OUTLINE OF A SYSTEM FOR *IN VITRO* PROPAGATION OF *SEQUOIA SEMPERVIRENS*

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Abstract. An outline system for the propagation of *Sequoia sempervirens* is described. Propagules were excised from juvenile tissue forced from epicormic buds, disinfected using a calcium hypochlorite and tincture of iodine solution and cultured on half-strength Shenk and Hildebrandt medium. Explant growth and development was stimulated by the inclusion of benzyl amino purine and kinetin in the medium. Shoot elongation occurred in their absence. *In vitro* rooting was inferior to that obtained *in vivo*.

INTRODUCTION

During the past decade, demand for timber and forest products has rapidly increased and, according to Thorpe and Biondi (16), consumption is now outpacing the rates with which forests are maturing. This pressure has led to demands: (a) to develop new methods for the mass production of trees with improved production indices (16), and (b) to grow more trees of all kinds (6). This is further substantiated by the number of symposia held or books published on this topic in recent years (2,3,8,10,14,17).

Most forest trees are sexually propagated and consequently exhibit tremendous variation, variation which cannot be assessed commercially for many years following seed sowing, because of their long life cycles. This variation could be reduced if it were possible to propagate selected clones vegetatively at a sufficient rate to satisfy re-afforestation programmes (11). Although tissue culture has the potential to propagate desirable genotypes rapidly on a large scale and has been applied to several coniferous species (9), in most instances only embryonic or seedling tissue has been satisfactorily propagated (1). However, there are reports where explants from mature trees have been successfully used (4,7,13).

This paper describes an outline procedure using epicormic shoot explants for micropropagating *Sequoia sempervirens*.

MATERIALS AND METHODS

A log measuring 250 × 150 × 100mm (l×b×h) and containing epicormic buds was obtained from a mature field-grown tree in March, 1987. It was placed in a dish containing water to a depth of 25mm and transferred to the laboratory where it was forced for four weeks at 20°C to develop juvenile tissue. Shoot tips measuring 10 mm in length were selected and cultured from the developing shoots, washed under running tap water for 20 min. and then disinfested. This was achieved by placing the shoots for 10 min. in a solu-

tion containing 7.5 percent w/v calcium hypochlorite plus a trace of Tween 20, followed by rinsing in sterile distilled water and re-disinfestation in tincture of iodine for a further 10 min. Finally, the shoot tips were rinsed in sterile, distilled water. All damaged tissue was aseptically removed and the shoot tip explants vertically implanted in a sterile nutrient medium adjusted to a pH of 5.6 ± 0.1 with 0.1 N potassium hydroxide or hydrochloric acid and solidified with 0.8 percent Difco Bacto Agar.

The nutrient medium was autoclaved for 15 minutes at 121°C and 103 kPa, allowed to cool, and approximately 30ml aliquots were poured into disposable 100ml sterilin containers. The cultures were incubated under low light conditions (13.3 μE m^{-2} sec^{-1}) with a 16-hr photoperiod provided by cool white fluorescent tubes. The temperature was maintained at 22°C ± 2°C.

Shoot initiation, multiplication, shoot elongation and rooting were stimulated on half strength Schenk and Hildebrandt (SH) 1972 nutrient medium (Table 1).

No hormones were added to the elongation medium. The auxins, *indoleacetic acid* (IAA), *phenylacetic acid* (PAA) and indolebutyric acid (IBA) were individually used at 1.0mg per litre to stimulate rooting.

Ten explants were initally cultured; thereafter 25 propagules were used. Subculturing occurred at four-week intervals when the numbers of shoots and buds produced were counted and the propagules re-inoculated onto the multiplication, elongation, or rooting media. A minimum of three subcultures were carried out prior to transfer to the latter two.

In vivo rooting and weaning of the microshoots was achieved in a 100 percent peat compost or maintained in a high humidity greenhouse enviroment provided by fog. The temperature of the rooting medium was maintained at 18°C, while that of the greenhouse fluctuated between 15°C and 25°C.

Table 1. *Constituents of the ½ dilution Shenk and Hildebrandt caulogenic medium.*

A. Mineral Salts			
Macro elements	Mg l^{-1}	Micro elements	mg l^{-1}
KNO_3	1250	$MnSO_4 \cdot H_2O$	5.0
$CaCl_2 \cdot 2H_2O$	100	$ZnSO_4 \cdot 7H_2O$	0.5
$Mg\ SO_4 \cdot 7H_2O$	200	H_3BO_3	2.5
Na_2 EDTA	7.5	KI	0.5
$Fe\ SO_4 \cdot 7H_2O$	10	$Na_2\ Mo\ O_4 \cdot 2H_2O$	0.25
$NH_4\ H_2\ PO_4$	150	$Cu\ SO_4 \cdot 5H_2O$	0.1
		$Co\ Cl_2 \cdot 6H_2O$	0.05
B. Organic constituents			
Sucrose	30,000	Niacin	2.5
Agar	8,000	Pyridoxine HCl	0.25
Myo-Inositol	500	BAP	1.1
Thiamine HCl	2.5	Kinetin	1.1

RESULTS AND DISCUSSION

Surface sterilization. The sterilization of explants from mature conifers grown under field conditions is regarded by many investigators as being difficult to achieve. For instance, Boulay (5) obtained less than 50 percent asepsis on *Sequoia sempervirens,* while Gupta and Durzan (7) obtained 90 percent for *Pseudotsuga menziesii* and *Pinus lambertiana* using a multistep sterilization procedure, one which was ineffective for mature field grown and immature greenhouse forced *Pinus contorta* shoots (O'Donnell 1987 unpubl.). In this work, 100 percent asepsis was achieved with explants derived from epicormic shoots, a factor being attributed to the double sterilization technique involving tincture of iodine, and forcing of the juvenile tissue.

Shoot initiation and development. Establishment and development of shoot tip explants occurred on the 50 percent dilution Schenk and Hildebrandt medium (Table 2).

After four weeks in culture each explant had developed two axillary buds, with a mean height of 2.4mm. When these were subcultured, growth and development occurred rapidly and after a further four weeks produced 5.2 shoots and 10.9 buds, respectively. The average length of these shoots was 19.8mm while bud size was similar to that of the developing explants.

These results are superior to those reported by Boulay (5) and reflect at least a 25 percent increase in growth rate compared with that obtained from the 50 percent dilution Murashige and Skoog (MS) (12) medium. When the propagules were subcultured onto the elongation medium (minus hormones) both extension growth and further shoot multiplication occurred. However, bud initiation was suppressed (Table 2). Shoot length ranged from a minimum of 10.0mm in length for the newly initiated shoots, to 67.0mm for those that were subcultured. The mean was 38.5mm.

Table 2. Influence of medium on explant establishment, multiplication, and elongation.

Medium	Mean No. of buds per explant/ propagule	Mean height of buds (mm)	Mean No. shoots per explant/ propagule	Mean shoot height (mm)
Inoculation	2.0	2.4	—	—
Multiplication	10.9	2.5	5.2	19.8
Elongation	0.0	—	3.0	38.5

The occurrence of rapid elongation in the absence of activated charcoal is in direct contrast to the work of Boulay (4,5) but is in agreement with that of Que (13) and demonstrates clearly that activated charcoal alone is not a prerequisite for inducing shoot elongation; 50 percent dilution SH medium is superior to the 50 percent dilution MS for micropropagating this species.

Rooting *in vitro* was unsatisfactory. In fact, indolebutyric acid (IBA) was the only one of the three auxins used individually in the rooting medium that stimulated root formation (Table 3).

Rooting was erratic and only 25 percent of the cultures rooted, even after eight weeks in culture. This is similar to the results obtained by Boulay (5) with IAA.

Contrary to expectation, PAA failed to induce root formation, even when used at higher levels than that recorded in Table 3. It is thus probable that its slow rate of degradation in the culture medium was insufficient to counteract its low activity levels.

Satisfactory rooting (at least 95 percent) was obtained when the *in vitro* produced shoots were inserted in a medium grade peat compost and maintained under fog. Root initiation was observed after seven days and the micro-cuttings were sufficiently rooted for potting-on after 28 days. Satisfactory *in vivo* rooting has also been achieved with Sequoia by other investigators (4,5,13).

Table 3. Effect of three hormones on rooting *Sequoia sempervirens* propagules after 8 weeks in culture.

Treatment	Percent rooting	No. roots/propagule	Mean root length (mm)
IAA	0	0	0
PAA	0	0	0
IBA	25	3.5	44.5

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MICROPROPAGATION: WHY WYEVALE NURSERIES TOOK THE PLUNGE

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In early 1988 Wyevale Nurseries entered the field of plant micropropagation by setting up a small self-contained laboratory next to its existing propagation facilities. This unit consists of a Portacabin with internal fittings supplied to our own specifications. It is divided into three areas:

1) The main work area comprised of a laminar air flow unit, media preparation area, and sink,

2) Growth room

3) Changing room that doubles as an airlock avoiding direct introduction of air, dust, and people from the outside.

We decided to go for a purpose-built unit because we believe this will reduce the chances of cross contamination and help us run a small lab efficiently. This will leave us more time to consider plant growth problems and thus reduce the lag time between start up and full production which many labs have encountered.