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A reproducible protocol for raising clonal plants from leaf segments excised from mature trees of *Betula utilis* a threatened tree species of Kashmir Himalayas

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Abstract: Tissue culture techniques such as micropropagation provide a fast and dependable method for production of a large number of uniform plantlets in a short time and offer potential means not only for rapid mass multiplication of existing stocks but also for the conservation of important, elite and rare plants. In this study, callusing was achieved from leaf explants of *Betula utilis* on MS medium supplemented with 2, 4-D (2.5mg/l). The callus was friable, nodular, having numerous embryoids which exhibited organogenesis on subculturing. Production of multiple shoots was achieved on MS medium supplemented with BAP (1.5mg/l). Rooting from these shoots was obtained within 10 days of inoculation on MS medium fortified with NAA (1.0mg/l). A combination of BAP (2.0mg/l) + NAA (0.5mg/l) proved to be the best auxin-cytokinin combination in which explants exhibited best morphogenetic potential in terms of development of shoots along with roots within 8-10 days of inoculation. The maximum frequency of root differentiation (8.2) roots was achieved on MS basal medium supplemented with NAA (1.0mg/l) after 10 days of inoculations in 80% cultures. The complete plantlets were produced which showed enhanced growth after transferring in the same media composition.

Keywords: Plant Regeneration, *Betula utilis*, growth regulators. morphogenetic potential

INTRODUCTION

It is an admitted fact that trees are the invariable resources for providing food, fuel, timber, medicines, gums, resins and other items of daily life. Since there are natural and anthropogenic pressures on these bio-resources, which have pleaded their depletion from the nature at an unprecedented rate, thus making them threatened. In this direction the *In vitro* techniques are being increasingly applied for large scale micropropagation to supplement the conventional methods. Tissue culture techniques such as micropropagation provide a fast and dependable method for production of a large number of uniform plantlets in a short time and offer potential means not only for rapid mass multiplication of existing stocks but also for the conservation of important, elite and rare plants. *In-vitro* propagation has revolutionized commercial nursery business [1]. Significant features of *in-vitro* propagation procedure are its enormous multiplicative capacity in a relatively short span of time; production of healthy and disease free plants; and its ability to generate propagules around the year [2].

The genus *Betula*, commonly known as birch, belongs to the family Betulaceae. *Betula utilis* D. Don (Himalayan birch) belongs to family *Betulaceae*. It is a deciduous tree attaining a height of 15-20 meters from the ground and is commonly known as 'Birch'. Phylogenetic analysis supports the division of the Betulaceae into two subfamilies, Betuloideae and Coryloideae [3]. Betuloideae includes *Alnus* (alder) and *Betula* (birch) and the subfamily Coryloideae is composed of *Corylus* (hazel), *Ostryopsis*, *Carpinus* (hornbeam) and *Ostrya* (hop hornbeam). There are approximately 50 species of *Betula* globally [4], all of which occur in the northern temperate zone. Clonal propagation of birch (*Betula* spp.) via tissue culture has been possible since the 1970's [5]. As reviewed by [6], research on tissue culture has been done with a number of species and varieties of birches. As birch plants regenerated from callus [7], leaf and roots seem to be genetically stable [8] in vitro regeneration could also represent a technique to improve mass propagation of this clone. Although micropropagation of birch can be routinely used in research, tree breeding and for large-scale production of reproductive material. Relatively few results concerning the performance of micropropagated birches in field conditions have been published [9]. In the nursery, the early development of micropropagated plants and seedlings of *B. platyphylla* var.

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szechuanica was followed by [10] and the performance of micropropagated material of *B. pendula* was reported by [11].

In this study, callusing was achieved from leaf explants of *Betula utilis* on MS medium supplemented with 2, 4-D (2.5mg/l). The callus was friable, nodular, having numerous embryoids which exhibited organogenesis on subculturing. When NAA was used at various concentrations, the leaf explants increased 2 fold in size upto 5-8 days of inoculation in 60% cultures, but these cultures turned pale yellow after 10-15 days of inoculation. The leaf explants however, failed to produce any response on MS medium supplemented with various concentrations of IAA and IBA. Production of multiple shoots was achieved on MS medium supplemented with BAP (1.5mg/l). Rooting from these shoots was obtained within 10 days of inoculation on MS medium fortified with NAA (1.0mg/l). A combination of BAP (2.0mg/l) + NAA (0.5mg/l) proved to be the best auxin-cytokinin combination in which explants exhibited best morphogenetic potential in terms of development of shoots along with roots within 8-10 days of inoculation. The maximum frequency of root differentiation (8.2) roots was achieved on MS basal medium supplemented with NAA (1.0mg/l) after 10 days of inoculations in 80% cultures. The complete plantlets were produced which showed enhanced growth after transferring in the same media composition.

MATERIAL AND METHODS

During the present investigation, Murashige and Skoog's basal mediumwas used [12]. For the preparation of a given volume of MS medium, the desired volume of the stock solution from macrosalts, microsalts, vitamins and iron source were added to known quantity of double distilled water. To this solution 30 gm/l sucrose and 0.8gm/l agar was added. This solution was heated till agar got dissolved completely. It was then cooled and the pH of medium adjusted between 5.6-5.8 using 0.1 N NaOH and 0.1N HCl. About 15 ml of this medium was dispensed in Boorsil culture tubes and about 25 ml in 100ml conical flasks which were plugged properly and tightly with sterilized non-absorbent cotton plugs.

Finally, these culture vials and flasks containing nutrient medium were sterilized by autoclaving at 15 PSI pressure at121^{0}C for 15-20 minutes in an autocleave. The explants used in the present study i.e., leaves, were excised from the field grown authentic plants of *Betula utilis* D. Don. These explants were firstly washed under a jet of running tap water in order to remove dust, dirt and other unwanted materials. They were then washed by a detergent solution (Labolenne) containing 2-3 drops of a wetting agent (tween 80). This was followed by washing with tap water to remove the detergent and finally washed 2-3 times with double distilled water under laminar air flow hood. Finally the explants were disinfected with 0.1% $HgCl_2$ solution for different time durations. The surface sterilized explants were then washed 5-6 times with autoclaved double distilled water so as to remove the last traces of the strilant. The sterilized plant material was then put into pre-autoclaved petri-dishes, cut into suitable size and finally aseptically inoculated into the culture medium. Each treatment involved about 10-20 explants and each experiment was repeated twice.

RESULTS

In vitro response of leaf explants

During the present study very young leaves of *B. utilis* were procured from the field grown plants of *Betula utilis* and were cut into 2-3 cms segments. These explants were inoculated on MS medium adjuvented with different concentrations and combinations of growth inducers on which the following results were achieved.

Induction of callus

The leaf explants when inoculated on MS basal medium adjuvented with different auxins viz (2,4-D, IBA, IAA and NAA) showed good responses to 2,4-D supplemented medium. 2,4-D was effective in concentrations ranging from (0.1-3.5mg/l) (Table 1). The concentration of 2,4-D (2.5mg/l) proved to be most effective for callus initiation. The explants produced green colour callus at the cut ends which registered 4 fold increase in size within 2-5 days of inoculation in about 80% cultures (Fig 1, 2). The callus was nodular and friable which also exhibited power of regeneration. At concentration (0.1-0.5 mg/l) 2,4-D induced callus formation from cut end of the explants in 30-40% cultures within 8-10 days of inoculations but after 10-15 days these cultures turned brownish in colour, remained quiescent and necrosed after 15 days of inoculation. Concentration greater than (2.5 mg/l) proved to be detrimental to callus growth. When NAA was used at various concentrations, the leaf explants increased 2 fold in size upto 5-8 days of inoculation in 60% cultures, but these cultures turned pale yellow after 10-15 days of inoculation. The leaf explants however, failed to produce any response on MS medium supplemented with various concentrations of IAA and IBA.

Table 1: Effect of 2, 4-D on leaf explants of *Betula utilis*

2,4-D (mg/l)	%age of cultures regenerated	Response of cultures forming callus	No. days taken
0.1	30	Callus formation took place from cut end of the callus but after 8-15 days, cultures turned brownish and showed signs of necrosis.	8-10
0.2	30	do	8-10
0.5	40	do	8-10
2.5	80	Explants produced green colour callus which registered 4 fold increase in size, callus was nodular and friable in texture	2-5
3.5	10	This concentration proved detrimental to the callus growth	-


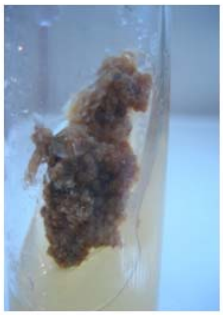

Fig 1 and 2; Callus at the cut ends which registered 4 fold increase in size within 2-5 days of inoculation in about 80% cultures

Effect of cytokinins
Subculturing of callus

The proliferated callus was subcultured on fresh MS basal medium adjuvanted with different concentration of cytokinins viz; (BAP and Kn) for shoot regeneration.

Shoot multiplication

The effect of cytokinins on dedifferentiation of subcutured callus was investigated **(Table 2).** The proliferated callus when inoculated on MS medium supplemented with Kn (0.5-1.0mg/l) produced elongated shoots after 20th days of inoculation in 20-30% cultures. Later on however, all the cultures turned brownish and remained quiescent and after 2nd week of inoculation the cultures showed signs of necrosis. When callus was subcultured on MS medium supplemented with BAP (0.2 mg/l) it proliferated into embroyids within 8-10 days of inoculation and these emboyids turned into shoots in about 60% cultures. On MS medium supplemented with BAP (0.5 mg/l) callus showed very good morphogenetic potential in terms of shoot differentiation in 70% cultures after 6-8th days of inoculation. When transferred to same medium, multiple shoot formation took place within 6-8 days of inoculation.

The best results in terms of shoot differentiation were obtained on MS medium supplemented with BAP (1.5mg/l) were 80% cultures exhibited morphogenetic growth. The differentiation of shoots started from 5-6 days of inoculation. After 15th day of inoculation the elongated shoots turned light green and got established as shoots (Fig 3, 4).

Table 2: Effect of cytokinin (mg/l) on subcultured callus of *Betula utilis.*

Cytokinin (mg/l)	Percentage of cultures forming usable shoots	Average no. of shoots/explant	Average length of shoots (cm)
MS basal medium (Control)	-	-	-
Kn			
0.5	20	2.1	2.0
1.0	30	3.0	2.1
BAP			
0.2	60	5.2	3.5
0.5	70	6.3	4.3
1.5	80	9.2	5.3


Fig 3 and 4: After 15th day of inoculation the elongated shoots turned light green and got established as shoots.

Effect of auxins
Induction of rooting

For the induction of rooting, the *in vitro* raised shoots were excised from shoot clumps and subcultured on MS basal medium augmented with different concentrations of auxins viz; (IAA, IBA and NAA) of which the effective ones are depicted in (Table 3). In MS basal medium augmented with IAA (0.5mg/l) these cultures started root initiation after 10th day of inoculation from basal cut portion of the shoots in 40% cultures but after further subculturing the callus remained quiescent and showed no further growth. MS basal medium fortified with IBA (1.0mg/l) induced root induction in 60% cultures after 12 days of inoculation. The sprouted roots increase in size in the same medium composition upto 4

weeks of inoculation. The maximum frequency of root differentiation (8.2) roots were achieved on MS basal medium supplemented with NAA (1.0mg/l) after 10 days of inoculations in 80% cultures.

Table 3: Effect of auxins on root induction from *In vitro* raised shoot clumps.

Auxins (mg/l)	Percentage of cultures forming useable roots	Average no. of roots/explants	No. of days taken for rhizogensis
IAA			
0.2	30	3.2	10
0.5	40	4.3	2.0
IBA			
0.5	50	5.1	25
1.0	60	6.2	12
NAA			
0.5	70	6.2	15
1.0	80	8.2	10

Effect of auxin-cytokinin combinations

For the production of complete plantlets the auxin-cytokinin combinations were also used of which the effective ones are given in (Table 4). *In vitro* raised shoot tips when inoculated on MS basal medium augmented with Kn (0.2-2.0mg/l) + IAA (0.5-1.0mg/l) exhibited shoot as well as root development after 2 weeks of inoculation in 30-40% cultures. When subcultured in the same media composition of same composition after a period of 4 weeks. The shoots as well as roots elongated but after words shoots turned yellow and finally decayed. Similar was the case when shoot buds were inoculated on MS basal medium supplemented with different concentrations and combinations of Kn+IBA. MS medium fortified with Kn (2.0mg/l) + NAA (1.0mg/l) induced shoot as well as root initiation in 60% cultures after 12 days of inoculation. These explants when subcultured in the same media composition after a period of 4 weeks produced an increased number of shoots which was accompanied by production of roots as well.

MS basal medium supplemented with BAP (0.5mg/l) +IAA (2.0mg/l) induced shoot as well as root production in 50% cultures upto 12^{th} days of inoculation. The sprouted shoots and roots increased in size in the same media composition upto 4 weeks of inoculation. MS basal medium fortified with BAP (0.2mg/l) + IBA (1.0mg/l) induced morphogenetic potential in 60% cultures. Shoots started differentiating 10 days after inoculation and in the next 4 weeks micro shoots as well as micro roots were produced in the same media composition. Shoot tips cultured on MS basal medium supplemented with BAP (2.0mg/l) + NAA (0.5mg/l) exhibited best morphogenetic potential as 80% cultures responded upto 8-10 days of inoculation. In the next 4 weeks the complete plantlets were produced which showed enhanced growth after transferring in the same media composition (Fig 5, 6).

Table 4: Effect of PGR's on *in vitro* raised shoot tip explants of *Betula utilis*

PGR's (mg/l)	%age of cultures regenerated	Average no. of shoots /explant	Average no. of roots/explant
Kn+IAA			
0.2+0.5	30	1.0	1.1
2.0+1.0	40	2.1	2.0
Kn+IBA			
0.3+1.0	50	5.0	3.0
1.0+2.0	50	4.2	4.0
Kn+NAA			
1.0+0.5	50	4.1	3.0
2.0+1.0	60	3.0	3.0
BAP+IAA			
1.0+0.5	40	5.1	3.1
0.5+2.0	50	6.0	4.0
BAP+IBA			
1.2+0.5	50	6.5	4.2
0.2+1.0	60	7.0	5.1
BAP+NAA			
1.0+2.0	70	8.2	8.0
2.0+0.5	80	10.5	10.2


Fig 5 and 6: The complete plantlets were produced which showed enhanced growth after transferring in the same media composition

Hardening and Transplantation

During the present work the *in vitro* raised rooted plantlets were carefully removed from the cultural vials and were washed thoroughly with running tap waters in order to remove agar adhering to their roots. They were treated with (0.5%) Baveston solution (Fungicide) for 10 minutes and transferred to small polythene bags or pots containing of either vermiculite or mixtures of sand and soil (1:1) or vermiculite and soil (1:1). The plantlets were covered with polythene bags so as to attain maximum humidity around the plantlets and were grown in shaded places under natural day lights with an average temperature of 25 ^{o}C (Fig 7 and 8).

These plantlets were watered every alternate day with tap water and ½ strength MS salt solution. Each treatment was repeated at least once. The bags covering the plantlets were cut open from the top after 2 weeks so as to bring the humidity to the level of ambient atmosphere. Finally the covers were removed after 2 weeks time. After 8 days these platelets were transferred to polythene bags or pots containing garden soil, Sand and farm yard manure (1:1:1). The survival percentage of micropropagated plantlets of *Betula utilis* D. Don was influenced by different planting out mixtures and irrigating solutions used. Maximum survival percentage of rooted plantlets was obtained after hardening in sand and vermiculite (1:1) mixture irrigated with ½ strength MS salt solution (Table 5). On vermiculite sprayed with ½ strength MS salt solution plantlets survived with 80% frequency. These plants showed normal growth and attained an average height of 34 cm after 2 months of transfer and finally showed vigorous growth in the field (Fig 9, 10).

Table 5: Effect of Planting out media and irrigating solutions on survival of micro propagated plantlets of *Betula utilis* D. Don during hardening.

Planting out media	Irrigating solution	Survival percentage	Plant height
Vermiculture	½ MS salt solution +tap water	50%	20 cm
Sand	½ MS salt solution +tap water	30%	25 cm
Soil	½ MS salt solution +tap water	30%	28 cm
Sand + vermiculture (1:1)	½ MS salt solution +tap water	80%	34 cm
Soil + vermiculture	½ MS salt solution +tap water	60%	32 cm
Sand + soil (1:1)	½ MS salt solution +tap water	40%	30 cm


Fig 7 and 8: Plantlets covered with polythene bags so as to attain maximum humidity around the plantlets and grown in shaded places under natural day lights with an average temperature of 25 ^{o}C


Fig 9 and 10: Plants showing normal growth and attained an average height of 34 cm after 2 months of transfer and finally showed vigorous growth in the field.

During the present study very young leaves of *B. utilis* were collected from the field grown trees and were cut into 2-3 cms segments. These explants were inoculated on MS medium adjuvented with different concentrations of auxins. Among various auxins 2, 4-D proved to be very effective in inducing callus regeneration from leaf explants. At concentrations of 2, 4-D (0.1, 0.2, 2.5 and 3.5) leaf explants showed no response but instead they showed signs of necrosis within 8 days of inoculation. However, 2,4-D (2.5 mg/l) was effective in the production of creamish colour callus in 80% cultures. Concentration greater than (2.5 mg/l) proved detrimental to the callus growth. Margareta and Barbara (1996) also achieved callus differentiation from nodal segments in silver birch (*Betula pendula*) on MS medium supplemented with different concentration of auxins. The best results in terms of shoot differentiation were obtained on MS medium supplemented with BAP (1.5mg/) were 80% cultures exhibited morphogenetic growth. The differentiation of shoots started from 5-6 days of inoculation. After 15th day of inoculation the elongated shoots turned light green and got established as shoots (Fig. 11). Similar results have been also achieved from leaf callus cultures of *Betula pendulla* Roth Fastigita [13].

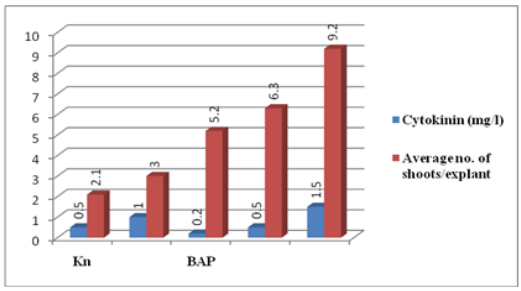

Fig 11: Effect of cytokinin (mg/l) on subcultured callus of *Betula utilis.*

The maximum frequency of root differentiation (8.2) was achieved on MS basal medium supplemented with NAA (1.0mg/l) after 10 days of inoculations in 80% cultures (Fig. 12). Elisa Sarkihita (1988) derived similar results from axillary buds of *B-Pendulla* Rooth on MS medium containing NAA (0.1mg/l). Rooting from the shoots of *Ulmus primula* was also achieved by Corehetee et al (1993) on half or full strength MS medium supplemented with (0.1mg/l) NAA.

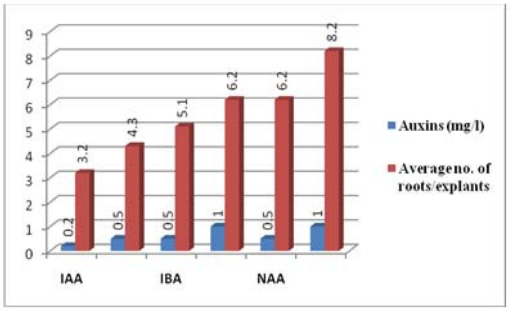

Fig 12: Effect of auxins on root induction from *In vitro* raised shoot clumps

Among various auxin-cytokikinin combinations the best results in terms of complete plantlet production was achieved in MS basal medium supplemented with BAP (2.0 mg/l) + NAA (0.5 mg/l) after 8-10 days of inoculation as 80% cultures responded and produced multiple shoots as well as microroots after subculturing the explants in the same medium. (Fig. 13).

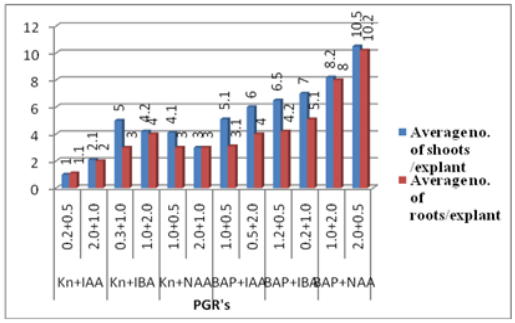

Fig 13: Effect of PGR's on *in vitro* raised shoot tip explants of *Betula utilis*

DISCUSSION

The leaf explants exhibited morphogenetic growth responses and increased many fold in size on MS medium adjuvanted with various growth regulators viz; 2, 4-D, NAA, IAA and IBA. These explants developed nodular and friable callus on MS medium supplemented with 2,4-D (2.5 mg/l). The best results in terms of shoot differentiation were obtained on MS medium supplemented with BAP (1.5mg/l) were 80% cultures exhibited morphogenetic growth. The differentiation of shoots started from 5-6 days of inoculation. After 15th day of inoculation the elongated shoots turned light green and got established as shoots. The maximum frequencies of root differentiation (8.2) roots were achieved on MS basal medium supplemented with NAA (1.0mg/l) after 10 days of inoculations in 80% cultures. Shoot tips cultured on MS basal medium supplemented with BAP (2.0mg/l) + NAA (0.5mg/l) exhibited best morphogenetic potential as 80% cultures responded upto 8-10 days of inoculation. In the next 4 weeks the complete plantlets were produced which showed enhanced growth after transferring in the same media composition.

CONCLUSION

. Tissue culture techniques such as micropropagation provide a fast and dependable method for production of a large number of uniform plantlets in a short time and offer potential means not only for rapid mass multiplication of existing stocks but also for the conservation of important, elite and rare plants like *Betula utilis* a threatened tree species of Kashmir Himalayas. *In-vitro* propagation has revolutionized commercial nursery business too.

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