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# DEFENSIVE SECRETION OF FIRST-INSTAR LARVAE OF ROOTSTALK BORER WEEVIL, *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae), TO THE FIRE-ANT *Solenopsis geminata* (F.) (Hymenoptera: Formicidae)

## INTRODUCTION

*Diaprepes abbreviatus*, commonly known as the rootstalk borer weevil, is an important pest of citrus and sugarcane in the Caribbean basin. This species has a broad host range, including fruit, vegetables, and ornamentals (Martorell, 1945; Wolcott, 1948; Woodruff, 1968) but the main problem concerns citrus groves and nurseries, especially in Florida (Schroeder and Beavers, 1977), Puerto Rico (Woodruff, 1964), and the French West Indies (Mauléon and Mademba-Sy, 1988).

The adult weevils feed on the young foliage and deposit their eggs in masses between mature leaves, which are held together with an adhesive secretion. After hatching, first-instar larvae drop and burrow into the soil where they spend all their larval stages. Larvae feed on roots, causing severe damage. After pupation, the adults emerge from the soil.

Among the natural enemies, several species of predatory ants can play a role. Egg mass predation in the trees by *Monomorium floricola* Jerdon, *M. ebinium* Forel, *Pheidolle fallax* Forel, and *Ectatomma ruidum* Roger is reported (Buren and Whitcomb, 1977).

In Florida and Puerto Rico, ants are the dominant predators on first-instar larvae (Richman et al., 1983); the major ant species involved are *Tetramorium similum* Roger, *Pheidolle dentata* Mayr, and *P. floridana* Emery in Florida and *P. fallax* and *Tetramorium bicarinatum* (Nylander) in Puerto Rico. In a qualitative evaluation of ants as predators on *Diaprepes* species in the French West Indies, Jaffé et al. (1991) report predation of egg masses, larvae, and adults by several species of ants, but in most cases, ants showed some kind of repulsion towards the first-instar larvae. In the literature, there is no report of such a phenomenon. In this study, we tested this repellency, using first-instar larvae or egg masses treated with first-instar larvae extracts and presenting it as prey to a common ant of Guadeloupe: *Solenopsis geminata* (F.). This species is an important predator in citrus groves (Jaffé et al., 1991). The aim of the present work is to determine whether there is any chemical basis for repellency. Gas chromatography, mass spectrometry, nuclear magnetic resonance, and infrared spectrometry were used for the detection and identification of the chemicals involved.

## METHODS AND MATERIAL

*Insects.* Fragments of ant colonies were collected in the field by digging up a nest and placing the soil containing large numbers of ants into boxes whose sides were coated with Fluon. The ants were separated from the soil by allowing water to slowly drip into the box and providing them with several wire bridges over which they could escape into a second Fluon-coated box containing the



nest. The nests consisted of plaster of Paris in standard Petri dishes, with a centrally positioned tube to permit the nest to be artificially moistened, and then covered with red plastic to simulate darkness. The ants were fed on larvae of the greater wax moth, *Galleria mellonella*.

The weevil species *Diaprepes abbreviatus* was collected in Guadeloupe and maintained in the laboratory at 26 ± 3°C, in large plastic cages. Adults were fed on young citrus foliage, and females oviposited readily between pieces of sulfurized paper stapled to a leaf, as well as between leaves. Egg masses were collected daily and placed in eclosion boxes. On hatching, the neonates drop through the gauze into a vial. They were collected daily, and some were placed in plastic vials with medium (supplied by Dr. W. J. Schroeder, USDA, Orlando, Florida) for rearing while others were utilized for extracts. The larvae used for extracts were kept in clean plastic vials with a piece of moistened paper towel to prevent dehydration and removed when required.

*Preparation of Extracts.* These were made by washing with hexane. For eggs, the number in a mass on sulfurized paper was counted and the paper cut to the smallest size possible. Larvae were counted and collected in a Pasteur pipet. Extracts were made from first-instar larvae up to 7 days old and also second instar larvae up to 30 days old. The eggs or larvae were placed in a Pasteur pipet that had a small plug of glass wool inserted. A volume of 500 μl was allowed to pass through the pipet and collected in a glass vial. After concentration under argon, extracts were stored at −18°C.

*Field Bioassay.* Before developing a laboratory bioassay, we simply observed the behavioral response of the ants near their nest on the soil of a citrus grove in Guadeloupe. Egg masses of *D. abbreviatus* were available to ants on the soil. Test and control stimuli consisted of 10 μl of first-instar larvae (FIL) extract at 1 L1 equivalent/μl (1 L1E/μl), 10 μl of second instar extract (1 L2E/μl), and 10 μl of hexane, respectively, applied to masses of approximately 10 eggs. Each stimulus was tested with 30 ants. The assays were conducted between 9:00 and 10:00 AM. When an ant came in contact with an egg mass, its behavior was recorded.

*Laboratory Bioassay.* A box containing the artificial nest of *S. geminata* was connected to a smaller box, containing the usual food of the ants, by three glass tubes (Figure 1). The stimulus (egg mass or larva) was placed in the central tube and when an ant entered this tube, the entrances were closed, using pieces of wrapped Parafilm as plugs. The ant was observed for a period of 5 min, and its behavioral response when it came in contact with the larva or egg mass noted. After 5 min, the plugs were removed and the ant was allowed to leave. This was repeated 20 times for each nest with the same test stimulus, and the glass tube and the stimulus were replaced each five replicates. An egg mass of 10 eggs or one larva was used for each test. For the tests that utilized extracts, 10 μl of a solution (concentration: 1 larva equivalent per microliter) were placed

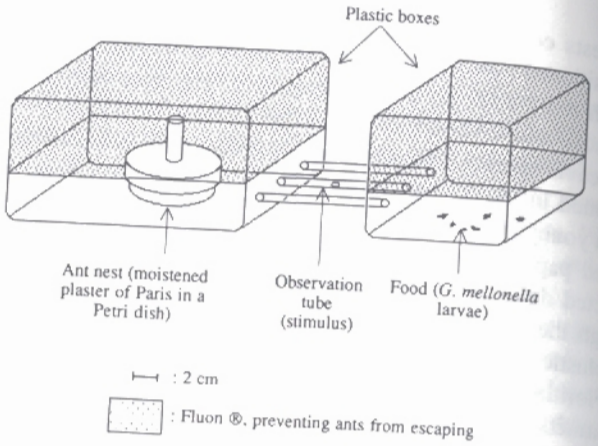



FIG. 1. Laboratory bioassay.

on the egg mass. Controls using 10 $\mu$l of pure hexane were also included. The following stimuli were tested: 5-day-old eggs, 5-day-old eggs treated with hexane, 5-day-old eggs treated with either a 0.5- or 5-day-old FIL extract and FIL of 0.5, 1, 2, 3, 4, and 5 days old. For statistical analysis, chi-square tests were carried out using the system STATITCF computer package for microcomputers.

Another laboratory bioassay was developed to test the activity of the two compounds (A1 and A2) isolated from the FIL extracts. One microliter of the tested solution was disposed on a piece of Bristol paper (1 × 1 cm), washed with a Soxhlet system. The exact place of the stimulus was delimited by a spot made with a pen. After a mechanical excitation of the ant nest, the piece of paper was placed on the Petri dish and the number of ants coming in contact with the source was recorded during 90 sec. The following stimulations were applied to four ant nests: compound A1 at 10, 100, 500 ng; compound A2 at 10, 100, 500 ng; a mixture of A1 (10 ng) and A2 (10 ng); and a mixture of A1 (100 ng) and A2 (100 ng) and a 12-hr-old FIL extract (10 L1E). The control consisted of 1 $\mu$l of *n*-hexane, and seven repetitions were made for each stimulus. For statistical analysis, the non-parametric U test of Mann and Whitney was used.

*Chemical Analyses.* Gas chromatography (GC) was performed with a HP 5890 Hewlett Packard chromatograph equipped with a Ross injector system fitted with a 25-m × 0.32-mm-ID CPSil8CB, WCOT; column temperature: 180°C (carrier gas: helium at 1 kg/cm$^2$). Gas chromatography–mass spectrometry (GC-MS) was carried out with a Nermag R-10-10C quadrupole mass spectrometer (electron impact at 70 eV) coupled with a Girdel 32 gas chromatograph equipped with a Ross injector. The system was fitted with a fused



silica capillary column (25 m WCOT CPSil5CB, 0.32 mm ID, Chrompack, Middleburg, The Netherlands). The column temperature was 200°C, using helium as carrier gas at 1 kg/cm$^2$. Chemical ionization–mass spectrometric data were obtained using ammonia as reactant gas at 92.5 eV.

To determine the number of unsaturations in the molecules, catalytic microhydrogenation was done, using $PtO_2$ in methanol.

Micropreparative capillary gas chromatography was performed on a Girdel 300 (Malosse, 1990) fitted with a wide-bore fused-silica capillary column (5 m WCOT HP-1, 0.53 mm ID, 160°C, Hewlett-Packard). The carrier gas was helium at 0.3 kg/cm$^2$. The split system was a Y glass tube (Chrompack) with capillaries giving a split ratio of 85:15. The different fractions were collected in 18-cm × 0.8-mm-ID glass capillaries and cooled with a mixture of acetone and Dry Ice.

Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker 600 MHz spectrometer. Samples separated by micropreparative GC were rinsed from collection tubes with $C_6D_6$ (99.93% CEA, France). After analysis, the different fractions were used to test their activity on ants.

Gas chromatography-infrared (GC-FTIR) was carried out with a IFS-85 Bruker coupled to a HRGC 5160 Carlo Erba chromatograph (on-column injector) fitted with a fused-silica capillary column 30 m WCOT DB-5, 0.32 mm ID, heated from 30 to 170°C (10°C/min) and from 170 to 220°C (5°C/min), with helium as carrier gas. The light pipe was heated at 220°C.

## RESULTS

*Field Bioassay.* Three kinds of behavior were observed when ants came in contact with the egg mass. First, predation of eggs: the ant stops, antennates the egg mass, and picks off an egg with its mandibles with a few seconds and carries it away. Secondly, repulsion: the ant avoids the egg mass or, after a short period of antennation (less than 2 sec), it quickly moves away from the egg mass, then stops and cleans its antennae with its forelegs. At last, indifferent behavior: the ant arrives close to the eggs but continues without stopping or antennating.

Table 1 shows that in most cases, ants refused the egg mass treated with FIL extract (1 L1E/egg). In contrast, when exposed to solvent control or to the 1 L2E/egg extract, ants always accepted eggs as prey after antennation. The proportion of indifferent behavior is less significant for the treated eggs. That means that a close contact is not necessary, indicating a relative volatility of the chemicals involved in this host–prey relationship.

*Laboratory Bioassay.* Figure 2 shows clearly the significant results of the tests. Eggs treated with FIL extracts (FIL of ages less than 12 hrs or 5 days)

TABLE 1. PREDATORY RESPONSE OF *Solenopsis geminata* TO LARVAL EXTRACTS OF *Diaprepes abbreviatus* DISPOSED ON EGG MASSES, AT PHYSIOLOGICAL DOSES

| Treatment[a] | Acceptance of eggs | Indifferent behavior | Repulsion |
|---|---|---|---|
| Solvent (a) | 15 | 13 | 2 |
| L1 extract (b) (1 L1E/egg[b]) | 2 | 5 | 23 |
| L2 extract (a) (1 L2E/egg) | 14 | 15 | 1 |

[a]Treatments followed by the same letter do not differ significantly ($\chi^2$ test, $P < 0.05$).
[b]One first-instar larva equivalent per egg.

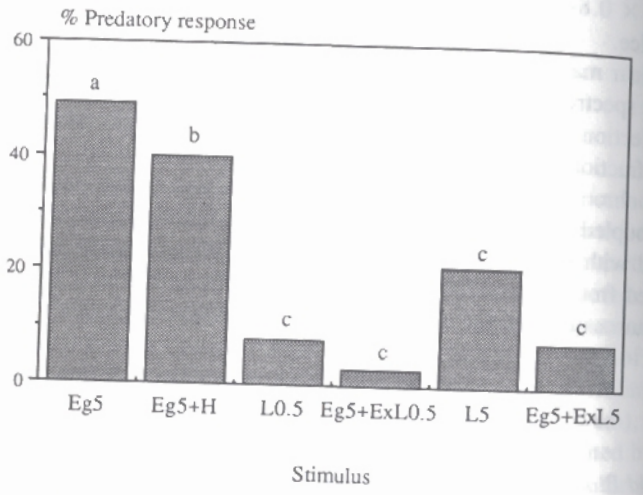



FIG. 2. Degree of predation by *Solenopsis geminata* upon *Diaprepes abbreviatus*. Results based upon the means of tests with 20 ants per nest, with three different nests per stimulus. Eg5: 5-day-old eggs; Eg5+H: 5-day-old eggs + hexane; L0.5: 0.5-day-old first-instar larvae; Eg5+ExL0.5: 5-day-old eggs + extract of larvae 0.5 days old; L5: first-instar larvae 5 days old; Eg5+ExL5: 5-day-old eggs + extract of larvae 5 days old. Histogram bars with the same letter are not significantly different ($\chi^2$ test, $P < 0.05$).

are less preyed upon than untreated eggs or eggs with hexane. Similarly, 0.5-day-old and 5-day-old first-instar larvae are preyed upon less than eggs. This confirms that first-instar larvae produce a chemical repellent that is active immediately after hatching.

There is a significant difference between the responses of ants to 5-day-old eggs and 5-day-old eggs treated with hexane. Hexane appears to make eggs less attractive. In the majority of the tests, predation seems slightly less on eggs +



larval extracts than on larvae of the corresponding age. It is possible that hexane dissolves phagostimulant compounds present at the surface of the chorions.

Figure 3 shows the evolution of the predatory response with the age of the larvae used for the tests or for the extracts. In both cases, we can see that direct predation is low (less than 30%) at any age. Repulsion varied from 30% to 70%. Indifferent behavior decreases with the age of the larvae. It can tentatively be stated that predation increases with larval age, but there is no clear pattern for repulsive behavior.

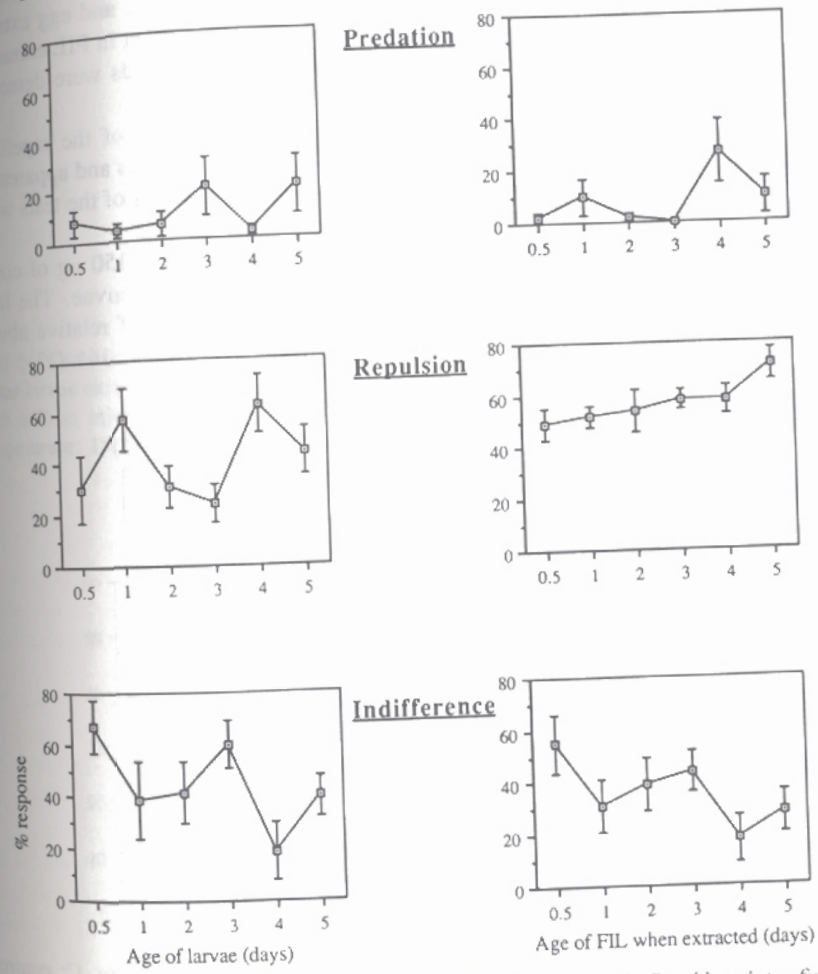



FIG. 3. Mean (±SEM) behavioral response of *S. geminata* towards *D. abbreviatus* first-instar larvae of different ages and egg masses treated with extracts of first instar larvae of different ages. An average of 170 ants were observed for each test.

The results with isolated compounds A1 and A2 are represented in Figure 4. A1 and A2 had no repellent activity at the dose of 10 ng. However, at 100 and 500 ng, their respective activities were significantly different from the control at the 5% level. The repellent activity of the whole extract was not different from the activities of each compound. The mixture of the two compounds was no more active than the compounds alone.

*Chemical Analyses.* Chromatograms of the *D. abbreviatus* first-instar larvae extracts showed the existence of two main peaks: A1 (65%) and A2 (35%) (Figure 5), which are nearly absent in the second-instar larvae and egg extract chromatograms. In addition to these specific compounds present in FIL extracts, hydrocarbons and saturated and unsaturated hexadecanoic acids were detected in all extracts.

According to the quantitative GC results, concentration of the repellent appears to decrease with the time, being at its highest in neonates and apparently absent from larvae after about four days (Figure 6). The results of the tests with *S. geminata* show some consistency with this result.

Micropreparative capillary GC led to the isolation of ca. 150 $\mu$g of compound A1 and 50 $\mu$g of compound A2 from 5000 first-instar larvae. The low resolution GC-EI-MS spectrum of A1 shows ions at *m/z* (% of relative abundance): 218 (M+, 38), 203 (29), 200 (5), 187 (52), 175 (21), 161 (13), 148

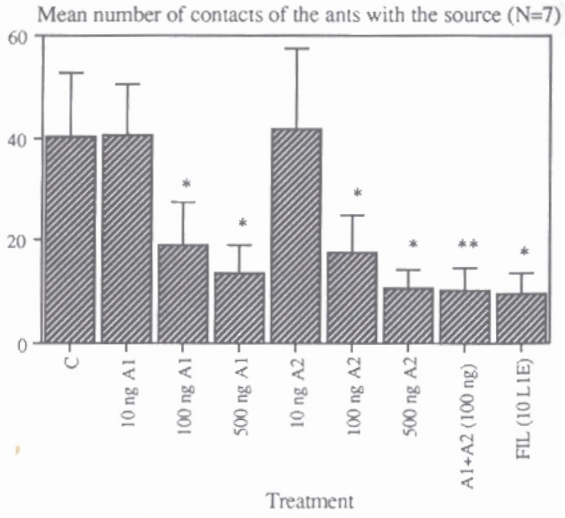



FIG. 4. Repellent activity of diaprepal A1 and A2 on *Solenopsis geminata*. C: control (1 $\mu$l of hexane); FIL (10 L1E): 1 $\mu$l of 12-hr-old FIL extract, corresponding to 10 FIL. Bars with asterisk are significantly different from the control, Mann and Whitney test ($*P < 0.05$, $**P < 0.01$).



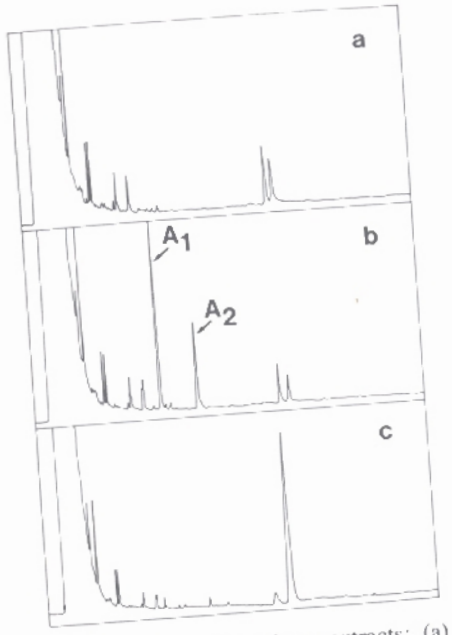



FIG. 5. Chromatograms of *Diaprepes abbreviatus* extracts: (a) egg extract; (b) first-instar larvae extract; (c) second-instar larvae extract. (HP 5890 chromatograph equipped with a Ross injector, system fitted with a 25-m × 0.32-mm-ID (CPSil8CB); column temperature: 180°C; carrier gas: helium at 1 kg/cm$^2$).

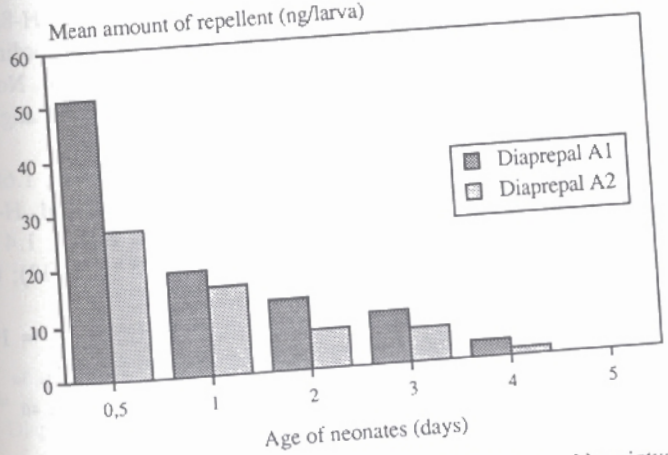



FIG. 6. Mean amounts of repellent extracted from *Diaprepes abbreviatus* first-instar larvae, at different ages.

(18), 147 (18), 133 (11), 119 (13), 107 (39), 91 (46), 79 (100), 67 (93), 55 (56), and 41 (73). GC ($NH_4$) CI-MS spectrum giving ions at *m/z* 236 ((M+$NH_4$)+, 31), 219 (MH+, 100), 201 ((MH−$H_2O$)+, 5), and 187 (4) confirmed the molecular formula as $C_{15}H_{22}O$ (HRMS calculated for $C_{15}H_{22}O$: 218,1658, found: 218,1658).

Catalytic microhydrogenation gave ions at *m/z* 222 (M+, 2), 204 (24), 191 (2), 179 (80), 161 (100), 119 (11), 109 (11), 105 (22), 95 (15), 81 (49), 69 (54), 55 (48), and 41 (56), indicating the occurrence of two unsaturations. The oxygen function was assigned to an aldehyde group regarding data from GC-FTIR showing absorption at 2800, 2708, 1705, and 1381 $cm^{-1}$. GC-EI-MS (loss of 43 amu) and GC-FTIR (absorption at 897 $cm^{-1}$) also confirmed the presence of an isoprenyl group. We proposed for the structure of compound A1, called diaprepal A1, a monounsaturated naphthane skeleton with three substituents: an aldehyde group, a methyl group, and an isoprenyl group. The final structure has been determined by $^1H$ NMR spectrometry (600 MHz). One-dimensional (1D) extensive decoupling and 2D COSY experiments led us to propose the structure represented on Figure 7.

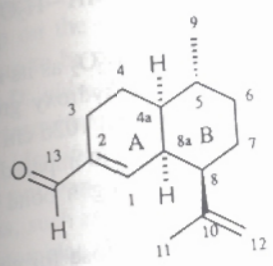
Stereochemistry of the *cis* decalinic system is surmised from the observation of a small coupling constant between H-4a and H-8a ($J$ = 3 Hz). Determination of the equatorial position on the B ring of the methyl and of the isoprenyl group is provided by measurements of characteristic *trans* diaxial coupling constants between H-5 and H-6$\alpha$, H-5 and H-4a, and between H-7$\beta$ and H-8. (9, 11, and 11.5 Hz, respectively). Proton H-8a is in an equatorial position in ring B, according to the coupling constant between H-8 and H-8a ($J$ = 2 Hz). Location of the double bond ($\alpha$ to the ring junction) is obtained by observation of two $^5J$ coupling constants characteristic of the cyclohexene ring between H-8a and both H-3 ($J$ = 6 and 3 Hz), the latter being in an allylic position according to their geminal coupling constant ($J$ = 21 Hz) and coupled with both H-4. No coupling constant is detected between H-8a and H-1 owing to a dihedral angle of 90° between the two bonds H-8a—C-8a and H-1—C-1 (Figure 8a).

$^1H$ chemical shifts ($\delta$ ppm): 1.12 (m, H-5); 1.2 (m, H-6$\alpha$); 1.68 (m, H-6$\beta$); 1.69 (m, H-7$\beta$); 1.00 (bt, H-7$\alpha$); 1.27 (dt, H-4a); 2.07 (bd, H-8); 2.65 (bs, H-8a); 2.22 (tm, H-3$\alpha$); 2.57 (bdd, H-3$\beta$); 2.00 (dm, H-4$\alpha$); 1.4 (tdd, H-4$\beta$); 6.55 (bs, H-1); 5.22 (s), 4.95 (s) (2H, H-12); 9.55 (s, H-13); 0.95 (d, 3H, H-9); 1.85 (bs, 3H, H-11).

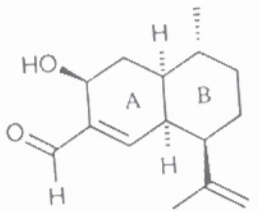
Coupling constants were: $J_{4a,5}$ = 11 Hz; $J_{5,6\alpha}$ = 9 Hz; $J_{6\alpha,6\beta}$ = 10.5 Hz; $J_{6\alpha,7\beta}$ = 10 Hz; $J_{7\alpha,7\beta}$ = 12 Hz; $J_{7\beta,8}$ = 11.5 Hz; $J_{8,8a}$ = 2Hz; $J_{8a,3\alpha}$ = 3Hz; $J_{8a,3\beta}$ = 6 Hz; $J_{3\alpha,3\beta}$ = 21 Hz; $J_{3\alpha,4\beta}$ = 11 Hz; $J_{3\beta,4\beta}$ = 6 Hz; $J_{4a,4\beta}$ = 2 Hz; $J_{4a,4\alpha}$ = 3Hz; $J_{4\alpha,4\beta}$ = 15 Hz.

The GC-EI-MS spectrum of the minor compound (A2) gives ions at *m/z* 234 (M+. 14), 219 (3), 216 (55), 205 (14), 201 (25), 191 (14), 187 (43), 173 (27), 159 (25), 146 (38), 131 (26), 119 (14), 107 (39), 91 (62), 79 (87), 68






Diaprepal $A_1$




Diaprepal $A_2$

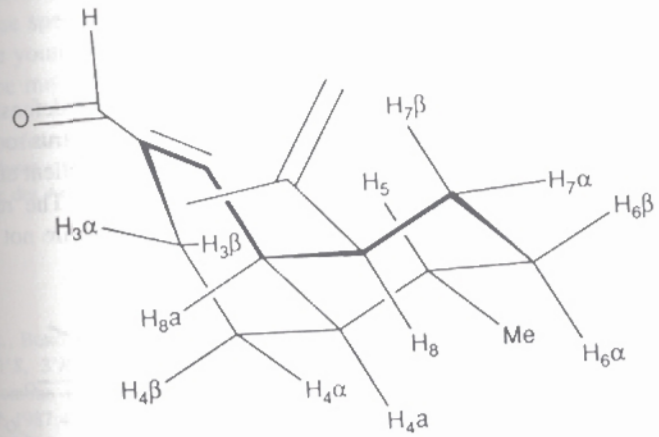



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FIG. 7. Diaprepal A1 and A2 from first instar-larvae of *Diaprepes abbreviatus* (*spatial representation of A1).

(90), 67 (100), 55 (88), 41 (84), and GC ($NH_4$) CI-MS, ions at *m/z* 252 [(M+$NH_4$+), 27], 235 (MH+, 27), 234 (M+, 46), 217 [(MH−$H_2O$)+, 100], 199{[(MH−$H_2O$)−$H_2O$]+,4} and 187 (4).

In agreement with these data, compound A2 has $C_{15}H_{22}O_2$ as its molecular formula. The second oxygen function was assigned as a hydroxy group. The GC-FTIR spectrum exhibiting absorptions at 3595 $cm^{-1}$ and 1026 $cm^{-1}$. Lowering of absorption at 3595 $cm^{-1}$ and also at 1695 $cm^{-1}$ (compared to 1705 $cm^{-1}$ for compound A1) indicates an intramolecular hydrogen bond between the hydroxy and the carbonyl group.

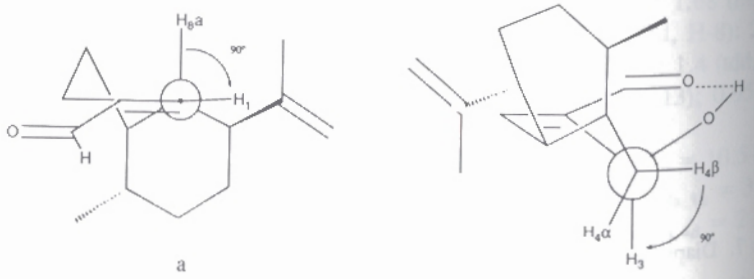
Close spectral NMR data of A1 and A2 led us to propose for compound A2 the same structure as diaprepal A1 with an additional hydroxy group at C-3 as represented in Figure 7. Owing to the fact that H-8a is in an equatorial position on the B ring and that H-3 on the carbon bearing the hydroxy group H-bonded to the aldehyde function has a coupling constant with only one proton H-4, the β position of the hydroxy group seems to be the more suitable (Figure 8b).

$^1$H chemical shifts (400 MHz, $C_6D_6$) $\delta$ (ppm) were as follows: 9.15 (s, H-13); 6.37 (s, H-1); 5.03 (s), 4.76 (s) (H-12); 4.48 (d, H-3); 2.3 (dd, H-4β); 2.27 (m, H-8a); 1.61 (s, H-11); 1.12 (s, H-9); and 0.82–2.1 (m, 8H, H-6, H-7, H-5, H-4α, H-4β).

Coupling constants were: $J_{3,4\alpha}$ = 5.8 Hz; $J_{4\alpha,4\beta}$ = 11 Hz; $J_{4\beta,4a}$ = 3 Hz.

## DISCUSSION

In this study, we report for the first time the presence of a defensive secretion produced by a curculionid larva: the first-instar larvae of a citrus root weevil, *Diaprepes abbreviatus*, produce semiochemicals that have a repellent effect upon an important predator in citrus groves, *Solenopsis geminata*. The repellency reduces predation by about 40%. These allomonal compounds are not detected




a b

FIG. 8. Spatial representation of diaprepal A1 (a) and A2 (b).



in the second instars, nor in the egg masses. In the citrus trees, egg masses are stuck between two leaves, and they are not easily preyed upon by ants. When they drop on the soil, the neonates are very vulnerable, and their secretions allow them to be protected against their main predators.

For *D. abbreviatus*, the secretion has been identified as a mixture of two bicyclic sesquiterpenes aldehydes, in the proportion 65% and 35%. They have not been identified previously and we call them diaprepal A1 and A2. Sesquiterpenes are very frequent in plants and insects, and they can have different functions, such as alarm pheromones in aphids (Blum, 1969) or sex pheromones in Pentatomidae (Baker et al., 1987). Moreover, caryophyllene and caryophyllene epoxyde (terpenoids produced by neotropical plants) are known to be deterrent to leafcutter ants (Howard et al., 1989). Tricyclic sesquiterpenes (zizanal and epizizanal) (Jain et al., 1982) and sesquiterpene aldehydes (cadinenal, dehydroxycadinenal) (Plavkan, 1985, in Davidson et al., 1990), present in the essential oil of *Vetiveria zizanoides*, have potent topical irritant activity on cockroaches and flies.

It would be interesting to extend the investigation by testing for the same or related repellents in other *Diaprepes* species or in other curculionids. The repellent appears to be effective against the predator as long as necessary under natural conditions, because, as already mentioned, the neonates generally remain on the soil surface and are subject to predation by ant species for less than 3 hr. In these species, all living stages are relatively protected against predators, except the young emerging adults on which natural regulation by predatory ants seem to be more effective than on neonates.

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