

Micropropagation of common yew using Embryo culture

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Abstract

Common yew *Taxus baccata* L. is an evergreen, slow growing tree, indigenous of hyrcanian forests in Iran. Discovery of Paclitaxel as an anti tumor agent in the bark of *Taxus* has increased importance of this plant for treatment of breast and ovary cancer, especially 7000 women die annually due to breast cancer in Iran. Regarding destructive method of paclitaxel production and protection of the yew as an endangered tree in Iran, micropropagation using embryo culture can be considered as an efficient method to increase *Taxus* resources in Iran. In this regard, effects of different culture medium compositions, active charcoal concentrations and gibberellic acid on embryo culture of *Taxus baccata* L. were studied. Murashig and Skoog culture medium at half and complete strengths of macronutrients and active charcoal at concentrations of 0, 2, 4 and 6 g/L were used. Based on experimental data MS/2 + 2 g/L active charcoal appeared to be the most effective treatment for successful embryo culture.

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Introduction

Taxus baccata L. an evergreen tree, indigenous of hyrcanian forests grows from Astara to Aliabad forests in the northern Alborz mountain with an altitude ranging from 900-1800 m. The main habitat of common yew is eastern Mazandaran and Golestan provinces [1]. The yew is an evergreen gymnosperm and slow-growing, long-lasting (>1000 years) tree which takes almost 70 years to get mature [2]. Yew is the only genus of Taxaceae with economical application which is used for carpentry, handicrafts and drug production [3].

Paclitaxel was first discovered in 1971 from the bark of *Taxus brevifolia* [4]. After passing the clinical trials in 1980s, FDA approved its application for treatment of acute ovary and breast cancers, as well as AIDS-related Kaposi's sarcoma. Paclitaxel market in 1999 could reach 1.5 billion US dollar and increased to 1.6 billion dollar in 2000 [5-7]. The yew tree is endangered due to wide harvest for paclitaxel production and increasing demand for this drug in future years and its very slow growth [8] which necessitates new approaches to protect and propagate this valuable plant. Current methods of *Taxus* multiplication are seed culture, cutting, grafting and layering [2]. Somatic multiplication increases the young trees, however, it needs one year for rooting and topophysis of axillary shoots is limiting for production of plant reservoirs [9, 10]. Natural seed germination alone is not satisfactory because most of it is eaten by birds and seed harvest is difficult [9]. In addition, yew seeds have strong dormancy lasting 1.5-2 years [11] and routine temperature treatments require a

few months to a year to break the seed dormancy [12]. Nevertheless, embryo culture is promising and can produce many plants in the shortest possible time. The percent of seed germination to seedling (30%-63%) [10] is lower than embryo culture compared to 70%-100% for embryo culture in *Taxus brevifolia* [13]. In *Taxus baccata*, seedlings can be produced using embryo culture within 8 weeks while 1-2 years is required for seed stratification [10] and it seems that seed dormancy is induced by abscisic acid or its derivatives [9]. Also low development of embryos in fresh seeds is considered a reason for strong seed dormancy [12]. Therefore, embryo culture is required to overcome seed dormancy and to shorten the breeding cycle in the yew tree.

Studies on embryo culture of *T. mairei*, *T. baccata*, *T. canadensis* and *T. cuspidata* has determined that composition of culture medium is a critical and important measure which Murashig and Skoog (MS)/2 + 0.8 g/L Polyvinylpyrrolidone (PVP) was the best medium supported germination of 90% of seeds to well developed seedlings. Next MS/2 + gibberellic acid (GA3) increased seed development up to 45% [9]. It was determined that Woody Plant Medium (WPM) + 5 g/L active charcoal (AC) + 20 g/L sucrose is the best medium for *T. baccata* embryo culture and caused 65% seed germination [10].

In this research modified concentrations of macronutrients and active charcoal was applied to optimize embryo culture conditions and its development to seedling.

Materials and methods

Sampling and Embryo Culture



The seeds were harvested from almost 100 years old trees in Doul Aram forest with an altitude around 1250 m in Sang Sourakh and 1480-1500 m in Jenni Darreh, nearby Siah Roudbar village, Ali Abad Katoul, Golestan Province, Iran.

To study the effects of medium composition, active charcoal concentration and also GA3 on embryo development into the seedling, different treatments of MS and MS/2 with full and half strengths of macronutrients, 0, 2, 4, 6 g/L concentrations of AC (Table 1) and also culture medium MS/2 + 1 mg/L GA3 was prepared. Concentrations of macro and micronutrients, iron and glycine was chosen according to Murashig and Skoog formulation [14]. 30 g/L sucrose and 7 g/L agar was added to the culture medium and the pH was adjusted to 5.8 ± 0.05 using 0.1 N NaOH. In total 9 treatments were applied.

Table 1. Combinations of AC and macronutrient concentrations of MS medium for embryo culture

AC (g/L)	0	2	4	6
MS	MS + 6 g/L AC	MS + 4 g/L AC	MS + 2 g/L AC	MS
MS/2	MS/2 + 6 g/L AC	MS/2 + 4 g/LAC	MS/2 + 2 g/L AC	MS/2

The seeds were washed with tap water after separating arils and air dried. 2 minutes were shaken in 70% ethanol and then dried under laminar air flow cabinet. The seed coat was broken and endosperm was removed. Then embryo with a little gametophyte tissue was separated and transferred onto culture medium. In each treatment 15 embryos, each one in a 120 ml screwed glass vial containing 20 ml culture medium was cultured. The seeds with red arils, opaque and developed embryos with visible cotyledonary buds and gametophyte tissue filled the seed cavity were considered mature and alive to be used for embryo culture [13]. The embryos were incubated 21 days in the dark at 24°C. After precocious growth of embryos they were transferred to 3000 Lux light intensity with a photoperiod of 14/10 light/dark condition.

Statistical Analysis

Data analysis were performed using Jump software (JMP®, SAS institute) with 95% confidence ($\alpha = 0.05$).

Results and Discussion

The embryo culture in MS/2 + 1 mg/L GA3 did not show any considerable growth. All embryos remained white in color and showed no change after transferring to light (after 35 days). MS/2 medium containing different AC concentrations, however, showed better embryo growth rather than MS medium with fewer abnormalities such as seedling twisting or browning. Embryo and seedling showed better growth in presence of AC because it decreases their browning, however, the concentration of 6

g/L AC causes abnormality of embryos. MS medium showed many twisted forms of embryo (Figure 1-D) that is a reason for more abnormalities of embryo growth and inconvenience of MS medium for embryo culture. The best embryo development was seen in MS/2 + 2 g/L AC (Figure 1-A & B) because seedlings were the longest and showed less abnormalities like lack of shoot growth or root, twisting or thickening the roots. MS/2 + AC 2 g/L treatment had the next best rank for embryo growth and higher rooting.

It was appeared that two weeks after transfer to light most of shoots cannot rise up from the medium surface and bleach or get brown mainly due to lack of root growth and penetration into the medium. It seems that root cannot penetrate the medium even in presence of AC (Figure 1-C) which can be due to high sugar concentration or lack of negative phototropism in roots or high humidity in culture vessel. Nevertheless, it seems 3 weeks dark period is enough for development of embryo because almost all embryos showed precocious growth while in two weeks darkness treatment many growth abnormalities were seen; most probably the main reason of disorder in embryo development into seedling is root growth stage because most of embryos show their precocious growth in almost all treatments and shoots develops but due to lack of root growth and inability to provide with nutrient requirements they show browning.

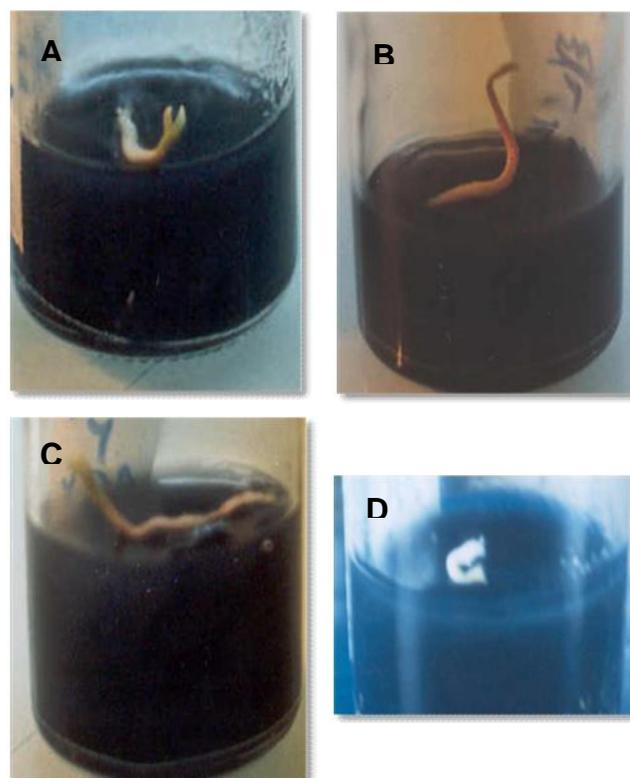


Figure 1. Shoot and Root growth in the medium 2 weeks after transfer of seedlings to the light (5 weeks culture on MS/2 + 2 g/L AC), D-Callogenesis and lack of embryo growth 5 weeks after culture on MS + 6 g/L AC.

In an study for *T. baccata* micropropagation it was appeared that MS/2 is better than MS because it produces

fewer abnormal forms which coincides with callogenesis effect of four media MS, MS/2, WPM and WPM/2 on twig explants of *T. brevifolia* [15], concluding if MS is the best for callogenesis it is proper medium for abnormal and irregular growth of cells and MS/2 should be a better option for embryo culture. Also it is known due to high salt concentration of MS medium it causes abnormal plants in embryo culture [16]. It is also reported that MS/2 is a better medium than MS for embryo culture [9]. It seems there are two steps in embryo culture: i. precocious growth (appearance of radicle along with greening and elongation of embryo after 3 weeks) ii. seedling development.

The first step is affected only by medium composition. It was reported that embryo culture of *T. mairei* in presence of PVP has shown the best development of embryo into seedling on MS/2 medium among MS, MS/2 and Gamborg's B5 media [9] which confirms our data with the only difference of AC application instead of PVP. To improve embryo growth and development into seedling GA3 were applied in the culture medium but had no evident effect but embryo elongation on embryo growth especially after precocious stage which is against its positive effect on *T. mairei* embryo [9], however, it has been reported that combination of GA3 + GA7 has been effective in germination of *T. mairei* seeds while GA3 had no effect alone [12].

Addition of 2 g/L AC showed satisfactory results in both stages of embryo culture, emphasizing importance of AC as an absorbent of embryo exudates and producer of the proper environment for rooting. It was reported that AC positively increases propagation rate of shoot and stimulates rooting [10]. AC also enhances growth and organogenesis in woody plants [13]. These results coincide with priority of addition of 0.8 mg/L PVP compared with 1 mg/L GA3 in MS/2 medium [9].

It seems the problem is with transition from precocious growth to seedling because in *T. mairei* more than 55% precocious growth occurs while less than 20% seedling is produced [9]. This also happens in *T. baccata* because precocious growth happens completely, however, shoot browning happens after a while due to lack of root growth. Addition of 2 and 4 g/L AC could solve the problem but still root showed no positive geotropism. Therefore, other factor than light should prevent root growth into the medium because radical appearance in darkness is faster than the light but functions independent of light [10]. On the other side it was determined 3 weeks darkness treatment is enough for precocious growth of embryo and increasing the dark period has no considerable effect on rooting. It was reported that increasing the dark period up to 6 weeks increases growth abnormalities and cause callogenesis even in the absence of plant growth regulators [15].

Sugar concentration has an important role in root formation and woody plants especially gymnosperms prefer low salt concentration for root formation [16]. Therefore, high osmotic potential should be considered as an inhibitor of root formation and penetration in the medium, can be avoided by using low salt medium such as MS/2 and decreasing sugar concentration. For example in embryo

culture of *T. mairei* and *T. baccata* optimum sucrose concentration were reported 25 and 20 g/L, respectively [9, 10]. Regarding above mentioned discussion it is recommended to apply low salt and sugar medium for embryo culture or apply other sugars than sucrose such as fructose or glucose.

Since the main problem of embryo culture is with rooting stage two different media should be used for precocious growth and rooting. The rooting medium can contains root formation stimulating compounds such as low concentration of weak auxins (0.1 mg/L), L-Tyrosine (up to 100 mg/L), AC (1-4 g/L) to darken the medium and absorbs the growth inhibitor exudates and also silver nitrate (100 mg/L) to prevent shoot browning and saves the yew seedling viability [1].

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