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Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*)

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Summary Controlled crosses of seven Scots pine (*Pinus sylvestris* L.) trees produced 49 families that included both reciprocals and selfings. Embryogenic cultures were initiated from immature megagametophytes and after 6 months in maintenance culture, mature somatic embryos were produced from the surviving 166 lines. The effect of parent genotypes on the cultures was evaluated at initiation of the tissue culture period, after 6 months in maintenance culture and at embryo maturation. The effect of the maternal parent was most pronounced at culture initiation. After 6 months in tissue culture, the maternal effect had decreased and the effects of both parents were significant. By the somatic embryo maturation stage, the maternal effect was still considerable but the paternal effect was no longer detectable. There was little correlation between the ranking of mothers and fathers, indicating that the maternal effect was caused by factors other than the paternal effect. No mother × father interaction was found, indicating that mothers successful at initiation and after 6 months in tissue culture, pollinated by any of the successful fathers, produced somatic lines and mature somatic embryos.

Keywords: elimination of lines, genotype survival, maternal effect, paternal effect, pine clonal propagation.

Introduction

Plantation forestry, based on successful breeding of selected genotypes, offers the possibility of growing and managing forests of high economic value and superior quality. In traditional tree breeding programs, the best genotypes are identified after time-consuming progeny tests. The best parents (backwards selection) and individuals from the best progenies (forwards selection) are then selected as parent trees for the next generation. Clonal multiplication of selected families from controlled crosses is a powerful method of capturing maximal genetic gain from the breeding population. Somatic embryogenesis is a potential method of clonal mass propagation applicable to coniferous species.

Somatic embryogenesis in conifers was first reported in 1985 in *Picea abies* (L.) Karst. derived from zygotic embryos (Hakman and von Arnold 1985) and in *Larix decidua* Mill. from megagametophytes (Nagmani and Bonga 1985). Somatic embryogenesis has since been initiated in other conifers, including several pine species. Pine embryogenic cultures have been initiated from immature (Becwar et al. 1988, Jain et al 1989, Lainé and David 1990, Bercetche and Pâques 1995, Arya et al. 2000) and mature zygotic embryos (Gupta and Durzan 1986, Hohtola 1995, Bozhkov et al. 1997) and from female gametophytes dissected (Arya et al. 2000) and intact (Lainé and David 1990, Nagmani et al. 1993, Keinonen-Mettälä et al. 1996).

Embryogenic tissue proliferates while still in a relatively undifferentiated, early embryonic phase. The proliferation of pine embryogenic tissue is similar to zygotic polyembryony (Gupta and Durzan 1986, Arya et al. 2000). Under suitable tissue culture conditions, the embryogenic tissue will develop mature embryos, capable of germinating and developing into somatic seedlings (Attree and Fowke 1993, Becwar and Pullman 1995).

The success of pine embryogenic cultures is affected by seed collection time and the developmental stage of the immature zygotic embryo (Finer et al. 1989, Jain et al. 1989, Lainé and David 1990, Jones et al. 1993, Bercetche and Pâques 1995, Chandler and Young 1995, Kaul 1995, Keinonen-Mettälä et al. 1996, Arya et al. 2000), cone storage time and temperature (Häggman et al. 1999), culture medium (Jain et al. 1989, Lainé and David 1990, Li et al. 1998) and the genotype of parent trees (Gupta and Durzan 1986, Jain et al. 1989, Nagmani et al. 1993, Handley et al. 1995, Keinonen-Mettälä et al. 1996, Lelu et al. 1999). Our objective was to determine how much parent genotypes affect the success of embryogenic cultures.

Materials and methods

Controlled crosses, seed collection and cold storage

Seven Scots pine elite trees P451 (A), K738 (B), K770 (C), K801 (D), K828 (E), K1005 (F) and K1011 (G), four of which were tested earlier as mother trees for their suitability for em-

bryogenic tissue culture (Keinonen-Mettälä et al. 1996) and resistance to *Gremmeniella abietina* (Lagerb.) Morelet (Terho et al. 2000), were parent trees in controlled crosses. The trees were crossed in the spring of 1998 at the Finnish Forest Research Institute, Punkaharju Research Station (61°48′ N, 29°20′ E, 88 m a.s.l.), where they are grafted on collections no. 21, 24 and 26. The mating design was a full diallele, including reciprocals and selfings (Table 1).

Initiation of embryogenic tissue cultures

On June 29, 1999, immature female cones (5–8 per family) were collected and stored at 4 °C. After 1–20, 21–32 and 42–62 days in cold storage, the immature female cones were dipped in 75% ethanol, surface sterilized with 4% NaOCl for 10 min and rinsed twice with sterile water before the seeds were dissected from the cones. Seed coats were removed and the whole megagametophytes with immature embryos were placed horizontally on 20 ml of initiation medium in 90-mm-diameter petri dishes, with five megagametophytes per dish. We used 1–3 cones per family for each cold-storage treatment.

In the shortest cold-storage treatment (1–20 days), 2329 explants were placed on one of three initiation media: (1) Modified MSG (Brown and Lawrence 1968, modification by Becwar et al. 1988) without asparagine and activated charcoal, in which the iron-chelate Fe-EDTA (61.5 mg l^{-1}) was replaced with Na-Fe-EDTA (39 mg l^{-1}) and 0.8% agar replaced with 0.325% Phytagel (Sigma). Growth regulators were 4.44 μM N^6-benzyladenine (BA) and 9.05 μM 2,4-dichlorophenoxyacetic acid (2,4-D). (2) Modified W-pat. (US Patent No. 5,034,325, G.S. Pullman & P.K. Gupta 1991) with the replacement of 1% sucrose by 1% maltose and 0.8% Difco Bacto agar by 0.325% Phytagel (Sigma). Activated charcoal (2 g l−1) was added to the medium and the pH adjusted to 5.8. (3) Modified DCR20 (Gupta and Durzan 1986) with reduction of the NH_4NO_3 concentration from 5 to 1.4 mM, replacement of DCR micronutrients and iron-chelate with those of W-pat., and addition of 500 mg l−1 casein hydrolysate. Sucrose was replaced with 2% maltose. Growth regulators were 2.22 μM BA and 13.6 μM 2,4-D, 0.325% Phytagel (Sigma) and pH adjusted to 5.9.

For the intermediate cold-storage treatment (21–32 days), 2154 immature embryos were placed on initiation media as described for the shortest cold-storage treatment. In the longest cold-storage treatment (42–62 days), 781 immature embryos were used for initiation. Modified MSG initiation medium was replaced by DCR15 medium, which was similar to DCR20 with 2% maltose reduced to 1.5%. The total number of immature zygotic megagametophytes in the experiment was 5264. The total number of explants per mother tree varied from 537 (mother F) to 1069 (mother C).

Maintenance and production of mature somatic embryos

Cultures were assessed for viability 2 weeks after initation. At this stage, the megagametophytes that had darkened and from which no growing tissues could be observed were discarded and the rest transferred to DCR proliferation medium (Gupta and Durzan 1986), supplemented with 500 mg l^{-1} casein hydrolysate, 13.6 μM 2,4-D, 2.2 μM BA and 2% maltose. The somatic embryogenic tissues were subcultured every 2 weeks, with nine embryogenic tissues subcultured on a 90-mm-diameter petri dish with 20 ml of culture medium. Only the proliferating parts about 0.5 cm in diameter from the upper and outer tissue were transferred to fresh medium, while the lower darker parts were discarded. The cultures were grown in the dark at 23 °C.

The cultures were evaluated after 2 months on proliferation medium, and all nongrowing and non-embryogenic cultures were discarded. In March 2000, after an average of 6 months of maintenance culture, mature somatic embryos were produced from the surviving 166 lines. For each line, we used two petri dishes containing nine 1.5-cm diameter somatic tissues. The following three-step maturation protocol was followed. Step 1: prematuration on modified DCR proliferation medium supplemented with 0.5% myo-inositol, 8% PEG 3500, 2.22 μM BA and 13.6 μM 2,4-D, 2% maltose, 0.325% Phytagel and pH adjusted to 5.9. After two 2-week subcultures, the cultures were transferred to the next medium. Step 2: Growth-regulator-free modified DCR proliferation medium with 0.1% activated charcoal and 2% maltose. After one week, the cultures were transferred to maturation medium. Step 3: Maturation medium, DCR with 8% PEG 3500 and 15.1 μM abscisic acid (ABA). The mature somatic embryos per line were counted every two weeks, when the tissues were subcultured.

Statistical analyses

Observed initiation percentages on modified MSG and DCR20 media were subjected to analysis of variance (AN-

Table 1. The full diallele mating pattern used to produce hybrid and self-pollinated families. Letters in parenthesis are codes assigned to the genotypes to identify the families.

Mother	Father						
	P451 (A)	K738 (B)	K770 (C)	K801 (D)	K828 (E)	K1005 (F)	K1011(G)
P451 (A)	AA	AB	AC	AD	AE	AF	AG
K738 (B)	BA	BB	BC	BD	BE	BF	BG
K770 (C)	CA	CB	CC	CD	CE	CF	CG
K801 (D)	DA	DB	DC	DD	DE	DF	DG
K828 (E)	EA	EB	EC	ED	EE	EF	EG
K1005 (F)	FA	FB	FC	FD	FE	FF	FG
K1011 (G)	GA	GB	GC	GD	GE	GF	GG

OVA) based on an additive model with the significant first-order interaction effects included. The level of significance was 0.05. Arcsine transformation was used to make binomial distributions (percentages) approximately normally distributed. The factors were mother, father and cold storage time effects. Selfed lines were excluded. Weighting with the explant frequencies was used, as there were unequal numbers of explants for different factor combinations. The effects of mothers and fathers were separated in the analyses so that non-genetic maternal influences (i.e., physiological variation among mother trees belonging to the same clone) would not bias evaluations of the parent trees as fathers, making it also possible to compare the similarity between the rankings of parent trees as mothers and as fathers.

Data on the survival of the lines after six months on proliferation medium were subjected to ANOVA as described for the initiation data. Analysis of variance for somatic embryo production was performed on the ranks instead of the numbers of mature somatic embryos per line because of the wide variation in numbers.

Results

Culture initiation

Embryogenic tissue was initiated from all of the mother trees tested. From the 49 families of the diallele crosses, 47 initiated embryogenic tissue. Two families, GB and GF, were unresponsive to the culture method and did not initiate any embryogenic lines after two weeks on initiation medium. In both of these families the mother's genotype was G, and the initiation success percentage of genotype G over all fathers was 4%, the lowest of all mothers. When genotype G was used in crosses as father, it shared the highest overall initiation percentage (17%) with genotype D (Table 2).

The overall percentage of culture initiation was 13% and the variation between families was 1–42%. The initiation success was about three times higher for cross-pollinated (14%) families than for self-pollinated (4%) families. There was more variation in initiation success among mothers than among fathers. Also, the ranking of a genotype changed depending on whether it was used as mother or father (Table 2).

The maternal effect was more significant than the paternal effect at the initiation stage. Cold storage time had no statistically significant effect on initiation success, but the mother × time interaction was significant. Neither the father × time nor the mother × father interaction effects were statistically significant for the initiation (Table 3).

The initiation success percentages for both cross-pollinated and self-pollinated families were highest on modified DCR20 after 21–32 days of cold storage. For the cross-pollinated families, the initiation percentage on the best medium (DCR20, 24%) was more than double that on the least effective medium (MSG, 9%), whereas the difference between the best (DCR20, 6%) and the worst medium (W-pat, 2%) was relatively larger for the selfings (Figure 1). In the 42–62 day cold-storage treatment, initiation success on initiation medium DCR15 did not differ from that on DCR20.

Survival of tissue cultures

After six months in maintenance culture, the genotype of both mother and father had a significant effect on survival (Table 4). From the 47 successfully initiated families, 33 survived in maintenance culture for 6 months. The number of lines per family varied between 1 and 27. There was a large variation in the survival rate between families; for example, compare father B, with the lowest survival percentage (6%), with father D, with the highest survival percentage (36%). With all mothers, father D yielded a higher survival percentage than father B (Figure 2).

After 6 months in maintenance culture, the proportion of surviving cross-pollinated lines was four times higher than with self-pollinated lines (25 versus 6%). The difference in survival percentage, however, was not statistically significant, because only 31 self-pollinated lines were originally initiated (Table 4).

Production of mature somatic embryos

Only the maternal genotype significantly influenced the production of mature somatic embryos (Table 5). Out of the 33 surviving families, 29 produced mature somatic embryos. The two surviving selfed families BB and DD had only one line each and they were mediocre in mature somatic embryo production (Figure 3).

Of the 166 embryogenic lines, 69% produced mature so-

Table 2. Initiation success percentages of Scots pine embryogenic cultures after 2 weeks in tissue culture. The letters in parenthesis are codes given to the genotypes. Number of explants per family: a = more than 100; b = 51–100; and c = less than 50.

Mother	Father							
	P451 (A)	K738 (B)	K770 (C)	K801 (D)	K828 (E)	K1005 (F)	K1011(G)	Mean
P451 (A)	1 a	10 a	7 b	14 b	2 c	6 b	1 b	6
K738 (B)	20 a	9 a	20 a	42 a	19 a	18 a	29 a	23
K770 (C)	3 a	20 a	2 a	6 a	5 a	11 a	10 a	9
K801 (D)	16 b	16 a	15 a	3 b	15 a	30 b	25 a	18
K828 (E)	17 a	12 a	20 a	27 a	6 a	9 b	18 b	16
K1005 (F)	24 a	20 c	9 a	21 c	17 c	2 a	15 b	13
K1011 (G)	13 b	0 b	7 b	4 b	2 b	0 b	4 b	4
Mean	13	13	12	17	10	11	17	13

Table 3. Analysis of variance for initiation success of Scots pine immature megagametophytes on modified MSG and DCR20 initiation media. The time effect for MSG initiation medium comprises two initiation periods: 1–20 (MSG-1) and 21–32 (MSG-2) days after cold storage. The time effect for DCR20 initiation medium comprises two initiation periods: 21–32 (DCR20-2) and 42–62 (DCR20-3) days after cold storage. The *F* and *P* values for the effects in parenthesis were calculated as if that effect was in the model, although it was excluded from the model in the calculation for the other effects. Asterisks indicate *P* values: *** = $P < 0.001$; and * = $P < 0.05$.

Effect	MSG initation medium[1]			DCR20 initiation medium[2]		
	Degrees of freedom	*F* value	*P* value	Degrees of freedom	*F* value	*P* value
Mother	6	9.59	< 0.0001 ***	5	6.52	< 0.0002 ***
Father	6	3.14	0.010 *	6	2.86	0.023 *
Time	1	0.12	0.73	1	1.40	0.25
Mother × time	5	5.77	0.0002 ***	3	3.37	0.030 *
(Father × time)	6	0.62	0.71	6	0.76	0.61
(Mother × father)	29	1.73	0.077	21	1.08	0.46

[1] Degrees of freedom for mother × time interaction effect was less than for father × time, because there were no explants of mother G on MSG-2 medium (after 21–32 days in cold storage).

[2] Degrees of freedom for mother effect is 5, because there were no explants of mother G. Degrees of freedom for mother × time interaction effect was less than for father × time, because there were explants of only four mothers on DCR20-3 (after 42–62 days in cold storage).

matic embryos. There was wide variation in mature embryo production among lines, with 31% of the lines producing no mature embryos and about 50% of the lines producing between 1 and 80 mature embryos. One line (BA1) produced over 500 mature embryos. There was large variation among lines within a family and the ranking of genotypes as mothers was different from that as fathers. The best mother at the mature embryo production stage was genotype E and the best father was genotype B (Figure 4). Among families, family EB produced the most mature somatic embryos (Figure 3), but the mother × father interaction had no statistically detectable effect on mature somatic embryo production (Table 5).

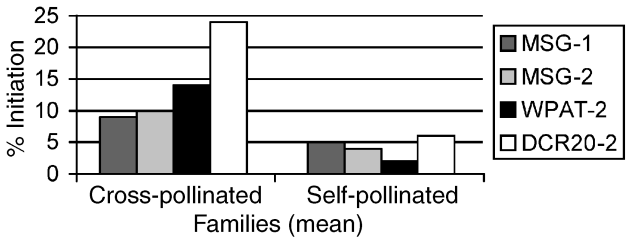

Figure 1. Mean immature megagametophyte initiation success percentage of cross- and self-pollinated Scots pine lines on different initiation media after 1–20 days (MSG-1) and 21–32 days (MSG-2, WPAT-2, DCR20-2) in cold storage.

Table 4. Analysis of variance for the survival of Scots pine embryogenic cultures after 6 months in maintenance culture. Results for mother and father effects were estimated from a model where self-pollinated lines were excluded. When the self-pollination effect was added to the model, it lacked statistical significance because of the low number of self-pollinated lines that survived. The asterisks indicate $P < 0.01$. The *F* and *P* values for the self pollination effect in parenthesis were calculated as if that effect was in the model. It was excluded from the model in the calculation for the Mother and Father effects.

Effect	Weighted		
	Degrees of freedom	*F* value	*P* value
Mother	6	4.00	0.0065 **
Father	6	5.22	0.0015 **
(Self-pollination)	1	2.06	0.16

Discussion

Initiation

In our study, proliferation started at the micropylar end of the megagametophyte by a process corresponding with Becwar and Pullman's (1995) description of extrusion of cleaved zygotic embryos followed by initiation of embryogenic tissue in *Pinus taeda* L. In Scots pine, the developmental stage of the zygotic embryo at the time of cleavage polyembryony coincides with simple polyembryony (Sarvas 1962) and may result in more than one genotype per embryogenic line, as has been reported with low frequency (8%) in loblolly pine (Becwar et al. 1991). The possible subordinate embryo extrusion followed by success or failure in initiating embryogenic culture is an event that should be investigated further.

In our study, the initiation percentage was 13%, which is slightly higher than the typically low initiation percentage reported for Scots pine (Keinonen-Mettälä et al. 1996, Häggman et al. 1999, Lelu et al. 1999). Four of the trees in our experiment (P451, K801, K828 and K1005) were included in earlier somatic embryo production experiments (Keinonen-Mettälä et al. 1996) and were the most responsive of 138 tested trees. This pre-selection of material may explain the high initiation percentage. Also, the survival of cultures at early stages of tissue culture does not necessarily correlate to embryogenic tissue formation.

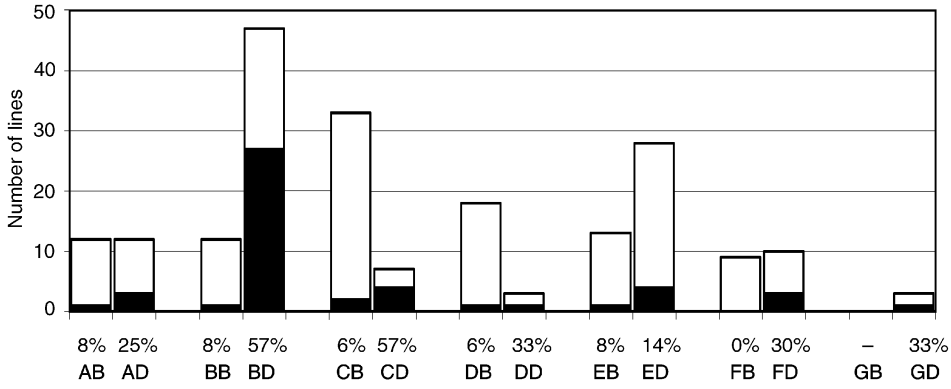

Figure 2. Comparison of the survival of Scots pine embryogenic lines for families where the father is B or D after 6 months in maintenance culture. The percentages are the proportions of surviving lines (dark part of the bars). Family codes assigned to genotypes are identified in Table 1.

Lelu et al. (1999) observed a significant mother tree effect in both *P. sylvestris* and *P. pinaster* Aiton at the initiation stage. We found that the maternal effect was greater than the paternal effect on culture initiation (Table 3). Even the mother × time interaction effect had higher *F* values than the paternal effect, suggesting that it is difficult to estimate the significance of the father's genotype, even if only one mother tree is used in all the crosses. Park et al. (1993) partitioned family variance based on the initiation of somatic embryogenesis in white spruce (*Picea glauca* (Moench) Voss) and found that over 20% of the variance was caused by general combining ability and that the specific combining ability was negligible. The low *F* values for the mother × father interaction effect in our experiment indicate that specific combining ability had no detectable effect on the initiation success of the families (Table 3).

Lelu et al. (1999) found a significant interaction between mother tree and culture medium at the initiation stage of Scots pine embryogenic culture. Häggman et al. (1999) also reported that both mother tree and culture medium significantly affected the initiation success of Scots pine, but found no interaction effect between the two. In our experiment, ranking of genotypes by culture initiation was affected by the genotype (mother versus father), initiation medium and cold-storage time. Häggman et al. (1999) concluded that cold treatment of the cones had no effect on initiation. Similarly, in our experiment, cold-storage time had no effect on initiation percentage; however, the ranking of the mother trees changed during the storage time, suggesting changes either in the physiological condition or developmental stage of the explants. That is, during cold storage some families may reach more optimal stages for culture initiation than others. Häggman et al. (1999) noted that a cold-storage temperature of 5 °C was high enough for the further development of the zygotic embryo in collected immature pine cones. Our results indicate that the maternal effect at the initiation stage can be explained by a combination of two factors: (1) the genotype and the developmental or physiological stage of the mother tree (with possible variation between different cones); and (2) the inherited maternal alleles of the zygotic embryo.

Table 5. Analysis of variance for the ranking of Scots pine lines according to the number of mature embryos per line. Mothers A and G and fathers B and F, from which only a few lines survived, were excluded from the analysis with self-pollinated lines. The asterisks indicate $P < 0.01$. The *F* and *P* values for the mother × father effect in parenthesis were calculated as if that effect was in the model. It was excluded from the model in the calculation for the mother and father effects.

Effect	Degrees of freedom	*F* value	*P* value
Mother	4	3.74	0.0064 **
Father	4	2.42	0.051
(Mother × father)	12	1.41	0.17

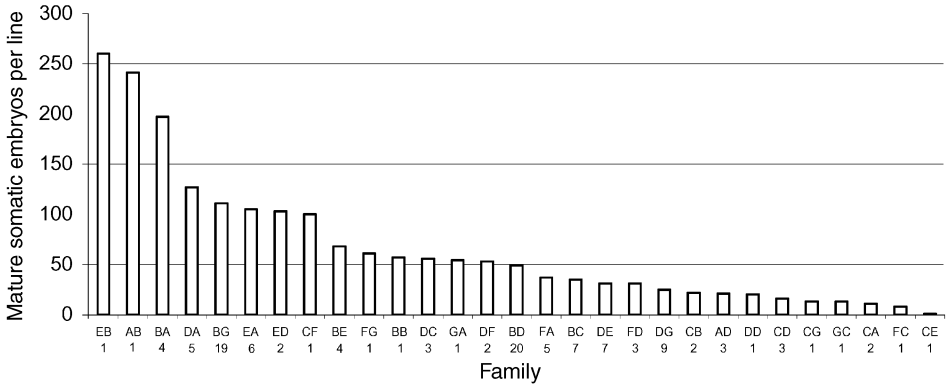

Figure 3. Mean number of mature embryos per line in the Scots pine families calculated from two 90-mm diameter petri dishes each containing nine embryogenic tissues. The number of lines per family is shown under the family code. Family codes assigned to genotypes are identified in Table 1.

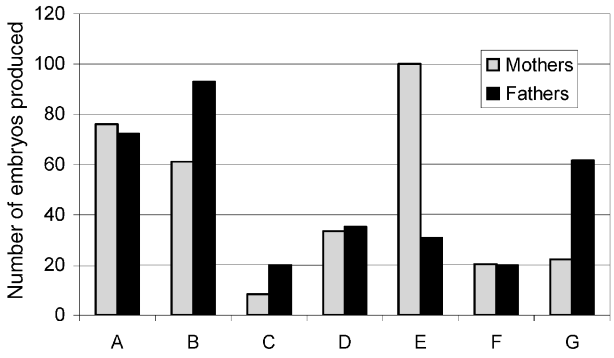

Figure 4. Mean number of Scots pine mature somatic embryos produced per line for different mothers and fathers.

Proliferation

Both maternal and paternal effects significantly affected survival of the lines during maintenance culture. Analysis of variance (Table 4) indicated that, during the prolonged maintenance culture period, the significance of the mother's genotype diminished and the significance of the father's genotype consequently became detectable.

The smaller percentage of lines eliminated during the proliferation stage compared with the initiation stage suggests that any genotype that becomes well established and proliferates will probably survive for several months. Garin et al. (1998) also noted large variation in embryogenic capacity among seed families; however, initiated embryogenic cultures of *Pinus strobus* L. from most of the families in low percentages, with variation in genotype survival occurring during proliferation. Finer et al. (1989) reported that two thirds of the initiated *P. strobus* lines survived the proliferation stage and Häggman et al. (1999) observed that 67% of *P. sylvestris* lines survived for at least 1 year in maintenance culture.

Survival of embryogenic cultures may partly depend on factors related to tissue culture techniques, e.g., the 2-week subculture period and culture medium may not have been optimal for all the genotypes. Chandler and Young (1995), working with *P. radiata* D. Don, and Lelu et al. (1999), working with *P. sylvestris*, reported successful proliferation on hormone-free culture medium. Further optimization of culture technique and culture media may increase the survival of the material used in our experiment.

Mature somatic embryo production

Analysis of variance (Table 5) indicated that the mother's genotype had a significant effect on mature somatic embryo production. There were, however, differences in somatic embryo production among lines within families. The variation among families was only about 10% higher than the variation within families, and the most and least productive lines were scattered in different families. Similar large variation in mature embryo production among lines has been observed in hybrid larch (Lelu et al. 1994).

The proportion of lines that produced mature somatic embryos was 69%, indicating that fewer lines was eliminated at the maturation stage than at either the initiation stage or the maintenance culture stage. A similar proportion of lines was eliminated during somatic embryo development in *P. strobus* embryogenic cultures (Garin et al. 1998). Häggman et al. (1999) reported that three of four lines of *P. sylvestris* tested produced mature embryos.

Because we used only seven genotypes in controlled crosses, our results were inconclusive. However, we demonstrated that parental effects varied at different stages of somatic embryo production. The maternal effect was considerable at the initiation stage, whereas the maintenance culture period was characterized by an increase in the paternal effect relative to the maternal effect. At the somatic embryo maturation stage, the maternal effect was still considerable, whereas the paternal effect was negligible. The ranking of the genotypes as mothers differed from their ranking as fathers, suggesting that the maternal effect was caused by factors other than the paternal effect. No mother × father interaction was found at any stage of somatic embryo production, indicating that successful mother trees pollinated by any of the successful father trees produced embryogenic lines and mature somatic embryos.

Acknowledgments

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