Embryogenic Callus Induction in Fraser Fir

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Abstract. Mass propagation of Fraser fir [*Abies fraseri* (Pursh) Poir], a valuable Christmas tree species in the United States, is problematic because methods currently used are inadequate. According to our results with somatic embryogenesis, the culturing methods used with other *Abies* species are applicable to Fraser fir. Stage 1 somatic embryogenic callus, characterized by suspensor cells and embryo heads, was obtained at low frequency using Schenk and Hildebrandt medium supplemented with 5 mM glutamine, 0.05% casein hydrolysate, 0.01% myoinositol, 2% sucrose, 5 μ M benzyladenine, and 0.6% agar. The developmental stage of the embryo was important; embryogenic callus was obtained only with immature, precotyledonary embryos, not with fully formed embryos. Cold storage of cones containing immature embryos inhibited callus proliferation. Genotype was significant in that 35 of 44 families tested proliferated callus; however, only one embryo within one family continued proliferation to produce stage 1 embryogenic callus. Fully formed somatic embryos were not produced because the callus did not continue to proliferate. Although these experiments met with only limited success, they demonstrate the potential for somatic embryogenesis in Fraser fir and the general applicability of methods used with other *Abies* species.

Fraser fir [*Abies fraseri* (Pursh) Poir] is a major Christmas tree species in the United States (Blazich and Hinesley, 1995) and is propagated commercially by seed. However, seeds are frequently in limited supply, often show low germination percentages, and yield plants with large variability. Commercial availability of more uniform, higher-quality transplants could be achieved if protocols for vegetative propagation were available. The use of grafts or cuttings with *Abies* species is not ideal because of their initial plagiotropic growth habit (Dirr and Heuser, 1987; Hinesley and Blazich, 1980; Wise, 1985). In vitro propagation, through organogenesis or somatic embryogenesis, would provide an alternative. An efficient somatic embryogenesis system that uses zygotic embryos would allow rapid multiplication of genotypes derived from crossing parents selected for superior performance and favorable specific combining ability.

Somatic embryogenesis has been demonstrated using mature or immature zygotic embryos in four *Abies* species and in several hybrids (Norgaard and Krogstrup, 1995). Somatic embryo formation has been observed using mature zygotic embryos of Fraser fir (Guevin et al., 1992). The objective of this research was to investigate the effects of genotype and zygotic embryo maturity on somatic embryogenic callus induction in Fraser fir.

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Materials and Methods

Cones were collected in June and Aug. 1994 from 44 and 19 unrelated trees (families), respectively, from a Watauga County, N.C., seed orchard located at $\approx$ 1200 m of elevation. The 19 trees harvested in August were a subset of the 44 harvested in June, selected because they had the highest frequencies of callus induction in June. June explants, consisting of immature embryos within megagametophytes excised from cone scales midway along the cone axis, were 1 to 3 mm long. August explants consisted of fully formed embryos within megagametophytic tissue. Cotyledonary development was nearly complete in all families by the August excision date. Cones were surface disinfested by washing them in 95% ethanol for 5 min followed by a 10-min soak in 6% calcium hypochlorite with dishwashing liquid as a surfactant.

A subset of the June-harvested cones was stored in plastic bags placed in a styrofoam cooler at 4 °C until August when explants were again taken to test the effect of cold storage on

callus induction. Cold storage is known to enhance embryogenic callus induction in *Picea glauca* (Moench) Voss (Hakman and Fowke, 1987).

Explants were cultured in 100 $\times$ 15-mm petri dishes containing 25 mL Schenk and Hildebrandt medium (1972) supplemented with five mM glutamine, 0.05% casein hydrolysate, 0.01% myoinositol, 2% sucrose, 5 μ M benzyladenine, and solidified with 0.6% washed phytagar (Gibco, Grand Island, N.Y.). The medium pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Randomized complete-block designs were used to evaluate June, August, and cold-stored June explants. Position within a laboratory cabinet served as the blocking factor. For each family, three replications of two plates each, and five explants per plate, were prepared. Thus, the total number of explants from the 44 families harvested and plated in June was 1320. The total number of explants from the 19 families plated in August was 1140: 570 from August harvested cones, and 570 from cold-stored, June-harvested cones. All cultures were incubated at $\approx$ 24 °C in the dark. Transfer to fresh medium was done every 4 weeks.

Explants were scored for presence or absence of callus after 4 weeks in culture. Data were subjected to SAS analyses of variance procedures (SAS release 6.11, SAS Institute, Cary, N.C.). Duncan's multiple range test was used to compare family means for callus induction frequencies. Tissue samples taken from proliferating callus were stained with acetocarmine per Gupta and Durzan (1987).

Results and Discussion

For June explants, callus induction frequency varied among families (Table 1). The callus produced by all families was friable, mucilaginous, and colorless. The three families with the highest callus induction rate ($\geq$ 62%) produced long, suspensor-like cells typical of conifer somatic embryogenic cultures (Norgaard and Krogstrup, 1995) after 5 weeks. These suspensor-like cells were evident in only one other family, A9, which had a callus induction frequency of 33%.

Although four families formed suspensor-like cells following the June excision date, only A9 produced somatic embryogenic callus during the second subculture. This callus visibly matched that previously designated as stage 1 embryonic cell masses in *Picea abies*

Table 1. Callus induction from immature embryo explants of 44 Fraser fir families harvested and plated in June, grouped by Duncan's critical range designation.

Family designation ^a	Callus induction frequency (%) ^b
F52	70 a
D33, D34	62 ab
H77, J97, J98	47 bc
A9, F53, G70, I83	41 cd
C30	23 e
D39, E44, E46, G69, J93, F56, H78, B17, H73, I90, J96	17 d-f
D37, J100, B15, B20, E47, G68, C28, D35, E45, E48, G63, G65, H72	7 ef
C21, C29, E43, F51, H71, H79, I85, I87, J94	0 f

^aFamily designations are from seed orchard position; similar letters or numbers do not represent genetic relatedness.

^bMean separation by Duncan's critical range at $P \leq 0.05$.

(L.) Karst. (von Arnold and Hakman, 1988) (Fig. 1A). The callus was colorless, glossy, mucilaginous, and consisted of bundles of long, suspensor-like cells terminating in compact masses of small cells (Fig. 1B and C). The

small compact cells stained readily with acetocarmine, a nucleic acid-specific staining method used to identify embryogenic cells (Gupta and Durzan, 1987). Only one of 30 explants within family A9 proliferated this

callus. The callus did not differentiate further and ceased proliferation during the second subculture.

Embryo maturity affected the frequency of somatic embryogenic callus induction. Mean callus induction frequency of August-harvested embryo explants was only 2%, even though the 19 families used were those with the highest callus induction frequencies in June. The callus produced was pale-green, dry, friable, and resembled organogenic rather than embryogenic callus. None of this callus proliferated readily or differentiated into embryogenic callus.

Cold storage also inhibited somatic embryogenic callus induction. Callus induction frequency from cold-stored, June-harvested explants was 10%, and none of the callus had suspensor-like cells or embryonic masses. Intense acetocarmine staining was absent in callus proliferated from August and June, cold-stored explants.

This is the first report of embryogenic callus induction from immature embryo explants of Fraser fir. Schenk and Hildebrandt medium supplemented with benzyladenine, a treatment that has been used successfully for somatic embryogenic callus induction in *Abies alba* Mull (Schuller et al., 1989), also worked well with Fraser fir. The treatment we used is similar to one of those found to induce somatic embryos from hypocotyls and mesocotyls of mature Fraser fir zygotic embryos (Guevin et al., 1992). However, it was successful only with immature material and not later-stage zygotic embryos. Our frequencies of somatic embryogenic callus induction were low, possibly due to confounding effects of explant genotype and developmental stage. Further work needs to be done to separate the effects of genotype and embryo developmental stage on callus induction before a true estimate of somatic embryogenic potential of Fraser fir can be determined. Once somatic embryogenic cultures are obtained more reliably, with a greater proportion of initial explants, the challenge will be to maintain actively growing cultures through the second subculture and beyond.

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Fig. 1. Embryogenic callus induced from a June-harvested zygotic embryo of Fraser fir. (A) Embryogenic callus mass proliferation after 12 weeks in culture. Bar = 1.7 mm. (B) Stage 1 somatic embryo with embryo head (eh) and long suspensor cells (s). Bar = 200 μ m. (C) Embryogenic cell mass dissociated in liquid with visible embryo head (eh) and suspensor cells (s). Bar = 280 μ m.