Assessment of Repellency of Nine Phthalates against Red Imported Fire Ant (Hymenoptera: Formicidae) Workers Using Ant Digging Behavior[1]

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Abstract Repellency of nine phthalates against red imported fire ant workers, *Solenopsis invicta* Buren, was evaluated using ant digging behavior. Test compounds included dimethyl, diethyl, dipropyl, dibutyl, dipentyl, dihexyl, diheptyl, dioctyl, and dinonyl phthalates. The active ingredient was incorporated into sand within a liquid scintillation vial with an entry hole on the cap. Fire ant workers dug and removed sand from the vial through the entry hole. The differences in amount of sand removed from the treated and control vials were used to evaluate chemical repellency. Of the 9 phthalates, dimethyl and diethyl phthalates were most repellant to red imported fire ant workers. The minimum repellant concentration within 24 h was 100 ppm for both dimethyl and diethyl phthalates.

Key Words *Solenopsis invicta*, repellency, dimethyl phthalate, diethyl phthalate

The red imported fire ant, *Solenopsis invicta* Buren, native of Mato Grosso in central Brazil (Buren 1972, Buren et al. 1974), was introduced into United States in 1930s (Vinson 1997). Since its introduction, the red imported fire ant has spread throughout nine southeastern states and to limited areas in Arizona, Oklahoma, Tennessee, New Mexico and California. The range of *S. invicta* distribution in the U.S. now covers 128 millions ha (Drees and Gold 2003). *Solenopsis invicta* is one of the most important medical and agricultural pest ants (Lofgren et al. 1975, Lofgren 1986, Adams et al. 1983, 1988, Drees and Gold 2003). Efforts to develop chemical and biological control measures for the red imported fire ants have produced numerous insecticide products (Williams et al. 2001) and the successful releases of *Thelohania solenopsae* Knell, Allen and Hazard, a protozoan parasite of fire ants (Williams et al. 1999, Williams and Brenner 2001), and *Pseudacteon* spp., small phorid flies that parasitize fire ants (Orr et al. 1995, Williams and Brenner 2001).

Increasing concerns of environmental impacts of insecticides demand development of nontoxic or less-toxic measures. One such alternative is the use of repellants (Vander Meer et al. 1993). Many materials have been reported as fire ant repellants (Blum et al. 1991, Kaakeh and Dutch 1992, Vander Meer et al. 1993, 1996, 1998, Oi and Williams 1996, Anderson et al. 2002). Blum et al. (1991) reported that combina-

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tion of decanoic and isovaleric acids was highly repellant to *S. invicta* workers. By using a Y-tube olfactometer, Vander Meer et al. (1996, 1998) also identified a number of fire ant repellants. Anderson et al. (2002) compared the number of ants on a filter paper treated with a leachate solution to that of ants on a control in a plastic tray. They found that sage (*Saliva* sp.), pine needle, and cedar shaving water suspension were repellant to *S. invicta* colonies. Oi and Williams (1996) found bifenthrin and tefluthrin in potting soil repelled red imported fire ants.

In laboratory studies, *S. invicta* shows digging behavior whenever adequate substrate, such as a layer of sand, is provided. In addition, in the field, *S. invicta* build nests and underground foraging tunnels by digging up surrounding soil. The extensive tunnel system gives foragers access to any point in their foraging territory without traveling long distances above ground (Markin et al. 1975). The tunnel system also makes it possible for workers to forage at a wider range of soil surface temperatures (Porter and Tschinkel 1987). Digging behavior is clearly essential to ecological dominance of most ant species, including *S. invicta*.

The objective of this study was to evaluate the repellency of nine phthalates against red imported fire ant workers by using fire ant digging behavior. Dimethyl and diethyl phthalates are common ingredients of mosquito repellants (Schreck 1991, Frances et al. 1993, Anonymous 1995, Frances and Cooper 2002). Dimethyl and dibutyl phthalates were listed as insect repellants in Compendium of Pesticide Common Names which was approved by International Organization for Standardization (Anonymous 2004). However, repellency of those phthalates against red imported fire ants has not been evaluated.

Materials and Methods

Test insects. *Solenopsis invicta* colonies were collected on 23 September 2003 in Washington Co., MS, and 25 March 2005 in Pearl River Co., MS. Ant mounds were collected by shovel and placed in a 19-L plastic bucket. The top of the bucket was coated with mild baby powder (Cumberland Swan Holdings, Inc., Smyrna, TN) to prevent ant escape. Each colony was maintained in a 44.5 × 60.0 × 13.0 cm plastic tray after ants, and brood were separated by using a water-drip method (Banks et al. 1981). The inside wall of the tray was coated with Fluon® (Ag Fluoropolymers, Chadds Ford, PA). Colonies were provided with water, 10% sugar solution, and cotton bollworm, *Heliocoverpa zea* (Boddie) and tobacco budworm, *Heliothis virescens* (F.) pupae. At the center of each tray were one to three 14.0 ± 2.0 cm Petri dishes with 1.0 cm of hardened dental plaster (Castone®; Dentsply International Inc., York, PA) on the bottom. Also, in the center of the Petri dish was a 5.0-cm diam brood chamber. Two 8-mm access holes were made on the wall of the Petri dish above the dental plaster. The Petri dish lid was painted black (1302 Gloss Black Spray Enamel, Louisville, KY) to block the light. All colonies were maintained at 25-30°C.

Test arena and apparatus. The test arena was a 33.5 × 6.5 cm round aluminum pan with no lid. The inner side of the pan was coated with Fluon to prevent ants from escaping. The apparatus was a capped Wheaton liquid scintillation vial (2.8 cm × 6.1 cm). At the center of the cap was a 3-mm diam access hole. In the no-choice tests, only one vial was placed at the center of the arena. In the choice tests, there was more than one vial in the test arena. Each vial was placed on a metal screen which covered a 6.0 × 1.5 cm Petri dish. In this way, most sand removed by ants was confined in the Petri dish. All vials, with Petri dishes on the bottom, were placed around another 10 × 1.5 cm Petri dish (center container), which was placed at the center of the test arena. The distance from each vial to center container was same, so that all vials were equally accessible to fire ants, which were released in the center container.

Optimum sand moisture for digging. Sand (Premium Play Sand, Plassein International, Longview, TX) with 0.11, 2.0, 4.0, 6.0, 8.0% moisture content was tested in the no-choice test. The moisture content of the sand initially was 0.11%. Sand moistures were adjusted to predefined levels by mixing with distilled water. In each vial a mean (±SD) 36.86 g (±1.64 g) sand was added. All experiments were conducted at the temperature of 25°C. Three hundred worker fire ants were released in the arena. After 24 h, sand in each pan was collected, dried at 350°C for 1 h, and weighed. The experiment was replicated 5 times for each level of moisture content. The general linear model analysis of variance and LSD test (PROC GLM; SAS Institute 1999) was used to compare the amount of sand removed by ants among treatments. Significance was determined at $P < 0.05$.

Cross-contamination check. Two vials were placed in each test arena. Each vial was placed on a metal screen with a Petri dish on the bottom. A 16 ml Congo Red (Fisher Scientific Company, Fair Lawn, NJ) water solution (0.1g/100 ml) was mixed with 250 g sand in an aluminum pan. A mean (±SD) of 36.02 g (±0.54 g) sand was added to one vial. Sand in the other vial was treated only with distilled water. Three hundred fire ant workers were introduced into the center container in each arena. Two colonies were used. The experiment was replicated 6 times for each colony. After 24 h, sand in each vial was collected and visually checked for the presence of any mixture of undyed and dyed sand. Sand in each vial was weighed after being dried at 350°C for 1 h.

Multiple-choice test on nine phthalates. Sand was washed with distilled water and dried at 350°C. A 10 ml acetone solution of test chemical was mixed with 200 g sand in an aluminum pan. The sand was stirred every 2 min to facilitate the evaporation of acetone. After acetone evaporated (10 min), 16 ml distilled water was added and mixed with sand. A mean (±SD) of 36.80 g (±0.8 g) sand was added to each vial with a concentration of 200 ppm for each test chemical. Sand in the control vial was treated only with acetone. Dimethyl, diethyl, dipropyl, dibutyl, dipentyl, dihexyl, diheptyl, dioctyl, and dinonyl phthalates were tested. Locations of vials were randomized. One thousand fire ant workers were introduced into the center container in each arena. The experiment was conducted at 25°C. After 24 h, sand in each vial was collected, dried at 350°C for 1 h, and weighed. Two colonies, colony #1 and colony #2, are used for this experiment. The experiment was replicated 5 times for each colony. The general linear model analysis of variance and LSD test (PROC GLM; SAS Institute 1999) was used to compare the amount of sand removed by ants among treatments. Significance was determined at $P < 0.05$.

Multiple-choice test on five phthalates. The experimental design and statistical analysis were the same as noted previously except the number of choices was reduced to six: dimethyl, diethyl, diheptyl, dihexyl, dipropyl phthalates and control for colony #1 and dimethyl, diethyl, diheptyl, dinonyl, dipropyl phthalates and control for colony #2. These chemicals were not statistically separated in the experiment with 9 phthalates. The purpose of this experiment was to check whether reducing the number of chemicals in one test would further separate test chemicals statistically. Six hundred fire ants were used for each test arena. Colonies were the same as those used in the previous test.

Two-choice test on dimethyl and diethyl phthalates. The experiment arena, apparatus, and procedure were similar as those described in the multiple-choice test, except only two choices, treatment and control, were presented in the arena. One vial was treated with either dimethyl or diethyl phthalate, and the other with only acetone. Three hundred fire ants were used for each test arena. Concentrations of 25, 50, 100 ppm were used. For each concentration, there were 5 replications. The experiment was conducted at 25°C. A paired *t*-test (critical *P* value = 0.05) was used to compare mean amount of sand removed in treated vial with that in control vial for each concentration. Two colonies, colony #3 and colony #4, were used for this experiment.

Results

General digging behavior. *Solenopsis invicta* showed digging behavior in all experiments. The digging preference was significantly affected by moisture content (Table 1) and the type of chemical incorporated in the sand (Tables 2, 3; Fig. 1). First, several sand particles were always placed around the entrance hole on the cap. At the end of experiment, that is 24 h after ants were released in the arena, most excavated sand was located around the bottom of the vial in no-choice tests. In choice tests, most excavated sand was located in the Petri dish under the vial.

Optimum sand moisture for digging. There was a significant effect of moisture content on the amount of sand removed from the vials ($F = 11.88$, $df = 4$; $P < 0.0001$) (Table 1). Ants dug the sand with only 0.11% moisture content; however, at this level, fire ants dug significantly less amount of sand from the vials than other moisture content levels. At the 6.0% moisture content level, a mean (±SE)1.67 g (±0.23 g) sand was removed from the vials 24 h after fire ants were released in the test arena. Although differences among 2.0, 4.0, 6.0 and 8.0% moisture contents were not significant, all repellency tests in further studies were conducted by using sand with 6.0% moisture content.

Cross-contamination check. For one colony, ants removed a mean (±SE) of 3.97 g (±0.87 g) dyed sand and 2.02 g (±0.32 g) undyed sand. For the other colony, ants removed a mean (±SE) of 2.08 g (±0.69 g) dyed sand and 0.65 g (±0.19 g)

Table 1. Effect of sand moisture on the mean (±SE) weight (g) of sand removed by *Solenopsis invicta* workers from vials 24 h after ants were released in the text arena

Sand moisure (%)	Mean (±SE) weight (g) of sand removed
0.11	0.12 ± 0.06 b
2.00	1.51 ± 0.13 a
4.00	1.59 ± 0.27 a
6.00	1.67 ± 0.23 a
8.00	1.53 ± 0.20 a

Means followed by the same letter are not significant different ($P = 0.05$; LSD test).

Table 2. Repellency of nine phthalates against *Solenopsis invicta* workers from two colonies, based on mean (±SE) weight (g) of sand removed by ants 24 h after release. There were five replicates for each colony.

Colony	Chemical	Mean (±SE) weight (g) of sand removed
#1	Control	3.83 ± 0.65 a
	Dibutyl phthalate	2.31 ± 0.57 b
	Dinonyl phthalate	1.93 ± 0.57 bc
	Dipentyl phthalate	1.61 ± 0.53 bcd
	Dioctyl phthalate	1.04 ± 0.23 cde
	Dihexyl phthalate	0.81 ± 0.27 cde
	Diheptyl phthalate	0.65 ± 0.08 ed
	Dipropyl phthalate	0.38 ± 0.25 e
	Diethyl phthalate	0.00 ± 0.00 e
	Dimethyl phthalate	0.00 ± 0.00 e
#2	Control	2.04 ± 0.49 a
	Dibutyl phthalate	1.42 ± 0.27 ab
	Dipentyl phthalate	1.37 ± 0.24 abc
	Dioctyl phthalate	1.35 ± 0.30 abc
	Dihexyl phthalate	1.17 ± 0.24 bcd
	Diheptyl phthalate	0.79 ± 0.21 bcde
	Dipropyl phthalate	0.57 ± 0.35 cde
	Dinonyl phthalate	0.54 ± 0.17 de
	Diethyl phthalate	0.00 ± 0.00 e
	Dimethyl phthalate	0.00 ± 0.00 e

Means followed by the same letter are not significant different ($P = 0.05$; LSD test).

undyed sand. No mixture of dyed sand and undyed sand occurred in any vials 24 h after fire ants were released in the center container.

Multiple-choice test on nine phthalates. There was a significantly effect of test phthalates on the amount of sand removed from vials for colony #1 ($F = 8.63$, df = 9; $P < 0.0001$) and colony #2 ($F = 5.66$, df = 9; $P < 0.0001$) (Table 2). There was no significant difference among arenas for both colony #1 ($F = 0.34$, df = 4; $P = 0.85$) and colony #2 ($F = 0.23$, df = 4; $P = 0.92$) Although dimethyl and diethyl phthalates were numerically most repellant to fire ant workers, few statistical separations were found. For colony #1, dimethyl and diethyl phthalates were statistically separated from dibutyl, dinonyl, dipentyl phthalates and control. Dibutyl, dipentyl, dioctyl, dihexyl phthalate and control were separated from dimethyl and diethyl phthalates for the colony #2.

Table 3. Repellency of five phthalates against *Solenopsis invicta* workers from two colonies, based on mean (±SE) weight (g) of sand removed by ants 24 h after release. There were five replicates for each colony.

Colony	Chemical	Mean (±SE) weight (g) of sand removed
#1	Control	2.08 ± 0.09 a
	Dihexyl phthalate	1.22 ± 0.20 b
	Diheptyl phthalate	0.70 ± 0.20 b
	Dipropyl phthalate	0.44 ± 0.18 c
	Diethyl phthalate	0.004 ± 0.004 d
	Dimethyl phthalate	0.00 ± 0.00 d
#2	Control	2.95 ± 0.70 a
	Dinonyl phthalate	1.19 ± 0.19 b
	Diheptyl phthalate	0.70 ± 0.17 bc
	Dipropyl phthalate	0.47 ± 0.38 bc
	Diethyl phthalate	0.00 ± 0.00 c
	Dimethyl phthalate	0.00 ± 0.00 c

Means followed by the same letter are not significant different (P = 0.05; LSD test).

Multiple-choice test on five phthalates. The effect of the tested phthalates on the amount of sand removed was significant for both colony #1 (F = 33.76, df = 5; P < 0.0001) and colony #2 (F = 9.18, df = 5; P = 0.0001) (Table 3). With six choices in the test arena, dimethyl and diethyl phthalates were statistically separated from the rest of test chemicals for colony #1. Dipropyl phthalate was also separated from dihexyl and diheptyl phthalates. For colony #2, dinonyl phthalate was successfully separated from dimethyl and diethyl phthalates. No significant difference among test arenas were found for both colony #1 (F = 1.17, df = 4; P = 0.35) and colony #2 (F = 0.22, df = 4; P = 0.92).

Two-choice test on dimethyl and diethyl phthalates. At 100 ppm level, there were significant differences between controls and treatments for dimethyl and diethyl phthalates (Fig. 1). Below the100 ppm level, the difference between control and treatment was not significant for both compounds.

Discussion

Dimethyl and diethyl phthalate are common ingredients of mosquito repellants (Schreck 1991, Frances et al. 1993, Anonymous 1995, Frances and Cooper 2002). This study showed that dimethyl phthalate and diethyl phthalates were also repellants to red imported fire ants. Both dimethyl and diethyl phthalates are inexpensive chemicals (dimethyl phthalate: $15.30 per liter; diethyl phthalate: $17.00 per liter, Fisher Chemicals, Fairlawn, NJ). It was reported that diethyl phthalate was used as ingre-

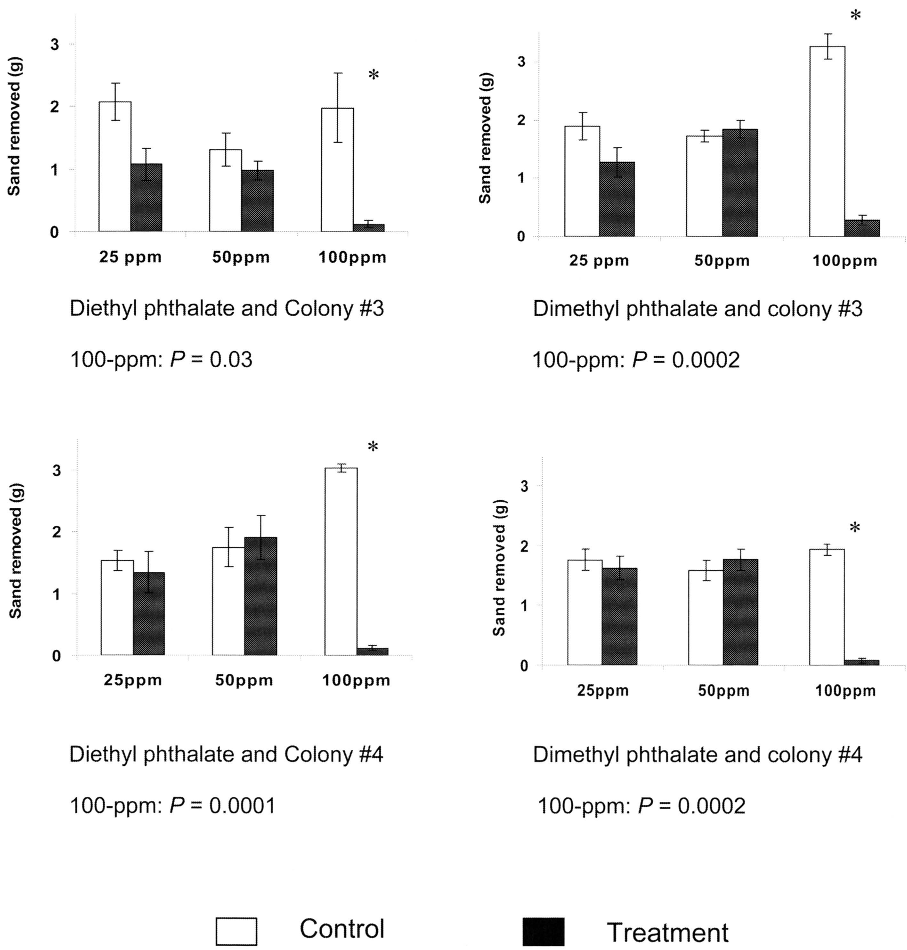

Fig. 1. Mean (±SE) weight (g) of sand removed from treatment and control vials in a series of two-choice repellency tests on dimethyl and diethyl phthalates against *Solenopsis invicta* workers. Concentration with an asterisk indicates significant difference between treatment and control (Paired *t*-test).

dient in 67 cosmetic formulations at concentration up to 50% (Anonymous 1995). Both dimethyl and diethyl phthalates are commonly used as polymer plasticizers.

Hubbard (1974) found fire ants preferentially dug in nest materials from their own colony when another choice of unnested soil or nest material from another colony was provided. The results of this research demonstrate that ant digging behavior is affected by the chemical nature of the substrate; such effect can be quantified by using the weight of the excavated substrate. Fire ant digging behavior can be successfully used to quantify the repellency of various treated substances.

Soil moisture affects the mound-building activity of *S. invicta* (Rhoades and Davis

1967). In this study, it was shown that sand with 2.0-8.0% moisture is adequate for digging. Other variables, such as temperature, group size and CO_2 content, might influence ant digging behavior. Evesham (1992) found a strong negative correlation between the time *Myrmica rubra* (L.) workers spend in the laboratory without soil and the amount of soil they excavated during an experiment. However, these variables can be easily controlled in the laboratory for the repellency tests.

With an adequate number of test chemicals in the arena, choice test can be used to screen multiple chemicals in a single test. Using this method makes a fast and direct comparison among potential fire ant repellants possible. For the multiple-choice test to be sensitive, the number of choices in a test must be carefully controlled. For example, with nine chemicals in the multiple-choice test for colony #1, dihexyl, diheptyl, and dipropyl phthalates could not be separated from dimethyl and diethyl phthalates; however, when the number of test chemicals was reduced to five, such separation was achieved.

Choice tests have been used in fire ant repellency tests, such as the Y-tube olfactometer method (Vander Meer et al. 1996, 1998). Y-tube olfactometer was designed for testing volatile compounds. However, not all ant repellants work through the olfactory receptors. Greater repellency may be attained by combining chemicals with different sensory input modes (Shorey et al. 1996). This new method can test repellency through both olfactory and gustatory receptors. Comparing number of ants on treated and control objects is common in fire ant repellency tests. It is technically difficult to count ants on an object without immobilizing them, especially when there are a great number of ants on the object. This digging bioassay provides a very easy method to quantify the repellant effect-weighing the excavated sand.

Due to different living conditions and ages, it is understandable that workers from different colonies may have different sensitivity to a particular chemical. Efficacy of one particular repellant has to be confirmed using multiple colonies. In some cases, increasing replicates, or decreasing the number of choices in multiple choice tests may be necessary to differentiate test compounds from each other.

In this study, the comparison was made only among 9 phthalates. The efficacy of dimethyl and diethyl phthalates, comparing to other known fire ant repellants, can only be revealed by further tests. This digging bioassay provides us with an easy tool to do such multiple comparisons.

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