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Inflorescence architecture, floral part movements and pollinator attraction by androecia – contrivance for successful mating in *Eremurus himalaicus* Baker

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ABSTRACT

Present study has been carried out to understand the mating strategies of *Eremurus himalaicus* Baker, important medicinal endemic plant, for its sustainable utilization. The inflorescence architecture, large numbers of attractive flowers, presence of sterile flowers which give specific arrangement to inflorescence, the coloured, attractive androecia and nectaries attract the pollinators. Protoandry, moderate to high pollen ovule ratio, unique movement of stigma away from dehiscing anthers, movement of pedicels and breeding experiment revealed that the species is primarily geitonogamous but xanogamy though rare is also operative. The exine sculpture and pollen size depicts that both anemophily and entomophily is operative in the species. Present study may provide important information for the conservation of this endemic medicinal plant species which has been categorized as threatened by various authors.

Keywords: endemic, medicinal importance, over-exploitation, mating system, conservation

РЕЗЮМЕ

Гани А.Х., Тали Б.А., Реши З.А., Начу И.А. Архитектура соцветий, подвижность частей цветка и привлечение опылителей мужскими цветками – приспособление для успешного опыления *Eremurus himalaicus* Baker. Исследование было проведено для понимания стратегий опыления *Eremurus himalaicus* в целях неистощительного использования его популяций. Архитектура соцветия, большое число эффектных цветков, наличие стерильных цветков, которые придают соцветию особую композицию, андроцеи и нектарники привлекают опылителей. Протандрия, умеренное и высокое соотношение яйцеклеток и пыльцы, уникальное удаление стигмы от раскрывающихся пыльников, движение цветоножек и эксперимент по размножению показали, что этот вид является в основном гейтогамным, хотя в редких случаях для него свойственна ксеногамия. Скульптура экзины и размер пыльцы показывают, что виду свойственны анемофилия и энтомофилия. Настоящее исследование может оказаться важным для сохранения этого эндемичного вида лекарственных растений, который был отнесен разными авторами к находящимся под угрозой исчезновения.

Ключевые слова: эндемик, лекарственное значение, чрезмерная эксплуатация, система опыления, сохранение

Floral and inflorescence characteristics are believed to have evolved to control the behaviours of pollinators (Iwata et al. 2011). Of the various floral and inflorescence characters, inflorescence architecture is one of important features which significantly affects pollinator behaviour and reproductive success. Inflorescence architecture has been evolve under pollinator-mediated natural selection in plant populations (Iwata et al. 2011, Harder & Prusinkiewicz 2012). It has also been demonstrated that presence of sterile flowers enhances pollinator attraction, supplementing the positive effect of the number of fertile flowers on the number of bees approaching the inflorescences (Morales et al. 2013). The orientation of flowers, the angle between a flowers' main and the horizontal axis affect pollinator attraction, foraging behavior and pollen transfer (Wang et al. 2014a). The movement of floral parts, including the pistil (style), stamen (filament, anther) and corolla have been observed in many angiosperms to affect successful pollination and mating (Ganie et al. 2015). The flower movements are also important to balance pollinator attraction and to protect the nectar and pollen (Haverkamp et al. 2019). It has been reported that styles do exhibit curvature movements either to promote outcrossing (Li et al. 2001, Verma et al. 2004); or to affect selfing or to achieve delayed selfing (Liu et al. 2006, Sun et al. 2007). Though the role of stylar movement in pollination in this species has been reported by Verma et al. (2004), little is known about role of inflorescence architecture, sterile flowers, movement of pedicle and attractive essential parts in accomplishment of pollination in this species. It is in this backdrop the present investigation has been carried out. In the present work we hypothesized that inflorescence architecture; floral part movements, production of sterile flowers and pollinator attraction by male parts are contrivance for successful mating in *Eremurus himalaicus* Baker.

MATERIAL AND METHODS

Study area

The present study was carried out in Kashmir Himalaya, which represents the main valley of Kashmir. The region falls within the biogeographic zone of the north-western Himalaya in India and lies between the latitudes 33°20' and 34°54'N and longitudes 73°55' and 75°35'E, covering an area of 15 948 sq. km. From the study area the target species was mainly collected and studied from Tangmarg (1673 m a.s.l.; 34°04'49"N 74°34'24"E), Dara-Srinagar (1680 m a.s.l.; 34°18'36"N 74°39'40"E) and Phalgam (2335 m a.s.l.; 34°02'84"N 75°20'50"E).

Sampling and measurements

Species sampled. *Eremurus himalaicus* (Xanthorrhoeaceae) is a perennial herb up to 190 cm in height; inflorescence (spike) long bearing white flowers; leaves all basal, leaves linear 30–90 cm long. The species is a Himalayan endemic medicinal plant, the tender leaves of the species are cooked and different recipes are used as tonic, against weak eye sight, urinary irritation and indigestion. e.g. the young leaves are fried with mutton and given to anemic and weak patients.

Flowering phenology. A random sample of 50 inflorescences was used for studying the morphology and flower development. The blossoming stages were observed / noted every day at regular time intervals. Subsequent events, such as fruit maturation were noted and recorded once a week. The micro-characters of inflorescence and flowers were analyzed and photographed with the help of trinocular stereo-zoom microscope (Model: Carl Zeiss Discovery V8).

Pollination. Presence of nectar at the base of ovary at different developmental stages was recorded by visual observations. Temporal activities of the floral visitors were recorded between 8:00 am to 14:00 pm over a one week period in each season. Each floral visitor was carefully observed during this period and legitimate pollinators were identified by bagging the flowers pollinated by different pollinators to record the fruit set

Stigma receptivity. Stigma receptivity was checked by fixing stigmas of different ages in Carnoy's fixative (3 alcohol: 1 acetic acid) for 3–4 hours. The stigmas were stained with aniline blue lacto phenol (Hauser & Morrison 1964) and scanned under light microscope (10× × 20× combination). The stigmas carrying germinating pollen grains were considered receptive.

Pollen features. Fresh pollen grains were collected from the dehiscing anthers by gentle shaking of flower. Immediately slides were prepared by dusting the collected pollen grains in a drop of lactophenol aniline blue. The shape and surface sculpture of pollen grains was observed at 10× eye piece and 100× objective lens of light microscope. Pollen fertility was estimated by two methods: 1) Dianne & Spicer's (1958) method, in which mature anthers ready to dehisce were squashed in 1 % aniline blue lactophenol and observed after 15 minutes; and 2) Stanley & Linsken's (1974) method, wherein mature anthers ready to dehisce were placed in 1 % tetrazolium chloride for one hour and squashed. Plump and well stained pollen in both cases were considered as viable.

Pollen to ovule ratio and pollen volume

Flowers ready to anthese were collected for estimating Pollen to ovule ratios (P/O). Pollen quantity was estimated by squashing one anther (several times) in 10 drops of distilled water in a cavity block and shaken with a glass rod. The following equations were used to calculate the number of pollen per flower:

$$\boldsymbol{r} = \boldsymbol{p} \times \boldsymbol{q} \quad \text{and} \quad \boldsymbol{t} = \boldsymbol{r} \times \boldsymbol{s},$$

where $\boldsymbol{p}$ is the mean pollen count per drop of water; $\boldsymbol{q}$ is the number of water drops taken initially in which one anther was squashed; $\boldsymbol{r}$ is the mean number of pollen per anther; $\boldsymbol{s}$ is the mean number of anthers per flower; $\boldsymbol{t}$ is the total count per flower.

Average ovule number per pistil was counted using dissection microscope.

Pollen to ovule ratio was calculated following Cruden's (1977) method as follows:

$$P/O = \frac{\text{Pollen count per anther} \times \text{Number of anthers per flower}}{\text{Number of ovules per flower}}$$

For calculating pollen volume Zhang's (2009) method was followed. The pollen grains from fifty randomly selected dehisced anthers were put in a drop of 2 % acetocrmine and diameter, polar axis and equatorial axis diameter were measured at 400× using ocular micrometer. The volume of pollen grain ($\boldsymbol{V}$) was calculated by following:

$$\boldsymbol{V} = \pi \boldsymbol{P}\boldsymbol{E}^2 / 6,$$

where $\boldsymbol{P}$ is the polar axis diameter and $\boldsymbol{E}$ is equatorial axis diameter.

Bagging experiments

The following experiments were conducted to ascertain breeding system operative in the species, which include:

I. Flowers were tagged and allowed to open pollinate.

II. Un-emasculated flower (only one flower/inflorescence) was bagged at bud stage and allowed to autonomous selfing.

III. Whole inflorescence was bagged to ascertain geitonogamy.

IV. The flowers were emasculated and allowed to open pollinate.

V. The tagged stigmas at its receptivity were pollinated with pollen grains from flowers of different plants to ascertain xenogamy.

The bagging experiments were carried out on 400 flowers/inflorescences in each case at all the selected sites (both *in-situ* and *ex-situ*) (n=20) for two consecutive years at each site within 3 years duration. However, in case of experiment no. II only one flower/inflorescence was tagged. The tagged flowers/inflorescences were later monitored for seed set.

Floral thinning experiment of inflorescence

Five inflorescences at each site also at transplanted site in Kashmir University Botanical Garden (KUBG) (n=20), were thinned by plucking flowers at the bud stages uniformly in order to check whether the number of sterile flowers will get reduced or the sterile flowers might not be produced in these thinned inflorescences.

Statistical analyses

The variation in different treatments were analysed using one-way ANOVA. Tukey tests were performed to determine post-hoc differences between means of traits. Data are presented as the Mean ± SE. Statistical significance was set at $P \leq 0.001$, and the results were adjusted by Bonferroni test. Statistical analyses were performed using SPSS (16) software (IBM SPSS, Bangalore).

RESULTS

The length of inflorescence of the studied plant species varies from 50–80 cm; the flowers are produced in acropetal manner, both sterile and fertile flowers are produced on the inflorescence. The quantitative characters of both the types of flowers (fertile and sterile) are depicted in Table 1. The blossoming of flowers starts from basal portion to upwards along the inflorescence, however; the sterile flowers remain un-opened. The fertile flowers are protandrous, and flowers of lowermost portion (10–15 cm) of this large inflorescence anthese first, as the anthesis precedes acropetally, the flowers of lowermost part of inflorescence heads from male to female phase. The lowermost portion of inflorescence bears 10–20 fertile and 10–15 sterile flowers as observer in the present study. When lower portion of inflorescence proceeds from male to female phase, the flowers of middle portion (8–12 cm) of the inflorescence at that time open and male phase commences in these flowers. In this portion there are 15–30 fertile flowers and 15–20 sterile flowers. It has been observed that at this particular time the middle portion (10–15 cm) of inflorescence is at male phase (with 25–35 fertile flowers and 15–23 sterile flowers). The flowers of upper most portion of inflorescence (30–35 cm) at that particular time remains un-opened; this portion bears 120–170 flowers (Fig. 1). Usually the flowers of upper most portion remain un-opened, however; sometimes few flowers from this portion anthese at the end of blossoming of inflorescence.

The present study revealed that at the time of anthesis in a particular flower the style is straight and starts downward movement immediately after anthesis, the stamens become fully mature during this period. As the anthers starts to dehisce, the style come to lie at an angle of 180º, downwards with respect to main axis of inflorescence i.e., away from dehisced anthers (Fig. 2A). When the anther dehiscence is completed, the style again becomes straight by upward movement and come to lie at angle of 85–90º with respect to main axis of the inflorescence and at that time stigma becomes receptive, which depicts commencement of female phase (Fig. 2B,C). After pollination, the style is directed upwards and is placed at an angle of 100–115º with respect axis of inflorescence which marks end of receptivity (Fig. 2D). The duration of stigma receptivity was also checked experimentally and experimentation revealed that at this developmental stage the stigma remains receptive (Table 2). It has also been observed that the pedicel which is directed at an angle of 170–180º towards inflorescence axis at bud stage, moves and come to lie at an angle of 80–90º at male and female stage and after pollination is accomplished, again move upward and come close to inflorescence axis at an angle of 170–180º (Fig. 1).

In present study it was observed that in an inflorescence when the flowers of upper whorls in an inflorescence are at male stage, the stigmas of flower of lower whorls of the same inflorescence just below the upper one becomes straight and consequently receptive. The pedicel at this stage becomes 80–90º with respect to main inflorescence axis and exposes the flowers fully so as to bring about successful pollination. At male stage of fertile flowers, particularly when anthers are fully developed and ready to dehisce, they are orange-red in colour and nectarines are also prominent, yellow in colour (Fig. 2E), pollinators particularly bees (*Apis cerana indica* (Fabricius, 1798)) start visiting these flowers. The pollinators are mainly attracted by orange-red coloured anthers; when anthers were emasculated manually (removal of mature anthers), it was observed that such flowers were not visited by the pollinators (Fig. 2F). Therefore, in *E. himilacus* pollinators are mainly attracted by anthers.

In the study it has been observed that when pollinators visit and land the flowers with mature anthers the pollen grains of the dehisced anthers of upper flowers fall under the influence of gravity/wind to the stigmas of lower flowers, the lower flowers at that time are at female phase and stigmas are receptive and thus successful pollination is ac-

Table 1: Quantitative characters of fertile and sterile flowers.

S. No.	Floral trait	Fertile flower		Sterile flower	
		(Mean ± SE)	**Range**	**(Mean ± SE)**	**Range**
1	Length of flower (cm)	0.52 ± 0.02^a	0.5–0.6	0.41 ± 0.02^a	0.4–0.5
2	Breadth of flower (cm)	2.28 ± 0.11^a	2–2.5	0.38 ± 0.07^a	0.2–0.6
3	Length of ovary (cm)	1.24 ± 0.11^a	1.0–1.5	0.56 ± 0.07^a	0.4–0.8
4	Length of style (cm)	1.71 ± 0.10^a	1.5–2	0.40 ± 0.03^a	0.3–0.5
5	Length of anther (cm)	0.60 ± 0.05^a	0.5–0.8	0.44 ± 0.04^a	0.3–0.5
6	Length of filament (cm)	1.44 ± 0.04^a	1.5	0.38 ± 0.02^a	0.4
7	Pollen volume (μm^3)	672.20 ± 6.72^c	665–675	197.40 ± 9.78^b	190–210
8	No. of fertile pollen (%age)	96.00 ± 3.72^b	94–98	0	0
P/F		**420.10/*****		**226.10/*****	

N=100. Values given in superscript alphabets (a–c) indicate means that are significantly different among different experiments. Same alphabet in superscript does not differ significantly. Tukey's test ($P < 0.001$); ***significant difference, $P < 0.001$. Significant differences ($a = 0.001$) in various experiments (Bonferroni test).

Table 2: Stigma receptivity of *Eremurus himalaicus* at different developmental stages

Days after anther anthesis	Pollen load on stigmatic surface (Mean ± SE)	Number and percent of germinated pollen (Mean ± SE)	
		Number	Percent (%)
0	0	0	0
1	0	0	0
2	49.00 ± 1.14	12.80± 2.68	26.12
3	37.00 ± 3.24	3.24 ± 1.14	8.75
4	0	0	0

complished. The self-compatible nature (geitonogamy) of the species was ascertained by bagging experiments during present investigation (Experiment III) (Table 3). The falling of pollen grains from upper flowers to the stigmas of lower flowers during the pollinator visitation and landing was ascertained by putting a clean glass slide just above the receptive stigmas of lower flowers and presence of pollen grains was observed under microscope on the glass slide put above the stigmas at the time of pollinator visitation. It has also been observed that bees mostly visit the flowers at male phase-with creamish white petals and orange-red anthers, and pollinators rarely visit the flowers at female stage. As pollinators visit the flowers at female stage rarely-this event may sometimes also bring about cross pollination as also has been proved experimentally. During the present study it was also observed that emasculated flowers (anthers removed) rarely produce fruits as pollinators seldom visit such flowers (Experiment IV) (Table 3).

Figure 1 Inflorescence architecture and different reproductive phases of flowers. A – whole inflorescence; B – flowers at budding stage; C – flowers at female phase; D– flowers at male phase; E – flowers at post female phase; a – bent style and stigma; b – sterile frowers

The production of sterile flowers are genetically controlled, thinning experiment revealed that the sterile flowers were also produced even in the thinned inflorescences as it is produced in normal inflorescences, and it does not vary significantly ($P \geq 0.05$) across the selected sites. Therefore, the production of sterile flowers (their position and number) in an inflorescence is per-determinant. The sterile flowers (Fig. 2G,H) never open and these flowers on the inflorescence are produced in such a manner that it gives geometry to inflorescence so that the fertile flowers i.e. flowers at female phase of lower whorl come to lie just below the flower at male phase of upper whorl in the same inflorescence. This architecture enables the upper flowers (at male phase) to pollinate 1–2 flowers of lower whorl-at female phase, at a distance of 2–4 cm below, the pollination is mediated mostly by pollinators and/or wind. In upper most part of inflorescence, the fruit set was rarely observed as there is lack of pollen grains to bring about pollination because the flowers are protandrous and at female stage there is not availability of pollen grains, however; sometimes these flowers set fruits by cross pollination but chances of cross pollination is very rare as pollinators rarely visit the flowers at female stage. Therefore, the present study revealed that the species is primarily geitonogamous but xanogamy though rarely is also operative in the species.

In the present study it has also been observed pollen grains are creamish white, rectangular-cylindrical, and with smooth exine sculpturing (Fig. 2I). The species produce 30,600±800 pollen grains which range from 28,200 to 33,000 per flower, and 16 ovules per flower. The pollen/ovule ratio for the species turned out to be 1913 and pollen volume is 672.20±6.72 μm^3 (Table 1).

The breeding experiments revealed that the bagged un-emasculated inflorescence set 65–75 % fruits and un-emasculated flowers which were bagged prior to anthesis do not set any seeds. The emasculated flowers which were tested for cross pollination also produce fruits but the fruit set was very low (5–6 %) (Table 3). The flowers of upper whorls in inflorescences which are at male phase and with orange-red anthers were tagged and allow opening pollinate results in low seed production (1–3 %).

DISCUSSION

In accordance with our hypothesis that inflorescence architecture; floral part movements, production of sterile flowers and pollinator attraction by male parts are contrivance for successful mating in *Eremurus himalaicus*. The present investigation revealed that the species produce large number of flowers which are showy, the attractive androecia and with nectaries present at the base of ovary, protoandry, moderate to high pollen ovule ratio, unique movement of stigma away from dehiscing anthers, movement of pedicels, and breeding experiment revealed that the species is primarily

Table 3: Seed production under different breeding experiments in *E. himalaicus.*

Experiment	Site	No of seeds produced (Mean ± SE)	Percent fruit production
Experiment I (Un-emasculated flowers to open pollinate)	Tangnarg	281.30± 12.27[c]	70.25 [1]
	Dara	285.31± 13.52[c]	71.25
	Phalgam	264.24± 10.25[c]	66.00
	Kashmir University Botanical Garden	292.61± 14.31[c]	73.00
Experiment II (Autonomous self-pollination- one flower/inflorescence).		NFF	NFF
Experiment III (Whole inflorescence was bagged to ascertain geitonogamy)	Tangnarg	87.36 ± 6.91[b]	21.75
	Dara	94.23± 5.96[b]	23.50
	Phalgam	82.32± 5.23[b]	20.50
	Kashmir University Botanical Garden	97.42± 4.82[b]	24.25
Experiment IV (Emasculated flowers allowed to open pollinate)	Tangnarg	4.19± 2.37[a]	1.00
	Dara	5.12± 2.33	1.25
	Phalgam	4.21± 2.34[b]	1.00
	Kashmir University Botanical Garden	6.23± 2.65[b]	1.50
Experiment V (Tagged stigmas pollinated with pollen grains from flowers of different plants to ascertain xenogamy)	Tangnarg	20.15± 0.83[a]	5.00
	Dara	21.32±078	5.25
	Phalgam	20.11±0.86	5.00
	Kashmir University Botanical Garden	23.12±0.91	5.75
P/F		**363.43/*****	

[1] Out of 400 flowers per inflorescence (n=20). NFF = No Fruit Formation. Values given in superscript alphabets (a-c) indicate means that are significantly different among different experiments. Same alphabet in superscript doesn't differ significantly. Tukey's test (P < 0.001); ***significant difference, P < 0.001. Significant differences (*a*= 0.001) in various experiments (Bonferroni test)

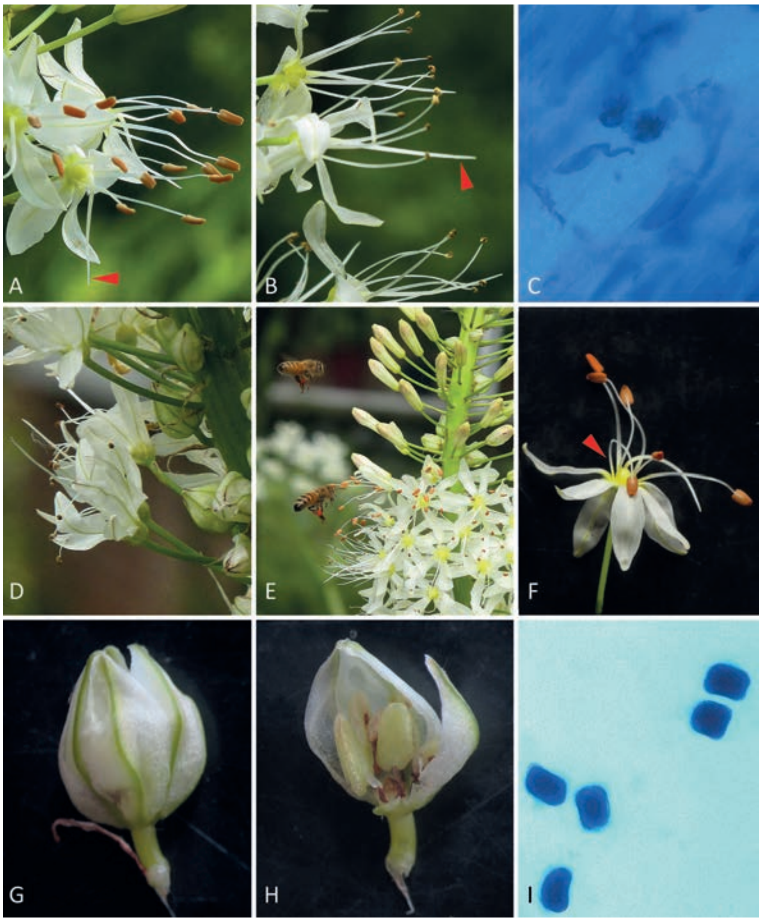

Figure 2 Flower development during the pollination process. A – flower at male phase with bent style and stigma-downwards (red arrow showing bent style and stigma); B – flower at female phase with straight style and stigma (red arrow showing position of style and stigma); C – germinating pollen on receptive stigma; D – flower at post female phase with style and pedicel bent upwards; E – pollinators visiting the flowers at male stage; F – flower at male stage with prominent yellow coloured nectary at the base of ovary (red arrow); G, H – sterile flower with shriveled essential and accessory parts; I – rectangular-cylindrical pollen grains

geitonogamous but xanogamy though rare is also operative. The exine sculpturing of pollen grains is smooth which suggests anemophily is operative in the species (Tanaka 2004), which has been brought about by wind; however, the rectangular-cylindrical pollen grains with large surface area/volumes favors entomophily (Wang et al. 2014b) in the species.

The inflorescence in which large number of flowers are displayed simultaneously attract more pollinators (Ohashi & Yahara 2001), however; in such inflorescences the chances of geitonogamy is more (Barrett et al. 1994, Karron et al. 2004) and are associated with reduction of pollen export (Harder & Barrett 1995, Karron & Mitchell 2012) as observed during the present study. It has also been demonstrated that floral densities and even helical angle of flowers on an inflorescence determine the pollinator visitation (Iwata et al. 2011).

It has been observed in the present study that pedicels move in such a way so as to keep the flowers at horizontal position with respect inflorescence axis therefore ensures consistency of pollinator movement on inflorescences (Wang et al. 2014a) and the movement of insects on inflorescence mediate pollen transfer, affecting geitonogamy and pollen export (Jordan et al. 2006). The stylar movement in the species is barriers for autogamy (Verma et al. 2004). The sterile flowers in an inflorescence keep intermediate floral densities and also increase long distance attraction of pollinators (Iwata et al. 2011, Morales et al. 2012). By enhancing pollinator attraction without the need to expose many fertile flowers simultaneously, inclusion of sterile flowers in floral displays improves pollen export and import, while enriching pollination quality by limiting both geitonogamy and autonomous selfing (Morales et al. 2012). However, during the present study it has been observed that usually pollinators directly visit the flowers at male phase and occasionally visit the flowers at female phase in an inflorescence; therefore the pollinator visitation increases geitonogamy, and to some extent the xenogamy as well. Present study revealed that *Eremurus himalaicus* has adapted a unique strategy by which geitonogamy may provide some reproductive assurance against limited cross pollination, and the xenogamy may limit the severe consequences of inbreeding depression. Further studies are needed to explain the evolution of pollination system operative in the species of the genus.

CONCLUSION

Present study is very important for the conservation of this important endemic medicinal plant species which has been categorized as threatened (Verma et al. 2004). The comprehensive knowledge of pollination mechanisms may contribute to improve our understanding to devise strategies for its conservation and sustainable development.

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