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Maturation capacity of somatic embryos of *Picea abies* after prolonged proliferation culture

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Abstract

Matured somatic embryos of different embryogenic cell lines of *Picea abies* formed as a response to abscisic acid and polyethylene glycol were categorised into three main groups: well-developed, less-developed and undeveloped type. Only the well-developed embryos were able to convert into normal plants. They were positively influenced by prolonged proliferation up to 4 months in contrast to the less-developed ones. Seed extract positively affected both well-developed and less-developed embryos, but only in the beginning of tested period. Intermediate cell lines after a peak of acquisition of maximum maturation competence decrease the maturation frequency with prolonged culture.

Additional key words: abscisic acid, *in vitro* cultivation, Norway spruce, seed extract.

Introduction

During the last decade progress of somatic embryogenesis in conifers was achieved (Jain *et al.* 1995). However, plant regeneration is still a problem, the least efficient step being the maturation. In some cell lines a high number of mature somatic embryos are obtained after one to two months treatment with abscisic acid (ABA). In the others the formation of mature somatic embryo is not easy, especially in pine species.

In previous studies we found that the ability to form mature somatic embryos in some embryogenic cell lines of *Picea abies* was retained during two years (Mo

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et al. 1989). On the other hand, in *Pinus pinaster* the regeneration ability decreased after five months (Barcetche and Pagues 1995). Also in *Pinus taeda* the embryogenic potential declined after prolonged culture, and this was suggested to be connected with ageing (Beckwar 1995).

We have separated our embryogenic cell lines of *Picea abies* into two main groups based on embryo morphology, growth characteristics and pattern of secreted proteins (Jalonen and von Arnold 1991, Egertsdotter *et al.* 1993, Egertsdotter and von Arnold 1995, Mo *et al.* 1996). Cell lines belonging to group A contain well-developed somatic embryos which are stimulated to mature after treatment with ABA. In contrast, cell lines belonging to group B contain relatively undeveloped embryos, which do not mature after treatment with ABA. The morphology of somatic embryos is similar within cell lines but differs among cell lines. The reason why the embryos in various cell lines have different morphology is not clear. However, we suppose that it is at least partly due to genetic differences. This is strengthened by the fact that when new embryogenic cell lines are established from mature somatic embryos from group A cell lines, they all belong to group A (Mo *et al.* 1996).

The current study aimed at investigating if group B cell lines are less stable than group A cell lines in the sense that they initially are formed as A but are converted into B cell line after prolonged culture.

Materials and methods

Plant material: Mature somatic embryos from the following cell lines of *Picea abies* (L.) Karst. were used: 1060 from 86&52 and 4280 from 86&47 (classified as group A), 800 from IA&13 and 400 from IA&62 (classified as intermediate group) and 300 B&41 and 800 from D1&7 (classified as group B) (Jalonen and von Arnold 1991). Previously initiated cell lines 86&52 (group A) and 88&37 and B93 (group B) were studied too (Mo *et al.* 1996).

Establishment of new embryogenic cell lines from mature somatic embryos: Embryogenic cultures of *Picea abies* were initiated from mature somatic embryos as described previously (von Arnold 1987). Twenty mature somatic embryos were grown in Petri dishes (diameter 9 cm) with 35 cm^3 half-strength LP medium (von Arnold and Eriksson 1981) containing 15 mM NH_4NO_3, 30 mM sucrose, 9 μM 2,4-dichloro-phenoxyacetic acid (2,4-D), 4.4 μM 6-benzylaminopurine (BAP), solidified with 0.35 % gellan gum and adjusted to pH 6.1 before autoclaving. The Petri dishes were incubated in darkness at 25 °C. The percentage of embryogenic callus initiated was estimated after 6 weeks.

Effects of proliferation time on maturation frequency: Cell lines were exposed to different period of proliferation - one, two, three and four months and transferred on the maturation medium. In addition, treatment with seed extract was applied according to Egertsdotter (1996).

The basal BMI-S1 medium containing 5 μM 2,4-D, 2.2 μM BAP and 2.2 μM kinetin, 3 % sucrose and 0.35 % gellan gum (pH 5.7 before autoclaving) was used during proliferation. Embryogenic tissue was cultivated for 24 h in the BMI-S1 growth-regulator free medium supplemented with 0.05 % activated charcoal, 3 % sucrose and 0.35 % gellan gum and then transferred to the BMI-S1 medium containing 3 % sucrose, 7.5 % PEG 4000 and 30 μM ABA.

The maturation frequency of the different cell lines was determined after 8 weeks by the mean number of produced mature somatic embryos per 1 g fresh tissue. Fixed model was used to compare the results - 15 calli for cell lines 86&47 and 86&52; 11 calli for cell lines IA&62 and IA&13 and 5 calli for cell lines D1&7 and B&93. Effect of seed extract treatment was evaluated by χ^2 test.

Plantlet development from mature somatic embryos: Well-developed (type I) and less developed (type II) mature somatic embryos, as well as nodules of group A, intermediate and group B cell lines were cultured on the growth-regulator free BMI-S1 medium (Krogstrup 1986) supplemented with 2 % sucrose and 0.35 % gellan gum. Ability of different types of mature somatic embryos to convert into plantlets was determined by the percent of the plantlets developing shoot and root. Group A is represented by 50 type I and 25 type II mature somatic embryos of cell line 86&52, 75 type I and 75 type II mature somatic embryos of cell line 86&47, 31 type I and 31 type II mature somatic embryos of cell line 86&52, and 125 type I and 25 type II mature somatic embryos of cell line IA&62. Group B cell lines is represented by 50 type I and 25 type II mature somatic embryos of cell line B&41 and 25 nodules of D1&7 cell line.

Histological observations: Embryogenic calli of the different cell lines were observed every month during proliferation under the light microscope after staining with acetocarmine and Evan's blue according to Gupta (1995) with the aim to test changes in their morphology. In addition, different types of mature somatic embryos were fixed in 2 % paraformaldehyde and 0.25 % glutaraldehyde, dehydrated in a graded series of ethanol and embedded in *Technovit 7100*. Sections were stained with 1 % toluidine blue. Five somatic embryos of type I and II from each cell line were analyzed.

Results and discussion

Plantlet development from mature somatic embryos: On maturation medium supplemented with ABA and PEG three different types of mature somatic embryos were observed: type I (well-developed), type II (less-developed) and abnormal nodules (nod). In the type I embryos (Fig. 2) the root with root cap (Fig. 4) and shoot meristems are well developed, as well as procambium (Fig. 3). The type II embryos (Fig. 5) lack well developed meristem and procambium and significant air spaces are visible (Fig. 6).

In the present study newly and previously initiated cell lines 86&47 and 86&52 categorised as A tissue type, IA&13 and IA&62 as intermediate and B&41 and D1&7 as B tissue type (Jalonen and von Arnold 1991, Egertsdotter *et al.* 1993, Egertsdotter and von Arnold 1995, Mo *et al.* 1996) were grown on proliferation medium for one month before being transferred to maturation medium for 6 weeks. Different types of mature somatic embryos of *Picea abies* were tested for their ability to convert into plantlets (Table 1).

Table 1. Ability of different types of mature somatic embryos of *Picea abies* cultured on the BMI-S1 medium with 2 % sucrose, 0.35 % gellan gum, and with or without seed extract to convert into plantlets.

Group	Type	Control number of tested embryos	conversion into plantlets [%]	Seed extract number of tested embryos	conversion into plantlets [%]
A	I	281	25	218	45
	II	156	4	162	5
	nod	50	0	0	0
B	I	-*	-	50	24
	II	-*	-	0	0
	nod	25	0	0	0

* - no type I and II formed

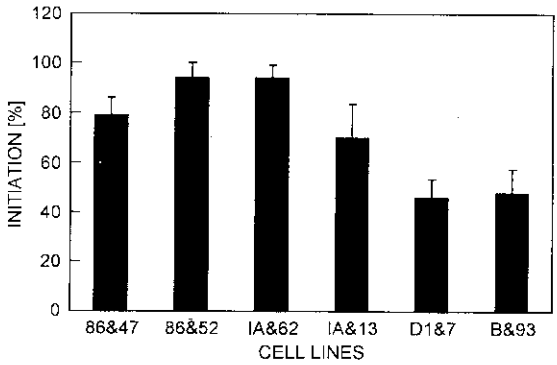

Fig. 1. Frequency of newly initiated embryogenic cultures of *Picea abies* from mature somatic embryos classified as group A (86&47, 86&52), intermediate (IA&62, IA&63), and as group B (D1&7, B&93). The embryos were grown on the medium (1/2 LP) with 9 μM 2,4-D and 4.4 μM BAP for six weeks. Means ± SE.

Plantlets can be developed from somatic embryos classified as type I and to a low extent from type II (Table 1). Under standard culture conditions type I embryos were only formed from group A cell lines. However, after one month treatment with seed extract type I embryos were also formed from group B cell lines. In addition to that

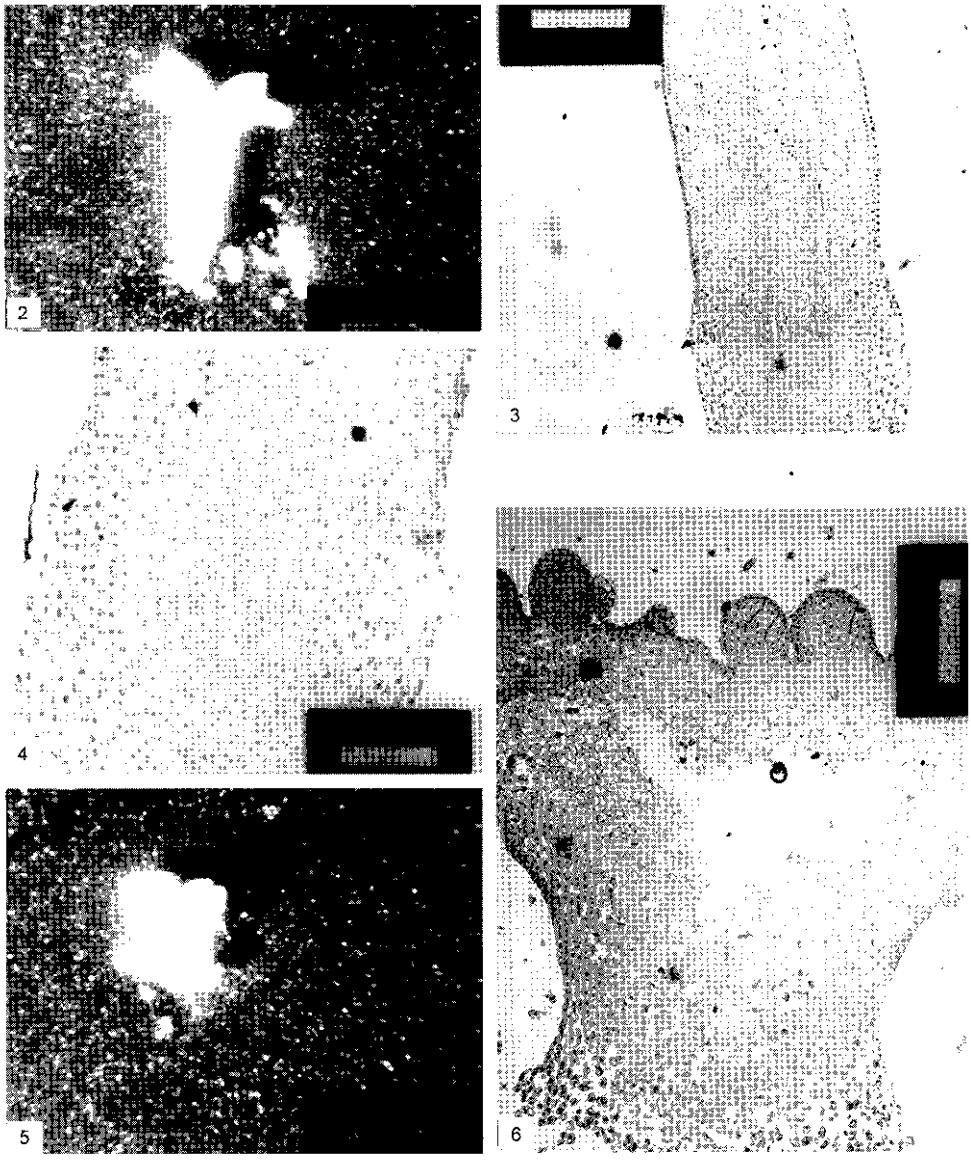

Fig. 2. Type I mature somatic embryo formed after 8 weeks on BMI-S1 medium with ABA and PEG (×16).
Fig. 3. Sectioned type I embryo. Note the procambium (*scale bar* 400 μm).
Fig. 4. Higher magnification of section shown in Fig. 3. Note the root meristem (*bar* 200 μm).
Fig. 5. Type II matured somatic embryos (×10).
Fig. 6. Sectioned type II embryo (*bar* 400 μm).

seed extract increased the number of type I embryos in group A cell lines (data not shown). The treatment also increased the regeneration frequency. This stimulating effect of seed extract is in accordance with the previous report (Egertsdotter and von Arnold 1998).

We have tried to regenerate plants from both type I and II embryos, but a high regeneration frequency was only obtained from type I. No plants could be regenerated from the nodules. An improved protocol including partial desiccation of mature somatic embryos of *Picea abies* and regeneration of plantlets has now been developed which allows regeneration of plantlets from almost all embryos (Bozhkov and von Arnold 1998).

Two forms of mature somatic embryos of *Pinus elliottii* were also obtained (Liao and Amerson 1995). The long embryos, corresponding to our type I, were morphologically normal. The short embryos, corresponding to our type II, were not completely developed; they lacked significant root cap tissue. The short type II resemble the abnormal embryos of hybrid larch reported to be formed in the absence of ABA in the maturation medium (Lelu *et al.* 1994a,b). On medium lacking ABA the embryos remained stubby as opposed the more cylindrically shaped embryos on ABA supplemented medium.

Establishment of new embryogenic cell lines from mature somatic embryos: Initiation of embryogenic cultures from zygotic embryos can give rise to either A or B cell lines. To test if this difference is due to physiological or genetic reasons we initiated 18 new embryogenic cultures from mature somatic embryos from A cell lines (Mo *et al.* 1996). All new embryogenic cultures were of A type. In the present study we obtained mature somatic embryos from group B cell lines after treatment with seed extract and could therefore establish new embryogenic cultures also from these embryos.

New embryogenic cell lines were produced from mature somatic embryos type I from cell lines classified as group A and group B as well as from intermediate cell lines (Fig. 1). The initiation frequency was significantly higher for cell lines classified as A than from those classified as B. The intermediate cell lines were in between. The new initiated embryogenic cultures were analysed in microscope every week during the first six weeks. The morphology of the proliferating somatic embryos was the same as for the original cell lines, *i.e.*, new embryogenic cultures from mature embryos originating from group A had typical A-morphology and those from group B had typical B-morphology.

Effects of proliferation time on maturation frequency: In order to test if the ability to form mature somatic embryos changes after prolonged culture we transferred samples from the new established cultures to maturation medium after various periods of proliferation.

The typical group A cell lines retained the ability to form mature somatic embryos of type I during the test period (Table 2). The best response of cell line 86&52 was observed up to the end. Increasing of the fresh mass twice every month strongly corresponded with the increase of the mean number of mature somatic embryos

Table 2. Effect of different proliferation time and seed extract treatment on the capacity of different cell lines of *Picea abies* to produce type I mature somatic embryos (number per 1 g tissue, mean ± SE) on the BMI-S1 medium with 30 μM ABA and 7.5 % PEG.

Line	1 month		2 months		3 months		4 months	
	control	seed ext.	control	seed ext.	control	seed ext.	control	seed ext.
86&47	10 ± 2	42 ± 6	18 ± 3	48 ± 4	44 ± 6	123 + 9	10 ± 2	21 ± 2
86&52	12 ± 3	77 ± 15	51 ± 3	74 ± 6	102 ± 2	15 ± 8	173 ± 38	20 ± 2
IA&62	46 ± 5	58 ± 4	98 ± 4	36 ± 3	49 ± 6	*	0	*
IA&13	22 ± 5	27 ± 3	0	0	*	*	*	*
D1&7	0	0	0	79 ± 4	*	*	*	*

* - cultures stop to grow; 0 - cell lines were still growing but did not form type I embryos

categorised as type I. Considering all distribution of converted plantlets by their length prolonged proliferation culture significantly influenced the formation of longer plantlets (more than 2 cm). Variation was observed in this group. With prolongation of the culture, some embryogenic cell lines (for example 86&47) grew variably, and changes in the morphology occurred. Simultaneously, the competence to form type I mature somatic embryos and production of smaller plantlets (1.0 - 1.5 cm) were observed. The typical group B cell lines (for example cell line 88&37) did not form type I, type II and abnormal nodules mature somatic embryos. In contrast, this cell line maintained high growth rate during the tested period, increasing the fresh mass twice or more. However, the intermediate cell lines formed mature somatic embryos of type I in the beginning but lost the ability after a prolonged culture period. Further, the somatic embryos acquire smaller embryo head and longer suspensor regions resemble variable growing A cell line 86&47. This shows that the group B cell lines are established already from the beginning but also that some cell lines lose their embryogenic potential after prolonged culture. Furthermore, a typical group A cell line retain stable. We have run some A-cell lines for more that ten years and they still produce mature somatic embryos of type I which regenerate plants (data not shown).

Addition of seed extract to the medium increased initially the frequency of mature somatic embryos of type I in cell lines belonging to group A (Table 2). However, prolonged treatment with seed extract significantly decreased the maturation frequency. The control cultures usually maintained higher competence to produce type I mature somatic embryos. In general, the pattern observed in group A was also obtained in a group B cell line, where a two months treatment with seed extract stimulated development of type I embryos. In contrast, seed extract had no positive effect on the intermediate cell lines.

Well-developed embryogenic cell lines belonging to group A can maintain a high maturation frequency. Less-developed embryogenic group B is not influenced in a positive way by prolonged proliferation culture. Intermediate cell lines lose their embryogenic potential after prolonged proliferation. Addition of seed extract during a short period can stimulate maturation frequency in both group A and B cell lines.

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