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Verbenone protects pine trees from colonization by the six-toothed pine bark beetle, *Ips sexdentatus* Boern. (Col.: Scolytinae)

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Hylurgus ligniperda, pest management, pine borers, repellents, *Temnoscheila coerulea*, *Thanasimus formicarius*

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Abstract

Verbenone and *trans*-conophthorin were tested against the aggregation pheromone of the six-toothed bark beetle (*Ips sexdentatus* Boern.) on two trapping bioassays. Two different release rates of verbenone (2 mg/24 h and 40 mg/24 h) gave similar significant catch reductions (by 73% and 82%, respectively), whereas *trans*-conophthorin lowered the catch by 45%. Joint release of both compounds reduced trap catches by 90%. Furthermore, the effect of these semiochemicals on *Thanasimus formicarius* L. and *Temnoscheila coerulea* Olivier, two important natural enemies of *I. sexdentatus*, as well as *Hylurgus ligniperda* Fabricius, a native associated scolytid, was evaluated. Both predators were significantly affected by verbenone, whereas the effect on *H. ligniperda* could not be concluded. Based on these results, verbenone was used in a third experiment to protect standing healthy *Pinus nigra salzmannii* J. F. Arnold trees from induced *I. sexdentatus* attack. The experiment consisted of two treatments and 20 paired-tree replicates. Treatments were pheromone-baited control trees and baited trees treated with verbenone (60 mg/24 h). These trees served as centres of 10 m radius plots from where a number of plot variables was recorded for local stand description. After the experimental period, all control trees had suffered attacks by *I. sexdentatus*, whereas trees treated with verbenone were significantly less attacked. Among other stand variables considered in the study, the following characterized the variation among plots most efficiently: (i) plot density, (ii) dominant height and (iii) the proportion of suppressed or (iv) intermediate dominance level trees in the plot. However, these variables did not significantly affect the repellent effect by verbenone. Further research should evaluate the use of verbenone on management strategies of ongoing attacks of *I. sexdentatus*.

Introduction

Widely distributed through the Eurasian continent, the six-toothed bark beetle (*Ips sexdentatus* Boern.), colonizes weakened, dying or recently dead trees when populations are endemic. Favourable circumstances that make breeding substrate more available, such as forest fires, climatic events or improper forest management, may promote populations to outbreak levels (Gil and Pajares 1986). At this stage, *I. sexdentatus* may become aggressive and attack healthy trees. Besides the active killing of trees, these beetles act as vectors of fungi carried on their body (Romon et al. 2007b). Piled logs in areas where *I. sexdentatus* is present are susceptible to infection with blue staining fungi, severely depreciating stored wood. This bark beetle has also been shown to carry spores of *Fusarium circinatum* Niremberg and

O'Donnell, a fungus causing pitch canker disease in many pine species, including *Pinus radiata* D. Don. (Romon et al. 2007a).

Fire damaged trees over 12 000 ha served in 2005 as breeding substrate for *I. sexdentatus* in Central Spain, and resulted in heavy damage of surrounding stands during following years (Sánchez et al. 2008). Similarly, trees torn down by 'Lothar' and 'Martin' storms in 1999 in south-western France, boosted *I. sexdentatus* populations to levels that allowed them to mass-attack standing trees during the following summers (Jactel et al. 2001), specially in the vicinity of log piles originated after sanitation removals (Rossi et al. 2009). In early 2009, 'Klaus' storm struck again the same area, leaving behind over 42.3 million cubic meters of fallen *Pinus pinaster* Aït. (Inventaire Forestier Nationale 2009). Although salvage management is being carried out, it is expected that *I. sexdentatus* populations will rise considerably increasing the risk of spill over to standing trees.

Bark beetles have evolved several olfactory mechanisms for finding suitable hosts, whereas non-hosts and unsuitable hosts are avoided (Zhang and Schlyter 2004). Most relevant is secondary attraction, by which blends of aggregation pheromones and attractive host kairomones mediate mass attack on appropriate host trees. In the case of *I. sexdentatus*, most of the work on its chemical ecology has been focused on the characterization of the pheromonal blend (Kohnle et al. 1992b; Francke et al. 1995).

Even if compounds involved in secondary attraction may directly signal for an appropriate breeding substrate bypassing primary cues (Borden 1997; Schlyter and Birgersson 1999), a number of other infochemicals have been reported to serve as landmarks for host suitability. Verbenone (4,6,6-trimethylbicyclo[3.1.1]-hept-3-en-2-one) was first discovered for *Dendroctonus ponderosae* Hopkins and has been found later associated with several aggressive bark beetles (Borden 1997). Several pathways result in the release of verbenone, namely the autooxidation of α-pinene in the absence of bark beetles (Flechtmann et al. 1999), the production by bark beetles themselves (Rudinsky et al. 1974), and the metabolic conversion of *trans*-verbenol by yeasts and gut microorganisms associated with adult beetles (Leufven et al. 1984). Verbenone might pose a general inhibitory effect by giving a signal of increased host unsuitability either by host decay or by intraspecific competition (Lindgren and Miller 2002b; Borden et al. 2003). Inconsistent results through different years, areas and host species have slowed down practical application for managing outbreaks (Amman et al. 1991). Recent studies, however, seemed to overcome these problems by using higher dosages of the compound (Borden et al. 2003; Bentz et al. 2005) or improving delivery methods (Gillette et al. 2009). Verbenone has been detected in six-toothed bark beetle hindgut extracts (Francke et al. 1986), and the insect has been shown to respond physiologically to the compound (Jactel et al. 2001). After initial positive results (Paiva et al. 1988), attacks could not be repelled in a number of tests (Kohnle et al. 1992a; b), and a very weak negative linear relationship between verbenone dosage and the number of captured *I. sexdentatus* has been shown (Romon et al. 2007a).

Recently, research on emissions by non-hosts has shown that dispersing bark beetles actively avoid trees releasing inadequate volatiles (Borden et al. 1998; Huber and Borden 2001). Green leaf volatiles (GLV), especially C_6 alcohols, from leaves (and partly from the bark) of non-host angiosperm trees, may represent non-host odour signals for the habitat level. Specific bark volatiles such as *trans*-conophthorin [(*E*)-7-methyl-1,6-dioxaspiro[4.5]decane; tC], C_8 alcohols and some aromatic compounds, may indicate non-hosts at the tree species level (Zhang and Schlyter 2004). Several studies have shown promising results for the management of some bark beetle species (e.g., Zhang and Schlyter 2003) and, particularly for *I. sexdentatus*, a blend of non-host volatiles (NHVs) capable of reducing the attraction to lured traps and logs has been reported (Jactel et al. 2001).

Anti-aggregative semiochemicals, such as verbenone, were the first to be used in management strategies involving repellence (Borden 1997), and some practical approaches have been studied (Amman et al. 1991; Kohnle et al. 1992a; Lindgren and Miller 2002a; b; Bentz et al. 2005). Although NHVs have only recently been taken into screen (Byers et al. 1998), promising results have been reported (Jactel et al. 2001; Jakus et al. 2003; Kohnle 2004; Zhang and Schlyter 2004). Although some experiments have been performed, consistent information regarding the usefulness of verbenone and tC, one of the most studied NHVs, for managing *I. sexdentatus* is still lacking. Thus our objectives for the present work were: (i) to explore the inhibitory effect of verbenone and tC on this species in trapping bioassays, (ii) to test if verbenone may inhibit *I. sexdentatus* attacks on standing trees and (iii) to report effects by these volatiles on kairomonally attracted predators and associated bark beetles.

Material and Methods

Trapping bioassays

In order to determine the repellent potential of verbenone and tC, four treatments were tested in 12-unit multiple funnel traps (Phero Tech, Inc., Delta, British Columbia, Canada; Lindgren 1983) at a site located in the vicinity of Otero de Bodas (Zamora, Castile and Leon, Spain), enclosed within UTM 29T 7350 4640 coordinates and with an altitude ranging from 900 to 1090 m AMSL. *Ips sexdentatus* population was considered to be at endemic level. The area is mainly reforested by pure ca. 40-year-old *P. pinaster*, with younger ca. 30-year-old *Pinus sylvestris* L. stands covering north faced hillsides. Firebreaks up to 60 m wide separating these formations were used for the establishment of the experimental blocks. Treatments were installed from the 27 of April 2006 to the 10 of August 2006, and consisted of a positive control releasing a major component of the aggregation pheromone (racemic ipsdienol; Francke et al. 1995), tC effect on aggregation, verbenone effect on aggregation at a low release rate and a negative control lured with verbenone at a low release rate. Limited availability of tC (Phero Tech Inc.) precluded the setting of a negative control for this semiochemical. Traps were suspended two meters above ground from metal poles and spaced > 75 m apart along the firebreaks. Semiochemical dispensers (table 1) were replaced after 6 weeks, at the end of *I. sexdentatus* first flight period. A replicate of each treatment was randomly placed within each of seven experimental blocks spaced > 200 m apart, in a complete randomized block design. Treatment positions within each block were re-randomized every 5 weeks to minimize positional effects. Catches of *I. sexdentatus*, its predators *Thanasimus formicarus* L. (Col.: Cleridae) and *Temnoscheila coerulea* Olivier (Col.: Trogossitidae), and associated scolytid, *Hylurgus ligniperda* Fabricius, were registered weekly.

Starting 10 of August 2006 and until 27 of September 2006, a second trial was carried out at the same site just before the onset of the third *I. sexdentatus* flight period. A higher releasing dosage of verbenone was tested this time (40 mg/24 h, instead of 2 mg/24 h), as well as the combination of verbenone and tC. Thus, treatment levels were: positive control (racemic ipsdienol), tC effect on aggregation, verbenone effect on aggregation at a high release rate, the combined effect of both repellents and a negative control. The setting, experimental design and sampling procedures were as in the first trial.

Tree protection

Between 20 and 21 of August 2008, the effect of verbenone inhibitting *I. sexdentatus* attack on standing European black pines (*Pinus nigra salzmannii* J.F. Arnold) was tested. The selected study area comprised a mixed afforestation of *P. nigra*, *P. sylvestris* and *P. pinaster* of ca. 40-year-old. Virtually no non-host and/or deciduous trees were present in the stand. The area is enclosed within UTM 30T 2540 4720 coordinates in a flat area at 1100 m AMSL next to Villarmeriel (Leon, Castile and Leon). At the time of the experiment, *I. sexdentatus* population was at an endemic level. Twenty blocks with homogeneous stand structure characteristics were defined within the selected stand. Within each block, two trees from the intermediate dominance class (see below) were selected as test trees, one of them was baited with ipsdienol to induce beetle attack (control or baited tree), whereas the treatment tree was baited with the attractant plus a high verbenone releasing pouch (60 mg/24 h, table 1). Experimental trees within a block were at least 75 m apart from each other and 150 m from the closest adjacent block.

Table 1 Description of semiochemical-releasing devices

Label	Semiochemical[1]	Dispenser	Source[2]	Release rate (mg/24 h)[3]
I	Ipsdienol (+50/ − 50)	Aluminium pouch	SEDQ	2.35
tC	*trans*-Conophthorin (+50/ − 50)	Closed polyethylene centrifuge tube (250 μl)	Phero Tech	0.3
V2	*S*-(-) Verbenone (+ 17/ − 83)	Polyvinyl bubble cap	Phero Tech	2
V40	*S*-(-) Verbenone (+ 17/ − 83)	Urethane pouch	Phero Tech	40
V60	*S*-(-) Verbenone (+ 17/ − 83)	Urethane pouch	Phero Tech	60

[1]All chemical purities > 98%.
[2]SEDQ LLC, Barcelona, Spain; Phero Tech Inc., Delta, BC, Canada.
[3]At 20 ºC.

It is known that tree vigour and stand characteristics may influence the performance of odour plumes and/or tree resistance/susceptibility (Schmitz et al. 1989; Reid and Robb 1999), and variation in sunlight might also affect the stability of verbenone (Kostyk et al. 1993). In order to control the influence of the surrounding stand on test trees, these were also used as centres of circular plots 10-m wide where each tree was characterized by conifer species, diameter at breast height (1.3 m; 'DBH'), individual tree height, and canopy dominance class (Kraft Crown Classification; Smith 1997). Dominant and co-dominant trees were those not restricted from above, and the largest in DBH and height was chosen as the dominant tree. An intermediate class was established for trees extending into the lower part of the highest canopy of the forest, but being clearly overtopped by the upper classes. Suppressed trees were rarely reaching the highest canopy. DBH of test trees did not differ between blocks or treatments [DBH ca. 15 cm; generalized linear model (GLM) procedures, $P > 0.05$].

Treatment effects were evaluated assessing tree damage level once the third flight of *I. sexdentatus* was over, on the 10 of September 2008. According to previous estimations on successful attack density thresholds (Fernandez et al. 2004), standing *P. sylvestris* started to decline around attack densities of 0.7 gallery systems per dm^2, whereas 1.5 gallery systems/dm^2 was the critical density threshold for tree survival. While evaluating test trees, these were classified into one of four levels of a categorical variable named as 'tree status' based on the maximum density of gallery systems at any region within the lowest 3 m of the tree trunk. The area with the highest density of entrance holes was gauged using an A4 size transparency film (6.23 dm^2). Attack density above which trees were not expected to survive was set at five holes per A4 (ca. 1 gallery system/dm^2). Thus the four tree status levels were defined as: 'Mass-Attacked', for test trees with densities above that threshold; 'Attacked', whenever an ongoing attack was found, but the threshold was not reached; 'pitched-out', if attacking beetles had been forced out by drowning or immobilization, resulting in an unsuccessful attack; and 'Alive', when no visible attacks by *I. sexdentatus* were detected.

Statistical analyses

Although data were collected on weekly basis, in order to avoid temporal pseudo-replication, the analysis on the results of the trapping experiments was performed with the total amount of beetles caught through each of the experimental periods as the response variable. The response was fitted against treatment and block factors and to a Poisson error distribution in a GLM with a log-link function. If significant treatment effects were detected, then means were separated using Tukey's HSD test, applying a Bonferroni correction to the value of α for the confidence intervals (Reeve and Strom 2004).

Fisher's exact test for count data was applied to account for frequencies with lower than four observations within the contingency table that summarized the results of the tree protection study (Crawley 2007). No attacks were registered in other than central trees, thus, observed frequencies for central tree status were then modelled under GLM procedures, following a Poisson family distribution, in order to test for interaction between treatment and resulting status after the experimental period.

To account for any possible effect by the stand structure of the plots on the registered response, measured stand variables (species composition, dominance class, density or height) or derived variables [standing wood volume, calculated as a function of DBH, height and site class (ICONA 1995); dominant height, that is, the height of the thickest tree in the plot; and basal area] per plot and conifer species were analysed. After an initial approach to detect correlated variables using principal component analysis (PCA), selected variables (density, dominant height and the proportion of suppressed or intermediate trees in the plot) were used in fitting a multivariate model by a canonical correspondence analysis (CCA). The resulting tree status was coded as a matrix of presence (1) or absence (0) of each of thee status classes, as the equivalent to species in a community data matrix. Starting from an unconstrained model, a stepwise model selection based on Akaike's information criterion (AIC) was carried out with selected stand variables forming the constraining matrix (environmental data matrix), providing the means for relating assessed tree status to any of the selected variables after PCA. All statistical computing has been carried out using the R language and environment (The R Development Core Team 2008).

Results

Trapping bioassays

Both tested repellents and their combination significantly interrupted the attraction of *I. sexdentatus* to ipsdienol-baited multiple funnel traps during the first (F = 22.79; d.f. = 3; P = < 0.001) and second bioassay (F = 44.48; d.f. = 4; P = < 0.001). Although the inhibitory effect by tC and low rate verbenone resulted in reductions of 45% and 73% of control catches, respectively, there were no significant differences between them. Verbenone alone caught almost no beetles (fig. 1a). On the second bioassay, catches on treatments which included verbenone released at 40 mg/24 h, either alone or with tC, were significantly reduced (82% and 90%, respectively), whereas tC alone interrupted *I. sexdentatus* in a similar fashion to the first bioassay, yielding catch reductions at approximately 45% (fig. 1b). It is noteworthy that although the combined release of tC and verbenone resulted in the lowest catch, the effect was not significantly different from the reduction prompted by verbenone alone.

The major component of the pheromonal blend of *I. sexdentatus*, ipsdienol, attracted high numbers of the bark beetle predators *T. formicarius* and *T. coerulea* during the first bioassay (table 2), in accordance with previously described kairomonal responses in these species (Pajares et al. 2004). Verbenone exerted a strong inhibition on the attraction of both predators, as catches of the clerid were lowered by 80% at a low release rate, whereas the effect at a high release rate could not be observed as the flight period of *T. formicarius* was almost over by the onset of the second bioassay at the end of July. On the contrary, *T. coerulea* catches by ipsdienol were reduced by approximately one-third when

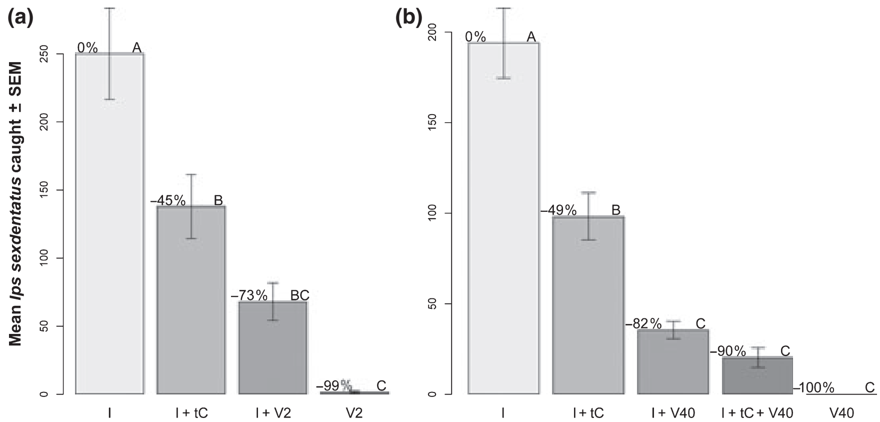

Fig. 1 Mean *Ips sexdentatus* catches ± SEM (n = 7) per treatment and experiment on multi-funnel traps baited with: (a) Bioassay 1: 'I', ipsdienol; 'I + tC', ipsdienol plus *trans*-conophthorin; 'I + V2', ipsdienol plus 2 mg/24 h verbenone; and 'V2', 2 mg/24 h verbenone. (b) Bioassay 2: 'I', ipsdienol; 'I + tC', ipsdienol plus *trans*-conophthorin; 'I + tC + V40', ipsdienol plus *trans*-conophthorin and 40 mg/24 h verbenone; 'I + V40', ipsdienol plus 40 mg/24 h verbenone; and 'V40', 40 mg/24 h verbenone. Mean percentage reductions of catches are shown. Bars designated by the same letter are not significantly different (Tukey's HSD, Bonferroni's adjustment, P < 0.05).

Table 2 Mean catches of predators and associated *Hylurgus ligniperda* in funnel traps baited with *Ips sexdentatus* lure and repellents during trapping bioassays 1 and 2

	Treatment[1]									
Bioassay 1	I		I + tC		I + V2		V2			
Hylurgus ligniperda	113.57 ± 22.00	A	94.43 ± 15.50	A	96.57 ± 17.51	A	1.43 ± 0.20	B		
Thanasimus formicarius	14.71 ± 1.30	A	19.57 ± 2.53	A	2.71 ± 1.13	B	0.57 ± 0.30	B		
Temnoscheila coerulea	121.86 ± 20.60	A	177.29 ± 37.90	A	82.14 ± 12.25	B	9.14 ± 5.69	B		
Bioassay 2	I		I + tC		I + V40		I + tC (+V40)		V40	
Hylurgus ligniperda	4.57 ± 1.07	AB	10.86 ± 2.79	AC	11.57 ± 2.62	AC	16.57 ± 2.82	C	0.00 ± 0.00	B
Temnoscheila coerulea	5.43 ± 1.51	A	5.29 ± 0.78	A	0.86 ± 0.46	B	2.57 ± 1.46	AB	0.14 ± 0.14	B

Treatments: 'I', ipsdienol; 'I + tC', ipsdienol plus *trans*-conophthorin; 'I + V2', ipsdienol plus 2 mg/24h verbenone; 'I + V40', ipsdienol plus 40 mg/24 h verbenone; 'V2', 2 mg/24h verbenone; 'I + tC + V40', ipsdienol plus *trans*-conophthorin and 40 mg/24 h verbenone; and 'V40', 40 mg/24 h verbenone.

[1]Treatment: mean catches ± SEM, n = 7.

Means within the same row designated by the same letter are not significantly different (Tukey's HSD, Bonferroni's adjustment, P < 0.05).

verbenone was released at 2 mg/24 h during the peak of its flight period and by approximately four-fifths with a 40 mg/24 h release rate later in the season (table 2). As for the effect of tC, although mean catches were slightly higher, in none of the bioassays did it significantly interfere on the attraction exerted by ipsdienol on its own (table 2). The attraction by ipsdienol to *T. coerulea* was also reduced when both repellents were tested together, but their effect was not significantly different to any other tested treatment. The low level of catches of the trogossitid during the second bioassay, although informative, calls for caution, as the low level of variance that arouse from small catches might increase apparent treatment effect (Reeve and Strom 2004).

Golden-haired bark beetle (*H. ligniperda*) was caught in high numbers in traps baited only with ipsdienol in bioassay 1, and catches were not significantly affected by the release of either verbenone or tC (table 2). Verbenone released alone at 2 mg/24 h attracted virtually no individuals of this secondary bark beetle. Similarly, the attraction of *H. ligniperda* to ipsdienol on the second bioassay did not significantly differ from treatments with verbenone or tC, but was significantly higher than the reference when both compounds were released together. Verbenone on its own was not attractive at all for *H. ligniperda* (table 2).

Tree protection

At the end of the experiment, all baited control trees suffered attacks by *I. sexdentatus*. Fifty-five percent of control trees were under mass attacks, whereas 30% had undergoing attacks. The remaining 15% of test trees had aborted all boring attempts, pitching out beetles (fig. 2). On the contrary, only 10% of verbenone treated trees were undergoing mass attacks when assessed, one tree (5%) was at the initial stage of attack, beetles had been pitched out on 25% of

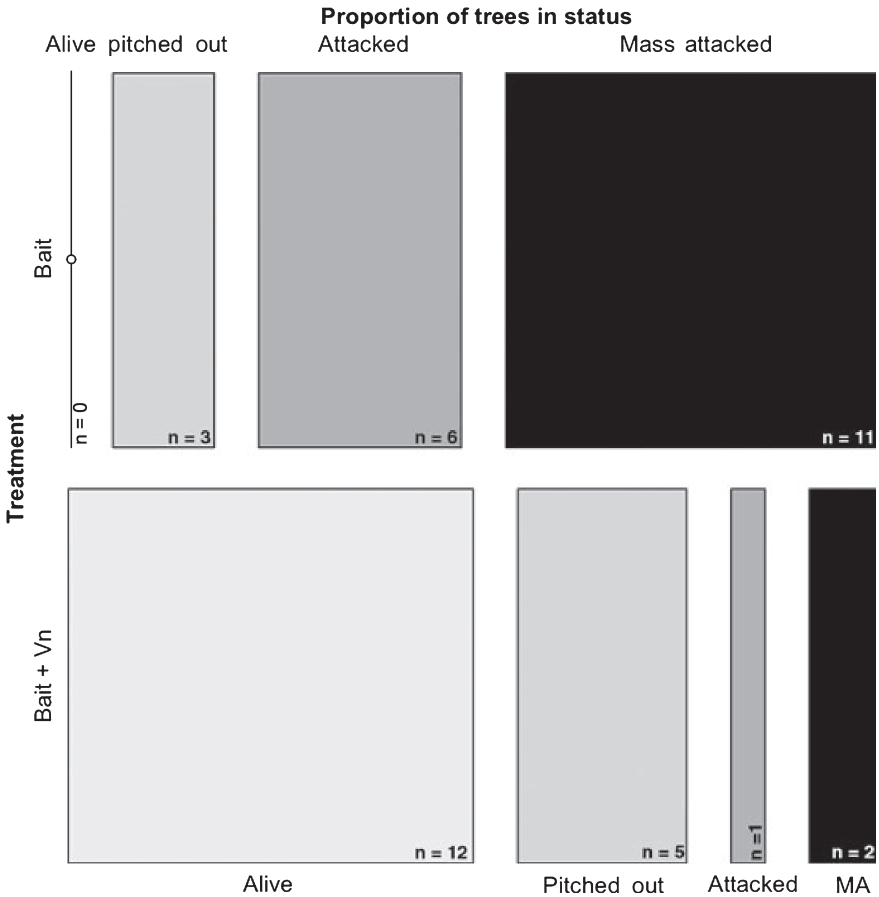

Fig. 2 Mosaic plot for the distribution of tree status at the assessment made after the tree protection assay. The area of each tile is proportional to the corresponding frequency of trees at each status and treatment after the experimental period. Bait, stands for ipsdienol-baited trees; Bait + Vn, stands for trees baited with ipsdienol and verbenone released at 60 mg/24 h. MA, stands for 'mass attacked'. See text for the definition of tree status categories.

Table 3 Mean ± 95% CI of plot variables in the experiment testing the effect of verbenone repelling *I. sexdentatus* attacks on standing *P. nigra* trees

	Bait (n = 20)	Bait + Vn (n = 20)	Overall (n = 40)
Density (trees/ha)	1317.80 ± 141.11	1362.37 ± 181.47	1340.08 ± 145.08
Basal area (m^2/ha)	37.60 ± 4.33	35.09 ± 5.14	36.34 ± 4.27
Dominant height (m)	13.15 ± 0.86	13.38 ± 0.84	13.27 ± 0.76
Standing volume (m^3/ha)	93.46 ± 12.98	84.55 ± 13.06	89.00 ± 11.49
Species composition[1]			
Pinus nigra	0.75 ± 0.11	0.82 ± 0.10	0.78 ± 0.09
Pinus sylvestris	0.13 ± 0.07	0.11 ± 0.09	0.12 ± 0.07
Pinus pinaster	0.13 ± 0.08	0.07 ± 0.05	0.10 ± 0.06
Dominance class[1]			
Dominants	0.19 ± 0.04	0.16 ± 0.04	0.18 ± 0.04
Co-dominants	0.34 ± 0.06	0.32 ± 0.05	0.33 ± 0.05
Intermediates	0.35 ± 0.06	0.38 ± 0.07	0.37 ± 0.06
Suppressed	0.12 ± 0.04	0.14 ± 0.04	0.13 ± 0.04

Control plots, 'Bait', test treatment; 'Bait + Vn', and overall means.
[1]Species composition and dominance classes given as the proportions of each category present in each plot set.

treated trees, and 60% of protected trees remained unattacked. The results of Fisher's exact test for the outcome of the tree protection study, based on 100 000 replicates, indicated that observed frequencies deviated significantly (P < 0.001) from expected mean frequencies. The analysis of deviance on the fitted GLM model showed a significant interaction between treatment and resulting tree status [$P(>|\chi|) < 0.001$; d.f. = 3].

Principal component analysis revealed best plot variables explaining the variation in stand characteristics, once correlated variables were removed from the ordination. These variables were: (i) density, (ii) dominant height and the proportion of (iii) suppressed or (iv) intermediate dominance level trees in the plot (table 3). However, the stepwise model selection showed that none of these variables improved the CCA model, treatment being the only constraining factor, and thus giving the lowest AIC number. Thus, no other variable than treatment with verbenone influenced the tree status output after the experiment.

Discussion

The spiroacetal *trans*-conophthorin is known to be produced by several bark beetles, both in angiosperm and in conifer twigs and cone species (Miller et al. 2000). It is also a major volatile in the bark of many broadleaves, such as *Betula pubescens* Ehrh. and *B. pendula* Roth (Byers et al. 1998; Huber et al. 1999). When plant extracts from these trees were placed close to aggregation pheromone components of the spruce bark beetles *Pityogenes chalcographus* L. and *I. typographus*, their orientation response was inhibited (Byers et al. 1998; Zhang et al. 1999). GC-EAD analysis reported that tC elicited antennal responses in *I. typographus* (Zhang et al. 2000). Similar antennal and field responses were found in five North American bark beetle species, *Dendroctonus ponderosae*, *D. pseudotsugae*, *D. rufipennis*, *Ips pini* (Say.) and *Dryocoetes confusus* Swaine (Huber et al. 1999, 2000). Jactel et al. (2001) provided the first electrophysiological evidence that *I. sexdentatus* detects some non-host leaf and bark volatiles, including tC. Our trapping results showed that tC inhibits *I. sexdentatus* aggregative response too. Surprisingly, Jactel et al. (2001) could not significantly reduce catches emitting a 16-times higher rate of tC. However, tC release rate was found to be the only significant source of variation when a number of repellent combinations were tested. Differences in composition and/or the release rate of the pheromone lures used may account for the disparities in the inhibitory effect found between these and our results.

It is known that verbenone interrupts the attraction to the aggregation pheromones in a multitude of bark beetle species (Borden 1997), for example, *I. typographus* (Zhang et al. 1999; Jakus et al. 2003) or *I. pini* (Lindgren and Miller 2002b). Previous trapping experiments evaluating the effect of verbenone on *I. sexdentatus* had resulted in either weak reductions (Paiva et al. 1988; Romon et al. 2007a) or non-significant differences (Kohnle et al. 1992a; b) of the attractive response to the aggregative bait. Our results clearly show that both tested formulations of verbenone significantly inhibited the response of this bark beetle to its major pheromonal compound ipsdienol. Dispensers releasing ca. 2 mg/24 h significantly reduced the response, but did not achieve a significantly lower effect than tC. A 20 times higher release rate of verbenone reduced catches by 82%, trapping significantly less beetles than tC (49%). The combined usage of tC and verbenone has shown strong synergistic inhibitory effect when trapping *I. typographus* (Zhang and Schlyter 2003). Although this blend elicited the strongest inhibition on the *I. sexdentatus* response to the aggregation pheromone component, differences were only significant to the effect of tC alone, but not to verbenone released alone at 40 mg/24 h. Thus no synergistic interaction of inhibitory effects of two repellent candidates became apparent.

Two of the most important natural enemies of the six-toothed bark beetle, *Thanasimus formicarius* and *Temnoscheila coerulea* (Pajares et al. 2008), are attracted by the aggregation pheromones of their bark beetle prey similarly as by host volatiles (Pajares et al. 2004). Verbenone strongly reduced the number of predators caught independently of the verbenone release rate. Both studied predators lay eggs on logs and trees attacked by the scolytid during the concentration phase, preying on *I. sexdentatus* adults that arrive on the breeding substrate. During this period, levels of verbenone gradually increase (Flechtmann et al. 1999), and whereas bark beetles seem to recon unsuitable hosts, verbenone can serve as an indicator of declining prey for early arriving predators. To our knowledge, these results are the first reports on the effects of tested inhibitors on European trogossitids or clerids, but very similar responses to verbenone have been reported for North American bark beetle predators (Lindgren and Miller 2002a). Release of tC did not significantly affect predator catches in any of the bioassays. *T. formicarius* preys also on bark beetles that feed on broad-leaf trees (Warzée 2005), thus it is likely that NHVs do not affect its kairomonal response towards ipsdienol.

The golden-haired bark beetle is generally considered a secondary species, although aggressive attacks on young trees have been reported (García de Viedma 1964). It has been recently established outside its native European range, causing some degree of damage (Hoebeke 2001). In a previous study, verbenone released at a low rate did not significantly affect *H. ligniperda* catches when compared with those caught with ipsdienol, ipsenol and myrcene (Romon et al. 2007a). In our first trapping trial, catches on traps baited with ipsdienol were not significantly affected by the release of verbenone or tC. This agrees with the formulated hypothesis on verbenone dose sensitivity in regard to beetle host requirements. Assuming that verbenone level is a function of microbial degradation of tissue, its inhibitory effect would be mainly on species colonizing fresh host tissues, thus responding to relatively low doses. On the contrary, species using aged tissues should not respond to verbenone, respond only to high doses, or even be attracted depending on their occurrence in the colonization succession (Lindgren and Miller 2002b). However, results from the second trial pointed to a trend of increasing catches with the release of both compounds; the low number of insects caught might have affected the statistical analysis because of the low variance (Reeve and Strom 2004).

A new verbenone pouch became commercially available by the time the third experiment was designed. Taking into account positive results by recent studies using high release rates (Borden et al. 2003; Bentz et al. 2005), the difficulties on procuring tC (e.g. Graves et al. 2008) and the lack of a significant difference between the high release rate of verbenone alone and the combination with tC (fig. 1), we decided to use the new pouch in the tree protection experiment. Although *I. sexdentatus* population was not at an epidemic level in the area, the fact that all the baited control trees were attacked indicated that the population level was sufficient for our goal, that is, to test the level of protection conferred by verbenone releasing devices to healthy trees withstanding an *I. sexdentatus* attack. The distribution of the proportions of non-attacked (alive), resistant, attacked and mass-attacked trees significantly deviated from randomness according to the outcome of Fisher's exact test (fig. 2). Even if the influence of some stand variables on verbenone and bark beetle behaviour has been shown (Schmitz et al. 1989; Kostyk et al. 1993), our results show that the release of verbenone was the only explanatory variable that accounted for the tree status at the end of the experiment. Although not many studies have focused on a tree level, these results support the findings of earlier works on *D. ponderosae* (Amman et al. 1991; Huber and Borden 2001), all of which showed that verbenone significantly reduced mortality of individual trees. As for *I. typographus,* trees were not fully protected in all experiences (Jakus and Dudová 1999), whereas attacks by *I. pini* on central trees were reduced in plots treated with verbenone released at 7.1 g/ha/24 h (Borden et al. 1992).

Damage caused by wood boring insects is expected to increase in the Mediterranean basin as global warming proceeds. Increased occurrence of climatic extremes will subject forests to a higher intensity and frequency of stress (La Porta et al. 2008), increasing in turn the availability of breeding material for beetles with similar behaviour as *I. sexdentatus*. Although in the long term, a change of current Mediterranean silviculture might be required (de Dios et al. 2007), methods protecting weakened stands are needed in the short term. Even if baited trees attracted more beetles than expected for a non-baited tree, verbenone successfully lowered the number of insects that begun boring test trees. The higher proportion of trees that 'pitched-out' *I. sexdentatus* attacks when verbenone was released might have arose from the overall reduction of the number of insects arriving on trees which were then not abundant enough to overcome tree defences. Thus, verbenone can provide a window of opportunity for defence to those trees that are weakened, for example, because of fire, drought or other abiotic stress. On the contrary, trapping bioassays showed the negative effect of verbenone on *I. sexdentatus* natural enemies, which could eventually result in a lower control of bark beetles in treated stands. Research is needed to determine if verbenone significantly affects predation by *T. formicarius* and *T. coerulea* in treated stands. The usefulness of a suitable repelling device can be further improved by the use of efficient attractants in the management strategy known as 'push-pull' (Lindgren and Borden 1993). This study presents the potential of verbenone for the protection of single trees. Future work should focus in the combination of a powerful aggregation lure and an effective repellent on the management of an ongoing attack of *I. sexdentatus*.

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