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Enhancement of plant formation from embryo cultures of *Taxus mairei* using suitable culture medium and PVP

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Abstract. Seedlings of *Taxus mairei,* of which seeds have proved difficult to germinate, were obtained by embryo cultures. Seed development in four stages was classified according to the visible characteristics of the fruits; viz, young fruits without aril, green fruits with undeveloped green arils, pink fruits with enlarging pink arils and mature fruits with fully developed red arils. The composition of the medium was the critical determinant. A 1/2MS medium with PVP supported the best embryonic growth and seedling development not only for *T. mairei* but also for *T. baccata, T. canadensis, T. cuspidata* and *T. media*. Only 15% of young embryos of *T. mairei*, in this medium, developed into plants. However, nearly ninety percent of embryos at green, pink, and mature stages developed into plants. A 90% survival rate of *in vitro* plantlets grown in the greenhouse was obtained.

Keywords: Immature embryo culture; Mature embryo culture; PVP; *Taxus mairei*.

Abbreviations: GA3, gibberellin acid; **PVP,** polyvinylpyrrolidone-40.

Introduction

Taxol, an effective anticancer drug for advanced ovarian and other cancers (NCI, 1990), has been found in the bark, root, and other parts of *Taxus brevifolia* (Wani et al., 1971). Other *Taxus* species have been extensively examined as additional potential sources due to the increasing demand for taxol (Fett-Netto et al., 1992). *Taxus mairei*, sparsely distributed in northern and central parts of Taiwan at altitudes of 1,000–3,000 m (Liu, 1980), is a slowly growing tree that reaches heights of up to 17 m and possesses good wood quality for furniture and sculpture. The species faces the threat of extinction because of strong demand for both wood and taxol production and difficulty in propagation. Most of the fruits are eaten by birds and are difficult to collect. Furthermore germination of mature seeds required a series of treatments. Alternative temperatures and cold stratification for 8.5 months produced a 50% germination rate (Chien et al., 1994). Vegetative propagation then is an alternative (Sheu, 1985); however, strong topophysis of lateral shoots limited its application for the production of planting stocks.

Embryo culture is a useful tool to overcome seed dormancy and to abbreviate the breeding cycle (Ho, 1987). Embryonic growth was first observed from cultures of immature and mature embryos of *T. baccata* (Le Page-Degivry, 1968; Le Page-Degivry and Garello, 1973). Immature embryo cultures, thought to contain less inhibitory compounds than mature embryos, were carried out in *T. baccata*, *T. brevifolia*, *T. cuspidata* and *T. media*. The fraction of seedling formation from immature embryos, however, was 15 to 40% (Flores and Sgrignoli, 1991). Mature embryo cultures were difficult to develop into seedlings (Flores and Sgrignoli, 1991; Hu et al., 1992). Many treatments to increase germination viz: GA3 added into the culture medium, a 14 h photoperiod during incubation, and an initial four weeks of culture in darkness were reported to improve embryo germination and growth into seedlings for *T. brevifolia*, *T. baccata* and *T. cuspidata* (Hu et al., 1992; Flores et al., 1993; Chee, 1994). The objective of our study was to find an optimal medium through which embryo dormancy could be bypassed and mass-production of *T. mairei* seedlings could be facilitated for conservation planting and taxol production.

Materials and Methods

Seed Material

Taxus mairei fruits were collected from Nan-Chuang, in the north-western part of Taiwan, ROC. Mature fruits were gathered in December 1992 and 1993. Immature fruits were collected from late September to December 1993. Seeds were sorted into the following groups according to the visible developmental characteristics of the fruits and aril: Y, young seeds from early gathered fruits without aril development; G, seeds from green fruits with undeveloped green aril; P, seeds from pink fruits with enlarging pink aril and M, mature seeds from red fruits with fully developed red aril. Fruits were rubbed over screens, washed thoroughly, and the light seeds were rinsed off. All seeds were mixed with wet sphagnum, placed in closed polyethylene bags, and stored in a refrigerator at 4°C. Fresh seeds were cultured within two weeks after collection. For comparison of storage effect, 6-month-old

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stored immature seeds and 15-month-old stored mature seeds were tested for their germination once a month for 6 to 15 months.

Mature seeds of *T. baccata*, *T. canadensis*, *T. cuspidata* and *T. media* were procured from Gruga Essen Park, the Netherlands. They were cultured in an 1/2MS medium containing 0.8 gl^{-1} PVP as described below.

Seed Sterilization

Seeds were surface disinfected by soaking in 70% (v/v) ethanol for one minute, followed by ultrasonic shaking in 5% sodium hypochlorite for 20 min. They were rinsed three times in sterile distilled water, and the seed coats were removed. Endosperms (including embryos) were immersed in 2% sodium hypochlorite for five minutes, and rinsed twice with sterile distilled water. Embryos were excised from longitudinally halved endosperm with a surgical blade and transferred onto the culture medium.

Culture Media and Conditions

Media used were: (1) basal media - Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962), 1/2 MS (half-strength macroelements of MS medium) or Gamborg's medium (B5) (Gamborg et al., 1968) containing 3% (w/v) sucrose, 0.75% (w/v) Bacto-agar (Difco); (2) basal MS and 1/2MS medium with 1 mgl^{-1} GA3 or 0.8 gl^{-1} PVP; and (3) liquid MS or 1/2MS medium without agar. All media were adjusted to pH 5.7 ± 0.05 and autoclaved at 121°C and 1.05 kg/cm^2 for 10 min. An embryo at each of the various seed development stages was placed onto a test tube containing 10 ml medium. Cultures in liquid medium were put into 100 ml Erlenmeyer flasks containing 15 ml medium and placed on a reciprocal shaker at 120 rpm for 7 days. Cultures were then transferred onto an agar medium. Every treatment consisted of three replications of ten embryos each at various stages. Cultures were incubated at 25°C under a 10-h photoperiod provided by Gro-Lux wide spectrum fluorescent bulbs at 600–700 Lux. Embryo germination was determined by two stages proceeding from precocious germination to seedling development. Percentages of precocious germination, defined as emergence of the radical accompanied by greening and elongation of the embryos, were recorded after three weeks in culture. All embryos, including precocious ones, were then subcultured on the same fresh medium and percentages of seedling development, defined as at least one pair of leaves sprouting out and root elongation of more than 0.3 cm, were determined at the end of the fourth week (seven weeks after initial cultures). The seedling growth, with time, in shoot and root length was also determined. Analysis of variance was conducted using General Lineal Models (GLM) of SAS (SAS Institute Inc., 1982). The binomial data of percentages of precocious germination, seedling development and rooting were transformed into arc sine before statistical analysis (Kwanchai and Arturo, 1984).

Out-Planting

The young in vitro plants were transferred to pots (6.5 cm height × 8.5 cm diameter) containing a mixture of vermiculite and perlite in volume ratio 2:1. A 100 ml beaker was placed on the pot as a lid to maintain humidity during the first week. Pots were then placed in a greenhouse with intermittent spray to maintain 85% humidity. A survival percentage was determined after 150 plants had been planted out for two months.

Results

Precocious Germination (Embryonic Growth)

Embryos of mature and immature seeds at different developmental stages of *T. mairei* appear to have varied features of color, shape, and size (Table 1). The mature seeds have a larger size (length ca. 6–7 mm) and a harder seed coat and endosperm (Figure 1) than immature seeds. Seeds of different developmental stages after seven days in culture all began precocious germination. Y embryos produced less than 20% of precocious germination on all media after three weeks in culture (Figure 2A). Embryos at G, P, and M stages gave varied responses depending on the media used; however, they produced almost the same percentages of precocious germination in the same culture medium. Comparing the effect of basal media, the 1/2MS medium produced a 78% precocious germination rate,

Table 1. Embryo color, shape and size of different developmental stages of *Taxus mairei*.

Seed stages	Embryo		
	Color	Shape	Size/mm
Young seed	Transparent	Torpedo	< 1
Green seed	White	Torpedo	1-1.5
Pink seed	White	Torpedo	1-1.5
Mature seed	White	Torpedo	1.5-2

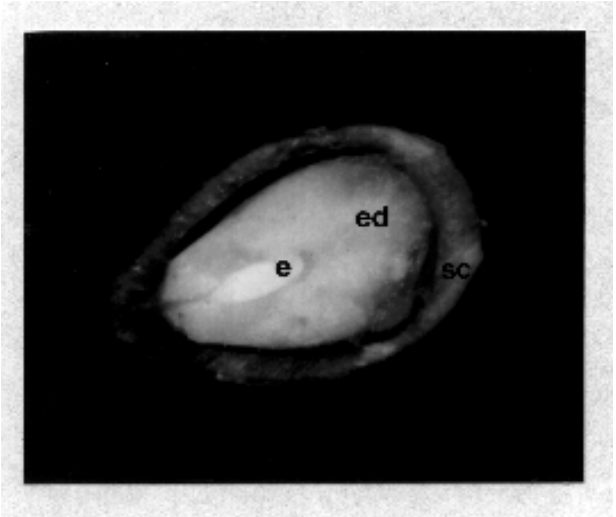

Figure 1. Longitudinal section of mature seed of *Taxus mairei*. sc: seed coat; ed: endosperm; e: embryo.

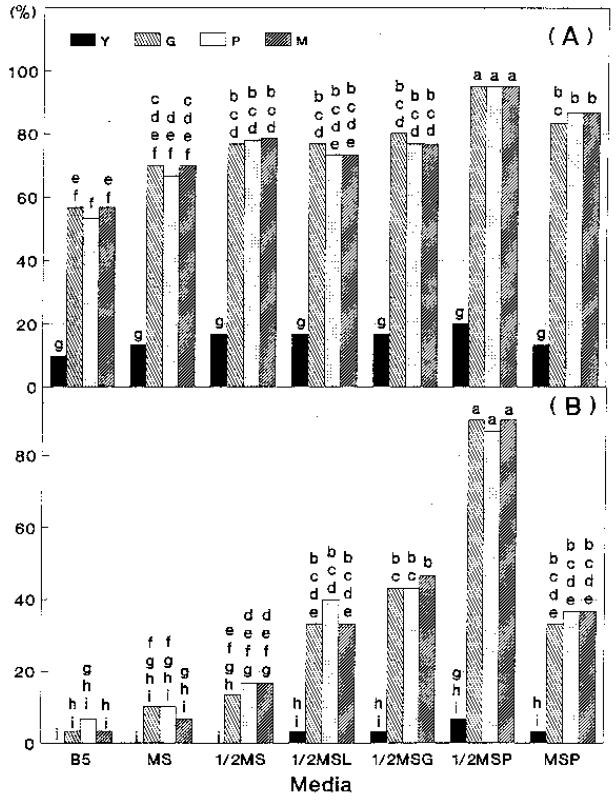

Figure 2. The effect of culture media on the percentage of precocious germination (PG) and seedling development (SD) of *T. mairei* embryos at various developmental stages (Y, G, P and M stages). (A) Precocious germination after 3 weeks in culture. (B) Seedling development after 7 weeks in culture. 1/2MS(L): liquid 1/2MS medium; 1/2MSG: 1/2MS medium plus 1 mgl^{-1} GA3; 1/2MSP or MSP: 1/2MS or MS medium plus 0.8 gl^{-1} PVP. Bars sharing the same letters do not differ at p= 0.05 by a Duncan test.

significantly better than the B5 medium, whereas it did not differ from the MS medium. The growth of embryo cultures on all these basal media was stunted, with brown spots occurring on seedlings after 20 days in culture. Liquid 1/2MS medium or solid 1/2MS and MS medium with addenda (PVP or GA3) kept embryos growing without browning. However, they did not increase the percentages of precocious germination except for the 1/2MS medium with PVP, which led to 95% of embryos precociously germinated.

Seedling Development

Seedling development did not readily occur following precocious germination of embryos. The ability to develop seedlings and their qualities depended on the degree of maturation of seeds, basal medium, and addenda used corresponding to the same response as precocious embryos. Y embryos were difficult to develop into whole plants in all basal media (Figure 2B). As in precocious germination, seedling development was affected by the medium used but not by the embryo development at G, P, and M stages. Among the basal media 1/2MS produced 16% seedlings, better than B5 (4%), but no significant differences from MS medium (9%) occurred. Liquid 1/2MS or solid 1/2MS medium containing GA3 increased the percentages of seedling development up to 45%. 1/2MS medium with PVP gave the best response to embryo development (Figure 3). Nearly 90% embryos developed into plants when they were grown in 1/2MS medium with PVP, but only 35% of embryos formed plants in MS medium with PVP.

Since root growth is an important factor in the development of seedlings, the temporal course for rooting fractions of G embryos cultured in MS or 1/2MS medium with PVP was investigated (Figure 4). 1/2MS medium with PVP produced 90% of rooting and better growth of roots (ca. 1 cm long) within six weeks. In contrast, 1/2MS and MS medium alone produced 37.5–40.0% of rooting and stunted roots less than 0.5 cm long after 12 weeks in culture. Furthermore, callus was also readily induced between hypocotyl and root, and some embryos then turned brown. Embryos cultured in MS medium plus PVP

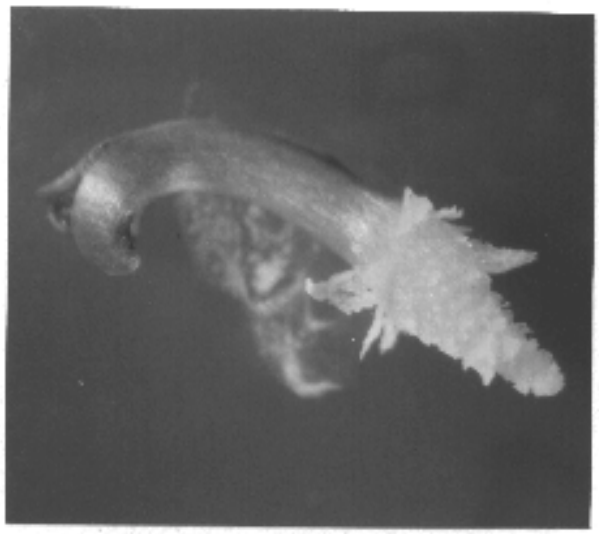

Figure 3. A germinating embryo of *T. mairei*.

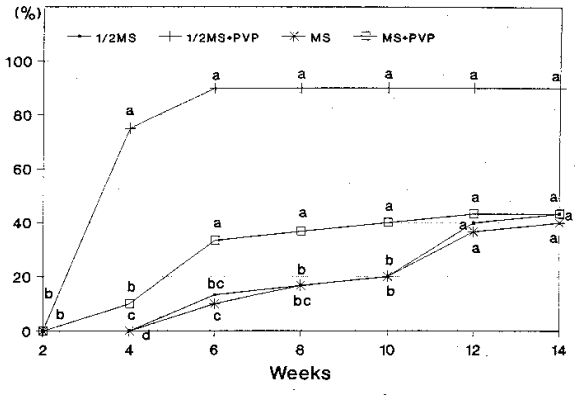

Figure 4. Effect of culture media on temporal course of rooting percentages for *T. mairei* embryos. Legends sharing the same letters do not differ at p=0.05 by a Duncan test.

reached the maximum rooting percentage with longer roots (ca. 1 cm) faster than those in MS and 1/2MS medium alone although their shoots produced callus and vitrification.

To estimate the seedling quality in terms of shoot and root length, G embryos cultured in Various media for 10 weeks were investigated (Figure 5). The growth of shoots did not exhibit significant difference between explants in 1/2MS and MS, but the growth of roots appeared to be inhibited in MS medium. Liquid cultures in both 1/2MS and MS media increased little in roots, but they did not increase in shoots. However, stunted plants with brown spots occurred when embryos were transferred into solid media after being in liquid ones for one week. Seedling growth in medium with GA3 was not significantly better than in liquid cultures, but they appeared to have longer hypocotyls with brown spots. G embryos in 1/2MS containing PVP produced much taller shoots and longer roots not only than those in MS with PVP but also than the explants in all other media. Seedlings at 14 weeks old cultured on 1/2MS medium with PVP were about 6 cm tall with 20 leaves and well developed roots (Figure 6).

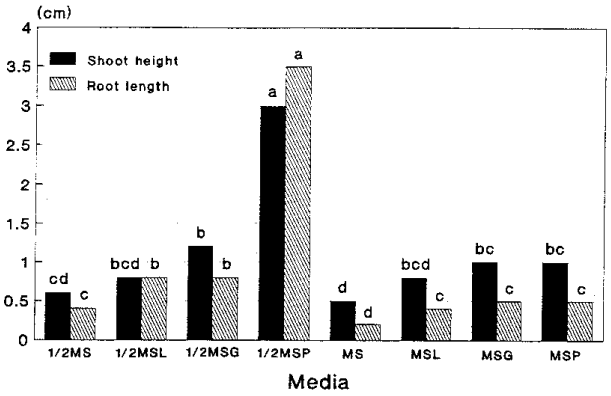

Figure 5. Effect of culture media on the growth of shoots and roots developed from *T. mairei* G embryos after 10 weeks in culture. Bars sharing the same letters do not differ at p= 0.05 by a Duncan test.

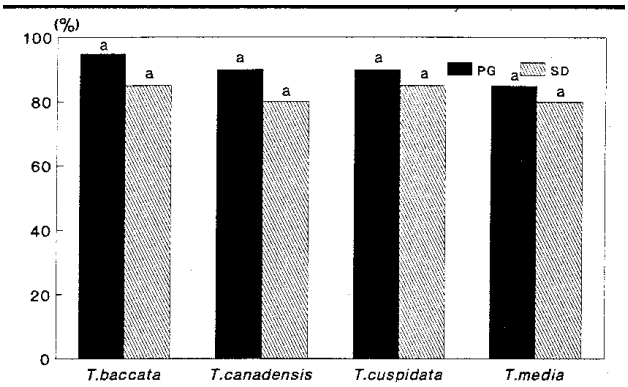

Figure 7. The percentages of precocious germination (PG) and seedling development (SD) for mature embryos of *Taxus* spp. cultured on 1/2MS medium with 0.8 gl^{-1} PVP after 3 (PG) and 7 (SD) weeks in culture . Bars sharing the same letters do not differ at p= 0.05 by a Duncan test.

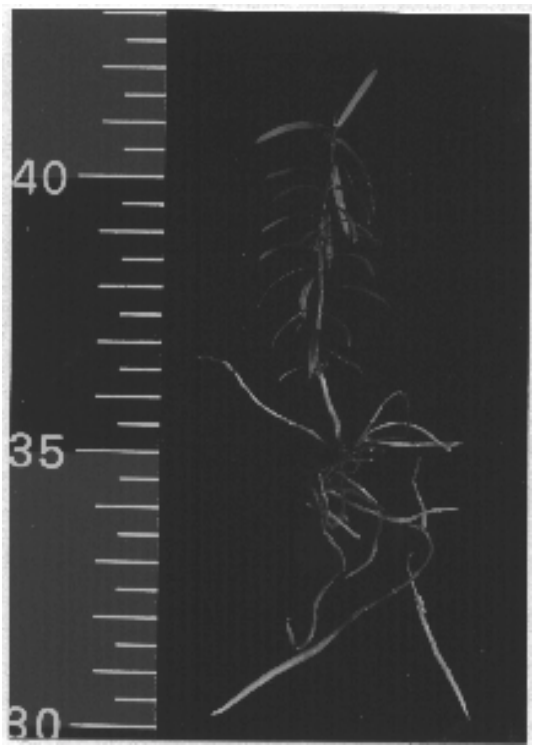

Figure 6. Full seedling of *T. mairei* developed from an embryo cultured on 1/2MS medium with 0.8 gl^{-1} PVP for 14 weeks.

Figure 8. Plantlet of *T. mairei* growing in the green house two months after planting out.

The 1/2MS medium with PVP proved to be capable of growing more than 80% of M embryos of various *Taxus* species, viz: *T. baccata*, *T. canadensis*, *T. cuspidata*, *T. media* and of subsequently developing them into full seedlings (Figure 7).

Embryo Viability After Storage

Embryos of G, P, and M seeds, after being stored for six and eighteen months, still maintained the same germination capacity (85–90%) as fresh seeds, and developed into normal plants when cultured in 1/2MS with PVP, whereas embryos from Y seeds lost viability after two months in storage.

Out-Planting

A total of 150 seedlings after being cultured for 12 weeks *in vitro* were planted in a greenhouse and 90% of them survived after having been grown for two months (Figure 8).

Discussion

Embryo dormancy is common in the *Taxus* species and requires cold stratification for one year to release it. The percentage of germinative capacity after stratification was 47 to 68% for *T. baccata*, *T. cuspidata* and *T. mairei* (Chien et al., 1994; Rudolf, 1974). Embryo culture is thus utilized to bypass stratification problems in forest tree species (Ho, 1987). The process of embryo cultures of *T. mairei* appeared to have two stages in germination, viz: precocious germination and seedling development. Difficulty in inducing root from precocious-geminated embryos occurred. These phenomena are consistent with those of other *Taxus* species as described in previous reports. The dormancy of *Taxus* seeds was hypothesized to be caused by ABA or an ABA-like compound in the embryos (Lepage-Degivry, 1968; 1973). It could be broken by washing out inhibitors or adding GA3 to obtain precocious embryos of *T. baccata* (Lepage-Degivry, 1973) and adding GA3 or NAA to improve the rooting percentage of *Taxus* embryos (Hu et al., 1992; Flores et al., 1993). In *T. mairei* more than 55% of precocious germination and less than 20 % of seedling development were obtained from the cultures of G, P, and M embryos on various basal media. This phenomenon suggests that the inhibition of embryo germination lies in the stage of seedling development and, as previous authors have pointed out, that it could also be overcome in some degree by application of GA3 or culturing in a liquid medium to provide a washing effect.

An observation was made by Flores and Sgrignoli (1991) that only immature embryos of *T. media* germinated and not mature ones. They suggested that embryo germination is not related to the culture media but to the developmental stages of the embryos. They hypothesized that at a certain stage of embryo development there may exist a "competence window" for immature embryos when they contain less inhibitory compounds that could result in a higher germination capacity. That window appears when the embryo development is at the 'G' stage of the fruit maturation process. However, in the latter report, Flores et al. (1993) pointed out that the percentages of *Taxus* embryo germination for both G and M embryos increased from 27% with no cold treatment to 30, 60, and 70% after storage at 4°C for 2, 3, and 6 weeks respectively. Their onset of germination also decreased from 32 days to 27, 24, and 15 days after storage at 4°C for the same period. The "competence window" appeared not to be obvious between G and M embryos after being cool stored for a certain period. The embryos of *Taxus mairei* seeds used in this study were stored at 4°C within two weeks, so they were still in dormancy. Their embryos from G to M stages also showed no "competence window" among them. The germination ability of G, P, and M embryos were the same at both precocious germination and seedling development when they cultured on the same medium. And this ability could be enhanced up to nearly 90% when 1/2MS medium containing PVP was used. 1/2MS medium with PVP also proved to have the same effect for the mature seeds of other *Taxus* species which have been considered difficult to germinate. Even for the difficult-germinating Y embryos, this medium could increase the percentage of seedling development. The type of basal media did not significantly affect embryo germination. However, when PVP was added, 1/2MS medium enhanced germination of embryos and growth of seedlings better than MS medium. From the fact that 1/2MS contains a lower concentration of salts than MS, we might hypothesize that a high concentration of salts inhibits the germination of *Taxus* embryos. The significant effect of PVP on embryo germination might be due to its ability to bind phenolics and some toxins to keep germinating embryos from turning brown. It is unnecessary to germinate *Taxus* embryos into plants by two stages as indicated by previous reports. Only one medium, 1/2MS medium plus PVP, is required to overcome the extreme dormancy of *Taxus* embryos and develop them into seedlings directly.

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台灣紅豆杉胚培養苗木發育之研究

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利用胚培養方法，可在短時間內培育出種子非常難以發芽的台灣紅豆杉苗木。依紅豆杉果實外形，將種子的發育分為四個階段：不具假果皮的年輕果實，具有發育中的綠色假種皮之綠果實，具增大的粉紅色假種皮之粉紅果實及發育完全的紅色假種皮之成熟種子。培養基的組成為胚培養成功與否，最重要的決定因子。1/2MS 添加 PVP 之培養基可以得到最好的胚生長及苗木的發育，此培養基不只對台灣紅豆杉的胚培養有效，同時對英國紅豆杉、加拿大紅豆杉、日本紅豆杉及英國與加拿大之雜交種紅豆杉也有相同效果。在此培養基，雖然只有 15% 年輕的台灣紅豆杉種子的胚可發育成小苗，但有接近 90% 的其它三個發育階段的胚都可發育成苗。將這些小苗宜入溫室中培養，90% 可以成活。

關鍵詞：未熟胚培養；成熟胚培養；PVP；台灣紅豆杉。