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Maturation of somatic embryos in two embryogenic cultures of *Picea morrisonicola* Hayata as affected by alternation of endogenous IAA content

Yue Ken Liao · Cherng-Kang Liao · Ya Ling Ho

Abstract Embryogenic culture lines T4 and T2 were initiated from two mature zygotic embryos of *Picea morrisonicola* Hay. Mature somatic embryos (SEs) were produced in culture line T2 but not in line T4 after 8-week abscisic acid (ABA) treatment. High performance liquid chromatography (HPLC) analysis of the endogenous indole-3-acetic acid (IAA) content has shown 7.5 times higher IAA production in T4 line than in T2 line during the proliferation phase. However, after ABA incubation the line T4 produced much less IAA than line T2. The application of an anti-auxin, 2,3,5-triiodobenzoic acid (TIBA) or 2-(4-chlorophenoxy)-2-methylpropionic acid (PCIB) induced culture line T4 to produce mature SEs. Both 1 μM TIBA and 5 μM PCIB increased the production of stage 2 SEs in T4 culture line when cultures were treated during the proliferation stage for 8 weeks. Occasionally cotyledonary (stage 3) SEs were even produced from treated T4 culture line. Both chemicals have also been demonstrated to significantly decrease the amount of IAA in the treated T4 and T2 embryogenic lines. However the decrease of the IAA level was not beneficial for SE production in the T2 embryogenic line. These results indicated the importance of endogenous IAA level in manipulating the process of SE maturation in spruce embryogenic cultures.

Keywords Anti-auxin · Embryonal-suspensor mass · HPLC · PCIB · Spruce · TIBA

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
BAP	6-Benzylaminopruine
BHT	Butylated hydroxytoluene
ddH_2O	Double distilled water
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
ICD	Improved cell development
PCIB	2-(4-Chlorophenoxy)-2-methypropionic acid
PGRs	Plant growth regulators
SE	Somatic embryo
TIBA	2,3,5-Triiodobenzoic acid

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Introduction

Since somatic embryogenesis in conifers were first successfully achieved in *Picea abies* (L.) Karst (Chalupa 1985; Hakman et al. 1985) and *Larix decidua* Mill. (Nagmani and Bonga 1985), the technique has been improved and widely applied to many other coniferous

species over these two decades (Stasolla and Yeung 2003). Although the procedure for the production of SE-derived seedlings has been established due to the efforts of many different laboratories, many restrictions still exist in the application of this propagation method. One of the major problems is that not all of the embryogenic cultures, although identically initiated, will produce the same yield of SEs (Becwar et al. 1987a; Jalonen and von Aronld 1991; Keinonen-Mettälä et al. 1996). This disadvantage limits conifer somatic embryogenesis to only certain genotypes or culture lines and unfortunately such genotypes or culture lines may not include the most desired ones from a breeding program.

Researchers have determined that there are several possible mechanisms involved in producing numerous mature SEs in conifer cultures. Unstable genome multiplication has been shown to be one reason for *P. abies* (L.) Karst (Fourré et al. 1997), *Abies alba* Mill. (Roth et al. 1997) and *Larix decidua* Mill. (von Aderkas et al. 2003). The prolonged proliferation period also affects embryo maturation frequency among culture lines (Gjuleva and von Arnold 1999). Egertsdotter and von Aronld (1993) demonstrated that it was possible to segregate embryogenic cultures at an early stage of development by making comparisons of their morphological characteristics. After further investigation they concluded that the extraction of certain proteins from mature seeds (Egertsdotter and von Arnold 1995) or from completely developed SEs (Mo et al. 1996) were important factors in improving SE production in loosely packed (less developed) cultures.

Different explanations for why plant cultures may produce less or no SE have also been provided by other researchers and have focused on the endogenous plant growth regulators (PGRs). Ethylene is considered to be one of the PGRs involved in SE formation. El Meskaoui and Tremblay (2001) suggested that in culture lines with high embryo productivity ethylene production as well as the 1-aminocyclopropane-1-carboxylic acid (ACC) accumulation are all less than that in the low embryo formation lines. However more attention has been placed on the endogenous IAA levels within embryogenic cultures. In species such as *Daucus carota* L. (Michalczuk et al. 1992) and *Triticum aestiyum* L. (Hess and Carman 1998), the IAA level must be kept to a very low concentration to ensure normal embryo formation. In coniferous species of *L. decidua* Mill., the embryogenic cultures will keep proliferating, rather than switching to a developmental phase, if the cultures contain a high amount of IAA (Korlach and Zoglauer 1995). It is apparent that endogenous IAA also plays an important role in controlling SE maturation in plant species.

In our laboratory several embryogenic culture lines were initiated from mature zygotic embryos of *Picea morrisonicola* Hay. (Liao 1999), using the method of Jain et al. (1988). Two lines exhibiting different pattern of early embryo development were used in this study. We have demonstrated that IAA content is the key factor affecting embryo development in these culture lines. The reduction of IAA activity by using two anti-auxins led to a partial improvement in the less developed culture line to produce mature embryos but this interfered with the ability to form SE in the so-called "normal" culture line.

Material and methods

Plant material

Embryogenic culture lines were initiated in our laboratory (Liao 1999) from mature zygotic embryos using solid LPG medium (LP medium as modified by Anderson (1990)) supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 μM 6-benzylaminopruine (BAP). The cultures were maintained for proliferation in the same medium by subculturing every 2 weeks. The morphology of these cultures was examined periodically using a saturated acetocarmine/acetic acid staining method. Two morphologically different embryogenic culture lines were characterized (Liao 2000) and used in the present study. Embryogenic culture line T2 consisted of typical early embryos of different development stages and those dominant ones were composed of embryonal masses and suspensors (Fig. 1a). On the other hand, fast growing T4 line contained large portion of abnormal early embryos with horizontally enlarged and flattened embryonal masses and screen-like shaped suspensors (Fig. 1b).

Culture clumps of 0.16–0.18 g fresh weight were transferred to LPG medium supplemented with 30 μM ABA, 6% (w/v) sucrose and solidified with 0.6% (w/v) agar (Sigma, type E) to induce embryo maturation. The maturation phase included two subcultures, each prolonged for a 4 week period (designated as
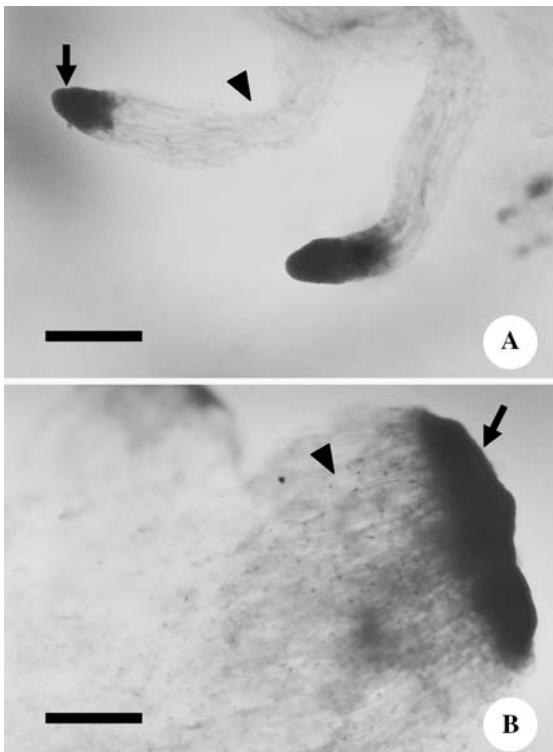
Fig. 1 A close-up view of two embryogenic cultures of *Picea morrisonicola* Hay. exhibiting the dominant early embryo development type in T2 culture line (**a**) and T4 culture line (**b**) under acetocarmine staining conditions. A typical structure of embryogenic cultures in conifers can be observed in (**a**) in which the suspensor cells (triangular marker) and embryonal masses (arrow head) are clearly seen. The same suspensor cells in (**b**) are found to be screen-like in shape (triangular marker) and with embryonal masses horizontally enlarged (arrow head). Bars = 0.3 mm

maturation I and maturation II). In culture line T2, an average production of about 15–33 mature SEs per clump was recorded, however in line T4, mature SEs were rarely obtained (Liao 1999; 2000).

Extraction procedure for IAA measurement

Culture clumps in three phases as mentioned previously (proliferation, maturations I and II) were collected respectively from these two culture lines. The samples were then freeze-dried for 18 h and stored at −80°C. Fifteen grams of freeze-dried cell material from the proliferation phase or 30 g from the maturation phases were mixed respectively with liquid nitrogen and ground with BHT (200 mg l^{-1})

containing 80% (v/v) cold methanol (10 ml per g dried material). The ground cell mass was kept at −20°C for 24 h and then vacuum-filtered through a Whatman No. 1 filter. Additional BHT-containing cold methanol was applied to wash debris left on the filter (5 ml for each 1 g of dried material). All the crude extract yielded was concentrated to a volume of 30 ml under reduced pressure at 35°C to remove the methanol. The extract was then filtered through a Millipore 0.22 μm filter, and the pH was adjusted to 2.5 by the addition of 1.0 M H$_3$PO$_4$ water solution. A Sep-Pak Cartridge (C$_{18}$, Waters) was activated by soaking it in 100% methanol, then pH balanced through an injection of 10 ml of ddH$_2$O, which was adjusted to pH = 2.5 by titration with 1.0 M H$_3$PO$_4$ solution. The previously prepared extract was then injected into the activated Sep-Pak with a flow rate of no more than 2 ml min^{-1}; it was eluted once by 1 ml 20% (v/v) methanol (pH = 2.5) and then again by 1 ml 80% (v/v) methanol (pH = 2.5). The IAA samples to be analyzed were then eluted in this final solvent before being ready to be loaded into the HPLC equipment.

HPLC analysis

For HPLC analysis, 20 μl of the purified IAA sample was injected into a 5 μm LiChospher 100 RP-18 column (Merck 250 × 4 mm); it was then eluted by a mobile phase composed of methanol (40)/H$_2$O (60; pH = 3 adjusted with acetic acid). The effluent was monitored at 254 nm through an UV absorbance detector.

The quantification of the HPLC results was made using an IAA calibration curve obtained over the range between 0.57 and 570.8 μM and with a correlation coefficient of 0.9984. The external standard, a mixture of 220 ml 80% (v/v) cold methanol with 1 ml 570.8 μM IAA solution, was used to estimate the recovery rate through out the extraction/purification procedures.

The influence of anti-auxin on SE development

To examine the anti-auxin effects on SE development, both culture lines were treated with TIBA or PCIB during the proliferation phase. The LPG medium was supplemented with 10 μM 2,4-D/5 μM BAP, 3% (w/v) sucrose, 0.6% (w/v) Sigma agar, and

TIBA or PCIB (0, 1, 5, 10 and 20 µM of each chemical) was tested in this experiment. The cultures were maintained in these media for 4, 6, 8 and 10 weeks with subculturing every 2 weeks. In each anti-auxin treatment regime, the tested factors were organized as a combination of concentration × incubation time, 20 cell clumps were cultured through out the assigned duration before being transferred to maturation phases I and II for 8 weeks for mature embryo production. The observation of early embryo development was first recorded at the end of the proliferation phase. The collected samples were stained with an acetocarmine/acetic acid solution and, 4 or 8 weeks later, the number of developing embryos appeared on the remaining cultures at the end of the maturation stages was counted (phases I and II, respectively).

During the staining examination, 0.1 g of cell sample was collected from each treated cell clump. These samples were then mixed homogeneously, stained with warm saturated acetocarmine/acetic acid solution. The stained cells were filtered and re-suspended in 15 ml ddH$_2$O. Two milliliter of suspension were randomly taken from this preparation, with additional dilution of 1.0 ml ddH$_2$O, and were poured into a Petri dish (5.5 cm in diameter) for microscopic examination. Three spots per dish were picked for examination through a Nikon SMZ 645 stereomicroscope (field of view 1.5 × 0.8 × 10). The Petri dish sample was prepared for five replications and 15 spots were examined from each treatment. Embryo development observed in each field of view could be classified into three types and counted individually. In addition to those embryo types observed in Fig. 1a (designated as type a) and Fig. 1b (designated as type c), we defined a third embryo development type (type b; see details for a, b and c type embryos in "Results"). The ratio of embryo development type was calculated by put each embryo type (numbers) into the formula: ICD = (a + b)/(a + b + c) from which the ICD values were recorded corresponding to the Improved Cell Development index. The ratio was further converted for statistical analysis by angle transformation.

To count mature embryos, a system for classification of embryo development (von Aronld and Hakman 1988) was applied. Stage 2 (embryos with a more prominent meristematic region; white or yellowish in color) and mature stage 3 (cotyledonary)

SEs in each clump were counted at the end of both maturation phases I and II.

One of the most effective anti-auxin treatments, as determined by the prior TIBA or PCIB experiments, was chosen and used again (with 2,4-D + BAP) to proliferate culture lines T2 and T4. At the end of this repeated proliferation treatment, the IAA content was extracted from the treated cultures (as well as from non-treated control ones) and was determined via the same HPLC procedures to clarify the relationship between IAA and the improvement in SE development.

Statistics

To compare IAA concentration among the three culture phases, the HPLC measurement was repeated three times for both culture lines and calculated for ANOVA. Once there was a significant difference detected among the culture phases; a further Duncan's new multiple range test was applied to clarify the difference.

To determine the anti-auxin effects, the angle transformed data of ICD values as well as the counting of stage 2 and stage 3 embryo numbers at the end of maturation phases I and II were all analyzed for ANOVA utilizing the statistical procedures (completely randomized factorial design) in which the effects of culture line × chemical type × incubation time were examined.

In the final experiment, the best dosage choice of TIBA or PCIB was again applied for culture proliferation in embryogenic lines T2 and T4. At the end of this incubation period, the IAA content extracted from these two culture lines was measured through HPLC and analyzed the same way as in the first experiment.
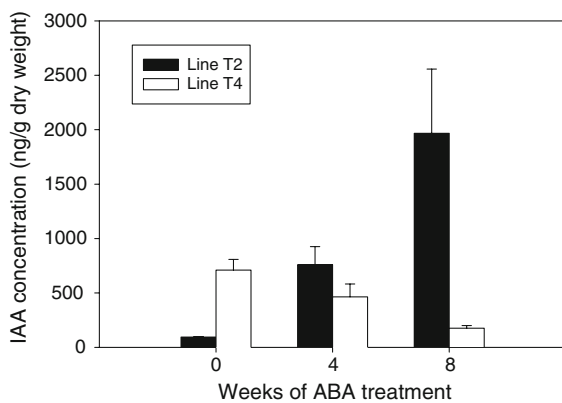
Culturing conditions

Cultures were kept in darkness during the proliferation phase. A 16/8 h photoperiod of cold white fluorescent light at 55.6 µmole m^{-2} s^{-1} was offered for SE maturation. The temperature in the culture room was set at 21 ± 1°C. The TIBA was first dissolved in 1.0 M NaOH then diluted by ddH$_2$O before being filter sterilized and added to the autoclaved medium. The PCIB was dissolved in 95% (v/v) ethanol and diluted, then added to the medium before autoclaving.

Results

IAA content in two culture lines examined at different culturing phases

Culture line T2 showed a statistically significant ($P < 0.05$) increase in IAA content between proliferation phase (94.9 ± 3.1 ng g^{-1}dw) and maturation phases I and II (760.1 ± 165.9 and 1966.5 ± 590.8 ng g^{-1}dw) (Fig. 2). We believe that this change reflects development of late SEs from embryonal masses. During sample collection at maturation phases, the gradually formed stage 2 and 3 embryos are also included in the sample ground for IAA extraction; the total amount of IAA thus increased as the SEs became more developed. However in culture line T4, IAA content decreased in a reverse pattern. The level recorded for T4 culture line was 710.3 ± 98.5, 464.5 ± 118.2 and 176.5 ± 24.6 ng g^{-1}dw in each culture phase (Fig. 2). There was a statistically significant difference ($P < 0.05$) between two maturation phases in this culture line. At the end of ABA incubation, no embryo formation was observed in T4 line and a very sticky culture was produced, in which puffy swollen suspensors had disappeared. In this experiment, a comparison of endogenous IAA level was conducted during proliferation phase; the morphologically abnormal T4 embryogenic line contained 7.5 times more IAA than did those in the T2 line (Fig. 2; 0-week of treatment). Hence we can speculate that a much higher IAA content in T4

culture line is one possible reason for its abnormal embryo development.

Application of anti-auxin for improved early embryo development during proliferation phase

After microscopic examination via the acetocarmine/acetic acid staining procedures, we defined embryo development type a corresponding to the dominant early embryo growth performance marked in the T2 culture line (Fig. 1a). This early embryo type was identified by its elongated and tightly coiled suspensors with bullet-shaped like embryonal masses. Type c embryo exhibited horizontal enlargement in both embryonal mass and suspensor regions, thus making the suspensor more like a screen (Fig. 1b). Type b embryo (Fig. 3), mainly been produced in T4 embryogenic line after anti-auxin treatments, could be distinguished from type c embryo due to its reduced size in the embryonal head region on tip of early embryos. Suspensor cells of type b embryo did not coil tightly so that they exhibited a less compact status as compared to type a embryo.

The ratio calculation data demonstrated that culture line T4 showed an improvement in the early embryo development. The ICD index achieved its maximum value at 1.0 μM TIBA or 1.0–5.0 μM PCIB (Fig. 4a, c). In this experiment, both the concentration and the culture duration led to a highly significant difference ($P < 0.01$). The application of 1.0 μM TIBA for 8-week proliferation was the best treatment, yielding an ICD index value close to 0.3 (Fig. 4a), much greater
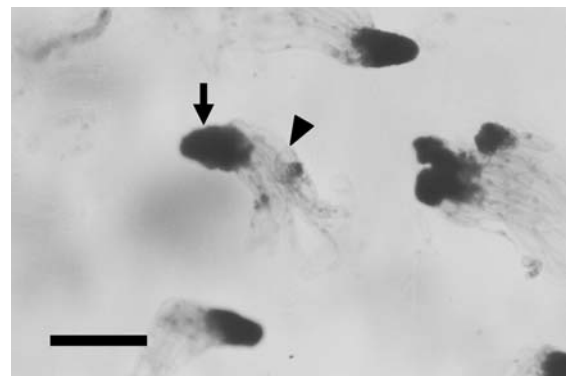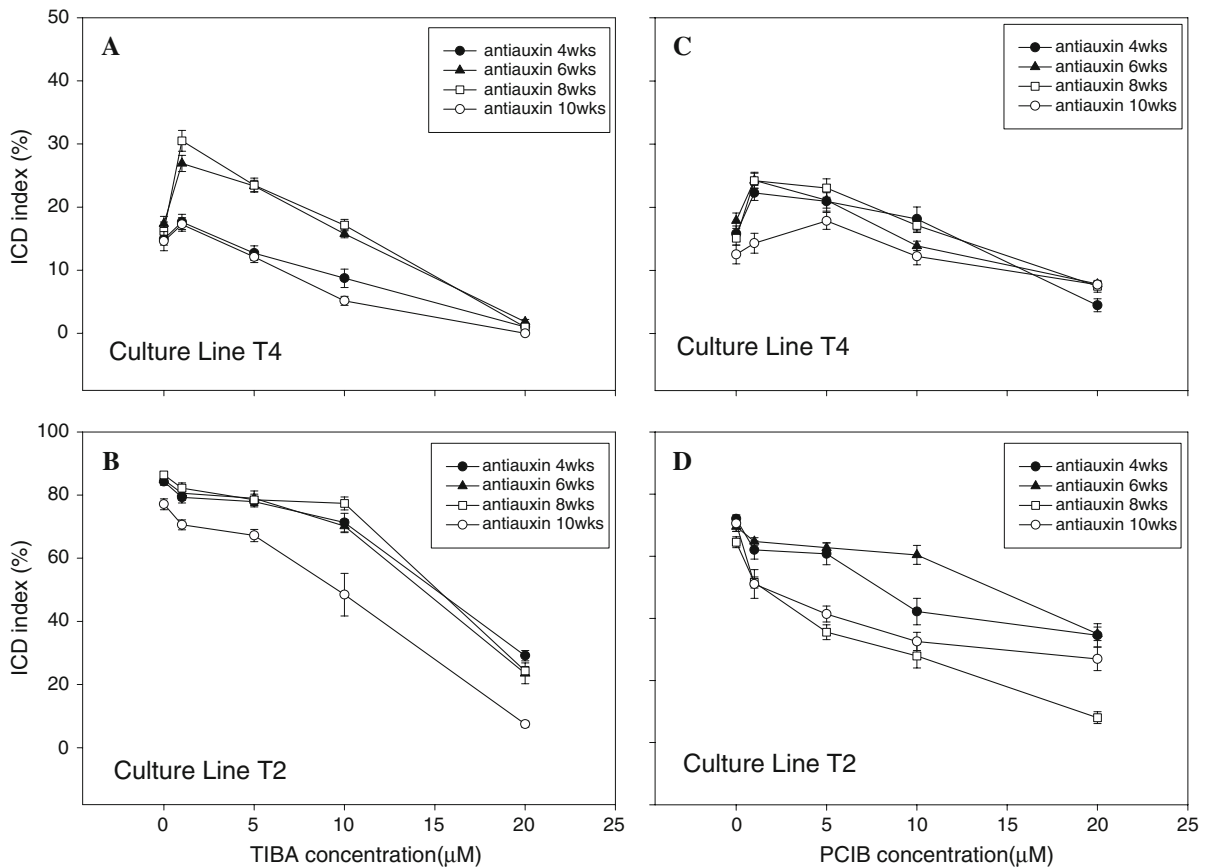

Fig. 2 IAA measurements in culture lines T2 and T4 throughout the proliferation phase (0 week) to maturation phases I and II (4 and 8 weeks respectively)

Fig. 3 The improved embryo development type (type b) showing loosely curled suspensors (triangular marker) and reduced embryonal masses (arrow head). Bar = 0.3 mm

Fig. 4 Influence of anti-auxin treatment on embryogenic culture development during the proliferation phase for both culture lines, data are presented as ICD percentages. Detailed descriptions for the ICD calculation are given in the text

than that at 4 or 10 weeks of treatment. PCIB at 5.0 μM and incubation for 6 or 8 weeks obtained a similar result (Fig. 4c). However in culture line T2, both chemicals had a negative effect on early embryo development (Fig. 4b, d). The ICD values decreased for all concentrations, after 10 weeks of culture duration with TIBA or 8–10 weeks of culture duration with PCIB, and severely inhibitory effects on early embryo development were observed ($P < 0.01$). These results were the first sign showing anti-auxin effect on embryogenic culture development.

The application of anti-auxin improves SE development in maturation phases I and II

At the end of maturation phase I, stage 2 embryos that were yellowish in color can easily be seen under the stereomicroscope. T4 line previously treated by TIBA (1.0 μM) or PCIB (5.0 μM) showed a promotive and prolonged effect on embryo development. Stage 2 embryo production was significantly ($P < 0.01$) increased by both chemicals at previously mentioned concentrations. An 8-week incubation period with PCIB prior to the ABA maturation treatment produced significantly ($P < 0.05$) better results than did the other culturing periods (Fig. 5c). In TIBA incubations the 8-week culturing period was also a better choice, however the improvement was not much distinguished from the 6-week period (Fig. 5a). In T2 line, none of the anti-auxin treatments improved stage 2 embryo development during this phase (Fig. 5b, d). The embryo counts decreased for all the treatment combinations and with this pattern similarly seen in the ICD index measurement experiment (Fig. 4b, d). The results obtained from this experiment further indicated that the anti-auxin
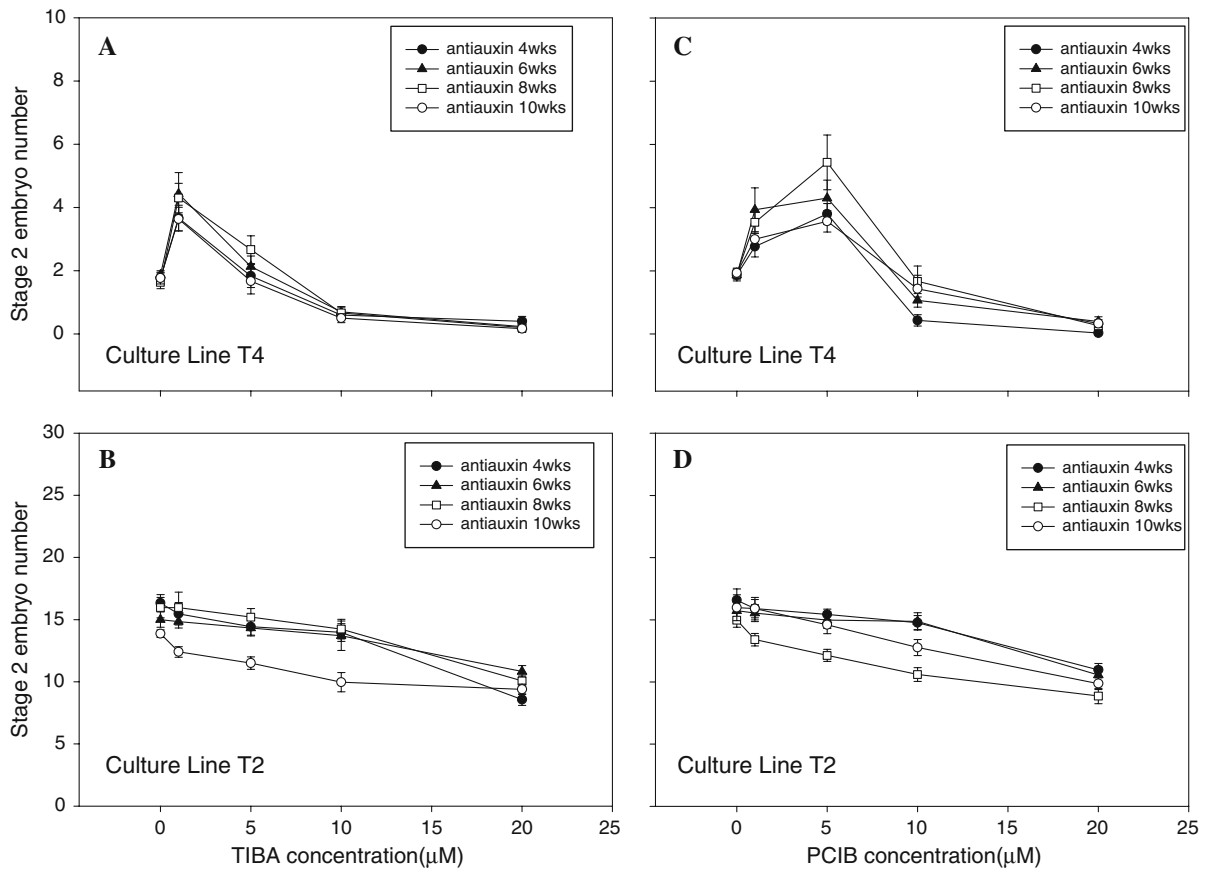
Fig. 5 Influence of anti-auxin treatment on SE development at maturation phase I in both culture lines; data is presented by counting the number of stage 2 embryos

effects detected in proliferation phase could be carried-over into the maturation incubation.

Production of mature stage 3 embryo as well as the number of stage 2 embryos, which were not converted into a more mature stage, were both recorded by the end of the maturation phase II (Fig. 6). Although both culture lines had previously yielded stage 2 embryos, only a few stage 3 embryos were found in T4 line (Fig. 6a1, c1). These cotyledonary stage embryos (Fig. 7) apparently developed under the prior influence of the anti-auxins, since they were all produced from the best treatment regime as determined in the earlier culturing phases. This result, the yielding of mature SEs from T4 culture line, was never achieved in our routine trials of ABA maturation procedures (data not shown) nor in other anti-auxin treatments in the present study. However, the fact that there were many stage 2 embryos which did not enter a mature stage was out of our expectation. The formation of

stage 3 embryos in T2 embryogenic line again decreased as the concentration of TIBA and PCIB gradually increased (Fig. 6b, d). This strongly suggested that in the so-called "normal" cultures, with the ability to form SEs, the anti-auxin putatively depressed the activity of the endogenous auxin and interrupted the whole developmental procedures.

Measurement of IAA content in the anti-auxin treated cultures

In the last experiment both culture lines were incubated again under TIBA (1 μM) or PCIB (5 μM) treatment, and proliferating culture samples were extracted for IAA analysis. We tried to demonstrate that the improvement of early embryo development in T4 culture line was directly correlated to the application of the anti-auxin and thus evidence should be
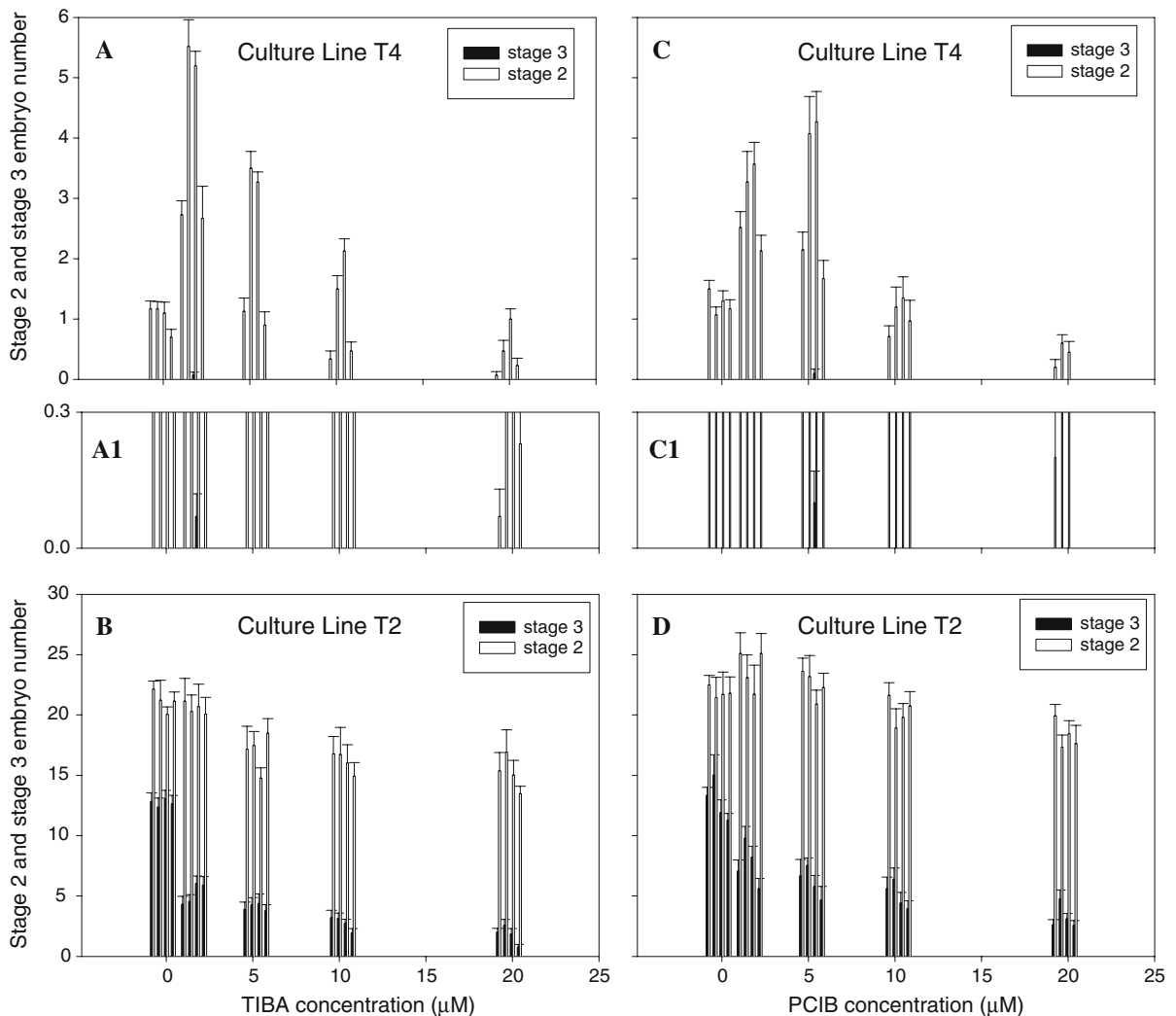
Fig. 6 Overall anti-auxin effects on SE development during the maturation phase II in both culture lines; data is presented by counting the number of both stage 2 and mature stage 3 embryos. Note **a1** and **c1** are partial enlargements of **a** and **c**, respectively, with the vertical scale values ranging between 0.0 and 0.3 only. The histogram (from left to right in each concentration) represents anti-auxin treatment duration at 4, 6, 8 and 10 weeks

presented in terms of having a much lower level of IAA in the treated embryogenic cultures. Furthermore, such analysis may also provide useful information to explain the inhibitory effect on embryo formation in T2 line. Based on our HPLC results, the endogenous IAA level in T4 embryogenic line treated with 5 μM PCIB or 1 μM TIBA decreased to 550.6 ± 12.0 and 648.9 ± 7.9 ng g^{-1}dw, respectively. This is a 36 and 25% decrease, as compared to the IAA level at 863.9 ± 89.7 ng g^{-1}dw, extracted from the non-treated cultures (Fig. 8). A similar result was also

obtained in T2 embryogenic line after PCIB treatment. The IAA content decreased by 40% (from non-treated samples at 127.2 ± 13.9 down to the level of 76.0 ± 8.9 ng g^{-1}dw; Fig. 8). Such analysis was not available for TIBA treated cultures since the IAA level was no longer detectable by our HPLC apparatus after 8-weeks of incubation. Although these two anti-auxin treatments significantly ($P < 0.05$) reduced the amount of IAA in T4 culture line, their IAA concentrations were still greater than those in T2 culture line. The decrease of IAA level in treated T2 line was also
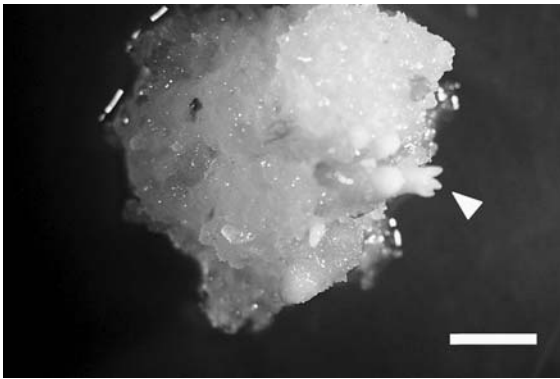
Fig. 7 A stage 3 embryo (triangular marker) developed from T4 culture line previously treated by 5.0 μM PCIB for 8 weeks and continuously cultured in an ABA-containing medium for eight more weeks. Bar = 0.25 cm
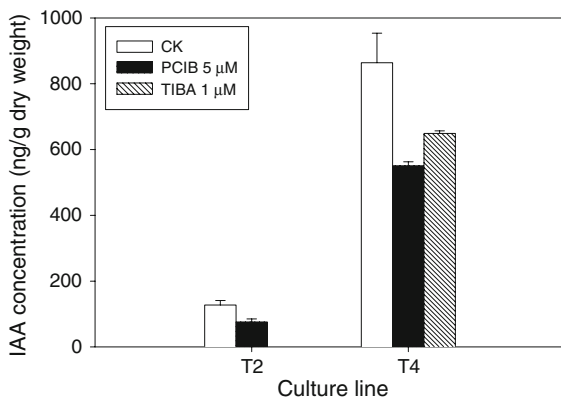
Fig. 8 IAA measurements of culture lines T2 and T4 specifically incubated for proliferation under PCIB (5.0 μM) or TIBA (1.0 μM) treatment

significant ($P < 0.05$) and such treatment was proved not to be beneficial for SE maturation.

Discussion

The two culture lines studied in this research were quite different in their morphology, as shown in Fig. 1, and were not as the same as those described by Jalonen and von Aronld (1991) or Egertsdotter and von Aronld (1993). Their observations indicated that the development of embryogenic cultures of *P. abies* (L.) Karst can be classified into several categories, mainly by their polar/solar development characteristics and growth habits. In the present study, both the

spruce embryogenic cultures maintained their bipolar conformation, but there was one abnormal culture in which early embryos with horizontally extended embryogenic head regions attached to loosely packed and uncoiled suspensor cells were formed. The polar/solar growth characteristics of embryogenic cultures were further correlated to the arabinogalactan protein (Egertsdotter and von Aronld 1995). However we conclude that the endogenous IAA level is the major event affecting embryogenic culture development and subsequent SE maturation.

The morphological difference in T2 and T4 culture lines directly influenced the pattern of SE development in the maturation phase. In T2 cultures, when mature SEs developed, the IAA content correspondingly increased. Such event was also reported both in the SE or zygotic seeds (von Aderkas et al. 2001; Silveira et al. 2004) in other coniferous species. This was probably also the reason why T4 cultures produced less IAA during the same culturing phases since there was no embryo production at all. Although the T4 embryogenic line successfully produced few cotyledonary SEs, its IAA level apparently did not decrease to a level as low as that in the untreated normal T2 cultures (Fig. 8). The data derived from this study further indicate that the anti-auxins also decreased the level of IAA in T2 culture line, and thus negatively influenced the SE maturation in that culture line. Based on these findings it seems safe to state that the IAA level during the culture proliferation phase should be adjusted to a suitable level before further receiving ABA stimulation. The embryogenic cultures such adjusted should then respond properly to ABA treatment and initiate SE maturation.

In plant tissue culture system, TIBA and PCIB were frequently applied to alter the activity or balance of endogenous PGR to approach specific differentiation or development in cultured cells or tissues. TIBA, due to its inhibition of polar transport of auxin, could be applied to achieve an auxin accumulation in certain explant parts. An unfavorable basal callus formation was eliminated from shoot cultures using TIBA to reduce the availability of IAA to the basal end of shoots (Lakshmanan et al. 1997; Lall et al. 2005). On the contrary, the same mechanism occurred in cultured root segments of *Cephaelis ipecacuanha* A. Richard leading to an auxin accumulation that suppressed adventitious bud formation around the cut end of root

tissue (Yoshimatsu and Shimomura 1994). In sugar-beet (*Beta vulgaris* L.) culture system, TIBA was well investigated and proven to be beneficial for shoot regeneration from epicotyl and leaf tissues (Roussy et al. 1996; Toldi et al. 1996). For in vitro rooting application, PCIB usually played a negative role (Bellamine et al. 1998) since it is commonly known as an inhibitor of IAA activity and it competes for IAA binding sites on the influx protein (MacRae and Bonner 1953). When we looked at somatic embryogenesis studies, the application of TIBA and PCIB were species and dosage-dependent since promotive and inhibitory effects were all presented (Choi et al. 1997, 2001; Moghaddam et al. 2000; Chen and Chang 2004; Agarwal et al. 2006).

In somatic embryogenesis studies related to conifers, TIBA has been tested for the embryogenic culture initiation (Ramarosandratana and Staden 2004) and for embryo maturation (Find et al. 2002), but both with negative results. In particular, in the latter case, TIBA was simultaneously applied with ABA during the embryo maturation phase to *Abies nordmanniana* Lk. The authors hypothesized that since the exogenous auxin is not essential for embryogenic culture initiation in the genus of *Abies*, the initiated cultures must be habituated to the plenty amount of endogenous auxin already presented. Using TIBA combined with ABA might therefore inhibit IAA activity and relatively enhance the promotion effect of ABA during SE maturation phase. This hypothesis can be generally accepted because we already know that to enhance SE formation in coniferous species the complete exclusion of PGR, but with ABA being maintained in the medium, is required during the SE maturation period (Gupta et al. 1993). This removal of PGR, mainly cytokinin and auxin, can also be achieved by washing the cultures with ddH$_2$O (Atree et al. 1989) or by pre-incubating the cultures on charcoal-containing medium (Becwar et al. 1987b). In the genus of *Abies*, it seems likely that it produces enough endogenous auxin, additional suppression of this auxin by TIBA during SE maturation stage would be a reasonable treatment. However the chemical did not work as the authors expected. We speculate that their protocols for adding the anti-auxin into culture medium might be too late. TIBA is well known as a non-competitive auxin transport inhibitor (Thomson et al. 1973), and usually induces abnormal embryo development if given to embryonal tissues early in the globular stage

or before the establishment of bilateral symmetry (Liu et al. 1993; Nakano et al. 2000; Choi et al. 2001). In the present study the application of TIBA was proceeded as early as in the proliferation phase, which was earlier than those in Find et al. (2002). Although it might lead to a risk of inducing abnormal cell development, we did not observe any abnormal embryo development in our spruce embryogenic cultures. As the best result of ICD index was achieved in embryogenic culture line T4 (TIBA 1.0 µM, 8 weeks; Fig. 4a), the pre-treated cultures further yielded more stage 2 embryos in the following maturation phase. This encouraging result was probably due to this good choice of timing for anti-auxin application. However such enhancement effects could not be prolonged throughout the whole culturing phase, among those induced stage 2 embryos only few cotyledonary stage embryos were formed (Fig. 6a1). For cultures like line T4 in which IAA was synthesized at such high levels; if anti-auxin can be applied during the proliferation phase, and continuously added to the following maturation medium, the efficiency of SE production might be further promoted.

In T2 culture line, once the endogenous IAA was inhibited by TIBA, neither a better ICD index nor a greater production of stage 2 embryos was found. Consequently the formation of cotyledonary stage embryos was also depressed as the incubation concentration was increased (Fig. 6b). Such a result implies that in a normal culture line with good embryogenic competence, its IAA concentration may have to be maintained above a certain minimal amount. Once the anti-auxin depresses the IAA activity down below the limit, the SE also ceases to develop.

Another anti-auxin used in this study was PCIB. Since it is not an auxin transport inhibitor, PCIB normally will not interrupt the bilateral development of early embryos in comparison with TIBA (Fischer and Neuhaus 1996). In our study, we could not identify any abnormal development of mature SEs after treatment with either chemical, in terms of asymmetrically developed cotyledons (Find et al. 2002) or shorter hypocotyls (Liao and Amerson 1995; Ramarosandratana et al. 2001). In the report of Find et al. (2002), it was found that PCIB was an effective chemical for enhancing SE maturation in *Abies nordmanniana* Lk. Although they used the same treatment model to apply this anti-auxin as for TIBA, the addition of PCIB during the maturation

phase had a positive effect. They concluded that this was due to the inhibition of continuous proliferation of embryogenic cultures during the embryo maturation phase. They also mentioned that a fast proliferating culture line (G3.8) must receive a stronger dosage of PCIB to promote SE formation. We suppose that the line G3.8 should be an embryogenic culture line with higher auxin content. Find et al. (2002) also reported that their PCIB treatment had no negative effect on SE maturation, even when applied to a normal, slow proliferating culture line (G35.8). This later conclusion was quite different as compared to our result in T2 culture line which was treated at an earlier stage. Our PCIB data demonstrated that this chemical works similar to TIBA except that it must be added in a higher concentration (5.0 μM) to achieve a better result.

The conclusions derived from our experiments indicate that the promotion effect of anti-auxin on SE maturation in spruce may perhaps be reinforced if the chemicals are used earlier and continuously applied, as suggested by Find et al. (2002), throughout the end of the maturation phase. This may possibly lead to better success especially for the T4 culture line. Accordingly, each in vitro initiated embryogenic culture may not need to receive the same auxin depression treatment to achieve better embryo maturation frequency. Our suggestion is that the application of anti-auxins to promote SE maturation in conifers should be carefully adapted to the species and to the different embryogenic culture lines.

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