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Electrophysiological and alarm behavioral responses of *Solenopsis invicta* Buren (Hymenoptera: Formicidae) to alkoxypyrazines

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ABSTRACT

The red imported fire ant, *Solenopsis invicta* produces an alarm pheromone component, 2-ethyl-3,6-dimethylpyrazine, and responds to its pyrazine analogs in a similar manner but at varied detection thresholds. Herein, the responses of fire ant workers by electroantennogram (EAG) and behavior were tested with twelve structurally-related oxygen-containing pyrazines (alkoxypyrazines) and the synthetic alarm pheromone. All tested compounds elicited a dose-dependent EAG response, with *S. invicta* responding greatest to the synthetic alarm pheromone. Chemical structure of pyrazines influenced the EAG response but not always alarm behavioral response. Among the 13 tested compounds, 7 compounds displayed significantly greater EAG response than 2-isopropyl-3-methoxypyrazine and 2-ethoxy-3-isopropylpyrazine at the dose of 1000 μg. Four of these 7 compounds, 2-ethyl-3,6-dimethylpyrazine, 2-methoxy-3-methylpyrazine, 2-ethoxy-3(5 or 6)-methylpyrazine, and 2-chloro-3-methoxypyrazine with characteristic substituents on pyrazine ring were further subjected to bait discovery bioassay. Hotdog bait containing pyrazines attracted significantly more fire ant workers in the first 15-min period, resulting in quicker recruitment to food block than hexane control. The potential of using alkoxypyrazines in fire ant control is discussed.

Introduction

In responding to disturbances or potential dangers such as a predator, ants emit alarm pheromones to alert or recruit nearby conspecifics, and often stimulate aggressive behavior. Aggressive behaviors may include frenzied running while attacking alien objects around the source of the disturbance (Blum, 1985). Reactions to alarm pheromones largely depend on the pheromone concentration. At low concentrations, alarm pheromone functions as an attractant where ants move towards the source of the pheromone, while at higher concentrations the typical alarm behavior is triggered (Blum, 1969).

Ant species with large, densely concentrated colonies cannot disperse readily and often attack the source of disturbance (Vander Meer and Alonso, 1998). The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), is an important pest throughout introduced regions, such as southern United States and China (Ascunce et al., 2011). Marked aggressive behavior, potent sting, a high reproductive potential, and lack of natural enemy have made this species a significant agricultural, medical, and urban pest (Fadamiro et al., 2009; Morrison, 2000). The exposed nest mounds of *S. invicta* may reach 30–50 cm in diameter and often over 35 cm in height, and contain 200,000–400,000 workers (Vinson, 1997). The ability to reach great population size and high population densities together with aggressiveness provides *S. invicta* notable invasive success, displacing native species (Fadamiro et al., 2009; Morrison, 2000).

Ants typically employ a complex of pheromones in colony communications (Ali and Morgan, 1990; Parry and Morgan, 1979; Vander Meer and Alonso, 1998). One example of which is the use of alarm pheromones to alert nestmates of colony disturbance. Wilson (1962) proposed that the source of the fire ant alarm pheromone was in the cephalic region, possibly in the mandibular glands as in other myrmicine species. 2-Ethyl-3,6-dimethyl pyrazine from the mandibular gland of *S. invicta* was identified as an alarm pheromone (Vander Meer et al., 2010). The incorporation of alarm pheromonal component from leaf-cutting ants (*Atta* spp.), 4-methyl-3-heptanone into baits has previously proved to substantially increase the attractiveness and harvest of the bait sachets (Hughes and Goulson, 2002; Hughes et al., 2002). A previous report screened the alarm activity of 14 analogs of 2-ethyl-3,6-dimethylpyrazine, demonstrating that 2,3,5-trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, and 2,3-diethyl-5-methylpyrazine elicited

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most significant alarm responses from *S. invicta* workers, and that incorporation of these alkypyrazines to food bait proved to enhance bait attractiveness (Guan et al., 2014).

In the present study, we aimed to examine both the electroantennogram (EAG) and behavioral responses of *S. invicta* workers to a synthetic fire ant alarm pheromone – 2-ethyl-3,6-dimethylpyrazine and 12 structurally-related commercially available oxygen-containing pyrazines (alkoxypyrazines) of relatively high volatility. Pyrazines yielding a positive response were further tested for enhanced attractiveness on baits.

Materials and methods

Ant sources and maintenance

Mature colonies of fire ant, *S. invicta* were collected from the campus of South China Agricultural University (Guangzhou, Guangdong Province, China). They were first transported to a local laboratory, separated from soil by flotation, maintained in fluon coated plastic trays while being provided with Milli-Q water and 10% honey solution ad libitum, and frozen *Gryllus testaceus* Walker every other day. Tests were carried out within a month of ant collection.

Test chemicals

The alarm pheromone component, 2-ethyl-3,6-dimethylpyrazine, was synthesized as described in Fang and Cadwallader (2013). Twelve structurally-related alkoxypyrazines were purchased from Sigma-Aldrich (Shanghai, China) or Acros Organics (Geel, Belgium) (Table 1: Compounds **2**–**13**). Each compound was diluted with HPLC-grade *n*-hexane to 100 μg/μL solutions. Further hexane dilutions ranging 10 μg/μL to 0.01 ng/μL were prepared for EAG and behavioral tests. All solutions were stored at −20 °C until needed.

EAG experiment

The antennal response of *S. invicta* workers to pyrazines was evaluated as described in previous studies (Chen and Fadamiro, 2007; Guan et al., 2014). Briefly, two glass electrodes filled with Ringer's solution were used, where one was connected to the base of an excised major worker head, and the other was kept in contact with the intact tip of one antenna. No cutting on the tip of the antenna is needed as olfactory sensilla are mainly distributed on the club, i.e. the last two antennal segments (9th and 10th segments) (Renthal et al., 2003). AgCl-coated silver wires were used to sustain the current between the antennal setting and a PRG-3 sensory probe. The analog signal was amplified with a data acquisition controller IDAC-4, and analyzed with the software EAG 2000 (all from Syntech, Kirchzarten, Germany). Stimuli were always in 10 μL of hexane solution applied to a filter paper strip (4 × 40 mm) inserted into Pasteur pipettes (15 cm long). Fresh stimulus pipettes were prepared before each set of experiments. Pure hexane was used as a control. Stimuli were delivered as 0.2 s puffs at a humidified flow rate of 1000 mL/min generated by an air stimulus controller CS-55 (Syntech, Kirchzarten, Germany). The front side of the antenna in preparation was oriented to the humidified airflow for EAG recordings.

A series of pyrazines at the same dose were applied to a single antennal preparation and the presentation order of these compounds was randomized. A blank stimulus (solvent control) was presented before and after testing the 13 compounds. A total of five doses (0.1, 1, 10, 100, or 1000 μg) were tested. The amplitude (mV) of the EAG response to each test stimulus was adjusted to compensate for solvent and/or mechanosensory artifacts by subtracting the mean EAG response of the two hexane controls. EAG recordings were obtained from 8 antennal preparations for each dose. The corrected EAG data were analyzed for each dose using one-way analysis of variance (ANOVA) to detect significant differences among treatments with each compound. Means across all 13 compounds for any given dose were compared by Tukey-Kramer HSD comparison test ($P < 0.05$) (SAS Institute, 2004).

Behavioral bioassay

The alarm response of workers to pyrazines was evaluated in a still-air tested arena as generally described by Vander Meer et al. (2010) with modifications described in Guan et al. (2014). At least 2 h prior to each bioassay, 0.1 g of workers (approximately 100–150) from a colony were added to a plastic cup (9 cm tall × 14 cm i.d.) painted from inside with Fluon to prevent escape. A 1-cm^3 sugar-agar block (10% sugar water + 1% solidified agar) was placed at the bottom of the cup to allow feeding. Water was provided in a 5-mL plastic tube clogged with a cotton ball at the open side. A filter paper strip (1 × 3 cm) folded into a triangle was put in an empty space in the bottom of the cup. Ten microliters of sample solution were gently loaded onto the filter paper strip. The response to a given stimulus was evaluated on the number of workers that were running out of the quiescent group and/or displaying disoriented alarm behavior during the recording time. New filter paper strips were used for each test. All individual replicates (i.e., cups containing subcolonies) were tested for a control (10 μL of hexane) and for all of the compounds at a same dose. Each individual replicate was evaluated in sequence, while the compounds presented were randomized in order. Workers of a test unit that were alarmed were given over 30 min of interval between tests to minimize the influence of pre-exposure to a different pyrazine. A series of pyrazines at the same dose (0.1, 1, 10, 100 or 1000 ng) was replicated 8 times with ants from 8 different colonies. Bioassay data was determined to be normally distributed and then analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD comparison test ($P < 0.05$) to establish significant differences among the treatments (SAS Institute, 2004).

Bait attraction bioassay

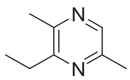
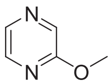
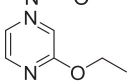
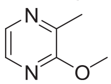
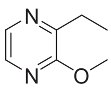
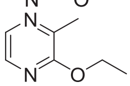
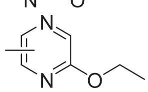
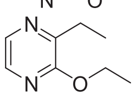
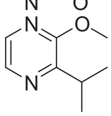
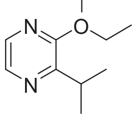
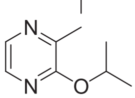
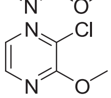
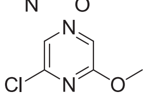
Four pyrazines with relatively high activity in both EAG and behavior bioassays were selected for further food block test as described by Guan et al. (2014). Briefly, 0.5 g of *S. invicta* workers were transferred into a plastic tray (55 cm diameter at the bottom) with the inside wall painted with Fluon to prevent escape. A moisturized flat cotton wad was placed in a Petri dish (d = 6 cm) to provide water at the center of the tray. Ants were always drawn to the cotton wad, where they remained for 2 h for acclimatization. Three aluminum foil disks (5 cm diameter) were then placed at 20 cm away from the center of the tray and at equal distance from each other. On top of each foil disk, a filter paper disk (4 cm diameter) was placed. Prior to the start of each evaluation, one block of hotdog (0.1 g) as a bait was placed onto the filter paper disk. Ten microliters of hexane were gently applied to both the filter paper disk and the hotdog block (5 μL each). For the other two hotdog blocks, 10 μL of compound solution (0.1 ng/μL) were loaded either onto the filter paper disk or onto the food block. The numbers of ants on a filter paper disk were counted 2, 5, 15, 30, and 45 min after the application of the test solution. Each sample solution was tested 8 times. Mean numbers of ants on different filter paper disks (i.e., hotdog bait on filter paper (hexane control), hotdog bait on treated filter paper and treated hotdog bait on filter paper) at each observation time period were compared by using Tukey-Kramer HSD comparison test ($P < 0.05$) (SAS Institute, 2004).

Results

EAG response

As expected, all tested compounds elicited dose-dependent responses from the antennae of *S. invicta* workers (Fig. 1). Statistical analyses (ANOVA) of the corrected EAG data revealed that both dose (F =

Table 1
Chemical parameters of pyrazines tested with *Solenopsis invicta* workers.

No.	Structure	Name	Molecular formula	Molecular weight	Purity
1		2-Ethyl-3,6-dimethylpyrazine	$C_8H_{12}N_2$	136	99%
2		2-Methoxypyrazine	$C_5H_6N_2O$	110	95%
3		2-Ethoxypyrazine	$C_6H_8N_2O$	124	98%
4		2-Methoxy-3-methylpyrazine	$C_6H_8N_2O$	124	98%
5		2-Ethyl-3-methoxypyrazine	$C_7H_{10}N_2O$	138	95%
6		2-Ethoxy-3-methylpyrazine	$C_7H_{10}N_2O$	138	98%
7		2-Ethoxy-3(5 or 6)-methylpyrazine	$C_7H_{10}N_2O$	138	99%
8		2-Ethoxy-3-ethylpyrazine	$C_8H_{12}N_2O$	152	98%
9		2-Isopropyl-3-methoxypyrazine	$C_8H_{12}N_2O$	152	97%
10		2-Ethoxy-3-isopropylpyrazine	$C_9H_{14}N_2O$	166	95%
11		2-Isopropoxy-3-methylpyrazine	$C_8H_{12}N_2O$	152	95%
12		2-Chloro-3-methoxypyrazine	$C_5H_5ClN_2O$	144.56	95%
13		2-Chloro-6-methoxypyrazine	$C_5H_5ClN_2O$	144.56	98%

707.39; df = 4, 455; $P < 0.0001$) and compound (F = 27.85; df = 12, 455; $P < 0.0001$) influenced EAG response. A significant interaction of dose × compound (F = 9.27; df = 48, 455; $P < 0.0001$) was also recorded on the EAG responses. However, no significant differences were recorded among EAG responses of ant workers to compounds at the lowest dose, 0.1 μg. Antennal responses to the synthetic alarm pheromone **1** varied 0.05–2.14 mV across all tested doses. Compared with Compound **1**, EAG responses to alkoxypyrazines **2**, **3**, **4**, **9** was significantly lower at the intermediary doses of 1 and 10 μg. At the dose of 100 μg, alkoxypyrazines **5** and **7** elicited EAG responses similar to Compound **1** while alkoxypyrazines **2**, **9**, and **10** displayed relatively low response. At the dose of 1000 μg, all alkoxypyrazines were significantly lower than Compound **1**, and alkoxypyrazines **9** and **10** generated the lowest EAG response. At doses of 1 and 10 μg, alkoxypyrazine **2** triggered the lowest EAG responses but a moderate response at the dose of 1000 μg.

Behavioral response

All pyrazines elicited observable alarm behavioral responses of *S. invicta* workers (Fig. 2). Similar to the results obtained for EAG response, significantly different effects from dose (F = 13.29; df = 4, 630; $P < 0.0001$) and compound used (F = 85.72; df = 13, 630; $P < 0.0001$) were recorded. However, there was no significant interaction of dose × compound (F = 0.94; df = 52, 630; $P = 0.5981$). Among the 13 tested pyrazine compounds, the synthetic alarm pheromone **1** elicited the significantly greater responses at every dose tested. Generally, the alarm responses induced by the 12 alkoxypyrazines were not significantly different at all test doses.

Bait attraction enhancement

Four pyrazines, 2-ethyl-3,6-dimethylpyrazine (**1**), 2-methoxy-3-methylpyrazine (**4**), 2-ethoxy-3(5 or 6)-methylpyrazine (**7**), 2-chloro-3-methoxypyrazine (**12**), which displayed significant EAG responses at high doses were selected for bait bioassays. Ant workers showed strong alarm response towards the odor sources, and more workers were observed on compound-marked food than on hexane-marked food (Fig. 3). Compound applied to the filter paper attracted slightly more workers than direct application to food block in all cases. However, the difference between the two treatments in all observation time periods was not significant. The number of workers on hexane-treated food increased continuously and stabilized around 45 min. However, the rate of increase was steeper within the first 15 min on the

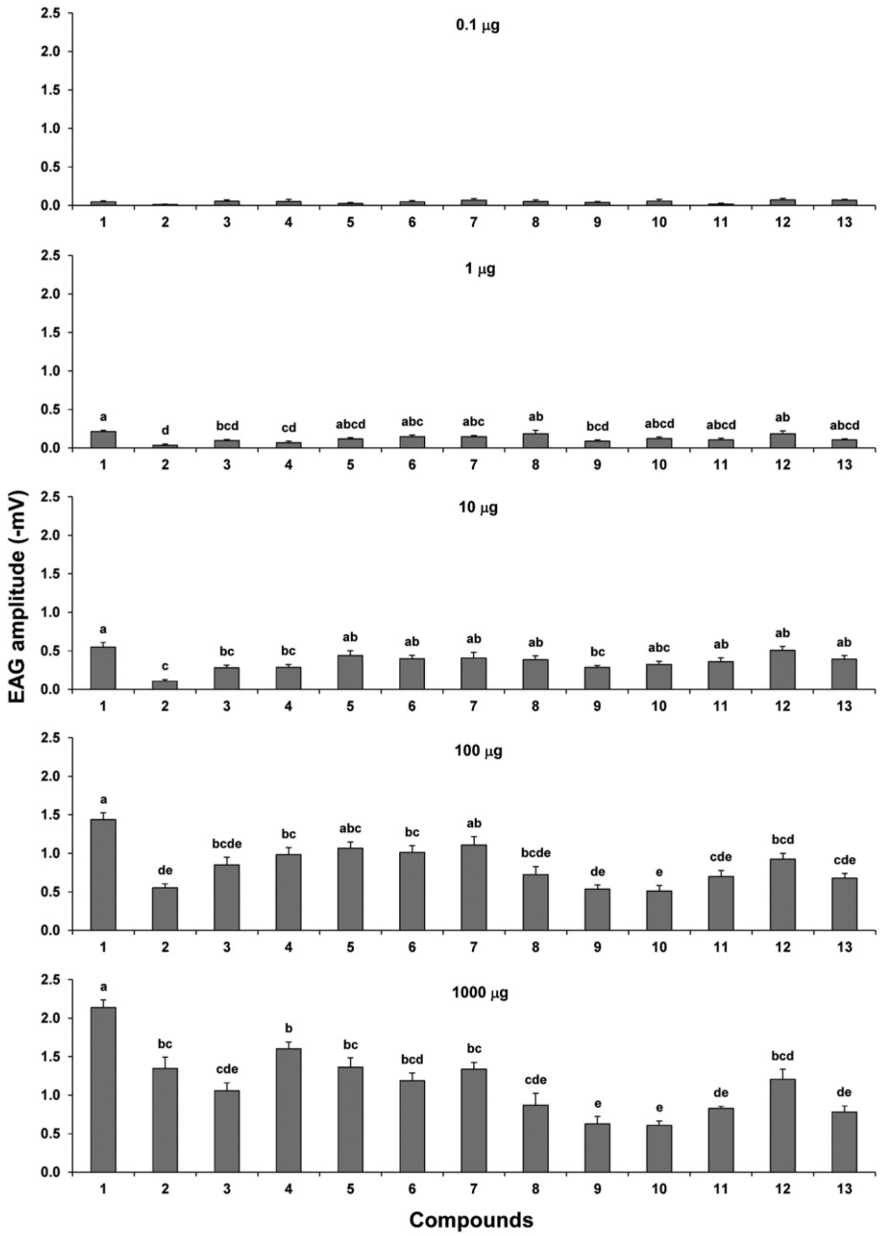


Fig. 1. EAG response (mV ± SE) of *Solenopsis invicta* workers to 12 alkoxypyrazines structurally related to an alarm pheromone. The doses shown are what were impregnated on the filter papers, not the actual dose delivered to the antennal preparation. Refer to referenced compounds' names on Table 1. Different letters within the same dose indicate significant difference at $P < 0.05$ by Tukey-HSD test, and no letters indicate no difference between responses.

compound-treated food blocks than controls. At the end of observation, no significant difference in the number of workers was observed among the pyrazine compound treatments.

Discussion

In previous studies a commercially available mixture of 2-ethyl-3,6(5)-dimethylpyrazine triggered significant EAG and/or behavioral responses from fire ant workers (Guan et al., 2014; Vander Meer et al., 2010). Here we demonstrated that synthetic 2-ethyl-3,6-dimethylpyrazine elicited the greatest EAG response at high doses and was always top-ranked at lower doses. Furthermore, synthetic 2-ethyl-3,6-dimethylpyrazine evoked significantly stronger behavioral response at all doses tested. There were wide variations in the EAG response from the 12 tested alkoxypyrazines. However, we did not observe significant difference in behavioral alarm response among the 12 alkoxypyrazines at the lowest and highest doses.

The six methoxy-substituted pyrazines, 2-methoxypyrazine, 2-isopropyl-3-methoxypyrazine, 2-chloro-6-methoxypyrazine generated lower EAG response than 2-methoxy-3-methylpyrazine, 2-ethyl-3-methoxypyrazine, and 2-chloro-3-methoxypyrazine at most doses. EAG responses to all five ethoxy-substituted pyrazines were not different at lowest doses. However, 2-ethoxy-3-isopropylpyrazine was much less active than the remaining four ethoxypyrazines at high doses. Isopropoxy substituted pyrazine, 2-isopropoxy-3-methylpyrazine was moderately active. Alkoxypyrazines tested in this study appear to be as active as the alkylpyrazines tested in a previous similar investigation (Guan et al., 2014).

Most methoxypyrazines possess flavors with very low odor threshold ranging from 0.024–400 ng/L (Maga, 1982). In general, alkoxypyrazines possess lower odor thresholds than corresponding alkylpyrazines, possibly due to hydrogen bond formation resulting from the methoxy group (Maga, 1992). Although it was judged to contribute the most to characteristic food flavor with an odor threshold as

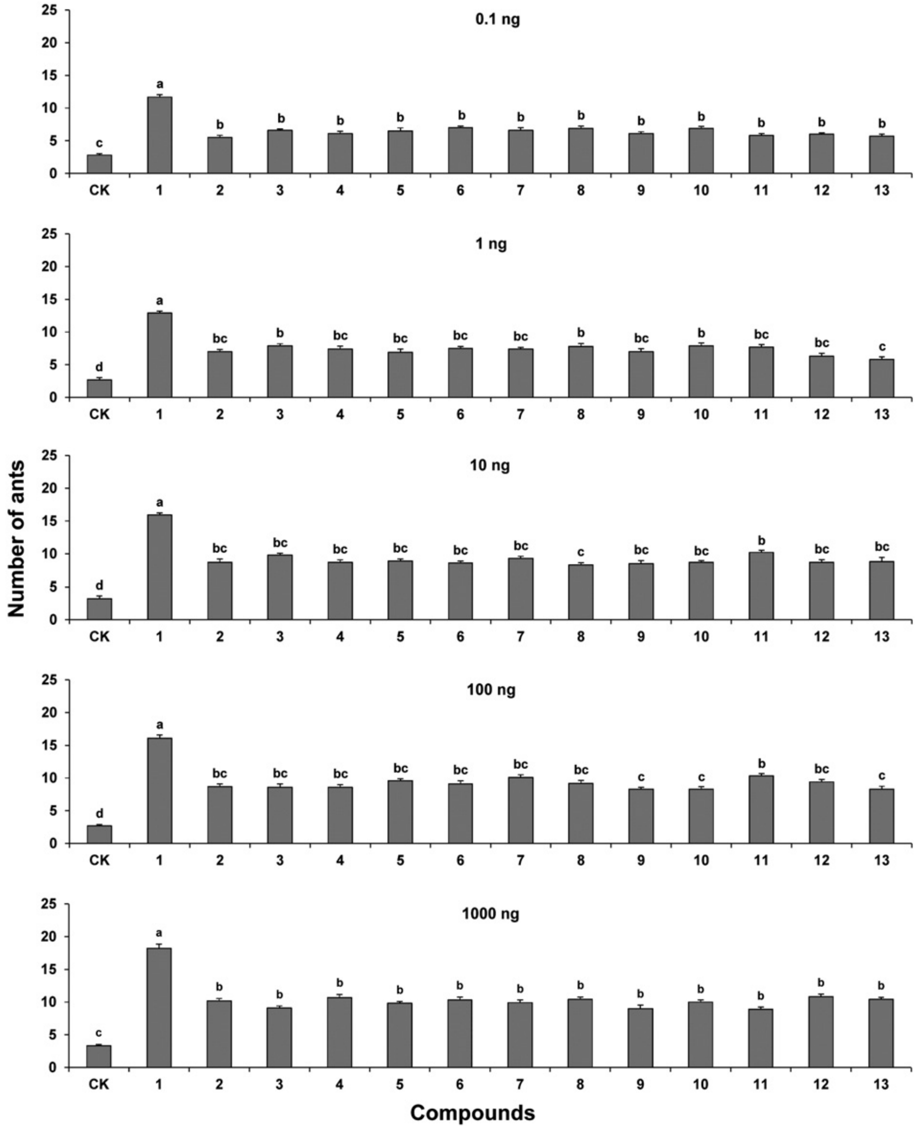


Fig. 2. Behavioral alarm response of *Solenopsis invicta* workers to 12 alkoxypyrazines structurally related to an alarm pheromone. The doses shown are what were impregnated on the filter papers. Refer to referenced compounds' numbers in Table 1. CK is hexane. Different letters within the same dose indicate significant difference at $P < 0.05$ by Tukey-HSD test, and no letters indicate no difference between responses.

low as 0.024 ng/L (Mihara and Masuda, 1988), 2-isopropyl-3-methoxypyrazine showed lower EAG activity than other alkoxypyrazines. As alkoxypyrazines are often present in various vegetables and plants (Maga, 1982, 1992; Maga and Sizer, 1973), fire ant workers might utilize these odorants for foraging, which represents a good opportunity for using these naturally occurring chemicals as insect attractants.

In contrast to dispersing behavior in aphids from feeding sites in response to (*E*)-β-farnesene (a main component of the alarm pheromone of many species of aphids) (Pickett and Griffiths, 1980), ants are generally attracted to emission sources of alarm pheromones (Blum, 1969). Therefore, such pheromones have the potential to rapidly recruit large numbers of nestmates. In this study, fire ant workers were observed to move rapidly towards pyrazines applied to food blocks and attack the bait intensely by biting, but not to feed on the food block at the initiation of the test. After few seconds, the workers started feeding on food, and more and more workers were attracted to the food block partially because of the attraction of alarm substance evaporated from food block. Part of workers was likely recruited out of their "nest" by food-fed nestmates. Even though nestmate recruitment by alarmed workers is likely confounded with attraction to the alkoxypyrazines, our observation demonstrated that alkoxypyrazines can trigger alarm behavior similar to fire ant alarm pheromone component, 2-ethyl-3,6-dimethylpyrazine. Statistically significantly more workers were attracted to food containing 2-ethyl-3,6-dimethylpyrazine and 2-chloro-3-methoxypyrazine in the first 5-min period than to bait incorporated with 2-methoxy-3-methylpyrazine and 2-ethoxy-3(5 or 6)-methylpyrazine, possibly due to lower activity of ants to controls. Compounds applied directly to filter paper showed similar attractiveness to that applied to food in the first 5-min period. The surface area of the filter paper, likely leading to increase in odor evaporation, did not change the recruitment rate significantly for that to the hot dog plus odor.

The application of pyrazines to food baits may have potential application to improving the efficacy of fire ant toxic baits; such possibility warrants specific investigations, e.g. outdoor longevity of the odor, from how far away the ants are attracted, enhancement on bait consumption. Although pheromone attractants may not always increase bait consumption (Troyer et al., 2009), we did not observe any negative effect of fire ant alarm pheromone and its pyrazine analogs on food consumption in the present study. Fire ant bait is a relatively safe, practical, and effective method commonly used for fire ant control. Synthetic alarm pheromone or analogs could be tested in combination with a

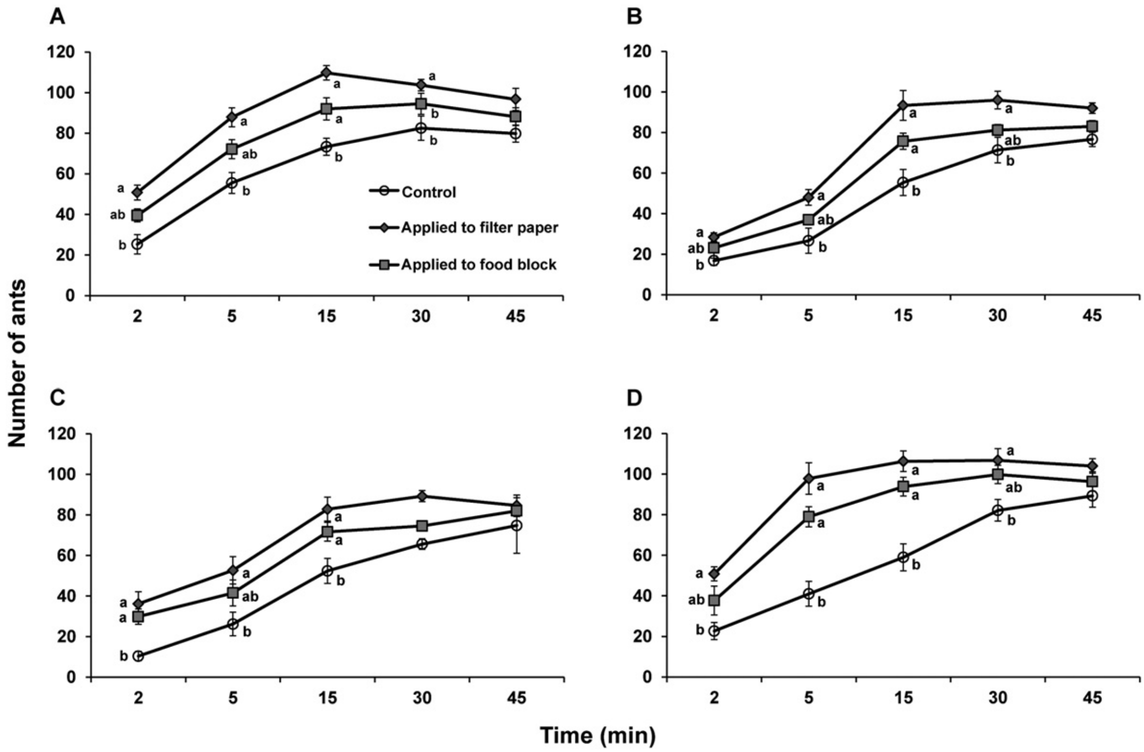


Fig. 3. Attractiveness of *Solenopsis invicta* workers to hotdog baits on filter paper (control), hotdog baits on treated filter paper and treated hotdog baits on filter paper with EAG-active pyrazines at the dose of 1 ng. (A) 2-Ethyl-3,6-dimethylpyrazine, (B) 2-methoxy-3-methylpyrazine, (C) 2-ethoxy-3(5 or 6)-methylpyrazine, (D) 2-chloro-3-methoxypyrazine. Means within the same time mark with different letters are significantly different at $P < 0.05$ by Tukey-HSD test, and no letters indicate no difference between treatments.

slow acting stomach-poison insecticide to test for increased efficiency against fire ant workers. The pheromones are expected to excite workers and young larvae to consume through trophallaxis more toxic bait taken back to the nest by foragers. Alkoxypyrazines may hold potential as an alternative in integrated pest management strategies for controlling fire ants.

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