCIB has been shown to inhibit auxin-induced b–glucuronidase (GUS) expression in transgenic *Arabidopsis* and physiological analysis has demonstrated inhibition of the auxin effect on the growth of *Arabidopsis* roots [45]. PCIB was also used successfully to inhibit auxin action in the ESM of fir [46] and was found to retard proliferation and promote the development of numerous high–quality mature embryos of *Abies nordmanniana* [46]. PCIB used in *Picea morrisonicola* proliferation increased embryo production in embryogenic culture with high auxin production [44]. The effect of PCIB on the maturation of *Abies alba* ESM in this study is strongly dependent on the concentration used. The complete destruction of embryos was found after one week of maturation on the medium supplemented with 20 μM PCIB. The effect of lower concentrations was less dramatic but a stimulating effect of PCIB on the development of somatic embryos was not found in the concentration range used. Our results are contrary to those of Find *et al*. [46] and Liao *et al*. [44] who obtained an increase in the yield and quality of somatic embryos of *Abies nordmanniana* and *Picea morrisonicola* by exogenous treatments with several auxin antagonists, including PCIB. They suggested that the effects of endogenously produced auxin may be one reason for low or failing maturation of embryogenic cultures. It is evident that the effect of auxin antagonists depends on the timing of the treatment and is strongly genotype-dependent.

Our results point to the importance of the process of proliferation during the development of fir embryos. It is very difficult to counterbalance the auxin treatment during proliferation with the same treatment during maturation. Early events in embryogenesis are crucial for the successful development of somatic embryos [44,47]. The same effect has been described in the process of embryogenesis of other plants such as *Lycopersicum* [48].

In this study, we have demonstrated a positive role for auxin on the proliferation of ESM of *Abies alba* and the negative effect of the treatment by 2,4–D on the maturation of embryos. Treatments with the auxin inhibitor PCIB during maturation did not increase the yield and quality of somatic embryos. At this stage we can only speculate about the likely effects of auxin treatments at the end of maturation or later. It is possible for example, that auxin could positively affect the development of apical meristems and cotyledons or the formation of leaves and roots in emblings.

Acknowledgements

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