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Genetic variation in somatic embryogenic response in open-pollinated families of black spruce

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Summary. Zygotic embryos from open-pollinated seeds of 20 black spruce (*Picea mariana*) families were used to investigate the proportion of genotypes that would give rise to embryogenic tissue (ET) and mature somatic embryos. Eighty-five percent of the maternal genotypes gave rise to embryogenic tissue. Within-family rates of ET induction ranged from 0 to 17%, with an average of 8%. The largest proportion of variation was among families, indicating the additive nature of the genetic variation. On a medium with 6% sucrose and 3.7 μM ABA, 90% of the embryogenic lines gave rise to abundant (>100/100 mg of ET), well-formed, mature somatic embryos. A medium with 2% sucrose, without 2,4-D, was used to germinate the mature somatic embryos. These were grown in the greenhouse and have now been established in field trials.

Key words: Somatic embryogenesis – *Picea mariana* – Biotechnology

Introduction

Somatic embryogenesis defines a process by which non-reproductive cells develop into differentiated plants through characteristic embryological stages (Thorpe 1988). Only since 1985 has embryogenesis been possible in the conifers (Hakman and von Arnold 1985; Nagmani and Bonga 1985). Since these first reports, however, the technique has been successfully extended to a number of different species including members in *Pinus* (Gupta and Durzan 1986, 1987), *Picea* (Hakman and Fowke 1987; Krogstrup et al. 1988), *Larix* (Klimaszewska 1989a; von Aderkas et al. 1990), *Pseudotsuga* (Durzan and Gupta 1987), and *Abies* (Schuller et al. 1989).

This has been an extremely important development in conifer biotechnology for two reasons. The first is that this system offers the potential to produce sufficient numbers of propagules, ultimately in the form of artificial seeds, for commercial reforestation programs for a larger number of species than is currently possible. This will permit the establishment of clonal forestry programs. Secondly, these cultures have been used as a source of protoplasts which have then been cultured to produce plants (Attree et al. 1989; Klimaszewska 1989b). This is the first successful regeneration of plants from protoplasts of conifer species in over 10 years of research (Kirby 1988).

Explants of immature zygotic embryos have been used to establish embryogenic lines in all responsive conifer genera to date. In addition, mature embryos and cotyledons have been used to successfully establish embryogenic lines in several different species of *Picea* (Gupta and Durzan 1986; Lelu et al. 1987; von Arnold and Woodward 1988). The positive response of mature embryos provides a convenient way to investigate levels of genetic variation in the population for this trait. Like more conventional asexual reproduction, the major initial advantage of these asexual systems in forestry will be to reduce the time required to have genetically improved material for reforestation programs (Hasnain and Cheliak 1985; Cheliak and Rogers 1990). Before serious consideration can be given to using this proposed propagation system in tree improvement, a number of questions need to be answered. One of these questions concerns the impact this system may have on the genetic structure of the regeneration population.

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In this paper, we report on patterns of genetic variation in the induction of somatic embryogenesis in a natural population of black spruce. We provide estimates of the proportion of genotypes and seeds within genotypes that are capable of producing an embryogenic tissue. Finally, we provide estimates of the proportion of embryogenic tissues that are capable of producing large numbers of well-formed, phenotypically normal somatic embryos.

Materials and methods

Open-pollinated seeds of black spruce (*Picea mariana* Mill B.S.P.) were obtained from collections made by the Ontario Ministry of Natural Resources. Cone storage and seed extraction were as per normal procedures for commercial seed lots. Seeds were kept in closed jars at 4 °C for approximately 2 years. Seeds were surface sterilized in 20% (v/v) Commercial Javex (sodium hypochloride, 6.1%) solution containing 2 drops of Tween 20 for 7 min, followed by three rinses in sterile, distilled water. Following sterilization, the seeds were imbibed in sterile, distilled water for 4 h prior to dissection of zygotic embryos. Ten embryos were cultured in each 100 × 15 mm petri dish. A total of 170 embryos were isolated from each of the 20 maternal families.

The culture medium used was LM (Litvay et al. 1981), modified to include half-strength major elements, 500 mg/l L-glutamine, 1,000 mg/l casein hydrolysate, 1% (w/v) sucrose, 2 μM, 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 μM 6-benzyladenine (BA). The medium was solidified with 0.9% (w/v) Difco Bacto-agar and the pH was adjusted to 5.8 prior to sterilization in the autoclave. Filter-sterilized L-glutamine stock solution was added to the sterile, molten media.

The cultures were maintained in darkness at 25 °C. After 4 weeks, embryos were subcultured onto fresh aliquots of the same medium, except for a higher concentration of 2,4-D (10 μM) and BA (5 μM). All subsequent subcultures were done in 2-week intervals. Every week the cultures were microscopically examined and initiation of callus and embryogenic tissue was recorded. Final observations were done 12 weeks after initiation of the cultures.

Seed germination test

Seeds from the same seed lots (100 seeds per lot) were germinated according to the standard ISTA method. This involved four replicates of 25 seeds each, with an 8-h light/16-h dark photoperiod at 30 and 20 °C, respectively. A seed was considered germinated when radicle extension was 5 mm beyond the seed coat.

Maturation and germination of somatic embryos

Pieces of embryogenic tissue, approximately 100 mg fresh weight (fw) (six per 100x15 mm petri dish), were placed onto LM medium, modified as above, containing 2 or 6% (w/v) sucrose with or without 2,4-D (0.5 μM) and abscisic acid (ABA, 3.7 μM). Media were gelled with 0.9% agar or 0.4% Gelrite gellan gum (K9A40, Kelco, San Diego/CA, USA). After 4 weeks the mature somatic embryos (most at cotyledonary stage) were transferred onto LM medium without growth regulators, containing 2 or 6% sucrose and solidified with 0.9% agar or 0.4% gellan gum. Somatic embryos that developed roots were subsequently placed in test tubes or magenta jars for further root and secondary needle development. The test tubes and jars contained paper plugs (Baumgartner Systems) saturated with 5 ml of liquid LM medium supplemented with 2% sucrose. [illegible] growth regulators, casein hydrolysate, or L-glutamine were added to the medium.

Results and discussion

Initiation of somatic embryogenesis

Excised embryos (Fig. 1 A) began to enlarge and produced unorganized, brownish-colored calli (Fig. 1 [illegible]) composed of small isodiametric cells. This callus could be observed as early as 2 weeks after explant culture initiation. Little new callus was produced after 4 weeks. About 55% (range: 15–82% among families) of the explants gave rise to calli.

The second type of tissue produced was translucent white in color (Fig. 1 B) and was composed of long cells and early embryogenic structures. Typically, this tissue did not appear until 4 weeks, and its initiation could be observed for as many as 12 weeks after onset of culture. Generally, the greatest proportion of embryogenic tissue appeared between weeks 6 and 10 (Fig. [illegible]). The range of family rates of induction of embryogenic tissue was 0 (Families 13, 18, 19) to 18% (Family [illegible]) (Fig. 3). In a study of induction of embryogenic tissue on different media with mature black spruce, Tautoras et al. (1990) found 8–10% of the explants to be responsive.

To successfully establish an embryogenic line, translucent callus had to be separated from most of the callus and cultured separately as soon as it reached sufficient critical mass. Several subculture cycles were generally required before a "pure" embryogenic line was established.

An ANOVA of embryogenic tissue and callus induction indicated significant differences among families (Table 1). The stand component was significant for induction of callus only. The among-family component, indicative of additive genetic variance, accounted for the majority of the variance in embryogenic tissue initiation and confirms the genetic basis for the variation. The biological significance of the among-stand component [illegible]

Table 1. ANOVA of induction of callus and embryogenic tissue from mature seeds of 20 families of black spruce

Source	*df*	Variance ratio	
		Callus	Embryogenic tissue
Reps	16	1.90	2.32
Stands	3	31.84*	2.96
Families/S	16	16.02*	4.08*
Error	304		

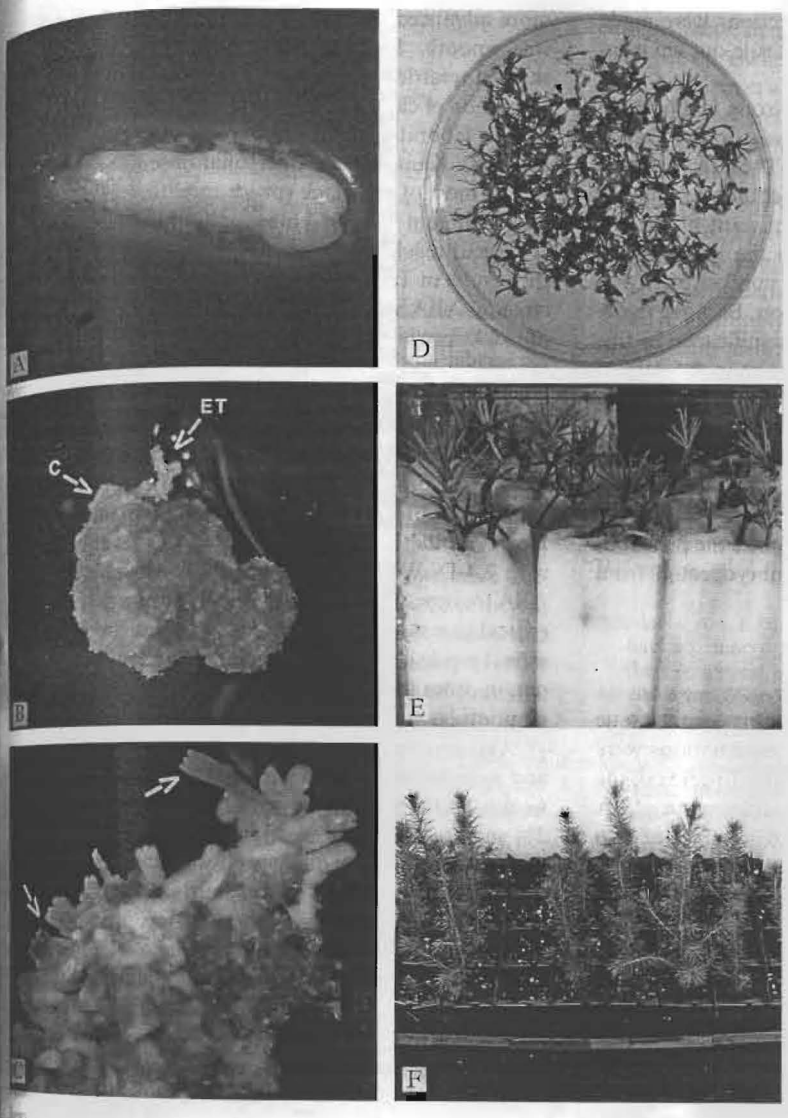

Fig. 1. Stages of embryogenic tissue initiation and somatic embryo development from isolated zygotic embryos of black spruce. **A** Freshly isolated zygotic embryo. 120×. **B** Initiation of embryogenic tissue (ET) and callus (C) after 4 weeks of culture. 100×. **C** Maturation of somatic embryos on medium with ABA and 6% sucrose after 4 weeks of culture. 80×. **D** Somatic embryo germination on medium with 2% sucrose and no growth regulators. 0.5×. **E** Development of germinated somatic embryos in paper plugs saturated with liquid medium (no growth regulators +2% sucrose). 1.2×. **F** Growth of somatic embryo-derived plants in a soil mix. 0.1×

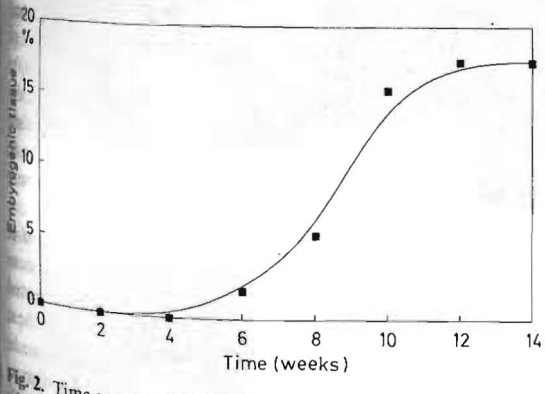

Fig. 2. Time course of embryogenic tissue initiation from isolated zygotic embryos of black spruce (Family 20)

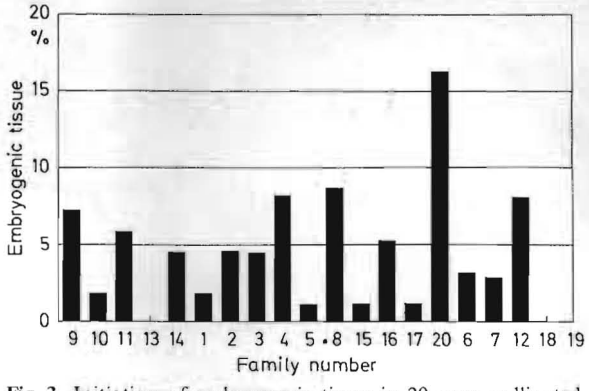

Fig. 3. Initiation of embryogenic tissue in 20 open-pollinated families of black spruce

not immediately clear. Although collections were made as consistently as possible, one cannot rule out environmentally induced variation.

As could be expected, there was a strong and positive correlation between the number of days it took to reach 50 and 90% germination (Table 2). There was also a positive correlation between the production of callus and embryogenic tissue. Although nonsignificant, there was an interesting negative trend between the induction of embryogenic tissue and callus and the number of days it took to achieve 50 and 90% germination. Because vigorous seed lots were used, the lack of significance in this case is probably due to the limited range in the rate of germination. However, one can infer from these results that seed lots which have either a reduced germinative capacity or are inherently slow to germinate, on average, will give rise to fewer embryogenic tissues. It therefore seems important to start with healthy vigorous seeds from any particular candidate, to maximize the probability of successfully inducing somatic embryogenesis from mature seeds.

Maturation and germination

In a set of experiments involving embryogenic tissue from three families, several medium combinations were tested for maturation of somatic embryos. In general, the best response was obtained on medium with ABA (3.7 μM) and 6% sucrose (Table 3). The effect of two gelling agents, agar versus gellan gum, was also tested. On media solidified with gellan gum, the embryos were more advanced in their development and the epidermis was smooth. In contrast, embryos matured on agar showed retarded growth and had a rough surface due to formation of callus. A similar phenomenon was observed in our laboratory with embryo maturation in hybrid larch (K. Klimaszewska, personal observation). After 4 weeks, most of the black spruce somatic embryos grown on gellan gum solidified medium with ABA and 6% sucrose had reached a cotyledonary stage (Fig. 1c). From the results in Table 3 it is clear that there is a synergistic effect of ABA and 6% sucrose on the maturation process of black spruce somatic embryos. Neither factor supplied separately had the same embryo maturation-promoting effect. The beneficial role of ABA together with 90 mM (3%) sucrose was observed in maturation experiments of white spruce somatic embryos (Hakman and von Arnold 1988), whereas Lu and Thorpe (1987) reported on the superiority of using 6% sucrose in the same species. However, the latter authors tested this level of sucrose with 2,4-D. We have observed this to be inferior to the ABA treatment for black spruce. In this work, it was also critical to remove the embryos from maturation medium after 4 weeks and transfer them onto germination medium, in order to achieve normal development of resulting plantlets.

The germination media contained 2 or 6% sucrose and were solidified with gellan gum or agar. After 4–6 weeks, all the somatic embryos cultured on media with 2% sucrose developed secondary needles and 60% of the shoots had simultaneously produced roots. In contrast, embryos germinated on medium with 6% sucrose showed stunted growth of shoots, slower root growth and had a high accumulation of antocyanins in the hypocotyl and radicle region. These plantlets never developed normally, even after subculturing onto medium with a lower concentration (2%) of sucrose.

There was a pronounced effect of the different gelling agents on germination. On medium solidified with gellan gum, we observed faster growth of plantlets as compared to agar gelled media. These early-germinated somatic embryos could be grown further in paper plugs saturated with liquid medium (Fig. 1E). After 4–6 weeks of growth, they could be transferred to a peat/vermiculite mixture in the greenhouse. In this soil-based medium, the plantlets developed normally (Fig. 1F). These plantlets underwent winter dormancy (1989/1990) and appear to be developing in the same way as their seed-derived counterparts.

Based on these experiments, tests on maturation of somatic embryos of all black spruce families were conducted on media solidified with gellan gum supplemented with 3.7 μM ABA and 6% sucrose. Germination of the embryos was carried out on gellan-gum-solidified medium with 2% sucrose. Randomly chosen lines of embryogenic tissue (a maximum of 2 per family), representing

Table 2. Correlation between germination characteristics and outgrowth induction from mature seeds of 20 families of black spruce

	50%	90%	ET[b]	C[b]
90%	0.89*	–	–	
ET	−0.05	−0.14	–	
C	−0.19	−0.31	0.66*	–

[a] 50%, 90% represent the number of days to achieve 50 or 90% germination in a seed lot
[b] ET and C refer to embryogenic tissue and callus
* Significant at 0.99

Table 3. Test for maturation of somatic embryos in three black spruce families

Hormones	Sucrose	
	1%	6%
None	none (0)	sporadic (1–20)
2,4-D (0.5 μM)	none (0)	sporadic (1–20)
ABA (3.7 μM)	sporadic (1–20)	abundant (>120)

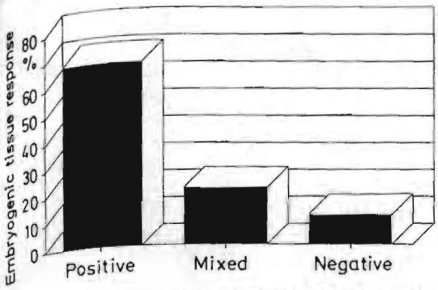

Fig. 4. Proportion of families giving positive, mixed, and negative response on the maturation medium. Positive: Families in which both lines produced high numbers (>100) of embryos per 100 mg fw of embryogenic tissue. Mixed: families in which the lines produced high and low numbers of embryos per 100 mg fw of embryogenic tissue. Negative: families in which both lines failed to produce high numbers of embryos per 100 mg fw of embryogenic tissue

the families that produced embryogenic tissue, were tested for their ability to develop mature somatic embryos.

Approximately 90% of the embryogenic lines tested were capable of producing abundant numbers (>100/100 mg fw) of phenotypically normal somatic embryos. Almost 70% of the lines from any particular family were consistent in forming large numbers of phenotypically normal embryos (Fig. 4). After 4 weeks on maturation media, these embryos could be transferred to a germination medium for further development, as previously described.

Black spruce, like other members of genus *Picea*, is capable of undergoing somatic embryogenesis from mature zygotic embryo explants. From this work, we have observed considerable genetic variation in the natural population for the ability of different genotypes to produce embryogenic tissue. This genetic variation has important implications when attempting to use this tissue culture technique in a tree improvement program. Most genotypes (85% in this study) can be successfully cultured through somatic embryogenesis. Therefore, if somatic embryogenesis were to be used as a means of propagating superior families, it is clear that the vast majority of parental genotypes would be represented. Thus, there would be no significant erosion of the genetic base in the production population. However, the proportion of explants that produced an embryogenic tissue from each genotype was low. This proportion could be increased in two ways. First, and most immediate, immature zygotic embryo explants, if available, have been shown to be more responsive in our laboratory. Secondly, due to the existence of additive genetic variation, the proportion of explants initiating an embryogenic tissue could be improved over time by selective breeding. Thus, possible concerns over loss of genetic variation within families can be readily addressed.

Advances in maturation protocols are overcoming many of the earlier problems in conversion of immature embryos (Jain et al. 1988; Becwar et al. 1989). The majority of embryogenic lines (90% in this study) were capable of forming abundant numbers of phenotypically normal somatic embryos on a single maturation media. From these results, it is evident that responsive genotypes can be identified early in the propagation phase for use in reforestation programs.

The germination medium was also important for speed of germination and subsequent normal plant development. On medium with 2% sucrose, the rate of germination was much faster than on 6% sucrose medium. In addition, if the osmotic potential of the medium was high (>6% sucrose in this work), it was not possible to recover normal plants, even when subcultured on to osmotically lower potential media.

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