

Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*)

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ABSTRACT

Opening of apical and axillary buds of mature Douglas-fir and sugar pine trees was obtained on a newly formulated basal medium (DCR) without growth regulators. Elongation of buds was observed on 1/2 strength DCR with 0.3% activated charcoal (DCR-1). In sugar pine, multiple shoots were obtained when explants on DCR with 0.5 mg/l BAP for 5-6 weeks were transferred to DCR-1 medium. On subculture, axillary buds again developed when shoots were cultured on DCR with 0.2 mg/l BAP for Douglas-fir and 0.5 mg/l BAP for sugar pine. These buds were again elongated on DCR-1 medium. By subculturing 7-10 shoots of Douglas-fir and 2-3 shoots of sugar pine, over 100 shoots can be obtained in a year.

ABBREVIATIONS

BAP, N⁶-benzylaminopurine
 KN, kinetin
 NAA, α -naphthalene acetic acid
 IAA, Indole-3-acetic acid
 MS, Murashige-Skoog medium
 WPM, woody-plant medium

INTRODUCTION

This paper describes a method for *in vitro* multiplication of shoots from 20 to 60-year-old trees of Douglas-fir (*Pseudotsuga menziesii* Franco.) and 8 to 30-year-old trees of sugar pine (*Pinus lambertiana* Dougl.). Douglas-fir and sugar pine are the most genetically diverse of the forest tree species. The clonal propagation of mature elite trees will contribute to the domestication of these trees.

Success with explants from mature trees has been achieved for the propagation of some hardwoods by tissue culture (Mascarenhas et al. 1982, Sommer 1983). There are also several reports on propagation of conifer from juvenile tissue (David 1982) but very little success has been achieved using explants from mature conifers (Bonga 1981, Boulay 1979ab, Franclet 1980). This study differs from the above by use of improved surface sterilization methods and by the development of a modified method and media that appear to be applicable to mature conifers for shoot multiplication.

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MATERIALS AND METHODS

Plant Materials

Lateral branches (10-20 cm length) containing preformed apical and axillary buds were collected from July to October from lower branches of 20 to 60-year-old Douglas-fir and 8 to 30-year-old sugar pine. Shoots were brought to the laboratory in plastic bags in a box cooled with ice. Trees were provided by the US Forest Service at the Institute of Forest Genetics, Placerville, California.

Surface Sterilization

Shoots (8-10 cm length) cut from lateral branches were surface-sterilized by the following method: 1) washed with running tap water (3-4 min); 2) treated with detergent (0.1% v/v Linbro) for 5-10 min and washed with distilled water (3-4 times); 3) treated with 30% H₂O₂ for 15 min and washed with distilled water (3-4 times); 4) treated with 10% v/v chlorox for 10 min and again washed with distilled water (3-4 times); 5) sterilized with 0.05% w/v HgCl₂ for 10 min and washed with sterile water (6-8 times) in a sterile cabinet.

Treatments 2 through 5 were carried out in glass flasks on a shaker rotating at 150 rpm. Shoots (3-4 cm) were excised and inoculated in different media.

Culture Media and Conditions

MS (Murashige and Skoog 1962), WPM (Lloyd and McCown 1980), and a modified basal medium (DCR) were used with cytokinins BAP, KN (0.1 to 2.0 mg/l) and auxins, NAA, IAA (0.01 to 0.1 mg/l) or without growth regulators. The DCR basal medium (final conc. mg/L) is composed of: NH₄NO₃, 400; KNO₃, 340; Ca(NO₃)₂·4H₂O, 556; KH₂PO₄, 170; MgSO₄·7H₂O, 370; CaCl₂·2H₂O, 85; H₃BO₃, 6.2; MnSO₄·4H₂O, 22.3; ZnSO₄·7H₂O, 8.6; CuSO₄·5H₂O, 0.25; KI, 0.83; FeSO₄·7H₂O, 27.8; Na₂EDTA, 37.3; CoCl₂·6H₂O, 0.025; NiCl₂, 0.025; NaMoO₄·2H₂O, 0.25; Thiamine·HCl, 1.0; Pyridoxine·HCl, 0.5; Nicotinic Acid, 0.5; Glycine 2.0; myo-Inositol, 200; Sucrose, 30g/L.

All media were adjusted to pH 6.0 at 24°C with KOH and HCl and solidified with Bacto agar (Difco) 0.6%. All media were autoclaved (1.1 Kg cm⁻² at 121°C for 20 min) after addition of growth regula-

tors as required. Activated charcoal 0.3% (w/v) (Baker Chemical Co., Phillipsburg, NJ) was used in elongation medium (DCR-1).

All cultures were incubated under continuous light at 22°C and ca. 60% relative humidity. The light source was cool white fluorescent tubes. Light intensity did not exceed 2.8, 0.5 and 2.0 $\mu\text{W cm}^{-2} \text{ nm}^{-1}$ in the blue, far-red and red spectrum, respectively.

Experimental Design

Combinations of culture media were treated with growth regulators in a Latin square design with 10 replications. The replicated combinations, treatments, and subsequent sequential treatments were repeated at least twice for each species.

RESULTS AND DISCUSSION

Surface Sterilization

We have obtained 90% asepsis among shoots from Douglas-fir and sugar pine over all seasons. We found that all steps listed were necessary to obtain optimal asepsis for shoots from different trees. With explants from mature trees, asepsis is essential to initiate tissue culture studies either for clonal propagation or genetic manipulation of forest trees.

The sterilization of explants from mature conifers grown under field conditions is difficult. Keathley (1984) used 25, 50, and 100% chlorox for sterilization of shoots from mature conifer trees but no treatment yielded sterile, viable shoots. Timmis and Ritchie (1984) also stated that Douglas-fir shoots from mature trees were extremely difficult to surface-sterilize without injury.

Bud Break and Callus Formation

Within 4-5 weeks, buds were opened on 70-80% of the shoots on DCR basal medium without any growth regulators both from Douglas-fir (Fig. 1A) and sugar pine (Figs. 1B,C). Opening of buds was also observed for 5-10% of the shoots on MC and for 30-40% of the shoots on WPM without any growth regulators. Callus always formed in the presence of cytokinins and auxins, alone or in combination. Under these conditions no opening of buds was observed.

When newly flushed buds were excised and transferred to 1/2 strength DCR basal medium with 0.3% activated charcoal (DCR-1), all the flushed shoots elongated and formed complete shoots within 5-6 weeks for Douglas-fir (Fig. 2A) and 7-8 weeks for sugar pine (Fig. 2B).

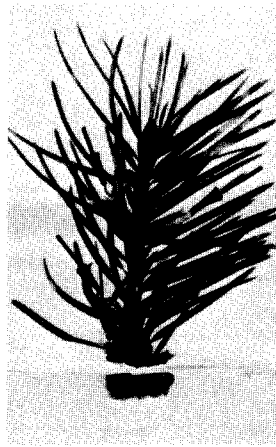
In sugar pine, multiple shoots (Fig. 3A) were obtained on top of the elongated buds after initial explants were cultured on DCR basal medium with 0.5 mg/l BAP for 6-7 weeks and then transferred to DCR-1 medium for 7-8 weeks.

These results extend methods used in earlier reports for *Pinus pinaster* shoots (Franclet 1980) and redwood tree shoots (Boulay 1979a).

Multiplication on Subculture

Flushed and elongated shoots of Douglas-fir were subcultured onto basal medium with 0.2 mg/l BAP. Within 4-5 weeks, again 2-3 axillary buds were

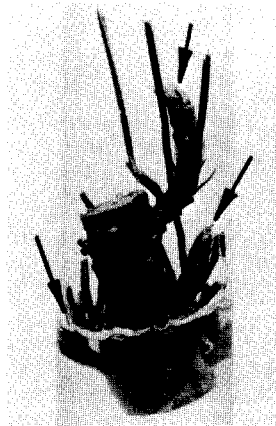
developed on each shoot (Fig. 3B). These buds were again elongated to form shoots on DCR-1 medium as described earlier.



A. Douglas-fir after 30 days on DCR



B. Apical buds of sugar pine after 35 days on DCR

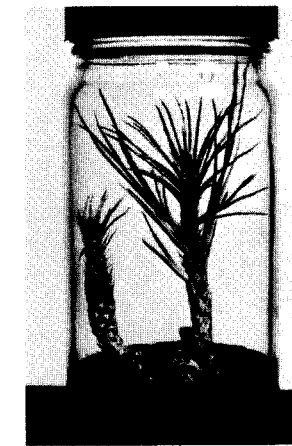


C. Axillary buds of sugar pine after 35 days on DCR

Fig. 1 Opening of apical and axillary buds (arrows) on explanted shoots from mature trees on DCR medium.



A. Elongation of axillary buds after 40 days on DCR-1



B. Elongation of apical bud of sugar pine after 50 days on DCR-1

Fig 2 Elongation of axillary and apical buds on DCR-1 medium.

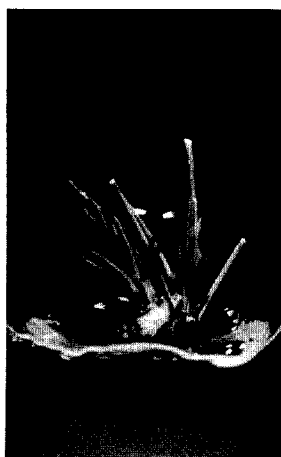
In sugar pine, multiple shoots were separated and cultured onto DCR-1 medium. Within 4-5 weeks these shoots elongated. Segments (10-15 mm) were also excised from elongated buds and cultured onto DCR basal medium with 0.5 mg/l BAP. Within 5-6 weeks each explant again developed multiple shoots (2-3) (Fig. 3C). These shoots were repeatedly elongated on DCR-1 medium as described earlier.



A. Multiple shoots of sugar pine on DCR-1 medium After 50 days



B. Development of axillary buds of Douglas-fir on subculture on DCR with 0.2 mg/l BAP



C. Multiple shoot formation on subculture of elongated buds of sugar pine on DCR with 0.5 mg/l BAP

Fig. 3 Axillary bud development and multiple shoot formation (arrows).

If the present rate of multiplication were maintained, approximately 100 viable shoots can be obtained from 7-10 shoots of Douglas-fir and 2-3 shoots of sugar pine in a year from mature trees. This rate is based on subculture at 8-to-10-week intervals.

As far as we are aware, this is the first report where a high percentage of sterile shoots and high multiplication rates have been achieved for mature trees.

Recently this technique was successfully applied to obtain multiple shoot formation from explants of 20-year-old disease ("blister rust") resistant clones growing at Badger Hill Breeding Arboretum, Placerville, California.

There are no reports on plantlet regeneration either from juvenile or mature tissues of sugar pine. Rooting of these shoots has not yet been tested, but that is the next step. Well-established methods for shoot culture are central to successful domestication of selected genotypes as well as to genetic improvement of trees using cellular techniques such as protoplast manipulation (Durzan 1980, McCown 1984).

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