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Pheromone-Assisted Techniques to Improve the Efficacy of Insecticide Sprays Against *Linepithema humile* (Hymenoptera: Formicidae)

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ABSTRACT Outdoor residual sprays are among the most common methods for targeting pestiferous ants in urban pest management programs. If impervious surfaces such as concrete are treated with these insecticides, the active ingredients can be washed from the surface by rain or irrigation. As a result, residual sprays with fipronil and pyrethroids are found in urban waterways and aquatic sediments. Given the amount of insecticides applied to urban settings for ant control and their possible impact on urban waterways, the development of alternative strategies is critical to decrease the overall amounts of insecticides applied, while still achieving effective control of target ant species. Herein we report a "pheromone-assisted technique" as an economically viable approach to maximize the efficacy of conventional sprays targeting the Argentine ant. By applying insecticide sprays supplemented with an attractive pheromone compound, (Z)-9-hexadecenal, Argentine ants were diverted from nearby trails and nest entrances and subsequently exposed to insecticide residues. Laboratory experiments with fipronil and bifenthrin sprays indicated that the overall kill of the insecticides on Argentine ant colonies was significantly improved (57–142% increase) by incorporating (Z)-9-hexadecenal in the insecticide sprays. This technique, once it is successfully implemented in practical pest management programs, has the potential of providing maximum control efficacy with reduced amount of insecticides applied in the environment.

KEY WORDS runoff, fipronil, bifenthrin, (Z)-9-hexadecenal, integrated pest management

Nationwide, ants consistently rank as one of the most important household pest species in the structural pest management industry (Klotz et al. 2010). Commercial pest control companies in California estimate that 65–80% of their pest control services deal with ants (Field et al. 2007). A recent nationwide survey indicated that ants are among the most difficult pest groups encountered by pest management professionals (PMPs), remaining the number one issue causing callbacks (Whitford 2013). The same survey also indicated that 62% of responding PMPs reported that ≥70% of ant management sales are from residential treatments. It is clear that managing pest ants in the urban residential settings is a significant challenge to both homeowners and PMPs.

Control strategies for urban pest ants have primarily focused on the application of barrier sprays, granules, and baits (Rust 2001, Rust et al. 2003, Silverman and Brightwell 2008, Klotz et al. 2010). Even with recent advances in bait technologies (Klotz et al. 2003), residual insecticide barriers are still widely used by PMPs to control urban pest ants (Rust et al. 2003, Klotz et al. 2008, Whitford 2013) partly because of the relatively high cost for baiting programs. For example, a cost analysis indicated that baiting increased the cost by 40% because of increased labor cost (Klotz et al. 2009). Pyrethroid sprays containing bifenthrin, cyfluthrin, cypermethrin, and permethrin, and phenylpyrazole spray containing fipronil are among the most common liquid treatments used by homeowners and PMPs to control ants in urban settings (California Department of Pesticide Regulation [CDPR] 2008, Greenberg et al. 2010). Consequently, pyrethroids, fipronil, and their toxic degradation products have been detected in urban waterways and aquatic sediments (Lao et al. 2010, Delgado-Moreno et al. 2011). The application of insecticides around structures to control ants and other pests by PMPs and homeowners are recognized as a major source of insecticides in urban waterways (Weston et al. 2005, 2009). Given the amount of insecticides applied to urban settings for ant control and their impact on urban waterways, the development of alternative integrated pest management strategies is critical to decrease the overall amounts of insecticides applied and found in urban waterways, while still providing effective control of target ant species.

Social insects such as ants, honey bees, and termites use a diverse array of pheromones for organization and coordination of all aspects of their colony development and maintenance, including defense, reproduction, foraging, and nest relocation (Hölldobler and Wilson 1990, Vander Meer and Alonso 1998). In par-

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ticular, the trail pheromones of ants are known to play critical roles in their foraging and nest relocation activities (Hölldobler and Wilson 1978, 1990; Wilkins et al. 2006; Witte et al. 2007, Cao and Dornhaus 2012). The trail pheromone of the Argentine ant, *Linepithema humile* (Mayr), has been the focus of numerous studies because of its significance in the species' mass recruitment behavior. Cavill et al. (1979, 1980) first isolated and characterized (Z)-9-hexadecenal from Argentine ants, and concluded that (Z)-9-hexadecenal might be a component of the trail pheromone complex based on the evidence that (Z)-9-hexadecenal was produced in a ventral gland and strongly attractive to workers in a multichoice olfactometer. Although the deposition of (Z)-9-hexadecenal on the trail by recruiting ants has been called into question (Choe et al. 2012), the chemical has been studied primarily as a trail pheromone component of the Argentine ant (Van Vorhis Key et al. 1981; Van Vorhis Key and Baker 1982a,b,c).

Several studies have explored the possibility of using synthetic (Z)-9-hexadecenal to develop practical management strategies for the species. For example, one study suggested that (Z)-9-hexadecenal might increase the consumption of sugar-based liquid baits by Argentine ant workers when it is mixed with the baits (Greenberg and Klotz 2000). However, this study did not use a toxicant or insecticide with the pheromone compound. Several studies have been conducted in Japan and Hawaii to test whether the application of synthetic (Z)-9-hexadecenal disrupted trail formation and foraging activity of Argentine ant populations in the field (Suckling et al. 2008, 2010; Tanaka et al. 2009; Nishisue et al. 2010; Sunamura et al. 2011). Even relatively large quantities of synthetic (Z)-9-hexadecenal had a negligible impact on Argentine ant populations when used as a stand-alone treatment (Nishisue et al. 2010), but produced a significant effect when used in conjunction with toxic baits separately applied in the field (Sunamura et al. 2011). Extermination of ants by the insecticidal bait coupled with inhibition of reinfestation by disruption of foraging by the applied (Z)-9-hexadecenal were attributed as possible mechanisms of the combination effect. These studies have only focused on "disrupting" trails by applying a large amount of synthetic (Z)-9-hexadecenal in the environment.

The current study explored the use of (Z)-9-hexadecenal to attract Argentine workers to an area treated with insecticide, thereby maximizing the efficacy of insecticide sprays applied in the urban environment. This "attract-and-kill" approach using the synthetic pheromone would be advantageous over conventional stand-alone applications of insecticide spray because the insecticide- and pheromone-treated surface (soil, cement, and wood) will attract foraging ants from nearby trails and even from the nest entrances and maximize the number of ants exposed to the treated surface while decreasing pesticide application. We discuss the potential implications for reducing the amount of insecticides applied to control ants and reducing potential pesticide runoff.

Materials and Methods

Three studies were conducted to determine the potential efficacy of pheromone-treated aqueous sprays. Experiment 1 was conducted in the field to determine whether the synthetic pheromone increases ant contact with treated surfaces by attracting them from an existing trail. Experiment 2 was conducted in the field to determine whether the synthetic pheromone increases ant contact with treated surfaces by attracting them from their nest entrance. Experiment 3 was conducted in the laboratory to determine whether the addition of synthetic pheromone will improve the efficacy of an insecticide spray compared with an insecticide only treatment.

Experiment 1: Effect of Pheromone Deposit on Workers From Nearby Trails. The study was conducted in the biological control grove at the University of California, Riverside, in April 2012. The site supports a large population of Argentine ants, primarily because of the absence of chemical insecticide use and the existence of honeydew-producing insects in the citrus trees. Five different trails of Argentine ants, which were relatively straight and at least 30 cm in length and originating from different nests, were located. Using these trails as "existing trails," we tested whether our "pheromone-assisted technique" would attract ants from the existing trails. For the pheromone treatment, two patches of sand (52.5 g of sand per patch), one on each side of the existing trail, were evenly spread in a square (20 by 20 cm). The distance between two patches was ≈5 cm. One patch served as a treatment, and the other one served as a control. For the treatment, 1 mg of the synthetic pheromone (>90% (Z)-9-hexadecenal, Bedoukian Research Inc., Danbury, CT) dissolved in 1 ml of 100% ethyl alcohol was mixed with 500 ml of deionized water. For the control, 1 ml of ethyl alcohol was mixed with 500 ml of deionized water. Both preparations were applied with sprays that deliver ≈0.8 ml of liquid at a time in a circular area of 130.9 cm^2. Each sand patch was treated with four sprays (each spray targeting 10- by 10-cm areas in one corner). This resulted in a rate of ≈12 ng/cm^2 for (Z)-9-hexadecenal on the treatment side. The final amount of (Z)-9-hexadecenal on the treated surface (12 ng/cm^2) was chosen because the rate was low enough so that humans could not detect the characteristic odor of (Z)-9-hexadecenal from the spray deposit, but the rate was high enough to attract Argentine ant workers from nearby areas. Van Vorhis Key and Baker (1982c) reported that Argentine ant workers were attracted to a linear source of (Z)-9-hexadecenal when it was applied at a rate of 0.2 ng/cm.

Both sand patches were photographed at 2 and 4 min after application of spray preparations with a digital camera equipped with a macro lens (Canon D60, Canon USA, Inc., Melville, NY). The number of ants on the 20- by 20-cm sand patches was counted at 2 and 4 min posttreatment from the digital photographs. The study was replicated five times using different trails.

Experiment 2: Effect of Pheromone Deposit on Workers from a Nearby Nest Entrance. The study was conducted in the biological control grove at the University of California, Riverside, in April 2012. Nest entrances of Argentine ant colonies were located, which had active trails associated with them (one nest entrance per nest). To determine whether our pheromone-assisted technique would attract ants from the nest entrance, two patches of sand were placed on both sides of the nest entrance. Approximately 52.5 g of sand were evenly spread in a square (20 by 20 cm). The distance between the two patches was ≈5 cm. The method and rate of application were identical with those of experiment 1. Both sand patches were photographed at 1, 2, and 3 min after application of sprays. The study was replicated 10 times using a different nest entrance for each replicate. The number of ants on the 20- by 20-cm sand patches was counted at 1, 2, and 3 min posttreatment from digital photographs.

Experiment 3: Effect of Pheromone on Efficacy of Insecticide Sprays. To determine whether the efficacy of an insecticide spray (i.e., fipronil and bifenthrin) could be improved by adding the synthetic pheromone, studies were conducted with small laboratory colonies. Ant nests were excavated from a citrus grove (biological control grove) on the University of California, Riverside, campus, and transported to a laboratory chamber where they were extracted from the soil. Laboratory stock colonies were maintained in plastic boxes (26.5 by 30 by 10 cm) with the inner sides coated with Teflon (fluoropolymer resin, type 30, DuPont Polymers) to prevent ants from escaping. Each colony was provided with two or three artificial nests constructed from plaster-filled petri dishes (9 cm in diameter by 1.5 cm in depth) formed with a 5-cm-diameter by 1-cm-deep cylindrical area in the center of the dish to serve as a nesting space. In addition to continuous access to water in a test tube, the colonies were provisioned with fresh water, 25% (wt:vol) sucrose water, and freshly killed American cockroaches (*Periplaneta americana* (L.) (Blattodea: Blattidae)) three times a week.

Argentine ants from the stock laboratory colony boxes were anesthetized with CO_2 and placed in an empty plastic box with the sides coated with Teflon to prevent them from escaping. This pooling process minimized the possible effect of colony difference (e.g., presence or absence of brood). About 0.5 g of ants were aspirated from the box and transferred into a large colony box (86 by 42 by 14 cm). Aspirating anesthetized ants resulted in relatively similar-sized colonies with brood as well as workers and reproductives. For example, the average number of ants in a colony was 731 ± 28.1 (mean ± SEM; n = 6; range 600–782) for six replications. All of the experimental colonies were provided at least one queen. The large colony box was provided with one artificial nest constructed from plaster-filled petri dishes (9 cm in diameter by 1.5 cm in depth), and a small plastic dish with 25% sugar water applied to a small piece of cotton. The nest and sugar water dish were placed on a same side of the box (Fig. 1). The entire bottom of the large colony box was covered with a thin layer of dry sand (400 g, play sand, The Quikrete International Inc., Atlanta, GA), providing a more natural substrate for insecticide treatment.

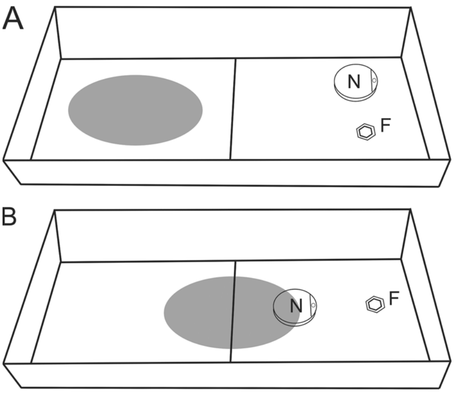

Fig. 1. Experimental settings for effect of pheromone on insecticide spray efficacy study. (A) Fipronil study. (B) Bifenthrin study. The shaded areas on bottom of the boxes indicate the locations where the insecticide sprays were applied. N, nest; F, feeder.

Two common insecticides used by PMPs were tested in the study: fipronil (Termidor SC, BASF, Research Triangle Park, NC) and bifenthrin (Talstar P, FMC Corporation, Philadelphia, PA). Three different spray preparations were tested: [a] pheromone + insecticide, [b] insecticide only, and [c] pheromone only. For fipronil, a 0.01% fipronil preparation was prepared, which was one sixth of the concentration recommended on the label for ant control. For bifenthrin, a 0.01% bifenthrin preparation was prepared, which was one third to one sixth of the concentration recommended on the label for ant control. For the spray combinations with pheromone (preparations [a] and [c]), we added 1 mg of synthetic (Z)-9-hexadecenal dissolved in 1 ml of ethyl alcohol into 500 ml of deionized water. For the preparation [b], we added 1 ml of ethyl alcohol only. All of the spray preparations were mixed well in the spray bottle by shaking vigorously before application. A circular area of 491 cm^2 (25 cm in diameter) either in the opposite side of the nest and sugar water dish (for fipronil) or in the center of the box (for bifenthrin) was treated using the hand sprayer, which delivers ≈3 ml of liquid at a time (Fig. 1). Bifenthrin spray was applied closer to the nest entrance than the fipronil spray, because preliminary experiments indicated that bifenthrin spray of this rate resulted in very low levels of control when it was applied in the same manner as fipronil spray. The amount of spray applied per unit area approximately followed the label recommendations for fipronil and bifenthrin (i.e., 40 and 60 ml/m^2, respectively). The application resulted in rates of ≈ 495, 764, and 12 ng/cm^2 for fipronil, bifenthrin, and (Z)-9-hexadecenal on the sand surface. The treated colonies were maintained at 21–25°C and 34–45% relative humidity (RH). Mortality of the treated colonies was recorded for 4 or 7 d for bifenthrin and fipronil treatments, respectively. Based on preliminary studies, the daily mortality stabilized after these dates and was not different from the controls. The dead ants were removed from the colonies after being counted. Each treatment was replicated six to nine times.

Statistical Analyses. For experiments 1 and 2, the count values were averaged from all posttreatment observations (i.e., two and three observations for experiments 1 and 2, respectively), and were compared between treatment and control patch with a paired one-sided *t*-test (Analytical Software 2008). For experiment 3, the total cumulative number of dead ants was compared between treatments with a one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) all-pairwise comparison test (Analytical Software 2008).

Results

Effect of Pheromone Deposit on Workers from Nearby Trails and Nest Entrance. The sand patches treated with pheromone spray attracted Argentine ant workers from nearby trails and nest entrance. In experiment 1, the number of ants on the sand patch treated with pheromone spray was more than two times higher than that of the control patch ($T = 2.52$; df = 4; $P = 0.033$). The average number on the treatment and control sand patches was 10.4 ± 4.0 and 4.6 ± 2.1 (mean ± SEM), respectively (Fig. 2A). In experiment 2, the number of ants on the sand patch treated with pheromone spray was more than five times higher than that of control patch ($T = 4.67$; df = 9; $P < 0.001$). The average number on the treatment and control sand patches was 18.1 ± 3.0 and 3.6 ± 0.7 (mean ± SEM), respectively (Fig. 2B).

Effect of Pheromone on Efficacy of Insecticide Sprays. The application of insecticide and pheromone combination sprays resulted in significantly higher mortality in the laboratory colonies of Argentine ants than did the application of insecticide only. For the fipronil, day 7 mortality levels were significantly different between the three different treatments ($F = 48.8$; df = 2, 13; $P < 0.001$). The cumulative number of dead ants in the "pheromone + fipronil" treatment was significantly higher than that in the "fipronil only" treatment (458 ± 36.8 vs. 291 ± 27.1, mean ± SEM, $n = 5$ and 6, respectively; Tukey HSD all-pairwise comparison test). The cumulative mortality of the "pheromone only" treatment at day 7 was 65.8 ± 11.0 (mean ± SEM; $n = 5$), being significantly lower than the other two treatments with fipronil (Fig. 3A). For the bifenthrin, the cumulative mortality at day 4 in the pheromone + bifenthrin treatment was significantly higher than that in the "bifenthrin only" treatment (402 ± 48.6 vs. 166 ± 38.4, mean ± SEM, $n = 6$, respectively; $F = 14.5$; df = 1,10; $P = 0.003$; Fig. 3B).

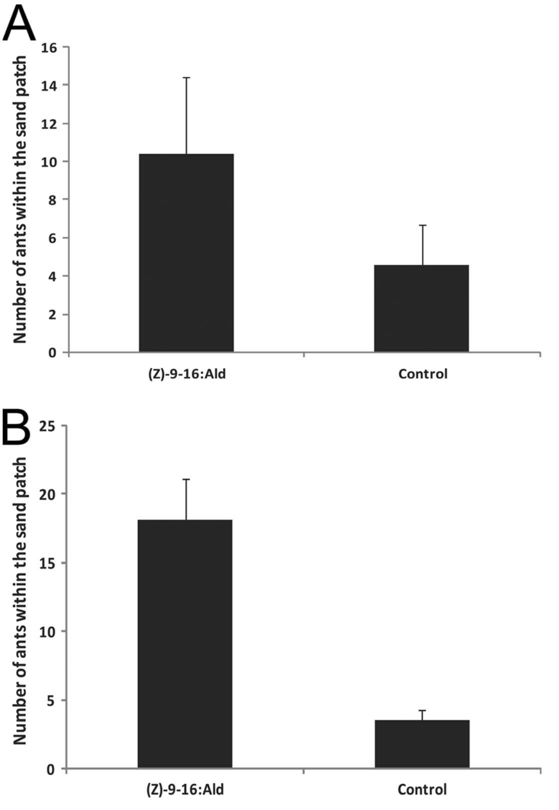

Fig. 2. Effect of (Z)-9-hexadecenal ((Z)-9–16: Ald) on Argentine ant foragers. (A) Number of ants attracted to the treated area from nearby trails. (B) Number of ants attracted to the treated area from nearby nest entrance. The height of each bar indicates the mean number of worker ants that are attracted to the treated patch from the nearby trails or nest entrance (±SEM). See text for the details of statistical analysis.

Discussion

Aqueous suspensions of (Z)-9-hexadecenal applied on the soil surface attracted Argentine ant workers from nearby trails and nest entrances. The number of foragers attracted increased by 126–403% when compared with the number of ants found in control areas. Argentine ant workers are extremely sensitive to low concentrations of (Z)-9-hexadecenal, and are attracted to the source of (Z)-9-hexadecenal from a distance, at least in part, via odor-mediated anemotaxis (Van Vorhis Key et al. 1981; Van Vorhis Key and Baker 1982a,b; Choe et al. 2012). However, high concentrations of (Z)-9-hexadecenal applied in point sources were repellent or disruptive to the foraging Argentine ants (Greenberg and Klotz 2000, Suckling et al. 2011). Thus, the concentration of the pheromone in the spray preparation may be critical to achieve the desired effect, the attraction of ants to the treated area.

Our method required 1 mg of synthetic (Z)-9-hexadecenal per 500 ml of spray preparation (0.002 mg/ml). Considering the typical amount of spray preparation applied in an average size house is ≈1.9–3.8 liters (0.5–1 gal), the total amount of pheromone required for treating a house would be <10 mg. Based on the current price of the synthetic pheromone (US $36.76 for 1 g), 10 mg of synthetic (Z)-9-hexadecenal

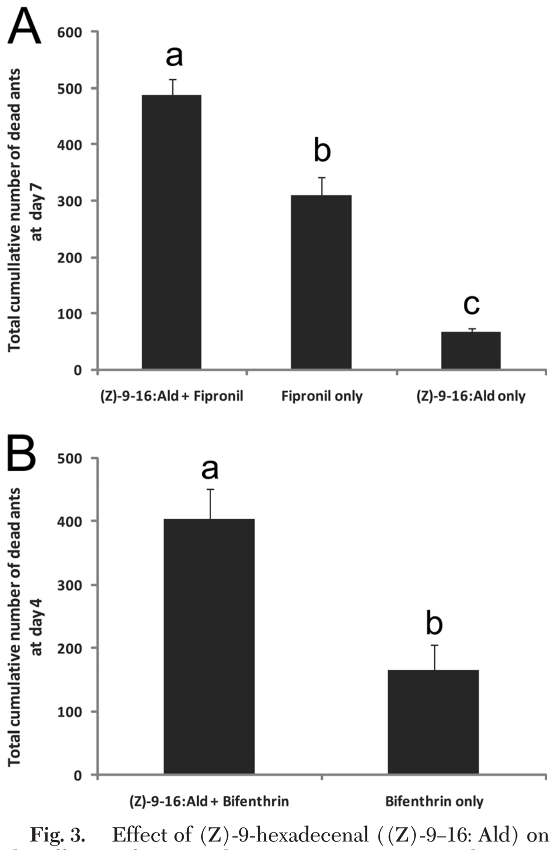

Fig. 3. Effect of (Z)-9-hexadecenal ((Z)-9–16: Ald) on the efficacy of insecticide sprays. (A) Comparison between pheromone + fipronil, fipronil only, and pheromone only. (B) Comparison between pheromone + bifenthrin and bifenthrin only. The height of each bar indicates the mean cumulative mortality of ants (workers and reproductives; ±SEM) by day 7 or 4 for fipronil and bifenthrin treatments, respectively. Within a trial, means with different letters are significantly different at the $\alpha = 0.05$ level. See text for the details of statistical analysis.

would cost ≈US$0.37. If the pheromone-assisted techniques are effective in reducing the amount insecticide that would be necessary to achieve a satisfactory level of control, homeowners or PMPs could reduce the amount of active ingredient applied in the environment and the cost of insecticides. For example, if the one sixth of label-recommended rate for Termidor would be sufficient when it is supplemented with the pheromone, this would save ≈US$1.80 per gallon of spray applied based on the current cost of Termidor SC (US$53 for 20 oz bottle). Thus, the pheromone-assisted technique would be economically viable for both general public consumers and PMPs.

Our laboratory studies with fipronil and bifenthrin sprays demonstrated that the addition of (Z)-9-hexadecenal in the insecticide sprays increased the insecticidal efficacy by 57–142% based on the cumulative mortality of ants. Because the pheromone was not insecticidal by itself (see Fig. 3A), the improvement in efficacy could be attributed to the attraction of ants from nearby locations, and subsequent exposure of the ants to lethal doses of the insecticides. We propose three possible advantages of this pheromone-assisted technique. First, with the pheromone-assisted technique, the insecticide spray deposits may be applied to porous surfaces and will still control the target ant species by attracting them to the insecticide deposits. This would interest many PMPs, particularly because of the recent regulatory and product label changes on several pyrethroid insecticides that prohibit perimeter applications of pyrethroids on hardscapes such as driveways and walks (CDPR 2012, United States Environmental Protection Agency [US EPA] 2013). Second, the insecticide and pheromone directly applied on active trails and the nest entrance might maximize the transfer of the insecticide to the target pests. Our preliminary observations from laboratory and field indicate Argentine ant workers attracted to the pheromone deposit stayed within the treated area for extended period of time (e.g., 10 min; D.-H.C., unpublished data). Saran and Rust (2007) showed a direct relationship between the length of exposure and the amount of insecticide picked up by termites. Increasing the time that ants contact the treated surface will reduce the quantity of insecticide applied in the environment without losing its efficacy to control the target pest ants. Third, the pheromone-assisted technique will increase the likelihood of ants contacting the insecticide residue before any significant degradation of the active ingredient of insecticide occurs.

From a practical standpoint, a future development of proper formulation of the (Z)-9-hexadecenal would improve its efficacy and usability. In the current study, pure synthetic (Z)-9-hexadecenal was dissolved in ethyl alcohol and mixed into the aqueous suspension. The sand sprayed with the pheromone aqueous preparations was attractive for <60 min. Field conditions, such as direct sunlight and extreme temperature would also negatively influence the persistence of the pheromone. The physicochemical characteristics of the pheromone formulation would be important factors in improving the persistence of the effect. Other solvent systems might help retard the loss of activity. Proper packaging may be necessary because the stability of pheromone might be negatively affected if it is mixed with the insecticide formulation for long-term storage. The pheromone and insecticide formulations might be prepared in the required concentrations in a solvent, packaged in separate containers, and mixed before use in the field. We are currently conducting field studies with a microencapsulated pheromone formulation with an industry collaborator to address these questions.

Further research is needed to determine whether other formulations of insecticide such as baits and granules might be combined with (Z)-9-hexadecenal. The pheromone might increase the likelihood that ants contact insecticidal granules or dusts. The discovery of insecticidal baits might also be facilitated by the addition of pheromone. Foraging ants would quickly ingest and transport the bait before degradation of the active ingredient or physicochemical changes of bait matrices occur, thus maximizing the efficacy. The pheromone can be also used to improve a previous novel baiting concept, "virtual baiting" (Choe et al. 2010). In the virtual bait station, foraging ants were permitted to cross surfaces treated with diluted insecticide en route to a sugar water feeder. Instead of using food items as attractants, the pheromone can be used to lure the target insect and expose them to a lethal dose of insecticide that is containerized in a station. The use of (Z)-9-hexadecenal with these control measures might also provide some levels of species specificity for urban integrated pest management program by attracting particular target species or groups of related species, while having little or no effect on nontarget ants or other invertebrates, but this potential advantage warrants further research.

Acknowledgments

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