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In vitro Regeneration of *Aconitum balfourii* Stapf: A Rare Medicinal Herb from Himalayan Alpine Through Root Explants

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ABSTRACT

Aconitum balfourii Stapf is a rare medicinal herb belonging to genus *Aconitum* and family Ranunculaceae. Excessive illegal collection from wild due to pharmacological significance along with quiescent nature of seeds and lengthy cultivation cycle are collectively responsible for its endangered status. Therefore, conservation applying tissue culture is a vital need and has been developed nowadays. In the present study, *in vitro* regeneration of *A. balfourii from* root explants has been attempted. Root explants were cultured on MS-Medium supplemented with different combinations of BA and NAA and callus induction took place at 13.4 μM NAA and 5.55 μM BA. Such induced calluses were transferred to shooting media. The best shooting were observed in 8.88 μM BA and 0.54 μM NAA. These shoots were subculture on 7.99 μM BAP. Furthermore the result showed that increasing the concentration of BA caused a reducing length of shoots; also presence of low concentration of NAA has been necessary for shoot induction. Some factors such as shoot length, no. of shoots and induction frequencies of shooting and rooting were also studied. The well developed micro shoots were cultured on rooting media for complete regeneration of plant. Rooting of micro shoots occurred in media containing 1.43 μM IAA and 1.23 μM IBA.

Key words: *Aconitum,* conservation, regeneration, pseudoaconitine

INTRODUCTION

The genus *Aconitum* belongs to the family Ranunculaceae is a diverse genus with nearly 300 species worldwide (Kadota, 1987) and has been found rich source of diterpenoid alkaloids. In, India, the genus is represented by about 26 species mainly distributed in subalpine and alpine zones of Himalayas from Kashmir to Uttarakhand and extending to the hills of Assam (Chaudhary and Rao, 1998). *A. balfuorii* Stapf. is an significant and highly valued herb of this genus. *A. balfourii* Stapf. is prevalent in different zones of Garhwal and Kumaon regions of Uttarakhand i.e., Valley of flowers, Kedarnath, Tungnath, Madhyamaheshwar and Panwalikantha on shady slopes at altitudes between 2800-4200 m. (Nautiyal *et al*., 2002; Chopra *et al*., 1984; Samant *et al*., 1998). It is an erect, glabrous shrub that becomes more than 1.5 m in height. Root tubers are 7-12 cm long and extraordinary heavy (Gaur, 1999) Tubers contain a crystalline toxic alkaloid called pseudoaconitine (0.4-0.5%) and aconitine, balfourine, bikhaconitine in small amount (Khetwal, 2007). The alkaloid isolated from the roots of this herb, has exhibited anti-inflammatory, vermifuge, anti-rheumatic, analgesic and cardio tonic activities

and used in all types of pains and inflammations (Ameri, 1998). Hence, it has been frequently used in Ayurvedic, Homeopathy and Unani system of medicine since long time so the commercial demand is very high. To accomplish the demand excessive illegal collection and sale of *Aconitum* have been continuously carried out. Due to over exploitation and destruction of natural habitats along with poor seed germination and seedling establishment under natural conditions, *A. balfourii* is identified as an endangered (Nautiyal *et al*., 2002) in Garhwal Himalaya and vulnerable in entire Uttarakhand by CMAP (2003). The importance of this plant, recalcitrant nature of its seeds and its endangered status has necessitated an urgent need to work on its proper regeneration. Therefore, in the present study an attempt has been made to establish *in vitro* regeneration protocol of *A. balfourii* Stapf, through root explants. Although, diverse sources of explants have been reported for regeneration, but root explants have some advantages over other explants in terms of higher regeneration potential, easy manipulation and higher susceptibility of *Agrobacterium rhizogene* transformation (Morton and Browse, 1991). This is first time report of regeneration protocol of *A. balfourii* Stapf. from root explants.

MATERIALS AND METHODS

Plant material: Actively growing mature plants and tubers of *Aconitum balfourii* Stapf. were collected from Tungnath region (3300 m). These plants and tubers were kept for establishment under controlled environment containment facility at College of Basic Sciences and Humanities, GBPUA&T, Pantnagar. The explants for *in vitro* regeneration were obtained from green house (25°C, 65.0% RH).

Callus induction: Callus induction was attempted with different explants viz., leaves, shoot tips and roots. All explants were excised in 0.5 cm length and pre-cleaned with double glass 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 on Murashige and Skoog (1962) basal medium containing 3% sucrose and 0.7% agar. The medium (MS) was supplemented with growth regulators in various combination of BA (0-6.66 μM) and NAA (0-13.42 μM) for callus induction. 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 and 1.05 Kg cm^{-2}. The cultures were maintained at 25±2°C under 14 h photoperiod (55 mmol m^{-2} sec^{-1}) from cool, white fluorescent tube lights. All explants were cultured in vessels each containing 50 mL of medium. To avoid blackening, due to release of phenolics, the medium was supplemented with 0.05% PVP. After eight weeks of culture, calli proliferated from the excised leaf explants, shoot explants and root explants as well and these were subculture in the fresh medium at a 2-weeks interval for continuous growth. Four explants were used per flask and four flasks were used per treatment. Callus of root explants was used for further shoot induction and multiplication.

Shoot proliferation: After callus was obtained from different explants, we have used root callus for further standardization of regeneration protocol. For Shoot bud induction and shoot differentiation, MS medium composed of BA (0-10.65 μM^{-1}) and combination with 0.54 μM NAA was employed. These shoot buds were subculture onto maturation medium supplemented with 7.99 μM BA, 0.05% PVP and 3% sucrose for three weeks. Every possible care has been taken to prevent any further contamination.

Rooting of microshoots: For root induction, excised micro shoots (1-2 cm length) were transferred to MS basal medium supplemented with different concentrations of IAA (0-4.52 μM and IBA (0-5.71 μM) individually or in combinations and 2% (w/v) sucrose. One excised shoot was placed in each culture vessel having 50 mL of the culture media.

Data analysis: All the experiments were performed in replicates. Four replicates in callus induction and shoot proliferation and ten replicates in root induction for each combination of hormone treatments were analyzed, respectively. Mean values of various treatments were subjected to one way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Effect of various concentrations of BA and NAA on callus induction: All the three explants viz., roots, stems and leaves used for callusing were respond at different concentration of growth hormones supplemented to MS-Medium along with 3% sucrose and 0.7% agar (Fig. 1a-c). The optimal callus from root explants were obtained at 13.42 μM NAA and 5.55 μM BA. In case of stem and leaf explants the best callusing was observed at 13.44 μM NAA and 4.44 BA and 16.11 μM NAA and 4.44 BA, respectively (Table 1). The callus induction from leaf explants was previously reported in different concentration of BA and NAA in *A. balfourii* (Pandey *et al*., 2004; Bist *et al*., 2011). Callus induction percentage was highest in leaf explants i.e., 90-91% followed by root and stem 80-85%. After obtaining callus from different explants sources the root callus was used for further regeneration, therefore sub culturing of root callus was performed to every 2 weeks of culture for minimizing the phenolics and increase the growth and mass of callus.

Table 1: Effect of different combinations of NAA and BA on callus induction from root, leaf and stem explants

Concentrations of PGR'S (μM)				
NAA	BA	Root	Leaf	Stem
0.0	0.0	-	-	-
2.68	1.11	33.33±0.408	33.33±0.40	33.33±0.577
5.37	2.22	33.33±0.577	-	33.33±0.408
5.37	4.44	50.00±0.645	-	50.00±0.288
8.05	2.22	41.66±0.478	16.66±0.288	41.66±0.478
10.74	3.33	50.00±0.288	16.66±0.500	50.00±0.288
13.42	3.33	25.00±0.478	25.00±0.478	25.00±0.478
13.42	4.44	66.66±0.408	58.33±0.288	83.33±0.288
13.42	5.55	83.33±0.288	50.00±0.288	66.66±0.408
13.42	6.66	66.66±0.408	41.66±0.288	50.00±0.645
16.11	1.11	33.33±0.408	33.33±0.408	33.33±0.408
16.11	2.22	41.66±0.478	41.66±0.478	58.33±0.629
16.11	4.44	66.66±0.408	91.66±0.478	50.00±0.250
1.34	-	-	-	-
2.69	-	16.66±0.00	16.66±0.288	16.66±0.288
-	1.11	-	-	-
-	2.22	-	-	-

SEM: 1.649, SEM: 1.451, SEM: 1.672. Cd at 5% = 4.740. Cd at 5% = 4.170. Cd at 5% = 4.807 in root, leaf and stem, respectively. Values are Mean±SE of four replicates and repeated three times. The data were recorded after eight weeks of culture

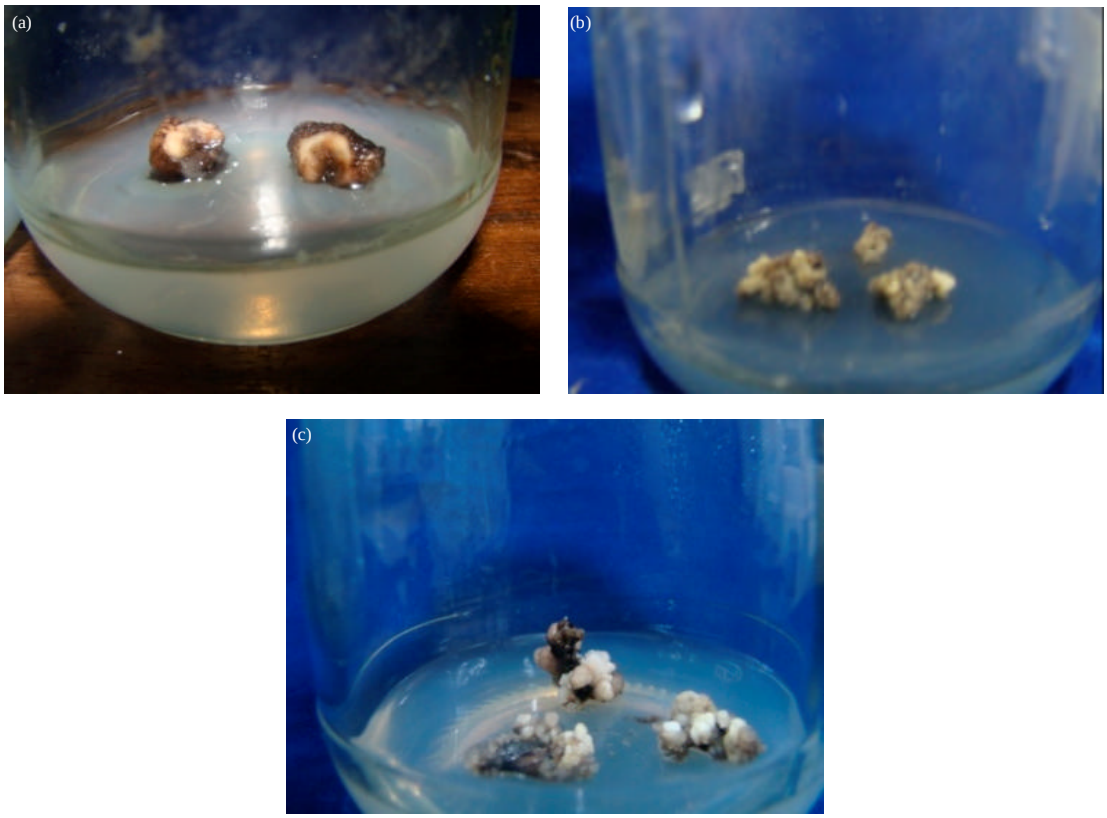

Fig. 1(a-c): Callus induction from different explants, (a) Root callus, (b) Shoot callus and (c) Leaf callus

Effect of different level of BA and NAA on shoot induction and proliferation: In Table 2 effect of different concentration of BA and interaction with NAA showed that the induction of shooting required a low level of NAA with BA. Different shooting stages of shooting are shown in Fig. 2(a-d). The maximum shoot induction 93.7% (14.50 shoots/flask) was obtained at 8.88 μM BA and 0.54 μM NAA. This finding is supported by earlier reported data on different species of the genus *Aconitum* (Giri *et al*., 1993; Watad *et al*., 1995). Furthermore, it was also observed that as we increased the concentration of BA from 0 to 10.65 μM, the shoot length was decreased and it also adversely affects the average no. of shoots. It might be due to that BA at high concentration causes decrease in apical dominance. This result is in line with what reported by earlier studies on this plant (Jabeen *et al*., 2006; Giri *et al*., 1993; Bist *et al*., 2011). For further sub culturing of micro shoots the medium supplemented with 7.99 μM BA alone was found to be optimum because shoot length was maximum (3.20 cm) at this concentration. Cytokinin as a plant growth regulator causes shoot proliferation by stimulating cell division (Ranjan *et al*., 2003).

Effect of different auxin concentrations on rooting: As saw in Table 3 two different auxins IAA and IBA individually and with various combinations were used to see the effect on rooting of excised shoots. The best rooting 72% was observed at 1.43 IAA and 1.23 IBA. However, the rooting was observed at every combination but with different level of response. Rooting of microshoots and

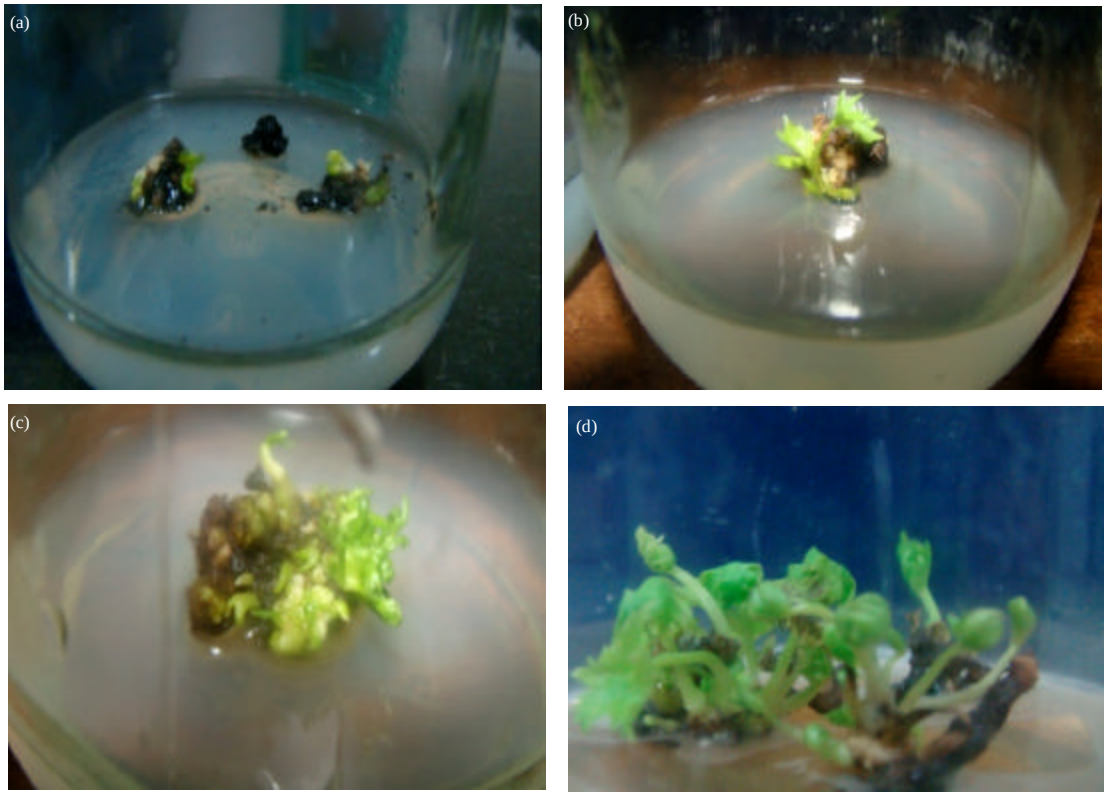

Fig. 2(a-d): Different stages of shoot induction and multiplication (a) Shoot initiation from callus, (b) Shoot differentiation (initial stage), (c) Shoot differentiation (later stage) and (d) Shoot multiplication

Table 2: Effect of BA and NAA on shoot induction

Concentration of PGR'S (µM)				
BA	NAA	% Shoot induction	Average shoots/flask	Approximate shoot length
0.00	0.00	-	-	-
3.33	-	-	-	-
3.33	0.54	-	-	-
4.44	-	37.5±0.28	1.33	1.6
4.44	0.54	-	-	-
6.21	-	50.00±0.40	3.66	2.4
6.21	0.54	43.75±0.62	2.00	2.4
7.99	-	87.5±0.28	12.66	3.20
7.99	0.54	68.7±0.25	11.66	3.0
8.88	-	62.5±0.28	10.60	3.06
8.88	0.54	93.7±0.25	14.50	2.16
10.65	-	56.25±0.25	8.33	1.66
10.65	0.54	50.00±0.40	6.20	0.33

SEM: 1.451. Cd at 5% = 4.218. Values are Mean±SE of four replicates. Data was taken after 8 weeks of culture

Fig. 3: Rooting of microshoots

Fig. 4: Complete regenerated plantlet

Table 3: Rooting response on different combinations of IAA and IBA

Concentration of PGR'S (µM)			
IAA	IBA	% Root induction	Days of rooting
0	0	30.5±0.2	20-22
0.570	-	40.5±0.6	20-22
1.43	-	55.55±0.4	20-23
2.85	-	43.24±0.6	20-22
-	0.492	31.50±0.2	25-27
-	1.23	58.15±0.5	22-24
-	2.46	52.25±0.2	20-22
0.570	0.492	45.4±0.6	20-24
1.43	1.23	72.35±0.7	20-25
2.85	2.46	58.50±0.1	20-22
4.52	5.71	50.60±0.2	20-22

SEM: 1.889. Cd at 5% = 5.540. Data was taken 3 weeks of culture and ±SE represents mean of 10 cultures per treatment

complete regenerated plant was depicted in Fig. 3 and 4, respectively. Considering Table 3, it showed that as we increase the concentration of individual auxin decrease the rooting response. But the combination of both auxins synergistically increases the rooting response. Our investigation is consistence with earlier reports on combined response of IAA and IBA improve the rooting in other plants (Samantary and Maiti, 2010).

CONCLUSION

Since *A. balfourii* Stapf. is a valued herb of the genus *Aconitum* due to presence of pharmacologically important alkaloids, in the present investigation an *in vitro* regeneration protocol was optimized through root explants. Although, *in vitro* regeneration protocols have been already reported but it is first time report of regeneration by using root explants. Based on the obtained result in this study, the protocol is helpful to regenerate whole plant after infection of *A. rhizogene* that is one of the most frequent approaches to enhance the production of important secondary metabolites from several medicinal plants.

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