

Pollination ecology of an important medicinal plant *Hellenia speciosa* (J.Koenig) S.R.Dutta of Asiatic tropics

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Abstract. Samanta A, Bera B, Karmakar P. 2023. Pollination ecology of an important medicinal plant *Hellenia speciosa* (J.Koenig) S.R.Dutta of Asiatic tropics. *Biodiversitas* 24: 3152-3161. Plants often show various floral traits that help them to achieve reproductive fitness. Floral adaptive traits help to maximize the possibility of outcrossing, even for clonally propagated plants. Furthermore, seed setting through successful pollination increases genetic diversity in clonal populations. This present study aimed to describe the flowering phenology, flower biology, pollinators, and breeding system of *Hellenia speciosa*, a perennial herb with rhizomes from the Costaceae family. According to our study, a pollen-ovule ratio of 210:1 indicates that the plant tends to facultative autogamy; however, the plant exhibits traits such as a large landing labellum for pollinators, bilabiate floral architecture, nototribic blossoms, a prominent nectar guide, abundant floral nectar ($27.64 \pm 2.661 \mu\text{L}/\text{flower}$), and high seed set through xenogamy (68.6 ± 3.07 standard error) to improve reproductive success through outcrossing. Here, the plant is pollinated by two hymenopteran members, namely *Xylocopa auripennis* and *Amegilla zonata*. Despite its ample nectar production, the plant is hardly visited by any honey bee species. GCMS analysis of floral nectar found 2-hexanol and derivatives of xylose were probably responsible for pollinator specificity and honey bee exclusion events, respectively.

Keywords: Breeding system, clonal propagation, floral biology, nectar, pollination

INTRODUCTION

In plant evolutionary biology, the interaction between floral characteristics and pollinators has long been discussed (Soares and Morellato 2018). The evolutionary force that drives the modification of flower traits is pollination, a significant plant-insect interaction (Krishna and Keasar 2018). Most flowering plants require insects for successful pollination (Syarifuddin et al. 2018). The relationship between a plant and its insect pollinators is an example of mutualism symbiosis, because flowers give insects nutrition mainly in pollen and nectar while also benefiting from pollination services (Koneri et al. 2021). Therefore, to maximize the attraction to pollinators, unrelated plants often show convergences in floral morphology (Gélvez-Zúñiga et al. 2018). The evolutionary and ecological relationship between a plant and its pollinator could be studied by pollination ecology.

Therefore, how pollinators influence the evolution of diverse flower shapes is a central subject in pollination ecology (Wilkins et al. 2022). Studying pollination biology assist in understanding reproductive success and identifying natural variables that affect genetic and population dynamics in species interactions (Arias-Coyotl et al. 2006). Plants' pollination ecology involves several critical aspects such as flowering phenology, floral biology, and breeding system that contribute significantly to their survival in changing environments. Phenology studies periodic biological events (Vilhar et al. 2013). Therefore,

flowering phenology and pollination biology in plants involve examining the recurrence of biological events such as flowering, pollination, and fruit set (Suhaida et al. 2018). Flowering phenology is a major factor determining the mutualistic connections between flowers and pollinators (Ahmad et al. 2021). The study of floral ecology focuses on examining flowers, starting from the maturation of floral organs such as the anther dehiscing and the stigma becoming receptive. It ended while all stamen had released their pollen and the stigma was no longer receptive. Floral longevity is a crucial reproductive characteristic that refers to how long a flower remains open and active (Song et al. 2022). Floral longevity plays an important role in plants' reproductive success by influencing the possibility of insect pollination (Zhang et al. 2021).

Moreover, understanding the reproductive system and the dependency on pollinators is crucial for managing and conserving plant species in their natural habitat (Becker et al. 2023). Plants use various sexual systems and asexual reproduction as part of their reproductive methods (Barrett and Eckert 1990). Therefore, precise scientific knowledge of all pollination ecological aspects is essential to manage and conserve any plant in its natural habitat. *Hellenia speciosa* (J. Koenig) S.R. Dutta. is a perennial herb in the family Costaceae, one of the most recognizable subgroups of the Zingiberales. Although *H.speciosa* has been found on several continents, such as Africa, Asia, North America, and Oceania, it is primarily indigenous to Malaysia and Southeast Asia (Sohrab et al. 2021). It is naturally

propagated through rhizomatous clones (Maji et al. 2020), propagated through seeds (Waisundara et al. 2015), and in-vitro micropropagation (Bharathi et al. 2022). The plant, particularly the rhizome, is a rich source of bioactive substances such as eremanthin, diosgenin, and dioscin (Maji et al. 2020).

Besides, *H. speciosa* has been proven in studies to have antioxidant and antihyperlipidemic (Shediwah et al. 2019), anti-microbial (Thambi and Shafi 2015), anti-inflammatory (Srivastava et al. 2013), anti-diabetic (Waisundara et al. 2015), and anti-cancerous properties (Malviya and Joshi 2016), making it a prospective for the creation of medicinal compounds option. However, the pollination ecology and breeding behavior of *H. speciosa* are surprisingly poorly understood despite their importance in the field of medicine. The study aims to determine the sexual reproductive strategies of a clonally propagated plant. Therefore, this study's specific objectives were to: (i) examine the plant's flowering phenology in the presence of environmental factors; (ii) determine the flower architecture and its modifications for pollinators and effective pollination; (iii) explore the plant's dependence on floral visitors for pollination; and (iv) testing an optimal breeding system for the species.

MATERIALS AND METHODS

Study species and study site

The study species *Hellenia speciosa* (J.Koenig) S.R.Dutta, is a clonally propagated, perennial rhizomatous herb. It is an erect, unbranched, occasionally branched herb with spiromonostichous phyllotaxy. The terminal spike inflorescence bears white zygomorphic and bisexual

flowers with a big labellum that resembles crepe paper. Seeds and clonal ramets naturally propagate the plant; it blooms from July to September, and the fruits fully develop into a dehiscent capsule by October to November. The study was performed in 2018 and 2019. Field experiments were carried out in the wild habitats of the plant in Kharagpur, Paschim Medinipur (latitude 22°20', longitude 87°17'), Vidyasagar University Campus, Medinipur (latitude 22°48', longitude 88°37') and Jhargram Raj College Campus, Jhargram (latitude 22°43', longitude 86°98'), West Bengal, India (Figure 1).

Flowering phenology and floral biology

We followed the suggested method by Belavadi and Ganeshaiah (2013) to study flowering phenology. For each sampling day, we calculated two flowering traits, flowering frequency and flowering intensity. Flowering frequency was calculated by observing the total number of plants in the flowering stage in the sampling population (n=20). The flowering intensity was calculated by observing the average number of flowers opening per inflorescence daily in the same sampling population. Finally, we computed the flowering phenology by multiplying each day's frequency and intensity. This approach allowed us to understand comprehensively the flowering patterns of the plant species under study. The data regarding environmental factors such as temperature in °C (Celsius degree) and precipitation were collected daily from the IMD (India Meteorological Department, Ministry of Earth Sciences, and Government of India) weather forecasting website and local weather and meteorological observations. Using a Metravi DL-TH01 Temperature and Humidity Data Logger, the temperature and humidity were carefully recorded, and the results were compared to those from the IMD website.

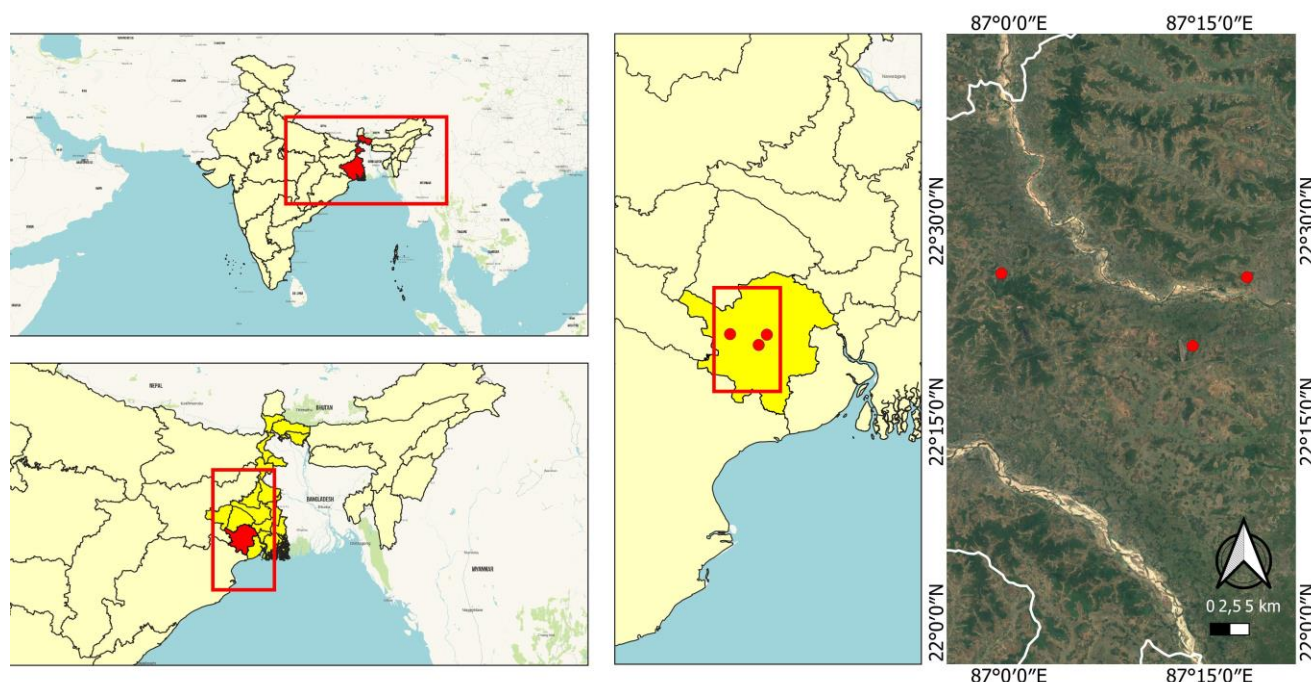


Figure 1. Map showing study sites of three different wild habitats of *Hellenia speciosa*

Inflorescences (n=20) from 20 different clonal ramets with unopened floral buds were randomly chosen to study floral longevity. Stigma receptivity (n=20) from 20 different clonal ramets was tested in-vivo by transferring pollen to the stigmatic surface at one-hour intervals. Pollinated stigma was collected after an hour, and the amount of germinated pollen was determined. The viability of the pollen was examined using acetocarmine staining (Yankova-Tsvetkova et al. 2013) and through pollen germination in the appropriate pollen germination medium (Shivanna and Tandon 2014). Ten flowers from ten separate inflorescences were used to calculate the number of pollen, ovules, and their ratio. Therefore, to investigate pollen production, the methodology of Bhaskara (2017) followed. The floral architecture was examined using ten randomly chosen open flowers. Furthermore, a Stereo binocular microscope (Zeiss Stemi 508) was used to make detailed morphological descriptions and morphometric data. A Canon 800D Digital SRL camera was used to capture the pictures.

Floral visitors

Observations were conducted during the peak flowering season, i.e., between the 3rd and 4th week of August, with a total observation time of 12 hrs to determine floral visitors' assemblage, frequency, and foraging behavior. Two observers recorded data at 30-minute intervals from the start of visitation until visitation ceased at 04:00 pm. Observations were made on warm, sunny days; a glass tube was used to catch visiting insects. Pollen loads were collected from insect bodies and studied. It has been known previously that many insect species experience a coma-like state when subjected to a crucially low temperature. The term "chill coma" refers to this comatose state, which is characterized by a complete cessation of movement (Findsen et al. 2014). By implementing this approach, the pollen could obtain from insects without causing them harm. Once the insects recovered from the chill coma, they returned to their natural environment. The pollen samples were then transferred onto a cavity slide and counted manually. Based on the work of (Layek et al. 2020), with some modifications, the equation for determining the relative abundance (RA) of visitors was developed.

$$RA (\%) = (\text{number of visits during peak visitation}) / (\text{total number of visits during}) \times 100$$

The Visitation frequency (V_f) of the pollinator was calculated using the formula given by Goverde et al. (2002):

$$\text{Visitation frequency} = \text{Number of visit} / \text{Number of flowers} \times \text{Observation time (h)}$$

Visitors were placed in glass vials with a piece of cotton soaked in ethyl acetate, and identification was made with the help of entomologists, Zoological Survey of India (ZSI), Kolkata. Visitors were grouped into potential pollinators and nectar robbers, as studied by (Gélvez-Zúñiga et al. 2018).

Floral rewards

The plant offers nectar and pollen as floral rewards to its visitors. The measurement of nectar volume involved collecting floral nectar from 30 randomly selected inflorescences from 30 clonal ramets with unopened floral buds, which were bagged using a cotton cloth to prevent nectar robbing and pollinator visits. Nectar was collected in 1-hour intervals from 06:00 am. to 06:00 pm. using a micropipette. Therefore, the petaloid stamen was lifted to avoid pollen contamination with nectar during collection, and the micropipettes were carefully placed at the petal base. Nectar samples were stored in sterile and dry Eppendorf tubes of 1.5 ml, with the collection time marked on each tube. As the nectar volume per flower was low in the early morning, nectar was collected from more than one flower for each sampling. Furthermore, a portable refractometer (Hanna-HI 96800-0-80% Brix) was used to determine the brix% of each nectar sample on the field to measure nectar concentration. The nectar concentration was then calculated using a known sucrose concentration method standard curve. GC-MS analysis of floral nectar using Joel GCMATE II GC-MS and an Agilent Technologies 6890N Network GC system attached to a mass spectrum component. GC column HP5 (50 m × 0.25 mm) was used during the analysis.

Breeding system

Studies on breeding systems were conducted to determine the mode of sexual reproduction during the peak flowering period. Inflorescences of each plant (n=10) underwent three hand-pollination treatments: (i) self-pollination using pollen from the same flower; (ii) geitonogamy or transfer of pollen from different ramets of the same genet; and (iii) xenogamy, or transfer of pollen between different genets. Nylon mesh bags were used to cover inflorescences before and after each treatment to avoid unintended pollination. The natural and open pollination system was considered the fourth (4) breeding treatment. Spontaneous autogamy on bagged flowers was regarded as the fifth (5) breeding treatment. Emasculation was performed before anther dehiscence in the case of geitonogamous and xenogamous treatments. Apomictic seed production was also tested by removing both the anther and stigma before maturity. The Shapiro-Wilk test was used to determine whether the number of seeds set by different breeding treatments was normally distributed.

The index of self-incompatibility (ISI) was computed using the method described by (Raduski et al. 2012):

$$ISI = 1 - (\text{Seed set in selfed success}) / (\text{Seed set in outcrossed success})$$

According to (Layek et al. 2023), we categorized the plant species into three groups using the ISI value: i. self-incompatibility ($ISI \geq 0.8$), ii. partial self-incompatibility ($0.2 < ISI < 0.8$), and iii. self-compatibility ($ISI \leq 0.2$).

Statistical analysis

A Shapiro-Wilk normality test was performed on the phenology and environmental data. Following that, phenology and environmental variables were correlated. Finally, the

percentage of seed setting in various breeding treatments was compared using ANOVA and Tukey's post hoc analysis to determine if there were any statistically significant differences between them. In addition, the R statistical tools were used to carry out the statistical analysis.

RESULTS AND DISCUSSION

Flowering phenology and floral biology

Hellenia speciosa blooms from August to October, and the flowering reaches its zenith in early September. After that, flowering declines, and the plant ceases at the end of October. Regarding flowering phenology, the peak flowering period has been demonstrated in Figure 2. The data on phenology and environmental variables were subjected to a Shapiro-Wilk normality test, which revealed that the phenology, precipitation, and maximum relative humidity were non-parametric. In contrast, the study's maximum and minimum temperatures and maximum relative humidity were parametric. Consequently, we used Spearman's correlation coefficient analysis to examine the relationship between the variables. The correlation analysis found no significant connection between phenological data and environmental variables; a comparison between the flowering phenology and the diverse variables of environmental factors in Figure 2.

Hellenia speciosa is a water-loving (Figure 3.A) rhizomatous perennial plant that produces 31.2 flowers per inflorescence per ramet (Figure 3.B) (terminal spike) with single or two blooms per day, occasionally three; these flowers are classified as single-day flowers. Pollinated flowers, whether through manual or natural pollination, tend to lose their freshness sooner than those left undisturbed or covered with a bag. In the open state, the flower's longevity is about 14-16 hours. The usual brownish discoloration of the nectar guide on the labellum base and petaloid stamen is a sign that the flower is no longer fresh. The closure of the flower commences with the inward folding of the labellum.

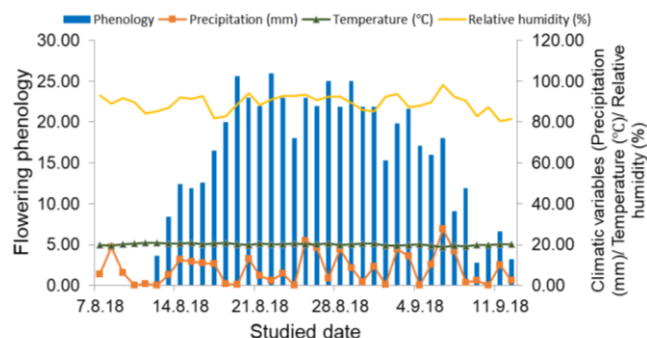


Figure 2. Showing a comparison between flowering phenology and environmental variables. Note: days begin on 7 August and end on 12 September, 2018

Flowers are bracteate and bracteolate (Figure 3.D), zygomorphic, hermaphrodite, campanulate, and have three white petals (Figure 3.E) that are around 5.5-6.0 cm long. A broad horizontally flattened labellum (Figure 3.F) (modified and fused staminodes) is also observed (7.5-5.4 cm long). Calyx comprises three reddish sepals, about 2.5 cm long, and persistent in nature. The androecium is represented by a single fertile petaloid stamen (Figure 3.H) positioned above the labellum as a lid. The lower surface is covered with dense, yellowish hair of varying lengths (1-5 mm) (Figure 3.H) and is a nectar guide. The stamen has two anthers measuring about 10.0 mm in length and 0.8 mm in width that dehisce through longitudinal slits (Figure 3.I). The anther dehiscence begins with the flower opening at around 01:00 am. Dehiscence takes place longitudinally; initially, a longitudinal slit of 1.0 mm forms from the anther lobe's upper portion. The slit gradually progresses downwards along with the connecting style. The plant produces ample pollen grains ($15,152 \pm 1083.69$) to ensure successful pollination. The pollen-ovule ratio was 210:1. Pollen grains are spheroid, psilate (Figure 3.J) with a diameter of 50 μ m (Figure 3.D). The IKI staining of pollen grains gives positive results, indicating the pollen surface is composed of starch. The staining technique of acetocarmine revealed that 98% of the pollen was viable when the stigma receptivity peaked. Therefore, to establish the ideal concentration of sucrose for pollen germination and pollen tube growth, various concentrations of sucrose were examined using Brewbaker and Kwack's medium. The optimal concentration was found to be 10%. In-vitro fertility of the pollen was tested from anther dehiscence until pollen germination ceases, and it was found that the highest rate of pollen germination, 73.42%, was observed between 09:00 and 10:00 am.

However, as time passed, pollen fertility gradually declined and by 4:00 pm., the pollen had lost its fertility completely. The ovary is inferior, brown-red, and 3-chambered, with multiple ovules arranged in axile placentation. The gynoecium consists of three carpels, syncarpous, an inferior ovary, a style about 4.0 cm long, and a stigma about 1.0 mm long and 2.0 mm wide. Stigma acquires its peak receptivity in the late morning at 11:00-12:00 noon on the day of flowering and loses its receptivity in the late afternoon to early evening, at 12:30-01:00 pm of the same day. Initially, stigma receptivity was found at the upper portion of the stigma. Later, receptivity reached the lateral sides of the lobe. The inferior, three-chambered ovary is 0.8 cm in length and 1.1 cm in breadth, with 19.59 ovules organized in axile placentation in each chamber (chamber diameter: 0.1-0.3 cm). The long, thin style carries the stigma at its tip and extends into the anther lobes. The stigma is wet, papillate (Figure 2) (Group-III type). Capsules dehisce through a longitudinal slit (Figure 3.M-N) and carried 50.04 ± 13.79 seeds. Dark black seeds ($3-5 \times 2-3$ mm) have a strong, four-sided seed coat. During dehiscence, the flat hilum region remains covered by a white and wet aril. In *H. speciosa*, the flower's stigma develops its receptivity in the late morning, between 10:30 and 11:30 am. and loses it on the same day at 1:00 pm.

Floral visitors

Altogether, three species of insects, namely *Xylocopa auripennis* Lepeletier. ♂ (Figure 4.A), *Amegilla zonata* Linnaeus (♂ and ♀) (Figures 4.B-C), and *Udaspes* spp. Cramer (Figure 3.I) have been found to visit the flowers. In addition, ants are observed on both the flowers and inflorescence during the entire reproductive phase, effectively deterring herbivory and oviposition by insects (Figure 4.H). The study on floral morphology and visitor behavior has revealed that pollen deposition on the stigma of *H. speciosa* is of the nototribic type, which involves the transfer of pollen to the stigma through the dorsal surface (Figures 4.E-G) of two hymenopteran insects, *X. auripennis* and *A. zonata*. The Lepidopteran species *Udaspes* sp. occasionally visits the flower as a nectar thief and collects nectar using its long proboscis without causing any contact with the stigma or anther (Figure 4.I). The collected data were subjected to a two-way ANOVA to ascertain if there was a noteworthy variation in the visitation duration of insects on flowers. The ANOVA test statistic value was calculated as 2.2, and the critical value was set at 3.368. As the test statistic value was below the critical value, the null hypothesis could not be rejected at the 0.01 significance level. Therefore, it was concluded that there was no significant difference in the timing of insect visits to flowers for pollination.

However, a significant difference was observed in the mean visit time of different insect species on the flower, with a test statistic value of 11.0 and a critical value of 5.849 at a 0.1 significance level. The pollination method was nototribic, meaning that most of the pollen ends up on the visitors' dorsal surface. When studying *X. auripennis*, it was found that they carried an average of 1378.2 ± 600.33 pollen grains on their first visit. In the case of *A. zonata* males and females, the pollen loads were 114.5 ± 59.13 and 64.5 ± 39.63 respectively. *X. auripennis* makes contact with the stigma nearly 100% of the time during the pollination process, whereas *A. zonata* comes into contact with the stigma for about 65% of its visiting time. *X. auripennis* was the most abundant species (RA=73.03 %), followed by *A. zonata* (♂ and ♀), whose relative abundance are (RA=25.84 %) and (RA=20.24 %) respectively. Visitation frequency during the peak period of stigma receptivity i.e. between 11:00 am. to 12:00 noon was found to be 0.86 visit/flower/hour for *X.auripennis*, 0.30 for *A. zonata* (♂) and 0.24 for *A. zonata*

Floral rewards

In *H. speciosa*, flower opening begins at 9:00 pm. and is completed by 03:30 to 4:00 am. of the next morning. Insect visitation to the flower starts at 6:00 am., and the nectar secretion begins at 5:00 am. The nectaries are located deep inside the corolla tube, just above the ovary. The entrance to the nectar source, i.e., the flower's mouth is closed by the petaloid stamen, which opens like a lid when pollinators enter. The reflexed apex of the petaloid stamen and corolla tube is provided with a yellowish nectar guide that directs pollinators toward the nectar source. During the commencement of insect visitation (06:00 am.), the mean nectar volume was 4.78 ± 1.07 µL/flower with a mean concentration of $45.71 \pm 0.04\%$. A notable increase in

nectar production was observed between 9:00 am. and 2:00 pm., with a mean nectar volume of 27.64 ± 2.661 µL/flower and a concentration of $40.27 \pm 8.18\%$. After 2:00 pm., nectar production gradually decreased with a moderate fall in concentration. The maximum nectar volume was observed at 02:00 pm with the lowest concentration of 28.03%, whereas the maximum nectar concentration (46.03%) was observed at 08:00 am when the nectar volume (7.77 ± 4.39 µL/flower) was found. A weak negative correlation ($r = -0.36$, $p > 0.05$) was found between nectar volume and concentration. A significant correlation was observed between the period and nectar concentration ($r = -0.84$, $p < 0.001$), foraging time of *Xylocopa auripennis* ♂ ($r = -0.86$, $p < 0.001$), foraging time by *Amegilla zonata* ♂ ($r = -0.69$, $p < 0.01$), and foraging time by *Amegilla zonata* ♀ ($r = -0.69$, $p < 0.01$). A positive correlation was found when comparing nectar concentration with insect mean visitation time ($r = 0.71$, 0.54 and 0.48 for *X. auripennis* ♂, *A. zonata* ♂ and *A. zonata* ♀ respectively). The result of GCMS analysis of nectar is shown in Table 1.

Breeding system

Pollination treatments significantly impacted seed sets in *Hellenia speciosa* ($F_{4,45} = 17.69$, $p = 0.000$, one-way ANOVA). Average seed setting per flower was found to be highest in the xenogamous treatment (68.6 ± 3.07 standard error), followed by geitonogamous treatment (51.40 ± 1.05 standard error), and lowest in spontaneous autogamy (37.8 ± 2.55 standard error). The apomixis treatment wasn't included in the analysis because apomixis didn't cause any seeds to be set. There were significant differences found in the average number of seeds set in the following breeding treatments, supported by Tukey's post-hoc test (Figure 6). i. spontaneous autogamy and open pollination ($p < 0.05$); ii. open pollination and xenogamy ($p < 0.01$); iii. spontaneous autogamy and xenogamy ($p < 0.01$); iv. spontaneous autogamy and geitonogamy ($p < 0.05$). v. self-pollination and xenogamy ($p < 0.01$); geitonogamy and xenogamy ($p < 0.01$).

Self-in-compatibility index (ISI) is relatively high (0.39), suggesting the plant is partially self-incompatible. The fruit-set percentage is about 92-93%, which is extremely high. After pollination, nine to ten days are needed for the ovary to resemble a juvenile fruit. The full maturation of fruit takes about 50-60 days.

Discussion

In our current research, we have explored the reproductive ecology of *Hellenia speciosa*. Our results support the idea that even though this plant typically reproduces through clonal propagation, it has evolved certain floral adaptations to promote outcrossing. Specifically, it has a bilabiate floral structure that facilitates pollen transfer on the dorsal side of a pollinator, a process known as nototribic blossoms. Interestingly, this type of flower structure is also found in ten other families of monocotyledonous plants, including Costaceae (Westerkamp and Claßen-Bockhoff 2007). The presence of labellum is considered to be an improved trait in terms of pollination. From an evolutionary standpoint, the labellum is extremely fascinating since it represents a special floral structure crucial for interacting with pollinators (Callens et al. 2018).

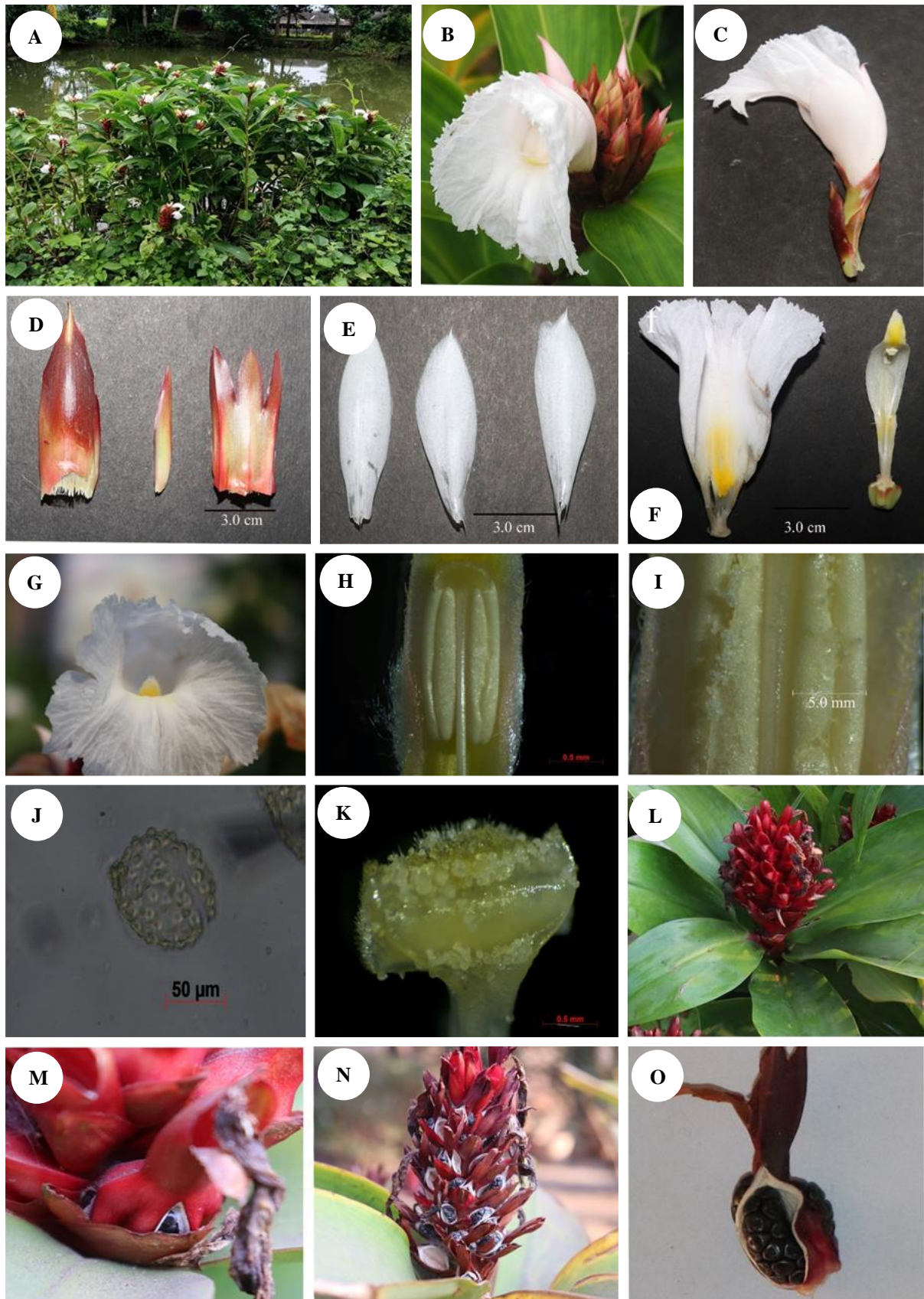


Figure 3. A. Wild habitat; B. Inflorescence; C. Single flower; D. Bract, bracteole and calyx (from left to right); E. Corolla; F. Labellum (left) and petaloid stamen with attached pistil (right); G. The reflexed apex of the petaloid stamen showing nectar guide; H. Co-occurrence of anther and stigma; I. dehiscenced anther; J. Single pollen; K. Pollen attached on papillae of stigma; L. Inflorescence matured into fruits; M. Dehiscence of capsule; N. complete dehiscence of fruits; O. Single capsule

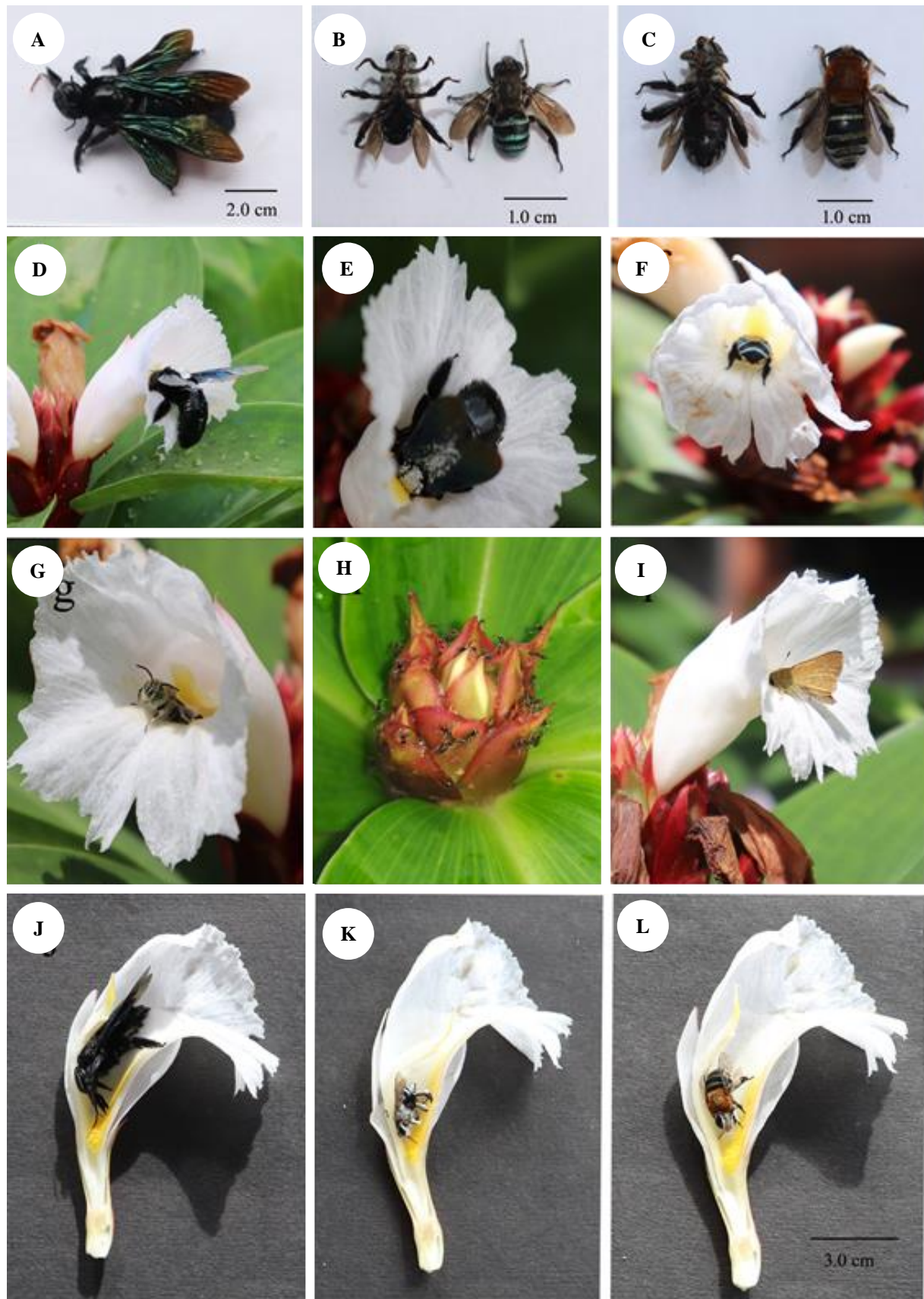


Figure 4. A. *Xylocopa auripennis* ♂; B. *Amegilla zonata* ♂; C. *A. zonata* ♀; D-E. Landing and entry of *X. auripennis*; F-G Entry and exit of *A. zonata* ♂ and ♀ respectively; H. Ants collecting extrafloral nectars on developing inflorescence; I. Nectar thief *Udaspes* spp. collecting nectar; J-L. The relative position of visitors inside the pollination chamber

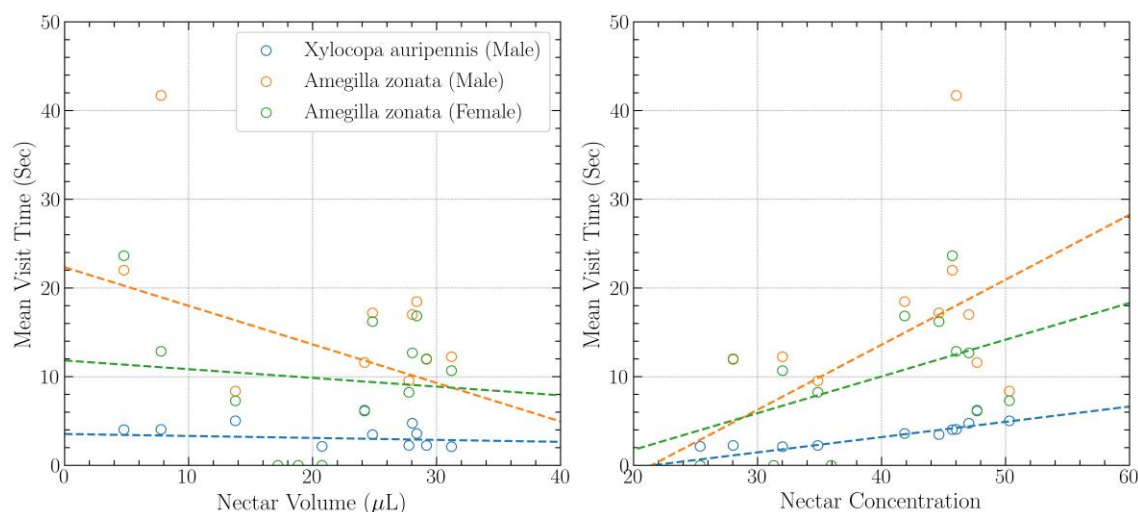


Figure 5. Showing a correlation between the mean visit time of floral visitors with nectar volume and concentration

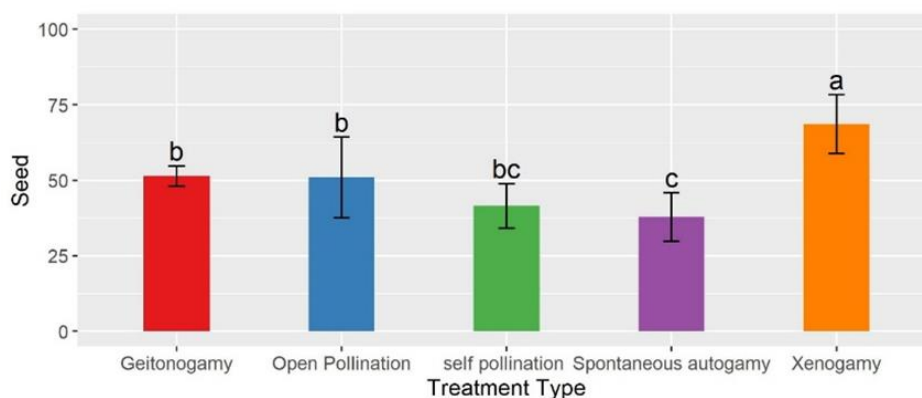


Figure 6. Tukey's post hoc analysis of the number of seed settings in different breeding treatments

Table 1. Compounds identified from GC-MS analysis of nectar from *H. speciosa* using methanol* and ethanol** as the solvent

Compound present in nectar	Empirical formula	Molecular weight	Retention time (min)
Paraldehyde*	C ₆ H ₁₂ O ₃	132	1.24
2-hexanol*	C ₆ H ₁₄ O	102	1.24
Dichlorine heptoxide*	O ₇ Cl ₂	182	1.25
2-Chloropropionamide*	C ₃ H ₆ ONCl	107	1.22
n-(2-Naphthyl)-23,24-dinor-5-cholen-22-amin-3beta-ol*	C ₂₁ H ₃₅ ON	317	1.22
Boric acid*	H ₃ BO ₃	62	1.22
Acetamide, n,n-dimethyl-*	C ₄ H ₉ ON	87	1.18
Guanidine, n,n-dimethyl-	C ₃ H ₉ N ₃	87	1.18
Acetamide, n-ethyl-*	C ₄ H ₉ ON	87	1.18
Oxalacetic acid*	C ₄ H ₄ O ₅	132	1.18
Ethanediamide*	C ₂ H ₄ O ₂ N ₂	88	1.18
Lyxopyranose, tetraacetate**	C ₁₃ H ₁₈ O ₉	318	1.18
d-Xylitol, 2,3,4,5-tetraacetyl-1-o-methyl-**	C ₁₄ H ₂₂ O ₉	334	1.18
Xylose, 1-o-(2-nitrophenyl)-**	C ₁₇ H ₁₉ O ₁₀ N	397	1.18
Xylopyranose, 3-deoxy-3-fluoro-, triacetate, beta-d-**	C ₁₁ H ₁₅ O ₇ F	278	1.18

The nectar guide is another floral trait for pollination. Traditionally, floral nectar guides show where pollinators will find rewards from flowers, such as nectar and pollen (Zhang et al. 2017). Pollen deposition efficiency on pollinators is typically thought to be influenced by anther position (Sapir et al. 2017). Here, the co-occurrence of stigma and anther and their placement at the entrance of the flower opening helps the pollen deposition on the pollinator's dorsal surface and subsequent transfer to the receptive stigma of another flower. Labellum, nectar guide, hidden nectar at the base of the floral tube, and the occurrence of both anthers and stigma, all indicate the plant's strong inclination towards cross-pollination. A previous investigation involving 16 plants from the same family demonstrated that the horizontally flattened labellum serves as a landing platform for insects (Kamer et al. 2016). Except for the yellow-flowered species such as *Costus gabonensis*, *C. giganteus*, *C. gracillimus*, *C. lateriflorus*, *C. macranthus*, *C. spectabilis*, all members of the Costaceae possess a nectar guide (Kamer et al. 2016). When an insect pollinator lands on the flower, it will trace the nectar guide towards the center of the flower, causing the reflexed apex of the fertile stamen to be lifted, granting access to the floral throat. All these special traits indicate insect pollination syndrome. The study's findings on the plant's breeding system reveal that there is a higher seed setting in geitonogamous and xenogamous treatments compared to autogamous treatments. Although the pollen-ovule ratio of 210:1 suggests that the plant tends facultative autogamy (Cruden 1977), further observations indicate that the plant exhibits adaptations that promote xenogamy, or cross-pollination.

From the GC-MS analysis of the methanol extract of nectar, a compound called 2-hexanol was detected, which is known to attract insects. A previous study conducted by Zheng et al. 2014 had also shown that 2-hexanol, present in the essential oil extracted from *Pterocarpus indicus* leaves, had a strong stimulatory effect on female *Aleurodiscus dispersus* and acted as an attractant for them. *H. speciosa* relies heavily on specific pollinators, namely *Xylocopa auripennis* and *Amegilla zonata*. Such pollinator-specific behaviour was previously studied by (Kessler and Baldwin 2007) in *Nicotiana attenuata*, where they found that the compound 1-hexanol present in *Nicotiana attenuata* flowers was able to attract only *Solenopsis xyloni* and no other floral visitors. This finding suggests that plants might use such compounds to filter and attract specific types of pollinators selectively. However, this pollinator specificity may pose challenges in terms of reproductive fitness. In addition to hexanol, our analysis of the nectar identified three other derivatives of xylose viz. d-Xylitol, 2,3,4,5-tetraacetyl-1-o-methyl-, Xylose, 1-o-(2-nitrophenyl)- and Xylopyranose, 3-deoxy-3-fluoro-, triacetate, beta-d- which were the only ones present. According to a previous study by (Jackson and Nicolson 2002) on *Protea* sp. and *Faurea* sp., the bee species *Apis mellifera capensis* did not prefer xylose as a reward during pollination. This could be a possible explanation for the absence of honey bees during the entire flowering period of the plant. Earlier research has shown that species of *Amegilla*, apart from *zonata*, have

been identified as pollinators of *H. speciosa* (Sakai et al. 1999). The strong relationship observed between the concentration of nectar and the frequency of insect visitation suggests that higher nectar concentration levels may facilitate the process of nectar concentration in *X. auripennis* (Portman et al. 2021). The results of pollen loads, relative abundance (RA) and visitation frequency (V_f) have revealed *X.auripennis* (♂) as the most effective pollinator for *H.speciosa*, followed by *A. zonata* (♂) and *A. zonata* (♀).

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