Pollination biology of *Impatiens cuspidata* Wight and Arn. (Balsaminaceae), a rare and endemic balsam of the Western Ghats, India

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Abstract: The pollination biology of *Impatiens cuspidata,* a rare and endemic balsam from the Western Ghats, has been studied with special reference to phenology, pollination, pollen-pistil interactions, breeding experiments and stigma receptivity. It flowers at night between 2330 and 0430 hr; flowering days extend up to 160 days in a year. The anther dehisced one day before anthesis, which confirmed the protandrous condition of the flower. Pollen-ovule ratio was calculated as 1729:1. Hawk moths, honeybees, flies and butterflies are the major pollinators of *Impatiens cuspidata*. Pollen grains are oval, having an average diameter of 28.24µm. Pollen viability by FCR test confirmed that 82% pollen grains are viable on the day of anthesis. Best pollen germination along with 1636µm tube development was achieved in Brewbakers medium. Stigma was more receptive (up to 80%) on the first day of flower opening. It chiefly reproduced by means of cross pollination, where the fruit set was only 40%, but artificial cross-pollination through xenogamy enhanced fruit set up to 80%. The plant is an obligate out-crosser and self incompatible, as confirmed by various hand pollination experiments. Seed germination in natural and controlled conditions was only 20%. Its dependence on a specialized habitat, bottlenecks in sexual reproduction, low percentage of seed germination and other abiotic factors could be reasons for its limited distribution and endemism.

Keywords: *Impatiens cuspidata,* phenology, pollination biology, stigma receptivity, xenogamy.

Tamil Abstact: அரிய மற்றும் குறிப்பிட்ட இடச்சூழல்களைக் கொண்ட மேற்குத்தொடர்ச்சி மலையின் இம்பேஸியன்ஸ் கஸ்ப்பிடேட்டா என்ற பால்ஸம் வகைத்தாவரத்தின் மகரந்தச் சேர்க்கை உயிரியல் பற்றி விரிவான ஆய்வு செய்யப்பட்டது மற்றும் இந்த ஆய்வில் முழு உடற்கூறு வளர்ச்சி, மகரந்தச்சேர்க்கை, மகரந்தம்- சூல் இடைவினை, மற்றும் இனப்பெருக்க அமைப்பு ஆகியவை பற்றி மிகுந்த கவனம் எடுத்துக்கொள்ளப்பட்டது. இதன் மலர் விரியும் நேரம் இரவு 10.00 மணி முதல் விடியற்காலை 04.30 வரை எனக்கணக்கிடப்பட்டது மற்றும் வருடத்திற்கு 160 நாட்கள் பூக்கும் என அறியப்பட்டது. மகரந்தம் பூ விரிவதற்கு ஒரு நாளுக்கு முன்பாகவே வெடிக்கத்தொடங்குகிறது இது முன்-ஆண்மை முதிர்ச்சி அடைதலைக் காட்டுகிறது. ஒரு மலரில் மகரந்த தூள்-சூல் வித்து, 1729க்கு ஒன்று என்ற விகிதத்தில் அமைந்துள்ளது. பருந்துப்பூச்சி, தேனீக்கள், ஈக்கள் மற்றும் வண்ணத்துப்பூச்சிகள் முக்கிய மகரந்தச்சேர்க்கைக் காரணிகளாகும். மகரந்தத் தூள் வட்ட வடிவமுடையதாகவும் சராசரியாக அதன் விட்டம் 28.24 நுண்ணளவு ஆகும். பூ விரிந்த நிலையில் மகரந்த தூள்களின் வாழுமை 80 சதவீதம் என்று எஃப்.சி.ஆர் என்ற ஆய்வு மூலம் உறுதி செய்யப்பட்டது. சிறந்த மகரந்தத் தூள் முளைப்புத்திறன் (82 சதவீதம்) மற்றும் மகரந்த குழாயின் நீளம் 1639 நுண்ணளவு என்பது புரூபெக்கர் எனப்படும் வளரளம் மூலம் பெறப்பட்டது. பூ விரிந்த முதல் நாள், சூல் முடியின் கருவுறும் திறன் 80 சதவீதம் வரைக் கணக்கிடப்பட்டது. இது அயல் மகரந்தச்சேர்க்கை மூலம் அதிகமாக இனப்பெருக்கம் செய்கிறது, இதன் மூலம் உண்டாகும் கனி உருவாதல் சதவீதம் 40 ஆகும் ஆனால் செயற்கை முறை அயல் மகரந்தச்சேர்க்கை (ஸீனொகேமீ) இதன் கனி உருவாதல் சதவீதத்தை 80 ஆக உயர்த்தியுள்ளது. இந்தத் தாவரமானது உறுதி செய்யப்பட்ட அயல்மகரந்தச்சேர்க்கையாளர் என்பதும் தன்மகரந்தச்சேர்க்கைவிரும்பாதது என்பதும்பல்வேறு செயற்கைஅயல்ம கரந்தச்சேர்க்கைஆய்வுகளின் மூலம்உறுதி செய்யப்பட்டது. இதன் விதை முளைப்புத்திறன் வெறும் 20 சதவீதம்மட்டுமே. இதன் தனிப்பட்ட இடச்சூழல்வாழுமை, பல்வேறு இனப்பெருக்கத் தடைகள், குறைந்த விதை முளைப்புத்திறன் மற்றும்பல்வேறு காரணிகள்தான் இதன் குறைவான இடச்சூழல்பகிர்மானத்திற்கும்குறிப்பிட்ட இடச்சூழி ஆவதற்கும்காரணம்ஆகின்றன.

INTRODUCTION

Pollination biology provides a framework to test a diverse array of paradigms in several subdisciplines of biology (Bawa et al. 1993). It also plays a critical role in the formation of seed and fruits. There are very few attempts made on pollination biology of *Impatiens* except by Rust (1977, 1979), Tian et al. (2004), Sreekala et al. (2007, 2008). A comprehensive study on pollination biology of *Impatiens* was fundamental to investigations into its reproductive syndrome, systematics and evolutionary biology.

The family Balsaminaceae comprises two genera; *Hydrocera* Blume and *Impatiens* Linn., which are commonly known as balsams, jewel weed, snap weed or touch-me-not (Willis 1973). The genus *Impatiens* is supposed to be one of the largest group among the flowering plants (Sreekala et al. 2007), comprising more than 1000 species (Grey-Wilson 1980; Clifton 2000; Pieter et al. 2006). It is essentially sub-cosmopolitan species and mainly distributed in montane areas in the Old World tropics and subtropics (Grey-Wilson 1980; Yuan et al. 2004), but several species occur in temperate Eurasia and North America (Pieter et al. 2006). Native species are absent from the South America and Australia. *Impatiens* is phytogeographically a unique genus, which has its greatest development in the Indian region and is found chiefly in moist sub-temperate areas.

In India, the concentration of *Impatiens* species is remarkably local and occurs in three major centers of diversity including the Himalaya in the north, Western Ghats in the south and parts of northeastern states. Although the altitude in southern India is lower compared with the Himalaya, conditions are favourable for the growth of *Impatiens* since the region gets rainfall from both southwest and northeast monsoons. There is no doubt that in respect of *Impatiens*, the Western Ghats are the second richest area in the Indian subcontinent and perhaps in the World (Bhaskar 1981). The genus contains over 206 species in India (Vivekananthan et al. 1997; Vishwanathan & Manikandan 2003; Bhasker 2006), half of which occur in southern India and more than 86 are endemic to the Western Ghats (Nair 1991). Due to their restricted distribution, nearly 30 species of *Impatiens* are already threatened with uncertain future (Vajravelu & Daniel 1983; Pandurangan & Pushpangadan 1997; Sreekala et al. 2008).

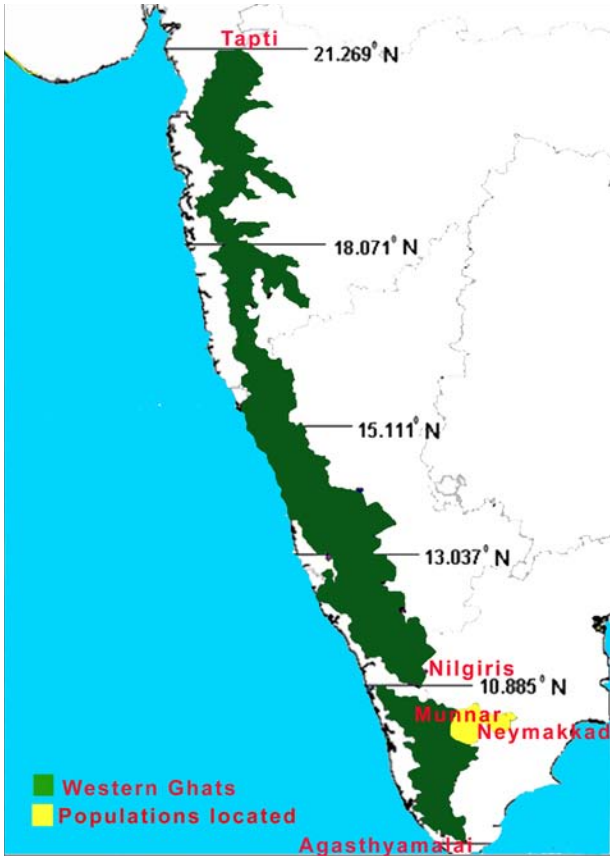

Figure 1. The study area in the Western Ghats

Though the ideal climatic conditions prevailing in the Western Ghats region provide suitable habitat for the balsams, their populations are rapidly declining due to various biotic and abiotic factors. A comprehensive study on pollination biology of endemic balsams has not been made so far due to their habitat specificity, island biogeography, delicate nature and explosive fruits. Against this background, one such wild ornamental balsam namely *Impatiens cuspidata* has been selected for the present investigation on pollination biology encompassing phenology, pollination, pollinator behaviour, pollen viability, stigma receptivity and breeding behaviour to find out possible reasons for its limited distribution in the Western Ghats.

MATERIALS AND METHODS

Impatiens cuspidata naturally occurs in evergreen, sholas and montane grasslands of southern Western Ghats (Fig. 1). The study was conducted on *Impatiens cuspidata* in natural conditions, which was located from

Neymakkad gap of Munnar, Idukki District, Kerala, India. The area is located between $9^0$15'–10^0 21'N and $76^0$15'–$77^0$25'E. *Impatiens cuspidata* is a shrub and may attain a height of 1.5m or even more in dense shola forests at an altitude of 1500–2000 m (Image 1a). This species is found to be associated with *Impatiens leschenaultii, I. coelotropis, I. henslowiana*, *Rubus* sp. *Alternanthera* sp. and *Rhodomytrus* sp. The area experiences rainfall from both southwest and northeast monsoons and receives an average rainfall of 3000mm except in Anaimudi where some times rainfall exceeds 7000mm. The region harbours vegetation types such as moist deciduous, semievergreen, evergreen, shola and montane grasslands. The study was conducted during March 2005 and December 2007. Five populations were selected for the present investigation in the natural condition. Twenty healthy plants were selected from each population and observations were made on a 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. Fifty flower buds were selected from different populations and observations were made between 2330 to 0830 hrs to study the time of flower opening (anthesis) and anther dehiscence. The number of pollen grains contained in each anther was determined by the method suggested by Cruden (1977) to determine P/O ratio of the candidate species.

Pollen fertility was assessed by acetocarmine and glycerin staining technique. 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). To study the pollen germination in vitro, pollen grains were incubated in sucrose medium of different concentrations (2, 5, 10, 15, & 20 %) and Brewbakers medium (Brewbaker & Kwack 1963) containing 2% sucrose for two hours. After two hours the percentage of pollen germination and tube elongation was noticed. 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 & Posluszny (1984). In vivo pollen germination was checked by using aniline blue (Aldrich chemical 86.102-2) florescence microscopic method as designed by Shivanna & Rangaswamy (1992). The preparations were observed under the fluorescent microscope (Lieca DME Germany). Percentages of pollen germination in the stigmatic surface and average tube length were calculated.

Continuous observations during July 2005 to March 2006 were made on behaviour of different pollinators. The pollinators were collected and identified with the help of experts from Kerala Agricultural University and KFRI (Kerala Forest Research Institute). The foraging period and the type of food collected by different visitors on daily basis were recorded by close observations. Different pollination systems such as autogamous self pollination (B), emasculation and hand crossing (S), emasculation and hand out crossing (O) and natural pollination (N) were tested in the field. Treatment 'S' was conducted to examine geitonogamy through artificial pollination using pollens from different flowers of the same plant. Treatment 'O' was conducted to examine xenogamy through artificial cross pollination by using pollens from flowers of different plants but within the populations. Twenty-five healthy flowers for each treatment from each population were chosen randomly and observed for fruit set. The developed fruits and seeds were collected and their numbers were recorded. In addition, the weight of each seed was also measured and recorded. Twenty mature capsules were selected randomly from five populations in the field for this experiment. The distance to which seeds ejected from the capsules were measured. In the laboratory 240 seeds, from capsules of each plant were taken and soaked overnight to

Table 1. Floral biology of *Impatiens cuspidata*

Floral characters	Observations
Flowering period	August–December
Flower opening time	1130–0430hrs
Flower colour	Light pink
No. of anthers/flower	5
Anther dehiscence time	One day before anthesis
Mean no. of pollen grains/flower	34,571
Pollen size	28.24µm
Stigma type	Wet and non-papillate type
Stigma receptivity	0800–0230 hr
Mean no. of ovules/flower	20
Fruit setting in natural condition	40%
Pollen fertility	80%
Pollen viability	82%
Pollen germination %	96%

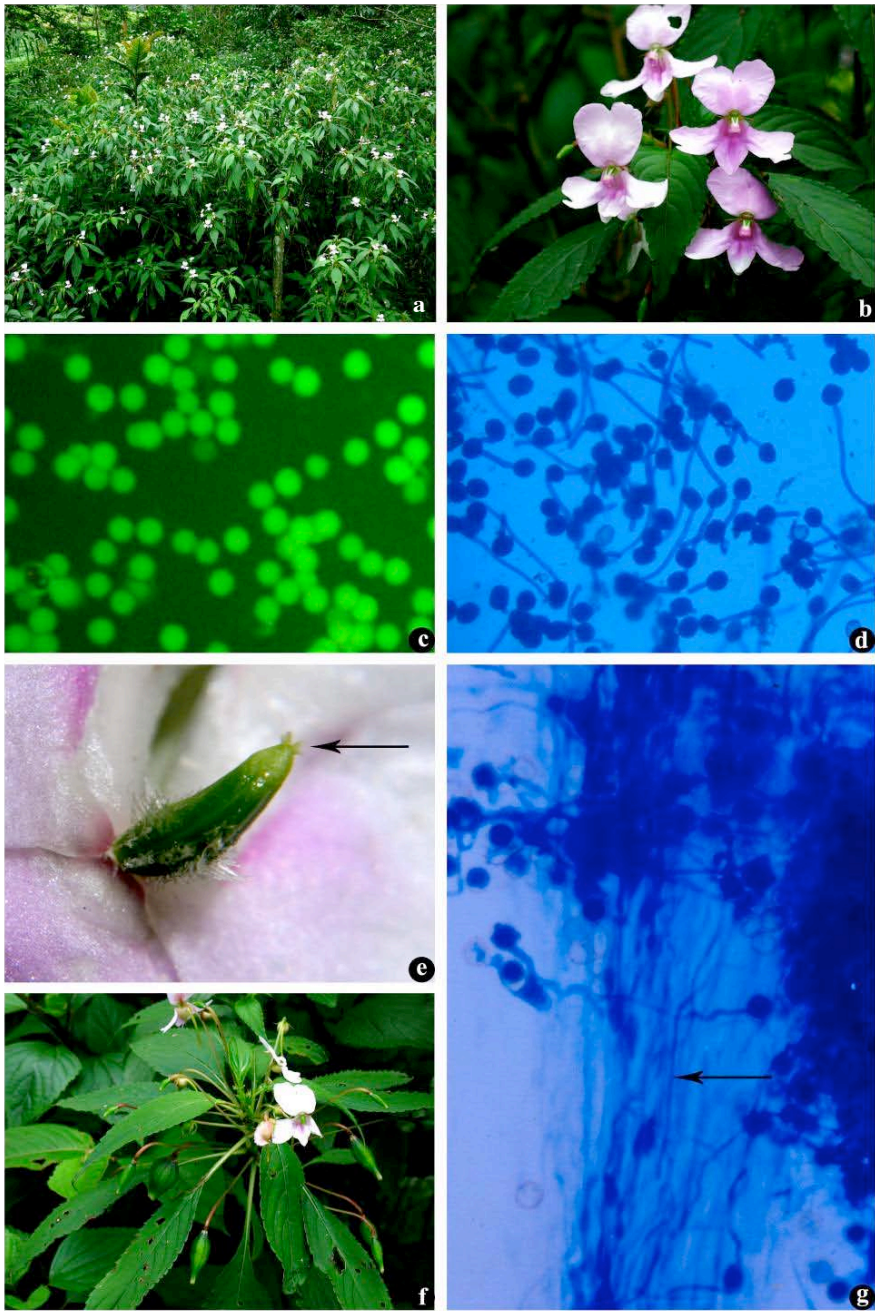

Image 1. Pollination biology of *Impatiens cuspidata*
a - population of *I. cuspidata*; b - full bloom flowers; c - pollen viability by FCR test; d - in vitro pollen germination by Brewbakers medium; e - receptive stigma with stigmatic lobes; f - in vivo pollen germination; f - fruit set in natural habitat

soften the seed coat. The soaked seeds were then placed on germination paper and incubated at 25^{0}C and germination percentage was recorded.

RESULTS

The flowers of *I. cuspidata* are light pink in colour (Image 1b) born at apical cyme in pair or solitary and spur is straight, oblong and glabrous. Capsules are ellipsoid and cuspidate in nature; contains 14–16 seeds in each capsule. The plant starts flowering in the month of August and it extends up to December with peak flowering during October. The flower buds take 6–11 days from initiation to full bloom. The flowering period is extended up to 160 days in a year and the average life span of the individual flower is 2–3 days. The flowers bloom in the night between 2330 and 0430 hr, confirming their nocturnal nature. Anther dehisced one day before flower opening which confirmed the protandrous condition of the flowers (Table 1). The mean number of pollen grains per flower was found to

Table 2. In vivo pollen germination of *Impatiens cuspidata*

Treatments	Period after flower opening (anthesis)			
	I day	II day	III day	Drooping stage
Total no. of stigmas observed	10	10	10	10
No. of stigmas showing germination	8	4	2	0
% of stigma receptivity	80	40	20	0
Total no. of pollen retained on stigma	28,520	3,430	165	11
Mean no. of germinated pollen	11,430	865	14	--
% of pollen germination	62	23	6	--
Pollen tube length (µm)	917	136	46	--

be 34,571 and the mean number of ovules was twenty. Therefore the pollen ovule ratio was calculated as 1729: 1 (Table 1).

Pollen grains are oval and have an average size of 28.24µm in diameter. The acetocarmine staining technique revealed that 80% of the pollen grains were fertile. Pollen viability by FCR test confirmed that 82% pollen grains were viable on the day of anthesis (Image 1c) and gradually reduced after second day of anthesis. In vitro pollen germination studies by using different concentration of sucrose and Brewbakers medium revealed that pollen grains were viable only up to the minimal period. Effect of sucrose on in vitro pollen germination studies revealed that 68% of pollen grains germinated and produced 1003µm tube in 5% sucrose medium. Best pollen germination (96%) along with 1636µm tube development was achieved in Brewbakers medium after four hours of incubation (Image 1d).

Receptivity of stigma is a critical factor for successful completion of post pollination events. The stigma is wet and non-papillate type (Heslop-Harrison & Shivanna 1977). Stigmas were more receptive up to 80% on the first day of flower opening (Table 2 & Image 1e); by showing 62% in vivo germinating pollens along with 917µm long pollen tube on the stigmatic surface (Table 2 & Image 1g). Subsequently, the receptivity percentages and in vivo germinating pollens were decreased on successive days after blooming. In *I. cuspidata,* pollen grains were well adhered on the stigmatic surface due to sticky nature 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.

When *I. cuspidata* flowers bloom in the night between 2330 and 0430 hr, two species of hawk moths: *Macroglossum corythus* and *M. variegatum* were found to forage actively. They helped in pollination by their rapid and frequent visit to flowers for nectar collection. Pollen loads were found on the head and long proboscis of hawk moths, which 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 were mainly during daytime from 0700 to 1600 hr. Honey Bees visited many flowers and spent an average of 2–4 seconds in each flower, whereas hawk moths spent slightly long duration from 3–6 seconds in each flower. Bees (*Apis cerana indica* & *Trigona* sp.) were found to be the most abundant visitors and they visited more flowers than any other pollinators. Butterflies were active during daytime and in fine weather, they actively visited the flowers, spending an average of 3–5 seconds on individual flower for nectar. However, when the weather was cloudy, butterflies were less active and in rainy days, they were completely inactive. Flies were intermittent visitors and found to be poor pollinators (Table 3). In general, hawk moths in night and honey bees in day time served as better pollinators, but they

Table 3. Pollinators and their characteristics in *Impatiens cuspidata*

Visitors	Visiting time	Foraging nature	Foraging hours
Apis cerana	Day	Nectar & pollen	0700–0400 hr
Trigona sp.	Day	Nectar & pollen	0730–0230 hr
Macroglossum variegatum	Night	Nectar	1140–0330 hr
M. corythus	Night	Nectar	1230–0300 hr
Butterflies	Day	Nectar	0630–0500 hr
Flies	Day	Nectar	0730–0330 hr

Table 4. Fruit set in different modes of pollination in *Impatiens cuspidata*

Treatments	No. of flowers pollinated	No. of flowers set fruit	% of fruit set
Bagged (B)/ autogamous-self	25	0	0
Emasculation and hand crossing (S)	25	14	56
Emasculation and hand out crossing (O)	25	19	76
Natural pollination (N)	25	10	40

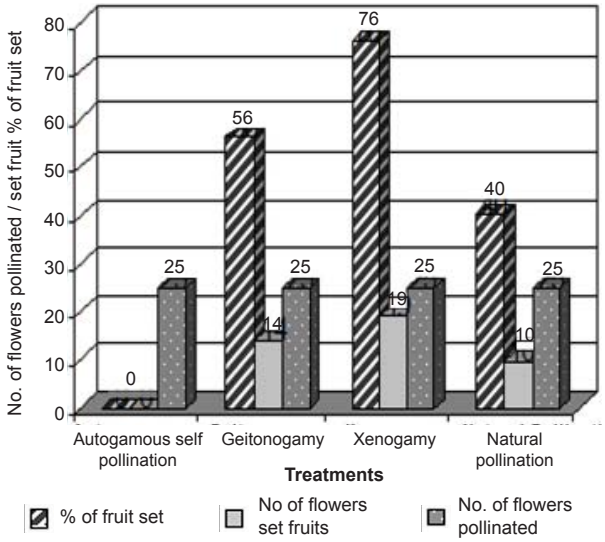

Figure 2. Fruit set in different modes of pollination systems in *Impatiens cuspidata*

were not sufficient to pollinate all the flowers in the selected populations.

In *I. cuspidata*, different breeding experiments were carried out to find the reproductive capacity of the plant. In natural condition, 40% fruit set was observed (Image 1f). The fruit set was not observed in autogamous self pollination. However, 56% fruit set was observed in geitonogamy and 76% in xenogamy (Table 4; Fig. 2). Breeding experiments like geitonogamy and xenogamy produced more fruits and seeds than the natural pollination. The average weight of the individual seeds produced by the treatments of natural, geitonogamy and xenogamy were 1.5, 1.7, 2.0 mg respectively which indicated that the seeds produced by artificial pollination were more healthy and viable than natural system because of combination of gametes from different flowers or plants. The fruit development took 25–30 days for attaining maturity after fertilization. As capsules mature, the fruit wall ruptured and the seeds were ejected up to 0.60–1.25 m away from the mother plant and this is the only way of dissemination of seeds in the present taxa. Seeds germinate after dehiscing from the capsule in a favorable place but very few of them established into seedlings in the natural condition. But seeds developed through xenogamy produced more seedlings. In the laboratory condition, the germination of seeds obtained through natural pollination was 20%.

DISCUSSION

The members of Balsaminaceae have their greatest development in the Indian region and are remarkably endemic. Endemism gives us clue that they are in restricted distribution, either due to their reproductive syndrome or by anthropogenic pressures. Knowledge on phenology and floral morphology are essential for conducting studies on breeding systems particularly on pollination syndrome if any. *Impatiens cuspidata* starts flowering in the month of August and continued up to December and reached a peak during October and anthesis commenced between 2330 and 0430 hr on the next day. 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). Bhaskar & 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 anther dehisced one day before anthesis, which in turn confirmed their protandrous condition. This observation was similar to that of *I. platypetala*, *I. korthalsii,* and *I. eubotrya* in Sumatra (Kato et al. 1991).

In *I. cuspidata,* pollen viability is highest on the day of anthesis and then gradually decreased on successive days after anthesis. This observation is similar to that of *I. reptans* in China (Tian et al. 2004). In vitro pollen germination test indicated that highest percentage of pollen germination and tube elongation was observed in Brewbakers medium. Sucrose acts as a nutritive material for pollen germination (Johri & Vasil 1961) and it helps in maintaining osmotic balance between the germination media and pollen cytoplasm (Mukerjee & Das 1964). Germination percentages were significantly low in higher concentration of sucrose medium.

According to Shivanna & Johri (1985), the optimum concentration of sucrose varies from species to species. In the present investigation, Brewbakers medium is the most suitable for pollen germination in *I. cuspidata*. Besides the medium contain carbohydrates, boron and calcium which are other important substances required for pollen germination and tube growth (Brewbaker & Kwack 1963). Pollen germination and subsequent post pollination events depend upon the receptivity of the stigma, its nature and compatibility.

It is well known that the flowers of *Impatiens* have enormous diversity and different pollinators. *Impatiens cuspidata* 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. cuspidata,* bees (*Apis cerana indica* and *Trigona* sp.) are the most important pollinators and visited more flowers than any other pollinators during day time for nectar and pollen gathering. There is a strong relationship between the weather and foraging activity of pollinators. When the weather is fine, butterflies are more active and spend on an average 2–6 seconds on a flower at each visit. But when the weather is cloudy and rainy, the butterflies and hawk moths are less active. The present investigation agrees with the findings on *I. coelotropis* (Sreekala et al. 2008).

In *I. cuspidata,* 40% fruit set was observed in natural pollination. However, artificial cross pollination (geitonogamy and xenogamy) enhanced the fruit set rate up to 76%. Pollination experiments demonstrated that, artificial cross-pollination enhanced the rate of fruit and seed set in *I. cuspidata*. The balsams are highly evolved members among the order Geranials as evident from their marked zygomorphic flowers and nectariferous spur. The arrangement of stamens, pistil and spur are markedly adapted for cross pollination in *Impatiens* (Bhaskar & Razi 1974) and hence most of the species of *Impatiens* reproduce by cross pollination (Schmitt & Gamble 1990; Lu 2000, 2002).

Stigma receptivity is a critical factor for successful completion of post pollination events. Usually it is highest soon after anthesis but it varies from species to species, depending upon the temperature and humidity (Shivanna & Johri 1989). But in *I. cuspidata*, stigmas remained receptive only after the shedding of androecium and gradually increased for 8–14 hr. The receptivity ends after 14 hours but at the same time its pollen viability reduced drastically. 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). In *I. cuspidata,* pollen grains are well adhered on the stigmatic surface. Pollen tubes penetrate the stigmatic surface and reached up to the ovary and successfully fertilize the ovules. The fertilized ovules developed into seeds. The percentage of seed set was only 60%.

Seeds are not dormant and they germinate immediately. Experimental results also substantiate the same. Very few germinated seeds were established into seedlings and remaining perished. This may be due to insufficient flow of nutrients into seeds. Therefore the study suggests that, absence of dormancy, protandry, self-incompatibility, pollinator limitation and perishing of considerable percentage of seedlings prior to establishment in combination with other abiotic traits are contributing factors for regulation of population size of *I. cuspidata* in its natural condition.

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