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REPRODUCTIVE BIOLOGY OF *DILLENIA SUFFRUTICOSA* (GRIFFTH) MARTELLI WITH EMPHASIS ON PROTANDRY

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Abstract

Protandry was investigated in *Dillenia suffruticosa* (Griffth) Martelli. The species is highly specialized for its herkogamous, nectarless, pollen - only flowers with sternotribic pollination. Flower nature is revolver type. Wind pollination is absent and replaced by insect pollination regarded as a transitional state of evolution of advanced pollination syndrome. Pollinators are a key factor in reproductive success of species. In *D. suffruticosa*, protandrous flowers with long pollen viability and spatial orientation of stamen and stigma found to be an adaptation to insect-mediated cross-pollination.

Introduction

Dillenia suffruticosa (Griffth) Martelli, a widespread species in Malay Peninsula, Borneo, Philippines (Hooker and Thomson 1872) and now naturalized in India, Kerala (Murthy 2000). The plant is traditionally used as a medicine to stanch bleeding wounds. Recent investigation showed that the solvent extraction from root, leaf, flower and fruit of the plant resulted antioxidant and cytotoxic properties (Armania *et al.* 2013).

Protandry is a dichogamous system characterized by early maturation of anther before stigma and recognized as a unique mechanism to prevent self-pollination. In general, dichogamy has been reinterpreted as a mechanism to reduce the effect of pollen-pistil conflicts on pollen transport (Routley and Husband 2003). There are no previous systematic reports on reproductive strategies of *D. suffruticosa.* The aim of the present study is to investigate reproductive assurance and constraints in reproductive biology of *D. suffruticosa* particularly to emphasize on floral biology, pollination biology, breeding system, fruit set and seed biology.

Materials and Methods

The present study was conducted at Calicut University Botanical Garden (CUBG), with 6 plants of *D. suffruticosa*. Altitude ranges from 40 - 50 m. The latitude is 11^{o} 25' - 45'N and longitude is 75^{o} 45' - 50'E. The average annual rainfall ranges from 250 - 300 cm occur during the months of June to September. The temperature varies between 17 and 35^{o}C. Field observations were done from December, 2011 - December, 2012.

Morphology of flowers (n = 30) was studied using a hand lens, CSM2 and LEICA M80 stereomicroscopes. The measurements of floral parts were made. The color was determined by direct visual observation. Presence of smell was detected by keeping some flowers in vials for 2 hrs. Phenological events like initiation of flower primordia, bud break, development of flowers, anthesis, anther dehiscence, flower longevity, peak period of flowering, shedding of leaves, fresh leaf emergence, fruit initiation and seed dispersal were recorded. Anthesis and anther dehiscence were observed in the field using a hand lens. Flower longevity was determined by recording the time of opening and shedding of flowers (Sihag and Wadhwa 2011).

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Freshly collected pollen grains preserved in 70% ethyl alcohol were mounted on a stub, air dried for three min and sputter coated with gold. Scanning electron micrograph of pollen grains was observed using Hitachi SU6600 Horiba-EMAX EDS (Japan) scanning electron microscope. The average size of pollen grains was measured from a random sample (n = 100) of pollen grains. Pollen production and number of pollen grains per flower were calculated following Cruden (1977). The biochemical analysis of pollen grains was done using IKI, sudan black B and coomassie brilliant blue R for the detection of starch, lipid, and protein, respectively. The viability of pollen grains was assessed by tetrazolium test (Zhou *et al.* 1999) and fertility was done by acetocarmine-glycerine technique (Shivanna and Rangaswamy 1992). The effect of organic and inorganic nutrients on *in vitro* pollen germination and pollen tube elongation were studied using Brewbaker and Kwack's medium, solutions of H_3BO_3 (100 - 500 µg/l), $CaNO_3$ (25 - 500 µg/l), $MgSO_4$ (25 - 500 µg/l), KNO_3 (25 - 500 µg/l) and $C_{12}H_{22}O_{11}$ (1 - 40 µg/l). The percentage of pollen germination and pollen tube elongation were observed under Lab. A1 ZEISS Axiolab phase contrast microscope (Zeiss, Germany). The average number of ovules per ovary was counted following Cruden (1977). Receptivity of stigma was noticed by cytochemical localization of non-specefic esterases on stigmatic surface, a method conducted by (Gosh and Shivanna 1984). Biochemical analysis of stigma was done for the detection of starch, lipid and protein using IKI, sudan black B and coomassie brilliant blue R, respectively.

Insects were identified by an entomologist at Trust for Animal Taxonomy, (C/o Zoological Survey of India), Kozhikode. To determine pollen load on insects (Dafni *et al*. 2005), visitors were trapped by a net and transferred them into a bottle containing a piece of filter paper dipped in ethyl acetate. Collected visitors were transferred to a glass slide and observed under a microscope. Pollination efficiency of different pollinators was studied by observing the pollen loads on different body parts according to the procedure given by Kearns and Inouye (1993). To check pollen load on stigmatic surface, stigmas (n = 25) were collected after each visit of insects and observed under a microscope. For scanning electron microscopy, stigmas mounted on a stub were air dried for 3 min and sputter coated with gold for 15 sec. Pollen load on stigmatic surface was studied by scanning electron micrograph.

Periodically slides coated with petroleum jelly were hung horizontally on branches of tree and collected after 24 hrs exposure to study the role of wind in pollination. Observations of slides were made under a microscope for pollen deposition. Apomixis was done in marked flower buds of one day before anther dehiscence. Marked flowers were emasculated in bud condition prior to anther dehiscence and observed for fruit and seed set. Autogamy was carried out to ensure whether the species was self-compatible or self-incompatible. Geitonogamy and xenogamy were performed to ensure the fruit set. Observations on natural (open) pollination were made on day to day basis to record fruit initiation, development and maturation. To record the fruit dehiscence mode and correct number of seeds of early opened fruits in *D. suffruticosa*, marked mature fruits were collected on the previous day of fruit dehiscence and allowed to open in laboratory. The parameters like fruit and seed type, shape, size, number and color were noticed.

Statistical tests (mean ± standard error) on pollen size, pollen count, pollen viability, pollen fertility, *in vitro* pollen germination, pollen-ovule ratio and visitation frequency of pollinators were applied to assess the variation and accuracy of observations using SPSS vs 20 software (USA).

Results and Discussion

Dillenia suffruticosa exhibited a continuous flowering throughout the year. Inflorescence was a raceme with pedicellate, actinomorphic, bisexual and pentamerous flowers. Heterantherous

androecium showed numerous, white, reflexed stamens with short-stout filaments and long anthers arranged in three whorls. Stamens in outermost whorl represented as staminodes. Gynoecium is represented by a syncarpous ovary with 6 - 8 carpels. Stylar branches with concave stigmas were present.

Anthesis occurred between 0245 and 0305 hrs. One-day opened flowers faded in afternoon and dropped out during 1500 to 1700 hrs. The flowers were protandrous. Anther dehiscence commenced 16 to 17 hrs before anthesis and dehiscence mode was apical pore slit (Fig. 1). In fact, protandry is regarded as a mechanism to avoid the interference in pollen dissemination and pollen lost to selfing (Sharma *et al.* 2008). In *D. suffruticosa* stamens are arranged connate to stylar branches and stigmas oriented outwardly 4 - 5 mm above and opposite to stamens. Recent study by Routley and Husband (2003) revealed the influence of close physical proximity of anthers and stigmas in enhancing autogamy or facilitated self-pollination. Present authors found that even though flowers were protandrous in bud condition with long pollen viability, the spatial separation of stigma above and opposite to stamens recognized as a herkogamous mechanism to prevent the self-pollination in *D. suffruticosa*. The period of peak flowering was observed from March to May. The flower bud started 30 to 33 days from initiation to full bloom.

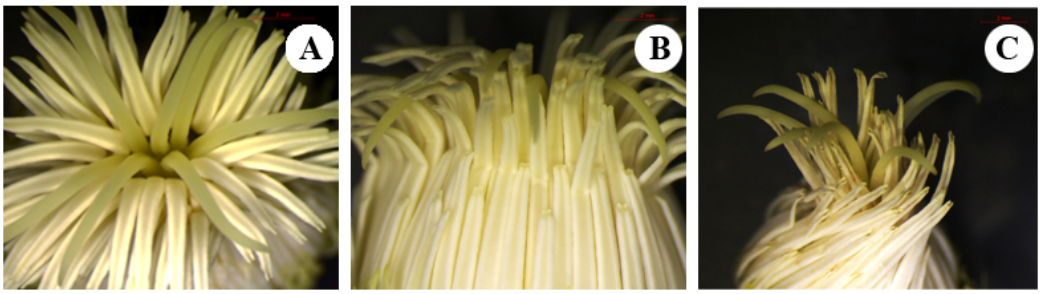

Fig. 1. Stages of protandry and herkogamy in *D. suffruticosa*. A, B: Bud stages. C: Anthesis day.

Pollen grains were round to spherical in equatorial view. Pollen types were tri- to tetracolpate and reticulate (Fig. 2). The average diameter of pollen grain was 25 ± 0.7 µm. The total pollen production in a flower was 16146 ± 1245%. The maximum viability shown by pollen grains on the day of anthesis was 92 ± 8 %. The viability decreased steadily during at the end of day of anthesis. Almost all pollen grains were fertile on the day of anthesis and the highest pollen fertility was 100 ± 0.0%. The biochemical analysis of pollen indicates the presence of protein, absence of starch and lipid when treated with coomassie brilliant blue R, IKI and sudan black B respectively. Among all concentrations of *in vitro* solutions, the average of 72 ± 6% pollen germination with maximum 250 ± 40 µm pollen tube length observed in 10 µg/l solution of KNO_3 for 6 hrs of incubation.

Cytochemical localization of esterases on stigmatic surface and receptivity of stigma using alpha naphthyl acetate test resulted a maximum color change from pinkish to reddish between 1000 to 1200 hrs and color diminished at end of the day of anthesis indicated decline of stigma receptivity. Biochemical analysis of stigma stained with IKI, sudan black B and coomassie brilliant blue R indicated the absence of starch, lipid and presence of protein, respectively. Pollen was the only reward offered by plant to pollinators (Table 1) and flower was nectarless.

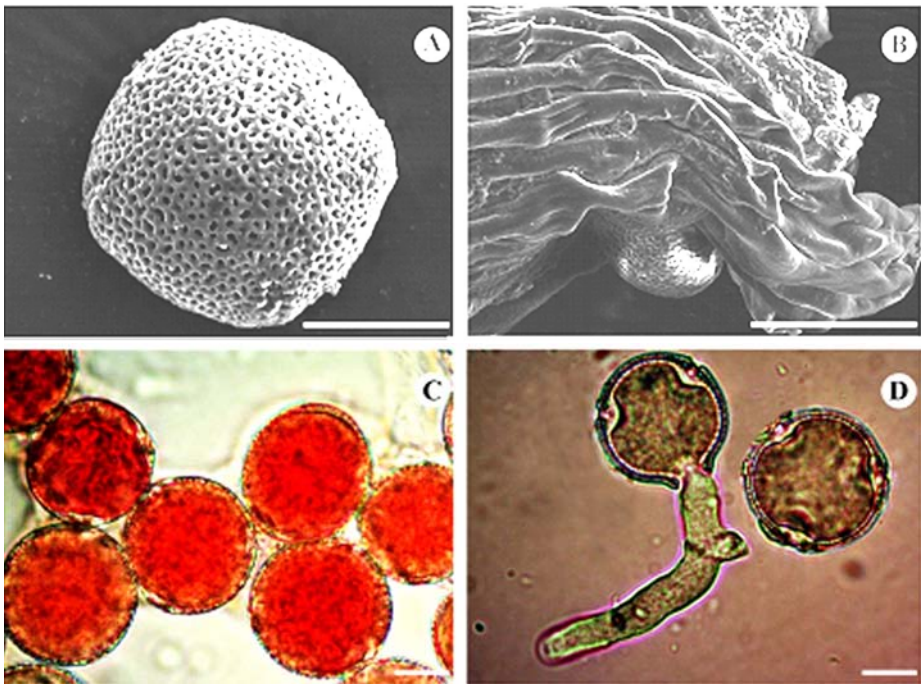

Fig. 2. Analysis of pollen grains in *D. suffruticosa*. A: Scanning electron micrograph of tetracolpate pollen grain. B: Pollen grain load on stigmatic surface. C: Tetrazolium test of pollen viability shows viable pollen grains. D: *In vitro* pollen germination in 25 µg/l solution of KNO_3. (Scale bars: A: 10 µm; B: 20 µm; **C**, D: 10 µm).

Table 1. Visitors of flower to *D. suffruticosa* include both pollinators and non-pollinators.

Name of taxa with family	Visiting time	Foraging mode	Foraging period (hrs)	Time spent in each flower (min)	Stigma touch
Xylocopa pubescence (Apidae)	d‡	Pollen	0600 - 1600	1 - 2	+++
Halictus sp. 1 (Halictidae)	"	"	0900 - 1600	1 - 3	+++
Xylocopa latipes (Apidae)	"	"	0800 - 1300	1 - 2	+++
Ceratina sp. (Apidae)	"	"	0800 - 1200	1	+++
Tetragonula irridipennis (Apidae)	"	"	0800 - 1200	1 - 2	++
Apis dorsata (Apidae)	"	"	0900 - 0200	1	+++
Amegilla sp. (Apidae)	"	"	1000 - 1200	30	++
Halictus sp. 2 (Halictidae)	"	"	0900 - 1100	1 - 2	++
Batocera rufomaculata (Cerambycidae)	"	nr¶	1400 - 1600	8 - 10	–
Componotus paris (Formicidae)	"	nr	0900 - 1500	15 - 20	–
Halyomorpha sp. (pentatomidae)	"	"	0900 - 1100	20 - 30	–
Oecophylla smargdina (Formicidae)	"	"	1000 - 1400	5 - 10	–
Paratrachea sp. (Formicidae)	"	"	1000 - 1500	10 - 15	–
Phalanta phalantha (Nymphalidae)	"	"	1000 - 1100	20 - 30	–

Stigma touch: +++: Effective; ++: Least effective; –: Ineffective. ¶: no reward; ‡: day.

Buzz pollination was the major pollination mechanism in *D. suffruticosa*. The carpenter bees, *Xylocopa* spp. and *Halictus* spp. were the effective buzzy pollinators. In each visit upon landing the connivent androecium pollinators revolved continuously on anthers producing an alerting vibration. The buzzing created a strong electrostatic force on anthers and enabled the anthers to release a mass number of pollen grains through apical spore slit. Ventral body surface of pollinators were sprayed with pollen grains effecting strictly sternotribic pollination mode. Pollination by *Xylocopa* spp. showed both geitonogamy and xenogamy. *Ceratina* sp. harvested pollen by squeezing on poricidal anther. *Tetragonula irridipennis* collected pollen by walking on the apical pores of anther. *Apis dorsata* drum the anther tip by its forelegs to release pollen from apical pores. *Halictus* spp. and *A. dorsata* had pollen bags to carry pollen grains and act as both pollinators and pollen feeders. The number of floral visits made by an insect and time spent in each flower were recorded using a stopwatch. The microscopic observation of pollinators showed pollen load on pollinator's ventral body surface. Frequency of visit of pollinators was shown in Fig. 3. Pollination mechanism of pollinator's was shown in Fig. 4. In *D. suffruticosa* wind had no role in pollination and no pollen grain deposition was noticed on slides coated with petroleum jelly under a microscopic observation. Taxa belonging to the order Hymnoptera were efficient pollinators and flowers thought to be oligophilic.

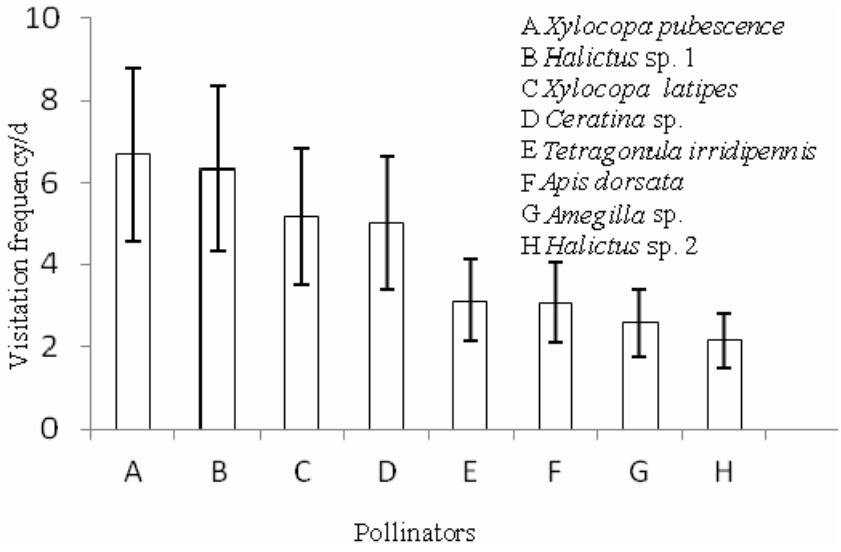

Fig. 3. Visit frequency of pollinators in *D. suffruticosa* (Mean ± SE). Frequency of visit: High: (5 - 30 $v^{\dagger}/d^{\ddagger}$); Intermediate: (1 - 5 v/d). Low: (< 1 v/d). †: Visit/s; ‡: day.

Significantly it has been reported that the insect and bird mediated pollination is considered as more advanced than wind pollination (Xiao *et al.* 2009). Furthermore, flowers with poricidal anther dehiscence and plant delivering only pollen are adapted to wind pollination (Stanley and Linskens 1974). In *D. suffruticosa*, we noticed the only reward offered by plant to pollinators is pollen and absence of wind pollination replaced by insect pollination considered as the evolution of entomophily (advanced) from anemophily (primitive). Synchronization in floral traits, pollinator abundance and availability, which together ensures pollination success in *D. suffruticosa*.

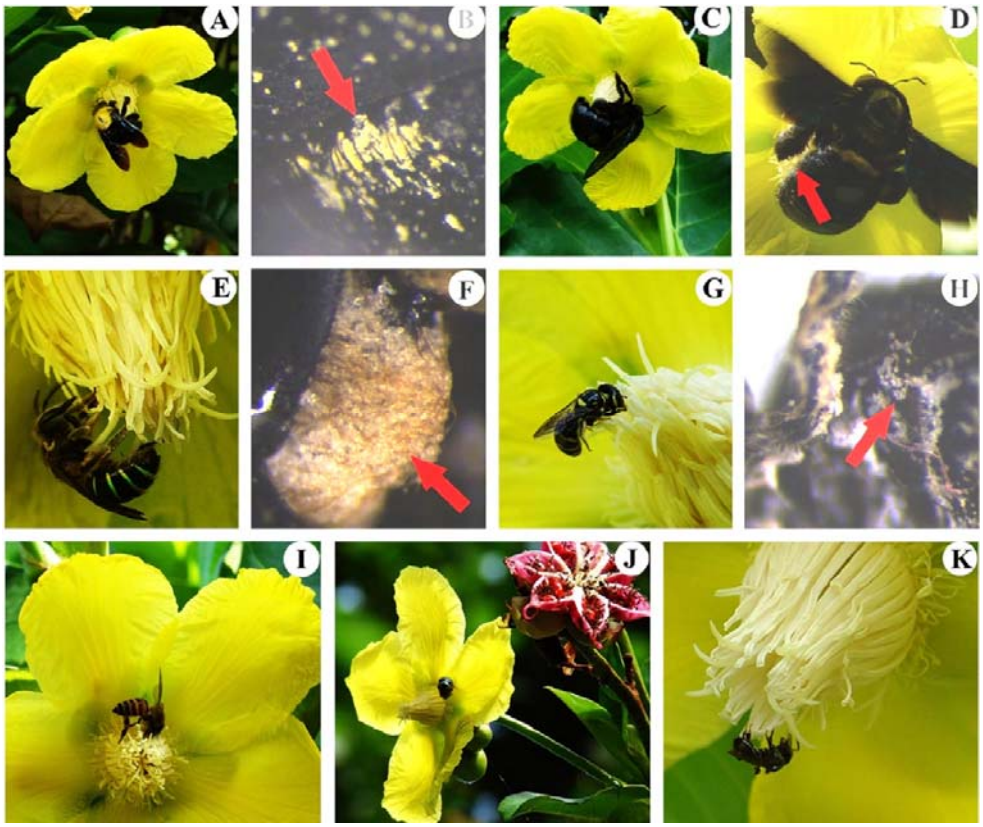

Fig. 4. Pollination mechanism and pollination efficiency of pollinators to *D. suffruticosa*. A, B: *Xylocopa pubescence*. C, D: *Xylocopa latipes*. E, F: *Halictus* sp. G, H: *Ceratina* sp. I: *Apis dorsata*; J: *Amegilla* sp. K: *Tetragonula irridipennis*. Arrows indicate pollen grains deposition on pollinator's body.

Table 2. Fruit set observed between 30 and 33 days after whole pollination treatments. All flowers were bagged after each pollination treatments except open pollination. Bags were removed by noticing the ovary swelling and color change.

Pollination treatments	No. of flowers treated	No. of fruits developed	% of fruit set
Natural/open pollination	50	50	100
Autogamy	50	0	0
Manual geitonogamy	50	40	90
Manual xenogamy	50	0	0
Apomixis	50	0	0

Open pollination resulted high fruit set. None of the fruits set has been observed on apomixis and autogamy (Table 2). Fruit type was an aggregate of follicle and red in color with green-pinkish tinge on green portion of persistent calyx. On ripening fruit splitted along the ventral line. Seeds were obovoid to reniform and dark brown to black in color, covered by a red colored fleshy aril. Fruit dispersal occurred within 1 to 3 days after dehiscence. Seeds were dispersed by ants.

Early protandry with long pollen viability, spatial separation of stamens and stigma and absence of anemophily were the specialized pollination syndrome that promoted the cross-pollination and high reproductive success in *D. suffruticosa*.

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