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Short Communication

Seasonal Terpene Variation in Needles of *Pinus radiata* (Pinales: Pinaceae) Trees Attacked by *Tomicus piniperda* (Coleoptera: Scolytinae) and the Effect of Limonene on Beetle Aggregation

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Abstract

Concentrations of four monoterpenes were determined in needles of *Pinus radiata* (D.Don) (Pinales: Pinaceae) trees that were attacked or nonattacked by *Tomicus piniperda* (L.) (Coleoptera: Scolytinae). Compounds were identified and quantified by gas chromatography–mass spectrometry. The mean ambient temperature was obtained using climate-recording data loggers. The effect of limonene on field aggregation was also evaluated at three limonene release rates using Lindgren attractant-baited traps and trap logs. Attacked trees produced less α-pinene in March, July, and November than nonattacked trees, less β-pinene in July and November, and less limonene from May to November. Limonene reduced the attraction of *T. piniperda* to attractant-baited traps and trap logs. Results were linked to better responses to high temperatures, with respect to terpene contents, by the nonattacked trees after the spring attack.

Key words: *Tomicus piniperda*, *Pinus radiata*, attractant, repellent, temperature

The pine shoot beetle, *Tomicus piniperda* (L.) (Coleoptera: Scolytinae), is a holarctic insect species that have been introduced into North America (Haack and Kucera 1993). It is known to be influenced by terpene composition of their host trees (Schroeder 1988, Byers 1992). In Europe, its main natural hosts are, in order of preference, *Pinus brutia* Ten., *Pinus nigra* Arnold, and *Pinus sylvestris* (L.), although a broad range of adaptation to other *Pinus* spp. has been recorded (Chararas et al. 1982, Sauvard et al. 1987). It causes some loss on *Pinus radiata* (D.Don) (Pinales: Pinaceae) due to direct damage and association with sapstaining and pathogenic fungi, mainly in the genera *Ophiostoma*, *Leptographium*, *Sydowia*, and *Fusarium* (Kirisits 2004, Bezos et al. 2015, Jordán Muñoz-Adalia et al. 2017).

After a bout of early spring shoot feeding by individual larva or adult that had not reached adult-hood in the previous autumn, adult beetles attack living trunks for mating and oviposition (Långström 1983). Regeneration shoot feeding occurs in early summer (May) when adults feed on the medulla of apical pine shoots after ovipositing, and cause yellowing and premature drooping of these shoots. After 3 mo of larval feeding and pupation, new adults emerge and feed on shoots in autumn (September) before overwintering under the thick bark at the trunk base (Salonen 1973).

Eleven compounds have been shown to be involved in the beetle's attraction, but α-pinene, β-pinene, and Δ^3-carene are considered to be the main volatile attractants as they are regulated directly by host-tree genetics (Lanne et al. 1987). Other compounds are produced by microbial activity within beetle galleries (α-terpineol, *cis*- and *trans*-carveol, myrtenol, ethanol, α-terpinolene, α-pineneoxide, and *trans*-verbenol) (Czokajlo 1998). Field tests with pine billets containing adult females initialy failed to prove the existence of a female-produced aggregating pheromone (Perttunen et al. 1970). The subsequent isolation of *trans*-verbenol from the hindgut of boring females; however, electroantenna and field bioassay responses led to the identification of this compound as an aggregation pheromone (Francke and Heemann 1976, Lanne et al. 1987, Poland et al. 2003).

In the present study, we assessed seasonal variations in four monoterpenes (α-pinene, β-pinene, and Δ^3-carene as attractants, and limonene as the potential repellent) in *P. radiata* needles from trees that were attacked or nonattacked by *T. piniperda*. We also tested for a relationship between monoterpene compositions and the

temperature, and evaluated the effect of limonene on *T. piniperda* aggregations in the field.

Materials and Methods

Terpene Analyses

The study site was located in a *P. radiata* cv. Año Nuevo 15-yr-old stand in Orozketa, northern Spain (UTM 30T 530.732, 4780.526). Samples were collected every 2 mo between March and November. The mean ambient temperature was obtained using climate recording USB-2 data loggers (Lascar Electronics, Salisbury, United Kingdom). Ten initially trunk-attacked and 10 nonattacked trees were marked in March. From each tree, using a 12 m pole pruner, ten 1-yr-old healthy needles were randomly selected from each of the three randomly pruned twigs. The samples were transported to the laboratory in a cooler, weighed, stored at −80°C for 1 wk, ground in an electronic mill, statically extracted for 24 h at 25°C in 3 ml of analytical-grade pentane containing 0.1% *p*-cymene, filtered through Whatman no. 44 paper and again stored at −80°C until analyses. *p*-Cymene was used as the internal standard because it is structurally similar to other monoterpenes but does not occur in *P. radiata*. A gas chromatograph with a cross-linked 5% phenyl methyl siloxane 30 m × 0.25 mm capillary column (HP-5MS) and an electron impact detector (Hewlett-Packard 6890) coupled to a mass spectrometer (HP5973) was used. The injection temperature of 70°C was maintained for 6 min, and the temperature was then ramped at 5°C/min to 150°C, and finally at 20°C/min to 250°C where it was kept for 10 min. The injector needle was washed five times in pentane between each injection and a solvent control was introduced at every 10 samples. The carrier gas was helium at 0.01 ml/s. Compounds were identified by retention time comparison with a standarized elution pattern of pure standards from Sigma-Aldrich, and by comparison of each spectrum with those of the Wiley library. Concentrations were determined by digital peak integration with the HP ChemStation software and the relative response of each analysed compound was compared with the internal standard.

Limonene Field Assays

Four 12-unit Lindgren funnel traps were set in six stands of *P. radiata*. Traps were spaced 50 m apart in grids of 2 × 2. Each trap was setup at least 5 m away from any tree and suspended such that the bottom was 0.5 m aboveground. Treatments were assigned randomly to traps: attractant blend alone or with 0.5 ml polyethylene tubes resulting in one of three limonene release rates: 1.2, 3.6 and 10.8 mg/24 h. The attractant blend comprised α-pinene polyethylene screw-cap bottle 15 ml 250 mg release rate/24 h, α-terpinolene polyethylene screw-cap bottle 15 ml 250 mg release rate/24 h, α-pineneoxide polyvinyl bubble cap 1 ml 0.2 mg release rate/24 h, and *trans*-verbenol polyvinyl bubble cap 1 ml 0.2 mg release rate/24 h (Romón et al. 2007). All release products were obtained from Pherotech Inc. (Delta BC, Canada). The same conditions were applied using 1 × 0.2 m (bark thickness about 2 cm) *P. radiata* trap logs instead of traps. Both funnel traps and trap-log assays were operated from March to November with a monthy count of captures (debarking the logs and replacing them) and replacement of the release devices.

Statistical Analyses

Terpene data were analysed by standard (GLM GEN function) and repeated-measures ANOVA (GLM REP), with month and tree type, respectively, as within- and across-subjects factors, using IBM SPSS Statistics Version 19. For the rest of the data, linear regression was represented, testing all possible combinations between several transformations (ln, log, square root) of both the dependent variable (terpene levels [n=300]/trap catches [n=24]) and the corresponding independent variable (temperature/limonene release rate), as needed for examination of residuals and to correct for heterocedasticity and nonlinearity.

Results

Terpenes Analyses

Retention times of α-pinene, β-pinene, Δ^3-carene, *p*-cymene, and limonene were 3.78, 4.56, 5.22, 5.53, and 5.62 min, respectively.

Considering nonindependent data derived from repeated samplings (repeated-measures ANOVA), the concentrations of α-pinene, β-pinene, and limonene were dependent on the tree type and previous months' concentrations. α-Pinene level differences between attacked and nonattacked trees changed over time in the overall interaction (Table 1). Attacked trees produced less α-pinene, β-pinene, and limonene between May and November than in March (Fig. 1), but limonene levels were lower than in nonattacked trees at the same months (Fig. 1C). In March, July, and November, levels of α-pinene were lower in attacked trees (Fig. 1A), and the same occurred with β-pinene at July and November (Fig. 1B). The levels of the three compounds were negatively related to increasing temperature in attacked trees (Fig. 2A–C), whereas they were not affected by temperature in nonattacked trees (Fig. 2D–F).

Limonene Field Assays

In March, limonene significantly reduced the attraction of *T. piniperda* to attractant-baited multiple-funnel traps (Fig. 3A) and trap logs at the two lowest limonene release rates (Fig. 3B). In contrast, limonene at the highest release rate did not reduce catches of *T. piniperda* in trap logs.

Table 1. Repeated-measures rm-ANOVA of the relationships of concentration (mg/g needle) of α-pinene, β-pinene, Δ^3-carene, and limonene with month and tree type within *Pinus radiata* trees attacked or nonattacked by *Tomicus piniperda*

Compound	α-Pinene			β-Pinene			Δ^3-Carene			Limonene		
Model term	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Tree type	1.97	7.04	**0.009**	1.94	9.93	**0.002**	1.111	2.91	0.091	1.96	8.65	**0.004**
Month	1.48	15.59	**0.001**	1.46	21.42	**0.001**	1.56	1.99	0.163	1.46	22.57	**0.001**
Tree type* Month	1.102	4.28	**0.041**	1.98	1.72	0.192	1.114	0.00	1.000	1.99	0.11	0.737

Bold values represent significant *P*-values.
df (degrees of freedom); *F* (*F*-value).

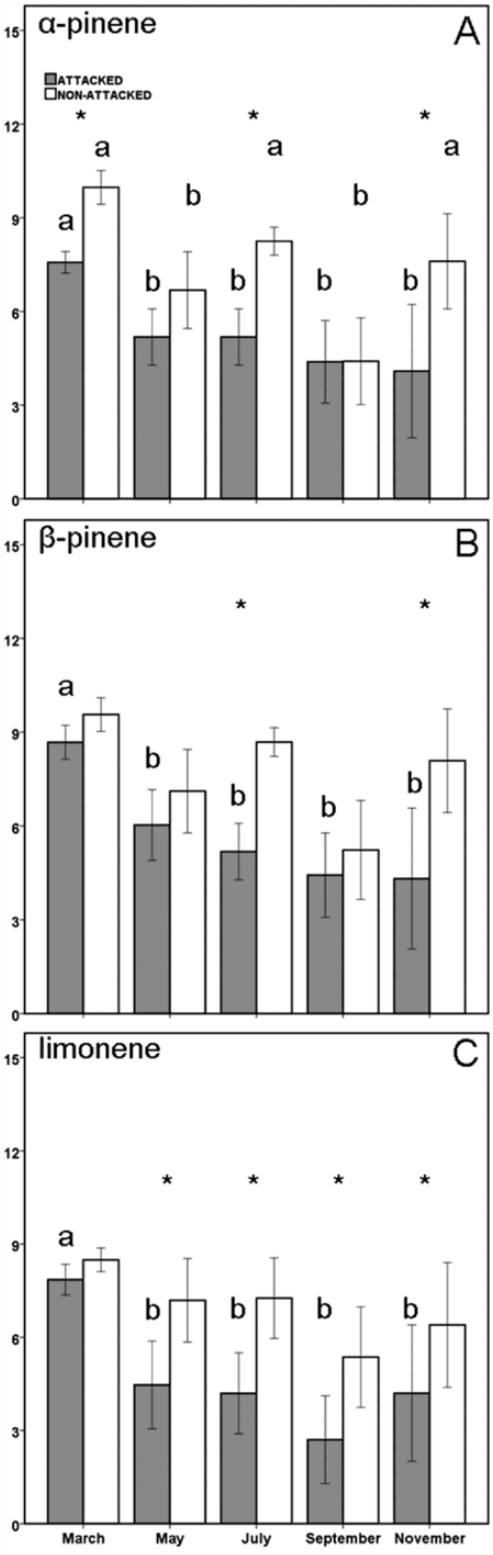

Fig. 1. Needle concentration (mg/g needle ± SE) of α-pinene (A), β-pinene (B), and limonene (C) in *P. radiata* trees attacked or nonattacked by *T. piniperda*. Letters denote significant differences between different months within a determined tree class. Stars indicate differences between the two tree classes at the same month (n=300).

Discussion

Our results do not explain why *T. piniperda* beetles selected attacked trees, because nonattacked trees in March had higher concentrations of α-pinene, a compound that is part of the attractant blend for this insect (Song et al. 2005). However, seasonal fluctuation of the concentration in terpenes was remarkable. The lower levels of limonene that we recorded in attacked trees between May and November could have implications for summer, autumn shoot-feeding periods or both. We found that attacked trees had 1) lower concentrations of α-pinene, β-pinene, and limonene in July and November; and 2) lower limonene. In the first case, nonattacked trees could offset higher concentrations of attractive kairomones by presenting higher levels of limonene. The second pattern was seen in May (regeneration shoot feeding) and September (callow adults sexual maturation period by shoot feeding). Further research is needed to discern whether individuals of this bark beetle species feed on apical pine shoots of trees where they have oviposited or trees from which they emerged. This seems to happen in *Tomicus* spp. colonizing *Pinus yunnanensis* in China where trunk attacks are caused by beetles coming from the crown of the same tree after an aggregation process that appears to occur during the shoot-feeding phase (Lieutier et al. 2003).

Levels of α-pinene in nonattacked trees returned to their March levels during July and November. In this sense, results were linked to better responses to high temperatures in the nonattacked trees. Overall, the results indicate that the concentration in needles of several compounds changes in response to attack at the trunk level, suggesting a distal effect. Similar distal results have been previously observed in other tree species–herbivorous insect interactions (Marpeau et al. 1989, Tomlin et al. 2000, Blande et al. 2009).

Previous studies have tested potential repellents against *T. piniperda*. For example, Byers et al. (1989) demonstrated that verbenone (release rate 0.32 mg/24 h) caused an 80% reduction in *T. piniperda* attraction to a 1:1:1 mixture of α-pinene, α-terpinolene, and Δ^3-carene released at the same rate than verbenone; however, Poland et al. (2004) showed that verbenone alone did not reduce the attraction to either attractant-baited traps or *P. sylvestris* logs. Similarly, Romón et al. (2007) observed that verbenone (0.01 to 3.1 mg/24 h) did not significantly affect catches of *T. piniperda* to traps baited with the same attractant blend as that used in the present study. On the other hand, limonene is one host monoterpene whose repellent properties have been demonstrated for some insects such as *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae) (Coyne and Lott 1976), *Scolytus ventralis* LeConte (Coleoptera: Curculionidae) (Raffa et al. 1985), *Ips calligraphus* (Germar) (Coleoptera: Curculionidae) (Cook and Hain 1988), *Hylobius abietis* (L.) (Coleoptera: Curculionidae) (Nordlander 1990) and *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Thaumetopoeidae) (Zhang et al. 2003). It is also related to host nonpreference as in the case of *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae) (West et al. 2016).

More assays should be directed to discern why the highest limonene release rate used in the present study was not significantly repellent in trap logs. In this sense, determining the natural amounts and proportions of terpenes emitted by different breeding materials (trunks, shoots, and logs) and sizes would be crucial. Initial work (Perttunen et al. 1970) showed that the ratios in which the attractant compounds occur may vary greatly between individual trees and between seasons, with marked differences between healthy and stressed trees, and that the variations in these proportions of

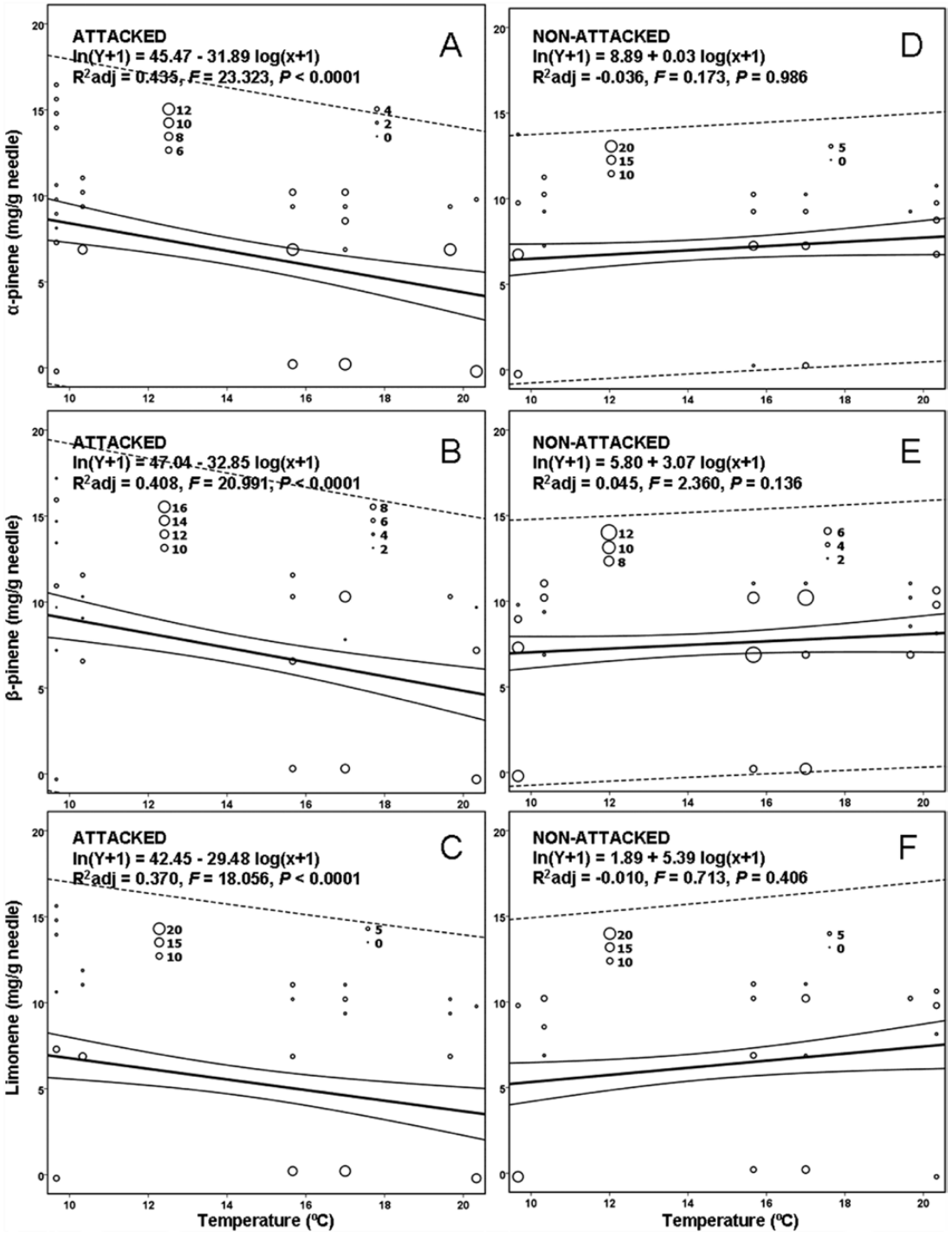

Fig. 2. Temperature effect on the needle concentration of α-pinene, β-pinene, and limonene within *P. radiata* trees that were attacked (A–C) or nonattacked (D–F) by *T. piniperda.* Mean confidence limits (thin solid lines) are associated with each regression line (thick solid line). Dashed lines represent individual confidence limits of 95%. Raw data are presented in the graphs.

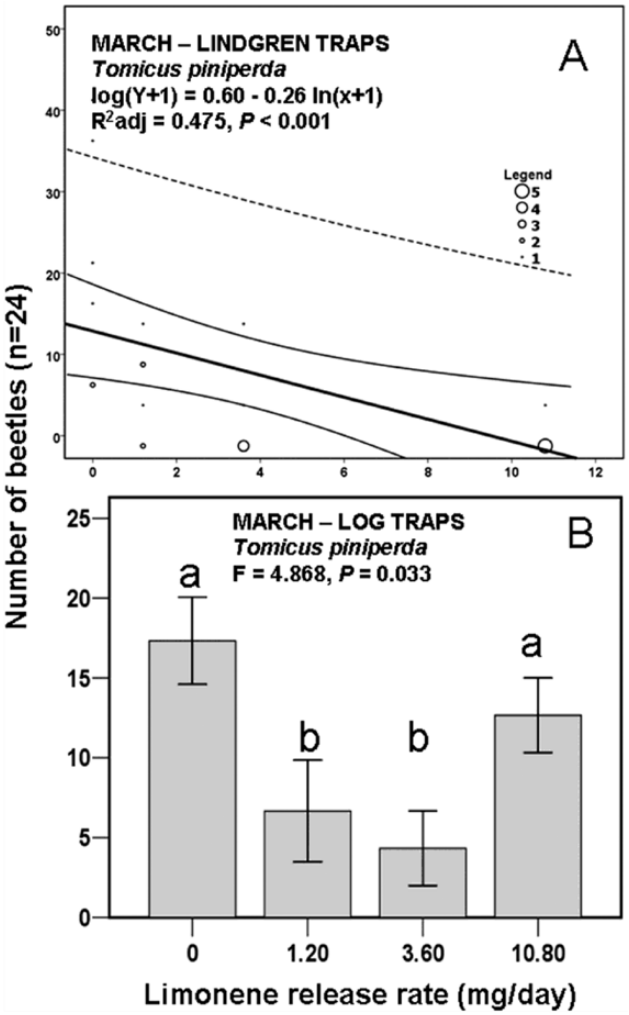

Fig. 3. Limonene effect, released at three rates (1.2, 3.6 and 10.8 mg/24 h at 22–24°C), on the aggregation of the bark beetle *T. piniperda* in Lindgren funnel traps (A) and baiting trap logs (B). Raw data are presented in graphs.

attractive and repellent compounds will guide the beetles to the most suitable breeding material.

All captured specimens were phenologically *T. piniperda* (Kohlmayr et al. 2002). However, co-ocurrence with *Tomicus destruens* has been previously reported in the vicinity of the sampled area. Further studies involving molecular differentiation (Gallego et al. 2004) should be directed to discern the effect of limonene on this other species. Assessing *T. destruens* will require the use of a kairomone blend (Gallego et al. 2008) optimized for that species and thus different from those tested in the present study.

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References Cited

Bezos, D., P. Martínez-Álvarez, J. J. Diez, and M. M. Fernández. 2015. *Tomicus piniperda* as a plausible vector of *Fusarium circinatum* in northern Spain. Ann. For. Sci. 72: 1079–1088.

Blande, J. D., K. Turunen, and J. K. Holopainen. 2009. Pine weevil feeding on Norway spruce bark has a stronger impact on needle VOC emissions than enhanced ultraviolet-B radiation. Environ. Pol. 157: 174–180.

Byers, J. A. 1992. Attraction of bark beetles, *Tomicus piniperda*, *Hylurgops palliatus*, and *Trypodendron domesticum* and other insects to short-chain alcohols and monoterpenes. J. Chem. Ecol. 18: 2385–2402.

Byers, J. A., B. S. Lanne, and J. Löfqvist. 1989. Host tree unsuitability recognized by pine shoot beetles in flight. Experientia 45: 489–492.

Cook, S. P., and F. P. Hain. 1988. Toxicity of host monoterpenes to *Dendroctonus frontalis* and *Ips calligraphus* (Coleoptera, Scolytidae). J. Entomol. Sci. 23: 287–292.

Coyne, J. F., and L. H. Lott. 1976. Toxicity of substances in pine oleoresin to southern pine beetles. J. GA Entomol. Soc. 11: 301–305.

Chararas, C., C. Revolon, M. Feinberg, and C. Ducauze. 1982. Preference of certain Scolytidae for different conifers, a statistical approach. J. Chem. Ecol. 8: 1093–1109.

Czokajlo, D. 1998. Semiochemicals for the larger pine shoot beetles (*Tomicus piniperda* L.) and its clerid predators. PhD dissertation. New York State University, New York, NY.

Francke, W., V. Heemann. 1976. The odour-bouquet of *Blastophagus piniperda* L. (Col., Scolytidae). J. Appl. Entomol. 82: 117–119.

Gallego, D., F. Canovas, M. A. Esteve, and J. Galián. 2004. Descriptive biogeography of *Tomicus* (Coleoptera: Scolytidae) species in Spain. J. Biogeogr. 31: 2011–2024.

Gallego, D., J. Galián, J. J. Diez, and J. A. Pajares. 2008. Kairomonal responses of *Tomicus destruens* (Col., Scolytidae) to host volatiles α-pinene and ethanol. J. Appl. Entomol. 132: 654–662.

Haack, B., D. Kucera. 1993. New introduction – Common pine shoot beetle, *Tomicus piniperda* (L.). *Pest Alert USDA*, FS, NA-TP-05-93.

Jordán Muñoz-Adalia, E., A. V. Sanz-Ros, J. A. Flores-Pacheco, J. Hantula, J. J. Diez, E. J. Vainio, M. Fernández. 2017. *Sydowia polyspora* dominates fungal communities carried by two *Tomicus* species in pine plantations threatened by *Fusarium circinatum*. Forests 8: 127–142.

Kirisits, T. 2004. Fungal associates of bark beetles, pp. 181–235. In F. Lieutier, K. R. Battisti, J. C. Grégoire and H. F. Evans (eds.), Bark and wood boring insects in living trees in Europe, a synthesis. Kluwer Academic Publishers, Dordrecht, Netherlands.

Kohlmayr, B., M. Riegler, R. Wegensteiner, and C. Stauffer. 2002. Morphological and genetic identification of the three pine pest of the genus *Tomicus* (Coleoptera, Scolytidae) in Europe. Agr. For. Entomol. 4: 151–157.

Långström, B. 1983. Life cycles and shoot-feeding of the pine shoot beetles. Stud. Forest. Sue. 163: 1–29.

Lanne, B. S., F. Schlyter, J. A. Byers, J. Lofqvist, A. Leufven, G. Bergstrom, J. N. C. van der Pers, R. Unelius, P. Baeckstrom, and T. Norin. 1987. Differences in attraction to semiochemicals present in sympatric pine shoot beetles, *Tomicus minor* and *T. piniperda*. J. Chem. Ecol. 13: 1045–1067.

Lieutier, F., H. Ye, and A. Yart. 2003. Shoot damage by *Tomicus* sp. (Coleoptera: Scolytidae) and effect on *Pinus yunnanensis* resistance to subsequent reproductive attacks in the stem. Agr. For. Entomol. 5: 227–233.

Marpeau, A., J. Walter, J. Launay, J. Charon, P. Baradat, and M. Gleizes. 1989. Effects of wounds on the terpene content of twigs of maritime pine (*Pinus pinaster* Ait.). Trees 3: 220–226.

Nordlander, G. 1990. Limonene inhibits attraction to α-pinene in the pine weevils *Hylobius abietis* and *H. pinastri*. J. Chem. Ecol. 16: 1307–1320.

Perttunen, V., H. Oksanen, E. Kangas. 1970. Aspects of the external and internal factors affecting the olfactory orientation of *Blastophagus piniperda* (Coleoptera: Scolytidae). Contr. Boyce Thompson Inst. Plant Res. 24: 293–297.

Poland, T. M., de Groot, P., Burke, S., Wakarchuk, D., Haack, R. A., Nott, R., Scarr, T. 2003. Development of an improved attractive lure for the pine shoot beetle, *Tomicus piniperda* (Coleoptera: Scolytidae). Agr. For. Entomol. 5: 293–300.

Poland, T. M., P. de Groot, S. Burke, D. Wakarchuk, R. A. Haack, R. Nott. 2004. Semiochemical disruption of the pine shoot beetle, *Tomicus piniperda* (Coleoptera: Scolytidae). Environ. Entomol. 33: 221–226.

Raffa, K. F., A. A. Berryman, J. Simasko, W. Teal, B. L. Wong. 1985. Effects of grand fir monoterpenes on the fir engraver (Coleoptera: Scolytidae) and its symbiotic fungi. Environ. Entomol. 14: 552–556.

Romón, P., J. C. Iturrondobeitia, K. Gibson, B. S. Lindgren, A. Goldarazena. 2007. Quantitative association of bark beetles with pitch canker fungus and effects of verbenone on their semiochemical communication in Monterey pine forests in Northern Spain. Environ. Entomol. 36: 743–750.

Salonen, K. 1973. On the life cycle, especially on the reproduction biology of *Blastophagus piniperda* L. (Col., Scolytidae). Acta Forestalia Fenicca 127: 1–72.

Sauvard, D., Lieutier, F., and Levieux, J. 1987. Répartition spatiale et dispersion de *Tomicus piniperda* L. (Coleoptera Scolytidae) en forêt d'Orléans. Ann. Sci. Forest. 44: 417–434.

Schroeder, L. M. 1988. Attraction of the bark beetle *Tomicus piniperda* and some other bark- and wood-living beetles to the host volatiles α-pinene and ethanol. Entomol. Ex. Appli. 46: 203–210.

Song, L. W., B. Z. Ren, S. H. Sun, X. J. Zhang, K. P. Zhang, C. Q. Gao. 2005. Field trapping test on semiochemicals of pine shoot beetle *Tomicus piniperda* L. J, Northeast For. Univ. 33: 38–40.

Tomlin, E. S., E. Antonejevic, R. I. Alfaro, and J. H. Borden. 2000. Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. Tree Physiol. 20: 1087–1095.

West, D. R., E. J. Bernklau, L. B. Bojstad, and W. R. Jacobi. 2016. Host defense mechanisms against bark beetle attack differ between ponderosa and lodgepole pines. Forests 7: 248–258.

Zhang, Q.-H., F. Schlyter, A. Battisti, G. Birgersson, P. Anderson. 2003. Electrophysiological responses of *Thaumetopoea pityocampa* females to host volatiles: implications for host selection of active and inactive terpenes. J. Pest Sci. 73: 103–107.