Behavioral Responses of Wiebesia pumilae to Volatiles from Female-Phase Syconia of Ficus pumila and Ficus pumila var. awkeotsang (Postprint)

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Abstract

Ficus pumila and Ficus pumila var. awkeotsang are both dioecious plants in the family Moraceae and genus Ficus, constituting a type-variety and variety relationship. They have respectively established obligate mutualistic relationships with their pollinator wasps (cryptic species to each other). Fig volatile compounds play a crucial role in maintaining the mutualistic relationship between pollinator wasps and their hosts. Using a Y-tube olfactometer, we assayed the behavioral responses of the Ficus pumila wasp (the pollinator wasps of both Ficus pumila and Ficus pumila var. awkeotsang) to volatiles from female-phase figs of the two plant types. The results demonstrated: (1) Fig size in the female phase had no significant effect on wasp behavioral response; volatiles from both large and small female-phase figs of both sexes of Ficus pumila exerted strong attractive effects on its pollinator wasp; (2) Fig volatile concentration significantly affected wasp behavioral response; a threshold response may exist for volatiles from female-phase figs of both sexes of both Ficus pumila and Ficus pumila var. awkeotsang, such that attraction is positively correlated with volatile concentration below the threshold, but decreases significantly once the threshold is exceeded, indicating that host fig volatile concentration influences pollinator host location; (3) The Ficus pumila pollinator exhibited neither attraction nor repellence to low-concentration volatiles from female-phase figs of both sexes of Ficus pumila var. awkeotsang, and the Ficus pumila var. awkeotsang pollinator showed similar neutral responses to low-concentration volatiles from female-phase figs of both sexes of Ficus pumila; the Ficus pumila pollinator displayed significant repellent behavior to high-concentration volatiles from female-phase figs of both sexes of Ficus pumila var. awkeotsang, while the Ficus pumila var. awkeotsang pollinator showed significant attraction to high-concentration volatiles from female-phase figs of both sexes of Ficus pumila, revealing asymmetric host specificity. Consequently, the Ficus pumila var. awkeotsang pollinator is more likely to enter Ficus pumila figs for pollination or oviposition, whereas the Ficus pumila pollinator in the Fuzhou area may have difficulty entering Ficus pumila var. awkeotsang figs. These findings provide a scientific basis for chemical ecological theory of fig-wasp mutualistic systems and for the cultivation of Ficus pumila var. awkeotsang.

Full Text

Behavioral Responses of *Wiebesia pumilae* to Female Phase Fig Volatiles of *Ficus pumila* var. *pumila* and *Ficus pumila* var. *awkeotsang*

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Abstract

Ficus pumila L. var. *pumila* (FPP) and *F. pumila* L. var. *awkeotsang* (FPA) are evergreen climbing dioecious fig plants that exist as an original variety and its variant, respectively. Each forms an obligate symbiotic relationship with its specific pollinating wasp, and these two wasp species are cryptic species. Fig volatiles play a crucial role in maintaining the symbiotic relationship between pollinating wasps and their hosts, as wasps use these chemical cues to locate host figs at the female phase for oviposition and pollination. Using a Y-tube olfactometer, we tested the behavioral responses of *Wiebesia pumilae* (Hill) Wiebes, the pollinating wasp of both FPP and FPA, to female phase fig volatiles from both varieties.

The results demonstrated that: (1) Fig size had no significant effect on the behavioral responses of *W. pumilae*. Both large and small female and male receptive phase figs of FPP released volatiles that strongly attracted its obligate pollinator. (2) Volatile concentration significantly influenced wasp behavior, with both FPA and FPP fig volatiles eliciting a threshold response in *W.*

pumilae. When volatile concentrations were below the threshold, pollinator attraction increased with concentration; however, when concentrations exceeded the threshold, attraction decreased. (3) At low concentrations, FPP pollinating wasps showed neither attraction nor repellence to FPA female phase fig volatiles, and FPA pollinating wasps showed similar neutral responses to low-concentration FPP volatiles. However, FPA pollinating wasps exhibited significant preference for high-concentration FPP volatiles, whereas FPP pollinators were significantly deterred by high-concentration FPA volatiles. These findings indicate asymmetric host specificity between the pollinating wasps of FPA and FPP. While FPA pollinating wasps may be able to enter FPP figs for pollination and oviposition, FPP pollinating wasps in the Fuzhou region appear to have difficulty locating and entering FPA figs. Our results provide a scientific basis for understanding the chemical signaling mechanisms underlying fig-wasp mutualism and for developing improved cultivation practices for FPA.

Keywords: dioecious; female phase; variant; cryptic species; Y-tube olfactometer

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Introduction

Ficus pumila L. var. *pumila* (FPP) is a dioecious fig species distributed throughout southern China south of the Yangtze River, including Hunan and Sichuan provinces, with natural populations also occurring in Japan and northern Vietnam. It is widely used in urban landscaping. *Ficus pumila* L. var. *awkeotsang* (FPA), known as "aiyuzi," is a specialty fruit crop with high economic value that is cultivated extensively in Taiwan and mainland China south of the Yangtze River. The type locality is Taiwan. While FPP primarily occurs at low elevations, FPA is mainly found in mountainous areas above 800 m. Chen et al. [5] reported sympatric distribution of wild FPA and FPP in low-elevation areas of Ningde, Fujian. The two varieties show similar plant morphology, differing only slightly in leaf shape and syconium characteristics [6].

Lin et al. [4] compared the chemical properties of pectin methylesterase from both varieties, finding that FPA had two isoforms with 28.5 activity units, while FPP had only one isoform with 25 units, suggesting they may be closely related but distinct species. With advances in molecular biology, molecular markers have been widely applied to species identification. Li and Hui [7] used three molecular markers—the chloroplast *trnT-trnL* intergenic region, the third intron of the nitrate reductase gene, and the internal transcribed spacer (ITS II) of nuclear ribosomal DNA—to conduct genetic analysis of FPP and FPA. Their results indicated no interspecific differentiation between the two varieties, and Wang et al. [8] found overlapping nuclear genes, suggesting possible gene flow. Jiang [9] demonstrated through interspecific cross-pollination that FPP and FPA could produce fertile hybrids, though with reduced reproductive capacity. These findings support the current classification of FPP and FPA as original variety and variant [10].

The pollinating wasps of FPP and FPA are morphologically extremely similar and were long considered the same species, *Wiebesia pumilae* (Hill) Wiebes [4, 11–12]. However, recent evidence from molecular biology, reproductive ecology, and morphology suggests they are either distinct species or cryptic species. Li and Hui [7] and Wu et al. [13] analyzed partial mitochondrial DNA sequences of the pollinating wasps and found genetic differentiation at the interspecific level. Analysis of the first intron of the mitochondrial *COI* gene (*mtCOI*) revealed substantial genetic divergence between the two wasp species with no overlap in nuclear markers [8]. Chen et al. [5] observed phenological differences in the life histories of wild FPA and FPP in Ningde, Fujian, indicating that the two pollinating wasp species have developed reproductive isolation. Jiang [9] identified differences in microscopic and submicroscopic morphology, finding seven female characteristics and two male characteristics that distinguished the wasps. Chen et al. [14] analyzed *Wiebesia pumilae* across the distribution range of FPP using nuclear microsatellites, concluding that the species complex comprises cryptic species. Molecular evidence suggests that the Taiwan FPA pollinating wasp belongs to *Wiebesia* spp. 3, while FPP pollinating wasps are *Wiebesia* spp. 1, 2, and 3. Currently, the pollinating wasps of FPP and FPA are recognized as cryptic species with similar morphology but distinct genetic structures.

Fig wasps typically live only 2–3 days and have limited visual capabilities [16], relying primarily on well-developed antennal sensilla to perceive volatile compounds released by figs for precise host location [17–18]. Volatile chemical signals are therefore critical for maintaining the fig-wasp mutualism [15]. Researchers have primarily used Y-tube olfactometers to test fig wasp responses to volatiles [19–20]. Yokoyama [21] found that the pollinating wasps of two closely related fig species, *Ficus nishimurae* and *F. boninsimae*, could distinguish between the compounds released by female phase figs of the two species, whereas *F. boninsimae* wasps could not, indicating asymmetric host specificity. Similar asymmetric host specificity was observed in the pollinating wasps of two varieties of *Ficus semicordata* [19].

This study investigates the behavioral responses of *Wiebesia pumilae* to female phase fig volatiles from both FPP and FPA, using the wasp as a test subject to explore the symmetry of host specificity and the potential for cross-pollination between the two systems.

Methods

2.1 Collection of Study Materials Pollinating wasps (*Wiebesia* spp. 2) and female phase figs of FPP were collected from Fuzhou National Forest Park (119°29 E, 26°15 N). Pollinating wasps (*Wiebesia* spp. 3) and female phase figs of FPA were collected from a Fujian FPA cultivation garden (118°55 E, 25°31 N). FPP plants approaching wasp emergence were bagged with 120-mesh nylon nets, brought back to the laboratory for hydroponic observation, and vigorous wasps emerging on the day of experiment were selected for testing.

2.2 Classification of Female Phase Fig Types We observed significant variation in the diameter of female phase figs on individual FPP plants and classified them into large and small types .

**** Comparison of fig types of female and male receptive phase figs of *Ficus pumila* L. var. *pumila* at different developmental stages (n = 20, mean ± SD)

Fig Type	Diameter (cm)	Wall Thickness (cm)	Height of Flowers (cm)	Diameter of Cavity (cm)
Large female figs	5.50 ± 0.22	0.90 ± 0.06	0.11 ± 0.01	2.57 ± 0.17
Small female figs	3.42 ± 0.14	0.57 ± 0.02	0.08 ± 0.01	1.99 ± 0.08
Large male figs	4.42 ± 0.25	0.93 ± 0.05	0.17 ± 0.01	1.86 ± 0.11
Small male figs	3.40 ± 0.15	0.66 ± 0.03	0.13 ± 0.01	1.93 ± 0.08

2.3 Y-Tube Olfactometer Setup and Experimental Design We used a custom-designed glass Y-tube olfactometer to test wasp behavioral responses to volatiles from female phase figs of FPP and FPA. The olfactometer had a 20 cm stem, with release tubes 1 cm in diameter at 15 cm and 10 cm lengths, each connected to an odor source bottle. Incoming air was filtered through activated charcoal and humidified in distilled water before entering the odor source bottles. Teflon tubing connected the stem to a vacuum pump with airflow regulated at 100–150 mL/min. Experiments were conducted in a completely darkened room at (25 ± 2)°C and 70–75% relative humidity.

Individual female wasps were introduced into the release tube and their choice reactions to the two arms were recorded within 5 minutes. A choice was recorded when a wasp crawled beyond the 10 cm mark on either arm. Wasps that did not make a choice within the time limit were recorded as non-responders. Each treatment group tested 30 wasps, with 10 wasps tested per day. After each treatment, the Y-tube stem, odor source bottles, and connecting tubes were replaced, and the apparatus was cleaned with ethanol and double-distilled water and dried in an oven at 50°C for 30 minutes. To control for directional bias, the positions of the two arms were swapped after every 10 wasps.

Figs were marked and tracked from bud formation. When buds developed to the female phase (indicated by the presence of pollinating wasps on the bag or detectable inflorescence odor), syconia were collected for experiments. The experimental design included both single-odor-source tests (odor source vs. clean air) and dual-odor-source tests (two odor sources against each other).

2.4 Experimental Treatments The experimental treatment groups are detailed in through .

**** Experimental treatment groups for behavioral tests of FPP pollinating wasps to female and male receptive phase fig volatiles of *Ficus pumila* L. var. *pumila* at different developmental states

**** Experimental treatment groups for behavioral tests of FPP pollinating wasps to female and male receptive phase fig volatiles of *Ficus pumila* L. var. *awkeotsang*

**** Experimental treatment groups for behavioral tests of FPA pollinating wasps to female and male receptive phase fig volatiles of *Ficus pumila* L. var. *awkeotsang*

**** Experimental treatment groups for behavioral tests of FPA pollinating wasps to female and male receptive phase fig volatiles of *Ficus pumila* L. var. *pumila*

2.5 Data Analysis All experimental data were analyzed using Excel 2007 and SPSS 17.0 software. Measurement data are expressed as mean ± SD. Significance of differences in wasp attraction among treatments was determined using chi-square tests.

Results and Analysis

2.1 Behavioral Responses of FPP Pollinating Wasps to FPP Female Phase Fig Volatiles The behavioral responses of FPP pollinating wasps to volatiles from female and male receptive phase figs at different developmental stages are shown in [Figure 1: see original paper]. Both small female and small male phase fig volatiles attracted their pollinators. Volatile concentration significantly affected pollinator behavior: within a certain concentration range, attraction to FPP pollinating wasps was positively correlated with volatile concentration, but decreased significantly when concentration exceeded a threshold.

In single-odor-source experiments, small female phase figs (1, 2, or 3 figs) showed significantly stronger attraction than larger quantities of small figs. In experiments using large figs, 1 or 2 large female phase figs attracted significantly more wasps than other groups. This suggests that volatile concentration per individual small fig may be higher than that from large figs. In dual-odor-source experiments, small male phase fig volatiles were significantly more attractive than large male phase fig volatiles, while small female phase fig volatiles were significantly less attractive than large female phase fig volatiles. No significant differences were observed among the remaining experimental groups.

Chi-square tests on single-odor-source data revealed no significant differences in attraction between female and male inflorescence volatiles ($^2 = 0.293$, $P = 0.588$; $^2 = 0.287$, $P = 0.592$), supporting the hypothesis that pollinators show no bias toward either sex. This suggests that when volatile concentration from individual small figs is high, increasing fig number to 4 does not enhance attraction, whereas for large figs with lower individual volatile concentration, increasing quantity to 4 maintains high attractiveness.

[**Figure 1: see original paper**] Behavioral responses of FPP pollinating wasps to different developmental stages of female and male receptive phase fig volatiles of FPP. Data are means; all data were analyzed by chi-square tests. * indicates significant difference ($P < 0.05$), ** indicates highly significant difference ($P < 0.01$), NS indicates no significant difference ($P > 0.05$). BFP: big female phase female fig of FPP; SFP: small female phase female fig of FPP; BMP: big female phase male fig of FPP; SMP: small female phase male fig of FPP; CA: clean air control. Numbers in rectangles indicate fig quantities.

2.2 Behavioral Responses of FPP Pollinating Wasps to FPA Female Phase Fig Volatiles The behavioral responses of FPP pollinating wasps to FPA female phase fig volatiles are shown in [Figure 2: see original paper]. Low-concentration FPA volatiles (1, 2, or 3 figs) elicited neither attraction nor repellence in FPP pollinating wasps, while high-concentration volatiles (4 figs) caused highly significant repellence. Chi-square tests revealed no significant difference in repellence between female and male inflorescence volatiles ($^2 = 0.348$, $P = 0.555$; $^2 = 0.118$, $P = 0.732$), indicating that within a certain concentration range, repellence is positively correlated with volatile concentration, but becomes independent of concentration once the threshold is exceeded.

[**Figure 2: see original paper**] Behavioral responses of FPP pollinating wasps to female and male receptive phase fig volatiles of FPA. FA: female phase female fig of FPA; MA: female phase male fig of FPA; CA: clean air control.

2.3 Behavioral Responses of FPA Pollinating Wasps to FPA Female Phase Fig Volatiles Compared with clean air, 1, 2, or 3 female phase FPA figs showed neither significant attraction nor repellence to FPA pollinating wasps. However, 4 female phase figs elicited highly significant attraction, demonstrating that volatile concentration significantly influences wasp behavior. Chi-square tests revealed highly significant attraction to both female and male inflorescence volatiles ($^2 = 72.900$, $P < 0.001$; $^2 = 92.564$, $P < 0.001$), with no significant difference between the sexes ($^2 = 0.047$, $P = 0.828$), supporting the hypothesis of intersex mimicry in volatile emission [3].

[**Figure 3: see original paper**] Behavioral responses of FPA pollinating wasps to female and male receptive phase fig volatiles of FPA. Data are means; all data were analyzed by chi-square tests.

2.4 Behavioral Responses of FPA Pollinating Wasps to FPP Female Phase Fig Volatiles Compared with clean air, low-concentration FPP volatiles elicited neither attraction nor repellence in FPA pollinating wasps, while high-concentration volatiles caused significant attraction. Non-parametric tests revealed no significant differences in attraction between female and male inflorescence volatiles (^{2} = 0.034, P = 0.926; ^{2} = 0.754, P = 0.457), indicating that within a certain concentration range, attraction is independent of inflorescence sex.

[Figure 4: see original paper] Behavioral responses of FPA pollinating wasps to female and male receptive phase fig volatiles of FPP. FP: female phase female fig of FPP; MP: female phase male fig of FPP; CA: clean air control.

Discussion

3.1 Potential for Interspecific Pollination Between FPP and FPA Cross-behavioral assays revealed that FPP fig volatiles attract FPA pollinating wasps, while FPA fig volatiles repel FPP pollinating wasps. This suggests that FPA pollinators can locate FPP figs, but FPP pollinators likely cannot locate FPA figs. These results indicate that using FPA pollinating wasps to pollinate FPP female phase figs is feasible in Fujian, whereas using FPP pollinating wasps for FPA pollination would have low success rates.

Jiang [9] reported that in Taiwan, the two pollinating wasps showed no host preference for the two varieties' female fig volatiles, but FPA pollinating wasps were attracted to FPP male fig volatiles while FPP pollinating wasps were not attracted to FPA male fig volatiles. This discrepancy between Taiwan and Fujian may reflect geographic variation in wasp populations.

Studies suggest that during glacial periods, *Ficus pumila* likely had refugia on both sides of the Nanling Mountains. Post-glacial expansion north of the Nanling range proceeded inland to higher latitudes, while southern populations spread along the Nanling and Daiyun Mountains. As fig populations expanded, their pollinating wasps underwent genetic differentiation at different rates. The *Wiebesia* spp. 3 population associated with FPA likely evolved more rapidly and is primarily distributed south of the Nanling range, while *Wiebesia* spp. 1 and 2 are mainly found in Taiwan and the Zhoushan Archipelago [6, 14, 24]. Liu et al. proposed that the Zhoushan *W. pumilae* spp. 1 population evolved from the *W. pumilae* spp. 3 population, and molecular evidence indicates that *W. pumilae* spp. 2 in Taiwan is closely related to *W. pumilae* spp. 3 [6, 25]. The *W. pumilae* spp. 2 population differentiated concurrently with the FPA variant at high elevations, while *W. pumilae* spp. 3 coevolved with FPP on Taiwan Island [6, 25]. Although these populations have partially overlapping distributions [6], the Taiwan Strait has isolated the *W. pumilae* spp. 2 population, preventing gene flow with FPA. Consequently, while *W. pumilae* spp. 2 in Taiwan can pollinate FPA figs [9], mainland *W. pumilae* spp. 3 cannot recognize FPA female phase figs, making it difficult for FPP pollinating wasps in Fujian to enter FPA figs.

3.2 Relationship Between Fig Size and Volatile Emission During Female Phase Development The maturation of florets inside the syconium marks the onset of the female phase [26], but florets open sequentially. Volatiles attracting wasps are released only when florets mature, and as florets open progressively, volatile concentration increases continuously. During FPP female phase development, syconium diameter increases. When all florets have opened, volatile concentration reaches its maximum. If wasps enter the fig, pollinated or oviposited florets alter their volatile quality and quantity to deter subsequent wasps [3, 27], preventing redundant pollination or oviposition and conserving energy and resources.

In late female phase development, if no wasps have entered, the fig continues expanding to maintain a loose ostiole for wasp entry. As florets gradually wither, attractive volatile concentrations decline. Our large female phase figs maintained attractiveness to FPP pollinating wasps for approximately 3-4 days. Patel et al. [28] reported similar patterns in *F. hispida*, where syconium attractiveness to wasps increased then decreased as diameter expanded during female phase development.

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Note: Figure translations are in progress. See original paper for figures.