

Article

The Similarity of Floral Scent Composition in Two *Breynia* Species Pollinated by the Same Host-Specific *Epicephala* Moth

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Abstract: Floral scent has been thought to play a critical role in the obligate pollination–seed consumption mutualism between *Breynia* (Phyllanthaceae) species and *Epicephala* moths. The two closely related *Breynia* species, *B. fruticosa* and *B. Rostrata* are jointly pollinated by the same moth species (*E. lativalvaris* and *E. mirivalvata*), which has led to an obligate pollination mutualism of ‘two-to-two’ species specificity. However, empirical data on host floral volatiles as chemical cues maintaining the plant–moth specificity are missing. In the present study, 27 volatile compounds were identified from floral scents of the two *Breynia* species. A total of 25 compounds were shared jointly by the two species, and only one compound, β -humulene, was species specific and exclusively produced by *B. fruticosa*. The two *Breynia* species clearly showed no interspecific floral scent profiles within both sexes, especially in male flowers. Male and female flowers of floral scent from these plants exhibited major qualitative differences, and male flowers produced more scent than female flowers. For male flowers of *B. fruticosa*, there was significant temporal variation in the volatile chemical profiles and total floral scent emission before and after pollination. Interestingly, β -phenylethyl butyrate and phenylethyl alcohol were present in maximal amounts during pollination, but they rapidly reduced in concentration after pollination. The findings suggest that the similar floral scent profiles of the two *Breynia* species reflect adaptations to the same pollinator, *Epicephala*, for their pollination. Sexually dimorphic floral scent between male and female flowers has been selected to provoke pollinator’s active pollination behavior on host flowers. Temporal changes in floral scent of male flowers before and after pollination may have evolved to limit the overexploitation of the ovaries by seed predators, *Epicephala* moths. We speculate that the two most abundant compounds in the male flowers, β -phenylethyl butyrate and phenylethyl alcohol, may be two signal compounds to attract *Epicephala* in a species-specific manner.

Keywords: *Breynia*; obligate pollination mutualism; floral scent; interspecific conservation; sexual dimorphism; post-pollination changes



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1. Introduction

Nursery pollination mutualisms exist between seed predators and their host plants, where seed predators lay eggs within the flowers after pollinating their host plants and, in return, the hosts offer seeds to nourish pollinator larvae. Presently, this is the most specialized case known in insect–plant interactions [1]. Among these mutualisms, the most thoroughly studied examples are the interactions between figs and fig wasps [2], and those between yuccas and yucca moths [3]. In addition, several analogous pollinating seed parasite systems have been documented more recently: *Epicephala* moths on Phyllanthaceae plants, mainly including *Glochidion* [4], *Phyllanthus* [5], and *Breynia* species [6,7], *Chiastocheta*

flies on globeflower *Trollius europaeus* [8], and the moth *Upiga virescens* on the senita cactus *Lophocereus schottii* [9].

Floral scents have been suggested to be an important signal for mediating chemical communication between flowering plants and their pollinators [10], ranging from antagonistic to mutualistic relationships [11–14]. In horizontally transmitted, highly specialized mutualisms, these chemical signals are often essential for guiding obligate pollinators to encounter their host plants [15,16]. Recently, several studies have highlighted the importance of specific sensory cues in nursery pollination mutualism. The floral volatiles have been chemically characterized for many host species of figs [17–23], yucca [24–26], and Phyllanthaceae [27–30], and the host floral scents for the attraction of obligate pollinators have been investigated for fig wasps [18,20,31–36], yucca moths [26] and *Epicephala* moths [27,28]; however, the specific attractants for obligate pollinators have only been chemically identified for *Ficus semicordata* [35] and *B. vitis-idaea* [28] in such mutualisms.

Plants of the family Phyllanthaceae are involved in obligate nursery pollination mutualisms with *Epicephala* moths. Pollination of the Phyllanthaceae species generally depends entirely on species-specific *Epicephala* pollinators [4–6]. At night, the female *Epicephala* moth actively collects pollen grains on the male flower, transports pollen grains on the female flower with its proboscises, and subsequently lays an egg within the pollinated female flower with its specialized acicular ovipositor. Single *Epicephala* larvae develop to maturity by consuming all seeds of each developed fruit, whereas a fraction of the fruits is left untouched in favor of plant reproduction [6,37]. Because female *Epicephala* moths are nocturnally active in pollinating their hosts, they use olfactory information to localize host plants and discriminate hosts from nonhosts [27,28]. To adapt to specific pollinators in these interactions, floral scents exhibit interspecific variation among host species [27,29]. In the *Epicephala*-pollinated Phyllanthaceae species, the active pollination behavior of their pollinating moths can promote divergence in floral scent between the male and female host flowers, where sexual dimorphism in floral scent may signal pollinator moths to facilitate alternative rewards in both flower sexes [28]. The post-pollination change in floral scent from host plants in this mutualism has led to the evolution of mechanisms to stabilize the interaction by avoiding the overexploitation of pollinated female flowers [30].

Breynia, a monoecious shrubs/trees genus of the family Phyllanthaceae, comprises 35 species mainly distributed in tropical and subtropical regions of Asia, Australia, and the Pacific Islands [38]. Recently, two closely related species, *B. fruticosa* and *B. Rostrata*, jointly pollinated by two *Epicephala* moths (*E. lativalvaris* and *E. mirivalvata*) at night, has led to ‘two-to-two’ obligate pollination mutualism [27,29]. Until recently, few studies have investigated floral scents guiding obligate pollinator moths to *B. fruticosa* and *B. Rostrata* species. Therefore, the following hypotheses were addressed in this study: (1) to attract the same pollinator, *Epicephala*, the two *Breynia* species may have selected a similar floral odor; (2) the two *Breynia* species are expected to show the sexual dimorphism in floral scents to signal alternative rewards from the two sexes for *Epicephala* moth; and (3) the changes in floral scent of male flowers of host plants *B. fruticosa* before, during, and after the pollination stage act as a mechanism to avoid already visited female flowers. To test these hypotheses, we first collected the floral scents of both male and flowers of the two monoecious *Breynia* species during pollination, and collected the odor bouquets of male flowers of *B. fruticosa* before pollination and after pollination, using the dynamic headspace adsorption technique. Secondly, we chemically identified the volatile composition using gas chromatography–mass spectrometry (GC-MS). Finally, we investigated the interspecific variations and intersexual differences in floral scents among the two *Breynia* species during pollination and tested the changes in the floral scents of male flowers of *B. fruticosa* before, during, and after pollination with non-metric multidimensional scaling (NMDS) and correspondence analysis (CA). Our study may provide a theoretical basis to further reveal the chemical and ecological mechanisms in obligate pollination mutualisms between Phyllanthaceae plants and *Epicephala* moths.

2. Materials and Methods

2.1. Plants

The two studied *Breynia* (Phyllanthaceae) species, *B. fruticosa* (L.) Hook. f. and *B. rostrata* Merr., usually grow in tropical and subtropical forests in south China [39]. These two plants are monoecious shrubs or trees with male flowers and female flowers arranged at the base and the apex of a branch. The small, inconspicuous flowers lack petals. *B. fruticosa* and *B. rostrata* share similar male floral morphology (Figure 1). Their male flowers are funnel shaped and have fused calyx lobes. With development of male flowers, green and small fused calyx at pre-pollination stage gradually change into green-yellow calyx at pollination stage, when the fused calyx crack into a small opening at the tip, and the three stamens fusing into a column and concealing in the calyx are exposed, then become faint yellow calyx at the post-pollination stage. This unique floral architecture of the male flowers of the two species makes pollen collection by floral visitors other than *Epicephala* moths very difficult. However, although female flowers of the two species bear free calyx and three styles, two *Breynia* species show morphological differences in calyx and stigmas (Figure 1): in *B. fruticosa*, female flowers have radial calyx sepals spreading into a disk and excurved stigmas; in *B. rostrata*, female flowers have reflex calyx sepals and erect stigmas. Because of their small size and lack of brilliant color, at night *Breynia* flowers' visuals may become less important for attracting *Epicephala* moths to visit. In contrast, flowers of *Breynia* species are fragrant to the human nose at night, suggesting that floral scent may be the key signal for attracting obligate pollinator *Epicephala* moths [39].

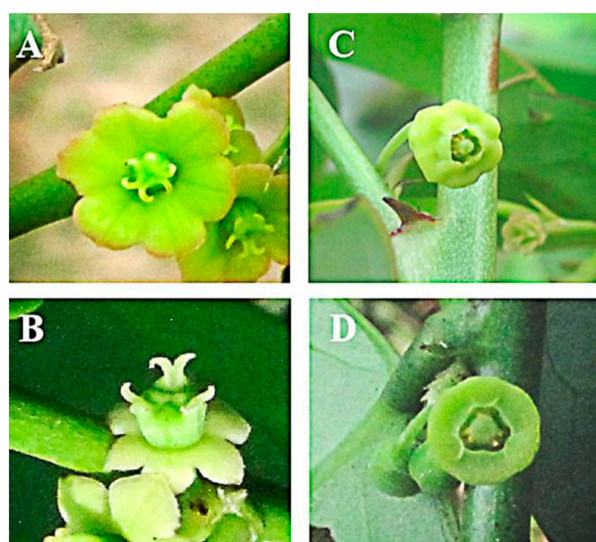


Figure 1. Flowers of the two *Breynia* species in this study: male flowers of *B. fruticosa* (A) and *B. rostrata* (B); female flowers of *B. fruticosa* (C) and *B. rostrata* (D).

B. fruticosa and *B. rostrata* are closely related and sister taxa in terms of phylogeny, and are exclusively and jointly pollinated by the same female moths of the *Epicephala* species (*E. lativalvaris* and *E. mirivalvata*). In this study, the two investigated *Breynia* species were grown at different study sites and were allopatric.

2.2. Floral Scent Collection

Floral scent was collected using the dynamic headspace adsorption technique from two *Breynia* species in Xiamen city of Fujian Province from China in October 2013 [29]. A total of 100 male and 300 female flowers per tree were cut from the plants and enclosed separately in polyvinylacetate oven bags for collection. Air was purified by an active charcoal filter (Sigma-Aldrich, St. Louis, MO, USA) and then pumped into the sample bag (entrance flux: 400 mL min^{−1}) with a mini air-pump, through a Teflon tube (VICI Jour Ltd., Schenkon, Switzerland). Volatiles were drawn out of the bag (exit flux: 400 mL min^{−1}) and

pulled into the glass cartridge (outer diameter, 6 mm; length, 75 mm) filled with 150 mg of Tenax-TA (mesh 60/80; Sigma-Aldrich) with a mini air-pump, a through Teflon tube, for odor adsorption. Synchronously, an air sample was also collected from an empty bag as a control to check for possible background contaminants from the odor collection system. Scent collection was performed for 5 h at night (from about 19:00 to 23:00) at ambient temperature (25–28 °C). After volatile collection, the filters containing captured flower volatiles were sealed with Parafilm, wrapped with silver paper, and stored at −20 °C until being used for analysis.

2.3. Chemical Analysis of Floral Scent

To analyze the chemical composition of floral scents of the two *Breynia* species, GC–MS was carried out using an HP 6890 gas chromatograph (Agilent Technologies Inc., California, PA, USA), equipped with an HP-5MS capillary column (30 m × 0.25 mm; film thickness, 250 µm; Agilent Technologies Inc., California, PA, USA), and linked to an HP 5975 mass spectrometer (Agilent Technologies Inc., California, PA, USA). Before GC–MS analysis, Tenax-TA filters adsorbing floral scents were eluted with 1.5 mL of hexane into 2 mL brown small glass vials (Agilent Technologies Inc., California, PA, USA), and we added 10 µL, methyl stearate (1 mg mL^{−1}) to each as an internal standard. The eluate was carefully concentrated down to 100 µL under N₂ flow. For GC, 2 µL of each sample was injected in split mode (the split ratio was 1:1) with an injector temperature of 250 °C. The solvent delay was set as 1 min to minimize the interference from solvents. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{−1}. The column temperature was programmed to be maintained at 40 °C for 5 min after injection, and then increased at 8 °C min^{−1} to 200 °C, where it was held for 5 min. For MS, mass spectra were obtained at 70 eV in the electron impact ionization mode, and the ionic source temperature was 230 °C.

For a preliminary identification of the compounds, we compared their mass spectra with those reported in the NIST 08 mass spectral libraries. Further compound identification was confirmed by comparison of retention indices with those reported in the Pherobase (<http://www.pherobase.com/>, accessed on 1 May 2015) [40] and the NIST Chemistry WebBook (<http://webbook.nist.gov/chemistry/>, accessed on 1 May 2015) [41]. Volatile compounds in the samples at higher or similar percentages in controls were considered as contaminants and excluded from our analysis. Their relative amounts were calculated with the peak area normalization method.

2.4. Statistical Analyses

All data are expressed as the mean ± SD. The total floral scent emission was compared between male and female flowers of each *Breynia* species using unpaired t-tests. One-way ANOVA was used to test the significant differences between floral development stage (at pre-pollination, pollination, and post-pollination stage) from male flowers of *B. fruticosa*. To compare floral scent composition (the relative proportions of all the compounds) of the different kinds of floral headspace samples, NMDS was performed using Canoco 5.0 for Windows (Microcomputer Power, New York, NY, USA). Proportions of odor compounds were first square-root transformed to better fit a normal distribution. Then, a data matrix of pairwise comparisons among floral samples was calculated based on the Bray–Curtis distance index to visualize the dissimilarity between floral samples. Finally, the Mann–Whitney U-test was used to check for significant differences among samples. Correspondence analysis (CA) was used to explore the relationship between the different floral development stages of the two *Breynia* species and the composition of their volatile compound blends using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). All figures were plotted with Graphpad Prism 8.0 (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Chemical Composition of Floral Scent

The floral scent profiles of male and female flowers of two investigated *Breynia* species are summarized in Table 1. In total, 27 volatile compounds were identified from two *Breynia* (*B. fruticosa*: male flowers at pre-pollination stage, M0_f, 10 compounds; male and female flowers at pollination stage, M_f and FM_f, 17 and 16 compounds, respectively; male flowers at post-pollination stage M1_f, 19 compounds; *B. rostrata*: male and female at pollination, M_r and FM_r, 17 and 15 compounds, respectively). The 27 compounds were assigned to four chemical classes: fatty acid derivatives (11), monoterpenes (3), sesquiterpenes (5), and benzenoid derivatives (8).

Table 1. Chemical composition of floral scents of both male flowers and female flowers of two *Breynia* species.

Compounds		RT ^a	<i>B. fruticosa</i>				<i>B. rostrata</i>	
			M0 _f ^d (N = 3)	M _f ^d (N = 7)	M1 _f ^d (N = 5)	FM _f ^d (N = 6)	M _r ^d (N = 3)	FM _r ^d (N = 4)
Fatty acid derivatives								
1	3-Hexenal	802	– ^c	–	–	2.04 ± 2.46	–	3.41 ± 2.42
2	(<i>E</i>)-2-Hexenal ^b	854	2.77 ± 1.01	–	3.05 ± 1.57	1.84 ± 2.19	–	14.86 ± 2.24
3	(<i>Z</i>)-3-Hexen-1-ol ^b	857	11.73 ± 2.65	0.51 ± 0.58	4.07 ± 0.40	3.56 ± 4.36	0.36 ± 0.42	6.09 ± 2.44
4	Decane ^b	1000	–	–	–	5.86 ± 1.13	–	1.53 ± 1.02
5	(<i>Z</i>)-3-Hexenyl acetate ^b	1007	50.96 ± 5.35	1.12 ± 0.39	6.64 ± 0.33	18.79 ± 6.06	0.70 ± 0.78	25.70 ± 3.93
6	Hexyl acetate	1011	1.51 ± 0.52	–	–	–	–	–
7	(<i>E</i>)-2-Hexenyl acetate	1012	–	–	–	0.76 ± 0.59	–	0.57 ± 0.14
8	3-Hexenyl acetate	1015	–	–	–	0.92 ± 0.57	–	0.97 ± 0.12
9	(<i>Z</i>)-3-Hexenyl butyrate ^b	1185	13.59 ± 1.64	3.52 ± 1.14	15.95 ± 1.42	0.97 ± 0.37	1.03 ± 0.45	14.04 ± 2.81
10	Hexyl butyrate	1192	–	0.50 ± 0.30	2.96 ± 0.36	–	0.12 ± 0.10	0.49 ± 0.12
11	(<i>E</i>)-2-Hexenyl butyrate	1195	–	0.72 ± 0.56	1.20 ± 0.51	–	0.06 ± 0.08	0.68 ± 0.10
Monoterpenes								
12	(<i>Z</i>)-β-Ocimene	1038	–	0.89 ± 1.00	1.21 ± 0.85	0.79 ± 0.50	0.23 ± 0.31	0.62 ± 0.08
13	(<i>E</i>)-β-Ocimene ^b	1048	3.51 ± 0.45	1.44 ± 0.78	2.43 ± 0.46	34.19 ± 8.42	0.47 ± 0.23	21.31 ± 1.35
14	cis-Linaloloxide(furanoid) ^b	1088	–	–	–	1.62 ± 1.95	–	8.69 ± 3.22
Sesquiterpenes								
15	β-Caryophyllene ^b	1418	6.40 ± 1.01	0.88 ± 0.67	13.44 ± 1.84	12.44 ± 8.85	0.01 ± 0.02	–
16	β-Humulene ^b	1454	1.42 ± 0.33	–	6.83 ± 3.64	4.74 ± 2.60	–	–
17	(<i>E,E</i>)-α-Farnesene ^b	1506	5.36 ± 1.31	0.88 ± 0.67	28.67 ± 4.34	10.42 ± 4.66	0.53 ± 0.51	–
18	(<i>Z</i>)-Methyl jasmonate ^b	1657	–	2.66 ± 0.66	2.50 ± 0.52	–	11.83 ± 1.29	–
19	[1.α,2.α,(<i>Z</i>)]-3-oxo-2-(2-pentenyl)-Cyclopentaneacetic acid, methyl ester	1685	0.55 ± 0.15	1.10 ± 1.58	1.63 ± 0.63	–	0.97 ± 0.14	–
Benzenoid dervatives								
20	Benzaldehyde	962	–	–	–	0.56 ± 0.33	–	0.59 ± 0.20
21	Phenylacetaldehyde ^b	1043	–	1.44 ± 0.78	0.59 ± 0.25	–	5.45 ± 0.10	–
22	Phenylethyl alcohol ^b	1116	–	10.37 ± 1.03	1.67 ± 0.32	–	10.13 ± 1.28	–
23	Phenylethanenitrile ^b	1143	–	5.88 ± 0.81	1.16 ± 0.66	–	0.04 ± 0.06	–
24	Methylsalicylate	1190	–	–	–	0.22 ± 0.30	–	0.15 ± 0.06
25	β-Phenylethyl acetate	1256	–	0.25 ± 0.20	2.25 ± 0.47	–	0.35 ± 0.22	–
26	Eugenol	1359	–	0.50 ± 0.98	1.80 ± 1.26	–	0.75 ± 0.09	–
27	β-Phenylethyl butyrate ^b	1447	–	66.79 ± 2.51	1.96 ± 0.57	–	66.99 ± 0.99	–

^a Retention index. ^b Compounds were used for correspondence analysis. ^c Compounds were not found in the scent. ^d M0_f, M_f, and M1_f represent the male flowers from *Breynia fruticosa* at pre-pollination, pollination, and post-pollination stage, respectively; FM_f represents the female flowers from *B. fruticosa* at pollination stage; M_r and FM_r represent, respectively, the male and female flowers from *B. rostrata* at pollination stage (the same below).

At pollination stage, 25 compounds were shared jointly by the two study species; only one compound, β-humulene, which was exclusively produced by *B. fruticosa*, is species specific. A total of five compounds, (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate, (Z)-3-hexenyl butyrate, and (Z)- and (E)-β-ocimene, were found in flowers of both species, regardless

of sex. More surprisingly, two benzenoid derivatives, β -phenylethyl butyrate (66.79 and 66.99%, respectively) and phenylethyl alcohol (10.37 and 10.13%, respectively) dominated the male flower scent of both *B. fruticosa* and *B. rostrata*, and together represented more than 80% of the total volatile signal, whereas the two compounds were absent in the female floral scent of the two species. Female flower scent of the two species (*B. fruticosa* and *B. rostrata*) predominantly contained (*E*)- β -ocimene (34.19 and 21.31%, respectively) and (*Z*)-3-hexenyl acetate (18.79 and 25.70%, respectively), whereas no such dominance was observed in the male floral scent.

The relative quantities of four chemical classes of compounds emitted by the male and female flowers of two species are shown in Figure 2. At pollination stage, the male floral scent of two species contained large quantities of benzenoid compounds, which accounted for 85.25 and 83.29% of the totals, respectively; however, sesquiterpenes (53.07%) and fatty acid derivatives (70.37%) dominated, respectively, the female floral scent of the two species. In addition, fatty acid derivatives (82.42%) and sesquiterpenes (53.07%) were included, respectively, in the male floral scent of *B. fruticosa* at pre- and post-pollination stages.

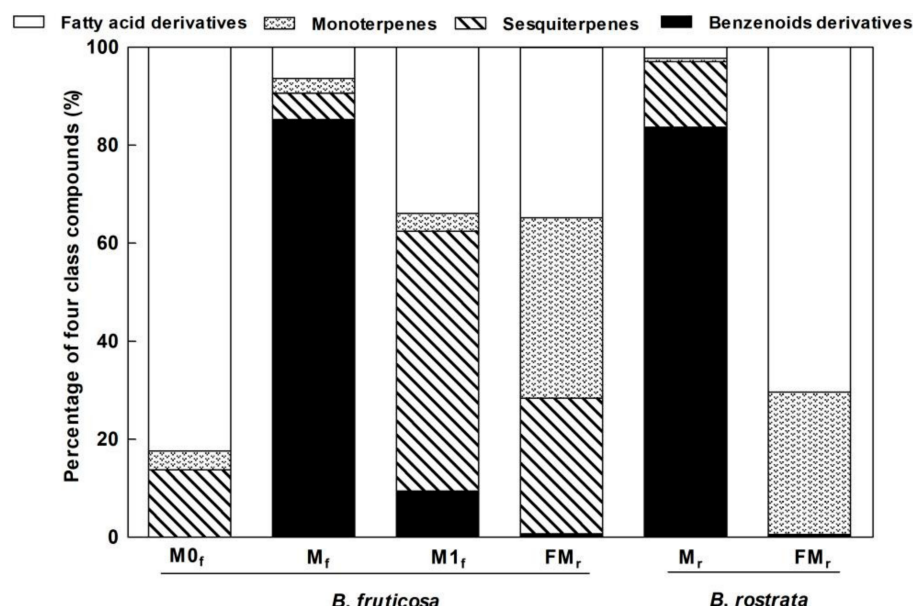


Figure 2. Relative quantity (%) of volatile compounds of four chemical classes emitted by the male and female flowers of the two *Breynia* species studied.

3.2. Interspecific, Intersexual, and Temporal Variation of Floral Scents

The dissimilarity of floral volatile profiles among individual samples was subjected to NMDS analysis. Scatterplots resulting from NMDS are shown in Figure 3. All floral samples were divided into four groups. For each sex, volatile samples of the two *Breynia* species at the pollination stage were closely spaced on the NMDS scatterplot. In particular, a significant overlap can be seen in male floral scents compared to female floral scents. In addition, the headspace samples of male and female flowers are clearly separated for both *Breynia* species on the NMDS scatterplot. For male flowers of *B. fruticosa*, volatile samples of the three developmental stages are evidently distinguished from each other on the NMDS scatterplot. These results suggest that the two *Breynia* species exhibited no obvious interspecific variation in the volatile chemical profiles for each sex. In particular, the male flower showed conservation of floral scent composition for the two *Breynia* species. However, sexual dimorphism of the volatile chemical profiles was very clear in both *B. fruticosa* and *B. rostrata*. Furthermore, there was significant temporal variation in the volatile chemical profiles produced by male flowers of *B. fruticosa* before and after pollination.

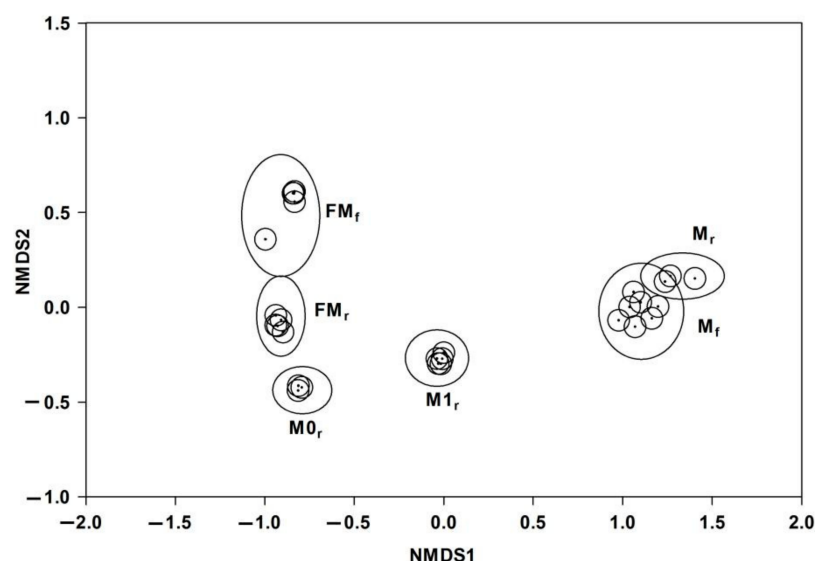


Figure 3. Score plots from nonmetric multidimensional scaling (NMDS) of floral scents in male and female flowers of the two *Breynia* species studied.

Fifteen compounds, each with >2% mean relative amounts of floral volatile profiles in two *Breynia* species, were included in the CA (see Table 1). Scatterplots resulting from CA are shown in Figure 4. The CA revealed that the chemistry profile of the two *Breynia* species was mainly dominated by a few of the main compounds. For example, the male flower scent of the two *Breynia* species predominantly contained (Z)-methyl jasmonate, phenylacetaldehyde, phenylethyl, phenylethanenitrile, and β -phenylethyl butyrate (compounds 18, 21, 22, 23, and 27); in particular, the two most abundant compounds in male flowers, β -phenylethyl butyrate (compounds 27) and phenylethyl alcohol (compounds 22), were located close to the male flower scent of the two *Breynia* species in the CA.

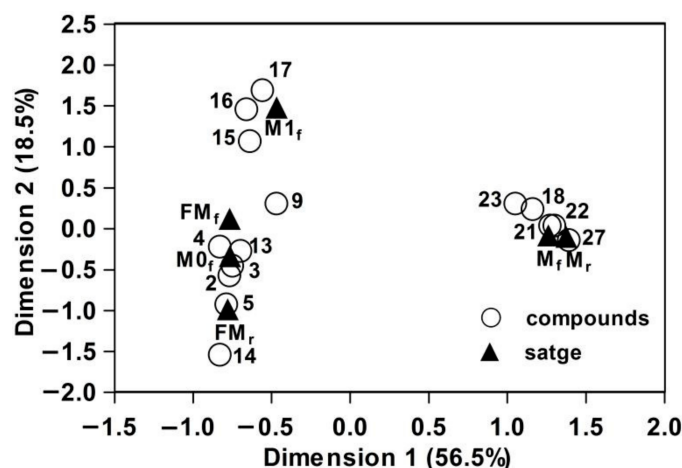


Figure 4. Score plots from correspondence analysis (CA) of floral scents in male and female flowers of the two *Breynia* species studied.

3.3. Emission of Floral Scents

The total emissions of floral scents produced by male and female flowers of both *Breynia* species are shown in Figure 5. For each investigated species, a male flower emitted significantly more scent than a female flower (*B. fruticosa*: $2.25 \pm 0.75 \text{ ng h}^{-1}$ vs. $0.17 \pm 0.11 \text{ ng h}^{-1}$, $t = 6.307$, $p < 0.001$; *B. rostrata*: $2.07 \pm 0.96 \text{ ng h}^{-1}$ vs. $0.22 \pm 0.03 \text{ ng h}^{-1}$, $t = 3.97$, $p < 0.05$). In male flowers of *B. fruticosa*, the amount of floral scent was significantly higher at the pollination stage ($2.25 \pm 0.75 \text{ ng h}^{-1}$) than at the pre-pollination stage ($0.43 \pm 0.15 \text{ ng h}^{-1}$) and at the pre-pollination stage ($1.04 \pm 0.17 \text{ ng h}^{-1}$) ($F = 15.28$,

$p < 0.001$). The emission of the nine main compounds ($>5\%$ mean relative amounts in one or more developmental stages) varied with the developmental stage (Figure 1), especially for β -phenylethyl butyrate ($F = 25.62$, $p < 0.0001$) and phenylethyl alcohol ($F = 32.63$, $p < 0.0001$). At pre-pollination, the male flower produced neither of these compounds. Until pollination, the release of these two compounds reached the maximum, whereas the two compounds significantly decreased at post-pollination. These results suggest that these *Breynia* species exhibit obvious quantitative differences in their sexes' floral scents. The total emission of floral scents appeared to change with the developmental stage of the male flower of *B. fruticosa*; in particular, the quantity of emission of β -phenylethyl butyrate and phenylethyl alcohol changed significantly between before and after pollination.

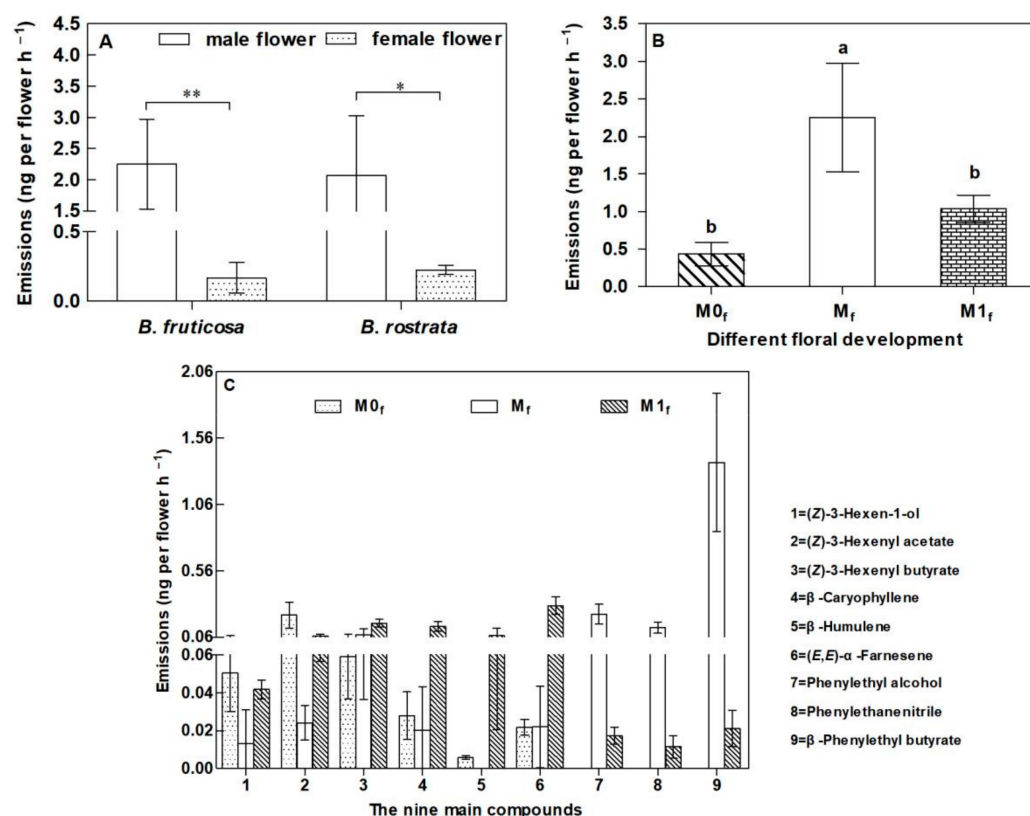


Figure 5. Total emission of scent compounds from male and female flowers of two *Breynia* species studied: (A) male and female flowers of two *Breynia* species studied at pollination stage, * and ** indicated respectively significant p values ($p < 0.05$) and extremely significant p values ($p < 0.01$); (B) male flowers of *B. fruticosa* during different floral development stages; (C) the nine main compounds from male flowers of *B. fruticosa* during different floral development stages, The different lowercase showed significant p values ($p < 0.05$).

4. Discussion

Overall, 27 volatile compounds were detected in both male and female flowers of the two monoecious *Breynia* species investigated in this study. These volatile organic compounds belong to three large chemical classes depending on the different biosynthetic pathways: (1) terpenoids (monoterpenes and sesquiterpenes) were synthesized by the mevalonate pathway; (2) fatty acid derivatives were synthesized from fatty acids; and (3) benzene derivatives were synthesized by the shikimate pathway [42]. Almost all identified compounds occur widely in the floral scents of many flowering plants [42]. In particular, the 25 identified compounds have been reported in floral odors of 13 *Epicephala* moth-pollinated *Phyllanthaceae* species [27–29,43]. Therefore, the two studied *Breynia* species are thought to attract the obligate pollinators via general floral compounds in the *Breynia*–*Epicephala* mutualisms; however, the physiologically active compounds that exclusively

attract host-specific pollinators in these interactions have not been further identified using electroantennographic detection analysis and behavioral testing with authentic chemicals. Interestingly, our findings are similar to scent data from other obligate pollination mutualisms between Phyllanthaceae species and *Epicephala* moths, which suggests that these interactions are mediated by the conventional floral volatiles [27,28]. The *Breynia* species–*Epicephala* moth associations contrast with the mutualism between *F. semicordata* and fig wasps, which has been demonstrated to be mediated by a single specific chemical compound (4-methylanisole) through ‘private channels’ to alone maintain the extreme species specificity [35].

In these specialized pollination systems, variation in the floral scent was found for almost all investigated species. This is an important isolation mechanism for closely related sympatric species to maintain the specificity of obligate pollinator attraction [15,44]. In highly species-specific *Epicephala*–Phyllanthaceae interactions, the reported floral scent profile of host plants is generally composed of several dominant compounds, rather than a single volatile constituting the attractive signals [27–29], and these major volatiles may account for variation in floral scents among species [27,29]. Similarly, our GC-MS analysis revealed that the male floral scent of the two *Breynia* species at the pollination stage was dominated by two compounds: β -phenylethyl butyrate (66.79 and 66.99%, respectively) and phenylethyl alcohol (10.37 and 10.13%, respectively), which are also known not to be largely emitted by the other sympatric Phyllanthaceae species studied here [27–29]. Conversely, 2-phenylacetonitrile (50.70%) and phenylethyl alcohol (29.66%) were the most abundant compounds in male floral scents of *B. vitis-idaea*, a close relative of the two investigated *Breynia* species, and which is also exclusively pollinated by host-specific *Epicephala* moths [6,28,37]. By comparison, based on dominant compounds from floral scents, the floral scent profile exhibited a significant variation between the two studied *Breynia* species and *B. vitis-idaea*. To facilitate host specificity in these mutualisms, strong divergent selection should act on the floral scent profiles among sympatrically occurring species involving pollinating *Epicephala* moths. Thus, our study confirmed the hypothesis that the chemical blend emitted from *B. fruitcosa* and *B. rostrata* is species-specific.

B. fruitcosa and *B. rostrata*, which are also closely related and sister taxa, share the same two *Epicephala* pollinators [37]. Our NMDS analysis showed the odor similarity in male flowers of the two investigated *Breynia* species. Similarly, *Glochidion obovatum* and *G. rubrum*, two species pollinated by the same two species of *Epicephala* moths, also produced similar floral odors [27]. In addition, other two *Glochidion* species, *G. hirsutum* and *G. zeylanicum*, sharing the same *Epicephala* moth, also had similar compositions of floral scent [29]. The phenomenon illustrated here for two *Breynia* species also occurred in several other highly species-specific pollination systems. For example, two southern Africa *Ficus* species, *Ficus natalensis* and *F. burkei* pollinated by the same two fig wasps emitted the similar floral volatile blends [45]. When different plant species are pollinated by the same group of pollinators, they may be expected to have floral volatiles of similar chemical composition [46]. Thus, selection for odor convergence between two *Breynia* species may be suggested to attract the same pollinator, *Epicephala*, for their pollination [45]. Imperfect chemical differentiation may facilitate a dynamic reticulate evolution of host–pollinator associations. Zhang et al. found that natural cross-pollination occurs in the two studied *Breynia* plants [37]. Due to the similar floral scent profiles among the two *Breynia* species, the *Epicephala* moth may not distinguish the scent signal of the two hosts, and may also favor natural interspecific hybridization among the two host plants.

The main purpose of insect pollination for the plant is that the pollinator obtains constant floral rewards from the flowers [47]. Generally, in unisexual plants pollinated by insects, both male and female flowers advertise the same floral rewards (e.g., floral nectar) for pollinators; thus, they are jointly selected to produce similar floral signals to ensure conspecific transfer pollen [48]. Conversely, if male and female flowers provide different floral rewards (e.g., pollen and ovule, respectively) for the pollinator, sexually dimorphic floral signals are evolved to guide pollinators to alternative rewards in male

and female flowers [49]. Our NMDS revealed clear sexual dimorphism in the floral scent profile of the two monoecious *B. fruitcosa* and *B. rostrata*. In addition, these male flowers emitted much higher quantities of volatile compounds compared with female flowers. A similar pattern has been observed in other *Epicephala*-pollinated Phyllanthaceae [28,29,43]. Recently, the role of sexual dimorphism in floral scent in these mutualisms was elucidated by Okamoto et al. [43]. In *Epicephala*-pollinated Phyllanthaceae, male flowers emitting more scent can attract more pollinator visits compared with female flowers, and then elicit active pollination behavior. Sexually dimorphic floral scent of male and female flowers can provoke the pollinator's legitimate altruistic behavior on host flowers, that is, pollen collection on male flowers and pollen deposition and oviposition on female flowers, and simultaneously signal an alternative reward provided by each sex for moths. Therefore, the two monoecious *Breynia* species studied may be selected to produce different floral scents to induce active pollination behavior from *Epicephala* moths. It is necessary to further analyze moth behavioral assays on sexual floral scent dimorphism in plant studies.

Floral scent production is a dynamic process. Pollinator attraction at the pollination stage is not just an important function of floral volatile compounds. With pollination, the functions of volatile compounds will change, and the floral scent profile is thus also likely to vary [21]. Our study also revealed that the floral scent produced by male flowers of *B. fruitcosa* changed both in terms of quantity (the chemical composition of volatile compounds) and quality (the emission of compounds) during their development. The chemical profiles of the floral scent from male flowers of this species exhibited clear differences before, during, and after pollination. Furthermore, the quantity of floral scents emitted in male flowers of this species was significantly higher at the pollination stage than at pre-pollination and post-pollination stages. In the obligate nursery pollination mutualisms, floral odor changes have been observed in several host plant species [21,30,35,50–53]. For example, *Epicephala*-pollinated *G. rubrum* exhibited significant changes in the floral scent of female flowers. The post-pollination change in the floral scent emission of *G. rubrum* may be optimized to reduce further visits and oviposition by the pollinator moth. A similar post-pollination change in floral scent has been observed in several *Ficus* species. Although figs produce volatile compounds during their entire developmental period, only the fig odor at the receptive stage is strongly attractive to fig wasps [21,35,54,55]. Therefore, the strongly reduced emission of floral bouquet and significant change in the chemical composition of male flower scents of *B. fruitcosa* after pollination may also function as post-pollination mechanisms to rapidly decrease pollinator attraction and further reduce flowers visits, thereby limiting the overexploitation of the ovaries by *Epicephala* moths that are also seed predators [56]. Due to the greater odor emitted by male flowers in *Epicephala*-pollinated Phyllanthaceae, the moth prefers the scent of male flowers compared to that of female flowers, resulting in pollen-collecting behavior [43]. Therefore, temporal changes in the odor production and composition of male flowers before and after pollination are important for maintaining the stability of the nursery pollination mutualisms between Phyllanthaceae species and *Epicephala* moths.

In several insect-pollinated plants, post-pollination changes in floral scent usually result from a decrease in the total amount of odor production and changes in the relative amounts of the main scent's compounds [21,35,51,54,57,58]. For example, the main compound, 4-methylanisole, is strongly predominant (94–98%) among the volatile compounds produced by *F. semicordata* during pollination, whereas it disappears totally after pollination. Further bioassays showed 4-methylanisole is the main signal compound in the floral scent of *F. semicordata*, which attracts its obligate wasps to the host figs [35]. As shown in this study, two benzenoid compounds, β -phenylethyl butyrate and phenylethyl alcohol, were not produced in male flowers at the pre-pollination stage. Interestingly, these two compounds were present in maximal amounts (66.79 and 10.13%, respectively) at pollination stage, but they rapidly reduced in concentration at the post-pollination stage. Thus, it is speculated that the two most abundant compounds, β -phenylethyl butyrate and phenylethyl alcohol, produced by the male flowers of *B. fruitcosa*, may be two signal

compounds that attract species-specific *Epicephala*. A combination of electrophysiological screening and behavioral experiments on these two active compounds should be carried out. Thus far, the identification of attractants mediating *Breynia*–*Epicephala* obligate pollination mutualisms has only been documented in *B. vitis-idaea* species. A blend of 2-phenylethyl alcohol and 2-phenylacetonitrile as attractants constituted the attractive signals for the obligate pollinator in *B. vitis-idaea*–*Epicephala* interaction [28].

5. Conclusions

Our results showed that the two *Breynia* species had no clear interspecific variation in floral scent profiles within both sexes, especially in male flowers. Both species exhibited obvious qualitative intersexual differences in floral scent profiles: male flowers produced more scent than female flowers. Both volatile chemical profiles and the total emission of floral scents from male flowers of *B. fruticosa* significantly changed between before and after pollination. Interestingly, β -phenylethyl butyrate and phenylethyl alcohol were present in maximal amounts during pollination, but they rapidly reduced in concentration after pollination. Thus, our findings suggest that the volatile similarity of floral scent among the two *Breynia* species reflects adaptations to the same pollinator, *Epicephala*. The sexually dimorphic floral scent of male and female flowers has been selected to provoke pollinators' active pollination for both host flowers. Temporal changes in the floral scent of male flowers before and after pollination may have evolved to limit the overexploitation of the ovaries by seed predators, *Epicephala* moths. In summary, we speculate that the two most abundant compounds produced by the male flowers from both species may be potential signal compounds that attract species-specific *Epicephala* moths. Floral scent has been thought to play a key role in the obligate pollination mutualism between two *Breynia* species and two *Epicephala* moths ('two-to-two'). Our study provides a theoretical basis to further reveal the chemical and ecological mechanisms in obligate pollination mutualisms between Phyllanthaceae plants and *Epicephala* moths.

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