CULTURE OF PINUS RADIATA EMBRYOS WITH REFERENCE TO ARTIFICIAL SEED PRODUCTION

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(Received for publication 9 January, 1986)

ABSTRACT

Embryos excised from mature **Pinus radiata** D. Don seeds were cultured in a small volume of a nutrient medium contained in a small aluminium capsule to form an artificial seed. The embryos developed with normal morphology, although not as large as those from natural seeds. All plants from artificial seeds formed roots in sterile soil. The growth of embryos placed radicle-down into the same agarified-medium was inferior in that the lengths of cotyledons, hypocotyls, and roots of the resulting plants were all markedly reduced. When embryos were cultured in liquid medium, development was aberrant, with virtually no root growth. Nutrient supply through the (natural) cotyledonary route appeared to be superior to entry through roots or hypocotyl.

Keywords: embryo culture; tissue culture; artificial seeds; mass propagation; **Pinus radiata**.

INTRODUCTION

Tissue-culture procedures offer great potential for forestry in two primary ways. Firstly, through shortening of production lead times and increasing the multiplication rates, elite genotypes may be mass propagated and planted with economic advantage over alternative clonal production methods. Secondly, tissue-culture methods will prove essential if novel and highly advantageous traits are to be introduced to forestry through genetic engineering techniques.

Standard micropropagation procedures used in research practice are 10-fold more expensive than seedling production in nurseries (Smith *et al.* 1981), whereas cuttings are typically produced for four times the price of seedlings. It is the labour costs of *in vitro* transfers which account for this.

Those transfers which are downstream of multiplication stages will most severely affect the economics of the over-all process. It is therefore desirable to have a minimum number of *in vitro* steps from multiplication through to nursery lifting, with a high

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survival rate. It is also apparent that successful transfer of small shoots or somatic embryos directly to nursery beds may offer considerable savings so as to be cost-competitive with cutting propagation. This note reports an evaluation of the growth of *Pinus radiata* plants from zygotic embryos encapsulated in a synthetic medium which substitutes for the endosperm (megagametophyte) nutrition of natural seeds.

MATERIALS AND METHODS

The medium SHR6 was used for embryo nutrition in all experiments. This was based on the formulation of Schenk & Hildebrandt (1972) but was modified by inclusion of additional $NH_4H_2PO_4$ (to 5.2 mM) and arginine hydrochloride (4.5 mM). In related embryo-culture experiments (unpubl. data) the SHR6 medium was found superior to the media of Schenk & Hildebrandt (1972), Murashige & Skoog (1962), or Greshoff & Doy (1972) for pine embryo development.

Medium was solidified with Coast PTC agar at 5 g/l – subsidiary studies have shown this agar is equivalent to agarose for pine embryo development at this concentration. Artificial seed capsules were formed from aluminium foil pressed over a dowel-end to make an open-ended capsule 8 mm in diameter and 12 mm long. Agarified medium (c. 0.6 ml) was set in presterilised capsules.

Mature embryos were excised from seeds which had been imbibed for 4 days at 2°C, then surface-sterilised in 100 volume H_2O_2 for 15 min, followed by three rinses in sterile water. Seeds (T1132) were obtained from controlled pollination between parents which were both listed on the Australian Plus Tree Register (50080 × 35132). Excised embryos were cultured under three experimental arrangements, each replicated 10 times. Firstly, the embryos were inserted, cotyledons down, into the gelled medium in the artificial-seed capsules so that only 1–2 mm of the radicle end protruded. The capsules were placed horizontally on to double layers of moist filter-paper in sterile screw-top containers (three to four per 50-ml container). Secondly, embryos were planted, radicle end down, into agarified medium: three to four embryos were placed into 5 ml of medium forming a shallow (c. 5 mm) layer in 50-ml screw-top containers.

The embryos were incubated at 25° ± 2°C with a 16-h daily photoperiod under a mixture of cool-white fluorescent and incandescent lights (30 W/m^2). Natural seeds (nine) were also incubated under conditions identical to the first treatment, i.e., that with artificial seed capsules.

RESULTS AND DISCUSSION

The excised embryos noticeably responded to all cultural treatments within the first 24 hours, primarily with development of some green pigmentation of the hypocotyl and slight opening of the cotyledons. Root emergence from synthetic seeds preceded that for natural seeds by 3–4 days. After 18 days, both the natural and synthetic seeds showed good growth (see Fig. 1(a) and (b)). The experiment was terminated at this time to permit assessment under non-sterile conditions.

Embryos cultured in liquid medium did not develop well (Fig. 1(a)). They showed almost no root growth and had a generally swollen, stunted appearance. Embryos planted in agar showed better development; the artificial seeds were clearly superior in all

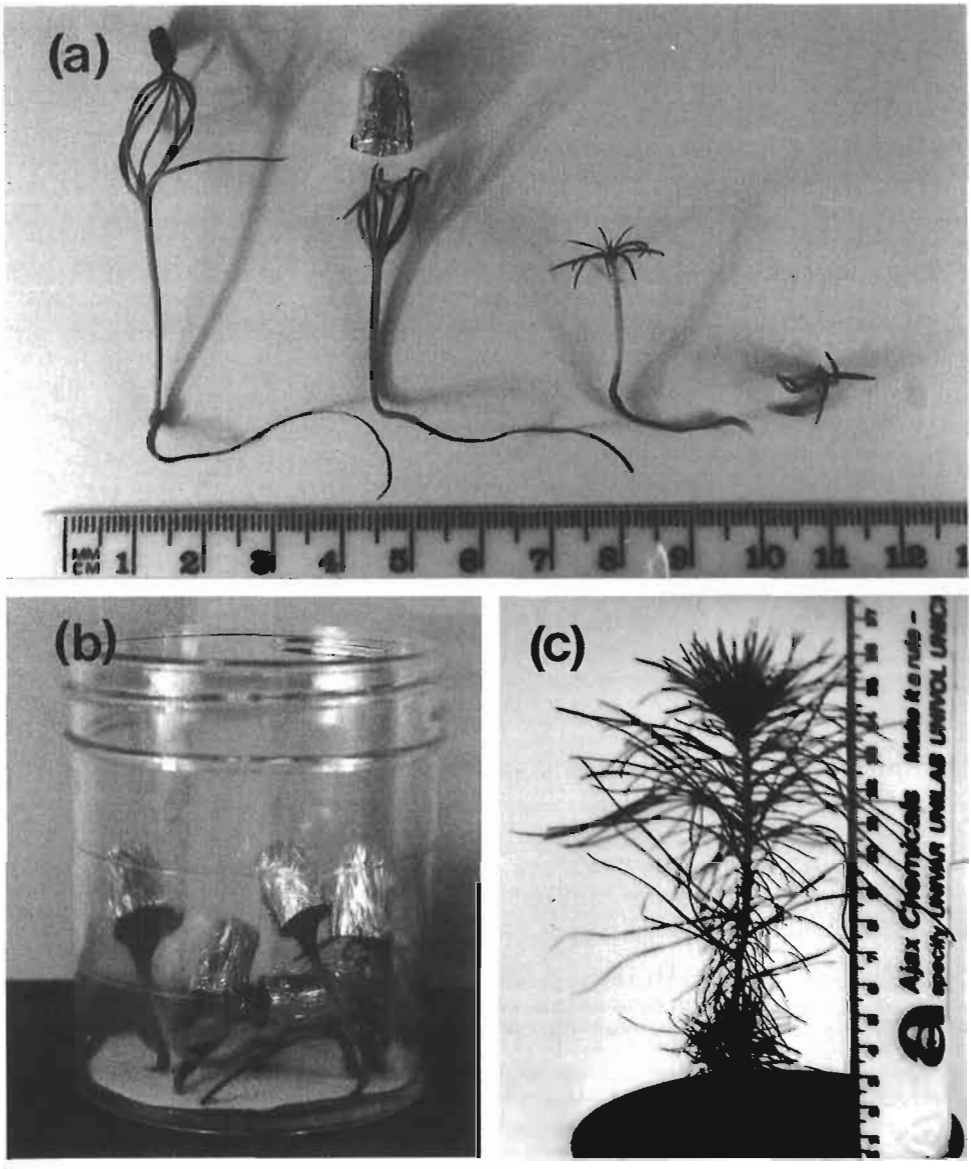

FIG. 1—The results of various culture regimes on medium SHR6 with mature **Pinus radiata** embryos.

(a) Plantlets after incubation for 18 days. Left to right: from a natural seed germinated on filter paper; from an artificial seed; from an agar-planted embryo; from a liquid-medium-cultured embryo.

(b) A group of four plantlets grown from artificial seeds on moist filter-paper. The aluminium caps raised on the cotyledons are the nutrient-containing seed capsules.

(c) A 14-month-old seedling grown from an artificial seed. Growth was commenced on sterile soil with transfer to a nursery pot at 9 weeks.

respects, although not totally equivalent to natural seeds. Measurements were made of wet weights of the plantlets, and of cotyledon, hypocotyl, and root lengths. Artificial and natural seeds did not differ significantly in their weights (Table 1), whereas agar-planted and liquid-cultured embryos were much smaller. Despite their similar weights, the natural seeds showed much greater elongation than the artificial seeds and accordingly were finer in cross-section. For all structural length measurements the plantlets followed the clear trend – natural seeds > artificial seeds > agar-planted > liquid-cultured.

TABLE 1—Comparison of the growth of embryos under various cultural regimes at 18 days

	n	Wet weight (mg)	Cotyledon length (cm)	Hypocotyl length (cm)	Root length (cm)	Total plant length (cm)
Natural seed	9	104.4 ± 35.2	2.73 ± 1.14	3.10 ± 1.01	4.35 ± 1.09	10.2 ± 2.5
Artificial seed	10	99.9 ± 12.4	1.72 ± 0.27	2.31 ± 0.34	2.40 ± 0.92	6.43 ± 1.27
Agar medium	10	37.5 ± 6.9	0.71 ± 0.19	1.57 ± 0.24	1.97 ± 0.57	4.25 ± 0.81
Liquid medium	10	48.3 ± 18.4	0.64 ± 0.16	0.91 ± 0.13	0.09 ± 0.14	1.64 ± 0.35

Errors shown are 95% confidence limits.

Artificial-seed plantlets were able to support and lift the capsule in the same way as does the natural seed coat, despite an initial mass of c. 0.8 g (Fig. 1(b)). In a separate experiment in which similar artificial seeds were incubated with sterile soil, the plantlets rooted with 100% frequency (n=12) to yield apparently normal seedlings (Fig. 1(c)). Thus, the performance of these artificial seeds can mimic that of natural seeds.

Very little medium remained in the capsules at the termination of the experiment. The small amounts left had become physically separated from the cotyledons. The superior growth obtained by nutrient supply through the cotyledons was gratifying. Such a route is the natural pathway by which the endospermal (gametophytic) nutrients are channelled. It is recognised that only mineral nutrients and not carbohydrates are normally supplied via the roots. This possibly accounted for the poorer performance of the agar-planted embryos. Bulard (1952) reported that *Ginkgo biloba* L. embryos would grow only if the nutrient medium was in contact with the cotyledons. Brown & Gifford (1958) similarly found with *Pinus lambertiana* Dougl. embryos that nutrient supply to the cotyledons was important, affecting both rate and duration of root growth.

In liquid-cultured embryos there is intimate contact between the cotyledons and the nutrient medium, so that nutrient entry route is not the cause of the very poor growth found here. Additional experiments (data not shown) have indicated that aberrant growth still occurs when the liquid volume is reduced to 1 ml per embryo. While there are insufficient data to determine the precise cause of this defective growth, the observations are consistent with the concept that normal polarity of growth is determined by gradients of growth factors which are lost through diffusion and mixing in liquid

medium. It appears that plantlet development *in vitro* depends on the way in which nutrients are presented to the embryo, and on the presence or absence of agar in the nutrient medium.

In conclusion, we are encouraged by the results that embryos may be transferred directly to soil in such simple artificial seeds, without the need to concentrate nutrients to obtain low weights. Further developments such as refinement of the nutrient composition and inclusion of fungicides and bactericides will no doubt improve the system. We are confident that this process will have a great potential for mass propagation in forestry, especially when a reliable procedure for somatic embryogenesis becomes available. These observations may have implications for application of tissue-culture methods outside embryo-culture, such as in micropropagation procedures.

ACKNOWLEDGMENTS

Thanks are given to Mrs B. Hooper for technical assistance. Financial assistance by APM Forests Pty Ltd is also gratefully acknowledged.

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