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In vitro propagation of *Aconitum balfourii* Stapf: A rare medicinal herb of the alpine Himalayas

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ABSTRACT

Small vegetative parts of *Aconitum balfourii* from different growth stages were excised for *in vitro* establishment. The callus induction was achieved from cultured leaf segment on MS medium fortified with NAA (2.5 mg/ l) and BAP (1 mg/ l). Calli upon transfer onto MS medium containing BA alone (0.5-4 mg/l) for shoot regeneration, multiplication and elongation. Up to 25 shoots per culture flask were obtained on 2 mg/l BAP after 7-8 weeks of culture. The optimum rooting on micro-shoots was observed on MS supplemented with IAA (1.8 mg/l). The *in vitro* developed plantlets with elongated roots were then subjected for further *ex vitro* rooting, acclimatization and hardening.

Key words: *Aconitum balfourii*, *in vitro* regeneration, micro-propagation.

INTRODUCTION

Aconitum balfourii Stapf. (Ranunculaceae) is the aconite of medicinal value (Kiritkar and Basu, 6) endemic to alpine and sub-alpine regions of Himalayas (grown above 3,000 m altitude). It is also known as *Meetha vish* and *Vatsnabh* whose tuberous roots are the source of several diterpenoid alkaloids which form the ingredient of several Ayurvedic and Homoeopathic systems of medicine (Chopra *et al*., 3). According to IUCN criteria, *A. balfourii* is an indeterminate species (Rao *et al*., 11). The plant is a perennial herb with fleshy, spindle-shaped root containing an alkaloid pseudoaconitine, a highly toxic principal component and benzylaconitine, picroaconitine and haemonepellene in traces (Anon, 1). Secondary metabolites in the form of alkaloids, amino acids, trisachcharides, amines and purines are stored in the underground parts after the aerial growth, thus forming a large group of medicinal plants in the alpine region (Nautiyal and Nautiyal, 9,10). *Aconitum* has other biological and pharmacological activites such as anti-fungal, anti-bacterial, insecticidal and brine shrimp cytotoxicity Anwar *et al.*, (2).

In the past decades, due to destructive anthropogenic activities and poor rate of natural regeneration, this high value plant species has acquired threatened status (Kuniyal, 7). The germination of seeds and seedling establishment is poor. The tubers derived from seeds result in large genetic variability and are found to be frequently infected with fungal diseases. Organized cultivation of *Aconitum* is therefore necessary to ensure the quality and continuous supply of drugs. The clonal propagation through tissue culture is highly desirable in this species to regenerate sufficient uniform plant material with similar aconite content. The present investigation describes a protocol for *in vitro* multiplication of *Aconitum balfourii* a high medicinal value and a rare plant facing extinction.

MATERIALS AND METHODS

Actively growing mature plants and tubers of *Aconitum balfourii* Stapf. were collected from Tungnath (3,300 m asl, Distt. Chamoli, Uttarakhand. These plants and tubers were kept for establishment under controlled environment containment facility at the College of Basic Sciences and Humanities, GBPUA&T, Pantnagar. Seeds from different identified populations (Kilpur, Yamunotri, Panwalikantha and Madhyamaheshwar) were mixed with carbendazim powder (1%) to check the fungal growth and also sown in earthen pots containing soil and farmyard manure (3:1, w/w) and kept inside greenhouse (25°C, 65.0% RH).

Excised shoot tips (0.5 cm) and matured as well as 10-15 days-old leaves after emergence were excised from plants raised in earthen pots and pre-cleaned with double distilled water containing 1% v/v Tween-20 for 5 min. The explants were surface sterilized with 0.1% aqueous mercuric chloride solution for 1 min. rinsed thoroughly 4-5 times in sterile distilled water and inoculated aseptically onto Murashige and Skoog (8) basal medium containing 3% sucrose and 0.7% agar-agar. Recent study indicates that *in vitro* grown healthy plants of *A. heteropyllum* were obtained with 0.1% (w/v) $HgCl_2$ at 5 min. significantly reduced both bacterial and fungal contaminations, while other sterilizing agents such as NaOCl and H_2O_2 did not give acceptable sterilizing outcome (Srivastava *et al.*,13). The medium (MS) was supplemented with

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growth regulators in various combination of BA (0.5-3.0 mg/l) and NAA (1.0-5.0 mg/l). The pH of the medium was adjusted to 5.8±0.1 with 0.1 N NaOH or 0.1 N HCl prior to autoclaving for 20 min. at 120°C (1.05 kg/cm_2). The cultures were maintained at 25±2°C under 14/10 h photoperiod (55 mM $m^{-2}s^{-1}$) from cool, white fluorescent tube lights. To avoid blackening, the medium was supplemented with 0.5 mg/l ascorbic acid. After seven weeks, calli proliferated from the excised mature leaf explant and these were sub-cultured onto fresh medium at a 3-week interval.

For root induction, microshoots (1-2 cm) were excised individually and transferred onto MS basal medium supplemented with different concentrations of IAA or IBA (0.1 to 1.0 mg /l) and 2% (w/v) sucrose. Complete plantlets were obtained and rooted micro-propagules were thoroughly washed to remove the adhering agar gel and transplanted in plastic pots (25 ml) containing a sterile mixture of sand, soil and vermicompost mixture in the ratio of 1: 1: 1 (w/w) and shifted to greenhouse for acclimatization.

RESULTS AND DISCUSSION

Only leaf explants (3-5 mm in length) from mature plant could induce callus after 20 days of culture at the cut ends or whole of the leaf segment into a mass of soft, green friable callus (Table 1). It was observed that calli were induced with combination of BAP (1.5 mg/l) and NAA (1.5 mg/l). No callus induction was observed with combination of NAA and BAP of the concentration ranging from 0-1.0 mg/l. Maximum response was observed in combination of BAP (1.0 mg/l) and NAA (2.5 mg/l) inducing the callus of 0.035 g fresh weight. In this combination number of shoots was observed to be maximum, *i.e.* 12.6±1.42. The present findings are supported by those of Giri *et al.* (4) who reported the callus formation on MS medium maintained on 1.0 mg/l level of NAA.

The callusing was low (45-50%) and frequent sub culturing at 2-week intervals could minimize the accumulation of phenolics and increased the rate of callus proliferation. The major problem was encountered during *in vitro* culture of *Aconitum balfourii* Stapf. was exudation of phenolic compound. Frequent subculture at a one-week interval was done to minimize the accumulation of phenolic compounds and increase the rate of proliferation. Inclusion of anti-phenolic substances, such as 0.5% PVP (Polyvinylpyrollidone), ascorbic acid (10 mg/l) and activated charcoal (2%) overcome this problem. The inclusion of anti-phenolic substances in the nutrient medium seems to alleviate this problem in several other plant species. The use of high concentration of NAA and lower concentration of BA for callus induction has also been reported in *A. heterophyllum* (4). In this study regular subculturing of callus at three week intervals prevented cessation of growth, generally caused by phenolic exudates, and promoted proliferation.

Table 1. Effect of BAP and NAA combination on growth of callus culture in *Aconitum balfourii*.

BAP (mg/l)	NAA (mg/l)	Mean No. of shoots	Callus formation	Fresh wt. (g)
1.5	1.5	2.3 ± 1.84	* +	0.009
2.0	1.5	2.5 ± 1.96	* +	0.004
3.0	1.5	2.4 ± 0.24	* +	0.014
0	2.5	-	-	-
0.5	2.5	-	-	-
1.0	2.5	12.6 ± 1.42	* +++	0.035
1.5	2.5	3.1 ± 0.26	* +	0.011
2.0	2.5	8.2 ± 1.24	* ++	0.028
3.0	2.5	2.6 ± 1.96	* +	0.014
0	3.0	-	-	-
0.5	3.0	2.6 ± 0.79	* +	0.008
1.0	3.0	2.78 ± 0.75	* +	0.006
1.5	3.0	2.06 ± 1.82	* +	0.004
2.0	3.0	1.2 ± 1.82	* +	0.005
3.0	3.0	1.02 ± 0.42	* +	0.007

Observations were taken after seven weeks of culture

Mean value ± SE of 10 replicates.

(-) No response, (+) Moderate, (++) Good, (+++) Very good, (*) Callus

The maximum shoot proliferation (70%) and multiplication was observed on MS medium supplemented with 1.8 mg /l BA and 0.5 mg/l ascorbic acid within 4 weeks of culture (Table 2). However, at higher concentrations of BA or kinetin, the rate of shoot proliferation declined per explant. The shoots proliferated and elongated to 1.0-1.5 cm within 4 weeks of culture. In some of the cases, the growth was inhibited and only 1-2 shoots elongated while in some there was formation of compact callus at the base of the explants. Prolonged culture on the proliferation and multiplication media resulted in the blackening of the basal ends of the developing shoots.

Micro-shoots (1-2 cm) were rooted individually on MS basal medium. Root initiation took place within 20-25 days of transfer to MS basal medium supplemented with 0.1-1.0 mg /l IAA or IBA. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of IAA or IBA. However, optimal rooting (60%) and growth of microshoots were observed on medium containing 0.25 mg /l IAA and IBA with 2% (w/v) sucrose (Table 3). Our studies were consistent with earlier report that MS medium supplemented with IAA or IBA will produce optimum rooting. In this report high percentage of rooted shoots produced with MS medium fortified with 0.1 mg/l IAA and IBA (12). However the root formation results reported by other findings in *A. heterophyllum* and *A. napellus* was best obtained on IBA (1.0 mg/l) and NAA (1.0 mg/l) (Giri *et al*., 4; Wattad *et al*., 14). The rooting ability was reduced with the increase in the concentration of IAA or IBA. Complete plantlets (5-10 cm) were recovered after 6-8 weeks on rooting medium. Rooted plantlets were washed thoroughly to remove the adhering gel and transplanted to 2.5 cm plastic sterile pots containing sand, soil and vermicompost mixture (1: 1: 1). About 96% of the rooted plantlets established in the greenhouse within 2-3 weeks of transfer. The plant grew well and attained 6-8 cm height within 4 weeks of transfer.

The previous study with shoot proliferation of *in vitro* grown *A. heterophyllum* revealed the potential to produce large scale multiplication through callus culture (Jabeen *et al*., 5). The present study too reveals the protocol developed, has the potential to be reproduced and utilized for large scale multiplication for its uninterrupted supply to herbal and neutraceutical industries and simultaneously conserving this medicinal herb, an indigenous rare medicinal plant.

Table 2. Effect of BA in MS medium on shoot multiplication using excised individual shoots of *A. balfourii*.

BA (mg/l)	No. of shoots/ flask	Shoot length (cm)
1.0	2.0 ± 0.5	3.6 ± 0.4
1.2	3.1 ± 0.6	3.7 ± 0.5
1.4	6.2 ± 0.2	4.3 ± 0.3
1.8	12.6 ± 0.5	6.2 ± 0.2
2.0	11.0 ± 1.0	5.2 ± 0.5
2.2	9.7 ± 0.7	4.8 ± 0.8
2.4	7.5 ± 0.5	4.3 ± 0.7
2.6	5.7 ± 0.7	3.7 ± 0.5
2.8	4.2 ± 0.2	3.6 ± 0.6
3.0	3.1 ± 0.6	3.6 ± 0.4

*Each value represents an average of 10 replicates (mean ± SE)

Table 3. Effect of IAA and IBA on rooting of excised shoots of *Aconitum balfourii*.

MS + growth regulators (mg/l)		Rooting (%)	Days of rooting
IAA	IBA		
0	0	30.6 ± 0.2	20-22
0.1	0.1	45.4 ± 0.6	20-23
0.25	0.25	60.2 ± 0.4	20-25
0.5	0.5	52.2 ± 0.7	22
1.0	1.0	50.6 ± 0.3	21

Data represent mean of 20 cultures/treatment (mean ± SE)

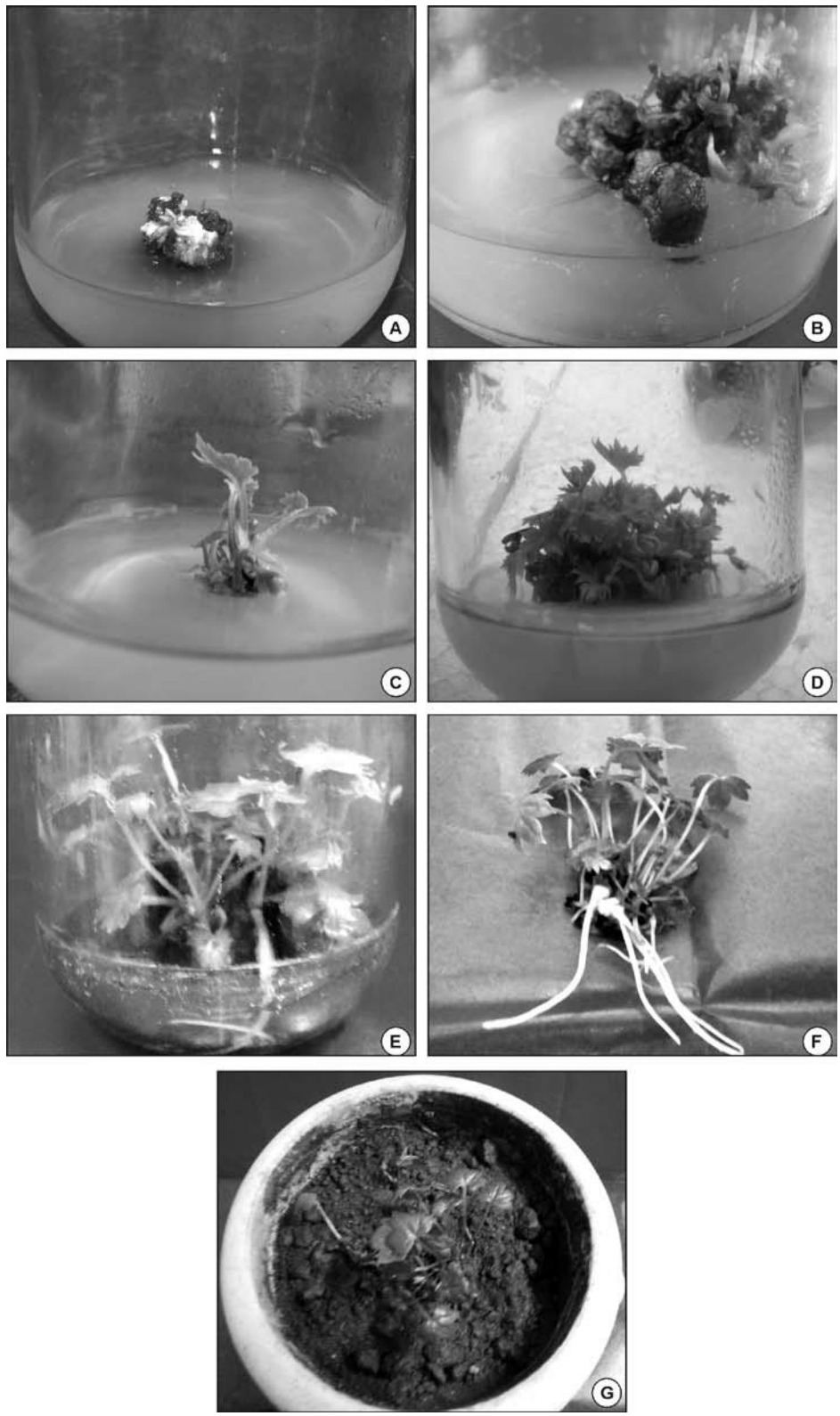

Fig. 1. Stages of *in vitro* regeneration in *Aconitum* sp. (a) Callus, (b) Shoot bud induction, (c) Shoot elongation, (d) Shoot proliferation, (e) Rooting, (f) Rooted plantlets, and (g) Hardened plant.

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