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RESEARCH ARTICLE

FLORAL BIOLOGY AND BREEDING SYSTEMS IN IMPATIENS GRANDIS HEYNE EX WALLICH (BALSAMINACEAE)

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ABSTRACT

Impatiens grandis Heyne ex Wallich is an evergreen, perennial shrubaceous balsam species facing a severe threat in the nature due to various factors. One of the factor is it own reproductive inefficiency and progeny recruitment. Hence the present investigation focused on floral biology and breeding systems of the species enlightening on phenology, pollen biology, pollen-pistil interaction, pollinators and breeding system to unravel the possible reasons for its rarity and endangerment. Flowers blooms between 0300-0500 h and anthers dehisced one day before anthesis confirmed the protandry. Pollen viability by FCR yielded 82% pollen grains are viable on the day of anthesis. Stigma showed 60% receptivity on the 3rd day of anthesis. Pollinators behavior in the wild confirmed honeybees made maximum visit (40%) followed by hawk moths (30%), butterflies (20%) and flies and ants (10%). The fruit set rate in natural pollination was very poor (30%) but artificial cross-pollination through xenogamy enhanced the fruit set up to 65%. Thus it is concluded that the plant is an obligate out-crosser and partially self-incompatible. Therefore, protandry, self-incompatibility, delayed stigma receptivity, pollinator's scarcity, bottlenecks in reproduction and other ecological factors could be the reasons for narrow distribution in the Western Ghats.

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INTRODUCTION

The family Balsaminaceae comprises about 1000 species under only 2 genera, viz. *Hydrocera* Blume ex Wight and Arnott and *Impatiens* L. (Grey-Wilson, 1980; Clifton, 2000; Yuan *et al.,* 2004; Tian *et al.,* 2004; Caris *et al.,* 2006; Mabberley, 2008). The genus *Hydrocera* is monotypic and all other species belongs to *Impatiens,* which is mostly distributed throughout tropical Africa, India, South East Asia and Japan (Grey-Wilson, 1980). In India the genus is represented by more than 210 taxa mainly distributed in the Eastern Himalayas, Hills of North Eastern states and the Western Ghats, which are the major centres of diversity and with each area being characterised by its own species group (Hooker 1908; Gamble, 1915; Vivekananthan *et al*., 1997; Augustine *et al*., 1999; Kulloli *et al.,* 2008; Sreekala *et al.,* 2008; Dessai and Janarthanam, 2008, 2011). Which represents approximately 21% of *Impatiens* species globally (Sreekala *et al.,* 2015). As far as this genus is concerned, the Western Ghats region is the main area of speciation in India.

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Interestingly, more than 90 species of *Impatiens* are endemic to the Western Ghats (Narayana *et al.,* 2013). Due to their restricted distribution and island biogeography, more than 40 species are already in threatened category with uncertain future (Vajravelu and Daniel, 1983; Pandurangan and Pushpangadan, 1997; Sreekala *et al.,* 2007).

Impatiens grandis Heyne ex Wallich is an evergreen, perennial shrubaceous balsam species found in the Southern Western Ghats and extended up to Sri Lanka. The flowers are large, orchid-like blossoms have bold splashes of peppermint red across pure-white petals. Due to its high impact on horticultural potentiality, this rare wild plant species has received much attention recently. But its presence in the nature is at alarming stage due to various issues hence limited population and even endangered in near future (Pandurangan, 1996; Sreekala *et al.,* 2007; 2008). Though the ideal climatic conditions prevailing in the Western Ghats region provide suitable habitat for the *Impatiens*, their populations are rapidly declining due to various factors. Meticulous information on the floral biology of the plant species is essential for plant breeding as well as for developing effective strategies for their conservation and

sustainable utilization. In this context, floral biology and breeding systems of *Impatiens grandis* has been investigated with special reference to phenology, pollination, pollen-pistil interactions, plant-pollinator interactions, breeding systems, stigma receptivity in order to understand causes of rarity and reasons for dwindling populations in the natural habitats.

MATERIALS AND METHODS

Plant Material and Study area

The plant material was located from Kallar valley of Munnar and Shantamparai of Kattapana, Idukki district, Kerala, India (Fig 2a). The area located between 9º 15'- 10º 18' N and 76º 15'- 77º 55' E. It is an evergreen, perennial shrubaceous balsam species belongs to family Balsaminaceae and endemic to the southern Western Ghats. The flowers are large, orchid-like blossoms have bold splashes of peppermint red across pure-white petals (Fig 2b). Its oversized leaves are unusually thick and robust, and the entire plant can grow to 5 feet tall or even more in dense shola forests at an altitude of 750-1500m asl (Kulloli, 2015). The study was conducted during 2005 and 2007 on selected 5 populations in the natural condition.

Phenology

Ten healthy plants were selected from each population and observations were made on day-to-day basis in natural habitats on flowering phenology, which include season, habit, development, anthesis etc. Floral morphology was also studied with the help of hand lens and dissection microscope. Flower openings were noted following the process of Mathur and Mohan Ram (1986). Almost mature anthers were sectioned and immediately covered with cover glass (Keijzer, 1987) before observation to recognize the anther dehiscence. Pollen grains per anther and per flower were quantified following the procedure of Mandal and Chanda (1981). Pollen per ovule (P/O ratio) was calculated by dividing the mean pollen number and ovule number of a flower following Crudens method (1977).

Pollen fertility and viability

Pollen fertility was assessed by acetocarmine and glycerin staining technique (Radford *et al.,* 1974). The stained pollen grains were treated as fertile and unstained pollens were counted as sterile. Pollen viability was checked by FCR (fluorochromatic reaction) test using fluorescein diacetate (FDA as a substrate) as suggested by Shivanna and Rangaswamy (1992). The observations were made under a (Lieca DME. Germany) fluorescent microscope using green filter at low magnification (10x × 10x). *In vitro* pollen germination was conducted to determine the effect of different nutrients like sucrose at various concentrations and Brewbakers medium (1963) containing different salts of various concentrations for 4 hour. After 4 hours of incubation the percentage of pollen germination and tube elongation was noticed.

Stigma receptivity and *in vivo* pollen germination

Stigma receptivity was studied visually with the help of hand lens and by hydrogen peroxide (H_2O_2) test according to the method of Scribailo and Posluszny (1984). *In vivo* pollen germination was checked by using aniline blue (Aldrich chemical 86.102-2) florescence microscopic method as designed by Shivanna and Rangaswamy (1992). The preparations were observed under the fluorescent microscope (Lieca DME Germany) at low magnification (10x × 10x). Percentages of pollen germination in the stigmatic surface and average tube lengths were calculated.

Pollinator observation

The observations of flower visitors were made for a total of approximately 72 hours during three consecutive days of high diurnal pollinator activity (8:00h to 15:00h). The types of insect floral visitors, purpose of visiting, time interaction with flowers and the foraging activity of insect floral visitors were observed during different periods of a day. They were observed with reference to the type of forage they collected, contact with essential organs to result in pollination and inter-plant foraging activity in terms of cross-pollination. They were thereafter classified either as pollinators or robbers (Dafni, 1992; Inouye, 1980). Some of these insect floral visitors were captured fixed in 70% alcohol for identification.

Breeding system

To determine the breeding system, pollination experiments were performed on randomly chosen from five trees in the population. Various types of breeding experiments including open pollination, autogamy, geitonogamy, xenogamy and apomixis were carried out during the two subsequent years with two flowering seasons (Radford *et al*., 1974; Dafni, 1992; Kearns and Inouye, 1993).

Open pollination (control): flower buds were tagged and observed the fruit set. Autogamy: mature flower buds were tagged and bagged with a cloth mesh bag, and fruit set at maturity was recorded, geitonogamy (manual self-pollination): mature flower buds were tagged and bagged, the buds upon opening were hand self-pollinated with pollen collected from the same plant, re-bagged and fruit set observed. Xenogamy (manual cross-pollination): mature flower buds were tagged and bagged, the buds upon opening were hand cross-pollinated with pollen collected from two or three other plants and then re- bagged and fruit set observed. Apomixis: mature flower buds were emasculated and bagged without pollination (Richards, 1986). The Index of self-incompatibility (ISI) was calculated using the method of Zapata and Arroyo (1978). The ratio of fruit set through manual self-pollination to those formed through manual cross-pollination was taken as the Index of self-incompatibility. The species with ratios <0.25 are considered self-incompatible and those with ratios >0.25 as self-compatible (Subasi and Guvensen, 2011; Mohandass, 2013; Bawa, 1974; Nayak and Davidar, 2010).

Statistical analysis

The statistical analyses were calculated for floral traits, and breeding behaviour. Mean and standard deviation was analyzed using mega stat model (Programmed by J.B. Orris, Version 9.1.).

RESULTS AND DISCUSSION

Plant material and phenology

Impatiens grandis is a large perennial, shrubaceous balsam reaching up to 1.5-2.5m height in the natural habitat. It prefers moist shady areas of evergreen and shola forests between altitudes of 750-1500m. Flowers are white with petals dotted or flushed dark red or magenta tinges (Fig 2b) born at apical cyme in pair or many and spur is thick filiform in the middle, slightly incurved, long and glabrous. Capsules fusiform in nature contain 10-14 seeds in each capsule (Fig 4a-b). *I. grandis* starts flowering in the month of August and extends up to January with peak during October. The flower buds take 6-11 days from initiation to full bloom (Fig 2c). The flowering days extended up to 120 days in a year and the average life span of the individual flower is 2-3 days. In *Impatiens grandis,* flowers bloom in the night between 0300h and 0500 h which confirmed the nocturnal nature. Another dehisced one day before flower opening which corroborated the protandrous condition of the flowers (Table 1). Protandry is the dichogamous pattern frequently observed in the Balsaminaceae. Some authors suggest that dichogamy is a mechanism that may reduce anther-stigma interface (Lloyd and Webb, 1986; Bertin and Newman, 1993). The mean number of pollen grains per flower of *Impatiens grandis* was ±48,620 and the mean number of ovules was 26. Therefore the pollen-ovule ratio was calculated as 1870: 1 (Table 1).

Knowledge on phenology and floral morphology are essential for conducting studies on breeding systems particularly on pollination syndrome if any. About 62% of *Impatiens* species in the Western Ghats flower during July-December, 16% during April–June and 15% during January-March. Interestingly 18% of the balsams flower throughout the year if conditions are favorable (Rajalal *et al.,* 1996). Bhasker and Razi (1974) had reported that majority of the wild balsams grown in the high altitude areas are night blooming and have a wide range of timing with regard to pollen germination. The average life span of each flower is 2-4 days and the anther dehisced one day before anthesis, which in turn confirmed the protandrous condition of the flower. This observation is similar to that of *I. platypetala*, *I. korthalsii,* and *I. eubotrya* in Sumatra (Kato *et al.,* 1991).

Pollen fertility, viability and *in vitro* pollen germination

Pollen grains are round and an average of 38.60µm in diameter. The acetocarmine staining technique revealed that 76% of the pollen grains were fertile. Pollen viability by FCR test confirmed that 82% pollen grains were viable on the day of anthesis and gradually reduced after 2nd day of anthesis (Fig 2e). *In-vitro* pollen germination by using different concentration of sucrose and Brewbakers medium (1963) revealed that pollen grains were remain viable up to transfer on pistil surface during the receptive period.

Table 1. Reproductive attributes of *Impatiens grandis*

Floral characters	*Observations*
Flowering period	August - January
Flower opening time	0300-0500 h
Flower type	Zygomorphic/Irregular
Odour	Minor
Nectar	Present (3-5µm)
Flower color	White with petals dotted or flushed dark red or magenta tinges
No. of anthers/ flower	5
Anther dehiscence time	One day before anthesis (0200-0500 h)
Mean no. of pollen grains/ flower	±48,620
Pollen size	±38.60
Pollen type	3-colporate
Pollen fertility	76%
Stigma type	Wet and non- papillate
Stigma receptivity	60%
Mean no. of ovules/ flower	26
Pollen ovule ratio	1870:1

Table 2. Pollinators and their foraging behaviours in *Impatiens grandis*

Visitors	Visiting time	Foraging nature	Foraging hours	% of visit	*M.N.I.V	Stigma touch
Apis cerana	Day	N & P*	0800-1300 h	29.10	12.32±1.08	+++
Trigona iridipennis	Day	P	0800-1500 h	23.25	9.84±1.20	+++
Apis dorsata	Day	N & P	0730-1600 h	15.45	6.54±0.97	+++
Ceratina cucurbitina	Day	P	0700-1330 h	7.568	3.65±0.25	+
Parantica aglea	Day	N	0700-1300 h	12.80	5.42±0.54	++
Badamia exclamationis	Day	N	0730-1120 h	9.80	4.15±0.99	++
Rpthima baladus	Day	N	0730-1600 h	5.63	2.38±0.28	++
Papilio demoleus	Day	N	0630-1200 h	3.97	1.68±0.54	+
Pachliopta aristolochiae	Day	N	0800-1500 h	4.56	4.38±0.98	++
Euploea core	Day	N	0830-1430 h	08.30	6.96±1.08	++
Macroglossum stellatarum	Night	N	0330-0430 h	12.56	5.68±0.42	++
Macroglossum corythus	Night	N	0340-0500 h	13.54	4.38±0.78	++
Oecophylla smaragdina	Day & Night	N	Throughout	2.36	8.68±0.21	+
Eristalinus arvorum	Day	N	0830-0130 h	2.69	2.68±0.69	+

*M.N.I.V- Mean no. of individuals visited per hour/ Population,
+ - Poor, ++ - Good, +++ - V. good, *N –Nectar, P-Pollen

Effect of sucrose on *in vitro* pollen germination studies revealed that 70% pollen grains were germinated and produced 673 µm tube in 5% sucrose medium. Best pollen germination (82%) along with 796 µm tube development was achieved in Brewbakers medium after 4 hrs of incubation (Fig 2d-f). In *I. grandis* pollen viability is highest on the day of anthesis and then gradually decreased on successive days after anthesis but the stigma is unreceptive on that day in self flower. This observation is similar to that of *I. reptans* in China (Tian *et al.,* 2004), *Impatiens hensloviana, Impatiens coelotropis* (Sreekala *et al.,* 2007; 2008) and *Impatiens campanulata* (Kulloli *et al.,* 2009) in India.

Successful seed sets and establishing newer population generally depends upon viable pollen grains. *In-vitro* pollen germination test indicated that highest percentage of pollen germination and tube elongation was observed in Brewbakers medium. It contains sucrose which acts as a nutritive material for pollen germination (Johri and Vasil, 1961) and helps in maintaining osmotic balance between the germination media and pollen cytoplasm (Mukerjee and Das, 1964). Germination percentages were significantly low in higher concentration of sucrose medium. According to Shivanna and Johri (1985), the optimum concentration of sucrose varies from species to species.

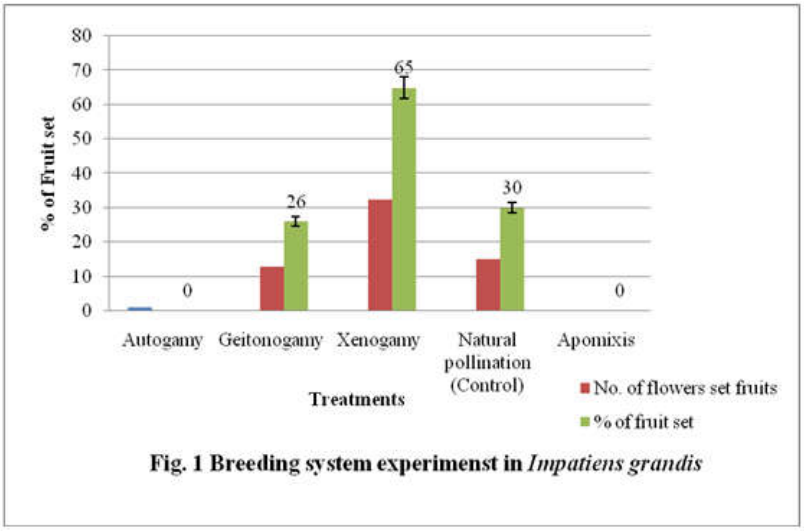

Figure 1. Breeding experiments in *Impatiens grandis*

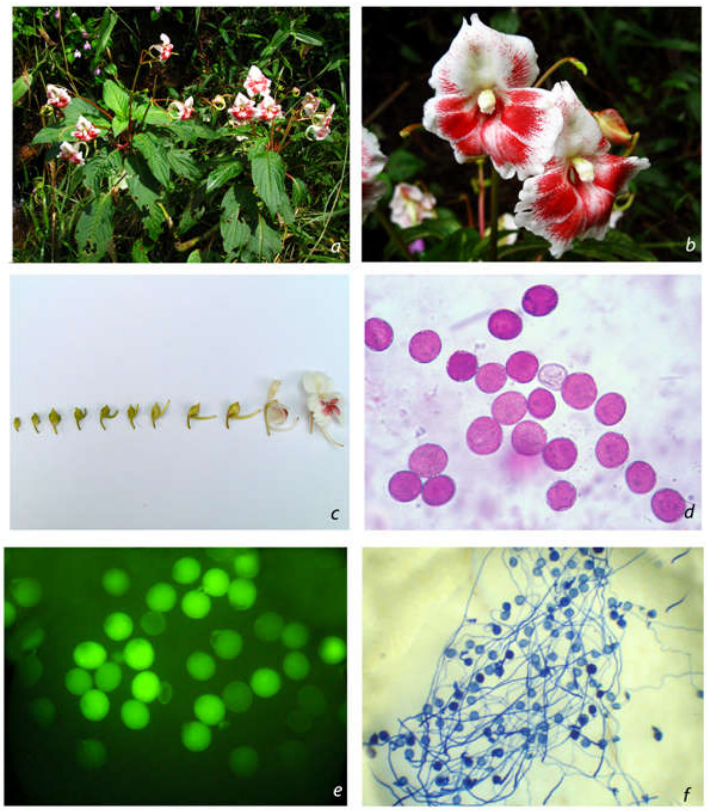

Figure 2 Floral biology and pollen viability of *Impatiens grandis*

Figure 2. Floral biology and pollen viability of *Impatiens grandis*
a. Habitat in the wild, b. Flower front view, c. flower development, d. pollen viability in acetocarmine test, e. Pollen viability in FCT test, f. In vitro pollen germination in Brewbakers and kwack medium

Figure 3. Stigma receptivity and Pollinator observation in *Impatiens grandis*
a. receptive stigma with star shaped lobes, b. *Ceratina cucurbitina* pollinating the receptive stigma, c. *Apis dorsata* landing on the colourfull ventral petal, d. *Apis cerana* visiting the receptive stigma with pollen load, e. *Oecophylla smaragdina* -foraging the extra floral nectaries, f. *Oecophylla smaragdina* entering into the spur to rob the nectar

Figure 4. Breeding experiments in *Impatiens grandis*

Figure 4. Breeding experiments in *Impatiens grandis*
a. Capsules developed through manipulated breeding experiments *in-situ,* b. Capsule with dehisced seeds

Table 3. Fruit set in different modes of pollination in *Impatiens grandis*

Treatments	No. of flowers observed	No. of flowers set fruits	% of fruit set	Mean ±SD
Autogamous self pollination	50	00	00	00±00.00
Emasculation and hand crossing -geitonogamy	50	13	26	26±18.23
Emasculation and hand out crossing-xenogamy	50	32.5	65	65±45.36
Natural pollination (Control)	50	15	30	30±15.56
Apomixis	50	00	00	00±00.00

In the present investigation, Brewbakers medium is the most suitable for pollen germination in *Impatiens grandis*. Besides the medium contain carbohydrates, boron and calcium are other important substances required for pollen germination and tube growth (Brewbaker and Kwack, 1963). Pollen germination and subsequent post pollination events depend upon the receptivity of the stigma, its nature and compatibility.

Stigma receptivity and *in vivo* pollen germination

The stigma is wet type and non-papillate type (Heslop-Harrison and Shivanna, 1977). Stigmas were more receptive up to 60% on the 3rd day after flower opening (Fig 3a); by showing 62% *in vivo* germinating pollens along with 875µm long pollen tube on the stigmatic surface. Subsequently, the receptivity percentages and *in-vivo* germinating pollens were decreased on successive days (4th day) after blooming. In *I. grandis,* pollen grains were well adhered on the stigmatic surface due to sticky and presence of pollen threads. Pollen tubes penetrate the stigmatic region and reached up to the ovary and fertilized the ovules. The fertilized ovules developed into seeds with only 20% germinability.

The stigma remains more receptive during the 3rd day after anthesis. Stigma receptivity is a critical factor for successful completion of post pollination events. Generally receptivity reaches a maximum soon after anthesis (Shivanna and Johri, 1985) but the period of receptivity may vary from species to species (Joshirao and Saoji, 1989), depending upon temperature and humidity. In *Impatiens grandis,* the delayed receptive period indicates supports cross pollination strategy. The adhesion of pollens on the stigma is a primary requirement for successful pollination. After landing on the stigmatic surface, pollen grains are subjected to hydration and then pollen wall proteins are released on to the stigmatic surface (Heslop-Harrison *et al.,* 1975).

Pollinator observations

In *Impatiens grandis,* flowers bloom in the night between 0300 h and 0500 h. During the night time two species of hawk moth such as *Macroglossum stellatarum* and *M. corythus* were actively foraged on the flowers (Table 2). They helped the flowers in pollination by their rapid and frequent visit to the flowers for nectar collection. The peak period of foraging of hawk moths are from 0330h-0430 h. Pollens are deposited on the back of its head and long proboscis of hawk moths. According to Fægri and Pijl (1980) this mode of pollen deposition is known for efficacy and economy in the utilization of pollen. Hawk moths transferred pollen grains from one flower to another from the same plant or another plant and thus favoring geitonogamy or xenogamy. The foraging periods of honeybees are mainly in daytime from 0730h-1600 h. Honeybees visited many flowers, spending an average of 2-4 sec. in each flowers, whereas hawk moths spent slightly long duration from 3-6 seconds in each flower. Honeybees (*Apis cerana* and *Trigona iridipennis*) were the most abundant visitor and visiting more flowers than any other pollinators for pollen and nectar collection (Table 2 and Fig 3b-d). Butterflies are active during daytime and in fine weather, they actively visited the flowers, spending an average of 3-5 seconds on individual flower for nectar only. Due to less nectar they spent very less time on each flower and visited more flowers. Butterflies visits rarely to the flowers due absence of enough scent, which is important cue signal for them to identify, subsequently, recognize and distinguish among rewardable plants. But they are considered as opportunistic visitors. They played an important role in pollination by touching the anthers and stigma while they take nectar with their proboscis. According to Fægri and Pijl (1980) colour and form of a plant play an important role in butterfly foraging. Flies and ants are intermittent visitor and serves poor pollination (Table 2 and Fig 3e-f). Of the total visits, bee made 40%, moths 30%, butterflies 20 % and flies and ants 10%. In general, hawk moths in night and honey bees in day time served as better pollinators but they are not sufficient to pollinate all the flowers in the selected populations.

It is well known that the flowers of *Impatiens* have enormous diversity and different pollinators. In the present study, *Impatiens grandis* is pollinated by honeybees, hawk moths and butterflies. In different climatic regions species of pollinators vary. In sub tropical regions of Africa the *Impatiens* species are pollinated by humming birds as well as by insects. In temperate zones, pollinators are bumblebees and humming birds (Rust, 1977, 1979; Heinrich, 1979; Kato *et al.,* 1989). In *I. grandis* honey bees (*Apis cerana* and *Trigona* sp) are the most important pollinators and visited more flowers than any other pollinators during daytime for nectar and pollen gathering. There is a strong relationship between the weather and foraging activity of butterflies. When the weather is fine, butterflies are more active and spend an average 2-6 seconds at each visit. But when the weather is cloudy and rainy, the butterflies are less active. The present investigation coincides with *Impatiens coelotropis* (Sreekala *et al.,* 2008) and *Impatiens trichocarpa* (Kulloli *et al.,* 2009).

Breeding systems

In *I. grandis*, different breeding experiments were carried out to find the reproductive capacity of the plant. In natural condition, 30% mean fruit set was observed. The fruit set was not observed in autogamous self-pollination. Which confirms the taxa is self-incompatible. However, 26% in geitonogamy and 65% mean fruit set in xenogamy was observed respectively (Table 3 and Fig 4a-b). Breeding experiments especially through xenogamy produced more fruits and seeds than the natural pollination. The relatively high pollen ovule ratio and specialized floral morphology which suggested *a priori* an out-crossing breeding system. When fruit production under natural conditions is lower than that obtained by artificial cross-pollination, the former may be interpreted as being pollen limited (Vaughton and Ramsey, 1995).

Open pollination was significantly higher fruit set than autogamy. However, no fruit set was observed in the emasculated and bagged flowers (apomixis), which fell soon after the treatment, indicating the absence of agamospermy of this species. The lowest mean fruit set were observed with geitonogamy pollination (26.00 ± 18.23%) and highest mean fruit set were observed with xenogamy (manual cross) pollination (65.00±45.36%) respectively.

Moreover, the ratio of percentage of fruit set between self and cross pollination showed 2.33 (more than >0.25 ratio). Thus, fruits were produced after the treatments of geitonogamy and xenogamy, indicating that *Impatiens grandis* was completely out crossing fertile and self-compatible (Table 3).

Based on the breeding experiments, it was observed that the flowers are morphologically and functionally hermaphrodite. Pollination is one of the prerequisites for fertilization and seed set in angiosperms (Faegri and Pijl, 1980). Open pollination experiments showed that it is self-compatible and out crossing fertile. Several factors may be responsible for the low fruit set under open- pollination (Tandon *et al.,* 2003). This finding was also reported in *Impatiens trichocarpa, Impatiens campanulata* (Kulloli *et al.,* 2009; Kulloli and Sreekala, 2009). In addition there is no apomixis occurred. Furthermore, because exclusion of pollinators resulted in the absence of fruit set, pollinators would seem to be necessary for the sexual reproduction of these species. Pollination experiments demonstrated that artificial cross-pollination enhanced the rate of fruit and seed set in *I. grandis.* The arrangement of stamens, pistil and spur are markedly adapted for cross-pollination in *Impatiens* (Bhasker and Razi, 1974) and hence most of the species of *Impatiens* reproduce by cross-pollination (Schmitt and Gamble, 1990; Lu, 2000, 2002).

Conclusion

The results suggest that *Impatiens grandis* flowers are hermaphrodite (bisexual), zygomorphic, protandrous and complete. Open pollination experiments showed that it is obligate out-crosser and self incompatible. The decline of this species in the wild is due to habitat loss, fragmentation of population narrow environmental niche. Less fruit production as compared to flowers may be due to delayed receptivity or unavailability of pollinators or pollen competition and pollen-ovule ratio. Therefore the study suggests that, protandry, delayed stigma receptivity, self-incompatibility, pollinator limitation in combination with other abiotic traits are contributing factors for regulation of population size of *Impatiens grandis* in its natural condition. Therefore, studies on the floral biology and breeding systems for developing strategies to preserve the genetic potential of these species which are crucial for restoration and re-introduction purpose. Thus, the present investigation definitely serves as an important model system not only for achieving the conservation of these RET species but also ensure sustainable utilization, which are otherwise on the road to extinction due to various reasons.

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