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In Vitro Multiplication of *Arnebia benthamii* Wall., a Critically Endangered Medicinal Herb of the Western Himalayas

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ABSTRACT

Arnebia benthamii (Wall. Ex G. Don) Johnston [Syn *Macrotomia benthamii* (Wall.) DC.] (family Boraginaceae), locally known as "Kahzaban", is a highly valued and critically endangered Himalayan medicinal plant, and ranks second in the list of medicinal plants prioritized for Western Himalaya and also figures among the 59 medicinal plants prioritized for conservation due to its high threat of extinction. An efficient *in vitro* multiplication and propagation system was developed for *A. benthamii*. Half-strength Murashige and Skoog (MS) medium augmented with different concentrations of 6-benzyladenine (BA) were used for shoot multiplication from shoot tip explants. The best response, i.e., multiple shoot formation, was with 5 µM BA. In another experiment, the combined effect of BA with 1 µM indole-3-butyric acid (IBA) was tested. The maximum number of multiple shoots was obtained on half-strength MS medium supplemented with 4 µM BA and 1 µM IBA. Different concentrations of IBA, indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) were used to induce roots from shoots. Roots formed best on half-strength MS medium supplemented with 4 µM IBA, and 80% of plantlets transferred to field conditions survived.

Keywords: multiple shoots, plantlets
Abbreviations: BA, 6-benzyl adenine; **IBA**, indole-3-butyric acid; **IAA**, indole-3-acetic acid; **NAA**, α-naphthalene acetic acid; **MS**, Murashige and Skoog

INTRODUCTION

Plants are one of the most important sources of medicine. The importance of medicinal plants lies in their biologically active principles, which are the real healers in the process of medication (Kumar 2004). These active principles may be present in different plant parts like roots, seeds, leaves, bark and wood. The inclination of people towards herbal rather than synthetic drugs during the last few decades has given rise to a large-scale collection of medicinal plants from the wild resulting in the depletion of resources and extinction of rare and endangered species (Mishra and Gupta 2006). The international market of medicinal plant products is estimated to be US$ 62 billion, which is poised to grow to US$ 5 trillion by the year 2050 (Garg 2007). The world trade figures suggest that India follows China by exporting 32,000 tonnes of medicinal plants raw material worth US$ 46 million annually (Fay 1992).

Arnebia benthamii (Wall. Ex G. Don) Johnston [syn *Macrotomia benthamii* (Wall.) DC.], family Boraginaceae and locally known as "Kahzaban", is a highly valued Himalayan medicinal plant, and ranks second in the list of medicinal plants prioritized for the Western Himalayas and also figures among the 59 medicinal plants prioritized for conservation (Sastry and Chatterjee 2000) due to its high extinction threat (Manjkhola and Dhar 2002). It is an alpine herb occurring normally in open slopes on stony or rocky substrates. In the Kashmir valley, it is confined to certain areas with rare distribution such as Gulmarg, Kishtwar, Zanskar and Nubra whereas in Karnah, Gurez, Lolab, Sonamarg and Kargil, it is found occasionally (Kaul 1997). It is classified as a critically endangered non–endemic plant of Kashmir (Kaul 1997; Ved and Tandon 1998; Dar and Naqshi 2001; Dar *et al.* 2002). *Arnebia* is considered to be useful in the treatment of tongue and throat diseases, fevers and cardiac disorders. In India, it is a traditional herb of Ayurvedic and Unani systems of medicine. Its flowers are reported to have a soothing effect on patients with heart ailments (Kaul 1997). Its roots are used as an antiseptic and antibiotic for healing wounds and are applied as a poultice. The paste of roots made in water was applied to fire burns for rapid healing (Chauhan 1999).

Tissue culture has been successfully used for commercial production of pathogen-free plants and conservation of the germplasm of rare and endangered species (reviewed in Teixeira da Silva 2006). The present study on *A. benthamii* aimed to develop a protocol for multiplying germplasm for conservation purposes.

MATERIALS AND METHODS

Plant material and surface sterilization of explants

A. benthamii shoot tips of one-year-old plants were collected from Sheri Kashmir University of Agriculture, Science and Technology-Kashmir (SKUAST-K) and thoroughly washed with 2-3 drops of a mixture of detergent (0.5% Cedepol) and Tween 20 (surfactant). Shoot tips were rinsed under running tap water then with double distilled water. Thereafter, shoot tips were surface disinfected with 0.1% streptomycin (an antibiotic; Himedia, Mumbai, India) for 20 min then by 0.1% $HgCl_2$ for 7 min and finally rinsed 3-4 times with autoclaved double distilled water to remove all traces of sterilants.

Culture media

The basal medium consisted of half-strength (i.e., half the concentration of mineral salts and organic nutrients) MS (Murashige and Skoog 1962) medium, 3% sucrose (Qualigens, India), 0.8% Difcobacto agar and different concentrations of plant growth regulators (PGRs; Himedia). When all the medium constituents were added, the pH was adjusted to 5.4 with 1 N NaOH or 1 N HCl and finally

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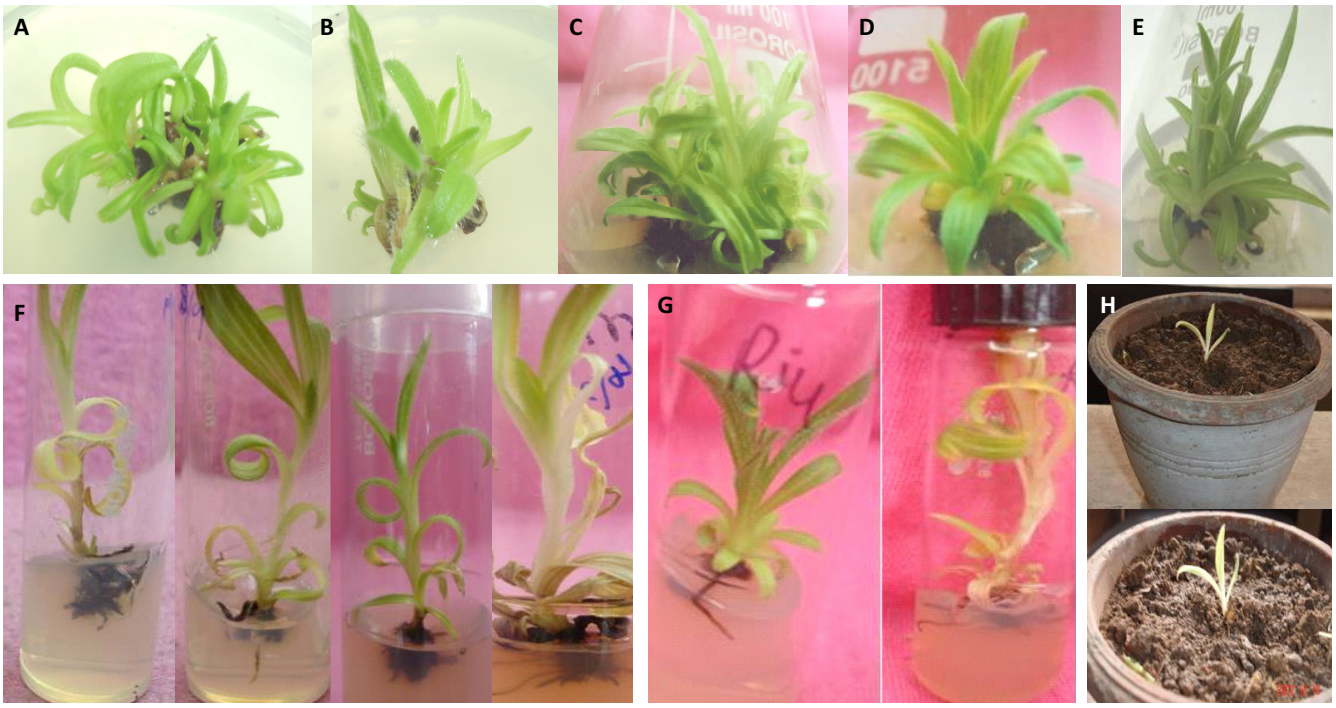

Fig. 1 ***In vitro*** **response of shoot tips of** ***Arnebia benthamii*** **Wall.** Multiple shoot formation on ½-MS + (**A**) 5 µM BA, (**B**) 20 µM BA, (**C**) 4 µM BA + 1 µM IBA, (**D**) 7 µM BA, (**E**) 4 µM BA + 3 µM IBA; *In vitro* root formation on ½-MS + (**F**) 4 µM IBA, (**G**) 4 µM IAA. (**H**) Plantlets in a 1:1 mixture of autoclaved sand: soil.

dispensed into 100-ml Erlenmeyer flasks (borosilicate glass) plugged with non-absorbent cotton prior to autoclaving at 121°C and 15 psi for 20 min.

Experimental design

Shoot tips were cultured on: a) ½-MS medium supplemented with different concentrations of 6-benzyladenine (BA) (2.5-20 µM); b) ½-MS medium + BA (1-7 µM) + indole-3-butyric acid (IBA) (1 µM); c) ½-MS medium supplemented with IBA, indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) at a concentration ranging from 1–5 µM each. Cultures were maintained in an incubation room at 25 ± 3°C under 16-hr photoperiod provided by cool white fluorescent tubes at 3000 lux (equiv. 42 µmole/m^2/s).

Statistical analysis

The experiments were carried out in completely randomized block design (CRD). Each treatment was repeated three times and each treatment had 10 replicates. A non-parametric Mann-Whitney test was applied to determine the difference in multiple shoots obtained on BA alone and BA + IBA. Significance was determined at $P < 0.001$. All statistical calculations were performed with Statsdirect, ver 2.5.6.

RESULTS

The effects of different concentrations of BA alone and in combination with IBA on shoot tips of *A. benthamii* are indicated in **Table 1**. Various concentrations of BA (2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 µM) induced multiple shoots, which proliferated and elongated slightly within 12 weeks of culture with a 100% response. Blackish, friable callus grew around the base of the explant. The number of shoots increased up to 5 µM BA, with an average of 9.7 ± 0.4 shoots/explant (**Fig. 1A**), but when the concentration of BA was increased to 20 µM the number of shoots decreased (**Fig. 1B**).

However, when shoot tips were transferred to different concentrations of BA (1, 2, 3, 4, 5, 6, 7 µM), each supplemented with 1 µM IBA, 100% formed multiple shoots with blackish, friable callus at the base of the main explant. The maximum number of multiple shoots (15.3 ± 0.4) with broad healthy leaves formed at 4 µM BA + 1 µM IBA after 12 weeks of culture (**Fig. 1C**). As the concentration of BA increased, shoot number decreased (**Table 1**); fewest average number of shoots (3.1 ± 0.8) formed with 7 µM BA + 1 µM IBA (**Fig. 1D**). Even though at this combination of PGRs the primary shoot developed well – albeit with callus at the base – further amendments in the concentration of BA and IBA also resulted in well-developed multiple shoots, also with light-brownish friable callus at the base, but shoot number never exceeding 15.3 ± 0.4, the best yielding 5.9 ±

Table 1 Shoot multiplication from shoot tip culture of *Arnebia benthamii* Wall. grown on half-strength MS medium supplemented with BA alone and with BA + IBA.

BA (µM)	IBA (µM)	Number of shoots/explant (mean ± SD) *
2.5	-	7.1 ± 0.7
5	-	9.7 ± 0.4
7.5	-	6.5 ± 0.6
10	-	5.1 ± 2.9
12.5	-	4.4 ± 0.4
15	-	3.2 ± 0.7
17.5	-	3 ± 0.0
20	-	2.5 ± 0.5
1	1	6.0 ± 0.7
2	1	8.2 ± 1.6
3	1	9.8 ± 0.9
4	1	15.3 ± 0.4**
5	1	8.5 ± 1.0
6	1	6.1 ± 0.7
7	1	3.1± 0.8
4	2	4.7 ± 0.6
4	3	5.9 ± 0.3
2	0.5	5.7 ± 0.4
2.5	0.5	4.6 ± 0.4
2.5	2	4.2 ± 0.8
5	2	3.2 ± 1.6
2.5	2.5	0 ± 0.0
5	5	0 ± 0.0
7.5	7.5	3.0 ± 0.4
10	10	2.9 ± 0.3

*Data scored after 12 weeks of culture period; 10 replicates/treatment.
Difference between mean values of maximum multiple shoots obtained on 4 µM BA + 1 µM IBA were found to be significantly higher than 5 µM BA at ** $P <$ 0.001 using the Mann-Whitney test. All other comparisons were insignificant.

Table 2 Effect of half-strength MS medium supplemented with different concentrations of IBA / IAA/ NAA on root regeneration of *Arnebia benthamii* Wall.

IBA (µM)	IAA (µM)	NAA (µM)	Response	Root No. Mean ± S.D.*	Percentage response
0.5	-	-	No response	-	-
1.0	-	-	-	-	-
1.5	-	-	Black hard tap root only	1.0 ± 0.0	70
2.0	-	-	Black hard tap root only	1.0 ± 0.0	80
2.5	-	-	Black hard tap root with lateral adventitious root initials	1.5 ± 0.2	80
3.0	-	-	Black hard tap root with lateral adventitious root initials.	2.5 ± 0.3	80
3.5	-	-	Black hard tap root with lateral adventitious root initials.	4.5 ± 0.2	80
4.0	-	-	Black hard tap root with lateral adventitious root initials.	7.5 ± 0.0[b]	80
4.5	-	-	No response	-	50
5.0	-	-	-	-	No response
-	0.5	-	-	-	-
-	1.0	-	-	-	-
-	1.5	-	Black hard tap root only	1.0 ± 0.0	70
-	2.0	-	Black hard tap root only	1.0 ± 0.0	70
-	2.5	-	Black hard tap root only	1.0 ± 0.0	70
-	3.0	-	Black hard tap root only	1.0 ± 0.0	70
-	3.5	-	Black hard tap root with lateral adventitious root initials.	2.0 ± 0.5	70
-	4.0	-	Black hard tap root with lateral adventitious root initials.	3.5 ± 0.5	80
-	4.5	-	No response	-	No response
-	5.0	-	-	-	-
-	-	0.5	-	-	-
-	-	1.0	-	-	-
-	-	1.5	Light brownish friable callus (+)	-	100
-	-	2.0	Light brownish friable callus (+)	-	100
-	-	2.5	Light brownish friable callus (++)	-	100
-	-	3.0	Light brownish friable callus (++)	-	100
-	-	3.5	Light brownish friable callus (++)	-	100
-	-	4.0	Light brownish friable callus (+++)	-	100
-	-	4.5	Light brownish friable callus (+++)	-	100
-	-	5.0	No response	-	100

*Data scored after 12 weeks of culture period: 10 replicates taken in each treatment; (+) low callus; (++) moderate callus; (+++) high callus. Difference between mean values of maximum multiple roots obtained on 4 µM IBA were found to be significantly higher than 4 µM IAA at ** $P < 0.001$ using Mann-Whitney test. All other comparisons were insignificant.

0.3 shoots/explant with 4 µM BA + 3 µM IBA (**Fig. 1E**).

Maximum multiple shoots from shoot tips of *A. benthamii* were significantly higher ($P < 0.001$) on 4 µM BA + 1 µM IBA compared to 5 µM BA.

The micro shoots of *A. benthamii* recorded from multiplication phase were transferred to half-strength MS medium supplemented with different concentrations of IBA, IAA and NAA (**Table 2**) for elongation and rooting. No response was noticed at least for 2 months after which only elongation was observed on IBA, NAA and IAA used independently. After a long period of 2½ months the basal zone of the elongated shoots turned black and hard with initiation of multiple rootlets. On 4 µM IBA 7-8 multiple black short thick rootlets were recorded from main black hard tap root like structure (**Fig. 1F**). Similarly elongation of shoots was also observed on 4 µM IAA and regeneration of 2-3 short rootlets were recorded (**Fig. 1G**). 4 µM NAA resulted in elongation of shoots but with intense friable callus formation at the base of the shoot. The healthy plantlets were deflasked and transferred in small pots containing sterile soil, sand, peat and vermiculite (1: 1: 1: 1, v/v) and showed 80% survival rate under field conditions (**Fig. 1H**).

DISCUSSION

Tissue culture technique is being increasingly exploited for clonal multiplication and in vitro conservation of valuable germplasm threatened with extinction. An efficient procedure for *in vitro* multiplication is an essential pre-requisite for employing *in vitro* techniques for germplasm conservation. The present investigation carried on shoot tips of *A. benthamii* offers a potential and efficient protocol for mass propagation and conservation of this medicinal herb. Scanning of literature revealed that there is scanty published report on *in vitro* studies of *A. benthamii* and the results obtained in the present study are discussed in light of high altitude medicinal plant species.

The main aim of the present study was to achieve maximum shoot multiplication in shoot tip culture through the use of different phytohormonal combinations and concentrations. Different concentrations of BA used alone resulted in multiple shoot regeneration but maximum average multiple shoots of 9.7 ± 1.1/explant was recorded on 5 µM BA and further increase in concentration resulted in decreased shoot number. Such results are quite similar to the earlier reports on *Rotula aquatica* (Martin 2003), *Hyoscyamus muticus* (Grewal *et al*. 1979), *Atropa belladonna* (Benjamin *et al*. 1987), *Swertia chirata* (Balaraju *et al*. 2009), *Glycyrrhiza glabra* (Vadodaria *et al*. 2007), *Withania* somnifera (Sen and Sharma 1991; Rani and Groover 1999; Ray and Jha 2001) and *Atropa baetica* (Zarate *et al*. 1997) where decreased shoot multiplication potential was registered with increased concentration of BA. In *Arnebia euchroma* maximal number of shoots from cotyledon and hypocotyl explants was noticed with low concentration of TDZ (Debergh and Maene 1981), a non purine derivative having cytokinin like activity, and is again in line with present studies. Hence, low BA has been found to be favourable for *A. benthamii* shoot multiplication from shoot tip cultures.

The most effective combination for shoot multiplication out of a number of trials given was 4 µM BA and 1 µM IBA resulting in 15.3 ± 1.6 shoots/explant. Similar results were recorded from nodal segments of *Rotula aquatica* under the influence of similar phytohormonol combinations (Martin 2003) and hence corroborate our findings. Similarly, organogenesis was also noticed from leaf derived callus of *Arnebia euchroma* (Manjkhola 2004) and adventitious shoot bud induction in leaf explants of *Rheum emodi* and their subsequent growth under the combined interaction of BA and IBA (Lal and Ahuja 2000). BA and IBA again favoured maximum multiple shoot regeneration in nodal culture of *Atropa acuminata* and *Solanum laciniatum* (Ahuja *et al*. 2002; Davies and Dale 1979) which strongly supports our results. Present findings however do not match some earlier

reports on nodal explants of *Gentiana kurroo* (Sharma *et al*. 1993), nodal explants of *Tylophora indica* (Sharma and Chandel 1992), epicotyl explants of *Saussurea obvallata* (Joshi and Dhar 2003) and rhizome explants of *Hedychium spicatum* (Koul *et al*. 2005) where combined interaction of BA + NAA, BA + NAA + ascorbic acid, Kn + NAA and BA + IAA were found effective for shoot multiplication respectively. The synergistic effect of BA and an auxin has been demonstrated in many medicinal plants like *Santoline canescens* (Casado *et al*. 2002), *Bupleurum fruticosum* (Fraternale *et al*. 2002) and *Curcuma longa* (Salvi *et al*. 2002) which indicates that low concentrations of an auxin in combination with a cytokinin positively modified the frequency of shoot induction and growth. In present study BA alone also resulted in multiple shoot formation but combined interaction of BA and IBA resulted in maximum multiple shoot regeneration suggesting that their interaction perhaps resulted in shifts in endogenous synthesis of BA and IBA thus making it either suboptimal or supra optimal hence resulting in varying needs in exogenous supply of both the hormones (Bhan 1998). Therefore in *A. benthamii* optimum level of exogenously supplied phytohormones for maximum shoot multiplication has been registered to be 4 µM BA and 1 µM IBA.

Rooting of isolated shoots was recorded on IBA followed by IAA and no rooting was observed on NAA. In earlier reports on *A. euchroma* rooting was noticed under the influence of IBA (Jiang *et al*. 2004 and Manjkhola *et al*. 2004), hence our results are in accordance with these. Published record reveals that the rate of auxin uptake has been found to be varied (De Klerk *et al*. 1997). NAA is taken up six times faster than IAA (Peeters *et al*. 1991), IBA four times faster than IAA (Vander Krieken *et al*. 1993). In present case IBA was found most effective for rooting as compared to IAA and NAA but in contrast to it NAA has been found to be the best rooting hormone in *Rotula aquatica* as compared to IBA and IAA (Martin 2003). The efficacy of IBA on rooting may be due to its faster uptake in present studies than NAA.

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