Evidence for (E)-pityol as an aggregation pheromone of Pityophthorus pubescens (Coleoptera: Curculionidae: Scolytinae)

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Abstract—We present evidence favoring the use of (E)-pityol as an aggregation pheromone in Pityophthorus pubescens (Marsham). (E)-Pityol was detected in effluvia of male and female P. pubescens, and antennae of both sexes responded to (E)-(+)-pityol in electroantennogram assays. In two-choice olfactometer tests, males significantly preferred (E)-(+)-pityol and (E)-(±)-pityol to blank controls at doses of 1, 10, and 100 ng, whereas females only showed a preference for (E)-pityol at the 1 ng dose.

Introduction

Most species of twig beetles, Pityophthorus Eichhoff (Coleoptera: Curculionidae: Scolytinae), are considered polygamous (Bright 1981; Wood 1982), but there is evidence of monogamy in a few species (Pfeffer 1976; Bright 1981; Dallara et al. 2000). In polygamous species, a male selects the host, starts constructing an egg gallery with a nuptial chamber, and attracts females, which extend the egg gallery (Bright 1981; Kirkendall 1983).

Little is known about pheromone-based aggregation in the genus Pityophthorus. Vité (1965); however, Chararas (1966, 1975) observed that males of P. confertus Swaine, P. annectans LeConte, and P. pityographus (Ratzeburg) attract conspecific females. Francke et al. (1987) identified (2R,5S)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran (E-(+)-pityol) and cis-1-(2-hydroxyethyl)-1-methyl-2-(1-methylethenyl) cyclobutane ((±)-grandisol) from males of P. pityographus, and showed that both compounds were active in the field. (E)-(+) Pityol was also found in males of P. carmeli Swaine and females of P. nitidulus Mannerheim and P. setosus (Blackman) (Dallara et al. 2000), and has been reported as the female-produced sex pheromone of the cone beetles Conophthorus resinosae Hopkins, C. coniperda (Schwarz), and C. ponderosae Hopkins (Coleoptera: Curculionidae: Scolytinae) (Birgersson et al. 1995; Pierce et al. 1995;
Miller et al. 2000). In addition to pityol, the spiroacetal (5S,7S)-(−)-7-methyl-1,6-dioxaspiro[4.5]decane (conophthorin) has been identified as a semiochemical in species of *Pityophthorus*. Conophthorin is a component of the aggregation pheromone emitted by males of *P. carmeli* (Dallara et al. 2000) and is a male-produced repellent in some other scolytines (Kohnle et al. 1992; Birgersson et al. 1995; Pierce et al. 1995; de Groot and DeBarr 2000). (E)-(−)-Conophthorin, by itself, is not attractive to *Pityophthorus* species, and significantly reduces catches of *P. setosus* (predominantly males) to (E)-pityol (Dallara et al. 2000), suggesting that it acts as a synomone to reduce intraspecific competition between *P. setosus*, *P. nitidulus*, and *P. carmeli*, three species that cohabit in *Pinus radiata* D. Don (Pinaceae) stands in central coastal California (Dallara et al. 2000).

*Pityophthorus pubescens* (Marsham) is the only *Pityophthorus* species known from *P. radiata* stands in the Basque Country (northern Spain) (López et al. 2007). It is associated with *Fusarium circinatum* (Niremberg and O'Donnell) (Hypocreales: Nectriaceae), the pathogen causing pitch canker disease (Romón et al. 2007). *Pityophthorus setosus* and *P. carmeli* have been associated with pitch canker-infected Monterey pines in California (Storer et al. 2004; Sakamoto et al. 2007). Our objectives were to identify the aggregation pheromone of *P. pubescens* and evaluate its biological activity in electroantennographic (EAG) and behavioral tests in the laboratory.

### Materials and methods

#### Beetles

Specimens of *P. pubescens* were collected from infested branches of *P. radiata* from a stand located at Gorosika (43°15′N, 02°42′W), Basque Country. Infested branches were maintained in an incubator at 25 °C and 65% RH under a 10L:14D photoperiod, and beetles were collected by dissecting the branches with a microscalpel under a binocular microscope.

#### Chemicals

Racemic (E)-pityol (93.4% chemical purity) was purchased from Contech Inc. (Delta British Columbia, Canada) and (E)-(−)-pityol (99%) (Mori and Puapoomchareon 1987) was provided by Prof. W. Francke (Institute of Organic Chemistry, University of Hamburg, Germany).

#### Collection of volatiles

Volatiles of *P. pubescens* were adsorbed on a Porapak Q column (50/80 mesh, Supelco, Bellefonte, Pennsylvania) using 200 beetles of each sex caged in a glass flask and exposed to a stream of charcoal-filtered air at a flow rate of 1 L/min for 40 h. Three independent sets of this system containing males, females, or no beetles (control) were operated simultaneously and two volatile collections and blanks (control) were made for each sex. Each column was extracted with 300 µL of dichloromethane and the extract was stored at −40 °C until used.

Additional volatile collections were obtained using a polydimethylsiloxane fiber (100 µm) for solid-phase microextraction (SPME) (Supelco) (Belardi and Pawliszyn 1989; Matich et al. 1996). Two hundred individuals of each sex were placed in separate 40 mL vials (29 mm × 81 mm) with a SPME fiber for 36 h under laboratory conditions (mean temperature 23 °C, 65% RH, 14L:10D). The fiber had been conditioned prior to use by inserting it into the injection port of a gas chromatograph (GC) for 15 min. Two replicates were done for each sex, using 200 different beetles each time.

Volatiles from both collection methods were analyzed on a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain) using helium (1 mL/min) as the carrier gas. The samples were introduced in splitless mode at 250 °C. The column used for analysis was a 30 m × 0.25 mm i.d. × 0.25 µm HP-5MS fused silica capillary (Agilent Technologies, Madrid, Spain). The following chromatographic conditions were used: injection at 60 °C for 5 min, increasing by 5 °C/min to 280 °C, and then maintained at this temperature for 10 min. Mass spectra were obtained
under electron impact ionization mode at 70 eV in the 40–400 m/z range.

EAG assays
The EAG instrument was obtained from Syntech (Kirchzarten, Germany). EAG recordings were performed using Ag-AgCl glass microcapillaries filled with Ringer solution. Each beetle was fixed upside down on a piece of double-sided sticky tape, and the head was excised using a microscalpel. The recording electrode was connected to the tip of an antenna and the reference electrode was inserted into the occipital foramen using MP15 micromanipulators (Syntech). Humidified pure air (1000 mL/min) was continuously directed over the antenna. The signals were amplified (100 ×) and filtered (DC to 1 kHz) with a IDAC-2 interface (Syntech), digitized on a PC, and analyzed with the EAG Pro program. The EAG system was set up in a Faraday cage (70 cm × 65 cm × 60 cm) to preclude external electric signals. A log dilution series of \((E)-(\pm)-\)pityol in hexane was prepared at doses of 0.1, 1, 10, and 100 ng/\(\mu\)L. Odor stimuli consisted of applying 10 \(\mu\)L to each concentration to a filter-paper strip (2.5 cm i.d.) that was then placed inside a Pasteur pipette. After evaporation of the solvent, puffs of 400 ms duration through the Pasteur pipette placed 2 cm from the antennal setup were pulsed with a stimulus controller, CS-01 (Syntech). The recovery time for the antenna between two consecutive stimuli was established at 1–1.5 min. Eight individuals of each sex were used with each dose of \((E)-(\pm)-\)pityol and only one antenna was used per beetle. Three puffs of each dose of \((E)-(\pm)-\)pityol were applied and the mean amplitude of depolarization was subtracted from that in response to puffs of the hexane control, before and after each stimulus. Stimuli were delivered in order of increasing dose.

Behavioral response
The behavioral responses of male and female \(P.\) pubescens to three different doses of \((E)-(\pm)-\)pityol and \((E)-(\pm)-\)pityol were evaluated using a Y-tube olfactometer designed for small beetles (5 mm i.d., main arm 5 cm long, short arms 4 cm long, 90° angle between short arms). Each short arm of the olfactometer was connected to a glass chamber containing the odor source. One of the arms contained 10 \(\mu\)L of hexane on a circle of filter paper (2.5 cm diameter) as a control, while the other contained a piece of filter paper of similar size treated with the test chemical. Different doses of the semiochemical were obtained from 10 \(\mu\)L of decadic dilutions in hexane containing 0.1, 1, and 10 ng/\(\mu\)L. Filter papers were replaced in each arm for every second beetle. Incoming air was filtered through activated charcoal and the airflow was maintained at 820 mL/min.

All tests were conducted at 23 ± 1 °C, 50 ± 9% RH, and beetles were acclimatized to conditions for 15 min before the assays. Each beetle was observed for a maximum of 5 min and was used only once. A response was considered positive when the beetle walked at least 3 cm into one of the arms. The arms were reversed after five beetles were tested, to avoid directional bias. After 10 individuals were tested, the olfactometer was cleaned first with soap and water and then with absolute ethanol, and left to dry until the solvent had completely evaporated. In total, 35–40 different beetles were used for each sex and dose.

Statistical analysis
Data on the EAG response to \((E)-(\pm)-\)pityol concentrations and sex were subjected to two-way ANOVA, followed by Tukey’s post-hoc tests at a significance level of \(\alpha = 0.05\). In olfactometer trials, the null hypothesis that \(P.\) pubescens showed no preference for either olfactometer arm (a response equal to 50:50) was analyzed by a \(\chi^2\) test.

Results
Volatile collections
GC-mass spectrometry analyses of volatile collections trapped on Porapak Q or by SPME revealed the presence of \((E)-\)pityol in both sexes of \(P.\) pubescens by comparing its retention time and mass spectra with those of
an authentic standard (Francke et al. 1987) (Figs. 1, 2). All spectra exhibited a base peak at m/z 59, suggesting a tertiary alcohol, and prominent peaks at 85, 102, and 129 m/z. The intense fragment ion of m/z 85 in the mass spectrum reflects the presence of a tetrahydropyran or methyl-substituted tetrahydrofuran ring as a partial structure. The amount of (E)-(+/C27)-pityol emitted was not compared between the sexes. As expected, no traces of (E)-pityol were found when the controls of both sexes were analyzed.

**Behavioral response**

Male *P. pubescens* significantly preferred the olfactometer arm containing either racemic (E)-pityol or (E)-(+) -pityol at all three doses tested (Fig. 4A). The lowest doses (1 ng for the racemic material and 1–10 ng for the chiral material) proved to be the most attractive. However, females were attracted only to the lowest dose (1 ng) of racemic (E)-pityol and (E)-(+) -pityol (Fig. 4B). Moreover, they significantly avoided racemic (E)-pityol at doses of 10 and 100 ng.

**Discussion**

We isolated and identified (E)-pityol in volatiles of male and female *P. pubescens*, and demonstrated an electrophysiological response to (E)-(+) -pityol in the antennae of both sexes, as well as a dose-dependent behavioral response of both sexes to racemic (E)-pityol and (E)-(+) -pityol in olfactometer bioassays. These results suggest that (E)-(+) -pityol may be a key compound in the chemical ecology of *P. pubescens*. Prior to our study, (E)-(+) -pityol had been detected in several *Pityophthorus* species but always in one sex only, e.g., males of *P. carmeli* and *P. pityographus* and females of *P. setosus* and *P. nitidulus*. In field tests, males of *P. setosus*, a monogamous species,
responded strongly to (E)-(+)‐pityol alone (Dallara et al. 2000), whereas *P. pityographus*, a polygamous species, was attracted by the combination of grandisol and (E)-(+)‐pityol (Francke et al. 1987). Another polygamous species, *P. carmeli*, was attracted only by the combination of (E)-(+)‐pityol and (E)-(−/C28)‐conophthorin (Dallara et al. 2000). Similarly, in species of *Conophthorus* Hopkins (a genus considered to be phylogenetically closely related to *Pityophthorus*) (Cognato et al. 2005), (E)-(+)‐pityol is known to be the major compound of its sex pheromone and has been found only in females (Birgersson et al. 1995; Pierce et al. 1995; Miller et al. 2000).

During dissection of naturally infested branches to collect beetles, we observed that all galleries contained a single mating pair of *P. pubescens*, and had a longitudinal pattern without a nuptial chamber, which is consistent with monogamy. However, it would be necessary to carry out a more extensive study of the gallery patterns from more naturally collected branches to allow us to draw accurate conclusions.

Although we found (E)-pityol in both sexes, the enantiomeric composition of the natural material has not been elucidated. Chirality plays an important role in determining pheromone specificity. In 60% of species and sex/aggregation systems studied to date, only a single enantiomer is bioactive, and its opposite enantiomer does not inhibit the response to the active stereoisomer in racemic blends (Mori 2007), but in some species the antipode can significantly reduce the attractive response to the active enantiomer (Birch et al. 1980; Leal 1996; Lacey et al. 2004). Owing to the lack of the (−)-enantiomer in our behavioral assays, its biological activity cannot be inferred. However, the racemic mixture was attractive, as was the pure (+)-enantiomer, to male *P. pubescens* when tested in the olfactometer, suggesting that (E)-(−)-pityol could be behaviorally inactive for males. This is consistent with the lack of response of other *Pityophthorus* species to (E)-(−)-pityol (Francke et al. 1987; Dallara et al. 2000;
Fig. 4. Responses of *Pityophthorus pubescens* males and females to different doses of \((E)-(+)\)-pityol and racemic \((E)\)-pityol in Y-tube olfactometer trials (*, \(P < 0.05\); **, \(P < 0.01\)). Numbers in parentheses denote the number of beetles that responded.

W. Francke, personal communication). In contrast, females were only attracted to the lowest dose in both cases, showing a significant preference for the blank arm when the racemic mixture was tested (and it is evident that increasing the \((E)-(+)\)-pityol dose leads
to an apparent and progressive decrease of the positive response, although this is not statistically significant). In light of our results we cannot assert that this behavior is caused by the presence of \( (E)-(\ldots)-\)pityol; further studies would be needed to determine the biological influence of each enantiomer on \( P. \) *pubescens*.

We report the first electroantennographic assays performed on a species of *Pityophthusor* and show that the antennae of both sexes of \( P. \) *pubescens* respond to \( (E)-(\ldots)-\)pityol. Interestingly, whereas female antennae showed the greatest response to the highest dose, the reverse was true for male antennae, *i.e.* greatest response to the highest dose, the presence of \( (E)-(\ldots)-\)pityol; further studies to an apparent and progressive decrease of the positive response, although this is not statistically significant). In light of our results we cannot assert that this behavior is caused by the presence of \( (E)-(\ldots)-\)pityol; further studies would be needed to determine the biological influence of each enantiomer on \( P. \) *pubescens*.

In summary, for the first time we detected the presence of \( (E)-\)pityol as a possible aggregation pheromone in male and female volatiles of \( P. \) *pubescens*, and demonstrated the biological activity of the \( (+)-(\ldots)-\)enantiomer and the race-mate in electrophysiological and behavioral studies. Studies should be carried out to confirm the attraction of both sexes of \( P. \) *pubescens* in the field for these chemicals.

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