Quantitative Association of Bark Beetles with Pitch Canker Fungus and Effects of Verbenone on Their Semiochemical Communication in Monterey Pine Forests in Northern Spain

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Environ. Entomol. 36(4): 743–750 (2007)

ABSTRACT The association between 11 species of bark beetles (Coleoptera: Scolytinae) and one weevil (Coleoptera: Entiminae) with the pitch canker fungus, *Fusarium circinatum* Nirenberg and O'Donnell, was determined by crushing beetles on selective medium and histone H3 gene sequencing. *Pityophthorus pubescens* (Marsham) (25.00%), *Hylurgops palliatus* (Gyllenhal) (11.96%), *Ips sexdentatus* (Börner) (8.57%), *Hypothenemus eruditus* Westwood (7.89%), *Hylastes attenuatus* Erichson (7.40%), and *Orthotomicus erosus* (Wollaston) (2.73%) were found to carry the inoculum. In addition, the root weevil *Brachyderes incanus* L. (14.28%) had the second highest frequency of occurrence of the fungus. The responses of the insects to a range of verbenone doses were tested in field bioassays using funnel traps. Catches of *P. pubescens*, a species colonizing branch tips of live trees, were significantly reduced in a log-linear dose-dependent relationship. Catches of *I. sexdentatus*, an opportunistic species normally attacking fresh dead host material, were also gradually reduced with increasing verbenone dose. Catches of *Tomicus piniperda* L., *O. erosus*, *Dryocoetes autographus* (Ratzeburg), *H. eruditus*, *Xyleborus dryographus* (Ratzeburg), *Hylastes ater* (Paykull), *Hylurgus ligniperda* (F.), *H. attenuatus*, and *B. incanus* were not significantly affected by verbenone. The effects of verbenone were consistent with differences in host-age preference. Semiochemical disruption by verbenone in *P. pubescens* and *I. sexdentatus* could represent an integrated pest management strategy for the prevention of the spread of pitch canker disease between different stands. However, several species associated with *F. circinatum* were unaffected by verbenone, not supporting this compound for prevention of the establishment of potential vectors in Northern Spain.

KEY WORDS *Pinus radiata*, bark beetles, verbenone, semiochemical communication, *Fusarium circinatum* epidemiology

Some species of bark beetles (Coleoptera: Scolytinae) are among the most destructive insects of coniferous forests representing a continuous threat (Ayres and Lombardero 2000). Economic losses caused by bark beetle infestations are difficult to quantify, and rough estimates exist for only a few species: during the 1970s, *Ips typographus* L. destroyed 2,000,000 m^3 of wood in Scandinavia (Bakke 1983); since 1895, *Dendroctonus ponderosae* Hopkins has caused an average annual loss of ≈1.5 billion board feet particularly in *Pinus contorta* Douglas ex Loudon in western North America (Wood 1982); from 1974 to 1980, outbreaks of *Dendroctonus frontalis* Zimmerman in southeastern Texas covered 3,200,000 ha with heavy economic losses (Carter et al. 1991); and during 1989, massive outbreaks of *Tomicus piniperda* L. caused losses of 92 million euros in *Pinus radiata* (D. Don) in Basque Country region in Spain (Amezaga 1993).

In addition to direct damage, bark beetles inoculate several economically important phytopathogenic and bluestaining fungi (Schowalter and Filip 1993). For example, pitch canker, caused by *Fusarium circinatum* Nirenberg and O'Donnell, a fungus endemic to the southeastern United States (Dwinell et al. 1985), is responsible for serious losses for the forest industry. More recently, pitch canker was identified and reported in California, predominantly in planted urban *P. radiata* and in native Monterey pine forest (Correll et al. 1991). Since being first reported in the United States, it has been also found in Japan (Muramoto et al. 1988), Mexico (Rodriquez 1989), South Africa (Viljoen et al. 1994), and Spain (Landeras et al. 2005), probably because of softwood lumber, seedling, and

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0046-225X/07/0743–0750$04.00/0

seed exports. In California, wounding has not resulted in transmission of the pathogen to cones (Correll et al. 1991) or branches (Fox et al. 1991), despite the presence of significant airborne inoculum. A complex of insects, however, seems to be capable of transmitting *F. circinatum* (Fox et al. 1991, Hoover et al. 1995, Storer et al. 2004). For example, *Ips paraconfusus* (Lanier), *Ips mexicanus* Hopkins, *Ips plastographus* (LeConte), *Pityophthorus carmeli* Swaine, *Pityophthorus nitidulus* Mannerheim, *Pityophthorus setosus* Blackman, *Conophthorus radiatae* Hopkins (Coleoptera: Scolytinae), and *Ernobius punctulatus* (LeConte) (Coleoptera: Anobiidae) have been shown to be phoretically associated with the fungus and are known to visit and infest nondiseased trees. This leads to the potential for development of integrated control strategies of pitch canker by preventing the establishment of bark beetle vectors in high value pine stands.

Bark beetles use plant compounds, predominantly the monoterpenes α-pinene or myrcene, as kairomonal precursors for their pheromonal components (Hendry et al. 1980). Many of the same pheromonal compounds are used by species in the same genus, e.g., ipsenol, ipsdienol, and *cis*-verbenol in the genus *Ips* (tribe Ipini) or *exo*-brevicomin, frontalin, *trans*-verbenol, and verbenone in the genus *Dendroctonus* (tribe Tomicini) (Borden 1982). Knowledge of the chemical ecology of bark beetle species colonizing conifers in southern Europe is very limited (Byers 1992, Schlyter et al. 2000).

Verbenone (4,6,6-trimethylbicyclo[3.1.1]-hept-3-en-2-one) is a simple oxidation product of *trans*-verbenol, which in turn is a biological oxidation product of α-pinene (Birgersson and Leufvén 1988), one of the most ubiquitous monoterpenes in the Pinaceae. α-Pinene is quite toxic to a number of coniferophagous insects, whereas *trans*-verbenol and verbenone seem to be less toxic (Lindgren et al. 1996). Thus, insects inhabiting environments high in α-pinene could be expected to have either a high tolerance or an effective detoxification system. Verbenone has been found in relatively large amounts (mg) in hindguts of *Dendroctonus ponderosae* (Pierce et al. 1987), *Dendroctonus frontalis* (Renwick and Vité 1968), *Dendroctonus brevicomis* LeConte (Byers et al. 1984), and *Dendroctonus pseudotsugae* Hopkins (Rudinsky et al. 1974), and in low amounts (ng) in *T. piniperda* (Lanne et al. 1987), but it seems to be absent in *I. paraconfusus, I. typographus,* and *Pityogenes chalcographus* L. (Byers 1983b, Birgersson et al. 1984, 1990).

Some bark beetle species of the formerly considered subfamily Hylesininae (tribe Tomicini) produce verbenone in relatively significant amounts early in colonization, so it has been suggested that it plays a role in reducing intraspecific competition and interspecific competition with other species (Byers et al. 1984). Therefore, some bark beetle species may have evolved to use α-pinene and *trans*-verbenol as kairomonal precursors, producing verbenone as an anti-aggregation pheromone that reduces intraspecific competition. Other sympatric bark beetle species on the same host might then evolve to avoid species that produced verbenone (as an allomone), and so avoid interspecific competition. However, verbenone is also produced in aging logs (Flechtmann et al. 1999), and it has been shown that verbenone increases with age of logs because of the activity of certain microorganisms (Leufvén et al. 1988).

Our first objective was to determine the association between bark beetles and *F. circinatum.* The possible role of bark beetles as vectors of pitch canker fungus has not been studied in Europe until now. Our second objective was to determine the ecological effect of verbenone on the aggregation of different bark beetle species in Spain to evaluate the potential for using verbenone in an integrated management strategy to reduce their indirect damages. There have also been no previous studies of the function of verbenone in the chemical ecology of bark beetles in the Iberian Peninsula.

Materials and Methods

Isolation and Identification of Fungi from Bark Beetles. Fungi were isolated from adult bark beetles collected from baiting logs in two stands of *P. radiata* localized in Morga and Muxika (Basque Country). In March 2004, 10 trap logs, 1.5 m long and 0.2 m in diameter (bark thickness ≈2 cm), were set out in each stand. One half of the logs from each locality were partially buried at an angle of 90°, and the other five were placed on the ground surface to capture bark beetles species with aerial and buried habitats. After 2 mo, all the logs at each site were inspected for the presence of beetle entrance holes. All beetles from a single gallery were removed using sterilized tweezers, placed individually in sterile bottles, and morphologically identified by using a LEICA MZ95 dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany) to determine taxonomic characters located in pronotum, scutellum, elytra, metapisternum, and antennal funiculum (Gil and Pajares 1986, Pfeffer 1995).

Beetles were removed from storage bottles using sterilized tweezers and squashed onto the surface of a selective medium for *Fusarium* species DCCP (15 g peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4$ 0.7 H_2O, 0.2 g chloramphenicol, 20 g agar, and 1,000 ml distilled water, amended with 0.002 g 2–6-dichloro-4-nitroaniline in 10 ml ethanol) (Burgess et al. 1988). After 2 wk of incubation at 25°C in the dark, fungal cultures were purified by transferring hyphal tips from the edges of individual colonies to fresh potato dextrose agar (Panreac). Pure, sporulating cultures were examined and classified into putative types using a LEICA DM4500B microscope according to characteristics of the fruiting structures produced by the anamorphic stage (Nelson et al. 1983, Britz et al. 2002). *F. circinatum* putative isolates were properly identified by DNA sequences comparison. DNA extractions and polymerase chain reaction (PCR) amplifications were performed as described by Steenkamp et al. (1999). Sequence alignments were performed by Sequence Navigator 1.0 (Applied Biosystems, Lincoln, CA) and properly identified by DNA sequences comparison using the services provided by the National Center for Biotechnology Information.

Table 1. Parameters for verbenone dose experiments in Basque Country (Northern Spain), in 2004, with description of semiochemical-releasing devices

Each experiment						
No.	Targeted insect species	Other collected insect species	Dates	Pheromonal–kairomonal attractant blend[a]	Description	Release rate (mg/24 h)[b]
1	*P. pubescens*	—	1–30 May	*trans*-Pityol	Polyethylene bubble cap	0.14
2	*H. palliatus*	*H. attenuatus*	1 June–	Ethanol	Polyethylene bottle (30 ml)	250.00
		H. ater	30 July	3-Carene	Polyethylene screw-cap bottle (15 ml)	250.00
		O. erosus		β-Pinene	Polyethylene screw-cap bottle (15 ml)	250.00
		H. eruditus		*cis*-Verbenol	Polyethylene bubble cap	2.10
		X. dryographus		*trans*-Verbenol	Polyethylene bubble cap	2.10
		B. incanus				
3	*T. piniperda*	—	1 Aug.–	Terpinolene	Polyethylene screw-cap bottle (15 ml)	250.00
			30 Sept.	α-Pinene	Polyethylene screw-cap bottle (15 ml)	250.00
				α-Pineneoxide	Polyvinyl bubble cap	0.20
				trans-Verbenol	Polyvinyl bubble cap	0.20
4	*I. sexdentatus*	*D. autographus*	1 Oct.–	Myrcene	Polyethylene screw-cap bottle (15 ml)	250.00
		H. ligniperda	30 Nov.	Ipsdienol	Polyvinyl bubble cap	0.20
				Ipsenol	Polyvinyl bubble cap	0.40

All experiments		
Chemical[a]	Description	Release rate (mg/24 h)[b]
Verbenone (+17/−83)	Closed polyethylene centrifuge tube (250 μl)	0.01
Verbenone (+17/−83)	Closed polyethylene centrifuge tube (400 μl)	0.20
Verbenone (+17/−83)	Polyethylene bubble cap	1.80
Verbenone (+17/−83)	Polyethylene bubble cap	3.10

[a] All chemical purities >98%.
[b] At 22–24°C.

All the beetles present in each gallery were treated as a single sample, recording only one isolate per gallery to avoid the overestimation of frequency calculations. Frequencies of occurrence of fungi were computed using the formula of Yamaoka et al. (1997) where F = (NF/NT)100 (%), and F represents the frequency of occurrence (%) of the fungus, NT represents the total number of samples from which isolation attempts were made, and NF represents the number of samples from which the fungus was isolated.

Semiochemical Experiments. Four separate experiments were conducted to evaluate dose effects of verbenone on the attraction of four targeted bark beetle species (Table 1). All release devices were obtained from Pherotech International (Delta, British Columbia, Canada; Table 1). Ipsenol and ipsdienol were formulated in 1,3-butanediol at a concentration of 80 mg/ml. Release rates for devices representing ipsenol and ipsdienol were determined by collection of volatiles on Porapak-Q and analysis by capillary gas chromatography. Release rates for all remaining devices were determined by weight loss.

In each experiment, five blocks (replicates) of five 12-unit Lindgren multiple funnel traps were set at least 100 m apart in stands of mature Monterey pine in Basque Country (Northern Spain). Traps were spaced 10–15 m apart in grids of 2 by 3 within each block. Each trap was at least 2 m from any tree and suspended by rope such that the bottom of each trap was 0.2–0.5 m above ground level. Each experiment was conducted in different *P. radiata* plantations areas (experiment 1, Lezama; experiment 2, Muxika; experiment 3, Morga; experiment 4, Orozketa) in which considerable population of the respective target insect species had been previously recorded (Goldarazena 2004), thus avoiding influencing subsequent experiments by the residual compounds and the obtained catches during the previous experiments.

In each experiment, treatments were assigned randomly to traps within each block as follows: attractants alone or with devices resulting in one of four verbenone release rates: 0.01, 0.2, 1.8, and 3.1 mg/24 h (at 22–24°C). The control trap in each experiment was a trap baited only with attractants. The optimal phenological season was selected for each target species (Goldarazena 2004), and known pheromonal and/or kairomonal blends (Vité et al. 1972, Volz 1988, Byers 1992, Czokajlo 1998, Dallara et al. 2000) were used as attractants (Table 1). Voucher specimens have been deposited at the Entomology Collection at NEIKER-Basque Institute of Agricultural Research and Development, Basque Country, Spain.

Data were analyzed by regression using SPSS 13.0 statistical software (SPSS 1999). All possible combination analysis between several transformations (ln, log, square root) of both trap catches and the independent variable were tested, as needed from examinations of residuals and to correct for heteroscedasticity and nonlinearity.

Results

A list of the results of the assays for the presence of *F. circinatum* (GenBank accession numbers DQ662828,

Table 2. Variation about frequency of occurrence of *F. circinatum* on several bark beetles (Coleoptera: Scolytinae) and weevils (Coleoptera: Entiminae) species in Northern Spain

Insect species	Total no. samples	NF	F (%)[a]
Pityophthorus pubescens	32	8	25.00
Brachyderes incanus	42	6	14.28
Hylurgops palliatus	117	14	11.96
Ips sexdentatus	35	3	8.57
Hypothenemus eruditus	38	3	7.89
Hylastes attenuatus	54	4	7.40
Orthotomicus erosus	73	2	2.73
Dryocoetes autographus	45	—	—
Hylastes ater	32	—	—
Tomicus piniperda	18	—	—
Xyleborus dryographus	9	—	—
Hylurgus ligniperda	5	—	—

[a] Frequency of occurrence F = (NF/NT)100 (%), where NT represents the total no. of samples from which isolations attempts were made, and NF represents the no. of samples from which *F. circinatum* was isolated.

DQ662829, DQ662830, DQ662831, DQ662832, and DQ352833) to determine potential transmission vectors is provided in Table 2. Six of the 11 species of bark beetles tested for fungal associates were identified as carriers of *F. circinatum.* These included *Pityophthorus pubescens* (Marsham) (25.00%), *Hylurgops palliatus* (Gyllenhal) (11.96%), *Ips sexdentatus* (Börner) (8.57%), *Hypothenemus* eruditus Westwood (7.89%), *Hylastes attenuatus* Erichson (7.40%), and *Orthotomicus erosus* (Wollaston) (2.73%). In addition, the root weevil *Brachyderes incanus* L. (14.28%) had the second highest frequency of occurrence of the pitch canker fungus. Thus, of 391 galleries examined from the seven potential vector species, 10.20% presented beetles carrying the fungus.

Verbenone significantly interrupted the attraction of *P. pubescens* to attractant-baited multiple-funnel traps ($F = 10.704$, df = 4, $P < 0.001$) in a log-linear dose-dependent relationship (Fig. 1A). Verbenone also reduced catches of *I. sexdentatus* significantly ($F = 9.299$, df = 4, $P < 0.001$). The relationship between catches of beetles and dose of verbenone was square root-linear for *Ips sexdentatus* (Fig. 1B). In contrast, verbenone did not significantly affect catches of *O. erosus* ($F = 0.917$, df = 4, $P = 0.473$; Fig. 2A), *Dryocoetes autographus* (Ratzeburg) ($F = 0.674$, df = 4, $P = 0.618$), *T. piniperda* ($F = 0.479$, df = 4, $P = 0.751$), *B. incanus* ($F = 0.703$, df = 4, $P = 0.599$; Fig. 2B), *H. eruditus* ($F = 0.240$, df = 4, $P = 0.912$; Fig. 2C), *Xyleborus dryographus* (Ratzeburg) ($F = 0.331$, df = 4, $P = 0.854$), *Hylastes ater* (Paykull) ($F = 1.200$, df = 4, $P = 0.341$), *Hylurgus ligniperda* (F.) ($F = 0.558$, df = 4, $P = 0.696$), and *H. attenuatus* ($F = 0.851$, df = 4, $P = 0.510$; Fig. 2D).

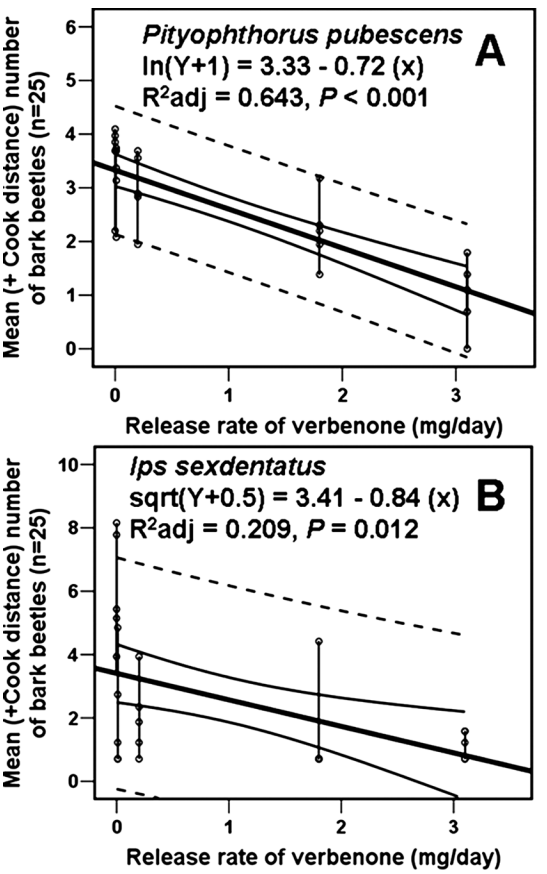

Fig. 1. Effect of verbenone, released at four rates, on the attraction of *P. pubescens* in experiment 1 (A) and *I. sexdentatus* in experiment 4 (B). See Table 1 for experimental details. Slopes of regression lines are significantly different from zero (*t*-test, $P < 0.001$). Confidence limits (95%; thin solid lines) are associated with each regression line (thick solid line). Dashed lines represent confidence limits (95%) for catches in control traps.

Discussion

The presence of *F. circinatum* on >10% of the potential vector species shows that these species are associated with pitch canker–diseased trees and that they may be vectors of the fungus. The consideration of management efforts directed at these species may therefore be justifiable.

Lindgren (1994) proposed the hypothesis that verbenone quantity increases with the level of microbial degradation of phloem tissue. If this hypothesis is valid, one would expect verbenone to have a strong inhibitory effect on species that need fresh host tissue, even at low doses. Species that use aged tissue should not respond to verbenone, respond only to high doses, or even be attracted depending on where they occur in the succession of insect colonization. Based on the hypothesis proposed by Lindgren (1994), one would expect the following ordering in verbenone dose sensitivity by the studied species: *P. pubescens* > *T. piniperda* > *I. sexdentatus* ≥ *O. erosus* ≥ *D. autographus* > *B. incanus* ≥ *H. eruditus* ≥ *X. dryographus* > *H. ater* ≥ *H. ligniperda* ≥ *H. attenuatus.*

Our data show partial agreement with Lindgren's hypothesis. *Pityophthorus pubescens* exhibited a log-linear dose-dependent relationship that is in concor-

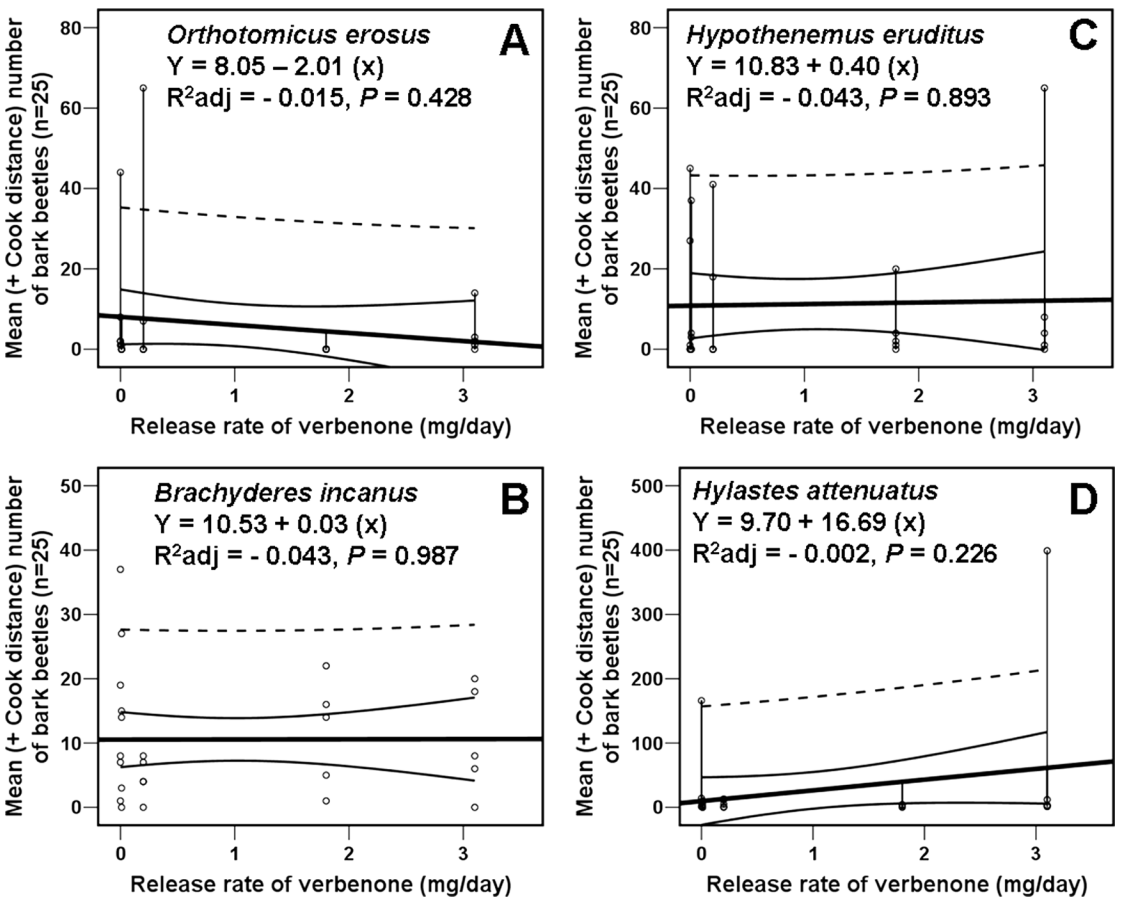

Fig. 2. Effect of verbenone, released at four rates, on the attraction of *O. erosus* in experiment 2 (A), *B. incanus* in experiment 2 (B), *H. eruditus* in experiment 2 (C), and *H. attenuatus* in experiment 2 (D). See Table 1 for experimental details. Slopes of regression lines are significantly different from zero (*t*-test, $P < 0.001$). Confidence limits (95%; thin solid lines) are associated with each regression line (thick solid line). Dashed lines represent confidence limits (95%) for catches in control traps.

dance with the primary characteristics of this bark beetle species, because it usually colonizes apical twigs of relatively large trees localized within wide forest areas. *Ips sexdentatus*, which still requires relatively fresh phloem and may mass attack live trees under favorable conditions, shows a square root-linear dose response to verbenone, resulting in an almost complete shutdown of attractiveness of the aggregation pheromonal blend. *H. palliatus* was not caught in high enough numbers to allow a representative evaluation of verbenone. This could be linked to the difficulty of monitoring populations of this bark beetle species with traps because of its main colonization of buried niches (Romón et al. 2007). Further research is needed to bioassay how the mentioned bark beetle responds to verbenone during the colonization of cut logs deposited in the forest floor. *Tomicus piniperda*, which feeds on pine shoots to complete sexual maturation and needs fresh phloem for breeding, would be expected to show relatively high sensitivity even to low doses of verbenone. However, under favorable environmental conditions, this species produces massive outbreaks in recently wounded trees, and it also can massively colonize stumps on the ground, so it may be adapted to moderate natural amounts of verbenone (Lanne et al. 1987), which is consistent with our results.

Orthotomicus erosus and *D. autographus*, which are pioneer competitors in wood colonization, did not respond significantly to verbenone dose, possibly because these species are associated with *I. sexdentatus* pioneer boring facilitating activity. As also expected, verbenone did not significantly affect catches of *X. dryographus* and *H. eruditus*, which are secondary species that seem adapted to mature stands and show strong feeding relationships with mutualistic fungi. Similarly, catches of *Hylastes* species and *H. ligniperda*, species often found in large numbers on stumps or logs in contact with the ground for relatively long periods, were not significantly affected by verbenone.

The catches of all nontargeted species could be considered incidental, so their responses to verbenone should be considered with caution because the knowledge about their semiochemical attractants is very

limited, and further studies are needed to discern the attractant ability of the tested compounds by experimental designs with the inclusion of negative control traps. Considering, however, the results obtained during this study, it is interesting to notice a trend of potential attractive effect of verbenone on *Hylastes attenuatus.* Future assays are thus also needed with the incorporation of higher verbenone release rates and comparative mathematical analysis of the obtained regression equations and significance values. In the case of confirmation of a significant attraction, studies dealing with the determination of whether this bark beetle is able to produce verbenone, de novo and/or from conifer host monoterpenes in the absence of microorganisms, should be performed to discern the source of its potential natural adaptation to high levels of verbenone.

In our experiments, both attractant compounds and verbenone release rates varied mostly only with temperature as a function of the vapor pressures of the tested compounds and release membrane properties. Under natural conditions, however, beetles are exposed to various doses and ratios of attractant and repellent compounds depending also on conifer species, tree genetics, bark-beetle associated microorganisms, free microorganisms, and direct autoxidations of semiochemicals by physical factors such as sunlight (Hunt et al. 1989). The significant regressions obtained for two bark beetle species, as well as medium flight temperature thresholds between 18 and 25°C (Bakke 1968, Kennedy and McCullough 2002), provided a high level of confidence about the observed verbenone dose-dependent effects for the targeted bark beetle species.

To our knowledge, very few temperature-dependent models for predicting release rates of volatile compounds from field dispensers have been developed (Bradley et al. 1995), and they cannot be applied to this study because they deal with other compounds and release devices material and/or shape. Similarly, knowledge is extremely limited about the compounds that are produced by direct oxidizing processes, as well as their different vapor pressures (Capouet and Müller 2005). Future laboratory studies are needed to determine the effect of the temperature and sunlight intensity on the release rates of the compounds from the release devices that were used during this study. This information would permit determining in the field a broader range of effects of verbenone at release rate variations caused by temperature and sunlight intensity. This objective will also need the development of an environmental sensor coupled to a digital sample fractioning system (Byers 1983a).

Based on the methodology used in this study, verbenone could be considered a component for an IPM strategy for preventing *P. pubescens* and *I. sexdentatus* aggregation, two potential insect vectors of the pitch canker pathogen. However, results in this study did not support verbenone as a tool to completely avoid the establishment of pitch canker fungus vectors in Monterey pine stands, because *B. incanus, H. eruditus, H. attenuatus,* and *H. palliatus* showed relatively high association percentages with *F. circinatum*, but catches of these bark beetle species were not significantly affected by verbenone.

During the past two decades, verbenone has been shown as a good natural repellent for the control of damages caused by several insect species such as *Hylobius pales* (Herbst) (Salom et al. 1994), *Hylobius abietis* L. (Lindgren et al. 1996), *D. ponderosae* (Lindgren et al. 1989), *Ips pini* (Say), and *Ips latidens* (LeConte) (Lindgren and Miller 2002). However, results in some subsequent experiments have been inconsistent probably because of some inadequate release rate standardizations and/or differences in effects on different conifer host subpopulations of the same insect species (Amman and Lindgren 1995). Generally, verbenone has shown relatively good results as an anti-aggregant only against pioneer bark beetle species probably because conifers have not evolved the capacity of converting α-pinene to verbenone. Future studies should be directed to evaluate the ability of several bark beetle species to directly vector *F. circinatum* into plant material, determine the efficacy of verbenone as potential inhibitor of *I. sexdentatus* aggregation in wood yards with high risk of pitch canker fungus establishment, and study the effect and genetically based variation of *P. radiata* primary monoterpenes to assayed different potential control compounds for other important primary bark beetle species such as *T. piniperda.*

Acknowledgments

We thank Forest Health Protection (USDA Forest Service) for providing test material needed for the development of this project and Pherotech International (Delta, BC, Canada) for technical assistance with release rate determinations and formulation of compounds; the Spanish Ministry of Education and Science (AGL2005–01711/FOR), partially funded by FEDER, and the Department of Agriculture and Fisheries of the Basque Country (DAP-VED2003016) for financial support; and the Department of Education, Universities and Research of the Basque Country for a grant to P. Romón to support a PhD. We also thank the management and employees of NEIKER and Bizkaia Government for the time and facilities to carry out this work, especially to P. Saint de Urturi, A. Isasmendi, and J. C. Pino.

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Received for publication 3 August 2006; accepted 27 March 2007.