



# Unique arrangement and temporal separation of essential organs promotes cross pollination in *Impatiens edgeworthii* Hook. f.: an endemic species of Western Himalaya

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## ABSTRACT

*Impatiens edgeworthii* is an important plant species endemic to Western Himalaya. In this species male reproductive organs conceal the stigma – seat of pollen reception during male phase therefore acts as barrier for self-pollination. In addition the stigma become receptive only after the androecium of the same flower is shed and then receptive stigma become exposed. This unique type of dichogamy and arrangement of essential organs are contrivance for cross-pollination in the species. The large showy flowers, production of large quantities of nectar, protandry, presence of nectar guides, ornamented exine sculpturing, high pollen to ovule ratio points towards cross-pollinated mode of reproduction is operative in this species. This was also established by breeding experiments. Our study may prove useful in conservation of this Himalayan endemic plant species and also in understanding the evolution of breeding system in the genus.

**Keywords:** endemic, self-pollination, unique dichogamy, cross-pollination

## РЕЗЮМЕ

Ахтер Ч., Решы З.А., Гани А.Х., Дар Г.Х., Хуру А.А. Уникальное расположение и временное разделение основных органов способствует перекрестному опылению у *Impatiens edgeworthii* Hook. f. – эндемичного вида Западных Гималаев. *Impatiens edgeworthii* – важный вид растений, эндемичный для Западных Гималаев. У этого вида мужские репродуктивные органы скрывают стигму – место приема пыльцы во время мужской фазы, поэтому она служит барьером для самоопыления. Кроме того, рыльце становится восприимчивым только после того, как андроцей того же цветка осыпается, и тогда восприимчивое рыльце становится открытым. Этот уникальный тип дихогамии и расположение основных органов являются приспособлением для перекрестного опыления у данного вида. Крупные эффектные цветки, производство большого количества нектара, протандрия, наличие нектаропроводов, орнаментированная скульптура экины, высокое соотношение пыльцы и яйцеклеток указывают на то, что у вида действует перекрестное опыление. Это также было установлено в ходе селекционных экспериментов. Наше исследование может быть полезным для сохранения гималайских эндемичных видов растений, а также может быть полезным для понимания эволюции системы размножения в роде.

**Ключевые слова:** эндемичность, самоопыление, уникальная дихогамия, перекрестное опыление

Переведено редколлегией *Botanica Pacifica*

To prevent deleterious effects of inbreeding, strong natural selection has promoted cross pollination in plants (Li et al. 2001). There are many contrivances for cross pollination in plants which include declivity, dichogamy, heteromorphy, herchogamy and self-incompatibility, and act as barriers for self-pollination (Cardoso et al. 2018). The temporal separation of essential organs (dichogamy) and their spatial separation (herkogamy) are widespread in outcrossing angiosperms and play a vital role in the successful functioning of flowering plants (Barrett 2003, Cardoso et al. 2018). These aforementioned mechanisms help in avoiding pollen–stigma interference to reduce the chances of self-fertilization, however; cross-pollination is promoted due to the non-simultaneous presentation of pollen and stigma (Cardoso et al. 2018, Ramírez & Hokche 2019). The

coordinated functioning of essential and accessory parts allows flowers to enhance pollination either autonomously or through interaction with pollen vectors (Harde et al. 2004). Floral architecture and temporal, spatial separation of essential organs are directly linked to pollination; the different floral characters have coevolved to maximize the successful pollination (Cruden 2000, Fenster et al. 2004). Although floral development in the genus with respect to phylogeny has been studied (Caris et al. 2006, Janssens), however, very little is known about the role of different floral part arrangements and their difference in temporal development in pollination system. Therefore, the present study was undertaken to study the pollination biology / breeding system of the *Impatiens edgeworthii*. In the present work we hypothesized that the male reproductive parts

itself acts as barrier of self-pollination and contrivance for cross-pollination in the studied species.

## MATERIAL AND METHODS

### Study sites

The present study was carried out in Kashmir Himalaya, which represents the main valley of Kashmir. The region falls within the biogeographic zone of the north-western Himalaya in India and lies between latitudes 33°20' and 34°54'N and between longitudes 73°55' and 75°35'E, covering an area of 15 948 km<sup>2</sup>. From the study area the target species was mainly collected from Dachigam, Gulmarg, Baramulla, Boniyar, Uri, Kangan and Sonamarg of Jammu and Kashmir, India. The species grows in damp shady montane habitats often along gullies and streams at an elevation range of 1800–3000 m a.s.l.

### Species studied

The species is annual herb up to 70 cm tall, robust, glabrous; stem erect, branched; leaves large, 10–14 × 3.5–6 cm, petiolated, ovate-lanceolate to ovate-elliptic, acuminate, crenate to serrate, glandular at base; inflorescence short, sub-terminal raceme; peduncles 2–7.5 cm long, sometimes keeled; flowers yellow, red streaked in the throat; bisexual; bracts large, keeled, often whorled, lanceolate, 1.5–2 mm long; lateral sepals ovate, acute, ca 2–5 mm long, mid-nerve prominent; lower sepal (lip) infundibuliform, gradually narrowed into a recurved spur, 2–3.5 cm long; standard or anterior petal orbicular, emarginate, crested, ca 1 × 1.5 cm long, lateral united petals 18–20 mm long, upper ones prolonged obliquely upwards, yellow-white; stamens 5, filament short and broad; carpels 5; capsule linear, erect 2.5–3 cm long; seeds brown, oblong, longitudinally rugose (Fig. 1a).

### Flowering phenology

A random sample of 50 flowers was used for studying the morphology and flower development. The blossoming stages were observed every day at regular time intervals. Subsequent events, anther dehiscence, stigma receptivity were noted and recorded at regular intervals of time for two years.

### Pollination

Presence of nectar at the base of ovary at different developmental stages was recorded by visual observation.

Temporal activities of the floral visitors were recorded at time between 8:00 am and 2:00 pm over a one week period in each season. Each floral visitor was carefully observed during this period and legitimate pollinators were identified by bagging the flowers pollinated by different pollinators to record the fruit set.

### Stigma receptivity

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

der to estimate duration of stigma receptivity, the stigmas of varying ages were pollinated manually by dusting on them the pollen obtained in bulk from freshly dehisced anthers with the help of a sterilized needle. The pollinated stigmas were fixed in 1:3 acetic alcohol and subsequently stained in aniline blue-lactophenol and studied periodically under a microscope. The data collected were recorded for determining the duration of stigma receptivity.

### Pollen features

Fresh pollen grains were collected from the dehiscing anthers by gentle shaking of flower. Immediately slides were prepared by dusting the collected pollen grains in a drop of lactophenol-aniline blue. The shape and surface sculpturing of pollen grains was observed at 10× eye piece and 100 (oil immersion lens) objective lens of light microscope.

### Pollen fertility, pollen to ovule ratio and pollen volume

Pollen fertility was estimated by two methods:

1. Dianne & Spicer's (1958) method in which mature anthers ready to dehisce were squashed in 1 % aniline blue-lactophenol and observed after 15 minutes.
2. Stanley & Linsken's (1974) method wherein mature anthers ready to dehisce were placed in 1 % tetrazolium chloride for one hour and squashed.

Plump and well stained pollen in both the cases were considered as viable.

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

$$p \times q = r,$$

$$r \times s = t,$$

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

Average ovule number per pistil was counted using dissection microscope.

P/O ratio was calculated following Cruden's (1977) method as follows:

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

For calculating pollen volume, Ganie et al. (2021) method was followed. The pollen grains from fifty randomly selected dehisced anthers were put in a drop of 2 % acetocarmine and diameter, polar axis and equatorial axis diameter were measured at 400× using ocular micrometer. The volume of pollen grain was calculated by following:

$$V = \pi PE^2/6,$$

where, P is polar axis diameter, E is equatorial axis diameter.

### In vitro pollen germination

The *in vitro* pollen germination was analysed in a medium containing sucrose, boric acid, calcium nitrate, magnesium

sulphate and potassium nitrate following Brewbacker & Kwach (1963) with and without modifications (Table 1) keeping the normality same as in the Brewbacker & Kwach solution. The pollen grains with tube length more than their diameter were considered germinated.

### 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. The tagged stigmas at its receptivity were pollinated with pollen grains of flowers of same plant to ascertain geitonogamy.
- IV. The tagged stigmas at its receptivity were pollinated with pollen grains of flowers of different plants to ascertain xenogamy.

The bagging experiments were carried out on 30 flowers in each case at the selected sites (both *in-situ* and *ex-situ*) for two consecutive years. The tagged flowers were later monitored for seed set. Pollen grains collected from anthers ready to dehiscence was used for manual pollination

### Scanning Electron Microscopy (SEM) studies

Double sided conductive tap was fixed to the stub and the pollen grains from mature undehisced anthers were dusted over it. The dusted material sputter coated with gold was observed under SEM (Hitachi S-3000H, MPN, Japan).

### Statistical analyses

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

## RESULTS

### Floral morphology and pollination

The species bears large, zygomorphic, yellow-white flowers with red coloured nectar guides towards the spur (Fig. 1b). The nectary is present at the base of ovary and

produces large amount of nectar till the sepals and petals are shed. The flowering period in the species is July–September.

It was observed that during flower development, at floral bud stage the style and stigma remain concealed by androecium; the filament forms a sheath around the stigma and style while the anther (a pad like structure) caps the stigma (Fig. 1c). After 2–3 days, anthesis (opening of flowers) starts. In open flowers the anthers are mature and anther dehiscence starts on the day of anthesis. During the anther dehiscence small gap becomes visible in the filamental sleeve which envelope the style and stigma (Fig. 1d); the gap widens as the anther dehiscence proceeds and another gap (two pore like structures), between anther and filament develops on lower side of anther which demarcates filament and anther regions (Fig. 1e).

It was observed in the present study that as the flower develops the filamental sheath starts rolling back from ovary side (Fig. 1f) and during filament rolling process the anther dehiscence continues and almost all the pollen grains are shed by the anther within 2–3 days. After anther dehiscence is completed whole androecia along with the anther pad (anther wall and other tissue) is shed on 3rd day of anthesis and consequently style and stigma becomes exposed (Figs 1g, 2a). The exposed stigma remains receptive on 3rd, 4th and 5th day; receptivity is lost after 5th day of anthesis (Table 2).

During male phase i.e., when anther conceals the stigma, it was observed that flower produce ample amount of nectar, therefore, the pollination visitation was maximum during this phase. Pollinators particularly honey bees and bumblebees were observed visiting these flowers (Fig. 1h). Pollinator visits were also observed in the flowers with receptive stigma i.e., flower at female phase (when anthers are shed). During the female phase accessory parts (sepals and petals) of flower are still intact to the floral axis (Fig. 1i) and nectar is also present to attract pollinators. Pollinators when visit the flowers at male phase get fully loaded with pollen grains and the subsequently visitation to flowers at female phase was also observed, therefore bring about pollination. It was observed that accessory parts of flower shed on 5th–6th day after anthesis and the pollinator does not visit the flower after 5th day as the floral organs become dry, the attractive petals are already shed and it also marks end of stigma receptivity as observed during the present study. The experimental study also revealed that stigma remains receptive on 3rd to 5th day of anthesis and receptivity is lost after 5th–6th day of anthesis.

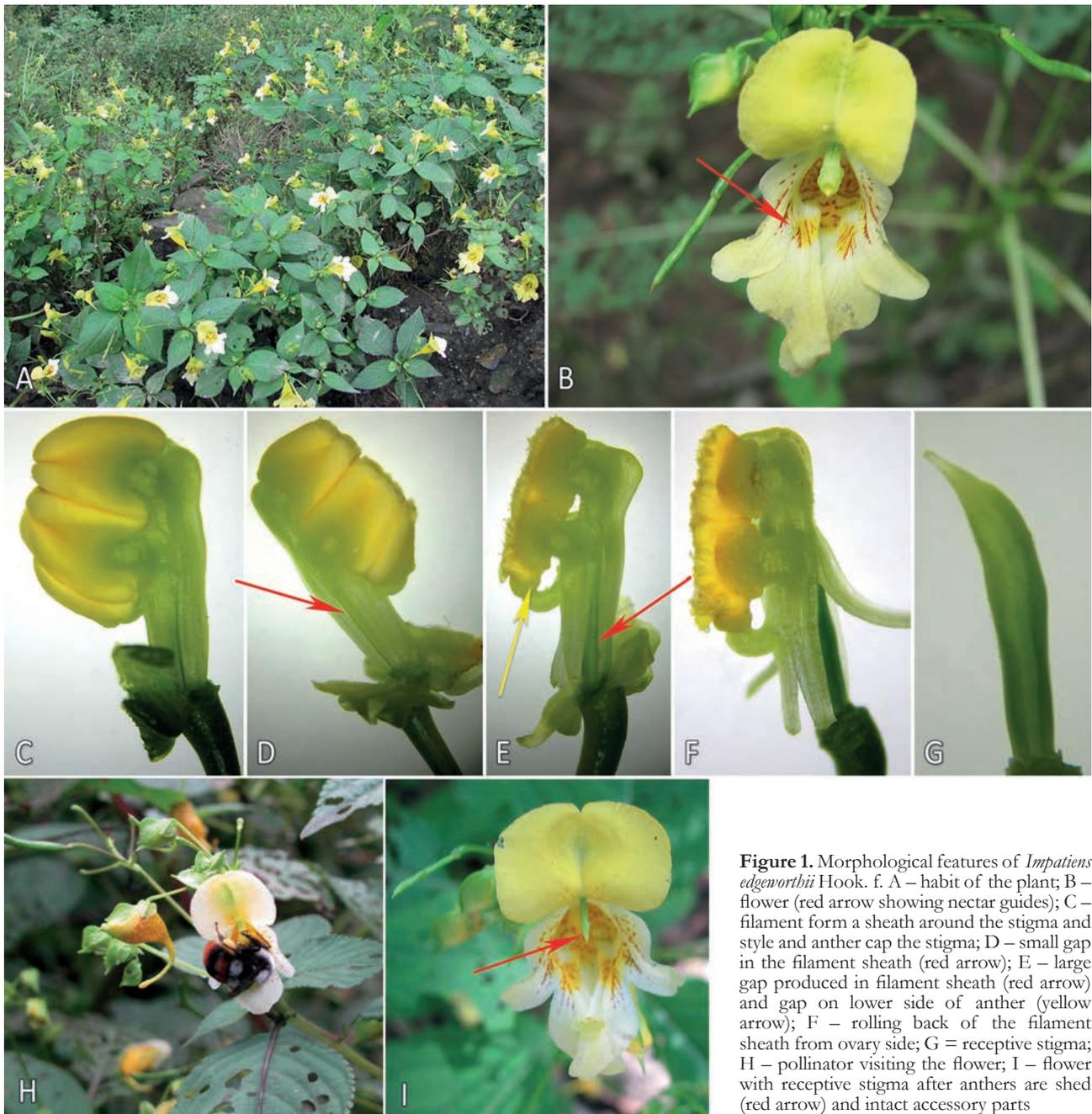
**Table 1.** Combinations of various nutrients used for pollen germination.

S. No. Nutrient combinations*	
1.	10% sucrose
2.	10% sucrose + boric acid 1 mg/ 10 ml distilled water (DW).
3.	10% sucrose + boric acid 1 g/ 10 ml DW + calcium nitrate 3g/ 10 ml DW
4.	10% sucrose + calcium nitrate 3g/ 10 ml DW.
5.	10% sucrose + magnesium sulphate 2g/ 10 ml DW
6.	10 % sucrose + boric acid 1 mg/ 10 ml DW + calcium nitrate 3 mg/ 10 ml DW + magnesium sulphate 2 mg/ 10 ml DW.

\* Final volume in each case was 30 ml

**Table 2.** Stigma receptivity of *Impatiens edgeworthii* at different developmental stages.

Days after anther pad is shed and stigma becomes exposed	Pollen load on stigmatic surface (Mean $\pm$ SE)	Number and percent of germinated pollen (Mean $\pm$ SE)	
		Number	Percent (%)
0	0	0	0
1	184.00 $\pm$ 4.40	33.00 $\pm$ 2.12	17.93
2	122.00 $\pm$ 2.88	63.00 $\pm$ 5.21	51.63
3	130.12 $\pm$ 4.48	19.00 $\pm$ 6.32	14.16 $\pm$ 2.55
4	0	0	0



**Figure 1.** Morphological features of *Impatiens edgeworthii* Hook. f. A – habit of the plant; B – flower (red arrow showing nectar guides); C – filament form a sheath around the stigma and style and anther cap the stigma; D – small gap in the filament sheath (red arrow); E – large gap produced in filament sheath (red arrow) and gap on lower side of anther (yellow arrow); F – rolling back of the filament sheath from ovary side; G = receptive stigma; H – pollinator visiting the flower; I – flower with receptive stigma after anthers are shed (red arrow) and intact accessory parts

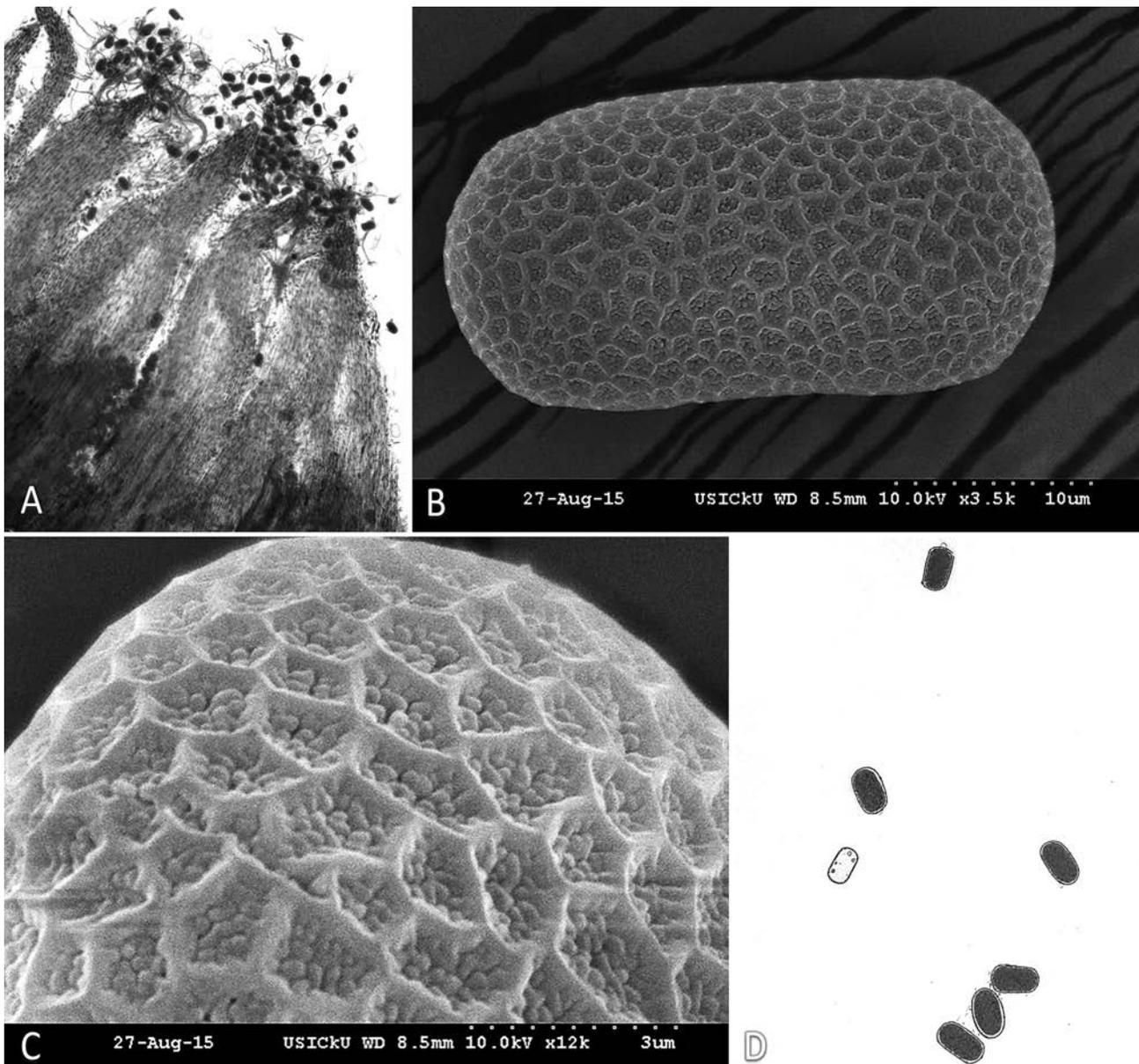
### Breeding system

The breeding experiments were carried out at both Kashmir University Botanical Garden and at collection sites. The study revealed that the tagged un-emasculated flowers set 85–90 % seeds and emasculated as well as un-emasculated flowers which were bagged prior to anthesis do not set seeds. By hand pollination experiments it was observed that in case of geitonogamy (Experiment III) 25–30 % seeds were set; while in xenogamy experiments (Experiment IV) 95–98 seed set was recorded. The seed production varied significantly ( $P \leq 0.001$ ) under different experimental conditions (Table 3).

### Pollen biology

The pollen grains are yellow in colour, oblate in shape (Fig. 2b) and with volume of  $798.58 \mu\text{m}^3$ . The exine orna-

mentation of pollen grains is reticulate and the muri enclose the lumina; in lumina the granules are also present (Fig. 2c). The species produces enormous quantity of pollen grains ( $145545 \pm 4275$ ) per flower as against  $9 \pm 1$  ovules per flower ( $N = 30$ ). Thus, P/O ratio of the species is  $9703 \pm 285$ . The high percentage of pollen grains is healthy and stained well in different stains (Fig. 2d), and in different media combinations good percentage of pollen grains germinates. Of the various media used to check the *in-vitro* pollen germination, the medium containing sucrose, boric acid, calcium nitrate, magnesium sulphate in (1:1:1:1) combination yielded highest percentage (97.2 %) of germinating pollen grains and the media containing 10 % sucrose yielded low percentage (34.93 %) of germinated pollen grains. The pollen germination varied significantly ( $P \leq 0.001$ ) in different treatments (Table 4).



**Figure 2.** Pollen features of *Impatiens edgeworthii* Hook. f. A – pollen load and germinating pollen grains on receptive stigma; B – oblate pollen grain; C – reticulate exine ornamentation of pollen with muri and granular lumina; D – viable and non-viable pollen grains

## DISCUSSION

In accordance with our hypothesis, the results of present study revealed that the male reproductive parts conceal the female stigma: the seat of pollen reception during male phase therefore, acts as barrier for self-pollination. In addition the stigma become receptive only after the androecium of the same flower is shed and this unique type of dichogamy are contrivance for cross-pollination in the species. In some species of the genus *Impatiens* the androecium is shed to expose the receptive stigma and the stamen appendages might have function to avoid self-pollination (Caris et al. 2006). The occurrence of dichogamy and herkogamy is a mechanism to avoid the interference of pollen–stigma and promote cross-pollination (Ramírez & Hokche 2019). This novel mechanism prevents self-pollination and increases the chances of cross-pollination to decrease the inbreeding

and to increase the fitness in plant species (Lu et al. 2000, Li et al. 2001).

In the large, zygomorphic, and coloured flowers with huge amount of nectar; the flowers of different species of the genus are known to produce large quantities of nectar (Rust 1977, Tian et al. 2004, Vervoort et al. 2011). Large showy flowers and production of ample amount of nectar are the contrivance for its pollinator attraction (Chittka & Schürkens 2001). The presence of nectar guides in the species attracts and increase foraging efficiency of pollinators (Chittka & Schürkens 2001). The nectar guides also increase the reproductive success (Leonard & Papaj 2011).

The present study revealed that honeybee and bumblebee are main pollinators of the species. It has also been reported that the nectar in the genus have sugar concentration within the range of bumblebee-pollinated plants (Chittka

& Schürkens 2001, Nienhuis & Stout 2009, Vervoort et al. 2011). According to pollinator-mediated stabilizing selection (PMSS) hypothesis specialized pollinator system of zygomorphic flowers favours stabilizing selection, and flower size variation would be less in zygomorphic compared with actinomorphic flowers (Wolfe & Krstolic 1999). The evolution of floral symmetry has occurred due to strong selection pressure exerted by specialized pollinators (Neal et al. 1998, Endress 2001], since zygomorphy promotes cross-pollination by transfer of pollen by appropriate pollinator (Lázaro & Totland 2014).

The high pollen-ovule ratio and high percentage of fertile pollen also depicts cross-pollinated nature of the species, Cruden (1977) observed that higher the degree of autogamy, the lower the pollen-ovule ratio. High percentage of pollen grains are viable as depicted by in vitro experimentation and can bring about successful pollination. The size and exine sculpturing also points towards insect pollination, the highly ornamented pollen grains are often insect pollinated (Proctor et al. 1996). The zygomorphic condition also favours cross-pollination (Lázaro & Totland 2014). All the aforementioned mechanisms favours cross-pollination in the species and may be the reason of huge diversity in the genus than its sister genus – *Hydrocera* (Janssens et al. 2012).

**Table 3:** Seed production under different breeding experiments. #Mean  $\pm$  SE (n=30); values given in superscript (a–b) indicate means that are significantly different among different experiments. Same character in superscript doesn't differ significantly; Tukey's test (P < 0.001); \*\*\* – significant difference, P < 0.001; significant differences ( $\alpha=0.05$ ) in various experiments (Bonferroni test)

Experiment	Site	No of seeds produced	Percent seed production
Experiment I (Un-emasculated flowers to open pollinate)	Dachigam	244.23 $\pm$ 5.14 <sup>b</sup>	90.37
	Gulmarg	242.31 $\pm$ 4.52 <sup>b</sup>	89.62
	Baramulla	244.24 $\pm$ 5.25 <sup>b</sup>	90.37
	Boniyar	242.61 $\pm$ 6.31 <sup>b</sup>	89.62
Experiment III (Geitonogamy)	Kashmir University Botanical Garden	246.12 $\pm$ 3.12 <sup>b</sup>	91.11
	Dachigam	70.15 $\pm$ 3.59 <sup>a</sup>	25.92
	Gulmarg	83.11 $\pm$ 4.21 <sup>a</sup>	30.74
	Baramulla	71.14 $\pm$ 3.33 <sup>a</sup>	26.29
	Boniyar, Kashmir University Botanical Garden	75.13 $\pm$ 1.12 <sup>a</sup>	27.77
Experiment IV (Xenogamy)	Dachigam	265.88 $\pm$ 2.51 <sup>b</sup>	98.14
	Gulmarg	263.12 $\pm$ 1.43 <sup>b</sup>	96.29
	Baramulla	260.81 $\pm$ 1.31 <sup>b</sup>	97.40
	Boniyar Kashmir University Botanical Garden	258.25 $\pm$ 2.12 <sup>b</sup>	95.55
P/F		113.60/***	

**Table 4:** In vitro pollen germination of *Impatiens edgeworthii* in different media combinations. **M1** – 10 % sucrose (30 ml); **M2** – 10 % sucrose + boric acid (15 ml each); **M3** – 10 % sucrose + boric acid + calcium nitrate (10 ml each); **M4** – 10 % sucrose + calcium nitrate (15 ml each); **M5** – 10 % sucrose + magnesium sulphate (15 ml each); **M6** – 10 % sucrose + boric acid + calcium nitrate + magnesium sulphate (7.5 ml each) (Brewbacker and Kwach solution); Mean  $\pm$  SE (n=50); different superscript characters (a–f) indicate means that are significantly different among different treatments (Tukey's test, P < 0.001); significant differences ( $\alpha=0.05$ ) in various treatments (Bonferroni test).

Composition of media	M1	M2	M3	M4	M5	M6
Pollen grains scanned	257.00 $\pm$ 15.34	196.53 $\pm$ 12.54	251.00 $\pm$ 19.32	201.34 $\pm$ 22.31	213.31 $\pm$ 14.21	285.21 $\pm$ 3.4
P/F			988.30***			
Percent pollen germination	34.93	72.32	96.01	60.45	59.10	97.20

## CONCLUSION

It can be concluded from the present study that the unique arrangement of essential floral parts and their temporal separation in *Impatiens edgeworthii* are contrivances for cross-pollination and the large showy flowers, production of large quantities of nectar, protandry, presence of nectar guides, ornamented exine sculpturing, high P/O ratio strengthens this view point that the species operate cross-pollination as the primary mode of pollination. The formation of anther pad and its role in evolution of breeding system of the genus needs further investigations.

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