

***In vitro* Germination and Seedling Development of *Taxus chinensis* var. *mairei* by Embryo Culture**

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ABSTRACT

Taxus chinensis var. *mairei* is a rare and endangered medicinal plant species distributed in China. In order to promote fast propagation and preserve the natural resources, conditions for *in vitro* germination and seedling development of embryos of *T. chinensis* var. *mairei* from Anhui or Zhejiang were investigated. Results showed that *in vitro* germination rate of excised embryos cultured under 14 hours photoperiod was higher than that in darkness. But, nearly all embryos germinated under 14 hours photoperiod failed to develop into seedlings. Comparatively, 23.3 and 36.3% of embryos from Anhui and Zhejiang, respectively, which germinated in darkness, developed into full seedlings. Addition of plant growth regulators [gibberellic acid (GA₃), indole-3-acetic acid (IAA), 6-benzylaminopurine (BA)] and organic additives (casein hydrolysate and yeast extract) in mediums promoted germination and seedling development. (Woody plant medium) WPM medium supplemented with 0.5 mg L⁻¹ GA₃, 0.5 mg L⁻¹ IAA, 0.5 mg L⁻¹ BA and 1 g L⁻¹ activated charcoal was optimal for the culture of embryos from Anhui, while WPM medium supplemented with 0.5 mg L⁻¹ GA₃, 500 mg L⁻¹ casein hydrolysate and 1 g L⁻¹ activated charcoal was optimum for embryos from Zhejiang. Moreover, the germination and seedling survival rate of embryos of *T. chinensis* var. *mairei* decreased with increasing maturity of the seeds. In conclusion, darkness during germination is necessary for subsequent seedling development and immature seeds are optimal for embryo culture of this species.

Keywords: Embryo culture, Germination, *In vitro* culture, Seedling development, *Taxus*.

INTRODUCTION

T. chinensis var. *mairei* is a valuable medicinal plant distributed in China (Ru *et al.*, 2008). In natural environment, characteristics such as dioecism, unisexual flower, and cross pollination result in low fruition rate of the plant. Natural germination of seeds from *T. chinensis* var. *mairei* usually take place in 1.5 to 2 years from collection, which leads to low germination rate, low survival frequency, and decreasing population quantity (Ru *et al.*, 2008). In recent years, this species has been excessively logged in order for production of taxol, the

most effective and expensive anticancer medicine in the world (Kumar *et al.*, 2010). The natural resource of *T. chinensis* var. *mairei* has suffered severe damage and face extinction (Ru *et al.*, 2008).

Embryo culture, in which embryos are excised aseptically and cultured *in vitro* on the medium, is a useful technique to overcome seed dormancy, shorten the breeding cycle (Ho, 1987), and rescue embryo and obtain plants from crosses between seedless cultivars (Razi *et al.*, 2013). In woody plants, the technique is also fit for generation cycle reduction when seeds require stratification period of 2-3 months for germination (Kaur *et al.*, 2006). Embryos of

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Taxus have been reported to germinate and grow into seedlings *in vitro*, which depended on the development degree of the seeds, culture medium components, and light condition (Flores and Sgrignoli, 1991; Flores et al. 1993; Zhiri et al., 1994). In *T. brevifolia* and *T. media*, embryos excised from immature seeds with undeveloped arils showed higher germination rates than those from mature seeds (Flores and Sgrignoli, 1991). In *T. baccata* L. cv. *Stricta*, after the embryos were washed with running tap water for 7 days and then cultured on modified Murashige and Skoog (MS) medium or Heller (H) medium for 7 days, the germination rate reached 100% (Zhiri et al., 1994). Moreover, embryos cultured on modified H (H⁺) or modified MS (MS⁺) media germinated and developed into full seedlings while those cultured on MS, H, Gamborg basal medium (B₅), and White and Risser basal medium (WR) medium germinated but no further development into full seedlings (Zhiri et al., 1994). In *T. X media* cvs. *Citation*, *T. X media* cvs. *Hicksii*, *T. brevifolia*, *T. baccata*, *T. cuspidata* cv. *Capitata*, Gupta and Durzan's medium was superior to W medium for embryo germination and root formation (Flores et al., 1993). Furthermore, a 14-hour photoperiod was found to improve embryo germination and growth into seedlings in *T. X media* cvs. *Citation*, *T. X media* cvs. *Hicksii*, *T. brevifolia*, *T. baccata*, *T. cuspidata* cv. *Capitata* (Flores et al., 1993).

The objective of this research was to elucidate the factors affecting embryo culture of *T. chinensis* var. *mairei* and establish rapid proliferation system for *T. chinensis* var. *mairei*, which is important for natural resource preservation of the medicinal plant.

MATERIALS AND METHODS

Plant Material

Seeds of *T. chinensis* var. *mairei* were collected in October and December 2011 from trees of natural habitats growing at

State-Owned Miaoshou Forest Farm, Jingde, Anhui and Tianmu Mountain, Linan, Zhejiang, respectively. The seeds were sorted into the following stages according to the characteristics of aril and the color of seeds. Immature (DG): Dark green to light brown seeds with green aril; Semi-mature (BO): Brown seeds with orange swollen aril; Mature (DR): Dark brown seeds with red swollen aril. Seeds were used immediately after collection. Mature seeds (DR) were used, except where mentioned otherwise.

Sterilization of Seeds and *In vitro* Embryo Isolation

After removing the arils, seeds were rinsed in running water for 1 hour and then surface-sterilized for 20 minutes in 5% sodium hypochlorite (w/v) followed by three times washing with sterile distilled water. Seeds were cut open with a sharp scalpel and the embryos were removed with forceps under stereo microscope. Then, the excised embryos were rinsed in sterile distilled water for three times to shake off the endosperm cells.

In vitro Embryo Culture

Excised embryos were cultured on WPM basal medium (Lloyd and McCown, 1980) (CK) or WPM basal medium supplemented with different concentrations of GA₃ (0, 0.5 mg L⁻¹), IAA (0.5, 2 mg L⁻¹), BA (0.5, 2 mg L⁻¹) in combination with casein hydrolysate (500 mg L⁻¹) and yeast extract (500 mg L⁻¹). All mediums were solidified with 0.75% (w/v) agar. On an average, 10 embryos were placed on each bottle (100 mL) containing 30 mL culture medium. All the growth regulators were filter sterilized. The pH of all media was adjusted to 5.8±0.1 prior to autoclaving at 121°C for 20 minutes. Each treatment was repeated three times and each replicate consisted of at least 120 embryos of *T. chinensis* var. *mairei*, half of which was from seeds collected from Anhui (A)

and the other half from seeds collected from Zhejiang (B). The embryos were sub-cultured out at 3 weeks interval.

In vitro Germination and Seedling Survival Rate Evaluation

Embryos were cultured at a light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ under 14-hours photoperiod (light-grown) or in darkness (dark-grown) at $25 \pm 2^\circ\text{C}$. Germination was defined as emergence of the radicle (about 1 mm long) accompanied by opening and greening of two cotyledons. After germination, embryos incubated in darkness were transferred to 14-hours photoperiod. The germination rate was the percent of germinated embryos in all embryos 30 days after culture. The seedling survival rate was determined by the percent of intact and normal seedlings in all germinant embryos 30 days after culture (60 days after initial *in vitro* culture).

Statistical Analysis

Each experiment was repeated at least three times. Analysis of variance (ANOVA) was performed by the statistical software

SPSS11.5. The means were compared using Duncan's multiple range test at the 5% level.

RESULTS

Effect of Light on *In vitro* Germination and Seedling Development of Embryos

Embryos cultured under 14 hours photoperiod or in darkness gradually swelled and elongated. Ten days later, two cotyledons were observed to open and gradually turn green. As shown in Figure 1(a-b), (Table 1) the germination rate of embryos from Anhui and Zhejiang incubated on WPM basal mediums under 14 hours photoperiod was 54.3 and 80.6%, respectively, while 44.3 and 45.3% in the dark, respectively.

After germination, all germinated embryos were transferred to 14 hours photoperiod. After fourteen days, some embryos began to initiate buds and roots and then developed into full seedlings, whereas some aborted after divergence and greening of the cotyledons. As shown in Figure 2 (a-b) 1.4 and 1.83% embryos from Anhui and Zhejiang, respectively, which germinated on

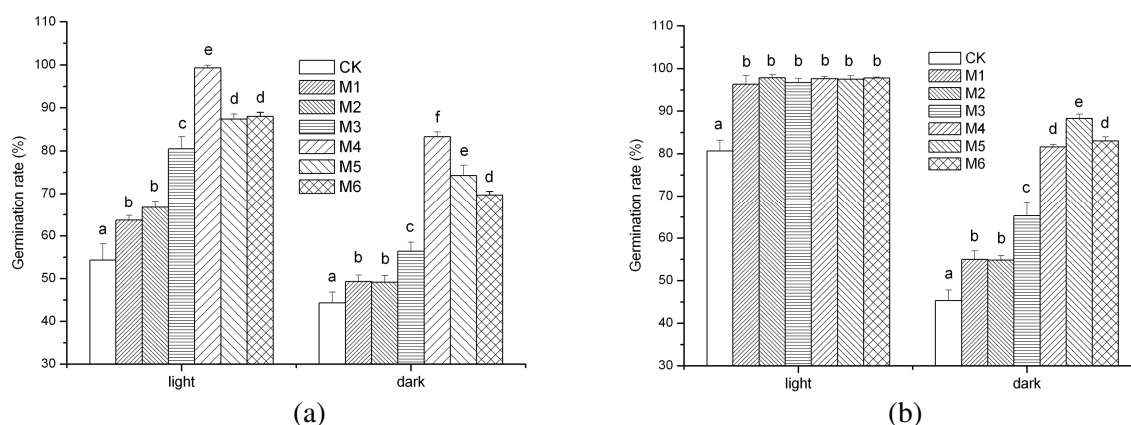


Figure 1. Effect of culture mediums and light situation on germination rate of embryos of *T. chinensis* var. *mairei* from Anhui (a) and Zhejiang (b). Embryos were cultured on WPM basal medium (CK) or WPM basal mediums supplemented with different concentrations of GA₃, IAA, BA, casein hydrolysate and yeast extract (named as M1, M2, M3, M4, M5, M6, respectively) at a light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ under 14 hours photoperiod (light) or in darkness (dark) at $25 \pm 2^\circ\text{C}$. Bars with different letters are significantly different at the 0.05 level.



Table 1. Plant growth regulators and organic additives supplemented into the WPM basal medium (with 20 g L⁻¹ sucrose +1 g L⁻¹ activated charcoal) used for embryo culture.

Medium code	GA ₃ ^a (mg L ⁻¹)	IAA ^b (mg L ⁻¹)	BA ^c (mg L ⁻¹)	Casein hydrolysate (mg L ⁻¹)	Yeast extract (mg L ⁻¹)
CK ^d	—	—	—	—	—
M1 ^e	0.5	—	—	—	—
M2 ^f	0.5	2	0.5	—	—
M3 ^g	0.5	0.5	2	—	—
M4 ^h	0.5	0.5	0.5	—	—
M5 ⁱ	0.5	—	—	500	—
M6 ^j	0.5	—	—	—	500

^a gibberellin A₃ ; ^b indole-3-acetic acid; ^c 6-Benzylaminopurine ; ^d WPM + 20g/L sucrose +1g/L activated charcoal ; ^e WPM + 0.5mg/LGA₃ + 20g/L sucrose +1g/L activated charcoal ; ^f WPM + 0.5mg/LGA₃ + 2mg/L IAA + 0.5mg/L 6-BA + 20g/L sucrose +1g/L activated charcoal ; ^g WPM + 0.5mg/L GA₃ + 0.5 mg/L IAA + 2 mg/L 6-BA + 20g/L sucrose +1g/L activated charcoal ; ^h: WPM + 0.5mg/L GA₃ + 0.5 mg/L IAA + 0.5 mg/L 6-BA + 20g/L sucrose +1g/L activated charcoal ; ⁱ: WPM + 0.5mg/L GA₃ + 500mg/L casein hydrolysate + 20g/L sucrose +1g/L activated charcoal; ^j: WPM + 0.5mg/L GA₃ + 500mg/L yeast extract + 20g/L sucrose +1g/L activated charcoal

WPM basal medium under 14 hours photoperiod, developed into full seedlings, whereas the others died ultimately. Comparatively, more than 23.3 and 36.3% embryos from Anhui and Zhejiang, respectively, which germinated on WPM basal medium in continuous darkness, grew into full seedling.

Effect of Hormonal Treatments on *In vitro* Germination and Seedling Development

As shown in Figure 1 (a-b), under 14 hours photoperiod, addition of GA₃ increased the germination rate of embryos from Anhui and Zhejiang to 63.6 and 96.3%,

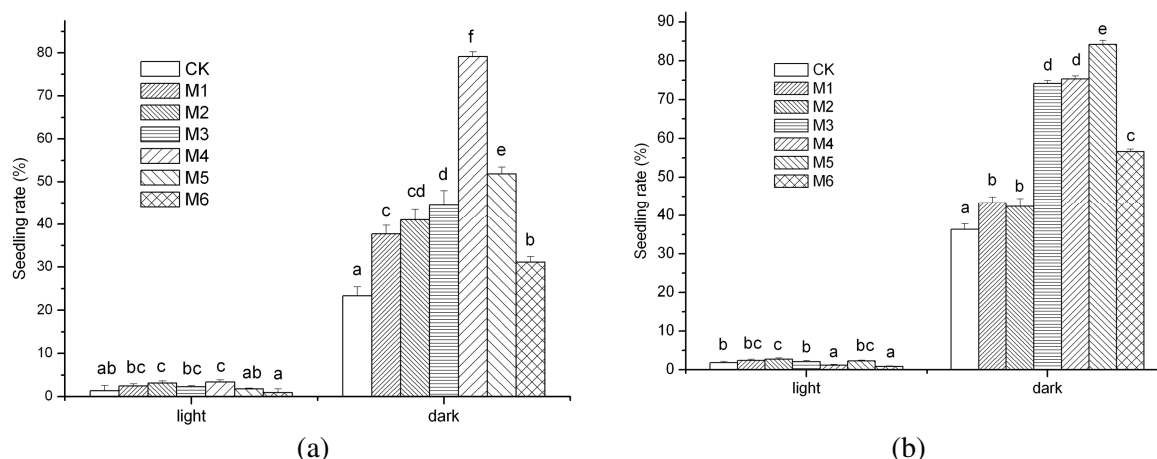


Figure 2. Effect of culture mediums and light situation on seedling survival rate of embryos of *T. chinensis* var. *mairei* from Anhui (a) and Zhejiang (b). Embryos were cultured on WPM basal medium (CK) or WPM basal mediums supplemented with different concentrations of GA₃, IAA, BA, casein hydrolysate and yeast extract (named as M1, M2, M3, M4, M5, M6, respectively) at a light intensity of 40 μmol m⁻² s⁻¹ under 14-hours photoperiod or in darkness (dark) at 25±2°C. Bars with different letters are significantly different at the 0.05 level.

respectively. Inclusion of casein hydrolysate or yeast extract in medium containing GA₃ further lifted germination rate of embryos from Anhui to 87.4 and 88%, respectively, and embryos from Zhejiang to 97.5 and 97.7%, respectively, suggesting promotion effects of GA₃, casein hydrolysate, and yeast extract on germination of *T. chinensis* var. *mairei*. Additionally, IAA and BA had positive influence on germination induction Figure 1(a-b). The germination rates of embryos from Anhui incubated on M2, M3 and M4 under 14 hours photoperiod were 66.7, 80.5, and 99.3%, and embryos from Zhejiang were 97.8, 96.7, and 97.6% respectively, implying that 0.5 mg L⁻¹ IAA in combination with 0.5 mg L⁻¹ BA was superior to other concentration proportions Figure 1(a-b).

For the dark-grown embryos from Anhui and Zhejiang, the germination frequency of embryos incubated on WPM basal medium was only 44.3 and 45.3%, respectively. Supplement of GA, casein hydrolysate, yeast extract, IAA and BA also significantly promoted germination Figure 1(a-b). M4 medium was the most optimal for germination induction of embryos from Anhui while M5 was the most appropriate for embryos from Zhejiang in the darkness Figure 1(a-b).

As shown in Figure 2(a-b), few embryos germinated on WPM basal medium under 14 hours photoperiod developed into full seedlings, but almost all embryos died ultimately. Addition of GA₃, casein hydrolysate, yeast extract, IAA and BA made no statistical difference, indicating the decisive role of light in seedling development. Furthermore, supplement of GA₃, casein hydrolysate, yeast extract, IAA, and BA significantly elevated seedling survival rate of embryos germinated in continuous darkness. M4 medium and M5 are optimal for *in vitro* culture of embryos from Anhui and Zhejiang, respectively Figure 1(a-b).

Additionally, we found that embryos from Anhui showed higher germination frequency when cultured under a 14 hours photoperiod

than in continuous darkness, despite the same culture medium, implying the promotion effect of light on germination induction Figure 1-a. The situation was similar in *T. chinensis* var. *mairei* from Zhejiang Figure 1- b.

Effect of Development Stage of Seeds on *In vitro* Germination and Seedling Development

The germination rates of the embryos obtained from immature seeds (DG), semi-mature seeds (BO), and mature seeds (DR) were determined on M4 and M5 medium in the darkness. Figure 3-a shows that the germination rate of embryos from Anhui excised from immature seeds was 98.6% on M4 medium. The germination rate of embryos from semi-mature seeds decreased to 89.8% while that from mature seeds further fell to 84.8%, suggesting that germination rate of embryos from Anhui decreased with increasing maturity of seeds Figure 3-a. The situation was the same on M5 medium. Likewise, the germination rate of embryos from Zhejiang also declined with increasing maturity of seeds on both M4 and M5 media Figure 3-b.

As shown in Figure 4-a, the embryos from Anhui excised from immature seeds showed the highest seedling survival rate, whereas those from mature seeds displayed the lowest on both M4 and M5 media. In *T. chinensis* var. *mairei* from Zhejiang, the situation was similar. Young embryos could develop into normal seedlings more than the semi-mature and mature embryos (Figure 4, b), demonstrating that seedling survival rate of embryos decreased with increasing dormancy of the seeds.

DISCUSSION

Light requirement for seed germination is diverse among different plant species. Light is necessary for the germination of some seeds while inhibitive for others. In our

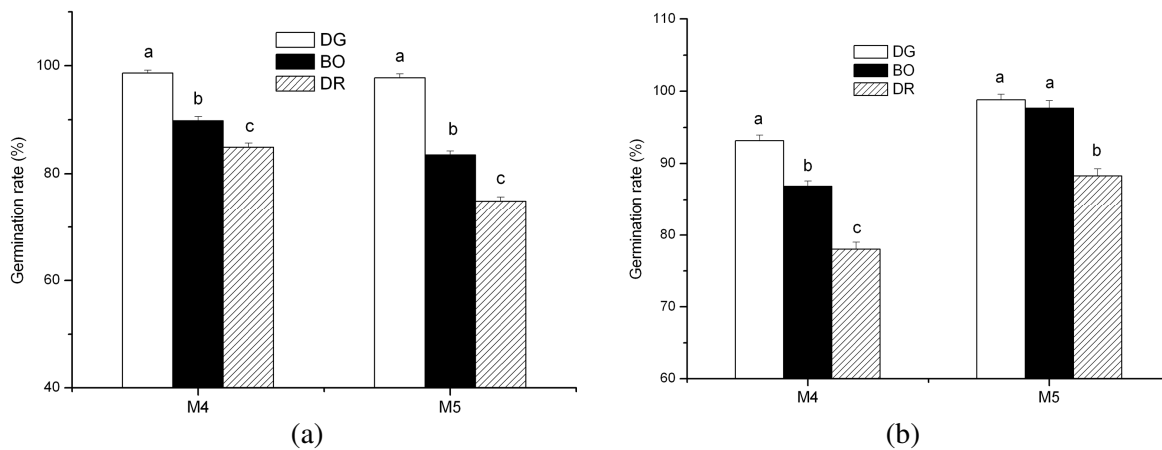


Figure 3. Effect of development stage of seeds of *T. chinensis* var. *mairei* from Anhui (a) and Zhejiang (b) on germination rate of embryos. Embryos were cultured on M4 and M5 mediums in darkness at 25±2°C. DG: Dark green to light brown seeds with green aril; BO: Brown seeds with orange swollen aril, and DR: Dark brown seeds with red swollen aril. Bars with different letters are significantly different at the 0.05 level.

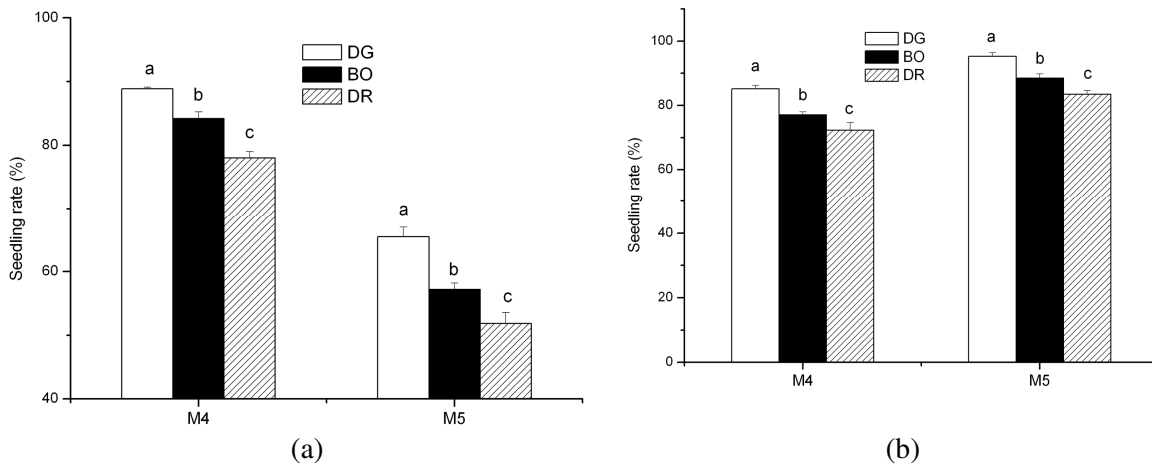


Figure 4. Effect of development stage of seeds of *T. chinensis* var. *mairei* from Anhui (a) and Zhejiang (b) on seedling survival rate of embryos. Embryos were cultured on M4 and M5 mediums in darkness at 25±2°C. DG: Dark green to light brown seeds with green aril; BO: Brown seeds with orange swollen aril, and DR: dark brown seeds with red swollen aril. Bars with different letters are significantly different at the 0.05 level.

research, germination rates of *T. chinensis* var. *mairei* embryos incubated under 14 hours photoperiod were evidently higher than those under continuous darkness, suggesting the promotion effect of light on embryo germination (Figure 1, a-b). Previous research showed that light might increase embryos germination by GA₃ regulation (Li et al., 2011). In our research, under either 14 hours photoperiod or in the darkness, GA₃ application significantly lifted the germination rate of embryos, indicating potential relationship between the

light and GA on embryo germination induction of *T. chinensis* var. *mairei* (Figure 1, a-b).

What was more interesting, however, was the effect of light during germination on seedling development. We found that few embryos germinated under 14 hours photoperiod developed into full seedlings whereas more than 20% embryos germinated in continuous darkness grew into full seedling, suggesting that photoperiod during germination induction was disadvantageous while darkness was

positive for further seedling development (Figure 2, a-b). These results were similar to those from *Paphiopedilum*, in which embryos germinated in continuous dark developed quite well while those germinated in the light developed poorly, and gradually turned brown and died off at the early stage of development (Pierik *et al.*, 1988; Tay *et al.*, 1988). Some amino acids supported seed germination, but inhibited shoot formation (Tay *et al.*, 1988). Light might affect seedling development through utilization of amino acid in the *in vitro* culture medium (Tay *et al.*, 1988). But this can't explain the situation without amino acid in culture medium. An understanding of contrary effect of light on germination and subsequent seedling development of *T. chinensis* var. *mairei* is currently lacking, which requires further research.

The WPM medium was demonstrated to be the optimal medium for germination and development of *T. chinensis* var. *mairei* (Zeng *et al.*, 2010), which was adopted in this research. Complex organic additives are known to improve growth and differentiation of *in vitro* plant culture (Al-Khayri, 2011). Our results approved the positive effect of casein hydrolysate and yeast extract on embryo germination and seedling development of *T. chinensis* var. *mairei* (Figure 1, a-b; Figure 2, a-b). But, in *Taxus baccata* (L), it was found that casein hydrolysate and yeast extract had no influence on *in vitro* germination of embryos (Zarek, 2007). The different effects of casein hydrolysate and yeast extract might be due to different species. Some emphases have been put on the effects of plant growth regulators on the embryo germination of *Taxus* species. Our results showed that a combination of GA₃, BA and IAA significantly improved *in vitro* germination of embryos from *T. chinensis* var. *mairei* (Figure 1, a-b). However, in the germination experiment of *T. wallichiana* embryos, BA or GA₃ was useless (Datta and Jha, 2004), suggesting that the effects of plant growth regulators on embryo culture differed depending on *Taxus* species.

In our experiments, embryos obtained from immature seeds showed higher germination rate and seedling survival rate than those from semi-mature or mature seeds (Figure 3, a-b; Figure 4, a-b). Another report on *T. chinensis* var. *mairei* suggested that the dormancy of seeds was not due to the rigid testa, which was water permeable, but primarily to the presence of germination inhibitory substances in the different parts of the seed, especially in embryo, which can inhibit embryo growth (Liu *et al.* 2011). It is possible that inhibitory substances in embryo prevented the germination of embryo. Investigation of germination inhibitory substances in seeds during different periods in the course of seed maturity will be helpful to elucidate why the germination rate of embryo from *T. chinensis* var. *mairei* decreased along with the increasing maturity of seeds, which remains a subject for further exploration.

In conclusion, immature seeds in combination with incubation in the darkness during germination period were optimal for embryo culture of *T. chinensis* var. *mairei*. WPM medium supplemented with 0.5 mg L⁻¹ GA₃, 0.5 mg L⁻¹ IAA, 0.5 mg L⁻¹ BA and 1 g L⁻¹ activated charcoal was optimal for the culture of embryos from Anhui, while WPM medium supplemented with 0.5 mg L⁻¹ GA₃, 500 mg L⁻¹ casein hydrolysate and 1 g L⁻¹ activated charcoal was optimal for embryos from Zhejiang.

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جوانه زنی درون شیشه و رشد گیاهچه *Taxus chinensis* var. *mairei* با کشت

جنین

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چکیده

گیاه دارویی *Taxus chinensis* var. *mairei* گونه ای کمیاب و در معرض انقراض در چین است. در این پژوهش، به منظور افزایش تکثیر سریع این گیاه و حفظ منابع طبیعی، شرایط جوانه زنی درون شیشه ای و رشد گیاهچه *Taxus chinensis* var. *mairei* از مناطق آنهویی و ژجیانگ بررسی شد. نتایج نشان داد که نرخ جوانه زنی درون شیشه ای جنین های جدا سازی شده ای که ۱۴ ساعت فتوپریود داشتند بیشتر از آنهایی بود که در تاریکی بودند. اما، تقریباً همه این جنین ها نتوانستند به گیاهچه تبدیل شوند. در مقایسه با آنها، ۲۳/۳ بئهد و ۳۶/۳٪ به ترتیب از جنین های مناطق آنهویی و ژجیانگ که در تاریکی جوانه زده بودند تا مرحله گیاهچه کامل رشد کردند. افزودن تنظیم کننده های رشد گیاه [جیبرالیک اسید (GA_3) و (IAA) و (BA) 6-benzylaminopurine] و افزودنی های آلی (casein hydrolysate و عصاره yeast) در محیط کشت موجب افزایش جوانه زنی و رشد گیاهچه ها شد. برای کشت جنین های آنهویی، محیط کشت WPM دارای مکمل های 0.5 mg/L GA_3 , 0.5 mg/L IAA, 0.5 mg/L BA در حالیکه برای جنین های ژجیانگ، محیط کشت WPM دارای مکمل های 0.5 mg/L GA_3 , 500 mg/L casein hydrolysate و یک گرم در لیتر زغال چوب فعال بهترین محیط بود. افزون بر این، بر پایه نتایج، نرخ جوانه زنی و بقای گیاهچه های *Taxus chinensis* var. *mairei* با رسیده شدن (بلوغ) بذر ها کاهش یافت. بنا بر نتایج این پژوهش، تاریکی در طی جوانه زنی برای رشد بعدی گیاهچه ضروری است و بذرهای نارس برای کشت جنین این گیاه بهترین اند.