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Plant-based compounds with potential as push-pull stimuli to manage behavior of leaf-cutting ants

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Abstract

Leaf-cutting ants are a serious pest of young forestry plantations. Currently, the main control method is the use of broad-spectrum insecticides, which have a negative effect on non-target organisms and the environment. In this work, plant-based compounds were evaluated in laboratory assays with *Acromyrmex ambiguus* Emery (Hymenoptera: Formicidae) for their potential use as repellent and attractant stimuli to be used in a push-pull strategy. Farnesol, a sesquiterpene present in many essential oils, was tested as a repellent at doses of 10, 50, and 100 mg. Its distance of action was studied by comparing the repellent effect of farnesol in a situation in which ants had to touch the farnesol in order to reach the food source in comparison to when ants could reach the food source without getting into direct contact with it. Different parts of the orange fruit (pulp and peel) were evaluated and compared as attractants, given that citrus-based baits are among the most popular attractants used. Results from laboratory bioassays indicated that farnesol is repellent at doses of 50 mg and acts upon contact or at a very short distance. Furthermore, orange pulp was more attractive than the peel, and volatile compounds were highly responsible for the attraction. When both stimuli were tested simultaneously in a laboratory experiment, repellency of farnesol was enhanced in the presence of orange pulp odor. When tested in a field push-pull experiment, the results also showed a good repellent effect of farnesol as well as an attractant effect of the orange pulp. These results encourage long-term studies with these substances in a field setting and suggest that repellents can be enhanced by the use of attractants to manage leaf-cutting ants behavior.

Introduction

Leaf-cutting ants are recognized as serious pests in the New World (Boulogne et al., 2012) and are amongst the most important forestry pests in South America, reducing wood production as well as affecting tree establishment (Blanton & Ewel, 1985; Cherrett, 1986; Fowler et al., 1986; Della Lucia et al., 1993; Folgarait et al., 1996). Distribution of *Atta* and *Acromyrmex* spp. (Hymenoptera: Formicidae: Myrmicinae: Attini) is limited to the American continent between latitudes 40°N and 44°S (Farji Brener, 1996). Mutualism with a fungus enables them to exploit a wide range of plant species. Until now, management strategies against these leaf-cutting ants have essentially focused on the use of traditional broad spectrum insecticides targeted at the ant workers (Antunes et al., 2000). Among control methods, toxic baiting is the most common method used for controlling *Acromyrmex* spp. ants. Toxic baits consist of an ant attractant, which is often dehydrated citrus pulp, impregnated with an insecticide (Robinson, 1979; Forti et al., 1998; Nagamoto et al., 2004). Sustainable agriculture demands new environmentally friendly pesticides that

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conform to strict international regulations. Research on new biorational pesticides based on plant compounds has increased considerably owing to their popularity with organic growers and environmentally conscious consumers (Boulogne et al., 2012; Regnault-Roger et al., 2012). Compounds that modulate behavior, and among these natural repellents, show promise (Howard et al., 1988; Salatino et al., 1998; Appel et al., 2004; Boulogne et al., 2012; Medina et al., 2012) and may prove useful in association with cultivated plants, thereby diversifying the crop system and minimizing the risk of attack (Della Lucia et al., 2014).

Many secondary plant metabolites are biologically active against phytophagous insects. Chemicals that are repellent to ants have been identified in plants that are not attacked by them (Hubbell et al., 1983; Okunade & Wiemer, 1985). Also, numerous terpenoid compounds which are major components of plant essential oils, are known to repel ants (Howard et al., 1988; Dos Santos et al., 2013). Among these, farnesol is present in many essential oils, such as *Pluchea dioscoridis* Cass and *Pittosporum undulatum* Ventenat (Grace, 2002; Medeiros et al., 2003), and it is commonly used in the fragrance and food industries. Farnesol has been shown to be very effective in protecting trees from the Argentine ant *Linepithema humile* Mayr at doses of 0.8 and 2 g per tree (Shorey et al., 1992, 1996). The application of repellent semiochemicals may help protect certain high-value crops or certain stages of plant development that are the most vulnerable, such as poplar, pine, or willow saplings, which can die from defoliation by foraging ants in commercial plantations (Pérez et al., 2011; Della Lucia et al., 2014). Repellents may prove particularly useful when used in combination with an attractant in a push-pull scenario. The stimulo-deterrent diversionary strategies exploit semiochemicals to repel insect pests from the crop (push) and to attract them into other areas (pull), such as trap crops, where the pest may be subsequently removed (Cook et al., 2007). This strategy, combining attractive and repellent compounds or crops to manipulate insect behavior, shows promise in integrated pest management (IPM) programs and, to our knowledge, has not yet been tested in leaf-cutting ants.

Citrus pulp is one of the most attractive substrates to the leaf-cutting ants (Mudd et al., 1978; Mudd & Bateman, 1979; Verza et al., 2006) and it is used as a base for insecticidal baits (Verza et al., 2011). Interestingly, citrus pulp contains *d*-limonene, a leaf-cutting ant repellent (Verza et al., 2011). Littledyke & Cherrett (1978) showed that ants did not collect fresh orange peel, although they did collect material when presented with whole oranges and dried pulp. Moreover, Almeida et al. (2013) report attractiveness of orange peel baits for *Acromyrmex niger* Smith and *Acromyrmex disciger* Mayr. Different species of ants may respond differently to attractants, as reported by Boaretto & Forti (1997). Thus, more detailed studies are needed to enhance plant-based attractive baits that target specific species of ants.

The aim of this study was to assess the repellency of farnesol in *Acromyrmex ambiguus* Emery, a species which is widely distributed in the north of Buenos Aires province (Farji-Brener & Ruggiero, 1994), and is a severe pest of young tree plantations, as well as to determine its potential use in a push-pull strategy as the push stimulus. Also, given the popularity of citrus pulp baits and the mixed results reported in the literature with regards to the response of ants to citrus, we investigated the behavior of *A. ambiguus* ants towards different orange parts (peel and pulp). For this purpose, we conducted preference bioassays and investigated whether preference is related to an odor cue. Our results could be used to enhance the attractiveness of baits and to develop plant-based repellents.

Materials and methods

Biological material

Acromyrmex ambiguus colonies were collected at the Delta Experimental Station, National Institute of Agropecuary Technology (INTA) Campana, Buenos Aires, Argentina, during 2013 and 2014. Once the nests were located, ants were collected and placed inside a plastic container (30 × 44 × 30 cm) acting as the foraging arena for the nest. The fungus material was placed in a rectangular plastic container (23 × 14 × 7 cm) inside the foraging arena.

Colonies were maintained in a chamber at 23–25 °C, 60% r.h., and L12:D12 h photoperiod. They were fed three times a week with either fresh leaves of ash, *Fraxinus* spp. (Oleaceae), or poplar, *Populus* spp. (Salicaceae), during spring and summer, or fresh leaves of primrose jasmine, *Jasminum meznyi* Hance (Oloaceae), during autumn and winter. Additionally, apple, oat flakes, maize flour, and rice were offered throughout the year.

Depending on the bioassay, ants were tested as sub-colonies or whole colony. The sub-colonies consisted of a portion of the colony's fungus and ca. 350 worker ants, with a mean (± SD) head width (between outer limits of the eyes) of 1.75 ± 0.1 mm, which were placed in a round plastic box (10.5 cm diameter, 10 cm high). This container was connected to a rectangular plastic box (23 × 14 × 7 cm), which acted as foraging arena, by three clear plastic tubes (1.2 cm diameter, 15 cm long). For the whole colony bioassays, the colony's plastic container was connected to a foraging arena (33 × 46 × 12 cm) by a bridge made of thin wood. The upper portions of the plastic containers' walls were coated with vaseline to prevent ants' escape.

Farnesol as a repellent

Preference bioassays using sub-colonies. The bioassay consisted of 12 filter paper discs (0.5 cm diameter) in a grid design, equidistantly spaced in four columns and three rows in the foraging arena. Half the discs were treated and half were untreated. The discs were randomly placed in the arena and the ants were allowed to forage. Then, the order and type of discs collected were recorded. Three doses of farnesol (Sigma-Aldrich, Buenos Aires, Argentina) were applied to the filter papers: 3, 15, and 30 μg. The doses were achieved by preparing solutions of farnesol and ethanol (treated discs) at concentrations of 1, 5, and 10 mg ml^{-1} and then applying 3 μl of each solution to the filter papers. Control discs were treated with 3 μl of ethanol. Six sub-colonies from each colony were tested, from a total of three colonies. The assay ended when half of the total discs were collected; if this did not happen within 2 h, the assay was finished and the results were not used. The number of discs collected from each treatment were analyzed using a χ^2 test for heterogeneity.

Preference bioassays using whole colonies. Willow cuts (*Salix babylonica* L. var. *sacramenta* 'Soveny Americano', Salicaceae) were used in choice tests to assess foraging preference when farnesol was applied to the cuts. The cuts consisted of twig portions of 22 cm long and 1.5 cm diameter. These bioassays were done with three different whole colonies. Two willow cuts were placed in the center of the arena, separated by 30 cm. Halfway up each cut (at 11 cm from the bottom) a cotton twine was wrapped. Both willow cuts had a small round container (2 cm diameter) with oat flakes at the top which was filled to allow ad libitum foraging (Figure 1). Three doses of farnesol were evaluated: 10, 50, and 100 mg. The doses were achieved by melting lanoline in a heating plate and mixing in farnesol. Once the mixture was prepared, it was allowed to cool down and it was applied to the cotton twine. Control twines were treated with lanolin in the same way, with no farnesol. Observations were conducted for a period of 5 days, and consisted of recording the number of ants on the willow cuts for 1 min. Observations started at 09:00 hours and were conducted every 40 min until around 16:00 hours, leading to a total of 10 observations per day. Ants were counted regardless of whether they were proceeding up or down the cut (Shorey et al., 1996). Each colony was tested with all the three farnesol doses. Data were converted to express percentage repulsion (PR) by the following formula: PR (%) $= (Nc - 50) \times 2$, where Nc is the percentage of ants present in the control cuts. Positive values expressed repellency and negative values attractancy (Talukder & Howse, 1994). Data (PR) were analyzed by using repeated measures ANOVA (Proc GLIMMIX) of SAS v.9.3 (SAS Institute, Cary, NC, USA). The PRs were compared for each dose and sampling time with the Tukey procedure. Dose and sampling time were included as fixed effects. Each colony tested was considered a random factor. Corrected denominator degrees of freedom were obtained using the Kenward-Roger adjustment (DDFM = KR option of the MODEL statement). Residual and normal quantile-quantile plots were used to examine data for evidence of heterogeneity of variance and non-normality of errors, respectively, which suggested that the model fit the data well.

Preference bioassays with or without contact with farnesol. Two different preference bioassays were performed to determine whether the effect of farnesol is

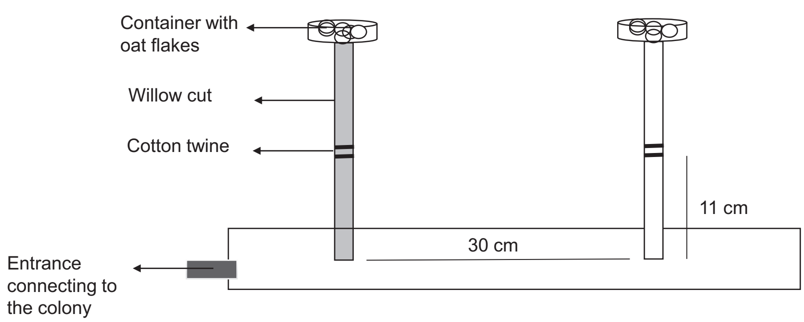

Figure 1 Diagram of the foraging arena used in the preference bioassays with whole colonies of *Acromyrmex ambiguus* ants. Willow cuts consisted of twig portions of 22 cm long and 1.5 cm diameter. Cuts were placed 30 cm from each other in a plastic container (33 × 46 × 12 cm) that served as the foraging arena. The treatment consisted of placing a cotton twine with lanolin and farnesol around the cut (grey). The control cut had a cotton twine with lanolin only (white). The container was connected to the colony through a wooden bridge via which the ants entered the arena and could choose to forage on the oat flakes (provided ad libitum) placed on top of each cut.

caused by its volatiles (odor cue) or upon contact. Ants could choose from two feeding stations, one treated with farnesol, the other a control. These stations consisted of small cages made of plastic mesh (2 cm diameter, 2.6 cm high) with a container with oat flakes on the top. Ants had to climb the cage to reach the oat flakes. The treatment consisted of a dose of 10 mg farnesol in 100 mg lanoline administered onto the cotton twine, and the control twine was treated with 100 mg lanoline alone. For the contact treatment, the cotton twines were wrapped outside the cages and the ants had to climb over them to reach the oat flakes. For the no-contact treatment, cotton twines were placed inside the cages. Ants could climb up the feeding stations to reach the flakes without touching the twine.

Each observation consisted of recording the number of ants touching the oat flakes container every 2 min during 40 min. Observations were conducted randomly between 09:00 and 16:00 hours, when ants were active. Each bioassay (contact and no contact) was repeated with six sub-colonies from three colonies. The numbers of ants in each treatment were analyzed using a χ^2 test for heterogeneity. For each bioassay (contact and no-contact), data were arranged in a 2 × 3 table, with control and treated options, and the results for each of the three colonies (each represented by the average of its six sub-colonies).

Orange as an attractant

Preference bioassays using sub-colonies were performed to determine which part of the orange (*Citrus sinensis* L. Osbeck, Rutaceae) was more attractive to foraging ants. Ants had three items to choose from: orange pulp, orange peel, and a combination of pulp and peel (half pulp, half peel). Each option consisted of a 1 × 1 cm portion of material. The options were placed 5 cm from each other. Ants were allowed to enter the arena and the number of ants in touch with each option was recorded every 2 min, during 1 h. Observations were conducted randomly between 09:00 and 16:00 hours, when ants were active. Given that orange pulp was preferred over the peel, another bioassay was conducted to identify whether this behavior was due to odor cues or whether it was elicited upon contact. Cages similar to those used to test the contact and no-contact bioassays with farnesol were used. The same three orange portions of 1 × 1 cm were placed inside the cages so that the ants could not touch them but could still detect the odor. The number of ants touching each cage was recorded every 2 min during 1 h. Observations were conducted randomly between 09:00 and 16:00 hours, when ants were active.

These bioassays were performed with six sub-colonies from three different colonies. The numbers of ants attracted to each option were analyzed using a χ^2 test for heterogeneity. For each bioassay (contact and no-contact), data were arranged in a 3 × 3 contingency table, with the three options of orange portions (pulp, peel, and pulp + peel), and the results for each of the three colonies (each represented by the average of its six sub-colonies).

Stimulo-deterrent diversionary bioassays

Bioassays using a stimulo-deterrent or push-pull strategy were used to determine whether the farnesol as repellent was more efficacious when used together with an attractant such as orange pulp.

Laboratory push-pull bioassays. The experiments were performed with whole colonies. Two groups of willow cuts, 25 cm apart from each other, were placed in the arena. Each group comprised four cuts, and each cut was wrapped with a 25-cm-long cotton twine attached in the middle. A small plastic cup (1.4 cm diameter, 1.4 cm high) containing oat flakes was placed on the top of each cut. While foraging proceeded, oat flake containers were refilled in order to maintain an ad libitum food source in each station. Two independent preference experiments were performed. In one experiment, preference between control or farnesol treatments was evaluated. The farnesol treatment consisted of cuts surrounded by cotton twines imbibed with 100 mg of farnesol in 100 mg of lanoline, or 50 mg of farnesol in 100 mg of lanoline. In both assays the cotton twines of the control group were treated with 100 mg of lanoline.

The push-pull experiment consisted of testing the preference of ants for farnesol against an attractant (orange pulp). The push treatment consisted of cuts treated with farnesol as explained for the preference experiment at the same doses of 50 or 100 mg of farnesol in 100 mg of lanoline. The pull treatment consisted of cuts which had an aluminum container with 1.5 g of orange pulp, inside a plastic mesh cage (1.4 cm diameter, 1.4 cm high), so that ants were unable to touch the orange, but were exposed to its odor. The number of ants walking across the cotton twine of each willow cut was recorded three times during a day at 09:00, 12:00, and 15:00 hours. Each observation lasted 30 s. Ants were counted regardless of whether they were moving up or down the cut. Six colonies were tested with each experiment. Statistical analyses were performed with MANOVA (Proc GLM) (SAS v.9.3, SAS Institute, Cary, NC, USA). The numbers of ants foraging in farnesol and control, or farnesol and orange-treated cuts, were the two dependent variables compared for each trial (farnesol vs. control or push-pull). Colony, treatment, and their interaction were the independent variables included in the model.

Field push-pull bioassays. The assays were performed in Delta Agropecuary Experimental Station in Campana district in Buenos Aires, on 13, 14, 25, 26, and 27 January 2016. The bioassays consisted of setting two willow cuts with two attached willow leaves (*S. babylonica* var. *sacramenta* Soveny Americano) in the top, presented at each side of the ant trail, at 5 cm apart. Three types of cuts were offered: (1) farnesol: 100 mg of farnesol applied to the cut at the middle of its length, forming a 2 cm band around it (no cotton twine); (2) orange: a portion of 1.5 g of orange pulp was set above the leaves at the top as an attractant; and (3) control: untreated cuts with willow leaves. Six *A. ambiguus* nests were identified and two foraging trails were chosen from each nest. Cuts were offered as follows. *Farnesol as a repellent vs. control (push)*: in one of the foraging trails ants could choose between farnesol or untreated control cuts. *Farnesol as a repellent vs. orange attractant (push-pull)*: in the second foraging trail from the same nest, ants could choose between farnesol or orange cuts. The number of ants above the leaves in each treatment was recorded after 30 min and 3 h.

The number of ants foraging in each treatment was analyzed with repeated measures ANOVA using the Proc GLIMMIX (restricted maximum likelihood) of SAS v.9.3. The numbers of ants foraging in each treatment and time were included as fixed effects. Colony was included as random variable and time was the repeated measure. Corrected denominator degrees of freedom were obtained using the Kenward-Roger adjustment (DDFM = KR option of the MODEL statement). Residual and normal quantile-quantile plots were used to examine data for evidence of heterogeneity of variance and non-normality of errors, respectively, which suggested that the model fit the data well. Where differences among fixed effects were indicated, differences among means were compared with the Tukey adjustment option of the LSMEANS statement.

Results

Farnesol as a repellent

Preference bioassays using sub-colonies. For all the doses tested, ants preferred to collect control discs, and as the concentration of farnesol increased, the differences between the number of control and treated discs collected were greater (3 μg: χ^2 = 4.19, P = 0.12; 15 μg: χ^2 = 8.04, P = 0.018; 30 μg: χ^2 = 9.47, P = 0.009, all d.f. = 2). Ants collected very few discs treated with 30 μg of farnesol (Figure 2).

Willow cuts preference bioassays using whole colonies. The overall PR across all doses and time averaged 25.9 ± 3.5, showing a marked repellent effect of farnesol (Figure 3). Analysis of variance indicated effects of time ($F_{4,28}$ = 5.14, P = 0.003) and dose ($F_{4,28}$ = 15.50, P<0.0001) on the percentage of ants in control cuts converted to PR, but not of their interaction ($F_{8,28}$ = 1.35, P = 0.3). Overall, the highest dose tested (100 mg) was more effective in repelling ants than 10 and 50 mg of farnesol (Tukey test: P<0.05).

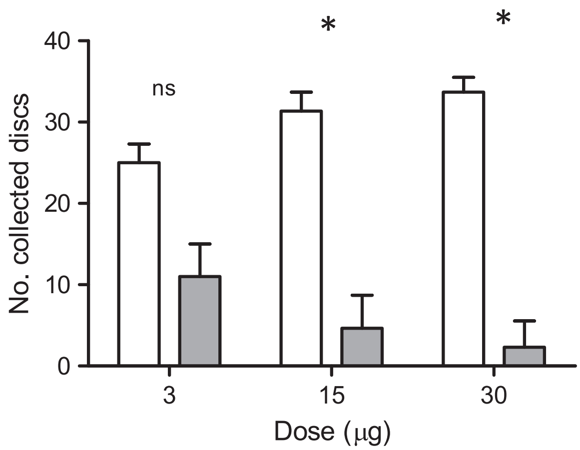

Figure 2 Mean (+ SE) number of discs collected by foraging *Acromyrmex ambiguus* ants in preference bioassays in which ants had a choice between control discs (white) vs. discs treated with 3, 15, or 30 μg farnesol (grey). Asterisk indicates a significant difference between treatment and control (χ^2 test: P<0.05; ns, P>0.05).

Preference bioassays with or without contact with farnesol. The number of ants foraging on the farnesol-treated feeding station was lower than in the control feeding station when ants had to get in contact with the repellent in order to reach the oats flakes (χ^2 = 78.74, d.f. = 2, P<0.0001). On the other hand, when ants were able to climb to the food source without touching the farnesol, there were no differences in the number of foraging ants between the feeding stations (χ^2 = 1.3, d.f. = 2, P = 0.52; Figure 4). These results suggest that the repellent effect of farnesol acts over a very short distance.

Orange as an attractant

There were differences among the three options (pulp, peel, or peel + pulp) in the contact (χ^2 = 60.50) as well as in the no-contact assay (χ^2 = 82.84, both d.f. = 4, P<0.0001). The number of ants recorded in the orange pulp portion was significantly higher than that in the peel or in the combination of both (Figure 5). These results indicate that ants had a similar preference for orange pulp, whether they could get in contact with the orange or not,

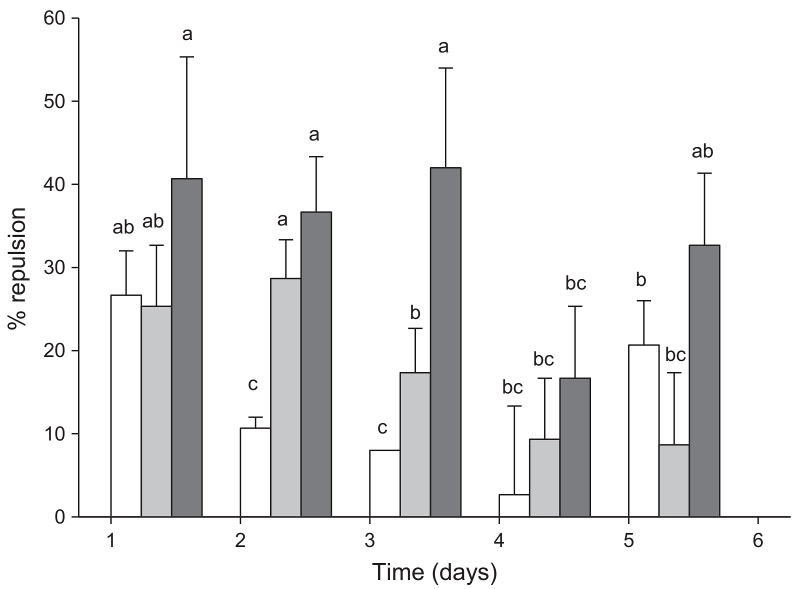

Figure 3 Mean (+ SE) repulsion (%) in bioassays in which *Acromyrmex ambiguus* ants could choose to forage on oat flakes placed at the top of willow cuts treated with solvent (control) vs. 10 (white), 50 (light grey), or 100 mg farnesol (dark grey). Means capped with different letters are significantly different (Tukey's test: P<0.05).

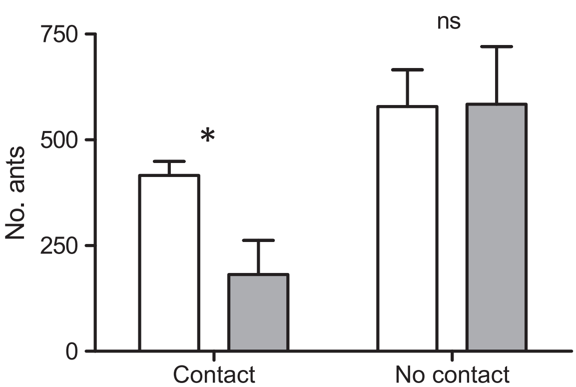

Figure 4 Mean (+ SE) number of *Acromyrmex ambiguus* ants in feeding stations, treated with farnesol (grey) or control (white), in two bioassays, one in which ants could come into contact with farnesol (contact) and another in which they were not in direct contact with farnesol (no contact). Asterisk indicates a significant difference between treatment and control (χ^2 test: P<0.05; ns, P>0.05).

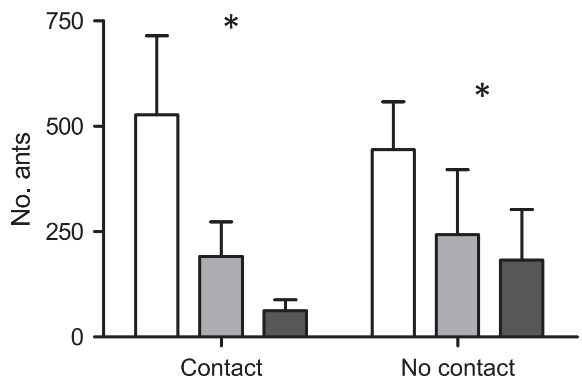

Figure 5 Mean (+ SE) number of *Acromyrmex ambiguus* ants recorded in the 'orange preference' assay, in which ants could choose between orange pulp (dark grey), peel (white), or a combination (light grey), after being in contact or not with the options. Asterisk indicates a significant difference between treatments (χ^2 test: P<0.05).

and that the attractiveness of the pulp is reduced in the presence of peel.

Stimulo-deterrent diversionary strategy

Laboratory push-pull bioassays. The number of ants foraging in the willow cuts differed in the experiments comparing farnesol vs. farnesol + orange pulp as an attractant (Figure 6). When the dose of farnesol was 50 mg, there was a treatment effect (Wilk's λ = 0.56, d.f. = 2, 35, P<0.0001), a colony effect on the number of ants foraging in the arena (Wilk's λ = 0.16, d.f. = 10,70, P<0.0001), and an interaction effect of colony and treatment (Wilk's λ = 0.37, d.f. = 10,70, P<0.0001). When the dose of farnesol was 100 mg, there was a treatment effect (Wilk's λ = 0.45, d.f. = 2,35, P<0.0001) and a colony effect on the number of ants foraging in the arena (Wilk's λ = 0.39, d.f. = 10,70, P<0.001), with no interaction effect of colony and treatment (Wilk's λ = 0.75, d.f. = 10,70, P = 0.4). Across all experiments and doses, the number of ants in the orange-treated cuts was greater than in the control cuts. The push-pull strategy was more effective than the push alone for all the colonies tested, given that there were more ants in the pull stimulus

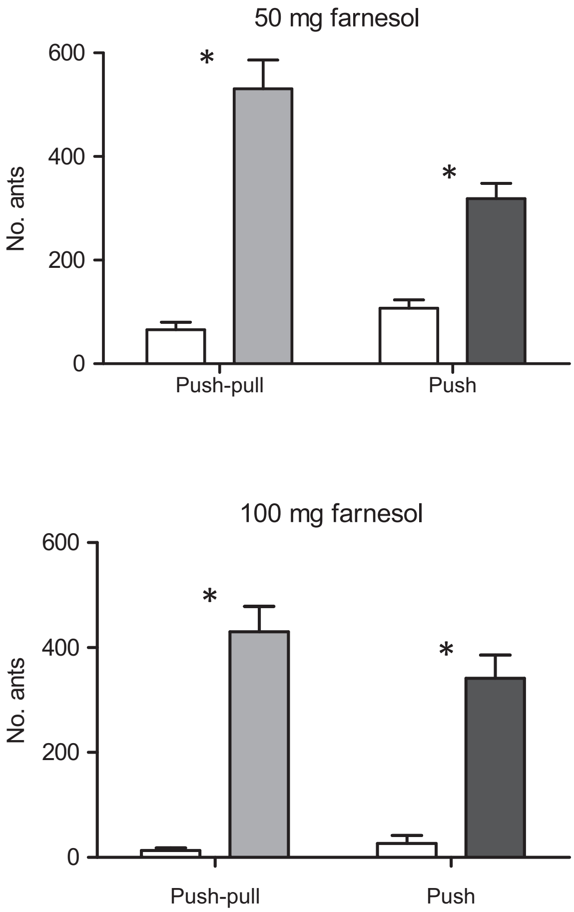

Figure 6 Mean (+ SE) number of *Acromyrmex ambiguus* ants foraging in experimental arenas in which willow cuts were treated with 50 or 100 mg of farnesol as repellent (white) vs. untreated willow cuts (dark grey; 'push'), or vs. willow cuts treated with orange pulp as attractant (light grey; 'push-pull'). Asterisk indicates a significant difference between treatments (χ^2 test: $P<0.05$).

and fewer in the push stimulus if the treatment had a pull stimulus with orange. The effect was more marked when 50 mg of farnesol was used as the push stimulus, given that when 100 mg of farnesol was used, both treatments (push or push-pull) had a much lower number of ants foraging in the oats.

Field push-pull bioassay. No ants climbed on the willow cuts treated with farnesol, after 30 min or 3 h (Table 1). Thus, the number of ants climbing the control was compared to the number climbing orange-treated cuts: the numbers differed, both for willow cuts with leaves only ($F_{1,16} = 29.54$, $P<0.0001$), and for cuts next to orange pulp ($F_{1,16} = 5.94$, $P = 0.02$). More ants foraged after 3 h than after 1 h since the beginning of the assay: more ants climbed the cuts ($F_{1,16} = 8.98$, $P<0.01$) and more plant material was removed ($F_{1,16} = 7.07$, $P<0.001$). The number of ants in the cuts treated with orange pulp was greater than in the control cuts after 1 and 3 h.

Discussion

Results from the current study indicate that farnesol is a potent repellent of *A. ambiguous*. Fresh orange pulp was found to be highly attractive and preferred over orange peel. A repellent response to farnesol, increasing with dose, was observed in various experimental designs. A repellent effect lasting at least 5 days was observed for the highest dose used (100 mg) under laboratory conditions. Shorey et al. (1992) reported a repellent effect of farnesol lasting up to 3 months for Argentine ants in a citrus plantation. Given that long-lasting repellence is desirable for a leaf-cutting ant management strategy, further studies should aim to determine the duration of the repellent effect of farnesol on *A. ambiguous* under field conditions.

Determining the distance of action of a repellent is needed to understand how it can be used in a push-pull strategy. Long-range stimuli represent the first line of defense, whereas short-range stimuli are powerful tools in preventing, e.g., feeding or oviposition once the insect is on the crop (Cook et al., 2007). Farnesol seems to act at very short distances, given that ants did not avoid climbing up the cages that contained farnesol inside, whereas they were more hesitant to climb them if they had to come into

Table 1 Mean (± SE) number of *Acromyrmex ambiguus* ants foraging on leaves on top of willow cuts in a push-pull reference experiment. The push treatment consisted of farnesol surrounding the stem cut and the pull treatment consisted of orange pulp next to the leaves

		Push vs. control		Push-pull	
Variable measured	Sampling time	Farnesol	Control	Farnesol	Pull
No. ants present	30 min	0.16 ± 0.41	8.16 ± 6.49	0	18.86 ± 13.60
	3 h	0	8.66 ± 8.38	0	43.33 ± 9.83
No. ants cutting leaves	30 min	0	3.33 ± 1.5	0	4.0 ± 2.36
	3 h	0	3.83 ± 3.37	0	7.15 ± 2.04

direct contact with farnesol. Thus, this compound could be used as a contact barrier to protect trees (Shorey et al., 1996). However, further evaluation of how the leaf-cutting ants perceive the farnesol is warranted.

Fresh orange pulp was found to be highly attractive and preferred over orange peel and laboratory assays clearly demonstrated that the repellent effect of farnesol was enhanced by the use of orange pulp as an attractant. This suggests that the odor of the pulp is the attractive cue for the ants and the presence of peel could have a negative effect in the attraction. An analysis of volatiles from pulp and peel showed mono and sesquiterpenes as major components (Table S1). The peel contained a great variety of sesquiterpenes. Interestingly most of these compounds are absent or present only in traces within the pulp volatiles. It is known that foraging leaf-cutting ants orient upwind to odor stimuli. Littledyke & Cherrett (1978) found that individual *Acromyrmex octospinosus* (Reich) respond positively to volatiles of dried citrus pulp and whole oranges. Our results suggest that ant preference for orange pulp was related to odor cues. Surprisingly, orange pulp odor lowered its attractive response when it was offered in conjunction with orange peel. (+)-Limonene has been reported as the main terpene found in both orange juice and orange peel oil (Qiao et al., 2008; Ruiz Perez-Cacho & Rouseff, 2008). Our qualitative volatile analysis showed that even when differential volatiles were found in peel, many of them appear as traces in the pulp, suggesting that it is possible that the repellent effect of the peel in comparison to the attractiveness of the pulp is related to the concentration of (repellent) secondary metabolites. Another possibility is that the peel is not as attractive as the pulp due to specific sesquiterpenes that are only present in the peel. These results warrant further research on the ants' response to individual volatile compounds in order to identify which ones are responsible for the citrus attractiveness. Such results would be useful to improve the attractiveness of citrus-based baits or to develop synthetic baits.

There is an abundance of recent research on the use of plant-based insect repellents, particularly volatile compounds found in essential oils (Maia & Moore, 2001; Isman, 2006; Regnault-Roger et al., 2012). However, one of the disadvantages of plant-based oils is that they are composed of a complex mixture of secondary plant metabolites, which varies with plant development, seasonality, and stress, among other factors. Registration issues are among one of the hindrances to the widespread use of products based on these compounds (Regnault-Roger et al., 2012). Furthermore, obtaining large quantities of an essential oil may not always be feasible, for example in the case of rare or protected plants that are not cultivated commercially. Thus, using synthetic semiochemicals such as farnesol, which can be standardized for purity and composition, can be advantageous.

Another issue that deserves consideration when using plant-derived volatile compounds is their volatility. Research on formulation of natural products is advancing at a fast pace and suggests that micro- and nanoencapsulation represent feasible and efficient approaches to modulating release, increasing physical stability of the active substances, protecting them from interactions with the environment, decreasing their volatility, enhancing their bioactivity, and reducing toxicity (Bilia et al., 2014; Werdin González et al., 2014).

The present study indicated that the repellent effect of farnesol was enhanced by the use of an attractant in laboratory bioassays showing potential for its use in a push-pull strategy for leaf-cutting ants.

Although field assays proved the attraction to orange pulp and repellency of farnesol, they could not demonstrate the enhancement of the repellent effect by the orange attractant because no ants were observed foraging in any of the farnesol treatments. The farnesol dose used was high enough to completely repel ants.

Further studies should aim to determine the duration of the repellent effect of farnesol and evaluate the use of push-pull strategy in a long-term field test. A slow-release granular formulation based on orange attractants could be developed as long-lasting attractive bait, in conjunction with a slow-release formulation of farnesol for use as a long-lasting repellent around homes or in quarantined plant material.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Total ion chromatogram obtained from analysis of volatiles collected from orange pulp and peel.