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Floral Biology of *Aconitum heterophyllum* Wall.: A Critically Endangered Alpine Medicinal Plant of Himalaya, India

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Abstract: *Aconitum heterophyllum* Wall. is a critically endangered wild medicinal herb of alpine Himalaya and cultivation is recommended owing to its large demand in the herbal market and to ensure the conservation of wild habitats. Therefore, observations on floral biology, pollen germination, pollination, and fruit and seed setting after implying different breeding systems were carried out for its successful domestication and improvement in cultivation practices. The study reveals that the plants grown in hothouse conditions showed considerable variation in the production of flowers and seeds. Flowering occurs from the second week of September to late October, with 20 days of peak flowering. Anthers dehisced longitudinally between 7:30 and 11:00 AM, strongly dependent on higher temperature. The pollen grains per anther varied between 2000 and 6000, which means an average of 80,000 pollen grains per flower. Nectar production begins at anther dehiscence and coincides with maximum stigmatic receptivity. Bees were observed as pollinators. Pollen germination and pollen tube elongation were maximum in 5% sucrose. Controlled pollination revealed that this species is self-incompatible, although few fruits developed from selfing. Such fruits were smaller than the fruits produced by open pollinated and from hand-crossed flowers and most aborted early in development.

Key Words: *Aconitum heterophyllum*, Ranunculaceae, critically endangered, anther dehiscence, pollination, pollen germination

Introduction

Aconitum heterophyllum Wall. (family Ranunculaceae) is an important medicinal plant of sub-alpine and alpine regions of Himalaya, distributed between 2800 and 4500 m asl (Stapf, 1905; Nautiyal et al., 2002). The root tubers of *A. heterophyllum* are used for various therapeutic actions such as anti-arthritic and anodyne. The marker ingredients for industrial use are *atisine* and *aconitine*. Its immense medicinal importance and high price in the market have led to an indiscriminate harvesting of tubers of this species from the wild and the species is identified as critically endangered in status (Nayar & Sastry, 1987; IUCN, 1993; Nautiyal et al., 2002; CAMP, 2003). Conservation of the natural populations and sustainable utilisation of this species via cultivation have been recommended by Nautiyal and Nautiyal (2004) and Nautiyal et al. (2006). However, the cultivation of this species is difficult (CAMP, 2003) due to poor seed availability and the lack of superior germplasm. In alpine conditions, low pollen germinability may be one of the several reasons for poor seed set (Cabin et al., 1991). Pollen fertility is an important aspect that can help in determining the successful adaptation of a plant species (Char et al., 1973; Qureshi et al., 2002). It is necessary to identify the factors influencing pollen germination and elongation of the tube. Following pollination, pollen grains absorb water and nutrients from the stigmatic surface and begin to germinate and produce long pollen tubes to

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fertilise the ovule. In any breeding programme, pollen germination studies are essential for estimation of the quantity of pollen required for controlled pollination studies (Khurana & Khosla, 1979; Chauhan & Katiyar, 1996). Physiological studies suggested a simple growth requirement consisting of water with dissolved carbohydrates and small quantities of Ca^{+} and boron for the germination of pollen grains (Brewbaker, 1963; Raghavan, 2000).

Knowledge of reproductive biology is essential for the conservation, management, and recovery of threatened species (Kuniyal et al., 2003; Murugan et al., 2006), and to improve desired traits or to develop new varieties. Despite the extensive medicinal utility, studies on the floral biology and breeding behaviour of *A. heterophyllum* have not been performed. Therefore, this communication reports the floral biology, pollination, and pollen germination of *A. heterophyllum*.

Materials and Methods

This study was conducted under hothouse conditions in the alpine garden of the High Altitude Plant Physiology Research Centre (HAPPRC), located at Tungnath (3550 m asl), Uttarakhand, India. The area lies at 30°14´ N latitude and 79°13´ E longitude in Western-central Himalaya. To study the reproductive biology, seeds of *A. heterophyllum* from 4 different seed sources, i.e. the alpine region of Tungnath, Dayara, Kilpur, and Panwali, were sown under hothouse conditions in May 2005. The reproductive phenology, viz. time of anthesis, anther dehiscence, stigma receptivity, pollination, and fruit and seed setting, were recorded daily from late August to October 2005 on 5 plants of each seed source selected randomly. In order to estimate flower production, the total number of flowers per plant was counted manually in all the selected plants of each seed source. The same procedure was carried out to quantify production of pods. Seeds per pod were estimated on 10 selected pods per plant (N = 5 plants). Pollen counts were made on 5 anthers from different flowers of the plant. The anthers were obtained from closed flowers prior to anthesis, placed in a small vial containing 1 ml of glycerine 1%, smacked, and the pollen grains were suspended. From this concentrate, five 10 µl droplets were removed and pollen grains were counted under the microscope. Production of pollen grains per flower was estimated by multiplying the number of pollen grains per anther by the number of anthers per flower.

Pollen viability tests by in vitro germination were done by taking pollen from different plants of each source and were germinated to optimise media composition. A factorial experimental design (Tuinstra & Wedel, 2000) was used to evaluate the effects of sucrose, indole 3 acetic acid (IAA), indole 3 butyric acid (IBA), gibberellic acid (GA_3), thiourea, and KNO_3 on pollen germination. Sucrose, boric acid, and calcium nitrate have been shown to be key substrates for pollen germination in other alpine species (Raina et al., 2003). Sucrose was initially tested at 1%, 5%, and 20%; IAA, IBA, GA_3, thiourea, and KNO_3 were tested at 1, 5, and 10 mg l^{-1}. The experiment was blocked in time with 5 replications in a randomised complete block design. Pollen was collected from undehisced anthers. Bulk pollen was distributed onto germination media in cavity slides and placed at a room temperature of 15 °C for 52 h. Germination was quantified as the percentage of germinated pollen grains per 100 evaluated. Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain (Tuinstra & Wedel, 2000). Pollen germination in different light conditions, viz. dark, blue, green, violet, and red, was also evaluated on optimal germination medium (5% sucrose) to test the effect of light on pollen germination. The same method was used to quantify the percentage of germinated pollen grains

The assessment of the breeding system involved 5 plants for each treatment and the following treatments were applied: (i) natural pollination, in which flowers were not manipulated; (ii) autogamous self-pollination, in which buds were bagged throughout their flowering period; (iii) hand self-pollination, in which bagged flowers were hand pollinated with their own pollens; (iv) open cross pollination, in which anthers were emasculated and stigmas left for open pollination; (v) hand cross pollination, in which emasculated bagged flowers were pollinated with pollens from another plant; and (vi) apomixis, in which anthers and stigma of buds were clipped. Fruit setting among hand-selfed and hand cross treatments, and among open pollinated and hand cross treatments was also compared. An indirect measure of self-incompatibility was obtained by dividing the average fruit set after self-pollination by the average fruit set after cross pollination (Lloyd & Schoen, 1992). The value of one indicates complete self-compatibility. All the experiments were carried out during 2005 and were repeated again during 2006 for more accuracy.

Results and Discussion

Floral biology

The height of the hothouse-grown plants was 3 times greater than that of the wild and garden plants with significant variation ($P < 0.001$). Seed production per fruit among the wild populations as well as hothouse plants was not significant ($P > 0.1$) although variation in the seed weight of wild populations as well as of hothouse-grown plants was significant ($P = 0.0$) (Table 1). In general, hothouse-grown plants under alpine conditions were phenotypically far more superior to wild ones, as reported earlier by Nautiyal and Purohit (2000). Floral display is summarised in Table 2 and the Figure. Flowers of *A. heterophyllum* are solitary, in lax arrangement (slender raceme), hermaphrodite (bisexual), hypogynous, and entomophilous. Pedicels erect and often adpressed to the rachis after maturation. Sepals more or less greenish blue with purplish veins, rarely whitish, glabrous, upper sepals almost navicular, obliquely erect and lateral sepals are very oblique and broadly obovate with dark tips. Pollen grains are 3-zonicolpate, prolate with size 24 × 18 µm. Exine 2.5 µm thick, sexine thicker than nexine, reticulate. Flowering starts from second week of September and ends in October with 20 days of peak flowering. The anthesis was observed between 7:00 and 10:00 AM and was highly dependent on the higher temperature. At that time, the temperature in hothouse conditions was recorded for 8:00-10:00 AM, 10:00 AM-12:00 PM, and 12:00-2:00 PM of the day and averaged 8.67 ± 0.48, 20.19 ± 0.88, and 18.09 ± 0.87 °C, respectively. The mean daily minimum and maximum temperature in alpine conditions was 5.48 ± 0.27 and 16.67 ± 0.67 °C, respectively at the same time. At low temperature, the corolla remains closed, and therefore is temperature sensitive. The opening and closing of the corolla at low temperature and during the night continues until fertilisation when the stigma lobes become dry and shedding of the corolla starts. Nectars are present, odourless, and glabrous with the hood leaning forward. The number of flowers per plant in natural populations varied from 5 to 10, whereas the plants grown under hothouse conditions produced massive flowers and the number was as high as 35 flowers/plant.

Anthers dehisced longitudinally between 7:30 and 11:00 AM, strongly dependent on higher temperature. Anthers numbered 20 per plant and the pollen grains per anther varied between 2000 and 6000, which means an average of 80,000 pollen grains per flower (Table 2). Pollen remains viable only up to 3 days after dehiscence. The anthers densely surround stigma up to 3 days before anthesis and move towards the corolla and attain maturity and dehisce to the discharge of pollen. A small gap thus separates the stigma and pollen, which provide a passage for insects (mainly bumble bees) for pollination. Colour pattern of the corolla attract insect vectors to effect pollination. Anther dehiscence was not synchronous, rather they dehisced at different times for 5-8 days. The stigmatic lobes at the time of anther dehiscence remain in adpressed conditions. After the completion of anther dehiscence, stigmatic lobes start opening until 2-5 days, becoming stigma receptive to pollen germination (Table 3). This has shown the existence of protandry in *A. heterophyllum*, with 1 week separation between male and female maturity

Table 1. Flowering and seed production potential of different wild populations and domesticated plants.

Populations/ microhabitats	Plant height (cm)	Length of floral axis (cm)	Flowers/plant	No. of seeds/fruit	Seed weight (mg/10 seeds)
Tungnath (3600 m)	35.1 ± 15.1	16.1 ± 8.1	8.2 ± 21	10.1 ± 2.5	12.0 ± 2.0
Kilpur (3500 m)	40.1 ± 5.1	18.5 ± 6.1	6.2 ± 3.1	9.5 ± 1.0	9.6 ± 1.5
Dayara (3400 m)	30.2 ± 10.2	12.2 ± 4.2	5.2 ± 5.0	8.5 ± 1.1	11.3 ± 3.0
Panwali (3400 m)	40.2 ± 6.1	20.2 ± 5.2	10.2 ± 4.1	11.5 ± 3.0	14.6 ± 2.5
Alpine garden	42.2 ± 10.2	22.2 ± 2.3	8.3 ± 4.2	12.1 ± 3.5	16.6 ±1.5
(Tungnath)	F = 7.1ns	F = 1.4ns	F = 0.9ns	F = 1.0ns	F = 4.7*
Hothouse	(P = 0.5)	(P = 0.2)	(P = 0.5)	(P = 0.5)	(P = 0.02)
(Tungnath)	125.3 ± 25.2	42.2 ±12.3	35.2 ± 15.2	15.5 ± 4.5	19.3 ± 1.1
	F = 20*	F = 6.1*	F = 8.7*	F = 2.2ns	F = 9.2*
	(P = 0.001)	(P = 0.005)	(P = 0.0)	(P = 0.1)	(P = 0.0)

*significant; ns-non significant

Table 2. Floral display of *A. heterophyllum.*

Floral characters	Observations
Flowering period	September-October
Inflorescence Type	Lax (Slender raceme)
Flower Type	Hermaphrodite, Hypogynous
Pollination	Cross pollination, Entomophilous
Colour	Greenish blue with purplish veins
Nectar	Present, glabrous
Odour	Odourless
Anthesis	7:00-10:00 AM
Time of anther dehiscence	7:30-11:00 AM
Mode of anther dehiscence	Longitudinal
Duration of stigma receptivity	2-5 days
Type of dichogamy	Protandry
No of anther/flowers	20
No. of pollen grains /anther	4000 ± 2000
No. of pollen grains /flower	80,000
Pollen shape	3-zonicolpate, prolate with size 24 × 18 m
Stigma type	Capitate, pentacarpellary, apocarpous
Ovary type	Pentalocular, axile placenta
Days taken for pod maturity	25-40
Seed	Obpyramidal, 3-4 mm long, blackish brown
Seed/plant	220-650

phases. Thus the protandrous flowering habit of *A. heterophyllum* makes individual flowers well adapted to out-breeding. The protandrous mechanism was also reported for other taxa (Bertin, 1993; Kalinganire et al., 2000). Variation in the time of anther dehiscence and stigma receptivity could be due to the rapid fluctuation in temperature, humidity, and light exposure (Khanduri & Sharma, 2003) as the weather of the study site during the flowering time remains cloudy with occasional rainfall.

Pollen germination and tube elongation

Tables 4 summarises the data on the effect of different growth regulators on pollen germination and tube elongation. The results revealed that pollen germination was higher in 5% and 20% sucrose, although the variation was not significant ($P > 0.1$). Among the growth regulator treatments, the maximum germination was observed in GA_3, 1 and 5 ppm, followed by IAA and IBA (1 mg l^{-1}).

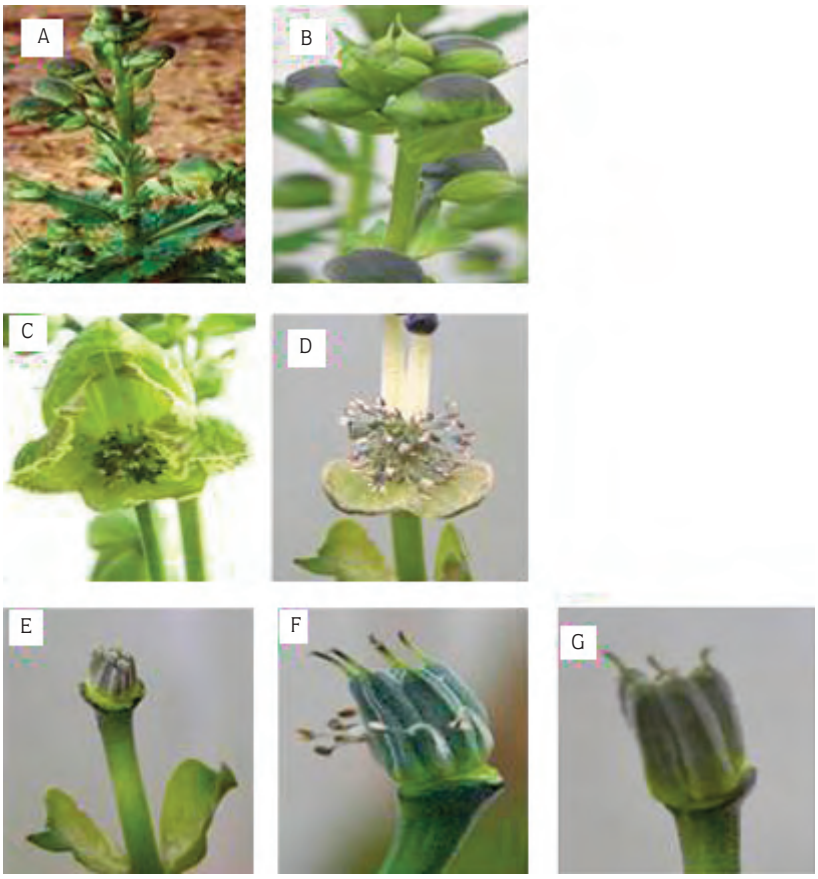

Figure. Flower and seed formation stages
(A) Initiation of floral buds, (B) Flower just before anthesis, (C & D) Stages of anther dehiscence, (E) Stage of pistil during anther dehiscence, (F) Receptive stage of stigmas, (G) Immature pod just after fertilisation.

Table 3. Effect of growth hormones and other medium on pollen germination and tube elongation.

Treatments	Pollen germination (%)	Tube elongation (µm)
Sucrose (%)		
1	43.3 ± 5.7	56.6 ± 2.0
5	53.3 ± 5.7	57.6 ± 2.5
20	53.3 ± 5.7	52.0 ± 2.0
	F = 1.3ns (P = 0.1)	F = 5.61* (P = 0.05)
IAA (ppm)		
1	40.0 ± 0.0	57.3 ± 1.1
5	36.6 ± 5.7	53.0 ± 4.1
10	26.6 ± 5.7	48.0 ± 2.3
	F = 6.5* (P = 0.002)	F = 15* (P = 0.002)
IBA (ppm)		
1	36.6 ± 5.7	54.3 ± 2.0
5	30.0 ± 0.0	51.0 ± 2.6
10	20.0 ± 0.0	49.3 ± 3.0
	F = 19* (P = 0.002)	F = 2.82ns (P = 0.1)
GA_3 (ppm)		
1	46.6 ± 5.7	51.0 ± 4.5
5	40.0 ± 0.0	49.3 ± 2.3
10	26.6 ± 5.7	52.0 ± 2.6
	F = 14* (P = 0.05)	F = 0.49ns (P = 0.5)
KNO_3 (ppm)		
1	36.6 ± 5.7	44.5 ± 1.3
5	30.0 ± 0.0	46.6 ± 4.1
10	23.3 ± 5.7	43.3 ± 2.3
	F = 6.0* (P = 0.03)	F = 1.05ns (P = 0.01)
Thiourea (ppm)		
1	26.6 ± 11.5	44.0 ± 3.4
5	13.3 ± 5.7	46.6 ± 2.0
10	10.0 ± 10.0	42.3 ± 2.0
	F = 2.62ns (P = 0.10)	F = 2.08ns (P =0 .20)
	F = 34.85* (P = 0.02) for treatment (T);	F = 25* (P = 0.01) for treatment (T);
	F = 20.38* (P = 0.02) for concentration (C)	F = 1.2ns (P = 0.2) for concentration (C)
	F = 2.36* (P = 0.02) for T × C	F = 3.37* (P = 0.001) for T × C

ns - not significant; * significant

Table 4. Effect of light on pollen germination and tube elongation.

Light Colour	Pollen germination (%)	Tube elongation (µm)
Dark	20.0 ±10.0	53.5 ± 0.8
Red	53.3 ± 5.7	54.3 ± 1.5
Green	33.3 ± 5.7	51.6 ± 1.5
Blue	26.3 ± 5.7	51.1± 1.5
Violet	16.6± 5.7	44.3 ± 2.0
	F = 2.5ns (P = 0.10)	F = 19.5* (P = 0.001)

Variation in germination was significant ($P < 0.02$) for all growth regulators, with higher germination achieved at low concentrations. Similar results were also recorded by Khanduri et al. (2001) for some temperate Himalayan trees, viz. *Acer caesium* Wall. ex Brand., *Aesculus indica* Coleb. ex Wall., *Celtis australis* L., *Juglans regia* L., and *Lyonia ovalifolia* (Wall.) Drude. The low concentrations of the nitrogenous medium also proved a better germination medium; however, the success rate of germination was

lower than the growth regulators and sucrose. ANOVA revealed a significant improvement in pollen germination due to different concentrations and treatments ($P < 0.02$). Tube elongation was a maximum of up to 57-58 µm in sucrose (1%) and IAA (1 mg l^{-1}) with significant variation. These concentrations represent the requirements for optimal pollen germination and pollen tube elongation. Even at optimal media composition, only 53.3% pollen germination was observed. It appears that different growth regulators (Khanduri et al., 2001) and nitrogenous compounds (Setia et al., 1985) influenced pollen germination and tube elongation differently. Chhabra and Malik (1978) stated that quiescent pollen contains RNAs and proteins needed for tube emergence. IAA pretreatments stimulate the synthesis of new RNAs and thereby increase proteins needed for tube growth. Mascarenhas and Mermelstein (1981) also emphasised the need for newly synthesised protein for tube growth. Over the years, an array of plant growth regulators and other chemicals have been empirically added to the culture medium to promote pollen germination and tube growth and the positive effects of some of these substances have led to speculation about their biochemical functions. Further, pollen germination and tube elongation are independent processes governed by separate sets of conditions (Malik, 1985).

The effect of different light colours on pollen germination and tube elongation was also observed (Table 5) as the alpine region experiences high intensity of solar radiation (Körner, 1999). Observations revealed that dark conditions and violet light inhibited pollen germination, whereas dark conditions enhanced tube elongation. The maximum pollen germination and tube elongation were observed in red light. However, the effect of light on pollen germination was not significant. Maximum germination and tube elongation in red colour suggest the involvement of phytochromes, as red synthesises phytochrome protein and its biological manifestation (Sharma & Malik, 1978; Katiyar, 1989).

Pollination and seed set

The results from controlled pollination are summarised in Table 6. Flowers used to test for apomixis did not set fruit. Fruit set differed significantly between the hand-selfed and hand-crossed treatments with an ISI value of 0.37 (Lloyd & Schoen, 1992). The results also revealed predominantly self-incompatibility in *A. heterophyllum,* as no fruit setting was observed from autogamous self-pollination, although few fruits developed from selfing. Such fruits were smaller than the fruits produced by open pollinated and from hand-crossed flowers and most aborted early in development. Thus the results demonstrated that *A. heterophyllum* possesses strong barriers to selfing with a predominating self-incompatibility system. This kind of breeding system has been reported in several other species, viz. *Lenotodon longirrostris* (Finch & P. D. Sell) Talavera (Ruiz de Clavijo, 2001), *Crepis sancta* (L.) Babc. (Cheplon et al., 2000), and *Senecio squalidus* L. (Brennan et al., 2003). Seed characteristics, viz. number of seeds and seed yield per pod and plant, were significantly at par with hand self-pollinated flowers. Nevertheless, the

Table 5. Different stages of development of anther and stigma receptivity.

Stages of anthesis	Anther development	Stigma development
5-3 DBA	All anthers in group at the centre of corolla tube densely surround stigma	Stigmatic lobes above anther level
1-5 DBA	anthers move towards corolla and increase in size, form gaps with stigma and anther dehiscence started, flower opens	Style as well as ovary starts increasing in size
1-5 DAA	Pollen dehiscence continued	Stigmatic lobes start opening
6-8 DAA	Dehiscence complete, pollen lost viability and many nonviable pollen remains in anther lobe	Stigmatic lobes opened, receptive and divide into 5 lobes
9-10 DAA	Anthers shrunken and fully dried	Lose receptivity and lobes start curling, pod formation starts

DBA = Days before anthesis; DAA = Days after anthesis

Table 6. Effect of different pollination methods on fruit/seed set in *Aconitum heterophyllum.*

Treatments	Fruit set (%)	Pod weight (mg)	No. Seeds /pod	Seed weight /pod (mg)*	No of seeds /plant**	Seed weight /plant (mg)
Natural pollination	80.0	41.2 ±1.6	14.6± 2.9	24.8 ± 5.0 (1.70)	511.0±83.0	868.1 ±176.5
Autogamous self-pollination	00.00	00.00	00.00	00.00	00.00	00.00
Hand self-pollination	30.0	32.5 ± 2.5	5.8 ± 1.7	6.38 ± 1.9 (1.10)	60.9 ± 18.7	223.3 ± 68.8
Open cross pollination	70.0	39.0 ± 2.3	11.2 ± 1.9	16.8 ± 2.8 (1.50)	274.4 ± 47.1	591.6 ± 97.8
Hand cross pollination	80.0	39.6 ± 2.2	11.6 ± 1.8	16.2 ± 2.5 (1.40)	324.8 ± 50.8	568.4 ± 89.01
Apomixis	0.00	0.00	0.00	0.00	0.00	0.00
F values	-	15.0^{+}	14.0^{+}	25.8^{+}	36.7^{+}	26.1^{+}
P level	-	0.00	0.05	0.05	0.05	0.05

* Data in parentheses are average weight of single seed; $^{+}$Significant

aim of this study was to observe floral biology, pollination behaviour, and seed production in *A. heterophyllum* so that effective breeding programmes could be undertaken in future for better in situ and ex situ conservation and cultivation for commercial purposes of the species, as IUCN has already indicated the critically endangered (CR) status of this species.

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