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Phenology and Reproductive Biology of *Aconitum violaceum* - A Threatened Endemic Medicinal Herb of the Northwestern Himalaya

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ABSTRACT

The study of reproductive biology provides essential paradigms for biodiversity conservation, reclamation, and restoration. The present study was undertaken with the aim of studying the phenology, floral biology, pollination ecology, and breeding behavior of an endemic threatened medicinal plant of Ladakh Himalaya. The present study revealed that *Aconitum violaceum* Jacq. ex Stapf is a biennial medicinal herb that completes its life cycle within 127–160 days. Flowers are typically hermaphrodite and partially protandrous. Pollens were spheroidal, monad, tricolpate, granulate, and exhibited 94–95% viability at the time of anther dehiscence. Boric acid and calcium nitrate at concentrations ranging between 100 – 500 ppm were optimal for *in vitro* pollen germination. The highest percentage of *in vitro* pollen germination was reported on the M7 medium (49.5%), followed by M4 (33.15%). The stigmas were highly receptive between 5^{th} – 8^{th} days after anthesis. The greatest pollen-ovule ratio was observed in the Khawous population with 3729.72 ± 48.92:1 pollen per ovule. Pollination indices, including foraging (FB), insect visiting efficiency (IVE), foraging speed (FS), visitor visitation frequency (VVF), and pollen load (PL) on the bodies of insect visitors, revealed that *Bombus* species were the chief pollinators of *A. violaceum*, accounting for 78.6% of pollination. The values of the self-incompatibility index (ISI), selfing rate (SR), greatest pollen ovule ratio, and breeding experiments revealed that *A. violaceum* is cross-fertile and partially self-compatible. The maximum proportion of fruit formation (86.66%) with a 94.87% seed set rate was recorded in open pollination, followed by induced xenogamy (68.88%) with 82.25% seed set, demonstrating that the species favoured cross-pollination. The present study first reported the phenological episodes and reproductive strategies operative in *A. violaceum* which will be crucial paradigms for its conservation, domestication, and sustainable utilization. It also aids in the development of specific techniques to overcome the challenges that limit its regeneration.

Key Message: This study elucidates the reproductive strategy of the threatened endemic medicinal plants *Aconitum violaceum*, revealing high pollen viability, peak stigma receptivity during 5–8 days after anthesis, and a breeding system favouring cross-pollination through *Bombus* spp. The findings establish critical ecological benchmarks for species recovery, domestication, and long-term conservation management.

Keywords

Aconitum violaceum · breeding behavior · conservation · Ladakh Himalaya · pollination biology · reproductive strategies

1. Introduction

India is considered one of the mega-biodiversity countries in the World. More than 5285 species of flowering plants belonging to 140 genera are endemic to the country (Moza and Bhatnagar 2007). However, this plant richness is disappearing at a fast pace due to habitat destruction, overharvesting, unscientific agricultural practices, invasive species invasion, pollution, uncontrolled tourism, and climate change (Rathcke and Jules 1993). Around 25% of higher plant species are anticipated to go extinct in the next few decades and another 25% are likely to go extinct by the end of the 21st century (Singh and Khurana 2002). Globally, about one million species of plants and animals are in danger of extinction (IBPES 2019) in the near future, and such a rapid loss of biodiversity necessitates immediate conservation planning and policy reforms. For the conservation and sustainable use of biodiversity, it is essential to comprehend the reproductive biology of endangered species in order to implement scientifically informed management approaches (Fiser et al. 2021).

Studies on the reproductive biology of threatened plant species could be essential for conservation, especially when the species is unable to thrive in nature due to regeneration failure (Marbaniang et al. 2018). It is essential to have in-depth knowledge of various aspects of reproductive biology, such as flowering phenology, mating system, pollinator availability, pollination and reproductive success, seed and fruit formation, germination capacity, the survival rate of seedlings, and seedling recruitment to determine the nature of species adaptation and comprehend the processes that contribute to the decline in the population structure of a species. It also provides information regarding patterns of genetic diversity, the extent of inbreeding depression, and the mechanisms of plant-pollinator interactions (Sletvold et al. 2012; Blambert et al. 2016; Rashid et al. 2023). Knowledge of reproductive biology aids in identifying bottlenecks that hamper the propagation of species in their natural populations. Therefore, deep knowledge about reproduction is vital for the establishment of appropriate conservation programmes for endangered species (Marbaniang et al. 2018; Rashid et al. 2023).

Pollination approaches, nectar production, and other reproductive attributes can also provide critical clues about the reproductive constraints of plants that require conservation (Marbaniang et al. 2018). Pollinator movements within a population are significantly influenced by factors like plant density and the spatial distribution of patch sizes (Cresswell 2000; Grindeland et al. 2005; Geslin et al. 2014). Moreover, floral nectar also plays a crucial role in plant reproductive fitness by enticing pollinators. The nectar characteristics of a flower can be directly influenced by plant traits and other environmental variables and exhibit extensive variations at the flower and plant level (Zimmerman 1988; Lu et al. 2015). Pollination

failure is mostly caused by disruption of mutualistic plant-pollinator interactions in several threatened taxa with small populations, leading to failure of seed set (Friedman and Williams 2003).

Modified mating tactics and pollinator behaviour contribute to genetic stochasticity effects such as inbreeding depression in small, scattered populations of endangered plant species. Self-incompatibility in some rare and threatened species also poses a survival threat under conditions of the unavailability of pollen from suitable mating types (Marbaniang et al. 2018). Successful pollination is a prerequisite for the survival of plants in natural ecosystems and is dependent on many biotic and abiotic variables. Plants and pollinators have coevolved, and major ecological alterations can decouple their coinciding flowering and breeding cycles (Gamba and Muchhala 2020; Ganie et al. 2021). Studies on reproductive biology will be helpful for developing strategies to conserve the genetic pool of rare and threatened species and are essential for restoration and reintroduction.

The Himalayas harbours a rich diversity of flora and fauna, including a high level of endemic species. (Tali et al. 2019; Dar and Khuroo 2020; Kaur et al. 2022). However, rapidly accelerated land use and climate change across the Himalayas put tremendous strain on its biodiversity (Hamid et al. 2020; Hassan et al. 2021). At present, more than 60 plant species in the Indian Himalayas are classified as threatened (Mehta et al. 2020; Rashid et al. 2023). *A. violaceum* is one of them. It is an endemic medicinal herb of the north-western himalayan region of India, Pakistan and Nepal (Chaudhary and Rao 1998; Rledl 1993; Jabeen et al. 2013; Hadi et al. 2022) and is categorized as vulnerable in the IUCN Red List (CAMP 2003; Ved et al. 2015).

The wild population of this species is declining day by day due to habitat destruction, overharvesting of rhizomes and climate changes. *A. violaceum* is one of the important medicinal herbs of the Tibetan and Amchi systems of medicine. Traditionally, it is used by various ethnic groups in mountainous regions to cure various ailments such as colds, coughs, asthma, boils, allergies, cuts, wounds, edema, and antidote against snake and scorpion bites (Hadi et al. 2023) and contagious infections, as the crude extract of underground tuber parts possesses antipyretic and analgesic, antioxidant, and antimicrobial properties (Miana et al. 1971; Bhattarai et al. 2010). Recently, the antiproliferative activity of the isolated alkaloids of *Aconitum*, was evaluated against human tumor cell lines, including ovarian and colon adenocarcinomas (Gao et al. 2006; Dall'Acqua et al. 2008).

An extensive literature survey revealed that there has been no such work reported on the reproductive biology of *A. violaceum*. To fill the knowledge gap, the present study was undertaken to study the reproductive strategies operative in *A. violaceum*. (i) What are the vital floral features that assist or hinder the sexual reproduction of *A. violaceum*? (ii) Do the pollen attributes (1.e., micromorphology, viability,

and quantity) influence its reproductive potential? (iii) Which types of mating strategies and pollination mechanisms are operative in *A. violaceum*? By comprehending these concerns, the study will bring a significant understanding of the reproductive mechanism operative in *A. violaceum* that are essential for the successful conservation, domestication, and sustainable utilization of *A. violaceum*.

2. Material and Methods

2.1. Study area and identification of target plant

The current study was undertaken from April 2019 to October 2022 in Ladakh-Himalaya, India. The entire region was explored and investigated to determine a suitable site for the study of phenology and reproductive biology. Based on accessibility, habitat structure, and attitudinal gradient, three sites were selected, I.e., Khawous (KH), Numsusru (NS) and Maan-man (MM). Khawous (N34°15′594 − N34°16′044; E75°95′887 − E75°96′172; 3200 − 3222m asl; Numsuru (N34°13′562 − N34°12′284; E75°96′715 − E75°96′340; 3280 − 3340m asl) are located on the eastern side of the Suru-river at Tehsil Taisuru; and Maan-man top (N34°40′157 − N34°39′284; E75°78′815 − E75°78′440; 3980 − 4010m asl) are located at the Umba-la and Lamochan mountain massifs of Drass, Kargil, Ladakh, India (Fig. 1). At each of the selected study sites, the species was evaluated throughout its various phenological episodes, and all the relevant data and samples were gathered for further investigation.

<<<< Fig.1. Map depicting study sites of *A. violaceum* in the Ladakh Himalaya **>>>>**

The specimens collected from various places in the Ladakh Himalaya were brought to Kashmir University and identified by consulting the herbarium specimens and taxonomic experts from the Centre for Biodiversity and Taxonomy (Biota) Department. The collected specimens were also authenticated by comparing the earlier submitted herbarium specimens (voucher No. *3693*; *Akhtar. H. Malik & Anzar. A. Khroo; Rangdum Zanskar, 9262-KASH; Vinod Gupta; Yousmarg, 7646; Gh, Hassan Dar; Pransheer, 838; Akhtar. H. Malik; Khardong-la, 116-KASH; Chadwell & Ramsey Pir Panjal, Kashmir*) and also confirmed by Dr. Anzar A. Khroo at the Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir, Hazratbal, Srinagar. Herbarium specimens were also submitted to the Kashmir University Herbarium (KASH) under specimen vouchers No. 3738-KASH and 3739-KASH, dated July 27, 2021. For corroboration, we also consulted previously published studies on its morphology and geographical distributions (Rledl 1993; Chaudhary and Rao 1998; Jabeen et al. 2013; Hadi et al. 2022).

2.2. Selected plant species

A. violaceum is a medicinal herb of the northwestern Himalaya with restricted global distribution (Ved et al. 2015; Chaudhary and Rao 1998). It is strictly distributed in moist rocky areas, alpine scrubs, alpine meadows, along the banks of springs, and continuous water flow irrigation canals in India, Pakistan, and Nepal at an elevation range of 3200 – 4800m asl (Ved et al. 2015). It's a biennial erect herb 25–90 cm tall with paired tuberous root. Inflorescence-short, lax, or dense raceme or corymb, or reduced to a solitary flower. Sepals– violet, rarely blue or yellowish-green with blue veins or variegated (Chaudhary and Rao 1998; Jabeen et al. 2013). Stamens –numerous (> 30), carpel 5, fruit – a bunch of 5 follicles (Fig. 2).

<<<< **Fig. 2.** Morphometric traits of *A. violaceum*: (a) Habitat; (b) Inflorescence; (c) Flower; (d) Follicle fruits; (e) Petals with nectaries; (f) Carpels; (g) Stamens; (h) Seeds; (i) Rhizomes. (Scale bar represents 0.5 mm).

2.3. Phenology and floral study

Data on all the phenological episodes were gathered soon after the sprouting of the underground rhizomes until senescence at all study sites. 10 plants were randomly selected from each population and marked with aluminium tags to assess the morphometric analysis of floral traits and record the onset and duration of various phenological events, viz., vegetative phase (sprouting of underground rhizome, initiation and duration of vegetative phase), sexual phase (initiation and duration of floral bud formation, anthesis, peak flowering, seed maturation), and senescence of aerial shoots (Smitha and Thondaiman 2016; Wani et al. 2022). Various floral traits, including the quantity, size, dimension and color of floral parts, were assessed. Moreover, the initiation and duration of anthesis, anther dehiscence, pollen shed, and stigma receptivity were also monitored. Photographs were taken under a trinocular stereo-zoom microscope (Make: Carl Zeiss Discovery V8) and Samsung a S22 ultra smartphone.

2.4. Anthesis time and pattern of anther dehiscence

The blooming time of individual flowers and inflorescences was assessed by making regular visits during the budding phase at all the study sites. Movement of filaments and pattern of anther dehiscence were observed on the same flowers with the help of a magnifying lens (Nautiyal et al. 2009; Li et al. 2018; Wani et al. 2022). Stylar-stigmatic movements from the day of anthesis to the phase of non-receptivity were also studied with the aid of a magnifying glass.

2.5. Pollen morphology and pollen viability

Pollen grains were collected from the dehisced anthers and squashed on slides with a drop of 1% acetocarmine stain and the morphological attributes were observed under 10X × 20X, 10X × 40X and 10X

× 100X of a light microscope (RTC-7-No. B-4422). Moreover, it was also observed under a scanning electron microscope (Zeiss Gemini SEM 500).

Pollen viability was assessed using both the staining approach and the *in vitro* germination method. Mature anthers were squeezed in 1% 2,3-5 triphenyl tetrazolium chloride, 1% acetocarmine, and 1% aniline blue-lactophenol (Hauser and Morrison 1964; Stanley and Linskins 1974; Swanson and Sohmer 1976; Shivanna and Rangaswamy 1992) and observed under the Radical compound microscope RTC-7 (model No. B-4422). Well-developed, fully stained pollen grains with defined membranes were considered viable, whereas unstained and diffused pollens were taken as non-viable. The percentage of pollen viability was estimated using the following formula:

$$\%\ of\ pollen\ viability = \frac{No.\ of\ viable\ pollen\ grains}{Total\ number\ of\ pollen\ grains\ observed} \times 100$$

The *in vitro* hanging drop culture method (Brewbaker and Kwack 1963) was employed to check the pollen viability. The freshly dehisced pollen grains were cultured in different compositions and combinations of Brewbaker and Kwack's medium with slight modifications (sucrose, boric acid, calcium nitrate, magnesium sulphate and potassium nitrate). The cavity slide technique (Shivanna and Ragaswamy 1992) was used to culture the pollen grains in different media (Table 1).

<<<< **Table 1.** Different nutrient media compositions used for *in vitro* pollen germination of *A. violaceum* >>>>

In this method, a drop of medium was put into the cavity slide and pollen was dusted and covered with a cover slip with its edges sealed with paraffin wax to maintain relative humidity. The slide is then incubated at 20°C for 4–24 hrs. The percentage of pollen germination was calculated by applying the following formula:

$$\%\ of\ pollen\ germination = \frac{Number\ of\ pollen\ grain\ germinated}{Total\ number\ of\ pollen\ grains\ observed} \times 100$$

2.6. Pollen count and pollen– ovule ratio

Pollen counts: The number of pollen grains per flower was estimated by following the method of Wafai et al. (2005). One anther from each flower bud about to dehisce was squeezed into a cavity block containing 10 drops of distilled water/ 1% acetocarmine stain and thoroughly mixed to make a homogenous pollen solution. One drop of this solution was placed on a glass slide and covered with a coverslip so that no stain leaked from the edges. The slide was scanned edge to edge in such a way that no pollen was missed or counted more than once. The number of pollen grains per flowers and per plant was estimated using the following equation:

$a \times b = c$

$c \times d = e$

Where "a" = Average number of pollen grains per drop dist. H_2O/ 1% acetocarmine stain

b = No. of dist. H_2O/ 1% acetocarmine drops taken initially in which one anther was squeezed,

c = No. of pollen grains per anther

d = No. of anthers per flower

e = total No. of pollens per flower

Ovule number: The ovaries were opened and ovules extracted using needles to ensure that no ovule was left inside the opening ovary, and the number of ovules in all five ovaries of a flower was manually counted to determine the number of ovules per flower. Observations were based on the average of 15 flowers from each population. The pollen-ovule ratio was calculated following the Cruden's method applying the following formula:

$$P/O\ ratio = \frac{Pollen\ count\ per\ anther \times No.\ of\ anthers\ per\ flower}{Number\ of\ ovules\ per\ flower}$$

2.7. Stigma receptivity

The stigma receptivity was evaluated on the basis of pollen load and pollen germination on the stigmatic surface. Hand-pollinated stigmas of different developmental stages were fixed in Carnoy's fixative (absolute alcohol plus glacial acetic acid in a ratio 3:1) for 3–4 hours. After a predefined time, it was transferred to 70% ethanol until further use. For microscopic study, pistils were stained with 1% lactophenol stain (cotton blue), and the stigmas were allowed to stain for an hour (Hauser and Morrison 1964) and observed under a fluorescence microscope (Make: Nikon Eclipse 80) with 10X × 40X resolution. Stigma bearing the germinated pollen grains was deemed to be receptive and the number of germinated pollen grains was employed to determine the degree of receptivity.

Hydrogen peroxide (H_2O_2) was also employed to determine the degree of stigma receptivity (Dafni and Mau´es 1998). For this purpose, the stigmas at pre-anthesis, anthesis and post-anthesis stages were collected and immersed in H_2O_2 for about 2–3 min and the emission of oxygen bubbles was observed under a microscope. The emission of bubbles was considered a positive result (Li et al. 2018; Wani et al. 2022; Rashid et al. 2023).

2.8. Pollination mechanism

The pollination process operating in *A. violaceum* was studied during the peak blooming season. Floral traits such as the pattern of anthesis and anther dehiscence, the position of androecium, and gynoecium,

and the nature and orientation of pollen and stigma were carefully scrutinized. To ascertain anemophily, glycerine-smeared glass slides were suspended from a wooden stain and placed near the inflorescence of plants for 24hr and finally examined the slide under a light microscope for pollen accumulation (Khajuria 2013; Yaqoob and Nawchoo 2016).

To assess entomophily, ocular observations were made from the onset of blooming until fruit set, and the foraging behaviors of insects approaching the flowers were tracked from 6:00 am. to 7:00 pm. The floral visitors were captured, anesthetized with chloroform, and examined under a stereo-zoom microscope (Model RSM-9; model No. 8436) to determine the number of pollens deposited on their bodies. The representative specimens of each insect visitor species were collected and identified by the Coordinator, Laboratory of Entomology, Department of Zoology, University of Kashmir, Hazratbal, Srinagar. Pollination indices like foraging behavior, insect visiting efficiency, foraging speed, visitor visitation frequency, and pollen load were assessed for each pollinator.

Foraging behaviour (FB) **(**Sajjad et al. 2009; Yaqoob and Nawchoo 2016): Time spent by a foraging insect species per inflorescence per visit, counted using a stopwatch.

Insect visiting efficiency (IVE) (Bingham and Orthner 1998): IVE was reckoned by the following formula.

$$IVE = \frac{No.of\ flowers\ visited\ by\ insects\ in\ one\ visit}{Total\ No.of\ opened\ flowers\ available}$$

Foraging speed (FS) (Pando et al. 2011): Number of flowers visited by a pollinator per minute. It was estimated using the following formula.

$$FS = \frac{Fi}{di} \times 60$$

Where, ‘Fi’ number of flowers visited during 'di' and
'di' is the total time of observation given by the stopwatch.

Visitor visitation frequency (VF) (Cosacov et al. 2008): Number of visitors per flower per hour. It was reckoned as follows.

$$VF = \frac{V}{F.T}$$

where ‘V’ is the total number of visits to flowers, ‘F’ is the total number of flowers in the patch and ‘T’ is the observation time in hours.

Pollen load (PL): It's the number of pollen grains carried by insect visitors on their bodies. It was assessed by capturing the visiting insects using foldable sweep nets and placing them in 15 ml falcon tubes filled with 10 ml of 70% ethanol. Tubes were vortexed in order to remove pollen grains from the insect bodies. Five samples of this pollen slurry were taken with a pipette, and the number of pollen grains was counted under a microscope (Delaplane et al. 2013). The average number of pollen grains in a sample was multiplied by the total volume of solution, or 10 ml, to determine the total number of pollen grains that were attached to the pollinator (pollen load).

2.9. Breeding behavior

To evaluate the mating behaviors operative in *A. violaceum*. A total of 70 healthy-looking individual plants were selected and marked with aluminium tags at Khawous, Taisuru, Kargil, Ladakh Himalaya. Seven different types of mating experiments were executed. 10 individual plants with an average of 90 flowers (@8–10 flowers from each plant) were randomly selected for each experiment.

(i) *Open pollination*: Inflorescences of tagged individual plants were allowed for open pollination without any artificial intervention

(ii) *Induced xenogamy*: Emasculated flowers were manually pollinated with pollens obtained from different individuals of the same population and then bagged with butter paper bags to protect them from exposure to self and geitonogamous pollination.

(iii) (iii) *Geitonogamy*: The flowers were emasculated and manually pollinated with the pollens of other flowers borne on the same plant and bagged with butter paper bags.

(iv) (iv) *Apomixis/ Agamosermy*: The flowers were emasculated at bud conditions and bagged with waxed paper bags to avoid anemophily and entomophily. The tagged individuals were regularly monitored for fruit sets.

(v) *Unassisted Pollination/ Spontaneous Autogamy*: The flower buds were bagged prior to anthesis and monitored for fruit set on completion of the sexual phase.

(vi) *Selfing/ Forced autogamy*: Flowers emasculated and manually pollinated with pollens from the same flower followed by bagging with waxed paper bags.

(vii) *Anemophily*: Emasculated flowers were bagged with mesh bags. The flowers used for mating tests were observed for fruit and seed set and the average seed set and seed mass of all breeding tests were examined and significant variations were evaluated using one-way ANOVA. The percentage of fruit and seed set was estimated by following the methods of Lubbers & Christensen (1986).

$$Percentage\ fruit\ set = \frac{Total\ No.\ of\ fruit\ formed}{Total\ No.\ of\ flowers\ treated} \times 100$$

$$Percentage\ seed\ set = \frac{Total\ No.\ of\ seeds\ produced}{Total\ No.\ of\ ovules\ borne\ by\ a\ plant} \times 100$$

The various reproductive indices such as Self-incompatibility index (ISI), Inbreeding depression (ID) and selfing rate (SR) were also reckoned.

Self-incompatibility index (ISI): It was estimated following the procedures of Zapata and Arroyo (1978) and Yaqoob and Nawchoo (2016). The ISI value indication was depicted in Table 2.

$$ISI = \frac{Average\ fruit\ set\ from\ manual\ self\ pollinaton}{Average\ fruit\ set\ from\ \ manual\ cross\ pollinaton}$$

<<<< Table 2. Self-incompatibility index (ISI) value indication for *A. violaceum* >>>>

Inbreeding depression (ID): It is estimated following the procedures of Ramirez and Nassar (2017).

$$ID = 1 - (\omega s/\omega o)$$

Where, ωs = average weight of self-pollinated seeds; ωo = average weight of cross-pollinated seeds

Selfing rate (SR): It's used to estimate the frequency of self-pollination (Charlesworth 1987)

$$S = \frac{Px-Po}{Px-Ps}$$

where "*Px*" seed obtained cross-pollination, "*Po*" seed obtained from open pollination and "*Px*" Seed obtained from self-pollination

2.10. Statistical analysis

In all the experiments, significant differences between each treatment were determined by Tukey's one-way ANOVA using IBM-SPSS statistical software (ver-23, Armonk, NY, IBM Corp.) and results were computed in MS-Excel (Office 19, Microsoft Corp.) Graphs were plotted in OriginPro (ver-21, Northampton, MA). Map was developed in ArcGis (Ver. 21, ESRI, America). Data were represented as mean ± SE (standard error) for each experiment and significance difference was set at $p \leq 0.05$. Photographs were taken using a Samsung S22 ultra smartphone, Olympus *EPL-5* DSLR camera.

3. Results

3.1. Floral morphology and phenology

A. violaceum is a biennial paired rhizomatous herb strictly grown along the banks of perennial water canals or on moist alpine slopes fed with snowmelt water on well-aerated gravel-loamy soils in Ladakh

Himalaya (Fig. 2a). The inflorescence of *A. violaceum* is a short lax/dense raceme/ corymb/ reduced solitary flower, and the average length of the inflorescence was 24.8 ± 4.27cm (6–50 cm). Flowers are zygomorphic, pedicellate and bracteate, and the average number of flowers borne on each plant was 18.8±3.24 (4–34). Sepals: pubescent, violet, rarely blue or variegated (Fig. 2b); petals (nectaries): glabrous, claw 1.3–1.9cm, hood gibbous dorsally with truncated recurved lips (Fig. 2e); Stamen: numerous (30–42), 0.5–1.2cm in length (Fig. 2c), filament hairy in the upper portion (neck region), lower portion winged and ended with tiny teeth (Fig. 2g); Carpel: 5, 0.3–0.6mm in length, densely covered with soft hairs, turns yellow when dry (Fig. 2f). Fruit: a bunch of 5 follicles, 1.0–1.5cm in length, oblong, trunicate, densely hairy (Fig. 2d). Seeds: obpyramidate with winged comers (Fig. 2h), 34–75 in number and 1.2–1.8mm × 0.4–0.9mm in dimension (Table 3).

<<<< **Table 3.** Floral traits of *A. violaceum* examined in Ladakh Himalaya >>>>

This was the first study reported on the phenological events of *A. violaceum*. The various phenological episodes starting from the sprouting of underground rhizomes up to the senescence of aerial parts were monitored. The plant initiates its life cycle usually from the sprouting of underground perennating rhizomes (vegetative propagule) or rarely from the germination of dormant seeds with the onset of the spring season immediately after snowmelts at all the study sites. Sprouting of underground perennating rhizomes starts in the 2nd week of April and lasts until June in all the populations of the selected study sites. It began to produce floral buds after investing > 2 months in the vegetative phase (leafing stage). However, the subsequent stages, including bud development, anthesis, and seed formation, partially overlapped one another (Fig. 3; S1). The population at lower elevations (KH, 3220m asl) displayed longer phenophases in contrast with the population that thrived at high elevations (MM, 4010m asl). Anthesis starts in the 2nd wk of July and reaches its peak (85%) in the 1st wk of August. Moreover, floral buds developing later in the 3rd week of August mostly failed to produce fruits as the ecological variables (temperature and photoperiods) become unfavorable. Furthermore, the floral buds were affected by an insect larva, which completely destroyed the floral anthers prior to anthesis. Withering of petals, shriveling of stigmas, and swelling of the pods were marked as indication for the initiation of seed formation. Senescence of aerial parts starts during the mid-session of seed development (3rd wk of August) and continues until 3rd or 4th week of September at all the study sites. The underground parent and collateral rhizomes, and mature dispersed seeds undergo physiological dormancy of > 6mo to overcome the extremely harsh climatic conditions and to maintain the continuity of their race. Hence, it is concluded from the above results that *A. violaceum* is purely an alpine plant with a shorter life cycle of 127–160d. The Populations of Maan-man Top exhibited a shorter life cycle, while the population of Khawous displayed a longer life cycle of 160 d. which is directly influenced by altitudinal gradients (Fig. 3; S1).

<<<< **Fig. 3.** Graphical representation of phenophases of *A. violaceum* across three different study sites >>>>

3.2. Anthesis and pattern of anther dehiscence

Anthesis is asynchronous, and the flowers usually bloom between 8:00 am.–11:30 am. in the 2nd week of July and continue up to the 3rd week of August. Flowers borne on basal branches bloom earlier than flowers borne on terminal branches. Flowers are hermaphrodite and protandrous in nature. Anther dehisces asynchronously 35.28 – 40.28 ± 4.5h after anthesis. The maximum anther dehiscence was observed on the 4th day of anthesis (Table 4).

<<<< **Table 4.** Pattern of anthesis and anther dehiscence in *A. violaceum* >>>>

Anther dehiscence initiated from the central portion towards the periphery (Fig. 4a, b). Subsequently, after anthesis, the individual flower takes 5 – 8d to shed complete pollen grains. From the day of floral bud formation until 3rd or 4th day of anthesis, the stigmas are enclosed by a bunch of stamens (Fig. 4c–e) consequently, it becomes visible and receptive to receiving pollens.

<<<< **Fig. 4.** Phases of anther dehiscence and stigma receptivity of *A. violaceum*. (a) Central anther dehiscence on 1DA; (b–c) Anther dehiscence on 3DA or 4DA; (d-e) Anther dehiscence on 5DA or 6DA; (f) Anther dehiscence and pollen discharge, 7DA or 8DA; (g) Position of stigma and style with respect to the ovary on 0DA; (h) Style bend slightly outward; (hd) Different phases of stigma and style position with respect to the ovary on 0DA–8DA; (i) The groove of stigma bifurcates after losing complete receptivity on the 9DA or 10DA. (Scale bar represents 5mm). >>>>

3.3 Pollen morphology and viability

Pollen morphology: The anther dehisces asynchronously along longitudinal ridges immediately after anthesis, and the clump of wet, sticky pollen grains aggregates on the surface of the releasing site (Fig. 4a-f). Microscopic analysis (light microscope and SEM) revealed that the pollen grains of *A. violaceum* are monad, spherical or ovoid in shape, tricolpate, isopolar with thick exine with scabrate granulate ornamentation. Polar axis 16.57 µm and equatorial axis 36.32 µm in diameter. P/E ratio 0.45. Granules are 85.42 nm and uniformly spread over the tectum (Fig. 5a-d).

<<<< Fig. 5. Pollen morphometry and viability of *A. violaceum* prior to anthesis or on the day of anthesis. (a-b) Polar and equatorial view of pollen gains (SEM), Mag = 6.00 KX; (c) Exine showing granulate ornamentation, Mag = 100.00 KX; (d) Granulate exine; mag =20.00 KX; (e) spheroidal or ovoid tricolpate

pollen grains; (f) Viable and non-viable pollen gains (1% aniline blue-lactophenol); (g) Viable pollen grain (1% 2,3-5 triphenyl tetrazolium chloride); (h-i) Viable and non-viable pollen grains (1% acetocarmine).>>>>

Pollen viability: The species produced an enormous quantity of healthy, plump, and stainable pollens (Fig. 5e-i). The highest percentage of pollen viability was reported by the Khawous population (95%) on the day of anthesis (0DA) in the lactophenol and acetocarmine tests, followed by tetrazolium test with 94% pollen viability (In all the populations, the percentage of pollen viability reduced over time and often took 10–12 days to loss complete viability (Fig. 6a, b).

<<<< **Fig. 6.** Line diagram depicting the pattern of pollen viability of *A. violaceum* at various study sites employing (a) 1% acetocarmine; (b) lactophenol. Bar represents SE for n = 15. >>>>

In vitro pollen germination: Among all the media compositions utilized, boric acid and calcium nitrate at a concentration range between 100–500 ppm were found to be ideal for *in vitro* pollen germination of *A. violaceum*. Maximum *in vitro* pollen germination was reported on M7 media (200 ppm calcium nitrate) with 49.5% pollen germination, followed by M4 (200 ppm boric acid) with 33.15% pollen germination (Fig. 7). The emergence of pollen tubes was considered an indication for *in vitro* pollen germination (Fig. 8a–f).

<<<< **Fig. 7.** Assessment of *in vitro* germination of pollen grains collected from the first day of anther dehiscence of *A. violaceum* >>>>

<<<< **Fig. 8.** *In vitro* pollen germination in different nutrient media: (a) Viable pollens; (b) pollen germination in 10% sucrose; (c) 100 ppm Boric acid; (d) 200 ppm Boric acid; (e) 200 ppm Calcium nitrate; (f) 500 ppm Calcium nitrate >>>>

3.4. Pollen count and pollen–ovule ratio

The maximum number of pollen grains per anther was observed in the Maan-man population with an average of 9313.66±145.23 pollens, followed by Khawous population with 9225.13±133.11 pollens per anther. However, the average number of pollen grains per flower was reported of highest in Khawous population with 3,46,864.8±1331.1 pollens, and it was due to the presence of more anthers per flowers than in the Maan-man population. Similarly, ovule number per flower was also reported highest in the Khawous population with an average of 93.0 ± 3.7 ovules, followed by Numsuru with 84 ± 3.3 number of ovules per flower. The Pollen-ovule (P/O) ratio was 3729.72b±48.92:1, 1324.65 ± 55.92:1 and 1278.89 ± 68.09:1 at Khawous, Numsuru and Maan-man populations respectively (Table 5).

<<<< **Table. 5** Pollen ovule (P/O) ratio of *A. violaceum* across different study sites >>>>

3.5. Stigma receptivity

The stigma undergoes various developmental phases, starting from floral bud formation until receptivity. Initially, the style, stigma, and ovary lie in a straight position and are enclosed by a bunch of stamens (Fig. 4h*a-d*). As it reaches the receptivity stage, the styles curve slightly and the stigma becomes bilobed with a sticky furrow, which marks the beginning of receptivity. Hand-pollinated stigmas were observed for receptivity from the day of anthesis. In the first couple of days after anthesis, no pollen was found to be deposited on the stigmatic surface (Fig. 9a-b). On the 3rd day, even pollens were deposited on the stigma, but none of them seemed to germinate, marking non-receptivity (Fig. 9c). The stigma becomes receptive between the 4th – 8th days after anthesis and reaches its peak on the 5th – 7th DAA, as confirmed by the germinated pollen grains on the stigmatic surface (Fig. 9d–f). Subsequently, the stigmatic surface developed morphological alterations, including flat pinkish coloration on the papillae and its ridge becomes highly dissected like the tail-fin of a fish, signaling the end of receptivity (Fig. 9g–h; Fig. 10).

<<<< Fig. 9. Pollen load and pollen germination on the stigmatic surface of *A. violaceum*: (a, b) No pollen loaded on the stigma from 0DA–2DA; (c) Pollen loaded but not germinated on the stigma on 3DA; (d–f) Pollen loaded and also germinated on the stigmatic surface after 5DA–8DA; (g-h); Stigma dissected like the tail-fin of a fish marks the end of receptivity (9DA–12DA); (i) Stigmatic surface emitting bubbles on the 6th day of anthesis demonstrating receptivity. *0DA*, on the day of anthesis; *DA*, day after anthesis **>>>>**

<<<< Fig. 10. Assessment of pollen germination percentage on the stigmatic surface after manual pollination to check stigma receptivity. *KH*, Khawous; *NS*, Numsuru; *MM*, Maan-man **>>>>**

Hydrogen peroxidase activity also demonstrated that stigmas were not receptive during the budding stage and remained as such for an initial couple of days after anthesis. Furthermore, they do not exhibit any secretions. On the 5th day after anthesis, the secretion of stigma considerably increased and reacted with H_2O_2, resulting in the emission of maximum O_2 bubbles. The number of oxygen bubbles determines the degree of stigma receptivity. The stigma emits the maximum number of O_2 bubbles between the 5th – 7th days after anthesis in the presence of H_2O_2 depicting the peak receptivity stage (Fig. 9i; Fig. 11).

<<<< Fig. 11. Number of oxygen bubbles emitted from the stigmatic surface of *A. violaceum* from the day of anthesis. The number of O_2 bubbles determines the degree of receptivity **>>>>**

3.6. Pollination system

A. violaceum produces a bisexual flower, offering nectar as compensation for pollinators. The nectar becomes accessible to pollinators when a flower approaches anthesis. One day after anthesis, anther dehiscence begins asynchronously from the center towards the periphery, releasing enormous pollen

grains. At this phase, the flowers were foraged by at least eight different types of insects, which include four species of *Bombus,* one species of *Apis,* one species of *Hycleus, Palomena prasina* and one species of *Papuaepilachna*. Typically, the *Bombus* species visit frequently, but the other species visit occasionally. *Bombus* species have higher visiting efficiency, foraging speed, visiting frequency and maximum pollen load than the other visitors; therefore, it is considered the primary pollinator that performed exclusive pollination in *A. violaceum* (Table 6; Fig. 12).

<<<< **Table 6.** Pollination indices of diverse insect visitors of *A. violaceum* >>>>

<<<< **Fig. 12.** Visitors of *A. violaceum*. (A) *Bombus trifasciatus*; (B) *Bombus asiaticus*; (C) *Bombus ladakhensis*; (D-E) *Palomena prasina*; (F) *Apis spp*; (H) *Hycleus* spp; (I) *Papuaepilachna* spp. >>>>

Based on the visiting efficiencies and pollen deposited on their bodies, *Bombus* species showed the highest percentage contribution to pollination (78.6%), followed by *Apis* species with 8.4%. The contribution of the other visiting insects is extremely minimal (Fig. 13).

<<<< **Fig. 13.** Percentage contribution of insect visitors in performing pollination >>>>

The lower flowers of an inflorescence bloom first and serve as females, while the top flowers bloom later and act as males. Thus, bumblebees transferred pollen grains from the upper flowers to the lower flowers of the same plant or other plants. The visitors approached the flower and inserted their proboscis into the nectar sac to suck the nectar; at the meantime, its body rubbed with the sex organs, getting heavily loaded with pollen grains and dispersing the pollens to other flowers, causing pollination (Fig. 12a, b). The environmental variables such as sunny, windy, rainy and cloudy conditions prevailing during pollination poses a significant influence on the foraging behavior of insect pollinators (Fig. 14). On a sunny day, foraging typically begins at about 9.40am., progressively reached to peak at 12:40pm. And again, decline until 4.00pm. The pollinator showed more activeness in between 10:30 am.–12:40pm. and 4:00pm.–5:30pm. The daily visitation efficiency of insect pollinators was reduced or absent during rainy or cloudy condition.

<<<< **Fig. 14.** Pollinator visiting frequency varies at different weather conditions >>>>

3.7. Breeding mechanism

It was confirmed from the breeding experiments that open pollination showed the highest percentage of fruit development (86.66%) with 94.87% seed set rate of the developed fruits, followed by induced xenogamy with a fruit formation of 68.88%. Geitonogamy contributed 46.33% of fruit formation with 41.92% of seed set. Autogamy and forced autogamy mostly failed to develop seed set; in a few cases, it did develop seeds, but the quantity of seed set was very low; only 41.92% of the ovules got converted into

seed. Apomixes test resulted in no fruit or seed formation. Moreover, the average number of ovules converted into seeds, and dry weight of developed seeds vary significantly under different breeding treatments (Table 7). The self-incompatibility index (ISI) value was reported between 0.2–1; indicating *A. violaceum* was partially self-compatible, similarly, ID = 0.37 (i.e., > 0) implies there exists inbreeding depression of 37% and selfing rate (SR) SR= -0.58 indication poor affinity for self-pollination. The negative value of selfing rate indicates that open cross results higher seed set as compared to controlled self or cross pollination

<<<< Table 7. Seed set in *A. violaceum* following different breeding treatments >>>>

4. Discussion

4.1. Phenology and floral biology

The study of cyclical biological events in plants such as sprouting, leaf unfolding, floral bud formation, anthesis, peak blooming, flowering, fruit and seed formation, seed dispersal, senescence of aerial parts and perennation of vegetative propagules in relation to climatic conditions is an important component for identifying the problem of regeneration (Davi et al. 2011). Phenology is undoubtedly one of the most effective ways to monitor and detect how an individual plant species interacts and responds to altering environmental conditions. Assessing flowering phenological events is also useful in quantifying the rate of reproductive fitness accumulated over generations in various environmental situations (Trunschke and Stocklin 2017; Mohan et al. 2019). In alpine regions, flowering phenology is critical for the reproductive fitness of a plant species, which is inextricably dependent on environmental variables including soil moisture, temperature, nutrients, and pollinator diversity (Cornelius et al. 2013; Lessard-Therrien et al. 2014).

The current study focused on the phenological events of *A. violaceum* across different altitudinal gradients. The plant starts its life cycle either from the sprouting of physiologically dormant rhizomes (vegetative propagule) or from dormant seeds (sexual propagule) with the onset of the spring season soon after the snowmelts in the 3rd week of April and lasts through July in all the presently studied areas. The sprouting phase is followed by floral bud formation, which partially overlaps with the sprouting process. It is then followed by anthesis, which fully coincides with the subsequent stages, including fruit and seed formation. Anthesis begins in the month of July and lasts through the first week of September. In an individual plant, anthesis occurs in an acropetal manner; the flowers borne on the basal stalk of the inflorescences open first, while the upper flowers open later. Pollen is mostly transmitted from the later-

opened flowers to the earlier-opened flowers. The same flower serves as a 'male' in the early stage of anthesis, and as 'female' in the later stage of anthesis.

The asynchronous anther dehiscence and pollen presentation assure pollen availability for a longer period of time to ensure successful pollination. A similar pattern of phenological events was also reported by Gojri et al. (2009) in *A. chasmanthum,* Utelli and Roy (2000) in *A. lycoctonum*, and a similar pattern of anthesis and anther dehiscence was also observed by Nautiyal et al. (2009) in *A. heterophyllum*. The temporal variations in the development of male and female phases prevent self-pollination within an inflorescence and facilitate outcrossing (Wyatt 1982; Wafai *et al*. 2005). Fruit development begins in the 1st week of August, and lasts until the end of August. By the end of the 1st week of September, the seeds are ready for dispersion, followed by the process of senescence of aerial parts of the plant. The dispersed seeds and underground rhizomes undergo physiological perennation for > 6 months to overcome the extremely harsh climatic conditions, as is true for many alpine plant species (Siddique 1991; Gojri *et al*. 2009). The phenology of *A. violaceum* exhibited a strong reliance on ecological variables including elevation, temperature, soil moisture (Cornelius et al. 2013; Kopp et al. 2020), precipitation, photoperiod, floral phenology, snowmelt impacts, and topography. Our observations were very much in conformity with the findings of Hartmann et al. (2013), Bliss (1971), Hulber et al. (2010), Ahmad et al. (2021). Plants in alpine ecosystems are constrained by a short growing season and harsh environmental circumstances, making them highly sensitive to limiting factor alteration (Thuiller et al. 2005). Hence, a clear understanding of phenological events is quite useful in planning conservation strategies and formulating measures for the commercial cultivation of threatened medicinal plants (Wafai et al. 2005; Gojri et al. 2009). In *A. violaceum*, the phenological attributes were more pronounced and early at low elevations and reduced with increasing altitude. Khawous population (3220m asl) completes its above ground life span within 160 days, while Maan-man population (4010m asl) completes within 127 days. This variation in phenological attributes along the altitudinal gradient is due to snow-capping, prolonged freezing temperatures, and other ecological variables.

The quantitative floral traits, mainly inflorescence length, flower number and dimension, displayed substantial variations across study sites. The Khawous population (3220m asl) exhibited a longer inflorescence and more flowers in contrast to Maan-man (4010) population. The variation in quantitative trait is due to varying environmental challenges such as temperature, precipitation fluctuations, nutrient composition, photoperiod and wind velocity (Pellerin et al. 2012; Rafferty et al. 2020). The occurrence of a short inflorescence and a smaller flower dimension enable alpine plants to endure the detrimental effects of strong winds (Korner and Cochrane 1983; Wani et al. 2022), and also assist in utilizing the existing resources efficiently (Wani et al. 2023).

A. violaceum bears a multiflowered inflorescence with the protandrous type of dichogamy. Anther dehiscence is centripetal; it begins asynchronously from central anthers and gradually proceeds towards peripheral anthers concurrently with anthesis. Particularly, protandry limits impulsive self-pollination, provides opportunities for outcrossing, and is more prevalent among self-compatible than self-incompatible taxa (Lloyd and Webb 1986; Barrett 2002). Protandry-type of dichogamy was also reported in other *Aconitum* species, such as *A. balfourii* (Nautiyal *et al*. 2009a), *A. chasmenthum* (Gojri *et al*. 2009), *A. heterophyllum* (Nautiyal et al. 2009b). After anthesis, it takes 4–6 days for most of the anthers of a single flower to shed their pollen grains. During the initial 4–5d, the stigmas were entirely obscured by a bunch of anthers, and they became prominent and responsive after 4–5d of anthesis. Pollens are tricolpate with thick granulate exine and exhibited a viability rate of > 94% at all the study sites, indicating the entomophilous nature of flowers and favoring the biotic mechanism of pollination. Moreover, a high percentage of pollen viability also indicates that there are no meiotic abnormalities in *A. volaceum*. The P/O ratio of *A. violaceum* was reported 3729.72b ± 48.92:1, 1324.65a ± 55.92:1 and 1278.89a ± 68.09:1 at Khawous, Numsuru and Maan-man respectively. Such types of pollen display and stigma receptivity have also been reported in other *Aconitum* species such as *A. columbianum* (Brink 1980; Siddique 1991), *A. heterophyllum* (Wafai et al. 2005; Nautiyal et al. 2009a), *A. lycoctonum* (Utelli and Roy 2000). The pollen-ovule ratio is a key indicator of the plant breeding system. According to Cruden, (1977), if the plant P/O ratio of a species goes from 2108.0 to 195525.0, the breeding system should be total xenogamy. These larger ratios correspond to usual out-crosser ratios, and hence indicate the cross-compatible nature of the species. Maximum *in vitro* pollen germination with healthy and vigorous pollen tubes were reported in calcium nitrate and boric acid. Similarly, calcium nitrate was proven to be an ideal nutrient medium for *in vitro* pollen germination of *A. chasmenthum*. (Gojri et al. 2009). Calcium ions have a synergistic function in pollen germination in various other angiosperm species (Brewbaker and Kwack 1963).

4.2. Pollination ecology

The genus *Aconitum* has a characteristic floral form. The exterior surface of flowers is composed of five violet sepals, with two nectaries concealed in the hood sepal (Fukuda et al. 2001). In *A. violaceum* inflorescences, the older flowers lie at the bottom, and successively the upper flowers are younger than the basal ones. The older flowers serve as females and the younger flowers act as males as a result of protandry, which is the key characteristic feature of the genus *Aconitum*. These flowers are typically pollinated by bumblebees. They usually star foraging near the base of the inflorescence and move successively upward (Kronfeld 1890; Loken 1949; Heinrich 1977). Among all the insect visitors, Bumblebees (*Bombus* spp) exhibited maximum foraging behaviour, insect visiting efficiency, foraging

speed, and pollen load, which clearly demonstrated that they are the primary pollinators of *A. violaceum*. A similar pattern of anthesis and the foraging behavior of Bumblebees in pollination were also reported from other aconites namely, *A. carmichalii* (Anton et al. 2014), *A. balfourii* (Nautiyal et al. 2009b), *A. chasmenthum* (Gojri et al. 2009), *A. columbianum* (Brink 1980), *A. gymnandrum* (Duan et al. 2009), *A. heterophyllum* (Nautiyal et al. 2009a; Wafai et al. 2005), *A. lycoctonum* (Anton et al. 2014) and *A. nagrum* (Langhu and Deb 2014). Pyke (1974; 1978) stated that the unique foraging behaviour of *Bombus* species fosters foraging efficiencies due to: (i) The older flowers generate more nectar and bumblebees tend to commence foraging on those flowers which contains most nectar; i.e., bottom functionally female flowers. The bees forage on progressively younger flowers as they move up the raceme, but they depart prior to reaching the topmost opened flowers, apparently due to decreasing nectar levels. A similar pattern of foraging behavior and nectar productions were also noticed in other Aconites such as *A. columbianum*, *A. chasmenthum* (Brink 1980; Gojri et al. 2009), bee pollinated *Delphinium* spp and in *A. violaceum* in the current study. Moreover, the foraging behavior of bumble in *A. violaceum* at Ladakh Himalaya is strongly influenced by environmental circumstances. Sunny day increases pollinator frequency whereas rainy and windy days reduces the number of insect visitors.

4.3. Breeding behavior

The mating system plays a critical role in gene flow, genetic diversity, and speciation. Cross-pollination promotes genetic diversity, increases the potential for genetic recombination, and can lead to improved vigor, adaptation, and reproductive success. Moreover, understanding the mating system of a species is crucial to managing the risk of inbreeding depression in conservation programmes and breeding efforts. Cross breeding taxa produce a high level of genetic diversity within populations (Brown 1989; Ritland 1989; Neel 2002).

The controlled breeding experiment revealed that *A. violaceum* is a partially self-compatible, partially protandrous, and cross-fertile plant. It developed the maximum percentage of fruits (86.66%) with a seed setting rate of 94.87% through open pollination and induced xenogamy, indicating that it favored out-crossing, which is strongly supported by floral structure and behavior, such as high pollen viability, pollen presentation, stigma receptivity, a high pollen-ovule ratio, a protandrous type of dichogamy, the progressive pattern of anthesis, flower architecture and intra-inflorescence foraging behavior. The plant also developed fruits through geitonogamy pollination, but the contribution of fruit development was comparatively less (37.77%). Spontaneous autogamy and forced autogamy developed only a few fruits. Such fruits were smaller and lighter-weighted than those developed by open pollination and induced xenogamy and were mostly aborted in the early stages of development. Similar breeding behavior has

been reported in other *Aconitum* species, such as *Aconitum helerophyllum* (Siddique 1991; Wafai et al. 2005; Nautiyal et al. 2009), *A. lycoctonum* (Utelli and Roy 2000), *A. kashmiriana* (Rashi et al. 2023). Duan et al. (2009) reported in *A. gymnandrum* that wind pollination offers extra pollen to unvisited and/or once-visited flowers, but contributes to only a small proportion of seed production in contrast to bumblebee pollination in natural conditions. In general, rare and small-population species suffer from inbreeding depression and reduced fitness because of homozygosity (Frankel & Soule 1981; Levin 1983; Barret & Kohn 1991). Genetic homogeneity makes the species more vulnerable to local extinction due to demographic and environmental stochasticity (Clegg and Brown 1983; Mitton and Grant 1984; Guerrant 1992).

5. Conclusions and implications for conservation

The present study revealed that *A. violaceum* is an endemic, threatened medicinal herb with restricted global distribution. It mostly perpetuates through underground perennating rhizomes and rarely through seeds and it completes its life cycle within a short growing season. The plant bears a bisexual flower with features like protandrous type of dichogamy, acropetal anthesis, asynchronous centripetal anther dehiscence, stigma receptivity, and P/O ratio favored outcrossing. The typical flower architecture, such as showy sepals, prominent nectaries, and exine orientation encourages entomophily, and Bumblebees were reported as the primary pollinators which perform exclusively pollination. The mating experiments as well as reproductive indices like ISI, ID and SR confirmed that *A. violaceum* is partially self-compatible, but the floral traits support outcrossing. The seeds had very low germination potential. Most of the seeds undergo juvenile mortality due to extreme diurnal temperature fluctuations at germination time. It only propagates and perpetuates through underground perennating rhizomes. *A. violaceum* faces a greater threat of decline from its natural habitat due to catastrophic disturbance (soil erosion, flood, drought, climate change) as well as human disturbance (over harvesting of rhizomes, pre-maturation harvesting pressure, construction of high-altitude roads, and agricultural practices). Therefore, the present study provides detailed information on the phenology and reproductive strategies operating in *A. violaceum* which ultimately determine its reproductive success and are also helpful in developing efficient *in-situ* and *ex-situ* conservation approaches. The exploitation of this rare, endemic and threatened species must be halted with the participation of the forest and ecology department and other law enforcement organizations. Biodiversity is a common human heritage, so loss of each individual limits our future options.

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Authors contribution

AH Generated the research idea, performed the experimental work, and drafted the original manuscript. SS and IA N Supervised the research work and reviewed the manuscript. SR Statistical analysis, helped in drafting the manuscript. MM and SA Assisted in field survey and data collection and helps in writing the manuscript. All the authors reviewed and approved the final draft of the manuscript for submission.

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Data Availability:

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable, as this is a plant- based study.

Consent for publication

Not applicable

Plant Reproducibility

Plant material of *Aconitum violaceum* Jacob ex Stapf were selected from three sites in the Kargil, Ladakh Himalaya, India: Khawous (N34°15′594 − N34°16′044; E75°95′887 − E75°96′172; 3200 − 3222m asl; Numsuru (N34°13′562 − N34°12′284; E75°96′715 − E75°96′340; 3280 − 3340m asl) and Maan-man top (N34°40′157 − N34°39′284; E75°78′815 − E75°78′440; 3980 − 4010m asl). The specimen was identified and authenticated by Dr. Anzar Ahmad Khuroo at the Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir, Hazratbal, Srinagar. A herbarium voucher specimens were also submitted to the Kashmir University Herbarium (KASH) under specimen vouchers accession number. 3738-KASH and 3739-KASH, dated July 27, 2021 for future reference.

The studied species (*Aconitum violaceum* Jacob ex Stap) is indeed enlisted in both IUCN Red List of threatened Species and CITES Appendices. To minimize any impact on natural populations, phenological observations and measurements were conducted on living plants in their natural habitats without damaging or removing plant parts. Breeding experiments were also performed in the wild without destructing the wild populations. Seed-related data were recorded only after natural seed dispersal. No destructive sampling of individuals was undertaken for the purposes of this study. All field investigations and experimental procedures were carried out in accordance with applicable institutional, national, and international guidelines and regulations governing biodiversity research and

the conservation of threatened plant species. The research was observational in nature and focused on documenting phenotypic variations across altitudinal gradients. As no protected plant material was collected, transported, traded, or subjected to destructive experimental procedures, the study posed negligible risk to the species or its habitat. Necessary permissions for field access and research activities were obtained from the relevant authorities prior to conducting the study. Moreover during the study with the help of agrotechniques many plants are restored in the natural habitats.

Competing interests:

The authors declare that they no competing interests.

References

Nogler GA (1984) Gametophytic apomixis. In: Johri BM (ed) Embryology of angiosperms. Springer-, Berlin, Heidelberg, New York, Tokyo, pp 475–518

Carman JG, Jamison M, Elliott E, Dwivedi KK, Naumova TN (2011) Apospory appears to accelerate onset of meiosis and sexual embryo sac formation in sorghum ovules. BMC Plant Biol 11:9. https://doi.org/10.1186/1471-2229-11-9

Carman JG, Crane CF, Riera-Lizarazu O (1991) Comparative histology of cell walls during meiotic and apomeiotic megasporogenesis in two hexaploid Australasian *Elymus* species. Crop Sci 31:1527–1532. https://doi.org/10.2135/cropsci1991.0011183X003100060029x

Ahmad M, Uniyal SK, Batish DR, Rathee S, Sharma P, Singh HP (2021) Flower phenological events and duration pattern is influenced by temperature and elevation in Dhauladhar mountain range of Lesser Himalaya. Ecological Indicators 129:107902. https://doi.org/10.1016/j.ecolind.2021.107902

Antoń S, Denisow B (2014) Nectar production and carbohydrate composition across floral sexual phases: contrasting patterns in two protandrous *Aconitum* species (Delphinieae, Ranunculaceae). Flora-Morphology, Distribution, Functional Ecology of Plants 209:464–470. https://doi.org/10.1016/j.flora.2014.07.001

Barret SCH, Kohn JD (1991) Genetic and evolutionary consequences of small population size in plants: implications for conservation. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, New York, pp 3–30.

Barrett SCH (2002) Sexual interference of the floral kind. Heredity 88:154–159. https://doi.org/10.1038/sj.hdy.6800020

Bhattarai S, Chaudhary RP, Quave CL, Taylor RSL (2010) The use of medicinal plants in the trans-Himalayan arid zone of Mustang district. Nepal J Ethnobiol Ethnomed 6:1–11. https://doi.org/10.1186/1746-4269-6-14

Bingham RA, Orthner AR (1998) Efficient pollination of alpine plants. Nature 391:238–239. https://doi.org/10.1038/34564

Blambert L, Mallet B, Humeau L, Pailler T (2016) Reproductive patterns, genetic diversity and inbreeding depression in two closely related Jumellea species with contrasting patterns of commonness and distribution. Annals of Botany 118: 93–103. https://doi.org/10.1093/aob/mcw014

Bliss LC (1971) Arctic and alpine plant life cycles. Annual Review of Ecology, Evolution, and Systematics 2:405–438. https://doi.org/10.1146/annurev.es.02.110171.002201

Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. American journal of botany 50:859–865. https://doi.org/10.1002/j.15372197.1963.tb06564.x

Brink DE (1980) Reproduction and variation in *Aconitum columbianum* (Ranunculaceae), with emphasis on California populations. American Journal of Botany 67:263–273. https://doi.org/10.1002/j.1537-2197.1980.tb07650.x

Brown AHD (1989) *Genetic characterization of mating systems*. In 'Plant population genetics, breeding and genetic resources. Eds ADH Brown, MT Clegg, AL Kahler, BS Weir, pp 145–162.

CAMP (2003) Threat assessment and management priorities of selected medicinal plants of Western Himalayan states, India. In: Proceedings of the Conservation assessment of medicinal plants workshop, Shimla, FRLHT, Bangalore, 22–26 May 2003.

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. Annual review of ecology and systematics 18:237–268. https://doi.org/10.1146/annurev.es.18.110187.001321

Chaudhary LB, Rao RR (1998) Notes on the genus *Aconitum* L. (Ranunculaceae) in North-West Himalaya (India). Feddes Repertorium 109:527–537.

Clegg MT, Brown AHD (1983) The founding of plant populations. In: Schonewald-Cox, S., Chambers, M., MacBryde, B., Thomas, L. (Eds.), Genetics and Conservation. Benjamin-Cummings, Menlo Park, California.

Cornelius C, Leing¨ artner A, Hoiss B, Krauss J, Steffan-Dewenter I, Menzel A (2013) Phenological response of grassland species to manipulative snowmelt and drought along an elevational gradient. Journal of Experimental Botany 64:241–251. https://doi.org/10.1093/jxb/ers321

Cosacov A, Nattero J, Cocucci AA (2008) Variation of pollinator assemblages and pollen limitation in a locally specialized system: the oil-producing *Nierembergia linariifolia* (Solanaceae). Annals of botany 102:723–734. https://doi.org/10.1093/aob/mcn154

Cresswell JE (2000) A comparison of bumblebees' movements in uniform and aggregated distributions of their forage plant. Ecological Entomology 25:19–25. https://doi.org/10.1046/j.1365-2311.2000.00230.x

Cruden RW, Lyon DL (2019) Facultative xenogamy: examination of a mixed mating system. The Evolutionary Ecology of Plants. CRC Press, pp 171–207

Dafni A, Mau´es MM, (1998) A rapid and simple procedure to determine stigma receptivity. Sexual Plant Reproduction 11:177–180. https://doi.org/10.1007/s004970050138

Dall'Acqua S, Shrestha BB, Gewali MB, Jha PK, Carrara M, Innocenti G (2008). Diterpenoid alkaloids and phenol glycosides from *Aconitum naviculare* (Bruhl) Stapf. Natural Product Communications 3: 985–1989. https://doi.org/10.1177/1934578X0800301209

Dar GH, Khuroo AA (2020) Biodiversity of the Himalaya: Jammu and Kashmir State. Springer Nature. https://doi.org/10.1007/978-981-32-9174-4

Davi H, Gillmann M, Ibanez T, Cailleret M, Bontemps A, Fady B, Lefèvre F (2011) Diversity of leaf unfolding dynamics among tree species: new insights from a study along an altitudinal gradient. Agricultural and Forest Meteorology 151:1504–1513. https://doi.org/10.1016/j.agrformet.2011.06.008

Delaplane KS, Dag A, Danka RG, Freitas BM, Garibaldi LA, Goodwin RM, Hormaza JI (2013). Standard methods for pollination research with *Apis mellifera*. Journal of Apicultural Research 52:1–28. https://doi.org/10.3896/IBRA.1.52.4.12

Duan YW, Zhang TF, He YP, Liu JQ (2009) Insect and wind pollination of an alpine biennial *Aconitum gymnandrum* (Ranunculaceae). Plant Biology 11:796–802. https://doi.org/10.1111/j.1438-8677.2009.00195.x

Fišer Ž, Aronne G, Aavik T, Akin M, Alizoti P, Aravanopoulos F, Bacchetta G, Balant M, Ballian D, Barazani O, & Zippel E (2021) Conserve Plants: An integrated approach to conservation of threatened plants for the 21st Century. Research ideas and outcomes 7:1–29.

Frankel OH, Soule ME (1981) Conservation and Evolution. Cambridge University Press, Cambridge.

Friedman WE, Williams JH (2003) Modularity of the angiosperm female gametophyte and its bearing on the early evolution of endosperm in flowering plants. Evolution 57:216–230. https://doi.org/10.1111/j.0014-3820.2003.tb00257.x

Fukuda Y, Suzuki K, & Murata J (2001) The function of each sepal in pollinator behaviour and effective pollination in *Aconitum japonicum* Var. montanum. Plant species Biology 16:151–157. https://doi.org/10.1046/j.1442-1984.2001.00059.x

Gamba D, Muchhala N (2020) Global patterns of population genetic differentiation in seed plants. Molecular Ecology 29: 3413–3428. https://doi.org/10.1111/mec.15575

Ganie AH, Tali BA, Reshi ZA, Nawchoo IA (2021) Inflorescence architecture, floral part movements and pollinator attraction by androecia–contrivance for successful mating in *Eremurus himalaicus* Baker. Botanica Pacifica 10:29.

Gao LM, Yan LY, He HY, Wei XM (2006) Norditerpenoid alkaloids from *Aconitum spicatum* Stapf. Journal of Integrative Plant Biology 48:364–369. https://doi.org/10.1111/j.1744-7909.2006.00178.x

Geslin B, Baude M, Mallard F, Dajoz I (2014) Effect of local spatial plant distribution and conspecific density on bumble bee foraging behaviour. Ecological Entomology 39:334–342. https://doi.org/10.1111/een.12106

Gojri GM, Nawchoo IA, Wafai BA (2009) Meiotic system and pollination mechanism of the critically endangered *Aconitum chasmenthum* Stapf ex Holmes- A novel species endemic to Kashmir himalyan region. International Journal of Plant Biology 1:153–162

Grindeland JM, Sletvold N, Ims RA (2005) Effects of floral display size and plant density on pollinator visitation rate in a natural population of Digitalis purpurea. Functional Ecology 19:383–3. https://www.jstor.org/stable/3599130

Guerrant Jr EO (1992) Genetic and demographic considerations in rare plant conservation. In: Fiedler, P.L., Jain, J.K. (Eds.), Conservation Biology. Chapman and Hall, New York.

Hadi A, Singh S, Nawchoo IA, Rafiq S, Ali S (2022) Impacts of habitat variability on the phenotypic traits of *Aconitum violaceum* Jacq. Ex Stapf. at different altitudes and environmental conditions in the Ladakh Himalaya India. Plant Science Today 9:546–554. https://doi.org/10.14719/pst.1734

Hadi A, Rafiq S, Singh S, Ali S, Nawchoo IA, Wagay NA (2023) Seed viability, seed germination, and micropropagation of *Aconitum violaceum* Jacq. ex Stapf—a rare and threatened medicinal plant of Ladakh Himalaya. In Vitro Cellular & Developmental Biology-Plant 59:315–328. https://doi.org/10.1007/s11627-023-10331-x

Hamid M, Khuroo AA, Malik AH, Ahmad R, Singh CP, Dolezal J, Haq SM (2020) Early evidence of shifts in alpine summit vegetation: a case study from Kashmir Himalaya. Frontiers in plant science 11:421. https://doi.org/10.3389/fpls.2020.00421

Hartmann DL, Tank AMK, Rusticucci M, Alexander LV, Bronnimann S, Charabi YAR, Soden BJ (2013) Observations: atmosphere and surface. In: Climate Change 2013. The Physical Science Basis: Working group I contribution to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press, United Kingdom

Hassan T, Hamid M, Wani SA, Malik AH, Waza SA, Khuroo AA (2021) Substantial shifts in flowering phenology of Sternbergia vernalis in the Himalaya: Supplementing decadal field records with historical and experimental evidences. Science of the Total Environment 795:148811. https://doi.org/10.1016/j.scitotenv.2021.148811

Hauser EJ, Morrison JH (1964) The cytochemical reduction of nitro blue tetrazolium as an index of pollen viability. American Journal of Botany 51:748–752. https://doi.org/10.1002/j.1537-2197.1964.tb06696.x

Heinrich B (1977) The game of the bees. Horticulture 55:39–45

Hülber K, Winkler M, Grabherr G (2010) Intraseasonal climate and habitat-specific variability controls the flowering phenology of high alpine plant species. Functional ecology 24:245–252. https://doi.org/10.1111/j.1365-2435.2009.01645.x

IPBES (2019) Global assessment report on biodiversity and ecosystem services of the intergovernmental science policy platform on biodiversity and ecosystem services. In Brondizio, ES, J Settele, S Díaz, & HT Ngo editors. IPBES secretariat. Bonn, Germany. p 1148.

Jabeen N, Kozgar MI, Dar GH, Shawl AS, Khan S (2013) Distribution and Taxonomy of Genus *Aconitum* in Kashmir: Potent Medicinal Resource of Himalayan Valley. Chiang Mai Journal of Science 40:173–186.

Kaur D, Tiwana AS, Kaur S, Gupta S (2022) Climate change: concerns and influences on biodiversity of the Indian Himalayas. In: Rani, S., Kumar, R. (Eds.), Climate Change. Springer Climate. Springer, Cham. https://doi.org/10.1007/978-3-030-92782-0_13

Khajuria A (2013) Conservation Biology of Three Overexploited Medicinal Plants of North-West Himalayan Region. Ph.D. thesis. Rajouri: Baba Ghulam Shah Badshah University, J& k, India

Kopp CW, Neto-Bradley BM, Lipsen LP, Sandhar J, Smith S (2020) Herbarium records indicate variation in bloom-time sensitivity to temperature across a geographically diverse region. International journal of biometeorology 64:873–880. https://doi.org/10.1007/s00484-020-01877-1

Korner C, Cochrane PM (1983) Stomatal responses and water relations of Eucalyptus pauciflora in summer along an elevational gradient. Oecologia 66:443–455. https://doi.org/10.1007/BF00378313

Kronfeld M (1890) Uber die biologischen Verltnisse der Aconitum-Blute. Engler's Botany. Jahrbher 1:1–20.

Loken A (1949) Bumble bees in relation to *Aconitum septentrionale* in central Norway. Nyt Magazin for Naturvidenskaberne 87:1–60.

Langhu T, Deb CR (2014) Studies on the reproductive biology and seed biology of *Aconitum nagarum* Stapf: a threatened medicinal plant of North East India. Journal of Research in Biology 4:1465–1474.

Lessard-Therrien M, Bolmgren K, Davies TJ (2014) Predicting flowering phenology in a subarctic plant community. Botany 92:749–756. https://doi.org/10.1139/cjb-2014-0026

Levin DA (1983) Immigration in plants: An exercise in the subjuntive. In: Sclionewald-Cox, S., Chambers, M., MacBryde, B., Thomas, L. (Eds.), Genetics and Conservation. Benjamin-Cummings, Menlo Park, California

Li T, Liu X, Li Z, Ma H, Wan Y, Liu X, Fu L (2018) Study on reproductive biology of *Rhododendron longipedicellatum*: A newly discovered and special threatened plant surviving in limestone habitat in Southeast Yunnan, China. Frontiers in Plant Science 9:33. https://doi.org/10.3389/fpls.2018.00033

Lloyd DG, Webb CJ (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms. I. Dichogamy. New Zealand Journal of Botany 24:135–162. https://doi.org/10.1080/0028825X.1986.10409725

Lu NN, Li X H, Li L, Zhao ZG (2015) Variation of nectar production in relation to plant characteristics in protandrous *Aconitum gymnandrum*. Journal of Plant Ecology 8:122–129. https://doi.org/10.1093/jpe/rtv020

Lubbers AE, Christensen NL (1986) Intra-seasonal variation in seed production among flowers and plants of *Thalictrum thalictroides* (Ranunculaceae). American Journal of Botany 73:190–203. https://doi.org/10.1002/j.1537-2197.1986.tb08520.x

Marbaniang EJ, Venugopal N, Verma S, Raina R, Khajuria A, Gautam K (2018) Floral biology and embryological studies are important for conservation of threatened plants having reproductive bottlenecks: a case study of *Illicium griffithii* Hook. f. & Thomson. Current Science 114:576–587. https://www.jstor.org/stable/26495111

Mehta P, Sekar KC, Bhatt D, Tewari A, Bisht K, Upadhyay S, Negi VS, Soragi B (2020) Conservation and prioritization of threatened plants in Indian Himalayan Region. Biodiversity and Conservation 29:1723–1745. https://doi.org/10.1007/s10531-020-01959-x

Miana GA, Ikram M, Khan MI, Sultana F (1971) Alkaloids of *Aconitum violaceum*. Phytochemistry, 10, 3320–22. https://doi.org/10.1016/S0031-9422(00)97417-X

Mitton JB, Grant MC (1984) Associations among protein heterozygosity, growth rate and developmental homeostasis. Annual Review of Ecology Systematics 15:479–499. https://doi.org/10.1146/annurev.es.15.110184.002403

Mohan JE, Wadgymar SM, Winkler DE, Anderson JT, Frankson PT, Hannifin R, Benavides K, Kueppers LM, Melillo JM (2019) Plant reproductive fitness and phenology responses to climate warming: Results from native populations, communities, and ecosystems. In: Mohan, J.E. (Ed.), Ecosystem Consequences of Soil Warming. Academic Press, USA, pp 61–102.

Moza MK, Bhatnagar AK (2007) Plant reproductive biology studies crucial for conservation. Current Science-Bangalore 92:1207.

Nautiyal BP, Nautiyal MC, Khanduri VP, Rawat N (2009a) Floral biology of *Aconitum heterophyllum* Wall.: a critically endangered alpine medicinal plant of Himalaya, India. Turkish Journal of Botany 33:13–20. https://doi.org/10.3906/bot-0805-8

Nautiyal BP, Nautiyal MC, Rawat N, Nautiyal AR (2009b) Reproductive biology and breeding system of Aconitum balfourii (Benth) Muk: a high altitude endangered medicinal plant of Garhwal Himalaya, India. Research Journal of Medicinal Plant 3:61–68.

Neel MC (2002) Conservation implications of the reproductive ecology of *Agalinis acuta* (Scrophulariaceae). American Journal of Botany 89:972–980. https://doi.org/10.3732/ajb.89.6.972

Pando JB, Fohouo FNT, Tamesse JL (2011) Foraging and pollination behaviour of *Xylocopa calens Lepeletier* (Hymenoptera: Apidae) on *Phaseolus coccineus* L. (Fabaceae) flowers at Yaounde (Cameroon). Entomological Research 41:185–193. https://doi.org/10.1111/j.1748-5967.2011.00334.x

Pellerin M, Delestrade A, Mathieu G, Rigault O, Yoccoz NG (2012) Spring tree phenology in the Alps: effects of air temperature, elevation and local topography. European Journal of Forest Research 131:1957–1965. https://doi.org/10.1007/s10342-012-0646-1

Pyke BH (1974) Studies in the foraging efficiency of animals. Ph. D. Dissertation, University of Chicago

Pyke, G.H., 1978. Optimal foraging in bumble bees and coevolution with their plants. Oecologia 36:282–293. https://doi.org/10.1007/BF00348054

Rafferty NE, Diez JM, Bertelsen CD (2020) Changing climate drives divergent and nonlinear shifts in flowering phenology across elevations. Current Biology 30:432–441. https://doi.org/10.1016/j.cub.2019.11.071

Ramirez, N, Nassar JM (2017) Breeding systems in Angiosperms: novel inferences from a new analytical approach. Plant Systematics and Evolution 303:119–137. https://doi.org/10.1007/s00606-016-1357-8

Rashid K, Rashid S, Ganie AH, Nawchoo IA, Khuroo AA (2023) Reproductive biology of *Trillium govanianum*, an endangered plant species endemic to the Himalaya: implications for conservation. Botany Letters 1–16. https://doi.org/10.1080/23818107.2023.2176355

Rashid S, Rashid K, Wani BA, Ganie AH, Nawchoo IA, Khuroo AA (2023) Reproductive biology of *Actaea kashmiriana*, an endemic medicinal plant species from the Himalaya: implications for conservation and sustainable utilization. Journal of Applied Research on Medicinal and Aromatic Plants, 34, 100461. https://doi.org/10.1016/j.jarmap.2023.100461

Rathcke BJ, Jules ES (1993) Habitat fragmentation and plant–pollinator interactions. Current Science 65:273–277. http://www.jstor.org/stable/24095130

Ritland CK, Ritland K (1989) Variation in sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). Imer Journal Botany 76:1731–1739.

https://doi.org/10.1002/j.1537-2197.1989.tb15163.x

Rledl H (1993) The genus *Aconitum* in Pakistan and adjacent territories. Linzer biologischen Beiträge 25:67–79

Sajjad A, Saeed S, Muhammad W, Arif MJ (2009) Role of insects in cross-pollination and yield attributing components of *Sesbania sesban*. International Journal of Agricultural Biology 11:77–80.

Shivanna KR, Rangaswamy NS (1992a) In vitro germination methods. Pollen Biology. Springer, Berlin, Heidelberg, pp 9–22.

Shivanna KR, Rangaswamy NS (1992b) Pollen Biology: A Laboratory Manual. Springer, Berlin Heidelberg New York.

Siddique MAA (1991) Germplasm Assessment of Some Rare and Threatened Medicinal Plants of Kashmir Himalayas. Ph.D. thesis. University of Kashmir, Srinagar, (J&K) India.

Singh JS, Khurana E (2002) Paradigms of biodiversity: An overview. Proceedings of the National Academy of Sciences, India-Section B: Biological Sciences 68:273–296. http://www.dli.gov.in/rawdataupload/upload/insa/IN...

Sletvold N, Grindeland JM, Zu P, Ågren J (2012) Strong inbreeding depression and local outbreeding depression in the rewarding orchid *Gymnadenia conopsea*. Conservation Genetics 13:1305–1315. https://doi.org/10.1007/s10592-012-0373-7

Smitha GR, Thondaiman V (2016) Reproductive biology and breeding system of *Saraca asoca* (Roxb.) De Wilde: a vulnerable medicinal plant. Springer Plus 5:2025. https://doi.org/10.1186/s40064-016-3709-9

Stanley RG, Linskens HF (1974) Viability tests. Pollen. Springer, Berlin, Heidelberg, pp 67–86.

Swanson SD, Sohmer SH (1976) The biology of *Podophyllum peltatum* L. (Berberidaceae), the May apple. II. The transfer of pollen and success of sexual reproduction. Bulletin of the Torrey Botanical Club 103:223–226. https://doi.org/10.2307/2484683

Tali BA, Khuroo AA, Ganie AH, Nawchoo IA (2019) Diversity, distribution and traditional uses of medicinal plants in Jammu and Kashmir (J&K) state of Indian Himalayas. Journal of Herbal Medicine, 17, 100280. https://doi.org/10.1016/j.hermed.2019.100280

Thuiller W, Lavorel S, Araújo MB, Sykes MT, Prentice IC (2005) Climate change threats to plant diversity in Europe. Proceedings of the National Academy of Sciences 102:8245-8250. https://doi.org/10.1073/pnas.0409902102

Trunschke J, Stocklin J (2017) Plasticity of flower longevity in alpine plants is increased in populations from high elevation compared to low elevation populations. Alpine Botany 127:41–51. https://doi.org/10.1007/s00035-016-0176-4

Utelli AB, Roy BA (2000) Pollinator abundance and behavior on *Aconitum lycoctonum* (Ranunculaceae): an analysis of the quantity and quality components of pollination. Oikos 89:461–470. https://doi.org/10.1034/j.1600-0706.2000.890305.x

Ved D, Saha D, Ravikumar K, Haridasan K (2015) *Aconitum violaceum*. The IUCN Red List of Threatened Species 2015, e.T50126562A79581679.

Wafai BA, Siddique MA, Nawhoo IA, Dar NA, Bhat MA, Mohi-ud-din GG (2005) Studies on reproductive biology of some endangered medicinal herbs as a prelude to their *ex situ* conservation. The Journal of the Indian Botanical Society 84:88–106.

Wani BA, Magray JA, Ganie AH, Qadir RU, Javid H, Nawchoo IA (2023) Understanding reproductive biology for realization of conservation and sustainable development of *Swertia thomsonii* CB Clarke-an endemic medicinal plant of Western Himalaya. Journal of Applied Research on Medicinal and Aromatic Plants 35:100493. https://doi.org/10.1016/j.jarmap.2023.100493

Wani IA, Verma S, Ahmad P, El-Serehy HA, Hashim MJ (2022) Reproductive biology of *Rheum webbianum* Royle, a vulnerable medicinal herb from alpines of North-Western Himalaya. Frontiers in Plant Science 13:10. https://doi.org/10.3389/fpls.2022.699645

Wyatt R (1982) Inflorescence architecture: How flower number, arrangement and phenology affect pollination and fruit set. American Journal Botany 69:585–594. https://doi.org/10.1002/j.1537-2197.1982.tb13295.x

Yaqoob U, Nawchoo IA (2016) Reproductive ecology of an endangered monocarpic herbaceous perennial, *Ferula jaeschkeana* Vatke. Tropical Ecology 57:849–864.

Zapata TR, Arroyo MTK., 1978. Plant reproductive ecology of a secondary deciduous tropical forest in Venezuela. Biotropica 1:221–230. Doi: 10.2307/2387907

Zimmerman M (1988) Nectar production, flowering phenology, and strategies for pollination. In: Doust J.L., Doust, L.L. (Eds.), Plant Reproductive Ecology. Oxford University Press., New York-USA, pp 157–78.

Figure 1. Map depicting study sites of *A. violaceum* in the Ladakh Himalaya

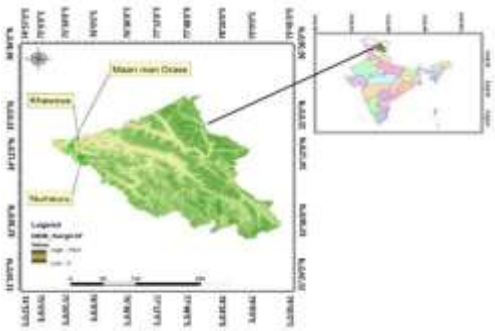
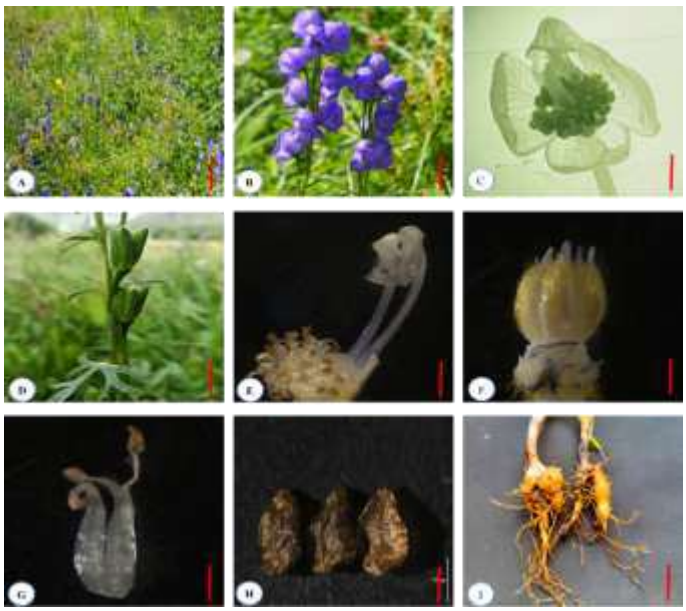


Figure 2. Morphometric traits of *A. violaceum*: (a) Habitat; (b) Inflorescence; (c) Flower; (d) Follicle fruits; (e) Petals with nectaries; (f) Carpels; (g) Stamens; (h) Seeds; (i) Rhizomes. (Scale bar represents 0.5 mm)



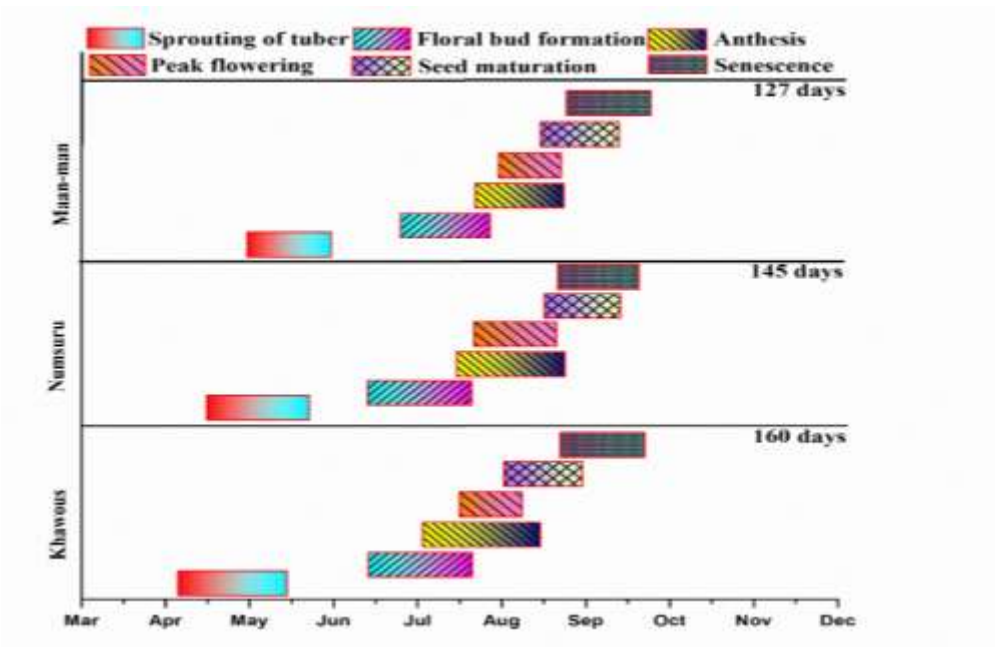


Figure 3. Graphical representation of phenophases of *A. violaceum* across three different study sites

Figure 4. Phases of anther dehiscence and stigma receptivity of *A. violaceum*. (a) Central anther dehiscence on 1DA; (b–c) Anther dehiscence on 3DA or 4DA; (d-e) Anther dehiscence on 5DA or 6DA; (f) Anther dehiscence and pollen discharge, 7DA or 8DA; (g) Position of stigma and style with respect to the ovary on 0DA; (h) Style bend slightly outward; (h^{a-d}) Different phases of stigma and style position with respect to the ovary on 0DA–8DA; (i) The groove of stigma bifurcates after losing complete receptivity on the 9DA or 10DA. (Scale bar represents 5mm).

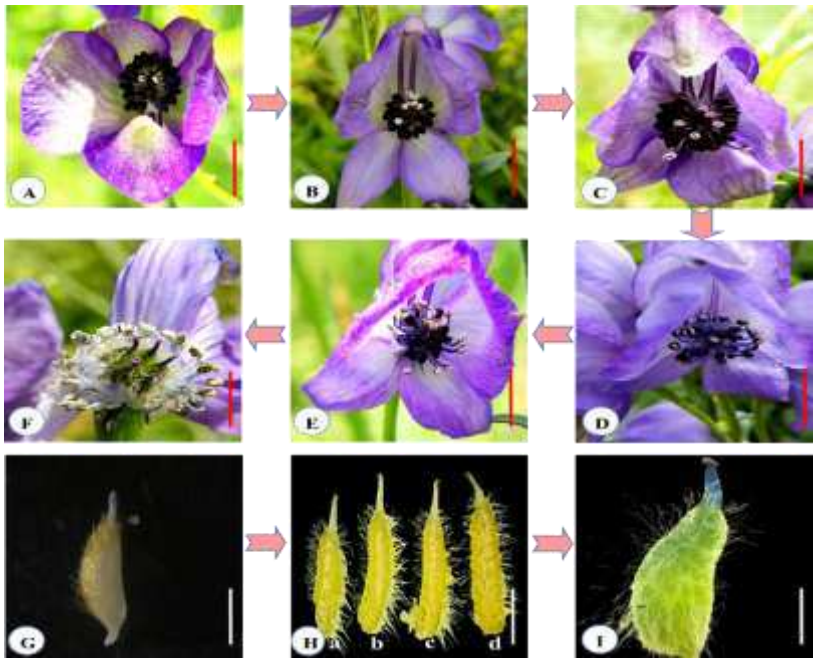


Figure 5. Pollen morphometry and viability of *A. violaceum* prior to anthesis or on the day of anthesis. (a-b) Polar and equatorial view of pollen gains (SEM), Mag = 6.00 KX; (c) Exine showing granulate ornamentation, Mag = 100.00 KX; (d) Granulate exine; mag =20.00 KX; (e) spheroidal or ovoid tricolpate pollen grains; (f) Viable and non-viable pollen gains (1% aniline blue-lactophenol); (g) Viable pollen grain (1% 2,3-5 triphenyl tetrazolium chloride); (h-i) Viable and non-viable pollen grains (1% acetocarmine).

Figure 6. Line diagram depicting the pattern of pollen viability of *A. violaceum* at various study sites employing (a) 1% acetocarmine; (b) lactophenol. Bar represents SE for n = 15

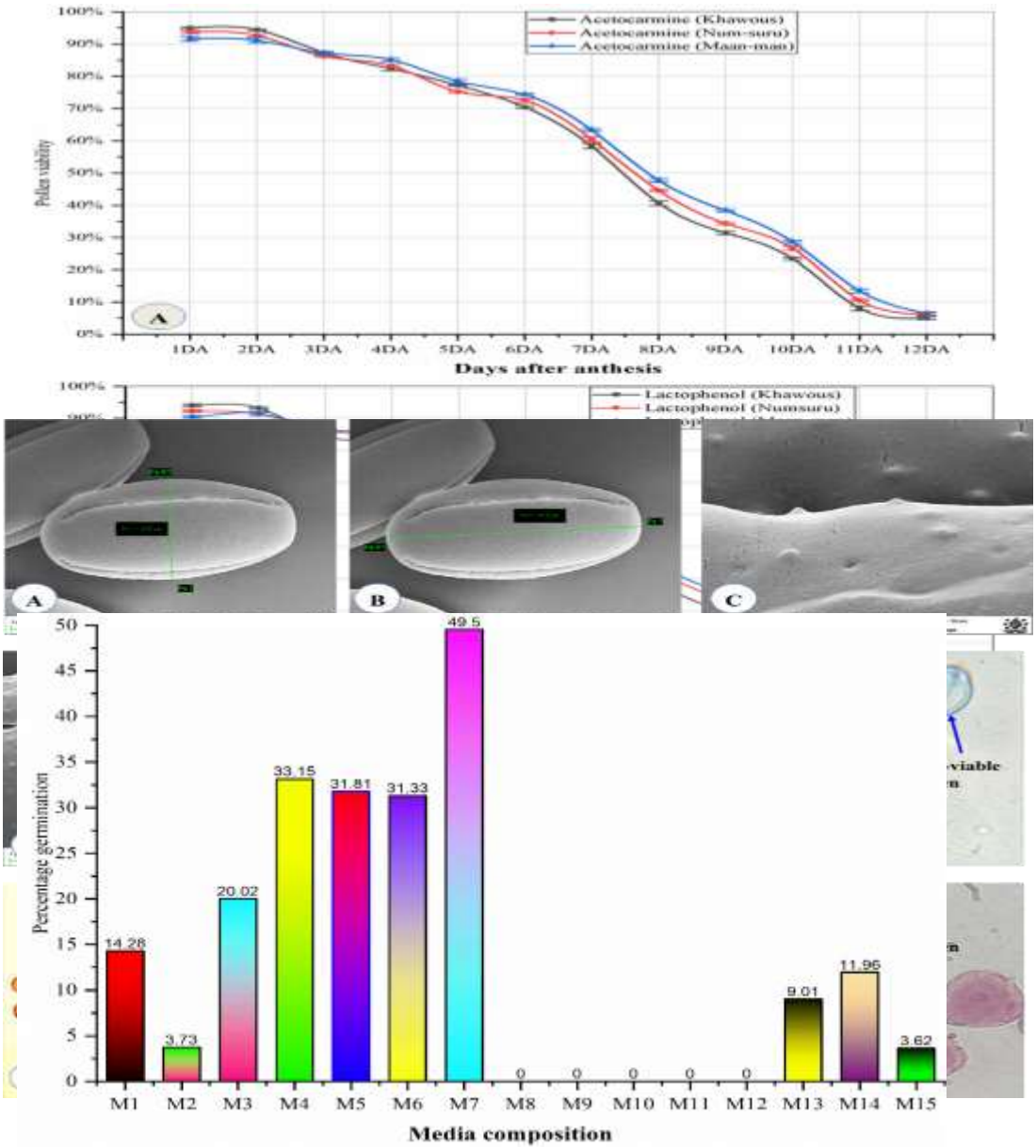


Figure 7. Assessment of *in vitro* germination of pollen grains collected from the first day of anther dehiscence of *A. violaceum*

Figure 8. *In vitro* pollen germination in different nutrient media: (a) Viable pollens; (b) pollen germination in 10% sucrose; (c) 100 ppm Boric acid; (d) 200 ppm Boric acid; (e) 200 ppm Calcium nitrate; (f) 500 ppm Calcium nitrate

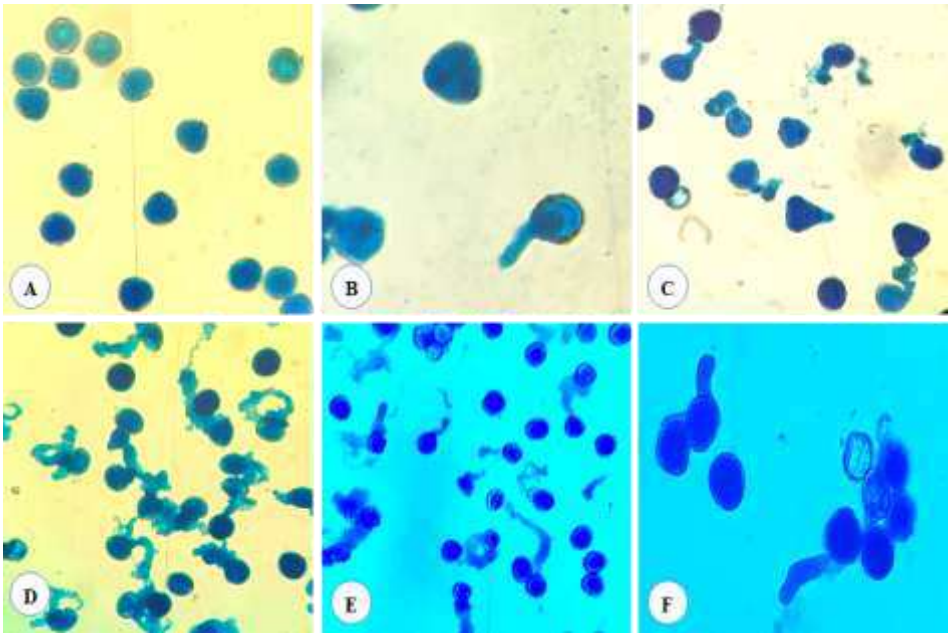


Figure 9. Pollen load and pollen germination on the stigmatic surface of *A. violaceum*: (a, b) No pollen loaded on the stigma from 0DA–2DA; (C) Pollen loaded but not germinated on the stigma on 3DA; (d–f) Pollen loaded and also germinated on the stigmatic surface after 5DA–8DA; (g-h); Stigma dissected like the tail-fin of a fish marks the end of receptivity (9DA–12DA); (i) Stigmatic surface emitting bubbles on the 6th day of anthesis demonstrating receptivity. *0DA*, on the day of anthesis; *DA*, day after anthesis

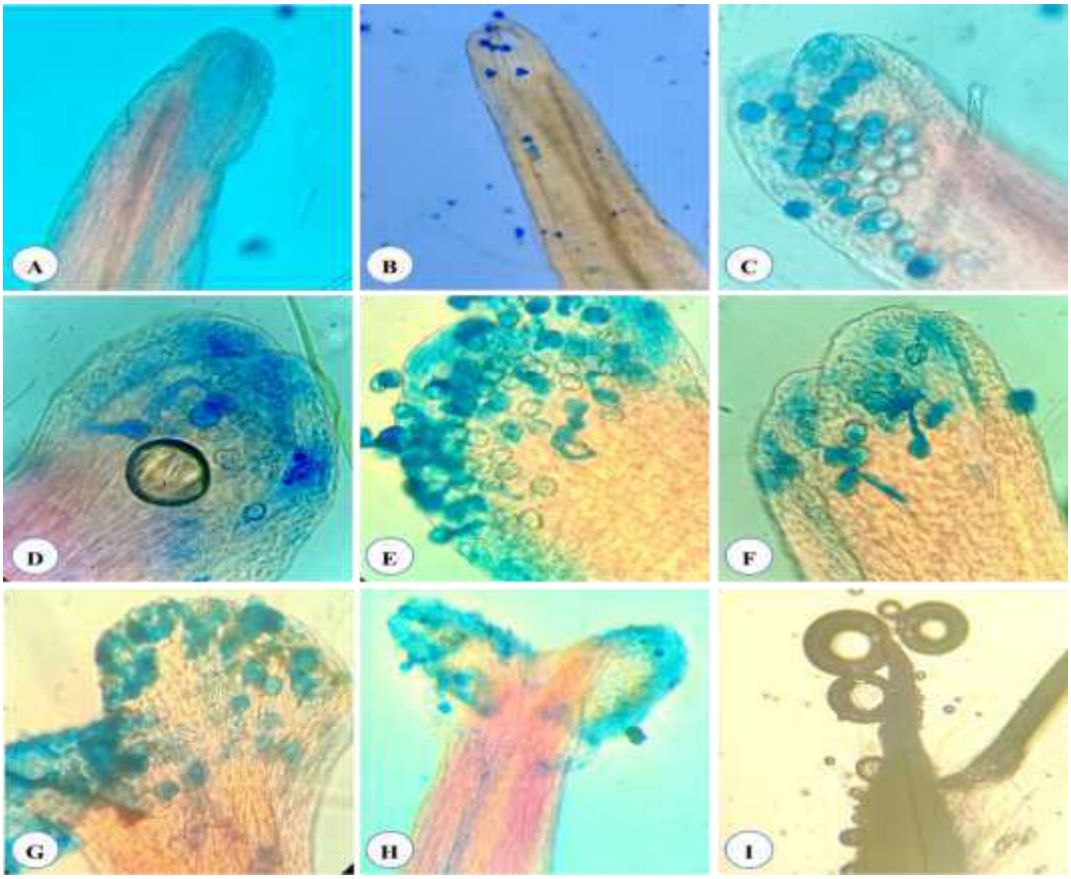


Figure 10. Assessment of pollen germination percentage on the stigmatic surface after manual pollination to check stigma receptivity. *KH*, Khawous; *NS*, Numsuru; *MM*, Maan-man

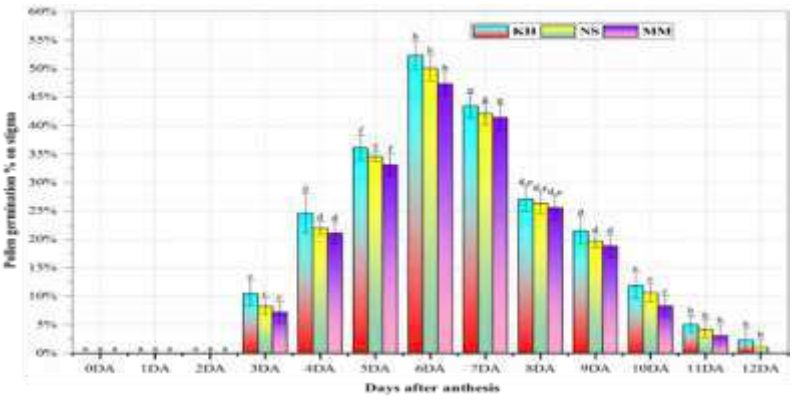


Figure 11. Number of oxygen bubbles emitted from the stigmatic surface of *A. violaceum* from the day of anthesis. The number of O_2 bubbles determines the degree of receptivity.

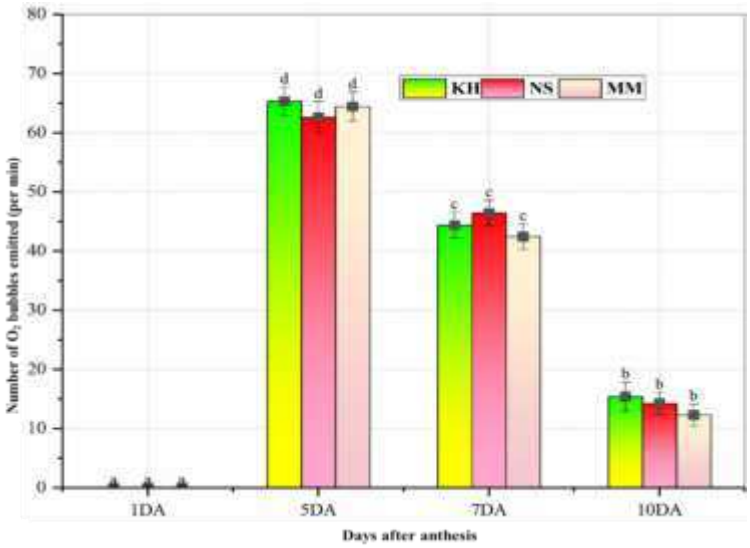


Figure 12. Visitors of *A. violaceum*. (A) *Bombus trifasciatus*; (B) *Bombus asiaticus*; (C) *Bombus ladakhensis*; (D-E) *Palomena prasina*; (F) *Apis spp*; (H) *Hycleus* spp; (I) *Papuaepilachna* spp.



Figure 13. Percentage contribution of insect visitors in performing pollination

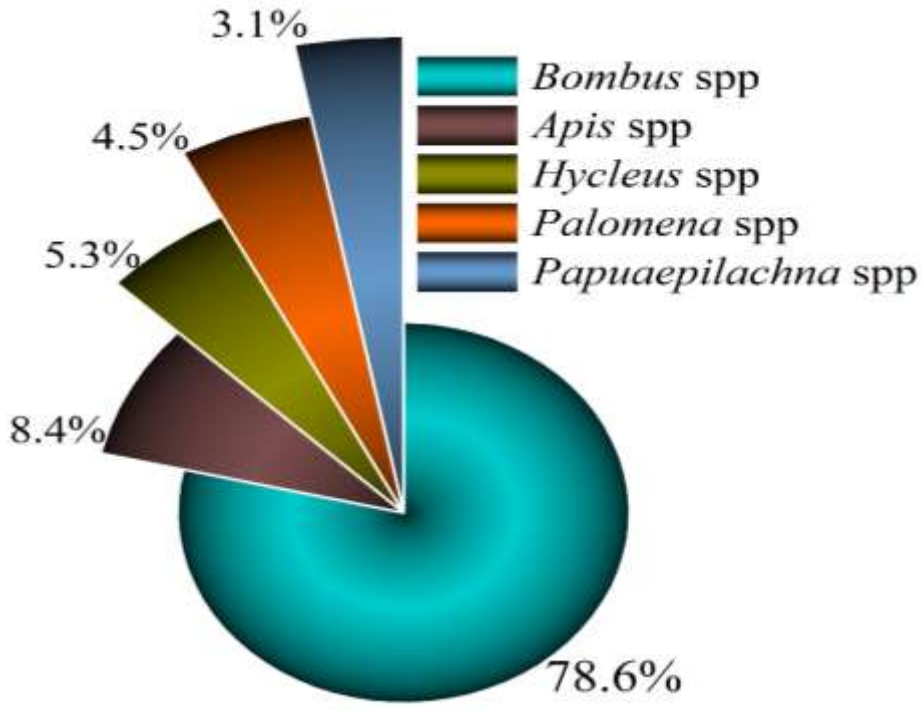
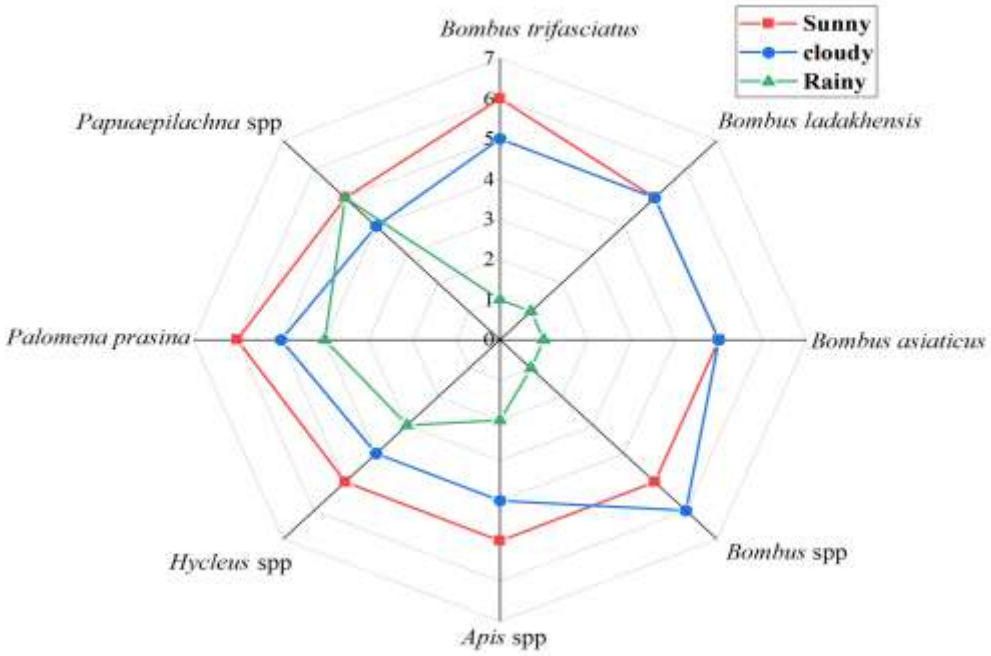


Figure 14. Pollinator visiting frequency varies at different weather conditions



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