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Insecticidal Activity of the Soil in the Rhizosphere of *Viburnum odoratissimum* against *Solenopsis invicta* (Hymenoptera: Formicidae)

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Abstract

Methyl salicylate produced by *Viburnum odoratissimum* is known to exert lethal or sublethal effects on insects. Replacing conventional pesticides with insecticidal plants is necessary for environmental protection. We evaluated the behavioral and toxicological responses of the red imported fire ant (RIFA *Solenopsis invicta*, Buren) (Hymenoptera: Formicidae) at different soil depths in the rhizosphere of *V. odoratissimum*. Results of insecticidal activity bioassays indicated that the mortality for minor and major ants in soil at depths of 0-10 cm at days 11 and 12 both ranged from 68.75% to 100.00%, with repellency rates of 83.54%-100.00% and 85.31%-100.00%, respectively. In behavioral ability tests, 85.45%-100.00% of minor ants and 86.74%-94.85% of major ants lost their ability to grasp after nine days, with crawling rates at only 0.00%-29.25% and 0.00%-55.77%, respectively. Therefore, we conclude from the result that the soil under *V. odoratissimum* at depths of 0-10 cm exhibited excellent insecticidal effect in controlling RIFA.

Introduction

The red imported fire ant (RIFA), *Solenopsis invicta* Buren, is an important invasive species native to South America. RIFAs are more aggressive than other ant species and have been identified to disrupt the balance of natural ecosystems (Porter, 1990). RIFAs also cause considerable harm in infested urban and agricultural areas. In the city, RIFA colonies are commonly found in gardens, lawns, parks, schoolyards, and golf courses. These insects are a threat to people's health because of their sting, which may cause inflammation, vesicles, sterile pustules, allergic phenomena, and even anaphylactic shock (Haddad et al., 2015). The venom of the RIFA is composed of various alkaloids, which can negatively affect the cardiovascular and central nervous system of vertebrates (Howell et al., 2005). The RIFA has spread over a number of countries and regions; it was detected in Taiwan in 2003 (Zhang et al., 2007) and mainland China in 2004 (Zhang et al., 2013). At present, the species can be found in South China and occupies an area of at least 71 km^2 (Lai et al., 2015).

RIFAs are usually controlled by applying traditional insecticides, such as richlorphon or clofenotane; both are harmful to humans and the environment. Thus, the search for natural, safe and non-polluting methods began to attract considerable attention, and many efforts have focused on exploring biological control (Appel et al., 2004; Vogt et al., 2002).

Methyl salicylate is an organic ester that is naturally produced by *Viburnum odoratissimum* (Wei et al., 2013). It is a volatile oil with a characteristic wintergreen odour and taste, and is used as a flavouring agent and as a topical counterirritant for muscle pain (Liu et al., 2013). The compound was first

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registered as a pesticide in 1972 for use as an animal repellent in impregnated twist tabs hung on plants to repel dogs and cats from flower gardens. In 1996, the Hygienic standards for uses of food additives approved methyl salicylate as an insect repellent to be used as a constituent of food and feed packaging material to repel insects in stored commodities.

In this study, methyl salicylate from *V. odoratissimum* leaves and soil were analyzed by using high-performance liquid chromatography (HPLC), and soil toxicity was determined to evaluate the effectiveness of the soil in the rhizosphere of *V. odoratissimum* in controlling the RIFAs.

Materials and methods

Plant

V. odoratissimum used in the study was planted in the Insecticidal Botanical Garden at South China Agricultural University. The height of the trees was approximately 2 m, and the basal stem diameter was approximately 3 cm. Grasses and other small plants within 5 m of the basal stem were cleared artificially within 30 days. All plants were cleared artificially within 30 days in a CK field (20 m^2), which is located approximately 30 m from the field of *V. odoratissimum*.

Chemicals

Methyl salicylate was purchased from Jingchun Chemical Reagent Co., Ltd., Shanghai, China. HPLC-grade acetonitrile and methanol, as well as analytically pure phosphoric acid, were purchased from Guangzhou Chemical Reagent Factory.

Insects

Adult workers of *S. invicta* colonies were obtained directly from nests in South China Agricultural University. Workers were reared in plastic cases coated with Teflon emulsion on the top, in which a test tube (25 mm × 200 mm) filled with water and plugged with cotton was used to provide water. A Petri dish (8.5 cm × 1.5 cm) that contained individuals of *Tenebrio molitor* was placed in containers for the food source. Individuals of *T. molitor* was purchased from the insect/fish market in Guangzhou, fed with wheat bran, and kept in a dry indoor environment at 25 ± 2 °C until use. Small worker ants were approximately 3 mm in length, whereas large worker ants applied in the experiment were approximately 6 mm in length (Cheng et al., 2008; Zhou et al., 2013; Zhang et al., 2013; Tang et al., 2013).

Collection of soil and leaf samples

The soil samples were collected at five points/tree, 20 cm from the basal stem, at depths of 0-5, 5-10, 10-15 and 15-20 cm. Soil samples from the same depth were combined and ground, and then sifted with a 40 mesh sieve. Each sample was replicated thrice, and a tree was used in each replication. Soil weight/replicate was set at >500 g. The soil samples were collected randomly at five points at the same depth. For the leaf samples, young and old green leaves, as well as yellow leaves, were picked from each tree. Leaf weight/replicate was obtained at >200 g. Half of the leaves and soil samples were placed in a dryer to dry for 12 h at 100 °C. The collected leaf and soil samples were stored in the refrigerator at -4 °C for analysis.

HPLC analysis

Graded soil (20 g) or mashed leaves (10 g) were mixed with 100 mL methanol in a beaker (500 mL) and then subjected to ultrasonic extraction for 30 min. This procedure was replicated thrice. The extract solutions were combined, and the soil or leaves were removed through filtration. Methyl salicylate contents in the extract solution were determined by HPLC.

HPLC was performed with an Agilent TC-C18 column (250 mm × 4.6 mm, 5 μm) by using a mobile phase of acetonitrile - 0.01% phosphoric acid solution (40:60), with a flow rate of 1.0 mL/min, column temperature of 30 °C, detection wavelength of 302 nm, and injection volume of 10 μL.

Toxicity bioassay

Toxicity bioassay was determined by using the method proposed by Zhang et al. (2013) with some modifications. Graded soil (10 g) was placed in a 200 mL beaker, with the vertical wall inside each glass coated with Fluon emulsion, and allowed to dry for 24 h to prevent the ants from escaping. Twenty individuals of each minor and major workers were laid at the bottom of a Bunsen beaker. Mortalities, walking rate, and grasping rate were observed after 5 h, 1 day, and daily thereafter until the 12th day after treatment. The treatments were replicated thrice. The ants were kept in the laboratory at 25 ± 2 °C and relative humidity of 80%.

The mortality statistics were determined as follows: Mortality was assessed after 5 h, 1 day, and daily thereafter until the 11th day after testing with different soils. All treatments were replicated three times. The following formula was adopted:

Mortality = (number of dead ants / number of total ants) × 100%

Corrected mortality = (the mortality of treatment group − the mortality of control group) / (1 - the mortality of control group) × 100%

The determination of repellency rate statistics is given as follows: The rationale for the bioassay is that the ants will always exhibit digging behavior whenever an adequate digging substrate, such as soil, is available. However, the ants do not dig or dig less when the substrate contains a repellent. Therefore, repellency was defined as suppression of ant digging behavior. Soil samples (20 g) were placed at the bottom of a 500 mL beaker with a vertical wall inside each glass coated with Fluon emulsion. Then, 20 g of control soil covered the upper part of the beaker, and 10 individuals of each minor and major workers were selected and placed in a beaker. In the experiment, four treatment groups and one control group were adopted. Each treatment was replicated thrice. We found that the ants preferred to inhabit the soil without repellent over the soil with repellent. The following equation was used to determine the repellency:

Repellency rate = (number of ants in the control group − number of ants in the treatment group) / number of ants in the control group × 100.

Behavioral observation on the ants' walking ability was conducted as follows: Observations on the walking ability of the workers were recorded after 5 h, 1 day, and daily thereafter until the 12th day after testing. Afterward, the workers were placed on a piece of A4 paper. The workers were observed to possess walking ability when they walked continuously for 10 cm without falling. Walking rate was obtained using the following formula:

Walking rate = (number of workers possessing walking ability/number of workers per replicate) × 100.

Behavioral observation on the grasping ability of ants was conducted as follows: The grasping ability of the workers was recorded after 5 h, 1 day, and daily thereafter until the 12th day after testing. The workers were placed on an A4 paper, which was gently rotated 180° after 10 s. The workers that did not fall from the A4 paper were considered to possess grasping ability. We used the following formula:

Grasping rate = (number of workers possessing grasping ability / number of workers per replicate) × 100.

Statistical analysis

The mortality and repellency rates were analyzed and transformed to arcsine square root values for ANOVA, followed by Duncan's multiple range test. All data were expressed as means ± standard error. Statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago). Differences between the values were considered significant when $P < 0.05$.

Results

Methyl salicylate content in the soil and leaf

A methyl salicylate standard was quantified with an external standard curve between 0.01 and 5 mg L^{-1} ($y = 36289x$, $R^2 = 0.9991$). Methyl salicylate content in fresh soil ranged from 0.13 to 4.88 mg/kg and 0.16 to 5.97 mg/kg in dry soil; a significant difference in contents was observed among depths of 0-5, 5-10, 10-15, and 15-20 cm ($F = 28.36$, $P < 0.01$). Methyl salicylate content (4.88 mg/kg) in the soil at depths of 0-5 cm was significantly higher than at other depths ($F = 14.56$, $P < 0.01$). The methyl salicylate contents in the fresh and dry soil showed little difference in every depth (Fig 1). The methyl salicylate contents in the fresh leaves were 133.3, 150.8 and 106.3 mg/kg, whereas those in the dry leaves were 486.6, 401.2, and 193.9 mg/kg. The methyl salicylate contents in the fresh yellow leaves (106.3 mg/kg) and in the dry yellow leaves (193.9 mg/kg) were significantly lower than

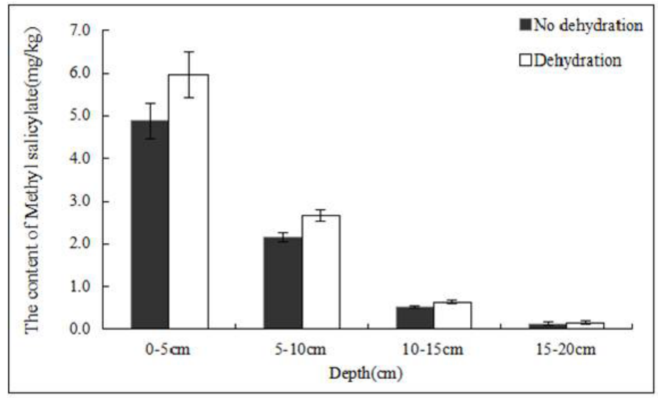

Fig 1. The content of methyl salicylate in soil in different depth.

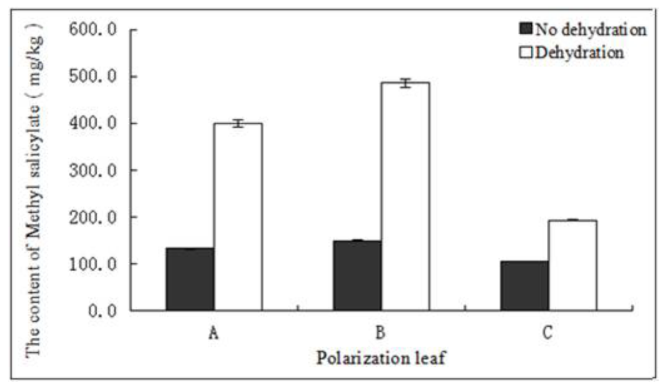

Fig 2. The content of methyl salicylate in *V. odoratissimum* leaves (A=younger green leaves B=older green leaves C=yellow leaves).

those of others ($F = 8.41$, $P < 0.05$) (Fig 2).

Insecticidal toxicity

The major ants were erradicated completely with the soil at 0-5 cm, whereas 30.75% were killed with the soil at 15-20 cm depth after 12 days. This finding indicates that the soil exerted toxicity against RIFA (Fig 4). For the minor ants (Fig 3), the soil at 0-5 cm depth required 11 days to achieve complete minor ant death. Soil samples at 15-20 cm depth

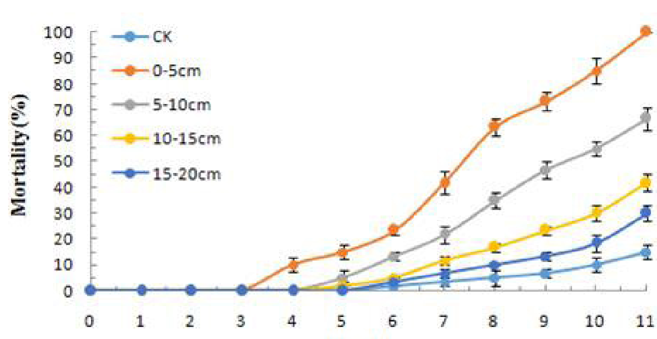

Fig 3. Mortality of minor red imported fire ants after open exposure to soil in different depth under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

caused 34.75% minor ant death. We also found that in all the treatments, no ants died on the first three days, but the mortality of both minor and major workers increased with exposure time ranging from 3-11 days. Mortality of the major and minor ants almost overlapped as the exposure interval was prolonged. No significant difference ($F = 1.58$, $P > 0.05$) in the mortalities between minor and major workers was found.

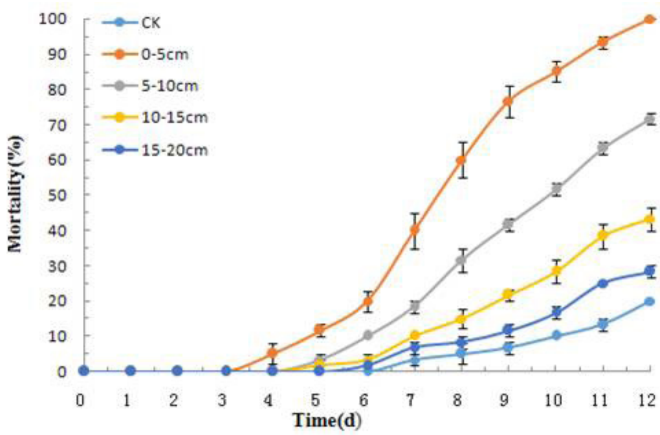

Fig 4. Mortality of major red imported tire ants after open exposure to soil in different depth under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

Repellent activity bioassay

When the major ants were placed in a beaker with soil samples at 0-5, 5-10, 10-15, and 15-20 cm depths, the repellency against major ants reached 100.00% (0-5 cm), 86.32% (5-10 cm), 55.56% (10-15 cm), and 35.31% (15-20 cm) after 10 days (Fig 6). At the 9th day of treatment, the repellency against the minor ants was 100%, 82.72%, 41.29%, and 29.54% (Fig 5). Both soils caused significant repellency against the major and minor ants.

Walking ability bioassay

We calculated walking abilities in accordance with Figs 7 and 8. Among the major ants, 100.00% (0-5 cm), 78.75% (5-10 cm), 48.75% (10-15 cm), and 30.00% (15-20 cm) lost

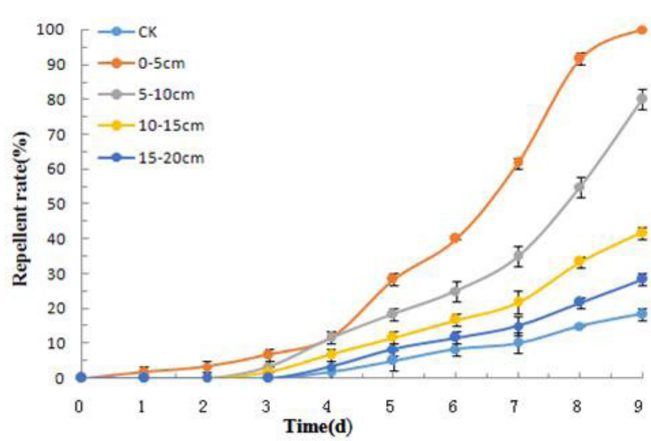

Fig 5. Repellent rate of minor ants after open exposure to soil under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

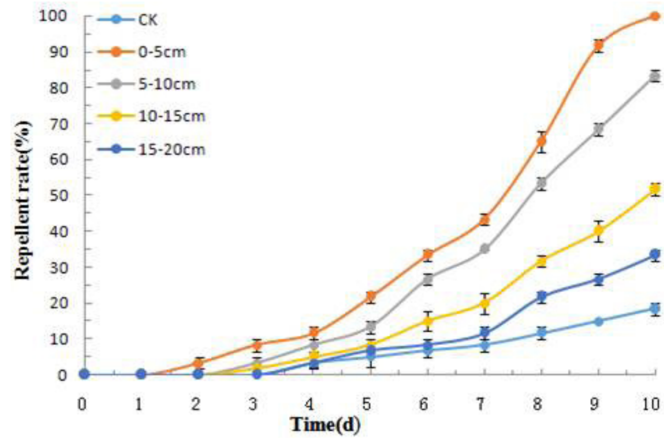

Fig 6. Repellent rate of major ants after open exposure to soil under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

the ability to walk after 12 days. Meanwhile, the values were 100.00% (0-5 cm), 85.72% (5-10 cm), 41.35% (10-15 cm), and 27.23% (15-20 cm) for the minor ants. All the major and minor ants lost walking abilities at the treated soil depths of 0-5 cm (Figs 7-8), which was significantly higher than the others ($F = 11.26$, $P < 0.01$).

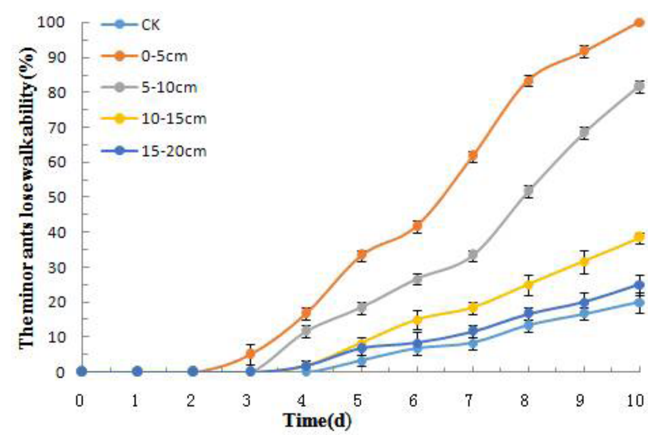

Fig 7. The rate of minor ants which lose walk ability after open exposure to soil under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

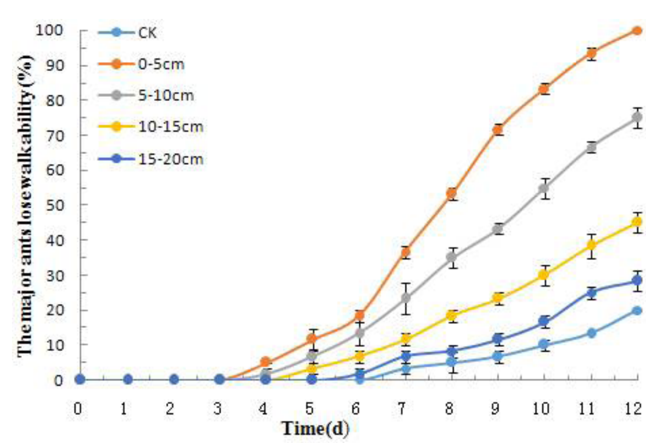

Fig 8. The rate of major ants which lose walk ability after open exposure to soil under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

Grasping ability bioassay

The soil in the rhizosphere of *V. odoratissimum* indicated a considerable grasping suppression (Figs 9-10). Among the major ants, 95.85% (0–5 cm), 70.75% (5-10 cm), 39.64% (10-5 cm), and 26.74% (15-20 cm) lost the ability to grasp after nine days. Meanwhile, when the minor ants were subjected to the test sequentially, the values were 100% (0-5 cm), 84.00% (5-10 cm), 38.29% (10-15 cm), and 26.45% (15-20 cm). However, almost none of the ants lost grasping ability on the first three days.

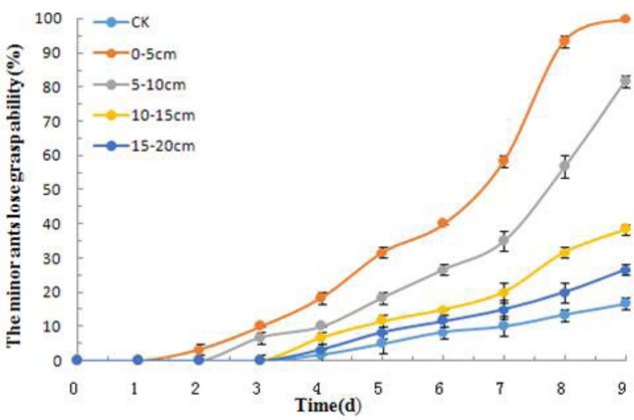

Fig 9. The rate of minor ants which lose grasp ability after open exposure to soil under *V. odoratissimum*. Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

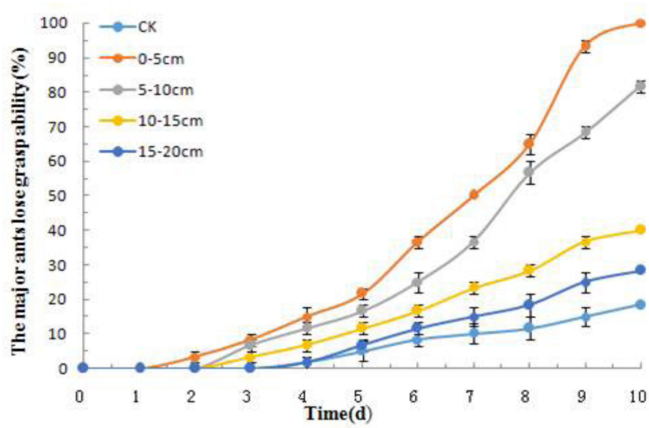

Fig 10. The rate of major ants which lose grasp ability after open exposure to soil under *V. odoratissimum.* Each data point represents mean ± SE of three replicate beakers each containing 20 ants.

Discussion

Methyl salicylate content in the leaves and soil

The leaf and soil both contain a certain amount of methyl salicylate, and the content in the fresh and dry leaves increased initially and then decreased. This result was achieved potentially because the leaf samples were in different growing stages. Hence, the methyl salicylate in plants may serve different functions in different growth stages. However, methyl salicylate was also obtained in the soil under trees. This finding may have been achieved because of fallen leaves. *V. odoratissimum* leaves drop across all four seasons, and numerous fallen leaves cover the ground every year. Methyl salicylate could be introduced into the soil in the natural decomposition process of fallen leaves. Therefore, the chemicals can be relatively stable in the soil. This investigation also revealed that soil at different depths had different toxicities and repellent activity against *S. invicta* workers. In this study, the soil at 0-5 cm depth, which contained higher methyl salicylate levels than other samples, showed more effective insecticidal and repellent activities against RIFA than the soil at other depths. The difference in mortality values may be attributed to the content of methyl salicylate.

Insecticidal and repellent activity

This study also showed that the soil in the rhizosphere of *V. odoratissimum* containing the methyl salicylate compounds showed effective insecticidal and repellent activity against RIFA. As a fumigant, methyl salicylate is potent against many arthropods, such as mosquitoes and mites (Delaplane et al., 1992). The importance of toxic and repellent influence of the soil may provide good practical significance if such effects can also be observed when applied to urban greening.

The use of chemicals from natural products in insect pest management is generally considered a safer alternative to using synthetic contact insecticides. Some components in plants have long been known as insect repellents. For example, camphor has been used as a repellent against many insect species (Abivardi et al., 1984). Eucalyptol has also been found repel various insects, such as American cockroaches (*Periplaneta americana* Linn.) (Maugh et al., 1982), mosquitoes (*Aedes aegypti*), and Colorado potato beetles (*Leptinotarsa decemlineata* Say) (Scheaper et al., 1984). Repellency of some natural products has been tested against the RIFAs, such as mint oil and water suspensions of pine needle (Chenet et al., 2008). However, the use of the plant itself to control pests was rarely reported. *V. odoratissimum* is an important garden landscape plant. Given its resilience, the plant has also been applied in forest fire prevention and urban greening. Based on its long history of topical application by humans, *V. odoratissimum* is considered safe for humans and other non-target organisms in its use in many scenarios for fire ant prevention. The dose-response relationships reported in this study provide a foundation for future investigations of *V. odoratissimum* as toxicant and repellent plant against adult RIFAs. The potential use of these selective and fully biodegradable materials in the management of invasive insects is promising. However, the success of a fire ant toxic and repellent plant depends heavily on its ecosystem. Therefore, additional knowledge is needed to use *V. odoratissimum* successfully.

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