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Differences in volatile composition and sexual morphs in rambutan (*Nephelium lappaceum* L.) flowers and their effect in the *Apis mellifera* L. (Hymenoptera, Apidae) attraction

Carla L. Aceves-Chong[a], Leopoldo Cruz-López[b], Daniel Sánchez-Guillén[b], Julieta Grajales-Conesa[a,*]

[a] *Universidad Autónoma de Chiapas, Instituto de Biociencias, Tapachula, Chiapas, Mexico*
[b] *Colegio de la frontera sur (ECOSUR), Tapachula, Chiapas, Mexico*

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ABSTRACT

We studied the volatile composition and sexual morphs of *Nephelium lappaceum* flowers from two orchards, and investigated the choice behavior of the honey bee, *Apis mellifera* toward the floral extracts from both locations. Our results showed significant differences in chemical composition and sexual morphs; only the hermaphrodite flowers from the Herradero orchard produced limonene and α-pinene and had longer peduncle and sepal than flowers from the Metapa orchard; on the other hand, the hermaphrodite flowers from the Metapa orchard had longer gynoecium. In the behavioral experiment the extracts from the Herradero orchard seemed to give *A. mellifera* foragers better cues for orientation to food sources, perhaps due to the presence of limonene and α-pinene, which are absent in the samples from Metapa. Such differences in both orchards could affect pollinator attraction and ultimately seed set and productivity.

Introduction

Pollination is an ecological interaction mediated by plants that produce flowers as a means to attract pollinators to reproductive organs. Actually plants exploit the learning capabilities of many insect pollinators by showing specific morphologies and producing particular floral volatiles that differentiate them from flowers of other species, triggering the so-called floral constancy behavior described in many insect species (Andrews et al., 2007; Whitney and Federle, 2012). For that reason, visually perceived characteristics are of utmost importance for pollination; for example, studies on the floral structure of *Geoffroea decorticans* (Gillies ex Hook. et. Arn) Burk, 1949 showed that variation in flower size and corolla length could affect pollinators' rate of visitation (Eynard and Galetto, 1999). Other studies have characterized floral volatile compounds in economically important crops, such as alfalfa (*Medicago sativa* L., 1753) (Pecetti et al., 2002; Pecetti and Tava, 2000), coffee (*Coffea arabica* L., 1753) (Vázquez et al., 2003), rambutan (*Nephelium lappaceum* L., 1767) (Mérida et al., 2003), onion (*Allium cepa* L., 1753) (Silva et al., 2003), pumpkin (*Cucurbita moschata* Duchesne ex Poiret, 1768) (Andrews et al., 2007) and canola (*Brassica napus* L., 1753) (Wright et al., 2002). Floral volatiles have very important roles in attracting bees to reproductive organs, in which rewards like pollen, nectar or oils are present and strength floral constancy; therefore, any change in flower's scent, and also shape, could potentially affect the rate of visitation of pollinators, and hinder reproductive fitness (Byers et al., 2013; Jürgens et al., 2000; Nunes et al., 2016; Schäffler et al., 2015).

Most flowering crop plants depend on insect pollination for fruit and seed set; bees pollinate almost 90% of wild vegetation and 35% of crops (Klatt et al., 2013; Klein et al., 2007; Slaa et al., 2006). Therefore, any knowledge on the effect of floral cues on pollinators would allow us to increase fruit set and crop yield; unfortunately, few studies have aimed to explore such issue (Byers et al., 2013). For example, studies with strawberries and its main pollinator the solitary bee *Osmia bicornis* L., 1758 have shown that foraging behavior is deeply influenced by floral scent, though floral display also plays a role; more interestingly yet, differences in volatile emission rates between varieties of strawberries influence bee visitation rates under field conditions (Chagnon et al., 1993; Howell and Alarcon, 2007; Klatt et al., 2013). Studies performed with alfalfa (*Medicago sativa* L., 1753) floral volatiles and *Apis mellifera* L., 1758 demonstrated that linalool was the only compound attractive to honey bees, among five other antennally perceived compounds, at the optimized concentration (Henning et al., 1992). Thus, to gather a better understanding about the importance of

* Corresponding author.
E-mail: jugrajco79@gmail.com (J. Grajales-Conesa).

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floral volatiles on pollination of economically important crops it is necessary to explore the chemical-morphological and behavioral interactions of pollinators and their hosts.

In the tropics rambutan, which is native to Southeast Asia, is a crop with major economic impact that is rapidly expanding into Southern Asia, Australia, the Caribbean, India, Sri Lanka, Florida, Hawaii and South and Central America (Rincón-Rabanales et al., 2015; Pohlan et al., 2007,). Rambutan plants are trioeecious with about 50% male and 50% hermaphrodite plants and in commercial orchards only hermaphrodite plants are grown, where most of them are functionally female. Recent studies in India found the stingless bee *Tetragonula iridipennis* Smith, 1854 and the Indian honeybee *A. cerana* Fabricius, 1793 to be the dominant visitors of *N. lappaceum*. In the Soconusco region, Chiapas, Mexico, rambutan was introduced during 1950–1970 as an economic alternative to coffee, mango and banana, but it has been successfully introduced into many more regions. Currently, rambutan is grown mainly in three municipalities: Cacahoatan, Metapa de Domínguez and Huehuetan (Rincón-Rabanales et al., 2015; Vanderlinden et al., 2004), in which *Scaptotrigona mexicana* Guérin-Meneville, 1845 a native bee is considered as the main pollinator, otherwise beekeeping of *A. mellifera* also occurs. Nonetheless, there is a lack of information on floral volatiles, morphological traits and their interaction with honey bees. Therefore, our study aimed to explore and understand such interaction by: (a) determining the volatile composition of rambutan flowers from two different orchards, (b) measuring morphological traits in flowers from both locations and (c) evaluating honeybee attraction to rambutan floral volatiles.

Materials and methods

Study site and volatile collection

This study was carried out in two orchards located 1.5 km from each other in Metapa de Dominguez in the Soconusco region: Metapa (14°50′ N, 92°11′ W, 100 masl) and Herradero orchards (14°49′ N, 92°11′ W, 100 masl). In both orchards the origin of plants can be traced back to one single donor plant; nonetheless despite the clonal origin, evident differences in morphological traits are present, such as flower shape and size. From February to March 2015 in each orchard we collected one panicle with hermaphrodite flowers per tree ($N = 6$); we wanted to investigate any potential difference between male and hermaphrodite flowers, thus in Herradero orchard we collected two panicles with male flowers per tree ($N = 2$), unfortunately we did not find male flowers in Metapa orchard. All sampled panicles were newly opened and randomly chosen by a blind (collector did not know the final fate of the flowers, either for morphometric or volatile analysis) procedure from the middle part of the canopy. All panicles were carefully removed, separately transferred in labeled polyethylene bags and transported in a cooler at 8 °C to the laboratory of Chemical Ecology in *El Colegio de la Frontera Sur* (ECOSUR). Twenty to 30 min after sampling, floral volatiles were collected with a solid phase microextraction (SPME) technique; the SPME syringe consisted in a polydimethylsiloxane-divinylbenzene fiber of 65 μm thick, the fiber was inserted for 30 min in a glass vial (7 mL) where flowers could not touch it. Next, the SPME fiber were immediately analyzed in a gas chromatograph coupled with a mass spectrometer Varian Saturn 2200 (Sandoval et al., 2007) with a DB5-MS nonpolar capillary column containing 95% of dimethylpolysiloxane and 5% of diphenyl siloxane, with a length of 30 m long by 0.25 mm inside diameter and 25 μm thick film, using helium as a carrier gas. Samples were analyzed by an initial program of 50 °C for 2 min with an increase of 15 °C/min to a temperature of 280 °C for 10 min. The injector temperature was 250 °C.

Volatiles were identified by comparing Kovats index, mass spectra and retention times of synthetic standards in conjunction with the National Institute of Standards and Technology library version 2.5 (NIST). All chemicals were acquired at Sigma-Aldrich Chemical, Parque Industrial Toluca 2000, street 6 north N° 107, P.C. 50200 Toluca, Mexico.

Morphological analysis

For this analysis we randomly chose one newly opened panicle per each of three plants in each orchard and were transported to the laboratory as described lines above; next, we randomly chose 50 flowers per site and removed them from the panicle. Immediately after separation from the panicle, sepal, gynoecium, peduncle length, calyx diameter and calyx width were measured using a calibrated scale and a digital camera (Carl Zeiss AXIO CAM MRC, E206588, Zen software 2011, LLC, United States) mounted on a stereomicroscope (Carl Zeiss model STEMI 2000c, LLC, United States).

Behavioral test

Foragers from two healthy queenright *A. mellifera* colonies were evaluated from November 2015 to January 2016 at 9–14 h in the campus of *El Colegio de la Frontera Sur*. The flower extracts used in this experiment were obtained by taking 800 hermaphrodite flowers of each orchard and separately washed for 30 min in 250 mL of hexane and taking it to a final volume of 100 μL using nitrogen; these extracts showed a chemical profile similar to the extracts obtained by SPME. The experiment consisted of two sequential phases. *Training phase*: Five to ten foragers were trained to collect 2 M sucrose solution from a feeder located at 10 m from the colony under study (Sánchez et al., 2008). The feeder consisted of a 1.5 cm diameter cotton ball soaked in the sucrose solution and placed on a plastic Petri dish, with a filter paper inside, which was used to release the floral volatiles. Trained foragers were distinctively paint-marked on their thorax with water-based paint to distinguish them from newcomers. Since there were feral colonies in the vicinity of our setup, we paint-marked the thorax of the foragers of our colony with a device that allowed us to distinguish them from those foragers coming from other colonies and from the trained ones (Mikery-Pacheco et al., 2013). *Test phase*: Once at the experimental distance, the training feeder was removed and two clean feeders separated from each other by 70 cm were showed to the foragers. One feeder contained a piece of filter paper with 100 μL of the extract of one of the orchards and the other one with a piece of filter paper with 100 μL of hexane. For 20 min any unmarked or device-marked forager that started collecting the sucrose solution was trapped using an entomological aspirator until the end of the experiments to avoid pseudoreplication, and its choice was registered; feeders exchanged places every 5 min to avoid site-learning. Besides, we registered the choices of the trained foragers, but they were not trapped to keep bringing newcomers to our setup. Overall, we carried out three replicates per colony per each extract orchard; each replicate was performed on a daily basis.

Data analysis

We built classification trees by analyzing the data from volatile composition, flower sex and study sites using the randomForest method from the randomForest package (Liaw and Wiener, 2002) implemented in R software; this analysis finds associations between individuals through regression using a permutation approach, which allows the analysis of small sample sets.

Morphometric data were analyzed by a Kruskal–Wallis test to compare sepal, petal length and gynoecium height between the

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