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Embryo Culture of *Taxus wallichiana* (Zucc.)

Mukul Manjari Datta, Sumita Jha*

Centre of Advanced Study, Department of Botany, Calcutta University, 35, Ballygunge Circular Road, Kolkata 700019, India

Abstract

Zygotic embryos were excised from immature and mature seeds of the Himalayan yew, *Taxus wallichiana*. The embryos germinated precociously when kept in darkness for 5 weeks and developed into full seedlings within 10-12 weeks. The highest rate of embryo germination (81%) was obtained in modified Lloyd & McCown's woody plant medium containing macro and micronutrients at half strength supplemented with 1% activated charcoal, which supported both the best embryonic growth (43%) and seedling development (32%). However, the supplementation of basal media with kinetin, thidiazuron, 6-benzyl aminopurine or GA_3 had no effect on the germination of the embryos. The embryos derived from immature seeds germinated but the frequency of embryonic growth was better in mature seeds. Stratification of seeds effected precocious germination of embryos. Seeds kept at 4℃ for 1 week germinated earlier and at a higher frequency irrespective of the stage of seed maturity, while the germination rate declined with prolonged cold treatment for 1 month at that same temperature. Analysis of taxanes in germinating seedlings revealed that root tissues contained high levels of taxol, 10-deacetylbaccatin III and baccatin III as compared to shoots. Thus embryo culture technique appears to overcome the lengthy dormancy requirement of *T. wallichiana* seeds.

Key words: Activated charcoal, embryo culture, Himalayan yew, Taxus wallichiana, taxol, taxane.

* Corresponding author, E-mail: sumita.jha@vsnl.com
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Introduction

Taxus wallichiana, a slow growing species of a maximum 30 m height is confined primarily to the temperate moist forests between 1500 m and 3500 m of the Himalayan region (Anonymous, 2002). Research interest in this plant has intensified with the discovery of taxol, a chemotherapeutic agent found in various parts of the tree (Miller et al., 1981). Due to the extensive use of young shoots, leaves and barks for taxol extraction, the species faces a threat of extinction. This has resulted in depletion of our forest reserves as over 90% of the Indian population has declined in recent years (Behera et al., 2000). Currently taxol and its analogue taxotere are being produced semisynthetically by the acylation of 10-deacetylbaccatin III and related compounds obtained from needles of Taxus (Hezari and Croteau, 1997).

Natural regeneration plays an important role in the conservation measures adopted for *T. baccata* (Rajewski et al., 2000) and in *T. wallichiana* the biomass yield, survival frequency and seed output are severely affected by the degree of canopy damage of the plant (Rikhari et al., 1998). Moreover seeds of *Taxus* are more difficult to germinate than most of the other coniferous species (Pilz, 1996 a, b) since the mature yew seeds require a lengthy seed dormancy period of 1.5-2 years (Chee, 1994).

Although the clonal propagation of forest tree species has progressed significantly over the decade, coniferous plants are still considered difficult to propagate (Ahuja, 1993). However, cutting and grafting techniques have been recently employed in the propagation of Himalayan yew (Mitter and Sharma, 1999; Saini, 2001).

Embryo culture is an useful technique to overcome seed dormancy and shorten the breeding cycle (Ho, 1987). Embryo culture could also serve as an alternative source of taxol and other related taxanes. Although embryo culture

of *T. chinensis* and *T. brevifolia* have been used to overcome the long dormancy period (Li, 2001), mature embryo cultures were difficult to develop into seedlings (Hu et al., 1992). Embryo culture studies in *T. brevifolia* (Flores et al., 1993), *T. media* (Flores et al., 1993), *T. chinensis* (Zhang et al., 1998; Li et al., 2000) and *T. mairei* (Chang et al., 1998) have been reported earlier, but this novel technique has not yet been explored in the micropropagation of *T. wallichiana*. The objective of the present study was to develop a protocol for *in vitro* propagation by embryo culture technique.

Materials and Methods

Plant material

Seeds of *T. wallichiana* were collected in October-November 2000-2001 from trees of natural habitat growing at Rimbik (altitude 2313 m), Darjeeling district of West Bengal. Materials were used for *in vitro* culture immediately after collection or upon arrival, except for some experiments, where the seeds were subjected to refrigeration at 4℃ for various periods.

Seed maturity and stratification

Seeds were sorted into the following stages according to the colour of seeds, size and characteristic feature of aril. Stage I (young): ≈ mm long, dark green to light brown seed with enlarged pink aril; Stage II (mature seeds): ≈ 6 mm long, brown seed with red and swollen aril. Seed stratification treatments consisted of the following: fresh seeds (no treatment), seeds stored at 4℃ for 1 week, or seeds stored at 4℃ for 1 month.

Embryo culture

Seeds of *Taxus* were surface sterilized for 10 minutes in concentrated HCl, rinsed thrice with sterile distilled water. Seeds were then kept in 0.1% $HgCl_2$ for 30 minutes and rinsed five times in sterile distilled water. After sterilization, the seeds were cut open and the embryos were excised aseptically, gently rinsed in sterile distilled water to shake off the endosperm cells. Sterile, zygotic embryos were cultured on different basal media (Table 1). All basal salt media were supplemented with 3% sucrose (w/v) and solidified with 0.75% agar (w/v). In experiments where the effects of growth regulators and activated charcoal on embryo germination were studied, embryos were cultured on WPM basal medium supplemented with growth regulators such as kinetin (0.5, 1 mg/l), TDZ (0.01, 0.05, 0.1 mg/l), GA_3 (0.5, 1 mg/l) or BA (0.5, 1 mg/l) and activated charcoal (0.1%, 0.5%, 1.0%, 2.0%). The pH of media were adjusted to 5.6-5.8 prior to autoclaving at 121℃ at 1.05 Kg/cm^2 for 15 minutes. On an average, 10 embryos were placed in each 100×20 mm Petri plate containing 20 ml of culture medium. After transferring the embryos to nutrient media, the Petri dishes were sealed with Parafilm™ and incubated under complete darkness for 5 weeks at 25℃. The cultures were then transferred to same basal media formulation in which they were initially cultured and maintained under 16/8h light/dark cycles at 25℃ ± 1℃ under artificial fluorescent light (80 mmol/m^2/sec^{-2}) at relative humidity of 55-60%.

Table 1. Schedule for germination of zygotic embryos from mature and immature seeds of *T. wallichiana*

Treatment	Basal medium
M1	MS (Murashige & Skoog, 1962)
M2	½ MS (macro and micronutrients at half strength)
M3	B5 (Gamborg et al., 1968)
M4	½ B5 (macro and micronutrients at half strength)
M5	WPM (Lloyd & Mc Cown, 1980)
M6	½ WPM (macro and micronutrients at half strength)
M7	½ WPMSH (WPM basal medium with vitamin supplementation as in Schenk and Hildebrandt, 1972)
M8	½ WPMB5 (WPM basal medium with vitamin supplementation as in Gamborg et al., 1968)
M9	M7 + activated charcoal

Taxane analysis

Seed and seedlings of *T. wallichiana* were analyzed by high performance liquid chromatography (HPLC) for taxol and other related taxane content. Twelve week old seedlings were cut at root/shoot interface. One gram (fresh weight) of *in vitro* root and shoot tissues was collected, cut into fine pieces with a scalpel, ground and dried in an oven (60℃). Fresh weight of seeds, shoot and root tissues was recorded. The extraction and analysis of taxanes and taxol was done following method published earlier (Jha et al., 1998). Quantitative analysis of taxol and related taxanes were performed in a Shimadzu liquid chromatograph (LC 10AD) employing a reverse phase ODS-Hypersil C-18 column (150 mm x 4.6 mm id), a Supelco guard column (Pelliguard™ LC-18 kit with 2 cm x 4.6 mm cartridge) and isocratic elution with acetonitrile: water: methanol (30:30:40). The flow rate was 1 ml/min and all chromatographs were plotted at 227nm, using a UV detector as published earlier (Miller et al., 1981). Identity of taxol and its related taxanes was confirmed by retention time and spiking with standard. Taxanes were isolated by

preparative HPLC and identified by mass spectra and 1HNMR.

Statistical analysis

Embryos were monitored at weekly intervals for at least 6 weeks. Precocious germination or breaking dormancy, defined as radicle emergence, accompanied by greening and elongation of the embryos, was recorded after 4 weeks in culture. All embryos were then subcultured onto the same fresh medium. Percentage of seedling development was defined as the number of embryos that ultimately developed into seedlings with at least two sprouting leaves and root elongation of 0.5 cms noted at the end of the 6th week. The experimental data were statistically analyzed with Analysis of Variance (ANOVA); compared at $p<0.05$ and Duncan's multiple range test (Gomez and Gomez, 1984). The mean of the treatments has been calculated with standard error.

Results and Discussion

Effect of different basal media on *in vitro* germination of embryos

The formulation of the basal medium has an effect on precocious germination of the embryos of the Himalayan yew (Table 2). In *T. wallichiana*, the germination frequency of the embryos cultured on B5 and WPM basal medium were found to be significantly better than in MS basal medium, irrespective of stage of embryos cultured (Table 2). Similarly, when macro and micronutrients of different basal medium were reduced to half, the best response was obtained in 1/2 WPM basal medium. This result indicates that germination of *T. wallichiana* embryos may be influenced by the salt concentration of the media, as reported in *T. mairei* (Chang and Yang, 1996) and *T. cuspidata* (Choi, 2000). In *T. brevifolia*, radicle emergence and seedling development was highest on B5 basal salt medium (Chee, 1994) while in *T. baccata* embryos placed in ½ MS basal medium showed the highest germination (Chang and Yang, 1996). In *T. wallichiana*, the growth of the embryos cultured on either ½ MS or B5 basal media were stunted with the appearance of brown spots after 3 weeks in culture. A brown halo was found to surround the embryos due to the leaching of phenolic substances into the medium.

The stage I and II embryos differed in their response depending on the basal media used. A higher precocious germination frequency was noted in embryos excised from stage II seeds, in contrast to embryos of stage I seeds, in all basal medium of different salt strength tried (Table 2). In *T. media*, Flores and Sgrignoli (1991) reported that immature embryos showed the highest germination rates as they contain less inhibitory compounds that result in a higher germination frequency. The present study shows that stage

Table 2. Precocious germination, radicle emergence and seedling development of *T. wallichiana* in relation to seed developmental stage, cultured on different basal media.

Stage of maturity	Basal medium	% response		
		Precocious germination	Radicle emergence	Seedling development
I	B5	20±3.8 bc	7±1.0 abcd	6±1.2 abc
	1/2B5	6±1.3 a	3±0.5 a	-
	MS	8±1.8 a	4±0.4 ab	-
	1/2MS	11±1.6 d	5±0.2 abc	4±0.8 a
	WPM	22±3.9 c	9±1.3 cdef	7±0.4 abcd
	1/2WPM	27±3.7 e	10±1.3 f	9±1.0 ab
	1/2WPMSH	35±4.0 f	13±1.2 g	12±1.5 abcd
	1/2WPMB5	16±2.3 b	7±1.0 abcde	5±0.9 d
II	B5	54±4.1 b	24±1.4 abefh	21±1.5 abc
	1/2B5	9±1.2 a	4±0.4 c	-
	MS	12±1.5 a	9±1.7 cdg	6±1.3 c
	1/2MS	22±1.8 e	11±1.8 cdeg	9±1.4 cd
	WPM	58±4.5 bc	29±2.1 abf	22±1.8 ab
	1/2WPM	63±4.7 cd	30±2.6 ab	25±1.9 ab
	1/2WPMSH	67±4.1 d	33±2.8 b	29±2.0 b
	1/2WPMB5	40±2.2 f	21±1.9 adefh	18±1.5 ade

Each treatment is based on four replicates, each of which consisted of 10 explants.
Comparison of the mean values ± S.E. obtained in treatments at respective stages was made using ANOVA and Duncan's multiple range test at 0.05 level of confidence. For each treatment, mean values followed by same letters are not significantly different from each other.

II embryos show the highest frequency of precocious germination.

Effect of activated charcoal on radicle emergence and seedling development

Radicle emergence and subsequent root growth is an important factor in the development of seedlings. The embryos placed in ½ WPMSH basal medium supplemented with 1% AC rooted (43%) within 6 weeks of culture and the result was significantly better than those cultured in ½ WPMSH basal medium supplemented with 0.1% or 2.0% activated charcoal. Frequency of radicle emergence in embryos cultured in other basal media formulations varied between 3-30%. In the latter cases, the roots were stunted and less than 0.3 cms in length even after 12 weeks of culture. On examining the effect of basal media on seedling development, it was revealed that 32% seedlings germinated in ½ WPMSH basal medium supplemented with 1% AC in contrast to 9% germination of the embryos in ½ MS basal medium (Table 2, Figure 3). The present study also indicates that while ½ WPMSH basal medium induced 29% of the embryos to develop into full seedlings, about 21% of the embryos developed into full seedlings when it was cultured on B5 basal medium. When embryos were cultured on ½ WPMSH basal medium supplemented with activated charcoal (1%), the germination frequency was significantly better than either lower (0.1%) or higher (2.0%) concentrations of activated charcoal used (Figure 3). The embryos developed without any sign of browning or retardation in growth. The inclusion of activated charcoal greatly enhanced the elongation and subsequent growth of the seedlings. When embryos were placed in WPM basal medium supplemented with either kinetin (0.5, 1 mg/l), TDZ (0.01, 0.05, 0.1 mg/l), BA (0.5, 1 mg/l) or GA_3 (0.5, 1 mg/l) without activated charcoal they did not germinate.

Effect of stratification

Embryos excised from stage II seeds precultured at 4℃ for 1 week showed an increase in germination frequency (69-81%) and was significantly better than freshly collected seeds or seeds kept at 4℃ for 1 month (Figure 1a). However, prolonged cold treatment (1 month at 4℃) was not favourable for germination of embryos. Radicle emergence and seedling development was better in embryos excised from seeds pre cultured at 4℃ for 1 week than in the freshly excised embryos excised from freshly collected seeds (Figure 1b, 1c). Embryo dormancy is common in *Taxus* species and requires cold stratification for one year to release it. The percentage of germination after stratification was increased from 47% to 68% in *T. baccata, T. cuspidata* and *T. mairei* (Chang and Yang, 1996). The short cold treatment may be able to break the dormancy of *T. wallichiana* seed and bring about the precocious embryo germination as reported for *T. mairei* (Chang and Yang, 1996). Adequate stratification periods however did not seem to vary among populations (Pilz, 1996) but the length of stratification required for growth was an important criteria which is related to seed maturity at the time of collection.

Germination patterns of Taxus embryos

The zygotic embryos of *T. wallichiana* cultured on ½ WPMSH basal medium supplemented with 1% AC, were white, intact, torpedo shaped during the first week of incubation (Figure 2a) during which the embryos showed no signs of growth. After this lag period, the embryos started to

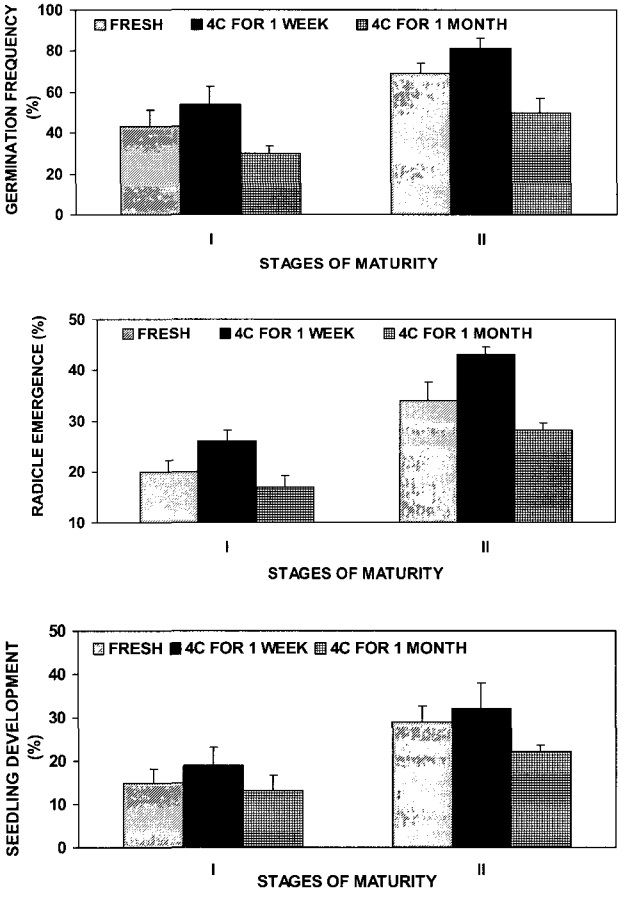

Figure 1. Effect of stratification on (1a) precocious germination (1b) radicle emergence and (1c) seedling development of embryos at different stages of seed maturity in *T. wallichiana*. The vertical bars indicate standard error of the mean data.

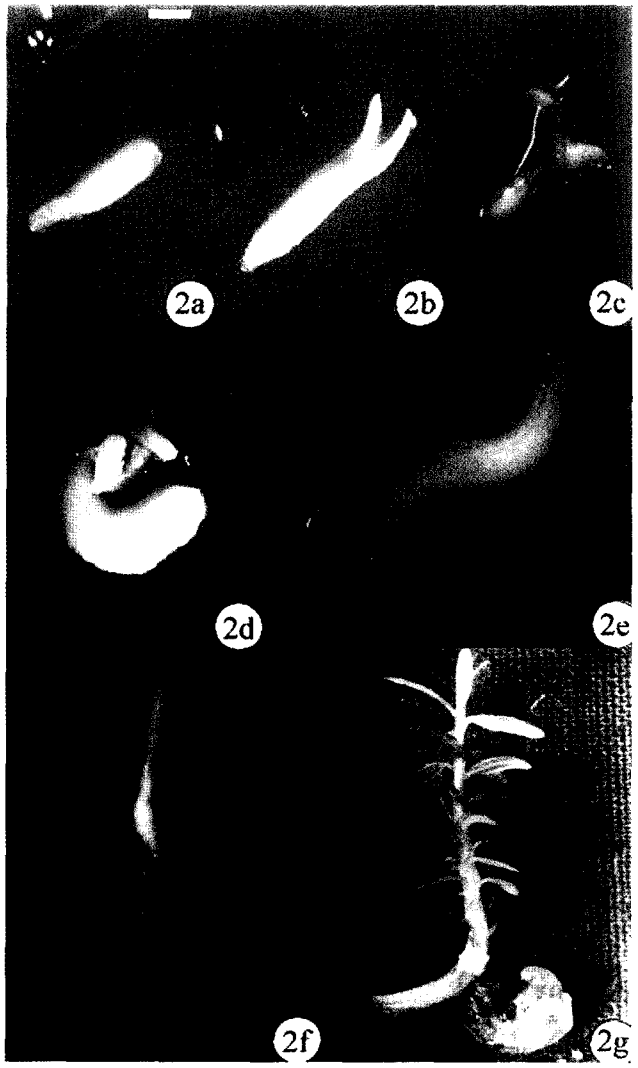

Figure 2. Embryo culture of *Taxus wallichiana*. (2a) Zygotic embryos excised from mature seeds and cultured on ½ WPMSH with 1% AC basal salt medium, after two (2b) three (2c) and four weeks (2d) of culture. Radicle emergence at the end of 5 weeks (2e), germinating seedling with secondary roots after 6 weeks of culture (2f) (2g). (Bar=1cm)

germinate precociously and enlarged at the end of 4 weeks (Figure 2b, 2c, 2d). Both the cotyledon and hypocotyl turned green and radicles had emerged by the end of 5 weeks (Figure 2e). Most seedlings had a single primary root, secondary roots were few in number at the end of 6 weeks of culture (Figure 2f). Seedlings of the Himalayan yew obtained through embryo culture were vigorous and were 5 cms tall with nearly 20 leaves after 12 weeks of culture. However, root growth was not satisfactory and further work is in progress on transfer of embryo derived plants to greenhouse.

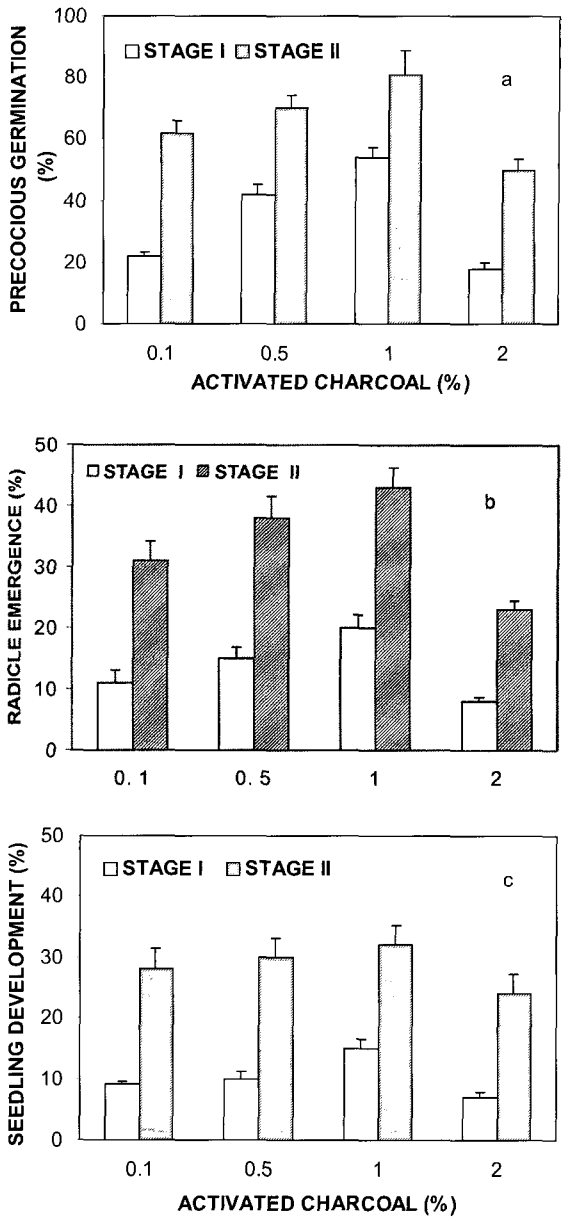

Figure 3. Effect of activated charcoal on precocious germination (3a), radicle emergence (3b) and seedling development (3c) of *T. wallichiana* in relation to seed developmental stage, cultured on ½ WPMSH basal medium. The vertical bars indicate standard error of the mean data.

Analysis of taxol and related taxanes

Taxol and other related taxanes were detected in twelve week old seedlings of *T. wallichiana*. The roots showed higher accumulation of taxanes as compared to shoots and seeds from which they germinated (Table 3). Thus embryo culture procedure allows the fast screening of individuals for producing higher levels of taxol and related taxanes as

Table 3. Taxane content *(mg/100gm dry weight) of seeds and of embryo derived seedlings

Plant material	Taxol*	Baccatin III*	10-DAB*
(1)Mature seeds	14±1.24	32±1.25	5±0.61
(2)Embryo developed seedlings (12 week old)			
(a)root	240±8.16	1580±64.97	600±26.43
(b)aerial shoots	80±4.99	210±12.31	20±2.87

reported in other species of *Taxus* (Shigemori et al., 1999; Majada et al., 2000; Shi et al., 2000).

It may be concluded from the present study that embryos of *T. wallichiana* undergo precocious germination at a higher frequency when excised from the appropriate stage of maturity of seeds. Thus the systematic approach to the formulation of culture media, along with cold stratification at 4℃ for 1 week had resulted in a high precocious germination frequency of embryos followed by the seedling development. Therefore, the embryo culture technique may provide a quick and reliable method for clonal propagation of *T. wallichiana* preserving their genetic diversity. Moreover, this *in vitro* technique will serve as an useful tool for the taxane production and biosynthetic studies in *T. wallichiana*.

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