- Short communication
ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **30**(1): 161-166, 2020 (June)

PTC&B

Conservation of an Endangered Medicinal Tree Species *Taxus wallichian* through Callus Induction and Shoot Regeneration

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Key words: Callus Induction, Shoot regeneration, *Taxus wallichiana*

Callus production and regeneration using leaf and internode as explant was carried out at different combinations of plant growth regulators. *Taxu wallichiana* leaf explant initiated significant callus on MS CIM_{K1} (1.7 mg/l 2, 4-D and 1.0 mg/l Kn) with 4.0 mm^2 callus area after 45 days. Similarly MS medium CIM_{B1}-CIM_{B4} (1.2, 1.5, 1.7 and 2.0 mg/l of BAP with 2.0 mg/l of 2,4-D) showed equally significant callus induction but with less callus area of 3.0 mm^2 compared to 2,4-D and Kn. For regeneration, shooting was optimized with MS SIM_{IA1}-SIM_{IA3} (1.5, 2.5, 3.5 mg/l IAA and BAP 2.0 mg/l). Maximum shoot length (1.5 mm) was obtained after 45 days from internode. However, there was no root formation in the above mentioned combinations.

Taxus wallichiana, a long lived evergreen tree species is known as Himalayan Yew (Poudel et al. 2012). It belongs to *Taxaceae* of genus *Taxus* and occurs at an altitude of about 1800 - 4400 m (Shaheen et al. 2015). *Taxu wallichiana* occurs in Asia including China, India, Iran, Pakistan, Korea Nepal, Philippines and Taiwan. It is also found in Thandiani, Dhongagali, Nathiagali, and the Himalayas region of Pakistan (Poudel et al. 2012). *Taxus wallichiana* is famous for its anti-cancerous, anti-convolunary and immune-regulatory effects (Dang et al. 2017). It is traditionally used for treatment of high fever, inflammation, tuberculosis and diarrhea (Juyal et al. 2014). It possesses antibacterial, anti-fungal and antioxidant properties (Adhikari et al. 2017).

Taxus wallichiana has been commercialized due to its immense ethno-botanical and medicinal properties (Dang et al. 2017). The leaves and bark have been explicitly used for the extraction of Taxol (Cusido et al. 2002). Taxol has unique property of preventing the growth of cancerous cells (Zhang et al. 2005). Plant tissue culture technique can be used to save this endangered tree species through micropropagation and callus culture.

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DOI: https://doi.org/10.3329/ptcb.v30i1.47801

(Datta et al. 2006). Callus culture cannot only result in plantlet production but it can also be used to enhance medicinal properties of such an important plant species. A large number of phytochemicals including taxenes, diterpenes, flavanoids, lignans and steroids derivatives have been isolated from several species of *Taxus* including *Taxus wallichiana* (Dand et al. 2017). In this study, *Taxus wallichiana* callus induction and shoot regeneration was optimized using different concentrations of growth regulators (Hussain et al. 2013) including cytokinin such as BAP and Kn and auxin such as 2,4-D and IAA.

Taxus wallichiana was collected from Dhongagali, Pakistan. Current work on regeneration and callus formation was conducted at Genetic Engineering laboratory of COMSATS University, Abbottabad, Pakistan. MS was prepared with 15 g of sucrose along with 2.2 g of MS powder (Murashige and Skoog 1962). Ph was adjusted to 5.8 and 4 g of agar was added to it. The medium was autoclaved at 120^0C/15 psi. Explants including leaf and internode were sterilized with 0.1% $MgCl_2$ and washed three times with distilled water. Later, explant was placed on different callus induction medium (CIM) and shoot induction medium (SIM; Table 1) under 8 hrs dark and 16 hrs light at 28^0C for callus growth and regeneration.

Table 1. Various concentrations of plant growth regulators including 2,4-D, NAA, IAA, BAP and Kn used for callus induction and regeneration.

Callus induction media	2,4-D (mg/l)	Kn (mg/l)
CIM_{K1}	1.5	1.0
CIM_{K2}	1.5	1.5
CIM_{K3}	1.7	1.0
CIM_{K4}	1.7	1.5
CIM_{K5}	2.0	1.0
CIM_{K6}	2.0	1.5
Callus induction media	2, 4-D (mg/l)	BAP (mg/l)
CIM_{B1}	2.0	1.2
CIM_{B2}	2.0	1.5
CIM_{B3}	2.0	1.7
CIM_{B4}	2.0	2.0
Shoot induction media	BAP (mg/l)	IAA (mg/l)
SIM_{IA1}	2.0	1.5
SIM_{IA2}	2.0	2.5
SIM_{IA3}	2.0	3.5

Data for *Taxus wallichiana* callus induction was collected after 15, 30 and 45 days by measuring callus area. In case of shoot regeneration, data were recorded for shoot length and number of leaves after 15, 30 and 45 days of culturing internode. Statistical analysis was performed based on ANOVA and the mean values were compared by performing LSD test using R Program (R Core Team 2018).

Callus appeared on MS with 2, 4-D and Kn from *Taxus wallichiana* leaf explant. Significantly high callus formation was recorded for CIM_{K3} (1.7 : 1.0 mg/l) with 2,4-D and

Kn after 15 days (Fig. 1, Table 2). Since optimal callus induction is important for metabolites production (Sarmadi et al. 2018), the effects of the different concentrations of 2,4-D and Kn on callus formation was determined after 15, 30 and 45 days. After 30 days, CIM_{K3} (1.5 : 1.0 mg/l) with 2,4-D: Kn was found to be most significant treatment along with CIM_{K6} (2.0 : 1.5 mg/l) based on statistical analysis. CIM_{K3} was the only treatment that showed best callus induction 45 days after culturing with 4.51 mm^2 callus area. Optimal callus formation was also achieved in MS with 2,4-D (2.0 mg/l): Kn (0.5 mg/l) from zygotic embryo and with 2,4-D, activated charcoal (2.0: 1.5 mg/l) from stem explant (Datta et al. 2006, Hussain et al. 2011). In another study callus formation with 2, 4-D and Kn was reported on B5 instead of MS (Jha et al. 1998). Present data however suggest CIM_{K3} (1.7 : 1.0 mg/l) with 2,4-D and Kn as optimal condition for callus induction with callus area 4.0 mm^2 (Fig. 1 and Table 2).

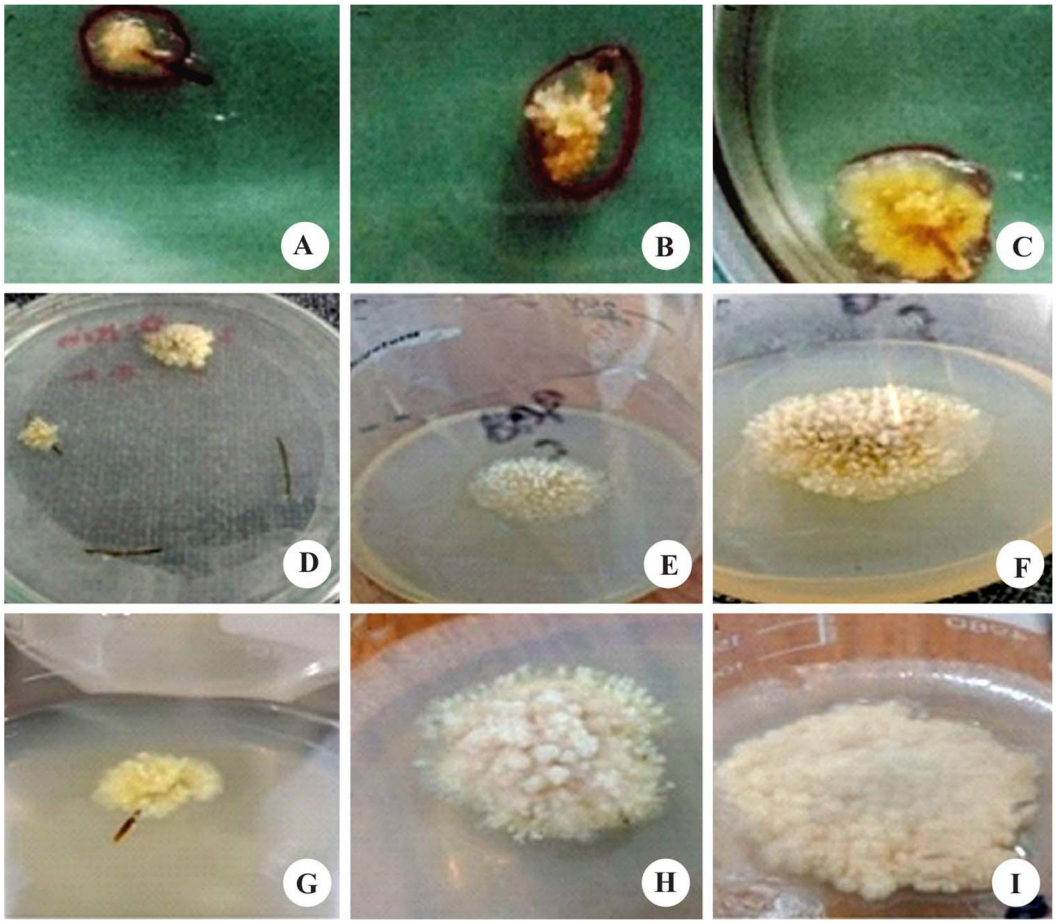

Fig. 1. *In vitro* grown *Taxus wallichiana* leaflets showing callus formation at different concs. of 2, 4-D and Kn. (A - C) CIM_{K1} after 15, 30 and 45 days of culturing (D - F) CIM_{K6} after 15, 30 and 45 days of culturing, (G - I) CIM_{K3} with 15, 30 and 45 days after callus formation.

Table 2. Statistical analysis of data recorded after 15, 30 and 45 days of *Taxus wallichiana* callus culture with 2, 4-D and Kn.

Sl. no.	Treatment	2,4-D : Kn (mg/l)	Callus area : mm^2 (A)	Callus area : mm^2 (B)	Callus area : mm^2 (C)
1.	CIM_{K1}	1.5 : 1.0	0.86 ± 0.5^{b}	1.78 ± 1.0^{ab}	3.75 ± 1.4^{ab}
2	CIM_{K2}	1.5 : 1.5	0.89 ± 0.7^{ab}	1.96 ± 1.0^{ab}	3.47 ± 1.4^{ab}
3.	CIM_{K3}	1.7 : 1.0	1.52 ± 0.2^{a}	2.60 ± 0.6^{a}	4.52 ± 0.7^{a}
4.	CIM_{K4}	1.7 : 1.5	0.35 ± 0.2^{bc}	1.07 ± 0.4^{b}	3.20 ± 1.4^{ab}
5.	CIM_{K5}	2.0 : 1.0	0.24 ± 0.1^{c}	0.98 ± 0.4^{b}	2.24 ± 0.6^{b}
6.	CIM_{K6}	2.0 : 1.5	1.46 ± 0.4^{a}	2.65 ± 0.4^{a}	3.53 ± 1.5^{ab}

Data are represented as means ± SE from 10 replicates. Different letters within each column represent significant differences between treatments at $p \leq 0.05$ LSD. (**A**) CIM_{K3} and CIM_{K6} were the most significant treatments for callus induction after 15 days of culturing. (**B**) CIM_{K3} and CIM_{K6} with 2,4-D: Kn gave significant result 30 days after culturing. (**C**) CIM_{K3} was found to be most significant for callus formation 45 days after culturing.

Different concentrations of 2, 4-D with BAP were used to determine their effect on callus formation (Table 1). Significantly result was recorded with CIM_{B2} (2.0 : 1.5 mg/l) for callus area based on data recorded after 15 days of culturing. Similarly $CIMB_2$ showed significant result after 30 days of culturing. $CIM_{B1,}$ $CIM_{B2,}$ CIM_{B3} and CIM_{B4} were all found to be equally significant for callus induction for 2, 4-D and BAP after 45 days of culturing (Table 3). BAP and 2, 4-D also showed callus formation at different concentrations in *Taxus baccata*. Thus different combinations of cytokinin and auxin were used for callus formation from leaf and stem explant (Ashrafi et al. 2010).

Table 3. Statistical analysis of data recorded after 15, 30 and 45 days of *Taxu swallichiana* callus culture with 2, 4-D and BAP.

Sl. no.	Treatment	2,4-D : BAP (mg/l)	Callus area : mm^2 (A)	Callus area : mm^2 (B)	Callus area : mm^2 (C)
1.	CIM_{B1}	2.0 : 1.2	0.27 ± 0.1^{b}	1.13 ± 0.4^{b}	2.32 ± 0.7^{a}
2.	CIM_{B2}	2.0 : 1.5	0.61 ± 0.1^{a}	1.66 ± 0.3^{a}	2.98 ± 0.6^{a}
3.	CIM_{B3}	2.0 : 1.7	0.33 ± 0.1^{b}	1.04 ± 0.2^{b}	2.30 ± 0.2^{a}
4.	CIM_{B4}	2.0 : 2.0	0.41 ± 0.2^{ab}	1.26 ± 0.4^{ab}	2.89 ± 0.5^{a}

Data are represented as means ± SE from 10 replicates. Different letters within the same column indicate significant differences between treatments at $p \leq 0.05$ LSD. (A) CIM_{B2} was found to be most significant for callus area. (B)CIM_{B2} showed significantly callus formation 30 days after culturing. (C) CIM_{B1}, CIM_{B2}, CIM_{B3} and CIM_{B4} were all found to be equally significant for callus induction after 45 days.

Table 4. Statistical analysis of data recorded for *Taxus wallichiana* shoot formation with BAP and IAA.

Sl. N no.	Treatment	BAP : IAA (mg/l)	Shoot length mm (A)	Shoot length mm (B)	Shoot length mm (C)
1.	SIM_{IA1}	2.0 : 1.5	0.68 ± 0.2^{a}	1.02 ± 0.2^{a}	1.30 ± 0.1^{a}
2.	SIM_{IA2}	2.0 : 2.5	0.80 ± 0.3^{a}	1.15 ± 0.3^{a}	1.43 ± 0.2^{a}
3.	SIM_{IA3}	2.0 : 3.5	0.48 ± 0.3^{a}	0.48 ± 0.2^{b}	0.82 ± 0.4^{b}

Data are represented as means ± SE from 10 replicates. Different letters within the same column indicate significant differences between treatments at $p \leq 0.05$ LSD. (**A**) SIM_{IA1}, SIM_{IA2} and SIM_{IA3} 15 days after internode culturing. (**B**) SIM_{IA1}, SIM_{IA2} and SIM_{IA3} 30 days after culturing. (**C**) Data recorded after 45 days of culturing with SIM_{IA1}, SIM_{IA2} and SIM_{IA3}.

Table 5. Statistical analysis of data recorded for *Taxus wallichiana* shoot and leaf formation with BAP and IAA.

Sl. no.	Treatment	BAP:IAA (mg/l)	No of leaves (A)	No of leaves (B)	No of leaves (C)
1.	SIM_{IA1}	2.0 : 1.5	3.14 ± 0.8^{a}	4.42 ± 0.5^{a}	6.14 ± 1.0^{a}
2.	SIM_{IA2}	2.0 : 2.5	4.16 ± 0.7^{a}	5.33 ± 0.8^{a}	6.83 ± 0.7^{a}
3.	SIM_{IA3}	2.0 : 3.5	3.40 ± 1.0^{a}	5.00 ± 1.0^{a}	6.00 ± 0.7^{a}

Data are represented as means ± standard errors from 10 replicates. Different letters within the same column indicate significant differences between treatments at $p \leq 0.05$ LSD (A) SIM_{IA1}, SIM_{IA2} and SIM_{IA3} 15 days after internode culturing. (B) SIM_{IA1}, SIM_{IA2} and SIM_{IA3} 30 days after culturing. (C) Data recorded after 45 days of culturing with SIM_{IA1}, SIM_{IA2} and SIM_{IA3}

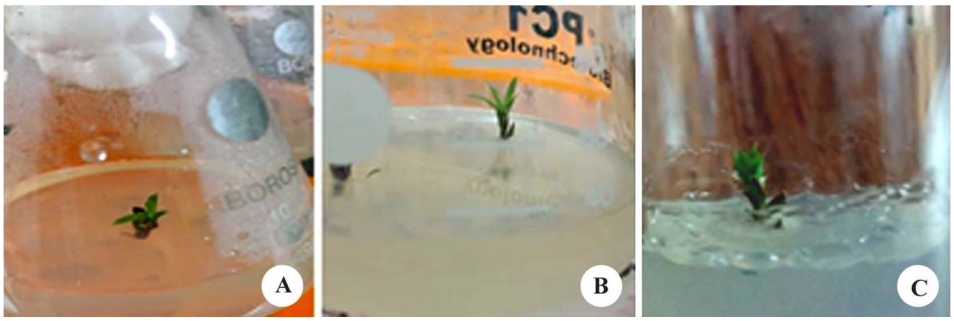

Fig. 2. *In vitro* culture of *Taxu wallichiana* node with shoot formation at various concentrations of BAP and IAA. (A) SIM_{IA1} after 45 days, (B) SIM_{IA2} after 45 days, (C) SIM_{IA3} after 45 days of shoot and leaf formation.

For regeneration, IAA (1.5, 2.5, 3.5 mg/l) was used in combination with BAP (2.0 mg/l). Data recorded after 15, 30 and 45 days of culturing (Fig. 2) showed shoot regeneration on shoot induction media (SIM_{IA1}-SIM_{IA3}) for all three treatments (Tables 4 and 5). No rooting was observed with different concentrations of IAA and BAP used.

Taxus wallichiana showed rooting on MS with BAP, IBA and IAA (Hussain et al. 2011), however different sources of explants may be the reason for no rooting in this study. 2,4-D with BAP on WP medium also showed *Taxu wallichiana* regeneration (Datta et al. 2006). This indicates that the composition of medium has important role in organogenesis and micropropagation.

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(Manuscript received on 30 December, 2019; revised on 7 May, 2020)