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Reproductive biology of *Peganum harmala* L. (Nitrariaceae) – an important medicinal plant of Kashmir Himalaya

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ABSTRACT

Peganum harmala L. is an important medicinal plant growing in Kashmir Himalaya. The species exhibits restricted distribution and the number and size of populations are declining continuously in the Kashmir valley. Therefore, the present study was carried out to understand the main cause of reduction in populations of their species and to unravel the bottleneck in its reproductive systems if any. The species produce bisexual flowers with large nectarines present at the base of the ovary. Protoandry and moderate to high pollen to ovule ratio depicts cross pollinated nature of the species. However, architecture of flower in which essential reproductive organs are not separated enough facilitates selfing in the species. The breeding experiments also indicate facultative xenogamous nature of the species. More than 80% seed germination was observed under controlled conditions which depict normal sexual system is operative in the species. The in vitro root sprouting studies revealed that moisture and soil type are important for the sprouting of these vegetative propagules. The present study bring in light that the species can be reclaimed and restored if the sexual seeds are not over harvested at the time of maturation and allowed to recruit, besides, if favourable habitats are also provided.

INTRODUCTION

Peganum harmala L. belongs to family Nitrariaceae. The species is commonly known as “Espand” in Iran, “Harmel” in North Africa and “African Rue”, “Maxican Rue” or Turkish Rue” in the United States [20] and “Izband” in Kashmir.The species possess immense medicinal potential with antimicrobial [1], anti-inflammatory, analgesic properties [21], antibacterial activity [7] and is considered to have anticancerous activity which could prove as a novel anticancer therapy [17]. The seeds of the plant are well known for fragrance and for insect killing properties when burnt. The smoke of burnt seeds of *Peganum harmala* is used as devil repellent and against evil eyes in Kashmir. Perhaps there is no family in Kashmir where the ‘Izband’ is not used at spiritual occasions. The species has been immensely exploited for its local use and commercial purpose, therefore facing the threat of extinction [25]. The studies on reproductive biology of *P. harmala* can prove helpful for its conservation and restoration. Knowledge of reproduction is crucial for understanding the

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causes of rarity and conservation of rare plant taxa [16, 15]. For biodiversity conservation, reclamation and restoration, such studies can provide important paradigms [23]. The knowledge about reproductive biology is important for determining barriers to seed and fruit set and for understanding pollination and breeding systems that regulate the genetic structure of populations [15]. Any conservation approach has to be based on an in-depth study of plant reproductive biology. Reproductive characteristics such as seed dispersal, germination capacity, survival rate of seedlings and adults, age at flowering, reproductive lifespan and number of flowers and seeds refer to a set of responses that allow a species to adapt to a particular environment. Besides these, the processes of gamete development, pollination, endosperm and embryo development and other reproductive features can provide important clues regarding the reproductive constraints of plants that need conservation [23]. Despite huge cultural and ethno-medicinal importance of *P. harmala* in Kashmir till date this important plant has not been scrutinized for its reproductive biology. Therefore, it was thought worthwhile to undertake the reproductive biology of this prized species so as to understand its rare nature and restricted distribution in Kashmir Himalaya.

MATERIAL AND METHODS

Plant material

The present study was carried out on *Peganum harmala* L. a perennial herb, 30 - 70 cm tall, erect to spreading, much branched from base, glabrous; leaves alternate, decompound, sessile to sub sessile; stipulate, multi-fid; leaf blade ovate, divided into 3-5 linear to lanceolate-linear lobes; flowers opposite to leaves.

Floral structure

Structure and arrangement of flower and its parts were studied following methodology of Kaufman *et al*. [14] and Nath [24]. Photographs were taken using stereo zoom microscope (Model: Carl Zeiss Discovery V8) and Trinacular microscope -Leica.

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 [10].The stigmas with germinating pollen grains were considered receptive.

Pollen viability and pollen - ovule ratio

Pollen viability was estimated by squashing anthers in 1% acetocarmine and 1% aniline blue – lactophenol [26]. The well stained, healthy pollen grains were considered as viable. Pollen - ovule ratio was calculated following Cruden's [5] method.

Pollen morphology

Pollen grains for morphological studies were collected from natural populations and were studied under light microscopy (LM) by mounting in 2% acetocarmine. Pollen diameter, shape in equatorial view, aperture type and sculpturing were recorded. For Scanning Electron Microscopy (SEM) double sided conductive tap was fixed to the stub and the pollen grains from mature un-dehisced anthers were dusted over it. The dusted material sputter coated with gold was observed under SEM (S-3000 H).

Pollination mechanism

Pollination mechanisms were studied by observing foraging behaviour of various insects visiting the flower for reward. The insect specimens were collected and identified in the Department of Zoology, University of Kashmir-Srinagar.

The number of flowers visited, time spent on a flowers and the total time spent per plant by an insect was also recorded.

Seed set and viability

Seeds produced per flower were calculated following Lubbers and Christensen [18]. Seed viability was analysed using Tz - Test [11].

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 flowers were bagged at bud stage to avoid cross pollination and allow autonomous selfing.

III. Emasculated flowers at bud stage were bagged to check the apomictic seed development.

The bagging experiments were carried out on 30 flowers in each case at all the selected sites

In vitro seed germination

For *in vitro* seed germination studies, the seeds were randomly collected from natural populations and washed with 0.1% mercuric chloride for 5-7 minutes, followed by 4-5 times washing with distilled water. These were then subjected to different physical and chemical treatments (Table 1). All the experiments were conducted in temperature, light and humidity controlled seed germinator. The treatment solutions were made using deionized water and analytical grade chemicals. Each treatment consisting of three replicates of 15 seeds were placed within closed glass petri-dishes on Whatman No. 1 filter paper moistened with 10 ml of the given treatment solution or distilled water (control). Seeds planned for dark treatment were moistened under green light and then quickly wrapped in aluminium foil to reduce the chances of inadvertent exposure of light. Emergence of radical was used as the indicator of germination, and total germination was recorded at culmination of the experiment in respect of all the treatments.

Table 1: Physical and chemical treatments employed to test seed germination in *Peganum harmala*

S. No.	Treatment	Concentration (mM)	Duration
1	Control	-	-
2	GA_3	1	-
3	Thiourea	1	-
4	IAA	1	-
5	Kinetin	1	20-30 seconds
6	Dip in H_2SO_4	-	-
7	Dip in HCl	-	-
8	Scarification (mechanical)	-	-
9	Dry Stratification	-	20, 40, 60, 80, 100 days
10	Wet Stratification	-	20, 40, 60, 80, 100 days

Sprouting of asexual propagules (roots)

Asexual propagules (roots) were used for *in vitro* germination studies. The roots were germinated at different moisture levels and soil types. Each treatment, with three replicates, included two roots of equal length placed in trays with 1.5kg of soil or sand only or mixture of both. Different moisture levels were ensured by adding desired quantity of water. These trays were kept inside automatic temperature and light controlled growth chambers at 25±2°C for 30 days. Total germination was recorded at the culmination of the experiment in respect of all the treatments.

RESULTS AND DISCUSSION

Floral morphology

The flower of the species is bisexual,solitary, pedicellate; sepels 5, green in colour, free and divided into lobes; petals 5, white to pale yellow in color; stamens 15, outer 5 pair arranged opposite to petals and inner 5 opposite to sepals, stamens are shorter than corolla; anthers are linear-oblong crescentic dithecal, dehisce by longitudinal slit, basifixed, dehiscence asyncronous; gynoecium tricarpellary syncarpous; stigma trifid; nectarines develop around the base of ovary, containing 40 - 50 ovules; ovules are anatropous with axile placentation.

The length of anther ranged from 0.47 ± 0.02 cm to 0.49 ± 0.01cm whereas length of filament recorded was 0.35 ± 0.01cm in all the studied population. The stigma, style and ovary length ranged from 0.30 ± 0.00 cm, 0.60 ± 0.02 cm and 0.20 ± 0.00 cm respectively (Table 2).

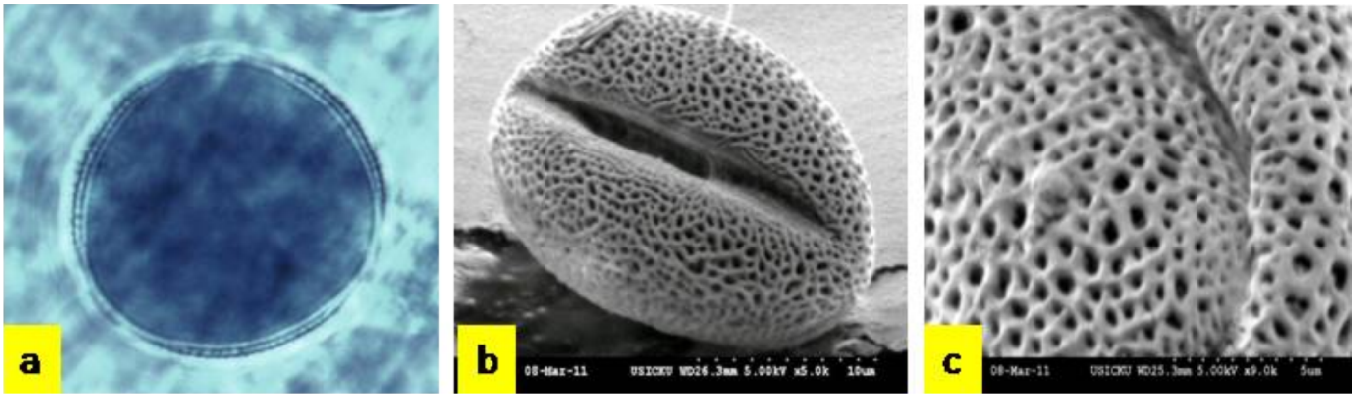


Fig. 1: Pollen morphology of *P. harmala*; (a) Tricolphate pollen, (b and c) Pollen with prominent colpi, muri and lamina (LM and SEM studies)

Table 2: Quantitative floral characters of *Peganum harmala* across different populations.

Floral characters	Populations		
	Srinagar	Pattan	Bijbehara
Length of stigma (cm)	0.30[a] ± 0.00*	0.30[a] ± 0.00	0.30[a] ± 0.00
Length of style (cm)	0.60[a] ± 0.00	0.60[a] ± 0.00	0.60[a] ± 0.00
Length of ovary (cm)	0.20[a] ± 0.00	0.20[a] ± 0.00	0.20[a] ± 0.00
Length of anther (cm)	0.49[a] ± 0.01	0.48[a] ± 0.03	0.48[a] ± 0.02
Length of filament (cm)	0.35[a] ± 0.01	0.35[a] ± 0.00	0.35[a] ± 0.01

*Mean±S.E; Tukey's test, similar figure 'a' indicated that means are not significantly different.

Pollen morphology

Pollen grains are spheroid, sub-prolate to prolate and tricolpate. The pollen diameter is 16.77 ± 0.18 µm. The SEM studies showed that the colpi are ectocolpus, long with acute ends. The muri are dense, elevated and thin enclosing irregular shaped lamina (Fig. 1).

Pollen–ovule ratio

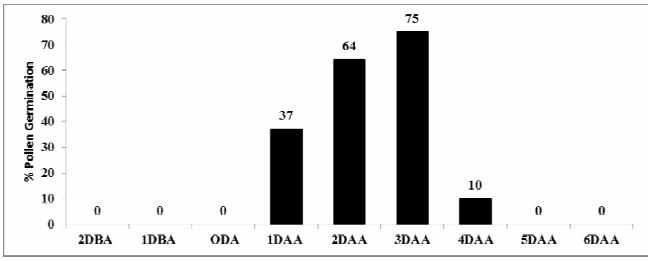
Peganum harmala L. produces enormous quantity of pollen grains. It was observed that flower produces 45.20 ± 0.96 ovules and 130177.50 ± 3731.78 pollen grains per flower. The average pollen-ovule ratio turned out to 2880.02 ± 112.98.

Pollen viability and stigma receptivity

Pollen viability was calculated to be about 90.46%. The stigma in the species is slightly exerted. Anther dehiscence took place before anthesis, at that time when the stigma was not receptive. As the anther dehiscence proceeds, the carpel develop further and stigmas become receptive one day after anthesis and the receptivity lasts for 4 days. The number of deposited pollen grains as well as the percentage of germinated pollen was highest on 3rd day after the anthesis. Hereafter, the receptivity of stigmas gradually decreases and is completely lost on the 5th day after anthesis (Fig. 2).

Pollination system

The species produces numerous flowers on each plant. However, flowers of same plant or different plants were at various developmental stages viz; male phase, female phase and fruiting stage and continue to advance from one phase to the other for three months. The flowers bear nectarines at the base of ovary. The white colored large flowers of *Peganum harmala* attract the pollinators of different insects (Hymenoptera and Diptera). The flowers of same plant or different



DBA= days before anthesis, **ODA=** on the day of anthesis and **DAA =** days after anthesis

Fig. 2: Comparison of stigma receptivity at different developmental stages in *Peganum harmala*

plants in a population behave functionally as male or female at particular period of time and are visited by pollinators to sip the nectar. In doing this the pollinators get heavily loaded with pollen and ensure transfer of pollen grains from one flower to another. Honey bees *(Apis mellifera*) accounted for the highest number and duration of visits thus were considered as main pollinator for the species. The mean number of flowers visited by honeybee per plant is 5.33±0.33. The mean foraging time spent by honeybee per flower is 40.00 ± 1.88 seconds. The time an individual honey bee spent foraging per plant and the mean foraging times per plant was 147.33 ± 4.05 seconds. The number of bees visiting per plant/minute ranges 13.00 ± 0.57.

Breeding behaviour

The breeding experiment revealed that un-emasculated flowers (Experiment 1) which were allowed to open pollinate produced 100% capsules with mature and viable seeds, while un-emasculated floral buds bagged to test selfing (Experiment 2) produced capsules only in 30% flowers and no capsule formation was observed in emasculated bagged flowers which depict that seeds are not apomictic.

Relationship between floral traits and pollination mechanism

Peganum harmala produces large number of bisexual flowers per plant. The species produces white-yellowish showy flowers which help the plant in attracting large number of pollinators. Floral display, shape, size and colour are the visual traits assumed to be attractive to pollinators [3, 28, 2, 13] and to accomplish successful pollination.

The pollen grains of the species are with reticulate ornamentation which is associated with biotic pollination because echinulate or reticulate pollen grains are associated with biotic pollination, particularly entomophily [19, 27]. The pollen viability in the species was very high (89-90%) which favors successful pollination and high seed set.

In *Peganum harmala*, just after anthesis, the anthers start to dehisce, while the stigma remains concealed within the flower. Once the anthers dehisce, the stigma starts to emerge out and becomes receptive within 2 days. Dichogamy (protandry) is the possible mechanism that prevents selfing in hermaphrodite flowers. Protandry is distinct in the species and is well known adaptation to out crossing [4]. However, there are possibility of selfing in the species as the essential organs i.e. androecium and gynoecium are not separated spatially enough thus facilitating selfing in the species. The receptive stigmas are slightly exerted but are well within the reach of dehisced anthers. The protogynous nature and floral architecture in the species also points towards mixed selfing- out crossing nature of the species. Hentrich *et al*.[12] also observed mixed selfing-out crossing strategy to ensure seed production in *Voyria caerulea* and *V. rosea.*

In *Peganum harmala* pollen to ovule ratio was recorded to be 2862.91 ± 90.78, the moderate P/O ratio indicates that facultative xenogamy is operative in the species [5, 6].

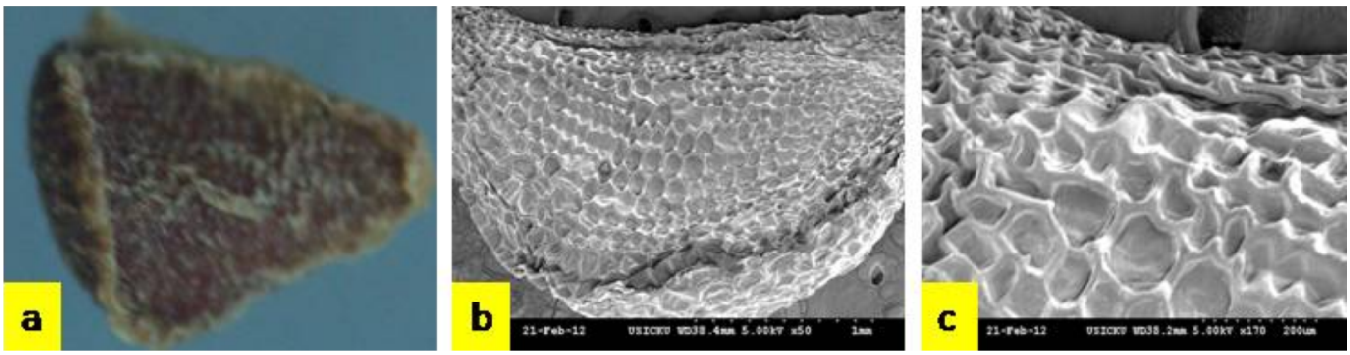


Fig. 3: Seed morphology of *P. harmala*; (a) Triangular shaped sees (b and c) Reticulatewith pentagonal or hexagonal shaped cells (LM and SEM studies)

The species was observed to be pollinated by few insect visitors belonging to different genera (Hymenoptera and Diptera). *Apis* was the major pollinator as it registered highest number of visits; Movafeghi *et al.* [20] also observed honeybee (*Apis mellifera*) as the main pollinator of *P. harmala*. The nectarines are present at the base of ovary which aids in attraction of pollinators and insects favored hexose rich nectar in the species [20].

Table 3: Effect of different physical and chemical treatments on seed germination of *Peganum harmala*

Treatment	Light regime	Germination	Percent germination
Control	Light	8.66 ± 1.20*	86
	Dark	8.00 ± 0.57	80
Thiourea 1mM	Light	9.00 ± 1.00	90
	Dark	8.00 ± 0.57	80
IAA 1mM	Light	4.66 ± 0.33	40
	Dark	2.33 ± 0.88	23
GA_3 1mM	Light	6.00 ± 1.52	60
	Dark	3.00 ± 0.57	30
HCl dip	Light	6.00 ± 1.52	60
	Dark	3.66 ± 0.66	36
HCl 1min	Light	7.66 ± 1.45	76
	Dark	5.66 ± 2.18	56
H_2SO_4 dip	Light	7.46 ± 0.88	74
	Dark	3.33 ± 0.88	33
H_2SO_4 1 min	Light	5.33 ± 0.33	53
	Dark	3.03 ± 0.30	30
Scarification	Light	8.33 ± 0.88	83
	Dark	5.66 ± 0.88	56
Wet chilling (20days)	Light	6.70 ± 0.57	67
	Dark	3.83 ± 0.33	38
Wet chilling (40 days)	Light	6.06 ± 0.33	60
	Dark	3.40 ± 0.57	34
Wet chilling (60days)	Light	5.50 ± 1.00	55
	Dark	2.33 ± 0.66	23
Dry chilling (20 days)	Light	5.30 ± 1.00	53
	Dark	4.03 ± 1.33	40
Dry chilling (40 days)	Light	3.50 ± 0.00	35
	Dark	2.50 ± 0.50	30
Dry chilling (60 days)	Light	3.33 ± 0.88	33
	Dark	3.10 ± 0.00	31

*(Mean ± S.E)

The present investigation revealed that the species produce large number of flowers which are showy with large nectaries present at the base of ovary, protoandry and moderate to high pollen ovule ratio depicts cross pollinated nature of the species. However, architecture of flower in which essential reproductive organs are not separated enough facilitates selfing in the species. It is thus concluded that the species is having mixed selfing-outcrossing strategy. The breeding experiments also indicate facultative xenogamous nature of the species.

Seed morphology

The seed coat surface of the species is regular, reticulate testa with pentagonal or hexagonal shaped cells; the cells are depressed with elevated cell boundaries (Fig. 3). The seed weight was calculated to be 2.66 gms/1000 seeds.

Seed viability and seed set

Peganum harmala produce an ample amount of seeds due to the presence of large number of ovules per flower and huge number of flowers per plant. On an average, the species produced 31.2±1.41 seeds per capsule. This seed set amounts to be 69.64%. The species showed a good percentage of seed viability which turned out to 90.30 %.

In vitro seed germination

In order to investigate *in vitro* seed germination, the seeds were subjected to several treatments such as application of thiourea, IAA, GA_3, kinetin, concentrated sulphuric acid and hydrochloric acid for different time durations, scarification, wet- chilling and dry-chilling, under alternating light and dark and continuous dark conditions. The data summarized in Table 3, reveal that the seeds of *Peganum harmala* germinate easily in distilled water (control), 80-86% seed germination was recorded in distilled water. However, among various treatments, the seeds treated with thiourea were observed to have promotory effect on seed germination (90%). Days taken by the first seed to germinate ranges from 1 to 4 days, while as total days taken for completion of germination ranges from 4 to 9 days in different media. The rate and overall germination was highest in Thiourea treatment.

Seed dispersal and germination

The more or less smooth seed coat and less weight may be associated with wind dispersal. Present study revealed that the seeds of *Peganum harmala* successfully germinate in distilled water (80-86%), which indicates that the species does not have seed dormancy. Among different treatments, Thiourea, HCl, H_2SO_4 and scarification yielded better result. Thiourea seems to be necessary, because it performs dual function of increasing the growth potential of embryo and to overcome the mechanical restrain conferred by the layers covering the embryo by weakening the tissue [9]. Therefore, the species does not have any difficulty with the sexual system and recruitment of new individuals, however, the over exploitation of the seeds for local use is the main cause of its restricted populations in Kashmir Himalaya.

Vegetative reproduction

Vegetative reproduction in the *P. harmala* takes place by means of roots. The species bears a crown of underground roots from which the main tap root grows up to 6 feet deep. Each crown of roots in turn bears a large number of buds which remain dormant during the winter. In the next growing season these buds develop into leafy shoots. The present experimental studies revealed that under different moisture content of soil, the sprouting of the root cuttings of the species responded differently at room temperature. It has been observed that at ½ the field capacity, the root showed good percentage of germination (83.33%). However, at double the field capacity the root does not germinate at all (Fig.4).

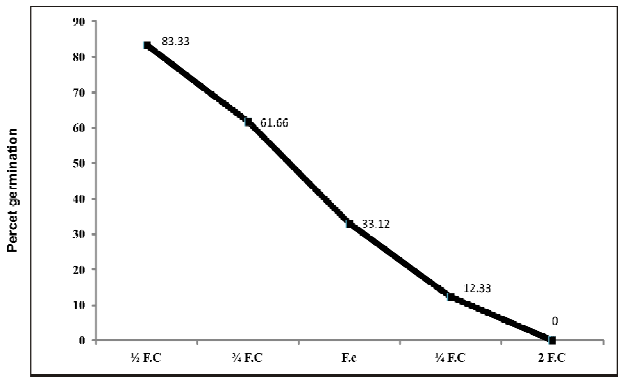


Fig.4: Effect of moisture on root sprouting of *Peganum harmala*

In another experiment, under different soil: sand mixtures it was observed that root sprouting was highest in sand: soil (2:1), followed by sand: soil (1:1) ratio. However the moisture content was maintained at field capacity and at room temperature (Fig 5).

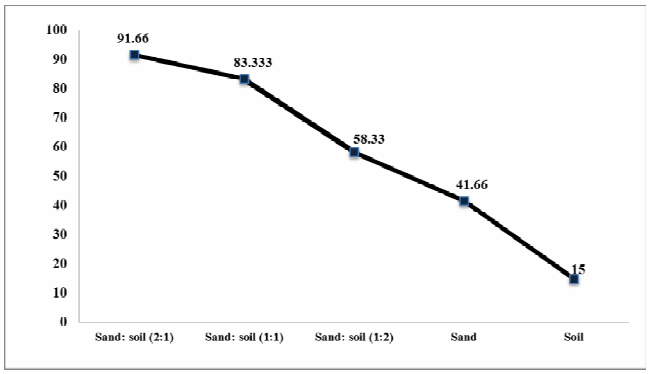


Fig.5: Effect of soil type on root sprouting of *Peganum harmala*

Percet germination

In vitro vegetative propagule sprouting

Vegetative methods employed by the species are the perennating buds borne on the underground roots. At the end of growing season, the root remains dormant during winter and starts to produce 50-150 perennating buds in next growing season. These buds give rise to new shoots which produce numerous flowering shoots in each growing season, this feature not only helps the plant to establish the population at a particular site but also ensures perpetuation even in absence of seeds. It has been observed that soil and moisture has a profound effect on root sprouting, the root show maximum sprouting at ½ the field capacity in sand: soil (2:1). It is clear that the plant grows best in soils with low moisture retaining capacity and mostly with sandy texture. The species mostly grows in semi-arid rangeland, steppe area and sandy soils with low moisture retention capacity [20, 8].

CONCLUSION

The present study revealed that there is no bottleneck in the sexual system of the species, the overharvesting of the sexual propagules for local use and the land use change which decline the

favoured habitats of the species are the main causes of its reduced populations in Kashmir Himalaya. The present study depicts that the species can be reclaimed and restored if the sexual seeds are allowed to recruit and favourable habitats are provided.

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