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Article

Identification of (1*R*, 2*S*)-Grandisal and (1*R*, 2*S*)-Grandisol in *Pissodes castaneus* Male-Produced Volatiles: Evidence of a Sex Pheromone

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O gorgulho-da-casca-do-pinus, *Pissodes castaneus* (De Geer, 1775) (Coleoptera, Curculionidae) é uma praga florestal que foi detectada no Brasil em 2001 e atualmente se encontra distribuída nos três estados da Região Sul, causando grande preocupação para o setor florestal. Os objetivos deste estudo foram isolar, identificar e avaliar a atividade comportamental dos voláteis produzidos pelos machos de *P. castaneus*. Nossos resultados indicam que a comunicação entre indivíduos desta espécie é mediada por feromônios, o que foi evidenciado pela atração significativa de fêmeas pelos voláteis produzidos pelos machos. Testes comportamentais realizados com os insetos e sua planta hospedeira, *Pinus taeda*, indicam que os compostos produzidos pelos machos atuam como feromônios sexuais e que os compostos liberados pela planta hospedeira não aumentam a atividade destes compostos. Os compostos produzidos pelos machos de *P. castaneus* foram identificados como (1*R*, 2*S*)-grandisal e (1*R*, 2*S*)-grandisol, em excesso enantiomérico superior a 95%.

The banded pine weevil, *Pissodes castaneus* (De Geer, 1775) (Coleoptera, Curculionidae) is a forest pest recorded in Brazil since 2001, which is already distributed in the three states of the Southern Region, causing great concern to the forestry sector. Objectives of our study were to isolate, identify, and assess the behavioral activity of male-produced volatiles of *P. castaneus*. Our results indicate that communication between conspecifics of *P. castaneus* is mediated by pheromones, as verified by the significant attraction of females to male-produced volatiles. Behavioral tests performed with the insects and the host plant, *Pinus taeda*, showed that male-produced compounds may act as sex pheromones and that the compounds released by the host plant did not enhance the activity of the male-produced volatiles. The chemical structures of the male specific volatile compounds produced by *P. castaneus* were determined to be (1*R*, 2*S*)-grandisal and (1*R*, 2*S*)-grandisol in an enantiomeric excess exceeding 95%.

Keywords: *Pissodes castaneus*, Coleoptera, Curculionidae, evidence of sex pheromone, *Pinus*

Introduction

Weevils of the genus *Pissodes* comprise an important group of pests of conifers in the family Pinaceae. The banded pine weevil *Pissodes castaneus* (De Geer, 1775) (Coleoptera, Curculionidae) is an important pest of *Pinus* species in Europe and is also present in Siberia, North Africa, Madeira, Canary Islands,[1,2] Turkey,[3] and South America.[4] *P. castaneus* has recently reached Brazil and caused great concern in the forestry sector. It was first

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recorded in 2001 in São José dos Ausentes, Rio Grande do Sul[5] and is currently distributed in the three states of south Brazil. The insect attacks mainly weakened trees. Adult weevils lay eggs under the bark. Subsequent damage is caused by the larvae feeding on the phloems of the trunk, interrupting the sap circulation which may kill the tree.[1] In 2002, 7.6% of the trees of a plantation of *Pinus taeda* L. grown in Cambará do Sul, Rio Grande do Sul State, Brazil, were attacked by *P. castaneus* while in São Joaquim, Santa Catarina State, Brazil, 16.53% of the trees were attacked.[5]

The first evidence of an aggregation pheromone of insects in the genus *Pissodes* was reported by Booth and Lanier,[6] who demonstrated in field tests that males of *Pissodes approximatus* (Hopkins, 1911) in the presence of the host plant were more attractive to both sexes than females along with the host plant or the host plant alone.[6] Males of *Pissodes strobi* (Peck, 1817) placed on the same host plant also attracted *P. approximatus*. Later, Booth[7] demonstrated that *P. strobi* males, caged on white pine (*Pinus strobus* L.) leaders, were attractive to conspecific males and females in field tests. However, females feeding on leaders and leaders alone were not attractive.[7]

Booth *et al.*[7] isolated two compounds, a monoterpene alcohol, grandisol (*cis*-2-isopropenyl-1-methylcyclobutane ethanol) and its corresponding aldehyde, grandisal, from males of both *P. strobi* and *P. approximatus*. In field tests, synthetic grandisol and grandisal together with volatiles from red pine (*Pinus resinosa* Aiton) bolts acted synergistically to attract both sexes of *P. approximatus*. This response was similar to that of males of *P. approximatus* feeding on the host plant. Males and females of *P. strobi* responded stronger to caged males feeding on leaders of white pine than to leaders alone. The combination of grandisol, grandisal, and leaders were less attractive to *P. strobi* than males on leaders, but more attractive than leaders alone. Investigations carried out during different seasons proved that males of both species produced grandisol and grandisal only when females were reproductively mature.

Fontaine and Foltz[8] showed that males of the deodar weevil, *Pissodes nemorensis* (Germar, 1824) feeding on slash pine (*Pinus elliottii* Engelmann) bolts produced an aggregation pheromone. Later, Phillips *et al.*[9] found that this aggregation pheromone was composed of grandisol and grandisal, which were isolated from extracts of volatiles from whole males and hindguts of males. A field test conducted in northeastern Florida showed that the combination of grandisol, grandisal, and slash pine (*P. elliottii*) bolts acted synergistically to attract a large number of males and females of *P. nemorensis.*

Hibbard and Webster[10] isolated and identified grandisol and grandisal as components of the aggregation pheromone of *P. strobi* and *P. nemorensis*. The presence of a male produced pheromone in *Pissodes schwarzi* (Hopkins, 1911) was also shown, but the corresponding chemical structure has not yet been determined.[11]

The objectives of this research were the isolation, identification and determination of the biological activity of the pheromone produced by *P. castaneus*, aiming at the application of this knowledge in Integrated Pest Management (IPM) programs against the banded pine weevil in *Pinus* cultivation areas.

Experimental

Insects

The insect colony was started with insects collected from log traps installed in *P. taeda* producing areas in the city of Cambará do Sul, in the State of Rio Grande do Sul, and Três Barras, in the State of Santa Catarina, Brazil. To obtain the insects for bioassays, the logs were removed from the field and placed in screened cages measuring 30 cm height by 30 cm diameter until adult emergence. Upon their emergence, the adults were sexed and kept for 5-10 days in an incubator at 22 ± 2 °C, 70 ± 10% relative humidity and 12L:12D light cycle. Females and males were selected for the trials and placed in plastic screened cages with ventilation at the sides under the same conditions as described above. Adults were fed a natural diet consisting of pieces of fresh pine branches. The cages were cleaned, and the food supply was replaced at regular intervals of five days.

Collection of volatiles from P. castaneus

Naive insects (30 males and 30 females, sexes aerated individually) were aerated in a 1 L glass chamber with fresh cuttings of *P. taeda* added for food. The pine branches were replaced every 48 h. Aerations were carried out under controlled conditions at 23 ± 2 °C, relative humidity of 70 ± 10% and a photoperiod of 12L:12D. Volatiles were trapped on a 0.4 cm long bed of Super Q resin (Alltech, Deerfield, Illinois, USA) held in place by glass wool plugs in a glass tube (4 mm ID). Volatiles were collected for two days (flow 1.0 L min^{-1}), then eluted with hexane (3 × 0.5 mL). Extracts were concentrated as required under Ar.

Laboratory bioassays

Bioassays were conducted with a vertical Y tube (2.5 cm ID, bottom arm 20 cm, top arms 20 cm) with ground glass female joints at the end of each arm, and matching male joints terminating in hose nipples. In the system, the speed of

humidified, charcoal filtered air was adjusted to 2.0 L min^{-1}. All connections were made of Tygon tubing (0.6 cm ID). After each trial, the glassware was washed with neutral soap, dipped in 70% ethanol, and placed in the oven for 30 min, at 100 °C, to avoid any residual volatile compounds. The Tygon tubings were not heated to avoid partial degradation and contamination of the aeration samples. Test insects were introduced individually into the bottom of the Y tube and allowed 20 min to respond. When the insect moved through the arm containing the stimulus walking at least 2/3 of the arm length, the result was considered positive, while the result was negative, when it moved through the control arm. However, when the insect did not move towards any of the arms within 20 min, it was considered non-responsive. Each insect was used only once in the bioassays. The insects used in the tests were given no food 12 h before the bioassays. The branches of *P. taeda* used as stimuli were collected one day before conducting the bioassays, placed in the thermal box and transported to the laboratory, where they were kept in the refrigerator at 10 ± 2 °C until the start of the tests. Bioassays were conducted in a temperature (23 ±2 °C) and humidity (70 ±10% relative humidity) controlled room. Each stimulus was tested with 40 sexually mature naive adult insects.

Chemical analysis

Extracts were analyzed by gas chromatography (GC) in the splitless mode with a Varian 3800 instrument equipped with a VA-5 capillary column (30 m × 0.25 mm × 0.25 µm), using a program of 50 °C for 1 min, 5 °C min^{-1} to 180 °C and held for 25 min. Extracts were also analyzed by coupled GC/MS with a Varian Saturn 2000 system equipped with an ion trap detector using a CP-Sil 8 low bleeding column under the same conditions as described above. Enantioselective gas chromatography was carried out by using a home-made 50 m, 0.25 mm ID fused silica capillary coated with a 1:1-mixture of heptakis-(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin and OV-1701, operated at 90 °C.

Chemicals

Racemic grandisol (98% purity) was purchased from Bedoukian Research Inc. (Danbury, CT, US). Pure (1*R*, 2*S*)-(+)-grandisol was prepared previously.[12]

Micro-reduction of natural grandisal to grandisol

The natural extract of *P. castaneus* (*ca*. 50 µL of an extract prepared by aeration of 20 beetles for 48 h) was concentrated to 1-3 µL and dissolved in 100 µL of diethylether. Lithium aluminum tetrahydride (1-4 mg) was added, and the mixture was stirred at room temperature for 20 min. Subsequently, 100 µL water was added and the organic layer was removed and dried over Na_2SO_4.[13] The sample was analyzed by GC/MS without further purification or derivatization.

Statistical analyses

The data obtained with insects that reached the corresponding odor sources were compared by the χ^2 test using the BioStat version 3.[14] Results showing $P \leq 0.05$ were considered statistically significant.

Results and Discussion

Identification of male-produced volatiles

Extracts of freshly emerged males and females *P. castaneus* showed the same qualitative composition until 21 days after they become adults. Subsequently, two sex specific compounds were observed in the aeration extracts of males: compound a (40-47%) and compound b (53-60%) (Figure 1). Investigations by GC/MS provided analytical data and fragmentation pattern that strongly suggested a and b to be grandisal [KI 1126, VA-5, ions of *m/z* 67 (base peak), 108 and 152 (M^+)] and grandisol [KI 1212, VA-5; ions of *m/z* 67 (base peak), 109, and 154 (M^+)], respectively.[13]

Using a modified β-cyclodextrin as the stationary phase, the grandisol enantiomers were base-line separated, showing an α-value of tr(1*S*, 2*R*):tr(1*R*, 2*S*) of 1.054 (Figure 2). Enantioselective gas chromatography of crude extracts of males revealed the produced grandisol to show (1*R*, 2*S*)-configuration of high enantiomeric purity, *i.e.*, *ca*. 95% enantiomeric excess. However, the enantiomers of grandisal could not be separated under these conditions. Therefore, one aliquot of a crude male extract was treated with lithium aluminum tetrahydride to convert grandisal into grandisol. Consequently, the peak representing grandisal disappeared, while the amount of grandisol increased.[13] The absolute configuration of the resulting grandisol was determined by enantioselective gas chromatography using the same GC-column as before.

The enantiomeric excess of grandisol obtained through the treatment of an extract of males with lithium aluminum tetrahydride was determined by enantioselective GC to be more than 95%. As a result, both the male produced grandisol and grandisal were shown to keep (1*R*, 2*S*)-configuration.

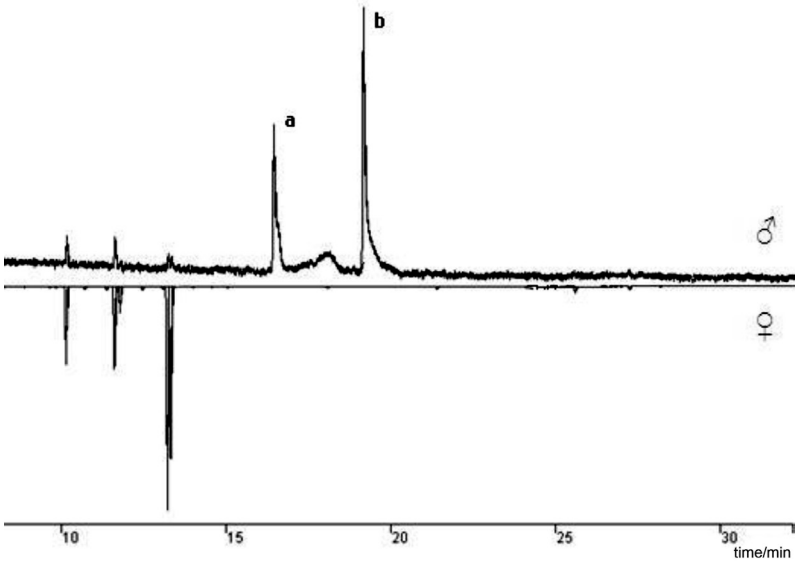


Figure 1. GC analysis of volatiles obtained from male and female *P. castaneus* insects, showing two male-specific compounds, a and b.

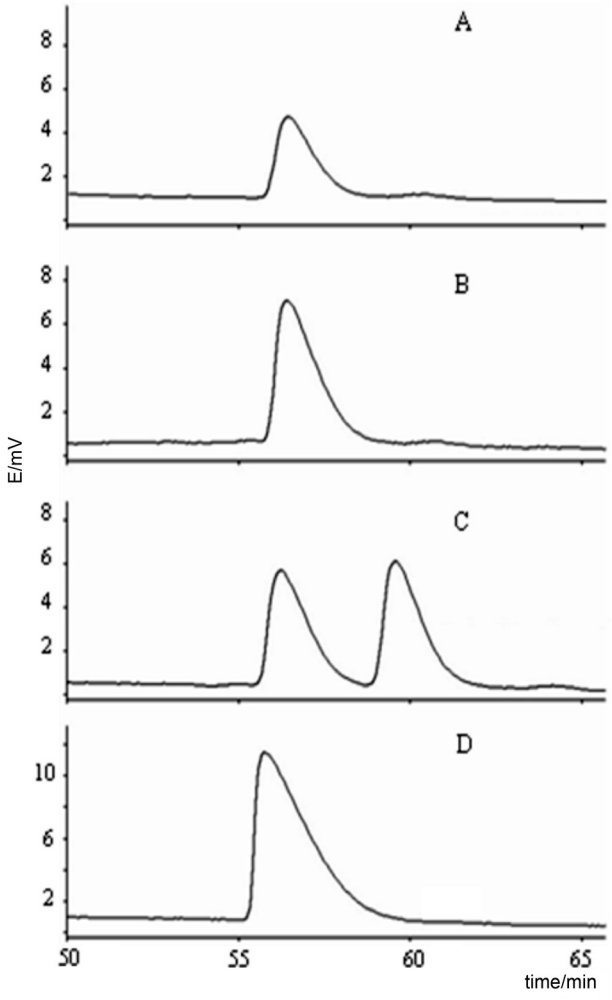


Figure 2. Chromatograms obtained with a fused silica column coated with a 1:1-mixture of heptakis-(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin and OV-1701 showing the area of elution of grandisol: (A), natural extract, (B), natural extract after treatment with $LiAlH_4$ (C), synthetic racemic grandisol, (D), synthetic (1*R*, 2*S*)-(+)-grandisol.

Laboratory bioassays

In Y-tube bioassays, test insects walked rather than flew when they responded to stimuli. In the first series of bioassays, females were significantly more attracted by males as compared to the control treatment ($\chi^2 = 4.90$, $p \leq 0.04$). Our results clearly prove that stimuli of males show biological activities on females, but not on conspecific males ($\chi^2 = 0.40$, $p \leq 0.63$) (Table 1).

A second series of bioassays proved that neither males nor females were significantly attracted to females (male $\chi^2 = 0.40$, $p \leq 0.63$; female $\chi^2 = 1.60$, $p \leq 0.27$).

The attractiveness of males and females to volatiles emitted by the host plant (fresh pieces of *P. taeda*) was determined (female $\chi^2 = 8.10$, $p \leq 0.01$; male $\chi^2 = 6.40$, $p \leq 0.02$) in another bioassay series. It was found that the presence of the host plant (pieces of *P. taeda*) associated with male-produced volatiles did not show a synergistic effect on the reaction of females.

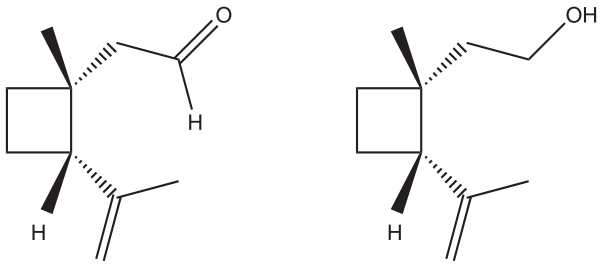
The good resolution obtained when racemic grandisol was directly analyzed by enantioselective GC using a modified β-cyclodextrin as the stationary phase, permitted the immediate determination of the enantiomeric composition of the naturally occurring grandisol without further derivatization. Corresponding data for grandisal were obtained indirectly after reduction of the natural product to grandisol.[13] Through this analysis we determined that males of *P. castaneus* produce both grandisal and grandisol with (1*R*, 2*S*)-configuration, (Figure 3) and in an enantiomeric excess of over 95%.

Although plant odors showed to be attractive to males and females they did not synergize the activity of male-produced volatiles in laboratory bioassays. These

Table 1. Responses of individual male and female *P. castaneus* adults to treatments in Y-tube olfactometer

Bioassay[a]	Source 1	Source 2	Sex responding	Response to		χ^2	*p*
				source 1	source 2		
1	Males	air	Female	27	13	4.90	0.04
			Male	22	18	0.40	0.63
2	Females	air	Female	24	16	1.60	0.27
			Male	18	22	0.40	0.63
3	Males + host plant	air	Female	27	13	4.90	0.04
			Male	22	18	0.40	0.63
4	Host plant	air	Female	29	11	8.10	0.01
			Male	28	12	6.40	0.02

[a]see experimental part for details.



a b

Figure 3. Structures of (1*R*, 2*S*)-grandisal (a) and (1*R*, 2S)-grandisol (b).

results differ from those found for *P. nemorensis*[9] and *P. approximates*[7,15] where the host plant showed additive effects, increasing pheromone activities.

Grandisal and grandisol, male-produced compounds in *P. castaneus*, are widespread components of weevil pheromones.[9] Male *P. nemorensis* produces nearly 100% of (1*R*, 2*S*)-grandisol and nearly 100% (1*S*, 2*R*)-grandisal, while male *P. strobi* produces approximately 99% of (1*R*, 2*S*)-grandisol and approximately 60% of (1*R*, 2*S*)-grandisal.[10] The specificity required in the mechanism of action of grandisol and grandisal as pheromones for these sympratic species certainly involves the stereochemical composition of the compounds as well their relative proportions, since for *P. castaneus*, for instance, grandisol is the major component while for *P. nemorensis* grandisal is the major one.[11]

Conclusions

Our results demonstrate that male *P. castaneus* very likely utilizes (1*R*, 2*S*)-grandisol and (1*R*, 2*S*)-grandisal as sex pheromone. The monoterpene alcohol and aldehyde are both produced in an enantiomeric excess of about 95%. No synergistic effects among host plant volatiles and the insect produced compounds were detected.

To the best of our knowledge, the work described here represents the first evidence of a sex pheromone for insects belonging to the genus *Pissodes*, while earlier work, carried out with other species, revealed the presence of aggregation pheromones.

The identification of plant-released volatiles showing attraction to male and female *P. castaneus* will be subject of a separate investigation, carried out in our laboratory.

Supplementary Information

Mass spectra of grandisol and grandisal are presented as supplementary data and are available free of charge at http://jbcs.sbq.org.br as a PDF file.

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