***Taiwania*, 49**(4): 237-249, 2004

Reproductive Behavior and Inbreeding Depression in Endangered *Eremostachys superba* Royle ex Benth. (Labiatae) in Dehra Dun Population, India

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(Manuscript received 13 July, 2004; accepted 1 October, 2004)

ABSTARCT: An assessment of reproductive behavior and inbreeding depression, if any, in critically endangered *Eremostachys superba* Royle ex Benth. (Labiatae) was made to unveil the factors playing vital role in it's reproductive biology and which may be responsible for the loss of fitness, viability and vigor of the species. Breeding experiments portrayed a failure of self-fertilization and a strong tendency towards out-breeding as seed set by xenogamy was highest (44.4%). However, the narrow restricted population of the type locality in Dehra Dun Siwaliks was just a ramet population sustained by clonal propagation of rhizomatous root stock, hence any out-crossing within these homozygous individuals also amounted to inbreeding. Further, there is no other population available within the range of normal seed dispersal mechanism or insect-pollinator-flight-range. The other populations reported are only from geographically distant region of Jammu and Kashmir state of India, which is too far a distance to be covered by the *Nomia rustica* West. and *Ceratina heiroglyphica* Sm., the oligophilic pollinators of *E. superba*, hence any crossing taking place also amounts to selfing in strict sense. Chances of induction of genetic variation by crossing between two different populations are remote. This was also supported by the data of seed production and germination experiments. Even the healthy seeds suffered from loss of fitness and failed to germinate under natural conditions. This strongly indicated prevalence of inbreeding depression and loss of fitness of the progeny right from the stage of germination, a phenomenon hazardous for sustenance and perpetuation of species leading to rarity.

KEY WORDS: Labiatae, *Eremostachys superba*, Inbreeding depression, Autogamy, Geitonogamy, Xenogamy.

INTRODUCTION

Inbreeding is generally defined as mating between genetically related individuals. Occurrence of inbreeding in species which are normally outbred results in loss of genetic diversity and becomes an inherent cause of reduced germinability of seeds, loss of viability, vigor and fecundity of progenys (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987) leading to an inbreeding depression. This effect is even more pronounced in highly endangered species with narrow distribution, often falling below the range minimum viable population size (Shaffer, 1981), facing a reproductive bottleneck, reaching the brink of extinction.

Inbreeding depression is a resultant of the exposure and expression of deleterious recessive alleles due to continuous selfing within populations and is most potent factor leading to species extinction. Amalgamation of various others factors to this, augment such an effect. These factors are: 1. increase in the number of individuals that are homozygous for

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deleterious recessive alleles as a result of continuous selfing, 2. decrease in the frequency of superior heterozygous genotypes and 3. decrease in genotypic diversity among individuals. The species that are normally self-fertilized exhibit lower levels of inbreeding depression than those that are normal outbreeders (Stebbins, 1950; Shields, 1982; Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987).

Clonal reproduction by creeping roots or stems, propagules such as bulbils and tubers, or agamous seeds occurs in many plant species (Wang *et al*., 2004a, b). These species normally continue to produce flowers and retain the potential for sexual reproduction (Arizaga and Ezcurra, 1995), but under extreme conditions it suffers a failure in sexual processes leading to a shift towards asexual reproduction (Wang *et al*., 2004a). The endangered species *Eremostachys superba* Royle *ex* Benth. (Labiatae), under investigation, is one such critically endangered highly ornamental species which propagates clonally by means of its thick deeply penetrating root stock. This endemic species inhabits the lower Siwaliks of Uttar Pradesh in the form of a narrow restricted population comprising of merely 33 (± 10) individuals struggling for survival. Recent surveys have revealed its fragmented occurrence in few other populations confined to Jammu and Kashmir state of India (Verma *et al*., 2003). The Dehra Dun population has already collapsed below the level of minimum viable population (MVP) size (Rao and Garg, 1994).

Earlier investigations made on *Eremostachys superba* (Rao and Garg, 1994) indicated that the species has evolved the survival strategies by means of its thick perennial rhizome-like root stock. The root stocks produce a rosette of leaves in winter, with long spike bearing the inflorescence of brightly colored showy flowers which come to bloom in spring. The flower structure was specialized for out-crossing. Each spike bears up to 100-150 flowers. However, the use of local people for medicinal values by digging out root stock has caused severe damage to its demography and perpetuation (Verma *et al*., 2003). The flowers were pollinated by oligophilic insect pollinators namely *Nomia rustica* West. and C*eratina heiroglyphica* Sm. (Garg and Rao, 1996, 1997a). Preliminary experiments on reproductive behavior in the species revealed 6.8% geitonogamous and 44.4% xenogamous seed production (Garg and Rao, 1997b) but a prevalence of pollen limitation which was one of the causes for failure in 100% seed production (Garg and Rao, 1997c), although the showy flowers produced enough insect attractants to lure insect visits (Garg and Rao, 2002). The light seeds are dispersed by wind as they possess pappus-like hairs at their distal end.

An assessment of detailed breeding mechanisms of *Eremostachys superba* was made to understand the reproductive behavior of this species which has drastically collapsed in the population of it’s type locality at Dehra Dun Siwaliks, from *ca.* 100 to 33 individuals within a decade and also unveil any malfunctioning in reproductive success or occurrence of inbreeding depression, if prevalent, for establishing the vital causes of loss of multiplication and expansion of this extant population. Further, the species was also found unable to counteract the stochastic perturbations enforced upon the struggling miniature population which is dwindling continuously, posing a challenge for botanists to save it from extinction.

MATERIALS AND METHODS

It was extremely essential to conduct all experiments with minimum possible material bearing in mind the highly endangered nature and miniature population size of the extant population of *Eremostachys superba* its natural habitat of Dehra Dun Siwaliks. Hence, only four plants of this endangered species were brought from the habitat along with their root stocks and grown in the experimental garden under controlled conditions of light and humidity (Fig. 1). Floral morphology and vegetative growth pattern were recorded. The experimental plants were carefully observed during the two consecutive years (1994-1995) for their phenological behavior based on which the experiments were conducted on breeding mechanisms. Simultaneously, observations were made in the natural habitat to record pollination methods (Fig. 2A) and any change in the demography of the population.

Fig. 1. *Eremostachys superba*. A: A mature plant in full bloom. B: Spike in close up showing bilipped corolla.

Seven types of breeding experiments were conducted in two successive flowering seasons in February-March, 1994 and 1995 in order to determine the levels of seed set to measure the breeding success.

All flowers used for pollination were checked for pollen viability. Pollen viability was determined by using Alexander's stain by sprinkling fresh pollen in Brewbaker and Kwak's medium (Alexander, 1980; Shivanna and Rangaswamy, 1993). The germinated pollen were counted after five hours. Seed abortions and loss of viability was assessed at seed maturity by counting the healthy viable seeds against the dry and empty ones.

The breeding experiments conducted were as follows:

Autogamous checking: insect exclusion test

Twenty six flowers at the stage of closed mature buds were bagged in order to assess for autogamous seed set in complete absence of pollinators.

Artificial selfing

Corolla of 18 mature buds was manually incised and carefully removed. Pollens from exposed anthers of these buds were applied to the stigma of the same bud with the help of a fine camel hair brush. The buds were then emasculated and bagged for seed set to assess the absence/prevalence of self-compatibility.

Geitonogamy: supplementary pollination

One full spike of 84 flowers was hand-pollinated by artificial transfer of pollens from one flower to the stigma of another flower borne on the same spike with the help of fine brush or even by detaching the entire stamens of one flower, puncturing its anther to expose the pollen and then transferring their pollens to a different stigma by gentle touch of the fractured anther. This was followed by emasculation of all flowers and then bagging them. Pollen were applied on the inner receptive surface of the stigmas. Care was taken to avoid contamination by self pollens.

Xenogamy: assisted crossing

Eighteen flowers of one spike were hand pollinated as above by applying pollens from flowers borne on one spike to the stigmas of emasculated flowers on a different spike. This was followed by bagging the flowers.

Natural pollination: in presence of vector

One spike bearing 69 flowers were left undisturbed for natural pollination in open field. Among the insects that visited the plants, the specific pollinators of the species namely *Nomia rustica* West. (Garg and Rao, 1996) acted as effective pollinator while the other insects avoided these flowers. Continuous field observations were recorded on the method of pollen collection and transfer. Field observations were also made in the type locality in Dehradun Siwaliks for pollination (Garg and Rao, 1997).

Natural pollination: in the absence of pollinators

During the second flowering season of 1995 the spikes consisting of 135 flowers were kept in enclosure amidst a number of cultivated flowering plants for natural pollination and seed set, where the specific pollinators of *Eremostachys superba*, ie. *Nomia rustica* and *Ceratina heiroglyphica*, were altogether absent.

Asexual seed set

Twenty flowers in one whorl were emasculated before anther maturation in order to deplete them from male reproductive units. These were bagged and left for asexual seed set.

Mature seeds were harvested after 20-25 days on termination of flowering. Healthy and aborted seeds were counted and then ratio of aborted seeds was determined against the ratio of healthy seeds. This was done for all the experiments conducted in the field in terms of breeding mechanism. The characters of healthy seeds which were expected to be viable, and of aborted or the expected sterile seeds were noted. The percentage of seed set against abortion was calculated for each experiment with the aim of evaluating the viability depression following different breeding systems.

A few healthy seeds were kept to record germination, following Ellis *et al*. (1985) methods viz. on soaked filter paper, in mud pots and in pots with sand as germination medium.

Rest of the healthy seeds were placed for germination in MS germination medium adding growth regulators to the medium but under controlled conditions of light temperature and moisture for recording seed germination.

The total number of seeds obtained by each experiment were calculated against the expected seed set as each flower bears four ovules and was expected to produce four nutlets or healthy seeds.

RESULTS

The single population of *Eremostachys superba* acquired only covers a small area of *ca*. 50 sq. m. on low lying slopes at the roadside in its natural habitat.

Vegetative growth pattern

Germination of the root srock commences after rains towards the end of September. A basal rosette of large (24-28 cm × 9-11 cm) radical leaves is produced by the middle of December and by January the plant attains a height of *ca*. 30 cm. A central stalk emerges by the end of January which reaches its maximum height of 4-6 feet towards mid-February. The stalk bears 3-5 pairs of cauline leaves with terminal inflorescence.

Flower morphology

Flowers bisexual. Calyx subcampanulate, broadly 5-lobed, truncate; mouth densely woolly. Corolla monopetalous, hypogynous, yellow, corolla tube included, 5-lobed, 2-lipped; upper lip 2-lobed, erect, hooded, bearded within; lower lip 3-lobed, midlobe largest. Stamens inserted on corolla tube, 4, didynamous, all perfect, ascending, lower pair largest, upper filaments fimbriate at the base; anthers conniving, cells divaricate. Ovary free, superior, syncarpous, carpels 2, each deeply lobed, appearing as 4; style simple, 4 cm long, gynobasic, bifid; stigmas 2. Nutlets 4, at the base of persistent calyx, golden brown to dark brown, with a tuft of pappus-like hairs at the distal end.

Flower phenology

Bud initiation occurs as soon as the spike emerges and flowering is initiated around the end of the first week of March when the temperature is considerably high *ca*. 30-35℃ with dry weather conditions. The spike terminates erect, densely wooly inflorescence up to 30 cm long. The flowers are borne in dense whorls of 8-10 flowers each, arranged in verticillasters of 5 on either side of the axis in the axil of a densely wooly bract. Each inflorescence produces ca 100-130 flowers, which bloom in acropetal succession. Sequence of flower maturation of the opposite bracts in each whorl is simultaneous.

The 1st flower appears on 13th / 14th March, and the last one on 27th / 29th March. Peak flowering time was from 19th till 22nd March when more than 70% flowers were simultaneously blooming. At this time the species appeared highly ornamental. Each flower continued to blossom for 3-4 days after which the corolla dried up leaving behind the persistent calyx with developing seeds. Anther dehiscence was longitudinal.

Pollen production was as high as 33,600 (SD = 300) per anther which amounts to ca 1,44,560 pollen per flower (SD = 1200) against 4 ovules per flower. All the four ovules are capable of developing into healthy seeds. Pollen fertility and germinability was recorded to be as high as 95% (SD = 3). All four seeds within each calyx were either all healthy or all sterile or both types mixed. Healthy viable seeds were 12-13 mm long, filled-up and thick with an average weight 28-32.5 mg/seed (Fig. 2B), whereas the sterile seeds were only 4-5 mm long, shriveled-up, empty and almost weightless (0.002-0.01 mg; Fig. 2C). Seed maturation and dispersal occur about 20-30 days after fertilization. It was observed that only those seeds kept in MS medium sprouted and produced juvenile cotyledons (Figs. 2D and 2E). Natural germination of seeds was altogether absent.

Viability depression was assessed on the basis of the number of viable seeds harvested at maturity following the seven types of experiments on different breeding mechanisms. These results are represented in table 1 and explained as follows:

Autogamous checking: insect exclusion test

Only 4 mature seeds were obtained out of expected 104 seeds, indicating 96.2% seed abortion against 3.8% seed set.

Artificial selfing

Only two ripe seeds were produced against the expected number of 72 nutlets, indicating 2.8% seed set against 97.2% seed abortion.

Geitonogamy: supplementary pollination

Out of the 84 flowers pollinated manually and thereafter expected to rear 336 seeds (4 seeds per flower), only 42 healthy seeds were obtained and the rest being all sterile ones. This data of geitonogamous seed production was fairly high (12.5%), when compared with autogamous seed set (2.8%), and in turn having lower seed abortions viz. 87.5% against 97.2% (from autogamy). This indicated that fertilization involving flowers of the same spike (which almost amounts to selfing) was relatively more successful than fertilization within male and female parts of the same flower (pure selfing), the degree of relatedness being slightly lesser in the former case.

Xenogamy: assisted crossing

Out of eighteen flowers outcrossed manually, 32 viable seeds were obtained against the expected 72 seeds, the remaining 40 seeds were aborted during maturation. Thus there was considerable downfall in seed abortion as a result of forcible outcrossing involving flowers of different spikes (xenogamy). The seed set was 44.4%, indicated that farther the degree of relation between the parent plants, higher is the percentage viability in the resultant progeny. The healthy seeds weighed *ca.* 31 mg.

It is known that in xenogamous mode of pollination the mating individuals belong to the same population, still little variation is expected to exist between individuals tending to result in more successful seed set. The lesser number of seed abortion (55.6%) following this method of breeding suggests a more ready acceptability of male unit from another plant in the same population by its female counterpart.

Natural pollination: in presence of vector

Seed set through natural pollination in the presence of a vector was observed in the year 1994. The control pots for natural pollination yielded as little as 6.9% seeds. The flowers are nectarless, therefore, insects visited them primarily for pollen food. Their visits for pollen

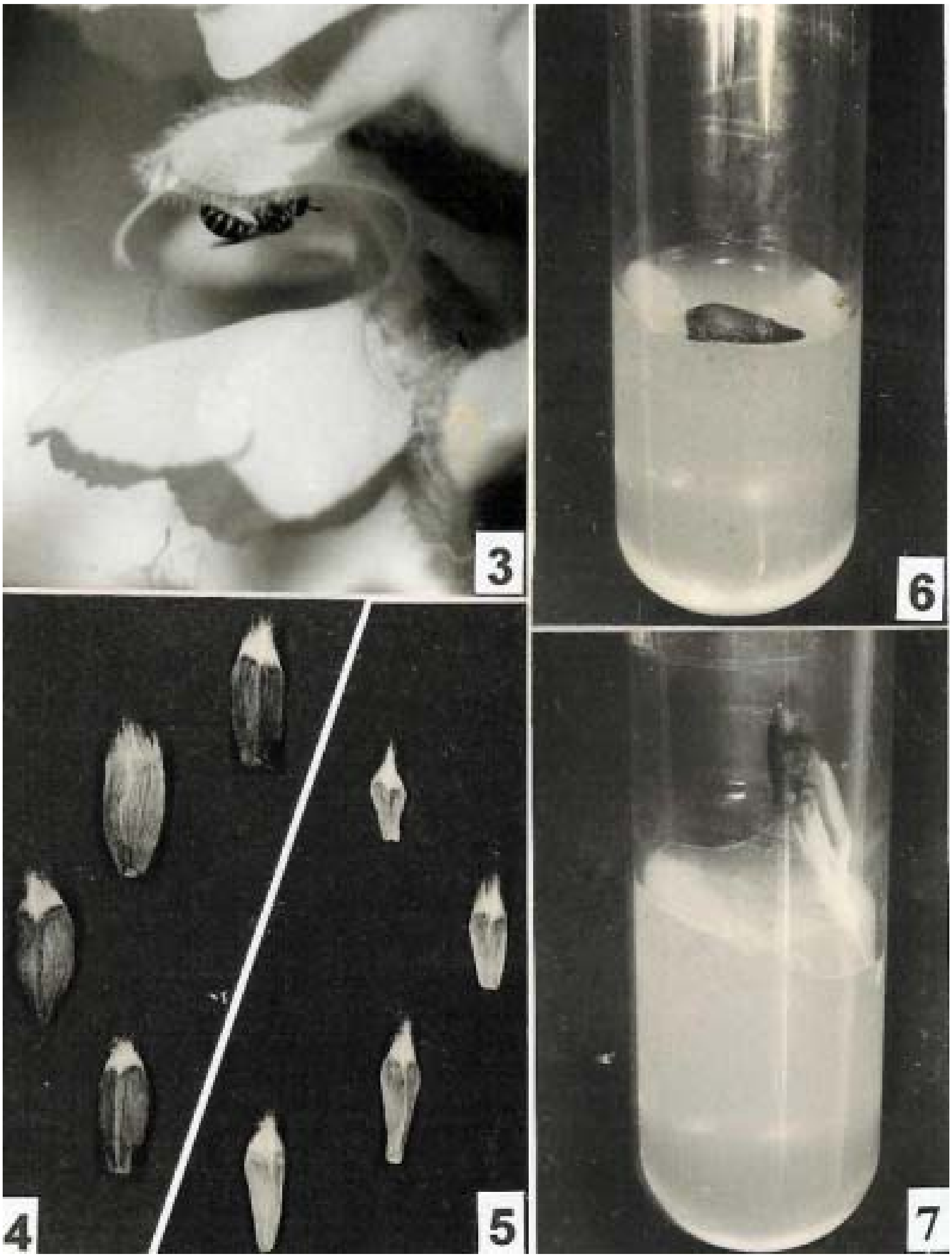

Fig. 2. A: *Nomia rustica*, pollinating a flower. B: Healthy viable seeds. C: Empty sterile seeds. D: Healthy seed in MS medium. E: Seed showing germination in MS medium.

collection were observed to be oligophilic with an ethodynamic approach and sternotribic mechanism of collection since only one insect species *Nomia rustica* West. visited the flowers, and collected pollen on the ventral surface of their body. Similar observation was recorded on pollination observation of the plants in their natural habitat.

Natural pollination: in the absence of pollinators

In the consecutive year 1995 when the experimental pots were kept for pollination in the absence of its potential pollinator *Nomia rustica,* many insect species such as aphids, bumble bees and large bodies honey bees. were seen to make only one or two stray visits to the plants perhaps in search of nectar. The seed set was negligible (0.3%) as only two out of expected 540 seeds from 135 flowers were viable rest being sterile. This implies that the species *Eremostachys superba* displays a biotic pollination syndrome and that pollinators namely *Nomia rustica* and *Ceratina heiroglyphica* (Garg and Rao, 1996) are essential for pollination leading to reproduction.

Asexual seed set

Eremostachys superba did not produce any asexual (parthenocarpic) seeds.

The flowers artificially outcrossed (xenogamously) resulted in relatively higher seed set (44.4%) and lesser number of seed abortions (55.6%). But when crossed with a different flower borne on the same spike (geitonomy) resulted in a lower output of 12.5% seeds. Autogamous output was always extremely low (2.8%-3.8%: Table 1). This suggests that with an increase in the spatial distance between the mating individuals, there is a simultaneous increase in seed production. Hence, the magnitude of inbreeding depression was found to be greatest in autogamous and geitonogamous (near outcrossed) flowers. The significant 3.7 fold increase in seed set between the near and distant outcrosses (geitonogamous and xenogamous respectively) is most significant, indicating that the species *E. superba* is self-incompatible and has a strong tendency towards out-crossing. The rate of survivorship of the resultant seeds increased with an increasing spatial distance occurring from autogamy to xenogamy through geitonogamy. This is because little variation is expected to increase with a decrease in relationship between the male and female reproductive units of a population involving a cross.

Table 1. Results of breeding experiments in *Eremostachys superba.*

Breeding mechanism	No. of flowers	Exptd. seeds	Viable seeds		Sterile seed No.	% viability	% abortion
			No.	Avg. wt. in mg			
Autogamy	26	104	3	32.5	77	3.8	96.2
Assisted selfing	18	72	2	28.0	70	2.8	97.2
Geitonogamy	84	336	42	31.4	294	12.5	87.5
Xenogamy	18	72	32	30.8	40	44.4	55.6
Natural Pollin. Vector +nt	69	276	19	27.5	257	6.9	93.1
Natural Pollin. Vector -nt	135	540	2	17.0	538	0.4	99.6
Asexual	20	80	0	---	---	---	---

Mean dry weight of viable seeds = 31.7 mg (range = 20-46 mg)
Mean dry weight of sterile seeds = 31.7 mg (range = 0.05-0.15 mg)
Size range of viable seeds = 12-13 mm
Size range of sterile seeds = 4-5 mm

DISCUSSION

In the clonally perpetuating population of *Eremostachys superba* at Dehra Dun Siwaliks, the overall seed set under natural conditions does not exceed 6.9% (natural pollination) and in any circumstance, 44.4%, even when forcibly out-crossing (xenogamy with assisted crossing). The low levels of seed set through various experiments could be attributed to one or more barriers encountered during the reproductive pathway of the species which are as follows:

1. Plucking of aerial spikes by Gujjars dwelling in the area, removes the fertile parts, wiping out chances of sexual reproduction and seed set (Rao and Garg, 1994).
2. Digging out of root stock for medicinal values dampens the chances of asexual propagation to a great extent (Verma *et al.*, 2003).
3. Scanty pollinator visits in it's type locality has negative effect on xenogamy success in this normally out-breeding species (Garg and Rao, 1996).
4. Strong pollen competition on stigma surface for good quality pollen (Garg and Rao, 1997c).
5. Discrete and sparse populations for successful out crossing. The other reported disjunct populations are from Jammu & Kashmir (Verma *et al*. 2003), a geographically distant area, which is out of reach of natural pollinators of this species namely *Nomia rustica* and *Ceratina heiroglyphica* (Garg and Rao, 1996) and is also too far for normal dispersal of the plant.

Differences in seed set between geitonomous supplementary pollination (12.5%) and assisted xenogamous crossing (44.4%) in the experimental plants demonstrates a severe malfunctioning in reproductive mechanism of the species in natural conditions. compared to the seed set by natural pollination in the natural habitat (6.9%). It is clear that *E. superba* benefits from xenogamy. Since the seed sets are six times higher (44.4% vs 6.9%; Table 1, Fig. 3), but still these seeds showed loss of vigor and germinability. Ovule survivorship or seed abortion after cross pollination is often related to life history (Wiens, 1984; Sutherland, 1986). The miniature ramet population of *E. superba* in it's type locality, is sustained by clonal propagation of rhizomatous root stock, hence comprises of homozygous individuals. This suggests that any out-crossing within these homozygous individuals also amounted to inbreeding.

Further, the only extant miniature population of endangered *E. superba* in the state of Uttar Pradesh, India, is it's type locality at Dehra Dun Siwaliks, which consists of only 33 (± 10) individuals. This number is far below the minimum viable population (Schaffer, 1981) required for species perpetuation and is not ample to buffer the severe stochastic perturbations which are more prevalent and drastic for small, restricted populations. The number of individuals were stagnant during the period under observation from 1993 till 1995 and there were no new upcoming plantlets observed. This evidence indicated a failure of seed germination. The other populations are disjunct in nature (in Jammu and Kashmir state) and far from reach of insect pollinators. For sexual reproduction which is negatively affected by various factors, pollinator availability is integral and is rarer in small fragmented populations with distantly scattered individuals. This pollinator scarcity adds on to inbreeding results in low seed set. A strong genetic drift leading to inbreeding depression (Levin, 1984; Charlesworth and Charlesworth, 1987; Charlesworth *et al*., 1990; Tsukamoto *et al*., 1999; Stephenson *et al*., 2000) thus prevails in the *E. superba* population. Inbreeding in normally out-breeding species brings about a reduction in fitness and loss of alleles which prevents the reversal of inbreeding depression. This renders the population almost devoid of variation and prone to demographic perturbations and thrust the species towards extinction.

A similar study in *Titanotrichum oldhamii* of Gesnariaceae (Wang *et al*., 2004a, b) and in *Coptis teeta* ssp. *lohitensis* of Ranunculaceae (Pandit and Babu, 2003) indicated lack of sexual reproduction in nature condition when outcrossing is rare. Wiens *et al*. (1989) suggests that the reproductive capacity clearly needs to be given careful consideration in management decisions regarding rare and endangered species, and this is particularly true in case of the

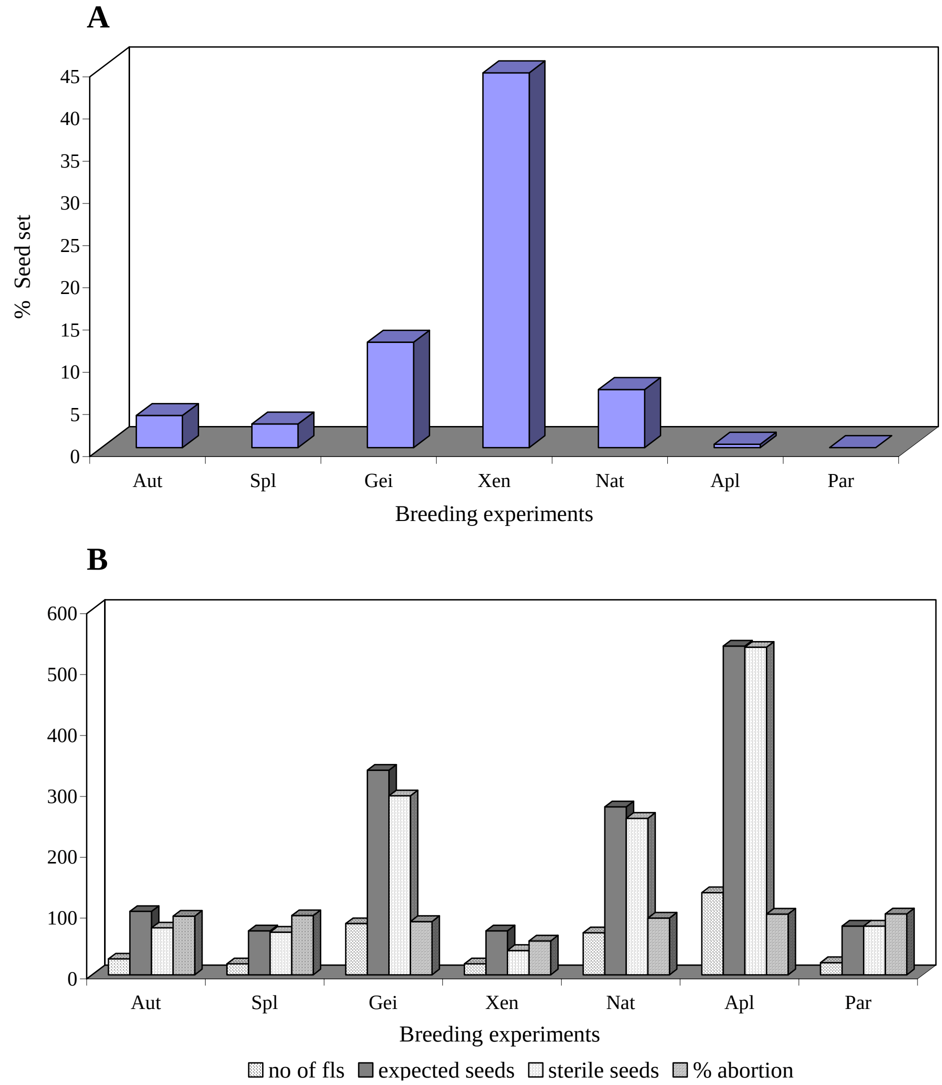

Fig. 3. Breedng results and seed abortion in *Eremostachys superba*. A: Breedng results. B: Seed abortion following inbreeding.

dwindling population of *E. superba*, which is facing a reproductive bottleneck and is currently at the brink of extinction.

It may finally be concluded that selfing in restricted populations increases homozygosity and more homozygous genotypes are more susceptible to the integrated stochastic effect detrimental to their existence. The above studies now establish that *Eremostachys superba* is certainly adapted for out-crossing, but the reduced and isolated nature of the population never permits out-breeding. Even when this is made to effect the genetic exchange is only between close relatives which is less different from inbreeding. This results in acute inbreeding depression and the resultant seeds reared by various inbreeding methods have a reduced viability, vigor and germinability and hence they succumb to environmental pressures. This augments the rate of elimination responsible for species extinction. Thus, the entire demography of the population gets adversely affected. The prevailing inbreeding depression is exemplified by the loss of competitive ability, and the loss of fitness of seeds and seedlings in the resultant progenies. This fact is strengthened by the absence of any juvenile seedlings in the natural habitat of the species during a span of 3 years from 1993 to 1995 as surveyed by the authors.

ACKNOWLEDGEMENTS

The author is thankful to the Director, Botanical Survey of India (BSI) and to the J. D., Central National Herbarium, BSI, Howrah – 3 for encouragement. I am greatly indebted to Dr. R. R. Rao, CIMAP, Bangalore, for his constant help and encouragement during 1993-1995 and to the Director, National Botanical Research institute, Lucknow, for providing facilities during the course of these studies.

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印度 Dehra Dun 地區唇形科瀕危植物 *Eremostachys superba* Royle ex Benth. 之生殖行為與近交衰敗之研究

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(收稿日期：2004 年 7 月 13 日；接受日期：2004 年 10 月 1 日)

摘　　要

本研究針對唇形科瀕危植物 *Eremostachys superba* Royle ex Benth.進行生殖行為與近交衰敗之探討，以顯示在其生殖生物學上所扮演之角色，並討論這些因子是否與該植物適應性，生存能力與活力的缺失有關。在授粉實驗中，自交成功的比例極低，而異株授粉的結種子率為最高(44.4%)，顯示可能具有外交的傾向。然而由於該植物在 Dehra Dun Siwaliks 的生育區域極為狹小，且多由無性繁殖方式繁衍其族群，故在此近均質的個體間的交配，也幾乎等同於自交。這是由於在其傳粉者的飛行距離，與正常狀況下種子的可傳播範圍內，都沒有任何其他同種植物的族群存在。該植物另外的族群產於印度的 Jammu 和 Kashmir 州，都遠超過它僅知的傳粉者 *Nomia rustica* West.和 *Ceratina heiroglyphica* Sm.的飛行距離。本研究中種子產量與發芽的結果也同樣支持這個論述。即使是外觀健康的種子，在自然狀況下也無法正常發芽。這些結果顯示該植物極可能受到自交衰敗的影響而喪失適應性，對於該瀕危植物族群的維持顯然有高度的嚴重性。

關鍵詞：唇形科、*Eremostachys superba*、近交衰敗、同花授粉、同株異花授粉、異株異花授粉。

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