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Volatile Component Analysis of *Michelia alba* Leaves and Their Effect on Fumigation Activity and Worker Behavior of *Solenopsis invicta*

DQ QIN[1], RL HUANG[1], ZH LI[1], SY WANG[1], DM CHENG[1,3], ZX ZHANG[1,2]

1 - Key Lab. of Nat. Pesticide and Chemical Biology, Ministry of Education, South China Agricultural University, Guangzhou, China,
2 - State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangzhou, China
3 - Department of Plant Protection, Zhongkai University of Agriculture and Engineering, Guangzhou, China

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Corresponding author
Zhixiang Zhang
Key Lab. of Nat. Pesticide and Chemical Biology, Ministry of Education
South China Agricultural University
Guangzhou, China, 510642.
E-Mail: zdsys@scau.edu.cn

Abstract

Volatile compounds from mashed (fresh, fallen, and dried) leaves of *Michelia alba* were collected via solid-phase microextraction and were then identified via gas chromatography-mass spectrometry. The results showed that linalool was the dominant component in different leaves, together with caryophyllene, β-elemene, and selinene, the contents of which vary across the samples. The fumigation bioassay results showed that the volatiles from *M. alba* leaves exhibited insecticidal activity against red imported fire ant workers, and the mortality of workers could reach up to 100% after the fallen leaves were treated for 16 h. Mashed fresh leaves could effectively reduce the aggregation and drinking ability of workers. The volatile substances released from the mashed leaves might kill the ants, or affect their behavior and weaken the activity by interfering transmit information between ants. A comprehensive consideration of the economic and ecological value of *M. alba* shows that fallen leaves might be a good resource to control red imported fire ant.

Introduction

Red imported fire ant (RIFA), or *Solenopsis invicta*, named by Buren in 1972, was first introduced to southern Alabama in the 1930s (Buren et al., 1974) and then rapidly spread to Australia (Moloney & Vanderwoude, 2002; McCubbin & Weiner, 2002), New Zealand (Ministry of Agriculture and Forestry of New Zealand, 2006), Malaysia (Na & Lee, 2001), Taiwan (Chen et al., 2006, Zhang et al., 2007), and mainland China (Zhang et al., 2007; Zeng et al., 2005; Ligon et al., 2012; Wang et al., 2015; Gutrich et al., 2007).

Red imported fire ant sting domestic animals and people. They cause significant economic losses in agriculture and other industries, and they also severely affect biodiversity (Vinson, 1997; Williams et al., 2001; Wojcik et al., 2001; Gutrich et al., 2007). Prior to the 1980s, attempts to eradicate fire ants by using early insecticides and formulations have failed (Drees et al., 2013). Environmental pollution from traditional insecticides, such as pyrethroid or organophosphate, has led to the development of natural, safe, and nonpolluting insecticides to control these ants (Appel et al., 2004; Vogt et al., 2002; Cheng et al., 2004; Cheng et al., 2008).

Botanicals might be a good candidate for developing these insecticides. Numerous studies have indicated that essential oils can be used to control red imported fire ant. Clove powder applied at 3 mg/cm^2 and 12 mg/cm^2 resulted in 100% ant mortality within 6 h and repelled 99% of ants within 3h (Kafle & Shih, 2013). Essential oils of mint (Appel et al., 2004), *Artemisia annua* (Wang et al., 2014), camphor, eucalyptus, mugwort, and wintergreen all showed strong insecticidal, fumigation, or repellent activity against red imported fire ant (Chen, 2009; Tang et al., 2013).

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Michelia alba (Magnoliaceae), named by DC in 1818, is an evergreen tree that is mainly distributed in the west and southeast of China (Zhu et al., 1982; Chen et al., 2008a). Previous studies have reported the chemical compositions of essential oils from flowers, leaves, and stems of *M. alba* (Huang et al., 2009; Qin et al., 1999; Chen et al., 2008b; Gu et al., 2014). These reports mostly focused on the seasoning properties and application of the said tree in the perfume industry. In fact, spices are also valued for their bioactive efficacy as bacteriostatics, fungicides, antioxidants, and nutrients (Hirasa & Takemasa, 1984). Jiang (2002) reported the insecticidal activity of *M. alba* essential oil to *Musca domestica* L; however, the effect of which against other insects has not been studied.

This study investigates the effects of volatile components from *M. alba* leaves on the fumigation activity and red imported fire ant worker behavior. The study aims to provide a new method for controlling ants.

Materials and Methods

Insects: *S. invicta* colonies were collected from a suburb in Guangzhou. The ants were reared with mealworms (*Tenebrio molitor*). The water source was a test tube (Φ25 mm × 200 mm) that was partially filled with water and plugged with cotton. The ants were maintained in the laboratory at 25±2 ºC and 75% relative humidity.

Plant Materials: Five *M. alba* trees were planted in the Insecticidal Botanical Garden at South China Agricultural University. The height and stem diameter were approximately 14.52 ± 4.39 m and 0.41 ± 0.1 m, respectively. Three kinds of *M. alba* leaves were used in the test: fresh, fallen, and dried. The fresh leaves were immediately sent to the laboratory once collected. Fallen leaves without dirt on the surface were selected. The fresh leaves were dried at 40 °C in an oven to a constant weight to obtain the dried leaves.

Fumigation Toxicity Bioassay: The fresh, fallen, and dried leaves of *M. alba* were mashed using a high-speed organization stamp mill for 3 min and then separately stored in a sealing bag at 4 °C for the application. Coating Fluon emulsion outside the vertical wall of a 100 mL beaker, then placed it in a 1000 mL beaker coated with Fluon emulsion inside a vertical wall. A total of 30 large workers (body length of about 4.52 ± 0.01 mm) or 30 small workers (body length of about 3.03 ± 0.02 mm) in the same colony were confined in the larger beaker and outside the small one. Approximately 30 g of mashed leaves were placed in the uncovered small beaker, and the 1000 mL beaker was sealed with plastic film. The deaths of worker ants were recorded after 6, 16, 40, and 64 h. A test tube (Φ10.5 mm × 75 mm), which was partially filled with water and plugged with cotton, was used as a water source. The workers were maintained at 25 ± 2 °C and 65% ± 5% relative humidity. All treatments were replicated thrice. The same operation was done in the control group without the mashed leaves. The following equation was used to calculate the mortality of ants:

$$M(\%) = \frac{N_a}{N_t} \times 100$$

where M is the mortality, N_d is the number of dead ants, and N_t is the total number of ants.

Behavior Observation on Aggregation of Workers: The test method was the same as described in the preceding section, more than two workers gathered together, and the distance between each worker was less than 0.5 cm, which is defined as aggregation, the aggregating level was observed after 6, 16, 40, and 64 h. (Depickère et al., 2004a; Depickère et al., 2004 b; Devigne et al., 2011). The following equation was used to calculate the aggregating level:

$$G(\%) = \frac{N_g}{N_t} \times 100$$

where G is the percentage of ants gathering together, namely the aggregating level, N_g is the number of ants aggregating, and N_t is the total number of ants.

Behavior Observation on Drinking Ability of Workers: The test method was the same as described previously. A water-soaked cotton ball (1 g) and 30 worker ants (the distance between the cotton ball and the ants was 25 cm) were placed on the midcourt line of a porcelain tray (20 cm × 30 cm × 5 cm) whose vertical wall was coated with a Fluon emulsion (the same as below). Worker ants were regarded as having water recognition ability if they continuously touched the cotton with its mouth for more than 10 s was regarded as drinking water (Zhang et al., 2013a). At each time point, the observation time was controlled within 30 min. The following equation was used to calculate the drinking water rate:

$$W(\%) = \frac{N_w}{N_t} \times 100$$

where W is the percentage of ants that can drink water, N_w is the number of ants drank water, and N_t is the total number of ants.

Extraction of Volatiles via Solid-Phase Micro extraction (SPME). Before the plant volatiles were extracted, 30 g of mashed fresh, fallen, and dried leaves were individually placed into a 250 mL conical flask sealed with tinfoil.

The manual SPME device (Supelco, USA) with a fiber precoated with a 100 µm thick layer of polydimethylsiloxane was used to extract the volatiles. The fiber was cleaned before use at the conditioning temperature (250 °C) for 30 min. The fiber was exposed to the headspace of the material for 60 min at room temperature after being pushed into the conical flask containing the mashed leaves. The fiber was then inserted into the injection port of the GC-MS where it was heated at 250 °C for 3 min, and the adsorbed volatile compounds were rapidly thermally desorbed into a capillary GC column for analysis.

Chemical Analysis via Gas Chromatography-Mass Spectrometry (GC-MS).The sample was detected directly using an Agilent 6890 gas chromatograph coupled with an Agilent mass spectrometer detector. A DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used to separate the compounds.

The injection temperature was 230 °C. The oven temperature was initially set at 50 °C for 1 min to 200 °C (3 °C/min) for 2 min, then to 230 °C (10 °C/min) for 2 min. The detector was operated at 280 °C. Helium was used as a carrier gas at a flow rate of 1 mL/min. The compounds were identified via mass spectroscopy and the spectra were then compared with those from computer mass libraries.

Statistical analysis

The mortality, aggregating level, and drinking ability were analyzed with *Duncan'S Multiple-Range test* for ANOVA. The data were expressed as means ± SD and evaluated using SPSS17.0. Differences at $P<0.05$ were considered significant. The figures were drawn using Microsoft Office Excel 2007.

Results

Results of ANOVA: Table 1 shows that the aggregation and drinking ability of workers varied in terms of plant material (M), exposure time (T), and worker size (S) ($P<0.05$), and the mortality of workers markedly changed at different plant materials and exposure times ($P<0.05$), except for worker size ($P=0.4970$). However, no difference was observed reading the effect of interaction T×S on mortality ($P=0.9759$), aggregation ($P=0.7988$), and drinking ability ($P=0.2842$) of the workers, as well as that of interaction M×T×S, respectively ($P=0.9173$, 0.0786, 0.2226).

Table 1. ANOVA for the main factors of the fumigation test affecting the behaviors of red imported fire ant.

Factors	DF	Mortality		Aggregation		Drinking ability	
		F values	P values	F values	P values	F values	P values
M	3	155.7554	0.0001	265.5776	0.0001	334.5175	0.0001
T	3	49.5313	0.0001	51.6354	0.0001	10.0814	0.0001
S	1	0.4666	0.4970	31.5410	0.0001	11.9353	0.0010
M×T	9	18.5101	0.0001	10.7687	0.0001	3.1519	0.0033
M×S	3	7.7240	0.0002	6.6313	0.0006	3.1400	0.0313
T×S	3	0.0697	0.9759	0.3367	0.7988	1.2936	0.2842
M×T×S	9	0.4245	0.9173	1.8361	0.0786	1.3654	0.2226

M: Plant material, T: Exposure time, S: Worker size (*P=0.05*)
(*Duncan'S Multiple-Range test,* SPSS17.0)

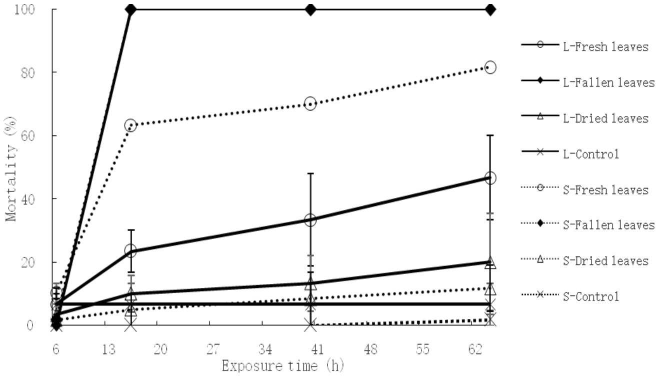

Fig 1. Mortalities of workers (L: large workers, S: Small workers) after treated with different mashed leaves of *M. alba* (*Duncan'S Multiple-Range test,* SPSS17.0).

Fumigation Toxicity Bioassay: From Fig 1, the exposure of both large and small workers to volatiles from mashed leaves of *M. alba* showed increasing mortality over time. The mortality of workers treated with fallen leaves could reach up to 100% after 16 h, which was significantly higher than those treated with dried and fresh leaves ($F = 223.05$, $df = 3$, $p< 0.05$). The mortality increased from 6.67% to 46.67% and 10.00% to 81.67% for large and small workers exposed to fresh leaves from 6 h to 64 h. The mortality rates were significantly higher than those of workers exposed to dried leaves, which were from 3.33% to 20.00% and from 1.67% to 11.67% for large and small workers($F = 223.05$, $df = 23$, $p< 0.05$). However, the mortality of the ants treated with dried leaves was not significantly different from that of the control group ($F = 223.05$, $df = 23$, $p> 0.05$).

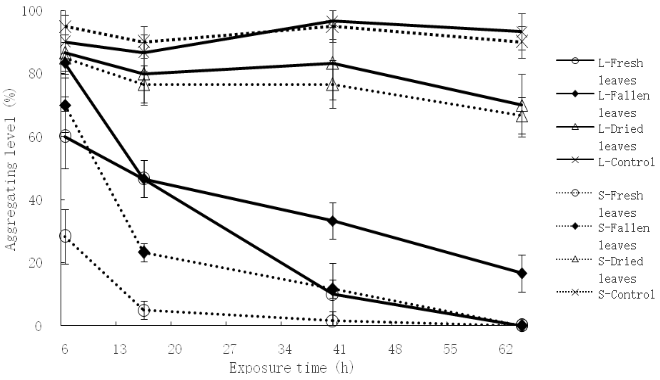

Fig 2. Effects on Aggregation Level of workers (L: large workers, S: Small workers) after treated with different mashed leaves of *M. alba* (*Duncan'S Multiple-Range test,* SPSS17.0).

Aggregating Level and Drinking Ability: Compared to the mortality, the aggregating level and drinking ability of red imported fire ant decreased over time after exposure to mashed leaves (Figures 2 and 3). Almost or more than 90.00% of workers in the control group always preferred to drink water and to aggregate.

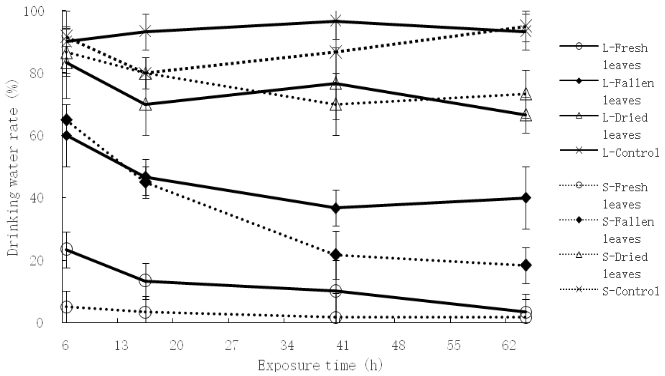

Fig 3. Effects on drinking ability of workers (L: large workers, S: Small workers) after treated with different mashed leaves of *M. alba* (*Duncan'S Multiple-Range test,* SPSS17.0).

Fresh leaves showed the highest inhibitory activity on drinking and aggregation of red imported fire ant. The aggregating level respectively decreased from 60.00% and 28.33% at 6 h to 0.00% and 0.00% at 64 h for major and minor workers treated with the fresh mashed leaves. The drinking ability also decreased from 23.33% and 5.00% to 3.33% and 1.67%. The aggregation levels of workers exposed to dried leaves decreased over time with no significant difference (*F* = 26.02, *df* = 23, *p*> 0.05), while always higher than those treated with fallen leaves. Similarly, after treated with dried leaves, the percentage of ants that drank water decreased from 83.33% and 86.67% at 6 h to 66.67% and 73.33% at 64 h for large and small ants, respectively. After 40 h exposure to fallen leaves, the drinking ability almost remained constant with time, which was observed among only 18.33% of minor workers after 64 h of treatment.

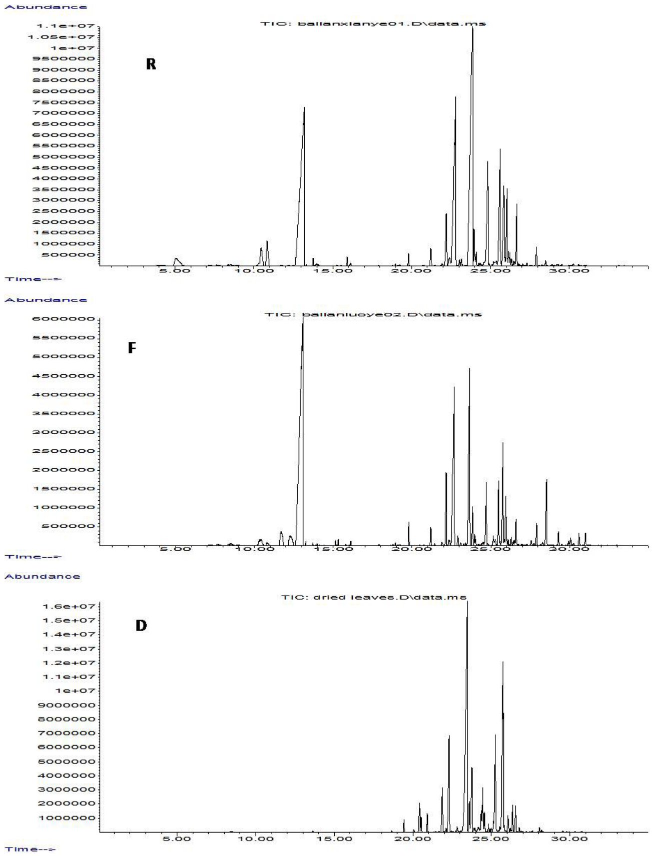

Fig 4. GC-MS total ion chromatograms of mashed fresh (R), fallen (F), and dried (D) leaves of *M. alba*.

Chemical Compositions of Mashed Leaves of ***M. alba****:* Fig 4 shows the GC–MS total ion chromatograms of the SPME extracts of fresh, fallen, and dried leaves of *M. alba*. The content of linalool was highest in volatiles from fresh, fallen, and dried leaves, which was 26.10%, 40.52%, and 36.52%, respectively. Other compounds like isocaryophyllene, aromadendrene, α-caryophyllene and (-)-γ-cadinene etc, which account for more than 40% of all the volatiles detected from fresh leaves. Volatiles from fallen leaves mainly contained linalool (40.52%), β-elemene (11.94%), β-caryophyllene (10.78%) and so on. Similarly, β-elemene, β-caryophyllene, α-selinene, α-cubebene, and 36.52% of linalool, which accounted for 70% of the volatiles from dried leaves.

Discussion

The results of the GC-MS analysis showed that in fresh, fallen, or dried leaves of *M. alba*, linalool was always the dominant component, together with common aromatic substances such as caryophyllene, β-elemene, selinene, and other alkene and terpenoids. The results were in accordance with previous findings (Qin et al., 1999; Li et al., 2000; Liu et al., 2001).

These compounds, especially those in fallen leaves, might kill the ants. Zhang et al. (2014) reported that linalool could absolutely kill minor workers at 5 mg/centrifuge tube within 24 h and 3 mg/centrifuge tube (48 h) after treatment. Few studies have reported on the insecticidal activity of caryophyllene. Caryophyllene oxide exhibits insecticidal and antifeed ant activity (Bettarini et al., 1993; Liu et al., 2012) and could function as a nerve poison for pests via sodium channel modulators (Balar & Nakum, 2010). In addition, β-elemene is a kind of structural isomer of elemenes that are used as pheromones by particular insects because of the floral aromas of particular plants.

Aggregation behavior is crucial in many aspects of animal life (reproduction, defense, and alimentation) (Depickère et al., 2008) and also regulates collective activities in nest digging (Rasse & Deneubourg, 2001). In addition, aggregation behavior is a strategy for workers to respond to adverse environments (Wang K., et al., 2014). Faced with stress factors such as floods or sudden changes in temperature, workers prefer to gather together for survival (Mlot et al., 2011; Hassall et al., 2010; Wang L., et al., 2014). Pheromones between individuals (Brown et al., 2006; Durieux et al., 2012) could affect the aggregation of insects; thus, the release of aromatic substances might interfere with the signals or pheromones between individual workers. The present study proved that the volatiles in *M. alba* leaves could effectively weaken the aggregation of red imported fire ant. Thus, the capacity response of the workers to their external environment is reduced. Under the condition of low aggregation rate, the ants' daily behavior cannot be carried out normally. For example, the food and water transport rate will be reduced, which will seriously affect the survival of ants and lead to the reduction of ants population. Which is conducive to control of ants.

In this study, volatiles from *M. alba* leaves showed an inhibitory effect on the drinking ability of the workers, and the order of effectiveness from high to low was fresh leaves > fallen leaves > dried leaves > control treatment. After treatment with fresh leaves for 6 h, almost all of the small ants lost their drinking ability, but the drinking rate in the control group always remained at approximately 90%. During foraging, the workers would receive odor signals or distinguish directions by constantly swinging their antennas (Xu et al., 2007). However, at extreme conditions, workers would have no food in their nest and no ability to move and recognize their surroundings. Thus, their survival rate is almost zero (Zhang et al., 2013a, b). The aromaticity of the volatiles might disturb the diffusion of odor signals between the individuals and food as well as weaken the water recognition or drinking rate of workers, which might indirectly threaten the survival of the species.

According to test results, more workers died after exposure to fallen leaves in a closed system. However, fresh leaves significantly affected the aggregation and drinking ability of the ants. Fallen leaves might be a better resource to control the ants and are also more cost-effective. Thus, a good method to control red imported fire ant is directly incorporating mashed fallen leaves *M. alba* into the soil where ants build their nest or stay for prolonged periods. Thus, the volatile substances could be constantly released from the leaves to affect the ants. In this way, people could avoid extracting essential oil through the complicated process, and also turn "waste" into wealth by collecting and reuse the leaves fallen from the tree, which reflect the economic and ecological value better. In the future , more and more plant materials, such as *M. alba* leaves, instead of chemical agents will be used to control red imported fire ant. And it's environmentally to develop fumigants to control red imported fire ant, in order to achieve green control.

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