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Floral biology, breeding system and pollination of *Pterocarpus marsupium* Roxb.

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ABSTRACT

Pterocarpus marsupium Roxb. is a medicinally important tree species distributed throughout India. In order to provide important information in relation to reproductive success, we investigated floral biology, breeding system and pollination mechanism which will help to conserve the plant. The flowering period in the trees ranged between 20 and 38 days. The bright yellow, showy flowers start to open from 05.30 h and continued upto 06:30 h during which they emit mild fragrance and nectar. A single flower produced around 35,180±127.9 pollen grains. After flower opening, different insects represented by Hymenoptera, Lepidoptera and Thysanoptera were found to forage the flowers. Among the flower visitors, bees were found to be the most dominant and effective one. Fruit-set in natural conditions was 32% and 4% through bagging while 12% was observed in netting condition. But in case of controlled pollinations 10%, 24% and 16% fruit set was noticed through autogamy, xenogamy and geitonogamy, respectively. Results from the breeding experiment suggested that, the trees of *P. marsupium* exhibits mixed breeding system with selfing and outcrossing.

Keywords : Floral biology, breeding system, pollination, *Pterocarpus marsupium*.

Pterocarpus marsupium Roxb. is a deciduous tree and commonly known as 'Malabar Kino' or 'Indian Kino tree', belonging to the family Fabaceae and native of Srilanka but found in deciduous forests throughout the India (Varghese 1996). It has its distribution in Andhra Pradesh, Bihar, Gujarat, Karnataka, Kerala, Madhaya Pradesh, Maharashtra, Orissa, Rajasthan, Tamilnadu, Uttar Pradesh, West Bengal and Goa (Sanjappa 2000). It is a medium to large tree reaching a height up to 15-20 meter with dark brown to grey bark having swallow cracks. The bark exudes a red gummy substance called 'Gum Kino' when injured. Flowers are yellow, odoriferous in terminal panicles. Fruits are circular, flat, winged pods with convex and bony seeds. It is well known for its versatile medicinal properties and a wide spectrum of biological activity (Badkhane *et al.* 2010). Now the populations of the plants are declining, as it is exploited for its timber, bark, latex and the plant is enlisted as a near threatened species [NT10.1] (Barstow 2017). Reproduction takes an important role in the survival and succession of plants. The pollination mechanism, pollinators diversity and breeding system of the species have not reported earlier. Information about reproductive needs of the plant is essential to manage its forest population.

MATERIAL AND METHODS

Present investigation was undertaken with the trees growing in an around our University Campus at Visva-Bharati, Santiniketan (26° 44' N to 27° 25' N and 77° 26' E to 78° 32' E) of West Bengal, India.

Flowering phenology—Different phenological events like initiation of flower primordia, flowering period, maturation of floral parts, anthesis, anther dehiscence, stigma receptivity, pollen dispersal etc. were observed. The observations were made both at individual and population levels. Flowering twigs were marked and bagged at bud stage. After anthesis, nectar was extracted from the flowers by capillary tube to measure the volume, and total sugar concentration was determined using a hand refractometer (Kearns and Inouye 1993).

Pollen production—Mature anthers were crushed in lactophenol-glycerine with aniline blue. A known dilution was placed on the grid and 10 replicate counts were made using a Hemocytometer (Barrett 1985). Pollen–ovule ratio was calculated following Cruden's (1977) method.

Pollen viability—Freshly collected Pollen grains from flowers were taken and viability test was carried out using 0.2% TTC (2, 3, 5-triphenyltetrazolium chloride) and acetocarmine solution in sucrose following the method of Hauser and Morrison (1964). To study *in vitro* pollen germination, pollens collected at the time of anthesis were germinated *in vitro* in the cavity slides containing sucrose solution of different concentrations (2%, 5%, 10%, 15%, 20%, 25%, 30% and 40%) in combination with that of boric acid (50, 100, 200, 300 and 400 ppm). For *in vitro* pollen germination the slides were then kept in Petridishes lined with moist filter paper at room temperature (25°C) and examined under Dewinter (Ultima) microscope at different time intervals to know the germination percentage and pollen tube length following the method of Shivanna and Rangaswamy (1992). The *in vitro* pollen germination test was carried out at different time intervals after anthesis.

Stigma receptivity— Stigma receptivity was examined by cytochemical localization of esterase, catalase on stigmatic surface. Receptivity of the stigma was examined by the method of Joshirao and Saoji (1989). The stigmas were fixed in acetic-alcohol (1:1), kept in 4N NaOH and stained with

aniline blue. For the localization of esterase on stigmatic surface, alpha- naphthyl acetate as a substrate, 0.15 M phosphate buffer (pH 6.8) and fast blue B Salt had been used following the methodology of Shivanna and Rangaswamy (1992).The evolution of bubbles per minute on the stigma as an indicator of catalase activity was recorded using hydrogen peroxide as substrate (Zeisler 1933).

Atmospheric pollen frequency—The hourly atmospheric pollen incidence was determined by operating 'Rotorod' sampler to know the potential role of air borne pollen grains in pollination and the frequency of trapped pollen grains were calculated (Mondal *et al.*1997).

Breeding system—Breeding behaviour of the trees was determined by autogamy, geitonogamy and xenogamy which were tested through controlled pollinations following the procedure of Aluri and Reddi (1994).

Pollination—Observations on the floral visitors and their behaviour were made following the procedure given by Faegri and van der Pijl (1979) and Dafni (1992). Their behaviour on the flower, time of visit etc. were recorded.

RESULTS AND DISCUSSION

Flowering phenology—The trees exhibited a brief deciduous phase of 25–35 days during April –May. Leaves defoliated in between 1st week of April and 1st week of May. Occurrence of new leaves commenced during the 3rd and 4th week of May and continued till June. Flowers were borne within 1st week of October and 2nd week of December. The proportion of anthesized flowers increased gradually in number and reached a peak in the 3rd and 4th week of October. The duration of flowering period ranged between 20-38 days i. e. with an average of 29 days. Each plant characteristically showed eight mass flowering days.

The inflorescences are profusely branched in axillary or terminal racemes. Flowers opened from 05:00 h to 06:30 h in the morning in an acropetal order. The flowers were bright yellow showy and emitted mild fragrance. The zygomorphic flowers are typically papilionaceous and bisexual. The flowers are medium in size, 2.5 cm in across. Stamens are monadelphous with ten anthers. Anthers are uniformly yellow. Stigma is slightly curved upward and extends beyond the length of anthers. Unilocular superior ovary contains two ovules. According to Faegri and van der Pijl (1979), the flowers are of 'flag blossoms' type. After flower opening the corolla became brighter and gradually turned into pale yellow with age. Anther dehiscence took place asynchronously. Aggregation of flowers in mass being one of the primary attractants has the various consequences with respect to pollinator attraction and successful pollination (Richards 1997). Mass blooming also facilitates the harvesting of foraging resources from large plants and increased the visitation rates per flower (Westphal *et al.* 2003).

Pollen production—Anther dehiscence occurred by means of longitudinal slits. The pollen grains are tricolporate and spheroidal. Each anther produces 3,518±12.79 pollen grains and each flower produces 35,180±127.95 pollen grains accordingly.

Pollen viability— Pollen viability was tested by acetocarmine (1%) and 0.2% TTC (2,3,5- triphenyl tetrazolium chloride) solution which exhibit 85 % (Fig.2A) and 82% (Fig.2B) viable pollen grains respectively. According to Dafni and Firmage (2000) pollen viability is considered as an important parameter of pollen quality. Staining with TTC is common technique used to determine pollen viability (Dafni and Firmage 2000). But *in vitro* pollen germination is the most reliable process to study the viability of pollen. In case of *in vitro* pollen germination maximum 75.65 % germinating pollen along with 325 µm tube length was recorded in 10% sucrose supplemented with 100 ppm boric acid after three hours of incubation (Fig. 2C). For *in vitro* pollen germination and tube growth sucrose is the best source of carbohydrates as it maintains the osmolarity and is a substrate for metabolism of pollen (Sari-Gorla *et al.* 1997). Boron is crucial for pollen germination along with pollen tube development in most species (Brewbaker and Majumder 1961), thus play an important role in fertilization of flowering plants towards the successful fruit and seed production. However, sucrose in combinations with boric acid promoted pollen germination as well as tube development because boron makes a complex with sugar and this sugar-borate complex is known to be capable of better translocation than non-borate, non-ionized sugar molecules (Vasil 1964 and Sidhu and Malik 1986). The conspicuous role of sucrose and boric acid on *in vitro* pollen germination is well established (Shivanna and Johri 1985). The present observation also gets support from earlier works (Bhattacharya and Mandal 2004, Ghanta and Mondal 2013, Pal and Mondal 2016). Reduced pollen viability was observed after 4 and 6 h of anthesis i.e 42.35% and 25.46%, respectively. Only 5% viable pollen grains were found after 8 h of anthesis and beyond that no pollen germination was observed.

Stigma receptivity— Successful pollination is determined by the flower opening time, anther dehiscence and also stigma receptivity (Renata *et al*. 2006). To study the receptivity of stigma, the style was divided into 4 stages based on time after anther dehiscence (2, 4, 6 and 8 hours after anther dehiscence) in the flowers. Maximum receptivity of stigma in terms of esterase and catalase activity was found after 4 hours of anthesis. The time of stigma receptivity is an important stage in the life cycle of the flowers for successful pollination towards the maturation of flower which may greatly influence the success of post-pollination events (Barrett 2002). Esterase and catalase expression during high receptive period becomes significant in that time after anther dehisences highlights the role of esterase and catalase toward the stigma receptivity. So, this study of stigma receptivity also gets support from earlier works (Bhattacharya and Mandal 2004, Choudhury *et al*. 2012).

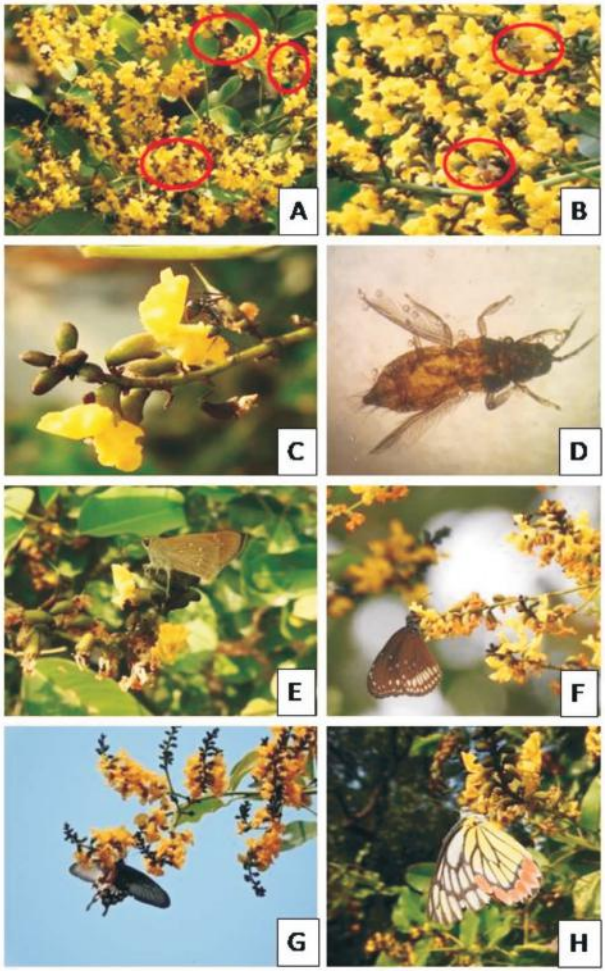

Fig. 1— Flower visitors of *Pterocarpus marsupium*; A. *Apis dorsata;* B. A. *cerana indica*; C. *Camponotus compressus*; D.Thrips; E. *Borbo cinnara*; F. *Euploea core*; G. *Pachliopta aristolochiae;* H. *Delias eucharis.*

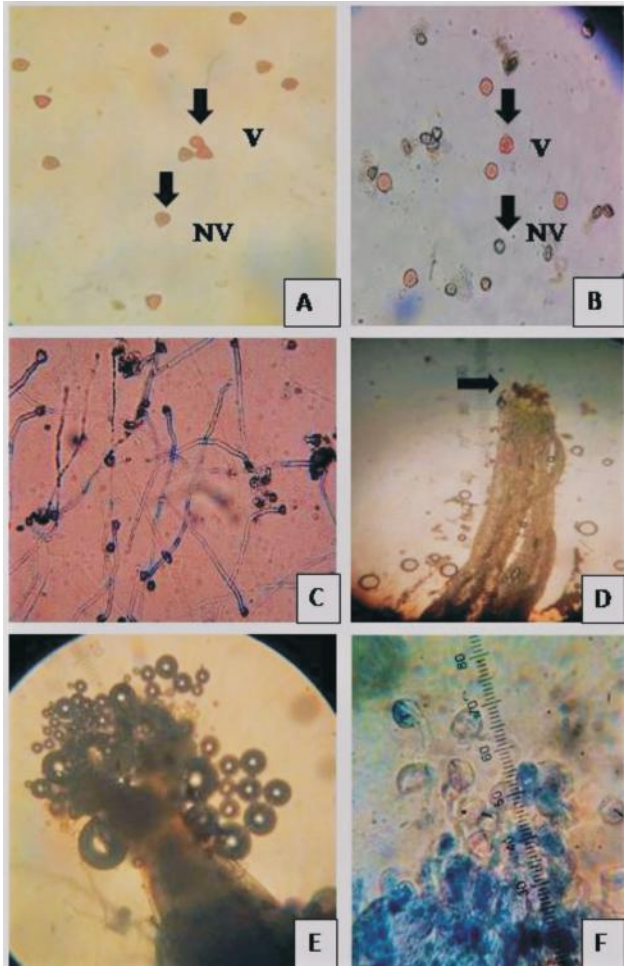

Fig.2—Pollen viability and stigma receptivity; A. Pollen viability in Acetocarmine (V-Viable; NV- Non viable); B. Pollen viability in TTC (V-Viable; NV- Non viable); C. *In vitro* germinating pollen; D. Presence of esterase over stigmatic surface; E. Bubbles over stigma showing catalase activity; F. *In vivo* pollen germination.

Breeding system—The fruit-set through open-pollinations was 32%, however only 4% fruit set was observed in the bagged flowers. In netting condition 12 % fruit set has been observed (Table 4). Fruit-set in bagging condition is due to selfing nature of plants which is enhanced by thrips as they help in auto deposition of pollen grains to the stigma. Thrips are the small fringed winged weak flayers and frequent in flowers for forage on pollen and nectar. They are able to enter the flower even in bud condition. They crawl through style to stigma and help in pollen deposition over the stigma thereby effect effortless selfing as stigma is extended beyond the anther level. Thrips visit flowers for their forage and also as a site of oviposition (Appanah and Chan 1981).The body surface carried considerable amount of pollen grains and come into intimate contact with stigmatic surfaces and help in pollination. Fruit formation in netted condition is due to air mediated pollen transfer (maximum 4.1% at 11:30) as well as by thrips. According to Ashton *et al*. (1988) movement between tree crowns and cross pollination appear to result from the thrips but limited flight largely promotes effortless selfing. But in case of hand pollination experiment 24% fruit set were observed through xenogamy which is better than fruit formation through geitonogamy (16 %) and autogamy (10%). Pollen ovule ratio is the indicator of plant's breeding system. Pollen ovule ratio is 17,900:1 which indicate that the plant is obligately outcrossing (Cruden 1977). Therefore, the breeding system is mixed with selfing and outcrossing. In the mating system outcrossing is predominant and self-pollination may assure the reproductive success (Zhand and Zi 2008, Ai *et al*. 2013).

Pollination—Both, nectar and pollen constitute rewards for the flower visitors. During the time of flower opening nectar was absent but nectar secretion increased gradually by the time. The amount of nectar was measured at hourly intervals between 06:30 h and 18:30 h on the flower opening day. The total amount of nectar collected in each flower was 10 µl in average. Maximum nectar production occurred between 09:00 h and 11:00 h. Freshly opened flowers are brightly coloured and attract a large number of visitors (Table 2). Maximum visitation occurred between 09:30 h and 11:30 h After flower

Table 1- Floral characters of *Pterocarpus marsupium*

Flower/Inflorescence		15 – 20
Colour		yellow
Shape		Zygomorphic
Size		2.5 – 3.0 c.m in across
Pollen production/flower		3518×10=35180±127.9
Pollen character		Tricolporate, spheroidal
Mode of anther dehiscence		Longitudinal
Number of Ovule/Flower		2
Pollen viability	Acetocarmine	85.0%
	TTC	82.0%
	In vitro pollen germination	75.65%
Average nectar production/ flower		10 µl
Sugar concentration of nectar		22 Brix
Anthesis period		05:30- 06:30 h

Table 2- Flower-visitors and their foraging behaviour in *Pterocarpus marsupium*

Flower-visitors	Mean abundance(N=5)	Foraging period	Time spent/flower(Sec.)	Reward
Apis dorsata (Apidae)	43.8±0.54%	06:00 - 15:00	12-14	Pollen and/ or Nectar
A. cerana indica (Apidae)	18.6 ±0.20%	06:00 - 15:00	10-12	Pollen and/ or Nectar
Borbo cinnara (Hesperiidae)	9.2±0.14 %	07:30 – 13:00	3-5	Nectar
Camponotus compressus (Formicidae)	4.2±0.34 %	Day and night	2-5	Nectar
Catopsilia pyranthe (Pieridae)	6.0±0.28 %	07:30 – 13:00	3-4	Nectar
Delias eucharis (Pieridae)	6.8±0.23 %	07:30 – 13:00	2-4	Nectar
Euploea core (Nymphalidae)	6.0±0.17 %	07:30 – 13:00	3-4	Nectar
Pachliopta aristolochiae (Papilionidae)	3.0±0.12%	07:30- 13:00	2-4	Nectar
Thrips (Thripidae)	2.4±0.16 %	Day and night	20-25	Pollen and/ or Nectar

opening insects (Fig.1) like *Apis cerana indica, A. dorsata,* and *Camponotus compressus* of Hymenoptera*, Borbo cinnara, Catopsilia pyranthe, Delias eucharis, Euploea core* of Lepidoptera were found to visit the flowers in day time (06:00 h to 17:00 h) for their forage while thrips (Fig.1D) of Thysanoptera visited throughout the day and night for their forage as well as brooding place. During, visit thrips carries considerable amount of pollen grains through their body parts and also help in pollination. The role of thrips has been established in different taxa (Ananthakrishnan 1982, Annadurai and Velaydhan 1986). The percentages of flower visitors of different categories of insects are presented in tabular form (Table 2). The flowers exhibit papilionaceous corolla and nectar is located at flower base. The study revealed that bees (*A. dorsata* and *A. cerana indica*) paid more visit than the other insects i.e. 62.4% of total foraging visit while the other insects constituted 37.6% visit in together (Table 2). The present findings of the study showed that the rockbee, *A. dorsata* spent more time to forage followed by *Apis cerana indica, Borbo cinnara, Catopsilia pyranthe, Euploea core, Camponotus compressus, Delias eucharis* and *Pachliopta aristolochiae*. Among the honey bees, *Apis dorsata* were more in number during forage and more efficient in probing the flowers than *A. cerana indica.* Bees probed the flowers in search of nectar from the base of flowers from an upright position and inserted into the flower through the floral gap between petal and staminal column. Bees are diverse in body size, length of proboscis and many other biological aspects (Roubik 1989). To get access the nectar from the flowers *A. dorsata* easily collect nectar but *A. cerana indica* took more than one attempts for nectar. During their forage, pollen grains get adhered to the body and transfer pollen grains sternotribically to the stigma. Among the bees *A. dorsata* are large in size than *A. cerana indica.* Being the largest bees among the *Apis* (Oldroyd *et al*. 2000) *A. dorsata* forage in the canopy which gets support from Perry (1984) and Bawa *et al*. (1985). Bee flowers are zygomorphic, mechanically strong having landing platform and semi closed petals (Faegri and van der Pijl 1979). According to Endress (1995) bees prefer yellow flowers. The role of Indian rock bee in pollination has been established (Rao *et al.* 2001, Sharma *et al*. 2006). Ants other than bees of Hymenoptera play minor role in pollen deposition to the stigma. The members of Lepidoptera have proboscis for collecting the nectar. When they forage on flowers for nectar they insert their proboscis in such way that it touches hardly the essential organs and act as nectar robber rather than pollinator.

Table 3- Incidence of atmospheric pollen

Time (h)	5:30	7:30	9:30	11:30	13:30	15:30	17:30
Pollen grains (%)	0.89	2.45	4.35	4.1	3.0	1.25	1.0

Table 4- Fruit sets in different conditions

Treatment		No. of flowers studied	No. of flowers set fruit	Fruit set (%)
Open		50	16	32
Netting		50	6	12
Bagging		50	2	4
Controlled	Autogamy	50	5	10
Pollination	Geitonogamy	50	8	16
treatments	Xenogamy	50	12	24

Studies on flower-visitors interaction indicated that the honey bees mainly *A. dorsata* are the major and legitimate pollinator and the occurrence of insect and wind mediated pollen transfer and fruit set in different conditions indicates that the plant is with mixed type of breeding system in nature. Understanding of such flower-visitors interaction will be helpful in the conservation of this near threatened plant in natural ecosystem.

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