TISSUE CULTURE OF CONIFERS

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Abstract. Three separate micropopagation systems are described: i) the establishment of multiple shoot and single shoot cultures with axilliary and lateral bud development using mature Douglas fir tissue; ii) the development of mature white spruce cultures exhibiting single shoots and shoot elongation with lateral bud development; iiia) enhancement of shoot growth exhibiting juvenile characteristics from mature yellow cedar tissue; iiib) the tissue and subsequent subcultures of secondary shoots.

Essential factors beneficial to enhancement of multiple shoots in Douglas fir and mature yellow cedar are: origin of source material, time of year for collection of source material, proximity of source material in relation to bole and crown of tree.

Proliferation of shoots in juvenile yellow cedar tissue trial resulted from pulse treatments involving a hormone enriched nutrient medium to one devoid of hormones. Primary rooting trials have been successful in transplanting juvenile yellow cedar plantlets, pre-rooted in-vitro, into soil in a greenhouse environment.

INTRODUCTION

Most mass plantings of forest trees are made from seedlings. The necessary seed is usually obtained through cone collection from selected stands. Most forest trees are grown from seed obtained as a product of open pollination and exhibit continuous variation of many characters. Desirable attributes due to non-additive gene effects are not preserved from one sexual generation to another, but could be maintained if it were possible to propagate individual trees vegetatively at a sufficient rate to produce clones for widespread re-forestation programs.

In practice, this conceivably means propagating mature trees, because superior growth of timber characteristics are often recognizable only after a prolonged period of growth. Because selection could be based upon small clones of trees rather than individuals, vegetative propagation of conifers would allow for a higher degree of genetic advancement in comparison with today's current breeding programs.

In vitro techniques clearly have the potential to provide ways of vegetatively propagating conifer species on a large scale.

The primary benefit from utilizing tissue culture is to have the ability to mass produce commercially valuable spe-

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cies that are genetic duplicates of trees with superior traits. With this capability one is able to realize increases in forest productivity through increased growth rates, better plantation survival, and increased resistance to disease and insects. In addition to benefits from genetic duplication there exists realizations of increases in wood supply available for harvesting, enhancement to existing tree improvement projects and reforestation programs, plus economic benefits to the forest industry. Through the use of micropropagation, seasonal weather restrictions, and seed and cone collection will no longer be of major concern.

From seeds or seedlings it is quite readily possible to initiate tissue cultures, but due to the fact most kinds of trees are heterozygous, this does not allow for individual trees to be cloned. Therefore, before *in-vitro* techniques can be used to produce large numbers of genotypes selected on phenotypic characteristics evident after several years of growth, methods must be found to obtain morphogenesis from tissues originating from mature trees. It is upon this statement that the following research has been based and its concurrent results herein presented.

For cultivation of the conifer tissue cultures three major British Columbian forest species; *Pseudotsuga menziesii* (Douglas fir), *Picea glauca* (white spruce), and *Chamaecyparis nootkatensis* (yellow cedar) were selected. All research work has been carried out at the laboratory facility of Les Clay and Son Limited.

METHODS AND MATERIALS

Douglas Fir and White Spruce Source of Explant Material. Terminal, subterminal, and lateral vegetative buds were collected from mature stands, at elevations from 1200 to 1400 meters. In the collection of the explant source material it was found that the proximity of the buds in relation to the crown and bole of the tree was of significant importance. Those buds gathered from the top and middle crown regions of the tree generated the highest degree of growth response within culture.

Time of year for bud collection was also crucial; just before spring flush when bud swelling is quite visible and overall bud size is approximately 1 to 2 cm. Stem sections 4 to 6 cm in length, with needles removed, exposed the buds for surface disinfestation. Meristematic bud tissue was excised from the surrounding bud scales following a quick-dip of the encapsulated buds directly into isopropyl alcohol. Ensuant to the above procedure the bud scales were removed with a sterile forcep and scalpel.

Since contaminants may not only come from the surface origin of the tissue, but from internal contamination from organisms trapped on the internal bud scales or due to diseased or necrotic tissue; it is thus important that upon excision of the explant the interior of the bud be exposed to a sterile surface where the bud and or bud scales have not previously touched.

This particular method of surface sterilization has minimized losses through bacterial and fungal contamination to 10 to 15% of a given test trial.

Source of Explants for Yellow Cedar. Along with vegetative shoot tips 1 to 3 cm in length gathered from mature tree stands, the implementation of juvenile shoot tip tissue 1 to 3 cm in length collected from yellow cedar seedlings (greenhouse grown) have been the primary sources for experimental trials. With regard to mature yellow cedar, collection of new spring growth material at elevations of 900 to 1000 meters generated more positive responses within culture than summer growth collections.

Yellow cedar explants undergo a more intense surface disinfestation procedure than either Douglas fir or white spruce. It begins with a 4-hr. soap bath with the addition of Tween 80 (soap water changed every ½ hr), followed by a 2-hr wash in a Benlate (fungicide) solution, then a 30-min 10% sodium hypochlorite bath, ending by rinsing 4-5× with sterile water. Each vegetative shoot tip has a small brown portion of basal tissue removed before inoculation.

Basic Culture Media and Environment. The basal salt nutrient medium for all experimental trials was agar solidified by the addition of 5.6 g. per litre Sigma brand agar and pH adjusted to 5.6 with NaOH and HCl. This was dispensed 15 ml per 25 × 150 mm culture tube unless otherwise noted. Many of the media formulas presented in this paper are proprietary to Les Clay and Son Ltd. (Table 1).

Douglas fir

Initiation of culture start material follows two pathways:

i) inoculation of explant onto a modified Murashige and Skoog basal medium with a 75% reduction in nitrate levels with organic constituents and concentrations labelled as Zr. Hormonal levels were high, ranging between 20 to 30 mg per litre 6-benzylaminopurine (BAP). Subsequent subculturing was onto Z1-1 medium devoid of phytohormones to allow for shoot elongation and proliferation.

ii) the second pathway followed an unusual methodology that proved very successful. The explant was placed onto two shoot initiation media, ZL-1 and FRBNH, FRBNH being a vari-

ation of MS medium. Both ZL-1 and FRBNH, respectively, were devoid of hormones. Test trials branched into two directions: i) initial trials were started on ZL-1 basal medium and continually subcultured onto the same; ii) additional set of experiments conducted saw the initiation of cultures onto FRBNH and subsequent subcultures onto ZL-1.

Table 1. A listing of proprietary media classification developed at Les Clay and Son Ltd.

	MEDIA CLASSIFICATIONS		
SPECIES	Shoot Initiation	Shoot Proliferation and/or elongation	Subcultures
Douglas fir	Zr,Z1-1 FRBNH	ZL-1,FRBNH	Z1-1
White spruce	SH,SH-1, SHNH	SHNH	SHNH
Yellow cedar (mature)	1/2MSP, 1/2MSP1	1/2 MSPNH	1/2MSP1, 1/2MSP, 1/2MSPNH
Yellow cedar (juvenile)	LMB,WPM-1' 1/2MSP, 1/2MSP1	WPMNH, 1/2MSPNH	WPMNH, 1/2MSPNH, LMB

White spruce

Initial inoculations of explant were placed upright with the basal portion pushed down into agar solidified media. The nutrient medium under primary investigation was that of Schenk and Hildebrandt (6). Various supplements were added including vitamins, amino acids, sucrose, and hormones. Final levels of additives were sub-categorized under formulas: SH, SHNH, SH-1. Although culture explants were inoculated onto all three SH modifications, it was found that those experimental trials that followed up with subculturing onto SHNH medium (no hormones) generated high degrees of growth response.

Yellow cedar (Mature and Juvenile)

On those test trials designed to run comparative studies on growth response between juvenile and mature tissue, identical media formulations were implemented for primary tissue inoculations. Modified MS basal nutrient medium at ½ strength salt concentration (½ MSP and ½ MSP1) supplemented with vitamins, amino acids, and a lower sucrose level were used. Inclusive was the addition of only auxins at low levels. Subsequent subculturing of explant for shoot initiation and proliferation took place on ½MSPNH medium without hormes.

Trials conducted to generate multiple shoots from juvenile yellow cedar explants included the implementation of Litvays medium (2) supplemented with a low level of BAP, written as LMB medium and continued subculturing onto Woody Plant

Medium (3) and 1/2MSPNH medium both containing no hormones.

The standard culture conditions were 2000 lux cool white fluorescent light, 18-hr, photoperiod at constant 20°C. Standard passage time between subculturing was every 3 to 4 weeks unless otherwise noted.

RESULTS AND DISCUSSION

Douglas Fir

Primary Shoot Formation. As was previously described, the guidelines set by pathway (i) found to induce multiple shoots, (Figure 1, left), in more than 35% of trial lots. This has been achieved by initiating pulse treatments for the first six weeks after explant inoculation from Zr medium, supplemented with an ever-decreasing level of cytokinin over the six week time period, to ZL-1 medium devoid of phytohormones.

Aside from cultures exhibiting multiple adventitious shoots, single shoot cultures were also attained. If exposure to cytokinins was prolonged over the initial six week period, the cultures exhibited signs of oxidation that led to eventual death of tissue. Rate of growth from time of inoculation to a growth plateau for both those cultures with noted shoot proliferations and cultures with only single shoots was just under four months with heights obtained up to 5 cm. In comparison, it was primarily suggested by McComb and Bennett (4) that explants from mature parts of trees may not start to multiply until they have been in culture for 6 to 10 months.

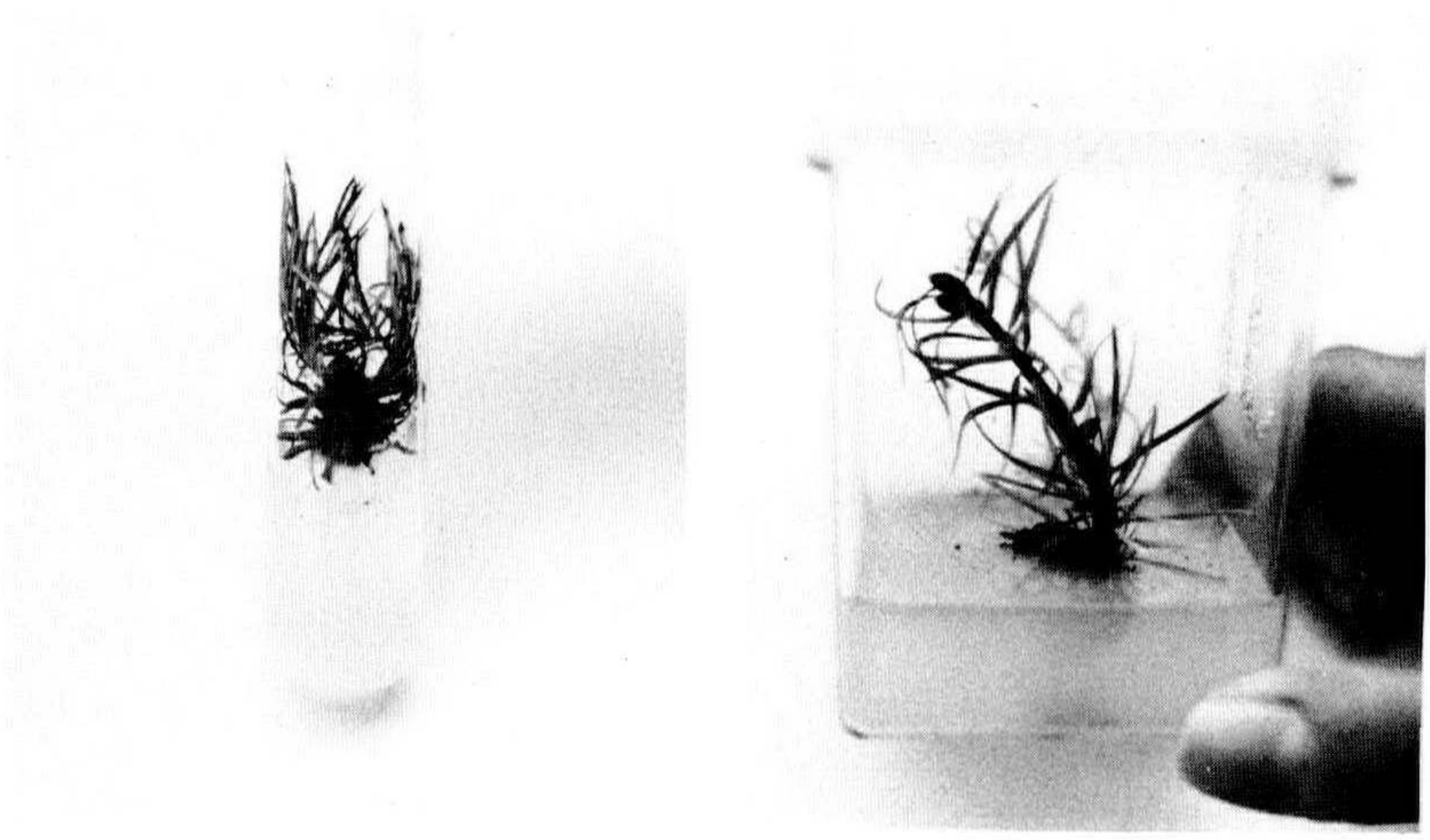

Figure 1. *(Left)*: Mature Douglas fir culture exhibiting multiple shoots. *(Right)*: Single shoot culture of mature Douglas fir with lateral and apical bud development.

Following pathway (ii), similar results were generated with a marked improvement of cultures exhibiting multiple shoots (50 to 60%). There was also a noted increase in growth rates.

Those trials initiated on ZL-1 medium and continually subcultured every 3 to 4 weeks onto the same medium, generated multiple shoots from the original explant within three months. All shoots displayed normal growth and needle development. Each new shoot, in turn, developed both axillary and lateral buds; apical bud formation was also noted.

Experiments conducted where FRBNH medium was the starting culture medium and subsequent transfers of explant onto ZL-1 medium followed, a slight increase in initial growth rate was observed. Here again, within three months proliferation of tissue had occurred with the development of multiple shoots. Better than 50% of test trials were indicative of these results.

Regardless of which pathway was taken, the end results were similar. By all rights inoculating and subculturing onto hormone-free media should, in fact, have hindered shoot proliferation, but instead it seemed to enhance multiple shoot development. A probable explanation may lie within the physiology of the plant itself or perhaps in the consistent nutrient supply made available to the tissue. Certainly the time of year for bud source material collection has proven to be critical. All conditions being equal, the types of responses just mentioned, are not obtained if the meristematic bud tissue is not at least 2 to 3 cm in size and in the final stages before flushing.

Secondary Shoot Development. Division of plantlets in those cultures showing a proliferation of tissue did not perpetuate continued multiple shoots. Instead, each excised plantlet grew only as a single shoot. Transfer to GA-3 culture vessels (from Magenta Corp.) became necessary as shoots outgrew the dimensions of the culture tube.

Removal of the shoot apex induced small, light-colored lateral and axillary buds that underwent typical development growth. Buds flushed and new shoots and lateral branching with continued bud development occurred.

Retention of the apical portion of the shoot also allowed growth, but larger, darker brown buds were produced that took a longer time to flush than the aforementioned smaller, lighter-colored buds (Figure 1, right). Here again both axillary and lateral bud development was present.

Shoot size and growth reached a plateau approximately 120 days after initial explant inoculation. Throughout early

spring and summer buds swelled and flushed and shoot growth was visible. Average height of plantlets at the end of 120 days growth period was 5 to 8 cm. Cultures exhibited good growth and tissue color. During the winter months, some signs of dormancy within the cultures became evident. There was a slight reduction in active growth accompanied by some browning of the basal needles. An increase in photoperiod to 18 hrs seemed to offset any tendency toward dormancy.

White Spruce

Primary Shoot Development. Initially several mineral salt formulations were selected for experimentation. Nutrient media included Murashige and Skoog salts (5), Woody Plant Medium salts, and B5 (1). All of the above were unsuccessful in generating growth responses and lead to eventual oxidation of the tissue.

Implementation of SH and SH-1 media for culture material proved very positive. Explant inoculation onto SH media, supplemented with various hormone levels, allowed for main shoot axis development. No adventitious shoots have been developed to date either through pulse treatments or repeated exposure to hormone-free nutrient media.

One to two week exposures of explants to hormone enriched SH medium (both SH and SH-1) were found to be optimum and the continued subculturing every 3 to 4 weeks onto hormone-free SHNH basal medium enhanced shoot elongation and lateral bud development along main axes (Figure 2, left). Problems of tissue vitrification were overcome by the incorporation of L-cysteine hydrochloride at 10 mg per litre into the nutrient medium.

Two very distinct types of shoot growth were observed in the white spruce cultures: (1) long, very slender, upright development, up to 4 to 5 cm in height with lateral bud production along main stem, and (2) short, bushy growth up to 2 cm in height with little to no lateral buds, only apical bud formation. (Figure 2, right). One explanation for the variance in shoot growth encountered in the white spruce cultures could be the variability in regional collection of source bud material. Bud development and growth is currently being monitored; to date none of the buds have flushed.

Yellow Cedar — Juvenile Tissue

Primary Shoot Development. The first set of experiments involved the inoculation of vegetative shoot tips 2 to 3 cm in length onto Litvay's medium with hormones. Pulse treatments alternating between a hormone-free medium (1/2MSPNH) to hormone-enriched media (WPM-1′ and LMB) were initiated at two-week intervals. This was essential for the first 4 weeks.

Forty-two days after initial inoculations, adventitious shoots were observed arising from the basal portion of the explant. (Figure 3, left). Slight basal callus formation was also noted. Postliminary subcultures repeated the initial pulse treatments and were able to continue generating multiple shoots.

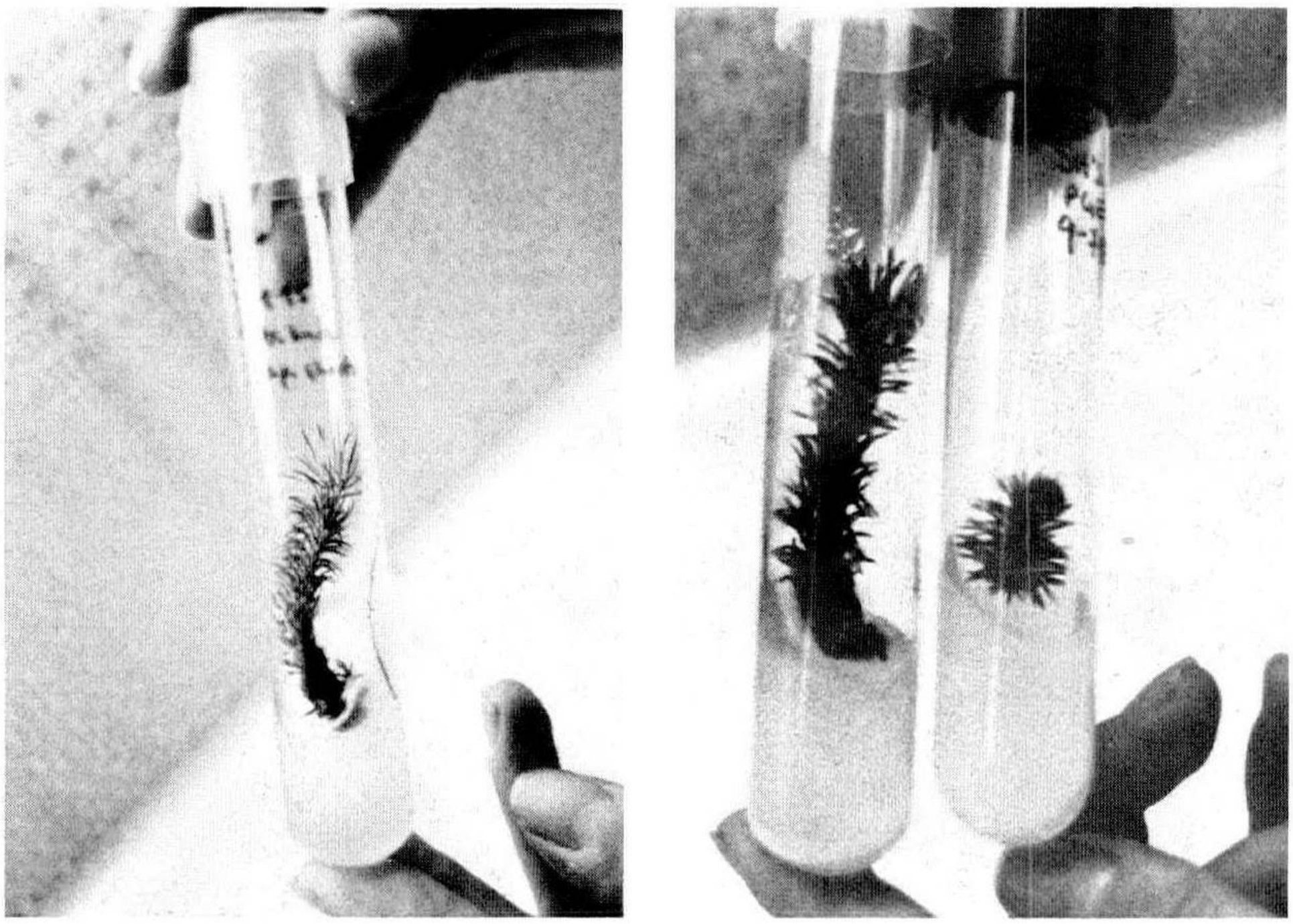

Figure 2. *(Left)*: Elongated single shoot axis of mature white spruce with lateral bud formation.
(Right): Contrast between two distinct types of shoot growth observed in mature white spruce culture.

Secondary Shoot Development. Prolonged exposure of secondary shoots to pulse treatments enabled a trial lot to undergo 1½ years of subculturing with an average increase of 2 to 3 culture tubes each transfer. After that time oxidation of tissue became noticeable. Attempts to reduce browning, which probably resulted from phenolic oxidation of the tissue, were made by using antioxidants singly and in combination at various concentrations and at different stages of secondary shoot formation. However, before a final treatment was selected the cultures had declined beyond the point of responsiveness.

Most recently, new juvenile yellow cedar cultures were established onto 1/2 MSP and 1/2MSP1 media and were then subcultured after 1 to 2 weeks onto 1/2MSPNH (a hormone-free medium), to initiate proliferation of tissue. To date some multiple shoots accompanied by axillary branching and lateral shoot development has occurred.

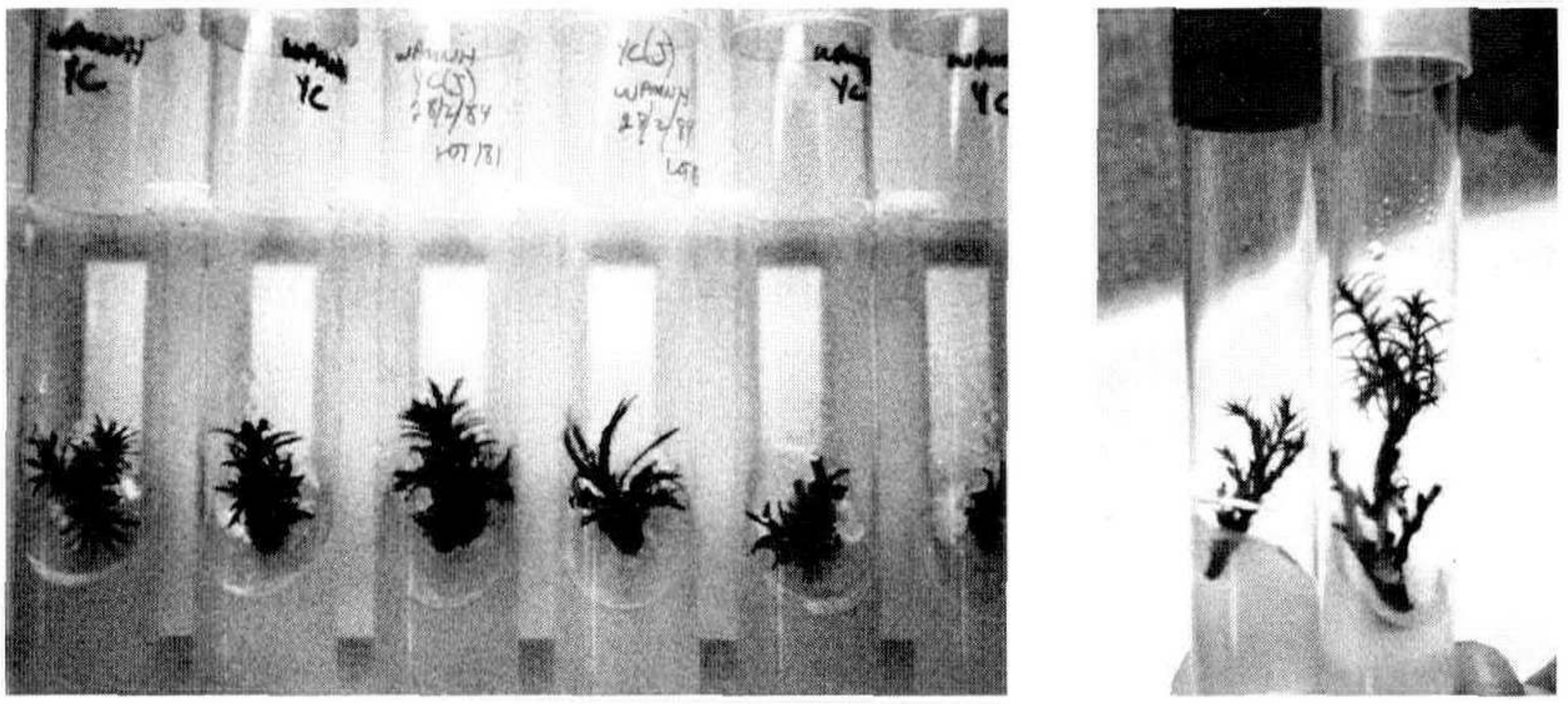

Figure 3. *(Left)*: Juvenile yellow cedar cultures exhibiting multiple shoot proliferation.
(Right): Both apical and lateral shoot growth displaying juvenile characteristics were generated from mature yellow cedar explants.

Growth Levels of Mature Yellow Cedar. Mature yellow cedar tissue was not as responsive when subjected to similar culture manipulations as the juvenile yellow cedar tissue.

Slight lateral branching was observed when the explant was inoculated onto ¼ strength MS mineral salts with low levels of auxins and cytokinin. When MS mineral salts were increased to 50% of full strength with a further reduction in cytokinin levels, apical shoot growth and axillary branch development was enhanced. New shoot growth exhibited distinct juvenile characteristics with dark green prickly scale leaves in comparison with the adult tissue of smooth scale leaves with light green tissue coloration. (Figure 3, right).

Rooting Studies of Juvenile Yellow Cedar Plantlets. Random in-vitro rooting of several juvenile yellow cedar cultures exhibited taproots 3 to 6 cm in length accompanied by lateral roots 1 to 3 cm in length 3 months after initial explant inoculation. (Figure 4, left). No conclusive comparisons can be drawn at this time between rooting of shoots and type of growth medium. Active vegetative growth was noted along with root development.

Greenhouse Care of Yellow Cedar Plants. *In-vivo* rooting trials of juvenile yellow cedar cultures have proven successful. To date only those cultures exhibiting some previous *in-vitro* root formation have survived transplanting to the greenhouse environment.

Upon removal of rooted plantlets from the culture tubes all traces of agar on the root system was washed off so as to prevent substrate sites for pathogen growth.

Figure 4. *(Left)*: A juvenile yellow cedar culture exhibiting *in-vitro* rooting. *(Right)*: One gallon containerized yellow cedar plantlets derived from tissue culture.

Clay's potting mix formula of a well proportioned ratio of peat to perlite provided the soil growing medium. It is essential that the potting mix provide good drainage and aeration otherwise the yellow cedar plantlets, being as susceptible as they are, succumb to basal rot. Plantlets were potted into 2¼ in. pots.

Acclimatization of plantlets included a 10 to 14 week time period under mist tent conditions with high humidity, increased light exposure, bottom bench heat of 20°C, and hand misting twice daily.

Within 3 to 4 months of transplanting, active top growth was observed, indicating an active root structure. One year after initial out-planting, yellow cedar plants were repotted in one gallon nursery containers (Figure 4, right). Root structure was healthy and compact and vegetative growth exhibited excellent color, form and lateral branching. Height of plants upon repotting to one gallon container was 14 to 20 cm. A fertilization program consists of weekly feedings of nitrogen, phosphorus, potassium — 10-52-10, respectively.

The research efforts outlined in this paper are continuing at Les Clay and Son Ltd. But before any method of mass propagation can be considered suitable for use in a tree improvement program, the findings set forth in this paper must be refined to a point where the cultured material can be easily established and maintained, plantlet regeneration can be obtained in a short while, and the newly-formed plants must prove true-to-type and must survive transfer to the field (7).

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PERENNIALS WORTHY OF CULTIVATION

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The University of British Columbia Botanical Garden operates a Plant Introduction Scheme of the Botanical Garden (P.I.S.B.G.) in an effort to introduce new cultivars and recommended plants to the public. The new plants are propagated by the Garden and released to particular nurseries in the province who agree to further propagate them and release them to the public at a later date. Thus far a number of woody materials and ground covers have been released through the P.I.S.B.G. or are now being tested or considered for inclusion. Only one perennial has been released to date, but with the comeback in popularity that perennials have enjoyed in recent years, we are considering other new and under-utilized perennials for inclusion in the P.I.S.B.G.

The first perennial in the P.I.S.B.G. is the blue pimpernel (*Anagallis monelli*). It has been grown to a limited extent in North American gardens, but most plants prove to be annuals or biennials at best. Our perennial plant which we have registered as 'Pacific Blue' is a selection from seed received from the Alpine Garden Society of England in 1980. Large gentain-blue flowers cover the low, spreading plants from late May