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IN VITRO CLONAL PROPAGATION OF *POPULUS CASPICA* (BORNM.) THROUGH NODAL STEM SEGMENTS

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ABSTRACT

A novel micropropagation system for *Populus caspica* (Bornm.) is developed for the first time using nodal stem segments from mature trees as starting material. Full strength Murashige and Skoog's (1962) revised medium containing a cytokinin (BA or Kn) was used for establishment of the cultures and a multiplication medium comprising of phytohormonal combination of cytokinin (BA or Kn) and an auxin (NAA or IAA) lead to the production of multiple elongated shoots. The combination of BA 5µM + NAA 2µM produced the highest number i.e. 34.1 shoots per explant followed by BA 5µM + IAA at 5µM which produced a mean of 21.2 shoots/explant. Increase in the concentration of BA lead to decline in the shoot number. The elongated microshoots were isolated and subcultured on different rooting media comprising of half strength MS basal medium supplemented with an auxin viz IBA/IAA/NAA at different concentrations(0.5-5 µM). The microshoots showed strong rooting potential and developed roots within 2-3 weeks of culture period. NAA (5µM) was found to be superior to IBA/IAA in terms of root number but IBA produced significantly longer roots. The microplants showed 80% survival upon their transfer to small pots containing vermicompost under controlled conditions of light, temperature and humidity.

Key words: *Populus caspica*, in vitro, nodal stem segment culture, shoot multiplication, phytohormones.

Abbreviations: BA: 6-benzylaminopurine; IAA: Indole-3-acetic acid; NAA: Naphthalene acetic acid; Kn: 6-furfuryl aminopurine; MS: Murashige and Skoog

INTRODUCTION

Populus caspica Bornm. belongs to Salicaceae family of dicotyledonous group of plants and is distributed in Europe, North Africa, South West and Western Central Asia including Kashmir and Pakistan. In Iran the species is considered to be an alive fossil found in Caspian forests of north Iran (Kartoolinejad *et al.*, 2007). This species has its origin in Europe and it grows well in Kashmir valley forests at altitudes varying between 1800 to 2600 m above mean sea level (Khuroo *et al.*, 2007) in association with *Cedrus deodara* and *Pinus wallichiana*. It is locally called as Dudha Fras or Kabuli Fras. The trees are large and grow very fast. It is used as small timber for construction purpose and also for production of match sticks, packing boxes etc. The species is a good soil binder and provides a moderate shade for upcoming germination of coniferous seeds. Over the last nine decades the species has been ruthlessly exploited for its great utility and presently the species is rarely found on Kashmir hills (Dar and Ahmad, 2007). Thus the species needs immediate attention to save it from becoming extinct. Natural regeneration of the species is found to be very low owing to

heterogeneity. Therefore, a modern propagation method must be evolved for its large scale production and in this direction the micropropagation of this species will be a promising step.

Scanning of the literature reveals that among the forest tree species Poplar was the first to be cultured in vitro and regenerated from callus (Mathes, 1964; Winton, 1968). Christie (1978) demonstrated shoot proliferation in aspen nodal explant culture. Later on other workers who used nodal explants for in vitro propagation of poplars include Kim *et al*., (1981),Ahuja (1983), Evers(1983), Xu (1987), Panetsos *et al*. (1987), Karkonen *et al*. (1999), Chaturvedi *et al*. (2004), Zhan *et al*., (2005) etc. Very recently Mofidabadi (2005) established a novel protocol for mass propagation of *Populus caspica* by in vitro culture of mature ovary. In light of this present work on in vitro propagation of *Populus caspica* through nodal stem segments was undertaken to establish a micropropagation protocol for its clonal multiplication and conservation.

MATERIAL AND METHODS

i) Explant source and preparation:

Fresh and healthy twigs of the tree growing in Botanical garden of the University of Kashmir were collected in the months of March-May and immediately swabbed with cotton moistened with 70% alcohol to remove all the cottony growth. From these twigs small 2-3cm sections of nodal segments containing an axillary bud were cut and immediately washed in running tap water using lab detergent 'Labolene' for atleast 30 minutes to reduce the effect of browning on the medium. This was followed by surface sterilization of explants in $HgCl_2$ (0.1%) for 20 minutes containing one or two drops of 'tween 20' (wetting agent). Finally the explants were rinsed in autoclaved double distilled water 3-5 times under the laminar air flow hood.

ii) Culture establishment:

The surface sterilized explants were cultured in agarified full strength MS (1962) medium fortified with cytokinin BA or Kn at varying concentrations of 1-10 μM to encourage bud burst and shoot growth so that aseptic shoot cultures are obtained,. The pH of the medium was adjusted at 5.8 and sterilization was done by autoclaving. The explant was transferred after every two days on to fresh medium 3-4 times to lessen the adverse effects of phenolic substances. The cultures were incubated under controlled conditions of light (3000lux), temperature (25 ± 5 °C), humidity (70%) and photoperiod (16 hr).

iii) Plantlet regeneration:

The established initial shoot cultures were subcultured on a multiplication medium comprising of full strength MS (1962) medium

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containing a hormonal combination of an auxin- NAA/IAA at 2 µM and a cytokinin BA/Kn at varying strengths of 2.5-10 µM. The individual shoots of atleast 2.5cm length from proliferating cultures were subcultured on rooting medium i.e. ½ MS (1962) basal medium enriched with an auxin IBA/NAA/IAA at 0.5-7.5 µM for inducing roots.

iii) Hardening:

The rooted in vitro raised plantlets were washed in running tap water to remove all the culture medium and planted in small pots having sterilized vermicompost under controlled conditions of temperature, light and humidity.

i)ii) Design of Experiment: All the experiments were carried out in completely randomized block design (CRD).Twenty/Twenty five replicates were taken for each treatment. Observations were recorded after 8 weeks. Non parametric Kruskal Wallis and Mann-Whitney tests were applied to determine the difference in maximum multiple shoots & roots obtained on different concentrations of BAP, Kn, NAA, IBA & IAA. Significance of results was ascertained at $P < 0.001$ level.

RESULTS

i) Culture establishment and shoot proliferation:

Nodal stem segments when cultured on MS medium containing BA or Kn showed a good and quick response and the buds developed into shoot within first week of culture. The percentage of culture establishment was found to be highest (100%) particularly with BA (5 µM and 7.5 µM) (Fig 1) and Kn (7.5 µM) (table 1)

Table 1: Culture establishment: Response of nodal stem segments to BA/Kn at different concentrations*.

Phytohormone	Concentration(µM)	Percentage culture
BA	1	80
	2.5	88
	5	100
	7.5	100
Kn	1	68
	2.5	76
	5	88
	7.5	100
Control (basal medium)	-	36

* Recording after 8 weeks; 25 explants/treatment

The established 8 week old shoot cultures were subcultured on different multiplication media for obtaining best shoot multiplication rate (Table 2). It was observed that MS medium supplemented with a combination of BA and NAA supported a good shoot proliferation as well as elongation, with highest average shoot number of 34.1 formed at a combination of BA (5 µM) and NAA(2 µM) with 100% response (Fig 2). Higher concentration of BA (beyond 5 µM) markedly reduced the shoot number and also decreased the elongation degree. When NAA was replaced with IAA multiple shoot formation was again noticed having comparatively higher elongation degree (Fig 3); however the number of shoot multiples formed was slightly less than what was obtained with the combination of BA and NAA at same strengths. The combination of Kn with NAA/IAA also proved effective in producing shoots in multiples with overall moderate elongation. However, it was found that shoot number formed was very low and maximum average number of 5.8 shoots/culture were obtained using Kn and NAA at 5 µM and 2 µM respectively (Fig 4). The use of BA or Kn at a high concentration of 7.5 µM and 10 µM with auxin- NAA/IAA produced shoots in rosette form with restricted elongation. The multiple shoots were found to be formed through enhanced axillary branching in all trials

ii) In vitro rooting :

The elongated microshoots of at least 3-4cm length from 8 weeks old multiple shoot cultures were subcultured on rooting medium which contained different auxins like NAA, IAA and IBA at various concentrations (0.5-5.0 µM) . Different effects on rooting response of micro shoots registered are depicted in Table 3. The rooting potential of invitro shoots was found to be very rapid and within 2 weeks root initials started differentiating from lower cut end of the shoots. It was observed that NAA at 5 µM and 2 µM produced a maximum mean number of 12.1and 8.4 roots respectively which were thick and moderately long with 100% response (Fig5). IBA (5 µM) and IAA (2.5 µM) were also effective in producing good rooting percentage with highest average root number of 6.1(Fig 6). A higher concentration of NAA/IAA/IBA used in the medium lead to mild callusing at the base of shoots.

The microplantlets were successfully transferred to small pots for hardening wherein 80% survival was observed after 8 weeks of transfer.

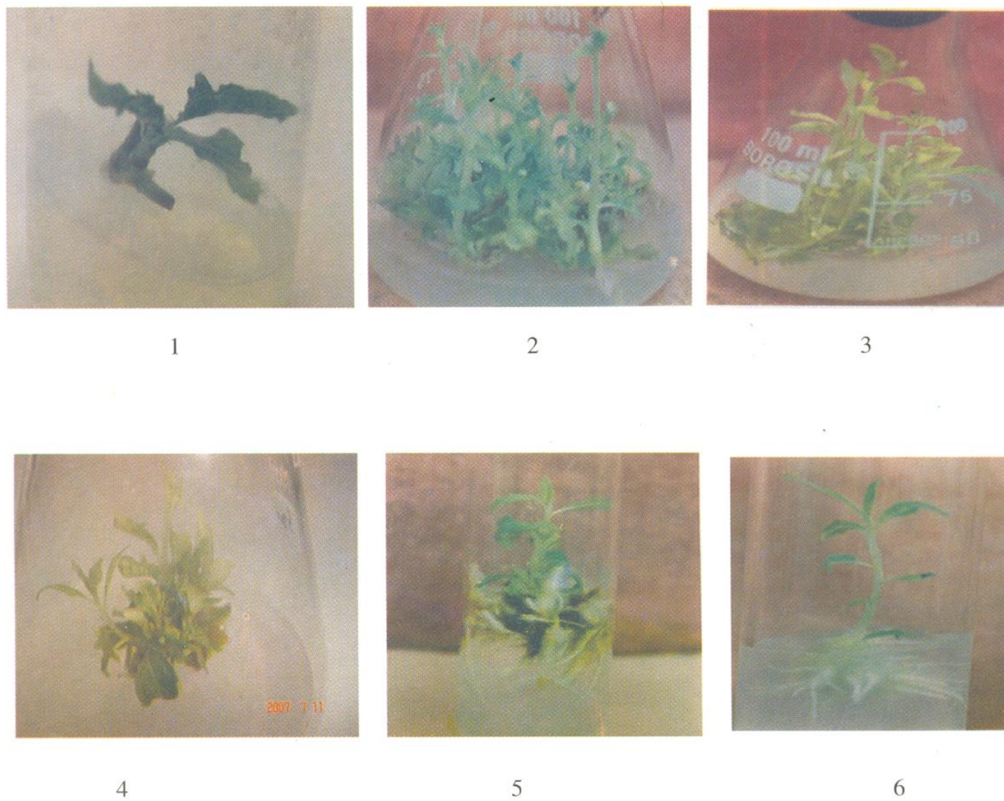

Fig 1-6: Nodal Stem Segment Culture of Populus caspica.

Fig 1: Growth of axillary bud on MS+BA5 µM; **Fig 2:** Shoot multiplication on MS+BA 5 µM+NAA2 µM; **Fig 3:** Shoot multiplication in MS+BA5 µM+IAA 2 µM; **Fig 4:** Shoot multiplication on MS+Kn 5 µM +NAA2 µM; **Fig 5:** Rooting of shoot on ½ MS+NAA 5 µM (after 8 weeks); **Fig 6:** Rooting of shoot on ½ MS+IBA 5 µM (after 8 weeks)

Table 2: Shoot multiplication and elongation response of subcultured shoots to various hormonal combination in MS medium*.

Phytohormone (µM)			Mean shoot number ±SD	Mean shoot length ±SD (cm)	Response %age
BA	NAA	IAA			
2.5	2	0	15.2±0.8	2.9±0.2	84
5	**2**	**0**	**34.1±0.76[a]**	**3.0±0.2**	**100**
7.5	2	0	17.0±0.9	2.9±0.2	92
10	2	0	15.2±0.8	1.5±0.5	88
2.5	0	2	10.1±0.8	3.2±0.8	100
5	0	2	21.2±0.7	3.1±0.3	100
7.5	0	2	14.2±0.4	2.9±0.2	100
10	0	2	12.6±0.4	1.8±0.3	92
Kn	NAA	IAA			
2.5	2	0	3.5±0.5	2.7±0.5	24
5	**2**	**0**	**5.8±0.3**	**3.1±0.3**	**36**
7.5	2	0	5.2±0.4	2.9±0.1	36
10	2	0	2.1±0.3	1.8±0.3	28
2.5	0	2	3.8±0.4	2.9±0.2	20
5	0	2	4.8±0.3	2.6±0.4	32
7.5	0	2	4.8±0.3	2.6±0.4	32
10	0	2	2.4±0.4	1.9±0.1	28
Control			-	-	-

* Recording after 8 weeks; 25 replicates /treatment; - no response
Difference between mean values of maximum multiple shoots obtained on 5µM BAP + 2µM NAA were found to be significantly higher than 5 µM Kn + 2µM NAA at aP < 0.001 level using Mann-Whitney test.

Table 3: Rooting response of microshoots to different hormones at varying strengths in ½ MS basal medium*.

Phytohormone (µM)	Mean root number ±SD	Mean root length (cm)±SD	Response %age
IBA			
0.5	2.0±0.3	3.4±0.7	40
1	3.2±0.5	5.1±0.5	60
2.5	6.0±0.7	4.9±0.4	100
5	**6.1±0.7**	**3.1±0.5**	**100**
NAA			
0.5	1.5±0.4	3.9±0.9	45
1	3.2±0.4	4.2±0.8	50
2.5	8.4±0.4	4.7±0.4	100
5	**12.1±0.6****	**4.1±0.8**	**100**
IAA			
0.5	2.1±0.7	2.4±0.4	55
1	3.1±0.3	4.3±0.6	75
2.5	**6.1±0.7**	**4.8±0.6**	**100**
5	5.9±0.7	4.8±0.4	100
Control	-	-	-

* Recording after 8 weeks; 20 replicates/treatment; - no response
Difference between mean values of maximum multiple roots obtained on 5µM NAA were found to be significantly higher than 5µM IBA & 2.5µM IAA at ** $P < 0.001$ using Kruskal Wallis test.

DISCUSSION

The establishment of axenic shoot cultures from mature tree explants has always been a difficult preposition owing to two critical factors-microbial infection because of long exposure in the environment and phenolic exudation in explants derived from mature trees which lead to browning of tissue and eventual death of explants. In present study the browning problem was overcome by washing the explants in running tap water for at least 30mts and transferring the nodal explants frequently on to the fresh medium 3-4 times in the culture establishment phase. Although the use of antibiotics to control the persistent infection of field explants has been demonstrated by some workers like Coleman and Ernst (1989), however as reported by Chaturvedi *et al.* (2004) the presence of antibiotics in the medium adversely affects the growth of explants and offshoots resulting in cultures with restricted growth. However, in the present study no antibiotic was used and the infection was demonstrated to be under control by effective surface sterilization using $HgCl_2$ alone.

Nodal stem segments were found to be very responsive in establishment with a high shoot culture percentage. The use of nodal explants

has been found to be ideal starting material for micropropagation of poplar species (Ahuja, 1983; Evers, 1983; Panetsos *et al.*, 1987). Earlier observations of Civinova and Sladky (1990) revealed that use of cytokinins is very effective in encouraging the rapid growth of shoots from field explants. Our studies showed that BA and Kn were very effective in encouraging shoot growth from the axillary buds particularly when used at concentrations of 5 µM and 7.5 µM. However, a high concentration of cytokinins proved less effective in production of new normal shoots instead it leads to rosette shoot formation with less elongation. These observations are similar to those of Christie (1978), Kim *et al.* (1981) and Chalupa (1983) who also found the high concentration of BA to be responsible for formation of cluster of stunted shoots and leaves in a range of Poplar clones. Cytokinins i.e. BA and Kn were used in the multiplication medium in combination with auxin to encourage shoot proliferation which proved effective.Watanabe *et al.* (1999) also noticed shoot multiplication and elongation with BA 0.25mg/l in combination with NAA 0.05mg/l in *Populus euphratica*. The combination of BA and NAA (0.1mg/l each) was also found to be effective in shoot proliferation of *Populus alba* var. pyrimidalis Bge. by Changli *et al.* (2004). Which is quite corroborative with our results thus showing that the *P. caspica* has a specific requirement for cytokinin (BA) and auxin (NAA) combination for optimum shoot proliferation. Kinetin has also helped in shoot proliferation but its effect was lower than BA.

The rooting potential of microshoots was found to be very high as the shoots showed rooting in almost all the auxins tried even at lower concentration. The rooting of microshoots was observed to be direct without any callus intervening phase, although a little callusing was observed at high concentration of the auxins used in this study. The ease of rooting was possibly due to juvenility of shoots-a finding corroborated by Noh and Minocha (1986) in *Populus tremuloides* Michx. In the present study 100% rooting was achieved in ½ MS medium containing IAA/NAA / IBA at 5 µM each, which are more or less to those of Rutledge and Douglas (1988) who have demonstrated 80% rooting in many *Populus* species using IAA at 5.7 µM. Out of these three auxins tried NAA (5µM) was found to be the best in terms of root number and moderate elongation because of the reasoning given by Peeters *et al.* (1991) who are of the opinion that NAA at a concentration of 5µM is taken up specifically faster for induction of rooting of in vitro raised shoots as compared to other auxins i.e. IAA and IBA. The invitro microplants were successfully transferred to small pots containing

vermicompost and 80% survival was noticed after 8 weeks of transfer under controlled conditions of temperature, light and humidity.

CONCLUSION

The present study demonstrates a complete protocol for the clonal micropropagation of *Populus caspica*. This technique, after refinement, can be used for mass propagation of this species to save it from going extinct and also in production of clonal planting stock for afforestation programmes

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