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Changes in Ultrastructure and Abscisic Acid Level, and Response to Applied Gibberellins in *Taxus mairei* Seeds Treated With Warm and Cold Stratification

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Seeds of *Taxus mairei* are known for their deep dormancy which can only be broken by a procedure involving warm stratification followed by cold stratification. Treatments with alternating temperatures of 25/15 or 23/11 °C (12 h light) for 6 months followed by 5 °C for 3 months were successful in overcoming seed dormancy. After 6 months of warm stratification, cytological changes observed included: enlargement of the embryo; a decrease in the number of lipid bodies; appearance of ER; and increases in mitochondria, plastids, dictyosomes, vacuoles and microbodies in the shoot apical meristem. Cold stratification following the warm treatment induced cell division, and one or two distinct nucleoli in the shoot apical meristem cells were observed. Both warm and cold stratification reduced endogenous ABA concentrations from the original 8888 pg per freshly harvested seed to 392 and 536 pg, respectively. Treatment with exogenous gibberellins after seeds had been warm-stratified showed that GA_4 and GA_7 were effective at promoting seed germination, but GA_3 was not. These results suggest that the strong seed dormancy of *T. mairei* could be caused by a high ABA content and underdevelopment of the embryos in freshly shed seeds. We conclude that warm stratification with alternating temperatures increases the growth of embryos by cell expansion and enlargement and decreases ABA content, but seeds still remain ungerminated. Cold stratification may induce the response to GAs and initiate cell division resulting in release from physiological dormancy and subsequent germination of *T. mairei* seeds.

Key words: *Taxus mairei*, ultrastructure, abscisic acid, gibberellin, seed dormancy, stratification, germination.

INTRODUCTION

Seeds with a combination of morphological and physiological dormancy have underdeveloped, dormant embryos, which must grow continuously to a certain length before seed dormancy can be broken. Six types of morphophysiological dormancy were described by Nikolaeva (1977). For example, a deep, simple morphophysiological dormancy, typified by seeds of *Fraxinus excelsior* L. (Villiers and Wareing, 1964) and *Jeffersonia diphylla* (L.) Pers. (Baskin and Baskin, 1989), requires warm followed by cold stratification before germination can occur. In these species, embryo growth is rapid during warm stratification and continues during cold treatment. However, some seeds with non-deep, complex morphophysiological dormancy such as *Erythronium albidum* Nutt. (Baskin and Baskin, 1985) and *Osmorhiza claytonii* (Michx.) C. B. Clarke (Baskin and Baskin, 1991), also require warm followed by cold stratification for germination, but embryo growth occurs during cold stratification and only in seeds that have first received warm treatment.

Taiwan yew, *Taxus mairei* (Lemee & Levl.) S. Y. Hu (Taxodiaceae), is a large, slow-growing tree, which can reach about 300 cm in diameter and is sparsely distributed in the natural forests of Taiwan between 1000 and 2700 m above sea level. This species has faced strong pressures of utilization in recent years. Thus, research interests are seeking to increase the *T. mairei* population within a framework of forest resource management and genetic conservation. In addition, the significant anticancer activity of taxol, found in the bark, twigs, leaves and other parts of *T. brevifolia* (Wani *et al.*, 1971) has led to protection of the species in Taiwan.

A dormancy-breaking method which employs a combination of warm and cold stratification has been established for seeds of *Taxus* species (Devillez, 1976; Suszka, 1985; Chien *et al.*, 1995). Seeds are treated with high alternating temperatures (25/15, 20/15 or 20/10 °C) in wet sand for 6 months or more and then transferred to a low temperature (3 to 10 °C) in wet sand. Devillez (1976) reported that warm stratification induced afterripening in underdeveloped embryos and overcame morphological dormancy, while cold stratification promoted embryo growth and physiological modification. Le Page-Degivry (1970, 1973) showed that when endogenous abscisic acid (ABA) present in *Taxus* embryos was leached out into a liquid medium germination occurred, but exogenous ABA inhibited embryo growth. ABA concentration in *T. baccata* L. seeds was quantified at approximately 710 μg per kg seeds (Le Page-Degivry, Bulard and Milborrow, 1969).

It has been shown that ABA and gibberellin (GA) can play an important role in the regulation of seed dormancy and germination (Luckwill, 1952; Hilhorst and Karssen, 1992). Cold stratification caused a decrease in ABA content in dormant seeds of *Acer* species (Pinfield, Stutchbury and

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Bazaid, 1987) and apple (Lee and Looney, 1978), but dormancy relief in apple seeds might also involve a decrease in sensitivity to ABA (Singh and Browning, 1991). Similarly, an increase in GA concentrations during cold stratification and/or a relation to GA sensitivity may induce seed germination in many dormant species (Karssen *et al.*, 1989; Bewley and Black, 1994). The fine differences between dormant and germinable seeds of *Taxus* species during warm and cold stratification treatments have not been investigated. It is not clear if the continuous increase in size of the embryo after seed shedding is due entirely to cell volume enlargement or to an increase in cell number. In this paper we describe ultrastructural changes in the shoot apical meristem, changes in endogenous ABA, and response to exogenous GA during the periods of warm and cold stratification necessary to break the dormancy of *T. mairei* seeds. The results increase our understanding of the physiological and biochemical processes occurring during warm and cold stratification.

MATERIALS AND METHODS

Collection and storage

Taxus fruits which consist of scarlet or green cuplike arils were collected in central Taiwan during November 1993 and 1994. Fruits were macerated by hand in water, and arils and empty seeds were floated off. The clean seeds were mixed with moist sphagnum then sealed into polyethylene bags (0·04 mm in thickness) and temporarily stored at 5 °C before treatment.

Warm and cold stratification

For warm stratification, seeds were placed in sealed polyethylene bags with moist sphagnum, and maintained at a constant temperature of 22 °C, or alternating temperatures of 30/15, 25/15 or 23/11 °C with 12 h fluorescent light (80–100 μE m^{-2} s^{-1}) during the higher temperature. Each treatment consisted of five replicates of 100 seeds each, and moisture levels of sphagnum were checked weekly during the period of stratification. Warm stratification was performed for both 4 and 6 months. These warm-stratified seeds were then given 3 months of cold stratification at 5 °C. It should be noted that the sphagnum usually suffered from fungal contamination during the long period of warm stratification and thus was replaced prior to cold stratification. Warm stratification for 9 months without a subsequent cold stratification was also performed as a control. The moisture content of sphagnum in both warm and cold stratifications was about 400 % of its dry mass.

Germination tests

Germination tests were performed at alternating temperatures of 25/15 °C (12/12 h) at a 12 h daily photoperiod (80–100 μE m^{-2} s^{-1}) during the 25 °C treatment. Germination, judged by radicle protrusion of at least 5 mm, was recorded weekly. Results were expressed as percentage germination after 60 d.

Scanning and transmission electron microscopy

Embryos were excised from (*a*) fresh-matured seeds; (*b*) seeds stratified at 25/15 °C for 6 months; and (*c*) seeds stratified at 25/15 °C for 6 months plus 5 °C for 3 months. Embryos from each treatment were prepared for scanning electron microscopy (SEM) (Kuo-Huang, 1990). For transmission electron microscopy (TEM), the shoot and root apices of embryos from each treatment were cut off and fixed for 8–12 h in 4 % paraformaldehyde and 2·5 % glutaraldehyde buffered with 1 % tannic acid in 0·1 M phosphate at pH 7·2. After washing in buffer three times, samples were post-fixed in 1 % osmium tetroxide in the same buffer for 12–16 h, rinsed three times with buffer, dehydrated through an acetone series, and then embedded in Spurr's resin (Spurr, 1969). Sections were made with a diamond knife and stained with uranyl acetate and lead citrate for TEM. Observations and photographs were made with a Hitachi H-7100 TEM or a Hitachi S-550 SEM (Tokyo, Japan).

ABA analysis

ABA was extracted from 15 seeds either (*a*) freshly-harvested; (*b*) after 12 months of cold-stratification; (*c*) after 6 months of warm-stratification; or (*d*) germinating seeds. The seeds were ground in darkness at 5 °C with 80 % acetone containing 1 % glacial acetic acid and 100 mg l^{-1} 2,6-di-tert-butyl-4-methyl-phenol. (^{3}H)ABA (Amersham, UK) was added to each extraction so that losses during the procedure could be determined. Three replications were performed. ABA was purified and quantified according to the method of Lin, Chen and Lin (1994).

Treatment of warm stratified seeds with exogenous GAs

To study the correlation between GAs and cold stratification, 100 warm-stratified and decoated seeds were soaked for 15 h in 0, 10, 50, 100 or 250 ppm of GA_3 or GA_4, or GA_4 and GA_7 mixtures with each chemical prepared to the same concentrations as above. Treated seeds were then separated into two replicates of 50 seeds and each was tested for germination as described above.

Chemicals

Gibberellins, t-zeatin and abscisic acid were purchased from Sigma (USA) and all other chemicals used were analytical grade.

RESULTS

Warm stratification at alternating temperatures of 23/11 °C resulted in higher germination than did the same treatment at 25/15 or 30/15 °C, and 6 months of warm stratification was more effective than 4 months (Table 1), giving a maximum of 88 % germination. Constant temperature, on

TABLE 1. *Effect of warm stratifications for 4 or 6 months followed by 5 °C stratification for 3 months on the germination of* Taxus *seeds*

Warm conditions (°C)	Germination (%)	
	4 months warm + 3 months at 5 °C	6 months warm + 3 months at 5 °C
22	1·6[d]	7·1[a]
25/15	30·2[e]	81·5[b]
30/15	48·9[f]	79·0[b]
23/11	60·5[f]	88·1[c]

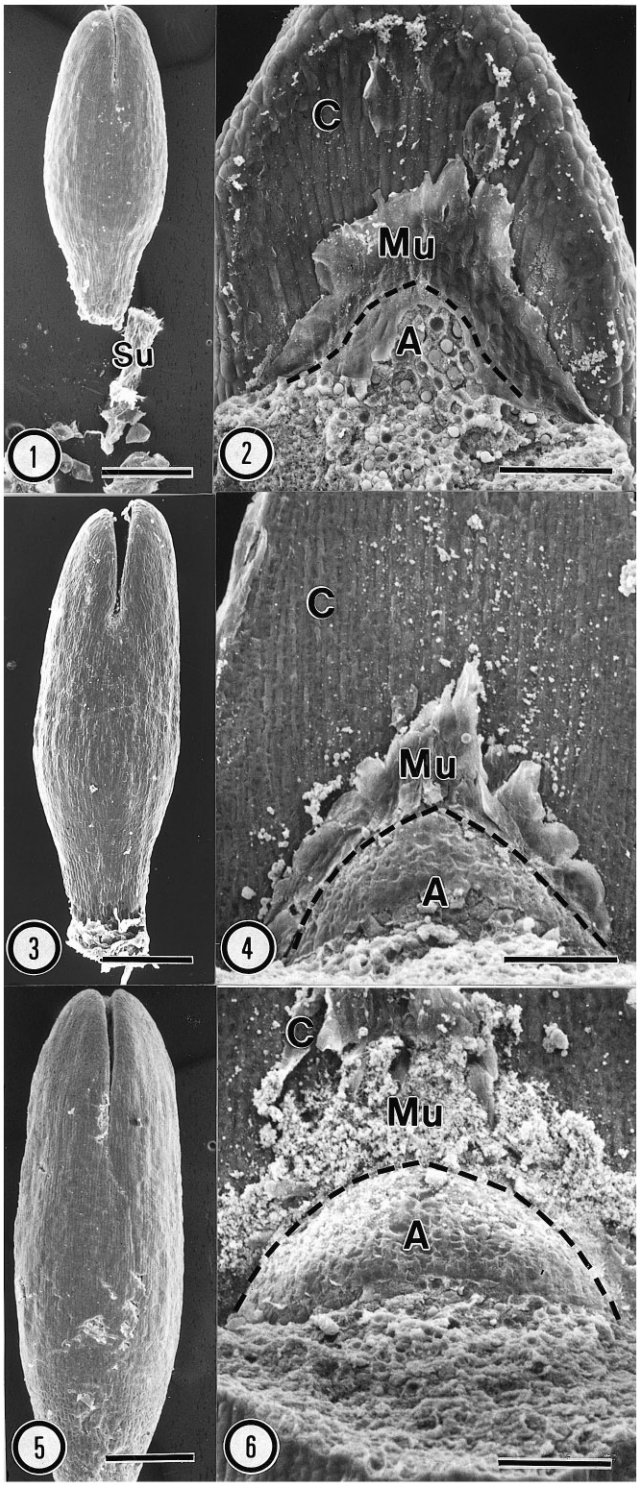
Seeds were stratified either at a constant 22 °C or alternating temperatures, and given light for 12 h during the higher temperature phase followed by 12 h in the dark. Data within columns with the same superscript are not significantly different at $P = 0{\cdot}05$.

the other hand, resulted in only 7% germination. We observed that either warm or cold stratification alone, even for a period as long as 12 months, failed to remove dormancy and the germination percentage was under 1% (data not shown). Cracked seed coats could be found after warm stratification, but such seeds failed to germinate under the same warm conditions.

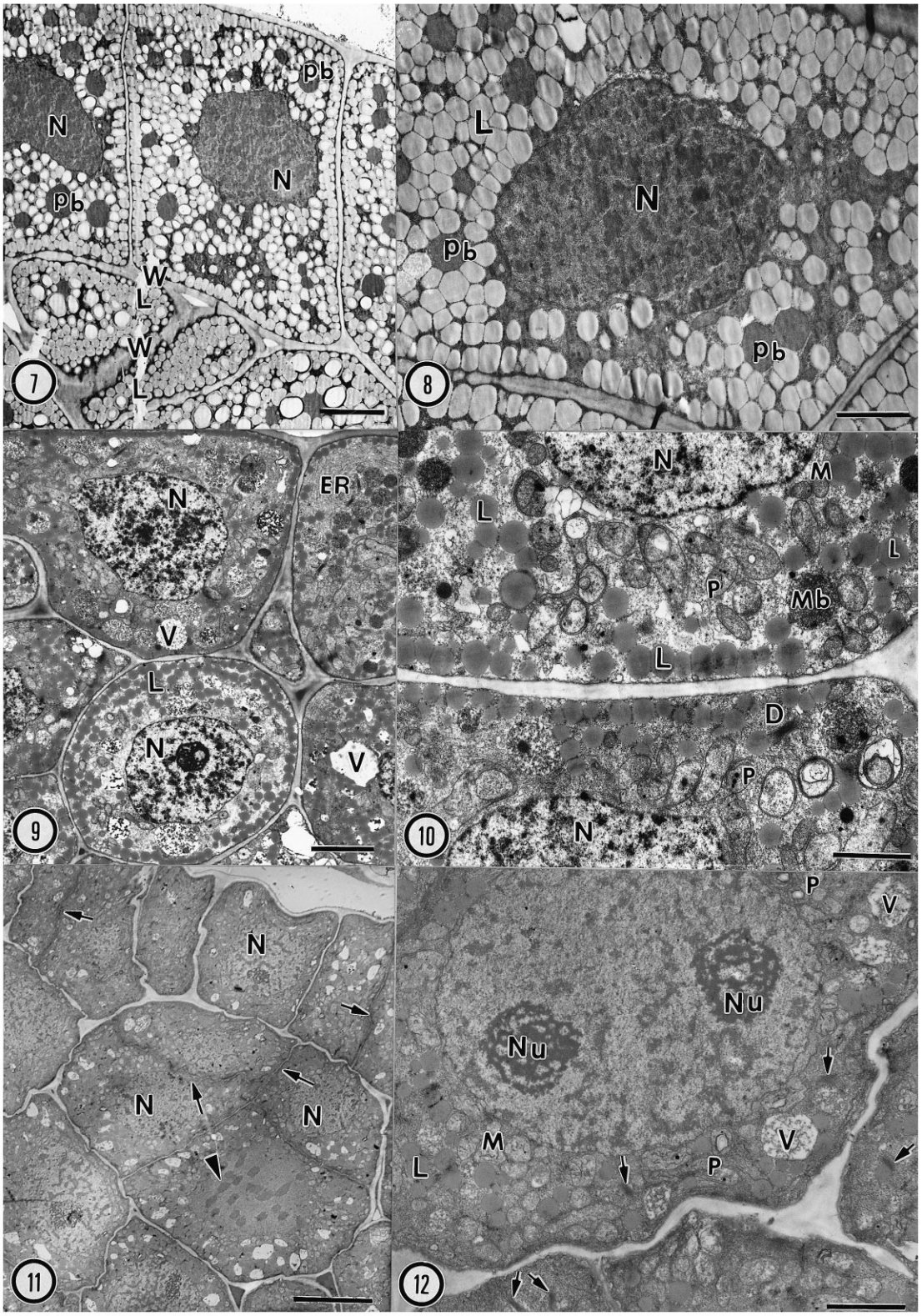
Fresh-mature seeds of *T. mairei* were 5·5–6·5 mm in length, and each contained a small embryo surrounded by a large female gametophyte. The embryo without suspensor was about 1·1–1·3 mm in length and had a small shoot apical meristem with two cotyledons (Figs 1 and 2). At this stage, the most noticeable feature was the large number of lipid bodies and their arrangement around the cell periphery (Figs 7 and 8). Protein bodies were also found in the fresh-mature seeds; they were limited by a single unit membrane and presented a homogeneous stroma of faint electron density. Few mitochondria, proplastids and endoplasmic reticulum (ER) were observed in the cytoplasm. Dictyosomes were rarely encountered in the cells of apical meristems. The nucleus of most meristem cells appeared spherical to ovoid and contained an obscure nucleolus.

Enlargement of embryos to nearly 1·5–1·7 times their original length was found after 6 months of warm stratification (Fig. 3). The cotyledons and shoot apical meristem were also enlarged (Fig. 4). At this stage of stratification, the lipid bodies in the apical meristem had dramatically decreased in number, but to a lesser extent around the cell periphery (Figs 9 and 10). Some sheet-like form of ER appeared and the nucleus had a distinct nucleolus. Mitochondria, plastids, dictyosomes, vacuoles and microbodies were also observed in cytoplasm.

After the combination of warm and cold stratification, the size of the embryo, cotyledons and apical meristem was slightly increased compared with warm stratification only (Figs 5 and 6). The process of cold stratification induced cell division in the shoot apical meristem of the embryo (Fig. 11). Each nucleus contained one or two distinct nucleoli. Cell division probably also occurred in the root apical meristem. Other changes observed included the development of dictyosomes, mitochondria, plastids and ER, plus a decrease in the number of lipid bodies (Fig. 12).



FIGS 1–6. SEM micrographs of embryos and shoot apical meristems of *T. mairei*. Figs 1 and 2. Two cotyledons (C) and suspensor (Su) (Fig. 1), and shoot apex (A) and mucilage (Mu) between the cotyledons (Fig. 2) in the embryo of fresh mature seed. Figs 3 and 4. The enlarged embryo, shoot apex and mucilage in seeds stratified at warm temperature for 6 months. Figs 5 and 6. The enlarged embryo, shoot apex and mucilage in seeds after warm followed by cold stratification. Scale bars = 300 μm (Figs 1, 3 and 5) and 100 μm (Figs 2, 4 and 6).



FIGS 7–12. TEM micrographs of shoot apical meristem in the embryo of *T. mairei*. Figs 7 and 8. Shoot apical meristem cells in the embryo of fresh mature seed showing many lipid bodies (L), a layer of lipid bodies beneath the cell wall (W), and protein bodies (Pb) which are surrounded by lipid bodies. Fig. 7. Bar = 4 μm; Fig. 8. Bar = 2 μm. Figs 9 and 10. Shoot apical meristem cells after 6 months of warm stratification showing lipid bodies around the cell periphery, and sheet-like form of endoplasmic reticulum (ER), nucleus (N) with a distinct nucleolus and vacuoles (V) (Fig. 9), mitochondria (M), plastids (P), dictyosomes (D) and microbodies (Mb). Fig. 9. Bar = 5 μm. Fig. 10. Bar = 2 μm. Figs 11 and 12. Shoot apical meristem cells after warm followed by cold stratification showing the dividing cell (arrowhead) and the newly formed cell walls (arrows) and nucleus (Fig. 11), and lipid bodies, vacuoles, mitochondria, plastids and dictyosomes (arrows) (Fig. 12). The nucleus contained one–two distinct nucleoli (Nu). Fig. 11. Bar = 10 μm. Fig. 12. Bar = 2 μm.

TABLE 2. *Abscisic acid concentrations in* T. mairei *seeds*

Treatments	Period (months)	ABA (pg per seed)		
		Seedcoat	Gametophyte + Embryo	Total
Fresh seeds	0	6696 ± 2931	2192 ± 933	8888
5 °C stratified	12	367 ± 92	169 ± 65	536
25/15 °C	6	198 ± 37	194 ± 84	392
Germinating (1 mm radicle)	9*	73 ± 22	203 ± 69	276

* Seeds were stratified at 25/15 °C (12 h light/12 h dark) for 6 months followed by 5 °C for 3 months.

Seeds were collected in 1993. Data are the mean ± s.e. of three replications with 15 seeds each.

Endogenous ABA concentrations were determined for the seedcoat, gametophyte and embryo under various pretreatments (Table 2). The ABA concentration was high in freshly-harvested seeds (8888 pg per seed), but decreased drastically after a 12-month cold stratification or 6-month warm stratification treatment. ABA concentration was low in germinating seeds, largely due to the low content of ABA in the seed coat.

An attempt was made to characterize the effect of GAs on the germination of *T. mairei* seeds which had been warm-stratified for 8 months. Seeds treated with GA_3 had a low germination percentage, whereas GA_4 and GA_7 brought about a considerable stimulation of germination (Table 3). The germination percentage increased as the concentration of GA_4 increased and reached a maximum of 43% at 250 ppm GA_4. GA_4 plus GA_7 also promoted seed germination; a maximum of 65% germination was observed at 250 ppm each of GA_4 and GA_7. Thus, it appears that the combination of GA_4 and GA_7 had a greater effect on seed germination than GA_4 alone. No germination was observed when t-zeatin (a common natural cytokinin) was applied to the warm-stratified seeds (data not shown).

DISCUSSION

In the present study, we have shown the effects of warm and cold stratification on the seed germination of *T. mairei*, and demonstrated that ABA and GAs might play a role in the regulation of seed dormancy and germination in this species. Since the seeds require warm followed by cold stratification before they will germinate, they have a type of dormancy known as deep, simple morphophysiological dormancy (Nikolaeva, 1977).

ABA has been implicated in the maintenance of dormancy and as an inhibitor of seed germination on the basis of experiments using genetic approaches (Karssen *et al*., 1983) and fluridone as an inhibitor of ABA synthesis (Le Page-Degivry and Garello, 1992; Wang *et al*., 1995). Chilling temperatures reportedly cause a decline in ABA (Powell, 1987). Also, Le Page-Degivry and Garello (1973) observed that embryos from *T. baccata* seeds germinated successfully in a liquid culture medium containing sucrose plus mineral salts because of the diffusion of ABA from the embryo to the medium. However, in the case of *T. mairei*, although the inhibiting effect of ABA on freshly-harvested seeds was apparent, loss of seed dormancy was not entirely due to a reduction in endogenous ABA concentrations. As shown in Table 2, ABA contents were low in seeds after either warm or cold stratifications but they still remained ungerminated. This agrees with the view that a reduction in ABA content in some dormant seeds is not sufficient to break dormancy (Walton, 1980).

Early works have indicated that the gibberellin contents of seeds increase during chilling. For example, cold stratification induced a rise in endogenous GA_{4+7} levels in apple seeds (Halińska and Lewak, 1987) and GA-like substances in peach seeds (Gianfagna and Rachmiel, 1986). However, an increase in sensitivity to GAs but not in biosynthesis of GAs *per se* during chilling has been demonstrated to remove seed dormancy in GA-deficient mutants of *Arabidopsis thaliana* (L.) Heynh. (Karssen and Laçka, 1985). Here, we do not attempt to quantify endogenous GAs from warm stratified or warm-plus-cold stratified seeds and it is not known if cold stratification of *T. mairei* seeds results in accumulation of GAs or an increase in sensitivity to GAs. But germination was improved by exogenous application of GA_4 and/or GA_7. Why GA_4 and/or GA_7 enhances *T. mairei* germination, but GA_3 does not, is unknown. One possible answer is that the lower polarity of GA_4 and GA_7 makes them more soluble in lipids at physiological pH levels, resulting in formation of GA_1 which, in many plants, appears to have high biological activity (Takahashi, Yamaguchi and Yamane, 1986).

The cytological changes in dormant under-developed embryos of *Fraxinus excelsior* were investigated during periods of warm and cold stratification (Villiers, 1971, 1972). After 6 months of warm stratification, the embryo was double its original size. The large decrease in lipid body numbers, and the formation of ER and appearance of nucleoli in the shoot apical meristem of *T. mairei* embryos in our work, were similar to the changes observed in the radicle meristem cells of *Fraxinus* embryos. The most significant difference for the *Taxus* embryo was that at low

TABLE 3. *Effect of gibberellins on the germination of* T. mairei *seeds*

	GA_3 (ppm)		GA_4 (ppm)				$GA_4 + GA_7$ (ppm)			
	100	500	10	50	100	250	20	100	200	500
Germination (%)	4	4	26	24	33	43	20	43	50	65

Control germination was 0%.

Seeds had been incubated under warm stratification (25/15 °C, 12 h light at 25 °C) for 8 months before the treatment with gibberellins. Seeds were germinated under 25/15 °C with 12 h light at 25 °C. Germination values are means of two replications with 50 seeds each.

temperature cell division occurred in the shoot apical meristem, and probably also occurred in the root apical meristem. Villiers (1972) reported that the large increases in nucleolar volume and total cellular RNA in embryos of *Fraxinus* were low temperature-dependent although no cell division was observed during that time. We suggest here that the stimulation of nucleolus activity and cell division probably led to the production of cellular RNA associated with plant growth substances, in particular, gibberellins, and resulted in the breaking of embryo dormancy.

Our results indicate that freshly-harvested seeds contain a high concentration of ABA and are dormant. The underdeveloped embryos continue to grow during warm stratification and ABA concentrations decline. However, a low germination percentage ($< 1\%$) was found after this treatment. Cold stratification may be necessary to induce accumulation of GAs, and/or to increase the sensitivity of seeds to GAs, thus resulting in removal of physiological dormancy. We note here that cold followed by warm stratification failed to remove dormancy of *Taxus* seeds. The most likely explanation for this phenomenon is that cold stratification can not provide a suitable environment for seed development. Previous studies on the germination of embryo cultures of *Taxus* species showed that excised embryos without any plant growth regulator in the medium could germinate well (Flores and Sgrignoli, 1991; Chang and Yang, 1996). This apparent discrepancy between the GA requirement of the embryo *in vivo* and *in vitro* may be due to the culture system used which provided nutrients, light and temperature for the dormant embryo.

Taxus seeds provide a model for the period marking the end of seed development and the start of germination. The germination phase of *Taxus* seeds can be traced to the period of cold stratification because of the occurrence of cell division. Cell division proceeds specifically during the histodifferentiation stage of seed development. Maturation of *Taxus* seeds can occur to some extent after harvest; this process is accompanied by complex changes in cells of the embryo where food materials are mobilized and organelles are developed. However, the seeds are still completely dormant during the maturation period unless chilled.

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