

Harvest Time and Nitrogen Source Influence In Vitro Growth of Apical Buds from Fraser Fir Seedlings

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Abstract. Information was obtained concerning appropriate bud harvest time and nitrogen source to be used in the tissue culture of Fraser fir [*Abies fraseri* (Pursh) Poir] apical buds from 2-year-old seedlings. April was the preferred time to harvest buds for culture, as summer buds had a high contamination frequency, and fall and winter buds did not develop well. Shoot elongation of buds collected in April (1.6 cm) was more than twice that of buds collected in February (0.7 cm) after 100 days in culture; during the same period, shoot fresh mass increased 5-fold (0.21 g in April, 0.04 g in February). Inclusion of a nitrate source reduced the frequency of bud browning, and glutamine was superior to ammonium as a source of reduced nitrogen. Litvay's basal medium containing 10 mM glutamine and 10 mM nitrate was the best nitrogen source combination tested when considering bud browning frequency and shoot fresh mass and length after 100 days in culture.

Fraser fir is a species indigenous to the southern Appalachian mountains, including southwestern Virginia, western North Carolina, and eastern Tennessee, found at elevations between 1200 and 2073 m (Liu, 1971). The species is important as a keystone species in the high elevation, spruce/fir ecosystem of the southern Appalachian mountains. Fraser fir is an important Christmas tree species in the United States; its value to North Carolina growers alone topped \$65 million in 1991 (W. Huxster, personal communication).

The spruce/fir ecosystem has been in steady decline since European settlement in the southeastern United States and now occupies <3% of its known, early settlement extent (McGraw, 1980; Dull et al., 1988).

The ability to vegetatively propagate Fraser fir would be of great value: 1) to conserve and multiply genotypes able to survive in the declining spruce/fir ecosystem of the southern Appalachians, 2) to circumvent problems of large yearly variation in seed availability (Franklin, 1974), poor seed germination rates (Henry and Blazich, 1988), and the long time needed to produce seedlings of sufficient size for field planting, and 3) to propagate individuals that exhibit desirable Christmas tree traits.

Rooted cuttings, somatic embryogenesis, organogenesis, and micropropagation have been used for asexual propagation of various conifer species. All but micropropagation have

been explored previously in Fraser fir. Rooting of cuttings is the lowest-cost and least technically demanding among potential vegetative propagation methods. Yearly pruning makes large numbers of lateral branch cuttings available. While 70% of these lateral branch cuttings produce roots (Hinesley and Blazich, 1984; Wise, 1985), they exhibit unacceptable plagiotropic growth. Saravitz (1990) showed that adventitious buds can be induced from hypocotyl explants through tissue culture. The low frequency of regeneration, about three shoots/explant, makes the method inefficient for mass propagation of elite Fraser fir selections.

Alternatively, micropropagation from dormant buds could be an attractive method for propagation of Fraser fir. Dormant buds can be harvested easily each year with the quality of the source tree known before bud harvest. Isolated dormant buds have been induced to elongate to produce plantlets directly and to form adventitious buds in several conifer species, including Norway spruce (*Picea abies* (L.) Karst.) (von Arnold and Eriksson, 1979), Sitka spruce (*Picea sitchensis* Bong.) (Selby and Harvey, 1985), and Balsam fir [*Abies balsamea* (L.) Mill.] (Bonga, 1981). The purpose of our study was to explore tissue culture responses of Fraser fir terminal buds to identify the best time of bud harvest and the nitrogen source and concentrations that would promote in vitro bud elongation.

Materials and Methods

Plant material. Two-year-old Fraser fir seedlings grown in nursery beds were collected at Crossnore, N.C. (elevation = 1100 m). Bare-root seedlings were wrapped in moist paper towels and placed in ice chests for transport to the laboratory. Seedlings were stored at

4 °C in the laboratory until terminal bud dissection.

Terminal 1.5-cm stem segments were excised from seedlings, needles were removed, and stem segments were soaked in 2% calcium hypochlorite for 30 min. Stem segments were rinsed three times with sterile distilled water. Terminal buds were then excised. Dissection was done on sterile filter paper moistened with 1% hydrogen peroxide. Buds were placed upright on agar-solidified medium.

Culture conditions. Litvay's basal medium (LM) (Litvay et al., 1981) was used because preliminary experiments indicated that it supported growth of Fraser fir terminal buds. All medium components, including 3% sucrose and 0.9% washed phytagar (Gibco, Grand Island, N.Y.), were combined before autoclaving, with the exception of glutamine, which was filter-sterilized and added to cooled medium. The pH of all media was 5.8 before autoclaving at 121 °C for 20 min.

Petri dishes (15 × 100 mm) containing 25 ml medium were used during the first 8 weeks of culture, and GA4 Magenta boxes (Magenta Co., Chicago) were used after 8 weeks to accommodate elongating buds. Transfers to fresh medium were made every 4 weeks. Culture vessels were placed in a growth chamber at constant 23 °C under a 16-h photoperiod provided by Gro-Lux wide spectrum fluorescent bulbs providing 40 μmol·m⁻²·s⁻¹ photosynthetic photon flux density.

Bud harvest time. A study was conducted to test the influence of bud harvest time throughout the year on in vitro performance of terminal vegetative buds from 2- to 3-year-old Fraser fir seedlings. Buds from seedlings that had been sown in June 1989 were collected during Apr., May, Sept., Oct., and Dec. 1991 and Feb. and Mar. 1992. The culture medium was LM with potassium nitrate and glutamine each provided at 10 mM.

Nitrogen source. Two trials were conducted to determine the influence of nitrogen source and concentration on in vitro performance of terminal vegetative buds from 2-year-old Fraser fir seedlings harvested in Sept. 1991 (sown in June 1989). The first trial examined the effects of reduced nitrogen type, either glutamine or ammonium, on in vitro responses. Ten nitrogen combinations were used to provide glutamine or ammonium at 0, 6, 10, or 20 mM (Table 1). Ion concentrations varied somewhat among media (Table 1). Nitrate was used as the common oxidized form of nitrogen.

A second trial examined the influence of nitrogen concentration and ratio of reduced to oxidized forms by excising buds from 2-year-old seedlings in Apr. 1992 (sown in June 1990) and culturing them on LM with glutamine and/or nitrate. Each nitrogen source was provided at 0, 5, 10, or 20 mM in a complete factorial. Except sulfate, all ion concentrations were consistent across treatments. Sulfate concentrations were 13.3, 10.8, 8.3, and 3.3 mM for media containing 0, 5, 10, or 20 mM nitrate, respectively.

Experiment design. A completely randomized design was used for all experiments. Each treatment in each experiment was represented

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by 12 buds, placed four to a culture vessel. Each experiment was run 3 times. Shoot fresh mass and length were measured 100 days after the initiation of all experiments. Because excised buds were very small (≈ 2 mm long) and difficult to handle, reported shoot mass is total fresh mass after 100 days rather than fresh mass after 100 days less initial bud fresh mass. Bud/shoot browning (yes or no) was noted in the glutamine : nitrate trial. SAS (SAS Institute, Cary, N.C.) was used to perform analyses of variance (PROC GLM) and to determine Duncan's critical ranges among means at $P \leq 0.05$. Predicted optimal values for glutamine and nitrate concentrations were determined from the full factorial glutamine : nitrate experiment by regression analysis of the response surface (PROC RSREG).

Results and Discussion

Bud harvest time. Month of bud harvest was found to influence in vitro bud response significantly. The culture of buds harvested in May and August was not possible because of heavy microbial contamination and the few buds which escaped contamination (from the August harvest only) turned brown and died within six weeks of culture establishment.

Shoot fresh mass after 100 days in culture differed ($P < 0.01$) among the six harvest times for which buds were successfully cultured (Fig. 1). Shoot length was influenced by month of bud harvest ($P < 0.01$) and followed the same trend as shoot fresh mass (Fig. 1). The lowest values for shoot fresh mass and shoot length occurred in February, the month during which buds would be expected to be in deepest dormancy. Maximum in vitro growth was obtained when buds were harvested in April, the period corresponding to budbreak on trees in the field. Results were consistent among the 3 replications. The observed differences among harvest dates were similar to data for in vitro shoot elongation in Balsam fir (Bonga, 1981), Sitka spruce (Selby and Harvey, 1985), and Douglas fir [*Pseudotsuga menziesii* (Mirb.) (Boulay, 1979)].

Nitrogen source. Shoot fresh mass and length varied among media ($P < 0.01$) (Fig. 2). Media with nitrate as the only source of nitrogen (i.e., media 1 and 2) were inferior to those containing some form of reduced nitrogen.

Overall, glutamine was a better nitrogen source than ammonium nitrate or nitrate alone, as judged by shoot fresh mass and length. Two other possible explanations for the observed differences among the 10 media were considered and dismissed: 1) varying potassium, calcium, and chloride ion concentrations in the various media, and 2) differing total nitrogen concentrations among the media. In neither case were the observed trends in bud response among the media consistent with these hypotheses. This experiment suggested that the ratio of reduced to oxidized nitrogen may be involved in in vitro Fraser fir bud performance, because shoot fresh mass and length increased from treatment 3 to 6 as the ratios of glutamine to nitrate increased from 3 : 7 to 20 : 1.

The second nitrogen study showed that the ratio of reduced : oxidized nitrogen sources is important in in vitro Fraser fir bud development. Shoot fresh mass and bud elongation differed ($P < 0.01$) among the 16 glutamine : nitrate combinations (Table 2). The regression analysis predicted optimal glutamine and nitrate concentrations to be 10.6 mM and 15.0 mM, respectively, for bud elongation and 11.9 mM and 16.3 mM, respectively, for fresh mass.

Large differences among treatments were noted in the frequency of bud/shoot browning (Table 2). Most noteworthy was the fact that when either nitrate or glutamine was absent from the medium, bud/shoot browning was more likely than if they were included (Table 2). The regression analysis predicted optimal glutamine and nitrate concentrations to be

13.0 mM and 11.9 mM, respectively, to minimize bud/shoot browning.

Buds taken in Apr. 1992 for the glutamine : nitrate study (10 mM glutamine:10 mM nitrate, Table 2) grew to a much greater extent than the Apr. 1991 buds placed onto the same medium (medium 5, Fig. 2). Such variation in in vitro performance may reflect differing physiological states in the explants resulting from unequal environmental conditions experienced by field-grown plants.

Nitrogen source was demonstrated to be an important factor in the development of Fraser fir buds in vitro. While this work showed glutamine to be superior to ammonium as a reduced nitrogen source, studies in other conifer tissue culture systems have met with mixed results. Cells in suspension cultures of white

Table 1. Ten media providing varying concentrations (0, 6, 10, 20 mM) of NH_4^+ and glutamine as reduced nitrogen sources, and the concomitant change in ion concentrations in the media, for in vitro culture of apical buds from 2-year-old Fraser fir seedlings. Concentrations are expressed in mM.

Constituent	Concentration (mM)									
	Medium ²									
	1	2	3	4	5	6	7	8	9	10
	<i>Compound</i>									
KNO ₃	10.0	20.0	7.0	0.0	10.0	1.0	7.0	0.0	10.0	1.0
KCl	0.0	0.0	0.0	10.0	0.0	9.0	0.0	10.0	0.0	9.0
Ca(NO ₃) ₂	5.0	0.0	3.5	5.0	0.0	0.0	3.5	5.0	0.0	0.0
CaCl ₂ ·2H ₂ O	0.3	3.3	0.3	0.3	3.3	3.3	0.3	0.3	3.3	3.3
NH ₄ NO ₃	0.0	0.0	0.0	0.0	0.0	0.0	6.0	10.0	10.0	20.0
Glutamine	0.0	0.0	6.0	10.0	10.0	20.0	0.0	0.0	0.0	0.0
	<i>Ion</i>									
K ⁺	10.0	20.0	7.0	10.0	10.0	10.0	7.0	10.0	10.0	10.0
Ca ²⁺	5.3	3.3	3.8	5.3	3.3	3.3	3.8	5.3	3.3	3.3
Cl ⁻	0.6	6.6	0.6	10.6	6.6	15.6	0.6	10.6	6.6	15.6
NO ₃ ⁻	20.0	20.0	14.0	10.0	10.0	1.0	20.0	20.0	20.0	21.0
NH ₄ ⁺	0.0	0.0	0.0	0.0	0.0	0.0	6.0	10.0	10.0	20.0
Glutamine	0.0	0.0	6.0	10.0	10.0	20.0	0.0	0.0	0.0	0.0

²All medium constituents are as per Litvay's basal medium (Litvay et al., 1981) with the exception of the noted modifications.

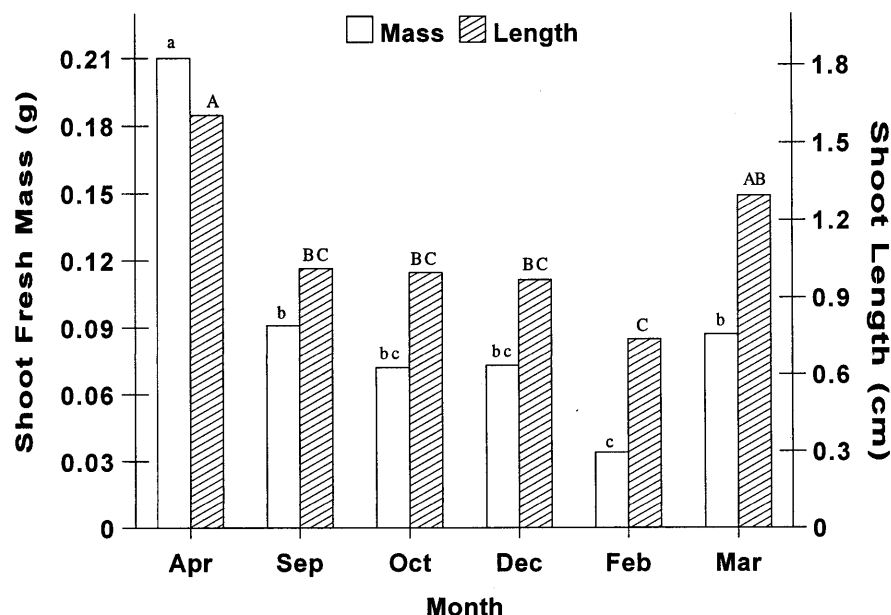


Fig. 1. Fraser fir shoot total fresh mass and length 100 days after placing excised apical buds from 2-year-old seedlings into culture, as influenced by month of field collection. Mean separation within each factor by Duncan's multiple range test at $P = 0.05$ (lower case for shoot mass and upper case for shoot length).

spruce (*Picea glauca* Moench) (Steinhart et al., 1961; White and Gilbey, 1966), jack pine (*Pinus banksiana* Lamb.) (Durzan and Chalupa, 1976) and Douglas fir (Kirby, 1982) grew better when glutamine was added to the culturing medium. Adventitious shoot formation from loblolly pine (*Pinus taeda* L.) embryos and cotyledons was enhanced when nitrogen was supplied as glutamine (Amerson et al., 1985). However, glutamine's effect on somatic embryogenesis was found to be quite variable. Glutamine inhibited formation of embryogenic cultures in *Picea abies* (von Arnold, 1987), enhanced somatic embryogenesis in *Abies nordmanniana* Stev. (Norgaard and Krogstrup, 1991), and had no effect on larch (*Larix occidentalis* Nutt.) somatic embryogenesis (Thompson and von Aderkas, 1992).

In this work, several glutamine : nitrate concentration combinations yielded quite similar responses, but it was clear that exclusion of nitrate or glutamine had the pronounced effect of dramatically increased bud/shoot browning frequency. Considering all of the observed variables together, medium containing 10 mM glutamine and 10 mM nitrate was best of the combinations tested for promotion of relatively high shoot fresh mass and bud elongation with relatively low bud/shoot browning frequency. The regression analysis showed that optimal concentrations, when considering all three factors, are ≈ 12 mM glutamine and ≈ 14 mM nitrate.

Buds harvested at different times of the year respond differently in culture. Presumably these differences in response reflect differences in initial physiological status. Durzan (1968) found that high amounts of glutamine (up to 60% of total soluble nitrogen) accumulate in white spruce dormant vegetative buds before spring growth. This association between high bud glutamine content and onset of spring shoot outgrowth suggests that supplying medium nitrogen as glutamine may partially mimic a physiological state that is conducive to bud growth. While glutamine may be necessary for shoot growth from buds placed into culture, glutamine alone is not sufficient, as evidenced by the fact that buds in deepest dormancy (February) did not exhibit much growth even though glutamine was provided in the medium.

Conclusions

The work presented here provides insights for the establishment of Fraser fir terminal bud tissue cultures. Methods were defined for achieving the goals of a low contamination rate, and the determination of appropriate harvest time and nitrogen forms and concentrations to establish buds in culture. April buds can be successfully disinfested, and the buds remain healthy and elongate well. Glutamine is preferred to ammonium as a reduced nitrogen source. When glutamine and nitrate are each included in the medium at 10 mM, browning can be limited to 28% of the cultures, and shoot fresh mass and elongation are among the highest observed in all our studies.

While this work was limited to 2- to 3-year-old seedlings, these findings might be extended to buds from mature trees selected as valuable phenotypes. One limitation may be the plagiotropic growth habit that is often observed when cuttings are taken from lateral branches of adult field trees or hedged stock plants. Alternatively, this information may prove useful in the development of a micropropagation system to be used with buds from seedlings resulting from proven superior crosses that produce limited quantities of viable seed.

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Table 2. Shoot characteristics 100 days after placing apical buds excised (Apr. 1992) from 2-year-old Fraser fir seedlings into culture, as influenced by varying concentrations of glutamine and nitrate.

Nitrogen source (mM)		Shoot characteristic		
Glutamine	Nitrate	Fresh mass (g)	Length (cm)	Browning frequency (%)
0	0	0.07	3.18	100
	5	0.43	6.93	91
	10	0.65	8.60	72
	20	0.49	8.31	100
5	0	0.09	3.50	44
	5	0.51	9.65	24
	10	0.47	8.16	28
	20	0.79	10.44	40
10	0	0.15	4.40	58
	5	0.42	8.88	33
	10	0.68	9.39	28
	20	0.53	8.64	29
20	0	0.22	5.12	57
	5	0.33	6.96	46
	10	0.60	9.08	47
	20	0.69	9.12	42

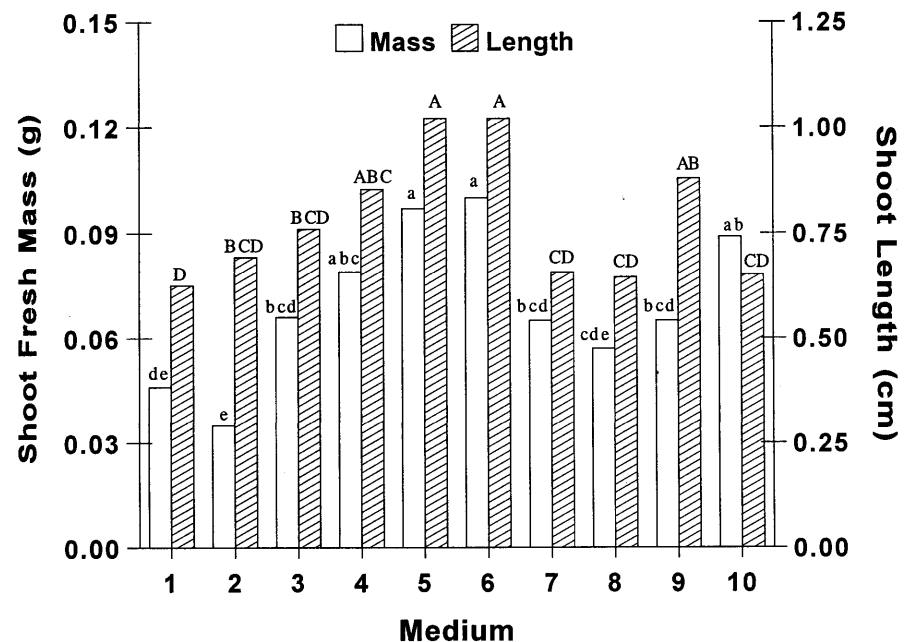


Fig. 2. Fraser fir shoot total fresh mass and length 100 days after placing excised apical buds from 2-year-old seedlings (Sept. 1991) into culture as influenced by media containing several nitrogen sources and concentrations. Differences among media are delineated in Table 1. Media 1 and 2 contained nitrate only, 3 to 6 glutamine and nitrate, 7 to 10 ammonium and nitrate. Mean separation within each factor by Duncan's multiple range test at $P = 0.05$ (lowercase for shoot mass and uppercase for shoot length).

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