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Identification of the Sex Pheromone of the Mealybug *Dysmicoccus grassii* Leonardi

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Supporting Information

ABSTRACT: Studies about the sex pheromone of the mealybug *Dysmicoccus grassii*, a main pest of Canary Islands banana cultivars, showed strong evidence that (−)-(*R*)-lavandulyl propionate and acetate in a 6:1 ratio are principal components of it. Volatile collection and GC-MS analysis from aeration of virgin females allowed the structural elucidation of the compounds. The activity and stereochemistry of both substances were established by means of relative attraction of mealybug males to synthetic standards in competitive Petri dish bioassays. (*R*)-Lavandulyl propionate induced a stronger attractive effect when compared to (*R*)-lavandulyl acetate. The attractiveness of the mixture of the two compounds at the original source ratio showed no statistically significant difference from that of the sum of each of the single compounds alone, suggesting that both components are not synergistic but additive.

KEYWORDS: *pheromone, semiochemical, terpene, mealybug, Dysmicoccus grassii*

INTRODUCTION

Dysmicoccus grassii Leonardi (Hemiptera:Pseudococcidae) is a polyphagous mealybug species with agriculturally important hosts including mango, pineapple, coffee, banana, papaya, and cacao.[1] This species is considered to be of neotropical origin and has been found in Africa, Europe, and several American countries.[2,3] In the past decade, its presence has been also reported in southern Asia.[4]

This pest is of major concern in the banana cultivars of Canary Islands, where it was incorrectly reported for the first time by McDoughall as *Pseudococcus comstockii* in 1929.[5] Subsequent studies in 1960 confirmed this species as belonging to the genus *Dysmicoccus*, by means of the synonym *D. alazon*.[6] The latter name was finally rejected in favor of the original description of the species made by Leonardi in 1913 for *D. grassii*.[7,8]

The attack of this pseudococcid causes hard damages over the plant and fruit, which becomes yellow spotted and covered with honeydew secreted by females, delaying banana filling. This sugary syrup usually supports fungal diseases, mainly sooty mold complex (also known as Fumagina), reducing the photosynthetic capability of the plant and leading to the production of small-sized bananas.[9] It is noteworthy that a fruit showing fungi or mealybugs on harvesting forces export rejection of the whole batch and blocks a profitable market for Canary Islands growers, causing important economical losses. Main direct damages caused to banana crops consist of plant weakening, productivity reduction, loss of growth rate, and drastic lowering of crop yields.[10]

Canary Islands banana cultivars have been traditionally protected from *D. grassi* attacks by the use of broad-spectrum insecticides, mainly organophosphates and pyrethroids.[11] However, the current regulatory restrictions and IPM implementation efforts demand the development of new tools to control this serious pest in a more environmentally friendly manner.[12] In addition, a fully organic production of banana crops in Canary Islands is a current challenge requiring new plant protection products.

Pheromones could be an emerging alternative for the management of agriculturally relevant Pseudococcidae species. Sex pheromone traps are eventually used for identification purposes, but few examples could be found in the literature for the efficient pest management of scale insects by means of pheromone-based control techniques.[13] Mating disruption appears to be an attractive possibility due to its specificity and low environmental impact. This study undertook the identification and biological assessment of the components of the sex pheromone of *D. grassi* as the necessary preliminary knowledge for the development of pheromone-based tools for the control of this pest in Canary Islands banana cultivars.

MATERIALS AND METHODS

Insects. *D. grassii* individuals were collected from synchronized colonies maintained on isolated young banana plants (*Musa acuminata* Colla. cv. Grand Nain) that were maintained at 25 ± 2 °C and a relative humidity of 70 ± 10%. The light period inside the insectary was set to 16 h light−8 h dark. The insect colonies were established starting from first nymphal instar individuals obtained from wild gravid

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females that were collected from banana plants in commercial groves located at Valle Guerra and Adeje (Tenerife, Spain). Newly hatched N1 individuals were collected over a banana leaf for a period of 24 h and then transferred to uninfested plants to set a new synchronized colony. Direct observation of colonies showed that females become sexually mature before male hatching for synchronized populations. This fact avoided the need to isolate females from the total population for volatile collection.

Chemicals. All reagents and solvents (reagent grade) were purchased from Sigma-Aldrich (Madrid, Spain) and employed without additional purification unless stated. Molecular sieves (3 Å, 4−8 mesh) (Sigma-Aldrich) were washed with acetone and dried under vacuum at 180 °C overnight prior to use. Flash column chromatography was performed on high-purity grade, 230−400 mesh silica gel (Sigma-Aldrich). Reactions were monitorized by silica gel F_{254} aluminum-precoated analytical thin-layer chromatography (Macherey-Nagel, Düren, Germany) using a mixture of hexane/ethyl acetate (9:1) as eluent.

Chromatographic and Spectroscopic Analysis. Gas chromatography coupled to mass spectrometry (GC-MS) analyses were performed on a Clarus 600T GC-MS from Perkin-Elmer (Wellesley, MA, USA). The column used was a 30 m × 0.25 mm i.d., 0.25 μm, Zebron ZB-5 (Phenomenex Inc., Torrance, CA, USA). One microliter of the different sample solutions was injected manually into the system. The injector temperature was set at 250 °C, and it was operated in splitless mode. The oven was held at 40 °C for 2 min, then programmed at 5 °C/min to 280 °C, and maintained at this final temperature for 1 min. Helium was used as the carrier gas with a flow of 1.2 mL/min. The detection was performed in EI mode (ionization energy, 70 eV; source temperature, 180 °C). The spectra acquisition was performed in scanning mode (mass range m/z 35−500). Chromatograms and spectra were recorded by means of GC-MS Turbomass software version 5.4.2 (Perkin-Elmer Inc.).

Infrared spectra (IR) were recorded on a Nicolet-iS10 FT-IR spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). Optical rotations were measured on a Jasco P-1000 series automatic polarimeter (Essex, UK). High-resolution mass spectra (HRMS) were carried out using an Acquity XevoQToF spectrometer instrument (Waters Corp., Milford, MA, USA).

Gas chromatography (GC-FID) analyses were performed on a Clarus 500 GC from Perkin-Elmer. The column used was a 30 m × 0.25 mm i.d., 0.25 μm, Zebron ZB-5 (Phenomenex Inc.). One microliter of the different sample solutions was injected manually into the system. The injector temperature was set at 250 °C, in split mode at a ratio of 1:40. The oven was held at 80 °C for 1 min, then programmed at 20 °C/min to 250 °C, and kept at this final temperature for 4 min. Helium was used as the carrier gas with a flow of 1.0 mL/min. The detection was performed by FID operating at 300 °C. Chromatograms and spectra were recorded by means of GC TotalChrom software version 6.2.1 (Perkin-Elmer Inc.).

Chiral gas chromatography (chiral GC-FID) analyses were performed on a 430-GC from Bruker-Daltonics (Fremont, CA, USA). The column used was a 30 m × 0.25 mm i.d., 0.25 μm, β-DEX 120 (Supelco Analytical, Bellefonte, PA, USA). One microliter of the different sample solutions was injected manually into the system. The concentration of standard samples was 1 mg/mL. The injector temperature was set at 250 °C, and it was operated in split mode at a ratio of 1:60. The oven was started at 50 °C, then programmed at 0.5 °C/min to 200 °C, and kept at this final temperature for 30 min. Helium was used as the carrier gas with a flow of 1.0 mL/min. The detection was performed by FID operating at 250 °C. Chromatograms and spectra were recorded by means of GC CompassCDS software version 2.0 (Bruker Daltonics, Inc.).

NMR spectra were recorded on an Avance 300 instrument from Bruker-Biospin (Karlsruhe, Germany) in $CDCl_3$ (^{1}H at 300 MHz and ^{13}C at 75 MHz) using tetramethylsilane (TMS) as the internal standard.

Synthesis. (*R*)- and (*S*)-lavandulol were prepared by enzymatic resolution from racemic lavandulol (Sigma-Aldrich) according to the procedure described by Zada and Harel.[14] Racemic and enantiomerically pure lavandulyl acetates or propionates were synthesized by adding 3 equiv of acetic anhydride or propionyl chloride to a stirred 0.5 M solution (dichloromethane/triethylamine, 5:3) of the corresponding alcohol at 0 °C, in the presence of 4-(dimethylamino)pyridine (0.01% mol) as catalyst. The reaction progress was monitored by TLC. After completion, the reaction mixture was diluted with dichloromethane, and the organic phase was successively washed with aqueous 1 M HCl, 1 M $NaHCO_3$, and brine solutions. Then the organic layer was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. Flash column chromatography with a product-to-phase load of 1:60 w/w using a 9:1 mixture of hexane/ethyl acetate as eluent afforded the corresponding acetate or propionate esters in 88 and 90% yield, respectively. Spectroscopic properties of synthesized lavandulyl acetates (^{1}H NMR, ^{13}C NMR, MS, and specific rotation) as well as specific rotation values for starting lavandulol isomers were in agreement with previously reported data.[15] Purity and enantiomeric excess of the esters were estimated by means of chiral GC-FID direct integration of the starting chiral alcohols (ee = 98.7% for (*R*)-lavandulol and ee = 99.5% for (*S*)-lavandulol).

Because lavandulyl propionate has not been fully characterized in the literature previously, we report here the complete spectroscopic data for the levorotatory enantiomer.

*(2R)-5-Methyl-2-(prop-1-en-2-yl)hex-4-en-1-yl Propanoate [(−)-(R)-Lavandulyl propionate] (**2**).* IR (KBr) ν_{max} cm^{-1}, 2970w, 2361w, 1738s, 1456m, 1377m, 1178s, 1082m, 891m, 808w; $[\alpha]^{26}_D$ = −4.7° (*c* 3.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$), δ 5.05 (tm, 1H, J = 7 Hz), 4.81 (m, 1H), 4.72 (m, 1H), 4.03 and 4.05 (AB system, 1H each, J_{AB} = 7 Hz), 4.05 (d, 1H, J = 7 Hz), 2.39 (m, 1H), 2.30 (qd, 2H, J = 7.5 Hz), 2.10 (m, 2H), 1.69 (3H, qd, J = 0.6 Hz), 1.67 (3H, d, J = 1 Hz), 1.59 (3H, s), 1.11 (3H, t, J = 7.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$), δ 174.41, 144.91, 132.85, 121.63, 112.30, 65.65, 46.13, 28.58, 27.62, 25.71, 19.92, 17.77, 9.14; EI-MS, m/z (int %) 136 (17), 121 (33), 107 (10), 93 (86), 80 (20), 69 (100), 57 (74), 41 (63); HRMS (ESI), calcd for $C_{13}H_{23}O_2$ (M + H$^+$), 211.1693, found, 211.1705.

Collection of Volatiles from Virgin Females. Cut banana leaves from synchronized colonies containing 1000−2000 virgin females at the late N3 instar (30−35 days old) were introduced in a 2 L round-bottom flask. The flask mouth was fitted to a gas-washing piece with the inlet coupled to an activated-charcoal filled glass tube. The outlet of this system was assembled to a glass tube filled with 250 mg of preconditioned type Q, 80−100 mesh Porapak (Sigma-Aldrich). The tube filled with the adsorbent was connected to a vacuum pump to provide an air flow of approximately 3 L/h. The extraction was continued for 90 h. Parallel collections were performed on uninfested banana leaves to yield plant background samples.

After collection, adsorbents were eluted with 10 mL of HPLC grade pentane (Chromasolv, Sigma-Aldrich), and the eluates were concentrated to ca. 100 μL under a gentle stream of nitrogen and then submitted to GC-MS analysis.

Detection and Identification of Volatiles. Comparison of GC-MS chromatograms from uninfested and infested leaves allowed the selection of candidate substances. The identity of a compound was tentatively assigned according to the NIST mass spectral library, version 2.0 (Thermo Electron Corp., Waltham, MA, USA) and then confirmed by comparing retention time and mass spectra of synthetic standard candidates. Bioactivity and absolute stereochemistry of the identified compounds were determined by competitive behavioral assays of enantiomers on *D. grassi* males.

Petri Dish Choice Bioassays. Activity bioassays are based on the methodology published previously by Arai.[16] The individuals were collected just after emergence from cocoons. Each experiment was repeated five times. Each replicate of the assay was performed by randomizing the relative position of test substances and control.

Experiment 1. Competitive Attraction between Plant- and Female-Aeration Eluates to D. grassi Males: Petri Dish Choice Bioassay. Pieces of filter paper (1 cm × 1 cm) were loaded with 10 female−day equivalents (FDE) or plant control aeration, and control paper was loaded with pentane. After evaporation of the solvent for 3 min, the pieces were placed near the inner edges of a polystyrene 100 mm × 15 mm Petri dish plate (Millipore, Madrid, Spain) forming an equilateral triangle. Then 15−20 *D. grassii* males were placed in the

center of the dish, and the number of individuals standing on each paper was registered after 24 h.

Experiment 2. Competitive Attractiveness of Pure Enantiomers to D. grassi Males: Petri Dish Choice Bioassay. Pieces of filter paper (1 cm × 1 cm) were separately loaded with 1 ng of (*R*)- or (*S*)-lavandulyl ester from a stock solution (1 ng/μL) in HPLC grade hexane (Sigma-Aldrich), and control paper was loaded with hexane. After evaporation of the solvent for 3 min, the pieces containing each enantiomer and control were placed near the inner edges of a polystyrene 100 mm × 15 mm Petri dish plate (Millipore), forming an equilateral triangle. Then 15−20 *D. grassii* males were placed in the center of the dish, and the number of males standing on each paper was registered after 24 h.

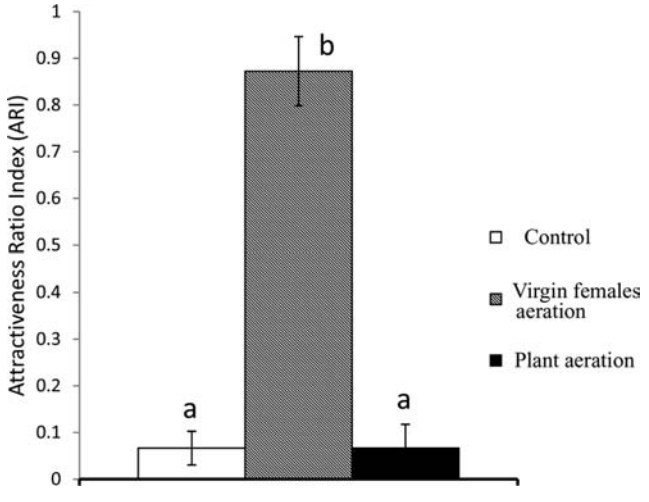

Figure 1. Mean (±SE, $N = 5$) attractiveness ratio index of competitive Petri dish bioassay for control and aeration extracts obtained from virgin females and from uninfested banana leaves. Bars with the same letters are not significantly different (Tukey−Kramer HSD test at $p = 0.05$).

Experiment 3. Competitive Attractiveness between Active Enantiomers and Racemic Material to D. grassi Males. The procedure used was equivalent to that stated for experiment 2 except that (*S*)-lavandulyl ester was substituted by the corresponding racemic ester. The concentration used for the stock solution of racemic material (2 ng/μL) balanced the amount of single enantiomers. For this experiment, the time spent by males on each paper piece was recorded for a 15 min period.

Experiment 4. Competitive Attractiveness between Major and Minor Components. The procedure used was equivalent to that stated for experiment 2 except that 1 ng of (*R*)-lavandulyl propionate or (*R*)-lavandulyl acetate was used for comparison. For this experiment, the time spent by males on each paper piece was recorded for a 15 min period.

Experiment 5. Relative Attractiveness between Enantiomerically Pure Single Components and the Natural Ratio Mixture to D. grassi Males. The procedure employed was equivalent to that stated for experiment 3 except for relative loading of each component. A load of 1 ng was used for the mixture of the two components at the original ratio of isolated pheromone (6:1), whereas (*R*)-lavandulyl propionate and (*R*)-lavandulyl acetate alone had a load of 6/7 and 1/7 of 1 ng, respectively.

The four paper pieces were located inside the plate in a manner similar to described in experiment 1 but forming a square in this setup.

Statistical Analysis. The attractiveness ratio index (ARI) was used to compare the attractiveness of each compound in the Petri dish bioassay experiments and normalize data. The values were calculated by dividing the number of mealybug males attracted to each compound to the total number of responding males in the Petri dish.

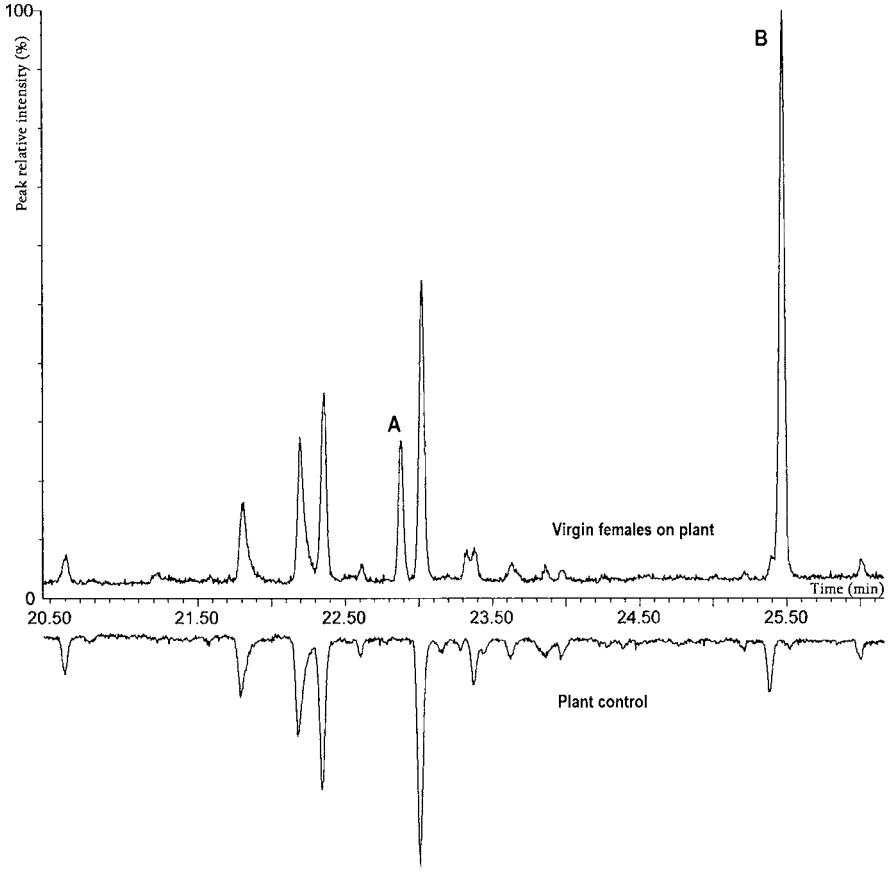

Figure 2. Gas chromatography profiles of representative aerations of virgin females over leaves of infested banana plants (upper trace) and uninfested leaves (lower trace).

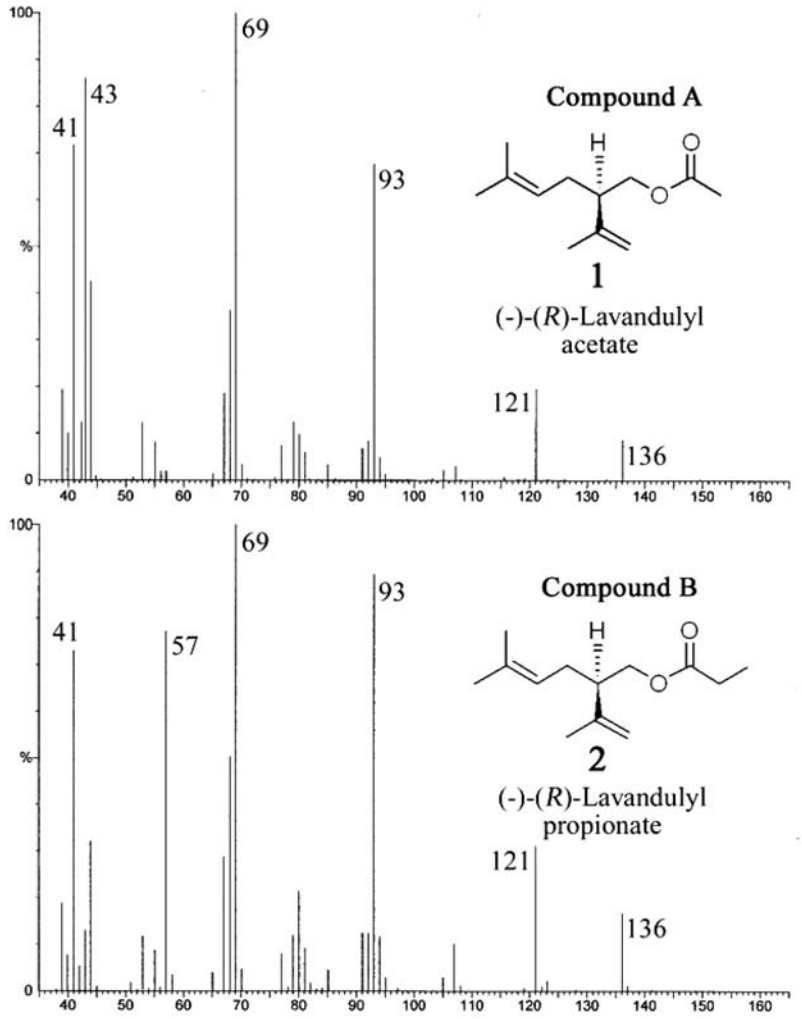

Figure 3. Mass spectra of compounds **A** and **B**, obtained from aeration of *Dysmicoccus grassii* virgin females, corresponding to (−)-(*R*)-lavandulyl acetate (**1**) and propionate (**2**), respectively.

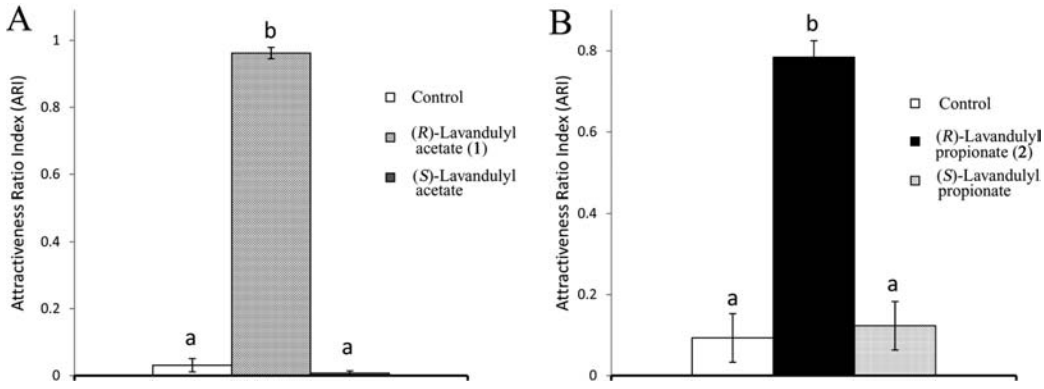

Figure 4. Mean (±SE, $N = 5$) attractiveness ratio index of competitive Petri dish bioassay for (A) control, 1 ng of (−)-(*R*)-lavandulyl acetate (**1**), and 1 ng of (+)-(*S*)-lavandulyl acetate; abd (B) control, 1 ng of (−)-(*R*)-lavandulyl propionate (**2**), and 1 ng of (+)-(*S*)-lavandulyl propionate. Bars with the same letters are not significantly different (Tukey−Kramer HSD test at $p = 0.05$).

For time spent measurements, ARI was calculated by dividing the accumulated time spent by individuals for each compound to the total time spent by all of the individuals on all of the paper pieces.

The ARI values were transformed to arcsine square root values for analysis of variance (ANOVA). The means were compared and separated by the Tukey−Kramer HSD test at $p = 0.05$. All statistical analyses were conducted using SPSS Statistics version 19 from IBM Corp. (Armonk, NY, USA).

RESULTS AND DISCUSSION

The data obtained from experiment 1 showed that *D. grassii* males were strongly attracted to collected volatiles emitted by virgin females against host plant volatiles alone (Figure 1). The conclusions derived from this initial experiment allowed us to prove the existence of a sex pheromone for this species.

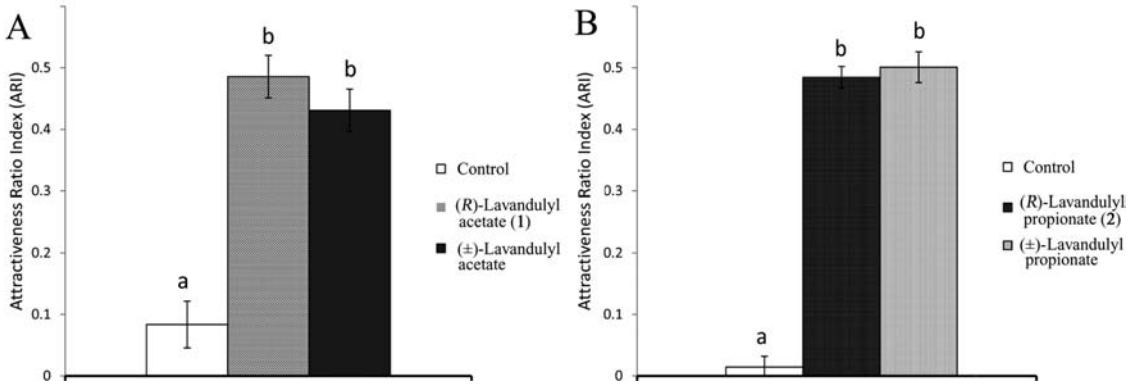

Figure 5. Mean (±SE, $N = 5$) attractiveness ratio index of competitive Petri dish bioassay for (A) control, 1 ng (−)-(*R*)-lavandulyl acetate (**1**), and 2 ng of (±)-lavandulyl acetate; and (B) control, 1 ng (−)-(*R*)-lavandulyl propionate (**2**), and 2 ng of (±)-lavandulyl propionate. Bars with the same letters are not significantly different (Tukey−Kramer HSD test at $p = 0.05$).

Subsequent differential GC analysis between volatile collections from aeration of virgin female infested and uninfested banana leaves highlighted two peaks that were assumed to originate from insects (Figure 2).

Mass spectra analysis by means of the NIST database pointed to an acyclic monoterpenic structure for both compounds. In principle, these preliminary results were in agreement with the structures of sex pheromones known to date for species within the Pseudococcidae family, which are acyclic or alicyclic monoterpenyl esters, with the exception of a *nor*-monoterpenyl ester for *Pseudococcus comstockii*.[17] The mass spectrum of the minor chromatographic peak (compound **A**, eluting at 21.61 min, KI 1282) afforded a high match factor for lavandulyl acetate. Separate injections and coelution of compound **A** with racemic synthetic lavandulyl acetate showed identical retention time and mass spectrum (Figure 3), confirming the tentative identification provided by the NIST database.

The fragmentation pattern of the mass spectrum of compound **B** and the NIST database strongly supported neryl or geranyl propionate as possible structures. Analysis of a standard sample of the aforementioned esters did not afford coincident retention times to compound **B** (eluting at 24.20 min, KI 1384). The fragmentation pattern of the mass spectrum was also very close to that of lavandulyl acetate, suggesting the homologue propionate (not included in the NIST database) as a reasonable structure. Synthesis of a standard sample of lavandulyl propionate confirmed the identity of the unknown compound **B** due to identical retention time and mass spectrum.

Once the structures of both compounds **A** and **B** had been established as lavandulyl acetate and propionate, respectively, further studies were required to determine their enantiomeric identity. Carrying out the necessary tests required the preparation of the corresponding pair of enantiomers of both esters. This was successfully achieved by enzymatic resolution from commercially available racemic lavandulol according to the work published by Zada and Harel,[14] followed by standard esterification procedures.

Attempts to compare the synthesized stereoisomers with the volatile collections on chiral GC-FID failed due to poor separation of enantiomers. Saponification of the minute amounts of material obtained from aeration led to complex mixtures that showed overlapping peaks in the retention times of (*R*)- and (*S*)-lavandulol. Attempts to optimize the separation performance by means of GC conditions did not improve the result. Therefore, the chromatographic approach was discarded.

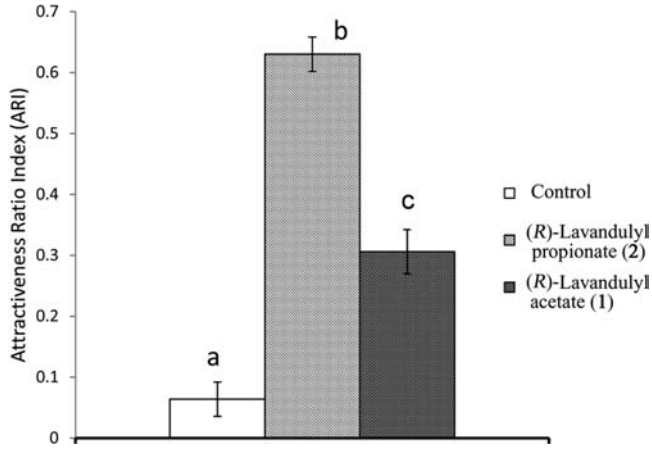

Figure 6. Mean (±SE, $N = 5$) attractiveness ratio index of competitive Petri dish bioassay for control, 1 ng of (−)-(*R*)-lavandulyl acetate (**1**), and 1 ng of (−)-(*R*)-lavandulyl propionate (**2**). Bars with the same letters are not significantly different (Tukey−Kramer HSD test at $p = 0.05$).

Bioassays on enantiomers of both lavandulyl propionate and acetate (experiment 2) strongly suggest that these substances are the main components of the sex pheromone of *D. grassii*. (*R*)-Isomers elicited an intense calling effect, whereas no significant attraction was found for (*S*)-isomers (Figure 4). Behavioral observations confirmed this fact because males insistently attempted to copulate with the (*R*)-isomer-loaded paper pieces after seeking and contacting them.

Additional Petri dish choice experiments (experiment 3) showed no modification over male attraction when (*S*)-isomers were loaded along with (*R*)-compounds (Figure 5). Differences in attraction were not significant for paper pieces loaded with 1 ng of the (*R*)-ester regardless of the stereochemical purity of its source. With respect to the change in quantitation of ARI for these experiments it is notable that in the case when more than one attractive source of (*R*)-lavandulyl esters was being assayed in the same dish plate, *D. grassii* males alternated between test pieces of paper, trying to copulate with them for some time before moving to the other paper piece. The observed behavior forced us to change the methodology of quantitation on bioassay by recording the cumulative time spent for all males on each paper piece.

On the other hand, the relative attractiveness of each pheromonal compound was evaluated by choice competitive assays. When (*R*)-lavandulyl propionate (**2**) was compared with the

same amount of (*R*)-lavandulyl acetate (**1**, experiment 4), the major component exhibited a significantly higher activity (Figure 6). Further studies revealed that a mixture of both compounds at a similar ratio to that found in aerations of virgin females (experiment 5) showed no statistically significant differences from the sum of the relative attraction of the separate substances in Petri dish experiments (Figure 7). These results

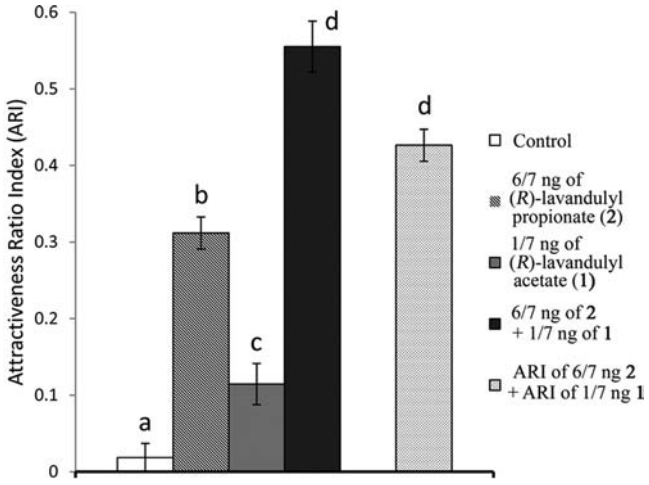

Figure 7. Mean (±SE, $N = 5$) attractiveness ratio index (ARI) of competitive Petri dish bioassay of single enantiopure compounds at isolated ratio against its corresponding mixture: control, 1/7 ng (−)-(*R*)-lavandulyl acetate (**1**), 6/7 ng of (−)-(*R*)-lavandulyl propionate (**2**), and 1 ng of a mixture of both compounds **1** and **2** at a ratio of 1:6. The bar separated from the group on the right represents the sum of ARI of individual compounds. Bars with the same letters are not significantly different (Tukey−Kramer HSD test at $p = 0.05$).

suggested an additive effect between both compounds, and no synergy was detected.

In conclusion, we have identified two terpenyl esters from virgin females of *D. grassii* that are responsible for chemical communication in terms of mate seeking and localization by males. The absolute stereochemistry of the naturally occurring substances, relative attractiveness, and possible synergistic effect have been established by means of competitive Petri dish bioassays. The structural elucidation of these compounds is the first step toward the development of new and ecological control methods for this important pest based on its sexual pheromone.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, ^{13}C NMR, DEPT, and mass spectra of lavandulyl acetate (Figures S1, S2, S3, and S4, respectively); ^{1}H NMR, ^{13}C NMR, DEPT, and mass spectra of lavandulyl acetate (Figures S5, S6, S7, and S8, respectively), tabular material for summarized statistical results of bioassay (Tables 1−7), and GC chromatogram for KI calculation of lavandulyl propionate and acetate (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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