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Effect of plant growth regulators and explant types on micropropagation of an endangered medicinal plant *Blepharispermum subsessile* DC.

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ABSTRACT

A reproducible protocol for *in vitro* plant regeneration of an endangered medicinal plant *Blepharispermum subsessile* was developed. The protocols were developed for establishment of secondary culture from shoot tips and nodal segments. They were cultured on MS medium supplemented with different concentration of BAP and Kn either alone or in combination with GA_3, IAA, IBA and NAA. Of the different cytokinin evaluated, bud break with multiple shoot proliferation in nodal segment and shoot tip explants was best achieved in MS medium supplemented with 1.5 and 2.5 mg/l BAP respectively. Percentage shoot development and the number of shoots per shoot tip was maximum in MS augmented with 1.5 mg/l BAP + 0.2 mg/l IAA, where *ca.* 91% cultures produced 6 shoots/explant with an average length of 2.43 cm over a period of 4 weeks. The maximum rooting regeneration was observed in ½ MS + 2% sucrose + 1.0 mg/l IAA, with a average 5.83 roots per regenerated shoots. Well rooted plants showed 39% survival during hardening under green house condition.

1. Introduction

Blepharispermum is a genus of the aster family Asteraceae, distributed in Africa, Madagascar, the Arabian Peninsula, India, and Sri Lanka. Out of the reported 16 species, *B. subsessile* DC. is found in moist deciduous forests of Odisha, Andhra Pradesh, Madhya Pradesh, Tamil Nadu, Karnataka and Maharashtra of India. It is known as "Rasnajhadi" in Odisha, "Naama banta" in Karnataka and "Adavi banti" in Andhra Pradesh and Telengana. This plant has been used in Indian system of medicine for formulation of a number of Ayurvedic drugs and also by tribals for ailment from various kind of diseases (Nayak and Kalidass, 2016). Under natural condition, the plant propagates only through seeds. Seed setting in this species is low and germinability of seeds is too poor. As this plant is an important medicinal species, local people harvest its aerial parts and rhizomes indiscriminately for their own use and trade. Human interference coupled with habitat degradation has been the causal factors for endangerment of the species in the wild. Further, no attempts have been made so far for *in situ* conservation of this rare medicinal plant species. However, a recent study reported multiple shoot regeneration from cotyledons of axenically grown seedlings of *Blepharispermum subsessile* (Nayak and Kalidass, 2016). In view of its threat status and problems associated with seed availability and germination, the present study aims at developing secondary cultures using nodal segments and shoot tips from already established primary cultures of this plant species.

2. Materials and methods

2.1 Collection of plant materials

Nodal segments and shoot tips of *B. subsessile* DC. were obtained from aseptically grown cultures of this plant species. The primary cultures were developed from cotyledonary nodes obtained by *in vitro* seed germination.

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The primary cultures were maintained in MS medium (Murashige and Skoog, 1962) supplemented with different concentration of BAP (6-benzyl aminopurine) and GA_3 (gibberellic acid).

2.2 Establishment of in vitro culture

The formulation of Murashige and Skoog's (1962) basal medium (MS) has been used in the present investigation. Shoot tips and nodal segments were aseptically excised from primary shoots and were inoculated in previously sterilized (autoclaved at 121ÚC and 15 psi for 15 – 20 min) MS medium supplemented with BAP (0.5 – 5.0 mg/l)or Kn (kinetin; 0.5 – 5.0 mg/l) either alone or in combination with Kn (0.5 - 2.0 mg/l), GA_3 (0.2 - 2.0 mg/l), IAA (indole-3-acetic acid), NAA (Naphthalene acetic acid) or IBA(Indole butyric acid) at 0.2 – 1.0 mg/l. Three explants were inoculated onto each culture vial containing 50 ml media. All the cultures were incubated in a growth room with a 16h photoperiod provided by cool, white fluorescent tubes (30 µmol m/s) and the temperature maintained at 25 ± 2°C, with 50-80% relative humidity.

2.3 Subculture of micro shoots and rooting

Proliferated micro shoots were separated and those measuring 2-3 cm and above were individually planted onto the full, half and quarter strength MS basal medium, with different sucrose concentrations and with or without the supplementation of auxins (0.2 - 2 mg/l) like IAA, NAA or IBA for rooting.

2.4 Hardening

Well rooted *in vitro* raised plants were carefully removed from the cultural vials and test tubes. These were washed thoroughly under running tap water to remove agar adhering to their roots. They were treated with (0.5%) bavistin (Bayar, India) solution for 10 minutes and plantlets were transferred to small plastic pots and thermo cool cups containing autoclaved vermiculite, soil and farmyard manure in 1:1:1 ratio. The plantlets were maintained in greenhouse condition and were watered every alternate day with tap water. Well-established and hardened plants were transferred to poly-pots containing growing media.

2.5 Data analysis

Experiments were set up in completely randomized design and repeated thrice with ten explants per replicate. Observations on the number of days taken for bud breaking, percentage response, the number of multiple shoots, and mean shoot length per culture were recorded at 30 days after inoculation in the shoot induction medium. Data on the number of days taken for root initiation, percentage response, the number of roots per shoot, root length and nature of roots were collected from the *in vitro* shootlets after 30 days of culturing in the rooting media. Data were statistically analyzed using analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) using SPSS 11.5; SPSS Inc., Chicago IL, USA) software. Significance differences were determined at the $p \leq 0.05$ level. The photographic illustrations were also provided wherever necessary.

3. Results and discussion

The most common approach in tissue culture is to isolate organized meristems like shoot tips or axillary buds and induce them to grow into complete plants. Out of the two explants taken for this study, shoot tips showed better response in culture. In the present study, shoot tips and nodal segments inoculated in MS basal medium devoid of growth regulators failed to show any regenerative response (Table 1 & 2). The addition of plant growth regulators especially cytokinins (BAP and Kin) to the basal culture medium resulted in bud break and shoot proliferation. About 91% shoot tips cultured in MS media augmented with BAP (1.5 mg/l) and IAA (0.2 mg/l) responded well producing an average of 6.33 shoots per explants with an average length of 2.43 cm (Table 1; Fig. 1A & B). On the other hand, about 88% nodal segments cultured in MS augmented with BAP (1.5 mg/l) alone responded producing an average of 4.67 shoots per explant with an average length of 3.17 cm per shoot (Table 2; Fig. 1C & D). This result is in conformity with the findings of Gnanaraj *et al.* (2011) in *Alternanthera sessilis* where shoot tips were more responsive than nodal segments. Sudharsan *et al.* (2015) observed that the type of explant markedly influenced the shoot organogenesis and growth of the regenerated shoots in *Hybanthus enneaspermus*. They found that the number of shoots formed per regenerative explant was greater with shoot tips in BAP (2.5mg/l) than nodal segments in BAP (2.0 mg/l). However, in *Sphaeranthus amaranthoides* nodal segments were found to be more responsive than shoot tips (Ravipaul *et al.*, 2008; Devika *et al.*, 2012). Out of the two cytokinins tested, BAP was most effective in inducing multiple shoots (Fig. 1E) in both the explants. The highest response was scored at the optimal concentration of BAP (2.5 mg/l) for shoot tips. Increasing concentrations of BAP beyond 2.0 mg/l reduced the number of shoot buds formed per explant. Highest elongation of regenerated shoots (average length 2.9 cm/shoot) was observed in BAP (1.5 mg/l). Shoot length decreased with increasing concentration of BAP beyond 1.5 mg/l. These shoots had narrow leaves and they showed stunted growth with minimum internodal elongation (Fig. 1F). Supplementations of Kn in the culture medium failed to show multiple shoot proliferation. The existing shoot bud

elongated producing a single shoot in both the explants (Table 1 & 2). Jawahar *et al.* (2008), Komalavalli and Rao (2000) and Reddy *et al.* (1998) reported that the MS medium containing BAP was more effective than Kn for induction of multiple shoots. Contrary to the findings of this study, Sudharsan *et al.* (2015) found that in *Hybanthus enneaspermus*, multiple shoots can be induced from explants treated with Kn. No significant variation was seen in multiple shoot induction and shoot elongation in all the combined concentrations of BAP & Kn (Table 1 & 2). In *Hybanthus enneaspermus,* shoot tips produced more shoot buds than nodal segments when BAP was used in combination with Kn (Sudharsan *et al.*, 2015).

A combined effect of a cytokinin BAP with GA_3 was also evaluated for multiple shoot induction. Addition of GA_3 (0.2 – 2.0 mg/l) along with BAP (1.5 mg/l) to the basal medium failed to show any significant morphogenic response (Table 1 & 2). Effect of the combination of cytokinin (BAP) with different auxins (IAA, NAA, and IBA) was also evaluated for multiple shoot induction. The addition of auxin (0.2 – 1.0 mg/l) along with a cytokinin BAP (1.5 mg/l) to the basal medium enhanced the morphogenic response significantly in the shoot tip explants and no significant variation was observed in case of nodal segments (Table 1 & 2). Percentage shoot development and the number of shoots produced per explant was maximum in MS basal medium supplemented with 1.5 mg/l BAP + 0.2 mg/l IAA, where *ca.* 91% cultures responded producing 6.33 shoots/ explant with an average length of 2.43 cm over a period of 4 weeks (Table 1 & 2). Shoots produced from shoot tip explants in MS basal medium supplemented with 1.5 mg/l BAP + 0.8 mg/l IAA attained highest shoot length of 3.93 cm/shoot. Among the three auxins tested, IAA at lower concentration was found most effective followed by IBA. NAA-treated explants showed callusing at the base of the regenerated shoots. In IBA treated cultures, the leaves were dark-green in color. Some successful plant growth regulator combinations used for multiple shoot induction have been reported earlier *viz.* BAP + IAA for *Desmodium gangeticum* (Behera and Thirunavoukkarasu, 2006); BAP + NAA for *Rauvolfia serpentina* (Mathur *et al.*, 1987), *Gomphrena officinalis* (Mercier *et al.*, 1992), *Gloriosa superba* (Hassan and Roy, 2005), *Costus speciosus* (Punyarani and Sharma, 2010), *Vernonia cinerea* (Seetharam *et al.*, 2007); BA + IBA for *Rheum emodi* (Lal and Ahuja, 1989); BAP + GA3 for *Saussurea lappa* (Arora and Bhojwani, 1989) and Kn + NAA in *Echinops kebericho* (Manahlie and Feyissa, 2014).

Rooting

Healthy and elongated shoots (2 -3 cm) were excised and cultured in different media for root induction. The effect of strengths of different media (full, half and quarter), sucrose concentration (3% and 2%) and types of growth regulators and concentration were assessed. Shoots cultured in full strength MS basal medium supplemented with 3% sucrose but without any growth regulator failed to produce any root (Table 3). In full strength MS basal medium supplemented with 2% sucrose but without any growth regulator, 42% shoots produced an average of 3.33 roots/shoot with an average length of 2.26 cm/root in 2 weeks time. In half strength MS basal medium supplemented with 2% sucrose but without any growth regulator, 100% of shoots produced an average of 4.14 roots/shoot with an average length of 4.28 cm/root in 2 weeks time. In quarter strength MS basal medium supplemented with 2% sucrose but without any growth regulator, 42% shoots produced an average of 2.67 roots/shoot with an average length of 2.96 cm/root in 2 weeks time. Augmentation of full strength MS basal medium containing 3% sucrose with auxins like IAA, NAA and IBA (0.2 – 2.0 mg/l) resulted in root formation (Table 3). About 100% of the shoots cultured in MS basal medium + 3% sucrose + IAA (1.0 mg/l) produced an average of 1.75 roots/ shoot with an average length of 2.2 cm/root in 2 weeks time. MS basal media supplemented with lower concentration of NAA (0.2 – 1.2 mg/l) failed to induce roots in shoots. Higher concentration (1.4 – 1.8 mg/l) of NAA induced rooting and was accompanied with callus formation at the base of the shoot. Shoots treated with IBA produced comparatively more number of roots, which were thick but failed to elongate further.

Augmentation of half strength MS basal medium containing 2% sucrose with auxins like IAA, NAA and IBA (0.5 – 2.0 mg/l) resulted in root formation (Table 3). But here more days were taken for root initiation compared to control (1/2 MS + 2% sucrose). Though more roots were produced here than control, the roots failed to elongate much in 2 weeks time. About 100% of the shoots cultured in 1/ 2 MS basal medium + 2% sucrose + IAA (1.0 mg/l) produced an average of 5.83 roots/shoot with an average length of 0.51 cm/root in 2 weeks time (Fig. 1G). Different growth regulators used for *in vitro* root induction have been reported *viz.* IBA for *Alternanthera sessilis* (Gnanaraj *et al.*, 2011), *Hybanthus anneaspermus* (Sudharsan *et al.*, 2015), *Phyllanthus urinaria* (Kalidass and Mohan, 2009); IAA for *Psoralea corylifolia* (Anis and Faisal, 2005); *Vernonia cinenea* (Seetharam *et al.*, 2007), *Vernonia amygdalina* (Khalafalla *et al.*, 2007), NAA for *Elephantopus scaber* (Rout and Sahoo, 2013).

Hardening

The plantlets transferred to root trainers/ poly pots containing vermiculite, soil and farmyard manure in 1:1:1

Table 1

Effect of different cytokinins BAP/Kn either alone or in combination with GA_3 and different auxins IAA/IBA/NAA on *in vitro* shoot regeneration from shoot tips of *Blepharispermum subsessile*

MS+3% sucrose+0.6% agar+ growth regulators (mg/l)						Days taken to shoot initiation	% response	No. of shoots/ explant	Shoot length /explant (in cm)
BAP	Kn	GA_3	IAA	NAA	IBA				
-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	5 – 7	71	2.00 fghij	2.03 cdefghi
1.0	-	-	-	-	-	4 – 9	66	4.33 bcd	2.10 cdefghi
1.5	-	-	-	-	-	4 – 6	70	4.00 cde	2.90 abcdef
2.0	-	-	-	-	-	6 – 7	87	5.00 abc	2.47 cdefghi
2.5	-	-	-	-	-	3 – 9	88	5.67 ab	1.53 ghi
3.0	-	-	-	-	-	4 – 9	76	2.67 defghij	1.63 fghi
3.5	-	-	-	-	-	3 – 9	66	3.00 defgh	1.70 efghi
4.0	-	-	-	-	-	5 – 6	67	2.67 defgh	1.53 efghi
4.5	-	-	-	-	-	3 – 7	70	2.33 efghij	1.47 hi
5.0	-	-	-	-	-	3 – 6	81	2.33 efghij	1.50 ghi
-	0.5	-	-	-	-	5 – 7	90	1.00 j	1.90 defghi
-	1.0	-	-	-	-	4 – 6	42	1.00 j	1.90 defghi
-	1.5	-	-	-	-	3 – 9	80	1.00 j	2.00 cdefghi
-	2.0	-	-	-	-	4 – 6	50	1.00 j	1.37 hi
-	2.5	-	-	-	-	3 – 7	86	1.00 j	2.46 cdefghi
-	3.0	-	-	-	-	2 – 8	90	1.00 j	2.88 abcdef
-	3.5	-	-	-	-	4 – 7	66	1.00 j	1.95 defghi
-	4.0	-	-	-	-	3 – 5	57	1.00 j	1.82 defghi
-	4.5	-	-	-	-	5 – 8	43	1.00 j	1.88 defghi
-	5.0	-	-	-	-	4 – 7	40	1.33 hij	2.43 cdefghi
1.0	0.5	-	-	-	-	5 – 8	80	2.67 defghij	2.67 cdefgh
1.0	1.0	-	-	-	-	3 – 5	88	2.67 defghij	3.20 abc
1.0	1.5	-	-	-	-	3 – 7	76	2.67defghij	3.00 abcde
1.0	2.0	-	-	-	-	2 – 6	66	2.33 efghij	3.00 abcde
1.5	-	0.2	-	-	-	4 – 6	67	3.33 defg	1.50 ghi
1.5	-	0.4	-	-	-	3 – 7	57	4.33 bcd	2.60 cdefghi
1.5	-	0.6	-	-	-	4 – 9	42	2.67 defghij	3.10 abcd
1.5	-	0.8	-	-	-	5 – 7	57	2.33 efghij	2.93 abcde
1.5	-	1.0	-	-	-	4 – 9	61	2.67defghij	2.43cdefghi
1.5	-	1.2	-	-	-	3 – 8	70	3.00 defgh	3.73 ab
1.5	-	1.4	-	-	-	4 – 7	76	2.00 fghij	1.30 i
1.5	-	1.6	-	-	-	5 – 6	81	2.00 fghij	1.43 hi
1.5	-	1.8	-	-	-	3 – 9	88	2.00 fghij	2.13 cdefghi
1.5	-	2.0	-	-	-	4 – 6	78	2.33 efghij	1.53 ghi
1.5	-	-	0.2	-	-	3 – 9	91	6.33 a	2.43 cdefghi
1.5	-	-	0.4	-	-	4 – 7	57	2.33 efghij	2.80 abcdef

1.5	-	-	0.6	-	-	2 – 8	78	5.00 abc	3.93 a
1.5	-	-	0.8	-	-	4 – 9	87	3.33 defg	3.93 a
1.5	-	-	1.0	-	-	4 – 8	76	2.67 defghij	1.37 hi
1.5	-	-	-	0.2	-	3 – 6	66	1.67 ghij	2.07 cdefghi
1.5	-	-	-	0.4	-	3 – 5	76	2.75 defghi	2.32 cdefghi
1.5	-	-	-	0.6	-	4 – 9	57	2.33 efghi	2.33 cdefghi
1.5	-	-	-	0.8	-	4 – 6	59	2.50 efghij	1.80 defghi
1.5	-	-	-	1.0	-	4 – 8	43	1.25 ij	1.32 i
1.5	-	-	-	-	0.2	5 – 7	55	2.67 defghi	1.47 hi
1.5	-	-	-	-	0.4	4 – 7	73	3.67 cdef	1.83 defghi
1.5	-	-	-	-	0.6	3 – 8	63	2.50 efghij	1.95 cdefghi
1.5	-	-	-	-	0.8	3 – 9	66	3.00 defgh	2.37 cdefghi
1.5					1.0	4 – 7	67	2.33 efghij	2.10 cdefghi

Data pooled from three independent experiments each with 10 replicates per treatment. Data presented of 4 weeks old cultures. Mean values within column followed by the same letter are not significantly different ($p<0.05$; Duncan's Multiple Range Test)

Table 2

Effect of different cytokinins BAP/Kn either alone or in combination with GA_3 and different auxins IAA/IBA/NAA on *in vitro* shoot regeneration from nodal segments of *Blepharispermum subsessile*

MS + growth regulators (mg/l)						Days taken to shoot initiation	% response	No. of shoots/ explant	Shoot length /explant (in cm)
BAP	Kn	GA_3	IAA	NAA	IBA				
-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	4 – 10	70	3.33^{bcde}	2.27 bcdefghij
1.0	-	-	-	-	-	3 – 6	78	4.33^{b}	2.47 abcdef
1.5	-	-	-	-	-	2 – 6	88	4.67 a	3.17 a
2.0	-	-	-	-	-	3 – 8	86	4.00 abc	2.53 abcde
2.5	-	-	-	-	-	4 – 6	87	3.67 abcd	1.93 defghijkl
3.0	-	-	-	-	-	6 – 9	63	2.00 fghi	1.8 defghijkl
3.5	-	-	-	-	-	4 – 6	57	3.00 cdef	1.57 ghijkl
4.0	-	-	-	-	-	3 – 12	81	3.67 abcd	1.3 l
4.5	-	-	-	-	-	5 – 9	87	3.67 abcd	1.73 efghijkl
5.0	-	-	-	-	-	3 – 7	69	3.67 abcd	1.5 hijkl
-	0.5	-	-	-	-	5 – 9	87	1.00 i	1.94defghijkl
-	1.0	-	-	-	-	4 – 7	81	1.00 i	2.32 bcdefgh
-	1.5	-	-	-	-	3 - 9	76	1.00 i	2.42 abcdefg
-	2.0	-	-	-	-	4 – 9	63	1.00 i	1.84 defghijkl
-	2.5	-	-	-	-	3 - 6	86	1.00 i	2.32 bcdefgh
-	3.0	-	-	-	-	4 – 6	64	1.00 i	2.41 abcdefg
-	3.5	-	-	-	-	5 - 7	62	1.00 i	1.98 defghijkl
-	4.0	-	-	-	-	4 – 7	70	1.00 i	2.84 abc
-	4.5	-	-	-	-	3 – 8	62	1.00 i	2.86 abc

-	5.0	-	-	-	-	3 – 6	76	1.00 i	1.86 defghijkl
1.0	0.5	-	-	-	-	4 – 8	66	2.67 defg	2.63 abcd
1.0	1.0	-	-	-	-	2 – 4	88	3.33 bcde	3.23 a
1.0	1.5	-	-	-	-	2 – 6	76	2.67 defg	3.00 ab
1.0	2.0	-	-	-	-	3 – 4	63	1.67 ghi	2.63 abcd
1.5	-	0.2	-	-	-	6 – 8	66	2.33 efgh	2.03 bcdefghijkl
1.5	-	0.4	-	-	-	4 – 6	76	1.67 ghi	1.5 hijkl
1.5	-	0.6	-	-	-	3 – 8	70	2.33 efgh	1.5 hijkl
1.5	-	0.8	-	-	-	6 – 9	76	2.33 efgh	1.4 jkl
1.5	-	1.0	-	-	-	4 – 5	84	2.33 efgh	2.3 bcdefghi
1.5	-	1.2	-	-	-	3 – 5	81	3.33 bcde	1.33 kl
1.5	-	1.4	-	-	-	3 – 7	57	2.00 fghi	1.77 efghijkl
1.5	-	1.6	-	-	-	4 – 8	86	2.33 efgh	1.83 defghijkl
1.5	-	1.8	-	-	-	3 – 9	76	2.67 defg	1.97 defghijkl
1.5	-	2.0	-	-	-	5 – 6	66	2.00 fghi	1.67 fghijkl
1.5	-	-	0.2	-	-	3 – 5	77	2.33 efgh	1.57 ghijkl
1.5	-	-	0.4	-	-	2 – 4	87	3.33 bcde	1.53 hijkl
1.5	-	-	0.6	-	-	4 – 7	67	1.67 ghi	2.2 bcdefghijk
1.5	-	-	0.8	-	-	3 – 6	63	2.00 fghi	2.23 bcdefghij
1.5	-	-	1.0	-	-	3 – 5	77	3.33 bcde	1.43 kl
1.5	-	-	-	0.2	-	4 – 8	66	2.33 efgh	1.9 defghijkl
1.5	-	-	-	0.4	-	4 – 6	70	2.33 efgh	1.63 fghijkl
1.5	-	-	-	0.6	-	3 – 7	76	2.25 efghi	1.45 hijkl
1.5	-	-	-	0.8	-	2 – 7	71	1.33 hi	1.33 kl
1.5	-	-	-	1.0	-	4 – 7	66	1.00 i	1.2 l
1.5	-	-	-	-	0.2	4 – 8	70	2.67 defg	1.53 hijkl
1.5	-	-	-	-	0.4	5 – 7	82	3.00 cdef	1.6fghijkl
1.5	-	-	-	-	0.6	5 – 8	63	1.67 ghi	2.3 bcdefghi
1.5	-	-	-	-	0.8	3 – 7	66	2.00 fghi	2.3 bcdefghi
1.5	-	-	-	-	1.0	6 – 8	87	3.33 bcde	1.57 ghijkl

Data pooled from three independent experiments each with 10 replicates per treatment. Data presented of 4 weeks old cultures
Mean values within column followed by the same letter are not significantly different ($p<0.05$; Duncan's Multiple Range Test)

Table 3

Effect of different media strength, sucrose concentration and concentrations of auxins IBA/NAA/IAA on induction of roots in *in vitro* regenerated shoots of *Blepharispermum subsessile*

MS+ 3% sucrose+ growth regulators			Days taken to root initiation	% response	No. of roots per shoot	Root length per shoot (in cm)
IAA	NAA	IBA				
-	-	-	-	-	-	-
0.2	-	-	-	-	-	-
0.4	-	-	-	-	-	-
0.6	-	-	10 – 12	60	1.00d	0.46ij

0.8	-	-	-	-	-	-
1.0	-	-	8 – 10	100	1.75bcd	2.22bcdefg
1.2	-	-	8 – 9	76	1.67bcd	1.30cdefghij
1.4	-	-	7 – 10	76	1.67bcd	2.30bcde
1.6	-	-	10 – 12	75	1.33cd	2.03bcdefg
1.8	-	-	9 – 10	75	2.67bcd	1.60cdefghi
2.0	-	-	8 – 10	60	1.67bcd	1.80bcdefgh
-	0.2	-	-	-	-	-
-	0.4	-	-	-	-	-
-	0.6	-	-	-	-	-
-	0.8	-	-	-	-	-
-	1.0	-	-	-	-	-
-	1.2	-	-	-	-	-
-	1.4	-	8 – 12	63	2.33bcd	2.40bcd
-	1.6	-	7 – 9	15	1.33cd	1.43cdefghij
-	1.8	-	10 – 12		1.50cd	1.00fghij
-	2.0	-	-	-	-	-
-		0.2	-	-	-	-
-		0.4	8 – 10	80	2.33bcd	2.50bc
-		0.6	8 – 12	88	3.00abcd	1.60cdefghi
-		0.8	-	-	-	-
-		1.0	7 – 9	80	1.67bcd	0.97ghij
-		1.2	8 – 9	69	3.00abcd	1.06efghij
-		1.4	9 – 11	66	2.67bcd	0.30ij
-		1.6	-	-	-	-
-		1.8	10 – 12	63	1.33cd	2.20bcdefg
-		2.0	7 – 9	50	2.00bcd	2.13bcdefg
MS+ 2% sucrose		-	8 – 10	42	3.33abcd	2.26bcdef
½ MS+2% sucrose		-	6 – 7	100	4.14abcd	4.28a
½ MS+ 2% sucrose+ growth regulators						
0.5	-	-	11 – 15	90	4.17abcd	0.60hij
1.0	-	-	8 – 12	100	5.83a	0.51ij
2.0	-	-	12 – 15	57	4.00abcd	0.46ij
-	0.5	-	10 – 12	90	4.40abc	0.40ij
-	1.0	-	10 – 15	28	1.33cd	0.26bcdefg
-	2.0	-	8 – 12	57	3.75abcd	0.37ij
-	-	0.5	10 – 15	42	3.67abcd	1.20defghij
-	-	1.0	10 – 12	42	3.33abcd	0.46ij
-	-	2.0	8 – 12	57	4.75ab	0.42ij
¼ MS+ 2% sucrose			13 – 15	42	2.67bcd	2.96b

Data pooled from three independent experiments each with 10 replicates per treatment. Data presented of 4 weeks old cultures. Mean values within column followed by the same letter are not significantly different ($p<0.05$; Duncan's Multiple Range Test)

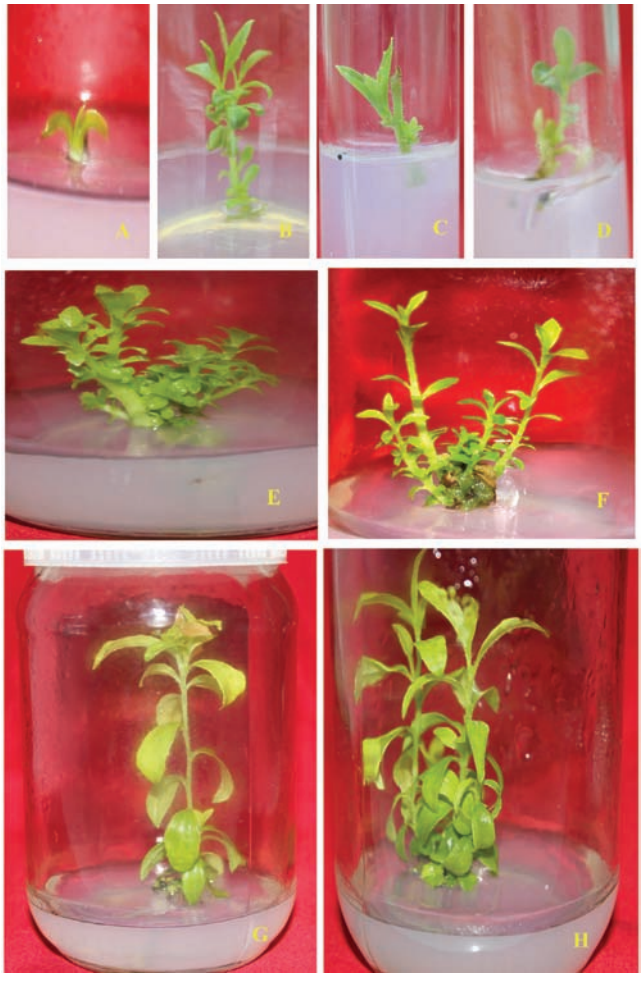

Fig. 1. Multiple shoot induction of *Blepharispermum subsessile* DC., A. Bud break in shoot tip in MS+BAP (1.5mg/l) + IAA (0.2mg/l); B. Shoot initiation in shoot tip in MS+BAP (1.5mg/l) + IAA (0.2mg/l) after 6 days; C. Bud break in nodal segment in MS+BAP (2.5mg/l); D. Shoot initiation in nodal segment in MS+BAP (2.5mg/l); E & F. Multiple shoot proliferation and G & H. Shoot elongation.

Fig. 2. Root induction of *Blepharispermum subsessile* DC., In *in vitro* rooted shoot in A. MS+IBA (0.6mg/l); B. MS+NAA (1.4mg/l); C. % MS+IAA (1.0mg/l); D. *In vitro* rooted shoots; E. An acclimatised plantlet in the polypot after 2 weeks of transfer and F. Acclimatised plantlets in root trainer.

ratio and maintained under greenhouse condition initially showed very slow growth. About 39% of micro-propagated plants survived hardening under greenhouse condition. It was observed that shoots with well-developed roots had a greater survival ability compared to those with shorter and slender roots (Fig.1H).

4. Conclusion

The results presented here suggest an efficient, reproducible and easy-to-handle protocol for *in vitro* regeneration of *Blepharispermum subsessile* using secondary cultures developed from shoot tips and nodes of primary shoots. The method has practical significance and the process has to be successfully exploited for large-scale production of cloned plants of this endangered medicinal plant species. The micro-propagation technique now developed will be helpful in producing planting materials in large-scale for raising commercial plantations and augmenting existing germplasm of this important and rare medicinal plant species in natural habitats.

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