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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 =