Chapter 5: PHENOLOGY AND REPRODUCTIVE BIOLOGY

5.1. Introduction

The term phenology is derived from the Greek word ‘phaino’ meaning to show or to appear and phenology is the study of the seasonal timing of life cycle events. For plants that occurring in riverine habitat, the seasonal timing of life cycle events can be critical to survival and reproduction. Reproductive biology covers a broad spectrum of events involved in reproduction of an organism (Shivanna, 2003) and their interaction with biotic and abiotic components of the environment. Effective conservation measures for rare and vulnerable species depend on our understanding of the nature of threats to the sustainability of the species and their effective remediation. As reproduction and seedling recruitment are the major threats in the final analysis of species stability, generation of baseline data on reproductive biology is a pre-requisite for effective management of our genetic resources. All aspect of pollination ecology, breeding system, seed biology and seedling recruitment are important in conservational aspects of plant genetic resources (Shivanna, 2012). Adequate knowledge on reproductive biology is essential for the conservation, management and recovery of rare and endemic overexploited species. Studies on reproductive biology encompasses phenology, foral biology, pollination and breeding systems.

5.2. Review of literature

The reproductive biology encompassing phenology, foral biology, pollination and breeding systems of *Butea monosperma* (Lam.) Taub. was studied by Tandon et al., (2003) and *Boswellia serrata* Roxb. ex Colebr. by Sunnichan (2005). Pollination ecology is the study of pollen transfer from the anther to the stigma through an understanding of interactions between plants and pollinators in relation to the prevailing habitat (Jones & Little, 1983; Herrero & Pellmyr, 2002; Roubik et al., 2005). In seed plants pollen is the gametophyte representative and they shed in a two celled or three celled stage (Shivanna, 1992). A number of specialised pollination systems have been described in global hotspots, where exceptional concentrations of endemic species are found, such as the Cape Floral Region of South Africa (Goldblatt...
Pollination biology was studied based on floral observations and the breeding system was determined using controlled crosses of *Psychotria tenuinervis* Muell. Arg. (Rubiaceae) in the Atlantic rain forest (Virillo et al., 2007). Comparative study of wild and cultivated populations of Cardamom to identify domestication syndromes showed significant changes in vegetative and reproductive traits and a shift in effective pollinators from native solitary bees to social bees (Kuriakose et al., 2009).

Recent investigations indicate that pollinator populations across the globe are declining, especially in biodiversity hotspots (Mitchell & Ashman, 2008). In fact, little is known about the reproductive biology of the majority of plants in species rich tropical countries where many such hotspots are located. Increasing evidence suggests that global change will have a significant impact on plant and pollinator interactions, and may result in biodiversity loss (Alonso et al., 2010). Studies on pollination and pollinator interactions and mechanisms on Areaceae family was extensively done in recent years (Barfod et al., 2011). Ramasubbu (2011) was studied reproductive ecology of *Impatiens platyadena* Fischer, a critically endangered Balsam of Western Ghats for conservation approach which based on an indepth study and understanding of plant and its environment, including reproductive biology which determines the fitness of the species in a given community.

The Western Ghats prevents the dry wind blowing from northern part of India and same time it brings the monsoon shower to Kerala. The monsoon indirectly plays an important role in the phenology of angiosperms inhabited to the Western Ghats geographic region. In addition, the exceptional concentration of endemic species present in such biodiversity hotspots suggests that these local species are facing a higher risk of extinction than more widespread taxa (Johnson, 2004; Vamosi et al., 2006). Therefore, understanding specialised plant pollinator interactions and floral adaptation is critical in a rapidly changing world from a conservation perspective (Kearns et al., 1998; Johnson, 2004). The genus *Rotula* is represented currently in India by a single species *R. aquatica* Lour. and its reproductive and pollination studies was not yet scientifically evaluated. According to Johnston (1951) “It is surprising that no detailed study of their habits and life history has yet been published”. So there
is need of understanding the reproductive biology, pollination ecology and seedling recruitment in the point of view of habitat specificity of this species to understand the species and also for its conservation.

5.3. Materials and methods

This study was conducted mainly in the R. aquatica growing Meenachil river at Vellani-Adukkam near Erattupetta of Kottayam district, Kerala, India. The study comprise of phenology, reproductive morphology and sexuality, pollen and pistil biology, pollination biology, pollen-pistil interaction and the breeding system, seed biology (seed production, dispersal and viability) and seedling recruitment.

5.3.1. Phenology

Phenology of the species was observed in the field studies conducted in tributary of Meenachil river in Kottayam district. Regular field visits were done in the peak flowering time in a season. The flowering phenology of the species was recorded through field visits. Studies on floral phenology and pollination studies were carried out during the peak of the flowering period. To study the floral phenology, flower buds (N= 50) that would open the next day were tagged and kept under observation to record anthesis, anther dehiscence, and other floral changes until they senesced. Number of buds opened after every 15 minutes interval was counted and anther dehiscence was also observed in the same way. For studies on floral morphology, details of the floral whorls were studied and all the floral parts were measured (N=25). The changes in plant population and habitat are carefully observed and recorded. 25 individual plants in particular locality at Vellani in Adukkam tributary of Meenachil river were selected for observation. Emergence of new flushes, flower buds, flowering peak and seed germination were studied. Voucher specimens and photographs were taken.

5.3.2. Reproductive morphology and sexuality

Morphological features of floral parts were observed and photographed. Number of flowers, the size of flower, male and female reproductive structures such as length of stamens, colour, structure and shape of stigma, length of style and ovary
were measured and photos were taken. Colour of floral parts was recorded during field studies. Pistils from freshly opened flowers and observed stigma under a stereomicroscope and recorded whether the stigma is of the wet or dry type depending on any visible secretion on the stigma or not. The number of ovules per ovary was estimated by taking cross section of ovary. A minimum of thirty flowers was examined under Stereo Microscope.

5.3.2.1. Receptivity of stigma

The methods followed to study the stigma receptivity were; first, the emerged out stigma was observed with hand lens to find wetness or exudations and colour changes. The observation was done in 30 flowers selected at random.

To determine the period of stigma receptivity, hand pollinations were conducted on bagged flowers at 2 hours intervals (N = 10 at each interval) starting with anthesis until 12 hours i.e., one day before anthesis, just before anthesis, soon after anthesis, 2, 4, 6, 9 and 12 hours after anthesis. Controlled pollination on each stigma with adequate amount of fresh pollen was carried out and after 12 hours incubation period pistils were fixed in formalin acetic acid alcohol (FAA; 5:5:90, v/v). Excised the stigma of each set, were stained with acetocarmine or using the aniline blue fluorescence method (Shivanna & Rangaswamy, 1992), gently teased them with a pair of fine needles, and observed under a (light/fluorescent) microscope. The stage that permitted pollen germination and pollen tube growth was considered to be receptive.

5.3.2.2. Pollen ovule ratio

The above data was used to calculate pollen-ovule ratio by dividing the mean number of pollen grains per flower with the number of ovules. The number of pollen was counted by using Haemocytometer from anthers collected from sample size of 10 flowers from different plants.

5.3.3. Pollen viability studies

Pollen viability was assessed through Acetocarmine staining test and in vitro pollen germination. Freshly collected dehisced anthers or pollen from fresh flowers,
were stained with Acetocarmine stain and fertile pollen grains were identified as those that take up deep and uniform stain. Sterile pollen grains are shriveled/incompletely filled or empty; they do not take up deep, uniform stain. The number of fertile and sterile pollen scored using at least 10 microscopic fields from at least two slides and calculated the percentage of fertility.

*In vitro* pollen germination is one of the most convenient and reliable methods used to test the viability of pollen grains. It can be used to understand the sexual reproduction in plants. Brewbaker and Kwak (1963) developed a medium, which is found to be suitable for more than 86 plant species (Table 7.).

Table 7: Composition of BK medium

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sucrose</td>
<td>10 %</td>
</tr>
<tr>
<td>2</td>
<td>Boric acid</td>
<td>100 mg/l</td>
</tr>
<tr>
<td>3</td>
<td>Calcium nitrate</td>
<td>300 mg/l</td>
</tr>
<tr>
<td>4</td>
<td>Magnesium sulphate</td>
<td>200 mg/l</td>
</tr>
<tr>
<td>5</td>
<td>Potassium nitrate</td>
<td>100 mg/l</td>
</tr>
</tbody>
</table>

*In vitro* pollen germination tests (sitting drop cultures) were conducted with different stages of flower bud in different pH conditions of BK medium Fresh anthers at -4, -2, -1, 0 (hours of anther dehiscence) of different maturity stages were collected and dusted on groove of clean cavity slides containing 0.5 ml germination medium having pH variations 5, 5.5, 6, 6.5, 7 and 8. While dusting, pollen grains of two anthers of same flower were mixed to account for the variation. 95 % to 98 % relative humidity was maintained by placing these slides on wet filter paper in petriplate covered with lid. Ambient room temperature of 28°C was provided. Half hour after inoculation, cavity slides were observed under trinocular light microscope (Magnus, MLX) at 10X resolution. Number of pollen grains germinated and total number of grains per field of view were recorded and photographs were taken. Mean value of 10 fields were taken. Pollen grains that produce pollen tubes longer than the diameter of the grains only were considered as germinated (Tuinstra & Wedel, 2000). Diameter of
the pollen grain and pollen tube length were determined using in micrometer in trinocular microscope under 10 x magnifications. Pollen germination was calculated and expressed in percentage as proposed by Guangchu (2002). 10 microscopic fields were randomly selected for taking observations.

Pollen germination % = No. of pollen germinated / Total No. of pollen grains X 100

5.3.4. *In vitro* pollen tube growth

*In vitro* germination and pollen tube growth of *R. aquatica* was carried out in standardized optimum conditions. BK medium having pH 6 in controlled physical conditions of RH 95 % temperature 28°C and white cool light in aseptic conditions used. Pollen grains from two mature and fresh anthers going to be dehisced are dusted in to 0.5 ml culture medium on a glass slide. First observations were carried out after half an hour and then every hour after were taken and recorded the pollen tube length of germinated pollen in the field. Pollen tube growth was computed from the growth of 10 pollen tubes.

5.3.5. Confirmation of breeding system and stigma receptivity

The breeding system was studied through controlled pollinations. Flower buds were emasculated and bagged one day before anthesis. On the day of anthesis, the bags were opened in the morning hours and the flowers were pollinated with self (pollen from another flower of the same plant) and cross (pollen from another plant) pollen and rebagged (N= 30 each). The bags were removed after 24 hours when the stigma was no longer receptive, and the flowers were kept under observation for fruit development. To assess seed set under open pollination, 30 flowers were tagged randomly and kept under observation for fruit development. To test the prevalence of apomixis, emasculated flowers (N=30) were bagged and scored for fruit set. Mature fruits were harvested before shedding and the number of seeds were counted.

5.3.6. Pollination studies

Studies on floral visitors, their visitation frequency and foraging behavior are important to document the visitors, their involvement in pollination and their efficiency. All floral visitors may not be pollinators. Many visitors may rob the nectar...
or pollen without bringing about pollination. The way the forager harvests the reward from the flower (whether legitimate or illegitimate) and patterns of their movements within and between flowers of the same and other plants would affect the type of pollen they deposit on the stigma. The frequency of visits (number of visits per flower per unit time) would indicate their possible involvement in pollination and also, serve as an indicator of pollinators abundance. Foraging time (the time pollinator spends on the flower) indicates the extent of reward available in the flower. Stopwatches, marking tape or flags, hand lenses, digital camera, data sheets, thermometer, butterfly net, ethyl acetate and marker pen were the basic requirements.

Plant *R. aquatica* in a convenient place for observation having 10 flower buds going to be open marked them with small tags or by twine. The number of flowers tagged so that the details of all the visitors can be conveniently recorded. The main consideration for selecting the plot is to ensure that the investigator can clearly see any visitor on any of the flowers of the selected plant. Record the number of flowers in the selected area. In *R.aquatica*, the study plot may be made up of a group of plants which can be kept under observation from the observation spot without difficulty. Sit at a comfortable place (about 2 or 3 m away from the selected plant or patch so that the visitors are not disturbed) so that all the flowers in the selected plot are in the vicinity. In general, especially when the number of visits is frequent, recording is confined to 30 minutes time slots are used starting from before 06.00 am covering the whole duration of the visits during the day till 18.00 hours at desk, and the results are extrapolated as the number of visits per flower per hour and mean time spent on flower. Observations are discontinued for the day after the visitors cease to visit the flowers or the flowers are shed or closed. During the data collection any type of perfumes, smoking, fruits, sweets and chocolate should be avoided because the smell of these things may influence the floral visitors.

A Preliminary study is necessary for finding out the rough range of pollination data, time slot setting, criteria to be recorded and measures to be taken to record the details of the following.
Study period of the day depending on the active period of the visitors. In this species the observation is extend to the whole day from morning to evening.

Suitable time slot: 30 minutes slot.

The identity of floral visitors: Identify each species that land on the flower or enter the flower in the selected patch. Unknown species considered as a morphospecies for later identification. A few individuals of each of the unknown species have collected by using a sweeping butterfly net and stored them in rectified spirit or ethyl acetate in air tight containers for later identification.

Mode of landing or entry: The visitor may land on the flower or enter the flower. The approach may be legitimate (when it comes in contact with the anthers and the stigma) or illegitimate when it robs the pollen or nectar without bringing about pollination. In symmetric flowers, visitors generally land on the top of the flower. In asymmetric flowers the visitors may land on one of the petals and move for foraging the pollen/nectar.

Contact with the anthers and the stigma: It is important to observe whether the visitor come in contact with the stigma and the anthers.

Behavior of the visitor after its exit from the flower: When the visitor leaves the selected patch of flowers, it would be desirable to note the number of flowers of the same plant visited in each, trip before moving to the flowers of the neighboring nonspecific plant. Sometimes it may move to the flowers of another species. These details would, indicate the quality of pollen deposited on the stigma. Since the above features are generally constant for each visitor. The details can be recorded during the preliminary studies can be used as original data.

5.3.7. Field data of floral visitors

The following features have recorded for each species for each visit in each time slot. It is prepared on a pre tabulated field data sheet before starting the observations (Appendix.B).
Number of visits in the time slot: The visit of each species for each flower in the observation patch has to be recorded. The visitor may visit some/all flowers in the selected patch.

Details of foraging: The visitor may forage only the nectar or only the pollen or both. Record the details of foraging. Many of the visitors forage pollen as well as nectar in the morning hours, but confine only to nectar in the afternoon and evening hours when pollen is no more available. Also record whether the visitor's body comes in contact with the anthers and/or the stigma during their entry or foraging or exit.

Duration of visit: Depending on the reward available, the duration of visits on each flower may vary from less than a second to several seconds, often minutes. The duration may change depending on the depletion of nectar/pollen in the afternoon and evening. Use of a stopwatch is the most effective method for recording the duration of visit. However it is cumbersome to record the time spent by each visitor on each flower. Alternatively start the stopwatch when the visitor lands/enters the first flower in the observation patch, keep counting stop the watch when the visitor leaves the last flower of the observation patch. Record the total time spent on these flowers. The results of all the foraging trips and calculated the average time spent on each flower. The observations for selected patch of flowers have continued until they remain fresh and continue to attract the visitor. In *R. aquatica* the flowers (stigma and pollen) remain fresh only for a day and the observations have extended until the flowers do not receive any more visitors or the flowers (corolla) start senescing in the evening. As the pollinators activity is affected by environmental conditions particularly temperature, light conditions and precipitation, these parameters have recorded preferably on each day of the study period.

The above studies have replicated at least for 3 days using different randomly selected patches of the population each day in the same area. The data was recorded in the peak (December-January) periods of flowering as the visitors for foraging at maximum.

The data analyzed after the completion of the studies on each patch of flowers. Calculated, visitors frequency (average number of visits per flower per hour), duration
of foraging (average foraging time per flower in seconds), and foraging pattern (nectar/pollen/both) at different time slots studied during the day. The data on replicates on different days of the same climatic conditions were combined to determine the mean value.

5.3.8. Confirmation of pollinators

5.3.8.1. Based on pollen load

The study plot was same described as above and observations were made at a convenient position to record floral visitors. It is confirmed that, before starting observations, none of those flowers should have received a visitor. This was ensured by bagging the flower buds/inflorescence in the previous evening. The bags were removed before starting the observation. Soon after a visitor exits a flower, excise the flower, label it with the name of the visitor and kept it in a container for later studies. The observations continued until getting 10 number of flowers for each visitors. The excised flowers taken were observed without disturbing their stigmas.

Each flower visited by potential pollinators was observed for confirming the presence of pollen grains. Whole flower is placed on a glass slide and the stigma observed under a stereomicroscope for the presence of pollen grains. Presence of conspecific pollen on a majority of the stigma confirms that the visitor is an effective pollinator. If the conspecific pollen is absent on the stigma of flowers visited by a particular visitor, is not a pollinator.

5.3.8.2. Based on seed set

Effective pollinator was also confirmed based on the fruit and seed set after one visit by a potential pollinator to a virgin flower. The same procedure was followed as described above. Instead of excising the flower after the visit, rebagged it and maintained it on the plant until it abscises or set fruit. Counting the visited flowers for setting fruits and seeds, the visitor is assumed as an effective pollinator. If none of them set fruit, the visitor is not a pollinator.
5.3.8.3. Based on pollination efficiency index (PE)

Pollination efficiency index of Spears (1983) of a visitor was calculated by using the formula:

\[ \text{PE} = \frac{(P_i - Z)}{(U - Z)} \]

Where \( P_i \) is the mean number of seed set per fruit by a plant population that received single visit by a pollinator \( i \), \( Z \) is the mean number of seed set per flower by a population receiving no visits and \( U \) is the mean number of seed set per flower that received unrestricted visits.

The procedure is the same as described above under confirmation of pollinators based on pollen load. In addition to the flowers visited once by a visitor \( (P_i) \), two other sets of flowers have to be bagged. One set of flower buds bagged before anthesis (in the previous evening). This will give information on pollen load on the stigma as a result of autogamy \( (Z) \). Another set of flowers which were allowed for open pollination (unrestricted visits, \( U \)). These three sets of flowers labeled and bagged were allowed for seed setting.

There are three sets of 30 flowers for seed setting in the field:

1. Flowers which were allowed one visit by a potential pollinator \( P_i \) (one set of flowers for each visitor).
2. Flowers which were allowed no visits by the pollinator \( (Z) \).
3. Flowers which were allowed unrestricted visits by the pollinator \( (U) \).

For 2 and 3 one set of flowers would be enough; the same data can be used to calculate pollination efficiency of all the visitors. Count the number of seeds on each tagged flowers.

5.3.9. Palynology

The stamens preserved were placed on a clean glass slide. The anthers were squeezed in to a drop of aqueous glycerine and a cover glass was placed on it. Then
the slide was viewed through the compound microscope. Pollen morphological details were recorded. Pollen size was measured attaching an ocular micrometer to the light microscope. Ocular micrometer was uniformly calibrated using a stage micrometer. Pollen grains were mounted in a drop of glycerine on a slide viewing through a dissection microscope. The grains were counted using a compound microscope. The total number of pollen grains per flower was calculated. Aperture type, shape of pollen and NPC classification were done based on Erdtman (1969).

5.3.9.1. Acetolysis method

The undehisced mature anther or whole flower bud was preserved in Acetone or in 70 % rectified spirit. The pollen grains were prepared for light and Scanning Electron Microscopy (SEM) by the standard methods described by Erdtman (1952) and Nair (1971). Light microscopic observations were done (Microscope- Olympus, CX 21i and Camera-Olympus, E-PL1) and SEM photographs (ZEISS, EVO 18) were analysed. Size and shape of pollen, exine thickness, number of colpus, position, shape, character of exine surface were studied. The protocol for acetolysis of *R. aquatica* pollen grain is as follows:

Wash the anther with glacial acetic acid

Crush the anther in a few drops of 70 % alcohol on a glass slide

Remove debris and collect the pollen grain in 3 ml of 70 % alcohol

Centrifuge at 2500 rpm for 3 min and decant the supernatant

Add glacial acetic acid 3 ml and centrifuge at 2500 rpm (3 min)

Decant the supernatant, add acetic anhydride + conc. H₂SO₄ (9:1 ratio)

Warm in 70°C to boiling point (1.5 min)

Centrifuge at 2500 rpm (3 min)

Add glacial acetic acid and centrifuge at 2500 rpm (3 min; brownish residue)
Decant the supernatant, add distilled water

Centrifuge at 2500 rpm (3 min, 3 times until colour clears)

Add 50 % glycerin and kept for 20 min

Centrifuge at 2500 rpm (3 min)

Decant the glycerin part and keep upside down for drying

Mount one portion of the acetolysed pollen on glycerin jelly

Seal the cover glass with paraffin wax (58-60°C) and label it.

One portion of pollen was used for SEM photographs.

The shape of pollen is calculated by Polar axis/Equatorial diameter x 100 (Erdtman, 1969; Bhattacharya, 2006).

5.4. Results

5.4.1. Phenology

5.4.1.1. Flowering phenology

*R. aquatica* inhabits in a riparian ecosystem, where it faces drastic seasonal changes in a year. The area selected for phenology and reproductive biology studies was a typical habitat of *R. aquatica*. The plant is adapted to monsoon floods and summer drought. During monsoon the plant become submerged in the water for more than five months from June to November. During this time plant has no leaves or short tiny branchlets on main flexible branches, which are floated in water current (Fig. 18.b). In submerged time the branches shows chlorophyll on surface give a greenish appearance. These adaptations help to survive the plant in adverse heavy monsoon floods in riparian habitat. Once the monsoon is over, new leaves sprouts on the branches and the plant is in active vegetative growth. Newly formed young branches have pinkish hue on tender stem. Later the colour of stem gradually turns to stony gray colour.
Post monsoon climatic conditions promote vegetative flourishing and lead to blooming. Numerous arrested branchlets and few trailing branches arise from the existing branches (Fig. 18.a). The plant produces two types of branches in a growing season; one type is of arrested, tiny branches with dense leaves and having inflorescence or flower and the later is trailing type. The arrested tiny branchlets are crowded on stem which later dry out in summer or decay during monsoon floods; trailing branches, which vigorously grows up to 1-1.5 meters in a growing season. The leaves are larger than those of tiny branchlets and not densely arranged. These trailing branches prefer to vigorous vegetative growth rather than producing inflorescence. After the vegetative growth, trailing branches bears inflorescence or produces roots and vegetative propagule (saunter type, which will discuss later) at the tip. Trailing branches which happens to touch in water produce roots at the nodes or tip of branch in which establishing to soil and axillary bud grows vigorously upright to become a new daughter clump. This type of vegetative multiplication is common in this plant and not observed among other riparian plants.

Figure 18: R. aquatica in flowering season (a) and in monsoon (b)

The southwest monsoon showers in Kerala starts from June marking the end of hot summer, and continues in July, August and September. Flooding and heavy water current occurs on rivers in hilly terrains. In September there is a repeat to rains last then the Northeast Monsoon showers starts in late October or in November leads again to flood and heavy flow in rivers. Moderate temperature, water availability and adequate sunlight sets in, after the monsoons until hot summer starts in March to June.
There are few summer showers in the end of January or in February. These summer showers mediate disperse of fruit or seeds to the river beds or rock crevices and trigger the germination.

5.4.1.2. Floral phenology and anthesis

Anthesis starts from early morning between 06.30 and 07.30 am in peak flowering season at normal climatic conditions. No anther dehiscence at the time of anthesis. The maximum number of flowers (86.34 % buds) opens in between 06.45 to 07.15 am in the morning (Fig.19). There is no anthesis in the evening or in night. Later period of flowering on March-April the anthesis and anther dehiscence become delayed by one hour approximately. In my observation on 2.3.2010 at Adukkam of Meenachil river, anthesis started at 8.15 am 34% and at 9.15 am 68 % but anther dehiscence and floral visitors was not noticed till 11.00 am.

Table 8: Anthesis in *R. aquatica* on peak flowering time

<table>
<thead>
<tr>
<th>Time</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.45-6.30</td>
<td>3</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>4.67</td>
<td>1.10</td>
</tr>
<tr>
<td>6.30-7.15</td>
<td>46</td>
<td>40</td>
<td>42</td>
<td>42</td>
<td>46</td>
<td>43</td>
<td>43.17</td>
<td>0.89</td>
</tr>
<tr>
<td>7.15-8.00</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2.17</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Figure 19: Anthesis in *Rotula aquatica*
Anther dehiscence starts only after anthesis. The basifixed anther dehescie is latrorse type in *R. aquatica* and happens in between 8.30 am to 10.30 am. In six days observation, 77.66 % anther dehiscence occure between 9.15-9.45am. Floral visitors come after anther dehiscence. The pollen grains are the floral rewards for pollinators and visitors. The petals acts as the landing place for the pollinators. The corolla become closed and decolourised in evening and shed by next day (Fig. 24.n). The persistent style extends out from the closed calyx. The covered persisting calyx protects the immature fruit in younger stage.

<table>
<thead>
<tr>
<th>Time</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Mean</th>
<th>SE</th>
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<tbody>
<tr>
<td>8.45-9.15</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>4</td>
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<td>8.33</td>
<td>0.99</td>
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<td>9.15-9.45</td>
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<td>38</td>
<td>38</td>
<td>43</td>
<td>40</td>
<td>38</td>
<td>38.83</td>
<td>0.98</td>
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<td>5</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2.83</td>
<td>0.70</td>
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</tbody>
</table>

**Figure 20: Anther dehiscence in *R. aquatica***

**5.4.2. Reproductive morphology and sexuality**

*R. aquatica* possesses bisexual flowers (hermaphrodite). Flowers are of pentamerous and actinomorphic, arranged in few flowered axial or terminal cymes.
The expression of gender of flower is hermaphrodite (bisexual) type which produces flowers having functional androecium and gynoecium. Flower bud shows imbricate aestivation. Bracts are lanceolate and two in numbers. Calyx consists of five lanceolate, acuminate and imbricated sepals which is hairy in nature. The corolla campanulate consists of five oblong, spreading pink petals. In bud stage the stigma is surrounded by anthers and not receptive. Flower is having 5 functional anthers inserted on the mouth of corolla tube. A mature anther consists of four wall layers. Pollen grains were shed at two celled stage.

Gynoecium consists of superior, oval, bicarpellary, incompletely 4 celled ovary, one ovule per each locule and a terminal style with slightly bifid and capitate stigma. Style is persistent and slightly longer (up to 3 mm) than anther. The style and filaments are pinkish in colour. The pistle has a length up to 6 mm. The globose ovary is pale coloured, style and stigma pinkish in colour. The ovule is anatropus type. Nectary was not prominent and nectar was very little to measure in the flower. Only one or rarely two flowers open in an inflorescence per day. In peak flowering time at least one flower opens from an inflorescence in every day. Flowers opens in the morning which is pink to purple colour but change hue to bluish violet at the desk is shed after 24 hours (next morning) of anthesis.

5.4.2.1. Receptivity of stigma

Stigma receptivity was observed at different stages of flower. The bifid stigma is wet and is receptive from the anthesis it extends to 5 hours in unpollinated flowers.

Table 10: Receptivity of stigma on different stages of anthesis

<table>
<thead>
<tr>
<th>Time</th>
<th>Time of Anthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
</tr>
<tr>
<td>Receptivity</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: Stigma receptive (+), Not receptive (-), (n=10)
After 6 hours of anthesis the stigmatic surface becomes dry. *In vivo* germination of pollen grains after controlled pollination showed stigma receptivity up to 6 hours after anthesis (Fig. 23.f,g,h).

### 5.4.2.2. Pollen-ovule ratio

The pollen count per flower was estimated as $1284.9 \pm 82.16$ and a single anther produces 256.98 pollen grains. Ovary consists of 4 ovules. The ovule pollen ratio was $1:321.225$

### 5.4.2.3. Pollen viability and germination

Under controlled conditions, mature pollen grains from dehiscing anthers in BK medium having pH 6 showed a maximum germination of 69.22 %. At pH 5.5 and pH 6.5 pollen germinated was 68.63 % and 44.43 % respectively (Table 11). At pH below 5.5 and above 6.5 has no pollen being germinated. Mean value of 10 microscopic fields under 10 x magnification were given on table (11).

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>pH</th>
<th>% of pollen germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5.5</td>
<td>68.63 ±3.11</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>69.22 ±4.72</td>
</tr>
<tr>
<td>4</td>
<td>6.5</td>
<td>44.43 ±2.08</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>36.71 ±2.88</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

On BK medium of pH 6, maximum pollen grains germinated (69.22%) was recorded from mature dehiscing anthers (Fig. 23.a). Pollen grains from anthers one hour prior to dehiscence showed 49.7 % germination and 64.32 % germination one hour after anther dehiscence. Pollen grains from anthers which are 2 hours and above prior to anther dehiscence does not have germination on BK medium (Table 12).
Table 12: Germination % of pollen grains at different maturity

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Anther maturity</th>
<th>% of pollen germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-4 hours</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-2 hours</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>-1 hours</td>
<td>49.70 ± 2.2</td>
</tr>
<tr>
<td>4</td>
<td>Anther dehiscence</td>
<td>69.22 ± 4.7</td>
</tr>
<tr>
<td>5</td>
<td>After 1 hour</td>
<td>64.32 ± 4.6</td>
</tr>
</tbody>
</table>

5.4.3. In vitro pollen tube growth and vigor of pollen

In standardized BK medium of pH 6 under controlled physical conditions the pollen tube growth was estimated. The pollen tube attained 76.992 μm, 116.596 μm

Table 13: In vitro pollen tube growth and vigour.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Pollen tube growth (μm)</th>
<th>Growth rate (μm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>76.992 ± 7.67</td>
<td>153.984</td>
</tr>
<tr>
<td>1.5</td>
<td>101.935 ± 5.71</td>
<td>24.943</td>
</tr>
<tr>
<td>2.5</td>
<td>144.586 ± 15.33</td>
<td>42.651</td>
</tr>
<tr>
<td>3.5</td>
<td>188.414 ± 11.50</td>
<td>43.828</td>
</tr>
<tr>
<td>4.5</td>
<td>219.101 ± 17.57</td>
<td>30.687</td>
</tr>
</tbody>
</table>

after half an hour and 158.887 μm, 200.637 μm and 227.650 μm after 30 min, 1.5h, 2.5g, 3.5h and 4.5h after one hour gaps respectively (Table 13). The rate of pollen tube growth was represented in Fig. (21). The pollen tube growth after 30 minutes observation was 76.99 μm lengths at a rate of 153.984μm/h. After 1.5 h the pollen tube growth was estimated to be 116.596 μm and the vigor of pollen tube growth was reduced to 39.604μm/h. After 1.5 hours the vigor of pollen tube growth was decreased but stable up to 3.5 hours with a growth rate of 0.687 μm/min (Fig. 22 & 23.a-d). Gradually the rate of growth of pollen tube was decreased by 0.450 μm/min after 4.5 hours observation (Fig.23.e).
Figure 21: Pollen tube growth in BK medium

Figure 22: Vigour of pollen tube in BK medium
Legend: a-germination of mature pollengrains on pH 6 BK medium, b,c,d&e-growth of pollen tube on BK medium, f,g&h- *in vivo* germination of pollengrains after controlled pollination.

Figure 23: *In vitro* and *in vivo* pollen germination
5.4.4. Confirmation of breeding system by controlled pollination

The maturation of androecium and pistil *i.e.*, anther dehiscence and stigma receptivity is on same time so the flower is homogamy. In controlled pollination 100% seed setting was found in both cross pollinated and self pollinated flowers. The stigma becomes dried and senescence occurs in those unfertilized flowers. Natural pollination is by melittophily. In open pollinated flowers 98.33 % seed setting was found. The seed formation by fertilization may be possible by self or cross pollination. The floral reward was the pollen grains and the nectar is very meager. The flowers have no distinctive odor for human sense.

There was no self incompatibility found in *R. aquatica* because both self and cross pollinated emasculated flowers produced viable seeds 100 % respectively. Open pollinated flowers were produced 98.33 % seeds. But unfertilized *i.e.*, emasculated, non pollinated stigmas fail to produce any fruit or seed set due to apomixes (Table 14). Bagged flowers without emasculation and no artificial pollination, few flowers produced seeds (20%). Naturally self pollination and cross pollination do occurring.

Table 14: Controlled and open pollination in *Rotula aquatica*

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Pollination type</th>
<th>Seeds (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Open pollinated</td>
<td>98.33</td>
</tr>
<tr>
<td>2</td>
<td>Cross pollinated</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Self pollinated</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Emasculated, non pollinated</td>
<td>00</td>
</tr>
<tr>
<td>5</td>
<td>Non emasculated for autogamy</td>
<td>20</td>
</tr>
</tbody>
</table>

5.4.5. Pollination studies

Stigma receptivity and anther dehiscence occurs after anthesis in the morning time. The stigma lay surrounded by anthers before one day of flower opening but the length of stigma become protruded out at the time before anthesis. The specific timing for anthesis starts when morning rays of Sun are visible. Soon after anthesis,
dehiscence of anther starts while stigma becomes receptive. Floral visitors come at this time. Homogamy and position of stigmas *i.e.*, surrounded by anthers in such a way that self pollination is ensured. The terminal or axial position of flower and spreaded corolla allows the easy visibility and landing of pollinator on flower.

### 5.4.5.1. Preliminary studies on pollination

Study period & suitable time slot was identified after the trials. For enumerating the data on pollination, the study period selected was between December to January after Monsoon raining over. In Kerala the flowering peak of *R. aquatica* was identified as the time after the North East Monsoon and before the summer showers *i.e.*, December to January. The time slot suitable for pollination studies was from morning to evening covering 12 hours observation because the flower opens in the morning and close or shed in the evening of the same day.

### 5.4.5.2. The identity of floral visitors

Collected and labeled floral visitors were identified as *Apis cerana*, *Trigona iridipennis*, *Xylocopa* sp. and as pollinators and, *Ceratina* sp, yellow crazy ant *Anoplolepis gracilipes* Smith and Ichneumon Wasp was also a robber visitor who does not involve in pollination.

### 5.4.5.3. Confirmation of pollinator based on pollen load

Pollen grains were found on the lower surface and legs of pollinator *Apis cerana* (Fig. 24.a) and on stigmatic surface of *Rotula aquatica* after the first visit on tagged flowers. *Trigona iridipennis* (Fig. 24.e) also collected in each visits. These pollen grains were identified under microscope as same as of *Rotula aquatica*.

### 5.4.5.4. Behavior of Pollinator

Floral visitors of each species behave differently. *Apis cerana* make vibrant movements on flower for collecting the pollen. This pollinator come and directly lands on the flower touching the anthers and stigma. *Apis cerana* actively forage and move to the next nearby flower of *R. aquatica*. *Trigona iridipennis* visits the flower and go around the anthers and pistil. It massively forages and collects the pollen.
grains and during this time, it touches the stigma. *Ceratina* sp only visits the flowers and immediately goes without landing. But Crazy ant and wasp come and make a touch corolla without disturbing the stigma or round dance, does not have vigorous foraging movement on floral surface. The wasp and ant go to the bottom of corolla. They were not taking the pollen.

Landing and foraging: The pollinator *Apis cerana* directly lands to top of flower (Fig. 24.b) and *Trigona iridipennis* after few rotations land to the petal and takes pollen by vigorous movements (Fig. 24.e). Active foraging after anther dehiscence when pollen grains are available. Pollinator *Apis cerana* lands on top of flower touching the stigma, anthers and petals and takes pollen in few seconds (Fig. 24.b-d). The *Trigona iridipennis* after landing rotates inside the corolla around stamens and in between anthers inside the corolla tube (Fig. 24. e-i). Both pollinators touches the anthers and stigma on landing on the flower or while foraging. The erect stigma becomes bended downwards after an active foraging. The pollen grains were deposited on the sticky stigmatic surface and also on body of pollinator (24.p).

Pollinators: *Apis cerana, Trigona iridipennis* and *Xylocopa* sp.

Floral visitors: Yellow crazy ant *Anoplolepis gracilipes*, Ichneumon wasp (Fig.24.q&r) and *Ceratina* sp.

Robber: Yellow crazy ant and Ichneumon wasp are the robbers. Crazy ant come and goes after a wandering but Wasp eats pollen and anther also. Some times ants cannot reach many of the flowers because the plants are in aquatic condition (Fig.24.r). The wasp is very minute one and it easily passes through corolla tube.

Pollen rewards: Pollen grains mainly and nectar.

5.4.5.5. **Based on seed set**

Seed setting of controlled pollination by single visit of *Apis cerana, Trigona iridipennis, Ceratina* sp, *Xylocopa* sp, Ichneumon Wasp, Crazy ant and flowers having no visits and open visits were studied (Fig.24.j-m). The mean number of seeds
per flower was calculated. Pollination efficiency of each visitor was calculated with
the Spears formula.

### 5.4.5.6. Pollination efficiency index

Pollination efficiency calculated by Spears pollination efficiency index PE =
Pi –Z / U –Z shows that *Apis cerana* performs the highest seed setting with PE 0.98
and by *Trigona iridipennis* (PE=0.38) and *Xylocopa* sp. by 0.25 PE (Table 13). Others
fail to pollinate on controlled pollination.

Table 15: Pollination efficiency of different floral visitors of *R. aquatica*.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Floral visitor</th>
<th>Mean seed set/flower (N=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pi</td>
</tr>
<tr>
<td>1</td>
<td><em>Apis cerana</em></td>
<td>3.86</td>
</tr>
<tr>
<td>2</td>
<td><em>Trigona iridipennis</em></td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td><em>Xylocopa</em> sp.</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td><em>Ceratina</em> sp.</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Crazy ant</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Ichneumon Wasp</td>
<td>0</td>
</tr>
</tbody>
</table>

### 5.4.5.7. Pollen morphology studies

Pollen morphology of *Rotula aquatica* was done by light microscopy and
scanning electron microscopy measurements and photographs were taken and
analysed (Table 16). The better preserving result was obtained from anthers stored in
Acetone. The shape of pollen is prolate (Polar diameter/equatorial diameter x 100= 145).
Legend: a-d *Apis cerana* & foraging, e-i *Trigona iridipennis* foraging, j-m controlled pollination, n-anthesis & senescence, o-pollinated flower, p-pollen load, q-Ichneumon Wasp, r-Crazy ant.

Figure 24: Pollination of *Rotula aquatica* flower.
As per NPC Classification based on aperture the number is three, position is zono i.e., equatorial (Fig.25.b,c & d), character and structure of aperture is colporate (Fig.25.a&c) and character of exine surface was foveolate (Fig.25.e). The SEM photographs of acetyolysed pollen (Fig.25.c & d), unacetyolysed pollen (Fig.25.a &b) and photomicrograph of fresh pollen grain (Fig.25.f, g &h) were shown on Fig. (25.f-h). Hence the pollen morphology of *Rotula aquatica* is described as 3-zono colporate.

Table 16: Pollen morphology of *Rotula aquatica*

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Characters studied</th>
<th>Position of view</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Size of pollen</td>
<td>Polar x Equatorial</td>
<td>Prolate P 29 µm x E 20 µm (P/E*100=145)</td>
</tr>
<tr>
<td>2</td>
<td>Shape of pollen</td>
<td>Polar, Equatorial</td>
<td>Triangular, obtuse, convex Angular, rhombic, truncate</td>
</tr>
<tr>
<td>3</td>
<td>Exine thickness</td>
<td>Polar</td>
<td>2.5 µm</td>
</tr>
<tr>
<td>4</td>
<td>Number of aperture</td>
<td>Polar view</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Position of aperture</td>
<td>Equatorial</td>
<td>Zono</td>
</tr>
<tr>
<td>6</td>
<td>Character of aperture</td>
<td>Zono (Equatorial)</td>
<td>Colporate</td>
</tr>
<tr>
<td>7</td>
<td>Exine surface</td>
<td>Polar</td>
<td>Foveolate</td>
</tr>
</tbody>
</table>
Legend: a\&b-SEM of unacetolysed pollen, c -SEM of acetolysed pollen equatorial view, d- polar view showing 3 colpate aperture, e-foveolate exine ornamentation, f,g\&h- pollengrains under 100x

Figure 25: Pollen morphology of *Rotula aquatica* Lour.
5.5. Discussion

Through the extensive field studies, and observation of three flowering seasons the phonological and reproductive system of *Rotula aquatica* was studied. On summer, the riverbeds of most of rivers in Kerala become dries up. Loss of periniability of river is not only due to the summer effect but also construction of reservoirs and river diversion enhances the scarcity of water. On Monsoon the river bed flood off and the entire habitat will be of heavy water flow. The loss of river beds and increase in depth of river basins happens in every year. Flowering happens after monsoon. The anthesis is diurnal which take place in between 06.30 and 07.30 am. Anther dehisces between 08.30 to 10.30 in morning. *Apis cerana, Trigona iridipennis* and *Xylocopa* sp. confirmed as pollinators based on pollen load and seed setting. These are the common pollinators in several species. Viability of mature pollen was studied in different pH of BK medium showed pH 5.5 and 6 are optimum. Mature pollen showed good response in pH 6 of BK medium. No sexual incompatibility was observed while conducting controlled pollination. Pollen viability and vigour, homogamy, possibility of cross and self pollination and presence of pollinators promotes more seed setting on this plant. Pollen morphology of *R. aquatica* was 3 zono colporate based on the SEM studies of acetolysed pollen.

5.6. Conclusion

Based on this investigation it is found that the plant has no sexual incompatibility or pollination difficulties. The reproductive syndrome of this plant favours the maximum fertilization. Pollen viability and vigour of pollentube, timing of anther dehiscence and stigma receptivity, presence of several pollinators and no specity on a particular pollinator, sufficient pollen rewards leads to high rate of seed production. Fruits produced were containing four seeds each. Fruits and seeds were dispersing by hydrochory. Because of hydrochoral mode of seed dispersal, the plant depends on the favouring optimal summer rains for the effective seed dispersal and thereafter seedling recruitments.