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Floral biology, breeding system and Pollination of *Oxystelma esculentum* (L.f.) Sm.

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ABSTRACT

The present paper reveals the floral biology, breeding system and pollination of *Oxystelma esculentum* (L.f.) Sm. which is a medicinally important plant distributed throughout the India. The flowering period of plants ranged from July to January and the beautiful, pale pink, nectariferous flowers start to open from 05:30 h and continued up to 06:30 h. A single flower produced 485±23.0 pollen grains in average. Different insects represented by Hymenoptera, Lepidoptera and Thysanoptera were found to visit the flowers for nectar. *Apis dorsata* were found as the most dominant and effective one among the flower visitors. The fruit set in natural open conditions was 8.8 %, however no fruit set was observed in netting and bagging condition. In case of hand pollination 12 % fruit set were observed through xenogamy which is better than fruit formation through geitonogamy (4 %) and autogamy (0). Results from the breeding experiment suggested that, the plants exhibits xenogamous breeding system.

Keywords : Floral biology, breeding system, pollination, *Oxystelma esculentum*

Oxystelma esculentum (L.f.) Sm. is commonly known as 'rosy milkweed vine'. The plant is slender, glabrous, lactescent climbing perennial herb of sub-family Asclepiadoideae (Apocynaceae). The species has a wide range of distribution from northeastern Africa to southwestern Asia. The plant has been recorded from Egypt, East through Iraq, Pakistan, Sri Lanka, India, Bangladesh, Myanmar and Nepal to Southern China (Guangdong, Guangxi, Yunnan) and South through Indochina and the Malay Peninsula to Indonesia. In India it is found to grow in the states like Uttar Pradesh, Punjab, Tamil Nadu, West Bengal, Gujarat, Rajasthan, Karnataka and Maharashtra (Lansdown 2011). The plant is distributed throughout plains and lower elevation areas of India, usually near water (Pandya and Anand 2011). The plants have versatile medicinal properties and a wide spectrum of biological activity (Boomibalagan *et al*. 2013). Among the dicotyledons, flowers of sub-family Asclepiadoideae are morphologically complex structured. The androecium and the gynoecium are joined into a complex structure and form gynostegium. Moreover a coherent mass of pollen grains are packed in a sac like structure known as pollinia. Two pollinia along with retinaculum and corpusculum is known as pollinaria. These complex structures extracted and transferred by pollinators from one flower to another flower. Studies on floral biology, breeding system and pollination of asclepiads have been done by several authors (Robertson 1886, Pant *et al*. 1982, Chaturvedi 1983, Kunze 1991, Chaturvedi 1995, Lipow and Wyatt 1998, Vieira and Shepherd 1999, Wang *et al*. 2011). Because of this remarkable floral morphology of asclepiads which is much more complex than usual flowers, breeding system and mode of pollination have been undertaken for investigation. Present studies provide the information on floral biology, flower-visitor interaction breeding system and mode of pollination of *O. esculentum* so as to manage its population as well as the population of flower-visitors.

MATERIAL AND METHODS

Study site—Present investigation was conducted at Santiniketan, Birbhum, West Bengal, India (26° 44' N to 27° 25' N and 77° 26' E to 78° 32' E). The study was carried out between July to January, 2015-2017. Different populations of investigated plant taxa were selected by random sampling to study the details about the floral biology, breeding system and pollination under natural conditions.

Flowering phenology—Flowering twigs were tagged to observe different phenological events like initiation of inflorescence, maturation of floral parts, initiation, duration, intensity of flowering, anthesis, post anthesis changes until senescence, stigma receptivity and pollen dispersal etc. Phenological studies have been carried out through regular field visits both at individual and population levels. Flowering twigs were tagged at bud stage. After anthesis nectar was extracted from flowers by capillary tube to measure the volume of nectar and sugar concentration of nectar was determined using a hand refractometer (Kearns and Inouye 1993). Observations on the flower-visitors and their behavior were made following the procedure given by Faegri and van der Pijl (1979) and Dafni (1992).

Pollen production and pollen-ovule ratio—To determine the average pollen production, from each flower (n=10), a single pollinaria was removed by dissecting needle. Each pollinaria was placed in a cavity slide and two to three drops of 2-aminoethanol were added to dissolve the pollinaria wall (Sreedevi 1979). The preparation was covered with a glass cover slip and incubated in an oven at 90ºC for 20 min. Excess 2-aminoethanol was blotted off and pollinaria were crushed in lactophenol-glycerine with aniline blue. Then a known

dilution (0.2 ml) was placed on the grid and 10 replicate counts were made using a Hemocytometer (Barrett 1985). Pollen-ovule ratio was determined following Cruden (1977) method. From each flower (n=10) ovaries were carefully dissected and placed in a drop of water on a glass slide and counted under microscope.

Pollen viability—Pollinaria were removed from freshly opened flowers and examined under microscope. Pollinaria were placed on several grooved slides containing sucrose and boric acid solution at different concentrations individually as well as in combinations. Pollinaria were crushed to release pollen grains in each groove containing solution with the help of sterile needle. Intact Pollinaria as well as pollen grains have been observed in case of *in vitro* pollinaria germination. For the *in vitro* germination slides were 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 (1993). The *in vitro* pollen germination test was carried out at different time intervals after anthesis.

Stigma receptivity—Ten stigmas were used at different stages of anthesis and the peak receptivity periods in terms of peroxidase activity following the method of Dafni (1998) was observed. Aqueous solution (6%) of Hydrogen peroxide (H_2O_2) was placed on the stigma and evolutions of bubbles were counted.

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

RESULTS AND DISCUSSION

Flowering phenology—The plants flower in late July and continue until early January. The flowers are arranged in branched axillary racemes and open from 05.30 hrs. to 06.30 hrs. in the morning in acropetal order. The flowers were pale pink with purple veined. The actinomorphic flowers are typically campanulate and bisexual. The flowers are medium in size, 2.5 cm in across. Each anther is bilocular and tipped with a whitish outgrowth that partially covers the stigmatic head. With regards to the flower architecture according to Endress (1994), synorganization of corolla and androecium resultants corona and gynostegium evolved due to androecium and gynoecium. 'Guide rails (slits)' formed due to the synorganization of neighbouring stamens. This slits is very important in view of pollen transfer as it help to attach the pollinaria to the pollinator's body and again from insect's body to stigmatic chamber. Bilocular superior ovary contains many ovules. Nectar is produced in between the narrow furrows of stamen, just below the 'guide rails'. Each flower secreted 4.9±0.17 µl nectar and sugar concentration is 32.2±1.28 ºbrix at the first day of anthesis but, the nectar volume (6.5±0.40 µl) increased in second day flowers (Table 4). Nectar volume has been reduced following the second day of anthesis. Nectar is one of the most important food resources offered by plants to potential pollinators (Proctor *et al.* 1996). Flowers of second day get visited mostly by the flower-visitors because of its nectar production and the production of nectar was continued until fourth day of anthesis and there was still little nectar available in the flowers and visitation decreased significantly. According to Zimmerman (1988) and Kearns *et al.* (1998), pollinators optimize their foraging pattern in terms of energetic costs. The corona is functionally involved with nectar production, nectar holding, guidance of pollinators for nectar and it also provides direction for pollination (Kunze 1993). Like other members of sub-family Aclepiadoideae nectar is present under the 'guide rail' and it is considered as to be a plesiomorphic character (Kunze 1997).

Table 1– Floral characters of *O. esculentum*

Anthesis period	05:30- 06:30 hrs.
Flower/Inflorescence	18 – 25
Colour	Pale pink
Shape	Actinomorphic
Size	2.5 c.m. in across
Pollen production/flower	485± 23.0
Number of Ovule/Flower	77.4±1.91
Pollen ovule ratio	6:1
Pollen viability (*In vitro* pollen germination)	92±1.18 %

Table 2— Flower-visitors and their foraging behaviour of *O. esculentum*

Flower-visitors	Mean Abundance	Foraging period	Time spent/ flower (Sec.)	Reward
Apis dorsata (Apidae)	56.4±1.36%	06:00–16:00	10-16	Pollen and/ or Nectar
Vespa sp. (Vespidae)	09.4±0.74	06:30–15:00	4-10	Pollen and/ or Nectar
Borbo cinnara (Hesperiidae)	23.2± 0.96%	06:30 – 16:30	12-18	Nectar
Thrips (Thripidae)	11± 0.94%	Day and night	20-25	Nectar

Table 3- Fruit sets in different conditions

Treatment		No. of flowers studied	No. of flowers set fruit	Fruit set (%)
Open		125	11	8.8
Netting		50	0	0
Bagging		50	0	0
Controlled	Autogamy	50	0	0
pollination	Geitonogamy	50	2	4
treatment	Xenogamy	50	6	12

Table 4—Nectar volume and sugar concentration in different flowering stages of *O. esculentum*

Different stages of flower opening	Nectar volume (μl) (n=5)	Sugar concentration (°Brix) (n=5)
1st day	4.9± 0.17	32.2±1.28
2nd day	6.5± 0.40	28.7±1.23
3rd day	3.9± 0.17	34.3±0.67
4th day	2.8± 0.14	35.6±0.85

Pollen production and pollen-ovule ratio—Each stamen has pendulous, flattened, oblong pollinial sac, each of which containing a mass of pollen grains. Each pollinial sac is attached to retinacula or translator arm and it is joined together to a brownish oval shaped structure i.e. corpusculum with dorsal middle slit. This brownish corpusculum is able to be seen on outer surface of stigmatic slits while the other parts are concealed within column. Each of pollinaria embedded in a hard matrix and a translator, which is developed from stigmatic secration and mechanically attached to the flower-visitors (Kunze 1993). Each flower produces 485±23.0 pollen grains and 77.4± 1.91 ovules and the pollen ovule ratio is 6:1.

Pollen viability—*In vitro* pollen germination is the most reliable process to study the viability of pollen. In case of *in vitro* pollen germination maximum 92±1.18% germinating pollen along with 845±14.25µm tube length was recorded in 20% sucrose supplemented with 100 ppm boric acid after three hours of incubation (Figs. 2C, D). For *in vitro* pollen germination and tube growth sucrose is the best source of carbohydrates as it maintains the osmotic pressure and is a substrate for metabolism of pollen (Shivanna and Johri 1998). Boron is crucial for pollen germination along with pollen tube development in most species (Brewbaker and Majumder 1961). However, sucrose in combinations with boric acid promoted pollen germination as well as tube growth 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, Sidhu and Malik 1986). Reduced pollen viability was observed after 2nd and 3rd day of anthesis i.e 73.8±1.51% and 37.4±1.99% respectively. Only 8.4±0.25% viable pollen grains were found after 4th day of anthesis and beyond that day no pollen germination was observed.

Stigma receptivity—The stigma attained its receptivity from the first day of anthesis but maximum stigma receptivity was observed on the second day of flower anthesis with a prominent peroxidase activity (33.3±0.61 bubble evolution /2 min; n=10) and stigma remained receptive up to 4th day. But the receptivity of stigma decreased gradually after the 2nd day of flower anthesis.

Breeding system—The fruit set in natural open condition was 8.8 %, however no fruit set was observed in netting and bagging condition (Table 3). But in case of hand pollination experiment 12 % fruit set were observed through xenogamy which is better than fruit formation through geitonogamy (4 %) and autogamy (0). Pollen ovule ratio is a conservative indicator of plant's breeding system. Pollen ovule ratio is 6:1 which indicate that the plant is selfing in nature (Cruden 1977) but the breeding experiment results showed that it is xenogamous. The plants are unique in possessing pollinia, which transfer large number of pollen grains at a time. Wyatt (1976) explained that, pollinia confer a fitness advantage by virtue of increased pollination efficiency. Pollen ovule ratios presumably also reflect the predictability of pollinators in a habitat and the efficiency of pollination (Cruden 1976). Sparrow and Pearson (1984) observed that *Asclepias syriaca* showed self-incompatibility while *A. tuberosa* found only 2% fruit set in self-pollinated flowers. Obligatory self-pollination was observed in *Tylophora hirsuta* (Chaturvedi and Pant 1986, Chaturvedi 1989). Late acting self-incompatibility has been reported in *Gonolobus suberosus* (Lipow and Wyatt 1998) and in *Asclepias* (Wyatt and Broyles 1994). In this present observation the plants were noted as exceptions to this overall pattern, being labelled by Cruden (1977) as plants of Asclepiadaceae with "sweepstakes reproduction". Therefore, the breeding system of this plant is truly xenogamous. It has never been observed that, both the ovaries get matured into fruits. Kunze (1991) explained it as the pollen tubes from one inserted pollinium are all towards the one ovary only, thus the plant can enable to allocate its resources to one carpel only.

Pollination—Nectar and pollen are the chief rewards to the flower visitors and nectar secretion increased by the time. The amount of nectar was measured at hourly intervals between 06:30 hours and 18:30 hours on the flower opening day. Maximum nectar production occurred in second day of anthesis. Freshly opened flowers are pale pink coloured and attract a good numbers of flower-visitors (Table 2). Maximum visitation occurred between 10:30 h and 13:30 h. After flower opening insects (Fig.1) like *Apis dorsata*, *Vespa* sp. of Hymenoptera, *Borbo cinnara* of Lepidoptera were found to visit the flowers in day time (06:00 h to 16:30 h) for their forage while Thrips (Thysanoptera) visited throughout the day and night. Thrips are the minute and fringed wing insect visited flowers for their forage as well as brooding place. The abundance of flower visitors of different order of insects are presented in tabular form (Table 2). The flowers are actinomorphic, saucer shaped and nectar is located under the

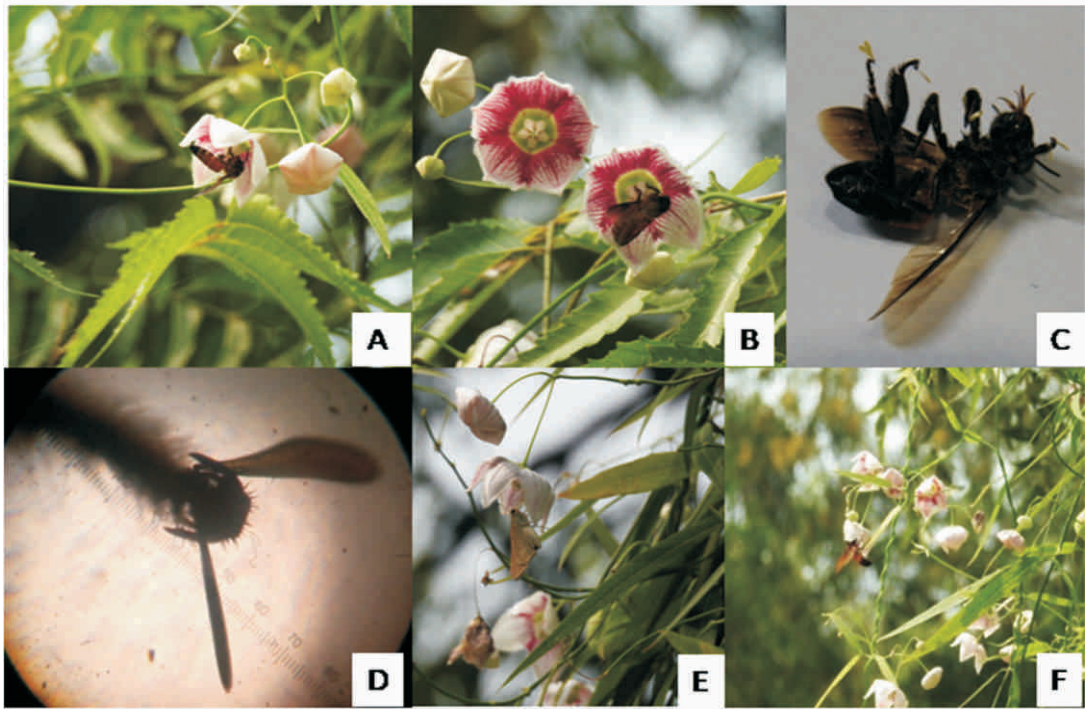

Fig.1— Flower visitors of *Oxystelma esculentum*; A –B. *Apis dorsata* visiting the flower C- D. Pollinaria attachment to the body parts of the *A. dorsata*; E. *Borbo cinnara*; F. *Vespa sp.*

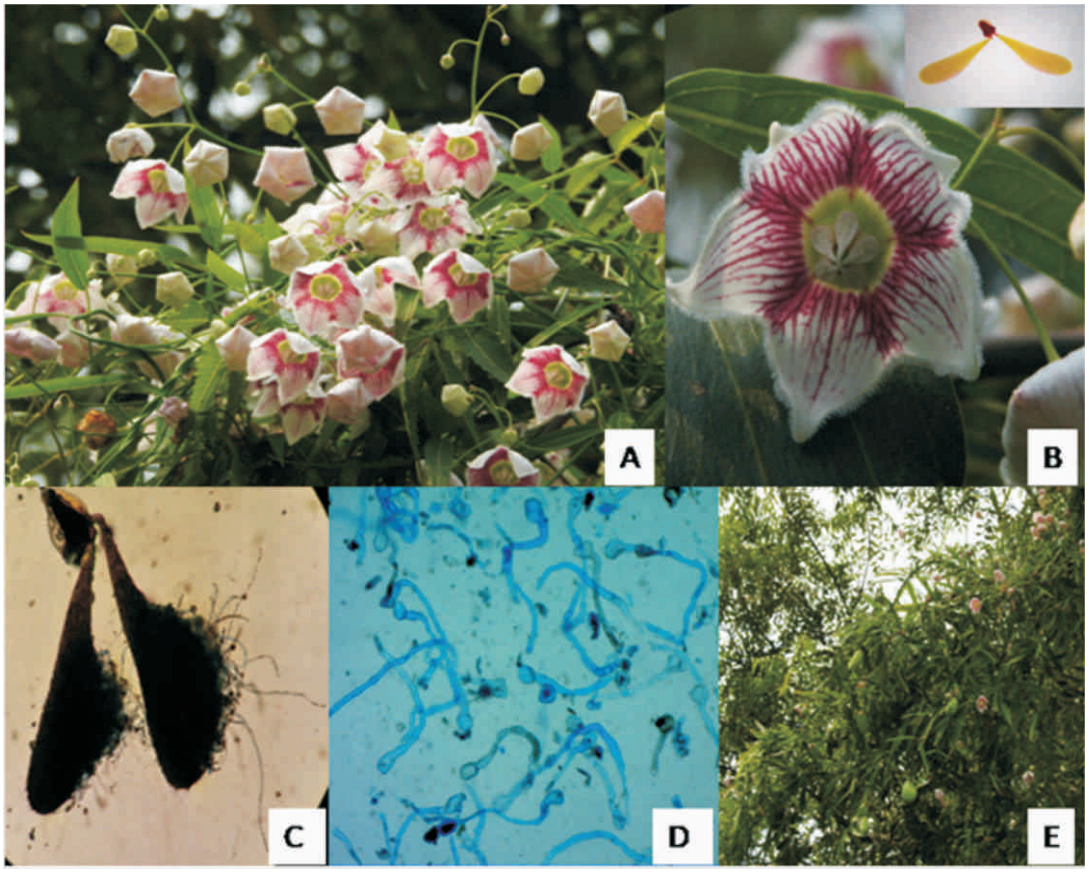

Fig. 2—A. Natural habit of *Oxystelma esculentum* B. A single flower and pollinaria; C. *In vitro* germinating pollinaria; D. *In vitro* germinating pollen; E. Natural fruit set

'guide rail'. Among the total foraging visit (Table 2) *Apis dorsata* (56.4±1.34%) paid more visit than *Vespa* sp. (9.4± 0.74%), *Borbo cinnara* (23.2±0.96 %) and Thrips (11±0.94 %). The present findings reveals that the *Borbo cinnara* spent more time to forage followed by *A. dorsata*, Thrips and *Vespa* sp. Among the flower-visitors, *A. dorsata* were more in number during forage and also more efficient in probing the flowers than other flower-visitors. During their visit both *A. dorsata* and *Vespa* sp. land on the petals and probed the flowers in search of nectar from the base of flowers. To get access the nectar from the flowers *A. dorsata* and *Vespa* sp. forcefully pushes their proboscis and during their visit, pollinaria get attached to their body parts. At the time of foraging due to upward movement of flower-visitor's leg, pollinaria get extracted and transferred to stigma surface in such a way that the germ farrow of pollinaria adhered to the stigma surface. It has also been observed that in case of *A. dorsata*, three to four pollinaria and in case of *Vespa* sp., one to two pollinaria were noticed on their legs. Subsequently, they carry the pollinaria from flower and help in cross pollination. When nectar is present under 'guide rails' the pollinia get removed by the legs of flower-visitors'. Not only legs but also proboscis also play vital role in pollinia removal (Pant *et al*. 1982, Eisikovitch 1986). *Borbo cinnara* has proboscis for collecting the nectar. But during their 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. Thrips are too small that they are not able to remove or carry pollinia, hence it also acts as nectar robber. Nectar removal by the illegitimate flower-visitor enhances the visitation rate of legitimate pollinators because of variability of nectar amount of flowers (Heinrich and Raven 1972, Soberon and Martinezd 1985, Cushman and Beattie 1991). Insufficient amount of nectar obliged the pollinators to visit another plant for their forage. Nectar robbing promotes flower-visitors in indiscriminate foraging in the robbed and unrobed flowers, which promotes fruit set (Singh *et al.* 2014) Generally among the asclepiads nectar, floral color and the corona serve as attractants to the flower visitors but corona not only serve as floral attractants but also act as flower-visitors exclusion and manipulation of pollinators behavior and position (Ollerton and Liede 1997, Ollerton and Liede 2003). Small insects like thrips have not been strong enough to remove the pollinaria and are trapped and die in the flower (Lied 1996). Pollination by bees was reported in both new and old world asclepiadiads but wasp pollination also found in some new world asclepiadiads (Michener 1979, Ollerton and Liede 1997).

Studies on flower-visitors interaction indicated that *A. dorsata* are the major and legitimate pollinators. The fruit set in different conditions indicate the flowers of *O. esculentum* would have been adopted for xenogamous breeding system in nature. Nonetheless, all of these features like pollen ovule ratio, and fruit set make sense in the broader context of the unusual reproductive system. Understanding of such flower-visitors interaction will be helpful in the conservation of this important medicinal plant in natural ecosystem.

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