In Vitro *Propagation and Phytochemical Assessment of* Aconitum ferox *Wall: A Threatened Medicinal Plant of Sikkim Himalaya*

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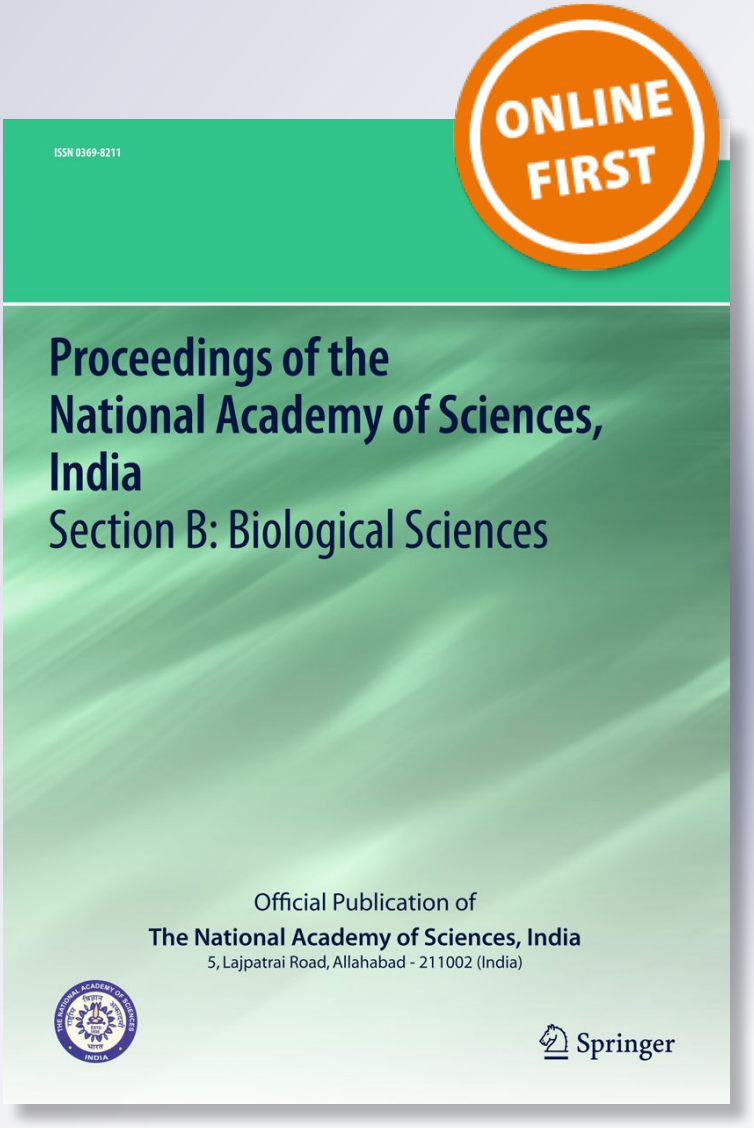

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RESEARCH ARTICLE

In Vitro Propagation and Phytochemical Assessment of *Aconitum ferox* Wall: A Threatened Medicinal Plant of Sikkim Himalaya

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Abstract This is the first report on *in vitro* propagation and phytochemical assessment of *Aconitum ferox* (Ranunculaceae), a threatened medicinal plant of Sikkim Himalaya. A simple and efficient *in vitro* propagation protocol through indirect shoot organogenesis has been established for *A. ferox* using root tip explants. Murashige and Skoog (MS) medium supplemented with 2.26 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) was found to be the best medium to induce and maintain the callus. Different concentrations and combinations of plant growth regulators (PGRs) were tested for *in vitro* shoot proliferation. Adventitious shoots were produced from callus when it was transferred to MS medium supplemented with N^6-benzylaminopurine (BAP). The synergistic incorporation of 3 µM indole-3-acetic acid (IAA) in 6 µM BAP containing medium-induced plantlets with well-developed roots and shoots. However, the best rooting responses were observed in shoots placed on paper bridge in liquid MS medium supplemented with 3 µM IAA + 6 µM BAP. The plantlets were successfully acclimatized in *ex vitro* conditions with 70% survival rate. Additionally, root extract of *in vitro*-raised plants showed antioxidant activity closer to wild plants. The study thus signifies the effectiveness of *in vitro* technique for *A. ferox* propagation and provides a method for sustainable utilization of this high-value medicinal plant at commercial scale in pharmaceutical industries.

Keywords Aconite · Threatened medicinal plant · Explant · *In vitro* propagation

Significance Statement This is the first report on *in vitro* propagation and phytochemical assessment of *A. ferox*. Study provides a sustainable technique for germplasm conservation and commercial cultivation of this threatened and high-value medicinal plant of Himalaya.

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Introduction

Indian Himalaya is known for its rich diversity of high-value medicinal plants; unfortunately, owing to unsustainable extraction pressures to meet the growing pharmaceutical demands for raw materials, many of these species are being threatened [1, 2]. Sikkim, located in the eastern part of Indian Himalaya, is not spared of this pressure and many medicinal plants of the state have been experiencing endangerment [3]. One of the highly used medicinal plant genus of Sikkim is the *Aconitum* (family: Ranunculaceae; common names: monkshood, aconites). It is used by ethnic communities of Sikkim to treat and cure various ailments. In view of its high medicinal value and decreasing population, the Indian Export-Import Policy

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2002-07 prohibited the export of plants, plant portions, their derivatives and extracts of any *Aconitum* species (http://www.forests.tn.nic.in/EximPolicy.htm; accessed on 22.10.2006), which highlights the conservation value of the genus. *Aconitum* genus comprises of 300 species, of which around 33 species are found distributed in the alpine and subalpine zones of Indian Himalaya from Kashmir to Uttarakhand and extending to the hills of Assam [4]. Out of them, 13 species showed promising medicinal properties [5]. Underground parts (root/tubers) of *Aconitum* are the source of natural neurotoxic alkaloid 'aconitine,' which attributes to the medicinal properties of the plant [6]. The genus has been used to treat tonsillitis, sore throat, gastritis and debility from antiquity and possesses analgesic, antioxidative and anti-inflammatory properties [7]. *Aconitum ferox,* also known as Indian aconite, locally named as Bikh and Bish in Sikkim, is a rhizomatous poisonous herb, but has great medicinal properties when used after vigorous purification in right amounts with certain constituents. The roots/tubers/rhizomes of this perennial plant are used in Tibetan medicine, Ayurveda, tribal and folk medical system [8, 9]. It is an important constituent of 'Swasabhairava Rasa,' 'Mahamrutyunjaya Rasa' and 'SankhVati' that are used for curing and treating diseases like asthma, constipation, fever, hyperacidity, ulcer, piles, fistula and hemorrhoids. Phytochemical studies revealed that the species is rich in active ingredients such as bikhaconite, pseudaconine, 14-O-acetylsenbusin A, lipobhikhaconitine, veratroylpseudaconine and pseudaconitine [10, 11]. The everyday growing popularity of *A. ferox* in both indigenous and modern system of medicine has generated a pressure on the plant. The commercial scale extraction of *A. ferox* from nature has pushed this species toward vulnerability [2, 12]. Furthermore, seed germination rate is very slow in *A. ferox* [1]. Though, *in vitro* propagation protocols have been developed for some *Aconitum* species viz. *A. balfourii, A. carmichaelii, A. heterophyllum, A. napellus, A. noveboracense* and *A. violaceum* [6, 13–17], but no such attempts have been made for *A. ferox*. Moreover, *A. ferox* has never been analyzed for its antioxidant activity. Considering the threatened status and excellent medicinal values of *A. ferox*, the objective of the present study was to develop an efficient micropropagation protocol for large-scale propagation of *A. ferox* from *in vitro*-developed root and comparative assessment of *in vitro* and wild plants for antioxidant activity. As far as the literature is concerned, it appears that this work is the first report regarding the *in vitro* culture of *A. ferox*, which could bring new avenues for the sustainable utilization of this species and re-evaluation of its conservation status.

Material and Methods

Plant Material

The seeds of *A. ferox* were collected from Tsomgo population, East Sikkim, India, and brought into the laboratory at the G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Sikkim Regional Centre, Pangthang, East Sikkim (longitude 27°4′46″ to 28°7′48″ North, and latitude 88°00′55″ to 88°55′25″ East, with an elevation of 2087 m asl). Seeds were first tied in a muslin cloth and washed under running water for 30 min followed by three times rinsing with distilled water. After washing, seeds were subjected to 10 min treatment of surfactant (Tween-20). The seeds were then sterilized with 0.5% fungicide (bavistin) for 30 min and 0.1% surface sterilant (mercuric chloride) for 5 min; both the treatments were followed by four times rinsing with sterile distilled water.

Pre-Sowing-Treatments and Explant Selection

The sterilized seeds (approx. 600 in numbers) were pre-treated with plant growth regulators (PGRs): indole-3-acetic acid (IAA), N^6-benzylaminopurine (BAP), gibberellic acid (GA_3) at 50, 100, 150 μM concentration and placed in dark for (8–10 h) at 24 °C. The pre-treated seeds were cultured in PGR-free MS medium [18] supplemented with 3% sucrose and solidified with 0.8% agar. Data related to seed germination were recorded after four weeks of culture. Seeds with radicle emergence of ∼ 2 mm were considered germinated *in vitro* (Fig. 1a). Initially, leaf (5 mm^2 size), nodal and root tip (∼ 1 cm) explants obtained from five-week-old plants were selected to develop propagation protocol for *A. ferox*. As leaf and nodal explants have not shown any response, they were abandoned to pursue further in this study.

Indirect Shoot Organogenesis and Culture Conditions

The MS medium supplemented with varying combinations and concentrations of auxins and cytokinins, including IAA, 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), BAP, 2-isopentenyl adenine (2-iP), N^6- furfuryladenine (KN) and thidiazuron (TDZ), was used to develop propagation protocol from the root explants. All media contained 3% sucrose and solidified with 0.8% agar, except the liquid media used during the *in vitro* rooting which lacks agar. After adjusting the pH to 5.8 ± 0.05 with 0.1 N NaOH or 0.1 N HCL, 60 ml medium was dispensed into 250-ml Erlenmeyer flasks. These

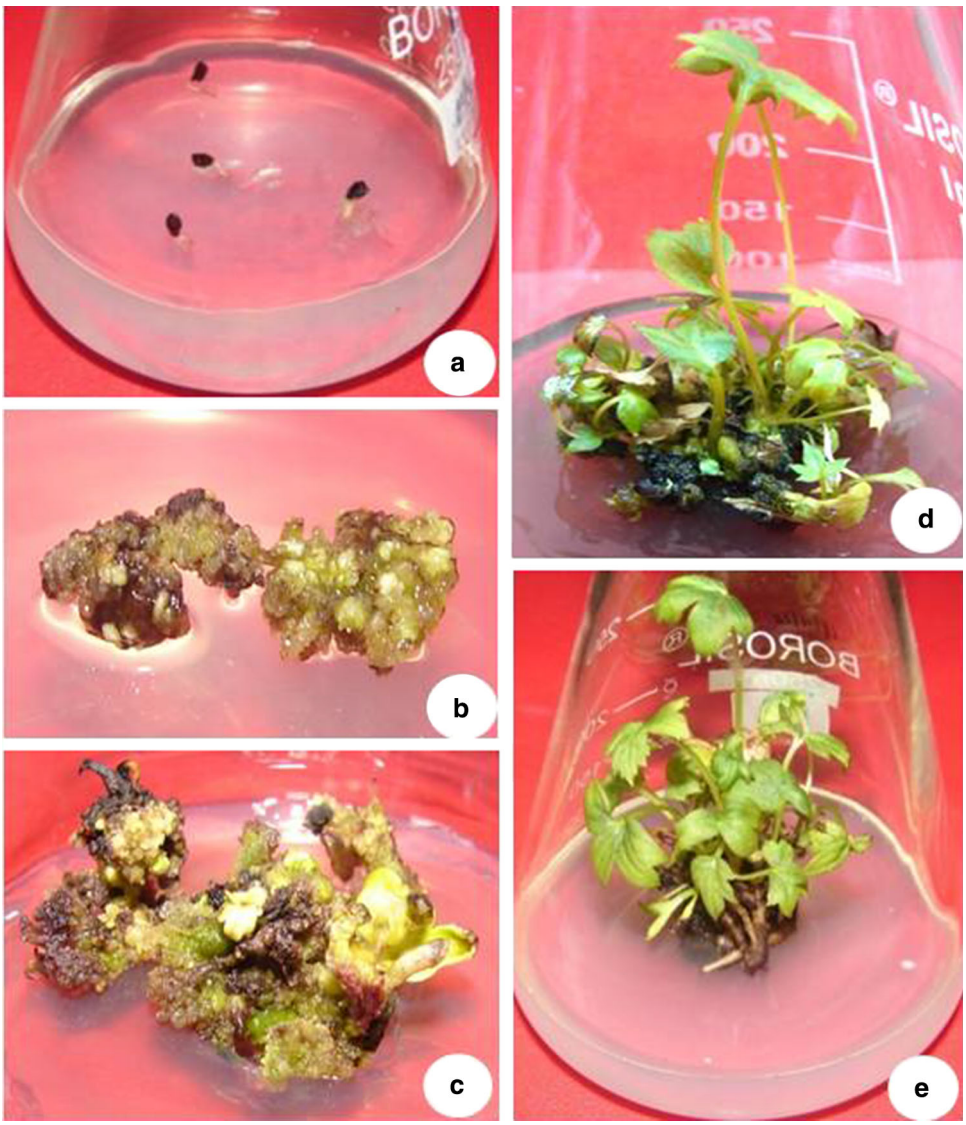

Fig. 1 **a** *In vitro* root emergence from pre-treated *A. ferox* seeds, germinated on MS basal medium, after four weeks **b** *in vitro* callus initiation from roots in MS + 2, 4D (2.26 µM) after six weeks of culture **c** shoot differentiation after two weeks of culture in MS + BAP (6 µM) **d** emergence of multiple shoots from callus in MS medium + BAP (6 µM) after six weeks of culture **e** signs of rooting in MS + BAP (**6** µM) + IAA (3 µM) after eight weeks of culture in the medium

flasks were plugged with non-absorbent cotton before autoclaving at 15 psi and 121 °C for 15 min. The cultures were maintained at 23 ± 2 °C temperature with 50–60% relative humidity and 16/8 h (light/dark) photoperiod provided with diffuse light (1000–2000 lx). All the chemicals used in the present study are of analytical grade and purchased from HiMedia, Pvt. Ltd., Mumbai, India. A total of twenty-four explants were cultured in each treatment. The experiments were repeated three times. Observations were recorded at weekly intervals and subcultured regularly into fresh medium at six-week intervals.

In vitro Rooting, Acclimatization and Hardening

For *in vitro* rooting, proliferated shoots (> 2 cm) of *A. ferox* were transferred in different media such as: MS basal liquid medium, MS medium with different percentages of sucrose, MS liquid medium supplemented with indole-3-butyric acid (IBA) and/or NAA and MS liquid medium supplemented with combination of both auxin (IAA) and cytokinin (BAP) (Table 2). To support plantlets in liquid medium, paper bridges were prepared using Whatman filter paper no.1 (Fig. 2a). Rooting response data were recorded after twelve weeks of culture in rooting medium.

Rooted plants were washed with sterile distilled water, then dipped in bavistin (0.15%, w/v; 20 min) and then planted in thermocol pots (125 ml) containing peat moss and autoclaved soil (1: 3). These pots were covered with transparent polybags with random holes to maintain its humidity. These potted plants were maintained at 23 ± 2 °C temperature with 50–60% relative humidity and a 16/8 h (light/dark) photoperiod provided with diffuse light (1000–2000 lx). After four weeks, well-developed plants with new leaves were transferred to pots containing

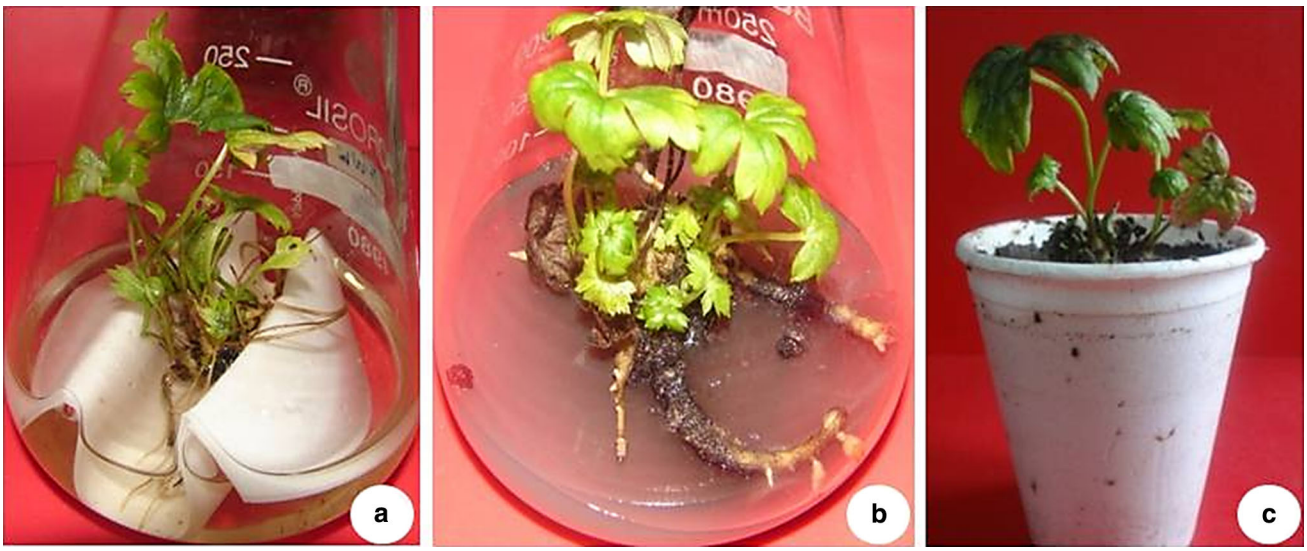

Fig. 2 **a** *In vitro* rooting of *A. ferox* in liquid MS medium supplemented with BAP (6 μM) + IAA (3 μM) after twelve weeks of culture **b** rooting of *A. ferox* in solid MS + BAP (6 μM) + IAA (3 μM) after twelve weeks of culture **c** well-acclimatized (four weeks) plantlet of *A. ferox* ready to field transfer

normal garden soil and maintained in greenhouse under 80% humidity and 25 °C temperature.

Extract Preparation

The roots of *A. ferox,* collected from wild (Tsomgo population), East Sikkim, and three-week-old *in vitro* growing plants were washed thoroughly under running tap water and then with distilled water to remove the traces of soil and agar. Thereafter, washed materials were dried at 40 ± 2 °C in hot air oven (MAC, India) until a constant weight was achieved. The dried plant samples were crushed using grinding mill to fine powder. The powder samples were packaged in polythene bags and kept in a cool dark place for further experimentations. To prepare the plant extract, 2 g of powdered sample was taken in a conical flask and soaked in 10 ml methanol solution for 24 h. The supernatant was transferred into a new tube, and the residue was re-extracted twice with 10 ml solvent. Extracts were filtered using Buchner funnel and Whatman no.1 filter paper. The accumulated filtrate was concentrated through drying in hot air oven at 40 °C. The concentrated extract was re-suspended in methanol to yield a 50 mg/ml stock solution.

Estimation of Total Phenolic Content (TPC)

The TPC was determined using Folin–Ciocalteu method [19]. Briefly, the plant extracts allowed to react with Folin–Ciocalteu reagent (FCR) and sodium carbonate (Na_2CO_3). In this spectrophotometric assay, the absorbance of the reaction mixture was measured at 760 nm. Gallic acid was used as a standard, and TPC of plant extracts was expressed in milligrams gallic acid equivalents (mg GAE/g extract).

Estimation of Total Flavonoid Content (TFC)

The TFC was determined by aluminum chloride ($AlCl_3$) calorimetric method, with some modifications [20]. Briefly, the individual test samples were dissolved in methanol, and 2 ml sample solution was properly mixed with 2 ml solution of 2% $AlCl_3$. After 10 min of incubation at ambient temperature, the absorbance of the solution was measured at 435 nm by spectrophotometer. TFC was expressed as milligram quercetin equivalent (mg QE/g extract).

Estimation of Antioxidant Activity

The antioxidant activity of plant extracts was compared using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2-azinobis-3-ethylbenzothiazoline-6 sulfonic acid (ABTS) radical scavenging assays. The effect of plant extracts on DPPH radical scavenging was determined using 0.135 mM methanolic DPPH solution. The absorbance of the mixture was measured spectrophotometrically at 517 nm, and radical scavenging activity was determined using following formula:

$$\text{DPPH radical scavenging activity}(\%) = [(\text{Abs control} - \text{Abs sample})]/[(\text{Abs control}) \times 100.$$

where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + extract/standard.

The 2, 2-azinobis-3-ethylbenzothiazoline-6 sulfonic acid radical scavenging assay was done by preparing stock solution mixing equal quantities of ABTS (7 mM) and ammonium persulfate [21]. The working solution was then prepared by diluting the previous solution with methanol until the absorbance at 734 nm was 0.706 ± 0.02. Fresh ABTS solution was prepared for each assay. Plant extracts (1.5 ml) were allowed to react with equal amount of ABTS working solution, and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity was estimated using the following formula.

$$\text{ABTS radical scavenging activity}(\%) = [(\text{Abs control} - \text{Abs sample})]/[(\text{Abs control})] \times 100.$$

where Abs control is the absorbance of ABTS radical + methanol; Abs sample is the absorbance of ABTS radical + extract/standard.

The radical scavenging activity of the extracts was determined by IC_{50} value. IC_{50} value is the concentration of extracts at which DPPH/ABTS radicals are scavenged by 50%. The lowest IC_{50} value indicates higher radical scavenging capacity and vice versa. Butylated hydroxytoluene (BHT) was used as standard.

Statistical Analysis

All the investigated parameters were analyzed using analysis of variance (ANOVA) and significance was determined at $p < 0.05$. The data were analyzed statistically using SPSS (version 16) software, and the significant differences among the mean values were assessed on the basis of the Duncan's multiple range test. Each experiment was repeated three times.

Results and Discussion

Indirect Shoot Organogenesis

To acquire rapid germination of *A. ferox*, various pretreatments were tested. Seeds treated with IAA (100 µM) were found to be germinated within four weeks of inoculation on the MS basal medium, while seeds under other treatments remained dormant and did not germinate. It is reported that genus *Aconitum* possesses the seed dormancy [6] and pre-sowing treatments would help in breaking of dormancy and initiate faster germination. For *in vitro* regeneration of plants, root explants were cultured on MS basal medium variously supplemented with growth regulators. MS basal medium alone did not show any response. Of the various treatments tested, non-regenerative callus was obtained on MS + 2, 4-D (2.26 µM). On this medium, after eight weeks, 73% root explants showed development of brownish-green hard callus (Fig. 1b, c). These calluses were multiplied on the same medium with frequent subculture in every six-week intervals. However, the callus remained in the non-regenerative state. To achieve shoot organogenesis, callus was transferred in different sets of media combinations. Among individual cytokinin/auxin treatments, BAP and 2-iP had induced shoot organogenesis from the callus. No shoot organogenesis occurred from callus cultured on MS medium containing KN and TDZ or any of the auxins viz. 2, 4-D, NAA or IAA at tested concentrations.

It was observed that 2-iP (25 µM) induced shoot proliferation in 45.83% cultures, but after 4–5 weeks of culture, the texture of the callus changed ultimately leading to necrosis of the plantlet. Callus cultured in different concentrations of BAP showed multiple shoot development with no change in the callus texture. Shoots developed in all BAP-supplemented medium, but 6 µm of BAP was found to be the best in terms of percent response (91.67%). Shoots developed in this medium were healthy, green and leafy (Fig. 1d). The frequency of shoot proliferation increased with an increase in BAP concentrations (2–6 µM; 52.78–91.67%), while further increase in BAP concentration declined the shoot proliferation rate up to 13.89% (Table 1). When calli were transferred in medium containing different concentrations of IAA, it had little or no effect on the shooting of *A. ferox,* but after 3–4 weeks of culture, it was observed that small whitish root-like projections were developed. Further, combined effect of cytokinin and auxin was evaluated. NAA (1.0 and 5.0 µM) was added in the BAP-containing medium as this combination induced full plantlets development in *A. balfourii* from leaf and bud explants [22]. But, in the present study, this combination did not induce shoot organogenesis from *A. ferox*. In comparison with BAP + NAA, incorporation of IAA in BAP-containing MS medium enhanced the number of shoot buds per explants after 10–12 weeks of culture. The rooting of the plant was also witnessed in the same media (Fig. 1e). The best response was obtained on MS medium supplemented with BAP (6 µM) + IAA (3 µM) where the plantlets developed with healthy shoots.

In vitro propagation protocols have been developed for other *Aconitum* species viz. *A. balfourii* [22], *A. carmichaelii* [15], *A. heterophyllum* [16, 23], *A. napellus* [14], *A. noveboracense* [14], *A. violaceum* [6, 17] but, so far, no attempt for micropropagation of *A. ferox* has been made. Generally, most of the *Aconitum* species micropropagated through callus formation in MS medium supplemented with 2, 4-D and medium having auxin and cytokinin combination supported shoot and root proliferation [16, 23]. Similar results were seen in the present study

Table 1 Effect of various growth regulators on shoot bud proliferation from root callus

Sl no.	Regeneration treatments	Response	
		Percent response	Change in callus texture and shoot bud differentiation
1	MS + 2-iP (10 μM)	4.17 ± 0.00 gh	No significant change in the callus texture
2	MS + 2-iP (15 μM)	11.11 ± 4.81 fg	Very few number of shoots (1-2) with limited number of leaves developed. No change in callus texture and color
3	MS + 2-iP (20 μM)	19.44 ± 2.41 e	Few shoots developed but darkening of the callus was observed
4	MS + 2-iP (25 μM)	45.83 ± 4.17 c	3-5 shoot buds differentiated but callus became dark brown. After three weeks of culture, the shoots and callus became friable and showed signs of necrosis
5	MS + BAP (2 μM)	52.78 ± 4.81 c	Healthy shoots (1–2) with leaves formed in this media. No change in callus texture and color
6	MS + BAP (4 μM)	87.50 ± 8.33 a	Profuse shoot bud differentiation was observed with no change in the texture of callus
7	MS + BAP (6 μM)	91.67 ± 7.21 a	Almost from whole callus shoot bud differentiation initiated. The shoots were green, healthy and leafy. No change in the texture of callus.
8	MS + BAP (8 μM)	65.28 ± 2.41 b	The developed shoots were green, healthy and leafy. No change in the texture of callus
9	MS + BAP (10 μM)	31.94 ± 4.81 d	The shoots were green, healthy and leafy. No change in the texture of callus
10	MS + BAP (12 μM)	13.89 ± 2.41 ef	1-2 Shoot buds differentiated from the callus with no change in the texture of callus
11	MS + IAA (1 μM)	5.56 ± 2.41 gh	Root organogenesis was observed
12	MS + IAA (2 μM)	5.70 ± 1.25 gh	Root organogenesis was observed
13	MS + IAA (3 μM)	5.00 ± 0.00 gh	Root organogenesis was observed
14	MS + BAP (6 μM) + IAA (3 μM)	69.44 ± 6.36 b	Almost from whole callus shoot bud differentiation observed. 5–7 Healthy shoots with roots were developed
15	MS + BAP (5 μM) + IAA (10 μM)	66.67 ± 4.17 b	3–5 Healthy shoots with roots were differentiated from the callus
16	Control	0.00 i	–

Mean values within a column sharing the same letter do not differ significantly ($p < 0.05$) according to Duncan's multiple range test. The data were recorded after eight weeks of culture. Control: MS basal medium

where micropropagation of *A. ferox* is achieved through callus formation in MS medium supplemented with 2, 4-D, and BAP alone or combination of BAP + IAA has induced shoot proliferation from the callus. At this point, it can be presumed that majority of *Aconitum* species multiply through indirect organogenesis and BAP is the best suited PGR for shoot growth.

The type and concentration of PGR used in the culture medium, interaction between endogenous and exogenous concentrations of hormones and cross talk between PGRs particularly auxin and cytokinin play pivotal role in determining the fate of cell in specific tissues. The pioneering work has shown that high and low ratios of auxin/cytokinin promote root and shoot development, respectively [24]. Moreover, requirement of auxin for triggering dedifferentiation in somatic tissue which further leads to callus development has been well documented. It has been reported that auxin pre-treatment leads to the upregulation of a cytokinin receptor gene *AHK4* expression during the callus formation [25]. Requirement of auxin along with cytokinin for obtaining optimal response of shoot bud differentiation is reported in a number of other plants including *Spilanthes acmella* [26], *Quercus serrata* [27], *Berberis aristata* [28], *Ficus religiosa* [29] and *Notopterygium incisum* [30].

In vitro Rooting, Acclimatization and Hardening

During shoot multiplication, it was already established that IAA favored rooting in *A. ferox*. Incorporation of IAA in BAP-containing medium induced complete plantlets with well-developed root systems, after twelve weeks of culture. The best rooting response was obtained in liquid MS + BAP (6 μM) + IAA (3 μM) medium with 91.67% rooting response (Table 2; Fig. 2a). However, the roots produced in similar solid medium were thick and dark brown in color with only 11.70% rooting response (Table 2; Fig. 2b). The *in vitro*-raised shoots were also transferred in MS media containing different concentrations of IBA and sucrose. In comparison with IAA + BAP-supplemented MS medium, IBA (1 μm) containing MS medium induced rooting but these were thin and fibrous, and the plant growing on these

Table 2 Effect of growth regulators and sucrose concentrations on *in vitro* rooting of *A. ferox* microshoots

Sl no.	Treatments	Rooting response (%)
1	MS + IBA (1 μM)	43.33 ± 5.77 b
2	MS + IBA (3 μM)	20.00 ± 5.00 c
3	MS + NAA (1 μM)	3.33 ± 2.89 e
4	MS + NAA (3 μM)	5.00 ± 0.00 de
5	MS + BAP (6 μM) + IAA (3 μM)	91.67 ± 2.89 a
6	MS + BAP (5 μM) + IAA (10 μM)	86.67 ± 7.63 a
7	Control	11.70 ± 1.80 d
8	MS + Sucrose (1%)	5.00 ± 0.00 de
9	MS + Sucrose (3%)	1.67 ± 0.49 e
10	MS + Sucrose (6%)	2.0 g ± 0.42 e

Mean values sharing the same letter do not differ significantly ($p < 0.05$) according to Duncan's multiple range test. All the microshoots were cultured on liquid MS medium, except control, the data were recorded after twelve weeks of culture. Control: MS medium supplemented with BAP (6 μM) + IAA (3 μM) and solidified with 8 % agar

medium shriveled turning brown and lost its shoots, whereas IAA + BAP-supplemented medium supported not only healthy rooting but also profuse shooting with no signs of necrosis. The rooted plants of *A. ferox* were successfully transplanted in to the greenhouse with 70% survivability rate (Fig. 2c). Elsewhere, the *in vitro* rooting in various *Aconitum* species such as *A. heterophyllum* was obtained in the MS medium supplemented with 4.90 μM IBA [16], whereas 100% *in vitro* rooting in *A. heterophyllum* was observed in MS medium supplemented with 5.71 μM IAA [23]. In *A. carmichaelii,* a medicinal species indigenous to China, the optimum rate of root formation was achieved in MS medium supplemented with 2.85 μM IAA [15]. It is perceived that IAA supports *in vitro* rooting and as BAP is best suited for shoot multiplication, optimizing the culture in the combination media of IAA + BAP would lead to formation of full healthy plantlets of *A. ferox*.

TPC and TFC of *In Vitro* Raised and Wild Plants

The extensive works, which have been done in a number of medicinal and edible plants all over the world, establish that the phenolics and flavonoids are the principal secondary metabolites responsible for plant's antioxidant property [31]. It is therefore essential to analyze plant extracts for total phenolic, total flavonoid contents and antioxidant activity. In the previous study (data not shown) conducted by the authors, different parts viz. leaf, stem and root of wild *A. ferox* plants were analyzed for TPC, TFC and antioxidant activity. Analysis results indicated that root

Table 3 TPC, TFC and antioxidant activity of *in vitro* and wild *A. ferox* plant's root

	In vitro	*Wild*
DPPH scavenging activity (IC_{50}, μg/ml)	833 ± 19.31 a	579 ± 8.54 b
ABTS scavenging activity (IC_{50}, μg/ml)	587 ± 9.84 a	481 ± 14.73 b
TPC (mg GAE/g extract)	18.52 ± 1.55 b	454.66 ± 1.52 a
TFC (mg QE/g extract)	5.06 ± 0.25 b	45.83 ± 2.66 a

Means in each row followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

is the main reservoir of phytochemicals in *A. ferox*. Therefore, in this comparative study, only roots of wild and *in vitro* plants were analyzed for TPC and TFC. The results of phenolic and flavonoid content of wild vs *in vitro* plant extracts are given in Table 3. The authors observed that wild plant's root contained significantly ($p < 0.05$) higher amount of TPC (454.66 ± 1.52 mg GAE/g) and TFC (45.83 ± 2.66 mg QE/g) than *in vitro* grown plant's root. The higher TPC and TFC of wild plants can be attributed to the stress conditions (high elevation; UV radiation; extreme cold winters) which these plants have faced in wild condition. The increasing radiation markedly increases total phenolic synthesis, because UV radiation causes strong universal induction of phenyl ammonia-lyase, the enzyme responsible for phenolic synthesis [32].

Antioxidant Activity of *In Vitro* and Wild Plant

To fully characterize antioxidant properties of *in vitro* and wild *A. ferox* roots, two *in vitro* assays viz. DPPH and ABTS that are based on different reaction mechanisms were used. The results of the antioxidant assays revealed that the wild plant methanolic extract has comparatively higher radical scavenging activity with IC_{50} values of 579 μg/ml and 481 μg/ml in DPPH and ABTS assays, respectively (Table 3). Nevertheless, *in vitro* root methanolic extract has shown low TPC and TFC values than wild plant extract, yet it has exhibited good antioxidant activity with IC_{50} values of 833 μg/ml and 587 μg/ml in DPPH and ABTS assays, respectively, suggesting that there could be some other phytocompounds present in the plant which has radical scavenging property. The phytochemical compounds found in plants are reported to have multiple biological effects, among which one such is the ability to scavenge free radicals resulting to its antioxidant activity. The genus *Aconitum* contains numerous phytochemical constituents like alkaloids, phenols, flavonoids and glycosides, but only some of its species are assessed for their antioxidant activity. Earlier, two *Aconitum* species *viz. A. heterophyllum* and *A. kashmiricum* have been

investigated for antioxidant property and their IC_{50} values were 57.36 μg/ml and > 1000 μg/ml, respectively [33, 34].

Conclusion

The present study first time reports (i) the *in vitro* propagation procedure and (ii) antioxidant potential of *in vitro* grown *A. ferox,* a threatened medicinal plant of Sikkim Himalaya. *A. ferox* is a habitat-specific species, which is hard to grow under *ex situ* habitats using conventional methods. In this study, an efficient micropropagation protocol has been established, with 70% survival rate, using root explants of *in vitro*-germinated plantlets. Additionally, *in vitro* plant root extract showed good antioxidant activity. The study indicates that other than phenols and flavonoids there are some compounds present in the roots which are responsible for antioxidant activity in *A. ferox*. Therefore, further intensive study is required to identify these compounds. Based on the results, this study promotes *A. ferox* as a source of natural antioxidant with firm belief that this propagation protocol will help in sustainable harnessing of the pharmaceutical potential of this high-value species of the Himalaya.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest to publish this manuscript.

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