

Development of *In-Vitro* Regeneration Protocol From Seedling Explants of *Populus Gamblei* Dode: A Difficult to Propagate Endemic Tree Species of Eastern Himalayas

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Abstract

This is the first report of *in-vitro* regeneration of *Populus gamblei*, an endemic and rare tree of montane to subtropical forest of Eastern Himalayas. Nodal segment from coppice shoot of young headed back seedling resulted in 100 % sprouting in MS + 1.0 mg L⁻¹ BAP. Each micro shoot proliferated and gave 7.06 micro shoots on MS + 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ IBA. *In vitro* multiplied shoots produced 100 % rooting in ½ MS + 0.5 mg L⁻¹ NAA. *In-vitro* regenerated plants recorded 80 percent survival during hardening.

Introduction

Populus gamblei, commonly known as Himalayan aspen, belongs the section *Populus* of genus *Populus* (Eckenwelder, 1996). The species is rare and endemic to Eastern Himalayas. In IUCN 11th technical committee meeting, *P. gamblei* was listed as rare and its conservation was suggested (Sahini 1970). The species has been reported from the states of West Bengal (Darjeeling), Arunachal Pradesh (Lower Subanshri and West Siang) and Nagaland (Kohima) (Naithani et al. 2001; Naithani et al. 2005; Naithani and Nautiyal 2012; Puran Chandra et al. 2015). The tree possesses commercially important traits such as fast growth (m.a.i 16.3–35.9 m³ ha⁻¹), deep penetrating roots, natural pruning ability and winter deciduous nature. Wood of the species is used for making huts in shifting cultivation fields and has been found suitable for plywood and match manufacturing (FAO, 2012). Attempts have been made since 1970s to propagate this species using wildlings (natural seedlings), cuttings and tissue culture, but with limited success. (Guhathakurta 1973; Gosh and Bhatnagar, 1977, Lahari 1979; Thakur et. al. 2010). Vegetative propagation and *in-vitro* regeneration protocols are unavailable. *In-vitro* regeneration protocol can efficiently contribute to the conservation of recalcitrant *Populus gamblei*, therefore, the present study was conducted.

Material And Methods

7–8 months old seedlings were collected from Kalimpong Forest Division West Bengal. The seedling was headed back and resulted stump was raised in polybags. Nodal segments were collected after 7-months old coppice shoot. These nodal segments were surfaced sterilized in 75 % (V/V) ethanol for 2 minutes. These segments were washed in *Tween 20* for 25 minutes followed by washing three times in autoclaved deionized water. The washed nodal segments were further treated with 0.2 % bavistin for 20 minutes. Surface sterilization in laminar air flow was achieved using 0.1 per cent mercuric chloride for 8 minutes. Nodal segments were inoculated in large size culture tubes, containing previously autoclaved inoculation medium with containing MS basal salts and vitamins (Murashige and Skoog 1962) + 3.0 % sucrose (w/v) + 6.7 g L⁻¹ agar + 1.00 mg L⁻¹ BAP, with a pH value of 5.8. After 3 weeks, 3.0–4.0 cm long sections of *in-vitro* regenerated micro shoots were transferred into shoot proliferation media consisting of MS (with basal salt, vitamins, sucrose and agar) supplemented with hormone combinations viz. 0.25 mg L⁻¹ BAP, 0.50 mg L⁻¹ BAP, 1.00 mg L⁻¹ BAP, 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ NAA, 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹

IBA, 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ IAA, 1.00 mg L⁻¹ BAP + 0.10 mg L⁻¹ NAA, 1.00 mg L⁻¹ BAP + 0.10 mg L⁻¹ IBA and 1.00 mg L⁻¹ BAP + 0.10 mg L⁻¹ IAA. A control (MS without hormone) was also maintained for comparison. Proliferated micro-shoots were cut into 2.5 to 3.0 cm sizes and transferred to a rooting medium consisting of ½ MS (half basal salts and vitamins) + 3.0 % sucrose (W/V) + 6.7 g L⁻¹ agar, supplemented with 0.25 mg L⁻¹ IBA, 0.50 mg L⁻¹ IBA, 1.00 mg L⁻¹ IBA, 0.25 mg L⁻¹ NAA, 0.50 mg L⁻¹ NAA, 0.50 mg L⁻¹ IBA + 0.50 mg L⁻¹ NAA and a control. The culture vessels were kept in an incubation room at 25±1⁰C ambient temperature and 47μ mol m⁻² S⁻¹ fluorescent white light with 16 hours light and 8 hours dark cycle. Plantlets were put in root trainers containing sterilized vermiculite and were acclimatized in mist chamber with 80 % relative humidity and 25° ± 3°C temperature. Observations on sprouting percentage during ex-plant inoculation, number of shoots explants⁻¹, mean shoot length during shoot proliferation and number of roots per micro-shoots, rooting percentage and mean root length during rooting were recorded. Completely randomized block design having four replications with three micro-shoots in each replication were used for analysis. Treatment means were compared with the help of least significant difference at 5% level of significance.

Results And Discussion

Axillary bud inoculation and sprouting

Sprouting was recorded 100 % in axillary buds inoculated in MS + 1.0 mg L⁻¹ BAP. Bud break started on the 3rd day of inoculation, all the buds sprouted in 7 days and in 3 weeks, single axillary bud produced multiple shoots after 3 weeks (Fig. 1). Maximum of 8 micro shoot per axillary bud (mean 4.29 micro shoots) was found. BAP has been found effective in bud break of *Populus* section of genus *Populus*. In *Populus gamblei*, Thakur et al (2010) achieved successful bud break in MS + 1.0–3.0 mg L⁻¹ BAP. Ahuja (1983; 1984) have reported 0.40–0.50 mg L⁻¹ BAP for bud break of *P. tremula* (European aspen) and *P. tremuloides* (American aspen). Huang and Dai (2011) have reported that 1.0 μM thidiazuron is effective in releasing bud dormancy and bud break of *P. tremula* var *erecta*. We exercised buds in active growth stage for inoculation and 100 % bud break was achieved in MS + 1.0 mg L⁻¹ BAP without using thidiazuron.

Shoot proliferation

Maximum shoot proliferation was recorded (7.06) in MS + 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ IBA (Table 1 and Fig. 1). The treatment was observed to be at par with 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ NAA (6.93), 0.50 mg L⁻¹ BAP (6.33), 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ IAA (6.30) and 1.00 mg L⁻¹ BAP + 0.10 mg L⁻¹ NAA (6.13). In MS without any hormone, shoot proliferation was low (1.40 shoots explants⁻¹) and the shoot growth was fast (mean shoot length 6.01 cm). The effect of BAP was positive in shoot proliferation and negative in the elongation of individual shoot. Results indicated, BAP in low concentration (0.5 mg L⁻¹) increased shoot proliferation whereas at higher concentrations (1.0 mg L⁻¹), it decreased shoot length drastically. Cytokinins induces maximum shoot proliferation at optimum level only, but higher level

of have shown stunting effect (Hartman, et al. 1997). Ahuja (1983; 1984) observed shoot proliferation *P. tremula* and *P. tremuloides* on media containing 0.40–0.50 mg L⁻¹ BAP and low level (0.02 mg L⁻¹) of NAA. Huang and Dai (2011) reported maximum shoot proliferation of *P. tremula* var *Erecta* in MS + 2.5 µM BAP. In present studies, the effect of auxins on shoot proliferation was found to be beneficial, but statistically non-significant. Mc Cown (1997) also have reported that the section *Populus* can be multiplied on MS medium supplemented with BAP and auxins are not required for *in vitro* proliferation.

In-vitro rooting

Rooting can be induced after 6 weeks of culture initiation. Effect of type and concentration of hormones was found to be significant on rooting. Rooting media supplemented with auxins resulted in 42.86–100 per cent rooting (Table 1 Fig. 1). Maximum rooting (100 per cent) was recorded in ½ MS + 0.50 mg L⁻¹ NAA and 0.50 mg L⁻¹ NAA + 0.50 mg L⁻¹ IBA. Number of roots shoot⁻¹ was maximum in ½ MS + 0.50 mg L⁻¹ NAA (14.17) which was at par with ½ MS + 1.00 mg L⁻¹ IBA (10.17), ½ MS + 0.50 mg L⁻¹ NAA + 0.50 mg L⁻¹ IBA (9.50), ½ MS + 0.50 mg L⁻¹ IBA (9.00) and ½ MS + 0.25 mg L⁻¹ NAA + 0.25 mg L⁻¹ IBA (8.83). Effect of auxin application on mean root length was found to be non-significant. Maximum mean root length was recorded in ½ MS + 0.25 mg L⁻¹ NAA (3.50 cm). Low concentration of NAA have been found effective in inoculation of rooting in aspens. In *P. tremula* var *erecta*, 86.9 % rooting was achieved in rooting media containing 0.50 µ M NAA (Huang and Dai 2011).

Hardening and acclimatization of regenerated plantlets

The regenerated plants were acclimatized in mist chamber using vermiculite as potting mixture (Fig. 1). After 6 weeks, these plants were transferred to polybags containing soil: sand (1:1) and kept in shed house. FYM was not used as *Populus gamblei* is a pioneer species and grows well in newly exposed sandy sites along river bank. The survival rate was recorded to be 80 %.

Successful *in vitro* shoot regeneration was achieved. Nodal segments were inoculated in MS + 1.0 mg L⁻¹ BAP, micro shoots were multiplied in MS + 0.50 mg L⁻¹ BAP + 0.10 mg L⁻¹ IBA and rooted ½ MS + 0.5 mg L⁻¹ NAA. Plants were hardened in mist chamber using vermiculate. In 6 months, nodal segments were regenerated to yield hardened plants. The protocol will supplement the *in-situ* conservation efforts of this rare endemic species of Eastern Himalayas.

Declarations

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Conflicts of interest/Competing interests: There is no conflict of interest

Availability of data and material: The authors confirm that the data supporting the findings of this study are available within the article

Code availability: Not applicable

Ethics approval: Not applicable

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Tables

Table 1: *In-vitro* shoot proliferation and rooting of *Populus gamblei* seedling explants.

<i>In-vitro</i> shoot proliferation						<i>In-vitro</i> rooting			
Hormone concentration (mg L ⁻¹)				Number of shoots	Mean Shoot length (cm)	Auxin concentrations (mg L ⁻¹)		Number of roots	Mean root length
BAP	NAA	IBA	IAA			IBA	NAA		
0.00	-	-	-	1.40	6.01	0.00	0.00	0.00	0.00
0.25	-	-	-	4.40	3.53	0.25	-	9.00	1.89
0.50	-	-	-	6.33	3.27	0.50	-	10.17	2.79
1.00	-	-	-	4.66	1.75	-	0.25	2.67	3.50
0.50	-	-	0.10	6.30	2.61	-	0.50	14.17	3.46
0.50	-	0.10	-	7.06	3.96	0.25	0.25	8.83	2.24
0.50	0.10	-	-	6.93	3.49	0.50	0.50	9.50	3.34
1.00	0.20	-	-	6.13	2.43				
1.00	-	0.20	-	4.60	1.69				
1.00	-	-	0.20	5.06	2.28				
LSD _{0.05}				1.05	0.99	LSD _{0.05}		5.75	NS

Figures



Figure 1

In-vitro plant regeneration of *Populus gamblei* using axillary buds. Bud break (top left), shoots after 3 weeks (top right), shoot proliferation (middle left), rooting (middle right), hardening (bottom right).