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Rapid and Efficient *in vitro* Germination of Embryos from *Taxus media* Rehder

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Abstract: An efficient protocol was established to obtain seedlings of *Taxus media* Rehder by embryo culture technique. No seedlings of *T. media* were obtained without the excision of endosperm and few embryos were able to germinate on half-strength MS media in the absence of plant growth regulators, so it was necessary for embryo germination to get rid of endosperms of the mature seeds and to add plant growth regulators to prompt embryo germination. The effects of three factors, namely BA, GA_3 and 2,4-D, on embryo germination were examined by $L_9(3^4)$ orthogonal design, the orthogonal array that corresponds to four levels and three parameters in nine treatments. The analysis of variance of the results of orthogonal tests showed that half-strength MS media supplemented with BA 1.0 mg L^{-1}, 2,4-D 0.1 mg L^{-1} were the optimized media for embryo germination of *Taxus media*. On the optimized media, 62% of the embryos could germinate and develop into seedlings.

Key words: *Taxus media*, embryo germination, orthogonal design, plant growth regulators

INTRODUCTION

Taxol®, a potent anticancer drug, has been found in the bark, root and other parts of various *Taxus* species plants[1]. *Taxus* species plants face the threat of extinction because of huge demand for both taxol and wood and difficulties in propagation. Fortunately, it has been found that *Taxus media* can produce a unique class of diterpenoid alkaloids, including taxol, even in needles which are the most important source of taxol used to treat cancer[2]. *T. media* has been approved to be the successive source for taxol production[3]. To plant *T. media* in a large scale is an efficient way to solve the shortage problem of taxol-producing materials. However, the germination of *Taxus* seeds has been very difficult. The germination of *Taxus* seeds required lengthy dormancy, often taking 2 or more years[4] and the rate of germination remained low[5-7]. Additionally, seeds of *Taxus* species were very difficult to develop into seedlings in the natural conditions[8]. All of the factors above restrict the germination of *Taxus* seeds and the production of seedlings. Embryonic culture is a useful tool to overcome seed dormancy and to abbreviate the breeding cycle. Many treatments have been employed to improve seed germination. Till now, embryos from some *Taxus* species such as *Taxus brevifolia*[4], *Taxus cuspidata*[6], *Taxus canadensis*[6] and *Taxus baccata* L. (English Yew)[9] were successfully cultured and the seedlings were got. However, the frequency of seed germination, only 2% for mature seeds[10], remains low and few emphases have been put on the effects of plant growth regulators on the embryo germination. The present study was mainly aimed at investigating the effects of plant growth regulators on *T. media* embryo germination and optimizing the culturing conditions by orthogonal designed tests.

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MATERIALS AND METHODS

Plant materials: Mature seeds of *T. media* were bought from Zhonglin Taxus Company (Yantai, Shandong, China) in 2002. The seeds were fresh and the seed viability given by 2, 3, 5-triphenyl tetrazolium chloride was more than (96±3)%.

Seed and embryo pretreatment: The pretreatments of the seeds were carried out as the following procedure. After being washed in running tap water for 7 days, the seeds were surface disinfected by soaking in 70% (v/v) ethanol for 2 min and 0.1% (w/v) $HgCl_2$ for 15 min followed by rinsing five times in sterile distilled water and the seed coats were removed with sharp surgical blades and tweezers; then embryos could be obtained by longitudinally excising seeds. Sixty seeds without seed coats and sixty embryos without endosperms were inoculated on half-strength MS medium[11] without plant growth regulators to investigate the effects of pretreatment (getting rid of endosperm or not) on the seedling initiation. For the orthogonal test, only the embryos were inoculated on media with different plant growth regulator combination. In each treatment of the orthogonal experimental test, 100 embryos were used.

Culture media and conditions: As Chang and Yang found that ½ MS was better than MS for *Taxus* embryo germination[6], in the present study, the half-strength MS medium was used as basal medium for embryo germination. All media contained 3% (w/v) sucrose, 0.26% (w/v) Phytagel® and 0.8% (w/v) PVP. The media were adjusted to pH 5.8 before being autoclaved at 121°C for 20 min. A culture bottle contained 30 mL semi-solid medium. All explants were cultured under a 12 h light/12 h dark photoperiod (55 μM m^{-2} sec^{-1} light intensity provided by daylight white fluorescent tubes) in an automatic incubator at 25°C. Germination percentage (%) was determined after 30 days. Each treatment in the orthogonal experimental test had 3 replicates with 100 embryos/replicate.

Plant growth regulators: The orthogonal test was used to identify the optimal plant growth regulators and their combination for improving *T. media* embryo germination. Three plant growth regulators, including BA, GA_3 and 2,4-D, were studied and each of the three was used at three different concentration levels. The orthogonal table of $L_9(3^4)$[12] was selected for studying the effects of three factors on the seed germination from *T. media* (Table 1); $L_9(3^4)$ is the orthogonal array that corresponds to at most four levels and three parameters in nine treatments. Nine treatments were arranged according to the design (Table 2). ANOVA was carried out to evaluate the effects of the three factors on the embryonic germination of *T. media*. Statistical analysis was carried out by applying Taguchi's method[12].

Table 1: The design form of orthogonal test $L_9(3^4)$

	Factors (mg L^{-1})		
Levels	6-BA	GA_3	2,4-D
1	0.5	0	0.2
2	1.0	1	0.05
3	2.0	2	0.1

Table 2: The effect of different concentrations of 6-BA, GA_3 and 2,4-D on the percentage of *Taxus media* excised embryo germination in the orthogonal test $L_9(3^4)$

Test No.	6-BA (mg L^{-1})	GA_3 (mg L^{-1})	2,4-D (mg L^{-1})	Excised embryo germination (%) (Means±SE)
1	0.5	0	0.2	11±1
2	0.5	1	0.05	29±3
3	0.5	2	0.1	36±4
4	1.0	0	0.1	62±6
5	1.0	1	0.2	39±3
6	1.0	2	0.05	30±2
7	2.0	0	0.05	47±2
8	2.0	1	0.1	32±3
9	2.0	2	0.2	18±1

RESULTS AND DISCUSSION

The effect of testa and endosperm on embryo germination: No seedlings were obtained on half-strength MS medium without plant growth regulators from the seeds without seed coat (but with endosperm). Only two embryos germinated out of the 60 embryos (without endosperm) that were placed onto half-strength MS medium without plant growth regulators.

The effects of plant growth regulators on embryo germination: Among the nine treatments, the treatment 4 (1.0 mg L^{-1} 6-BA, 0 mg L^{-1} GA_3 and 0.1 mg L^{-1} 2,4-D) showed the highest germination, while the other combinations all increased germination percentages significantly compared to the control treatment (no growth regulators).

The best medium for embryo germination was the combination of $A_2B_1C_3$ (the treatment 4, 62% germination), that was 1.0 mg L^{-1} 6-BA, 0 mg L^{-1} GA_3 and 0.1 mg L^{-1} 2,4-D. 2,4-D and BA had significant effects on the germination of *Taxus media* ($p<0.01$) and GA_3 had no important influence on *Taxus media* embryo germination ($p>0.05$) on half strength culture medium. 2,4-D was the most influential of the three factors on embryo germination (Table 2).

Mature seeds or embryos of *Taxus* species plants are difficult to germinate and develop into seedlings in natural

conditions[5]. This is mostly due to the physical limitation of thick seed coats, the presence of inhibitors and the dormancy that is a common phenomenon in *Taxus* plants[8]. Many treatments have been reported to improve embryo germination and growth into seedlings. The thick seed coats could be cut away to expose the embryos; the inhibitors such as ABA and ABA-like compounds that block germination could be partially washed away by running tap water[9]; the dormancy of *Taxus* seeds could be released by alternative temperature and cold stratification which is time-consuming[13]. In this study we used the method reported by Zhiri *et al.*[9] but found that only a small percent of seeds of *Taxus media* could germinate on basal media. Flores and Sgrignoli[5] found that only immature embryos of *Taxus media* germinated and not mature ones. In fact, the seed materials used in the present study were mature ones that contained much more inhibitors than immature ones. The thick seed coats of *Taxus* species prevent the seedling formation. After seeds are carefully split in half with sharp blades and scalpels, the embryos can be examined. The present results showed that no seedlings were obtained from the seeds without seed coats but with endosperm; two embryos germinated out of the 60 embryos without endosperm that were placed onto half-strength MS medium without plant growth regulators. So, removing the embryos from seeds and culturing them on nutrient medium could result in embryo germination and without long-term waiting but was not enough for efficient germination of *Taxus* embryos. The dormancy of *Taxus baccata* seeds may also be broken by adding plant growth regulators into culture media[4]. Earlier research suggested that the dormancy of *Taxus* embryos was caused by ABA or an ABA-like compound in the embryos[14] and could be broken by adding plant growth regulator GA_3[15]. Results showed that a combination of 2,4-D and BA could be used for breaking the dormancy of *Taxus* embryos in mass production of *Taxus media* seedlings for conservation planting and taxol production.

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REFERENCES

1. Wani, M.C., H.L. Taylor, M.E. Wall, P. Coggon and A.T. Mcphail, 1971. Plant antitumor agents, VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. Am. Chem., 93: 2325-2327.
2. Rowinsy, E.K., L.A. Cazenave and R.C. Donehower, 1990. Taxol: A novel investigational antimicrotubule agent: A review. J. Natl. Cancer Inst., 82: 1247-1259.
3. Miller, R.W., 1980. A brief survey of *Taxus* alkaloids and other taxane derivatives. J. Natl. Prod., 43: 425-437.
4. Flores, T., L.J. Wagner and H.E. Flores, 1993. Embryo culture and taxane production in *Taxus* spp. *In vitro* Cell. Dev. Biol. Plant, 29: 160-165.
5. Flores, H. and P.J. Sgrignoli, 1991. *In vitro* culture and precocious germination of *Taxus* embryos. Cell. Dev. Biol. Plant, 27: 139-142.
6. Chang, S.H. and J.C. Yang, 1996. Enhancement of plant formation from embryo cultures of *Taxus mairei* using suitable culture medium and PVP. Bot. Bull. Acad. Sin., 3: 35-40.
7. Anderson, E.D. and J.N. Owens, 2001. Embryo development, megagametophyte storage product accumulation and seed efficiency in *Taxus brevifolia*. Can. J. For. Res., 31: 1046-1056.
8. Hu, L.Y., L. Wang and B. Wu, 1992. *In vitro* culture of immature *Taxus* embryos. HorticSci., 27: 210.
9. Zhiri, A., M. Jaziri, J. Homes, M. Vanhaelen and K. Shimomura, 1994. Factors affecting the *in vitro* rapid germination of *Taxus* embryos and the evaluation of taxol content in the plantlets. Plant Cell Tiss. Org. Cult., 39: 261-263.
10. Chee, P.P., 1994. *In vitro* culture of zygotic embryos of *Taxus* species. HorticSci., 29: 695-697.
11. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15: 473-479.
12. Ross, P.J., 1989. Taguchi Techniques for Quality Engineering: Loss Function, Orthogonal Experiments, Parameters and Tolerance Design. Mc Graw Hill, New York, pp: 88.
13. Chien, C.T., J.J. Yang, Y.I. Chuang and T.P. Lin, 1994. Germination promotion of *Taxus mairei* seed. Taiwan For. J., 20: 46-47.
14. Le Page-Degivry, M.T., 1968. Mise en evidence d'une dormance associ une immaturity, I'embryon chez *Taxus baccata* L. (The title in English: The evidence of dormancy associated to the immaturity of the embryos of *Taxus baccata* L.). Comptes Rendus De L Acad. Des. Sci., 266: 1028-1030.
15. Le Page-Degivry, M.T., 1973. Intervention d'un inhibiteur lie dans la dormance embryonnaire de *Taxus baccata* L. (Intervention of inhibitor in the embryonic dormancy of *Taxus baccata* L.). Comptes Rendus De L Acad. Des. Sci., 277: 177-180.