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Reproductive biology of *Alstonia scholaris* (L.) R.Br. (Apocynaceae)

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ABSTRACT

Reproductive biology of *Alstonia scholaris* (Apocynaceae) a medium to large evergreen ornamental tree was studied. It is commonly known as saptaparna or devils tree. Flowering commenced in September and continued till the end of January with the maximum was during the months of November to December. Flowers were protandrous and large amount of pollen and nectar attracted a wide variety of insects during the entire flowering period. Honeybees (*Apis dorsata* and *Apis indica*), small bees (*Mellipona* spp.), butterflies (*Danaus genutia, Eurema laeta,* and *Parantica aglea*), black ant (*Componotus compestris*), wasps (*Polistes hebraeus* and *Vespa* spp.), beetle, moth (*Achoria grisella*) and white and yellow spiders forage either for both nectar and pollen or nectar alone. *Apis dorsata*, *A. indica* and *Mellipona* were the main pollinators of this ornamental tree as they obtained both pollen and nectar by their frequent inter-plant movements to facilitate cross-pollination. The other insects were nectar thieves. Fruit formation started in December and mature fruits dehisced in February. The pollen: ovule ratio and hand pollination experiments indicated facultative geitonogamy. The fruits were green, long double follicles. The mature follicles turned brown and dehisced longitudinally from the base into two halves and large number of compressed and small seeds with a tuft of brown hairs on both the ends were liberated.

Keywords : floral biology, honey bees, butter flies, moth, geitonogamy

Understanding the mode of reproduction in plants has been a fascination area of multidisciplinary research for not only extending fundamental knowledge but also for genetic improvement of plants, optimal utilization and conservation (Tandon *et al.* 2005, Koul-Moza and Bhatnagar 2007). Knowledge on reproductive biology has been utilized in largely in herbaceous crops and trees have been neglected. Trees have received little attention due to numerous problems e.g. large size, long life cycles, in frequent flowering and inaccessible flowers. A systematic study of reproductive biology of any particular tree species requires collection of comprehensive data on phenology, floral morphology, pollination ecology, breeding system, pollen-pistil interaction, fruit and seed set and their dispersal and seed germination and seedling recruitment (Shivanna 2003).

Alstonia scholaris (L.) R. Br. is a medium to large, evergreen tree of the family Apocynaceae. The family comprises nearly 5,100 species in five subfamilies, Apocynoideae, Asclepiadoideae, Pteriplocoideae, Rauvolfioideae and Secamonoideae (Nazar *et al.* 2013, Endress *et al.* 2014). The subfamily Asclepiadaceae, now known as Asclepiadeae which contains 411 genera and 4,650 species (Heywood *et al.* 2007). In India there are about 30 genera and over 60 species of this family (Jones and Luchsinger 1987). The genus *Alstonia* comprises about 45 species inhabiting tropical and subtropical Africa, Central America, Southeast Asia, Polynesia and Australia. *A. scholaris*, commonly called as saptaparna or satpatiya or Devil's tree, is native of tropical and subtropical Africa and now present in India, China, and South-east Asia and Australia (Pratap *et al.* 2013). It is planted in the gardens and avenues for its dense umbrella-like crown of dark green leaves and several branches of creamish-white flowers with sweet fragrance. The bark containing diamine, echitenine alkaloids is used as an alternative to quinine (Dey 2011, Kaushik *et al.* 2011).

The reproductive biology in some members of the family Apocynaceae has been studied earlier (Herrera 1991, Galetto 1997, Torres and Galetto 1998, 1999, Lopes and Machado 1999, Barrios and Koptur 2011). Lopes and Machado (1999) studied the pollination and reproductive biology of *Rauvolfia grandiflora* (Apocynaceae) and according to them, it is self-incompatible and melittophilous and pollinated by only one species of long-tongued bee. Barrios and Koptur (2011) studied the breeding system in *Angadenia berteroi* a tropical perennial subshrub. The floral arrangement is complex (the anthers form a conical structure surrounding the stigma, which has a secondary pollen presentation) that promotes out crossing by visits of long-tongued pollinators. Hand pollination experiments showed however, that *A. berteroi* is mostly self-incompatible, with greatest fruit set, fruit length, and seedling emergence resulting from crosses between unrelated individuals. The low fruit set observed in natural populations may be due to low visitation by pollinators, mating between closely related individuals, or both.

Present communication is based on various aspects of reproductive biology of *Alstonia scholaris* until now never studied in detail.

MATERIAL AND METHODS

Study sites—Present investigation was conducted on 30 plants of *Alstonia scholaris* growing at different parts of cities of Agra and Delhi from 2013 to 2016 (Table 1).

Table 1- Study sites.

City	Locality
Agra (28° 11' N to 28° 25' N and 78°' E to 78° 2' E)	1. Lajpat Kunj. 2. Lawyers' colony. 3. Bank colony
Delhi (28°372 N to 28.61°N and 77°142 Eÿþ to ÿþ 77.23°E)	4. Bharti Nagar (Lodhi estate) 5. All India Institute of Medical Sciences.

Five plants more or less of same age and cultivated through cuttings were marked at above mentioned sites. Three sets of herbarium specimen of selected plants (one each from each site) were prepared and were sent to the Botanical Survey of India (BSI), Salt Lake, Kolkata and Regional Station, BSI, Dehradun for identification and one was deposited as voucher specimen No. Agra-11/2006 in the herbarium of Academy of Life Sciences, Agra.

Phenology– Phenoevents (time of leaf fall, leaf renewal, flowering and fruiting period) were recorded during the period between 2013-2016 by semi-quantitative (SQT) method based on a monthly estimation of each phenophase incidence through a visual inspection of thirty whole plants as described by Castro-Diez *et al.* (2003).

Flowering phenology–The time of commencement, peak and decline in flowering was recorded from marked plants growing at different sites. Flowering intensity (average number of flowers/inflorescence x average number of inflorescence/plant) was recorded following the procedure of Dafni (1992). The inflorescence were selected at random from different individuals and tagged before the initiation of flowering. Observations were recorded every day between 05:00-18:00 h during the flowering period (September-January) for three consecutive years (2013-2016). These individuals were followed daily and the number of open flowers/inflorescence was recorded. The open flowers were then removed to avoid recounting on the next day. The tagged inflorescences were followed until flowering ceased. One hundred flowers were sampled for recording the floral morphology. The time of daily anthesis and anther dehiscence was recorded.

Floral Biology–Flowering period, evaluation of the intensity of blooming and dimensions were measured with the help of Vernier's calliper on every alternate day. Young flowers and their parts were observed under Nikon-DS-Fitc Stereozoom SM 2800.

Anthesis–To study the time of opening of flowers, 50 flowers of each plant were tagged when these were at their largest size. Observations were made at an interval of 2 h for five days. Flower longevity was determined by marking 50 buds on different branches/ plant (Gill *et al*. 1998). Time was recorded when new flowers opened i.e. when the petals refluxed to expose the androecium and gynoecium. The flowers were observed at regular intervals until the corolla withered. These changes were observed every day for a week.

Scanning electron microscopy (SEM) – Floral buds at different stages of development were fixed in 3% glutaraldehyde, dehydrated through aqueous acetone series and dried with CO_2 in Jumbo Critical Point Dryer (Polaron). The samples were coated with gold (20 mm) in a Balzer Union SCPO 020 sputter coater and observed in a EVO-18 (Zeiss, UK) with Acc. voltage: given on the picture (30 kV); and EDS Image analyzer system at All India Institute of Medical Sciences, Delhi.

Pollen morphology – Size and shape of pollen grains, characteristics of surface, presence of aperture (shape, number and position) and exine sculpture were recorded in acetolyzed pollen grains under light and scanning electron microscope. Acetolysis of pollen grains was done by suspending them in a mixture of acetic anhydride and concentrated sulfuric acid (9:1), and the suspension was heated to boiling. The acetolysis mixture was removed by centrifugation, the pollen grains were rinsed first in glacial acetic acid and then in water by low speed centrifugation and mounted in glycerine.

Number of pollen/anther/flower–Number of pollen/ anther/ flower was determined from 100 flowers following the procedure reported by Cruden (1977). Mature anthers were crushed in lactophenol-glycerine with aniline blue. A known dilution was placed on grids and 10 replicate counts were made using a hemocytometer (Barret 1985).

Pollen viability–Pollen viability was assessed by FCR (flurochromatic reaction) test according to Heslop-Harrison and Heslop-Harrison (1970), 1% TTC (2,3,5-triphenyl tetrazolium chloride) in 0.15 M tris buffer at pH 7 (Hauser and Morrison 1964) and *in vitro* pollen germination by hanging drop culture method using Brewbaker and Kwack's (1963) medium.

Number of ovules/flower– Ovules/flower were counted by the method reported by Stelly *et al.* (1984). Floral buds fixed in F.A.A. were hydrated and stained in Mayer's hemalum (using Sass's modification of 20 g instead of 50 g of alum per liter), for 1-2 days. Destained with 2.0% acetic acid for 1-2 days and rinsed with tap water for 2-24 hours. Samples were dehydrated, infiltrated with xylene and cleared with methyl salicylate in the series 2:1, 1:2 (xylene: methyl salicylate) for 15+ minutes each. The number of ovules from the cleared ovaries were counted using stereomicroscope.

The pollen-ovule ratio–In order to find out pollen-ovule ratio the number of pollen grains/flower was divided with the number of ovules/flower (Cruden 1977).

Stigma receptivity– Cytochemical method according to Mattsson *et al.* (1974) localizing esterases was used to assess

stigma receptivity. Fresh unpollinated stigmas were collected from fully developed flowers. These were placed in a drop of solution A consisting of α-naphthyl acetate, 0.15 M phosphate buffer, 10% sucrose and fast blue B on a slide. For control, the stigmas were placed on another slide in a drop of the similar solution B, minus α-naphthyl acetate. Stigmas kept on both the slides were incubated at 25-35°C in a humid chamber for 10-20 minutes, washed with same phosphate buffer. The esterase activity in stigmas was observed by mounting them in glycerin and observing under light microscope. Esterase activity appeared pinkish/reddish in the stigma mounted in solution A.

Nectar—Volume of nectar from individual flowers (25 from each marked plant) was measured using 20 µl microcapillary tubes during both the flowering periods. Nectar volume was calculated using the procedure after Cruden and Hermann (1983). During anthesis, sugar concentration in the nectar collected from unprotected and protected flowers at different hours was evaluated by a light refractometer (Bausch & Lomb) after the method of Dafni (1992). The values on the refractometer were considered as sucrose equivalents as given by Inouye *et al*. (1980). Sugars were separated using Whatman No. 1 filter paper (Kearns and Inouye 1993). Three descending solvent systems used were: a. Ethyl acetate-pyridine-water (8:2:1, v/v), b. n-butanol-glacial acetic acid-water (3:1:1 v/v) and c. l-propanol-ethyl acetate-water (7:1:2 v/v). The dried chromatograms were treated with p-amino benzoic-acid to detect sugars.

Pollination Biology– Observations on the floral visitors and their foraging behavior were made following the procedure after Faegri and van der Pijil (1979) and Dafni (1992). Flower visitors on the flowers were observed to settle as potential pollinators using a binocular. Their behaviour on the flower, flying pattern across inflorescences, time of visit and kind of resource collected were recorded. Pollination efficiency of different insects was checked by observing the pollen load on different body parts under light microscope as per procedure described by Kearns and Inouye (1993). Butterflies were identified with the help of "The book of Indian Butter flies" (Kehimkar 2008) and other insects were identified by Dr. Girish Maheshwari, Head, Department of Entomology, St. John's College, Agra.

Breeding system–Breeding behavior by autogamy (bagged and hand pollinated), geitonogamy and xenogamy was tested by controlled pollination studies. In order to observe the rate of natural fruit-set, fifty inflorescence on different trees were tagged and were followed until fruit development. The daily foraging schedules, forage collected and probing behaviour of different foragers were recorded.

Statistical analysis– Data obtained from various studies were subjected to analysis of variance (ANOVA)

OBSERVATIONS AND DISCUSSION

Phenology–*Alstonia scholaris* is an evergreen, medium to large tree of 30-50 m with corky grey to brown bark (Fig.1a). Branches were whorled and arising from the main trunk at the same height. Young leaves containing anthocyanin appeared at the apex of the branches in the month of February (Fig.1a1). The mature leaves 5-9 in numbers were arranged in a whorl, and are leathery, petiolate and simple. Upper surface of mature leaves is dark green with lower greenish white surface (Figs.1b,c,d). Leaf fall and renewal was simultaneous. Flowering period varied from plant to plant at the same site or at different sites. Optimum flowering was observed during the months of November to December (Figs. 1b-e) and sometimes flowering continued till February and a limited number of trees flowered even in the months of April-May also. Fruit formation started in December (Fig.1f) and mature fruits dehisced in February-March and released large number of hairy seeds (Figs.1g, h).

Floral biology–The inflorescences were compound due to branching (Figs. 1b-e). They were compact, terminal pannicle or compound umbel developed at the tip of the shoot (Figs.1b-e, Figs. 3a,b) and each inflorescence was up to 10±2 cm long and consisted of 480±23 strongly perfumed 8±2 mm long tubular flowers (Fig.1e). The corolla tubes were hairy; lobes sparsely or densely pubescent and 1.5-4 mm long (Fig. 1e, 2a). There were 170±10 open flowers and 300±10 floral buds of different sizes on randomly selected inflorescence (Fig. 2a,b). The flowers were white or cream or greenish white flowers of 1.0±0.25 cm in size (Fig.1e). They were bracteates, pedicillate and peduncle was 0.4±01 cm long, hermaphrodite, pentamerous, actinomorphic, hypogynous and tetracyclic, ending in a somewhat bi-lobed, conical (0.22±0.07 cm) (Fig. 3c, d, Fig. 2a 2) wet stigma (Heslop-Harrison and Shivanna 1977). Calyx consisted of five green gamosepalous sepals. Corolla consisted of five white or greenish white tube-like, gamopetalous strongly perfumed petals (0.8±0.2 cm) overlapping to left (Fig.1e). At the throat of the funnel, there were downwardly pointed hairy collars or corona (Fig. 2a). Androecium consisted of five epipetalous, didynamous, polyandrous stamens (0.26±0.1 cm) with basifixed and introse anthers (0.12±0.02 cm) which dehisced by a longitudinal slit (Fig.4a, b, 2a1). Gynoecium was bicarpellary and syncarpous with separate bilocular, superior, distinct and pubescent ovaries with axile placentation (Fig. 2a 4). The style two filiform, (Fig.2 a3) and united into a bilobed wet stigma (Fig. 2a 2) (0.22±0.07 cm).

Flowering phenology–Anthesis took place between 18:00-20:30 h. Anthers dehisced through a longitudinal slit between 20:00-21:30 h. The stigma became receptive between 20:30-22:00 h and remained so until next morning (20:30-9:00 h).

Trichomes-The surface of calyx and corolla was covered with large number of trichomes (Fig. 3a,b).Inside the throat of

Fig.1 a-h. Phenological events of *Alstonia scholaris*. a: Tree; a1: emergence of young leaves containing anthocyanin; b: floral initiation; c: mature floral buds; d: mature flowers in inflorescence; e: flowers at anthesis; f: fruiting tree; f1: bunch of young fruits; g: dehisced fruits; h: hairy seeds.

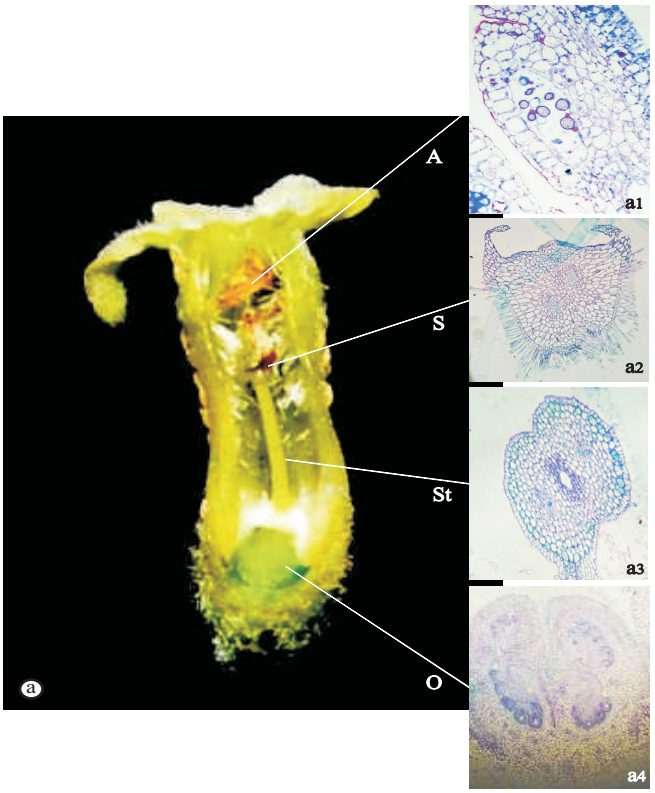

Fig. 2. a: L.S. floral bud showing different parts. a1-a4: T.S. floral parts. a1:mature anther showing pollen and developed endothecium; a2:stigma covered with unicellular trichomes; a3: hollow style; a4: bilocular ovary showing parietal placentation. (A: anther; O: ovary; S: stigma; St: style).

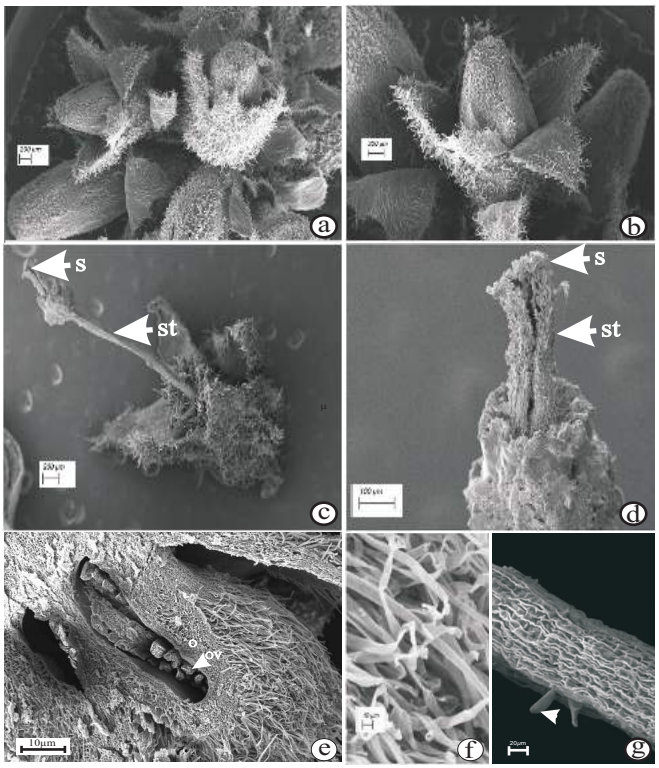

Fig.3. Scanning electron microscopic (SEM) pictures of floral parts. A: young hairy buds; b: a mature floral bud; c: pistil with stigma, style and hairy sepals; d: magnified view of upper part of Fig. c showing conical stigma and hollow style; e. L.S. ovary covered with large number of trichomes and ovules inside; f: unicellular trichomes; g: trichomes (arrow) on style. (O: ovary, Ov: ovules; S: stigma; St: style).

the corolla funnel also there were large numbers of downwardly pointed trichomes (Fig.2a). The trichomes were also present on the style and ovary surface (Fig.3e). These trichomes were much elongated, unicellular with slightly swollen tips (Fig.3f).

Fruits- The ovaries after fertilization developed into a pair of distinct and linear follicles (Fig.1f). Young fruits were pendulous green, long two-lobed follicles (40.3±5.34 cm long and 0.75±0.05 cm broad) produced in pairs (Fig.1f1) which turned brown on maturity in 25±5 days. The mature follicles dehisced longitudinally from the base into two halves (Fig.1g). There were 50±20 seeds/fruit. The seeds were compressed and small (0.9±0.1 cm) with a tuft of brown hairs (10±3 mm long) on both the ends on maturity (Fig.1h). The seeds failed to taper to a point at either end. The fruits dehisced on the tress and the seeds with the tuft of silky hairs at each end were dispersed by wind.

Nectar-At initial stages of the opening of flowers at 18:00 h, nectar secretion commenced and the quantity was 55±5.5µL/flower. Quantity increased gradually with age of the flower and at the time of anther dehiscence (20:00-21:30 h) it was 78±7.5µL/flower and by the time stigma became receptive (20:30 h), the quantity of nectar was 132±20.25µL/flower. In the morning hours (04:00 h) of the day two, the quantity of nectar was maximum (155±31.5 µL/flower). As the day progressed, nectar secretion gradually declined after 12:00 h and became dilute. Nectar was sugar (fructose, glucose and sucrose) dominating. Galetto (1997) and Torres and Galetto (1998) studied the flower morphology, nectar features in an Argentine population of *Mandevilla laxa, M. pentlandiana* and *M. petraea*). Nectar was sucrose dominant and secreted large amount of nectar during bud-stage.

Pollen biology- Anthers dehisced between 19:00-20:00 h in September-November and 18:00-19:00 h in December-January (Fig.4b). The pollen grains were spherical, 45±5 µm in diameter and tricolporate with smooth exine (Fig. 4 c,d). There were 70±5 ovules per flower and there were 980±25 pollen grains/anther and 4900±68 pollen/flower with 27.1:1 pollen-ovule ratio. Mondal *et al*. (1998) recorded production of 5175 pollen grains/flower in plants

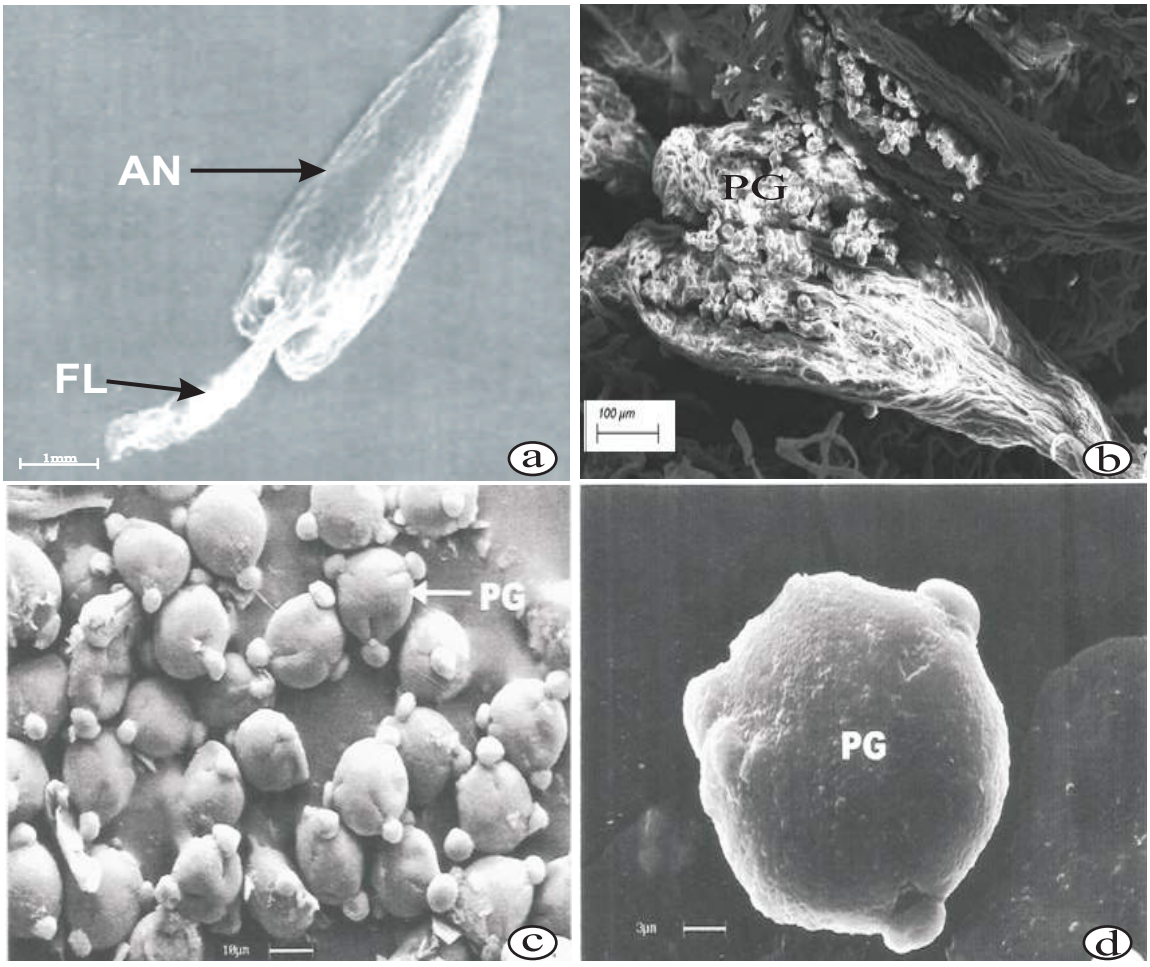

Fig. 4. SEM pictures of stamen and pollen grains. a: mature stamen; b: dehisced anther with large number of pollen grains; c: pollen grains; d: single tricolpate pollen. (AN: anther; FL: filament; PG: pollen grain).

Table 2-Floral visitors, their foraging period, pollen load and percentage of visits.

S. No.	Visitor	Foraging period (h)	Pollen load	Total visits (%)
1.	Bees			
	Apis dorsata	04:00-19:00	435±61	98
	Apis indica	04:30-19:00	238±45	81
	Mellipona spp.	05:00-19:00	189±51	78
	Wild bees	05:00-19:00	101±23	97
2.	Wasps			
	Polistes spp.	11:00-17:00	24±09	28
	Vespa spp.	11:00-17:00	21±07	20
3.	Butterflies			
	Eurema laeta (Boisdural)	12:00-20:00	36±17	72
	Danaus genutia (Cramer)	12:00-20:00	30±13	67
	Parantica aglea (Stoll)	12:00-20:00	27±11	19
4.	Black ants			
	Componotus compestris	05:00-19:00	19±8	26
5.	Moth			
	Achoria grisella	19:00-04:00	16±6	18
6.	Spiders	05:00-19:00	nil	7-8
7.	Unidentified insects	Different periods of the day.	nil	Not recorded

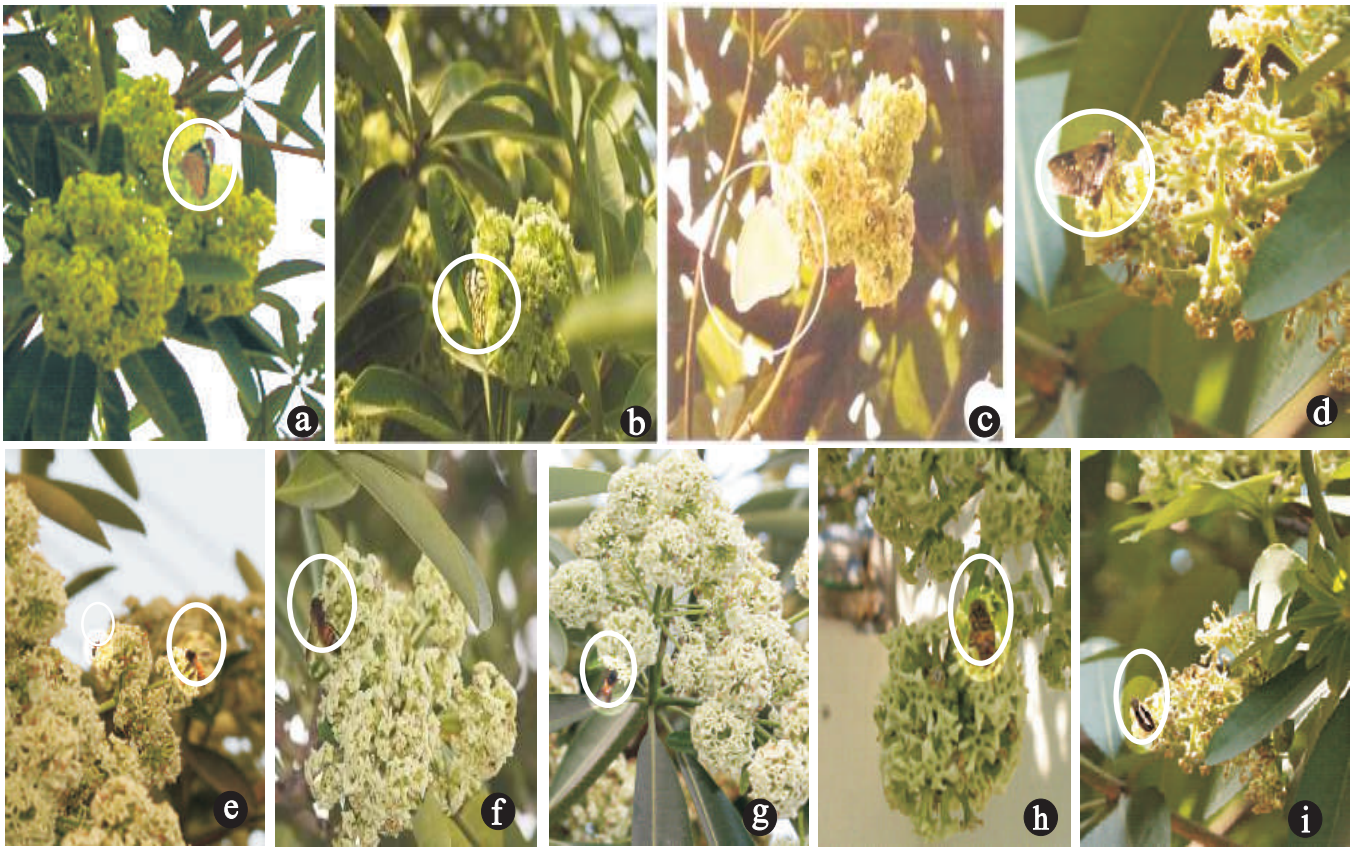

Fig.5. Floral visitors. a. butter fly *(Danaus genutia)*; b: butter fly (*Parantica aglea*); c: butter fly *(Eureama laeta)*; d: moth *(Achoria grisella)*; e: honey bees, *Apis indica* (small) and *Apis dorsata* (large); f: *Apis dorsata*; g-i: unidentified insects.

of this species growing in Eastern India. The pollen grains (45µm) were tricolporate with smooth exine. Kuijt and Van der Ham (1997) have studied pollen morphology of actolysed pollen in five species of *Alstonia* by light and scanning electron microscopy. According to them, the pollen grains are always colporate and 2 aperturate. Recently, Isabella *et al.* (2016) have studied morphology of pollen of 25 species among 14 genera in the family Apocynaceae from Brazil by light and scanning electron microscopy. According to them, the pollen grains were porate to colporate, with exine ornamentation varying from psilate or scabrate to microreticulate.

Pollination biology- White or greenish white flowers with sweet scent and large amount of pollen and nectar attracted a wide variety of insects, honey bees, butterflies, wasps, moth and wild bees during the entire flowering period. The insect visitors with their foraging period and pollen load are listed in Table 2.

It is evident from Table 2 that honeybees (*Apis dorsata* and *Apis indica*) (Figs.5e, f), small bees (*Mellipona* spp.) were first to visit flowers between 04:00-19:00 h and collected both nectar and pollen. On the other hand, a wide range of butterflies (the spotless grass yellow [*Eurema laeta* (Fig. 5c)] of family Pieridae and subfamily Colladinae commonly known as yellows), stripped tiger [*Danaus genutia* (Fig.5 a)] and glassy tiger [*Parantica aglea* (Fig. 5b)] of family Nymphalidae and subfamily Danainae (commonly known as milkweed butterflies), black ant (*Componotus compestris*), wasps (*Polistes* spp. and *Vespa* spp.), beetle, moth (*Achoria grisella*) and white and yellow spiders, while moths visit between 11:00-20:00 h. Honey bees make maximum visits flowed by butterflies, wasps, ants and ants. Butterflies are the next to visit during the day followed by wasps and moth. The number of visits and pollen loads on their body parts clearly indicated that *Apis dorsata* which arrives first on the trees followed by *A. indica* which arrives later were the main pollinators of this ornamental tree as the carried maximum amount of both pollen and nectar and their frequent inter-plant movements facilitated cross-pollination. Several other unidentified insects were also seen to visit for nectar (Figs.4 g, h, i).

Galetto (1997) and Torres and Galetto (1998) observed that the flowers of Argentine population of *Mandevill pentlandiana* (Apocynaceae) were visited by bumblebees, honeybees and hummingbirds. Their interesting observations showed that the greater the number of open flowers and nectar variance, the more the mean reward quantity per flower was available in the inflorescence. The nectar variability in *M. pentlandiana* seems to be linked with both the female function (nectar resorption, nectar cessation) and the male one (early and comparatively large nectar availability, variation in nectar production with the age of flower, nectar secretion stimulation by nectar removals).

Breeding behaviour- There are 70±8 ovules/flower and the pollen-ovule ratio is 27.1:1.There is 69% fruit formation in open pollinated flowers and the bagged un-emasculated flowers failed to produce fruits indicating that the tree is self-incompatible. The begged and emasculated flowers on pollinating with pollen from the fresh dehisced anthers of flowers of the same plant produced 79.9% fruits (geitonogamy) while there was only 20.1% fruit set in emasculated begged flowers pollinated with the pollen from flowers of different plants (xenogamy). This data about pollen/ovule ratio and hand pollination experiments clearly indicate facultative geitonogamy.

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