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Floral Phenology and Breeding System of *Aponogeton appendiculatus* V. Bruggen (Aponogetonaceae)

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ABSTRACT

Present study was undertaken with the aim to study the floral phenology and breeding system of *Aponogeton appendiculatus* a critically endangered aquatic species endemic to Southern Western Ghats. It shows small, actinomorphic bisexual flowers, with maximum percentage of buds (8.1±0.7) open between 02:00 to 02:30 h. The highest percentage (3.2±0.6) of anthers dehisced between 09:00 to 11:00 h. The maximum percentage of stigma receptivity (95±0.1) and pollen viability (69±0.13) were observed on the day of anthesis. Pollen grains were oval in shape and monosulcate, and 10,000±1000 pollen grains were produced per flower. Seed set percentage was low (23±8.6), and the survival of seedling was also poor. While analyzing the breeding system, the highest pod set percentage (1.1±1.84) was brought about by xenogamy followed by geitonogamy (1.03±0.4), autogamy (0.68±0.73) and lowest in open pollinated flowers (0.4±1). The pollen ovule-ratio and pollination experiments indicate facultive xenogamy.

Key words : breeding system, endemic, phenology, pollen viability, sigma receptivity

Aponogeton appendiculatus V. Bruggen is a member of the mongeneric family Aponogetonaceae. It is a rare, endemic, threatened, red listed aquatic submerged perennial herb species of Southern Western Ghats (IUCN 2011). Its roots are edible and part of the diet in certain region of Kerala. It has become vulnerable due to severe habitat loss. Studies on effective conservation strategies have not been reported for the species so far. In order to conserve this plant species it is necessary to have a detailed knowledge of its reproductive biology (Kaul-Moza and Bhatnagar (2007). The genus is known to have 45 species and mainly distributed in the tropics and subtropics of the Old world (Van-Brugggen 1985). In India, the genus is represented by 7 species (Yadav and Gaikwad 2003) of which *A. appendiculatus* is narrow endemic and critically endangered (Mishra and Singh 2001, Yadav and Gaikwad 2003) and are included in Indian Red Data Book (Nayar and Sastry 1987, 1988). This species is distributed in Wayanad, Thrissur, Alappuzha and Ernakulam districts of Kerala (Sasidaran 2011).The species occurs mostly in brackish water especially at a depth of 1.5-3 meters. Its tuber produces the tuft of completely submerged leaves and spike inflorescence, which are carried above water level. Most *Aponogeton* species produce tuber and go through an annual resting stage or dormancy period (Sainty and Jacob 1994). Apart from some morphological and taxonomical studies (Van Bruggen 1969 and Aston 1973) significant studies were not carried out on the reproductive biology of *A. appendiculatus* till date. A detailed study of floral phenology and breeding system is an essential prerequisite to formulate the strategies for the conservation of this critically endangered plant species.

MATERIALS AND METHODS

The study was conducted from June 2015 to December 2017. Natural habitat area of *Aponogeton appendiculatus* are located in North paravoor of Ernakulam district of Kerala, India and positions were marked by using GPS (Longitude : 10^0 9'12.95"N and Longitude : $76^0$13' 40.78"E) .

Floral phenology and morphology—The phenological studies were recorded from the beginning and end of vegetative period, fruiting and flowering period of the plant and it is determined by direct observation in each natural habitat. Morphological and reproductive parts were observed with the help of stereo zoom- microscope (Nikon SMZ 745T-Japan and Nikon-H600L Eclipse Ci-L –Japan) (Kearns and Inouye 1993).

Pollen production—Total number of pollen per anther was calculated by the aid of Haemocytometer. Pollen –ovule ratio was estimated as per the standard procedure of Cruden (1977) and Dafni (1992).

Viability of pollen— Viability test of pollen was done by MTT staining method (Rodrigues and Dafni 2000).

Stigma receptivity— To ensure the stigma receptivity, artificial pollination of flowers were carried out under controlled condition in different time duration such as one day before anthesis, on the day of anthesis and one day after anthesis and two days after anthesis (Dafni 1992).

***In vitro* germination of pollen—** provides quantitative measure of pollen viability. *In vitro* pollen germination was evaluated in Brewbakers and Kwack's (1963) lmedium supplemented with varied sucrose concentrations of 5-50% (Dafni 1992).

Artificial breeding experiment—Various types of breeding experiments were carried out during the two subsequent flowering seasons (Dafni 1992). All quantitative values are represented as the mean ± standard deviation.

RESULTS AND DISCUSSION

Flower morphology—*A. appendiculatus* is a perennial aquatic herb of 12 cm- 170 cm height. Leaves are completely submerged with long petiole. Lamina oblong-lanceolate, midrib distinct with parallel nerved**.** Inflorescence is spike on long peduncle, solitary or 2-10 spikes, enclosed on a caducous or persistent spathe when young. Flowers sessile spirally arranged along rachis and turned towards all directions. Flowers are bisexual, hypogynous, and pinkish white in colour. Tepals 2 to 4, persistent. Stamens 4 to 8, free, persistant. Anthers didynamous, extrose, and basifixed. Pollen grains subglobose or ellipsoid monosulcate. Carpel 2 to 3, free, sessile ovules 1 or 2 with basal placentation. Style short, stigma discoid with stigmatic ridge on ventral suture, persist. Follicles distinct with lateral appendage with curved beak. Seeds without endosperm, testa with double membrane (Fig.1). The individual flowers weighed 0.012 g. Flowers spirally arranged on spike were born on long peduncle (22-145 cm) and are solitary. Fruiting spike 3-35 cm long, densely flowered (40-165) and flower percentage is (90.7±35.8). The flower were small in size and were 01.5-02.5 cm long white with pink colour bearing short stamen (01.5-02cm x 0.1.5-0.3cm) and pistil (0.2-0.25cm x 0.5-015cm)

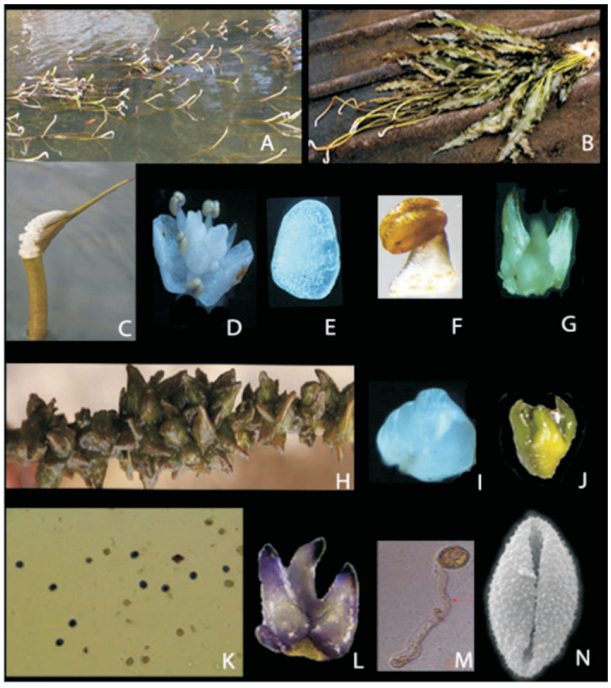

Fig.1–Aponogeton appendiculatus. A. Habitat, B. Habit, C. Inflorescence, D. Single lower, E. Tepal, F. Stamen, G. Stigma, H. Fruit, I. Ovule, J. Embryo, K. Viability of pollen viability tested by MTT. L. Receptive stigma, M. Germinating pollen, N. SEM photograph of pollen.

Floral phenology—The vegetative growth in *A. appendiculatus* takes place in the middle of June during this period water is non saline, followed by flowering, fruiting and seed production up to November. It flower only once in a year and flowering is at its peak during August and usually continue for five months from July to November. Flowers open in early morning from 13:30 h with maximum number of floral buds open between 14:00 – 14:30 h (8.1±0.7%) and continue between 14:30 to 15:00 h (1.2±0.6%). Rain and cloudy weather delay the blooming process. The range of life span of a single flower was 3-4 days (3.8±0.63). The average days for completion of its flowering phase ranges from 14 to 16 (15.3±0.8). The flowers on the bottom of the spike open first and the top youngest flowers were rudimentary and delicate and damaged before pollination. However, as the inflorescence grows the spike filament extends. After anthesis anther dehiscence occurs through longitudinal slit. The anther dehiscence varies from 7am to 1pm. In a single flower maximum number of anthers dehisced between 09:00 to 11:00 h (3.2±0.6) was observed. Moderate number of anther dehisced between 07:00 and 09:00 h (1.7±0.4). Minimum anther dehiscence noticed in between 11:00 h and 13:00 h (0.9±0.6). Atmospheric conditions influence pollen release, which usually occurs under dry conditions that favor pollen dispersal (Whitehead 1983). In same way, *A. appendiculatus* shows pollen release under dry conditions which is favorable for pollen transfer. During the tidal condition when water comes in contact with anther, it absorbs water and do not dehisce widely and arrest the release of pollen grains. After absorbing water pollen grains become sticky thus pollination is inhibited (Table1). Fruiting starts in July and the peak of fruiting period was in September and October.

Table 1–Reproductive parameters.

S. No.	Characters	Observation
1	Flowering period	July to November
2	Flowering peak	August
3	Fruiting period	July to November
4	Fruiting peak	September-October
5	Flower type	Spike
6	Flower colour	Pinkish white
7	Anthesis	2 am to 2.30am (8.1±0.7)
8	Average number of anther/flower	6
9	Anther dehiscence time	7 am to 11 am (3.2±0.6)
10	Average number of pollen/anther	8000 to 10,000
11	Mean number of pollen grains/flower	60,000
12	Mean number of ovules/ flower	3 or rarely 4
13	Pollen/ovule ratio	20000:1
14	Breeding system	Facultative xenogamy
15	Stigma	3 or 4, apocarpous
17	Stigma receptivity	First day(95±0.1) and second day, (43±0.22)
18	Pollen germination	69±0.13
19	Fruit -set	23 ± 8.6

Pollen – Ovule ratio—The floral analysis indicated that each flower has 6 anther and 3 ovules. The average range

of pollen number of a single anther is 8000-10,000, and the individual flower has approximately 60,000 pollen grains. Cruden (1977) postulated that, generally greater the propensity for xenogamy higher the pollen quantity produced per ovule. Hence P/O ratio of was 20000 pollen per ovule or (20000:1). As per the value given by Cruden (1977), the observations on aerial flowers shown that it should be facultative xenogamous and correlation of pollen ovule-ratio and mating system positively significant in this study. 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. Their results indicated that P: O ratios vary more strongly with pollination mode and life form than with the mating system.

Pollen viability and germination—*A. appendiculatus* is an aquatic macrophyte, so the pollination process is likely to be affected by the presence of water. The pollen viability as tested by MTT method indicated that high percentage pollen viability (94±0.01) was observed between 10:00 to 11:00 h and percentage of viability (47±0.1) declines by 18:00 h. The pollen grains remain viable for 7-9 hours/day. However, pollen viability is retained when they are in contact with water, but viability is reduced gradually with time.

Pollen viability was evaluated by Brewbaker and Kwack's (1963) medium supplemented with varied concentrations of sucrose solution (5-50%). The result indicated that 20% sucrose is suitable for rapid pollen germination and long pollen tube growth. Poor pollen germination is noticed in sucrose solutions below 20% and the all pollen grains burst in 30% and above sucrose solutions (Table 2). The pollen grains in Brewbaker and Kwack's medium (100 ml) supplemented with 20% sucrose solution showed highest percentage of germination (69±0.13) and long pollen tubes (13µm) were seen at 10:00 h (Table 2). Poor pollen germination (8±0.03) and pollen tube growth (9µm) was seen at 18:00 h. In this species, the pollen viability test (MTT) was not positively correlated with *in vitro* germination tests. The use of MTT stain test for pollen viability in the present study may have led to over estimation of pollen viability, since staining capacity depends not on the viability but on the protoplasm content of the pollen grains, thus this measure of pollen stain ability may depart considerable from real value of pollen viability (Dafni 1992). MTT stains may therefore be used to determine pollen viability in this species to provide only a rough idea of viability.

Stigma receptivity—MTT stain was used to determine the stigma receptivity (Table 3). After staining and incubation period, the pink colour stigma became dark blue, indicating stigma receptivity. To determine the period of stigma receptivity, stigma at different time of pollination were pollinated under the controlled condition. The result indicated that the maximum percentage of stigma receptivity was observed on the day of anthesis (95±0.1) between 0:07 to 00:11 h. However, stigma receptivity continues up to next day after anthesis but low percentage of receptivity (43±0.22),

Table 2–*In-vitro* pollen germination in Brewbaker and Kwack's medium

S. No.	Time	Sucrose + Brewbaker & Kwack's medium	% of pollen germination			% of Pollen germination (M ± STDEV)
			Slide No 1	Slide No2	Slide No 3	
1	7am	20%+100ml	53%	63%	66%	61±0.06
2	8am	20%+100ml	63%	59%	59%	60±0.02
3	9am	20%+100ml	68%	67%	63%	66±0.23
4	10am	20%+100ml	66%	57%	83%	69±0.13
5	11am	20%+100ml	66%	68%	66%	67±0.01
6	12am	20%+100ml	64%	60%	64%	63±0.02
7	1pm	20%+100ml	45%	57%	59%	54±0.07
8	2pm	20%+100ml	56%	58%	58%	57±0.01
9	3pm	20%+100ml	46%	48%	43%	46±0.41
10	4pm	20%+100ml	24%	24%	16%	21±0.04
11	5pm	20%+100ml	19%	6%	12%	12±0.06
12	6pm	20%+100ml	8%	11%	4%	8±0.03

Table 3- Sigma receptivity.

S. No.	Time of pollination	Hours	Number of flowers pollinated	Number of pod set	% of pod set	MeanSTDEV
1	One day before anthesis	7am – 9am	10	0	0%	0
2		9am –11am	10	0	0%	
3		11am -1pm	10	0	0%	
4		1pm – 3pm	10	0	0%	
1	On the day of anthesis	7am – 9am	10	10	80%	95±0.1
2		9am –11am	10	10	80%	
3		11am -1pm	10	10	80%	
4		1pm – 3pm	10	8	70%	
1	One day after anthesis	7am – 9am	10	5	50%	43±0.22
2		9am –11am	10	6	60%	
3		11am 1pm	10	5	50%	
4		1pm – 3pm	10	1	10%	
1	Two day after anthesis	7am-9am	10	0	0%	0
2		9am-11am	10	0	0%	
3		11am-1pm	10	0	0%	
4		1pm-3pm	10	0	0%	

for comparatively less duration. Stigma was lost receptivity on third day. The stigma receptivity as compared to pollen viability showed long receptive period than pollen viability on the day of anthesis (Fig.2).

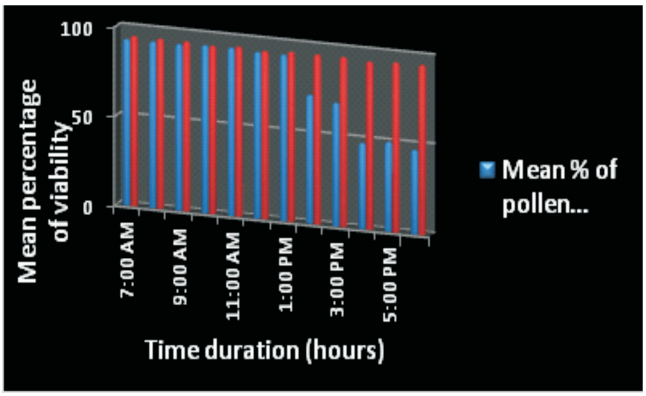

Fig.2– *Aponogeton appendicualatus* : stigma viability and pollen viability on the day of anthesis.

Breeding system—Hand pollination treatments established that the species is self incompatible (Table 4). Among the breeding experiment artificially cross-pollinated flowers produced maximum number of fruit set (1.1±1.84) by xenogamy. Moderate percentage of fruit set (1.03±5.8) was observed by geitnogamy. Low rate of fruit set percentage (0.68±0.73) was noticed in self pollination. In natural open condition fruit set was only 0.4±1. There was no fruit set in the emasculated bagged flowers indicating absence of apomixes in this species. Breeding experiments of this plant revealed that high fruits produced by artificial crossing than open natural pollination.

Fruit set and seed set—The number of flowers/ plant was 97.2±35.08 and only 23±8.6 percentage of fruits produced indicating poor fruit set.

Present study provides data about the floral morphological and reproductive characters of *A. appendiculatus*. In natural condition the propagation of this plant is limited due to the

Table 4- Breeding system in

Treatment	Number of flowers observed	Number of flowers fruit set	Average of Total number of Fruit set	% of fruit set (M±STDEV)
Open pollination	100	15	7	0.4±1
Xenogamy	100	62	68	1.1±1.84
Getinogamy	100	63	65	1.03±0.4
Autogamy	100	72	49	0.68±0.73
Apomixis	100	0	0	0

slow growth rate of tuber. Comparing with flower number, the rate of fruiting and seed setting was very low. The highest percentage of fruit set observed in hand cross pollination than natural pollination. Above observations on poor pollen germination leads to narrow distribution in its natural habitat and may leads to the present status of *A. appendiculatus* as endemic and endangered.

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