Micropropagation and Phase Specificity in Mature, Elite Douglas fir

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Abstract. Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) plantlets, micropropagated from axillary buds of nodal segments from invigorated stems and adventitious buds from young needles of two 60-year-old trees, have been established in soil. Stem tips with half-pruned-back needles from the lower crown, when placed on DCR medium, sprouted buds in 90% of explants. Shoots from bud sprouts were plagiotropic when collected from explants of shoots with a horizontal and downward growth habit. By contrast, upright growth of micropropagated shoots was observed in all explants from branches with an upright habit. After four subcultures (3- to 4-week intervals on 0.5 mg-liter\(^{-1}\) BA in DCR), sprouted shoots of nodal segments from all explant sources produced four to six multiple buds. With excised needles of sprouted buds, numerous (>10) adventitious buds formed after 11 to 12 weeks on needle surfaces after the fifth subculture on DCR with 0.5 mg-liter\(^{-1}\) BA. All adventitious and axillary buds elongated on 0.5 DCR lacking plant growth regulators. Rooting of the elongated shoots was induced in 20% of the explants with 0.2 mg-liter\(^{-1}\) each of NAA and IBA. In all instances, buds programmed for the development of female reproductive cones continued their phase-specific development even under conditions conducive to rejuvenation of vegetative tissues. Chemical names used: N-(phenylmethyl)-1H-purin-6-amine (BA); 1-naphthaleneacetic acid (NAA); 1H-indole-3-butyric acid (IBA).

This study describes a method for adventitious bud formation, high shoot-multiplication rates, rooting, and establishment of plantlets in soil for explants of two 60-year-old elite Douglas firs. In seed orchards and in nature, Douglas fir is reproduced sexually. Seed years are variable and cones are susceptible to considerable insect damage. Micropropagation offers an asexual method to capture the additive and nonadditive genetic variance in the laboratory. Micropropagation is currently limited to juvenile material by high costs and by poor success with explants from mature elite trees (1–3). Some of the more important genetic traits (e.g., heavy seed production, tree shape) are identifiable only at maturity, and, for this reason, many breeding programs rely on juvenile–mature correlations for progeny selection (4). Previously, we reported on shoot multiplication with mature Douglas fir (6) and provided comparative data on shoot and root development in juvenile and mature trees (9). Our study extends that work to include invigorated micropropagated shoots from two 60-year-old trees. It also shows that conditions conducive to rejuvenation of vegetative tissues do not change the determination of buds already programmed for female cone production.

Materials and Methods

Explant selection. Lateral branches (20 to 30 cm) at 1.8 to 2.5 m in height of two 60-year-old trees were collected the first week of the month over the year. Trees represented elite Douglas fir grown at the El Camino progeny test by the U.S. Forest Service, Institute of Forest Genetics, Placerville, Calif. (lat.38°54′4.2″N, long.120°6′W, elev. 1220 m). Branches with horizontal habits, branches oriented upwards, and branches oriented downwards were brought into the laboratory at Davis, Calif. (lat.37°32′N, long.122°5′W, elev. 18.3 m) sealed in plastic bags with wet paper towels.

Surface sterilization and culture. Nodal segments (2 to 3 cm long) of branches were surface-sterilized and inoculated on DCR medium as described (6). Methods for axillary bud multiplication and elongation of shoots up to the fourth subculture (3- to 4-week intervals) were identical to those reported earlier (9).

All media were prepared and treated as follows. Freshly prepared DCR basal media (6) with growth regulators were adjusted to pH 6.0 at 24°C with KOH and HCl, autoclaved (1.1 kg-cm\(^{-2}\) at 121°C for 7.5 min), and solidified with 0.7% Difco Bacto-agar. Cultures were incubated at 23°C ± 1°C and ~60% RH, continuous cool-white fluorescent light, 25 cm above the culture vessels. Light did not exceed 5.0, 2.0, and 0.5 \(\mu\)W·cm\(^{-2}\)·nm\(^{-1}\) in the blue, red, and far-red spectrum, respectively.

Experimental design. All the experiments were carried out with 100 stem segments and collected three times (2nd, 5th, and 7th day of every month). Results are means of stem segments collected on three dates. Experiments were repeated at least twice in two years and found reproducible. This design included the effect of needle integrity on the sprouting of axillary buds, crown position, adventitious bud development, shoot multiplication rates after the fourth subculture, and rooting of micropropagated shoots.

Results

Effect of needles on sprouting of axillary buds. Stem segments were cultured on DCR medium (6) in three ways: a) with all needles removed, b) half-pruned-back needles, and c) intact needles. Ninety percent of the axillary buds sprouted from each stem segment with half-pruned needles (Fig. 1), in contrast to 40% with intact needles. Axillary buds did not sprout on explants with all needles removed. Season was a very important factor for mature tree tissue culture. As reported previously (9), spring (March–April) was found to be the best time to initiate tissue cultures from mature trees. Hence, all subsequent experiments were carried out in March and April.

Plagiotropic growth of shoots was obtained in culture from stem segments collected from horizontal branches with a downward habit. Shoot growth was orthotropic in culture from stem segments collected from branches that were growing upward (Fig. 2).

Bud multiplication after fourth subculture. After the fourth
subculture, axillary buds sprouted after 10 weeks as compared to second and third subcultures, when sprouting occurred after 3 to 4 weeks. Different BA concentrations were tested at the fourth subculture to improve sprouting of buds. Within 4 to 5 weeks, each nodal segment, excised from in vitro shoots, developed four to five axillary shoot buds on DCR medium with BA (0.5 mg-liter\(^{-1}\)) (Fig. 3). Within 5 weeks, these buds elongated to 30 to 40 mm on 0.5 DCR basal medium without activated charcoal (Fig. 4). Charcoal was essential for elongation at initial and early stages (9). So far, 10 subcultures have been carried out without diminishing shoot multiplication and elongation. Four to five shoots were obtained from a single explant at each subculture, thus showing that tissues were stabilized in culture. We have maintained cultures by subculturing shoots every 4 to 5 weeks for 2 years.

Adventitious bud development. After the fifth subculture, needles from sprouting buds touching the medium developed adventitious buds (Fig. 5). Multiple adventitious buds developed on DCR medium with BA (0.5 mg-liter\(^{-1}\)) when incubated for 11 to 12 weeks. These adventitious buds were elongated on 0.5 DCR basal medium. Elongation occurred after 9 to 10 weeks, in contrast to axillary buds, which required 4 to 5 weeks.

Female cone development. Three percent to 4% of the nodal segments developed female cones after 11 to 12 weeks on 0.5 DCR medium with 0.25% (w/v) activated charcoal (Figs. 8 and 9). Nodal segments were excised from elongated shoots growing on DCR medium before subculture to a medium containing BA (0.2 mg-liter\(^{-1}\)). Longer incubation on the medium with charcoal is necessary for cone development.
Rooting. After the fifth subculture, shoots elongated to 30 to 40 mm on 0.5 DCR medium and were harvested for rooting. For rooting, shoots were treated with NAA and IBA (0.2 mg-liter\(^{-1}\) each) added to the medium on 0.5 DCR medium for 15 days in dark at 22\(^\circ\)C. After 15 days, they were transferred to 0.5 DCR medium with 1% sucrose without plant growth regulators and incubated in white light. Twenty percent of shoots were rooted after 12 weeks (Fig. 6). Earlier, 5% rooting was obtained after the third subculture (9). Following root initiation, plantlets that continued to grow were transferred to plastic pots containing 1 sterile peatmoss: 2 vermiculite: 1 perlite (by volume) (Fig. 7).

Discussion

Our results are based on widely spaced, mature Douglas fir trees under intensive cultivation and in full sun. Here, explants taken from the base of cultivated trees are different from explants of trees in natural stands, where the lower branches are heavily shaded. Branches from our trees provided vigorous vegetative explants and represent extremes in topophyll (position), periphysis (local environment), and cyclophysis (age).

An unexpected observation with explants from horizontal branches with downward or upright habits was that the pruning back of needles played an important role in sprouting of buds. Another factor improving the performance of the micropropagation method, especially for the production of orthotropic vs. plagiotropic shoot development, was explant position in the crown (5). Shoots derived from upright branches and not from lateral basal sprouts grew upright. Results reaffirm earlier observations with *Sequoia sempervirens* (D. Don) Endl (1) and *Cunninghamia lanceolata*, Hook. (5). A more intense degree of plagiotropic growth was seen in *Cunninghamia*, even after 10 subcultures (5).

Shoot multiplication depended on cytokinin invigoration of shoots by repeated sprouting and subculture. In Douglas fir grown at 1220 m, the threshold that permitted micropropagation, was expressed at sea level at Davis, Calif. and was independent of time of year. Since complete rejuvenation was not achieved, further improvements in micropropagation of mature trees are required. Results show the importance of explant choice and the physiological state of cells during growth-regulator-directed expressions of totipotency.

Over the past 2 years, our results indicate that micropropagation of mature Douglas fir, while difficult, can be improved. Because of the wide genetic variation of this species, we believe that elite clones may be identified that improve micropropagation and rooting, as has been accomplished with loblolly pine (12). Further, with Douglas fir (unpublished data), we now have invigorated shoots from this study that may be amenable to phase changes and somatic embryogenesis (7, 8, 10).

The determination of buds programmed for reproductive growth was not reversed by our procedures. The opportunity arises to study cone development and meiosis in vitro under conditions that permit reproductive development. All of the above raises questions as to the expression of mature characteristics in vitro, especially since these have been observed earlier in tissue culture plantlets derived from embryos of loblolly pine (12). In the interim, our technique can be used, as appropriate, to establish high-value individual elite trees for progeny tests and in seed orchards. Trees cloned in this way are useful for breeding in tree improvement programs.

Literature Cited