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In Vitro Germination and Propagation by Embryo Culture of *Taxus cuspidata* for the Taxol Production

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Key words: *Taxus cuspidata*, zygotic embryo, GA_3, propagation, taxol, precocious germination

Abstract

To develop an efficient propagation method for yew tree, zygotic embryos were cultured under various conditions. When dissected embryos were cultured on GA_3 containing media, the highest germination frequency was observed on WPM medium contaning 1.0 mg/L GA_3. For germination of the embryos, two different conditions were compared; culturing embryos with endosperm (Method I), and 2) culturing embryos only (Method II). Maximum germination was achieved in 0.5 mg/L GA_3 when embryos with endosperm were cultured on the media. Of the media tested, White and WPM medium were the most suitable on germination of embryos. The abnormality of yew embryos found was observed when it cultured on GA_3 or culture media. About 40% of the precociously germinated embryos could be developed into full seedlings. Seedlings contained taxol in high quantity (535 μg/g dry weight). *In vitro* techniques will be served as a useful tool for the development of transformed root cultures and biosynthesis studies.

Introduction

Cultured cells and tissues may offer an alternative source for producing secondary metabolites in plants. Many workers have demonstrated that tissue culture techniques could be used for the micropropagation of drug-producing species for field planting, an in selection programmes aimed at increasing natural levels of valuable secondary products (George and Sherrington, 1989). However, these still exist many difficulties to overcome before such techniques can be applied in a commercially feasible scale.

The importance of the tissue culture approach for clonal propagation of woody plant including conifers, for tree improve, reforestation etc., have been discussed by several workers (Patel and Thorpe, 1983; Boulay, 1987; Choi et al., 1993). As softwoods are more recalcitant than hardwoods in culture, much emphasis has to be given to developing methods for their *in vitro* propagation. It has been suggested that the use of embryonic or seedling tissues of conifers has a potential application for the *in vitro* regeneration of plants from scarce and costly seeds that become less available (Bornman, 1991).

The clonal propagation of forest tree species has progressed significantly over the past fifteen years (Boulay, 1987). However, coniferous species are still considered difficult to propagate. Especially, *in vitro* propagation methods are not established for *Taxus* species. These include somatic embryogenesis, organogenesis, and multiplication. *Taxus* spp. have been propagated mainly through cuttings (60 to 90% efficiency). One of the reasons might be its requirement for germination that needs at least 1.5 to 2 year stratification (Flores and Sgringnoli, 1991). Another risk of exploiting yew tree for the drugs may be destroying the already dwindling natural populations of *Taxus*. Dormancy of seeds can be caused by numerous factors including endogenous inhibitors, specific light requirements, low temperature, dry storage requirements, and embryo immaturity (Raghaven, 1980). An advantage of employing em-

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bryo culture is the possibility of breaking seed dormancy.

Taxol, a diterpene taxane, originally isolated from the bark of Pacific yew is currently considered the most promising chemotherapeutic agent for the treatment of ovarian cancer (Wani et al., 1971). Therefore, the supply of the method has been limited and thus limited the supply of the compound to patients. However, since *Taxus* spp. in wild populations are small in number and are growing very slowly.

Kwak et al. (1995) reported that whole seeds and immature embryos contained taxol in high quantity. The levels of taxol and its related compounds appeared to be fluctuated according to embryo developmental stage. Choi et al. (1995) also reported that Korean native yew contained taxol in high quantity comparable to those of Pacific and European yew. Tissue and organ culture of *Taxus* has been suggested as an alternative system for the production of taxol (Christen et al., 1989). *In vitro* based systems could also contribute to further propagation methods for *Taxus* spp. (Flores and Sgringnoli, 1991). This study was done to induce precocious germination of immature embryo, and to establish *in vitro* propagation techniques by embryo culture.

Materials and Methods

Plant materials

The seeds of *Taxus cuspidata* and *T. cuspidata* var. *latifolia* were collected from natural yew habitats in Mt. Sobaek (S) and Ullung Island (U) in 1992 and 1993. The seeds of ornamental yews (T) were collected near KAIST in Taejon, Korea. The seeds of Pacific yew (*T. brevifolia*) collected from the Rogue River National Forest in Jackson County, southwestern Oregon in September 1992 and English yew (*T. baccata*) were gifts from Dr. P.W. Owston (Forestry Sciences Lab., Pacific Northwest Research Station, USDA) and Dr. K.H. Han (University of Washington), respectively.

Embryo culture

Seeds of *Taxus* were disinfected with 70% (v/v) ethanol for 30s and then with 5% (v/v) sodium hypochlorite solution for 10 min. After throughly washing in sterile distilled water the seeds were soaked for 4 days at 4℃ prior to embryo culture. For germination of the embryos, two different conditions were compared; culturing embryos with endosperm (Method I), and 2) culturing embryos only (Method II). Surface sterilized seeds were decoated, after embryos were dissected out, they were cultured on several shooting containing different level of hormones. These included B5 medium (Gamberg et al., 1968), SH (Shenck and Hidebrandt, 1972), White (White, 1943), WPM (Llyod and McCown, 1980), and MS (Murashige and Skoog, 1962) medium. All media contained with 3% (w/v) sucrose, 0.4% (w/v) gelite. All the cultures were maintained under dark at 25℃.

After 3 weeks on the shooting medium, the explants bearing shoot buds were transferred to hormon-free medium for 4 weeks prior to being maintained on half-strength B5 medium for 8-12 weeks. Elongated shoots were further used for either rooting experiments or taxane analysis. All cultures were maintained at 25℃ and 16 hr photoperiod with a fluorescent ramp of 80 $\mu M/m^2 sec$.

Taxane analysis

Endogenous taxanes were extracted, purified and quantitatively analyzed as described previously in Choi et al. (1995). One gram of *in vitro* seedling was collected, and dried in dry oven. Taxanes were extracted twice with 5 mL methylene chloride-methanol (1:1) for 12 h after hexane treatment to remove nonpolar components. The combined extract was evaporated to dry, which was partitioned two times between methylene chloride and water (5 mL each). The methylene chloride soluble fraction was dried *in vacuo* and redissolved in methanol and filtered through a 0.5 μM FH-type Millipore filter. The sample was loaded onto a Curosil G column (3.2x250 mm, Phenomenex) connected with Spectra-Physics HPLC system, and eluted with mixture of 10 mM ammonium acetate (pH 4.0) and acetonitril (55:45) at a flow rate of 0.6 mL/min. The taxol and related compounds were detected at 228 nM. The quantitative analysis was carried out by comparing the peak areas of the samples with those of the authentic taxanes

Results and Discussion

Effects of GA_3 and culture methods on germination

Germination of *Taxus* embryos varied with the culture methods and GA_3 concentrations applied (Figure 1 and 2). GA_3 treatment proved to be effective on germination regardless of culture methods tested. When dissected embryos were cultured on GA_3 containing media, the highest germination frequency was observed on WPM medium contaning 1.0 mg/L GA_3. However, maximum germination was achieved in 0.5 mg/L GA_3 when embryos with endosperm were cultured on the media (Figure 1). Although the rate of germination using the Method I

was higher than that of the Method II, growth of the embryos was better when only embryo culture were cultured (data not shown). While low concentrations of GA_3 are favorable on germination rate, high concentrations of GA_3 caused callus initiation at the shoot base.

Figure 2 shows the growth of shoots in presence of GA_3. The growth of shoots was optimized in the embryos cultured in the presence of GA_3. Incorporation of 5 mg/L GA_3 was effective on the shoot growth. Many buds formed callus even after they had been isolated from mother explants. Once they formed the callus, they did not grow well. Mature seeds of *Taxus* have been reported to contain dormancy compounds which may hinder germination of embryos. According to Lepage-Degivry (1973), it was necessary to double treat embryos either leaching, GA_3 treatment and chilling to obtain a high percentage of germination.

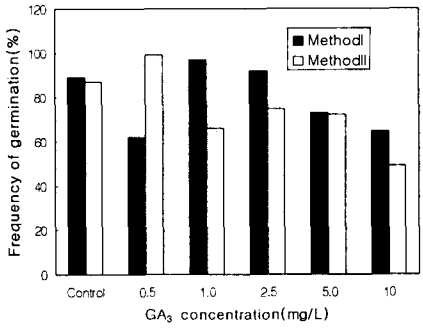

Figure 1. Interaction of GA_3 concentrations and culture methods *in vitro* germination of *Taxus* embryos.
Dissociated embryos were cultured on GA_3 containing WPM medium. The Method I culturing embryos with endosperm, and Method II culturing embryos only.

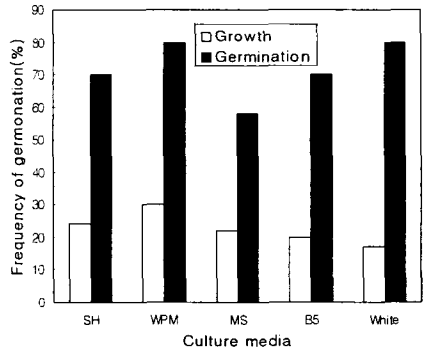

Figure 2. Effects of culture media *in vitro* germination and shoot growth of *Taxus cuspidata* embryos.
Dissociated embryos were cultured on several culture media contained with 3% sucrose, 0.4% gelite. All the cultures were maintained under dark at 25℃ for four weeks.

Germination patterns of Taxus embryos

The initial explants were intact white embryos of about 1mm in size (Figure 3A). During the first few days of culture in the presence of GA_3, the embryos slightly elongated and both cotyledons and hypocotyls turned to green (Figure 3B). Swelling of both the cotyledons and hypocotyls were swollen and were yellow green in color. After 10 days of culture, they continued to swell, and developed the hypocotyl to contyledons. In the case of hypocotyls, only segments near to the shoot apices developed a few cotyledons, while other damaged segments accumulated phenolics and became senesced.

Effect of culture media

There were no differences among the culture media tested on germination rate. Of the media tested, White and WPM medium were the most suitable on germination of embryos (Figure 2). This results suppose that germination of yew embryo may be strongly influenced by the salt concentrations. SH, White, and WPM media do not contain much salt. Petal and Thorpe (1983) reported that a half strength MS medium was optimum for the bud induction from the zygotic embryos of *Pinus contorta.*

Frequency of abnormal embryos

Five types of germination patterns of embryos was observed when it cultured on GA_3 or culture media (data not shown). First, the embryos looked morphologically normal, and developed normal cotyledons and root radicle (Type I). Second, they can be germinated, but further growth of embryo was impaired, because root initiation was retarded. Third,

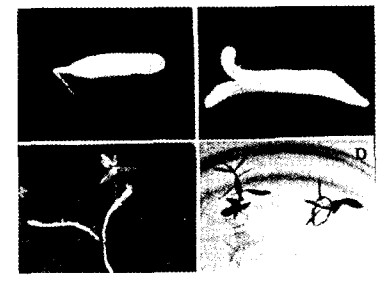

Figure 3. Germination and shoot elongation of immature embryos. A: freshly isolated immature embryos; B: germination of zygotic embryo on SH medium with 2.0 mg/L GA_3 after 1 weeks of culture; C: A shoot and root elongation on SH medium without growth regulators after 4 weeks of culture; D: Secondary shoot elongation on half strength SH medium without growth regulators after 10 weeks of culture.

the small embryos were so small (ca., less than 5 mm) but frequently showed cotyledon development and root growth (Type III). The embryo developed callus in the base of hypocotyl or cotyledon tissue (Type IV). However, this type did not develop cotyledons and roots. And the last, the embryo never germinate (Type V). When the embryos were cultured on 0.5 mg/L GA_3, they showed less germination. When the concentration of GA_3 was increased to 1 mg/L, more embryos were germinated. The Type II was found in the presence of low concentrations of GA_3. The abnormality of yew embryos may arise due to GA_3 salts and culture conditions. Petal and Thorpe (1983) reported that abnormality could be reduced by decreasing the exposure time to growth regulators.

Elongation of shoots

After 4 weeks of culture on GA_3 containing media, the embryos were transferred to a hormone free SH medium with 2% sucrose. Upon transfer, the buds started to develop. The growth of the buds were enhanced by using half strength SH basal medium. About 40% of the precociously germinated embryos could be developed into full seedlings (Figure 3 C and D).

A number of woody plants have now been propagated by in vitro embryo culture. Embryo culture techniques can be a useful propagation tool for most of coniferous species which are hard to propagate (Boulay, 1987). Although this method has a great potential for propagation, several problems are remained. First of all is the problem of obtaining true to type clone or trees.

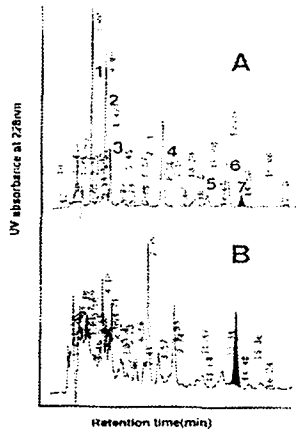

Figure 4. HPLC spectrums from *in vitro* seedling extracts of *Taxus*. A: authentic taxane compounds (1:10-deacetyl baccatin III, 2:7-epi-deacetyl baccatin III, 3:baccain III, 4:10-deacetyltaxol, 5:cephalomannine, 6:7-epi-10-deacetyltaxol), 7:taxol, B: in vitro seedling extracts.

Analysis of taxol and related compounds

Taxol and related taxane compounds were detected from the plants after 10 weeks in culture (Figure 4). Seedling contained taxol in high quantity (535 μg/ g dry weight). Jaziri et al. (1991) reported that extracts from shoot tips showed variation ranging from 0.3 to 4.6 mg/100g fresh weight. *In vitro* seedling obtained from zygotic embryos could be used for establishing cell and organ cultures. Also, *in vitro* techniques will be served as a useful tool for the development of transformed root cultures and biosynthesis studies.

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