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The floral scent of *Ficus pumila* var. *pumila* and its effect on the choosing behavior of pollinating wasps of *Wiebesia pumilae*☆

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ABSTRACT

Floral scent is an important vehicle of communication between plants and their pollinators. In an obligate mutualism between fig and fig wasps, the chemical regulation plays a crucial role in their encounter. Pollinating wasps mainly rely on their olfactory sensation to the volatile organic compounds (VOCs) emitted by their host plants to localize their hosts.

In this study, we collected the VOCs from male and female figs of *Ficus pumila* var. *pumila* by in vivo dynamic headspace adsorption (VDHA) before and after pollination, and analyzed the VOCs using gas chromatography–mass spectrometry (GC–MS). We examined the *Wiebesia pumilae* pollinators' behavior in response to the VOCs at different developmental phases in Y-tube experiments.

The results: (1) The fig volatiles of *F. pumila* var. *pumila* contained a variety of compounds, in which Linalool appears to be the dominant one to pollinating wasps, suggesting that Linalool may be used for the long-range localization while the short-range localization may rely on the chemical profile containing specific compositions and concentrations. (2) Although the quality and quantity of VOCs were different between receptive male and female syconia, they shared a few compounds which account for >50% of total volatiles. In addition, the proportion of the three classes of compounds including terpenes, benzenoids, and fatty acid derivatives was similar, suggesting that a simulating interaction occurs between the VOCs of receptive male and female syconia. Furthermore, the Y-tube experiments showed no preference of pollinating wasps to the male and female syconia. Therefore a pollinator is unlikely to discriminate male and female figs via the VOC variations. (3) The VOCs of syconia were changed after pollination or oviposition, with some compounds decreased (e.g. Linalool) or even disappeared, while some increased (e.g. Longifolene) or new compounds formed. In terms of the chemical composition, terpenes and fatty acid derivatives are decreased, while benzenoids are increased, and nitrogens arisen. The results derived from Y-tube experiments showed that the VOCs of receptive male and female figs played a very significant role in attracting pollinating wasps. In contrast, the fig volatiles of inter-floral phase (5 days after pollination or oviposition) were significantly repellent to the wasps. Figs may thus express their flower developmental and pollinating or ovipositing status by changing their VOC quantity and composition in order for pollinating wasps to receive the chemical signals, choose the right hosts, and recognize the right developmental phases of their hosts.

1. Introduction

Many herbivorous insects localize their host plants primarily through their olfactory sensation of the odors emitted by plants. Plants emanate specific odors consisting of different types and different amounts of compounds. Meanwhile, herbivorous insects can discriminate different volatile mixtures and choose the right plant. Both the changes of composition and relative amount of plant odors may affect the insects' behavioral responses to their hosts [1–6]. The emission of plant odors is affected by many factors including development, temperature, light, mechanical damage, pests, etc. Plants such as apples, cucumbers, beans, corn, rice, and others are able to release volatile compounds. Studies on the mechanism and application of plant volatiles have been carried out on the multiple dimensions including plant community, population, individual, cellular and molecular levels. However, no details regarding the fig and

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fig wasp mutualism have been described. In 1970, Ramírez proposed a hypothesis that figs might release unique VOCs to attract the specific fig wasps when the syconia are ready to be pollinated. However, due to the low concentration and complexity of fig volatiles, this hypothesis has not been carefully examined. To date, studies on the fig and fig wasps mutualism have been focused on population ecology, reproductive biology, comparative biology, biorhythm and phenology, pollination biology and new fig-wasp pair discovery. However, very little data have been reported regarding the chemical attraction and recognition between figs and fig wasps.

The genus *Ficus* comprises about 750 species, among which <50 species have their receptive fig volatiles being tested [7–11]. Most fig species release complex volatile mixtures, with only very few releasing a single volatile (e.g. *Ficus semicordata*) [12]. The quantity and composition of fig volatiles are changed dynamically after pollination (or oviposition) [13, 14]. But the chemical composition remains similar between male and female fig volatiles. The information of fig semiochemical is mostly derived from indirect evidence, with no clear chemical attractant found except for *F. semicordata* [15–17]. It's certain that insects rely on volatile compounds to localize their hosts. However, due to the composition complexity of plant volatiles, it is still unclear if the pollinators is attracted by a specific compound or a few in the mixture [1,18], or by a chemical profile consisting of specific composition and concentration of volatile compounds [19–25]. With the improvement of modern analytical technology, many evidences regarding the involvement of plant volatile semiochemicals in the plant-insect interaction have been provided. This topic has become one of the most attractive studies in this field.

Ficus pumila var. *pumila* (Moraceae, *Ficus*) is a functionally dioecious and creeping evergreen shrub widely distributed in the provinces to the south of the Yangtze River in China (including Taiwan and Hong Kong), as well as in Japan (Ryukyu) and northern Vietnam [26]. *F. pumila* var. *pumila* has an obligate pollinating mutualism with *Wiebesia pumilae* (Hill) Wieb. *F. pumila* var. *pumila* relies on wasps to be pollinated, syconia on the male phase open the ostiole and female wasps flying out. Meanwhile, both receptive male and female syconia release VOCs to attract wasps to pollinate or oviposit in the figs. *F. pumila* var. *pumila* depends on wasps to produce seeds. Conversely, wasps must develop in galls of male figs to fulfill their life cycle. Both the number of seeds and wasp offspring in the syconia, and figs without wasp visitation aborted are dependent on the number of foundress entered in the receptive phase [27]. Therefore, the precise location of pollinators to the host is a premise to their interaction. The complex environmental conditions, special flower phase restrictions, together with the short lifespan of pollinators (survive for 3 days at room temperature once emerged) [28] determine the quick and correct location of pollinators to the hosts, which is crucial for both the reproduction of pollinators and their hosts. Many studies show that special fig volatiles are closely related to the chemical recognition and location of pollinating wasps [29–31]. However, it is difficult to find the attractive compounds from the complicated VOCs. The composition of VOCs changes in different developmental phases and the difference between VOCs of male and female syconia should be studied. In our research, we collected the VOCs of male and female figs of *F. pumila* var. *pumila* before and after pollination by VDHA, analyzed the VOCs by using GC–MS to identify the specificity of fig volatiles, and compared the VOC variations between male and female figs as well as figs in the different developmental phases. We used a Y-olfactometer to test the pollinator behavior in response to the fig volatiles of receptive and inter-floral phases. The results may suggest a novel mechanism underlying the obligate plant-insect mutualism.

2. Materials and methods

2.1. Study sites and organisms

F. pumila var. *pumila* syconia were collected from three locations in Fujian province, China: Fuzhou Cangshan (119°31′E,26°04′N), Beifeng Mountain (119°29′E,26°15′N) and Putian Dayang (118°55′E,25°31′N). The syconia of 3 male and female fig trees in pre-female phase was marked and covered with a mesh bag to prevent wasps from entering until receptive phase (with wasps walking on the fig surface or floral scent smelled). The morphological characters of the syconia were observed in order to collect the VOCs and the figs timely. When the syconia reached male phase before wasps emerging, the figs were harvested by cutting off the branches and the new-emerged *Wiebesia pumilae* were collected.

2.2. Collection of volatiles

Volatiles of figs were collected from January 2011 to October 2012. Both receptive volatiles of male and female figs of *F. pumila* var. *pumila* were collected. The wasps were then introduced one by one (10-min interval) into the receptive syconia (10 wasps per syconia) and the figs with wasps were covered again to prevent extra wasps from entering. The inter-floral volatiles were collected 5 days later.

The collection of VOCs was done by using the in vivo DHA technique, with two pumps (Type QC-2, BMILP, Beijing) working in a circulatory way. Both the connecting tubes and bags were PTEE material (PL, America). 200 mg Tenax TA (60 to 80 mesh, SUPELCO, America) was activated and dried before packing in the column. The air flow rate was controlled at 100–150 ml/min and all samples were stored at −18 °C before assay. The collection was usually conducted for 8 h (10 figs), between 6:00 am and 18:00 pm, at temperature of 22–28 °C in the sunny day. The experiment was repeated three times. There were two control groups: (1) the same branch without figs; and (2) an empty collection bag without branches and figs. The same volatile compounds in the control groups were removed before the analysis.

2.3. Chemical analysis of volatiles

The extracts were analyzed by gas chromatography–mass spectrometry (GC–MS; TraceDSQ; Finnigan, USA). The column temperature was programmed as the following: pre-purge for 2 min with trap temperature of 180 °C, purge for 2 min at trap temperature of 180 °C, purge drying 2 min at trap temperature of 35 °C, pre-desorption for 1 min with temperature raised to 200 °C; desorption for 2 min and maintaining at 200 °C, and then drying for 5 min at 300 °C. GC conditions were: the GC was equipped with TR-35 ms silica capillary column (30 m × 0.25 mm × 0.25 μm), with injector temperature of 240 °C and the initial column temperature was maintained at 100 °C for 5 min, and then increased by 5 °C/min to 210 °C and maintained for 13 min. Helium with purity >99.999% was used as the carrier gas at the velocity of 1 ml/min, splitless. MS conditions were: the mass spectra was recorded at 70 eV in the electron impact ionization mode, with the trap temperature of 180 °C, trap jacket temperature of 40 °C, and transfer line temperature of 250 °C. The full spectrum scan was employed with of 50–650 *m*/*z*. The compounds were identified based on the comparison of their mass spectra with NIST Library supplied by the computer. The reports of retention time were saved in the document. Chromatographic peak area normalization was used to calculate the relative amount of each volatile compound.

2.4. Behavior analysis of Wiebesi pumilae

Glass Y-olfactometer was used to test the responses of *Wiebesi pumilae* to volatiles released by the figs of different developmental stages and different sexes of *Ficus pumila*. Each arm of the Y-tube olfactometer (internal diameter 1 cm, stem 20 cm, length of each arm 15 cm, 90° angle between the arms, and a 5 cm release tube 10 cm away from the angle) was connected to a glass container (500 ml) inside which an odor source was located. Flow rates through each arm were maintained at 100–150 ml/min using a flow meter. The airflow through each arm was drawn through Teflon tubes by an air pump and passed through a charcoal filter and distilled water. The tests were carried out in a dark room with only three 40-W cool white fluorescent tubes placed above the arms of the