INITIATION, ELONGATION, AND REMULTIPLICATION OF LARIX DECIDUA MICROPROPAGULES

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ABSTRACT

Larix decidua Mill. was micropropagated **in vitro** using juvenile tissues from young seedlings as well as tissues from **in vitro**-cloned propagules. Wounds made to seedling hypocotyls stimulated adventitious bud initiation at wound sites. Initiation frequency was best using 2-week tissue incubation on medium supplemented with 4.4×10^{-5} molar cytokinin. A cytokinin pulse treatment of non-growing older adventitious shoots stimulated renewed growth. Clone population sizes were increased **in vitro** using successive generations of propagules as source tissues for initiation of adventitious buds. Clones were further rapidly augmented by bud break on plantlets horizontally affixed in soil; apices had been excised from these plantlets.

Keywords: micropropagation; **in vitro** culture; tissue culture; **Larix decidua**.

INTRODUCTION

Larches *(Larix* species) offer considerable opportunity for genetic improvement. They are highly variable in growth rate, form, cold hardiness, and pest resistance. Furthermore, they are readily amenable to hybridisation. However, several factors restrict improvement of the species by conventional means. Firstly, larches do not begin to produce large amounts of seed in an orchard until they are 10 to 15 years old or older. Secondly, larches are inconsistent seed producers and have years of very low seed yields. Thirdly, larch seed has notoriously poor viability. Attempts to generate hybrids are hampered both by differences in flowering times of the various larch species, even when grown side by side, and by the small number of seeds obtained per pollinated strobilus (Campbell 1983). This combination of difficulty in seed production and good potential for improvement makes larches excellent candidates for asexual propagation. For this reason, our laboratory is concentrating on the development of methods for rooting cuttings and for *in vitro* micropropagation of larches. We have shown that adventitious buds may, for example, be induced *in vitro* from juvenile tissues of *L. decidua* (European larch) (Karnosky & Diner 1984; Karnosky *et al.* 1984).

The micropropagation system for *L. decidua* shows considerable promise for production of plantlets for tree improvement research, and may eventually be used for commercial production of planting stocks for reforestation purposes. Here we present

results of studies on (1) determining environmental conditions necessary for maximising bud initiation and improving shoot elongation, and (2) developing techniques for remultiplication of plantlets to increase population sizes of established clones.

MATERIALS AND METHODS

Initiation

N^6-benzyladenine (BA) has been commonly used to stimulate adventitious bud induction from conifer juvenile tissues. Both concentration and duration of exposure to BA have been shown to influence induction frequency (Biondi & Thorpe 1982; von Arnold 1982; von Arnold & Eriksson 1985; Patel & Thorpe 1984). To determine any effects of these variables on our larch system (Karnosky & Diner 1984), seed from an Austrian orchard (Weinerwald provenance) were first surface-sterilised by two successive soaks in 30% aqueous hydrogen peroxide separated by 6 hours' storage at room temperature. Seeds were rinsed and then germinated for 6–7 days at room temperature on 2% agar-solidified water in sealed petri dishes. Seed coats were then removed and discarded. Seedlings partially enclosed by gametophytes were soaked for 5 minutes in 5% aqueous commercial bleach, then rinsed. The effects of induction period length were determined (Experiment 1) using cotyledons excised and placed for 1, 2, 3, 4, or 5 weeks on a 1% agar-solidified, pH 5.5, BLG mineral salts medium (Brown & Lawrence 1968) supplemented with glutamine (10^{-2}M), sucrose (3×10^{-2}M), and BA (4.4×10^{-5}M) in petri dishes. Dishes then sealed with parafilm were incubated at 20°C under constant 4500 lux mixed cool-white fluorescent (70%) and incandescent (30%) illumination (standard conditions).

Optimum growth regulator levels for use in *L. decidua* adventitious bud initiation were determined using juvenile tissues as described above, incubated 2 weeks on 1% agar-solidified BLG medium (Experiment 2). Cytokinin (BA) at levels of 2.2×10^{-5}, 4.4×10^{-5}, or 6.6×10^{-5}M was incorporated into the medium. In a fourth test, the medium was prepared containing both BA (4.4×10^{-5}M) and alpha-naphthalene acetic acid (NAA) (5.4×10^{-7}M).

Preliminary experimentation suggested that both BA concentration and length of induction period exerted effects on bud initiation frequency. Therefore, Experiment 3 was undertaken to compare the usefulness of a 2-week induction period for tissues on a medium supplemented with 4.4×10^{-5}M BA, *v.* a 4-week induction period on medium supplemented with 2.2×10^{-5}M BA.

Recently there has been considerable interest in endogenous growth regulator control of morphogenesis in tissue culture systems (Albersheim & Darvill 1985; Tran Thanh Van *et al.* 1985). Since we had often noticed clusters of adventitious buds developing near cut ends of hypocotyl segments placed on bud induction media, we examined the effects of various types of wounds on adventitious bud production by *L. decidua* hypocotyl and cotyledon sections (Experiment 4). Cotyledons as well as intact or variously sectioned hypocotyls were incubated for 2 weeks on induction medium supplemented with 4.4×10^{-5}M BA.

All tissues (Experiments 1–4) were transferred from bud induction medium to 1% agar-solidified half-strength Gresshoff & Doy (GD 1/2) mineral salts medium (Mehra-

Palta *et al.* 1978) (pH 5.5) supplemented with 3×10^{-2}M sucrose and 1% (w/v) activated charcoal (Sigma Chemical Co.). After this, tissues were transferred for shoot elongation to freshly prepared, 1% agar-solidified LMG (Litvay *et al.* 1981) mineral salts medium supplemented with 3×10^{-2}M sucrose for two successive 2-week periods, whereupon clonal population sizes were determined.

To optimise both the clonal population sizes and rates of shoot growth, we examined (1) the efficacy of different mineral salts media used to elongate propagules initiated from juvenile tissues of various conifers, (2) different concentrations of the most commonly used carbon source (sucrose), and (3) different frequencies of tissue subculture for shoot development and progressive elongation, since the frequency of tissue transfer to fresh nutrient medium has major impacts both on tissue health and on those materials and labour costs implicit in tissue culture propagation programmes. To these ends, tissues were used from Experiment 3, after their 2-week treatment on half-strength, charcoal-supplemented, GD medium.

In Experiment 5 tissues representing each clone were collectively transferred to one of each of three 1% agar-solidified mineral salts media. These included LP (Quoirin & LePoivre 1977), full-strength Gresshoff & Doy (GDI), and Litvay's as previously described. Each medium (pH 5.5) was supplemented with sucrose to levels of either 3×10^{-2} or 9×10^{-2}M.

Of the clones on each medium variation, half were transferred fortnightly for 8 weeks to freshly prepared medium of the same composition. The other half were transferred at the end of 4 weeks, for a second 4-week period. Thus, all tissues were incubated for a total of 8 weeks on elongation media, though provided with fresh nutrients at either 2- or 4-week intervals. Clonal population sizes were determined after a total of 8 weeks of shoot elongation.

Elongation

While induction of recognisable adventitious buds requires only about 1 month using *L. decidua* juvenile tissues, elongation of these buds into shoots of size sufficient for rooting can take several months.

Three media (LP, LMG, GD 1/2) were compared (Experiment 6) for the ability of each to support elongation of adventitious shoots which had been grown from buds induced for 2 weeks on BLG medium with 4.4×10^{-5}M BA, then developed for 2 weeks on GD 1/2 medium with 1% charcoal. Each of the three elongation media (pH 5.5) was supplemented with 3×10^{-2}M sucrose and no charcoal. Shoot heights (exclusive of apical needle length) were determined after 5 weeks (2 1/2 successive 2-week treatments on freshly prepared medium).

At approximately 16 weeks of age (12 weeks elongation), many adventitious buds are of sufficient stem length (minimally 1 cm) to root in the greenhouse. However, smaller shoots are common among populations of this age. While we have been able to root these short shoots *in vitro* (unpubl. data), their small size (Fig. 1) jeopardises their successful transfer to rooting media under intermittent mist in the greenhouse. Thus, the growth-stimulating effects of various media were determined using these short 16-week propagules (Experiment 7). The 1% agar-solidified media combinations

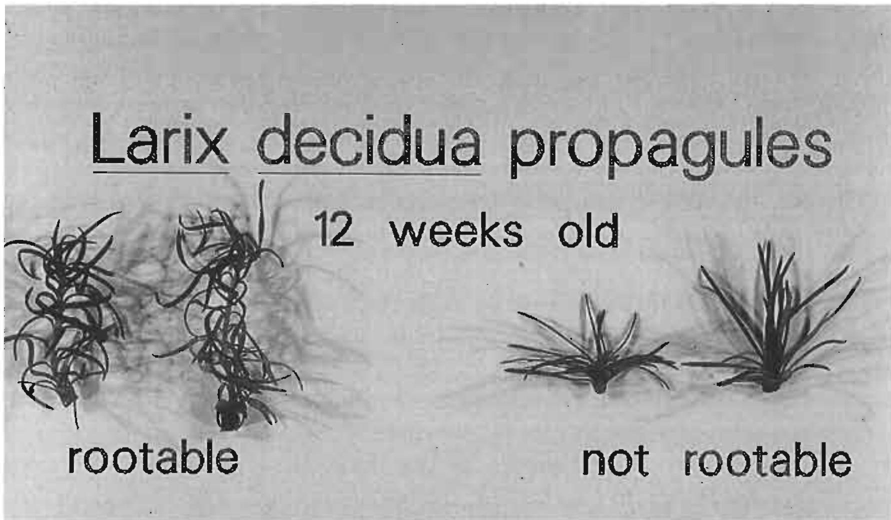

FIG. 1—Morphologies of four random genotypes of **Larix decidua** propagules. The short "bushy" propagules at right are more difficult to root than the elongate propagules at left. However, "bushy" propagules may be induced to elongate to a "rootable" form by a pulse treatment with cytokinin ($\times$ 1).

selected for study included GD1 with 6×10^{-2}M sucrose $\pm$ 1% charcoal, LMG with 3×10^{-2} or 6×10^{-2}M sucrose, and LP with sucrose (3 or 6 or 12×10^{-2}M $\pm$ 1% charcoal.

In a further attempt (Experiment 8) to stimulate elongation of these short and recalcitrant shoots, and in response to a report (J. Aitken-Christie, pers. comm.) of *Pinus radiata* D. Don short propagule growth-stimulation by BA pulse treatment, *L. decidua* adventitious short shoots (16 or more weeks of age) were measured and incubated for 4 weeks (two 2-week treatments) on 1% agar-solidified LMG media supplemented with either 2.2×10^{-6} or 4.4×10^{-6}M BA. Shoots were then transferred through four successive 2-week treatments on LMG agar medium devoid of growth regulators. Heights were determined at the end of this period.

Remultiplication

A possible opportunity to increase clonal population sizes is attractive, particularly if seed is limited as is common in most crosses between genetically improved selections, or in a hybridisation programme. The advantages of recycling individuals from selected clonal populations for use as source tissues or organs to develop progressively larger populations (remultiplication) are obvious. Some success has been reported for *Pinus* spp. using cuttings from elongating, branching micropropagules (Aitken-Christie & Gleed 1984; Aitken-Christie *et al.* 1982; Kolevska-Pletikapic *et al.* 1983).

To determine whether adventitious buds could be induced from tissues of *L. decidua* propagules, needles which were peeled from propagule (8–16 weeks of age) stems were incubated for 2 weeks on 1% agar-solidified BLG medium supplemented with 3×10^{-2}M sucrose and 4.4×10^{-5}M BA (Experiment 9). Residual stripped stems were also so treated. Tissues were then transferred for 2 weeks to GD 1/2 with 1% charcoal, thence to LMG for successive 2-week shoot elongation treatments. Needles and stems were examined for development of adventitious shoots. Shoots generated by this process, and then elongated for 4 to 12 weeks, were themselves cycled through this remultiplication process for two further successive generations.

Another recently reported remultiplication process (J. Aitken-Christie, pers. comm.) employs conifer tissue culture plantlets grown to 30 cm or more in height in the nursery. The top third of each shoot is discarded; the residual stem is then affixed horizontally in the nursery bed. Within weeks, multiple fascicle bud break along the stem length is followed by branch growth to lengths amenable to cutting, then rooting. To determine the efficacy of this process in our larch micropropagation programme, nine *L. decidua* tissue culture plantlets, 45 to 60 cm in height ($1\frac{1}{2}$–2 years of age), were treated as described above but placed in flats in the greenhouse (Experiment 10).

RESULTS AND DISCUSSION

Data from testing the effect of initiation period duration on initiation bud frequency (Experiment 1) are shown in Table 1. Optimum initiation period was 2 weeks, resulting in an average of 7.2 adventitious buds from each seedling. Although treatments of 1 through 3 weeks generally resulted in appreciable (5+) bud production, periods of 3 weeks or more resulted in increasing proportions of vitreous buds. Vitreous buds have been reported for plantlets of numerous species generated in tissue culture, and generally show poor survival.

TABLE 1—Effects of initiation period on adventitious bud production from juvenile **Larix decidua** tissues

Initiation period (weeks)	Number of genotypes tested*	Average number of adventitious shoots produced per genotype
1	64	5.4 ± 4.0†
2	65	7.3 ± 4.3
3	46	5.7 ± 3.8
4	43	2.2 ± 2.2
5	60	1.0 ± 1.2

Initiation medium: 1% agar-solidified BLG with 4.4×10^{-5}M BA

* Germinated seedlings

† Standard deviation

Effects of growth regulator concentrations on adventitious bud induction (Experiment 2) are shown in Table 2. A 4.4×10^{-5}M concentration of medium BA was the most effective of those levels used, and evolved an average of 8.2 buds per clone. A very low concentration (5.4×10^{-7}M) of NAA added to this medium resulted in both a decline in bud initiation and an increase in callus production.

TABLE 2—Growth regulator concentration effects on adventitious bud initiation from juvenile tissues of **Larix decidua**

Growth regulator* concentration (M)	Number of genotypes tested†	Average number of buds formed per genotype
BA 2.2×10^{-5}	44	6.0 ± 3.4‡
BA 4.4×10^{-5}	51	8.2 ± 5.8
BA 6.6×10^{-5}	44	2.5 ± 2.1
BA 4.4×10^{-5} + NAA 5.4×10^{-7}	49	3.8 ± 2.4

* BA = N^6-benzyladenine; NAA = naphthalene acetic acid

† Germinated seedlings

‡ Standard deviation

Data comparing the effect of initiation medium BA concentration (2.2 *v.* 4.4×10^{-5}M) and duration of initiation treatment (2 *v.* 4 weeks) on bud initiation (Experiment 3) are given in Table 3. Also provided in Table 3 are data on the relative efficacies of (1) 2- *v.* 4-week sub-culture frequencies, and (2) selected basal nutrient media and sucrose concentrations (Experiment 5). Greatest productivity resulted from the use of a 2-week initiation treatment on BLG medium supplemented with 4.4×10^{-5}M BA. This productivity (6.1 growing shoots per clone) was obtained by a 2-week subculture frequency on GD elongation medium containing 3×10^{-2}M sucrose. However, great variability occurred in numbers of shoots produced in each clone (standard deviation of the mean was 6.1).

Wounds made to hypocotyls of juvenile *L. decidua* generally resulted in greater bud initiation (Experiment 4). However, wounded cotyledons became necrotic and generated no buds. Generally, cotyledons intact to their point of excision from the hypocotyl apex generated approximately six buds per clone. Similar results have recently been reported for wounded stem segments of *Torenia fournier* Lind. (Takeuchi *et al.* 1985). However, in contrast to *Torenia* stem segments, adventitious buds of larch originated only from tissues at or directly adjacent to the wound site. And, although longitudinally wounded *Torenia* stem segments generated buds, similarly wounded larch hypocotyls did not, and became necrotic within the 2-week initiation period. A single bisection of the isolated *L. decidua* hypocotyl resulted in twice as many adventitious buds being produced (19 1)

TABLE 3—Effects of cytokinin concentration, subculture frequency, elongation medium, and medium sucrose concentration on adventitious shoot production from **Larix decidua** juvenile tissues

Initiation treatment*	Subculture frequency on elongation medium (weeks)	Elongation media†	Sucrose conc. (M)	Average number of shoots produced per genotype‡
BLG(4.4) to GD 1/2	2	LP	3×10^{-2}	5.2 ± 3.9§
	2	LP	9×10^{-2}	1.7 ± 2.5
	2	GD	3×10^{-2}	6.1 ± 6.1
	2	GD	9×10^{-2}	3.6 ± 4.2
	2	LMG	3×10^{-2}	2.8 ± 3.1
	2	LMG	9×10^{-2}	0.8 ± 1.7
BLG(2.2)	4	LP	3×10^{-2}	2.9 ± 3.5
	4	LP	9×10^{-2}	1.0 ± 1.4
	4	GD	3×10^{-2}	4.7 ± 3.7
	4	GD	9×10^{-2}	1.2 ± 1.7
	4	LMG	3×10^{-2}	4.1 ± 3.4
	4	LMG	9×10^{-2}	1.3 ± 2.1

* Initiation treatments: BLG(4.4) to GD 1/2 refers to 2-week initiation on Brown & Lawrence salts medium supplemented with 4.4×10^{-5}M BA and 10^{-2}M glutamine, followed by 2 weeks on half-strength Gresshoff & Doy salts medium with 1% Sigma charcoal. BLG(2.2) refers to initiation for 4 weeks on Brown & Lawrence salts medium supplemented with 2.2×10^{-5}M BA, followed by 2 weeks on half-strength Gresshoff & Doy salts medium with 1% Sigma charcoal.

† Elongation media: LP = Quoirin & Le Poivre; GD = Gresshoff & Doy; LMG = Litvay's medium with 10^{-2}M glutamine.

‡ Each treatment was represented by 40 genotypes.

§ Standard deviation.

as when the entire hypocotyl was plated intact (9.6) on similar cytokinin-supplemented medium (Table 4). Buds proliferated near both cut ends of the top half-section of hypocotyl (Fig. 2) and near the top cut end of the lower section. Smaller (< 2 mm) cross-sections of the hypocotyl became necrotic and generated no buds.

Five-week preliminary elongation of young and developing adventitious buds was compared on LMG, LP, and GD 1/2 media (Experiment 6). At the start of the experiment, the adventitious buds were too small for stem height measurements. Average measurements after 5 weeks' growth on each medium are given in Table 5. Litvay's medium with glutamine was the most effective of the three media used, and supported

TABLE 4—Effects of wounding on adventitious bud production from **Larix decidua** juvenile tissues

Embryo sections placed on initiation medium	Average number of buds produced per genotype†
C* + top 1/2 H*	11.6 ± 7.1‡
C + entire intact H	9.6 ± 6.7
C + top 1/2 H + bottom 1/2 H	19.1 ± 9.0
C + H cut to 2-mm cross-sections	6.4 ± 3.6
C + H cut in 2 lengthwise sections	5.2 ± 3.1
C cut in 2-mm-cross sections + bottom 1/2 H	6.8 ± 3.7

* C = cotyledons; H = hypocotyl.

† Average of 30 genotypes per treatment.

‡ Standard deviation.

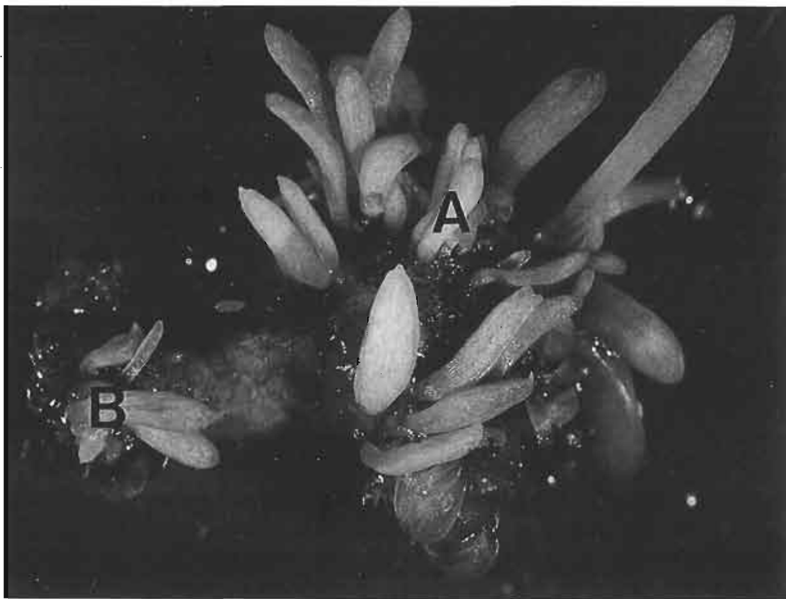

FIG. 2—Adventitious buds developing from transverse wounds made to apical (A) and basal (B) sites of the upper half of the **Larix decidua** seedling hypocotyl. Note the characteristically large number of buds developing from the apical wound site (× 10).

TABLE 5—Comparative 5-week elongation of **Larix decidua** adventitious shoots on Litvay's medium with glutamine (LMG), Quoirin & Le Poivre medium (LP), and half-strength Gresshoff & Doy medium (GD 1/2)

Medium	Number of shoots*	Average final stem height† (mm)
LMG	100	6.4 ± 1.2‡
LP	50	5.7 ± 3.1
GD 1/2	50	2.9 ± 1.04

* One to three shoots per genotype.
† Initial stem heights did not exceed 1 mm
‡ Standard deviation.
Elongation determined for a 5-week period subsequent to a post-initiation charcoal-medium treatment.

6.4 mm stem growth. However, *L. decidua* propagules incubated on LMG for prolonged periods (4+ weeks; 2½ times 2-week subcultures) tended to become chlorotic. Propagules of similar ages incubated on GD 1/2 medium did not show this tendency, but did show relatively poor elongation (2.9 mm).

Small, 16-week-old, *L. decidua* adventitious shoots showed generally poor elongation on the several media (Experiment 7) employed to stimulate growth during 4 weeks of further incubation (Table 6). The one exception to this general response occurred using

TABLE 6—Influence of selected media on 4-week elongation and mortality of short 16-week-old **Larix decidua** adventitious shoots

Medium*	Number of shoots†	4-week stem height increment (mm)	Mortality (%)
GD1 0.06 M sucrose, 1% charcoal	70	2.3 ± 4.7‡	13.9
GD1 0.06 M sucrose	186	1.1 ± 1.58	3.7
LMG 0.03 M sucrose	180	1.7 ± 2.2	3.9
LMG 0.06 M sucrose	79	1.4 ± 1.7	2.7
LP 0.03 M sucrose	301	2.0 ± 2.1	5.6
LP 0.06 M sucrose, 1% charcoal	83	4.1 ± 4.0	7.3
LPG 0.09 M sucrose	277	1.6 ± 2.3	11.4

* Media: GD1 = Gresshoff & Doy; LMG = Litvay's with 0.01 M glutamine;
LP LP = Quoirin & Le Poivre; LPG = Quoirin & Le Poivre with 0.01 M glutamine
Media contain 1% agar.
† One to three shoots per genotype.
‡ Standard deviation.

LP medium with 6×10^{-2}M sucrose and 1% (w/v) activated charcoal. Stems of propagules on this medium averaged 4.1 mm incremental growth. Foliage remained green and apparently healthy. Mortality of shoots ranged from a low of 2.7% (LMG with 6×10^{-2}M sucrose) to 13.9% (GD with 6×10^{-2}M sucrose and 1% (w/v) charcoal).

Older short shoots which had been pulse-treated for 4 weeks with 2.2×10^{-6}M BA (Experiment 8) showed average stem growth of 1.3 mm when measured 12 weeks after exposure to cytokinin (Table 7). Short shoots treated instead with 4.4×10^{-6}M BA averaged 3.1 mm elongation, while untreated controls did not grow. Although pulse treatments using cytokinin may prove useful to stimulate elongation in slow or non-growing propagules, the many weeks (12) required to effect so small an elongation as 3 mm may prove prohibitive.

TABLE 7—Growth effects of a 4-week N^6-benzyladenine (BA) pulse treatment on short 16-week-old **Larix decidua** adventitious shoots

Medium*	Number of shoots	Average stem height increment† (mm)
LMG	66	0.0 ± 0.0‡
LMG + (2.2×10^{-6}M) BA	64	1.3 ± 2.0
LMG + (4.4×10^{-6}M) BA	86	3.1 ± 3.8

* All media contained 0.03 M sucrose.

† Initial stem heights did not exceed 1 mm.
Shoot elongation measured by change in shoot length from start of 4-week BA treatment to 8 weeks after treatment concluded.

‡ Standard deviation.

Adventitious buds were induced on needles and stripped stems of elongating propagules (Experiment 9) which themselves had been developed from adventitious buds on juvenile seedling tissues. This process was repeated through two further generations. Approximately five buds formed from the detached organs of each propagule, through each of three generations (Table 8). Neither the morphologies of these remultiplied propagules, nor their general rates of growth appeared unusual. Short non-growing shoots had not been used as source tissues for first-generation initiation, nor did they develop among the progeny through the three-generation remultiplication sequence. Such short shoots are fairly common (see above), grown from adventitious buds induced on juvenile tissues of *L. decidua.* This suggests that our choice of "normally" elongating propagules for remultiplication may have constituted a positive selection process for morphologically and developmentally favourable individuals.

TABLE 8—Remultiplication of **Larix decidua** through four generations*

Explant source	Number of genotypes tested	Genotypes producing buds (%)	Average number of buds formed per genotype†
7-day-old embryos	51	94	8.7 ± 4.2‡
Needles from first-generation shoots	123	60	4.6 ± 1.8
Needles from second-generation shoots	24	100	5.3 ± 2.1
Needles from third-generation shoots	17	88	5.9 ± 2.3

* Approximately 42 weeks.

† Average number of new buds formed on those genotypes forming buds.

‡ Standard deviation.

An additional remultiplication process for *L. decidua* employed branch stem cuttings taken from greenhouse-grown plantlets (which had been induced to collective bud break (Experiment 10)). Nine plantlets (one of which is shown in Fig. 3) produced 500 shoots 70–75 cm tall, for an average of 55.5 shoots per plantlet, during 5 months

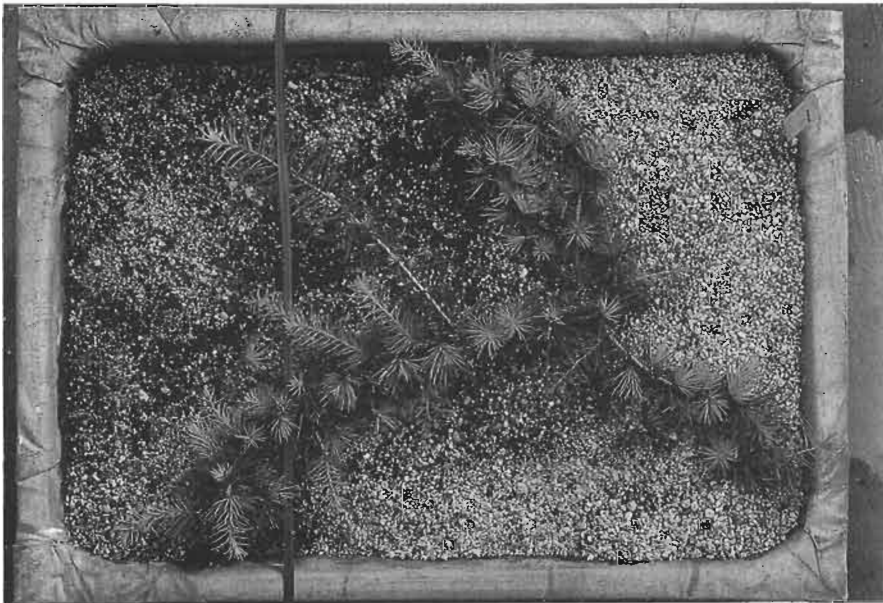

FIG. 3—A 1-year-old **Larix decidua** plantlet which has been trained to a horizontal growth habit and had its apex removed to stimulate axillary buds to break and elongate for remultiplication purposes (× 1/5).

These remultiplication processes offer opportunities to increase the population sizes of selected individual genotypes showing early rapid growth and suitable form. Experimental verification of the fidelity of genetic responses to root initiation treatments may be made using clonal populations evolved through successively remultiplied generations.

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