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MICROPROPAGATION OF YEW THROUGH TISSUE CULTURE TECHNIQUE; A CONSERVATION APPROACH

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Abstract

Yews are an important medicinal plant species in the genus *Taxus*. It has high importance due to the presence of Taxol, a well-known anti-cancer drug, used for the treatment of ovarian and breast cancer. Due to its slow growth and long seed dormancy period of 1.5-2 years, its natural regeneration is slow. In the current research, we developed an *in-vitro* propagation system to aid in conserving this species. The various plant tissues were excised from field-grown trees and kept moist at 4℃ until they were brought to the lab. We conclude that the best sterilization is possible only by treating explants with 70% ethanol for 2 min followed by 35% of bleach for 10 min. Indole Butyric Acid (IBA) 100 and 200 mg/L were the best dozes that showed a high number of rooting with IBA 200 mg/L→Days to Root Initiation (DTRI)=87 days, and IBA 100 mg/L→DTRI=52 and 55 days. In comparison, the growth of the shoots was found as? IBA 200 mg/L→ Days to Shoots Initiation (DTSI)=14d, while that of IBA 100 mg/L→DTSI=14d was noticed weekly up to the sixth week of culturing. Hence it is proved that viable, rooted plantlets of H. yew can be produced *In vitro* using MS media supplemented with IBA, which shows the emergence of the adventitious roots by keeping the explants for a longer time in media.

Key words: Micropropagation, Conservation, Endangered, Yew, and Tissue culture.

Introduction

Himalayan yew (*Taxus wallichiana*) is a slow-growing tree with poor germination (Rikhari *et al.*, 1998) and has a lengthy seed dormancy period of 1.5–2.0 y (Chee, 1994). Yew is difficult to root and requires a longer time (Fordham and Spraker, 1977). Due to its illegal cutting and other anthropogenic pressures, Himalayan yew is endangered and threatened with extinction in Himalaya. Slow growth rate, lack of awareness, transformation, construction, agriculture, forest fires, habitat loss, grazing, over-harvesting, medicinal use, ornamental use, accidental mortality, and lack of management policies are the major threats to the species (Iqbal *et al.*, 2020; Pant & Samant, 2008). Due to 87% decrease in its population, this species has recently received considerable attention. Conservation and restoration efforts for this medicinal plant species have been hampered by its slow growth rate and long seed dormancy period (Stenfield, 1992; Haq, 2012; Joshe, 2009; Vishnu-Mittre, 1984). To help the long-term survival of this species, it is important to develop approaches for its propagation and the production of Taxol (Schneck, 1996). In Pakistan, a prominent technique employed for the in-situ conservation of *T. wallichiana* involves the application of hormones to the plant cuttings (Iqbal *et al.*, 2022). Furthermore, the quantification of paclitaxel content in different parts of the species from the moist temperate forests of Swat and Hazara districts has been reported (Ghaffar *et al.*, 2023). Sharma *et al.*, (2021) reported successful micropropagation of the endangered Himalayan yew (*T. wallichiana*) using axillary bud explants. The study optimized various growth regulators and cultural conditions for the *In vitro* regeneration of the plant. Majumdar *et al.*, (2021) developed an efficient micropropagation protocol for the Indian yew (*Taxus baccata*) using nodal explants. The study used different concentrations of cytokinins and auxins to induce multiple shoot proliferation. Huang *et al.*, (2020) investigated the genetic stability of micropropagated yew plantlets using ISSR and SRAP markers. The study found that the micropropagated plantlets showed a high degree of genetic stability compared to the mother plants. Qu *et al.*, (2020) investigated the effect of different plant growth regulators on the *In vitro* propagation of the Chinese yew (*Taxus chinensis*). The study found that the combination of NAA and TDZ promoted the highest shoot proliferation. Piotrowska *et al.*, (2021) explored the potential of using plant growth regulators and culture conditions to improve the rooting of micropropagated yew plantlets. The study found that the combination of IBA and sucrose in the rooting medium significantly increased the rooting percentage and root length of the plantlets. Huang *et al.*, (2020) investigated the genetic stability of micropropagated yew plantlets using ISSR and SRAP markers. The study found that the micropropagated plantlets showed a high degree of genetic stability compared to the mother plants.

The species is very important because of the presence of the secondary metabolite taxol, which is an effective chemotherapy that is used to treat ovarian and breast

cancers (Holmes *et al.*, 1991; Trimble *et al.*, 1993). Moreover, the species treat many other ailments, including bronchitis, epilepsy, asthma, internal injuries, diabetes, and various lung diseases, and has also been used as an aphrodisiac (Rikhari *et al.*, 1998; Sharma *et al.*, 2014). The growing demand for paclitaxel and other taxanes is a significant cause that is produced in various parts of yew (Miller *et al.*, 1981; Behera *et al.*, 2000). Thus, interest in the *In vitro* culture of the species has been intensified.

Natural regeneration has played a significant role in the conservation methods used for English yew (*T. baccata*) (Rajewski *et al.*, 2000); seed production, survival frequency, and biomass yield are severely affected by the canopy damage of *T. wallichiana* (Rikhari *et al.*, 1998). Furthermore, in comparison to other coniferous species, the seeds of *Taxus* are very difficult to germinate (Pilz, 1996a, b). Few publications describe the regeneration of *Taxus* spp. or any other member of the Taxaceae (non-cone-bearing) family. In contrast, regeneration has been successfully done in different families of Coniferophyta that have well-developed seed-bearing cones, such as Taxodiaceae (Bourgkard & Favre, 1988) and Pinaceae (Attree *et al.*, 1990; Becwar *et al.*, 1989; Lu *et al.*, 1991). Although forest clonal propagation has developed significantly over the decades, coniferous tree species are still considered difficult to propagate (Ahuja, 1993). To overcome the seed dormancy of *T. brevifolia*, embryo culture methods could be of great importance (Flores & Sgrignoli, 1991; Chee, 1994). Vegetative propagation (i.e., rooting stem cutting) can also be used to aid in the restoration of this species (Eccher, 1988; Chee, 1994). Grafting techniques have been currently applied for the propagation of *Taxus baccata* Linn. (Mitter & Sharma, 1999; Saini, 2001).

It has been shown that Himalayan yew can be propagated using an embryo culture technique (Datta & Jha, 2008). Regeneration of Pacific yew (*Taxus brevifolia*) via direct organogenesis from zygotic embryos has been reported (Chee, 1995a). Adventitious bud development from calli derived from hypocotyls of germinated zygotic embryos has also been documented in Chinese yew (*T. chinensis* var. *mairei*) (Bhuju & Gauchan, 2018). At present, there are no publications on *in-vitro* regeneration of Himalayan yew via direct organogenesis, and a limited number of literatures are available for English yew (Chang *et al.*, 1998).

Micropropagation via *In vitro* culturing can be a reliable way to propagate yew trees because it results in the production of higher-quality plantlets. The resulting plants can be used for restoration work. The present experiment was designed to develop a quick and reproducible method to regenerate yew using explants derived from mature trees.

Material and Methods

Explant collection: Explants were collected from mature trees growing at the Purdue University Campus. Recently emerged shoot tips and stem segments were collected as explant sources.

Explant sterilization: Explants were rinsed with tap water to remove particulate impurities and reduce the microbial load before being soaked in 70% ethanol for 2 min. Explants were then sterilized using 35% commercial bleach (5.25% sodium hypochlorite) treatment for 10 min. Following the bleach treatment, explants were rinsed five times with deionized, distilled water in a laminar-flow hood to prevent contamination. The favorable conditions found in this experiment are shown in the (Table 1).

Media propagation: Basal Murashige and Skoog (M-S) media (Murashige and Skoog, 1962) was supplemented with L-glutamine, Myo-inositol, MES, vitamins (500×FV vitamins), and 20 g/L of sucrose. Different concentrations (25, 50, 75, 100, 150, and 200 mg/L) of indole-3-butyric acid (IBA) were used. Media was supplemented with 2.40 g/L PhytoBlend agar, 2 g/L Gelrite and the final pH was adjusted to 5.8. The media were autoclaved at 121°C for 25 min and then poured into sterile tubes.

Explant culture: The sterilized shoot tips and stem segments were cut into small pieces ranging from 2-3 cm using sterilized scalpels or scissors. The explants were aseptically inserted and cultured in the test tubes containing M-S media; all explants were sub-cultured using the same media supplemented with various concentrations of IBA. Cultures were maintained under a 16-h day and 8h dark photoperiod at 25±5°C.

Statistical Analyses

Statistical analyses were performed using SPSS version 25.0. Mean values of all the parameters (number of roots, root length, the maximum number of shoots, shoots length) were calculated to determine which IBA concentration led to the best shoot development. Multivariate Analysis of Variance (MANOVA) was used to compare responses to various IBA concentrations, including the time spent on each medium and the amount of growth (roots and shoots).

Results

The MS media was supplemented with various concentrations of IBA. The DTSI, maximum and average sprout numbers, growth of shoots (cm), DTRI, number of roots, and roots growth (cm) of explants were evaluated after 7 days of inoculation. Buds became apparent as early as 14 days following the establishment of the cultures (Fig. 1; Table 2). Six weeks after emergence, number of shoots and roots along with the length were evaluated (Figs. 2, 5 and 7). The explants cultured in M-S media supplemented with 100 and 200 mg/L IBA showed root emergence after 54 and 87 days, respectively, after cultures were established (Figs. 3-4; Table 2). The largest number and length of roots were seen with 200 mg/L IBA. The weekly average root length is shown in Fig. 7. These explants were sub-cultured to MS media having IBA 100 mg/L once after a month up to the final root emergence. Root emergence was observed on explants exposed to 100 mg/L IBA after a single sub-culturing. The maximum numbers of roots were 3, and the maximum root length was 2.5 cm (Figs. 4, 6). The weekly averages are shown in (Figs. 7-9).

Table 1. Favorable condition for explant sterilization, ethanol concentration, time for ethanol treatment (min), bleach concentration (%), time for bleach treatment (min), contamination (%), condition favorable.

Ethanol Conc (%)	Time for ethanol treatment (min)	Bleach Conc (%)	Time with bleach treatment (min)	Contamination (%)	Condition favorable
70	2	25	15	50	+
70	2	25	10	50	+
70	2	35	15	40	+
70	2	35	10	20	++

(+) favorable, (++) very favorable

Table 2. Details of explants growth observed till the root emergence.

Conc. IBA (mg/L)	Media	Days to shoot initiation (DTSI)	Shoots	Days to root initiation (DTRI)	Roots
25	MS	14	Yes	No	No
50	MS	14	Yes	No	No
75	MS	14	Yes	No	No
100	MS	14	Yes	54	Yes
150	MS	14	Yes	No	No
200	MS	14	Yes	87	Yes

Statistical Analysis

Assumptions of MANOVA

Normality: P-value of root growth and shoot growth is 0.200 and 0.156, respectively (Table 3) which do not reject the hypothesis of normally distributed data. Hence, the normality assumption was fulfilled.

Table 3. Tests of Normality.

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
Root growth	0.124	18	0.200*
Shoot growth	0.174	18	0.156

*This is a lower bound of the true significance

P-value of Wilks Lambda for IBA concentrations and time (weeks) is <0.05 (Table 4) therefore, null hypothesis was rejected, and it was concluded that there is significant difference between average root growth and number in response to different concentrations and length of exposure to IBA. In other words, there is a significant effect of IBA concentration and length of exposure on root growth and number.

The results discussed previously can also be confirmed by the above (Table 5). The effect of IBA concentration and time interval (weeks) is significant for root growth and number of roots. After getting the significant effects of IBA and time (weeks) on the root's growth and the numbers of roots, LSD (Least Significant Difference) test was applied for checking the pairwise significant differences.

Table 4. Multivariate Tests[a].

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.972	175.947[b]	2.000	10.000	0.000
	Wilks' Lambda	0.028	175.947[b]	2.000	10.000	0.000
	Hoteling's Trace	35.189	175.947[b]	2.000	10.000	0.000
	Roy's Largest Root	35.189	175.947[b]	2.000	10.000	0.000
IBA	Pillai's Trace	0.878	36.040[b]	2.000	10.000	0.000
	Wilks' Lambda	**0.122**	**36.040[b]**	**2.000**	**10.000**	**0.000**
	Hoteling's Trace	7.208	36.040[b]	2.000	10.000	0.000
	Roy's Largest Root	7.208	36.040[b]	2.000	10.000	0.000
Time (weeks)	Pillai's Trace	1.024	2.306	10.000	22.000	0.049
	Wilks' Lambda	**0.081**	**5.033[b]**	**10.000**	**20.000**	**0.001**
	Hoteling's Trace	10.073	9.065	10.000	18.000	0.000
	Roy's Largest Root	9.943	21.874[c]	5.000	11.000	0.000

a. Design: Intercept + IBA + time (weeks)

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level

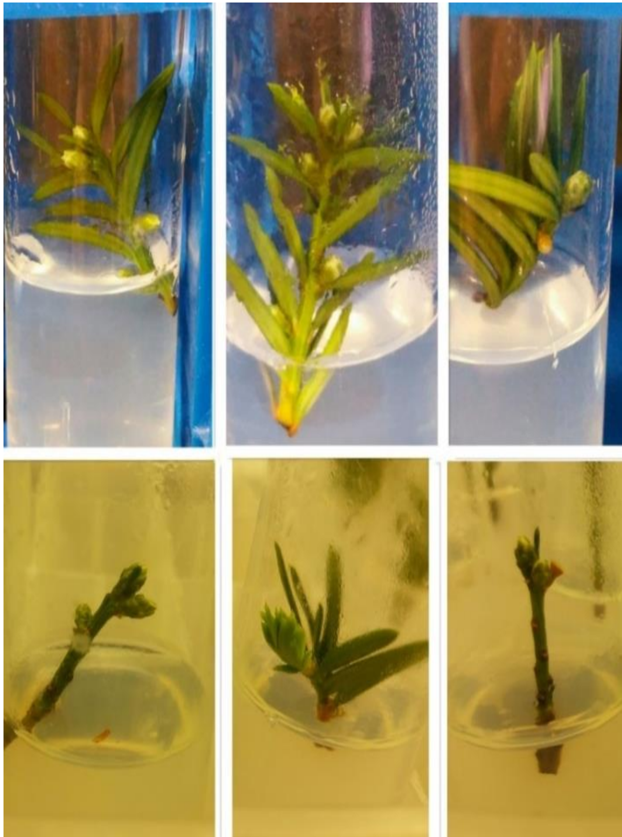

Fig. 1. The appearance of buds and shoots on explants after 14 days of culturing on M-S media supplemented with IBA.

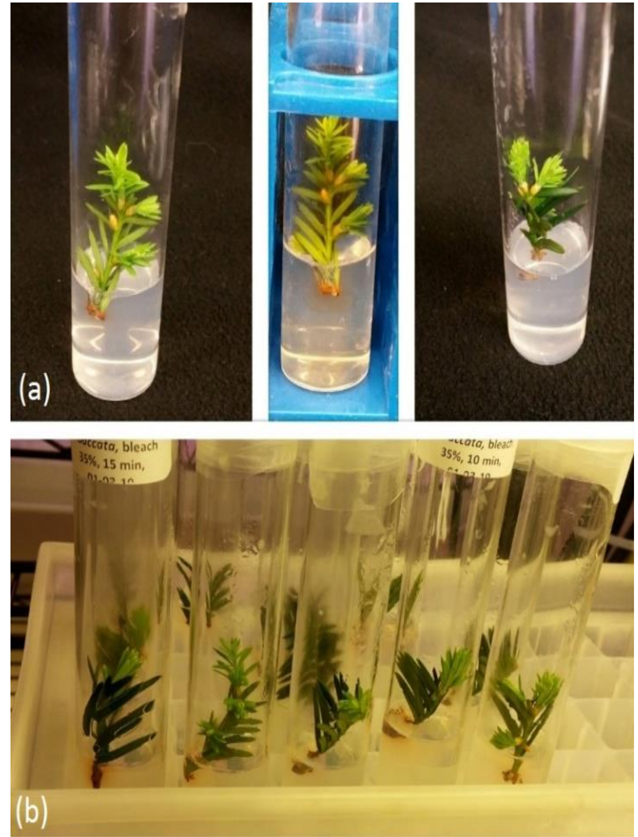

Fig. 2. Shoot development on explants during the (a) 3rd and (b) 4th week of inoculated on MS media supplemented with IBA.

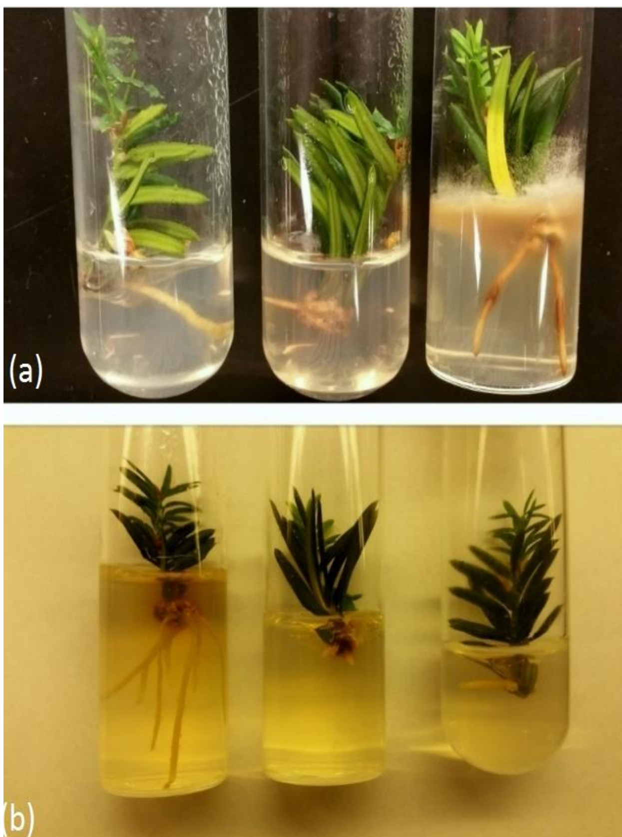

Fig. 3. Explants showing roots emergence successively after culturing on the MS media supplemented with IBA (a) 100 mg/L, and (b) 200 mg/L.

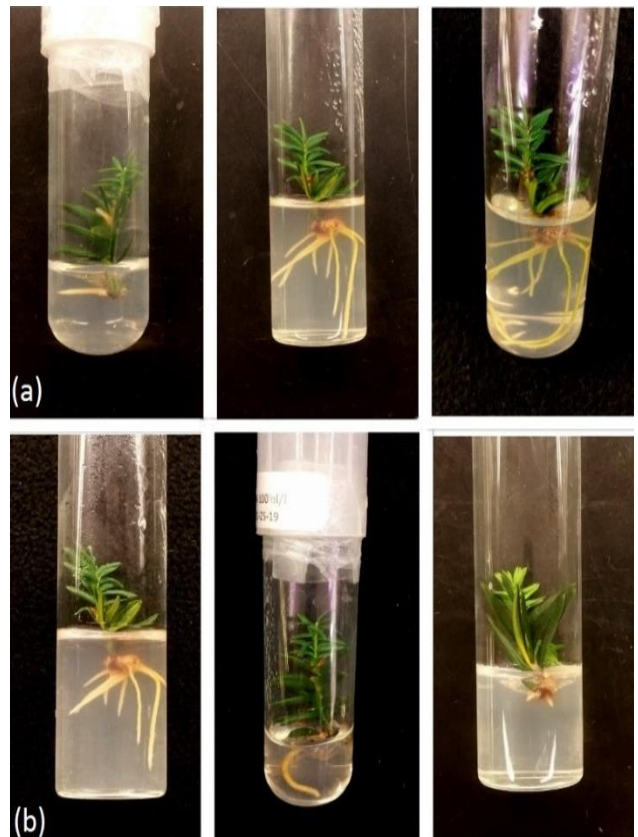

Fig. 4. Shoot development and root growth by culturing explants on MS media supplemented with IBA 200 mg/L and IBA 100 mg/L.

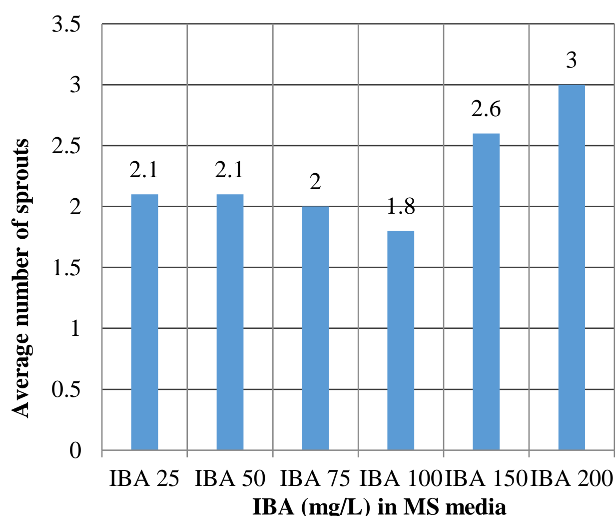

Fig. 5. Average number of sprouts of explant up to six weeks of culturing by the MS media supplemented with various concentration of IBA.

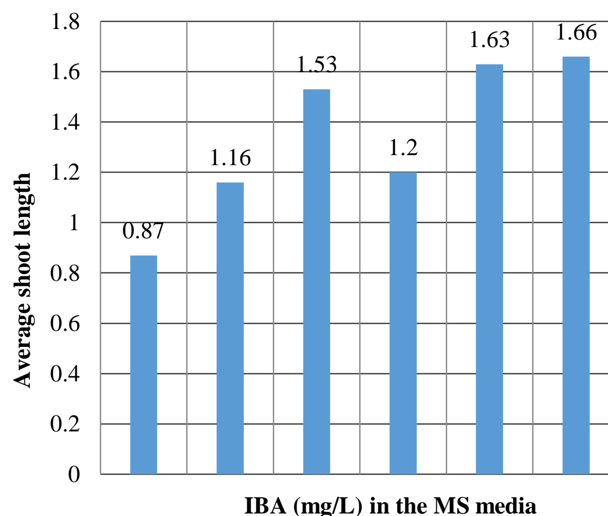

Fig. 6. Average shoot length increase up to 6 weeks of culturing in MS media supplemented with the various concentration of IBA.

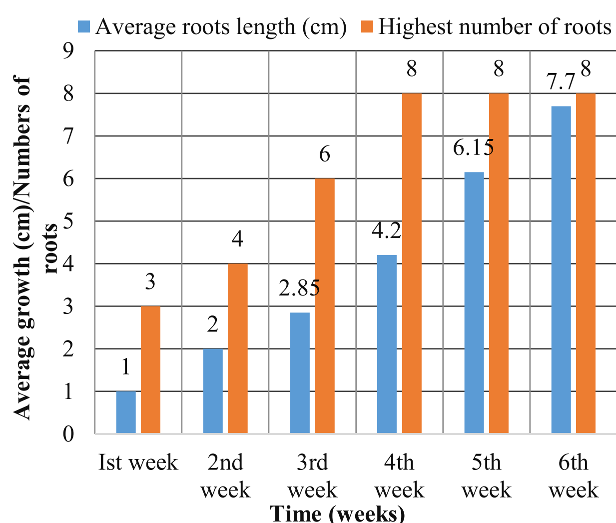

Fig. 7. Average roots length (cm) and highest number of roots observed up to six weeks of successively root initiation (DTRI 87) after culturing on MS media concentrated with IBA (200 mg/L).

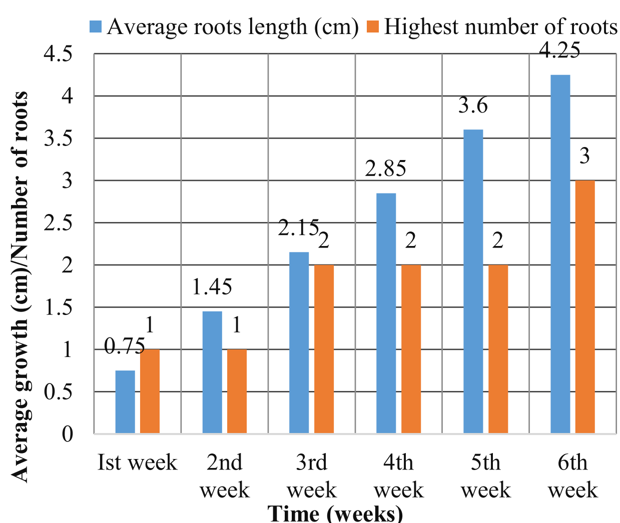

Fig. 8. Average roots length and the number of roots observed in one of the successful trials up to six weeks of successively root initiation (DTRI 52) after culturing on MS media concentrated with IBA (100 mg/L).

Table 5. Tests of between-subject effects.

Source	Tests of between-subjects effects					
	Dependent variable	Type III Sum of squares	df	Mean square	F	Sig.
Corrected model	Root growth	54.575[a]	6	9.096	19.868	0.000
	No of roots	101.806[b]	6	16.968	15.590	0.000
Intercept	Root growth	170.520	1	170.520	372.459	0.000
	No of roots	240.250	1	240.250	220.740	0.000
IBA	**Root growth**	**8.266**	**1**	**8.266**	**18.054**	**0.001**
	No of roots	**84.028**	**1**	**84.028**	**77.204**	**0.000**
Time (weeks)	**Root growth**	**46.310**	**5**	**9.262**	**20.230**	**0.000**
	No of roots	**17.778**	**5**	**3.556**	**3.267**	**0.047**
Error	Root growth	5.036	11	0.458		
	No of roots	11.972	11	1.088		
Total	Root growth	224.323	18			
	No of roots	288.000	18			
Corrected total	Root growth	59.611	17			
	No of roots	113.778	17			

a. R Squared = 0.916 (R^2= 0.869)
b. R Squared = 0.895 (R^2= 0.837)

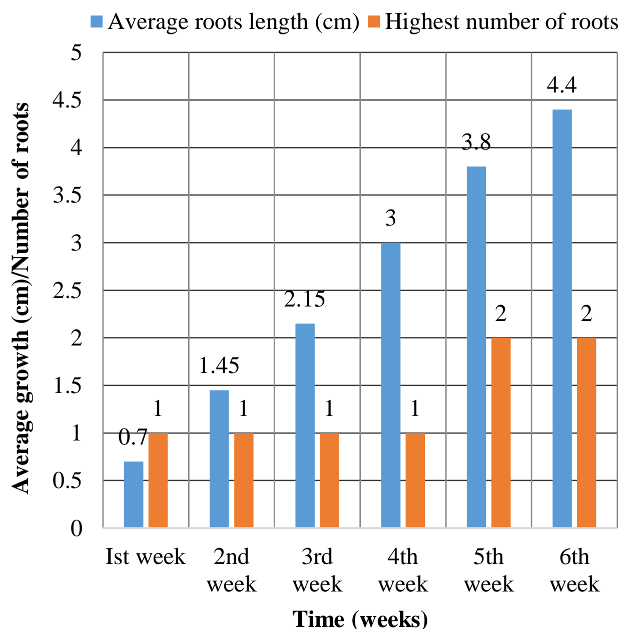

Fig. 9. Average roots length (cm) and the number of roots observed up to six weeks of successively root initiation (DTI 55) after culturing on MS media concentrated with IBA (100 mg/L).

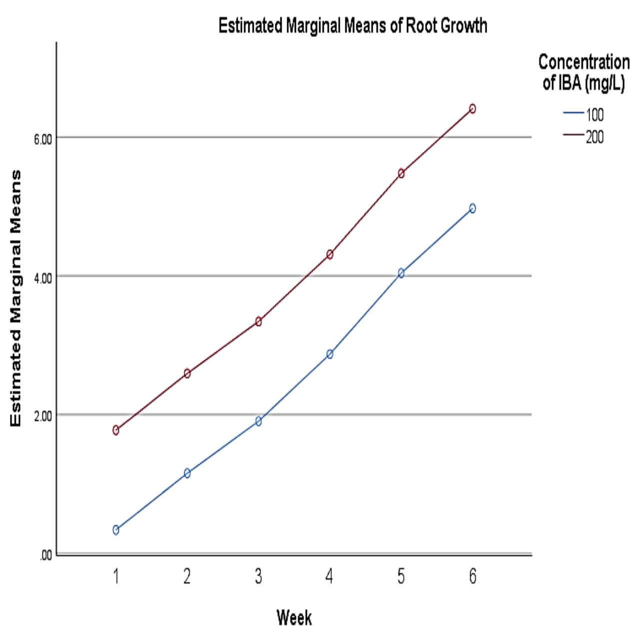

Fig. 10. Estimated marginal means of roots growth (cm) in M-S media supplemented with 100 and 200 mg/L IBA.

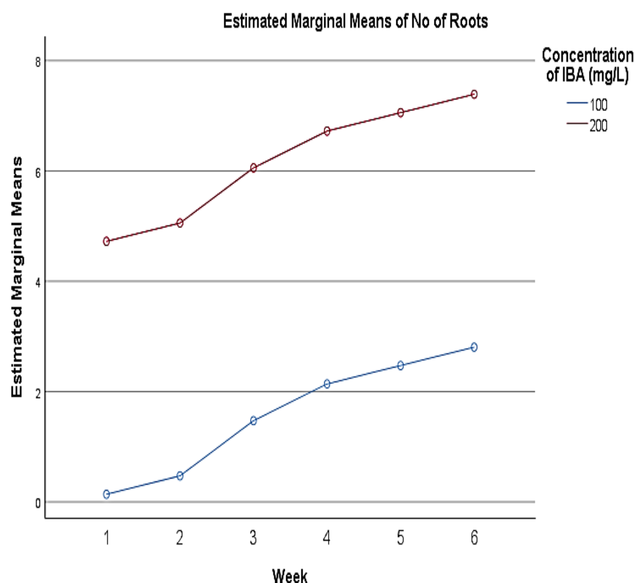

Fig. 11. Estimated marginal means of numbers of roots in MS media in the presence of IBA=100 & 200 mg/L.

From the LSD test (Table 6) it was concluded that there is significant difference between root growth and numbers of roots for various concentration level of 100 and 200 mg/L. Furthermore, negative sign between mean difference of 100 and 200 mg/L shows that average root growth and numbers of roots are higher/more for 200 mg/L as compared to 100 mg/L (Table 7).

Pairwise of differences for both concentration and length of exposure to IBA revealed that root growth and number were significantly different at weeks 4, 5, and 6.

Number of Sprouts and Shoot growth of Explant in the MS media in the presence of different concentrations of IBA: P-value of Wilks Lambda for IBA concentrations and increasing time intervals (weeks) is < 0.05 (Table 8) therefore, the null hypothesis is rejected, and it was concluded from the statistic tests that there is a significant difference between shoot growth and the maximum number of sprouts for various concentrations of IBA and time intervals (weeks). It means there is a significant effect of IBA concentrations and time (weeks) on shoot growth and the number of sprouts.

While checking the pair of significant differences for both IBA various concentrations and time (weeks), it is found that based on the estimated marginal means, the means differences are significant at 0.05 level (Table 11).

Table 6. LSD (least significant difference) test.

Dependent variable	(I) Conc. of IBA (mg/L)	(J) Conc. of IBA (mg/L)	Mean difference (I-J)	Std. Error	Sig.[b]
Root growth	100	200	-1.438*	0.338	0.001
	200	100	1.438*	0.338	0.001
Numbers of roots	100	200	-4.583*	0.522	0.000
	200	100	4.583*	0.522	0.000

Based on estimated marginal means

*The mean difference is significant at the .05 level

Table 7. LSD test for pairwise differences between root growth and number over time.

Dependent variable	(I) Time (weeks)	(J) Time (weeks)	Mean Difference (I-J)	Std. Error	Sig.[b]
Root growth	1st	2nd	-0.817	0.552	0.167
		3rd	-1.567*	0.552	0.016
		4th	-2.533*	0.552	0.001
		5th	-3.700*	0.552	0.000
		6th	-4.633*	0.552	0.000
	2nd	1st	0.817	0.552	0.167
		3rd	-0.750	0.552	0.202
		4th	-1.717*	0.552	0.010
		5th	-2.883*	0.552	0.000
		6th	-3.817*	0.552	0.000
	3rd	1st	1.567*	0.552	0.016
		2nd	0.750	0.552	0.202
		4th	-0.967	0.552	0.108
		5th	-2.133*	0.552	0.003
		6th	-3.067*	0.552	0.000
	4th	1st	2.533*	0.552	0.001
		2nd	1.717*	0.552	0.010
		3rd	0.967	0.552	0.108
		5th	-1.167	0.552	0.058
		6th	-2.100*	0.552	0.003
	5th	1st	3.700*	0.552	0.000
		2nd	2.883*	0.552	0.000
		3rd	2.133*	0.552	0.003
		4th	1.167	0.552	0.058
		6th	-0.933	0.552	0.119
	6th	1st	4.633*	0.552	0.000
		2nd	3.817*	0.552	0.000
		3rd	3.067*	0.552	0.000
		4th	2.100*	0.552	0.003
		5th	0.933	0.552	0.119
Number of roots	1st	2nd	-0.333	0.852	0.703
		3rd	-1.333	0.852	0.146
		4th	-2.000*	0.852	0.039
		5th	-2.333*	0.852	0.019
		6th	-2.667*	0.852	0.010
	2nd	1st	0.333	0.852	0.703
		3rd	-1.000	0.852	0.265
		4th	-1.667	0.852	0.076
		5th	-2.000*	0.852	0.039
		6th	-2.333*	0.852	0.019
	3rd	1st	1.333	0.852	0.146
		2nd	1.000	0.852	0.265
		4th	-0.667	0.852	0.450
		5th	-1.000	0.852	0.265
		6th	-1.333	0.852	0.146
	4th	1st	2.000*	0.852	0.039
		2nd	1.667	0.852	0.076
		3rd	0.667	0.852	0.450
		5th	-0.333	0.852	0.703
		6th	-0.667	0.852	0.450
	5th	1st	2.333*	0.852	0.019
		2nd	2.000*	0.852	0.039
		3rd	1.000	0.852	0.265
		4th	0.333	0.852	0.703
		6th	-0.333	0.852	0.703
	6th	1st	2.667*	0.852	0.010
		2nd	2.333*	0.852	0.019
		3rd	1.333	0.852	0.146
		4th	0.667	0.852	0.450
		5th	0.333	0.852	0.703

*The mean difference is significant at the 0.05 level

Table 8. Multivariate testsa.

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.986	857.531[b]	2.000	24.000	0.000
	Wilks' Lambda	.014	857.531[b]	2.000	24.000	0.000
	Hoteling's Trace	71.461	857.531[b]	2.000	24.000	0.000
	Roy's Largest Root	71.461	857.531[b]	2.000	24.000	0.000
Concentration of IBA mg/L	Pillai's Trace	1.001	5.013	10.000	50.000	0.000
	Wilks' Lambda	**.235**	**5.095[b]**	**10.000**	**48.000**	**0.000**
	Hotelling's Trace	2.245	5.163	10.000	46.000	0.000
	Roy's Largest Root	1.627	8.133[c]	5.000	25.000	0.000
Week	Pillai's Trace	1.262	8.550	10.000	50.000	0.000
	Wilks' Lambda	**.022**	**27.827[b]**	**10.000**	**48.000**	**0.000**
	Hotelling's Trace	32.098	73.824	10.000	46.000	0.000
	Roy's Largest Root	31.684	158.420[c]	5.000	25.000	0.000

a. Design: Intercept + conc + week
b. Exact statistic
c. The statistic is an upper bound on F that yields a lower bound on the significance level

Table 9. Tests of between-subjects effects.

Source	Dependent variable	Type III Sum of squares	Df	Mean square	F	Sig.
Corrected model	Highest number of sprouts in explants	67.611[a]	10	6.761	21.055	0.000
	Shoot growth	40.363[b]	10	4.036	45.199	0.000
Intercept	Highest number of sprouts in explants	191.361	1	191.361	595.934	0.000
	Shoot growth	65.618	1	65.618	734.797	0.000
Conc. of IBA	**Highest number of sprouts in explants**	**5.806**	**5**	**1.161**	**3.616**	**0.013**
	Shoot growth	**2.940**	**5**	**.588**	**6.583**	**0.000**
Time (weeks)	**Highest number of sprouts in explants**	**61.806**	**5**	**12.361**	**38.495**	**0.000**
	Shoot growth	**37.424**	**5**	**7.485**	**83.815**	**0.000**
Error	Highest number of sprouts in explants	8.028	25	0.321		
	Shoot growth	2.233	25	0.089		
Total	Highest number of sprouts in explants	267.000	36			
	Shoot growth	108.214	36			
Corrected total	Highest number of sprouts in explants	75.639	35			
	Shoot growth	42.596	35			

a. R Squared = .894 (Adjusted R Squared = .851)
b. R Squared = .948 (Adjusted R Squared = .927)

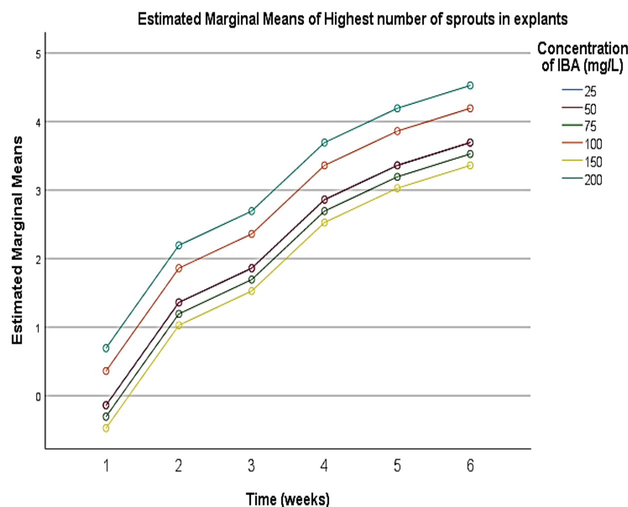

Fig. 12. Estimated marginal means of highest numbers of sprouts on explants cultured on MS media supplemented with various concentrations of IBA i.e. 25, 50, 75, 100, 150, and 200 mg/L up to six weeks of culturing.

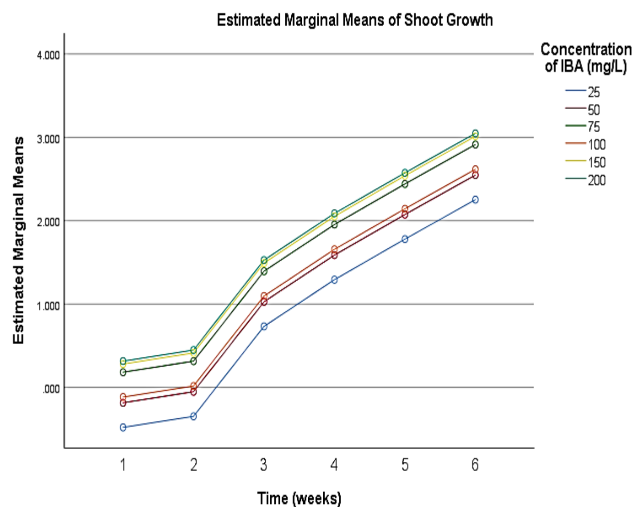

Fig. 13. Estimated marginal means shoot growth (cm) of explants cultured on MS media supplemented with various concentrations of IBA i.e., 25, 50, 75, 100, 150, and 200 mg/L up to six weeks of culturing.

The results discussed previously can also be confirmed by the above (Table 9). The effect of IBA concentration and time intervals (weeks) is significant for shoot growth and highest number of sprouts in explants cultured in MS media. After getting the significant effects for both the parameters, the LSD (Least Significant Difference) test was applied to check the pairwise significant differences (Table 10).

Table 10. LSD Test for the Highest numbers of sprouts and Shoots growth (cm) on Explants cultured on MS media supplemented with different concentrations of IBA (25, 50, 75, 100, 150, and 200 mg/L).

Dependent variable	Pairwise comparisons						
	(I) Conc. of IBA (mg/L)	(J) Conc. of IBA (mg/L)	Mean difference (I-J)	Std. Error	Sig.[b]	95% Confidence interval for difference[b]	
						Lower bound	Upper bound
		50	0.000	0.327	1.000	-0.674	0.674
		75	0.167	0.327	0.615	-0.507	0.840
	25	100	-0.500	0.327	0.139	-1.174	0.174
		150	0.333	0.327	0.318	-0.340	1.007
		200	-0.833*	0.327	0.017	-1.507	-0.160
		25	0.000	0.327	1.000	-0.674	0.674
		75	0.167	0.327	0.615	-0.507	0.840
	50	100	-0.500	0.327	0.139	-1.174	0.174
		150	0.333	0.327	0.318	-0.340	1.007
		200	-0.833*	0.327	0.017	-1.507	-0.160
		25	-0.167	0.327	0.615	-0.840	0.507
		50	-0.167	0.327	0.615	-0.840	0.507
	75	100	-0.667	0.327	0.052	-1.340	0.007
		150	0.167	0.327	0.615	-0.507	0.840
Highest number of sprouts on explants		200	-1.000*	0.327	0.005	-1.674	-0.326
		25	0.500	0.327	0.139	-0.174	1.174
		50	0.500	0.327	0.139	-0.174	1.174
	100	75	0.667	0.327	0.052	-0.007	1.340
		150	0.833*	0.327	0.017	0.160	1.507
		200	-0.333	0.327	0.318	-1.007	0.340
		25	-0.333	0.327	0.318	-1.007	0.340
		50	-0.333	0.327	0.318	-1.007	0.340
	150	75	-0.167	0.327	0.615	-0.840	0.507
		100	-0.833*	0.327	0.017	-1.507	-0.160
		200	-1.167*	0.327	0.001	-1.840	-0.493
		25	0.833*	0.327	0.017	0.160	1.507
		50	0.833*	0.327	0.017	0.160	1.507
	200	75	1.000*	0.327	0.005	0.326	1.674
		100	0.333	0.327	0.318	-0.340	1.007
		150	1.167*	0.327	0.001	0.493	1.840
		50	-0.295	0.173	0.100	-0.650	0.060
		75	-0.662*	0.173	0.001	-1.017	-0.306
	25	100	-0.359*	0.173	0.048	-0.714	-0.003
		150	-0.760*	0.173	0.000	-1.115	-0.405
		200	-0.795*	0.173	0.000	-1.150	-0.440
		25	0.295	0.173	0.100	-0.060	0.650
		75	-0.367*	0.173	0.044	-0.722	-0.011
	50	100	-0.064	0.173	0.715	-0.419	0.292
		150	-0.465*	0.173	0.012	-0.820	-0.110
		200	-0.500*	0.173	0.008	-0.855	-0.145
		25	0.662*	0.173	0.001	0.306	1.017
		50	0.367*	0.173	0.044	0.011	0.722
	75	100	0.303	0.173	0.091	-0.053	0.658
		150	-0.098	0.173	0.574	-0.454	0.257
Shoot growth (cm)		200	-0.133	0.173	0.447	-0.489	0.222
		25	0.359*	0.173	0.048	0.003	0.714
		50	0.064	0.173	0.715	-0.292	0.419
	100	75	-0.303	0.173	0.091	-0.658	0.053
		150	-0.401*	0.173	0.028	-0.757	-0.046
		200	-0.436*	0.173	0.018	-0.792	-0.081
		25	0.760*	0.173	0.000	0.405	1.115
		50	0.465*	0.173	0.012	0.110	0.820
	150	75	0.098	0.173	0.574	-0.257	0.454
		100	0.401*	0.173	0.028	0.046	0.757
		200	-0.035	0.173	0.841	-0.390	0.320
		25	0.795*	0.173	0.000	0.440	1.150
		50	0.500*	0.173	0.008	0.145	0.855
	200	75	0.133	0.173	0.447	-0.222	0.489
		100	0.436*	0.173	0.018	0.081	0.792
		150	0.035	0.173	0.841	-0.320	0.390

Based on estimated marginal means

*. The mean difference is significant at the 0.05 level

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments)

Table 11. LSD Test for the effect of Time (weeks) on Highest numbers of sprouts and shoots growth (cm) of Explants cultured on MS media supplemented with different concentrations of IBA.

Dependent variable	Pairwise comparisons						
	Time (weeks)	(J) Time (weeks)	Mean difference (I-J)	Std. Error	Sig.[b]	95% Confidence interval for difference[b]	
						Lower bound	Upper bound
Highest number of sprouts on explants	1st	2nd	-1.500*	0.327	0.000	-2.174	-0.826
		3rd	-2.000*	0.327	0.000	-2.674	-1.326
		4th	-3.000*	0.327	0.000	-3.674	-2.326
		5th	-3.500*	0.327	0.000	-4.174	-2.826
		6th	-3.833*	0.327	0.000	-4.507	-3.160
	2nd	1st	1.500*	0.327	0.000	0.826	2.174
		3rd	-0.500	0.327	0.139	-1.174	0.174
		4th	-1.500*	0.327	0.000	-2.174	-0.826
		5th	-2.000*	0.327	0.000	-2.674	-1.326
		6th	-2.333*	0.327	0.000	-3.007	-1.660
	3rd	1st	2.000*	0.327	0.000	1.326	2.674
		2nd	0.500	0.327	0.139	-0.174	1.174
		4th	-1.000*	0.327	0.005	-1.674	-0.326
		5th	-1.500*	0.327	0.000	-2.174	-0.826
		6th	-1.833*	0.327	0.000	-2.507	-1.160
	4th	1st	3.000*	0.327	0.000	2.326	3.674
		2nd	1.500*	0.327	0.000	0.826	2.174
		3rd	1.000*	0.327	0.005	0.326	1.674
		5th	-0.500	0.327	0.139	-1.174	0.174
		6th	-0.833*	0.327	0.017	-1.507	-0.160
	5th	1st	3.500*	0.327	0.000	2.826	4.174
		2nd	2.000*	0.327	0.000	1.326	2.674
		3rd	1.500*	0.327	0.000	0.826	2.174
		4th	0.500	0.327	0.139	-0.174	1.174
		6th	-0.333	0.327	0.318	-1.007	0.340
	6th	1st	3.833*	0.327	0.000	3.160	4.507
		2nd	2.333*	0.327	0.000	1.660	3.007
		3rd	1.833*	0.327	0.000	1.160	2.507
		4th	0.833*	0.327	0.017	0.160	1.507
		5th	0.333	0.327	0.318	-0.340	1.007
Shoot growth (cm)	1st	2nd	-0.132	0.173	0.450	-0.488	0.223
		3rd	-1.206*	0.173	0.000	-1.562	-0.851
		4th	-1.772*	0.173	0.000	-2.127	-1.416
		5th	-2.258*	0.173	0.000	-2.614	-1.903
		6th	-2.732*	0.173	0.000	-3.087	-2.376
	2nd	1st	0.132	0.173	0.450	-0.223	0.488
		3rd	-1.074*	0.173	0.000	-1.429	-0.718
		4th	-1.639*	0.173	0.000	-1.995	-1.284
		5th	-2.126*	0.173	0.000	-2.481	-1.770
		6th	-2.599*	0.173	0.000	-2.955	-2.244
	3rd	1st	1.206*	0.173	0.000	0.851	1.562
		2nd	1.074*	0.173	0.000	0.718	1.429
		4th	-0.565*	0.173	0.003	-0.921	-0.210
		5th	-1.052*	0.173	0.000	-1.407	-0.697
		6th	-1.525*	0.173	0.000	-1.881	-1.170
	4th	1st	1.772*	0.173	0.000	1.416	2.127
		2nd	1.639*	0.173	0.000	1.284	1.995
		3rd	0.565*	0.173	0.003	0.210	0.921
		5th	-0.487*	0.173	0.009	-0.842	-0.131
		6th	-0.960*	0.173	0.000	-1.315	-0.605
	5th	1st	2.258*	0.173	0.000	1.903	2.614
		2nd	2.126*	0.173	0.000	1.770	2.481
		3rd	1.052*	0.173	0.000	0.697	1.407
		4th	0.487*	0.173	0.009	0.131	0.842
		6th	-0.473*	0.173	0.011	-0.829	-0.118
	6th	1st	2.732*	0.173	0.000	2.376	3.087
		2nd	2.599*	0.173	0.000	2.244	2.955
		3rd	1.525*	0.173	0.000	1.170	1.881
		4th	0.960*	0.173	0.000	0.605	1.315
		5th	0.473*	0.173	0.011	0.118	0.829

Based on estimated marginal means
*. The mean difference is significant at the 0.05 level
b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments)

Discussion

In this study, we have developed a way to propagate *Taxus sps.* using micropropagation technique. In our study vegetative buds became apparent after 2 weeks of culturing. Similar work has been done to develop an efficient regeneration technique for Himalayan yew via organogenesis of shoot from callus cultures derived from zygotic embryos. In this work ½-strength basal media supplemented with ½ SH vitamins (Schenk & Hildebrandt, 1972) and a specific concentration of auxin (2.5 mg/L IBA). In this case, explants produced roots that were 2.15 cm in length after 4 weeks of culturing (Datta *et al*., 2006). Hussain *et al*., (2013) also developed an in-vitro regeneration system for Himalayan yew. They reported shoot tip elongation of 10-14 cm after sub-culturing 3-4 times on M-S media supplemented with 2 mg/L 6-Benzylaminopurine (BAP) and 1 mg/L IBA. In our work, we obtained the best shoot elongation after 14 days without the use of BAP. Hussain *et al.* also reported roots being initiated on the elongated shoots after 60 to 80 days on the M-S media supplemented with 3.5 mg/L IBA and on ½-strength, M-S media supplemented with 8 mg/L IBA. We also left our explants on the initial medium for one month, to achieve good shoot growth before sub-culturing the explants on fresh media. After successive transfers, root emergence occurred between 55 and 87 days. Hussain *et al.* concluded their work by saying that shoot elongation and root induction in shoot tip culture is an effective means of multiplying Himalayan yew. Abbasin *et al*., (2010) also studied micro-propagation of English yew. They were successful using IBA (8 mg/L) and ½-strength M-S salts combined with forests soluble soil. As in our research, Kishor *et al*., (2015) used IBA for in-vitro micropropagation of Himalayan yew.), but to achieve rooting, they successively used 1,000 μM IBA for air-layered shoots. Similar work has also been conducted by many others with varied approaches and results. Some have applied more than one auxin and used different media (Attree *et al*., 1990; Flores & Sgrignoli, 1991; Lu *et al*., 1991; Chee, 1994; Chee, 1995a; Chee, 1995b; Chang *et al*., 1995; Datta *et al*., 2006; Datta & Jha, 2008; Abbasin *et al*., 2010; Ahuja, 2013; Hussain *et al*., 2013).

Conclusions

Micropropagation of explants from mature *Taxus* trees poses challenges due to plant tissue recalcitrance and contamination issues in *In vitro* cultures. Moreover, the species exhibits a lengthy seed dormancy period of 1.5-2.0 years. Micropropagation serves as a crucial technique for mass-propagation of yew plantlets in nursery operations. This study presents a simple and successful procedure for in-vitro clonal propagation of *Taxus baccata* using mature shoot tips. The resulting plantlets can be economically produced to meet the demand for commercial-scale planting stock, thereby mitigating the risk of extinction for this valuable medicinal plant species. Further research is required to address contamination concerns *In vitro* and to minimize the genotypic impact on micropropagation. These efforts are essential to ensure the success of yew restoration programs.

References

Abbasin, Z., S. Zamani. S. Movahedi. G. Khaksar and B.S. Tabatabaei. 2010 *In vitro* micropropagation of yew (*Taxus baccata*) and production of plantlets. *Biotechnol.*, 9: 48-54.

Ahuja, M.R. (Ed.). 2013. Micropropagation of woody plants. *SSBM*., Vol. 41. Springer Science & Business Media.

Attree, S., M. Budimir and L.C. Fowke. 1990. Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings from stored seeds of black and white sprees (*Picea mariana* and *Picea glauca*). *Can. J. Bot.*, 68: 30-34.

Becwar., M.R., T.L. Noland and J.L. Wyckoff. 1989. Maturation, germination, and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants. *In vitro cell. Dev. Biol.*, 25: 575-580.

Behera, M.D., S. Srivastava. S.P.S Kushwaha and P.S. Roy. 2000. Stratification and mapping of *Taxus baccata* L. bearing forests in Talle Valley using remote sensing and GIS. *Curr Sci.*, 78: 1008-1013.

Bhuju, S. and D.P. Gauchan. 2018. *Taxus wallichiana* (Zucc.), an endangered anti-cancerous plant: a review. *Int. J. Res.*, 10: 21.

Bourgkard, F. and J.M. Favre. 1988. Somatic embryos from callus of Sequoia sempervirens. *Plant Cell Rep.*, 7: 445-448.

Chang, S., S. Sen. C.R. McKinley, J. Aimers-Halliday and R.J. Newton. 1995. *In vitro* plant regeneration from embryogenic explants of Pinus *pinea*. *L. Plant Cell Rep.*, 31: 202-206.

Chee, P.P. 1994. *In vitro* culture of zygotic embryos of *Taxus* species. *Hort Sci.*, 29: 695-697.

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

Chee, P.P. 1995b. Stimulation of adventitious rooting of *Taxus* species by thiamine. *Plant Cell Rep.*, 14: 753-757.

Datta, M.M. and S. Jha. 2008. Plant regeneration through somatic embryogenesis in *Taxus wallichiana*. *J. Plant Biochem. Biotechnol.*, 17: 37-44.

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

Eccher, T. 1987. Response of cuttings of 16 *Taxus* cultivars to rooting treatments. In: *International Symposium on Vegetative Propagation of Woody Species,* 227: 251-253.

Flores, H.E. and P.J. Sgrignoli. 1991. *In vitro* culture and precocious germination of *Taxus* embryos. *In vitro Cell. Dev. Biol. Plant.*, 27: 139-142.

Fordham, A.J. and L.S. Spraker. 1977. Propagation manual of selected gymnosperms. *Arnoldia.*, 37: 1-88.

Ghaffar, N., L.S. Lee. B. Khan. H. Khan. J. Iqbal. I.A. Mian and S. Khalid. 2023. Paclitaxel content in various parts of *Taxus wallichiana* from moist temperate forests of Swat and Hazara, Pakistan. *Pak. J. Bot*., 55: 1851-1856.

Haq, F. 2012. The critically endangered flora and fauna of District Battagram Pakistan. *Adv. Life Sci.*, 2: 118-123.

Holmes, F.A., R.S. Walters. R.L. Theriault. A.U. Buzdar. D.K. Frye. G.N. Hortobagyi and M.N. Raber. 1991. Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *JNCI.*, 83: 1797-1805.

Huang, Y., Y. Zou. J. Liu. X. Fang and Y. Zhang. 2020. Genetic stability assessment of micropropagated plants of *Taxus cuspidata* Sieb. et Zucc. using ISSR and SRAP markers. *Physiol. Mol. Biol. Plants*., 26: 1897-1905.

Hussain, A., I.A. Qarshi. H. Nazir. I. Ullah, M. Rashid and Z.K. Shinwari. 2013. *In vitro* callogenesis and organogenesis in *Taxus wallichiana* ZUCC, The Himalayan Yew. *Pak. J. Bot.*, 45: 1755-1759.

Iqbal, J., B. Khan. S. Khan. N. Ghaffar. A.I. Mian, I. Ahmad, N. Yousaf, I. Aamir, I. Ahmad and S. Manzoor. 2022. Effects of hormones (IBA & IAA) on the propagation of Himalayan

yew in Pakistan: a conservation approach. *Pak. J. Bot*., 54(4): 1237-1243.

Iqbal, J., R. Meilan and B. Khan. 2020. Assessment of risk, extinction and threats to Himalayan yew in Pakistan. *Saudi J. Biol. Sci*., 27: 762-767.

Joshi, K. 2009. *Taxus wallichiana* (Himalayan Yew; Loth salla) in Nepal: Indigenous uses, conservation and agenda for sustainable management. *Ethnobot. Leafl.*, 12: 8. 2009.

Kishor, K., B.M. Upreti, Y.P.S. Pangtey, A. Tewari and L.M. Tewari. 2015. Propagation and conservation of himalayan yew (*Taxus baccata* L.) through air layering: A simple method of clonal propagation. *Ann. Plant Sci*., 4: 1064-1067.

Lu, C.Y, I.S. Harry. M.R. Thompson and T.A. Thorpe. 1991. Plantlet regeneration from cultured embryos and seedling parts of red spruce (*Picea rubens* Sarg.). *Bot. Gaz.*, 152: 42-50.

Majumdar, S., S. Roy P. Mukherjee and U. Dey. 2021. An efficient *In vitro* propagation protocol for *Taxus baccata* L. (Indian yew). *Physiol. Mol. Biol. Plants*., 27: 113-123.

Miller, R.W., R.G. Powell, C.R. Smith Jr., E. Arnold and J. Clardy. 1981. Antileukemic alkaloids from *Taxus wallichiana* Zucc. *The J. Organ. Chem.*, 46: 1469-1474.

Mitter, H. and A. Sharma. 1999. Propagation of *Taxus baccata* Linn. by stem cuttings. *Ind. For.*, 125: 159-162.

Pant, S. and S.S. Samant. 2008. Population ecology of the endangered Himalayan Yew in Khokhan Wildlife Sanctuary of North Western Himalaya for conservation management. *J. Mount. Sci*., 5: 257-264.

Pilz, D. 1996a. Propagation of Pacific Yews from Seed, (Part I). *Amer. Conifer Soc. Bull*., 13: 13-18.

Pilz, D. 1996b. Propagation of Pacific Yews from Seed, (Part II). *Amer. Conifer Soc. Bull*., 13: 80-83.

Piotrowska, A., A. Bajguz and A. Wojtania. 2021. The effect of plant growth regulators and culture conditions on the rooting of micropropagated yew (*Taxus baccata* L.) plantlets. *In vitro Cell. Dev. Biol. Plant*., 57: 309-318.

Qu, L., X. Chen. W. Zhao. L. Liu and X. Liu. 2020. *In vitro* propagation of *Taxus chinensis* var. mairei. *PCTOC.,* 143: 423-431.

Rajewski, M., S. Lange and H.H. Hattemer. 2000. Problems of reproduction in the genetic conservation of rare tree species: the example of common yew (*Taxus Baccata* L.). *For. Snow Landsc. Res.*, 75: 251-266.

Rikhari, H.C., L.M.S. Palni, S. Sharma and S.K. Nandi. 1998. Himalayan yew: stand structure, canopy damage, regeneration and conservation strategy. *Environ. Conserv.*, 25: 334-341.

Rikhari, H.C., L.M.S. Palni, S. Sharma and S.K. Nandi. 1998. Himalayan yew: stand structure, canopy damage, and regeneration and conservation strategy. *Environ. Conserv*., 25: 334-341.

Saini, R.P. 2001. Vegetative propagation in silviculture (hills) division, Darjeeling (West Bengal). *Ind. For.*, 127: 389-408.

Schenk, R.U. and A.C. Hildebrandt. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot*., 50: 199-204.

Schneck, V. 1996. Studies on the influence of clone on rooting ability and rooting quality in the propagation of cuttings from 40- to 350-year-old *Taxus baccata* L. ortets. *Silvae Genetica.*, 45: 246-249.

Sharma, P., P.L. Uniyal and J. Slowik. 2014. Community involvement and conservation of *Taxus Baccata* in Pangi valley, Himachal Pradesh. *Nat. Areas J.*, 34: 470-474.

Sharma, R., S. Verma V. Sharma and A. Kumari. 2021. *In vitro* propagation of *Taxus wallichiana*, an endangered medicinal plant. *J. Plant Biochem. Biotechnol.*, 3: 17-27.

Sharma, S. and R. Thokchom. 2014**.** A review on endangered medicinal plants of India and their conservation. *J. Crop Weed.,* 10: 205-218.

Vishnu–Mittre. 1984. Floristic change in the Himalaya (southern slopes) and Shiwliks from the mid tertiary to recent times. In: (Ed.): Whyte, R.O. The *Evolution of the East Asian Environment*., 483-503.

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