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BREEDING SYSTEM AND SEED BIOLOGY OF *HUMBOLDTIA VAHLIANA* WIGHT. (FABACEAE) –A WESTERN GHATS ENDEMIC

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ABSTRACT
Humboldtia vahliana Wight. is an endemic and medicinally important tree species belongs to the family Fabaceae. In order to understand the narrow distribution, breeding system and seed biology of this plant, an investigation was carried out during 2014-2017. The floral visitors include honey bees, stingless bees, butterflies and ants. Highest percentage of fruit set was observed in xenogamy flowed by open pollination and geitonogamy. Seeds are recalcitrant and seed germination is registered as 80 ±0.9% at the time of dehiscence. Habitat destruction, environmental factors, over exploitation of the species, recalcitrant nature of the seeds, seed infestation and poor seedling recruitment in the natural habitat could be the reasons for its narrow distribution.

KEY WORDS: Breeding system, Endemic, Honey bees, *Humboldtia vahliana*, Western Ghats

INTRODUCTION

The reproductive biology of flowering plants is important for determining barriers to seed and fruit set for conservation, pollination and breeding systems that regulate the genetic structure of populations (Tandon *et al.*, 2003). Reproductive characters such as seed dispersal, germination capacity, survival rate of seedlings, flowering, reproductive life span and number of flowers and seeds refer to a set of responses which allows a species to adapt to a particular environment (Aswani & Sabu, 2015). Besides these, the process of gamete development, pollination, endosperm and embryo development and other reproductive features can provide important clues regarding the reproductive constraints of plants that need conservation. Adequate knowledge on reproductive biology is essential for conservation, management and recovery of endemic and endangered plants. Therefore, detailed information on the reproductive biology of rare, endangered and threatened plants is essential for developing effective strategies for their conservation and sustainable utilization.

Humboldtia Ruiz. & Pav. is a genus of legume in the family Fabaceae which includes 9 species in Kerala. *H. vahliana* Wight. belong to the subfamily, Caesalpinioideae is a large tree growing up to 20 m tall, usually found adjacent to rivers/streams in semi-evergreen and evergreen forests up to an altitude of 750m. The tree is endemic to Southern Western Ghats of Tamilnadu and Kerala (Sasidharan, 2004 ; Nair *et al.*, 2006). The hardwood of the tree is used for making match boxes and also for fuel. Bark powder or decoction is used to cure biliousness, impure blood, ulcers and epilepsy (Sanjappa, 1986). A comprehensive study on the reproductive biology of this species has not so far been made. In this background, a study has been conducted on breeding system and seed biology of *Humboldtia vahliana*, an endemic tree of Southern Western Ghats with an intension of understanding the causal factors that lead to population reduction.

MATERIALS & METHODS

Study area

Humboldtia vahliana is an endemic tree species belongs to the family Fabaceae, growing along with the river banks of evergreen forest areas of South Western Ghats (Fig. 1. a-c) out of this we selected Aryankavu of Kollam and Chittar river basin of Thiruvananthapuram for the present study which is at an altitude of 436m asl and 86m asl respectively. Population in Aryankavu forest areas consists of more than 150 individual species, only few individual (<20 individuals) were located in Chittar river basin. It is a large tree up to 15-20m height having dark brown bark with 5-6mm thickness and mottled with white; blaze is pink in colour. The plant grows in association with other wet evergreen tree species such as *Baccaurea courtallensis, Vateria indica, Xanthophyllum arnotiianum, Syzigium occidentale, Artocarpus hirsutus, Blachia umbellata, Lagerstroemia speciosa etc.* The study was carried out during 2014-2017.

FIGURE 1: *Humboldtia vahliana*- a. Habitat, b and c. Habit of the species

METHODOLOGY

Twenty healthy plants were selected in the community and observations were made on day to day basis in natural habitat on flowering phenology which includes season, habit, development, time of anthesis and anther dehiscence. Fifty flower buds were selected at random and observations were made between 1800 - 2200h to study the time of anthesis and anther dehiscence. Peak flowering time was noted when maximum number of flowers opened.

The average number of ovules per ovary was determined by dissecting the ovaries under the microscopes. The pollen-ovule ratio was calculated as suggested by Cruden (1977).

$$\text{Pollen} - \text{Ovule ratio} = \frac{\text{Mean number of pollen grains/flower}}{Me\quad number\ of\ ovules/flower} + \cdots$$

Pollen fertility was assessed by Acetocarmine-glycerine staining technique (Radford *et al*., 1974). The number of stained and unstained pollen grains was counted. The stained pollen grains were considered as fertile and the unstained pollens as sterile. Pollen viability was assessed by FCR (fluorochromatic reaction) and TTC (2, 3, 5-Triphenyl Tetrazolium Chloride) tests as per procedure proposed by Shivanna and Rangaswamy (1992). In FCR test, the observations were made under (Leica DME, Germany) fluorescent microscope using blue filter, the bright green fluorescent pollen grains were scored as viable. The brownish red pollen grains were counted as viable in TTC test. Stigma receptivity was studied visually with the help of hand lens and hydrogen peroxide test according to the method of Scribailo and Posluzky (1984) and cytochemical localization of stigma esterase using α-naphthyl acetate (Dafni *et al*., 2005). Floral visitors were observed in all the two sites. During the peak flowering period, the pollinators were observed at the time of anthesis to the next day evening itself. The number of floral visitors, foraging behavior, foraging hour, time spent on each flower and the frequency of visit are recorded. All the visitors were collected and identified with the help of experts and insect taxonomy manual.

Different breeding experiments such as autogamy, geitonogamy and xenogamy were conducted in the field along with open pollination. The study of fruit development was gone underway from the day of pollination until maturation and dehiscence. Matured fruits from candidate species were harvested and seeds were collected. The average number of fruits developed in each population and average number of seeds developed per fruit/capsules were also calculated. Seed biology of the

candidate species that include seed moisture content, viability and germination was studied as per ISTA 2008.

RESULTS AND DISCUSSION

Flowering phenology

Humboldtia vahliana starts flowering in the month of February and extends up to May and reaches a peak during March. Fruit initiation was noticed in last week of March. The plants took 3-4 months from initiation to full maturity and dehiscence of seeds. Seasonal variation affects the flower size, anthesis, anther dehiscence, pollinator behavior as well as fruit setting. In the present study (2 study sites), *Humboldtia vahliana* starts blooming in February and extends up to May and fruit maturation was noticed during July-August. Fifty flower buds were selected randomly and observations were made between 1800-2200h to study the time of anthesis and anther dehiscence. Peak flowering was noted when maximum number of flowers opened. The flower buds take 15-20 days from initiation to full bloom. The flowering period extends up to 120 days in a year and life span of each flower is 1-2 days. The flowers opened in the evening between 1800-2100h and anther dehiscence was noticed 2hr after anthesis. The fruit dehisced and seeds are scattered around the floor premises and water courses of parent plant at a distance of 2.5m, later these get cycled to vegetative phase of the species. Flowering and fruiting patterns ultimately determine the reproductive success in plants. These phenological events are strongly controlled by climatic factors and evolutionary processes. The timing of flowering and fruiting has major influence on biological process of the organism in the ecosystem niche (Primack, 1980; Bawa *et al*., 1990).

Pollen biology

Floral analysis indicated that, each flower has five anthers and four ovules. A single anther consists of 1056 pollen grains and thus a flower has around 5280 pollen grains hence the pollen ovule ratio had been worked out as 1320:1. This clearly indicates that, an external agency is required for effective pollination, therefore the species favors cross pollination. The Acetocarmine staining technique revealed that 84.66 ±1.36 % pollen grains are fertile. Pollen viability by FCR and TTC tests confirmed that 83.27 ± 2.01 % and 85.76 ±2.07 % pollen grains were viable respectively on the day of anthesis and its viability gradually decreased on successive days after anthesis Table 1. This observation was similar to that of *Humboldtia decurrens* and *H. sanjappae* (Jayalakshmi *et al*., 2015, 2016).

TABLE 1: Pollen viability under different treatments

Treatments	Percentage of pollen viability		
	One day before Anthesis	On the day of Anthesis	Second day of Anthesis
Acetocarmine	62.92±1.86	84.66±1.36	53.83±1.42
FCR	58.07±1.42	83.27±2.01	44.64±1.66
TTC	59.82±1.08	85.76±2.07	42.39±1.23

Pollen viability is a critical factor, which is considered as an important parameter of pollen quality (Dafni and Firmage, 2000). Treating pollen grains with stains such as Acetocarmine and aniline blue in lactophenol essentially imparts colouration to the contents of the pollen as well as fixed/ dead pollen. It may be useful to determine the degree of pollen stability in plants of hybrid origin or those grows under favourable conditions (Alexander 1969). From time to time, the pollen viability test has been constantly upgraded. In the present investigation, fluorochromatic reaction test (FCR) and 2, 3, 5-Triphenyl Tetrazolium chloride (TTC) test were used for assessing the pollen viability as suggested by Shivanna and Rangaswamy (1993) and Dafni and Firmage (2000).

Stigma receptivity

Microscopic study revealed the stigma is capitate, wet and papillate. Stigma receptivity is an important factor for successful pollination. Hence to know the intrinsic factor of stigma the receptivity of stigma was studied by the cyto-chemical localization of stigma esterase using α naphthyl acetate and also supports H_2O_2 tests for peroxidase activity. The stigma become receptive soon after anthesis and receptivity extends up to 20 hours after anthesis and the anther dehiscence occur 2h after anthesis by releasing pollen grains which indicates a protogynous nature. Visual observation indicates that the stigma secrets exudates at their surface only after flower opening. Maximum percentage of stigma receptivity was noticed at the time of anthesis which indicates that the highest percentage of pollen germination and pollen tube growth on the receptive surface of the stigma. The receptive stigma showed maximum bubble activity (50 bubbles/ minute) during the peak receptive period. However, the receptivity declined gradually with time. The presence of esterase on stigma coincides with its receptivity (Stone *et al.,* 1995; Bhattacharya and Mondal, 2003). This shows a positive correlation between esterase activity and stigma receptivity of candidate species.

Pollination

The flowers are white in colour with pleasant fragrance. The pollinators were attracted by the mass blooming and fragrance of the flowers. The major floral visitors are honey bees, stingless bees, flies, butterflies and ants (Table 2). The flowers open in the evening between 1800-2100h and anther dehiscence was noticed 2h after anthesis shows protogyny. Several floral visitors were noticed in between 0600-1300h which indicated that floral visitors are attracted by mass flowering of white flowers with fragrance. Primary floral rewards are pollen and nectar which attract the visitors giving cues such as visual signals and brooding sites. Majority of the insects forages in the day time except the ants. Where in, the ants show throughout activity *i.e*. 24 hrs in the flowering branch of *H. vahliana*. The ants such as *Oecophylla smaragdina* and *Vombisidris humboldticola* inhabiting in the domatia of the plants. Domatia provides shelter for ants, which is a special structure developed inside the stem and internodes of ant inhabiting plants like *Humboldtia*. Some reports showed that (Bentley 1977; Heil and Mckey, 2003*) H. brunonis and H. laurifolia* possess such domatia which

gives brooding sites for ants. They also have extra floral nectaries in order to attract the ants to prevent herbivory. Chanam *et al.* (2014) reported that the inhabitants of domatia produced organic wastes that are absorbed by the plant and produced better fruit set. The ant *Vombisdris humboldticola* inhabits in the domatia of ant plants such as *H. decurrense. H.brunonis* and *H.vahliana* (Jayalakshmi, 2018). Ant-plant interactions are regulated by extra floral nectar which supports the ant visitation rate.

The principle pollinators are honey bees; which include *Apis cerana, A. dorsata* and stingless bees such as *Trigona iridipennis*. They collect both pollen and nectar from the flowers subsequently and transfer the viable pollen grains from one flower to another of the same plant or flowers of different plants. *Apis cerana* and *Apis dorsata* forage during the day time between 0630-11.30h for nectar and pollen collection, while *Trigona iridipennis* gather its food during 0600-1300h. Butterflies are also active during day time for nectar collection only (0730-1300h) and they spend 2-4 seconds in each flower. In fine weather they are very active, their interplant movement facilitates pollination. However, during the rainy season the butterflies are completely inactive. The major visiting groups include *Euploea core, Eurema blanda, Pachliopta aristolochiae* and *Danaus genutia.*

The plant exhibit a semi-myrmecophyte adaptation, *Vombisidris humboldticola* inhabiting in the domatia of the plant. This is because of secretion of extra floral nectar by the plant which attracts these ants for getting their reward easily and as well as providing brooding site. The plant offers both nectar and brooding sites as reward for ants acting as predator/ defender by destroying the floral parts which affected the visitation rate of the pollinators. But only a small percentage of them effected pollination. This ant species has been predominantly residing in the domatia of other species of *Humboldtia* such as *H. decurrense* and *H. brunonis*, as they are mutually benefitted. Some *Xylocopa* species rarely visiting the flowers of *H. vahliana* for collecting nectar and accidently damages the floral parts (corolla tube inside which nectaries are present) without affecting pollination. In *H. vahliana,* the associated plants are tree forms itself and thus provide a mutual tight competition for energetics resulting in low percentage of fruit set.

TABLE 2. Pollinator behaviour of *H. Vahliana*

Visitors	Family	Visiting status	Visiting time	Foraging nature	Foraging hours
Apis cerana	Apidae	Regular	Day	Pollen+ nectar	0630-1100
Apis dorsata	Apidae	Regular	,,	Pollen+nectar	0800-1100
Trigona iridipennis	Apidae	Regular	,,	Pollen+ nectar	0600-1300
Euploea core	Nymphalidae	Occasional	,,	Nectar	0730-1200
Eurema blanda	Pieridae	occasional	,,	Nectar	0700-1300
Pachilopta aristolochiae	Papilionidae	occasional	,,	,,	0800-12.30
Danaus genutia	Nymphalidae	occasional	,,	,,	0830-1300
Oecophylla smaragdina	Formicidae	Resident	Day and night	Nectar	Through out
Vombisidris humboldticola	Formicidae	Resident	,,	Nectar	Through out

Breeding system

Flowering plants showed different types of breeding systems from autogamy to outcrossing (Bawa *et al.,* 1985). The breeding system of the plant has a correlation to the genetic structure of the population (Richards, 1986) Detailed knowledge regarding the biology of a species especially on breeding system of endemic and threatened plants are essential for their management and conservation (Bernardello *et al.*, 2001). Breeding experiment was conducted on the flowering twig which was chosen during the peak period of stigma receptivity. A minimum of 50 flowers were selected in 2 populations for breeding experiments. *viz*, autogamy, geitonogamy, xenogamy and open pollination. Highest percentage of fruit set was observed in xenogamous pollination, followed by open pollination and geitonogamy in both the study sites. Fruit-set was not observed in autogamous pollination in all the study areas. The style curvature in buds and freshly opened flowers act as a mechanism to prevent autogamous pollination resulting into no fruit set. Maximum percentage of fruit set through xenogamous pollination was noticed in Aryankavu study site (74 ±0.22%) (Table 3).

TABLE 3. Breeding experiments in *H. Vahliana*

Treatments	No. of flowers pollinated	Aryankavu		Chittar river basin	
		No. of flowers set fruit	% of fruit set	No. of flowers set fruit	% of fruit set
Autogamy	50	0	0	0	0
Geitonogamy	50	16	32 ±0.12	11	22 ±0.02
Xenogamy	50	37	74 ±0.22	34	64 ±0.14
Natural pollination	50	28	56 ±0.116	22	44 ±0.16

Values mean± SD

Seed biology

The fruit development, seed dehiscence and dispersal of seeds are the three major phenomenons in flowering plants and these are the prime factors in the life cycle of plants (Levin *et al.,* 2003). After fertilization, pod attains maturity about 12-16 weeks. The 4 ovules get developed into 3-4 seeds, separated by thick sutures. The fruit reaches a maximum of 15-20 x 3.7-6cm, elliptic, compressed, sutures thick valves prominently veined; seeds 3-4, almost orbicular and glabrous (Fig. 2 a &b). The fertilized ovules developed into seeds but some of

them were aborted due to the attack of certain beetles (Fig. 2d). The number of seeds vary from 3-4.

Average number of flowers produce per plant during the peak period of flowering was about 1200 ±2.64. The number of fruits developed from these flowers was limited to 98 ±3.78. Therefore flower fruit ratio in natural condition had been calculated as-13:1. The mean number of ovules in each ovary was 4, but only 2-3 seeds were developed. Hence the ovule-seed ratio had been calculated as 2:1. This may be due to the ovule abortion, insufficient pollen transfer, infestation, pollinator scarcity *etc*.

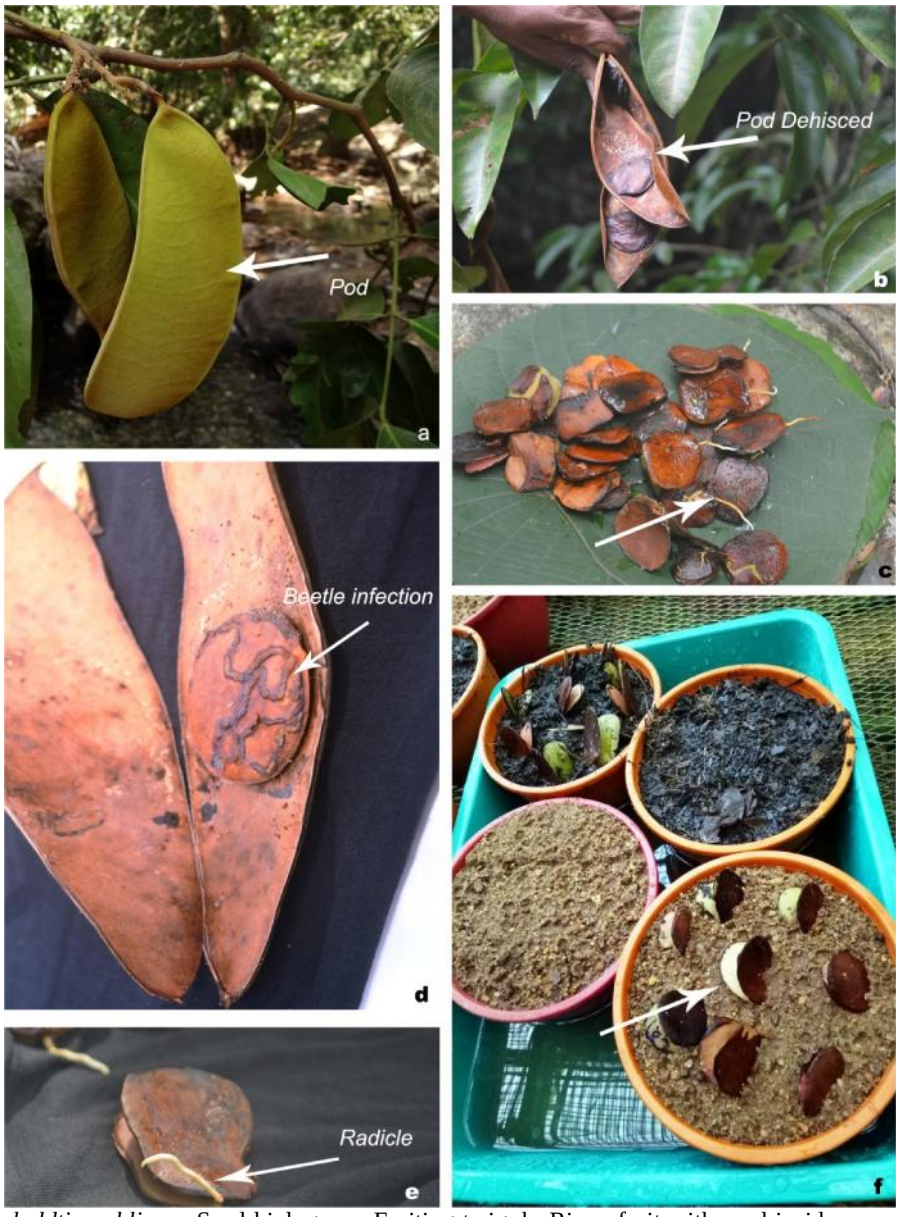

FIGURE 2: *Humboldtia vahliana*- Seed biology- a. Fruiting twig, b. Ripen fruit with seed inside, c. e & f Germinating Seeds, d. Infected seeds.

Seed dispersal

The maturity of fruit is determined by observing the fruit size as well as by monitoring the hardness of pod. After reaching maturity of the pod, the inner pressure and temperature strained by the cell wall made the 4 seeded pods to rupture in a unique explosive manner by flinging the seeds into the water course and waterlogged rock crevices. This type of seed dispersal (Ballistic) is reported in many Leguminosae members (Vander pijil, 1982). he explosive type of seed dispersal was noticed in some trees belongs to Caesalpinioideae in the forest areas of Africa. Seeds took15-16 days for natural germination and then develop into seedlings. Before germination, the seeds were split open. In *Humboldtia vahliana* epigeal type of seed germination was observed. Wherein, most of the seeds germinated immediately which shows recalcitrant type. The above results are supported by many authors (Ganeshiaiah and Uma Shanker, 1988; Bawa and Buckley, 1986) in Fabaceae members itself. Fruits are dehisced during the monsoon; they did not show any dormancy and germinated quickly.

Seed germination:

The seed germination percentage is maximum at the time of dehiscence and observed to be reducing in a span of one month. Fruits are dehisced during the monsoon season. Seeds didn't exhibit any dormancy and the seed coat becomes thinner quickly causing the seeds to split open and gets germinated in most cases. Through observation, it is inferred that seeds in water course germinate to a great extent compared to seeds falling in soil (Fig. 2c, e & f).

Germination test was conducted in seed germinator without light having wet rolled paper towels placed, maintaining the temperature at 30℃ +2℃ and 80% RH (Relative humidity). Moisture content at the time of seed received was 51.5 ±1.7%. When the seeds were tested the germination registered is of 80 ±0.9%. In which the germination started within 5 days and completed up to 17 days. When seeds were kept for desiccation in open laboratory condition (28 ±2℃/70%RH), some of the seeds found to split open, which were removed. The same removed seeds were tested for germination and exhibited 40% germination. In open desiccation, within 5 days moisture content was reduced to 48.3±1.3% and germination percentage was 65 ±2%. Within 20 days moisture content reduced to 39 ±1.1% and where most of the seeds lost their viability.

CONCLUSION

In *H. vahliana* less floral longevity, stylar curvature of bud as well as freshly opened flowers before anther dehiscence and exposed versatile anthers prevents self pollination and parallel decreases the durability and percentage of cross pollination. In the natural condition noticed unseasonal rainfall which has made negative ecological effect on the plant in which full bloomed flowers were washed out which is limiting the species expansion and progeny regeneration. In *H. vahliana* the associated plants are tree forms itself and thus provides a mutual tight competition for energetics resulting in low percentage of fruit set. The plant provides brooding sites for ants such as *Oecophylla smaragdina* and *Vombisidris humboldticola*. The ants act as predator or defender by destroying the floral parts which affected the visitation rate of the pollinators. Later during fruit development stage, some unidentified insects and beetles infect the tender fruits which adversely affected the fruit production. Thus in totality, all these factors clearly indicate that gene flow between the natural populations is restricted within that area gradually leads to less reproductive success.

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