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Somatic embryogenesis and plant regeneration from callus cultures of *Aconitum heterophyllum* Wall

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Abstract

Plants were obtained via somatic embryogenesis in callus derived from *in vitro* raised leaf and petiole explants of *Aconitum heterophyllum* Wall. Callus was induced on a Murashige-Skoog medium supplemented with either 2,4-dichlorophenoxy acetic acid (2,4-D 1 mg l^{-1}) and kinetin (KN 0.5 mg l^{-1}) with coconut water (CW 10% v/v) or naphthalene acetic acid (NAA 5 mg l^{-1}) and benzylaminopurine (BAP 1 mg l^{-1}). Somatic embryos appeared after 2–3 months or 2 subculture passages when 2,4-D or NAA induced source of the callus was transferred to a MS medium containing BAP (1 mg l^{-1}) and NAA (0.1 mg l^{-1}). For successful plantlet formation, the somatic embryos were transferred to a medium containing 1/4 strength MS nutrient with indole-3-butyric acid (IBA 1 mg l^{-1}). Alternatively, the somatic embryos were dipped in a concentrated solution of IBA for 5 min and placed on a hormone free medium. Complete plantlets were formed after 4 weeks and were transferred successfully to soil.

Introduction

Aconitum heterophyllum Wall., (Ranunculaceae) is an important perennial medicinal herb distributed between 1830 m to 4575 m in the alpine and subalpine regions of the Himalayas. The tuberous roots of this plant contain aconites i.e. atisine, heteratisin and hetasin (Khorana & Murti 1968). The crude white root powder is used as a bitter tonic and febrifuge for controlling debility after fever and diarrhoea. The roots are also used for curing hysteria, throat infection, dyspepsia, abdominal pain and diabetes (Bhatnagar et al. 1948; Satyavati et al. 1976). The tubers of *A. heterophyllum* are collected in large quantities from the natural habitat because of the increase in demand of crude drugs by modern pharmaceutical industries and the resurgence of Ayurvedic and Unani systems of medicines. This indiscriminate exploitation has reduced the species towards rarity, so endangering its survival.

In its natural habitat, *A. heterophyllum* multiplies by seeds as well as through root tubers. However, the seeds require a constant moist and low temperature regime (15°C) for germination and thus bear a high loss of viability. Under these circumstances tissue culture of *A. heterophyllum* assumes importance. Propagation of *A. carmichaeli* has been achieved via shoot tips and embryogenesis from anther derived callus (Hatano et al. 1987, 1988). *A. noveboracense* and *A. napellus* (Cervelli 1987) have been successfully reported from shoot tips

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or axillary buds. The latter two species were also mentioned as threatened species. Thus, as a part of an overall strategy for the conservation of endangered plants enlisted in Indian plant red data book (Jain & Sastry 1984), the present work elucidates the propagation of *Aconitum heterophyllum* via somatic embryogenesis from callus cultures.

Materials and methods

Plants of *Aconitum heterophyllum* Wall. were collected from Milam (3,500 m Pithoragarh, U.P., India) and maintained in earthen pots under controlled environmental conditions (22 ± 2°C; 14: 10 h light: dark regime and 70% relative humidity, rh). Complete shoot tips (0.5 cm in length) and leaves at 10–15 days after emergence were excised from these plants and pre-cleaned with double glass distilled water containing 1% v/v Tween-20 (Sigma, USA) for 5 min. They were surface sterilized with 0.1% $HgCl_2$ for 1 min, rinsed thoroughly 6–7 times in sterile distilled water and inoculated aseptically on Murashige & Skoog's (1962) basal medium containing 3% sucrose and 0.7% agar. The medium (MS) was supplemented with growth regulators singly or in combination (naphthaleneacetic acid, 2,4-dichlorophenoxy acetic acid, 6-benzylaminopurine and kinetin) with concentrations specified in the results, with a minimum of 5 replicates per treatment.

The pH of the medium was adjusted to 5.8 ± 0.1 with 0.1 M NaOH or 0.1 M HCl before autoclaving. The cultures were maintained at 25 ± 2°C under continuous light (3000 lux).

Callus induction and somatic embryogenesis

Shoot cultures obtained from *in vivo* grown excised shoot tips were maintained on MS medium with 0.25 mg l^{-1} BAP. New leaves from such shoots were used as explants for callus induction. Subsequently, callus was induced from these leaf and petiole sections on MS medium supplemented with 2,4-D: KN and CW 10% or on NAA: BAP (Table 1). The callus was subcultured at 6 or 8 weeks depending upon the plant growth regulators used.

Somatic embryos were prepared for scanning electron microscopy by fixing in 3% gluteraldehyde for three h at room temperature, then washing thoroughly with water for 0.5 h. Fixed material was dehydrated in a graded acetone series, critical point dried, mounted on stubs, gold plated and viewed under SEM. For light microscopy the callus was fixed in formalin: acetic acid: 70% ethanol (1:1:8) (Johansen 1940) then dehydrated in ethanol:xylene series and embedded in paraffin wax. Specimens were sectioned at 10 μm and stained with safranin and fast green.

For plantlet formation, somatic embryos were placed on full, half and one fourth strengths of the MS nutrients with varying concentrations of IBA (0.5, 1 and 2 mg l^{-1}). The somatic embryos were also dipped in a concentrated solution of IBA (1 mg ml^{-1}) for varying times and placed on a hormone free MS medium for complete plantlet formation.

In vitro raised plantlets were transferred to earthen pots containing a mixture of soil, vermiculite and farm yard manure (1:1:1) and were maintained under controlled temperature (24 ± 2°C) and humidity (80–90%) in a glass house for hardening.

Results and discussion

Establishment of aseptic shoot cultures

Initially, experiments for the induction of callus used sections of leaves and petioles obtained directly from plants that were maintained in growth chambers. However, early senescence of the explants due to the harshness of the sterilizing procedure inhibited callusing. Subsequently complete shoot tips were excised and aseptically grown *in vitro* on a hormone-free MS medium and on the same medium with 0.25, 0.5, 1 and 2 mg l^{-1} BAP. The explants failed to respond on a hormone-free medium but shoot cultures survived and grew best on a 0.25 mg l^{-1} BAP containing medium to give rise to fresh explants. Thus, shoot tips were routinely subcultured on MS medium with 0.25 mg l^{-1} BAP to obtain material for experiments on callus induction and regeneration.

Induction and maintenance of callus

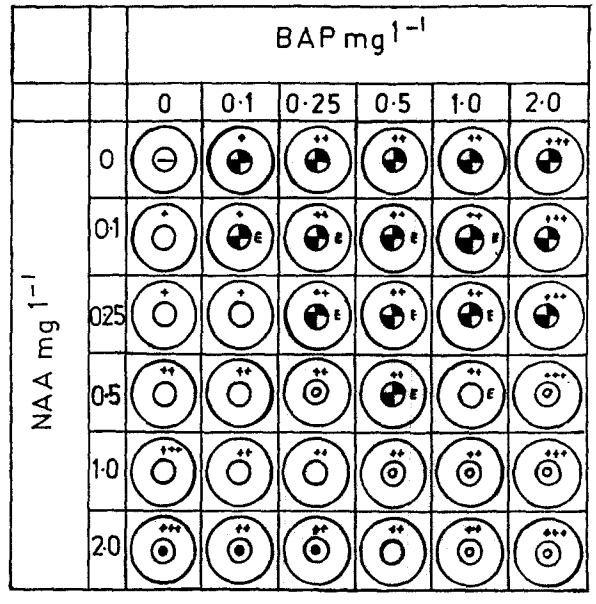
Leaf and petiole segments from shoot cultures were placed on a MS medium supplemented with NAA (0.5, 1, 2, 3, 4 and 5 mg l^{-1}). Swelling was apparent after 4 d in all the NAA containing media and callusing was observed within 12–14 d of culture initiation. 60% of leaf explants and 90% of petiole explants responded to form callus. A cell mass with suppressed root like structures was observed on all the treatment combinations. Thus for inducing a friable callus, BAP was incorporated in the MS medium at 0.5 1 mg l^{-1} in combination with 1, 2, 3, 4 and 5 mg l^{-1} of NAA. Optimal callusing response was obtained on a medium with 5 mg l^{-1} NAA and 1 mg l^{-1} BAP. The subculture interval on this medium was 6 weeks.

Petiole and leaf segments were also placed on MS medium containing 2,4-D (1 mg l^{-1}) and KN (0.5 mg l^{-1}) with or without CW (10%). Friable callus could only be induced when CW was added to the medium. Callus maintenance during subculture was, however, difficult due to excessive leaching of apparent phenolic compounds. The presence of exudate was a general feature of *Aconium* cultures, but the problem was more acute on this hormone combination. Inclusion of antiphenolic substances such as ascorbic acid (50 mg l^{-1}), polyvinylpolypyrrolidone (PVP, 0.5 and 1%) and activated charcoal (2%) did not overcome this problem. Hence, callus induced on 2,4-D supplemented medium was transferred to NAA (0.1–2 mg l^{-1}) containing media. The optimal growth of callus was observed on MS medium with 1 mg l^{-1} of NAA.

The callus obtained on MS medium containing 2,4-D; KN and CW and maintained on 1 mg l^{-1} level of NAA (hereafter referred to as MS1 medium) was first used in plant regeneration experiments. Callus was transferred to MS medium supplemented with NAA (0.1, 0.25, 0.5, 1 and 2 mg l^{-1}) and BAP (0.1, 0.25, 0.5, 1 and 2 mg l^{-1}), and growth responses are summarised in Table 1.

Somatic embryogenesis was evident within a range of NAA:BAP concentrations from 0.1 to 0.5 mg l^{-1} NAA, in combination with 0.1 to 1 mg l^{-1} BAP. An increase in the level of BAP up to 1 mg l^{-1} progressively improved the frequency of somatic embryogenesis. A maximum frequency of 16.6 ± 1.6 visible embryos as observed from callus cultures placed on a MS medium with 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} of BAP by 8 weeks of culture in a 100 ml Erlen-

Table 1. Effect of BAP and NAA combinations on growth of callus cultures in *A. heterophyllum*.



Observations were taken after 8 weeks of culture
⊖ No response
O White callus
◕ Green callus
◎ Pale green callus
◉ White callus with rooting
E Somatic Embryogenesis
+ = Moderate; + + = Good; + + + = Very Good

Table 2. Effect of hormone concentration and combination on the frequency of induction of somatic embryogenesis in callus cultures of *Aconitum heterophyllum*.

Hormone level (mg l^{-1})		Mean number* of somatic embryos per culture in 100 ml flask with standard deviation
BAP	NAA	
0.1	0.1	3.80 ± 0.6
0.25	0.1	8.50 ± 0.5
0.50	0.1	9.60 ± 0.7
1.00	0.1	16.60 ± 1.6
0.25	0.25	4.16 ± 0.6
0.50	0.25	5.33 ± 0.4
1.00	0.25	8.83 ± 1.3
0.50	0.50	9.80 ± 0.9

meyer flask (Table 2). In (120 × 25 mm) culture tubes, 7–10 embryos were visible on the same medium (Fig. 1C). Somatic embryogenesis was confirmed by scanning electron microscopic and histological studies (Fig. 1A–B). Histological studies revealed sequential development from globular to well developed somatic embryos with cotyledons and roots. SEM studies further affirmed the occurrence of somatic embryogenesis. When callus with somatic embryos was sub-cultured on fresh medium profuse secondary embryogenesis was observed (Fig. 1A).

The callus obtained on MS medium with 1 mg l^{-1} BAP and 5 mg l^{-1} NAA responded similarly and produced somatic embryos within 6 weeks of culture. Although profuse embryogenesis was readily induced in the callus culture growth was subsequently arrested. Attempts to overcome this using manipulations of growth regulator levels or osmotica proved unsuccessful, but when

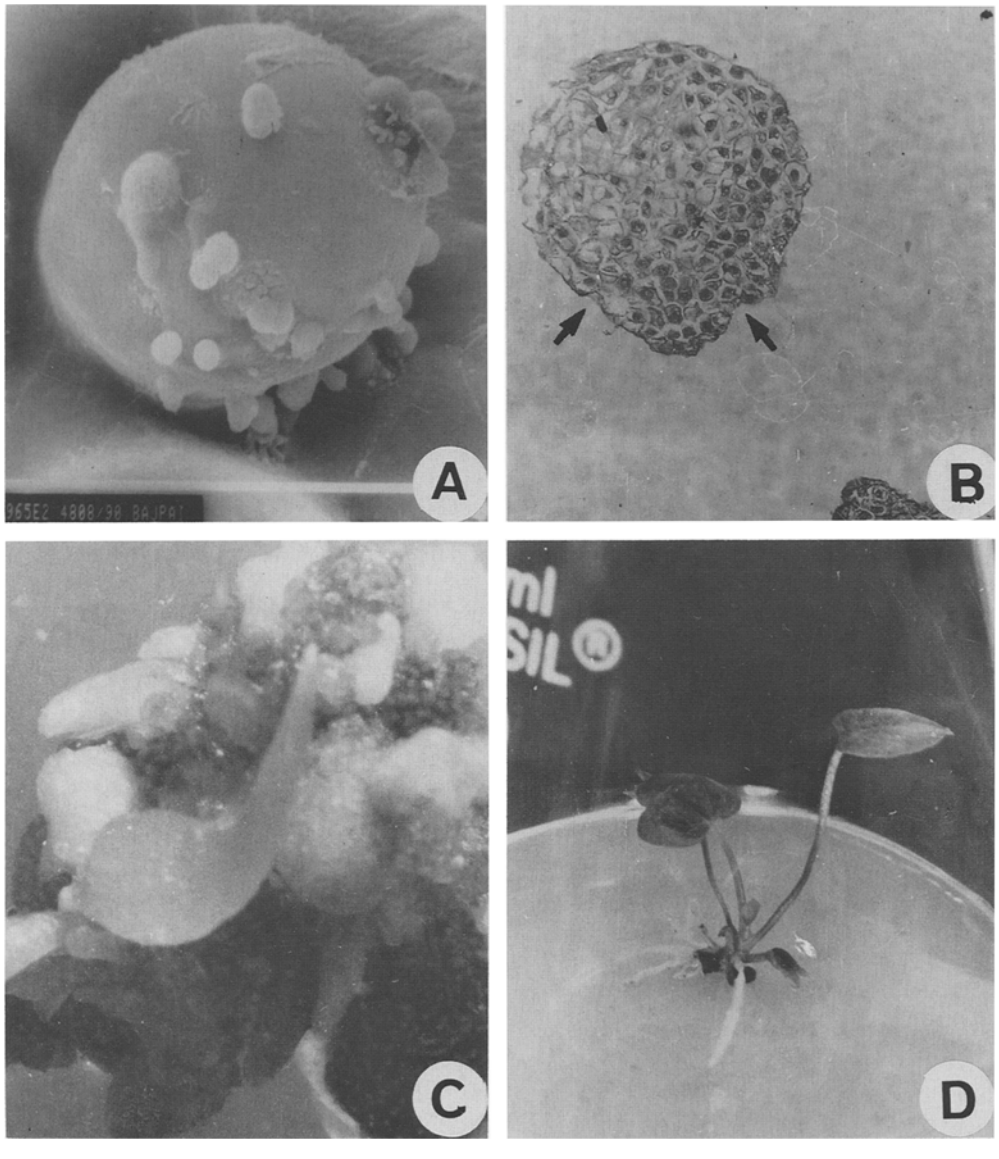


Fig. 1. (*A*) Somatic embryo showing secondary somatic embryogenesis, ×825. (*B*) LS of somatic embryo with cotyledon primordia initiation (arrow), ×324. (*C*) Close up of a somatic embryo, ×16.3. (*D*) complete plantlet from somatic embryo, ×.75.

somatic embryos were placed on full, one-half, one-fourth strengths MS medium and doubled glass distilled water, gelled with 0.7% agar supplemented with 1 mg l^{-1} IBA, 80% of the somatic embryos formed roots on one-fourth strength MS nutrient although the growth of these plantlets was very slow. When after 3 weeks these plants were again cultured on a hormone-free (full strength) medium the plantlets showed vigorous growth. Alternatively, somatic embryos were dipped in a 1 mg ml^{-1} IBA solution for 5 min and cultured on a hormone free medium. This resulted in high frequency (60%) plantlet formation and was subsequently adopted, routinely followed, because this procedure requires only one step for root induction and plantlet development thus reducing time required for complete plantlet formation.

Establishment of plants in soil

Plantlets were transferred to pots containing a mixture of soil, vermiculite and farmyard manure (1:1:1) and successfully hardened at 25 ± 2°C, 90–95% rh and 10–15 d under glass house conditions.

The initial success of hardening of the plantlets was only to an extent of 25%, however, upon altering the soil mix to leaf compost, maintaining a relatively lower temperature of 20°C and observing general precautions against infections the percentage establishment was raised to 50%. The hardening phase solicits attention for further improvement.

Thus from the above mentioned experiments a protocol emerged that give rise to repeatable induction of somatic embryogenesis leading to plantlet development and plants capable of establishing under controlled environmental conditions. A minimum of 27 weeks was required to obtain plants via somatic embryogenesis from petiole or leaf derived callus (Fig. 2). This embryogenic callus has retained its morphogenetic potential even after 2 years of its initiation. These somatic embryos develop into complete plantlets. (Fig. 1D).

The present study with *Aconitum heterophyllum* Wall. provides an alternative morphogenetic pathway leading to plant propagation of this generally threatened but economically

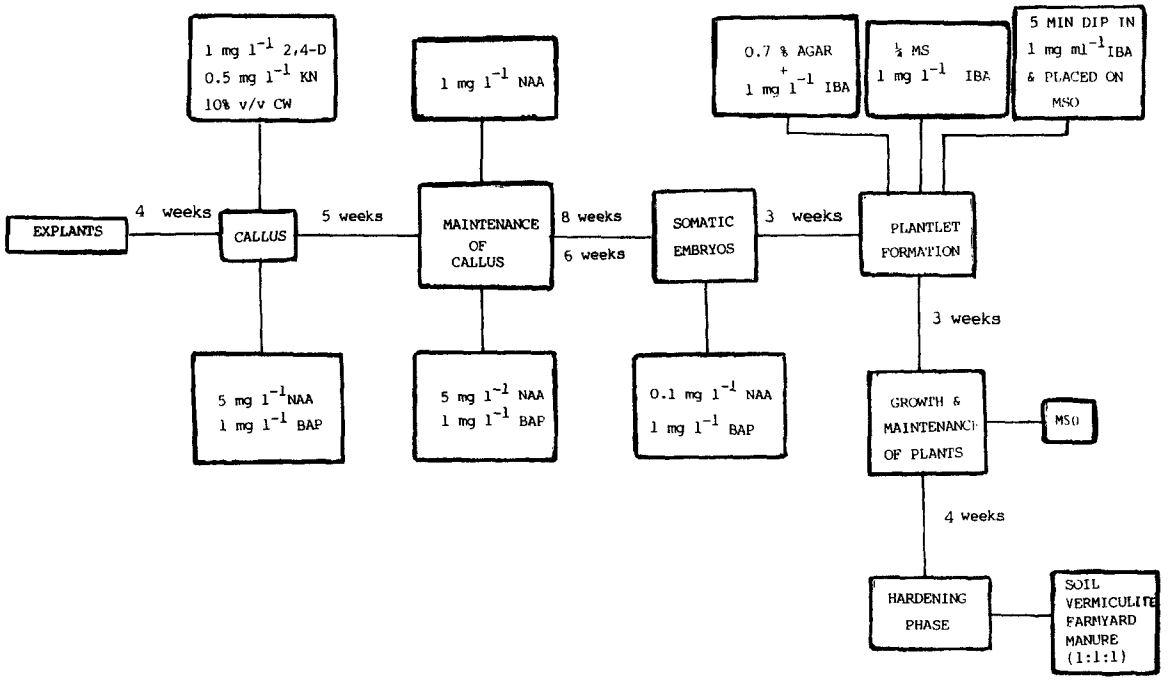


Fig. 2. A 27 week schedule from explant to plantlet in field via somatic embryogenesis in *A. heterophyllum*.

important species. The procedure of obtaining plantlets from somatic embryos by using low or no salts in the culture medium with 1 mg l^{-1} IBA or by dipping the embryos in a concentrated solution of IBA (1 mg ml^{-1}) is important as an approach to propagation via callus induced somatic embryogenesis. Similar studies in medicinal plant species namely *Foeniculum vulgare* (Miura et al. 1987) and *Bupleurum falcatum* (Hiraoka et al. 1986) have shown that the coefficient of variation between plants obtained via embryogenesis was lower when compared with plants obtained from seeds. Further, the productivity in terms of quantity as well as quality of secondary metabolites was enhanced in plants derived from somatic embryos. It normally takes 2–3 years of growth before *Aconitum heterophyllum* plants are harvested for extraction of aconites. Hence, the details on comparative performance are not yet available, however, considering the published results the present work bears relevance in the asexual propagation of the endangered plant species for higher productivity.

Further, the work shows that even petiole and leaf explants are capable of generating somatic embryogenesis from callus in *Aconitum heterophyllum* because it has been earlier reported that only anthers of *Aconitum carmichaeli* could form somatic embryos.

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