VEGETATIVE PROPAGATION OF RADIATA PINE BY TISSUE CULTURE: PLANTLET FORMATION FROM EMBRYONIC TISSUE

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ABSTRACT

Numerous adventitious buds were induced when fully developed embryos of **Pinus radiata** were placed on a nutrient agar medium containing cytokinin. The adventitious buds formed directly from the cotyledons and hypocotyls, and often also from meristematic tissue proliferating from these. The meristematic tissue has been maintained in culture for 6 months and still gives rise to many more adventitious buds. The buds, when separated and grown individually on a medium without cytokinin, developed into well-formed shoots. These rooted after approximately 6 months in culture and have developed into sturdy plants.

INTRODUCTION

Vegetative propagation plays an important part in the genetic improvement of *Pinus radiata* D. Don (radiata pine) in New Zealand. It is used for the preservation of special genotypes and the establishment of seed orchards. There is also interest in the clonal propagation of superior trees for plantation establishment (Thulin and Faulds, 1968). In the past, grafting of scions from parent trees on to seedling root stock was the main means of asexual propagation. However, incompatibility between root stock and scion may occur with time, resulting in the death of the graft (Sweet and Thulin, 1973). More recently, rooted cuttings have been used but, with radiata pine and many other species, this technique has its problems. Cuttings from older trees do not form roots as readily as those from young trees, and some genotypes fail to root at all.

Tissue culture methods may offer an additional or alternative way of propagating selected genotypes. For a number of plants, e.g., orchids, gerbera, and African violets, tissue culture has proved to be a practical means of rapidly multiplying selected varieties (Murashige, 1974; Pierik *et al.,* 1975; Start and Cumming, 1976).

Until recently there have been few reports of organogenesis in tissues of gymnosperms grown in culture. Among the conifers, varying degrees of success have been obtained with *Sequoia sempervirens* (D. Don) Endl. (Ball, 1950), *Biota orientalis* L. (Konar and Oberoi, 1965), *Pinus gerardiana* Wall. (Konar, 1975), *Cryptomeria*

japonica D. Don (Isikawa, 1974), *Pinus palustris* Mill. (Sommer *et al.*, 1975), *Pseudotsuga menziesii* (Mirb.) Franco (Cheng, 1975; Sommer, 1975), and *Picea glauca* (Moench.) Vos. (Campbell and Durzan, 1975; 1976). In this paper we describe, for the first time, the differentiation of plantlets from cotyledonary and hypocotyl tissue of fully developed embryos of radiata pine.

MATERIALS AND METHODS

Seeds of radiata pine were placed in a cheesecloth bag and surface-sterilised in a saturated solution of calcium hypochlorite for 15 minutes. After being rinsed in running water for 24 hours, the seeds were placed in a plastic bag and refrigerated for 2 days at 5°C. They were surface-sterilised again, this time in 5% hydrogen peroxide for 4 minutes, and rinsed in sterile water. The embryos were dissected out from the seed either under sterile conditions in a laminar flow hood, or in the laboratory (and then surface-sterilised in 5% H_2O_2 containing a trace of Tween 80 for 2-3 minutes), then rinsed in sterile water. The embryos were cultured in petri dishes (five embryos per 90 × 15-mm dish) containing nutrient media adjusted to a pH of 5.6-5.8 and gelled with 3.7 g/litre of Difco purified agar. The nutrients were autoclaved for 15 minutes at 1.4 kPa. Petri dishes were sealed with plastic film and placed under cool white fluorescent lights. The temperature varied from 20° to 25°C. Although a range of media was tested, two main ones were used for the cultural procedure. Shoot initiation and elongation were stimulated on a Schenk and Hildebrandt (1972) nutrient medium (SH) containing (per litre): 2500 mg KNO_3, 400 mg $MgSO_4.7H_2O$, 300 mg $NH_4H_2PO_4$, 200 mg $CaCl_2.2H_2O$, 10.0 mg $MnSO_4.H_2O$, 5 mg H_3BO_3, 1 mg $ZnSO_4.7H_2O$, 1 mg KI, 0.2 mg $CuSO_4.5H_2O$, 0.1 mg $NaMoO_4.2H_2O$, 0.1 mg $CaCl_2.6H_2O$, 15 mg $FeSO_4.7H_2O$, 20 mg Na_2EDTA, 100 mg myo-inositol, 5.0 mg thiamine HCl, 5.0 mg nicotinic acid, 0.5 mg pyridoxin-HCl, and 30 g sucrose. Depending on the experiment, the culture medium was supplemented with one or more of the following: glutamine (filter-sterilised) 200 mg/litre, the auxins, indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), and the cytokinin, N_6benzylaminopurine (BAP) at varying concentrations. In another experiment embryos were placed on a medium containing one-fifth the concentration of nutrients except for sucrose which was at a concentration of 10.0 g/litre. At various time intervals adventitious shoots were transferred to a "root-initiating" medium. One of the successful nutrient media (GD), a modification of Gresshoff and Doy's (1972) nutrients, contained (per litre): 200 mg $(NH_4)_2SO_4$, 150 mg $CaCl_22H_2O$, 250 mg $MgSO_4.7H_2O$, 1000 mg KNO_3, 90 mg $NaH_2PO_4.H_2O$, 30 mg $NaHPO_4$, 27.8 mg $FeSO_4.7H_2O$, 38.3 mg Na_2EDTA, 10 mg $MnSO_4.H_2O$, 3 mg $ZnSO_4.7H_2O$, 3 mg H_3BO_3, 0.75 mg KI, 0.25 mg $CuSO_4.5H_2O$, 0.25 mg $NaMoO_4.2H_2O$, 10 mg inositol, 20 g sucrose, 1.0 mg thiamine HCl, 0.1 mg nicotinic acid, 0.1 mg pyridoxin, 0.5 mg NAA, and 2.0 mg IBA.

After roots had been induced on adventitious shoots, the plantlets were rinsed under tap water to remove agar adhering to the roots and were placed in non-sterile water in test tubes. Two to three weeks later they were planted in pots containing a mixture of garden soil, vermiculite, fine pumice, and peat (4 : 1 : 2 : 1).

For microscopic examination, hand-cut sections of cotyledons and outgrowths were stained with acetocarmine, gently heated, and examined under the light microscope.

RESULTS

Bud Initiation

Embryos placed on SH medium containing BAP (at concentrations varying from 0.05 ppm to 25 ppm) became swollen and succulent after 5 days in culture (Fig. 1A). The cotyledonary and hypocotyl portions turned green and the radicle formed a bright red callus except that at 0.05 ppm BAP the radicle did not form callus but elongated and grew into the agar. Embryos dissected in the laminar flow hood survived better (95% survival) than did embryos that required surface sterilisation in hydrogen peroxide (72% survival). The former technique was employed in most experiments.

Two to three weeks after embryos were placed on the agar, small buds and clusters of small primary leaves were visible on the cotyledons and on some hypocotyls. Two different types of responses were noted depending on whether the cotyledons were in contact with the medium. Cotyledons touching the medium became very swollen and often smooth-surfaced tissue proliferated from them (Fig. 1B). Microscopic examination showed such cotyledons and tissue to be composed of small meristematic cells with large nuclei. Some of this tissue gave rise to masses of tiny buds with clusters of small scale-like primary leaves (Fig. 1C). In contrast, cotyledons not in contact with the medium swelled very little and only one or two individual buds at the tips of the cotyledons were formed (Fig. 1D). These quickly grew into small shoots.

The effect of BAP concentration on bud formation (percentage of embryos forming buds; 25 or more embryos per treatment) did not vary appreciably, results being as follows:

BAP (mg/litre)	0.05	0.1	0.25	0.5	1.0	5.0	10.0	25.0
Embryos (%)	29	61	55	86	74	92	76	80

At high concentrations of BAP (i.e. 10 ppm and 25 ppm) it was noted that cotyledons not in contact with the agar formed buds at the cotyledonary tips more frequently than did the cotyledons in treatments with lower BAP concentrations. The total number of buds formed per treatment was difficult to estimate since the clumping together of many tiny leaves and buds made counting extremely difficult.

On one-fifth SH medium the cotyledons did not swell and there was no proliferation of meristematic tissue. Instead, tiny leaves were formed directly on the cotyledons in contact with the medium and a few small buds developed on the tips of cotyledons not touching the agar.

When filter-sterilised glutamine was added to the SH medium containing 5 ppm BAP, the buds and needle primordia were greener than in control treatments without glutamine.

Bud Proliferation and Elongation

On the shoot-inducing media (SH + BAP) the clusters of buds formed from the swollen meristematic cotyledons did not develop beyond 1 mm in length. In fact, when left on the same medium for more than 8 weeks, callus grew over the buds and the cultures eventually died. However, when portions of the cotyledons bearing the bud masses and smooth meristematic swellings were dissected from the embryos and transferred to SH medium without cytokinin (SHO) the leaves elongated and after 3 weeks individual shoots could be recognised. When separated and placed on fresh

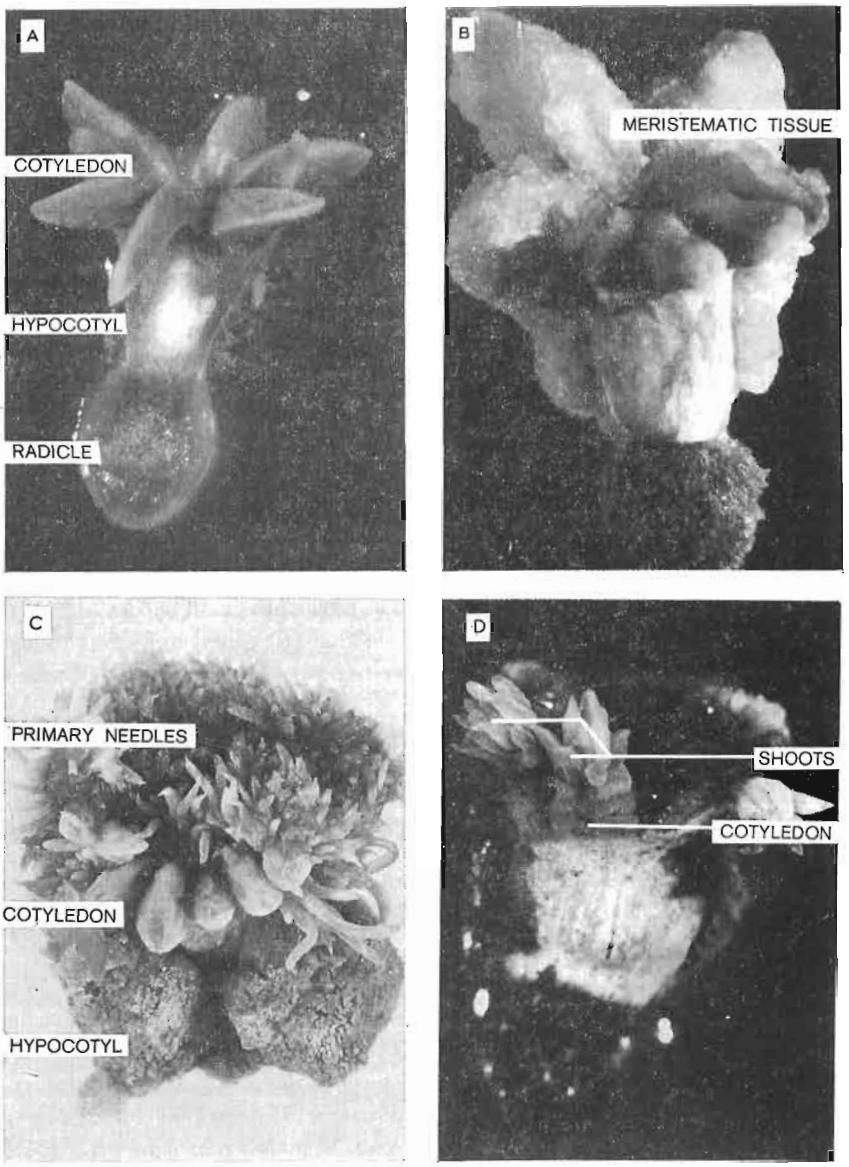

FIG. 1—(A) Embryo after 5 days in culture on SH 5 ppm BAP. The cotyledons have become green and the radicle swollen and red. X10. (B) Smooth-surfaced meristematic tissue forming from cotyledons and hypocotyl after 2½ weeks in contact with agar. X10. (C) Clusters of primary leaves originating from the cotyledonary meristematic tissue. X5. (D) Whole embryo showing shoots formed on tips of cotyledons. X10.

medium (SHO) these grew into well-formed shoots (Fig. 2). The remaining meristematic tissue was also subcultured on to SHO and this proliferated to form more meristematic tissue and buds. These were also separated and the process was repeated every 4 weeks. In this way over 200 shoots from the one embryo have been obtained over a period of 6 months.

Root Formation

The response of shoots placed on a medium containing auxin depended on the "history" of the shoot. Small shoots, which were dissected from embryos 4-6 weeks after first being initiated, formed only green friable callus at their base when placed on SH medium containing 1 to 25 ppm IBA. When shoots were allowed to elongate on SHO medium for 3 months and then placed on SH + auxins (as above) little callus formed, nor were roots initiated. However, some shoots which grew vigorously on SHO for 6 months formed roots without the addition of auxin. Others developed roots when transferred to a modified Gresshoff and Doy medium containing 0.5 ppm NAA and 2.0 ppm IBA.

Generally, when NAA was included in the medium the shoots became greener, but once roots were formed all the shoots greened considerably. After the roots had extended 1-4 cm into the medium, the planlets were placed in test tubes of non-sterile water (Fig. 2) which allowed further growth and elongation of the roots to occur.

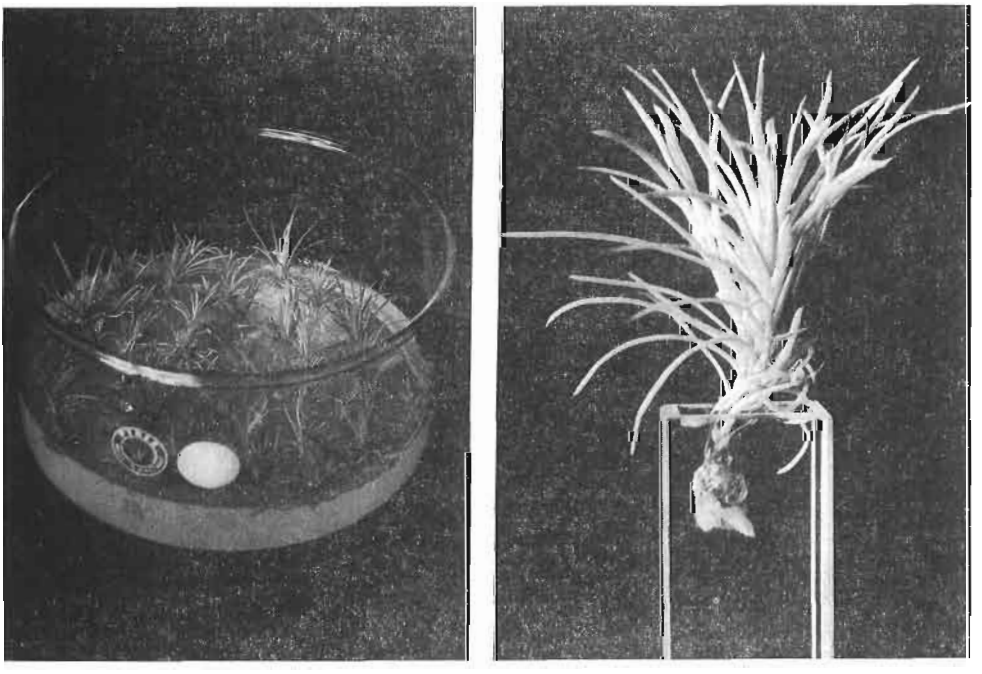

FIG. 2 (left)—Shoots dissected from cotyledonary tissue and placed on SH (no cytokinin) for elongation. (Right) Roots emerging from base of shoot, X1.

Two to three weeks later they were planted into soil in pots. The shoots continued to grow and are now (10 months after root formation) approximately 25 cm in height. The plantlets have developed a good mycorrhizal root system (Fig. 3).

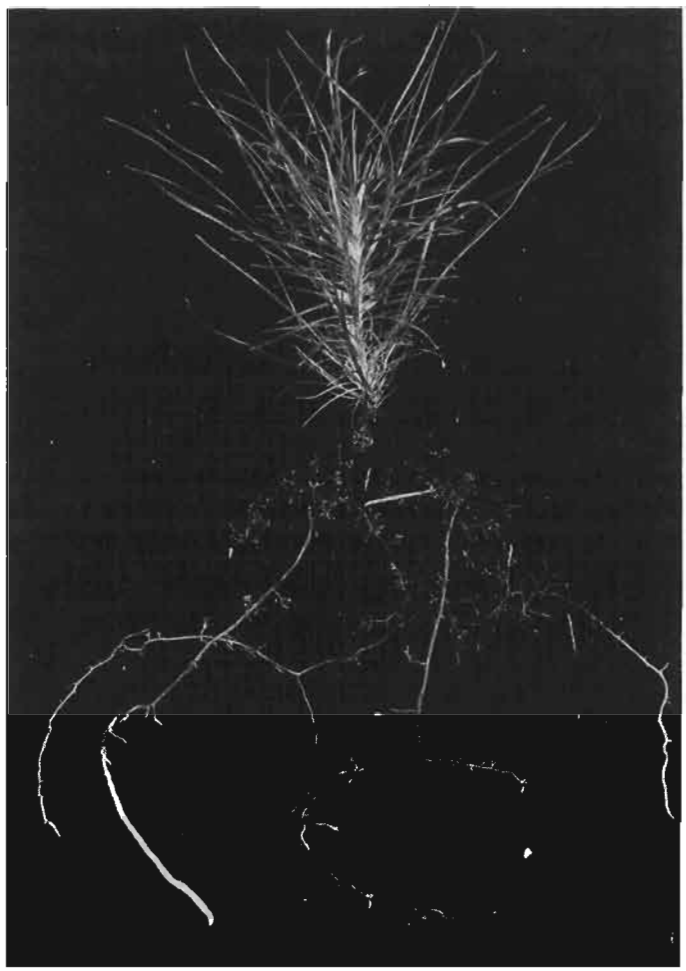

FIG. 3—Plantlet 4 months after transfer to soil with well-developed mycorrhizas. X½.

CONCLUSIONS AND DISCUSSION

These experiments showed that numerous adventitious buds can be induced by placing radiata pine embryos on a nutrient medium (SH) containing cytokinin. The buds may be formed directly from the cotyledons and hypocotyls or from meristematic tissue proliferating from them. Elongation and proliferation of the buds occurred when the tissue was placed on SH medium without cytokinin. When separated out individually, the buds grew into well-formed shoots. Roots formed on these after they were maintained

without hormones for 6 months. A medium (GD) containing 0.1 ppm NAA and 2.0 ppm IBA induced roots most consistently.

These results differ from those which describe organogenesis in some other conifer species. Sommer *et al.* (1975), working with longleaf pine, found that 2.0 ppm NAA and 1.0 ppm BAP induced adventitious shoots. Roots were often induced during the first subculture of shoots although buds often remained inhibited. Cheng (1975) found that organogenesis in Douglas fir (*Pseudotsuga menziesii*) was achieved by flooding cells with a 0.5-1 mM (100-200 ppm) solution of cytokinin and subsequently transferring them to a cytokinin-free medium. She suggested that this allowed the concentration of cytokinin within the cells to decrease as cellular proliferation continued and eventually the optimal condition for triggering shoot formation was reached. However, using radiata pine we found that exogenously applied cytokinin at high concentrations very frequently killed the tissue and that it was simpler, and yielded better results, to place embryos on an agar medium containing a known concentration of cytokinin. Campbell and Durzan (1975; 1976) using *Picea glauca,* and Sommer (1975) working with *Pseudotsuga menziesii* embryos, obtained results similar to ours, although proliferation of large numbers of shoots from the one embryo is not described by them.

Although nutrient concentration does not appear to play a major role in the initiation of adventitious buds directly on the cotyledons, it is important in the proliferation of meristematic tissue. Cotyledons not in contact with the nutrient medium, or cotyledons in contact with diluted nutrients failed to form meristematic outgrowths although some shoots formed directly on the cotyledons. The balance of salts in the nutrient medium does not appear to be critical since similar although less consistent results have been obtained using other types of medium, e.g., Murashige and Skoog's (1972) salts. Benzylaminopurine may be replaced by other cytokinins such as zeatin and isopentyladenine (K. Reilly, unpublished results).

There is room for improvement in the plantlet formation system described. Although a high percentage of embryos formed numerous shoots in a few weeks, root formation has been more erratic and has occurred only after the shoots have elongated. Possibly a well-developed vascular system is necessary for root formation or, as has been proposed by Quoirin *et al.* (1974), leaf and shoot elongation may play an important part in root formation and the subsequent survival of the plant. They found that in *Prunus* sp. healthy plants were obtained from apical meristems only when production of new leaves was followed by stem elongation and root formation.

In our experiments shoots formed roots after six months in culture. It is conceivable that to obtain the optimum conditions for shoot growth and root formation some modification may be necessary. Murashige (1974) suggested that an increase in light intensity may be necessary for the successful rooting of shoot cuttings and for the hardening-off of plants before the successful transfer of propagules from tissue culture to soil. We are at present investigating this, and the effect of other physical conditions on plantlet formation.

When this work is completed, the clonal propagation of seeds may facilitate the progeny testing of selected crosses. Since a number of plants with the same genotype would be obtained, site-genotype interactions could be examined very precisely. The production of large numbers of plants from a limited number of seeds could also be

used to accelerate the propagation of selected genotypes for use in forest plantations. It is visualised that tissue culture could provide a faster method for the buildup of propagules than the use of conventional cuttings.

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