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Screening of large numbers of seed families of *Pinus strobus* L. for somatic embryogenesis from immature and mature zygotic embryos

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Abstract The somatic embryogenic process was investigated using immature and mature zygotic embryos originating from 13 open-pollinated seed families selected for their embryogenic capacity from over 128 seed families of *Pinus strobus*. In a first step, intact megagametophytes with precotyledonary embryos from these families were placed on modified Litvay medium. Embryogenic tissues (ETs) were obtained for 12 of them, with initiation rates varying from 2.6% to 23%. On average, 14% of the ETs (36/258) formed stable embryogenic cell lines (ECLs) after 4–6 months of subculture. Mature somatic embryos were produced for 30 out of 52 cell lines, and plants were regenerated. Later, initiation of ETs from mature zygotic embryos was also tested for the selected families. ECLs were obtained for five of them, with a maximum initiation rate of 2.7%, and plants were produced for four ECLs.

Key words White pine · Conifers · Initiation · Open-pollinated seed families · In vitro plant regeneration

Abbreviations *ABA* Abscisic acid · *BA* Benzyladenine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *ECL* Embryogenic cell line · *ET* Embryogenic tissue · *SE* Somatic embryogenesis

Introduction

The use of somatic embryogenesis (SE) in eastern white pine (*Pinus strobus* L.) improvement programs will depend on the capacity to apply this process to a broad range of genotypes. Effectively, in pine species, low embryogenic initiation rates are generally observed which may limit the number of genotypes that can be regenerated through SE. However, some studies on other conifer species have shown that the capacity of zygotic embryos to initiate SE varies among individuals and families (Cheliak and Klimaszewska 1991; Isabel et al. 1997).

Another major problem with the use of SE in pine species is that the developmental stage of zygotic embryos seems to be critical for initiation of embryogenic tissue (ET). Indeed, immature zygotic embryos at the precotyledonary stage of development in intact megagametophytes are the most responsive type of explant (Finer et al. 1989; Becwar et al. 1991; Klimaszewska and Smith 1997), limiting the period for initiation to a few weeks per year. Furthermore, the use of immature seeds as explants may result in a single embryogenic cell line (ECL) composed of multiple genotypes, as was observed in *Pinus taeda* L. using isozyme markers (Becwar et al. 1991). In conifers, simple polyembryony resulting from fertilization of more than one egg cell within a single ovule may occur and lead to initial development of multiple zygotic embryos with different genotypes (Singh 1978). Eventually, only one zygotic embryo becomes dominant but the subordinate embryos remain viable and initiation of ET may originate from one or more subordinate zygotic embryos (Becwar et al. 1991).

In this study, the proportion of genotypes within and among seed families of *P. strobus* capable of producing ET from immature zygotic embryos was determined, followed by maturation, germination and acclimatization. Using the more responsive seed families, we were also successful in initiating SE and regenerating plants from mature zygotic embryos.

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Materials and methods

Plant material

Open-pollinated cones of *P. strobus* L. were collected from trees located in the clonal bank of the Canadian Forest Service in

Cap Tourmente (Québec, Canada). These trees are part of a large breeding population maintained by the CFS, Laurentian Forestry Centre.

The immature cones were collected from 128 trees between 20 July and 3 August 1994, and from 13 of them on both 24 and 28 July 1995, when zygotic embryos were at the precotyledonary stage of development. Cones were stored in paper bags at 4 °C until dissection, for a maximum of 2 months after collection in 1994, and for a maximum of 10 days in 1995. Seeds were extracted and surface disinfected for 1 min in 70% (vol/vol) ethanol, then 15 min in 1% (vol/vol) sodium hypochloride with one drop of Tween 20, followed by three rinses (10 min each) in sterile deionized water. The immature seeds were dissected and intact megagametophytes were placed onto the initiation medium.

Mature cones were collected in 1993, 1994 and 1996 from 13 trees (immature seeds were previously tested from 12 of them in 1995). Seeds were stored at –20 °C for 1–4 years until the time of dissection. Unless otherwise specified, mature seeds were imbibed in sterile deionized water for 4–6 h. Seeds were surface disinfected with the same procedure as immature seeds except that a 12-min submersion in 1% (vol/vol) sodium hypochloride was used instead of a 15-min one. The mature seeds were dissected and excised zygotic embryos were placed onto the initiation medium.

Initiation of ETs

Immature zygotic embryos

In 1994, initiation of ET was tested using intact megagametophytes derived from 128 open-pollinated seed families. The DCR medium (Gupta and Durzan 1985) and a modified HLM medium (Tremblay 1990), HLM-PB, were tested with 30 megagametophytes per seed family each. Ten megagametophytes were placed per Petri dish.

In 1995, 18–420 megagametophytes were collected for 13 open-pollinated seed families selected from the previous experiment. Except in a few cases, ten megagametophytes were placed per Petri dish. The HLM-PB medium consisted of Litvay's salts (Litvay et al. 1985) used at half-strength, supplemented with 250 mg l^{-1} glutamine, 1 g l^{-1} casein hydrolysate, 10 g l^{-1} sucrose, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 μM benzyladenine (BA), and 7 g l^{-1} purified agar (Sigma, St. Louis, Mo.). The pH of media was adjusted to 5.7 before autoclaving for 15 min at 121 °C. Glutamine was filter-sterilized and added to the cooled medium. Transfers to fresh medium were carried out every 4 weeks. ET extruding from the micropylar end of megagametophytes was separated from the explant and multiplied on initiation medium with a 2-week subculture. Except for initiation of ET, which was performed in darkness at 25±1 °C, cultures were maintained under a 16-h light/8-h dark photoperiod. Multiplication was done at a light flux of 5 μmol m^{-2} s^{-1} light, maturation and germination were performed at 20 μmol m^{-2} s^{-1} and 80 μmol m^{-2} s^{-1}, respectively. The total number of zygotic embryos producing ET was evaluated after 12 weeks. The effect of the culture medium on SE initiation, and initiation frequencies among seed families were compared using logistic analysis with the Catmod procedure (SAS Institute, Cary, N. C.).

Mature zygotic embryos

Initiation of ET from mature zygotic embryos was performed in two separate experiments using 13 open-pollinated seed families selected according to their initiation rates previously obtained from immature zygotic embryos. In the first experiment, five open-pollinated seed families collected in 1994 were tested. Excised zygotic embryos were placed onto modified HLM-PB medium. One hundred zygotic embryos were cultured per family with ten zygotic embryos per Petri dish.

In the second experiment, 12 seed families collected in 1993, 1994 and 1996, and three pretreatments were evaluated. Seeds were either imbibed in sterile deionized water and zygotic embryos were placed onto HLM-PB medium (treatment 1), or they were imbibed in liquid growth-regulator-free HLM-PB medium and embryos were placed either onto HLM-PB medium (treatment 2) or HLM-PB medium supplemented with 4.5 μM BA without 2,4-D (treatment 3). After 1 week of culture, all zygotic embryos were transferred onto HLM-PB medium. For each seed family and treatment, 30–50 zygotic embryos were cultured, with 10 zygotic embryos per Petri dish. Transfer of explants to fresh medium was carried out every 4 weeks during initiation. ET produced was grown on the initiation medium by subculturing every 2 weeks. Culture medium was solidified with 4 g l^{-1} gellan gum (Phytagel, Sigma).

The total number of zygotic embryos producing ET was evaluated after 12 weeks and after 16 weeks, for the first and the second experiment, respectively. ET was characterized by the presence of at least one immature somatic embryo attached to a suspensor.

Maturation, germination of somatic embryos and cryopreservation

ECLs derived from immature zygotic embryos (subcultured for 1–3 months) were tested for their capacity to produce mature somatic embryos. Depending on the material available per line, approximately 220–2200 mg of fresh weight ET was put on the maturation medium. Two to six pieces of ET weighing 110 mg each were placed per Petri dish. Tissues were placed onto embryo maturation medium EMM1 (Smith 1994) containing 6 g l^{-1} gellan gum for 2 weeks and were then transferred to EMM2 medium containing 4.5 g l^{-1} gellan gum. Both EMM media contained 30 g l^{-1} sucrose, 80 μM abscisic acid (ABA) and a mixture of several amino acids (mg l^{-1}): 7300 glutamine, 2100 asparagine, 700 arginine, 79 citrulline, 76 ornithine, 55 lysine, 40 alanine and 35 proline (Smith 1994). The pH of media was adjusted to 5.7 before autoclaving for 15 min at 121 °C. ABA and amino acids were filter-sterilized and added to the cooled medium.

ECLs derived from mature zygotic embryos were tested for their maturation capacity after 2 months of maintenance. The maturation medium consisted of Litvay's salts used at half strength, supplemented with 30 g l^{-1} sucrose, 80 μM ABA, EMM amino acids and 10 g l^{-1} gellan gum (Klimaszweska and Smith 1997).

Maturation capacity for an ECL was defined as the production of at least one mature somatic embryo, and this was evaluated after 10 weeks of maturation. Cotyledonary somatic embryos that were white to yellow with normal-shaped cotyledons were picked and placed onto germination medium consisting of Litvay's salts used at quarter-strength, supplemented with 20 g l^{-1} sucrose, 250 mg l^{-1} casein hydrolysate, and 4 g l^{-1} gellan gum, in magenta boxes. Plantlets were transferred into the substrate (peat moss : vermiculite 3 : 1) in the greenhouse when epicotyls had reached at least 1 cm. The survival rate was established after 2 months under greenhouse conditions.

ETs were cryopreserved after 3 months of subculture according to the modified procedure of Klimaszewska et al. (1992). The modifications consisted in adding the DMSO solution to the cell suspension at one time. The tissue suspension was then distributed in 1-ml quantities in cryovials and allowed to sit on ice for 2 h. Vials were then transferred into a prechilled freezing container (Nalgene) and placed at –80 °C for 2 h before being transferred to the liquid nitrogen freezer.

Results and discussion

Initiation of ET from immature zygotic embryos

In a preliminary initiation experiment from immature zygotic embryos in 1994, 128 open-pollinated seed families had been tested for their embryogenic capacity using two initiation media, HLM-PB and DCR medium. No significant difference between media was found (*P*-value 0.8179), therefore data on initiation frequency for both types of media were pooled. In total, 88 seed families pro-

Table 1 Embryogenic response of 13 open-pollinated seed families using immature zygotic embryos in *Pinus strobus*. The number of immature zygotic embryos forming ET from intact megagametophytes after 12 weeks of initiation (8 weeks in 1994), number of ECLs (subcultured for 1–3 months) that produced mature somatic embryos, as well as the number of ECLs proliferating after 4–6 months of subculture are shown

Maternal tree	Seeds collected in 1994	Seeds collected in 1995			
	Number of explants forming ET	Number of explants forming ET	Number of ECLs producing mature somatic embryos	Number of ECLs producing acclimatized plants	Number of explants with proliferating ET after 4–6 months
76509	9/60 (15.0)	17/115 (14.8)	4/6	2/4	4/115 (3.5)
78501	3/60 (5.0)	2/33 (6.1)	–	–	0/33
79501	4/60 (6.7)	0/18	–	–	–
82531	6/60 (10.0)	5/194 (2.6)	0/1	–	1/194 (0.5)
84536	11/60 (18.3)	10/100 (10.0)	3/6	2/3	4/100 (4.0)
84548	3/60 (5.0)	50/240 (20.8)	3/5	2/3	5/240 (2.1)
84550	8/50 (16.0)	24/120 (19.2)	0/4	–	2/120 (1.6)
84552	4/60 (6.7)	4/30 (13.3)	1/1	1/1	1/30 (3.3)
84553	4/60 (6.7)	21/174 (12.1)	4/8	3/4	5/174 (2.9)
84568	6/60 (10.0)	98/420 (23.3)	13/16	10/13	11/420 (2.6)
84569	15/60 (25.0)	8/50 (16.0)	1/1	1/1	1/50 (0.5)
84580	11/60 (18.3)	9/113 (8.0)	0/1	–	0/113
84598	8/30 (13.3)	10/75 (12.0)	1/3	1/1	2/75 (2.7)
Total	92/770	258/1682	30/52	22/30	36/1682

Note: Values in parentheses are percentages

duced ET and initiation rates varied from 3 to 50% (data not shown).

From this experiment, 13 seed families showing the highest initiation rates were selected for further initiation experiments. Thus, in 1995, intact megagametophytes originating from these seed families were placed onto HLM-PB medium (Table 1). The majority of ETs (89.1%) were produced during the first 6 weeks of culture. No ET was observed for the seed family derived from maternal tree 79501. However, in this case, only 18 megagametophytes had been tested. Significant differences in response were observed among seed families (*P*-value 0.0001), as was the case for the 1994 experiments. Initiation rates varied from 2.6 to 23.3% (Table 1), with an overall mean rate of 15.3%, which was comparable to that obtained in 1994, 11.9%.

In total, 258 ETs out of 1682 megagametophytes were separated from the initial explants (Table 1). A drastic decrease was observed in the number of ETs proliferating after separation. Effectively 20% (52/258) of the ETs continued to proliferate after 1–3 months of subculture, and 14% (36/258) after 4–6 months. Stable ECLs were obtained for 10 of the 13 families after 4–6 months of subculture. After 3 months of subculture, 42 ECLs were cryopreserved, 40 successfully (data not shown).

The frequency of SE from immature zygotic embryos in our study was consistent with previous reports on eastern white pine. Initiation rates were lower than those reported by Finer et al. (1989), where the mean initiation rate was 54%, and similar to that obtained by Klimaszewska and Smith (1997), although these authors did not mention the number of seed families or maternal trees tested nor the initiation rates per family. On the other hand, Kaul (1995) obtained a 6.3% initiation rate of ET, but in this case only four open-pollinated seed families had been tested. Since we had tested a large number of seed families, the wider range of initiation rates (2.6–23.3%) obtained here in comparison with the previous studies may reflect the genetic variability for embryogenic capacity that exists in the natural population. Such differences were also reported in spruce species (Cheliak and Klimaszewska 1991; Isabel et al. 1997) where the largest proportion of variation was found to be among families.

A decrease in the number of ECLs that proliferated on multiplication medium has been reported both in *Pinus* and *Picea* species, and this rate varied among species (Finer et al. 1989; Webb et al. 1989; Tautorus et al. 1990; Mo and von Arnold 1991). However only a few studies reported on the number of stable ECLs produced. In *P. strobus*, higher rates of ECL establishment were observed by Klimaszewska and Smith (1997) compared with our study, with 42% of ETs continuing to proliferate after 2 months of subculture. In *P. taeda*, 80% of initiated ETs formed stable ECLs (Becwar et al. 1990). In our case, a low frequency of stable ECLs was observed 6 months after initiation (4% for the best family) in comparison with initial initiation rates (23% for the best family). This decrease may reflect an inadequate formulation of the culture medium. A higher growth rate of ECLs of *P. strobus* was observed when purified agar was replaced by gellan gum (K. Klimaszewska, personal communication) and this should be used in future initiation experiments.

Production of mature somatic embryos and recovery of plants

A maturation protocol developed for *Pinus radiata* (Smith 1994) was employed to investigate the capacity of somatic embryos to mature for 52 ECLs (11 families) after 1–3 months of subculture. Development of somatic embryos to

the cotyledonary stage was observed after 10 weeks of maturation. Mature somatic embryos were produced for 30 ECLs (57.7%) originating from eight seed families (Table 1). The production of mature somatic embryos varied from 1 to 154 somatic embryos per gram fresh weight (data not shown). However, the low amount of tissue tested per ECL did not make it possible to directly compare the production of mature somatic embryos among the various ECLs. In addition, stage 2 somatic embryos (as defined by von Arnold and Hakman 1988) were obtained for eight other ECLs (1 cell line from an additional seed family), suggesting that with improved culture conditions these lines might form mature somatic embryos, since this protocol was not developed for *P. strobus*.

Mature somatic embryos from the different ECLs of *P. strobus* were isolated and transferred to the germination medium. Depending on the ECL, 1–147 mature somatic embryos were transferred for a total of 819. After 1 month, 91.2% somatic embryos (747) germinated (elongation of somatic embryos as defined by Bewley and Black 1985), and after 3 months on germination medium, 51.4% had developed an epicotyl. However, variation in quality of the germinants was observed among genotypes. For example, 95 somatic embryos from genotype PSS-2 germinated but 93 of them presented hyperhydricity, and did not develop an epicotyl. For some other genotypes, the epicotyl developed but callusing of the root was observed, decreasing further survival in the greenhouse. In total, 349 plantlets derived from 23 ECLs and eight families were transferred to the greenhouse and 73.5% of them grew into plants (22 ECLs). The acclimatization rates varied among genotypes from 47.2% (17/36) to 96.0% (73/76), and this variation was possibly due to the different rates of root callusing among genotypes. The mean conversion rate, that is, conversion of somatic embryos into acclimatized plants growing under greenhouse conditions after 2 months, was approximately 31%, varying from 0 to 50% depending on the genotype.

Up to now, most reports on conifer species have not specified the rate at which ECLs produce mature somatic embryos in pines, except in *Pinus caribae*, where six ECLs out of nine produced mature somatic embryos (Laine and David 1990). In *P. radiata*, 27% of the ECLs (39 of 144) gave rise to plants in the greenhouse (Smith et al. 1994), which is lower than this study. However, more indications related to the capacity of ECLs to produce mature somatic embryos are available in spruces. In *Picea mariana*, 90% of the ECLs produced a high number of mature somatic embryos (Cheliak and Klimaszewska 1991). However, in this case, only one or two ECLs were tested for each of the 17 families available. Other extensive studies have shown, in *Picea abies*, that 78% (47 of 60) of ECLs derived from five full-sib families produced mature somatic embryos (Norgaard et al. 1993), whereas in *Picea glauca*×*engelmannii*, 94% (67 of 71) of ECLs originating from six open-pollinated families were able to produce mature somatic embryos (Webster et al. 1990).

In *P. strobus*, the conversion rate of somatic embryos into plants was lower than in *P. radiata* (31% compared with 67%) (Smith et al. 1994). With standardized conditions during acclimatization, up to a 70% conversion rate was obtained for some genotypes, but differences were still noticed between genotypes (data not shown). Such differences were also reported in *P. radiata* (Smith et al. 1994) and *P. mariana* (Khlifi and Tremblay 1995). It thus seems important to test ECLs from maturation to acclimatization in the greenhouse to evaluate the real potential of individual genotypes.

Recovery of ECLs from mature zygotic embryos

Thirteen seed families showing moderate to high initiation rates of ET from immature embryos in 1994 and 1995 were selected for initiation experiments using mature zygotic embryos as explants. Initiation was first performed using stored mature seeds from five seed families collected in 1994 (experiment 1), and then using 12 seed families collected between 1993 and 1996 (experiment 2). In both experiments, callusing was observed during initiation. A brown callus was first produced in the radicle zone but rapidly stopped proliferating. Enlargement of cotyledons and hypocotyls was observed during the first weeks of culture followed by the formation of a hard creamy callus that turned brown. A white callus composed of dissociated spherical cells was also produced. Formation of callus was observed for 39–90% of embryos after 4 weeks of culture, and for 94–100% after 8 weeks depending on the family. After 8 weeks of initiation, a soft translucent tissue was observed on the surface of the callus that developed in the hypocotyl-cotyledon region (Fig. 1A–C). Observation of this translucent tissue under a microscope clearly demonstrated the presence of somatic embryos with dense meristematic cells attached to a few suspensor cells (Fig. 1D). New ET could be produced until the 16th week of culture on initiation medium. A total of nine mature zygotic embryos produced ET from five seed families, with initiation rates varying from 0.7% to 2.7% (Table 2). No pretreatment effect was observed.

From the nine zygotic embryos, four stable ECLs have been established after 4 months of subculture. Mature somatic embryos were produced for the four ECLs (Fig. 1E) and subsequent germination and development of plantlets was observed for all of them (Fig. 1F). The conversion rate observed for genotype PSZ-22 (seed family derived from maternal tree 84553) was 56% (44/78).

To our knowledge, this is the first report of SE from mature zygotic embryos in *P. strobus*. Up to now, only a few studies had explored the genetic variability of SE initiation capacity and it was shown, for different species, that this capacity may vary among genotypes and families (Becwar et al. 1990; Cheliak and Klimaszewska 1991; Isabel et al. 1997). In *P. strobus*, to increase our chances of obtaining ET from mature zygotic embryos, seed families that produced ET from immature zygotic embryos were tested. As had already been observed for *P. glauca* (Park et al. 1993), *P. strobus* families producing ET from mature embryos were not necessarily the ones with the

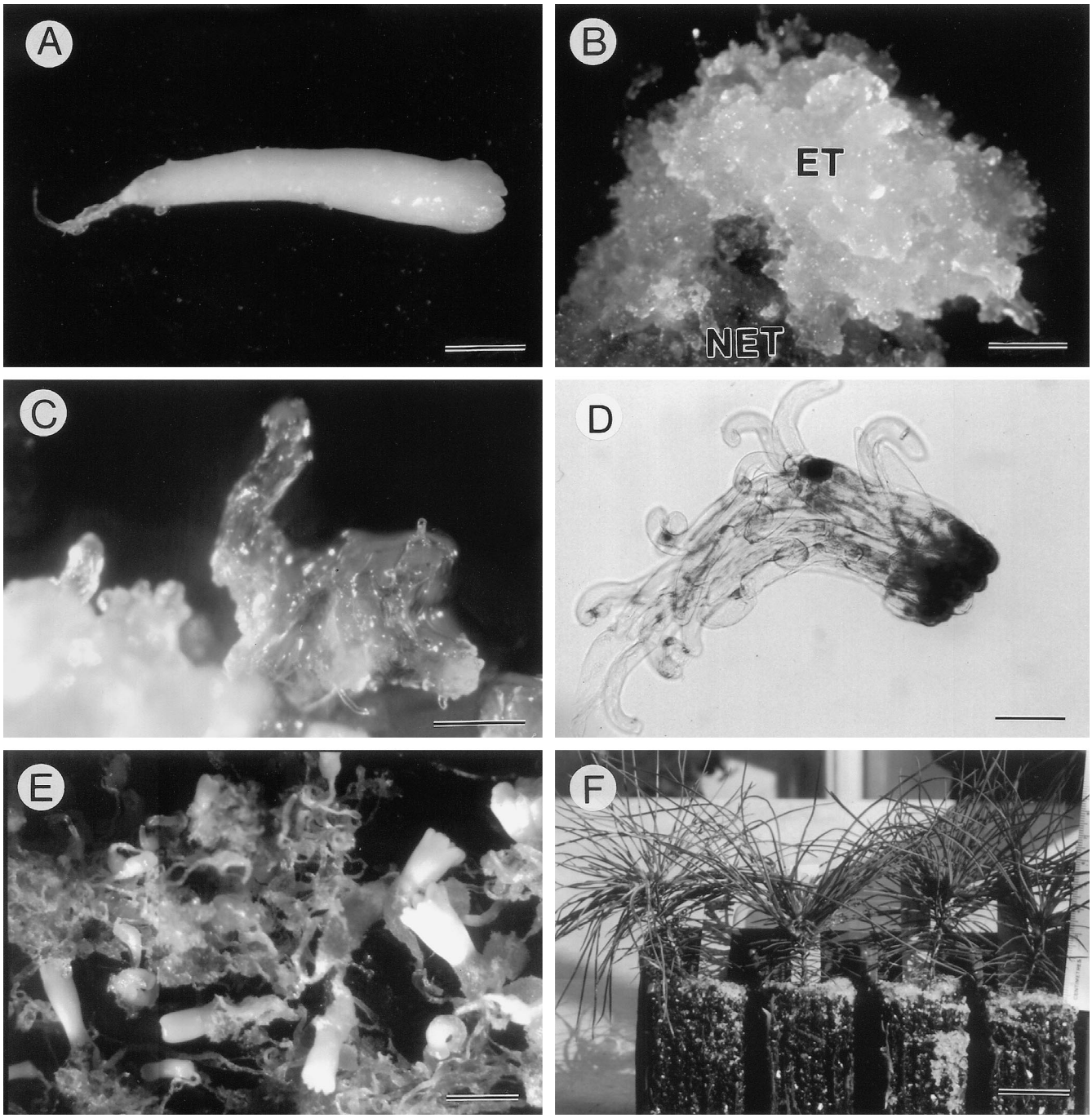

Fig. 1A–F Somatic embryogenesis from mature zygotic embryos in *Pinus strobus*. **A** Excised mature zygotic embryo (*bar* 1 mm). **B** Development of embryogenic (*ET*) and non-embryogenic tissue (*NET*) (*bar* 2 mm). **C** Group of somatic embryos with suspensors (*bar* 500 μm). **D** Staining with acetocarmine showing somatic embryos consisting of densely cytoplasmic cells subtended by suspensor cells (*bar* 150 μm). **E** Mature somatic embryos produced from ECL PSZ-22 (family 84553) (*bar* 2.5 mm). **F** Regenerated plants from ECL PSZ-22 acclimatized in the greenhouse (*bar* 3 cm)

highest initiation rate from immature embryos. The seed family derived from maternal tree 84553, from which an ECL was obtained in the first experiment, did not produce ET in the second experiment. However, seedlots used for these experiments were collected in two different years, 1993 and 1994, and it was shown in white spruce that year-to-year variation may affect significantly initiation rates when mature zygotic embryos are used as explants (Isabel and Beaulieu, in preparation).

Initiation of ET in pines was mostly restricted to immature zygotic embryos (Becwar et al. 1990; Tautorus et al. 1991) at the precotyledonary stage of development, while SE was obtained from both mature and immature zygotic embryos in spruces. In general, lower initiation rates are observed for mature zygotic embryos compared with immature zygotic embryos in *Picea* species (Tautorus et al. 1990; Park et al. 1993). Similarly, in *Pinus lambertiana*

Table 2 Somatic embryogenesis from mature zygotic embryos in *Pinus strobus*.The number of zygotic embryos forming ET after 12 weeks for the first experiment and 16 weeks for the second experiment, and number of ECLs that produced mature somatic embryos (SE) after 4 months of subculture

Maternal tree	Number of zygotic embryos forming ET		Number of ECLs producing mature SE
	Experiment 1	Experiment 2	
76509	0/94	0/102	–
77121	0/96	0/150	–
78501		1/143 (0.7)	–
79501		0/145	–
82531		0/150	–
84536	0/99	0/125	–
84548		2/90 (2.2)	2/2
84552		0/140	–
84553	1/97 (1.0)	0/120	1/1
84568		1/150 (0.7)	1/1
84569	0/100		–
84580		0/80	–
84598		4/149 (2.7)	–
Mean	1/486	8/1544	4/4

Note: Values in parentheses are percentages

(Gupta and Durzan 1986) and *Larix×leptoeuropaea* (Lelu et al. 1994), the frequency of ET initiation was greatly reduced when mature zygotic embryos were used.

To date, obtaining ET from mature zygotic embryos in *Pinus* species has been reported only in *P. lambertiana* (Gupta and Durzan 1986), *Pinus massoniana* (Huang et al. 1995) and *Pinus koraiensis* (Bozhkov et al. 1997), and regeneration of plants from such ECLs was reported in only *P. massoniana*. Restriction of SE to immature zygotic embryos in pines could be related to the origin of ET, since in pines it usually appears in the suspensor region (Gupta and Durzan 1986; Becwar et al. 1991; Bercetche and Pâques 1995). In spruces, the origin of ET is different, with initiation originating from the hypocotyl-cotyledon region either in immature or mature zygotic embryos (Webb et al. 1989; Tautorus et al. 1990; Mo and von Arnold 1991). In *P. strobus*, initiation of ET was observed after the formation of a callus in the hypocotyl-cotyledon region of mature zygotic embryos, which is similar to *P. mariana* (Cheliak and Klimaszewska 1991) and *P. massoniana* (Huang et al. 1995), whereas in *P. koraiensis* (Bozhkov et al. 1997), initiation was mainly observed in the suspensor region (up to 14.7%) after 3 weeks of culture. The production of ET was observed later in *P. strobus* (after 8 weeks) in comparison with *P. koraiensis*, but was similar to the report in *P. glauca* (Park et al. 1993).

In conclusion, despite the genetic variability that exists among different seed families for embryogenic capacity, initiation rates remained low in *P. strobus* compared with spruces. However, plants were regenerated from somatic embryos for the majority of the families tested. A decrease in the proportion of genotypes able to finalize the entire SE process occurred at each step of the process. This was particularly critical during the proliferation of ET, and to a lesser extent during maturation of somatic embryos, while no major limitation was observed during the germination and development of plants. Improved proliferation protocols for *P. strobus* are expected to greatly increase the proportion of genotypes that can be regenerated through SE, and higher production of mature somatic embryos per ECL should be obtained with improved maturation media.

Finally, mature zygotic embryos may be used to produce ECLs and regenerate plants in *P. strobus*. However, as initiation rates using immature zygotic embryos are higher than those observed using mature seeds, and proliferation rates are similar for both types of explant, immature zygotic embryos remain the preferred type of explant to produce embryogenic culture lines in a large number of seed families. Despite this, work should still be made on initiation using mature seeds since they provide several advantages over immature zygotic embryos. Stored mature seeds are available throughout the year and from year to year, thus allowing independence both from irregular flowering and from the restricted competence window of immature embryos during the year. Furthermore, excised zygotic embryos from mature seeds avoid any risk of multiple genotypes in individual ECLs. Further studies are needed to understand and determine the degree of genetic control of SE initiation in *P. strobus*, using both immature and mature zygotic embryos. The development of a complete diallel with maternal trees whose seed families have presented high, intermediate and low initiation rates, to enable us to determine the genetic components and their effects on SE initiation, is in progress.

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References

Arnold S von, Hakman I (1988) Regulation of somatic embryo development in *Picea abies* by abscisic acid (ABA). J Plant Physiol 132:164–169

Becwar MR, Nagmani R, Wann SR (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can J For Res 20:810–817

Becwar MR, Blush TD, Brown DW, Chesick EE (1991) Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly pine seeds. Plant Cell Tissue Organ Cult 26:37–44

Bercetche J, Pâques M (1995) Somatic embryogenesis in maritime pine (*Pinus pinaster*). In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol 3. Kluwer, Dordrecht, pp 221–242

Bewley J, Black M (1985) Seeds: physiology of development and germination. Plenum, New York

Bozhkov PV, Ahn IS, Park YG (1997) Two alternative pathways of somatic embryo origin from polyembryonic mature stored seeds of *Pinus koraiensis* Sieb et Zucc. Can J Bot 75:509–512

Cheliak WM, Klimaszewska K (1991) Genetic variation in somatic embryogenic response in open-pollinated families of black spruce. Theor Appl Genet 82:185–190

Finer JJ, Kriebel HB, Becwar MR (1989) Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). Plant Cell Rep 8:203–206

Gupta PK, Durzan DJ (1985) Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep 4:177–179

Gupta PK, Durzan DJ (1986) Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technology 4:643–645

Huang J, Wei Z, Xu Z (1995) Recovery of somatic embryos and plantlets from callus of mature masson pine embryos. Chin Sci Bull 40:1196–1200

Isabel N, Jeandroz S, Beaulieu J, Park YS, Plourde A, Bousquet J (1997) Genetic characterization of SE initiation in white spruce. Joint meeting of the IUFRO working parties somatic cell genetics and molecular genetics of trees, August 12–16 1997, Quebec, Canada

Kaul K (1995) Somatic embryogenesis in eastern white pine (*Pinus strobus* L.). In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol 3. Kluwer, Dordrecht, pp 257–268

Khlifi S, Tremblay FM (1995) Maturation of black spruce somatic embryos, part I. Effect of L-glutamine on the number and germinability of somatic embryos. Plant Cell Tissue Organ Cult 41:23–32

Klimaszewska K, Smith DR (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiol Plant 100:949–957

Klimaszewska K, Ward C, Cheliak WM (1992) Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix×eurolepis*) and black spruce (*Picea mariana*). J Exp Bot 43:73–79

Laine E, David A (1990) Somatic embryogenesis in immature embryos and protoplasts of *Pinus caribaea*. Plant Sci 69:215–224

Lelu MA, Klimaszewska K, Charest PJ (1994) Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of *Larix*. Can J For Res 24:100–106

Litvay J, Verma D, Johnson M (1985) Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep 4:325–328

Mo LH, Arnold S von (1991) Origin and development of embryogenic cultures from seedlings of Norway spruce (*Picea abies*). J Plant Physiol 138:223–230

Norgaard JV, Duran V, Johnsen O, Krogstrup P, Baldursson S, Arnold S von (1993) Variations in cryotolerance of embryogenic *Picea abies* cell lines and the association to genetic, morphological, and physiological factors. Can J For Res 23:2560–2567

Park YS, Pond SE, Bonga JM (1993) Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. Theor Appl Genet 86:427–436

Singh H (1978) Embryology of gymnosperms. Borntraeger, Berlin

Smith DR (1994) Growth medium for plant embryogenic tissue. Australia/Canada patent no PM5232

Smith DR, War A, Grace LJ (1994) Somatic embryogenesis joins the plantation forestry revolution in New Zealand. Proceedings TAPPI 1994 biological sciences symposium, Minneapolis, 3–6 October 1994

Tautorus TE, Attree SM, Fowke LC, Dunstan DI (1990) Somatic embryogenesis from immature and mature zygotic embryos, and embryo regeneration from protoplasts in black spruce (*Picea mariana* Mill.). Plant Sci 67:115–124

Tautorus TE, Fowke LC, Dunstan DI (1991) Somatic embryogenesis in conifers. Can J Bot 69:1873–1899

Tremblay FM (1990) Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can J Bot 68:236–242

Webb DT, Webster F, Flinn BS, Roberts DR, Ellis DD (1989) Factors influencing the induction of embryogenic and caulogenic callus from embryos of *Picea glauca* and *P. engelmanii*. Can J For Res 19:1303–1308

Webster FB, Roberts DR, McInnis SM, Sutton BCS (1990) Propagation of interior spruce by somatic embryogenesis. Can J For Res 20:1759–1765