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Effect of explants, salts concentration medium and hormone treatments on *Taxus baccata* in vitro culture

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Abstract

Taxus baccata is an endangered forest tree species with low regeneration. The highest Callus induction (96.67%) was occurred on ½ MS medium which had one-fourth nitrogen (KNO_3, $NH4NO_3$) supplemented with glutamine, 1 mg/l 2,4-D and 1 mg/l Kin from stem. The maximum callus size (80.67 mm^2) was obtained from leaf culture on ½ MS medium in combination with glutamine, 2 mg/l NAA and 0.2 mg/l Kin. In order to observe cells meristematically, the tissue was transferred to the ½ MS medium supplemented with 0.4 mg/l 2,4-D and 3 BAP for 7-8 weeks. In micropropagation, adding activated charcoal (2 g/l) to the medium increased the average number of new leaves and shoot elongation. The maximum shoot elongation (2.66 cm) and growth new leaves were observed in the MS medium supplemented with 3 mg/l Kin after 1 month. The best Rooting of elongated shoots was obtained in the WPM medium without growth regulators.

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Introduction

Taxol, a unique drug employed for the treatment of cancers, was first identified in 1954. This effective agent was extracted from the bark of Taxus species. European yew (*Taxus baccata*) is a slow growing tree with regeneration which is endangered and prone to extinction due to the small size and senescent status of most populations. Moreover seeds of Taxus are more difficult to germinate than most of the coniferous species (Pilz, 1996 a, b).

Many attempts have been devoted to produce Taxol by chemical synthesis, but to date the availability of this anticancer compound is not sufficient to satisfy the commercial requirements. On the other hand, reduced pools of natural adult trees available for the extraction, and low levels of paclitaxel and related taxanes in Taxus tissue, underline the need for an alternative source of taxanes, such as plant cell and tissue culture (Mihaljevic *et al*., 2002). Vegetative propagation elite yew can serve as a renewable and economic tissue source for increasing taxol production (Ho *et al*., 1998), But several years are still required to masspropagation these clones. However, cutting and grafting technique have been employed in propagation of Himalayan yew recently (Saini 2001). Chee (1995) and Eccher (1988) reported methods on large scale propagating of Taxus spp. In addition, Wickremesinhe and Arteca (1993) reported methods on initiation of callus cultures and maintenance of suspension cultures of Taxus species. Young stem cutting of adult trees were commonly used as primary explants sources for callus induction (Mihaljevic *et al*., 2002). This result encouraged us to attempt to optimize the induction and selection of *T. baccata* callus lines on modified MS medium in combination with hormonal treatments and two type explants for fast growing culture. Micropropagation might be a very useful tool to use for the mass propagation of superior yew trees and the production of high-quality plantlets for nursery operation.

Material and methods

Plant materials

In this study were used three explants (leave, stem and shoot tip), which obtained from 10 years old plants. The explants were surface sterilized by soaking in 70% ethanol for 5 min and disinfected by a 7-min treatment with 1% $Hgcl_2$ and then rinsed three times with sterilized distilled water. Sterilized explants were cut into 1cm long pieces and cultured on medium.

Culture medium for callus induction

For callus induction, explants (stem and leaf) were cultured on 4 modified MS media (E, F, G and H), which differ in nitrogen sources (KNO_3, $NHNO_3$) supplemented with 2.5% sucrose and Glutamine (Table 1), three growth regulators 0 - 4 mg/l NAA, 2,4-D and 0 - 1 mg/l Kin were used for callus induction with different levels and 1% insoluble poly vinil pyrolydone (pvp). Ten explants were used in each Petri dish (treatment) and were replicated three times and cultures were grown in the dark at 24 c°. After 8 weeks, the tissues were transferred to a secondary medium comprised ½ MS medium + 0/4 mg/l 2,4-D + 3 Kin for 7-8 weeks. All cultures were maintained for 16 hr photoperiod at 26 c°.

Histological techniques

Tissues were fixed in 5cc neutral buffered formalin + 5cc Acetic Acid for 30 min, dehydrated in ethyl alcohol, tertiary butyl alcohol series, and were embedded in paraplast at 56 c°. Embed tissues were cut, thick section and stained for 24 hr with 1% safranin, then dissolved in 50% alcohol, followed by 20 seconds exposure to 1% fast green dissolved in 100% alcohol.

Culture medium for micropropagation

For micropropagation, shoot tip explants with the length of 1- 1.5 cm with either one or three nodes were cultured on MS and half strength MS nitrate (MS (½ KNO_3, $NH4NO_3$)) media supplemented with 2.5% sucrose which has different levels of 3 growth regulators (2-5 mg/l BAP, 2 mg/l Kin, 2 mg/l Zeatin and 0-1 mg/l IBA) and presence or absence of 2 g/l activated charcoal were used. One explant was used in each tub and the experiment was repeated three times.

Root induction

After 1 month, shoots were excised and cultured in test tubes containing MS, ½ MS, WPM and ½ WPM supplemented with 25 g/l sucrose and NAA or IBA at 1,2,3 mg/l for 2 months (subculture monthly) and also all of them were transferred to MS, ½ MS, WPM and ½ WPM free growth regulators for 2 months.

Results

Callus induction and callus size

Analysis of variance for callus induction and callus size showed significant differences among each treatment (Hormone combination, medium and explant) and their interactions (Table 2). The earliest visible sign of callus growth from stem explants was noticeable between 6 and 8 days of incubation, while callus formation on leaf explants were observed after 3 weeks culture. Callus morphology also differed. While the calluses from stem explants were creamish and friable, calluses derived from leaf became brown and compact. The best callus induction in stem (96.69%) was obtained using H medium supplemented with 1 mg/l 2,4-D and 1 mg/l Kin, While the less efficient callus induction (13.33%) was obtained using H medium supplemented with 4 mg/l NAA and 0/4 mg/l Kin. from leaf (Table 3A). The frequency of callus induction from stem segments was significantly higher than that from leaf. Combination of 2,4-D with Kin was the most effective for consistent callus induction. The effect of types of explants depends on different combinations of auxins, cytokinins and the application of medium types on callus induction and callus size. The effect of 2,4-D and NAA were quite reducible when used in higher concentration. The explants inoculated on without growth regulators showed no callus induction. The best callus size in leaf (85/67 mm^2) was obtained using F medium supplemented with 2 mg/l NAA + 0/2 mg/l Kin. Among the auxin analyzed 2,4-D + Kin was more effective than NAA in callus production and NAA + Kin was more effective in callus size. Callus induction and callus growth was observed in low concentration of auxin in combination with cytokinin (Table 3B). proliferated calli of the two explants was occurred on the callus induction medium after two subculture.

Table 1. Nitrogen source of MS and 4 MS modified media (cited amounts and mg/l).

N-source/ Media	MS	E	F	G	H
Macro elements	Full	½	½	½	½
Micro elements	Full	½	½	½	½
KNO_3	1900	950	950	475	475
NH_4NO_3	1650	825	825	412.5	412.5
Glutamine	0	0	100	0	100

Histological

The tissue was transferred to the secondary medium for 8 weeks anatomical studies of the stem and leaf calluses that showed meristematical cells in stem callus. It also observed that localized groups of cells (growing centers), otherwise called meristemoids were scattered throughout the callus. Some of the parenchyma cells were found empty and dead and finally, many of the parenchyma cells were seen to have accumulated starch grain-like structures within their cytoplasms. The grains were mainly seen within the callus tissues especially in meristemoid areas (Fig. 1).

Shoot elongation

To establish the in vitro propagation system of *T. baccata,* medium constitutive factors were studied. No differences were observed in survival growth of new leaf and shoot elongation. In the using MS and half strength MS nitrogen media supplemented with BAP, Kin (0-5 mg/l) and IBA (0-1 mg/l) without activated charcoal. Data obtained from cultures showed growing of explants in MS and half strength MS nitrogen media with activated charcoal (2 mg/l) after 1 month. Representing the interaction of two factors medium and hormonal treatments were differed significantly ($p<0.01$) on both traits growth

new leaf and shoot elongation (Table 4).

The shoots were subcultured on the same medium every 14 days. Maximum number of leafs (22) were obtained with 3 mg/l BA within 4 weeks in MS medium. The lowest number of leafs (5) were obtained in MS medium supplemented with Kin 5 mg/l and IBA 0.5 mg/l (Fig. 2).

Table 2. ANOVA (Analysis of variance) for callus induction and callus size.

Source of variation	df	Mean square	
		Callus induction	Callus size
A (hormone combination)	7	1588.302 **	372.842 **
B (medium)	4	2112.517**	483.674**
A × B (interaction)	28	857.683 **	328.103**
C (explant)	1	13320.600**	67.416
A × C (interaction)	7	1275.648**	298.558**
B × C (interaction)	4	1545.600 **	153.438**
A × B × C	28	979.576 **	233.259**
Error	160	169.554	30.012
Coefficient of variation (%)	-	21.88	10.35

The best shoot elongation (2.67 cm) was obtained using MS medium supplemented with 3 mg/l Kin also in combination BA 3 mg/l (Fig 3). Increasing in cytokinin level to 3 mg/l reduced the mean length of shoots. Kin or BA was found to be comparatively more effective than Zeatin for growth new leaf and shoot elongation. Survey results will be referred to the fact that high concentrations of hormones are fine for seedling growth, and activate charcoal which is one of the requisite growth factors was stimulated growth (Fig. 4).

Table 3 A. Callus induction from stem and leaf cultured media (MS, E, F, G and H) with different concentration of auxins in combination with Kin.

treatment	Con.(mg/l)	Callus induction (%)									
		Stem					Leaf				
		MS	E	F	G	H	MS	E	F	G	H
NAA & Kin	0.5 & 0.4	90	33.33	26.67	60	90	43.33	40	65	70	50
NAA & Kin	1 & 1	50	66	50	55	60	55	13.33	50	53.33	60
NAA & Kin	2 & 0.2	53.33	55	70	93.33	90	36.67	73.33	46.67	83.33	23.33
NAA & Kin	4 & 0.4	45	53.33	70	46.67	36.67	18.33	65	70	76.67	13.33
2,4-D & Kin	0.5 & 0.4	90	76.67	86.67	80	56.67	56.67	56.67	43.33	71.67	63.33
2,4-D & Kin	1 & 1	60	66.67	86.67	70	96.67	70	85	66.67	50	25
2,4-D & Kin	2 & 0.2	56.67	60	76.67	90	50	70	50	80	70	46.67
2,4-D & Kin	4 & 0.4	86.67	63.33	66.67	83.33	83.33	26.67	20	40	66.67	20

**Significant at 1% level.

Table 3 B. callus size from stem and leaf cultured media (MS, E, F, G and H) with different concentration of auxins in combination with Kin.

Treatments	Con. (mg/l)	Size callus (mm)									
		Stem					Leaf				
		MS	E	F	G	H	MS	E	F	G	H
NAA & Kin	0.5 & 0.4	20.17	26.37	7.93	12.43	23.37	13.97	30.87	17.03	11.07	18.07
NAA & Kin	1 & 1	14.90	17.47	16.40	14	10.03	38.60	10.83	23.43	26.23	10.17
NAA & Kin	2 & 0.2	26.83	9.4	20.77	20.57	20.20	39.50	10.97	80.67	13.97	20.20
NAA & Kin	4 & 0.4	38.37	20.67	29.97	25.57	8.13	20.87	19	22.97	18.23	14.17
2,4-D & Kin	0.5 & 0.4	17.73	14.83	19.33	13.27	18.90	17.77	12.13	6.4	18.07	22.30
2,4-D & Kin	1 & 1	18.20	12.97	21.33	22.23	19.50	12.97	26.23	11.63	18.80	15.60
2,4-D & Kin	2 & 0.2	25.802	25.20	18.73	16.63	18.33	13.80	15.80	21.80	15.37	19.07
2,4-D & Kin	4 & 0.4	21.43	22.53	12.93	9.66	11.17	16.77	25	22.60	5.33	3.93

Rooting

Shoots (> 2 cm) were excised to test the effects of various media and auxin treatments on root induction, after 2 months rooting of *T. baccata* was obtained on WPM medium in absence of growth regulators, while no other treatments resulted in induction of roots in elongated shoots of *T. baccata* (Fig. 4).

Table 4. ANOVA (Analysis of variance) for micropropagation.

Source of variation	df	Mean square	
		Number of leave	Shoot elongation
A(medium)	1	36.214	0.244
B(hormonal combination)	6	33.151	1.219 **
A × B (interaction)	6	103.325**	0/935**
Error	28	24.857	0/265
Coefficient of variation (%)		28.96	28.26

**Significant at 1% level.

Discussion

T. baccata is a very valuable medicinal plant. There is no study about callus induction and in vitro propagation of this plant. Maximum callus induction was observed from the stem explants in H medium with 2,4-D plus Kin combination. Overall, percentage of callus induction was lower for all explants on media with NAA plus Kin when compared to 2,4-D plus Kin combination (Table 3A). The best callus size was observed from the leaf explants in F medium supplemented with NAA/Kin combination. Of two auxin/cytokinin combination tested, NAA/Kin was found more effective than 2,4-D/Kin combination with callus size (Table 3B). These results clearly show that 2,4-D played a central role in the callus induction. Different hormonal combination of auxin (2,4-D-NAA) and cytokinin (KT) at varying concentrations have been previously tested in basal MS medium (Trolinder and Goodin, 1987; Kumria *et al.*, 2003; rajasekaran *et al.*, 2000; leelavathi *et al.*, 2004).

The selection of 2,4-D or NAA for callus initiation from stem and leaf in *T. baccata* was based on earlier reports which revealed that callus was induced from zygotic embryos and female gametophyte in *T. wallichiana* (Datta and Majumder, 2006) and *T. media* (Goleniowski, 2000). Results of this study indicated that the presence of 2,4-D and Kin in the medium is necessary for the optimum callus formation from various explants of *T. baccata*. Moreover, earlier studied indicated that callus was induced from stem and needle leaf explants cultured on B5 basal medium supplemented with 2,4-D and Kin (Jha *et al.*, 1998); however, the stem showed significantly higher frequency of callus initiation than leaf explants (Table3a). Stem and leaf explants of *T. baccata* cultured on MS basal medium did not from callus. These results indicate that plant growth regulators especially auxins, were absolutely necessary for callus induction. In culture of many woody species full strength MS salts have inhibitory effected on organized growth. For example Toxicity can be reduced by lowering amount of ammonium or total nitrogen (Bonga and Von Aderkas, 1992). Cultured on MS basal medium supplemented with 2,4-D, NAA and BAP callus was also induced from zygotic embryos cultured on MS basal medium supplemented with NAA and Kinetin in *T. baccata* (Mihaljevic *et al.*, 200.

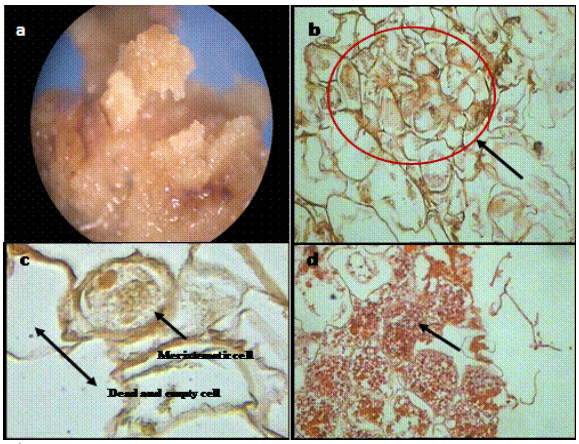

Fig. 1. A) Stem callus b) indicating meristematic c) Dead and empty parenchyma cells and meristematic cell d) and e) Starch grain-like structures within parenchyma cell.

In this study, the effects of organic nitrogen in callus production indicated that callus production and growth callus increased by supplementation of basal media (E, G) with 100 mg/l glutamine (Table 3), high callus induction percentage treatments and growth callus was observed in media containing half strength MS salts and ½ MS (4750 mg/l KNO_3, 4125 mg/l $NH4NO_3$) alone or in combination with 100 mg/l glutamine. According to these reports, reduction of nitrates and application of glutamine proliferate callus (Haq and Zafar, 2004). Conifers and other forest trees species require a combination of both nitrate and ammonium for good growth stimulation. Moreover, glutamine is required for cell division and callus proliferation from protoplasts of Douglas-fir and maritime pine (*Pinus pinaster*) (Salehi Shanjani, 2003).

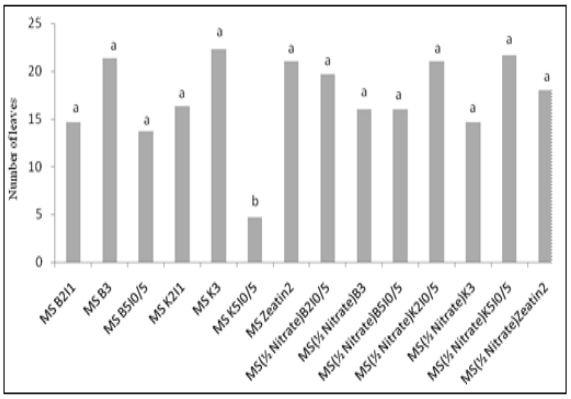

Fig. 2. Interaction medium and hormonal combination in number of leaves.

Secretion of some pigments or phenolic compounds into the medium has been shown to have a deleterious effect on the growth and viability of Taxus cell. As the addition of 1/5% insoluble pvp, a phenolic complexing agent to B5 medium partially relieved this problem with no impact on the growth of stem, derived from *T. cuspidate* callus (Mihaljevic *et al.*, 2002). In our experiment the addition of 1% pvp to MS medium had no growth effect on stem and leaf derived callus. Stem callus showed meristematicalls cells after transfering to the secondary medium for 8 weeks (Fig. 1).

Akaneme and Eneobong (2008) reported meristematicall cells on *Pinus caribaea* by calluses induction from zygotic embryo in hormonal combination auxin and cytokinin. Moreover, George and Sherrington (1984) reported that, friable calluses admit more oxygen because their cells are loosely arranged and therefore full respiration occurs under this condition. Among the cytokinins, BA is the most commonly used for the induction organogenesis of the pinaceae family such as Lodgepole pine (Patel and Thorpe 1984). Earlier studies indicated that green colored callus cultures were also induced from stem and needle leaf explants in *T. xmedia* cv. *Hicksii* (Wickremsrihe and Arteca, 1993). In previous study, regeneration of plantlets was also reported from such callus cultures of *T. wallichiana* (Datta and Majumder, 2006). Results from this study have given evidence that *T. baccata* (woody plant) should be listed as a species with the ability for organogenesis.

In mocropropagation of *Taxus baccata*, medium supplemented with activated charcoal increased the shoot elongation and growth new leafs than medium without activated charcoal (Fig. 4). This study showed that two media in combination with BAP and Kin are better than two media in combination with Zeatin for shoot elongation. We found no significant difference in effect between the two media (Fig. 3). Shoots elongated better on media containing lower concentrations of plant growth regulators that BA–induced multiple shoots needed to be transferred in to PGR – free medium to promote elongation (change *et al* 2001). BA was also found to be effective in inducing shoots from stem cultures of *T. mairei* seedlings (Chang *et al.*, 1998). Chee (1995) reported that shoot regeneration from embryo cultures of *T. brevifolia* was better under BA induction than under thidiazuron or Kinetin induction. Spanos *et al* (1997) reported large numbers of shoots of both *Cupressus sempervirens L*, and *Chamaecyparis lausoniana* were recovered from explants placed on medium lacking BA. In comparison to medium lacking BA; however, increasing concentration of cytokinin induced higher numbers of shoots, but increasing the BA concentration above 0.1 mg/l reduced the number of shoots recovered to compare with media containing 0.01 mg/l or 0.01 mg/l BA. Adding of cytokinins to medium is important for development of axillaries buds in the coniferous genera, such as

picea (Kunze *et al.*, 1993). Several studies have indicated that the medium composition and culture environment have important role in the modulation of morphogenic reaction in micropropagation (Amancio *et al.* 1999, Baker *et al.*, 1999).

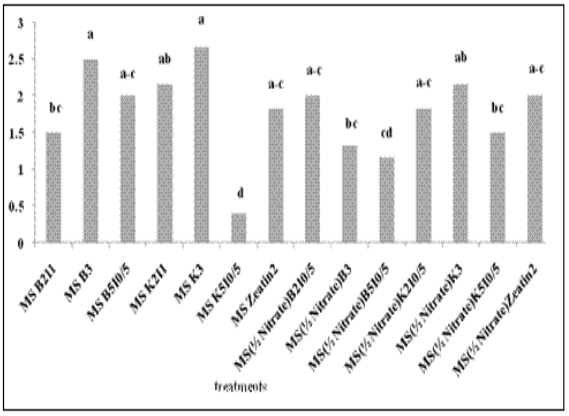

Fig. 3. Interaction medium and hormonal combination in shoot elongate.

The most difficult stage of micropropagation in woody species is the induction of roots on elongated shoots. In agreement with previous study (Datta and Majumder, 2006) on *T. wallichiana,* rooting was not obtained when the micro shoots were cultured on ½ WPMSH basal medium supplemented with different auxins either singly or in combinations; however micro shoots rooted on modified MS basal medium in which the concentration of nitrates was reduced to one-fifth the normal concentration (MS-N basal medium) after 4 months of culture. The elongated shoots of *T. mairei* could be rooted with the application IBA (1/5 mg/l) but *T. baccata* which treated with IBA did not produce any roots (Chang *et al.*, 2001). Addition of indol acetic acid to media was necessary to stimulate rooting in C. dupreziana (Hrib and Dobry, 1984).

In many other coniferous genera, the presence of auxin in rooting media is perquisite for root initiation (spanos *et al.*, 1992). Ho *et al.*, (1998) reported less than 10% rooting obtained of stem cutting derived from adult trees after 6 months of green house cultivation therefore suggest that micropropagation can improve rooting efficiency and thus, clone propagate of adult yew trees. H medium with 1 mg/l 2,4-D and 1 mg/l Kin are the most effective medium for callus induction of *T. baccata*, and F medium with 2 mg/l NAA + 0.2 mg/l Kin are the most effective medium for callus size. Also MS medium supplemented with 3 mg/l Kin in combination with BA 3 mg/l is the most effective for shoot elongation.

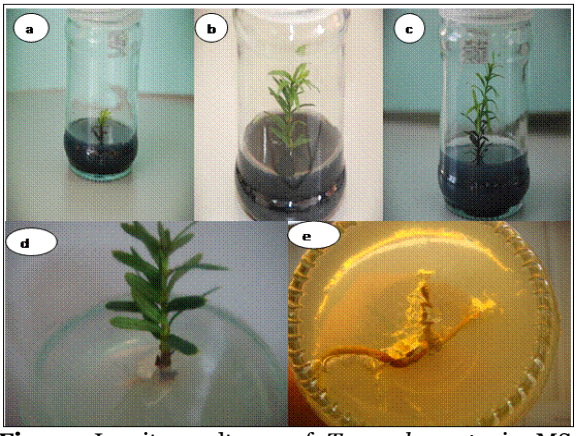

Fig. 4. In vitro cultures of *Taxus baccata* in MS medium with Kin 3 mg/l. a) elongated shoot after 12 days. B) shoot after 22 days c) shoot after 31 days (2.67 cm) d) initiation of root from the base of the elongated shoot cultured on WPM without growth regulators after 2 months e) root elongation after 3.5 months.

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