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Reproductive biology of *Gynochthodes umbellata* (L.) Razafim. & B. Bremer (Rubiaceae)

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ABSTRACT

Gynochthodes umbellata (Syn: *Morinda umbellata*), belonging to the family Rubiaceae, is a climber with bright orange fruit having potent medicinal properties. This plant is a rich source of many biologically active compounds. Traditionally it is used for treating dysentery and diarrhoea. The leaves are valued for their antimicrobial and antioxidant properties, and they are used to expel intestinal worms. Fruit juice expels toxins and regulates menstruation cycle. The ripened fruits of *G. umbellata* are eaten raw. Unripe green fruits are used in curries. Wild populations of *G. umbellate* are severely depleted owing to its over exploitation. Natural regeneration through root suckers is slow. Propagation through seeds is beset with problems of poor viability and germination of seeds. Therefore, a comprehensive knowledge on reproductive biology is required for effective management, conservation and sustainable utilization of *Gynochthodes umbellata*. To achieve this, flowering phenology, floral biology, pollen production per anther, pollen-ovule ratio, pollen viability, stigma receptivity, pollen visitors and pollination and breeding system were analysed. The plant is with two morphotypes: 'staminate' and 'pistillate' flowers are born in separate plants in terminal umbel inflorescence, with flowering during February-May. Fruit is infructescence, reddish orange at maturity, fleshy and edible. Pollen viability and fertility was found to be high. Number of pollen grains per flower was higher in 'staminate' flowers compared to the 'pistillate' flowers. Percentage of *in vitro* pollen germination was 28% in staminate flowers in 5% Brewbaker's medium. No *in vitro* pollen germination was noticed in pistillate flowers. Common wasps belonging to the order Hymenoptera are the floral visitors in this species. The breeding system studies indicated that self and cross pollination occur in 'staminate' and only cross pollination in 'pistillate' morphotype.

Keywords : *Gynochthodes umbellate*, pistillate, staminate, morphotypes

Gynochthodes umbellata (L.) Razafim. & B, a woody climber belonging to the family Rubiaceae, is native of Southern India and the Deccan peninsula to Burma, China, Srilanka, Southeast Asia, Philippines, Northern Australia, Japan and in some regions ascending to altitudes of 1500m (Petelot 1953, Anonymous 1962).It is commonly known as Common Indian Mulberry or Vomit vine or climbing noni or nino and in India, it may be called nuna or Chotaalka (Drury 1873, Perkin and Everest 1918, Quisumbing 1951). In India, it is seen in East Bengal, Khasia hills upto1300m, Madras State, Eastern Ghats, Vishakhapatnam, Deccan, Chengalpettu, N.Coimbatore, and the Western Ghats and this species is seen in the plains of Kerala, especially in sacred grooves (Sasidharan 2004, Vijayaraghavan 2011). Earlier this plant was included in the genus *Morinda*, however, based on the molecular studies by Razoafimandimbison et *al.*(2009) and Razafimandimbison and Bremer (2011), this species has been excluded from the genus *Morinda* and included in the genus *Gynochthodes.*

This plant is a rich source of many biologically active compounds. Traditionally it is used for treating dysentery and diarrhoea. The leaves have antimicrobial and antioxidant properties and they are used to expel intestinal worms (Burkill 1935, Vijayaraghavan 2011). Fruit juice expels toxins and regulates menstruation cycle. The ripened fruits of *G. umbellata* are eaten raw. Unripe green fruits are used in curries (Anonymous 1962). In India and Vietnam, the leaves and roots are employed for treating dysentery and diarrhoea (Kritikar and Basu 1935, Petelot 1953). The roots are reported to be utilized as a drastic purgative in American medicinal practice (Quisumbing 1951). The roots are applied for dropsy. The leaf powder was found to possess high medicinal properties and the root bark contains colouring constituents - anthraquinones. *G. umbellata* is used for leukemia, gonorrhea and syphilis (Ismail and Sultahana 2008). The curry made up of the green fruit is useful in removing phlegm, against common cold, herpes, tumours, leprosy, gonorrhea, and dysuria. Fruits are rich in carbohydrates, proteins, fibre, fatty acids, vitamins (C, A, B_1, B_2) and minerals (Anjusha and Gangaprasad 2016).

The family Rubiaceae shows a wide spectrum of floral mechanisms characterized by different types of gynoecium and androecium organization. Despite this diversity, Robbrecht (1988) pointed out the presence of three reproductive strategies common in Rubiaceae, which are related to certain groups within the family. Reproductive failure, as a result of constraints in one or several reproductive events, is the driving force for species extinction. Therefore, a comprehensive knowledge on reproductive biology is required for effective management, conservation and sustainable utilization of *Gynochthodes umbellata* In the present study a detailed analysis on the floral characters and breeding systems were carried out to find out the reasons of rarity of this plant in the wild and also to formulate strategies to conserve the plant.

MATERIALS AND METHODS

Present study was carried out in the Kerala university Campus, Kariavattom,Thiruvananthapuram, Kerala from July 2014 to September 2016. Feild data were collected during the peak flowering time of February to May for the two years.

Flowering phenology and floral morphology—Phenological data on initiation of flowering, peak flowering period, time of anthesis, anther dehiscence, stigma receptivity and time of fruiting was recorded. Ten flowers each from ten individuals were monitored from anthesis to flower abscission. In order to estimate the number of flowers per plant, inflorescence number per plant and flower number per inflorescence from random samples of 20 plants of both 'staminate' and 'pistillate' were counted. Total number of flowers per plant was calculated. The details of flower morphology were analyzed and measured with the aid of stereomicroscope (Olympus SZ61).

Determination of pollen production per anther—To determine the number of pollen grains per anther, 20 flowers were collected before anther dehiscence, and the two anthers from each flower (40 anthers) were gently removed and vortexed in a tube containing 100 µl of 0.5 M sucrose. The pollen suspension was injected into a haemocytometer and the total numbers of grains within the 25-square counting area were determined. The number of pollen grains per anther was calculated by using the formula; (mean number of pollen grains per square x 10t x 0.1)/40 where 0.1 is the volume of sucrose solution in ml and 40 is the number of anthers used. To estimate the number of pollen grains per flower, the number of pollen grains per anther was multiplied with the average number of anthers per flower (Barret 1985).

Determination of pollen-ovule ratio—Pollen/ovule ratio was calculated according to (Cruden 1977) For counting all pollen grains (stained by acetocarmine) from a half of a thecae of anthers from 20 flowers and all the ovules from 20 pistills in both cases one flower was used per plant, and the observation made under a microscope .

Pollen viability studies

Fluorochromatic reaction test—Pollen viability study by using FCR test was conducted according to Heslop-Harrison and Heslop-Harrison (1970). For this, fluorescein diacetate (FDA) in acetone (2mg/ml) and sucrose solution (20%) were prepared separately as stock solutions. In a glass vial, 5ml of sucrose solution was taken and FDA was added drop by drop until it became turbid. Drops of this mixture were taken on a micro slide and sufficient quantity of pollen grains were placed and uniformly spread. The preparation was incubated in a humidity chamber for 5-10 min and observed using fluorescence microscope under UV excitation. The viable pollen grains showing intense fluorescence were counted and the percentage of viability was calculated.

***In vitro* germination studies**— *In vitro* germination of pollen grains was carried out in Brewbaker and Kwack's medium (1963) supplemented separately with different concentrations of (5-50%) freshly prepared sucrose solution. For this, pollen grains were collected from the plants just before anther dehiscence and dusted on a drop of culture medium placed on a clean slide. The slides were incubated in a germination chamber consisting of a pair of petri dishes lined with moist cotton. The slides were kept undisturbed for 2h. at room temperature. Pollen grains which germinated were taken as viable while others were non- viable. The experiment was repeated using pollen grains from ten plants .Percentage of pollen viability was calculated.

Stigma receptivity—Aniline blue fluorescence method (Martin 1959) was also used to locate the pollen tubes on the pistil. For this, pistils were fixed for about 24 hr. and stored them in 70% ethanol. The fixed pistils were transferred to 0.8% NaOH for 12 hr. to make the tissue soft. Then the tissues were thoroughly washed in distilled water and stained with 1% of decolourised aniline blue. Stained pistils were mounted on a drop of glycerin and gently pressed without scattering the tissue too much. The preparations were observed under light microscope using the "image analyzer software" (Leica DM 200) and photomicrographs were taken. Stigma receptivity was also tested with stigmatic peroxidase activity (SPA) according to the method of Kearns and Inouye (1993). The intact styles of emasculated flowers were placed on a glass slide in a drop of 3% hydrogen peroxide and covered with a cover slip. Stigmas that produced bubbles within 2-3 minutes were considered as receptive.

Floral visitors—Different flower visitors were found to visit the flowers soon after flower opening. In the field, period of visiting and the behavior of floral visitors were examined during the flowering February–May and the observations were recorded.

Pollination and breeding system—Percentage of fruit-set in the following pollination experiments were estimated both for'staminate' and 'pistillate' plants. For each pollination experiment 50 flowers each were used.

1. Open pollination: flower buds were tagged and fruit set at maturity was recorded.
2. Autogamy: Mature flower buds were tagged and bagged with a cloth mesh bag, and fruit set at maturity was recorded.
3. Cross pollination: Mature flower buds were bagged prior to anthesis and pollen grains were transferred from staminate to pistillate flowers and reciprocal crosses were also made, flowers were rebagged and fruit set was observed.

Fruit maturation period and period of detachment from the parent were recorded for each pollination experiment separately. The number of seeds per fruit were determined for 10 open-pollinated fruits from each plant. All seeds from each

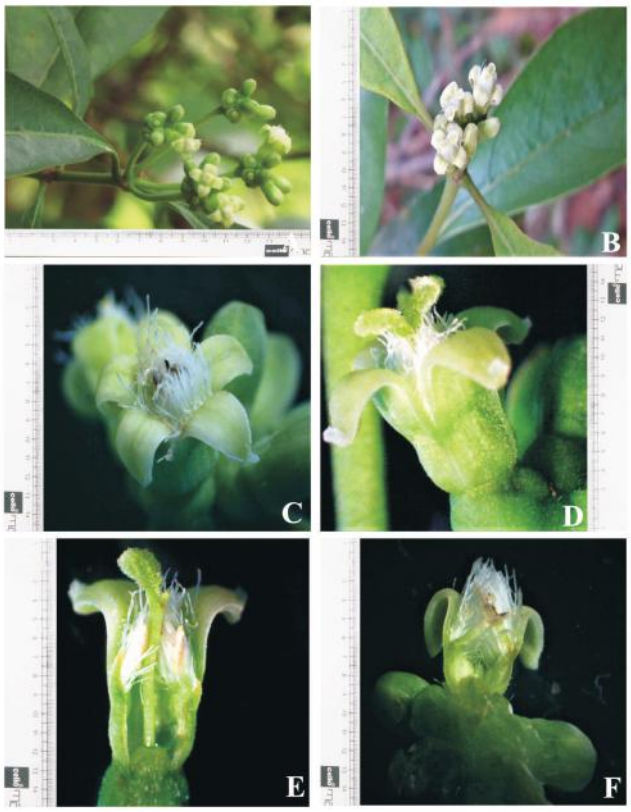

Fig. 1 A-F: Floral morphology. A. Inflorescence with staminate flowers, B. Inflorescence with pistillate flowers, C. Staminate flower, D. Pistillate flower, E. L.S of pistillate flower, F. L.S of staminate flower.

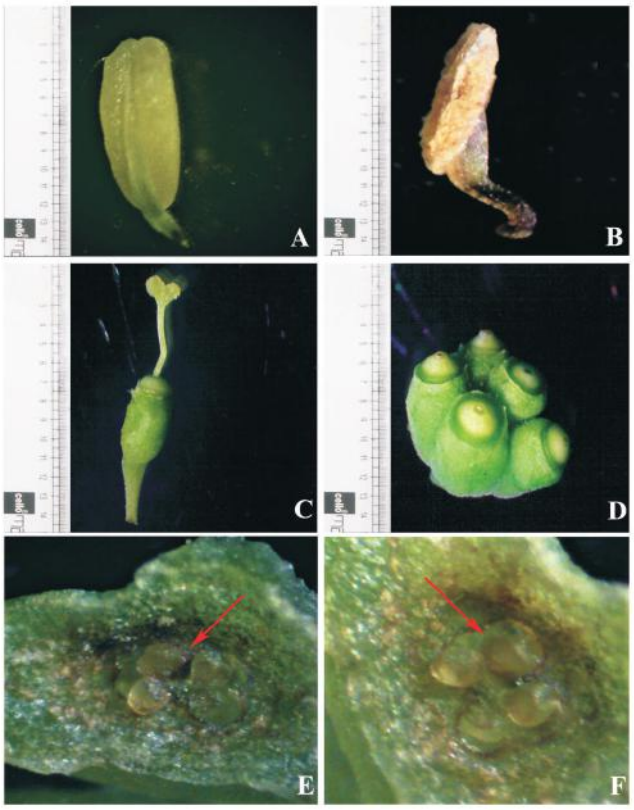

Fig. 2 A-F: Floral parts. A. Anther from staminate flower, B. Anther from pistillate flower, C. Gynoecum, D. Mature ovary, E. C.S of ovary with ovules in staminate flower, F. C.S of ovary with ovules in pistillate flower.

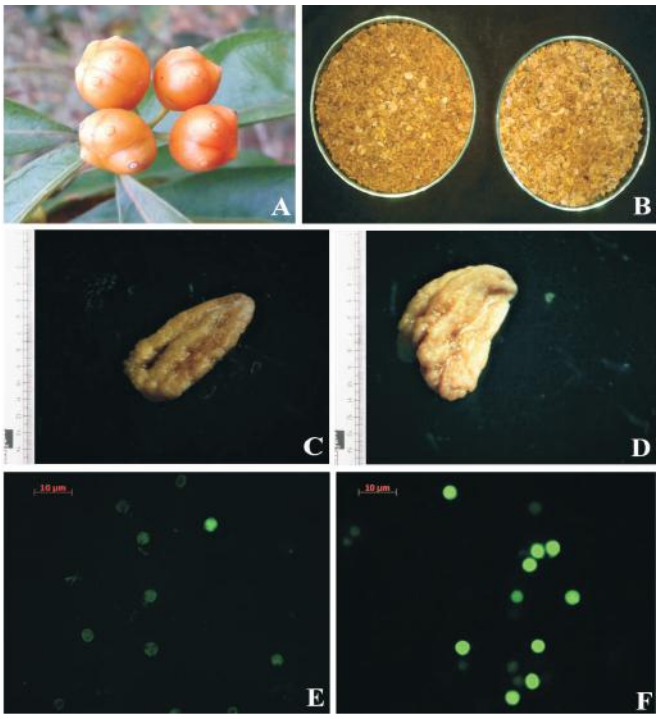

Fig. 3A-F: Fruit, seed and pollen. A. Ripe fruits; B. Seeds from staminate and pistillate plants; C. Single seed from staminate flower; D. Single seed; E &F. Pollen viability tested by FDA ; E. Pollen from pistillate flower; F. Pollen from staminate flower.

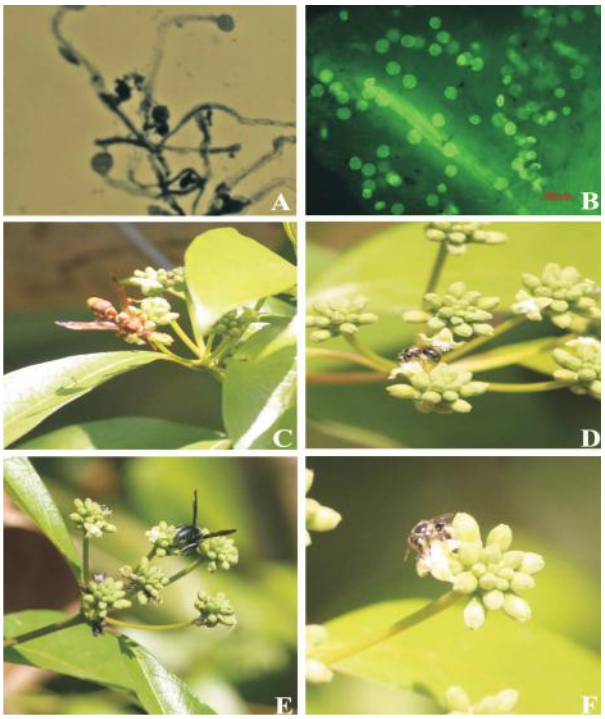

Fig. 4A-F: Pollen viability and pollinators**.** A. *In vitro* germination of staminate pollen; B. *In vivo* pollen germination on stigma of pistillate flower; C, D, E & F Insect visitors.

fruit were kept in separate paper envelopes and used for germination study.

RESULTS AND DISCUSSION

Flowering phenology and floral morphology—*Gynochthodes umbellata* flowers during February–May. The inflorescence is a terminal umbel with 7-10 flowers per inflorescence (Figs. IA and B).Two floral morphotypes viz. 'staminate'(Fig. IC) and 'pistillate'(Fig. ID) flowers are found in separate plants. 'Pistillate' flowers have a style with two stigmatic lobes and four or five abortive stamens (Fig. IE) while staminate' flowers have four or five stamens and style and stigma were absent (Fig. IF). Flowers open in the morning 7:00 -8:00 h and anther dehiscence occurred between 07:30 to 08:30 h. Flowers last only one day after anthesis. Difference in the size of anthers was noticed between staminate and pistillate flowers (Figs. 2A and B). Stamens are epipetalous, anthers oblong with short included filament. The stigma of 'pistillate' flower is protruding beyond the corolla tube and it positioned above the anthers. Stigma is bifid and style is slender with an average 2mm long (Fig.2C). Ovary is start to develop immediately after withering of corolla (Fig.2D).Ovary is inferior with two locules (Figs. 2E and F). Fruit is infructescence (syncarpium) and green in colour which became reddish orange at maturity (Fig. 3A).Seeds are yellow in colour with size difference was noticed between different morphoforms (Figs. B,C and D). Major difference in the floral features of two morphotypes are given in the Table 1.

Table1– Major differences between 'staminate' and 'pistillate' plants of G.umbellata

'Staminate'	'Pistillate'
7-10 flowers/ Inflorescence	4-5 flowers / Inflorescence
White hairs in the corolla tube more in number	White hairs in the corolla tube less in number
Stigma and style absent	Stigma and style present; stigma protruding outside
Ovary present (inferior ovary with 2 locules)	Ovary present (inferior ovary with 2 locules)
Stamens present ; larger in size	Stamens present but smaller in size
Pollen grains are more in number	Pollen grains are less in number
Pollen viability – 90%	Pollen viability – 13.3%

Total pollen production—The average number of anther per flower was found to be four and in 'staminate' the number of pollen grains per anther was 0.105. The total number of pollen grains per flower was calculated to be 0.42. Pollen ovule ratio was found to be 0.10. In 'pistillate', number of pollen grains per anther was 0.07The total number of pollen grains per flower was calculated to be 0.28. Pollen ovule ratio was found to be 0.07 Pollen grains are 3-zonocolporate.

Pollen viability studies—Pollen viability test using FCR showed 13.3±5.12% viability in 'pistillate' flowers (Fig. 3E) and 90.66±2.34% viability in 'staminate' flowers (Fig. 3F) and Pollen viability records at different time intervals showed a significant decline in viability after four hours of anther dehiscence.

In vitro the pollen germination study using Brewbaker and Kwack medium supplemented with 5% sucrose showed 28.12±2.2% pollen germination. (Figs. 3E and F).

Stigma receptivity—No pollen germination was observed on the surface of the stigma after *in vivo* Periodic examination of stigma by using stigmatic peroxidase activity test, the stigma after 2 h. of anthesis showed rapid bubbling of oxygen from added H, O, . Thus it is assumed that stigma has maximum receptivity by 10.00 am**.**

Flower visitor—Wasps (Hymenoptera sp.) and Weaver ants (*Oecophylla smaragdina)* are found to be the frequent floral visitors.

Breeding system—In order to understand the breeding system operative in these species, different pollination experiments like open pollination and cross pollination were carried out. Observations were recorded and shown in Table 2.

Table 2- Types of pollination and fruit set percentage.

Type of pollination	Fruit set	
	'Staminate'	'Pistillate'
Open pollination	100% (25)	100% (25)
Autogamy	100% (25)	0% (25)
Cross pollination pollination	0% (PxS) (25)	80% (PxS) (25)

Determination of natural fruit and seed-set—Natural fruit set in 50 staminate and 50 pistillate flowers were analyzed and was found that in 'staminate' plants it was 52.8 ±4.65 and 33± 6.40% in 'pistillate' plants. After pollination the floral parts became black and dry. The ovary enlarge and develops into fruits within 30-35 days. Fruit and seed characters of different morphotypes are given in the Table 3

Table : 3 – Comparisons of fruit and seed characters of 'staminate' and 'pistillate' plants.

Characters	'Staminate '	'Pistillate'
No. of fruits /umbel	5.80 ±1.32	3.00 ±1.05
Weight of fruit (g)	0.77 ±0.09	0.69 ± 0.15
Fruit length (mm)	13.07 ±0.9	11.10 ±1.91
Fruit width (mm)	12.13 ± 0.75	11.58 ± 1.91
Number of seeds per fruit	14.10±1.79	12.30 ±1.06
Weight of seed (g)	0.02±.001	0.01±.005
Seed length (mm)	5.07 ± 0.52	4.76 ± 0.53
Seed width (mm)	2.42 ± 0.28	2.27±0.24

The reproductive biology of plants is essential for developing effective strategies for their conservation and sustainable utilization. It is important for determining barriers to seed and fruit set for understanding pollination and breeding systems that regulate the genetic structure of populations. Considering the ever increasing demand for phytochemicals present in the plant parts of *G. umbellata* and its dwindling population in the wild, development of suitable conservation strategies is the only alternative to utilize its astonishing medicinal properties. Any conservation approach requires an in-depth study of the phenology and reproductive biology (Moza and Bhatnagar 2007).The phenological studies in general and flowering in particular are useful in planning the conservation strategies as well as formulating measures for cultivating such species on large scale (Bernardello *et al.* 2001).

In oder to understand the reproductive biology in angiosperms it is essential to have a thorough knowledge of phenology and flowering morphology. *G. umbellata* flowers during February –May. . Anthesis and anther dehiscence is most important event in the process of flower development. Flowers open in the morning from 7:00-8:00 h and anther dehisced from 07:30-08:30 h. Pollen grains are 3-zonocolporate. Flowers last only one day after anthesis. In the present study, it was observed that the 'staminate' and 'pistillate' plants produce flowers which are morphologically different, however, the two morphotypes cannot be distinguished until the flowering time.

The number of pollen grains per flower, pollen: ovule ratio and pollen viability revealed the sufficiency of pollen traits for fertilizing the ovules. The use of pollen-ovule ratio as a method of estimating breeding system, developed by Cruden (1977), is relatively quick, low cost method of estimating breeding system compared to molecular and crossing techniques.

In the present study, Brewbaker and Kwack's medium containing 5% of sucrose was found to be more suited for *in vitro* germination of pollen. A maximum of 28.12+2.2% germination of pollen in 'staminate' flower was observed. Thus, in the present study, it can be inferred that failure of pollen germination in high sucrose concentration is due to the lack of normal osmotic gradient for pollen rehydration and germination.

Pollen viability is generally tested by FCR test which has been suggested by Heslop–Harrison and Heslop–Harrison (1970). FCR test revealed that in 'pistillate' flowers 13.33±5.12% and in 'staminate' flowers 90.66±2.34% pollen grains were viable and *in vivo* pollen germination was not observed.

Stigmatic receptivity is known to be an essential stage in flower maturation and it may significantly influence the rate of self-pollination and pollination success (Dafni and Maues 1998). In the present study periodic examination of stigma was carried out by using stigmatic peroxidase activity test. The stigma after 2 h of anthesis showed rapid bubbling of oxygen from added H, O,. Thus it is assumed that stigma has maximum receptivity by 10.00 am.

. Pollination success in plants is determined by the timing of flowering, anther dehiscence and stigma receptivity (Renata *et al.* 2006). It was observed that insects were attracted by freshly opened flowers. Pollinators were active during day time especially 09:00 – 11:00 h. The sudden decrease in the insect visit may be due to rise in temperature. Average time spent by an insect on a flower was 10-15 seconds. This implied that the foraging speed was very fast. However, during rainy season insects were less active than on sunny days.

The breeding system of a species or a population is determined by a large number of pre- and post- fertilization factors, either biotic or abiotic (Barret 1998). The biotic factors include pollination efficiency, energy resource allocation for fruit and seed production, natural abortion rates, flower, fruit and seed predation, as well as germination capacity (Wiens 1984, Wiens *et al.*1987, Beardsell 1993). Among pre-fertilization factors, nutrient and pollen availability are of paramount importance (Haig and Westoby 1988, Parra-Tabla *et al.* 1998). Present breeding system experiments revealed that 'staminate' flowers are autogamous as well as xenogamous in nature. But the 'pistillate' flowers are only xenogamous in nature because their pollen grains are not viable. Based on the population study, it was found that 'pistillate' plants are less than the 'staminate'. Because of this, the 'staminate' plant shows the autogamous nature to overcome the reduction in the number of 'pistillate' plants.

According to Cruden (1977), the pollen/ovule (P/O) ratio is used as indicator of the reproductive system of angiosperms (lowest P/O values correspond to autogamous species while the highest correlate with xenogamous species). In the present study, P/O values are low in both the morphs. It was observed that natural fruit set percentage was higher in 'staminate' (52.8±1.2 %) than in 'pistillate' (33.4±4.1%) flowers. The number of seeds per fruit was also higher in 'staminate' (14.10±1.79%) than in 'pistillate' morphs (12.30±1.06%). The presently studied flowers showe dimorphism in the their characters. But staminate flowers produced fruits after open and self-pollination indicating that rudimentary pistil is functional and produce fruits and seeds. In pistillate flowers only the pistil is functional and show 80.22±3.1% of fruit-set by cross between "pistillate" x "staminate" flowers, whereas there was no seed-set in the reciprocal crosses.

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