In Vitro Culture of Zygotic Embryos of *Taxus* Species

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Abstract. An embryo culture method overcomes the lengthy dormancy requirement of *Taxus* L. spp. (yew) seeds. When zygotic embryos excised from mature *T. brevifolia* L. seeds were cultured in darkness for 4 weeks on one of three basal salt media (B5, Litvay, and Murashige and Skoog), radicle emergence and seedling development was highest on B5 basal salt medium. After 1 to 2 weeks on B5 basal salt medium, seedling development of *T. brevifolia*, *T. cuspidata* L., *T. baccata* L., and *T. baccata stricta* L. ranged from 2% to 36%. BA at 2.25 μM had no effect on radicle emergence; 22.5 μM prevented it. Embryos excised from mature or nearly mature seeds had the highest frequency of radicle emergence and seedling development. Cultured embryos developed seedlings in only 8 to 10 weeks. Chemical name used: *N*⁶-benzyladenine (BA).

Taxus brevifolia is a slow-growing evergreen species occurring primarily in ancient forests of the Pacific Northwest in the United States. Interest in the culture of this and other *Taxus* species has intensified with the discovery of taxol, an anticancer drug found in various parts of the tree (Vidensek et al., 1990; Witherup et al., 1990). The total synthesis of taxol has recently been reported (Nicolaou et al., 1994); however, because of the complexity and numerous steps involved, large-scale synthesis is not now feasible.

Because *T. brevifolia* grows slowly and has a lengthy seed dormancy requirement (1.5 to 2 years), the supply of taxol is limited (Steinfeld, 1992). Embryo culture may overcome the problems posed by lengthy dormancy (Collins and Grosser, 1984). Flores and Sgrignoli (1991) reported an embryo culture method for *Taxus* that could overcome the dormancy requirement. However, their method was based on experiments that included few excised zygotic embryos. Therefore, the objective of the present research was to evaluate the response of excised *T. brevifolia* embryos to three basal salt culture media, i.e., B5 (Gamborg et al., 1968), LV (Litvay et al., 1985), and MS (Murashige and Skoog, 1962). Two additional objectives were to determine embryo responses of *T. brevifolia*, *T. cuspidata*, *T. baccata*, and *T. baccata stricta* in B5 culture medium, and whether seed maturity influences the frequency of radicle emergence and seedling development in *Taxus*.

Materials and Methods

Plant material and embryo isolation. Mature *T. baccata*, *T. cuspidata*, and *T. baccata*

stricta seeds were supplied by F.W. Schumacher Co., Sandwich, Mass. *Taxus brevifolia* seeds were provided by Special Trees, Portland, Ore. Seeds were surface-sterilized for 10 min in concentrated HCl, and then rinsed five times in sterile, distilled water. After sterilization, seeds were soaked in sterile, distilled water for 24 h. Embryos were excised aseptically from surrounding gametophytic tissue under a binocular microscope using fine forceps and a scalpel.

Embryo culture. Zygotic embryos were cultured on B5, LV, or MS basal medium. All basal salt media were supplemented with 3% sucrose and solidified with 0.7% Phytagar (Gibco-BRL, Gaithersburg, Md.). In experiments where the effects of a growth regulator on embryo germination were studied, embryos were cultured on B5 basal medium supplemented with BA at 0, 2.25, or 22.5 μM . The pH of all media was adjusted to 5.8 before autoclaving at 121°C for 20 min. Generally, 20 embryos were placed in a 100 × 20-mm petri dish containing 25 ml of culture medium. In cases where 20 embryos were not obtainable, some replicate dishes contained either more or fewer embryos. After transferring embryos to nutrient media, petri dishes were sealed with parafilm and incubated for 4 weeks at 26°C in darkness. Subsequently, cultures were transferred to fresh medium of the same formulation and maintained under a 16-h photoperiod provided by cool-white fluorescent lamps (80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Seedlings were transferred to soil after they developed leaves and branched root systems.

Developmental stages of seeds. Seed maturity was classified into three stages according to the color of the seeds and status of fleshy arils. Stage I (young seeds): seeds were ≈2 mm long and light to medium green; the pre-aril sheath of young seeds was unswollen and green. Stage II (intermediate seeds): seeds were ≈4 mm long and dark green or light brown; the pre-aril sheath had begun to swell and was light pink. Stage III (mature seeds):

seeds were ≈6 mm long and brown; the aril was swollen and red.

Statistical analyses. Germination or breaking dormancy was defined as radicle emergence, accompanied by embryo greening and elongation. Percent seedling development was defined as the number of embryos that ultimately developed into seedlings. The total number of embryos cultured in each treatment of each experiment is reported in Tables 1–5. For each treatment, data were entered into a contingency table. The response or non-response of different treatment groups was compared using the statistical procedure CATMOD (SAS Institute, 1989).

Results and Discussion

Formulation of basal salt. Radicle emergence frequency of *T. brevifolia* embryos cultured on B5, LV, and MS basal salts media was between 50% and 60% (differences nonsignificant), but final seedling development was lowest with LV, with MS being intermediate (Table 1). The effectiveness of the tested media on plant development was ranked as B5 > MS > LV.

Light vs. darkness. Radicle emergence of excised *T. baccata stricta* embryos was not affected by light; *T. baccata* radicle emergence was superior in darkness (Table 2). Darkness effected a greater response on seedling development for both species. Overall, *T.*

Table 1. Radicle emergence and seedling development of *Taxus brevifolia* embryos cultured on B5, LV, and MS basal salt media.

Medium ²	No. embryos	Radicle emergence (%) ³	Seedling development (%) ⁴
B5	118	58	36
LV	127	56	2
MS	106	53	20

²B5 (Gamborg B5), LV (Litvay), MS (Murashige and Skoog).

³B5 vs. LV ($\chi^2 = 0.07$, $P = 0.79$); B5 vs. MS ($\chi^2 = 0.52$, $P = 0.47$); LV vs. MS ($\chi^2 = 0.22$, $P = 0.64$).

⁴B5 vs. LV ($\chi^2 = 26.5$, $P < 0.001$); B5 vs. MS ($\chi^2 = 7.4$, $P = 0.01$); LV vs. MS ($\chi^2 = 13.5$, $P < 0.001$).

Table 2. Comparison of radicle emergence and seedling development of *Taxus baccata stricta* and *T. baccata* embryos under light and in darkness.

Species	Condition	No. embryos ²	Radicle emergence (%) ³	Seedling development (%) ⁴
<i>Taxus b.</i>				
<i>stricta</i>	Light	341	89	29
	Darkness	6172	91	63
<i>Taxus baccata</i>				
<i>baccata</i>	Light	1296	65	9
	Darkness	864	78	16

²Embryos cultured 4 weeks on B5 basal salt medium under light (16-h photoperiod) or in darkness for 4 weeks; subsequently, all cultures transferred to a 16-h photoperiod.

³Light vs. dark: *T. b. stricta* ($\chi^2 = 1.35$, $P = 0.25$); *T. baccata* ($\chi^2 = 41.5$, $P < 0.001$).

⁴Light vs. dark: *T. b. stricta* ($\chi^2 = 136.2$, $P < 0.001$); *T. baccata* ($\chi^2 = 23.4$, $P < 0.001$).

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Table 3. Radicle emergence of *Taxus cuspidata* embryos cultured on B5 basal medium with or without BA.

Culture medium	No. embryos	Radicle emergence (%) ^z
B5	184	41
B5 + BA (2.25 μ M)	231	39
B5 + BA (22.5 μ M)	200	0

^zB5 vs. B5 + BA (2.25 μ M) ($\chi^2 = 0.33$, $P = 0.57$); B5 vs. B5 + BA (22.5 μ M) ($\chi^2 = 15.08$, $P < 0.001$).

baccata stricta responded better than *T. baccata* ($\chi^2 = 156$, $P = 0.001$) with respect to radicle emergence and seedling production.

Growth regulator. In many cases, exogenously supplied growth regulators are not required for embryo culture. Monnier (1978) suggested that embryos can be considered plants with their own endogenous hormones. However, there are several cases in which adding either cytokinin, auxin, or gibberellin has greatly facilitated embryo culture (Collins and Grosser, 1984). Radicle emergence of *T. cuspidata* embryos on B5 was similar with or without 2.25 μ M BA; however, no radicles emerged on B5 + 22.5 μ M BA (Table 3).

Species comparison. Embryos from *T. brevifolia*, *T. baccata*, *T. baccata stricta*, and *T. cuspidata* seeds cultured on B5 appeared similar after 1 week of incubation (Fig. 1A). After this period, embryos from all species

Table 4. Radicle emergence and seedling development of embryos from various *Taxus* species cultured on B5 medium.

Species	No. embryos	Radicle emergence (%) ^z	Seedling development (%) ^y
<i>T. brevifolia</i>	3,541	61	14
<i>T. cuspidata</i>	3,553	64	14
<i>T. baccata</i>	3,800	58	17
<i>T. baccata stricta</i>	19,784	63	11

^z*Taxus brevifolia* vs. *T. cuspidata* ($\chi^2 = 6.8$, $P = 0.01$); *T. brevifolia* vs. *T. baccata* ($\chi^2 = 6.8$, $P = 0.01$); *T. brevifolia* vs. *T. b. stricta* ($\chi^2 = 5.1$, $P = 0.02$); *T. cuspidata* vs. *T. baccata* ($\chi^2 = 27.7$, $P < 0.001$); *T. cuspidata* vs. *T. b. stricta* ($\chi^2 = 1.3$, $P = 0.25$); *T. baccata* vs. *T. b. stricta* ($\chi^2 = 33.3$, $P < 0.001$).

^y*Taxus brevifolia* vs. *T. cuspidata* ($\chi^2 = 0.001$, $P = 0.98$); *T. brevifolia* vs. *T. baccata* ($\chi^2 = 12.5$, $P < 0.001$); *T. brevifolia* vs. *T. b. stricta* ($\chi^2 = 26.7$, $P < 0.001$); *T. cuspidata* vs. *T. baccata* ($\chi^2 = 12.7$, $P < 0.001$); *T. cuspidata* vs. *T. b. stricta* ($\chi^2 = 26.4$, $P < 0.001$); *T. baccata* vs. *T. b. stricta* ($\chi^2 = 107.2$, $P < 0.001$).

began to enlarge (Fig. 1 B and C), and radicles had emerged by the end of 4 weeks. Radicle growth of embryos within a species was not synchronized, and showed much variation in morphology and size. Excised embryos did not turn brown. Most seedlings had a single, slender primary root with secondary roots developing from these primary roots during

Table 5. *Taxus brevifolia* radicle emergence and seedling development of embryos from seeds of different developmental stages cultured on B5 and MS basal salt media.

Stage	Basal salt	No. embryos ^z	Radicle emergence (%) ^y	Seedling development (%) ^x
I	B5	40	0	0
	MS	20	0	0
II	B5	1900	26	21
	MS	960	24	15
III	B5	1680	69	52
	MS	1380	52	31

^zStage II, B5 vs. MS ($\chi^2 = 1.4$, $P = 0.24$); stage III, B5 vs. MS ($\chi^2 = 136.5$, $P < 0.001$).

^yStage II, B5 vs. MS ($\chi^2 = 14.8$, $P < 0.001$); stage III, B5 vs. MS ($\chi^2 = 135.3$, $P < 0.001$).

the first two culture passages (Fig. 1D). Plants derived from embryo culture were vigorous and adapted to transplant within a few days (Fig. 1E). Radicle emergence after 4 weeks for the four species ranged from 58% to 64%, and 11% to 17% developed into seedlings (Table 4).

Development stages of seeds. Developmental stage of the excised embryo has an important effect on germination frequency (Table 5). Embryos from stage I seeds did not survive in culture, but radicles emerged and seedlings developed from embryos of stage II and III seeds, with best results from the more mature seeds.

The interaction between maturity stage of seeds and basal salt formulation was highly significant for stage II and III seeds ($\chi^2 = 29.47$, $P \leq 0.001$) (Table 5). B5 and MS basal salts gave similar results for stage II seeds, but B5 produced significantly more germinating embryos with stage III seeds than did MS. Embryos of stage III seeds produced a higher percentage of germinating embryos than stage II seeds for both basal salts ($P < 0.001$ in both cases). Embryos from stage II and III seeds had better seedling development on B5 than MS ($P \leq 0.001$), which is consistent with data in Table 1.

Flores and Sgrignoli (1991) observed that germination frequencies of *Taxus* embryos depended on seed maturity; germination diminished to 0% as seeds reached maturity. Differences in results between the present study and the earlier one may be due to the use of different seed lots and culture media [Flores and Sgrignoli (1991) used White basal salt (White, 1934)].

Results herein indicate that lengthy seed dormancy of *Taxus* species can be overcome by culturing excised embryos from relatively mature seeds. The best culture condition was in B5 basal salt medium in darkness for 4 weeks and subsequent transfer to fresh medium under a 16-h photoperiod. Seedlings can be obtained in 8 to 10 weeks compared to 1 to 2 years for stratified seeds.

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Fig. 1. Embryo culture of *Taxus brevifolia*. Embryos were excised from mature seeds of *T. brevifolia* and cultured on B5 basal salt medium. (A) One-day-old embryo culture, 2 mm long. (B) Two-week-old embryo culture, 5 mm long. (C) Three-week-old embryo culture. (D) Regenerated 2-month-old seedling from embryo culture. (E) Three-month-old seedling. (F) Two-year-old plant.

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