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Effect of Growth Regulators on *In vitro* Cultures of *Aconitum heterophyllum*: An Endangered Medicinal Plant

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ABSTRACT

Aconitum heterophyllum Wall. an endangered medicinal plant contains alkaloids that have significant antipyretic and analgesic role. An efficient protocol was developed for its mass shoot multiplication which can meet the requirements of pharmaceutical industries. The most effective treatment for sterilization of explant was three step sterilization method where 70% ethanol for 30 sec + $HgCl_2$ (0.1%) for 2 min + $KMnO_4$ (1ppm) for 3 min was used, leading to 73.3% of uncontaminated explants. The MS medium supplemented with 1.0 mg/l BAP, 0.5 mg/l Kn and 1.0 mg/l GA3 resulted in shoot growth of 46.50% with an average number of shoots 2.7± 0.48 and average shoot length 2.63± 0.35. Six different combinations were used for callus induction and high frequency of embryogenic callus was obtained in MS medium supplemented with 2.0 mg/l 2,4-D.

Key words: *Aconitum heterophyllum, in vitro, growth regulators, callus, endangered*

INTRODUCTION

Aconitum heterophyllum Wall. also called as 'Atis' is an endangered and important medicinal plant of Western Himalayas and is found in Kashmir province[6,13]. The roots are tuberous which contain alkaloids have significant antipyretic, antibacterial and analgesic role[10,15]. The plant is used for the treatment of nervous system, rheumatism, throat infection and digestive system[2,4,6]. The plant has been indiscriminately exploited due to restricted distribution, unskilled harvesting and constant pressure from the herbal market, so now this plant has been affirmed as endangered medicinal plant by IUCN (http://www. iucnredlist.org) and listed in Red Data Book.

Conservation of this plant under natural conditions through vegetative propagation is slow and time consuming but *in vitro* conservation using micropropagation offers another tool for rapid multiplication and conservation in a short period, and thus facilitates continuous supply of raw material. Different growth regulators play an important role in achieving high success rate during *in vitro* plant propagation. Growth regulators especially cytokinins and auxins are often added to culture media for controlling the different physiological responses *in vitro*, thereby resulting in production of different tissues like callus, shoots, roots or whole plants[1,12]. The objective of the present work is to study the effect of different growth regulators on *in vitro* propagation, multiplication and callus induction of this endangered medicinal plant.

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MATERIAL AND METHODS

Collection and preparation of plant material

The plant material was collected from Galhar (Kishtwar) in Jammu and Kashmir at an altitude of 2238 meters above sea level during the months of August to October. The plants were brought to the laboratory, wrapped in moist papers, in plastic bags and were used for *in vitro* propagation studies.

Different parts of plants (leaves, nodes, and auxillary buds) were taken and used as explants, from healthy and profusely growing parts of *Aconitum heterophyllum*. To prepare explants, the plants were washed under running tap water so as to completely remove all the soil debris. Then all the leaves were removed. Explants i.e. auxillary buds and internodal segments were excised with the help of scalpel blade and were collected in a beaker containing distilled water.

Surface sterilization

The explants collected in beaker were washed 3-4 times in running tap water, with 2-3 drops of Tween 20 and then washed 3-4 times with distilled water. Henceforth further sterilization of explants was done using ethanol, $HgCl_2$, Sodium hypochlorite and $KMnO_4$ in laminar flow chamber. The different sterilants were used either singly or in combination for the surface sterilization of explants. Different surface sterilants used are given in Table-1.

Media preparation

MS medium (Murashige and Skoog)[8] was prepared with 3% Sucrose and 0.7% agar was added to the solution before autoclaving. The pH was maintained 5.8. For all the *in vitro* experiments, the medium used was MS basal or modified MS supplemented with different combination and concentration of hormonesas given in table-2.

Inoculation of explants

For the inoculation of cultures, the explants of *A. Heterophyllum* were surface sterilized and inoculated on basal MS medium. The cultures were then maintained at 25^{o}C and 16 hrs photoperiod in culture room for the establishment of explants. The proliferation of buds was indicated by unfurling of small leaves and elongation of petiole. The established cultures were transferred to modified MS medium supplemented with growth regulators.

Inoculation of leaf explants on solid media for callus initiation

Young leaves of *Aconitum heterophyllum* were surface sterilized with 70% alcohol for 30 sec and 0.1% $HgCl_2$ for 2 minutes. The leaves were than washed thrice with distilled water, cut into small pieces with sterilized blade and inoculated on MS media supplemented with different concentrations of 2,4-D (1.0 mg/l to 4.0 mg/l) either singly or containing BAP, IAA and NAA.

RESULTS AND DISCUSSION

Surface Sterilization

The plants collected from the fields contain large quantity of microorganisms in the form of fungal and bacterial spores, so a suitable sterilization treatment was required which can result in maximum sterilization of explants[7]. In total, ten different treatments were carried out alone or in combinations. The surface sterilization of explants was carried out using mercuric chloride 0.5% and 0.1% for delicate explants like leaf, intermodal segments and auxillary buds for 2 minutes along with 70% ethanol. The various treatments applied in combination for surface sterilization of explants and the percentage survival has been indicated in the following table (Table-3). The most effective treatment was three step sterilization method where 70% ethanol for 30 sec + $HgCl_2$ (0.1%) for 2 min + $KMnO_4$ (1ppm) for 3 min was used, resulting in 73.3% of uncontaminated explants, followed by 70% ethanol for 30 sec + $HgCl_2$ (0.2%) for 2 min + $KMnO_4$ (1ppm) for 3 min resulting in 60.0% uncontaminated explants. As the concentration of mercuric chloride was increased it resulted in browning and ultimately death of the explants because at high concentration of steriliants, the soft tissues are severely affected. Falkiner[3] observed that efficient aseptic techniques with correct concentration of antibiotics are required for the reduction of contamination caused by bacteria. Use of 5% and 10% of sodium hypochlorite alone resulted in 26.6% and 13.3% of uncontaminated explants respectively. Even Srivastava *et al*[11]., observed that the

sterilizing agent NaClO did not give acceptable sterilization even on increasing concentration in *A. heterophyllum* because the nodal explants are more open to the external environmental elements, so they require reasonably strong sterilizing agents.

Inoculation of explants and shoot multiplication

Sterilized explants of *A. heterophyllum* were inoculated on basal MS media. Though the plants remain green but no growth was observed so the explants were then transferred on to modified MS media containing different growth hormones (Table-2). The proliferation of leaves from the explants was observed after three weeks and after four weeks there was increase in petiole length (Fig 1a). For shoot establishment and multiplication twelve treatments with different combinations and concentrations of growth hormones (BAP, Kinetin and GA_3) were used. Best shoot multiplication was observed after six weeks in MS supplemented with 1.0 mg/l BAP, 0.5 mg/l Kn and 1.0mg/l GA_3. The number of shoots per explant and the length of shoots were measured after six weeks. (Table 4; Fig 1b). During micropropagation cytokinin promotes the growth of axillary buds by reducing the apical dominance of buds[14].

The percentage shoot growth was 46.50% only with an average number of shoots 2.7± 0.48 and average shoot length 2.63± 0.35 in MS medium supplemented with 1.0 mg/l BAP, 0.5 mg/l Kn and 1.0mg/l GA_3 whereas no shoot initiation was observed in MS medium supplemented with 0.5, 1.0 and 1.5mg/l Kn. Jabeen *et al*[5]., reported multiple shoot regeneration from MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA.

Inoculation of leaf explants for callus induction

For callus induction six different combinations were tried. MS medium when supplemented with 2.0 and 3.0 mg/l of 2,4-D resulted in callus induction after six weeks. No callus was observed in MS medium containing 1.0 mg/l 2,4-D. However MS medium supplemented with 2.0 mg/l 2,4-D resulted in creamy white, friable callus but the growth rate was not so good. As the concentration of 2,4-D was increased to 4.0 mg/l, it resulted in browning of the explants (Table 5, Fig1c). Rawat *et al*[9]., observed callus formation in MS medium supplemented with 2, 4-D and Kinetin in *Aconitum violaceum.*

Table 1: Concentration and combination of different sterilants used for surface sterilization of explants

S. No.	Treatment s	Time duration
1	70% ethanol	30-60 sec.
2	$KMnO_4$ (1ppm)	3 min
3	70% ethanol + $HgCl_2$ (0.05%)	30 sec. + 2 min
4	70% ethanol + $HgCl_2$ (0.1%)	30 sec. + 2 min
5	70% ethanol + $HgCl_2$ (0.2%)	30 sec. + 4 min
6	70% ethanol + $HgCl_2$ (0.5%)	30 sec. + 2 min
7	Sodium hypochlorite (5% v/v)	5 min
8	Sodium hypochlorite (10% v/v)	5 min
9	70% ethanol + $HgCl_2$ (0.2%) + $KMnO_4$ (1ppm)	30 sec. + 2 min + 3 min
10	70% ethanol + $HgCl_2$ (0.1%) + KMnO4 (1ppm)	30 sec. + 2 min + 3 min

Table 2: Different concentrations and combinations of growth hormones used in MS media

Culture media	Concentration in mg/l
MS + BAP	0.5, 1.0 ,1.5
MS + Kinetin	0.5, 1.0 ,1.5
MS + BAP + Kinetin	i. 1.0 mg/l BAP+ 0.5.0mg/l Kn ii. 0.5 mg/l BAP+ 1.0mg/l Kn iii. 1.0 mg/l BAP+ 1.0mg/l Kn
MS + BAP+Kinetin+GA_3	i. 0.5 mg/l BAP+ 1.0 mg/l Kn + 1.0 mg/l GA3 ii. 1.0 mg/l BAP+ 0.5mg/l Kn + 1.0 mg/l GA3 iii. 1.0 mg/l BAP+ 1.0 mg/l Kn + 1.0 mg/l GA3

Table 3: Effect of different sterilants on surface sterilization of explants in *A. heterophyllum*

S. No.	Treatments	Time duration	Percent survival of explants
1	70% ethanol	30-60 sec.	Nil
2	$KMnO_4$ (1ppm)	3 min	Nil
3	70% ethanol + $HgCl_2$ (0.05%)	30 sec. + 2 min.	06.6
4	70% ethanol + $HgCl_2$ (0.1%)	30 sec. + 2 min.	26.0
5	70% ethanol + $HgCl_2$ (0.2%)	30 sec. + 4 min.	23.3
6	70% ethanol + $HgCl_2$ (0.5%)	30 sec. + 2 min.	15.9
7	Sodium hypochlorite (5% v/v)	5 min.	26.6
8	Sodium hypochlorite (10% v/v)	5 min.	13.3
9	70% ethanol + $HgCl_2$ (0.2%) + $KMnO_4$ (1ppm)	30 sec. + 2 min + 3 min	60.0
10	70% ethanol + $HgCl_2$ (0.1%) + $KMnO_4$ (1ppm)	30sec. + 2 min + 3 min	73.3

Table 4: Effect of different concentrations of plant growth regulators in the culture medium on *in vitro* shoot multiplication in *A. hetrophyllum*

S.No	Concentration of growth regulators (mg/l)	Percentage Shoot growth	Average no. of shoots/explant	Average shoot length (cm)
1	MS + 0.5 BAP	--	--	---
2	MS + 1.0 BAP	23.32	2.7± 0.57	1.19 ± 0.71
3	MS + 1.5 BAP	14.19	2.3± 0.41	1.32± 0.32
4	MS + 0.5 Kn	--	--	--
5	MS + 1.0 Kn	--	--	--
6	MS + 1.5 Kn	--	--	--
7	MS + 1.0 BAP + 0.5 Kn	43.52	2.3± 0.63	2.12± 0.54
8	MS + 0.5 BAP + 1.0 Kn	31.41	1.7± 0.35	2.23± 0.67
9	MS + 1.0 BAP + 1.0 Kn	---	---	---
10	MS + 1.0 BAP + 0.5 Kn+ 1.0 GA_3	46.50	2.7± 0.48	2.63± 0.35
11	MS + 0.5 BAP + 1.0 Kn+ 1.0 GA_3	28.54	1.8± 0.62	2.52± 0.72
12	MS + 1.0 BAP + 1.0 Kn+ 1.0 GA_3	33.14	1.2± 0.55	2.14± 0.43

*Data is from thirty replicates and is represented as mean±SD

Table 5: Effect of plant growth hormones on callus induction after 6 weeks in *A. heterophyllum*

S. No.	MS+Growth hormones (mg/l)	Type of Response in *A. heterophyllum*	Rate of callus induction
1	MS+1.0 2,4-D	No callus	---
2	MS+2.0 2,4-D	Creamy white , friable, small in size	++
3	MS+3.0 2,4-D	Callus initiation but no proliferation	+
4	MS+4.0 2,4-D	Browning of the explant	---
4	MS+2.5 2,4-D+ 1.0 BAP	Callus initiation	+
5	MS+2.5 2,4-D+ 1.0 BAP+1.0 IAA	No callus	---
6	MS+2.0 NAA+0.5 IAA	No callus	---

---- Absent, + Less callus with very slow growth, ++moderate growth

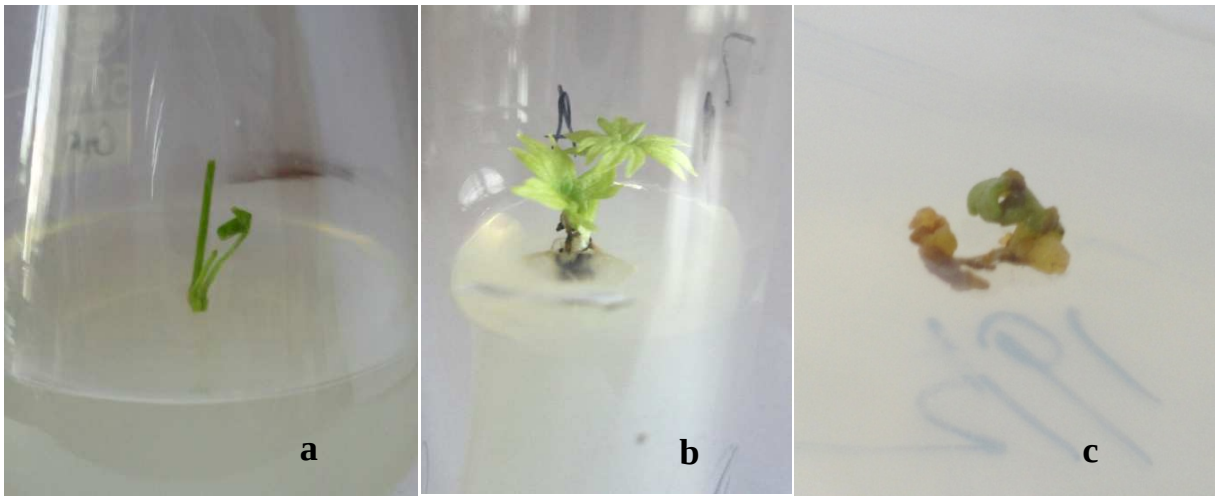

Fig 1: (a) Establishment of explant (b) Shoot multiplication in *A. heterophyllum* in MS medium supplemented with BAP, Kn and GA_3 after six weeks of inoculation (c) Callus induction from leaf explants inoculated on MS supplemented with 2,4-D

CONCLUSIONS

In conclusion, our present investigation shows that micropropagation of *A. Heterophyllum in vitro* is a reliable method for the rapid multiplication and conservation of this endangered medicinal plant. The callus formed from leaf explant can be used for cell suspension and also in creating variations and transformation studies.

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