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Original Paper

Floral characteristics support nocturnal pollination and pollination syndrome in *Barringtonia racemosa*

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Abstract

To test for pollination syndrome and to complement records of foraging visitors of an associate mangrove, *Barringtonia racemosa* (L.) Spreng., aspects of its floral biology were investigated. Phenology and floral rewards (pollen viability, pollen histochemistry and nectar sugar concentration) were focused on owing to their role in plant-pollinator interaction. During the floral opening at 19:00, pollen viability, lipid presence and nectar sugar concentration were highest at 88.05 ± 0.98%, 80.69 ± 10.51%, and 68.98 ± 1.09% mg flower^{-1}, respectively. Starch presence in pollen was rather low (mean 24.95 ± 14.58%), due to hydrolysis of starch occurring during anthesis. These floral characteristics, which reflected the highest reproductive activity, coincided with nocturnal foraging of previously recorded moth visitors. Although bats were rarely observed in previous studies, nocturnal anthesis complemented with the recorded large amount of sugar in nectar (68.98 ± 1.09% mg flower^{-1} = 1158 J) might be sufficient to provide the average needs of nocturnally foraging bats. Investigation of floral characteristics to complement observation of floral visitors is highly recommended to give a better understanding of the pollination system. Moreover, this study supports the pollination syndrome concept, emphasizing the need for investigation of the role of floral rewards in plant-pollinator relationships.

Key words: anthesis, *Barringtonia racemosa*, nectar sugar content, pollen histochemistry, pollination syndrome.

Introduction

Aspects of floral biology like morphology, anthesis, and biochemistry of floral rewards can infer relationships with possible pollinators (Faegri, van der Pijl 1979; Perez et al. 2006; Whittall, Hodges 2007; Fleming et al. 2009). The suites of floral characteristics suggestive of an associated pollinator are referred to as the controversial pollination syndrome concept. Despite challenges to the concept (Ollerton et al. 2009; Wang et al. 2020), various studies have provided strong support to its reliability, with certain modifications with regard to plant-pollinator groups and with caution in providing conclusions (Fenster et al. 2004; Rosas-Guerrero et al. 2014; Aguilar et al. 2015; Abrahamczyk et al. 2017; Vanderlook et al. 2019; Dellinger 2020). Abrahamczyk et al. (2017) particularly emphasized the importance of studying specific traits relevant to functions of plant-pollinator interactions i.e. signals and rewards.

Floral rewards, including nectars and pollen are highly interrelated to, or directly influencing, the foraging behaviors of pollinators such as humming birds (Waser, Price 2016), bumble bees (Brunet et al. 2015; Ceulemans et al. 2017; Mountcastle et al. 2015) and bees (Pelabon et al. 2012). Studies on floral rewards across plant species have also shown how variation in floral rewards is related to foraging behavior of the pollinator (Thomson et al. 1989; Waser, Price 2016; Yan et al. 2016). The above emphasizes the importance of floral rewards, including pollen and nectar, in the establishment of evolutionary relationships between the plant and the pollinator (Diaz, Kite 2006; Farré-Armengol et al. 2015).

Despite such importance, floral rewards are not sufficiently studied among tropical plants, even for widespread and economically important species like *Barringtonia racemosa* (L.) Spreng. (Dichoso 2000; Deraniyagala et al. 2003; Orwa et al. 2009; Musman 2010; Prance 2012; Osman et al. 2015). This mangrove associate species, which inhabits areas near sea, and along streams, and freshwater swamps, is valued as a fuel and timber source (Orwa et al. 2009), for production of soap (Prance 2012), medicine (Deraniyagala et al. 2003; Osman et al. 2015), and molluscides (Musman 2010), as a living fence, and as a source of oil for illumination (Dichoso 2000). Limited data are available on its ecology, more so on its

pollination ecology (Mao et al. 2012; Lin et. al. 2013; Australian National University 2018; Aluri et al. 2019). Marshall (1983) mentioned the plant as chiropterophilous based on position of the inflorescence. On the other hand, moth species have been documented to forage on the plants in two different studies: in Iriotome Island, Japan (Tanaka 2004) and in Andhra Pradesh, India (Aluri et al. 2019).

Considering the indefinite pollination system and limited studies on the plant, this study focuses on the floral biology of the plant through investigation of floral rewards, including pollen biochemistry, nectar sugar concentration, and phenology. These traits capture the essential dimension of pollination and can provide reasonable support to the hypothesis of pollination syndrome (Abrahamczyk et al. 2017). The aim of the study was to investigate floral dynamics to verify whether reported visitors of *Barringtonia racemosa* L. (Spreng.) are complemented with plant rewards, determined by biochemical and physiological parameters. Following the reports of Marshall (1983), Tanaka (2004), and Aluri et al. (2019), it was hypothesized that floral dynamics would show the highest reproductive activity nocturnally; and that pollination syndrome based on floral rewards would point to either bat or moth pollination. The hypothesized complementary information on floral rewards and reported visitors would enable to confirm the pollination relationship.

Materials and methods

The study was conducted at Barangay Maligaya, Palanan, Isabela (17°6'43.254"N, 122°27'55.7676"E; altitude 12 m) during April and May 2018. Three *Barringtonia racemosa* stands with robust flowering were randomly chosen, where four mature flowers from each tree were harvested hourly. These 12 flowers were used as replicates for each histochemical test per sampling hour. The floral samples were collected hourly within a 24-h period to monitor floral dynamics (pollen histochemistry and floral phenology) from floral opening to dehiscence.

Pollen histochemical tests followed protocols from Dafni (1992). Specific histochemical stains included aniline-lactophenol blue stain and acetocarmine stain to test pollen viability test, Sudan Red 7B stain to estimate lipid presence, and I2KI to estimate starch presence (Dafni 1992; Munhoz et al. 2008; Ge et al. 2011). All implemented histochemical tests were colorimetric, where acetocarmine, aniline, I2KI and Sudan Red 7B stained red, blue, brown to black, and red, respectively. Stained pollen recorded as positive indicated presence, and unstained (negative) indicated absence of the biochemical component of interest. A freshly collected anther was macerated on a slide with the respective stain. Each slide is covered with a cover slip with a 10×10 grid and sealed with dissolved nitrocellulose. This was conducted within no more than 15 min after plant collection. Immediate fixation after collection was vital to preserve the time-specific biochemistry of the sample. Pollen (stained and unstained) were manually counted from 10 grids, using the grided coverslip as guide, using a compound microscope and recorded as either positive (stained) or negative (not stained). Results were presented as percentage values determined by dividing the number of positively stained pollen grains over the total number of pollen grains counted from the 10 grids. All collection and staining were conducted on site, as was also microscopy to perform counting for the lipid test using Sudan Red 7B, immediately after fixation due to the short life of the dye. All slides prepared for the other histochemical tests were counted for positively stained pollen later in the laboratory (Dafni 1992).

Nectar samples were collected from flowers during floral opening (19:00) until dehiscence the next day (6:00). Due to the interfering voluminous stamen adnate at the floral base, the washing method was most effective for nectar collection (Morrant et al. 2009; Power et al. 2018). Collected nectar samples were kept in an ice chest during collection and transportation, and then were transferred to a freezer upon reaching the laboratory to prevent fermentation. From the 100 µL aliquot nectar washings, total nectar sugar concentration was determined using the phenol sulfuric acid method (de Toledo et al. 2012) with 5% phenolic acid (0.5 mL) and 2.5 mL 95% sulfuric acid for sugar hydrolysis. Vortex mixing was conducted prior to addition of each reagent and the final solution was allowed to stand for 30 min prior to spectrophotometry. Positive results of phenol and hydrolyzed sugars appear yellow to orange with absorbance at 490 nm using a pre-calibrated 752S UV-Vis spectrophotometer (de Toledo et al. 2012). Standards were prepared using 1:1 glucose-fructose solution (de Toledo et al. 2012). Known concentrations were determined and were prepared through serial dilution and the calibration equation ($y = 0.1386x + 2.9299$; $R^2 = 0.9362$) was used to calculate for unknown concentrations of the nectar samples (Thermo Fisher Scientific Inc. 2012). Similar standards for sugar hydrolysis were used for the nectar samples. Absorbance was as well read at 490 nm. The energy equivalence of 1 mg of sucrose (4 calories = 16.8 joules) was used to calculate the amount of energy provided by the nectar for its floral visitors (Dafni 1992).

Individual floral phenology was studied by monitoring the flowers hourly from bud stage until dehiscence (Dafni 1992). Two inflorescences per tree, for a total of six inflorescences, were tagged and monitored.

The relevant physicochemical parameters (relative humidity and temperature) were recorded hourly using a handheld digital meter (Neda 1604).

Statistical analyses were carried out using R Statistical Software (2018). Normality and tests for heterogeneity were conducted as pretests. The non-parametric Welch's test and Games-Howell post ad hoc test were used to test for significant differences between sampling times.

Correlation analysis using the Pearson method was used to determine presence of any linear relationship between dynamics of floral biology and the physicochemical parameters measured (relative humidity and temperature).

Results

Floral phenology

Details of the development of an individual flower of *B. racemosa* are presented in Fig. 1. The bud stage took a week to develop, starting from a small oval-elliptical green (Fig. 1A) to a larger, red brown and rounder bud (Fig. 1B). Onset of floral opening started with breaking of sepals into two distinct parts and their color becoming lighter red brown; the petals (folded) were well exposed at this stage (Fig. 1C). In the last stage of the bud before fully breaking, the sepals divided into four parts while the enclosed petals and coiled stamen were in a much larger coil (Fig. 1D). Buds as shown in Figure 1D are about to open on the following night. At about 18:00, the stamens began to uncoil as the floral opening began (Fig. 1E as shown by the arrow). The flowers were mildly dichogamous and specifically protandrous. After more than an hour, full floral opening was observed with the stigma visible while the stamen slightly extended (Fig. 1F–H). Flower maturation continued until the stamens were fully extended as shown in Fig. 1I. The flower remained for almost the entire night and fell the following morning. Fig. 1J shows the floral base with female parts only remaining and Fig. 1K shows basally connate stamen detached from the floral base, which was about to fall off. Within an inflorescence, floral opening was noted to be asynchronous for *B. racemosa.* Fig. 1G–I show inflorescences having both dehisced flowers and floral buds.

Pollen biochemistry

From six replicate flowers, an average of 656.67 ± 260.92 pollen grains per anther (range 416 to 1084 grains per anther) of *B. racemosa* were observed.

Pollen histochemical tests reflected positive results (presence) of a specific compound when the resulting sample was darkly stained; otherwise, an absence of the compound was recorded (Fig. 2). Percentage pollen viability estimated by acetocarmine and lactophenol-aniline blue staining over time is presented in Fig. 3. Pollen viability significantly differed over time: aniline-lactophenol blue

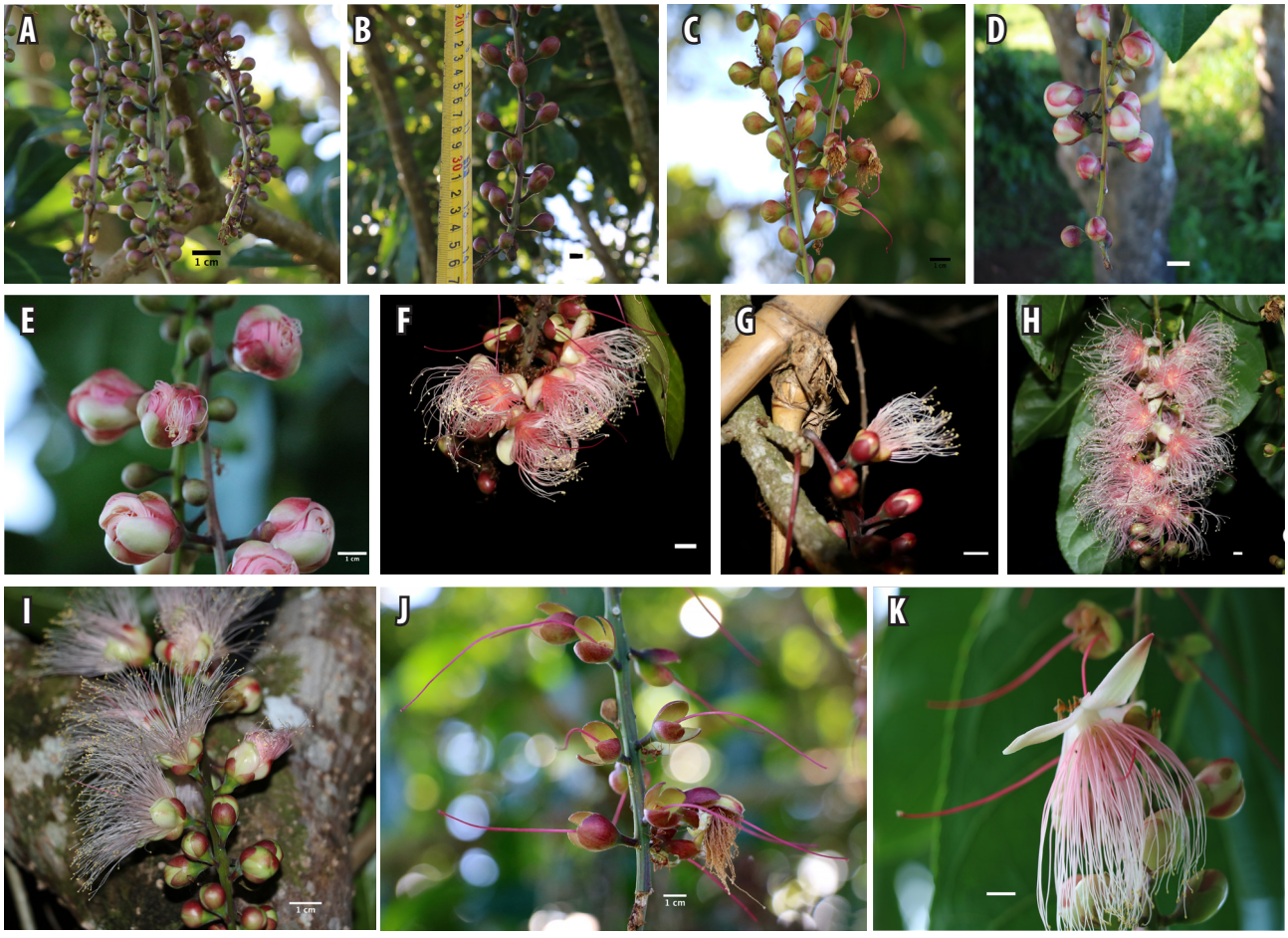

Fig. 1. Individual floral phenology of *Barringtonia racemosa* observed from bud stage (A – D) to breakage (E,) full opening (F – I), until dehiscence (J – K).

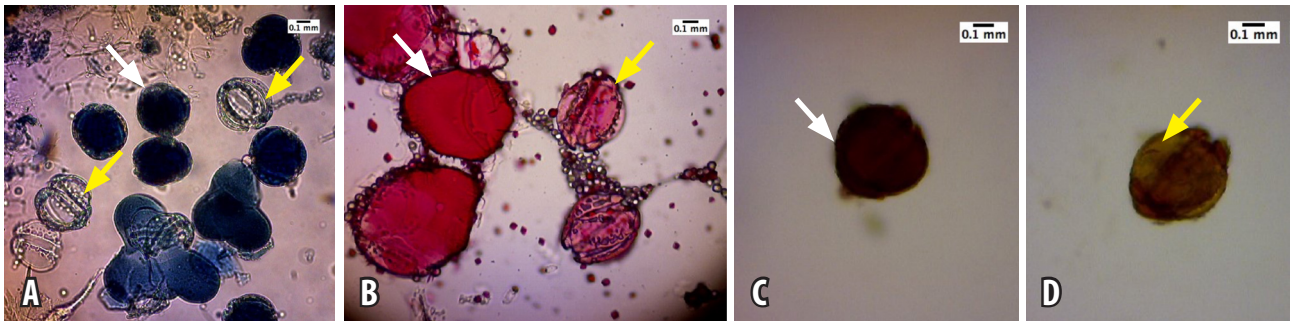

Fig. 2. Pollen viability test using aniline-lactophenol (A), acetocarmine (B) and (C – D) starch content test using IKI (C – D). Darkly stained (pointed by white arrow) indicate viability (A – B) and presence of starch (C). Unstained (pointed with yellow arrow) pollen grains indicate non-viability (A – B) and absence of starch (D).

stain ($F_{20,70}$ = 37.58, $p < 0.001$) and acetocarmine stain ($F_{19,65}$ = 4.95, $p < 0.001$). There was evidence that pollen viability was not stable and that the *B. racemosa* gametophyte may be viable only for a specific period of the day. Specifically, the gametophyte was observed to be most viable from 19:00 to 22:00, and around 4:00, based on the post ad hoc test (Fig. 3). The results showed the highest pollen viability during the first few hours when the flower was open (19:00 to 22:00) and then it decreased at noon on the day after it opened.

Presence of lipids and starch over time are presented in Fig. 4. Occurrence of lipids was lowest at 1:00 (0.69 ± 1.95%) and highest at 22:00 (80.91 ± 28.16%). Presence of starch ranged from 8.83 ± 23.57% at 2:00 to 40.98 ± 6.63% at 1:00. Percent presence of both lipids ($F_{19,61}$ = 63.98, $p < 0.001$) and starch ($F_{20,75}$ = 4.34, $p < 0.001$) differed significantly over time. The level of lipids sampled between 19:00 and 22:00 was similar to that sampled between 4:00 and 5:00, and significantly differed for that sampled at midnight (00:00) and in morning (8:00 and 10:00). Presence of lipids followed the trend of pollen viability, where higher percent presence occurred during floral opening and was very low the following morning. In contrast, presence of starch followed a different trend with lower percentage of stained samples during floral opening and higher after the flower has aged. The post ad hoc test showed significant differences in presence of starch only between 20:00 and 2:00, and between 9:00 and 3:00. No other significant differences in presence of starch over time were found, indicating that presence of starch was generally similar across time after floral opening.

Consequently, all histochemical tests on pollen samples (high viability, high lipid percent presence, low starch percent presence) suggested an active male gametophyte during the night at floral opening and at dawn around 2:00. Twelve hours after opening, histochemical tests (low viability, low lipid) highly suggested loss of vigour in the male gametophyte.

Nectar sugar content

Sugar concentrations in nectar samples ranged from 8.09 ± 0.99 to 68.98 ± 1.09 mg flower^{-1} (Fig. 5) which across time revealed significant differences ($F_{4,4.87}$= 11.02, p = 0.0119). Nectar sugar concentration collected during floral opening at 19:00 to 21:00 was significantly higher compared to nectar samples collected from old flowers. A continuous

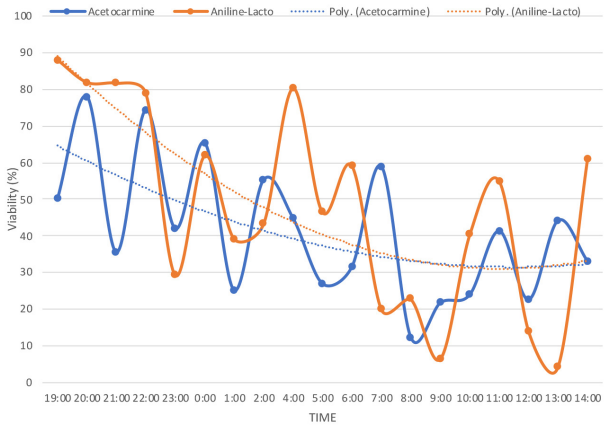

Fig. 3. Percent viability of *Barringtonia racemosa* pollen across time using aniline-lactophenol blue and acetocarmine staining method.

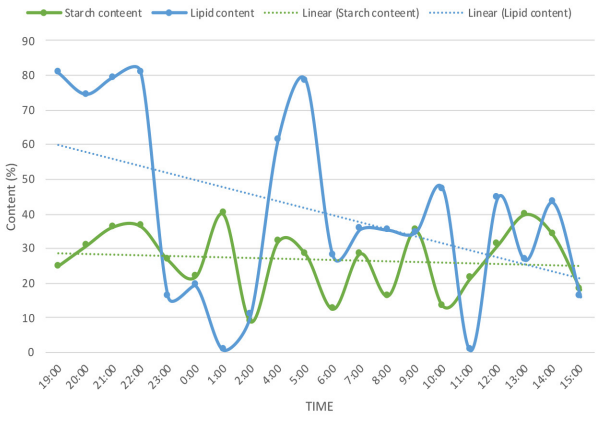

Fig. 4. Lipid content and starch content of *Barringtonia racemosa* pollen across time using Sudan Red and IKI staining method, respectively.

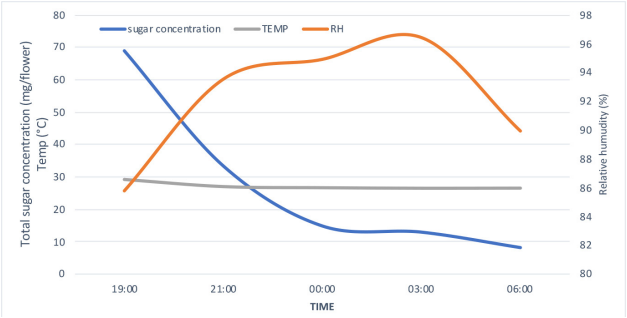

Fig. 5. Total nectar sugar concentration per flower of *Barringtonia racemosa* sampled across time.

decrease in nectar sugar concentration was observed from 24:00 until next morning at 6:00 as the flower aged (Fig. 5). Consistent with pollen biochemistry, the floral reward in the form of nectar was highest during floral opening.

In situ, total sugar concentration was highest (68.98 ± 1.09 mg flower^{-1}) when relative humidity was low (85.77%) and temperature was relatively warm (29.12 °C) (Fig. 5). Correlation analyses revealed that there was strong but non-significant negative linear relationship between relative humidity and nectar sugar concentration (r = –0.725077; p = 0.1657). On the other hand, a significant and strong positive linear relationship between temperature and nectar sugar concentration (r = 0.9741406; p = 0.004972) was observed.

Based on the sugar concentration in nectar, a single flower of *B. racemosa* was calculated to provide maximum energy of 1158.91 J during floral opening, while an aged flower could still provide about 135.84 J.

Discussion

Floral phenology

With a mean number of 656.67 pollen grains and eight ovules (Payens 1967; Chantanarothai 1995), the pollen to ovule ratio reflected the *B. racemosa* exhibited facultative autogamy (Cruden 1977). The observed floral phenology supports that anthesis occurs during the night starting at 19:00. Floral longevity was less than a day, as the flowers that opened at night fell by morning. The same floral phenology was also observed by Aluri et al. (2019) for *B. racemosa* in Andhra Pradesh, India. While the short life span of the flower is associated with Lecythidaceae (Prance 1976), records of their time of opening varies. *B. racemosa* is observed to open at night (Tanaka 2004; Orwa et al. 2009), while other members of Lecythidaceae open during the day (Prance 1976; Maués 2002). Opening in an inflorescence was also asynchronous for *B. racemosa*, as seen in Fig. 1G–I, inflorescences had both dehisced flowers and closed floral buds. This agrees with the observation of Orwa et al. (2009), who noted that only half of the inflorescence opens at the same time. Such asynchrony in floral opening was considered to contribute to reproductive success by allowing more visitation from pollinators (Sun et al. 2009; Gates, Nason 2012). This observed floral phenology of the plant occurs in parallel to changes in pollen histochemistry and nectar physiology.

Pollen histochemistry

The number of pollen grains ranged from 416 to 1084 grains per anther. Similarly, wide variation in number of pollen grains was observed in the same species by Aluri et al. (2019). Wide variance in pollen grain number per anther was also observed among other plant species, with pollen production known to be related with anther size and length and number of anthers per flower (Piotrowska 2008; Bhowmik, Datta 2013) and with cultivar (Godini 1981; Vidal et al. 2006). Being a brush-type flower, variation in staminal length, anther number, and anther size in each flower is expected.

Pollen histochemistry confirmed nocturnal anthesis, demonstrated by active pollen activity during the opening hours. Aniline-lactophenol blue is a stain for cytoplasmic and nuclear material while acetocarmine is a stain that indicates chromatin integrity; thus, the two stains are widely used in pollen viability tests (Rodriguez-Riano, Dafni 2000; Ferrara et al. 2007; Munhoz et al. 2008; Yeamens, et al. 2014). For *B. racemosa*, both lactophenol-aniline blue and acetocarmine tests showed that pollen was most viable during floral opening. Significantly higher pollen viability from both stains occurred during 19:00–22:00 and was significantly lower at midday, 12:00–13:00 (Fig. 4). This indicates the plant's high reproductive activity, especially of the gametophyte, during floral opening at 19:00–22:00.

High pollen viability during anthesis, assessed using the same stains, was also observed for other species. Tests using aniline-lactophenol blue solution on *Panicum virgatum* (Ge et al. 2011), *Beta vulgaris* (Hecker, McClintock 1989), *Campanula carpatica* (Bengston 2006), *Isatis tinctoria* (Asghari 2000), *Oryza sativa* (Khatun, Flowers 1995) and *Cucumis sativum* (Vizintin, Bohanec 2004) similarly high viability (~80%). On the other hand, tests using acetocarmine stain on other Lecythidaceae members such as *Couroupita guanensis* (Ormond et al. 1981) and *Eschweilera nana* (de Moraes de Potascheff et al. 2014) showed 88.0 and 97.9% viability, respectively. High percentage viability was also observed for other coastal species including *Canavalia rosea, Premna serratifolia* and *Cerbera manghas* (Rigamoto, Tyagi 2005). Also, *Olea europea* cultivars had high viability ranging from 48 to 96% while *Carica papaya* recorded 98.2% viability with acetocarmine used as stain (Ferrara et al. 2007; Munhoz et al. 2008). This study, along with previously conducted studies, confirmed the efficient use of aniline-lactophenol blue and acetocarmine for rapid viability tests on pollen samples.

Nevertheless, high pollen viability has been observed using acetocarmine and aniline blue stain even with pollen

samples that were heat killed, raising doubt to the reliability of the stain for testing viability (Yarsick et al. 1986; Parfitt and Ganeshan 1989; Khatun, Flowers 1995; Soares et al. 2016). However, the variable results of pollen histochemical tests depend on the specific reaction of the pollen across species (Soares et al. 2016); thus, these stains are still widely used with cautious interpretation of results. For *B. racemosa*, high pollen activity reflected through viability tests agrees with floral phenology, and both suggest high floral activity or floral anthesis of the plant at around 19:00. This supports the use of lactophenol-aniline blue and acetocarmine stains as suitable for viability tests in *B. racemosa*.

Other histochemical tests included Sudan Red 7B and I_2KI stains for lipid presence and starch presence, respectively (Fig. 2). Previous studies using Sudan Red for pollen viability tests showed overestimated results, making the stain as a better indicator of pollen lipid alone; while a positive test result may be related to pollen viability, interpretation needs caution (Munho et al. 2008; Vieira et al. 2012; Souza et al. 2017). However, for *B. racemosa*, the results for lipid presence coincided with pollen viability across time, using the aniline-lactophenol and acetocarmine stains (Fig. 2), as well as with floral phenology. Thus, Sudan Red 7B can be recommended as an additional pollen viability rapid test for *B. racemosa*. Lipids provide structural integrity to the membrane of pollen, which indicates that lipid content can be associated with pollen viability (van Bilsen, Hoesktra 1993). Similarly, high percent presence of lipids during anthesis of other plant species was observed in Bromeliaceae species (79 to 100%) (Souza et al. 2017), and in *Manihot* sp. (62.7 to 97.3%) (Vieira et al. 2012) and in *Carica papaya* with 93.3% lipids (Munhoz et al. 2008). The lipid test results of this study contribute to the pool of data on plant species tested using Sudan Red 7B.

Significantly higher lipid content in pollen during floral opening and anthesis at 19:00–22:00 (Fig. 4) can be explained by its role in pollen germination and stigmatic penetration. Various classes of lipids accumulate during pollen maturation, and each class is utilized depending on pollen development (Piffanelli et al. 1997). The importance of lipids in pollen development, especially regarding the exine, was described by Zhang et al. (2016). Also, many authors have shown the importance of lipids in pollen tube growth and penetration to the stigma, emphasizing the importance of lipids in pollination (Mascarenhas 1993; Wolters-Arts et al. 1998; Edlund et al. 2004; Ischebeck 2016). There is a diversity of lipid groups in different parts of pollen (Evans et al. 1991; Bashir et al. 2013; Sidorov et al. 2016). Studies have further examined response of lipid classes to environmental changes and how this affects pollination, to further determine changes in reproductive biology of plants in achanging climate (Edlund et al. 2004; Ischebeck 2016). Such studies are highly recommended for tropical species like *B. racemosa*.

Presence of starch in *B. racemosa* pollen was observed to be rather lower, compared to lipid presence and pollen viability. Percent starch presence was observed to be higher in previous studies on *Carica papaya* (91.2%) (Munhoz et al. 2008), *Eriobotrya japonica* (98.6%), *Prunus* spp. (87.74%) (Bolat, Pirlak 1999) and *Pistachia teribenthus* types (64.73 to 88.24%) (Gunver-Dalkilic, Dayi-Dogru 2011), than in our study on *B. racemosa* (8.83 to 40.98%) (Fig. 5). Presence of starch in pollen is related to the metabolism and maturity of the pollen (Bellani et al. 1985; García 2007). Mature pollen of *Solanum* had starchless pollen at maturity during anthesis, and among wild tomato species, starch was found to accumulate during the microspore bicellular stage and decreased by anthesis (García 2007). The loss of carbohydrates at maturity was due to high metabolic consumption during anthesis (Bellani et al. 1985). Loss of starch during anthesis explains the low detection rate of starch in pollen in this study, as sampling was conducted starting from full floral opening and prospected anthesis of *B. racemosa* until the flower aged (Fig. 4).

Starch in pollen grains is known to play an important role in the hydration of the gametophyte (Franchi et al. 1996; Pacini 1996; Roulston, Buchmann 2000). Carbohydrates in pollen grains may be classified into polysaccharides such as starch, disaccharides such as sucrose, and monosaccharides such as glucose or fructose (Pacini 1996). These carbohydrates can be found in amyloplasts or plastids or dispersed in the cytoplasm or in both (Franchi et al. 1996; Pacini 1996). During dispersal, pollen tend to become dehydrated or partially hydrated. As an adaptive mechanism, large starch molecules are hydrolyzed into simpler sugars including sucrose and glucose to aid in retention of pollen membrane stability, by maintaining turgor pressure at low water levels (Hoekstra et al. 2001; Pacini, Hesse 2004). This may allow starch to become undetectable by simple stain tests during anthesis.

Nectar sugar concentration

Similar to pollen biology, a baseline study on nectar analysis of *B. racemosa* had been lacking, except that of Aluri et al. (2019). Studies are available on a neotropical relative, *Bertholetia excelsa*, which was observed to have a lower total sugar content, ranging from only around 5 to 12 mg per flower (Cavalcante et al. 2018). Other studies on nectar of members within the family were focused on other neotropical species (Mori, Kallunki 1976; Mori et al. 1978; Ayres, Prance 2013). Studies on nectar sugar composition, especially of sucrose, have been conducted on Lecythidaceae species across different pollination guilds (Freeman et al. 1991; Cavalcante et al. 2018). Sucrose-rich nectars were observed among megachiropteran-pollinated flowers, to which *B. racemosa* is hypothesized to belong (Baker et al. 1998). Studies on neotropical chiropterophilous flowers showed that floral nectar samples were more hexose-rich instead of sucrose-rich (Baker et al. 1998; Rodriguez-Pena et al. 2016). For *B. racemosa*, a hypothesized paleotropical

bat-pollinated plant, further studies are needed to establish whether nectar composition is similar to that of Lecythidaceae species or other chiropterophilous plants.

Sugar concentration ranged from 8.09 to 68.98 mg flower^{-1} (Fig. 5), and this is much higher than 0.86 mg sugar flower^{-1} reported for *B. racemosa*, estimated extracted nectar using a refractometer (Aluri et al. 2019). The difference in extraction method, or the timing of extraction (post anthesis) can explain this difference. The highest recorded nectar sugar concentration (68.98 mg flower^{-1}) observed at 19:00 (Fig. 5) indicates high floral activity at this time, which coincides with floral opening (Fig. 1) and pollen viability (Fig 2). Later, at 24:00, there was a decline in sugar concentration (Fig 5). Nectar is a luxurious product of a plant, and is produced as a reward to pollinators; thus, decreasing the total sugar concentration or reabsorption at an idle or non-anthesis period is economical for the plant (Faegri, van der Pijl 1979).

Although it requires more data for support, the nectar sugar concentration was observed to be inversely proportional to relative humidity across time (Fig 5). Studies have shown that with high relative humidity, the evapotranspiration rate decreases due to already high water vapor; in such a case, the sugar concentration decreases as volume of nectar increases with a lower evapotranspiration rate (Oertel 1946; Corbet et al. 1979; Wyatt et al. 1992). On the other hand, ambient temperature showed a direct linear relationship with nectar sugar concentration of *B. racemosa* (Fig. 5).

Available literature, however, reports variable effect of temperature on nectar sugar concentration. Contrasting results were observed on a neotropical species, *Bertholetia excelsa*, where nectar sugar concentration had a negative correlation with temperature (Cavalcante et al. 2018), with induced heat stress attributing to the negative relationship (Wyatt et al. 1992; Descamps et al. 2017). The difference in other environmental factors between neotropical and paleotropical conditions might account for the contrasting differences. However, studies of Oertel (1946) on recorded ambient temperature and nectar sugar concentration of goldenrod and white clover nectar showed similar results to those of our study. The nectar sugar concentration was positively correlated with ambient temperature, and the temperature was within the range of viable reproductive activity of the plant; however, at higher temperatures that may be stressful to plant reproductive physiology, the sugar concentration may be nectars lower.

During anthesis, a single flower of *B. racemosa* was calculated to provide energy equivalent to 1158.91 J, capable of supporting reward-based pollination from visitation of either bat (Marshall 1983; Fleming et al. 2009) or moth (Tanaka 2004; Aluri et al. 2009). Compared to other plants of same pollinator guild, *B. racemosa* nectar provides more energy than for species of Gentianales – only 37.26 J for moth-pollinated species and 15.98 J for bat-pollinated species (Wolff 2006). However, this amount of energy was still much lower than for other bat-pollinated flowers – 1380 J in *Helicteres baruensis* (Malvaceae) and 15 210 J in Ceiba aesculifolia (Malvaceae) (Rodriquez-Peña et al. 2016). Such variation in nectar and energy amounts of different plants within the same pollinator guild has also been observed for hummingbird-pollinated plants (Fonesca et al. 2015). Bats can efficiently utilize sugars in nectar through direct and sequential oxidation of sugars in muscles (Suarez, Welch 2017). Although torpor occurs in Old World phytophagous bats (Geiser, Stawski 2011), high sugar and energy requirement of bats to sustain a large body mass and long-distance flights has been found (Amitai et al. 2010; Raghuram et al. 2011; Gonzalez-Terrazas 2012). This high demand of energy among bats can be provided by *B. racemosa*, with amounts close to a high level of energy in established chiropterophilous plants (Rodriquez-Peña 2016). On the other hand, documented visitation of moths provides evidence that *B. racemosa* is able to provide energy for smaller pollinators (Tanaka 2004; Aluri 2019).

Pollination syndrome

Based on results of pollen viability, pollen histochemistry and nectar sugar concentration of *B. racemosa*, it can be deduced that its floral anthesis occurs from floral opening at 19:00, and lasts until 21:00, with another high activity at early dawn around 4:00. The observed anthesis for *B. racemosa* in this study coincides with studies of Lin et al. (2013), found that floral scent *in situ* from *B. racemosa* was secreted at around 20:00 and was actively secreted at 22:00 to 2:00. All these activities – phenology, pollen biochemistry, nectar content and floral scent – indicate high reproductive activity during the night. This highly suggests nocturnal pollination, as confirmed by moth visitors during the night (Tanaka 2004; Aluri et al. 2019). The observed pollination syndrome that includes nocturnal anthesis, brush-type floral morphology (Fig. 1), drab coloration (pinkish white flower) (Fig. 1), pendulous inflorescence or flagelliflory (Fig. 1), and sugar-rich nectar (Fig. 5) also highly supports bat pollination in *B. racemosa*, although very difficult to document.

Pollination syndromes were established in relation to starch and pollen content, as low starch but high lipid pollen content are indicative of bee-pollinated plants (Baker, Baker 1983). However, *B. racemosa*, despite having low starch and high lipid pollen content (Fig. 4), has nocturnal anthesis when most bees are diurnal. Bees, as limited by their apposition eyes (Kelber et al. 2006), are more likely scavengers during the day than pollinators during the nocturnal anthesis of the plant. Records of tropical nocturnal bees actively foraging at night are not neglected, but the limitations in their vision being not fully adapted for nocturnal foraging is as well recognized (Warrant 2008). Thus, such generalizations on pollen histochemistry and pollination syndrome may be inapplicable for *B. racemosa*

(Baker, Baker 1983). The floral characteristics of *B. racemosa* are highly suggestive of moth and bat pollination. However, there is still a need to conduct more extensive and detailed work on observation of bat pollination to establish the pollination system of the plant.

Conclusions

Aspects of floral biology of *B. racemosa* have been investigated, and all results support nocturnal pollination. The nocturnal anthesis of the plant as evidenced by phenology, high pollen viability, low starch and high lipid pollen content, and high sugar concentration in nectar, which coincide with known nocturnal foraging time. These investigated floral characteristics, especially floral rewards, provide robust support to pollination syndrome, supporting and complementing visitation in *B. racemosa*, as recorded by other studies. Baseline data on reproductive biology of lesser studied paleotropical species like *B. racemosa* were obtained. These data will hopefully contribute to future research not only on predictive capability of floral characteristics for pollination syndrome, but also on evolutionary studies of floral characteristics among paleotropical plants for pollination ecology.

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