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IN VITRO CALLOGENESIS AND ORGANOGENESIS IN *TAXUS WALLICHIANA* ZUCC. THE HIMALAYAN YEW

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Abstract

Taxus wallichiana Zucc., is a medium sized temperate forest tree species of Asia ranging from Afghanistan through the Himalayas to the Philippines. It has been heavily exploited for its leaves and bark which are used to produce the anti-cancer drug Taxol. Due to its long seed dormancy period, its natural regeneration from seeds is very low. In the present study, biotechnological method is applied to grow the plant *In vitro* through plant tissue culture techniques. Different plant growth regulators (2, 4-D, NAA, IBA, BAP, Kin) were used to regenerate it through direct or indirect route of organogenesis in the *In vitro* conditions. Callus was induced successfully both in stem and leaf (needle) explant on MS media supplemented with 1 mg/L, 1.5 mg/L, 2 mg/L, 2.5 mg/L and 3.0 mg/L, 2,4-D. The best callus was induced on MS media supplemented with 2 mg/L 2,4-D and 5 mg/L Activated Charcoal (AC) within 2 weeks of culture in stem explant, but we did not succeeded in the regenerations of shoot in both type of callus culture. The shoot tip meristem was elongated on MS media supplemented with 2 mg/L BAP and MS media supplemented with 1mg/L IBA up to 10-14 cm after 3 to 4 subculture. Roots were induced in the elongated shoot tips in 60-80 days on MS media supplemented with 3.5 mg/L IBA and on half strength MS media supplemented with 8 mg/L IBA. It is concluded from the present study that callus culture from stem and needle explant is not suitable for organogenesis in *Taxus wallichiana*, however shoot elongation and root induction in shoot tip culture is feasible and suitable for the multiplication of *Taxus wallichiana* through tissue culture.

Introduction

Taxus wallichiana Zucc. commonly known as Himalayan Yew belongs to family-Taxaceae is a temperate Himalayan multipurpose tree species of high medicinal value and ethnobotanical importance. Wild medicinal plant species form an important component of livelihood strategies in Asia and collection of medicinal and aromatic plants providing a critical source of income in many areas. This is particularly true in areas such as the high alpine regions of the Himalayas, where agricultural outputs are low and there are very few opportunities for income generation. The increasing demand for medicinal plants and the consequent increase in the rate of collection has created a negative impact on the wild populations of many species; as a result some species are now considered to be threatened and are at the verge of extinction (Shinwari & Qaisar, 2011). The leaves and bark of *Taxus wallichiana* has been exploited for the extraction of Taxol. It has unique property of preventing the growth of cancerous cells, and being used in the treatment of breast and ovarian cancer (Kovacs *et al.,* 2007). It inhibits cell proliferation through inhibiting microtubule dissociation, due to its tubulin binding affinity. (Ashrafi *et al.,* 2010). It is obtained from all the Taxus species and for the first time it was obtained from the bark of *Taxus brevifolia*. Its anticancer activity was discovered in 1971 (Wani *et al.,* 1971). The compound, used in treating cancer was subsequently identified in *Taxus wallichiana* prompting a rapid increase in wild collection of the needles and bark of this Himalayan species for paclitaxel extraction. Natural regeneration in *Taxus wallichiana* is very low due to long seed dormancy and even in the controlled conditions the pericarp of the seed acts as the barrier for seed germination. Based on the current bark extraction procedures, nearly 7, 000-10,000 kg of bark is needed to produce 1 kg of Taxol (Cragg *et al.,* 1993; Wann & Goldner, 1994). The estimated need of Taxol per year is 250 kg of the purified drug that need 750,000 trees. The ever increase demand of Taxol in the treatment of cancer need a large source of plants for extraction. Therefore, *Taxus wallichiana* is exposed to the risk of extension (Liao *et al.,* 2006). Many studies on in-vitro regeneration of Taxus species viz. *Taxus cuspidata, Taxus baccata, Taxus media, Taxus canadensis* (Globa *et al*., 2009), *Taxus brevifolia* (Chee, 1995a), *Taxus wallichiana* ZUCC (Hien *et al*., 2004) have been undertaken earlier. So, alternative biotechnological method such as cell suspension culture for the production of Taxol and tissue culture for the rapid propagation and conservation of *Taxus wallichiana* should be considered as demonstrated by Hussain *et al*., (2011). In the present investigation, efforts were made to develop protocols for the *In vitro* establishment of callus and direct shoot elongation and root induction in the shoot tip culture of *Taxus wallichiana.*

Materials and Methods

Preparation of explant: Explants were taken from *Taxus wallichiana* tree growing in the Herb garden of Qarshi Industry at Hattar, Pakistan. Juvenile meristem, stem and leaf were used as explants. The explants were washed with tape water up to 15 min to remove any mud or dust particle and reduce the microbial load. Then washed with distilled water and sterilized with 0.1% mercuric chloride for 1min. after sterilization with mercuric chloride, the explant were washed 3 times with autoclaved distilled water to reduce the toxic effect of mercuric chloride.

Propagation media: MS basal media (pH 5.8) containing MS mineral and vitamins (Murashige & Skoog, 1962) supplemented with 30g/L sucrose as energy source was used. Different concentration of 2, 4-D, IBA, BAP and a photoperiod of 16 hr light/8 hr dark condition at 25±5˚C, relative humidity 40-70% was used throughout the experiment. Activated charcoal was also used in some experiment to reduce the browning effects of exudates. Media was solidified with 8 g/l agar, added before autoclaving.

Inoculation of explant: The sterilized explant (Leaf, stem and shoot tips) were cut into small pieces and aseptically placed in the test tubes/flasks containing MS media under laminar air flow hood. Culturing was carried out in 50 ml test tubes or 250 ml Erlenmeyer flasks. All the cultures were sub-cultured using same media supplemented with aforementioned growth regulators and carbohydrates to regenerate the species.

Results and Discussion

Pakistan's population mainly depends on wild plant to treat their ailments (Shinwari, 2010). To have sustainable availability of herbs and quality assurance of herbal products in the market, modern disciplines have to be employed. Fist for correct identification, we need to use DNA data to ensure usage of exact species (Mahmood *et al*., 2010; Shinwari & Shinwari, 2010). Secondly to conserve the biodiversity and ensure high quality herbs, *In vitro* regeneration and cultivation of wild herbs are adopted (Shinwari *et al.,* 2012). *In vitro* regeneration and propagation of the slow growing woody plant species like *Taxus wallichiana* is very essential for rapid growth and production of secondary metabolites. Taxus the Himalayan Yew is very important plant for Taxol production. Various protocols have been developed for the *In vitro* production of Taxol in Taxus culture. In the present study, protocols were developed for the initiation of callus from leaf and stem explant and direct organogenesis from shoot tip meristem culture.

For callus formation the explant were inoculated on MS media supplemented with different concentration of 2, 4-D, NAA, IBA, Kinetin and activated charcoal (AC) alone or in combination (Table 1). Callus formation from stem and leaf explant was achieved on MS medium supplemented with 2 mg/L 2, 4-D. The best callus was obtained in the stem explant on MS media supplemented with 2 mg/L 2, 4-D + 5 mg/L activated charcoal (AC) as shown in Fig. 1. However with time it becomes brown and fragile, so it was sub cultured after every 2-3 weeks to reduce the browning. The callus developed from stem explant was healthy, green and compact. The callus formation starts within 2 weeks of culturing the stem explant. Similar results were also reported by Ashrafi *et al*., (2010). Callus develop from leaf explant was also green, but it take longer time than stem explant as reported by earlier study conducted by Fett-Neto *et al*., (1992). The other problem with the leaf induced callus is that it becomes fragile early and it is difficult to subculture and increase the callus biomass on MS agar solidified media. Earlier studies conducted by Jha *et al.,* (1998), indicates that callus induced from stem and needle leaf explants cultured was efficient on B5 basal medium supplemented with 2,4-D (2.0 mg l^{-1}) and Kin (0.5 mg l^{-1}). However callus induced from stem or leaf explants in *T. wallichiana* has not been reported to show shoot organogenesis (Wickremesinhe & Arteca 1993; Datta *et al*., 2006). Different concentrations of BAP, Kinetin and IBA were used for shoot regeneration from callus but failed to succeed in the regeneration of shoot from both types of callus. However the callus induced from the stem explant multiply very rapidly and it would be a very suitable source of Taxol production in the cell suspension culture of Taxus. The callus developed from zygotic explant material as described by Datta *et al*., (2006) is very efficient in the formation of shoot bud primordia on ½ WPMSH basal medium. It was observed that as the concentration of 2, 4-D increased in the MS media, the response of callus formation increased. The same results was reported by Mehaljevic *et al*., (2002) and Das *et al*., (2008), when cultured on B5 media supplemented with hormone combination of 5μM 2, 4-D and 1.0 μM Kin.

The process of shoot elongation and root induction in Taxus is very slow. The shoot were elongated up to 10 cm on MS media supplemented with 2mg/l BAP and MS media supplemented with 2 mg/L IBA in two months of culturing and sub culturing as shown in Fig. 3(a & b). Lower concentration of BAP @ 0.5 mg/L and 1.0 mg/L was also efficient in the elongation of shoots and formation of new shoot bud primordia Fig. 2. The elongated shoot tip was cultured both on full strength and half (½) strength MS media supplemented with different concentration of IBA (Table 1). In both type of media the rooting response was observed on ½ MS media supplemented with 8mg/L IBA and full strength MS media supplemented with 3.5 mg/l IBA after 60 - 80 days of culturing Fig. 3 (c, d & e). Similar result was reported by Chee (1996) in the *In vitro* culture of *Taxus brevifolia* and Das *et al*., (2008) in *Taxus wallichiana*. Similarly Ewald, (2007) successfully induced roots in shoot tips on nutrient medium L9 containing 2 mg/L IBA. However, Datta *et al*., (2006) did not succeeded in obtaining roots in *Taxus wallichiana* microshoots when cultured on ½ WPMSH medium supplemented with different auxins added either singly or in combinations. In most of the cases, auxin treatments produced callus at the base of the microshoots. Similar results of callus formation were reported in rooting of cuttings taken from both male and female trees of *Taxus baccata* (Nandi *et al*., 1996). It is clearly evident that IBA is the most efficient hormone for root formation in the shoot tip culture of Himalayan Yew, but its response in different Medias is different.

Table 1. Effects of different hormones on callogenesis and organogenesis of *Taxus wallichiana* Zucc.

Media + hormones	Concentration (mg/L)						Response*		
	Kin	2,4-D	IBA	NAA	BAP	AC	Callus	Shoot	Root
MS + Kinetin	1.0						-	+	-
	1.5						-	+	-
	2.0						-	+	-
	2.5						-	+	-
	3.0						-	+	-
MS + 2,4-D		1.0					++	+	-
		1.5					+++	++	-
		2.0					+++	++	-
		2.5					+++	+	-
		3.0					+++	+	-
MS + 2,4-D + AC	1.0					3.0	++	-	
	1.5					4.0	+++	++	
	2.0					5.0	++++	+	
	2.5					5.0	++++	+	
	3.0					5.0	++++	+	
MS + Kinetin + 2,4-D	1.0	1.0					-	-	-
	1.5	1.5					+	-	-
	2.0	2.0					++	+	-
	2.5	2.5					+++	++	-
	3.0	3.0					++	+	-
MS + BAP					0.5		-	+	-
					1.0		-	++	-
					2.0		+	++++	-
					2.5		++	++++	-
					3.0		++	+++	-
					3.5		++	+++	-
					4.0		+	+	-
MS + IBA			0.5				-	+	-
			1.0				+	++	-
			2.0				+	+++	-
			2.5				+	++	+
			3.0				+	+	++
			3.5				+	+	++++
			4.0				+	+	++
1/2MS + IBA			4.0				-	-	-
			5.0				-	-	+
			7.0				-	-	++
			8.0				-	-	++++
			9.0				-	-	+++
MS + NAA				1.0			+	-	-
				1.5			+	-	-
				2.0			++	-	-
				2.5			+	-	-
				3.0			+	-	-

* (-) No, (+) Poor (++) Fair, (+++) Good, (++++) Excellent

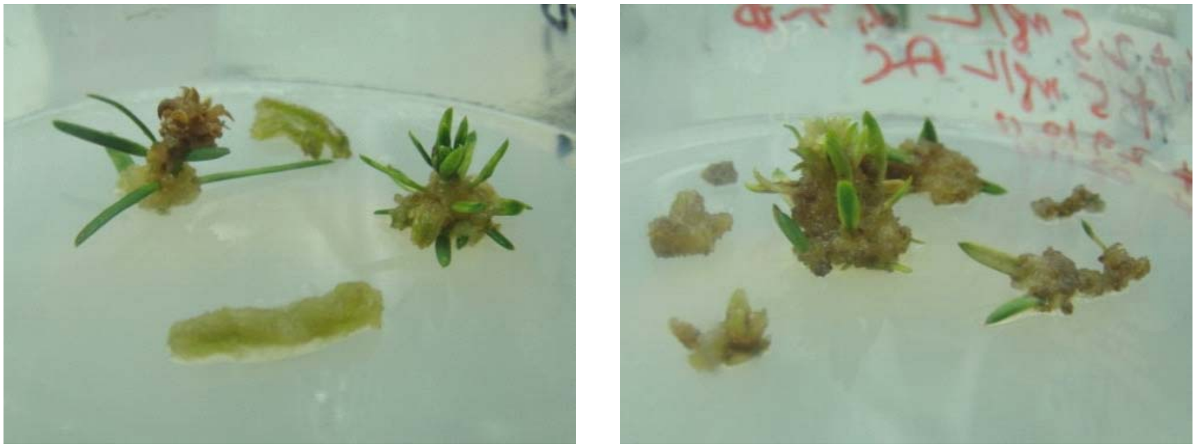
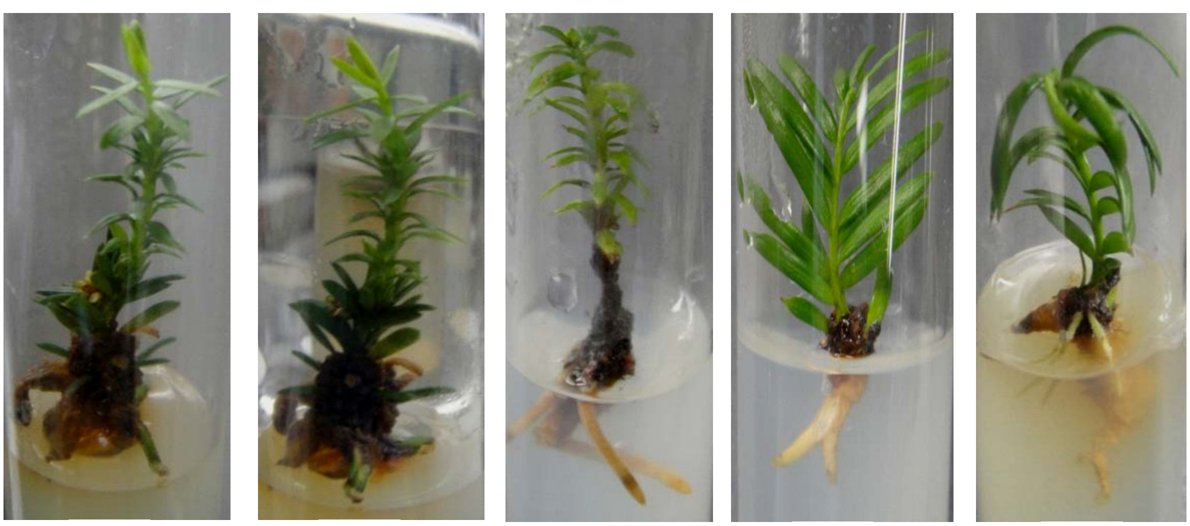


Fig. 1. Callus induction in stem explants of *Taxus wallichiana*.

Fig. 2. Formation of new shooting bud in the shoot tip culture.

According to Red List category of IUCN, 2012, the species is categorized as endangered. To reduce the pressure in the future on wild populations, cultivation of *Taxus* on large scale is deem necessary. This species continues to be over-exploited throughout its range of distribution for Taxol production. Therefore tissue culture is one way to rapidly propagate this species for replanting and *In situ* conservation of remaining population.

From the present investigation it can be concluded that callus culture from stem and needle explant is not a suitable option for organogenesis in *Taxus wallichiana,* however shoot elongation and root induction in shoot tip culture is feasible and suitable for the multiplication of *Taxus wallichiana* through tissue culture. The growing demand for taxol and its derivatives, due to a specific action mechanism and the scarcity of the taxane ring in nature, Taxol is most interesting targets for biotechnological production. Therefore, the authors suggest producing Taxol in the cell suspension culture as described by Wang *et al*., (2001), Khosroushahi *et al*., (2006) and Tabata (2006) instead of over exploiting its leaves and bark.



a b c d e

Fig. 3. *In vitro* shoot elongation (a) & (b), root Induction (c), (d) & (e) in the shoot tip meristem culture of *Taxus wallichiana.*

References

Ashrafi, S., M. R. Mofid, M. Otroshi, M. Ebrahimi and M. Khosroshahli. 2010. Effects of plant growth regulators on the callogenesis and Taxol production in cell suspension of *Taxus baccata*. *Trakia J. Sci.*, 8(2): 36-43.

Chee, P.P. 1995a. Organogenesis in *Taxus brevifolia* tissue cultures. *Plant Cell Rep*., 14: 560-565.

Chee, P.P. 1996. Plant regeneration from somatic embryos of *Taxus brevifolia*. *Plant Cell Report*, 16: 184-187.

Cragg, G.M., S.A. Shepartz, M. Suffness and M. Grever. 1993. The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *J. Nat. Prod*., 56: 1657-1668.

Das, K., R. Dang, N. Ghanshala and P.E. Rajasekharan. 2008. *In vitro* establishment and maintenance of callus of *Taxus wallichiana* Zucc. For the production of secondary metabolites. *Natural Product Radiance*, 7(2): 150-153.

Datta, M. M., A. Majumder and S. Jha. 2006. Organogenesis and plant regeneration in *Taxus wallichiana* (Zucc.). *Plant Cell Reports*, 25: 11-18.

Ewald, D. 2007. Chapter 11. Micropropagation of yew (*Taxus baccata* L.). (Eds.): Jain and H. Haggman. Protocol for micropropagation of Woody Trees and Fruits, 117-123.

Fett-Neto, A., F. Dicosmo, W.F. Reynolds and K. Sakata. 1992. Cell culture of Taxus as source of the anti neoplastic drug Taxol and related taxane. *Biotechnol*., 10: 1572-1575.

Globa, E. B., E. V. Demidova, V. V. Turkin, S. S. Makarova and A. M. Nosov. 2009. Callusogenesis and formation of cell suspension cultures of four Taxus species: ***T. canadensis***, ***T. baccata, T. cuspidata*** and ***T. media***. ***J. Biotekhnologiya***, 3: 54-59.

Hien, N.T.T., D.V. Khiem, N.H. Vu, N.T. Don and D.T. Nhut. 2004. Primary study on the induction and growth of callus of *Taxus wallichiana* Zucc., a valuable medicinal plant in lam dong province. ***J. Agri. Sci. and Technol.***, 4: 79-85.

Hussain, A., S. Naz, H. Nazir and Z.K. Shinwari. 2011. Tissue culture of black pepper (*Piper nigrum* L.) in Pakistan. ***Pak. J. Bot***., 43(2): 1069-1078

Jha, S., D. Sanyal, B. Ghosh and T.B. Jha. 1998. Improved taxol yield in cell suspension culture of *Taxus wallichiana* (Himalayan yew). *Planta Med*., 64: 270-272.

Khosroushahi, A.Y., M. Valizadeh, A. Ghasempour, M. Khosrowshahli, H. Naghdibadi, M.R. Dadpour and Y. Omidi. 2006. Improved Taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biol. Int*., 30(3): 262-9.

Kovacs, P., G. Csaba, E. Pallinger and R. Czaker. 2007. Effects of taxol treatment on the microtubular system and mitochondria of Tetra hymena. *Cell Biol. Int*., 31:724-32.

Liao, Z., M. Chen, X. Sun and K. Tang. 2006. Micropropagation of endangered plant species. *Methods Mol. Biol*., 318: 179-185.

Mahmood, T., S. Muhammad and Z.K. Shinwari. 2010. Molecular and morphological characterization of *Caralluma* species. ***Pak. J. Bot***., 42(2): 1163-1171.

Mihaljevic, S., I. Bjedov, M. Kovac, D.L. Levanic and S. Jelaska. 2002. Effect of explant source and growth regulator on *In vitro* callus growth of Taxus baccata L. cv. Washingtonii, *Food Technol. Biotechnol*, 40: 299-303.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*., 15: 473-479.

Nandi, S.K., L.M.S. Palni and H.C. Rikhari. 1996. Chemical induction of adventitious root formation in T. baccata cuttings. *Plant Growth Reg*., 19:122-177.

Shinwari, Z.K. 2010. Medicinal Plants Research in Pakistan. ***Journ. Med. Pl. Res***., 4(3): 161-176.

Shinwari, Z.K., S.A. Gilani and A.L. Khan. 2012. Biodiversity loss, emerging infectious diseases and impact on human and crops. ***Pak. J. Bot***., 44: 137-142, Special Issue May 2012.

Shinwari, Z.K. and M. Qaisar. 2011. Efforts on conservation and sustainable use of medicinal plants of Pakistan. ***Pak. J. Bot***., 43 (Special Issue): 5-10.

Shinwari, Z.K. and Shehla Shinwari. 2010. Molecular data and phylogeny of family smilacaceae. ***Pak. J. Bot.,*** Special Issue (S.I. Ali Festschrift) 42: 111-116

Tabata, H. 2006. Production of paclitaxel and the related taxanes by cell suspension cultures of *Taxus* species. ***Curr. Drug Targets***, 7(4): 453-61.

Wang, C., J. Wu and X. Mei. 2001. Enhanced taxol production and release in *Taxus chinensis* cell suspension cultures with selected organic solvents and sucrose feeding. *Biotechnol Prog*., 17(1): 89-94.

Wani, M., H. Taylor, M. Wall, P. Coggon and A. McPhail. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. ***J. Am. Chem. Soc***., 93: 2325-7.

Wann, S.R. and W.R. Goldner. 1994. Iduction of somatic embryogenesis in taxus and the production of Taxane ring containing alkaloids there from. United states patent, 5310672.

Wickremesinhe, E.R.M. and R.N. Arteca. 1993. Taxus callus cultures: Initiation, growth optimization, characterization and taxol production. *Plant Cell Tissue Organ Cult*., 35: 181-193.

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