

Electrophysiological and behavioural responses of *Pityophthorus pubescens* (Coleoptera: Scolytinae) to (*E,E*)- α -farnesene, (*R*)-(+)-limonene and (*S*)-(–)-verbenone in *Pinus radiata* (Pinaceae) stands in northern Spain

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Abstract

BACKGROUND: Some twig beetles in the genus *Pityophthorus* (Coleoptera: Scolytinae) may vector pitch canker disease *Fusarium circinatum* (Nirenberg & O'Donnell) of *Pinus* spp. (Pinaceae). Because *Pityophthorus pubescens* (Marsh.) has been found to be associated with *F. circinatum* in the Basque Country (northern Spain), various experiments were conducted to assess the beetle's behavioural responses to (*E,E*)- α -farnesene, (*R*)-(+)-limonene and (*S*)-(–)-verbenone to develop a potential inhibitor to host attraction. These experiments comprise electroantennographic and double-choice olfactometer tests, as well as field assays in *Pinus radiata* D. Don stands.

RESULTS: Both sexes of *P. pubescens* showed similar electroantennographic responses to different doses (from 1 ng to 1 μ g in decadic steps) of each individual compound, with depolarisations to (*S*)-(–)-verbenone (100 ng) being similar to those of the aggregation pheromone (+)-*trans*-pityol. In olfactometer assays, both sexes were significantly attracted to (+)-*trans*-pityol, but the attraction was reduced when increasing amounts of the chemicals were added to the pheromone. Particularly relevant was the repellent effect induced by (*S*)-(–)-verbenone at 1 ng dose and higher. In the field, (*E,E*)- α -farnesene, (*R*)-(+)-limonene and (*S*)-(–)-verbenone reduced significantly the number of beetles attracted to (+)-*trans*-pityol and racemic *trans*-pityol, with (*S*)-(–)-verbenone being the most effective.

CONCLUSIONS: (*S*)-(–)-Verbenone showed an interesting potential for use in the protection of *P. radiata* stands. A potentially effective strategy, which could be implemented in further, more in-depth studies, could involve the use of this semiochemical as repellent and (+)-*trans*-pityol-baited traps as attractant in a 'push-pull' strategy.

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Keywords: *Pityophthorus pubescens*; Scolytinae; behaviour; olfaction; inhibitor; management; push-pull strategy

1 INTRODUCTION

Several species of bark beetles (Coleoptera: Scolytinae), including twig beetles of the genus *Pityophthorus* Eichh., have been shown to be phoretically associated with *Fusarium circinatum* (Nirenberg & O'Donnell) (Hypocreales: Nectriaceae), the causal agent of pitch canker disease. The insects may vector the pathogen or create wounds through which the pathogen can enter the tree.^{1–4} Beetles of the *Pityophthorus* spp. complex rarely cause mortality of trees, but attack weakened or dying trees, where they breed under the bark or in the pith of small twigs.⁵ Indeed, *Pityophthorus carmeli* Swaine, *P. setosus* Black. and *P. nitidulus* (Mann.) have been shown to be associated with pitch canker fungus, and to infest Monterey pine *Pinus radiata* D. Don (Pinaceae) in California.^{2,3} In addition, an association has recently been reported⁴ between *Pityophthorus*

pubescens (Marsh.) and *F. circinatum* in Monterey pine stands of this pine species in the Basque Country (northern Spain),

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where about 163 000 ha have been planted. The existence of *P. radiata* monocultures and the association between *P. pubescens* and *F. circinatum* make the study of the epidemiology of pitch canker disease and its relationship to *P. pubescens*, as well as the development of possible management options, highly desirable.

Knowledge regarding the chemical ecology of beetles of *P. pubescens* is limited. Francke et al.⁶ identified (2*R*, 5*S*)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran [(+)-*trans*-pityol] and *cis*-1-(2-hydroxyethyl)-1-methyl-2-(1-methylethenyl)cyclobutane [(±) -grandisol] from males of *P. pityographus* Ratz., and showed that both compounds were active in the field. (+)-*Trans*-pityol was later found in males of *P. carmeli* and females of *P. nitidulus* and *P. setosus*.⁷ Regarding *P. pubescens*, *trans*-pityol has been recently reported as a component of the aggregation pheromone of this species.⁸ In addition to *trans*-pityol, the spiroacetal (5*S*, 7*S*)-(-)-7-methyl-1,6-dioxaspiro[4.5]decane (conophthorin) has been identified as a component of the aggregation pheromone released by *P. carmeli* males.⁷ It is well known that host monoterpenes of conifers, e.g. from *Pinus* spp. (Pinaceae), play an important role in the behaviour of many Scolytinae, including host seeking and selection.^{9,10} However, unlike most conifer-attacking scolytids, *Pityophthorus* spp. appear not to be attracted to volatiles from cut branches, and it is hypothesised that they may recognise a suitable host through a process of random searching and 'exploratory tasting'.^{11,12}

(4*S*)-(+)-Limonene [(4*S*)-4-isopropenyl-1-methyl-cyclohexene, hereafter (+)-limonene] is considered to be one of the most toxic monoterpenes for the bark beetle species *Dendroctonus brevicomis* Lec.,¹³ *D. frontalis* Zimm.¹⁴ and *Scolytus ventralis* Lec.,¹⁵ and the (-)-enantiomer inhibits the attraction of *Pityophthorus puberulus* (Lec.) to racemic *trans*-pityol.¹⁶ However, Miller¹⁷ reported later (in 2007) that both enantiomers act as attractant kairomone in *Conophthorus coniperda* (Schwarz).¹⁷ As the genus *Conophthorus* is closely related to *Pityophthorus* spp., the present authors considered it worth testing the effect of (+)-limonene on *P. pubescens*. (1*S*, 4*S*)-(-)-Verbenone {4,6,6-trimethylbicyclo-[3.1.1]hept-3-en-2-one, hereafter (-)-verbenone} has been shown to inhibit the response of several species of bark beetles to pheromone-baited traps and to deter attacks in conifer stands when used alone^{4,18–22} or in combination with non-host volatiles.^{23–25} In addition, in preliminary assays, (-)-verbenone reduced catches of *P. pubescens* and *Ips sexdentatus* (Boern.) but not of other beetles according to host-age characteristics.⁴ (*E*, *E*)- α -Farnesene (3,7,11-trimethyl-1,3,6,10-dodecatetraene, hereafter α -farnesene) is a sesquiterpene produced especially when conifers are infested by acarid species of the genus *Nalepella* Keifer (Acari: Eriophyoidea: Nalepellidae)²⁶ or attacked by the white pine weevil *Pissodes strobi* Peck (Coleoptera: Curculionidae).²⁷ However, its possible activity on insects has not yet been demonstrated. Therefore, α -farnesene was included along with (+)-limonene and (-)-verbenone in this study to evaluate their potential as possible aggregation inhibitors of *P. pubescens* in the laboratory and in the field. This work complements and enlarges the authors' previous study on the effects of (-)-verbenone on bark beetles associated with the pitch canker fungus, *Fusarium circinatum*, among them *P. pubescens*.⁴

2 MATERIALS AND METHODS

2.1 Insects

For the laboratory bioassays, *P. pubescens* adults were collected weekly from infested branches of *P. radiata* in a mature (nearly 30 years) stand located at Gorosika (43° 16' N, 2° 43' W) (Basque

Country, northern Spain). After identification, the insects were maintained in an incubator at 25 °C and 65% RH under a 10:14 L:D photoperiod until their use 2 days later.

2.2 Chemicals

(*E*, *E*)- α -Farnesene (67%) in closed polyethylene centrifuge tubes (release rate 0.8 mg day⁻¹), (*R*)-(+)-limonene (99%) in polyethylene screw-cap bottles (release rate 5.0 mg day⁻¹) and (*S*)-(-)-verbenone (98%) in polyvinyl bubble caps (release rate 2.0 mg day⁻¹) were purchased from Contech Inc. (Delta British Columbia, Canada). (+)-*Trans*-pityol (99%) and the racemic *trans*-pityol (99%) were kindly provided by Prof. Dr Wittko Francke (University of Hamburg, Germany). Propylene glycol (99%) was purchased from Panreac (Barcelona, Spain). Serial dilutions tested in electrophysiological and walking bioassays were obtained from all of these compounds, as described below.

2.3 Electrophysiological assays

Owing to the very small size of the beetle *P. pubescens* (1.0–1.2 mm long),²⁸ the entire EAG preparation and recordings were done under a stereomicroscope. Recordings were achieved using Ag–AgCl glass microcapillaries filled with Ringer's solution. To hold the microelectrodes, glass micropipettes were pulled from 2.0 mm capillary glass (World Precision Instruments, Sarasota, FL) on a PE-2 pipette puller (Narishige, Tokyo, Japan), and tips of the micropipettes were broken to 30 μ m diameter. Each beetle (male or female) was fixed upside down on a double-sided sticky tape, and the head was excised using a microscalpel. Subsequently, the recording electrode was put into contact with the tip of the antenna (ca 0.30 mm long), and the reference electrode was inserted into the insect club (ca 120 μ m long, 95 μ m wide) using MP15 micromanipulators (Syntech, Hilversum, The Netherlands). Only one antenna of each insect was used. In each assay, (+)-limonene, (-)-verbenone, and α -farnesene were puffed individually over the antenna ($n = 7$ for each compound) at four different doses (from 1 ng to 1 μ g in decadic steps). For comparison purposes, 100 ng of each compound and (+)-*trans*-pityol were also applied to a similar filter paper and then 'puffed' to male and female antennae ($n = 6$). The required amounts of the compounds were obtained by dilution of 10 mg of the commercial compound [synthetic in the case of (+)-*trans*-pityol] in 10 mL of hexane, followed by a log dilution series in hexane to obtain solutions of every compound at 0.1, 1, 10 and 100 ng μ L⁻¹. A quantity of 10 μ L of each solution was applied to a round filter paper (2.5 cm diameter), the solvent was allowed to evaporate and the filter paper was placed inside a Pasteur pipette with the tip at 2 cm from the head of the beetle. Each dose was delivered as 400 ms puffs three consecutive times over the antenna, and two puffs of hexane were applied just before and after the test compound. The net electroantennographic responses were calculated by subtracting the mean response of hexane before and after each stimulus from the mean response of each dose of the test compound. Stimuli were delivered in increasing order of doses to avoid saturation of the antennal receptors. The recovery time for the antenna between two consecutive stimuli was 1–1.5 min. The EAG system was enclosed in a Faraday cage (70 \times 65 \times 60 cm) connected to the ground to prevent extraneous electrical signals.

2.4 Behavioural response

Walking responses of *P. pubescens* to α -farnesene, (+)-limonene and (-)-verbenone were recorded using a Y-tube olfactometer

adapted to the very small size of the beetle. The main tube was 5 cm long \times 5 mm ID, the arms were 4 cm long \times 5 mm ID and the angle between arms was 90°. The terminal tubes of the arms were connected to two glass chambers, which contained the odour source. One of the arms held 10 μ L of hexane on a 2.5 cm diameter filter paper as control. The other arm contained another filter paper of the same size with 1 ng of (+)-*trans*-pityol either alone (reference) or with one of the five different doses of the test compounds. The doses consisted of 0.01–100 ng in decadic steps and were obtained from 10 μ L solutions of the required concentrations in hexane. The insect response was evaluated versus that of 1 ng of (+)-*trans*-pityol alone. Filter papers were renewed in each arm after every second insect was tested. Incoming air was filtered through activated charcoal, and the airflow was maintained at 70 cm s⁻¹ in the olfactometer.

All tests were conducted in a room at 23 \pm 1 °C and 50 \pm 9% RH, and, before the assays, insects were allowed to acclimatise in a petri dish for 15 min. Insects were allowed to respond for 5 min; if there was no response after this time, the insect was discarded. A response was considered positive if the beetle walked at least 3 cm into one arm. After testing five insects, arms were switched over to avoid unidirectionality, and, after ten insects, the entire olfactometer was washed, first with soap and water, then with absolute ethanol, and left to dry. A total of 35–40 insects were used for each sex and dose.

2.5 Field assays

During May–June 2007, 2008 and 2009, three field experiments were conducted to evaluate the effect of the terpenoids as potential disruptants of *P. pubescens* aggregation. Only a single compound at five different release rates was tested each year, as follows: α -farnesene in 2007, (+)-limonene in 2008 and (–)-verbenone in 2009. Field assays were implemented based on previous research conducted to find the flight period of the species in the Basque Country.^{4,29,30} The experiments were done in four mature Monterey pine stands at the following localities: Llodio (43° 08' N, 2° 59' W), Amurrio (43° 03' N, 2° 57' W), Gorosika (43° 16' N, 2° 43' W) and Olabarrieta (43° 01' N, 2° 22' W). At each locality, two blocks (with two replicates each) of six 12-unit Lindgren multiple-funnel traps were set out (Table 1). One trap in

each block, baited only with the attractant [either with (+)-*trans*-pityol or racemic *trans*-pityol], was considered as positive control, whereas the other traps were baited with attractant plus the test compound at different release rates (0.8–4.0 mg day⁻¹ for α -farnesene, 5.0–25.0 mg day⁻¹ for (+)-limonene and 2.0–10.0 mg day⁻¹ for (–)-verbenone) (Table 1). Release rates higher than the initial 0.8, 5.0 and 2.0 mg day⁻¹ for the three compounds, respectively, were obtained by adding new commercial devices as needed; for instance, to achieved a release rate of 15.0 mg day⁻¹ of (+)-limonene, 3 \times 5.0 mg day⁻¹ of polyethylene bottles was required. Traps were spaced 20 m apart in a 3 \times 2 grid within each block, and the distance between blocks was greater than 100 m. Each trap was hung between two adjacent trees 2 m above ground. Because traps were not rerandomised during the trapping period, all traps were placed unbaited in the field 2 weeks before the assay to discard the hypothesis that the possibly obtained catches would be due to a spatial effect of traps. Control attractants (2.5 μ L of racemic and chiral *trans*-pityol) were dispensed in 1.5 mL capped plastic microcentrifuge vials that were attached to the outside of the middle funnel. After sealing them, the caps were pierced with a pin to allow evaporation of the chemical. The vials were refilled every week. The dispensers of α -farnesene allowed a release rate of 0.8 mg day⁻¹, (+)-limonene was dispensed at 5.0 mg day⁻¹ and (–)-verbenone was dispensed at 2.0 mg day⁻¹. Propylene glycol was added to each trap-containing jar to preserve captured insects. The experiments lasted 2 months.

Insects were collected weekly and identified on the basis of their characteristic anatomical features of the elytra and elytral declivity using a LEICA MZ9.5 stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany).^{28,31} Insects were separated by sex on the basis of the yellowish hairs on the frons, an exclusive feature of females. For identification purposes, reference specimens were obtained from the British Natural History Museum (London, UK) and the Museo Nacional de Ciencias Naturales (Madrid, Spain). Voucher specimens have been deposited at the Entomology Collection of the NEIKER-Basque Institute of Agricultural Research and Development (Basque Country, Spain).

Table 1. Parameters for each field experiment performed in 2007–2009

| Experiment | Date | Block ^a | Attractant | Volume (μ L) | Tested compound | Release rate (mg day ⁻¹) of test compound |
|------------|--------------------|--------------------|---------------------------------|-------------------|-------------------------------------|-------------------------------------------------------|
| 1 | 1 May–30 June 2007 | 1 | (+)- <i>Trans</i> -pityol | 2.5 | (<i>E,E</i>)- α -Farnesene | 0 (control) 0.8–4 ^b |
| | | 2 | (\pm)- <i>Trans</i> -pityol | 2.5 | (<i>E,E</i>)- α -Farnesene | 0 (control) 0.8–4 ^b |
| 2 | 1 May–30 June 2008 | 1 | (+)- <i>Trans</i> -pityol | 2.5 | (<i>R</i>)-(+)-Limonene | 0 (control) 5–25 ^c |
| | | 2 | (\pm)- <i>Trans</i> -pityol | 2.5 | (<i>R</i>)-(+)-Limonene | 0 (control) 5–25 ^c |
| 3 | 1 May–30 June 2009 | 1 | (+)- <i>Trans</i> -pityol | 2.5 | (<i>S</i>)-(–)-Verbenone | 0 (control) 2–10 ^d |
| | | 2 | (\pm)- <i>Trans</i> -pityol | 2.5 | (<i>S</i>)-(–)-Verbenone | 0 (control) 2–10 ^d |

^a In duplicate experiments.

^b In dispensers releasing 0.8, 1.6, 2.4, 3.2 and 4 mg day⁻¹.

^c In dispensers releasing 5, 10, 15, 20 and 25 mg day⁻¹.

^d In dispensers releasing 2, 4, 6, 8 and 10 mg day⁻¹.

2.6 Statistical analysis

Electroantennographic responses and field catches were analysed by analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$, followed by Tukey's *post hoc* test for separation of means (SPSS v.13.0 for Windows). For field trapping assays, several transformations (ln, square root) of trap catches and the independent variable were tested, as required from examinations of residuals, to correct heteroscedasticity and non-linearity. In olfactometer tests, the results were analysed by the chi-square test (χ^2) at a significance level of $\alpha = 0.05$. Males and females showed no preference for either olfactometer arm (a response equal to 50 : 50) in the absence of any stimuli.

3 RESULTS

3.1 Electroantennographic assays

Antennae of both sexes of *P. pubescens* responded slightly to all doses of the three compounds tested, although no significant differences were found between different concentrations within sex and compound (α -farnesene: males $F_{3,28} = 1.119$, $P = 0.360$; females $F_{3,28} = 0.581$, $P = 0.633$; (+)-limonene: males $F_{3,28} = 0.772$, $P = 0.772$; females $F_{3,28} = 0.053$, $P = 0.984$; (-)-verbenone: males $F_{3,28} = 0.474$, $P = 0.860$; females $F_{3,28} = 0.474$, $P = 0.860$) (Fig. 1). Depolarisations elicited by 100 ng of (+)-*trans*-pityol were higher than those induced by the other compounds in males and females, the differences being significantly different to those elicited by (+)-limonene (males $F_{3,24} = 0.642$, $P = 0.772$; females $F_{3,24} = 0.053$, $P = 0.984$) and α -farnesene (males $F_{3,24} = 11.683$, $P < 0.001$; females $F_{3,24} = 12.391$, $P < 0.001$) (Fig. 2). (-)-Verbenone elicited an intermediate response, not significant relative to (+)-*trans*-pityol or the other two semiochemicals.

3.2 Behavioural response

As expected, males and females were significantly attracted to 1 ng of (+)-*trans*-pityol when tested alone. However, attraction of both sexes towards the (+)-*trans* pityol arm was reduced when increasing amounts of α -farnesene were added to the pheromone (Fig. 3). Neither males nor females walked preferentially to any arm in the presence of mixtures (+)-*trans*-pityol: α -farnesene 1 : 1 and higher. A similar effect was observed with (+)-limonene. Males and females still preferred the arm containing (+)-*trans*-pityol plus (+)-limonene when the doses of the monoterpene were 0.1 ng or lower, but higher doses (1–10 ng) reduced the attraction to the synthetic attractant (Fig. 4). The effect of (-)-verbenone was more notable. Attraction of males to the arm containing 0.01–0.1 ng of (-)-verbenone became practically null, and insects significantly preferred the control arm when the amount of the chemical at the attraction source was 1 ng and higher (Fig. 5). Females behaved similarly, but preference for the control arm was already noticed at 0.1 ng of (-)-verbenone.

3.3 Field assays

In 2007, a total of 50 212 beetles (39 903 males and 10 309 females) were caught. α -Farnesene significantly inhibited the attraction of *P. pubescens* to (+)-*trans*-pityol (males $F_{5,186} = 2.689$, $P = 0.023$; females $F_{5,186} = 2.651$, $P = 0.024$) and (\pm)-*trans*-pityol (males $F_{5,186} = 2.354$, $P = 0.042$; females $F_{5,186} = 2.727$, $P = 0.021$) (Fig. 6A). Catches of both sexes were significantly lower in traps baited with (+)-*trans*-pityol plus α -farnesene at a release rate of 2.4 mg day⁻¹ and higher. With (\pm)-*trans*-pityol, the effect of α -farnesene was more pronounced, 1.6 mg day⁻¹ being sufficient to

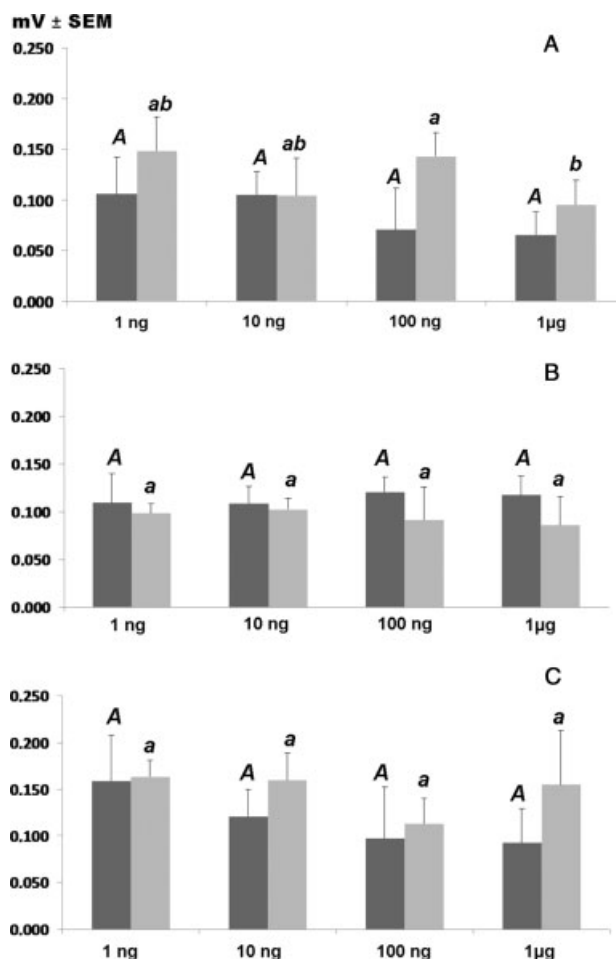


Figure 1. Absolute net EAG (mV \pm SEM) responses of *P. pubescens* males (black bars) and females (grey bars) to different doses (1 ng to 1 μ g in decadic steps) of (*E,E*)- α -farnesene (A), (*R*)-(+)-limonene (B) and (*S*)-(-)-verbenone (C). The compounds were deposited on a filter paper and puffed. Bar means followed by different letters within sex are significantly different by ANOVA followed by Tukey's multiple test ($\alpha = 0.05$).

promote partial but significant inhibition of captures. Maximum reduction in male catches relative to control traps containing pheromone alone was 44.8 and 51.4% for the (+)-enantiomer and (\pm)-*trans*-pityol respectively, whereas female catches were reduced by 49.7 and 58% respectively (Fig. 6A).

In experiment 2, 16 371 beetles (11 765 males and 4606 females) were trapped. (+)-Limonene significantly reduced the number of catches to (+)-*trans*-pityol when released at 10 mg day⁻¹ (males $F_{5,186} = 8.909$, $P < 0.001$; females $F_{5,186} = 5.716$, $P < 0.001$) and higher and to (\pm)-*trans*-pityol when released at 15 mg day⁻¹ (males $F_{5,186} = 5.538$, $P < 0.001$; females $F_{5,186} = 2.775$, $P = 0.018$) and higher (Fig. 6B). The maximum reduction in catches for both sexes was 68% with regard to the (+)-*trans*-pityol-baited traps alone and 59% relative to the (\pm)-*trans*-pityol-containing traps.

In 2009, 22 159 *P. pubescens* (18 706 males and 3453 females) were caught. (-)-Verbenone also significantly inhibited attraction of beetles to traps baited with chiral *trans*-pityol when released at 2 mg day⁻¹ (males $F_{5,186} = 9.716$, $P < 0.001$; females $F_{5,186} = 7.620$, $P < 0.001$) or racemic *trans*-pityol when released at the same dose (males $F_{5,186} = 10.562$, $P < 0.001$; females $F_{5,186} = 8.313$, $P < 0.001$) (Fig. 6C). Higher doses did not further increase the inhibitory effect. The maximum decrease in catches

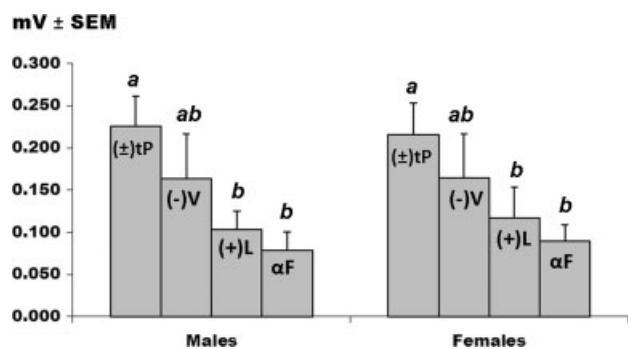


Figure 2. Absolute net EAG ($mV \pm SEM$) responses of *P. pubescens* males and females to 100 ng of (\pm)-*trans*-pityol, (*E,E*)- α -farnesene, (*R*)-(+)-limonene and (*S*)-(-)-verbenone. The compounds were deposited on a filter paper and puffed. Bar means followed by different letters within sex are significantly different by ANOVA followed by Tukey's multiple test ($\alpha = 0.05$). Legend: (\pm)tP = (\pm)-*trans*-pityol; α F = (*E,E*)- α -farnesene; (+)L = (*R*)-(+)-limonene; (-)V = (*S*)-(-)-verbenone.

was 66.6% in males and 75% in females at 10 mg day⁻¹ of (-)-verbenone in racemic and chiral pheromone traps respectively (Fig. 6C).

4 DISCUSSION

Electroantennography is a useful technique for isolating volatiles that insects use to locate suitable host plants.^{32–34} In the first electroantennographic recordings performed on the genus *Pityophthorus*, α -farnesene, (+)-limonene and (-)-verbenone elicited slight antennal responses in both sexes of *P. pubescens* (Figs 1 and 2), in line with the small number of olfactory sensilla (300–800 antenna⁻¹ in scolytids)³⁵ with regard to Lepidoptera [10⁵ sensilla in *Manduca sexta* (L.)].³⁶ Slight responses have also been previously reported in other Scolytinae species, e.g. the depolarisations elicited on *Tomiscus destruens* (Woll.) by different pine shoot and bark volatiles ranged from 0.06 to 0.12 mV.³⁷ In the present case, the depolarisations were smaller than those elicited by racemic *trans*-pityol, one component of the aggregation pheromone, although with regard to (-)-verbenone the differences were not significant at 100 ng dose. Females responded better than males, but the difference was not significant, and neither sex responded in a dose-dependent fashion, in contrast to the electrophysiological responses of female moths to host odours.³⁸

In dual-choice bioassays, α -farnesene, (+)-limonene and (-)-verbenone inhibit the attraction of pityol (racemic or chiral) to males and females. The effect of (-)-verbenone is particularly noticeable, with doses of 1% relative to (+)-*trans*-pityol being sufficient to abrogate the attraction of the synthetic attractant to either sex. Moreover, doses of 10% and higher of (-)-verbenone completely switch attraction of females to the control arm. On males, the repulsive effect is evoked when placing the same amount of the inhibitor [1 ng of (-)-verbenone with 1 ng of chiral pityol] in the corresponding arm.

Field experiments confirmed the results obtained in the laboratory bioassays. The three terpenoid compounds caused a reduction in the number of *P. pubescens* caught, with (-)-verbenone and (+)-limonene having a stronger effect. Previous research has demonstrated that (-)-limonene inhibited the attraction of *P. puberulus* to traps baited with (-)- α -pinene, (-)- β -pinene or mixtures of both.¹⁶ The inhibitory effect of (-)-

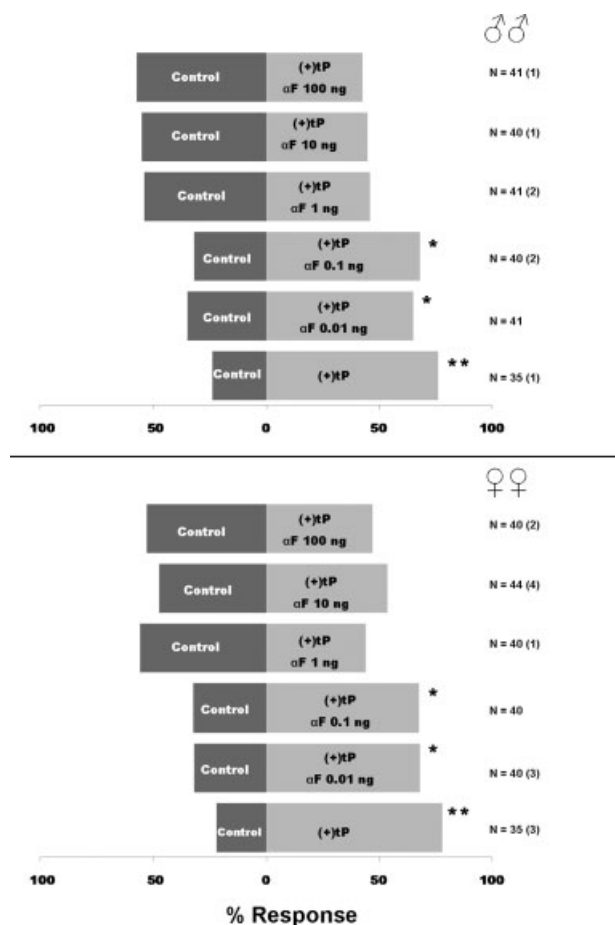


Figure 3. Response of *P. pubescens* males (♂♂) and females (♀♀) to different mixtures of (*E,E*)- α -farnesene and 1 ng of (+)-*trans*-pityol in Y-tube olfactometer trials. Hexane was used as control. One and two asterisks indicate significant differences at $P < 0.05$ and $P < 0.01$ (chi-square test) respectively. Number in parentheses represents number of beetles not responding. Legend: (+)tP = (+)-*trans*-pityol; α F = (*E,E*)- α -farnesene.

limonene was not evident, however, when racemic *trans*-pityol was added to various mixtures of monoterpenes, except when the pheromone was combined with (-)- α -pinene. The inhibition followed a dose-dependent trend.¹⁶ Because (-)- α -pinene and (-)- β -pinene appear to be attractive to *P. puberulus*, the inhibition elicited by (-)-limonene suggests that this species is able to discriminate among terpenes to locate suitable hosts. It should be noted that the (-)-limonene release rates used by Brauner and De Groot¹⁶ (34–234 mg day⁻¹) were considerably higher than the rates used in the present tests with (+)-limonene. In the present case, there was no need of (-)- α -pinene for the (+)-enantiomer to elicit a disruptant effect of the pheromone action.

(-)-Verbenone shows promise in reducing *P. pubescens* aggregation in the field. The present results partially agree with those of a previous study in which a negative linear relationship between (-)-verbenone dosage and the number of insects caught was found.⁴ However, higher release rates were tested than those used by Romón *et al.*⁴ (2–10 mg day⁻¹ versus 0.01–3.1 mg day⁻¹), and it is known that different release rates may greatly influence the level of catches.²¹ This is the case, for instance, with *Dendroctonus ponderosae* Hop., catches of which were unaffected relative to the control [no (-)-verbenone] at release rates of 0.2 mg day⁻¹ or less, but were significantly reduced at 1.8 mg day⁻¹ of (-)-verbenone or

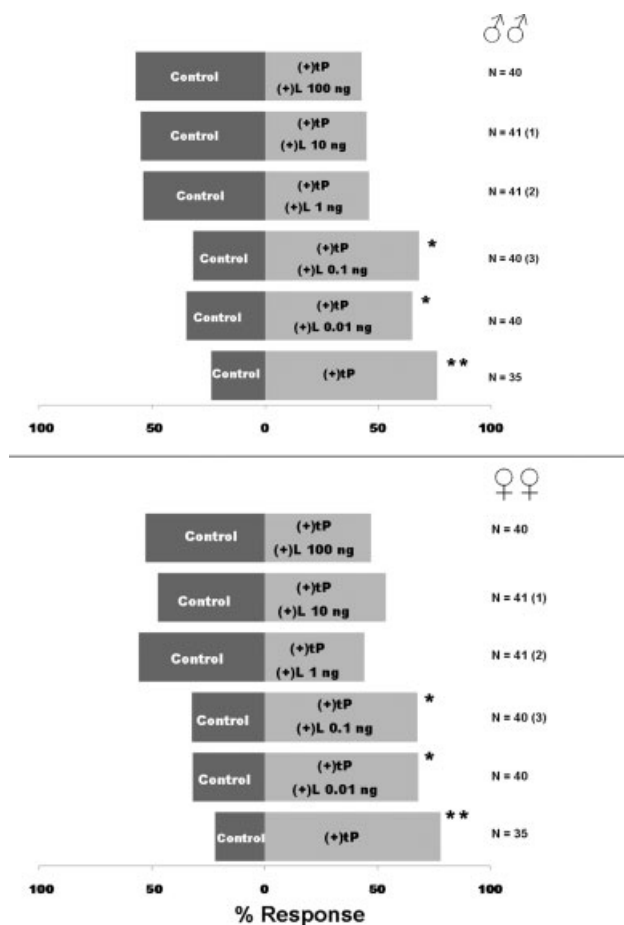


Figure 4. Response of *P. pubescens* males (♂♂) and females (♀♀) to different mixtures of (*R*)-(+)-limonene and 1 ng of (+)-*trans*-pityol in Y-tube olfactometer trials. Hexane was used as control. One and two asterisks indicate significant differences at $P < 0.05$ and $P < 0.01$ (chi-square test) respectively. Number in parentheses represents number of beetles not responding. Legend: (+)tP = (*trans*)-(+)-pityol; (+)L = (*R*)-(+)-limonene.

higher.²¹ In the present case, the results suggest a threshold-type response rather than a dose-dependent relationship,⁴ as there are no significant differences in catches among traps when baited with (–)-verbenone at any release rate tested. If it is assumed that (–)-verbenone is a good indicator of the nutritive quality of plant material owing to its generation via microbial activity,^{17,39} this chemical could be expected to have a strong inhibitory effect on species primarily feeding on fresh host tissue,²¹ whereas species inhabiting aged tissues would have higher tolerance to (–)-verbenone and/or a more efficient detoxifying system. In light of the present results, it can be hypothesised that *P. pubescens* might avoid colonisation of hosts with aged tissue, as previously suggested for other bark beetles, such as *D. ponderosae ips pini* (Say) and *I. latidens* (Lec.), but not *Hylurgops porosus* (Lec.) and *Hylastes longicollis* Swaine, two species that usually feed on aged phloem tissue below or at ground level.²¹ (–)-Verbenone effectively reduced catches of *D. ponderosae* in a threshold-type pattern and catches of *Ips* spp. in a negative dose-dependent relationship, but did not affect catches of the two latter species.²¹

On the other hand, although (–)-verbenone is an antiaggregation pheromone for a number of scolytid species,¹⁰ it did not reduce catches of the white pine cone beetle *Conophtho-*

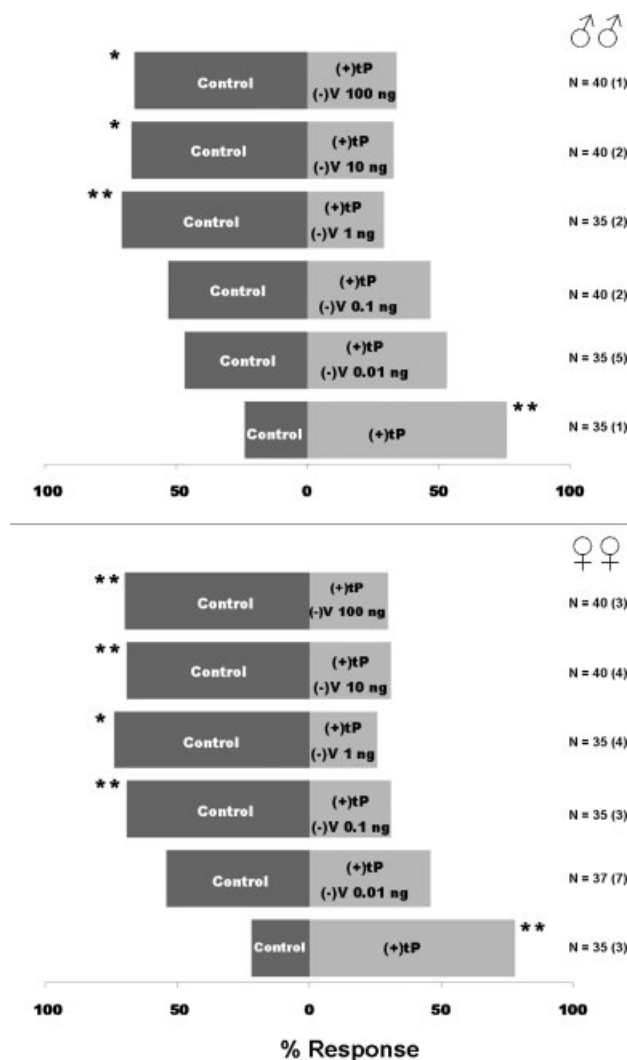


Figure 5. Response of *P. pubescens* males (♂♂) and females (♀♀) to different mixtures of (*S*)-(-)-verbenone and 1 ng of (+)-*trans*-pityol in Y-tube olfactometer trials. Hexane was used as control. One and two asterisks indicate significant differences at $P < 0.05$ and $P < 0.01$ (chi-square test) respectively. Number in parentheses represents number of beetles not responding. Legend: (+)tP = (+)-*trans*-pityol; (-)V = (*S*)-(-)-verbenone.

rus coniperda when added at 0.6 mg day⁻¹ to a (±)-*trans*-pityol (0.2 mg day⁻¹) and α-pinene (12 mg day⁻¹) attractive blend.⁴⁰

α-Farnesene appears to be the least disruptive of the three compounds tested. In spite of the fact that the three highest release rates used caused a reduction of almost 50% in catches, it is unclear whether the use of even higher release rates would have a stronger repellent effect, or whether its disruptive potential is limited, as indicated by the present results.

5 CONCLUSIONS

The results provide basic knowledge for the possible development of IPM strategies to protect *P. radiata* stands from pitch canker disease. A ‘push-pull’ strategy is proposed as potentially effective against *P. pubescens*, using (–)-verbenone as a repellent (‘push tactic’) within a landscape, and either (±)-*trans*-pityol- or (+)-*trans*-pityol-baited traps (‘pull tactic’) on the perimeter to capture repelled beetles. Future research could be focused on testing

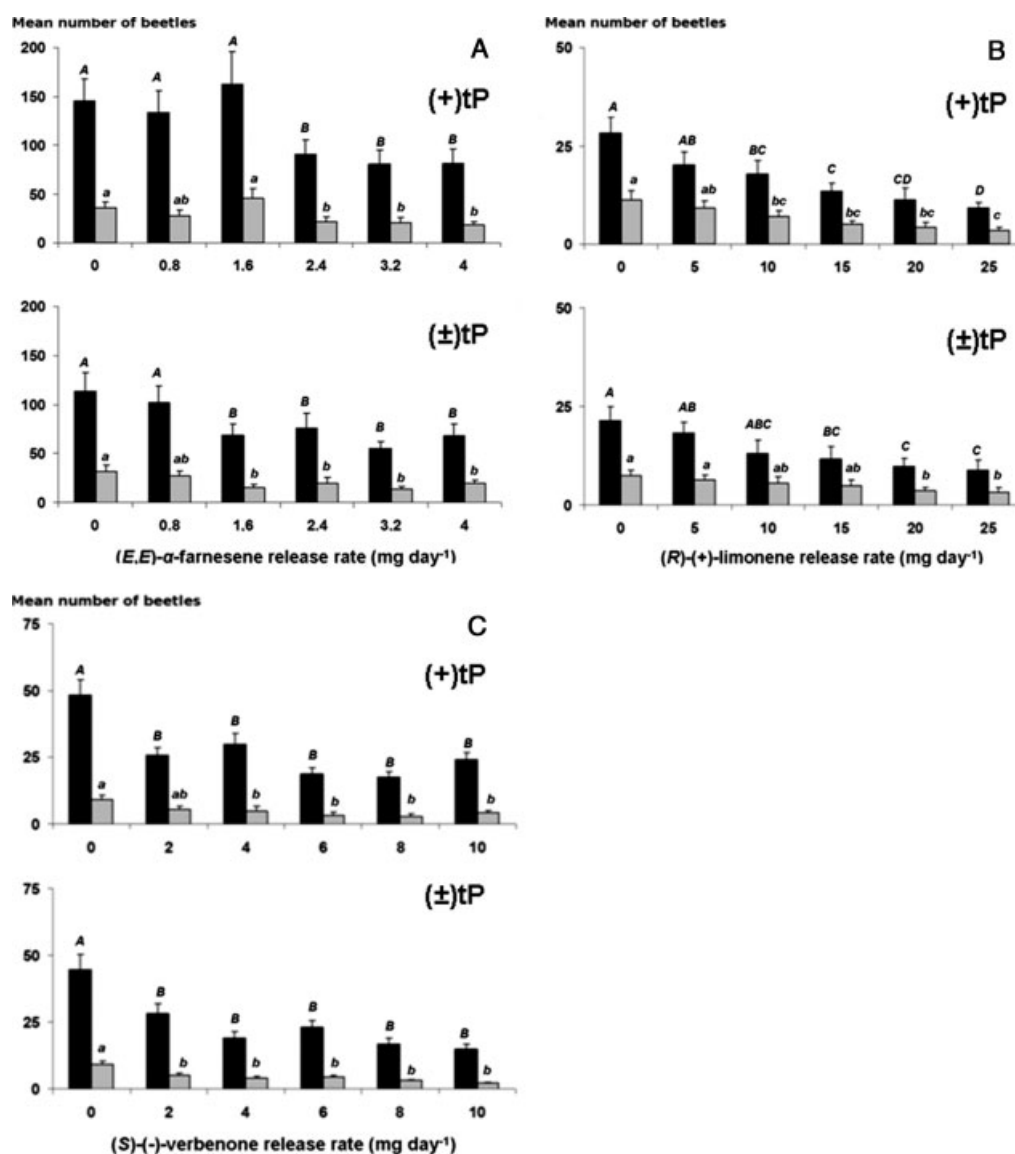


Figure 6. Mean number (\pm SEM) of *P. pubescens* males (black bars) and females (grey bars) caught in traps containing 2.5 μ L of (+)-*trans*-pityol [(+)-tP] or (\pm)-*trans*-pityol [(\pm) tP] and different release rates of (*E,E*)- α -farnesene (A), (*R*)-(+)-limonene (B) and (*S*)-(-)-verbenone (C). Means with the same letter are not significantly different at $P < 0.05$ (Tukey's multiple comparison test).

this strategy in greater depth, as already proposed against *D. ponderosae*.⁴¹

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