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STUDIES ON REPRODUCTIVE BIOLOGY OF SOME ENDANGERED MEDICINAL HERBS AS A PRELUDE TO THEIR EX SITU CONSERVATION

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Podophyllum hexandrum, *Aconitum heterophyllum* and *Saussurea costus* are among highly priced medicinal herbs of Kashmir. The flowers are hermaphrodite and solitary in the former but aggregated in racemes or capitula in the remaining two taxa. In *P. hexandrum* the pollen transfer is not insect mediated, but in *A. heterophyllum* and *S. costus* it is accomplished mainly by bumble bees. All the three herbs are protandrous. Flowers of *P. hexandrum* are mostly homostylous and few heterostylous (pins and thrums). In the absence of pollinators, contact between the essential organs is made possible only in homostylous flowers by closure of petals at dusk. Pollen are released in groups of four which minimizes pollen loss during pollination and ensures landing of maximum pollen on stigmatic surface. Rarely, among the heterostylous flowers, "pins" may produce fruit, suggesting cross-compatible potential of the species as a primary breeding option, having resorted to autogamy in response to pollinator absence . *A. heterophyllum* and *S. costus* are self-sterile exclusive out-breeders. While the apical flowers (in the former) and central florets (in the latter) enter into "male phase" , the basal (in the former) and the peripheral ones (in the latter) – being the oldest flowers in the inflorescence enter the "female phase" . Pollen are transferred from "functional male" flowers of one plant to "functional female" flowers of the other. Seed set is fairly high. Despite following an efficient breeding system these herbs are vanishing from the region owing to several factors.

Key Words : endangered Himalayan medicinal plants, *Podophyllum hexandrum*, *Aconitum heterophyllum*, *Saussurea costus*, reproductive biology, *ex situ* conservation

Van vangun or Bankakri (*Podophyllum hexandrum* Royle; Podophyllaceae); Patees or Patris (*Aconitum heterophyllum* Wall. ex. Royle; Ranunculaceae) and Kuth (*Saussurea costus* (Falc.) Lipschitz; Asteraceae) are some important and highly priced medicinal plants of Kashmir. On account of indiscriminate extraction and uncontrolled smuggling for pharmaceutical purposes, coupled with multitude of other factors, these herbs are dwindling fast (Siddique,1991), and have been included in "high risk group" of plants by the Botanical Survey of India and the conservation biologists (Henifin *et al.*, 1981a,1981b; Gaur and Semwal,1983; Jain,1983). Consequently, their conservation and multiplication are put on top priority. Development of conservation strategies for endangered taxa necessitates detailed study of their reproductive biology (Massey and Whitson, 1977; Ayensu, 1981; Rao, 1994) . In their Himalayan abode, these herbs are confined to highly specific and extremely stringent ecological niches located at cold, alpine-subalpine altitudes (Siddique and Wafai, 1998 a,b,c) and have evolved unique pollen presentation mechanisms in order to optimize their fitness (Siddique and Wafai, 1998a,b,c) .

The present communication summarizes the salient features of the reproductive strategies of these herbs, and the potential threats of their survival in the region. This information may be of immense use in planning long-term conservation strategy.

MATERIALS AND METHODS

Studies on flowering phenology were conducted during the growing seasons (March-September) from 1996-2004. Phenological behaviour of the plants was closely observed in 34 Kashmir Himalayan populations located in 12 distant regions ranging from 2125- 5655m in altitude (Table 1).

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For studies on the onset and duration of stigma receptivity hand pollinated stigmas were fixed in ethanol-acetic acid (3:1) at regular intervals (24h, 48h, 96h, after anthesis), stored in 70% ethanol and stained with a mixture of 2 ml 1% aqueous light green, 2 ml 1% aqueous acid Fuchsin, 40ml lactic acid and 46 ml distilled water (Lewis, 1979) for an hour. Stigmas carrying germinating pollen were treated as receptive. Some of the pollinated stigmas were subjected to fluorescence microscopy to detect the receptivity of the stigmas by the presence or absence of germinating pollen on the stigmatic surface and pollen tubes in the stylar tissue and embryo sac.

The pollen to ovule ratio was calculated by dividing the pollen count by the number of ovules per flower. Pollen count per flower was estimated by squashing an anther in 10 drops of water and counting the pollen in several drops taken at random with the help of a pipette. The approximate number of pollen grains per flower was computed by multiplying the mean number of pollen per drop of suspension with the number of water drops taken initially and the number of anthers per flower.

For controlled pollination studies, flowers of healthy plants were covered with bags until mature fruit stage to workout the type of pollination. The number of seeds per plant and per fruit was estimated from a random sample of 10 plants per population and 10 flowers per plant. Pollen viability was tested by germinating the pollen in 2% sucrose in glass cavity blocks and/or staining the pollen in 1% acetocarmine, aniline blue-lactophenol or tetrazolium salt. Stainable pollen were considered viable and fertile as recommended by Swanson and Sohmer (1976).

Seed viability was tested by the following methods:

Table: I Characteristics of the population sites explored in Kashmir for the endangered medicinal herbs

Place visited	Location	Population sites scanned	Altitude in metres	Status *A. hetero-phyllum*	Status *P. hexand-drum*	Status *S. costus*	Stress to which exposed
A. KASHMIR							
Tangmarg	34°04 N 74°20 E	-Drang forest region	2125-2150	R	R	AB	b, e, g
Gratnar	34°30 N 75°30 E	-Gratnar	2350-2450	LF	LF		a
Ahrabal	33°34 N 75°20 E	-Gurwatan	2400-2450	LF	LF	AB	b ,e, g
		-Kungwatan	2400-2450	VR	VR	AB	
		-Kousarnag	2400-2450	LF	LF	R	
Naranag	34°30 N 75°30 E	-Naranag Main	2450-2500	LF	LF	R	b, g
Gulmarg	34°04 N 74°20 E	-Akadpatri Nala	2310-2650	LF	LF	AB	
		-Kandwa Forest			LF		
		-Baba Reshi to Gulmarg		AB	R		
		-Kangdori Forest		AB	AB		
		-**Gulmarg-I and Gulmarg-II			AB		
Upper Dachigam	34°10 N 75°55 E	-Satro Nagbairan	2750-2850	AB	VR	R	d , g
		-Nagbairan medow	2850	VR	VR	R	b
		-Hangalmarg	3000	VR	VR	R	a
		-Sangerloo	3000	VR	VR	R	a
Gurez	34°32 N 74°55 E	-Chorven	2950	LF	LF	F	b, g
		-Koragbal	3000	R	R	R	b, g
		-Razdhani Pass	3000	R	R	AB	b,e,f, g
		-Khandyal	3000	LF	LF	AB	
Pahalgam	34°02 N 75°20 E	-Phalgam main	2200	VR	VR		
		-Aru	2500	R	R		
		-Sarabal	2800	VR	VR		
		-Chandawani	2900	VR	VR		
		-Dum Pattar	3500	VR	VR		
Sonamarg	34°30′N 75°30′E	-Baltal	2750	VR	VR	R	b, e
		-Lidervas	3000	VR	LF	F	b, g
B. LADAKH							
Kargil	33°30′N 76°06′E	-Sankoo	3100	R	LF	AB	g
		-Sankara	3100	LF	F		
		-Panikhar	3280-3296	LF	F	AB	
		-Tangole	3300	R	R		g
Zanskar	33°30′N 76°40′E	-Padam	3614	R	AB	AB	
		-Phey	3630	AB	R		
Nobra	34°28′N 77°40′E	-Diskit	3225	R	AB	AB	
		-Khardungla	5655	R	LF		

- Status AB: absent, R: rare, VR: very rare; LF: less frequent F: frequent ;
- a= well protected; b = under grazing stress; c= frequently trampled; d= plants mowed for fodder purposes; e= site subjected to tourism development work; f= strategically important area hence disturbed by extensive road construction ; g= extraction for medicinal purposes

• ** Gulmarg –I and Gulmarg-II : Two distinct populations of *P. hexandrum* in the same region

i) Germination test: 100 seeds were sown in plastic trays containing moist clay mixed with sand under laboratory and field conditions at temperatures varying from -5° to 25°C. Few such samples were sown in beds. Some of these beds were covered with polythene sheets during the entire winter period from November (previous growing season) till March (next growing season) to create green house effect and enforce germination.

ii) Tetrazolium test: The embryos in the vertically cut moist seeds were stained with tetrazolium chloride. Intense stainable embryos were treated viable.

The SEM studies of floral organs were carried out at the Instrumentation Facility for Electron Microscopy, AIIMS, New Delhi and Scanning Electron Microscopy Division, NBRI, Lucknow.

The pollinators were identified and voucher specimens deposited at Zoological Survey of India (Identification report No: 47, lot No. 16/89).

RESULTS

Floral morphology

Podophyllum hexandrum

Each plant produces a solitary homostylous or heterostylous (pin or thrum) hermaphrodite flower (2.56 ± 0.09 to 25 ± 0.14cm in diameter) (Fig.1) bearing 6-9 anthers (Figs 2,3), cylindrical ovary without style and a large highly convoluted stigma close to the ovary.

Aconitum heterophyllum

The main inflorescence (having 3-14 flowers) may bear upto 8 laterals (having upto 83 flowers);flowers hermaphrodite, dull green, blue or greenish purple with deep purple veins, beaked upper petals form 17-29 mm high and 6-12 mm deep hood (Fig. 4), lateral petals oblique, broadly obovate with dark tips, lower ones elliptic and green; nectaries two, sac like (Fig.5), stamens numerous, aggregated at the base of the stalks of nectaries (Figs.6,7), filaments 7-9 mm long; ovary pentacarpellary with adpressed hairs; follicles linear-oblong,11-24 mm long.

Saussurea costus

Plants bear 3-10 capitula; but majority (64.10-86.66%) have 5-8 capitula of which 3, 4 or 5 are clustered at the top of the stem (Fig.8) , while the remaining exist in the leaf axils along the stem; capitula are stalked or sessile, having 74.2 ± 1.70 - 85.5 ± 2.38 florets per capitulum and 496- 581 (543.4 ± 7.79) per plant; florets are hermaphrodite and purple; involucral bracts many, ovate lanceolate, with recurved tips.

Flowering phenology

Initiation and duration of phenological events viz; sprouting of the subterranean perennating organs, anthesis, petal fall and senescence of the aerial shoots were studied.

P. hexandrum

The floral bud matures underground and is fully developed when aerial shoot sprouts. In low altitude populations (<2500 m) sprouting begins in first week of March while in high altitude ones (above 2500m) it initiates in early May inferring a difference of almost 60 days in the two types of populations in the initiation of fresh vegetative phase after over wintering. Complete sprouting takes 8-21 days. Anthesis occurs within 1-3 weeks after sprouting . Within 8-20 days entire population blooms. Different plants and populations take 8-20 days from sprouting to anthesis.

Flowers of high and low altitude plants vary from 14-30 days and 9-15 days in age respectively. Correspondingly, the petal fall starts in the 1st and 2nd weeks of April and June in these populations. The petals fall within 8-17 days exposing the juvenile fruit which remains green for 45-54 days followed by "red fruit stage" which lasts for one to two months (Fig. 9), (end of May to August/September). The red fruits are shed on the ground. Being indehiscent, the seeds are burried in the pulp, hence do not disperse. Senescence of the aerial shoot proceeds from July to September and is completed within 10-20 days in a

Fig. 1. Part of a population of *Podophyllum hexandrum*. Note a solitary flower on each plant

Figs. 2 & 3 . Anthers are almost in line (homostyly) or much below the stigma (heterostyly-pin type) in *P. hexandrum* . Petals have been removed to expose the sex organs. Note 6 anthers and highly convoluted stigma **Fig. 4** . Side view of a flower of *A. heterophyllum*. Note the beaked hood at the top of a wide mouth through which the bumble bee enters **Fig.5**. SEM picture of a sac like nectary of *A. heterophyllum*. (Scale 1000μm) **Fig.6.** An opened flower of *A. heterophyllum*. Note the staminal group at the base of two stalks carrying one nectary each at its top **Fig.7.** Stamens clustered at the base of the stalks of the nectaries in *A. heterophyllum* revealed after removing petals. The young carpels are still hidden inside the stamenal group **Fig.8.** Apical clustered capitula of *S. costus*. Note anthesis in the peripheral florets of two capitula plant. The total life span of the aerial shoots varies between 136 -163 days.

A. heterophyllum

The plants overwinter as dormant subterranean tubers for 5-6 months from October to March-April. Sprouting initiates from mid-March to April and completes within 17-32 days. Vegetative growth proceeds for two months and plants switch over to the sexual phase around early May at low altitudes (2125-2450m) and late June at high altitudes (above 2500 m). The young floral buds which are available for 10-18 days anthese within 4-50 days. Anthesis continues from 3rd week of June till early September. Flowers of an inflorescence anthesce acropetally within 7-17 days (Fig.10), remain open for 10-15 days and subsequently shed their petals. Between late August and mid September more than 90% fruits mature. The aerial shoots senesce from mid-August (lower altitudes) to late September (high altitudes).

S. costus

Sprouting proceeds from April through May for 15-30 days and in June –July the plants switch over to the sexual phase. Florets begin to open from July and anthesis completes within 4-8 days in a capitulum and 13-22 days in a plant. The peripheral florets of the apical head are the first to open (Fig.8); anthesis proceeds gradually towards the centre of a head. About 2-73 florets of a head open in a day. The capitulum blooms for 5-10 days and entire plant for 15-24 days. The clustered capitula on shoot apices open first followed by non-clustered ones. The basalmost capitula may not open at all. The florets remain open for 4-5 days, thereafter the corolla and stigmas shrivel which marks the beginning of seed development. The seeds are ready for dispersal in September and the aerial shoots whither in October.

BREEDING SYSTEM

Anther dehiscence and stigma receptivity

P. hexandrum

Development of sex organs is completed underground. Anthers dehisce asynchronously usually a day or two after anthesis; dehiscence is completed in 4-7 days in a flower. The lobes of the same anther may or may not dehisce simultaneously. Pollen are available for 8-10 days. Initially the ovary is cyclindrical and the stigma just a shallow depression at its top. The mature stigmatic surface is wide and highly convoluted (Fig. 11). The controlled crossing experiments reveal that the stigmas are receptive on the first day of anthesis (20.5 seeds per fruit);receptivity attains peak 48 hours after anthesis, (42 seeds per fruit) and declines 96hrs after anthesis, (18.6 seeds per fruit) (Table 2).

A. heterophyllum

The species is protandrous. The basalmost flowers in an inflorescence are first to mature and enter "male phase". Before anthesis, the turgid anthers stand upright and conceal the young carpels. Pollen are discharged through two longitudinal slits (Fig. 12) 1 or 2 days after anthesis. Dehiscence continues for 4-5 days in a flower and 15-20 days in a plant. The stigma elongates and after complete pollen shedding the filaments bend outwards exposing hitherto concealed stigmas which elongate further, diverge fully and cleave to expose the convoluted receptive surface (Figs.13, 14). The basal flowers now enter the "female phase". Shift from "male" to "female" phase in the basal flowers is accompanied by initiation of "male phase" in the upper flowers . Stigmas remain receptive for 3-4 days (Table 3). Peak receptivity is attained on second and third day of anthesis and the entire pistil is abundantly impregnated with pollen tubes (Figs. 15,16). The fruits develop in the basal flower first.

Table 2: Results of hand pollination depicting duration of stigma receptivity in *Podophyllum hexandrum.*

Time of hand pollination	Seeds produced per fruit**
Day of anthesis	20.5
+24 hrs	25.4
+48 hrs	42.2
+72 hrs	34.4
+96 hrs	18.6

\+ = as many hours after anthesis
** = figures in this column represent an average of 4 plants

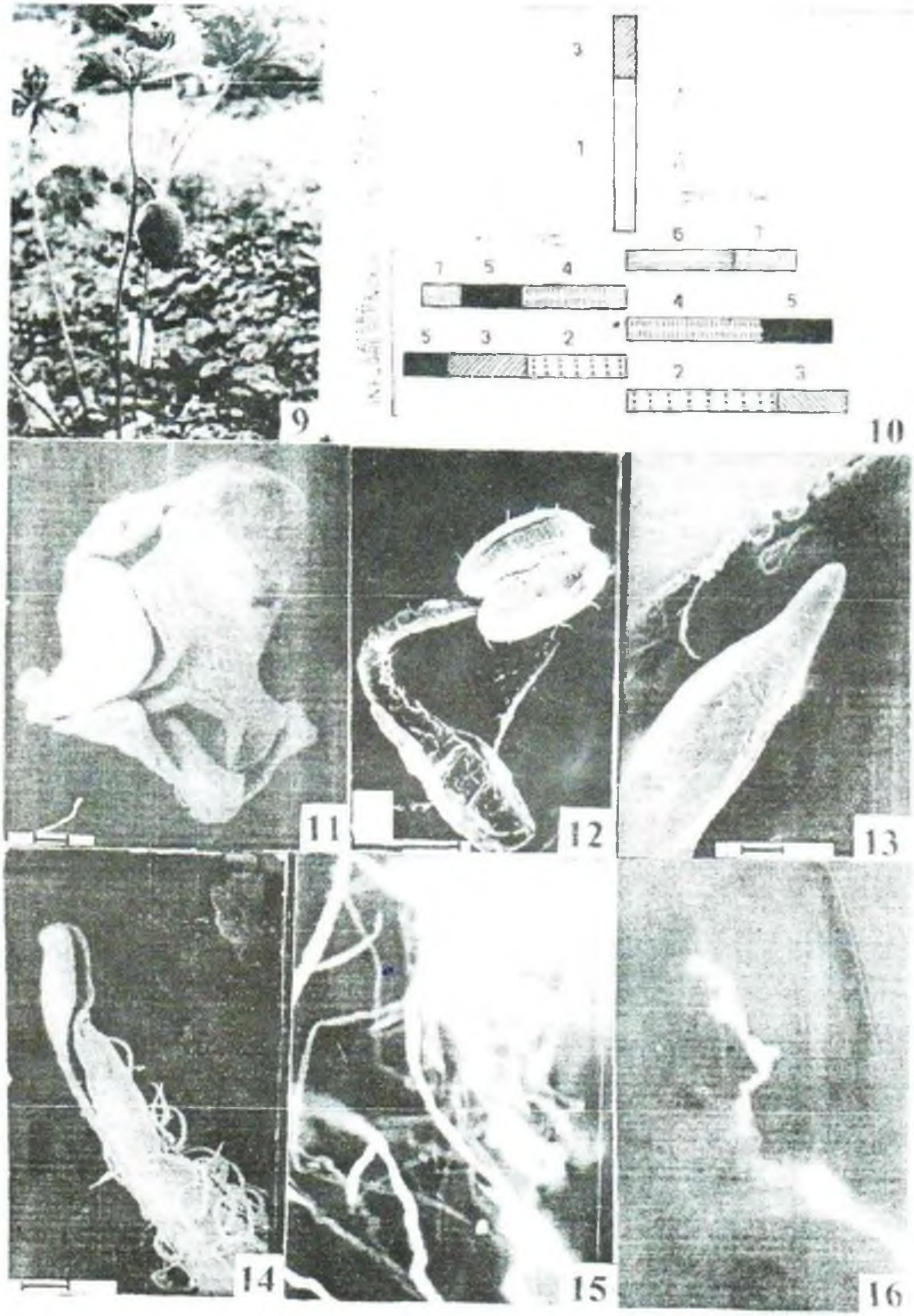

Fig. 9. Each plant of *Podophyllum hexandrum* bears a drooping solitary red berry **Fig. 10.** Pattern of flower opening in main and lateral inflorescences of *A. heterophyllum*. The sequence of flower opening is indicated by the increasing order of the digits. 1= flowers open first; 7= flowers open last of all **Fig. 11.** The stigmatic surface of *P .hexandrum* is highly convoluted (Scale100μm) **Fig.12.** Note two lateral longitudinal slits in the anther of *A. heterophyllum* (Scale 1000μm) **Figs.13 & 14.** Young and receptive carpels of *A. heterophyllum*. The stigmatic surface becomes cleaved in receptive stigmas (Fig 13. Scale 100μm, Fig .14 Bar = 100μm) **Figs. 15& 16.** Pollen tubes moving down the style (Fig .15) into the ovule (Fig .16) in *Aconitum heterophyllum* (x1000)

Table 3. Results of hand-pollination depicting duration of stigma receptivity in *Aconitum heterophyllum*.

Stage of stigma	Stigmas hand- pollinated	Stigmas with germinating pollen(%)
On the day when anther dehiscence was complete	10	-
+ 1	100	25 (25 %)
+ 2	100	70 (70 %)
+ 3	100	92 (92 %)
+ 4	100	8 (8 %)

+1, +2, +3, +4, = No. of days after complete anthesis.

S . costus

In a capitulum anthesis proceeds from periphery to the centre. The peripheral florets are the first to mature followed by the inner whorls; the central florets being last to open. The anther dehiscence begins while the stigmatic branches of the bifid stigmas (Fig.17) are still clasped together .After most of the pollen are released, the style elongates , pierces its way through the dehisced anthers and within an hour, the stigmatic branches diverge to expose the receptive surfaces (Fig. 18). The receptive stigmas now available to the insect visitors get heavily impregnated with pollen. The receptivity lasts for 3-4 days.

Pollen to ovule ratio

The P/O ratio is believed to be an integral part of a plant's breeding system and tends to increase from auto – to allogamous taxa (Cruden, 1976, 1977; Schoen, 1977; Miller, 1978; Cruden and Jensen, 1979; Spira, 1980; Wyatt, 1984; Preston, 1986) hence provides an index of the nature of breeding system operative in a species.

P . hexandrum

Pollen are shed in groups of four (Fig. 19) of which all the four, or only three, two, one or none are richly cytoplasmic and intensely stainable (Figs.20,21,22). When germinated in 2% sucrose, they produce four (Fig. 23), three, two, one or no pollen tubes. More than 90% pollen are viable (Table 4).

Table 4: Pollen viability in *P. hexandrum* soon after anther dehiscence

Intensely stainable and healthy cells per pollen tetrad	Frequency of such pollen tetrads (%)
4	15,964 (87.1)
3	468 (2.5)
2	1,189 (6.4)
1	375 (2.0)
0	328 (1.7)
Total No. of pollen tetrads scanned (pollen grains)	18,324 (73,296)
Total No. of viable pollen grains (%)	69,613 (94.9)
Total No. of nonviable pollen grains (%)	3,683 (5.0)

There are 6-9 anthers per flower. The pollen grains are shed as tetrads whose number per anther and per plant varies from 5,433 to 13,110 and 32,598-78,660 respectively (130,392 - 314,640 pollen grains per plant). The number of ovules per plant varies between 35 and 145. The P/O ratio is 2,599.51 - 4,129.14

A. heterophyllum

The flowers have 42-62 anthers ; each anther has 8,947-13,850 pollen grains (451,000-744,806 pollen grain per flower or 4,285,008- 23,948,064 pollen grains per plant). The number of ovules ranges between 200-1,780 per plant. The P/O ratio equals 8,257.5 - 14,234.2.

S. costus

The species produces 4-8 capitula per stalk and copious quantities of pollen (Table 5) of which > 99% are viable. Increase in the number of pollen grains with increasing number of capitula is associated with increase in the number of ovules per plant. This explains why the P/O remains almost constant despite changes in the number of capitula.

Table 5: Pollen production in *S. costus*

Capitula per plant	Average pollen per plant	Average ovules per plant	Average pollen: ovule ratio
4	383,901.1	294.50	13,022.12
5	5,392,11.7	397.37	13,573.12
6	5,949,680.1	432.12	13,761.87
7	7,613,950.7	548.62	13,895.37
8	8,947,236.1	639.50	13,993.87

Maximum number of pollen and ovules are produced by the topmost and the least by the basal capitula (Table 6). Evidently the gamete producing potential

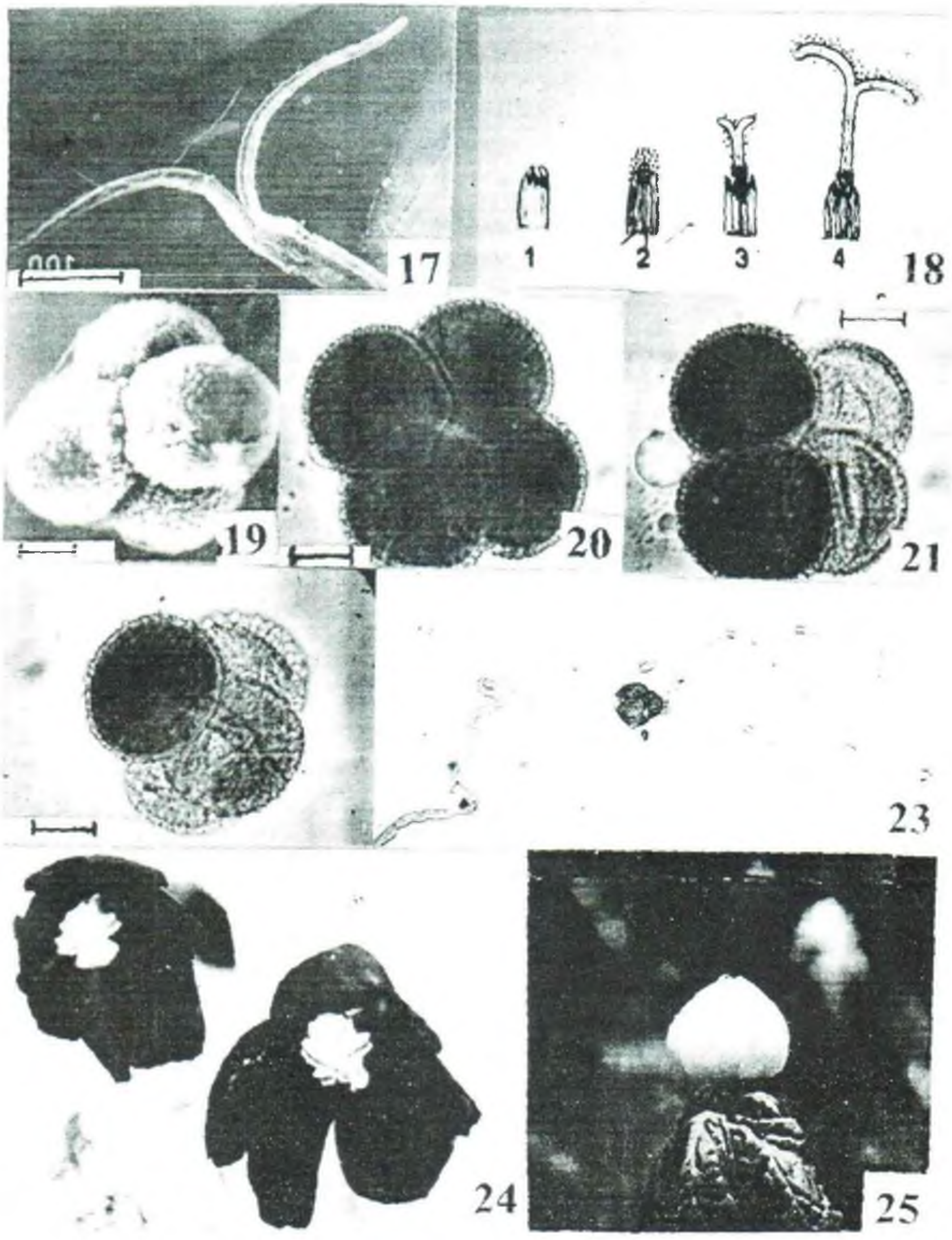

Fig. 17. Bifid Stigma of *S. costus* (Scale 1000μm) **Fig.18.** Different developmental stages in the bifid stigma of *S. costus*. The receptive stigmas (4) are heavily loaded with pollen **Fig.19.** SEM picture of a pollen tetrad of *P. hexandrum* (Scale 10μm) **Figs. 20, 21 & 22.** Note 4,2 and 1 microspores stained intensely with aceto-carmine in *P. hexandrum* (Scale 10μm) **Fig .23.** Pollen tetrad artificially germinated in 2% sucrose. Note four pollen tubes (x1000) **Figs. 24 & 25.** Note the physical contact between anthers and stigma in *P. hexandrum* brought about by closure of petals in the dusk (x 1). (The Photograph No. 24 has been printed in black and white from a colour picture for the sake of contrast)

decreases towards the basal capitula.

Table 6:Inter-capitular variability in the pollen and ovule production in *S.costus*

Position of capitulum within the plant	Average pollen grains		Ovules per capitulum
	per floret	per capitulum	
upper	13,729.33	1,099,208.00	80.00
middle	12,874.66	931,552.33	72.66
basal	12,308.00	855,902.66	69.50

Pollination system

P. hexandrum

Pollination is not insect mediated. Due to spatial isolation of sex organs in heterostylous flowers transfer of pollen usually does not occur. Pollination is achieved in homostylous flowers by physical contact between sex organs (Fig. 24) brought about by the rythmic opening and closure of the petals (Fig.25).The anthers adhere to stigma and the pollen tetrads are deposited on it (Fig. 26).

A. heterophyllum

The species is insect pollinated. The nectar is available from the time the flower opens. The visitors (Hymmopterans: Bombidae and Bremidae - the bumble bee, *Bombus spp.* being the major pollinator, Fig. 27) may be pollen/nectar collectors (actual pollinators) or "nectar thieves" which pierce the hood to sip out the nectar without coming in contact with the essential organs. The pollinators align on the floral opening, turn upside down and penetrate the proboscis into the nectaries to sip the nectar for a few seconds (Fig. 28). During this process the insect's body gets heavily loaded with pollen which are tricolpate (Fig.29). The bees forage from the basal flowers upwards in an inflorescence, often neglecting the small apical flowers. The pollen are transferred from upper "functionally male" flowers of one plant to basal "functionally female" flowers of the other (Fig. 30). Foraging starts around 9.00hrs, attains peak by 13.30 hrs and continues till 18.00 hrs. on a sunny day.

S. costus

The species offers enormous quantity of pollen (Fig.31) as reward to the visiting insects. The florets are regularly visited by honey bees, bumble bees , *Syrphus* sp. and butterflies between 9.00 hrs to 18.00 hrs. The floret opening is asynchronous which ensures pollen availability for a longer time to effect pollination of as many heads as possible. The plants are taller than the surrounding vegetation which facilitates frequent insect visitations. The species is protandrous, self-incompatible and an exclusive cross breeder. Presentation of many single ovuled florets clustered together in heads for fertilization over an extended period of time probably leads to much higher rates of outcrossing than in many ovuled single flowers (Brutt, 1961).The basal heads are usually neglected by insects or become available to the pollinators by increase in their stalk length.

Breeding behavior

In order to understand nature of the breeding system operative in these species, the following experiments were conducted (Table 7; Chart 1).

P. hexandrum

The open pollinated unemasculated homostylous flowers produced maximum seeds. The "pin" type flowers forced to self-pollinate or hand-pollinated after emasculation with pollen from a different plant also seeded well. However, the emasculated flowers left open or bagged without pollination failed to produce seed. Crosses within and between plants of Tangmarg, Gulmarg I, Gulmarg II, Gratnar and Sangergloo were successful, the intrapopulational crosses resulted into higher seed set (28-60 seeds per fruit) than the inter-populational crosses which yielded only 22-50 seeds per fruit (Table 8).

A. heterophyllum

Flowers emasculated prior to dehiscence or umesmaculated when allowed to open-pollinate produced >40 seeds. The controlled crosses in which pollen were transferred geitonogamously or xenogamously produced 28-30 seeds per fruit. Unpollinated or self-pollinated flowers produced no seed.

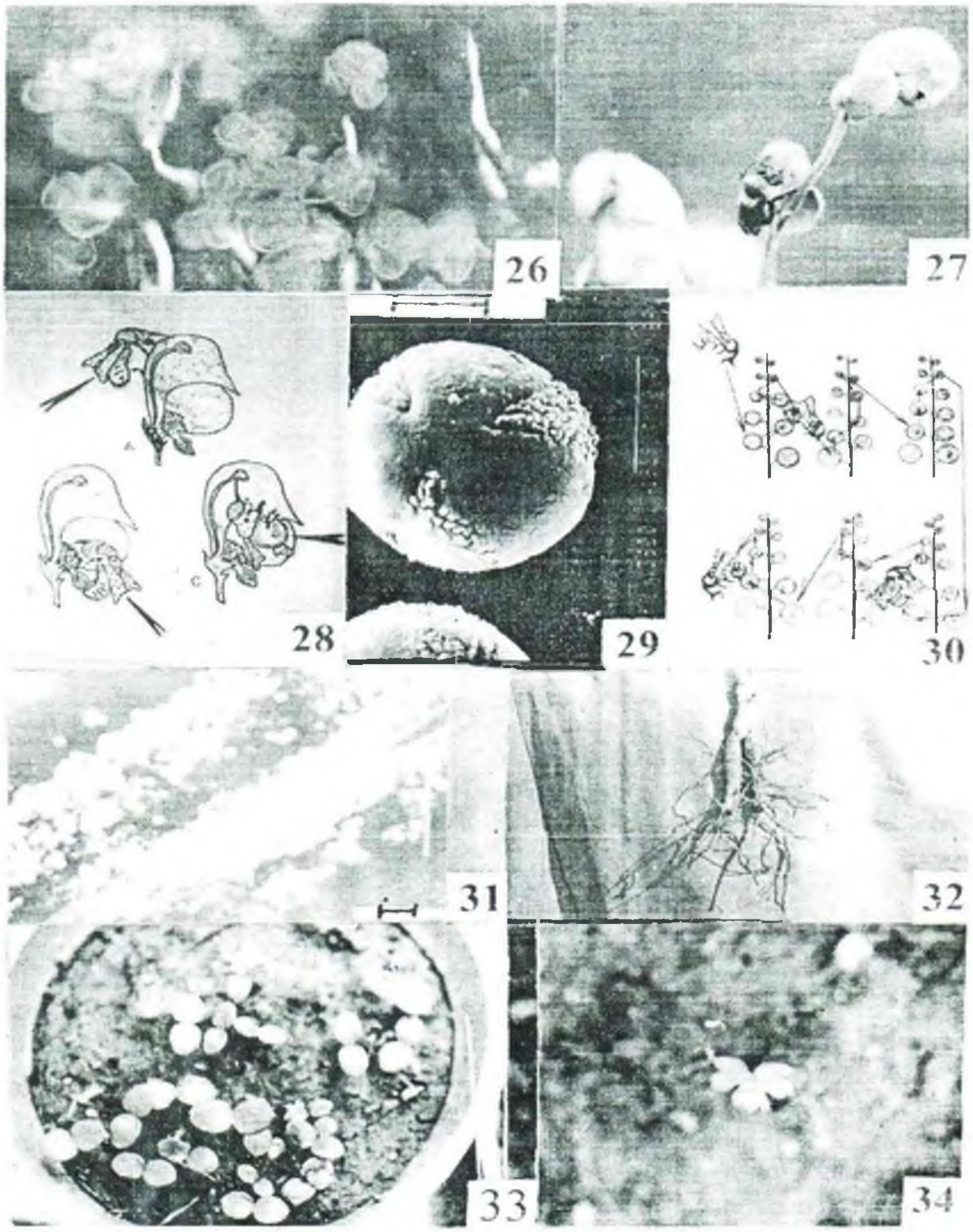

Plate: 4

Fig. 26. Fluorescence photo-micrograph of the pollen tubes moving down the style in *P. hexandrum*. Note the emptied "Pollen tetrads" on the stigmatic surface. (x1000) **Fig. 27**. Bumble bee landing on a flower of *A. heterophyllum* **Fig. 28**. Pollination mechanism in *A. heterophyllum* A = "Nectar thief" on the hood to sip the nectar B, C = Pollinator landing on the floral mouth where the stamens are assembled and taking a turn upside down (C) to sip the nectar **Fig. 29.** SEM picture of tri-colpate pollen of *A. heterophyllum* (Scale 10μm) **Fig. 30**. Foraging behaviour of a bumble bee on the inflorescence of *A. heterophyllum* from base upwards **Fig. 31**. Receptive stigma of *S .costus* richly impregnated with pollen (Scale 100μm) **Fig. 32.** In *A. heterophyllum*, previous years tuber (source of the drug) is steadily consumed and replaced by a single tuber **Fig. 33**. Seeds of *P. hexandrum* being germinated in plastic trays kept indoors **Fig. 34 .** Seedlings of *A. heterophyllum*. As the embryonic leaves fall towards the end of the growing season, photosynthetic leaf appears

S. costus

Plants allowed to open pollinate produced 25.6±2.65 – 33.6 ± 2.6 seeds per capitulum and those bagged before anther dehiscence to self-pollinate produced no seed suggesting self-incompatible nature of the species.

Table 7: Controlled crossing experiments in some endangered Himalayan medicinal herbs and their results.

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Experiment	*Podophyllum hexandrum*		*Aconitum heterophyllum*		*Saussurea costus*	
	No. of flowers	Seeds per fruit	No..of flowers	Seeds per flower	No. of capitula	Seeds per capitulum
Un-emasculated flowers/capitula bagged before anthesis to enforce selfing	15	49.6 ±3.27	20	—	21	—
Unemasculated flowers/capitula tagged for open-pollination	15	50.4 ± 4.20	36	46.81±4.2	44	396.68±2.23
Emasculated flowers left open to pollinate	17	-	38	42.0±3.8	*	*
Emasculated flowers hand pollinated with pollen from a different plant (xenogamy)	10	44.16±8.31	25	28.5±3.00	*	*
Emasculated flowers bagged and not allowed to pollinate	7	-	14	-	*	*
Emasculated flowers hand pollinated with pollen from a different flower of same plant (geitonogamy)	**	-	16	30.7± 2.7	*	*

*Not done because removal of anthers from the small florets by hand is not possible.

**Not possible since the plants have a solitary flower.

Table 8: Intra- and inter-populational cross compatibility in *P. hexandrum.*

No. of crosses made	Pollen parent / Ovule parent	Seeds per fruit (Range x̄ ± S.E)			
		Tangmarg	Gulmarg I	Gulmarg II	Gratnar
12	Tangmarg	(28-50)* 44.16±6.12	NR	NR	NR
7	Gulmarg II	NR	(30 - 48) 39.74±4.22	NR	NR
6	Gulmarg I	NR	NR	(25-40) 28.68±3.18	NR
6	Sangerloo	NR	NR	NR	(22-50) 38.0±2.7
7	Gratnar	NR	NR	NR	(29-60*) 49.51±4.31

*Note relatively higher seed output in the intra-populational crosses.

NR : Not recorded

Modes of propagation

P. hexandrum

1-6 winter buds produced by the underground rhizome remain dormant during winter. Growth is resumed during February - March. Usually only one of the winter buds is sexual and is composed of a small stalk, two leaves and a floral bud. The vegetative bud comprises a shoot primordium which produces a stem with a single leaf. The winter bud matures underground, sprouts in March with a minute stalk clasped by the two leaves crowned by a nodding floral bud. The flower opens before the leaves spread out. Each new sexual shoot produces a fruit and new buds on its rhizome. These buds undergo differentiation in the next growing season in much the same way as the previous buds. After 2-3 cycles of growth, the rhizomes attain sufficient maturity to produce the sexual bud. A single rhizome may produce even six shoots within a few years.

The sexual shoot produces a single indehiscent fleshy fruit which is eaten by birds viz. *Columba laevia* and *Carvus* spp. While threshing out the fruit, they disperse the seed. The fruit often falls prey to black bear or monkeys and the undigested seeds are ejected with faeces. Seed set ranges between 69.48 – 92.67%. The seeds are reddish or blackish, globular. 75.8-85% viable, exhibit prolonged seed coat dormancy and germinate in a protracted manner at 15 - 25°C.

A . heterophyllum

The plant grows for 4-6 weeks and the resources begin to allocate towards the development of a single new tuber (Fig.32), which grows fully upto September . The new tuber bears a solitary apical bud to produce new shoot in the next growing season.

The species produces 374.76±33.0 - 799.46±41.44 seeds per plant of which 74.2-83.8% are viable and weigh about 400-405 seeds per gram. They remain dormant for five to six months and germinate optimally in a protracted manner from March through June.

S. costus

Chart : 1 Controlled crossing experiments in *Podophyllum hexandrum, Aconitum heterophyllum* and *Saussurea costus* and their results

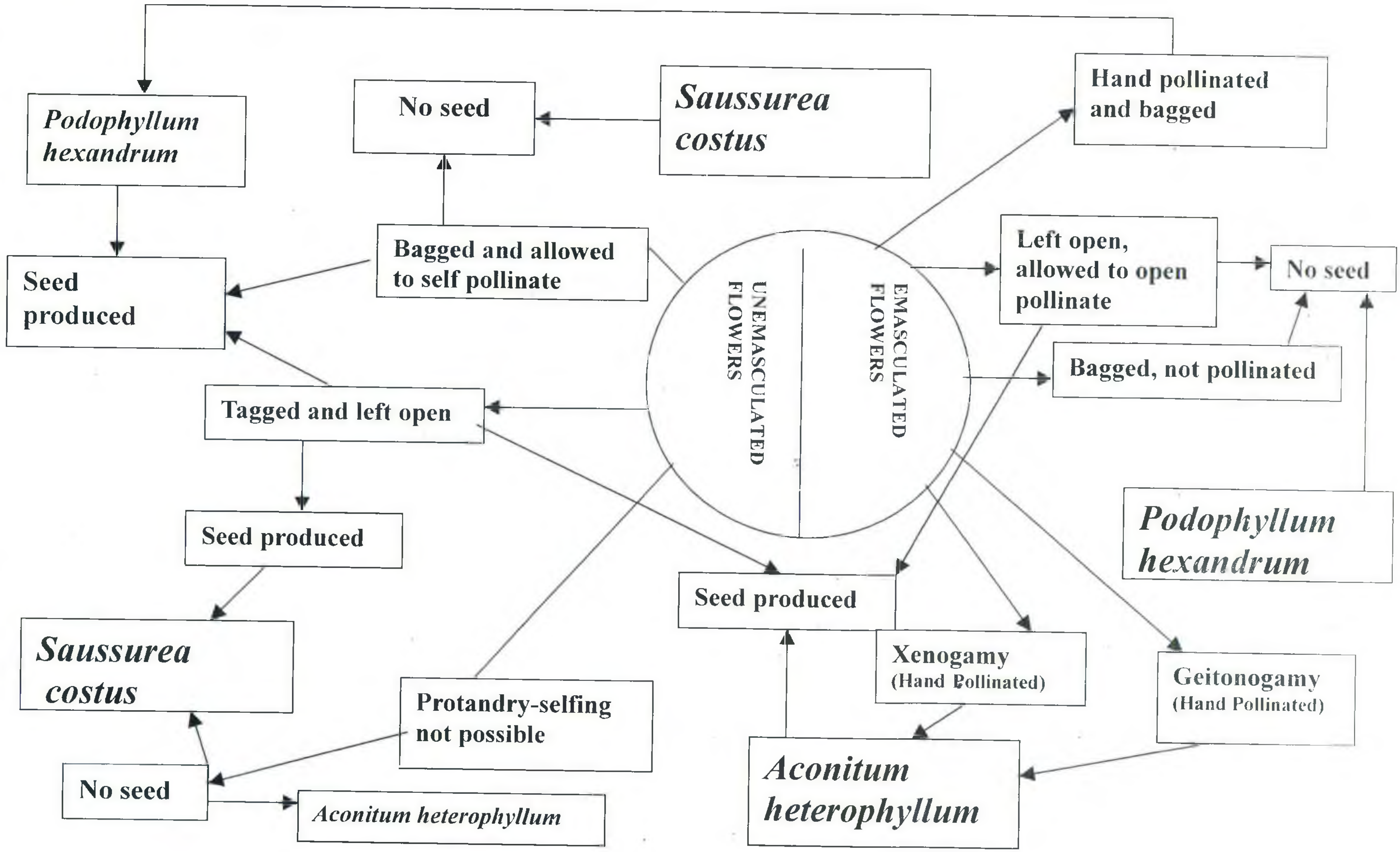
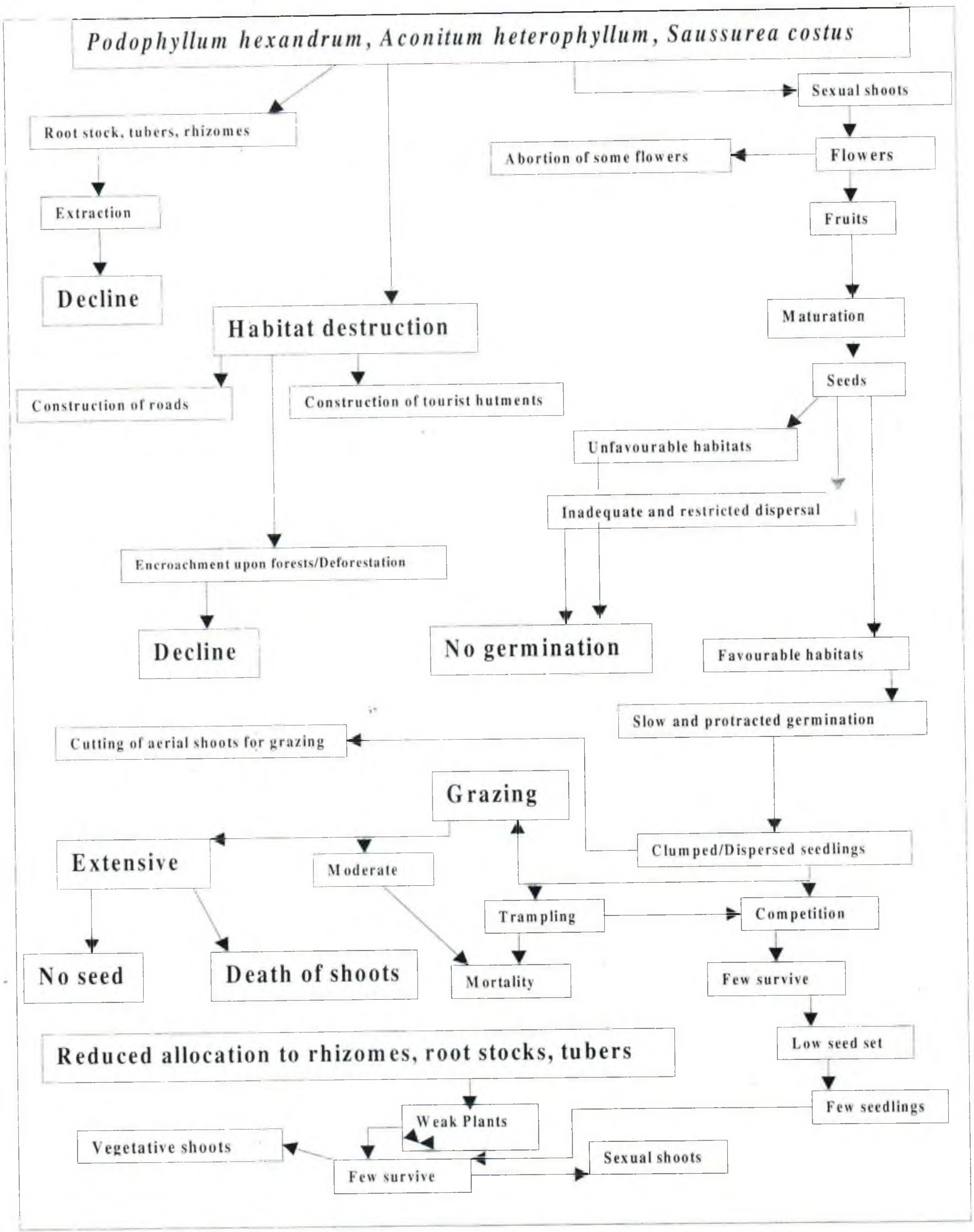


Chart 2. Causes of decline of the important medicinal herbs in Kashmir Himalayas *Podophyllum hexandrum, Aconitum heterophyllum, Saussurea costus*



The rhizome produces 1-3 vegetative buds after first year, and remains dormant during the winter months. Only a few buds develop into leafy shoots which switch over to the sexual phase in the second growing season . The mature rhizome is branched and bears 5-8 shoots. The species can be propagated through cuttings of the rhizome.

The plants produce 81.66-90.711 seeds per 3-capitulate shoots and 265.75-284.5 per 9 capitulate ones. The total number of seeds per capitulum ranges between 25.6±2.65 and 33.6±2.6. More than 80% seeds are viable.

The seeds germinate immediately under favourable conditions. The young seedlings, however, succumb to frost. The seeds that germinate in the next growing season constitute the only means for new recruitments.

The species' sexual system is, therefore, of a limited value and the plants have to rely mainly on the vegetative mode.

***In vitro* seed germination**

Seeds of these herbs were grown in plastic trays and subjected to different treatments to initiate germination (Table 9). Chilling before sowing resulted into maximum germination (Fig.33). The seeds germinated in a protracted manner in one's and two's between March and July. Seedlings raised under laboratory conditions were transferred to the experimental plots, most of them flowered and produced seeds. As the embryonic leaves fall the photosynthetic leaf appears (Fig.34).

Table 9: % germination of *Podophyllum hexandrum (P. h)*, *Aconitum heterophyllum (A .h)* and *Saussurea costus* (*S. c*) seeds obtained from Gulmarg (Kashmir) and Kargil (Ladakh) in response to different treatments under field (F) and laboratory (L) conditions.

	Collection site											
	Gulmarg (Kashmir) Germination %						Kargil (Ladakh) Germination %					
	F			L			F			L		
Treatment	*P.h*	*A.h*	*S.c*	*P.h*	*A.h*	*S.c*	*P.h*	*A.h*	*S.c*	*P.h*	*A.h*	*S.c*
15 day chilling	08	02	44	28	18	11	18	41	14	35	40	00
30 day chilling	36	00	45	16	18	22	44	02	04	16	52	01
60 day chilling	00	00	46	00	18	30	09	00	09	03	00	00
90 day chilling	60	00	46	52	27	35	10	00	03	18	34	00
120 day chilling	20	17	04	12	22	00	11	16	04	01	32	00
Seed coat puncturing	00	00	22	00	03	11	00	00	08	00	22	00
Scarification	00	00	16	00	00	04	00	01	10	02	02	00
Conc.H_2SO_4	00	00	25	00	00	05	05	00	11	00	02	00
Hot water treatment	00	00	11	00	00	02	11	00	09	00	00	00
GA_3 Treatment (100ppm)	--	--	40	--	--	32	--	--	20	--	--	05

DISCUSSION

P. hexandrum, A. heterophyllum and *S. costus* are principally temperate Himalayan taxa inhabiting altitudes between 2000m and >5000m. Having highly specific ecological requirements; their phenological behaviour seems to be greatly affected by altitude and temperature. Higher altitudes (>2500m) in the region register much colder climates all the year round because of which onset of various phenophases from sprouting till senescence are delayed by about 30 – 40 days compared to those inhabiting the lower altitudes (1490 – 2500m).

Onset and duration of flowering, relative maturation of male and female sex organs and the number and arrangement of flowers in a plant profoundly influence the pollinator visitation pattern in the taxa under reference which has a direct bearing on the success of their sexual cycle (Siddique,1991).

Floral morphology and pollination mechanisms

All the three taxa are hermaphrodite. While the flowers of *P. hexandrum* are solitary and non-showy , those of *A. heterophyllum* and *S. costus* are borne in aggregates of multiple racemes and capitular heads for pollinator attraction (Siddique and Wafai, 1998a, Wafai *et al.*, 1999) . In *P. hexandrum* the absence of pollinators is compensated by the presence of homostylous flowers in which pollen transfer is achieved through prolonged physical contact between the sex organs caused by rhythmic evening closure of petals ("ecological cleistogamy" sensu Percival, 1965). The heterostylous flowers may produce small sized fruit.

The pollen are released in groups of four in which each cell represents an independent pollen grain. This is a unique presentation mechanism which in a single event of pollen discharge ensures deposition of at least four times the number of pollen grains onto the stigmatic surface to which the anthers cling (Siddique *et al.*, 1997; Wafai *et al.*,1998, Siddique and Wafai, 1998c). The stigmatic surface being wide, complex, highly convoluted and richly impregnated with exudates is able to entrap copious quantities of pollen tetrads. This system maximizes pollen deposition on the stigmatic surface and minimizes the pollen loss.

Pollination in aconites is principally mediated by bumble bees (Löken, 1949, 1950; Pyke,1974; Heinrich, 1979; Brink, 1980, 1982). The floral structure is so highly specialized for these insects that ancestors of the present day bumble bees are believed to have been largely responsible for the development of *Aconitum* flower therefore, nicknamed "bumble bee flower" (Leppik, 1964; Heinrich, 1977). The species is protandrous .The stigmas remain concealed within the staminal group emerging at the completion of the "male phase". Perfect temporal isolation of the "male" and "female" phases within a flower occludes selfing . After completing the "male function" the basal flowers enter the "female phase", the upper ones enter into "male phase". This system creates possibilities of geitonogamous pollen flow from upper "functionally male" flowers to lower "functionally" female" flowers in an inflorescence and also promotes chances of xenogamy in the species (Siddique and Wafai, 1998b; Wafai *et al.*, 1996) as is typical of protandrous aconites *A. septentrionale* and *A. nepellus* (Epling and Lewis, 1952; Percival, 1965; Percival and Morgan, 1965; Benham, 1969; Faegri and Vander Pijl, 1971; Proctor and Yeo, 1972). The chances of geitonogamy are, however much less because the pollinators forage from base upwards in an inflorescence in favour of decreasing nectar production up a raceme. This system is known to enhance the foraging efficiency of the bees and maximize the out crossing rates (Pyke, 1974; Wyatt, 1982).

The florets in *S. costus* are aggregated in compact capitula. Several workers contend that the heads of composites represent single flowers (Good, 1931, 1956; Leppik, 1960).However, this is true only in terms of pollinator attraction. Presentation of many single ovuled flowers for pollination over a greater period of time results in much higher rates of outcrossing than in many ovuled single flowers (Brutt, 1961). *S. costus* is a protandrous species and exclusively cross-pollinated. The opening of florets is highly asynchronous which ensures availability of pollen and receptive stigmas over long periods, enhances pollination efficiency, and possibilities of higher fruit and seed set (Wyatt, 1982).

The anthesis starts from the peripheral florets. "Male phase" commences first followed by "female phase". As the bifid stigmas push through the anther collar, they get loaded with pollen. Also, the pollinators impregnate stigmas richly with cross pollen. The self-pollen fail to germinate as revealed by the present bagging experiments.

Breeding system

The taxa under reference are protandrous. Homostyly, however, creates opportunities of inbreeding in *P. hexandrum,* where as protandry and self-incompatibility favour outbreeding in *A. heterophyllum* and *S. costus.*

P. hexandrum

Although protandrous to begin with, the "male" and "female" phases in a flower overlap at later stages to ensure selfing in absence of pollinators. The few heterostylous flowers that exist probably suggest the species to be primarily an outbreeder having shifted to inbreeding in response to pollinator absence as also indicated by high P/O ratio which increases from auto to allogamous behaviour (Cruden, 1976, 1977; Schoen, 1977; Miller, 1978; Cruden and Jenson, 1979; Spira, 1980; Short, 1981; Wyatt, 1984; Koul, 1985; Preston, 1986). In many angiosperm taxa selection is believed to favour inbreeding in response to pollinator non availability (Hagerup, 1951). Despite practising autogamy, the species has allogamous potential as demonstrated by intra- and inter-populational crosses made during the present investigations. The flowers hand pollinated with cross pollen set almost the same amount of seed as those forced to self pollinate (Table 8). The results point to a stronger autogamous tendency in the species which might have evolved in course of its adaptation to the pollinator absence.

A. heterophyllum

The specialized floral architecture, high P/O ratio, protandrous nature and entomophillous pollination indicate cross-compatible breeding system operative in the species. The emasculated flowers (male function removed), if allowed to open-pollinate produce almost the same quantities of seed as by the flowers hand pollinated with cross pollen. The self-pollinated flowers failed to produce seed. In response to the pollinator scarcity the species can utilize geitonogamous mode as revealed through controlled crossing experiments which resulted into more than 30 seeds per flower hand pollinated geitonogamously.

S. costus

The species is exclusively cross compatible. Heads allowed to open pollinate produced large quantity of seed while those bagged to exclude the foreign pollen and hand pollinated by self pollen failed to produce seed thus confirming self incompatible nature of the species. Self incompatibility is known in several genera of Asteraceae including genus *Saussurea* (Brewbaker and Majumdar, 1961; Lewis,1966; Lawrence, 1985).

Modes of propagation

These taxa operate vegetative as well as sexual modes. While the latter generates high degree of variability, the former fixes it.

In *A. hetrophyllum*, the vegetative mode is of a limited potential because the tuber is consumed during the ensuing growing season and replaced only by a single tuber. This method represents one to one replacement of the vegetative propagule, hence essentially only a means of year to year perpetuation.

The sexual system is however, efficient and yields copious seeds which are viable. The potential of seed as sexual propagule is limited because of inadequate dispersal and slow germination protracted over several months. The seedlings suffer mortality owing to numerous biotic or abiotic factors, hence the seedlings are usually scant. In *P. hexandrum*, the seed dispersal is inadequate as happens in *P. peltatum* (Gleason and Cronquist, 1964; Swanson and Sohmer, 1976).The seedlings are produced in clumps, face precocious competition and most of them collapse.

The herbs presently investigated are endemic to parts of central Asia, Afghanistan , Pamirs and the adjoining areas including north west and parts of mid Himalayas and Kashmir where they are restricted to specific high altitude niches which experience extremely severe climates. Several anthropogenic factors viz. habitat destruction, indiscriminate extraction of the drug (rhizomes and tubers) without any cognizance of the limited regeneration potential of these species, unchecked grazing and detoping of the aerial shoots for fodder, precocious floral and fruit drop and fruit predation threaten the very survival of these herbs (Chart 2) in the entire Himalayan region. Besides , slow and protracted seed germination reduces their potential of sexual multiplication .

Although mass awareness through media for legislative protection and effective control on ruthless extraction of the raw material, identification, demarcation and protection of the germplasm reserves and *in situ* conservation of vulnerable habitats are some of the remedial measures, *ex situ* conservation under simulated conditions in herbal gardens at appropriate and easily approachable sites and development of cost effective protocols for large scale multiplication are immediate need of the hour .

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