SHOOT PRODUCTION AND ELONGATION ON EXPLANTS FROM VEGETATIVE BUDS EXCISED FROM 17- TO 20-YEAR-OLD PSEUDOTSUGA MENZIESII

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ABSTRACT

Improvement in shoot primordia production was achieved on explants from vegetative buds excised from a 17- to 20-year-old **Pseudotsuga menziesii** (Mirb.) Franco (Douglas fir) during the dormant season. Seventy-five percent of inoculated explants produced an average of 8.3 shoot primordia each when grown on a modified Boulay medium containing benzyladenine (1 mg/l) and ammonium nitrate (800 mg/l). The optimum duration of exposure to ammonium nitrate was 6 weeks. Approximately 66% of these shoot primordia grew to a length of 1.0 to 1.5 cm, and were suitable for subsequent excision.

A comparison of shoot primordia initiation amongst nine randomly selected trees showed that distinctly superior and inferior trees occurred, with a range from 100% to 0% of explants showing the shoot primordium response. The average number of shoot primordia per responsive explant showed similar differences with a range from 8.8 to 0. Five out of the nine trees responded favourably with primary multiplication factors from 8.8 to 4.1 primordia per inoculated explant. The origins of shoot primordia and their location on the bud explant were found to be dependent upon the donor tree.

Keywords: micropropagation; vegetative bud explant; tree-to-tree variation; primary multiplication; ammonium nitrate; benzyladenine; **Pseudotsuga menziesii**.

INTRODUCTION

Douglas fir is an economically important lumber species with a wide distribution in the Pacific Northwest (Allen & Owens 1972). The constant demands placed upon this species in Canada are resulting in the depletion of phenotypically superior individuals. Vegetative multiplication, by plant tissue culture techniques, may have a valuable role to play in the provision of phenotypically or genotypically elite clones for seed or breeding orchard establishment and for reforestation.

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A variety of coniferous forest tree species have been subjected to tissue culture techniques, and the explants that have proved most responsive have been from germinated seedlings or from embryos. The techniques are exemplified by those reported for *Pinus radiata* D. Don (Aitken-Christie & Thorpe 1984), *P. taeda* L. (Amerson *et al.* 1985), Douglas fir (Cheng 1977), and *Sequoia sempervirens* (D. Don) Endl. (Boulay 1979a) – for a more detailed discussion *see* Dunstan & Thorpe (1986).

There have been few reports of vegetative propagation of explants of trees older than 1 or 2 years (Bonga 1982; David 1982; Thorpe & Biondi 1984). In the absence of demonstrably elite seed, it remains desirable to multiply older trees which have reached an age when phenotypically elite traits can be determined. For Douglas fir in British Columbia the minimum age is judged to be 12 to 15 years (K. Illingworth, British Columbia Ministry of Forests, pers. comm.).

Several authors have reported the successful production of shoot primordia on vegetative buds of older trees, e.g., from 15- to 20-year *Abies balsamea* (L.) Mill. (Bonga 1977), 10-year *Picea abies* (L.) Karst. (Jansson & Bornman 1983), 26-year *Picea abies* (von Arnold 1984), and up to 50-year-old *Pinus radiata* (K. Horgan, New Zealand Forest Research Institute, pers. comm.). In many experiments only a low frequency production of shoot primordia was recorded (e.g., up to 50% of explants – von Arnold 1984) and usually no details were reported on the continued development of shoots, or their remultiplication. With particular reference to Douglas fir, Boulay (1979b) reported slow growth and some adventitious bud production on entire embryonal shoot axes collected between September and April from trees older than 2 years. Subsequently Thompson & Zaerr (1981) obtained adventitious shoot production on 40–80% of entire embryonal shoot axes collected from 20- to 30-year-old trees during the spring, and in a more recent report Gupta & Durzan (1985) indicated that it was possible to obtain 100 new shoots from 7–10 *in vitro*-flushed shoots in 1 year for 20- to 60-year-old Douglas fir. We believe that this paper will be the first to present data about the number of shoot primordia and shoots than can be produced on bud explants from older Douglas fir trees.

Variations in donor-to-donor explant response during *in vitro* culture have been reported in the past, e.g., for Douglas fir cotyledons (Cheng 1977), and for buds from 10-year-old (Jansson & Bornman 1983) or 26-year-old *Picea abies* (von Arnold 1984). Such variation has been correlated to donor genotype (von Arnold 1984) and donor environment (Durzan 1982). Variations in response *in vitro* may be reduced by cytokinin applications to the donor (David *et al.* 1982; Inglis 1984). To our knowledge, similar donor-to-donor variations for buds of older trees of Douglas fir have not been previously reported.

This paper provides data from experiments with 17- to 20-year-old Douglas fir. Experiments 1 and 2 were designed to determine the quantitative and qualitative effects of exogenous supplements of ammonium nitrate and benzyladenine (BA) upon shoot primordium production on the basal section of the embryonal shoot axis (bud-base explant) and upon shoot primordium elongation. This was based on the previous use of the compounds *in vitro* (Thompson & Zaerr 1981) and on evidence that endogenous levels of total nitrogen and of cytokinin in collected plant material increased prior to

bud flush (Proebsting & Chaplin 1983; Ross *et al.* 1983). Experiment 3 was designed to provide data on any tree-to-tree differences as shown by variations in explant response during culture *in vitro*.

MATERIALS AND METHODS

Plant material

All collections were made in the dormant season, from randomly selected 17- to 20-year-old Douglas fir trees growing in a natural stand in the Penticton Forest District of southern interior British Columbia. Portions of first- and second-order branches, bearing dormant vegetative buds, were harvested from the lower two-thirds of each tree. In Experiments 1 and 2 buds were collected from one tree (No. 6) on 3 February and 6 March 1985. In Experiment 3 buds were collected on 20 November and 3 December 1984, and 4 January and 3 February 1985 from nine trees (No. 4, 6, 8, 10, 11, 12, 14, 15, and 16) growing at the same elevation within a 15-km radius. The data presented for each experiment are pooled from each collection date because no significant differences in response were detected amongst the collections.

Sterilisation

Buds, together with 3 mm of subtending stem tissue were excised from the branches and sterilised for 15 min in a 1.2% chlorine solution (prepared from 5.25% sodium hypochlorite). Post-sterilisation, buds were rinsed three times with sterile double-distilled water.

Explant preparation

Only buds with an axis of 4 mm or longer were used. Their bud scales were removed by a circumferential cut at the widest part of the bud. The terminal 0.25 to 0.5 mm of exposed embryonal shoot axis (comprising the meristem dome and several needle primordia) was excised and discarded. The final explant was the remaining bud base (bud-base explant), which was approximately 50% green embryonal shoot axis and 50% subtending crown and stem tissue.

Nutrient media

The nutrient formulation B (described by Boulay 1979b) was chosen as the preferred medium. Boulay B is closely related to MS (Murashige-Skoog), with the exception that ammonium nitrate is omitted, and in our experiments aluminium chloride and activated charcoal were also excluded (modified B).

In Experiment 1, growth on modified B was compared with three derivative media: B-1, modified B with 1 mg BA/*l*; B-2, modified B with 1 mg BA/*l* and 400 mg NH_4NO_3/*l*; and B-3, modified B with 1 mg BA/*l* and 800 mg NH_4NO_3/*l* (Table 1). Ten explants were inoculated on to each medium on each of two collections.

In Experiment 2, ammonium nitrate was removed, or added, at different times in culture to determine its influence upon shoot primordia production and upon shoot elongation (up to 1 cm long). Using the same media (B-1, B-2, and B-3), 120 explants on each of two collections were distributed as follows: 40 explants were inoculated on

TABLE 1—B-3 medium. A modified Boulay B medium with incorporation of ammonium nitrate and benzyladenine, and exclusion of aluminium chloride and charcoal (agar 5.0 g; pH 5.5)

Compound	Weight/litre mg	Molarity
$CaCl_2.2H_2O$	440.0	2.99 mM
NH_4NO_3	800.0	10.00 mM
KNO_3	1 900.0	18.79 mM
$MgSO_4.7H_2O$	370.0	1.50 mM
KH_2PO_4	170.0	1.25 mM
KI	0.83	5.00 μM
H_3BO_3	6.2	0.10 mM
$Na_2MoO_42H_2O$	0.025	0.103 μM
$MnSO_4.4H_2O$	22.3	0.076 mM
$CuSO_4.5H_2O$	0.025	0.064 μM
$ZnSO_4.7H_2O$	8.6	29.89 μM
$FeSO_4.7H_2O$	27.85	0.10 mM
Na_2EDTA	37.25	0.10 mM
Inositol	100.0	1.8 mM
Thiamine HCl	10.0	0.03 mM
Sucrose	30 000.0	0.088 M
Benzyladenine	1.0	4.44 μM

B-1 at Day 1, 10 being transferred to B-2 on Days 21, 42, and 63; similarly, 40 explants were inoculated on to B-2 and 40 on to B-3, with 10 from each being transferred to B-1 on Days 21, 42, and 63. In Experiment 3, only B-2 was used, and 20 explants were inoculated on each of four collections.

For each experiment sucrose was present at 30 g/*l* and agar at 5 g/*l*, and the pH was adjusted to 5.5 (using sodium hydroxide). All media were dispensed into 150 × 25-mm test tubes at 20 ml per tube, capped with Kaputs, and autoclaved at 138 kPa for 15 min. In later stages when individual shoots were excised, 75 ml medium per jar was dispensed into 375-ml-capacity glass universal jars which were loosely capped with screw metal lids. Explants were transferred to their respective fresh medium every 21 days, and benzyladenine was always removed at 42 days – therefore, after 42 days medium B-1 and modified B were identical because of the removal of benzyladenine from B-1.

Growth conditions

Cultures were incubated at 25°C ± 2°C with an incident photosynthetically active radiation (Krizek 1982) of 110 $\mu E/m^2/s$ from high output, cool white, fluorescent lights during a 16-h photoperiod.

RESULTS AND DISCUSSION

Nutrient medium

The nutrient formulation B was chosen as the preferred medium as a result of preliminary comparisons between MS (Murashige & Skoog 1962) at one-third, two-thirds, and full strength; Boulay B, and E (Boulay 1979b); LP (Quoirin & Lepoivre

1977); WPM (Lloyd & McCown 1980); SH (Schenk & Hildebrandt 1972) and Cheng (1977). Initially only LP and WPM could support the development of shoot primordia on Douglas fir cultures, though these media subsequently proved inferior to B-2 in terms of quantity of primordia produced and ability to sustain growth (Dunstan *et al.* 1987). The other media did not promote similar shoot primordia formation.

Response and choice of explant

Explant size had increased to 4–5 mm before shoot primordia were observed. Shoot primordia were produced on explants on all the benzyladenine-containing media tested (Table 2) and were evident from the sixth week onward. The primordia developed into small shoots asynchronously from the ninth week.

The shoot apical meristem *in vivo* is an organ which perpetuates itself and the embryonal shoot axis of the coming year (*see* Allen & Owens 1972). The shoot apical meristem may not be necessary for the *in vitro* production of shoot primordia because lateral buds arise on the current year's embryonal shoot axis at about the time of flushing, in the year after that axis was initiated by the apical meristem. Original experimentation compared three explant types – entire axes, bud-bases (axis with shoot apex removed as described), and excised apices. During the comparisons the bud-base explants had a higher potential for survival and shoot primordium production, and were selected for further study. While excised apices could show low levels of shoot primordia induction, the shoot apices of entire bud axes were found to undergo only a limited extension growth on cytokinin-containing media. Selby & Harvey (1985) also reported that the meristems of buds from *Picea sitchensis* (Bong.) Carr. trees usually remained undeveloped.

TABLE 2—Responses of bud-base explants grown on four nutrient media varying in their content of benzyladenine and ammonium nitrate (20 explants were inoculated per treatment). Experiment 1

Parameter	Medium			
	Modified B	B-1	B-2	B-3
A. Survival (6 wk)	17/20	16/20	15/20	16/20
B. Explants with shoot primordia* (12 wk)	none	8/16 b	12/15 ab	15/16 a
C. Shoot primordia per explant, mean (± S.E.)† (12 wk)	none	6±0.4 b	8.2±0.4 a	8.3±0.3 a

Figures with the same letter are not significantly different at the 1% level.

* Chi-square test for independence.

† Fisher's least significant difference.

Response of Tissues to Culture

Requirement for benzyladenine and ammonium nitrate

Results from Experiment 1 (Table 2) indicate that it was possible to produce 75–85% contaminant-free explants of Tree 6. The number of these which had produced shoot primordia by Week 20 was found to be influenced by medium, being best on Media B-2 (80%) and B-3 (94%). The average numbers of primordia per responsive explant were also greatest on these media (8.2 and 8.3 per explant, respectively) and were higher than the numbers produced on Medium B-1. In Experiment 1, using explants excised from Tree 6, shoot primordia were found to occur only in the axils of pre-formed needles at the base of the explant (Fig. 1a), presumably from pre-formed meristems in similar relative positions to those reported by Gupta & Durzan (1985).

Exposure to ammonium nitrate

The total numbers of shoot primordia and of 1.0-cm shoots produced in Experiment 2 (Table 3) reveal the influence of medium and duration of treatment. The most successful results were obtained when explants were grown for 42 days on B-3 (with ammonium nitrate) before transfer to the ammonium nitrate-free B-1 (equivalent to modified B). An average of 8.6 shoot primordia were produced on 100% of inoculated explants, and 66% of these primordia elongated to small shoots. Similarly, cultures grown on B-2 for 42 days gave a relatively high frequency of shoot primordium initiation (an average of 8.4 on 80% of inoculated explants) and subsequent shoot elongation (65.2%). By contrast, cultures grown on the ammonium nitrate-free B-1, with or without subsequent transfer to B-2 with ammonium nitrate, were significantly inferior in shoot primordium production and in the subsequent elongation of these into shoots. This indicated that ammonium nitrate was most necessary during the initial culture period with an optimum at 42 days for Media B-2 and B-3.

Studies on total nitrogen and cytokinins from tissues of temperate woody species *in vivo* (Proebsting & Chaplin 1983; Ross *et al.* 1983) assisted in the development of Experiments 1 and 2. For each class of compound, endogenous concentrations *in vivo* were at a low steady state during the dormant season, with subsequent increases at the onset of the spring bud-break. This role (i.e., an association between rising concentrations and bud-break) has effectively been simulated *in vitro* by the provision of related exogenous compounds for explants collected from trees in the dormant season. Further, the requirement for exogenous ammonium nitrate and benzyladenine can be expected to decrease with the advent of the spring bud-break. By experimenting with the concentrations of exogenous supplements, it may eventually be possible to achieve morphogenetically competent cultures in most months of the year.

Tree-to-tree variation

A comparison of bud-base explants from nine donor trees showed tree-to-tree differences in culture response *in vitro*. For example, survival at Week 6 (Table 4) was 100% for explants from Trees 6, 8, 10, and 16, 95% from Tree 15, and 91% from Tree 11. Explants from Trees 4, 12, and 14 frequently turned brown and died.

TABLE 3—The influence of duration of exposure to ammonium nitrate upon shoot primordium elongation from bud-base explants (20 explants were inoculated per treatment). Experiment 2

Treatment			Plant response			Elongation of primordia forming shoots (%)
First medium	Duration (days)	Second medium	Shoot primordia (12 wk)	Responsive explants (12 wk)	1.0 cm shoots* (20 wk)	
B-3	21	B-1	128	16	66 c	51.6
B-3	42	B-1	173	20	115 a	66.5
B-3	63	B-1	135	17	67 c	49.6
B-3		Continuous	124	15	64 c	51.6
B-2	21	B-1	125	15	60 d	48.0
B-2	42	B-1	135	16	88 b	65.2
B-2	63	B-1	96	12	50 e	52.1
B-2		Continuous	98	12	50 e	51.0
B-1	21	B-2	26	6	5 f	19.6
B-1	42	B-2	21	4	7 f	33.3
B-1	63	B-2	19	4	3 f	16.9
B-1		Continuous	48	8	10 f	20.8

* Figures followed by the same letter are not statistically different at the 5% level (Chi-square test for independence).

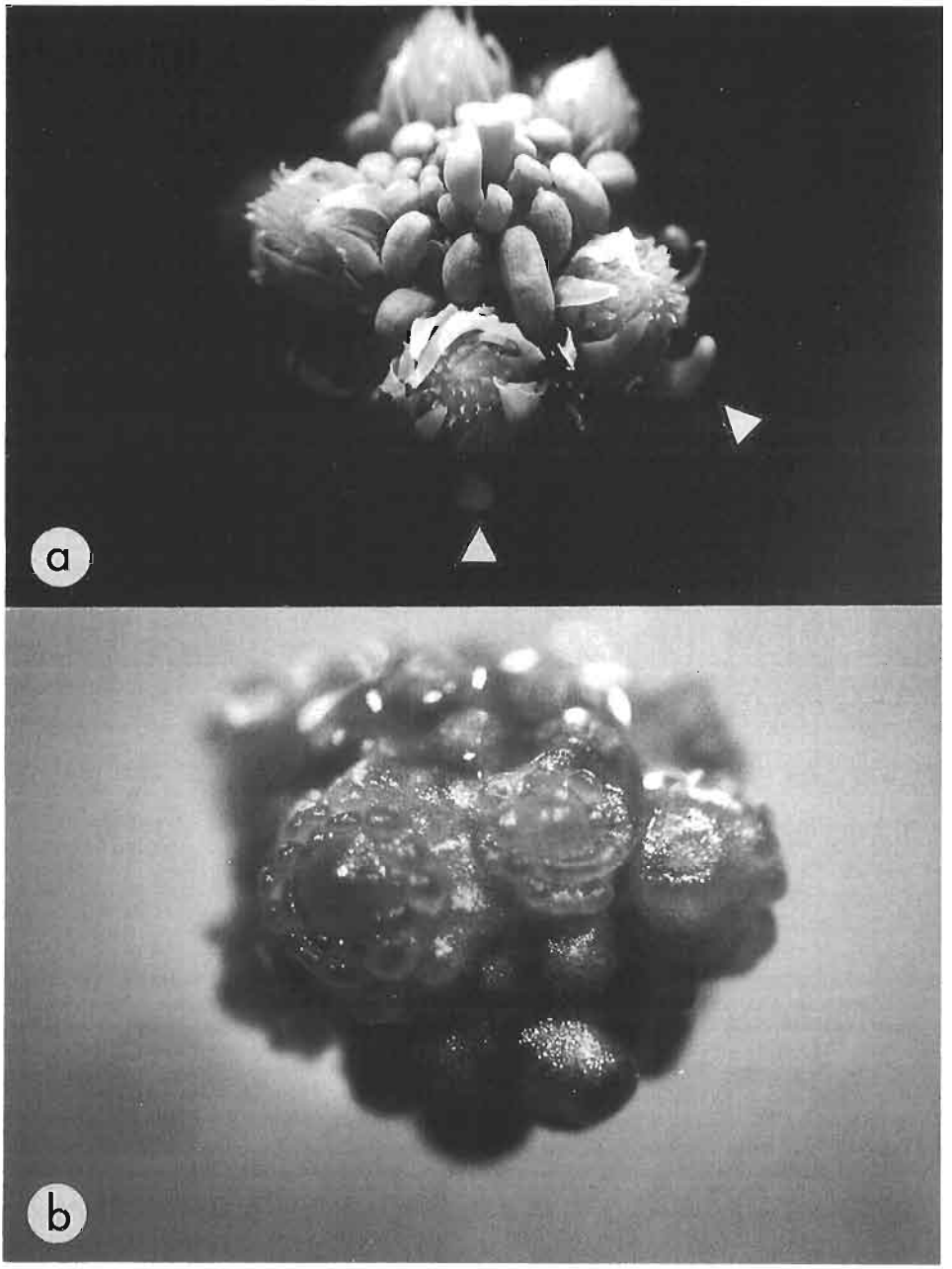

FIG. 1a—Shoot primordia formed toward the base of the bud-base explant, viewed from the distal end of the embryonal axis. Arrows denote needles on the explant which subtend two of the shoot primordia. Tree 6, Week 6 (× 6).
1b—Shoot primordia formed directly upon the needles of the explant's embryonic shoot axis, viewed from a lateral angle. Such primordia usually occurred in positions flanking the needle apex. Tree 8, Week 8 (× 30).

TABLE 4—A comparison of the **in vitro** responses of bud-base explants excised from nine donor trees (80 explants were inoculated per tree). Experiment 3

Parameter	Tree number								
	4	6	8	10	11	12	14	15	16
Survival (6 wk)	24/80	80/80	80/80	80/80	73/80	32/80	8/80	76/80	80/80
No. of explants producing shoot primordia* (12 wk)	0	66/80 b	80/80 a	75/80 ab	73/73 a	13/32 c	0	0	76/80 ab
Mean number of shoot primordia per responsive explant (±S.E.)† (12 wk)	0	8.5 (±0.25) a	8.8 (±0.23) a	6.3 (±0.22) b	6.2 (±0.2) b	4.1 (±0.47) c	0	0	4.3 (±0.17) c
Primary multiplication factor	0	7.01	8.80	5.91	5.66	0.66	0	0	4.08

Figures with the same letter are not significantly different at the 1% level.

* Chi-square test for independence.

† Fisher's least significant difference.

Formation of shoot primordia and their elongation was as described above, except that shoot primordia arose not only in the axils of needles (Fig. 1a) but also directly upon the needles in a position immediately flanking the needle apex (Fig. 1b). A similar needle origin has been noted by others (e.g., for Douglas fir (Thompson & Zaerr 1981) and *Picea abies* (Jansson & Bornman 1983)). The loci of shoot primordia was influenced by the donor tree, as shown for explants from Trees 6, 8, 10, 11, and 16 (Table 5). Explants from Trees 6 and 16 produced shoot primordia exclusively in needle axils, whilst those of Tree 8 were predominantly borne upon needles. Explants from Trees 10 and 11 displayed both types of shoot primordia origin. Shoot primordia in needle axils were most often confined to basal positions on the explant (Fig. 1a), whilst primordia borne upon needles occurred in a more scattered distribution (Table 5).

TABLE 5—The influence of donor tree upon the origin and loci of shoot primordia on bud-base explants (12 wk). Data are the total numbers recorded for Experiment 3, reported in Table 4

Parameter	Tree number				
	6	8	10	11	16
Number of shoot primordia in needle axils	561/561	71/708	236/472	180/450	327/327
Number of shoot primordia on modified needles	0	637/708	236/472	270/450	0
Number of shoot primordia at explant base	561/561	0	94/472	45/450	327/327
Number of shoot primordia in other positions	0	708/708	378/472	405/450	0

The production of shoot primordia occurred on 100% of inoculated bud-base explants from Tree 8 (Table 4). Production was also recorded for the surviving explants from Trees 6, 10, 11, 12, and 16. The number of shoot primordia that were produced on these responsive explants also varied with tree. Explants from Tree 8 showed the best response (average 8.8 shoot primordia on 100% of inoculated explants) followed by those of Tree 6 (average 8.5 on 82% of inoculated explants). The lowest value was recorded for Tree 12 (average 4.1 shoot primordia on 16% of inoculated explants). The calculation of a primary multiplication factor from these results gives a relative ranking of each tree's potential for vegetative multiplication, taking into account all losses arising during *in vitro* culture. Tree 8 was superior to Tree 6 in this regard (average 8.8 versus 7.01 shoot primordia per inoculated explant respectively). Three other trees (10, 11, and 16) also showed a positive primary multiplication (i.e., greater than 1).

The range of *in vitro* responses observed amongst the nine Douglas fir trees may be due to the individual physiological conditions of each tree, to their genotypic makeup

(von Arnold 1984) or to their localised environments (Durzan 1982). Differing degrees of variations in response have been reported for explants from older trees of other species. For example, von Arnold & Eriksson (1979) reported no differences in adventitious shoot primordium production for *Picea abies* trees of varying ages (5 to 50 years) in natural stands; 30% of all buds from all trees showed adventitious bud production. Jansson & Bornman (1983), however, in a comparison of buds from three 10-year-old *P. abies* trees, noted that only one gave rise to buds which showed a 100% capacity for adventitious shoot primordium production (at 10 per explant). A second could increase in capacity (to 50% of buds at two per explant). A second could increase in capacity (to 50% of buds at two per explant) following a 2-month cold treatment. In 1984, von Arnold compared four different 26-year-old *P. abies* clones, grown for vegetative cutting production, and noted marked differences in the ability of their buds to produce adventitious shoot primordia in culture (from 34% to 2%) and in the numbers of such primordia per explant (from 11.2 to 4.4). A culture method for eventual use with vegetative multiplication of older trees, at an age at which phenotypically elite traits can be judged, must accommodate the observed variation in tree-to-tree response. Because explants from different trees may have different optima for ammonium nitrate or for benzyladenine, such accommodation may be made by changes in culture technique to elicit maximum shoot primordium production and subsequent elongation. However, for practical applications it may be more convenient to derive protocols which will permit positive responses in explants from a majority of trees.

Shoot Elongation and Remultiplication

Shoots were excised from the original explant material in Week 21 when they were 1.0 to 1.5 cm long (from base to shoot meristem) (Fig. 2a). Shoots continued to grow on modified B indefinitely, though their responses were often variable with the progeny from some bud-base explants showing a greater propensity to grow than others (Fig. 2b). This may be related to the topophysical position of each original bud, some of the effects of which have been indicated by Evers (1981). Such shoots occasionally developed two to three lateral buds per 1.5 to 2.0 cm length of stem. These appear similar to those produced after benzyladenine treatment by Gupta & Durzan (1985). Lateral buds elongated into side-shoots spontaneously or could be induced to grow by removal of the terminal 1.0- to 1.5-cm portion of shoot axis (Fig. 2c). This may be analagous to topping used by others (Aitken-Christie & Thorpe 1984). Experiments are presently under way to amplify lateral bud production by combined "topping" and benzyladenine treatment. Topped shoot apices (1.5 cm long) have been recycled on to modified B for continued elongation, while the decapitated shoot-bases (1.0 to 2.0 cm long) were placed for 21 days on modified B containing 3 mg Ba/*l*. During preliminary experiments using randomly chosen shoots collected in Week 24, 10 out of 16 decapitated shoots produced one to three new lateral buds (Fig. 2a). Experiments will continue in this direction to determine if it is possible to achieve a perpetuating shoot culture system analagous to that used for fruit trees and other woody species (Dunstan & Thorpe 1984; Dunstan *et al.* 1985). Experiments are also under way to increase the percentage of shoot primordia which elongate from the primary explant, and light microscope analyses will be performed to determine the origins of the shoot primordia which arise in needle axils.

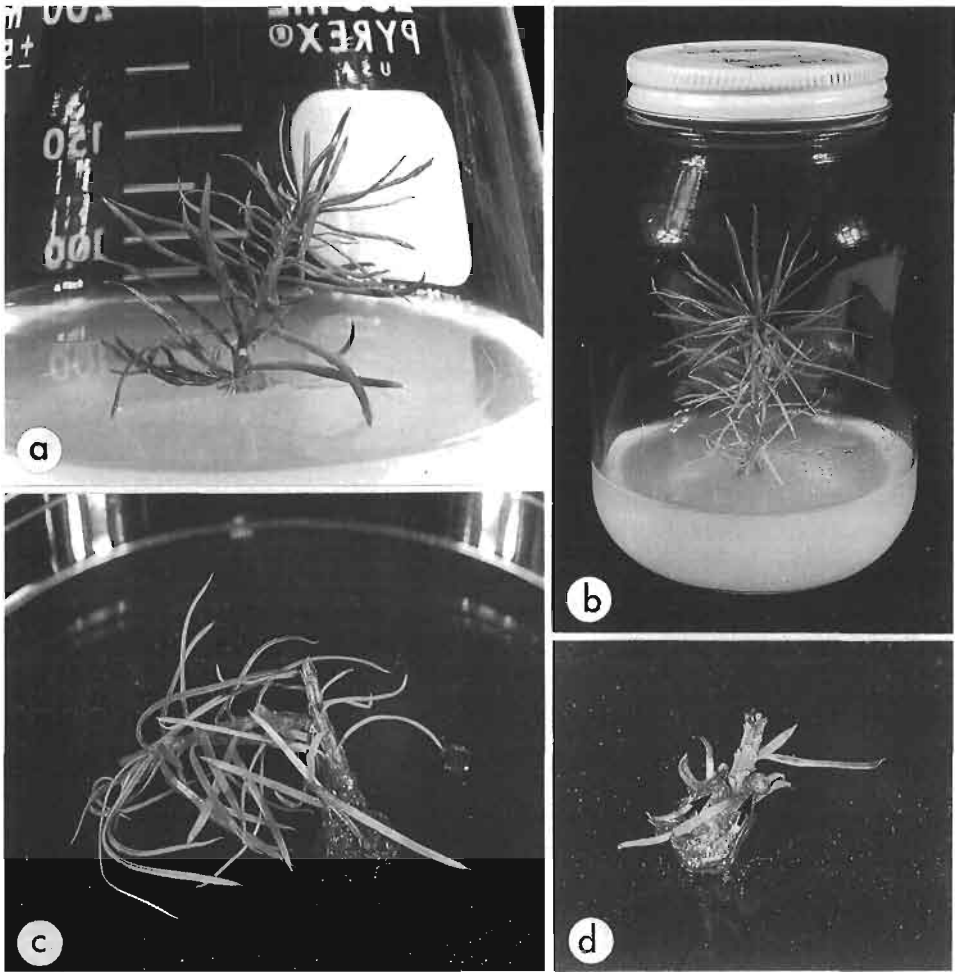

FIG. 2a—Elongated shoot (1.5 cm long) excised from the original bud-base explant in Week 20, growing on modified B. Tree 6 (×1.6).
2b—Elongated shoot (4 cm long) in Week 24, growing on modified B. Tree 6 (×0.5).
2c—Elongated side shoot (1.5 cm long) induced to grow after removal of terminal 1.5 cm of main axis in Week 24, shown in Week 27 growing on modified B. Tree 6 (×1.3).
2d—Induction of two lateral buds (arrows) upon main axis after removal of terminal 1.5 cm in Week 24, shown in Week 27 after 21 days on modified B with 3 mg BA/l. Tree 6 (×1.3).

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