The International Journal of Plant Reproductive Biology 10(2) July, 2018, pp.166-171
DOI 10.14787/ijprb.2018 10.2.

Pollination biology of *Osbeckia wynaadensis* C. B. Clarke (Melastomataceae)- an endemic plant in Southern Western Ghats.

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Received : 20.02.2018; Revised: 02.04.2018; Accepted: 01.01.2018; Published online: 01.06.2018

ABSTRACT

Osbeckia wynaadenis is a rare endemic large shrub restricted to marshy area of Southern Western Ghats. The present study on pollination biology of *O. wynaadenis*, and was conducted at Vagamon, Kottayam-Idukki district, Kerala, for a period between 2014 to 2016. It flowers between November to April with peak in the middle of December. Anthesis takes place between 06.00 – 09:30 h accompanied with anther dehiscence. The life span of single flower is one day. The flower has poricidal anthers and external agency is needed for pollen removal. Buzz pollination was observed in *O. wynaadensis*, and insects visitors were *Xylocopa pubescence, X. latipes, Amegilla zonata, Ceratina* spp. and *Andrena* spp. Fruit set in flowers pollinated by hand is higher than in natural pollinated plants. Fruit development until maturation takes 25–30 days. As capsules ripened, the fruit wall dehisced loculicidaly. Seed germination was 1.8±0.7% and seed viability was 5.6 % ± 2.3. Seeds germinate only in soil saturated with water. There was no special mechanism of seeds dispersal. Human activity, inbreeding depression, poor seed germination and viability, low seedling sustainability, lack of pollinators during humid conditions, low seed dispersal distance seems be the reason for its limited distribution and endemism. The results of the study are valuable for the conservation of natural population.

Keywords : Buzz pollination, poricidal anther, autogamy, geitinogamy, xenogamy

INTRODUCTION

Endemism is the major threat to biodiversity, caused either due to reproductive syndrome or by anthropogenic pressures, and that may lead into extinction (Sreekala and Pushpandagan 2004). Studies on pollination biology and breeding systems of rare and endangered species are essential for successful management and conservation (Spira 2001, Tandon *et al.* 2005, Kaul-Moza and Bhatnagar 2007). This information may also provide insights into the vulnerability of a species (Carlsen *et al.* 2002).

Osbeckia wynaadensis C.B. Clarke (Melastomataceae) is a rare endemic large shrub located in marshy area of semi evergreen and evergreen forest in Southern Western Ghats at about 4500 f above the sea level. It is wild plant usually seen in swamps below tea plantation near to small freshwater streams. The plant is ecological indicator of pure underground water (Sujina and Subban 2012). It is an underutilized herbal plant, but has several medicinal importance for curing inflammation, urinary tract infection, hemorrhage, menorrhagia, hemorrhoids and leucorrhea and raw fruits are eaten by tribal people in Nilagiries (Ramachandran and Udhayavani 2013). Flower and fruits of plant are used for preparation of dye by Kani tribal in Ponmudi hills (Bosco *et al*. 2015). Methanolic extract of *O. wynaadensis* is used against human cervical cancer cell line (HeLa), mouse embryonic fibroblasts cell line (NIH 3T3), and showed good activity against murine embryonic fibro blast cell line (Sujina and Subban 2012).

Present study deals with pollination biology of *Osbeckia wynaadensis* with a conservation perspective. The objective was to understanding of the reproductive biology of this species and identifies factors which can be related to its rarity and endemism, as well as to examine the implications of the results on its conservation.

MATERIALS AND METHODS

Study material and Study site—*Osbeckia wynaadensis* C.B. Clarke is a large shrub with purple flowers in sub terminal corymbs. The distinguishable features of plant are comb like hairs on the lower surface of the calyx cup. Study was conducted between October 2014 and April 2016 at Vagamon ($9^0$41'11.80''N-$76^0$54'15.63''E), which is a hilly tourist spot situated 1100 meters above the Sea level in Kottayam-Idukki border of Kerala. The average temperature, rainfall, humidity during the study period was 22ºC, 93.3mm, 64% respectively.

Flowering phenology—Five plants in their natural habitat were selected for the study. During the flowering period field visits were conducted weekly. Ten flower buds at pre anthesis stage were selected and tagged, and morphological changes of floral parts, anther dehiscence and stigmatic status were observed at one hour interval. The time of initiation time of anthesis, duration and termination and dimensions of different floral parts were measure by Varner's calliper (Kearns and Inouye (1993).

Pollen biology—For studying pollen morphology, the anthers from open flowers were collected from five flowers of five different plants. These were fixed in 1:3 glacial acetic acid and ethanol and acetolyzed by the method after Erdtman (1952). Pollen size was measured by using ocular stage micrometer (Willis 1999). Histochemical localization of starch, lipid and protein in the pollen grains was made by

different methods as described by Pearse (1972). Number of pollen grains was counted using hemocytometer as described by Kearns and Inouye (1993).

Pollen viability—Viability of pollen at regular at intervals was checked in five flowers each of five selected plants by Alexander's staining method (Alexander 1980).

In vitro* pollen germination—In vitro* pollen germination was studied using pollen grains collected from fresh flowers incubated for one hour in sucrose solution (20%) with Brewbaker and Kwack's (1963) medium. The pollen grains producing pollen tubes longer than the diameter of the pollen grains were counted as viable (Tuinstra and Wedel 2000), and percentage of pollen germination was calculated by counting the number of germinated and total number of pollen grains in 5 microscopic fields (Kearns and Inouye 1993).

***In vivo* pollen germination—**In order to check *in vivo* pollen germination on the stigmatic surface, the virgin stigmas were pollinated with pollen from another plant. All the flowers were covered by the net. The pollinated stigmas at regular intervals were fixed in 45% glacial acetic acid, 70% ethanol (3:1) for 1 h and then transferred to 70% ethanol. They were hydrolyzed in 45% acetic acid at 60ºC for 10-60 m to make the tissue soft. The stigmas were splitted longitudinally and stained with hot dye (150 mg safranin O, 20 mg aniline blue in 25 ml hot glacial acetic acid) for 5-15 m. The stained tissue was placed on a slide and squashed under a cover slip and observed under microscope (Dafni 1992).

Stigma receptivity—Receptivity of stigma of five flowers/plant was checked at regular intervals in five selected plants in the field using a Peroxtesmo esterase indicator paper liquid (one paper+1 ml water) (Dafni and Mause 1998).

Ovules/flower and pollen-ovule ratio—Number of ovules/flower were counted after the dissection of ovaries under a stereomicroscope (Bhojwani *et al.* 1967). The pollen–ovule ratio (P/O) was calculated (Cruden 1977).

Fruits were collected and percentage of fruit set was calculated. Time of fruit initiation, development, maturation and dispersal of seeds were carefully observed. The seeds were collected and preserved for further studies. The viability of the seeds was analyzed by using tetrazolium chloride test (Dafni 1992). Percentage of seed germination was observed by sowing the seeds on moist cotton lined petridishes, on potting mixture, and on soil collected from natural habitat.

Pollination biology—To study the behavior of different floral visitors, continuous observations were made from November 2014 to March 2016. These were collected and identified with the help of experts from Entomology Department, Kerala Agricultural University and Kerala Forest Research Institute (KFRI). The foraging period and the type of resource collected by different visitors were recorded by visual observations daily. Pollination efficiency was checked by observing pollen loads on different body parts under a stereomicroscope according to the procedure given by Kearns and Inouye (1993).

Pollination experiments—In order to ascertain the breeding system, hand pollination experiments were conducted on four randomly selected plants. Inflorescences selected for the study were tagged, and flower buds subjected to hand-pollination were bagged before anthesis and re-bagged after hand pollination and were observed 5 days after each treatment to monitor fruit set. The pollination experiments consisted of: 1. Flowers were left for open pollination (Control); 2. Flower bud enclosed with loosely woven bags to allow only wind pollination; 3. Enclosed in nylon bags to ascertain self-pollination; 4. Pollinated by pollen from the same flower; 5.Cross-pollinated using pollen from different flowers of the same plant (geitonogamy); 6. Cross pollinated using pollen from other plants (xenogamy) and 7. In order to check apomixis, emasculated flower buds before anthesis were checked for fruit set. These experiments were repeated three times during the flowering period.

RESULT AND DISCUSSION

Flowering Phenology—Floral buds appear at the end of October and plants bloom between November, and optimum in mid December and was continued till April. At the time of peak flowering 2.4±0.5 flowers blooming/day/cyme and 27.2±1.9 flowers bloom on a single plant. Life span of the individual flower is one day. The anthesis starts at 06:00 h and extends up to 09:30 h. The flowers start closing at 17:30 h and completely closed at 19:30 h. It varies depending up on the climatic conditions and day length. Fruiting started 4.2±0.4 weeks after the onset of blooming. The fruits mature within 35±5 days after pollination.

Anthers are yellow and poricidal. In young floral buds, anthers bent downwards with the commencement, they started bending slowly upward towards the upper side of the corolla. Anthers dehisce simultaneously with corolla movement. In buds, the styles were 7-9 mm long and stigmas were capitate. During the initial stage of anthesis, anthers dehisce through apical pores to release pollen featuring protandry. As anthesis progressed, style elongated (12-14 mm). This is followed by bending of style towards the anthers, while stigmas become receptive. In *Monolena trichopoda*, another member of the family Melastomataceae, automatic selfing has been reported (Warner 1981, Renner 1983). On the other hand, in *O. wynaadensis*, the spatial separation of stamens and stigma, and absence of receptive stigma at the time of pollen release prevents self-pollination. Breeding experiments conducted in the field supported this observation.

Pollen biology—Pollen grains are heterocolporate with alternating colpi, exine scabrate and have an average size of 13.4±1.1ìm in diameter. Pollen viability as checked by Alexander's (1980) staining method was 82.3%±8.7 on the day of anthesis, but viability declined gradually and was lowest at end of anthesis (16.7%±5.2).

Pollen production—The number of pollen grains produced per flower was 324961±345.

***In vitro* pollen germination**—In 20% sucrose medium, pollen germination was 61.51±10.34% with 58.84±3.36µm long pollen tubes. Pollen germination (87.5±1.08%) along with 110.96±9.5µm tube tubes were observed in Brewbaker and Kwack (1963) medium after one hours of incubation.

Stigma receptivity—Stigma receptivity was tested by using a Peroxtesmo esterase indicator paper liquid (one paper+1 ml water) (Dafni and Mause 1998). The stigmas become receptive at 09.00 h and receptivity reached peak at 12.00 h.

***In vivo* pollen germination**—Germination percentage of pollen grain on stigma also determines the pollen viability as well as stigma receptivity. Stigma receptivity was studied at different time intervals on the day of anthesis. After landing on the stigmatic surface pollen grain are subjected to hydration and then pollen wall proteins are released on to the stigmatic surface (Heslop-Harrison *et al*. 1975).The stigmatic pellicle act as a receptor of pollen wall proteins. After the pollen is accepted, the pollen tube emerges out and grows towards the stigmatic pellicle by penetrating the cuticle and move downwards (Shivanna 2003). However, if the pollen is incompatible it fails to germinate and rejected (Dickinson and Lewins 1973). Present experiments showed 80% stigma receptivity and 70.7% *in vivo* pollen germination taking place at 13:00-14:00 h. On the basis present observations it was revealed that between 12:00 -14:00 h, stigma receptivity and *in vivo* pollen germination was maximum and most suitable for effective pollination.

Number of ovules/flower—There were 78.8–78.5±9 ovules/flower and the pollen ovule ratio was 4.309.

Breeding system-Different breeding experiments were carried out in *O. wynaadensis* to record the breeding system of the plant (Fig. 1B). In open pollinated flowers there was 65% fruit set. No fruit set was observed by self pollination indicating self-incompatibility. There is no wind pollination and apomixes. However, 40% fruit set was observed in flowers pollinated by its own pollen (selfing), 90% fruit set was observed in flowers pollinated with the pollen of a different flower of the same plant (geitonogamy) and 80% fruit set with 58.2±4.6% seed set was recorded in flowers pollinated with the pollen of a flower from different plant (xenogamy). Thus, pollination experiments indicate facultative xenogamy. Production of large number of pollen grains and high pollen ovule ratio are characteristics of buzz pollinated plants (Buchmann 1983). Renner (1983) reported that Melastomataceae is characterized by buzz pollination.

However, P: O ratio is indicating obligate xenogamy (Cruden 1977). However, Michalski and Durka (2009) compiled P: O ratios and outcrossing rates for 107 angiosperm species and analyzed the relation between these traits considering pollination mode, life form and phylogenetic relatedness among species. They concluded that P: O ratios in general correlated significantly with outcrossing rates and when taking additional factors into account, the relation became ambiguous. The correlation was significantly positive in wind pollinated species, but only marginally so in animal

Fig-1– A. Habitat, B- Breeding Experiment, C- Xylocopa pubescence, D-Xylocopa latipes, E-Amegilla zonata, F- Andrena sps, G- Ceratina sps.

pollinated species. According to them, wind pollinated species had higher P: O ratios than animal pollinated taxa. Their results indicated that P: O ratios vary more strongly with pollination mode and life form than with the mating system.

Pollination biology—Anthers dehisce by apical pores. Pollination is possible only through mediation by buzzing insect. Buzz pollination is wide spread among 20000 species of flowering plants e.g. tomato, egg plant and red pepper (Raw 2000) and it is characteristic of the family Melastomataceae (DeLuca and Vallejo-Marín 2013). Flowers are visited by *Xylocopa pubescence, Xylocopa latipes*, *Amegilla zonata, Ceratina* species, *Andrena* species, some beetles and bugs (Figs.1C-G). *Xylocopa* (Figs.1 C-D), a large buzzing bee, *Amegilla zonata* (Fig.1E), a blue banded bee is effective pollinators of *O. wynaadensis*. *Xylocopa* visited flowers at around 10:00 h and touched the flower to produce vibration and buzzing. A single bee visited all flowers of a plant and then visits next plant. Number of bees visiting flowers and visiting frequency increases as flowering reaches peak season. Two *Xylocopa* spp. namely, *X. latipes* and *X. pubescence* were observed. The former is large carpenter bee with shiny, fully black in colour with fuscous metallic blue green or purple wings in sunlight. *X. pubescence* forage only on warm days and also avoid revisiting the same plants visited earlier by marking the plant with pheromones (Raju and Rao 2006). Most of the visits made by *Xylocopa* spp. are short, lasting for about only 1 second. Usually one individual bee, rarely two or three, visited a plant at any given time. *Xylocopa* produce high frequency vibration by which large number of pollen ejected from the poricidal anther. According to Raju and Reddi (2000), *Xylocopa* belongs to the buzzing genera Anthropodiae usually prefer zygomorphic large size, showy flowers and oil rich pollen. Therefore, floral morphology of *Osbeckia wynaadensis* is suited for Xylocopal pollination. Some of the studies have shown that body size of the bee is important that it determines the ability of visitors to contact the stigma of visited flowers and thus become potential pollinators (Duncan *et al.* 2004). Large size of the *Xylocopa* is suitable to make contact with stigma and thus it becomes an efficient pollinator for fruit production. One unknown bee was observed after the visit of *Xylocopa* and it eats away pollen without touching stigma. *Amegilla zonata* a blue banded medium sized bee is also a vibrating bee and is highly sensitive and is usually one or two are seen during the flowering peak time. They vibrate around the flower and beating the anther with the front legs which lead to emptying the anther. The smooth, dry and fine pollen then sprayed from the fine aperture at the tip and they also touch the stigma along with pollen. A small carpenter bee *Ceratina* spp. (Fig. 1G) also visited the plant and landed on the stamens bundle. They arched their body over the stamens, clasping the anther and squeezing it with legs to release the pollen grains. They spent lengthy period on stamen. *Andrena* spp. (Fig.1F) while sitting on the stamens buddle inserts their proboscis and extracts pollen from the anthers. This bee stays for 20-30 m on a single flower. Some beetles and bugs were also observed. They chew the anther sac and destroy the stamen. *Ceratina* and *Andrena* are small sized bees and do not touch stigma. According to Liu and Pemberton (2010) halictid bees (*Ceratina*) has low pollination efficiency because, the size of the flower and bee is mismatched and they often fail to contact the stigma. However, maximum pollen load was observed on the body of *Xylocopa* species and they are the effective pollinator. *Xylocopa* and *Amegilla* are considered as legitimate visitors because they touch the stigma and anthers. Floral visitors and their foraging activities are illustrated in Table 1. Buzz pollinating insects usually prefer nectar less flowers, the main and only reward of the insects is pollen (Vallejo-Marín *et al.* 2009). .

Fruit and seed- Mature fruits develop in 25–30 days. The mature capsules dehisce loculicidaly. Seeds are minute and muricate. Seeds released from the capsules germinate in water saturated soil, but a few of them grow into seedlings under natural conditions. *In vitro* seed germination studies revealed that, a limited number of seeds germinated within 7 days while, a few seeds germinate after two months. Seed germination is very poor as only 1.8±0.7% germinates. Viability of seeds as tested by tetrazolium chloride test is only 5.6±2.3%. Sujesh and Indira (2010) while analyzing breeding system and reproductive constraints in *Dipterocarpus bourdillonii*, reported that low seed germination is due to the lack of efficient pollinators. According to Renner (1983) forty percent members of Melastomataceae bear capsular seeds,

Table 1- Floral visitors in *Osbeckia wynaadensis*

Name of Taxa with family	Visiting time	Resource	Foraging hours (h)	Time spend on Each flower	Stigma touch	Frequency of visit
Xylocopa pubescence	Day	pollen	10:00 to 14:00	3 sec.	+++	High
Xylocopa latipes	Day	pollen	11:00 to 15:00	3 sec.	+++	High
Amegilla zonata	Day	pollen	11 :00 to12: 30	3-4 min.	++	Intermediate
Ceratina spp.	Day	pollen	09:00 to 15:00	30-45 min.	_	Intermediate
Andrena spp.	Day	pollen	09:00 to 15:00	20-30 min.	_	High

which disperse by wind. But there are no data on dispersal distance. *Osbeckia wynaadensis* produce numerous minute seeds, but their mode of dispersal mechanism has not been observed. Most of the seeds fall within 1m.

It is concluded on the basis of present observations that pollination in *Osbeckia wynaadensis* is brought about by buzzing insects. During the cloudy and rainy days pollinator activity was less, and sometimes it also lead to the failure of pollination. There were five types of insect visitors of which only three species were effective pollinators and others were pollen thieves. However, during buzzing process large quantity of pollen is wasted. Hand pollination experiments indicated facultative xenogamy which leads in inbreeding depression and plants fail to cope up with the change in environmental conditions. Poor fruit set and poor seed germination, low seedling sustainability and reduction in seed dispersal are responsible for the limited distribution of the plant.

Acknowledgements—The authors are thankful to DST for providing research facilities through FIST programme and also grateful to Department of Botany, S. N. M. College, Maliankara.

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