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INHIBITORY EFFECT OF MONOTERPENES ON RESPONSE OF *Pityogenes bidentatus* TO AGGREGATION PHEROMONE RELEASED BY PIEZOELECTRIC SPRAYER FOR PRECISION RELEASE OF SEMIOCHEMICALS

ASHRAF M. EL-SAYED* and JOHN A. BYERS

Department of Plant Protection Sciences
Swedish University of Agricultural Sciences
SE-230 53 Alnarp, Sweden

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Abstract—A piezoelectric sprayer for dispensing semiochemicals was developed and used for a field test of bark beetle semiochemicals. The sprayer consists of a geared pump that pushes a syringe slowly to dispense semiochemicals in solvents through a microtube to a glass micropipet fixed to a piezoelectric high-frequency vibrator. The frequency is adjusted via a function generator to about 120 kHz until the harmonic properties of the glass micropipet, drawn by an electrophysiological pipet puller, cause vibrations that atomize the solvent from the micropipet tip. The sprayer, syringe, pump, function generator, and power supply were hung on one arm of a rotating trap pair (traps 6 m apart) that was slowly rotated at 2 revolutions per hour (rph) to even out the position effects on trap catches. The aggregation pheromone components of *Pityogenes bidentatus*, grandisol and *cis*-verbenol, were released by standard tube dispensers in one trap and compared to the release of similar amounts by the sprayer in the other trap. No significant differences in catch were observed. No effect of the solvent hexane on aggregation could be observed. The trap pair also caught approximately equal numbers of bark beetles when the baits were identical. The release of (+)- and (−)-α-pinene, (+)-3-carene, and terpinolene, monoterpenes of host Scotch pine, *Pinus sylvestris*, at increasing rates from 0.01 to 10 log-equivalents in decadic steps (each at 0.1–100 μg/min) resulted in decreasing responses to aggregation pheromone (only 9% at highest rate). Inhibition by the individual monoterpenes tested at the 100 μg/min rate was significant for (+)- and (−)-α-pinene and terpinolene (12, 13, and 15% of control, respectively). The inhibition by the host Scotch pine monoterpenes may allow *P. bidentatus* to avoid resistant trees that release large amounts of toxic monoterpenes in their

* To whom correspondence should be addressed at present address: Agricultural and Agri-Food Canada, P.O. Box 6000, Vineland Station, Ontario, Canada L0R 2E0. e-mail: elsayeda@em.agr.ca

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resin and instead colonize dying and diseased limbs or slash, the usual host substrate. The piezoelectric sprayer should prove generally useful to dispense precise amounts of semiochemicals in field and laboratory experiments.

Key Words—Host selection, dispenser, release rates, Coleoptera, Scolytidae, *Pityogenes bidentatus*, *Pinus sylvestris*, Scotch pine, conifers.

INTRODUCTION

Bark beetles (Coleoptera: Scolytidae) attack trees by boring through the bark and tunneling in the phloem and cambium layers surrounding the sapwood. Conifers such as pines and spruce usually produce copius amounts of resin to defend against the penetration by bark beetles (Raffa and Berryman, 1987). These resins have long been known to contain monoterpenes with toxic properties as well as being viscous and sticky, causing entrapment and suffocation of beetles (Webb, 1906; Smith, 1961, 1965; Hodges et al., 1979; Raffa and Berryman, 1982, 1987; Byers, 1989; Werner, 1995; Klepzig et al., 1996). Therefore, it could be expected that bark beetles have evolved olfactory mechanisms and behaviors for the avoidance of specific volatile monoterpenes in tree resins. Pitman et al. (1966) reported that gas chromatographic effluents of frass from male, pine bark beetles, *Ips paraconfusus* Lanier, containing the monoterpenes α-pinene, myrcene, β-pinene, 3-carene, and limonene, elicited "strong negative klinotactic movements" by walking beetles. However, most other earlier studies found that certain monoterpenes enhance the attraction to pheromone components in some of the most "aggressive" bark beetles that kill living trees (Bedard et al., 1969; Werner, 1972; Rudinsky et al., 1972).

The role of monoterpenes in the ecology of bark beetles is further complicated since some host monoterpenes (α-pinene and myrcene) have been implicated or proven as precursors of aggregation pheromone components of several bark beetle species (Hughes, 1974; Renwick et al., 1976; Hendry et al., 1980; Klimetzek and Francke, 1980; Byers, 1981, 1989). However, more recent studies have indicated that the pheromone components that can be synthesized by the beetles from myrcene, e.g., ipsenol, ipsdienol, and (*E*)-myrcenol, are mostly made de novo (Byers and Birgersson, 1990; Ivarsson et al., 1993; Seybold et al., 1995). In addition, monoterpenes appear to aid in host selection since certain host monoterpenes increase the proportion of beetles entering holes or attractive to flying beetles (Byers et al., 1985, 1988; Phillips et al., 1988; Byers, 1989, 1992). The roles of host monoterpenes in the chemical ecology of even the most studied bark beetle pests are not fully understood, while in many other species little is known.

Progress in elucidating the functions and interactions of insect semiochemicals has been hindered by the lack of a dispenser that can be easily adjusted to

release semiochemicals at practically any rate from among the wide range of rates desired in the laboratory and field. The first objective of this study was to modify the piezoelectric sprayer designed for laboratory wind tunnels (El-Sayed et al., 1999a,b) to be portable for field use. The second objective was to release exact amounts of aggregation pheromone components of the bark beetle *P. bidentatus* from the sprayer in a rotating trap pair and compare the catches to similar releases of the neat components. In addition, the release of host Scotch pine *P. sylvestris* monoterpenes at various rates would indicate whether they increased or decreased attraction to pheromone components of *P. bidentatus* when compared to baits with only the neat compounds.

METHODS AND MATERIALS

Piezoelectric Sprayer for Field Use. A custom-made gear pump delivers a specific amount of semiochemicals through a microtube (0.12 mm ID, CMA/100, Carnegie Medicine AB, Stockholm, Sweden) to a glass capillary of 1.4 mm OD and 0.62 mm ID (ABS, Zürich, Switzerland), which were drawn out and broken to tip diameters of about 55 μm OD and 40 μm ID. The capillary tube was fixed to a piezo disk (Type Nr 4322020, Valvo, Hamburg, Germany) of 10–25 mm OD and ca. 1–2 mm thickness by a U-shaped wire. The piezo disk was driven at its vibration mode resonance by a sine or square wave of about 12 V peak to peak (see below). The U-shaped wire clip transfers the oscillations from the piezo disk to the microtubing and the glass capillary tip that oscillate at about 120 kHz. This produces an aerosol of the semiochemical solution that disperses and immediately evaporates. Due to their small size, the droplets evaporate completely within a small distance from the capillary tip. Smooth tips that are created by a micropipet pulling and cutting device tend to release one droplet at each half-oscillation. Normally we used tips that were pulled manually with the ignition flame (disposable micropipets from ABS). This yielded an irregular tip that tended to release one droplet at only one of the extreme positions of the oscillating tip. For example, a flow of 1 ml/hr is dispersed into droplets of ca. 4.3 pl (corresponding to a droplet diameter of ca. 25 μm) that are sprayed into the ambient air. A sprayer kit was adapted for field use by using a hand-wired circuit for generating sine or square waveforms and a portable syringe pump with low power consumption.

Driving Signal. The sine or the square waveform signals used to drive the piezo disk were taken from hand-wired circuits (Figure 1). The main element of the circuit is a frequency-tunable oscillator chip XR-2206 monolithic IC (Exar Corp.). This circuit provides two basic waveforms: sine and square waves. There are four overlapping ranges of 100 Hz to 200 kHz, and the desired range was obtained by changing the capacitor (C) connected between pins 5 and 6 or by

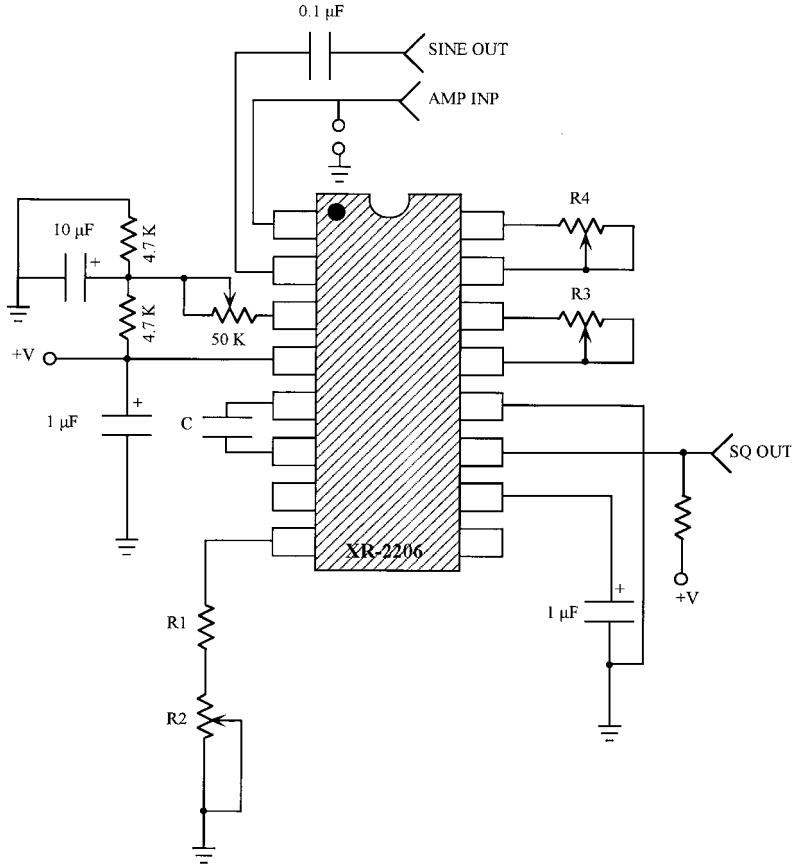

FIG. 1. Circuit used to generate a sine or square waveform with minimum harmonic distortion for driving the piezoelectric disk.

changing the value of resistor (R1). In this set-up, the frequency is inversely proportion to the value of the capacitor connected between pins 5 and 6, $(F) = 1/RC$, where C is the capacitance in farads, and R = R1 + R2 in ohms, C was set to 0.001 μF in our design, which produces a frequency range of 90–160 kHz. The amplitude of the waveform output can be varied from 0 to 12 V (peak to peak). The distortion in the output waveform was minimized by changing the value of R3 and R4 and observing the sinusoidal waveform in an oscilloscope. This circuit is designed to operate with a power supply of 15 mA at 12 V DC, which makes it ideal for battery-based field work due to its lower power consumption.

Syringe Pump. We constructed a gear syringe pump to deliver the semio-

chemicals in this study (Figure 2). The syringe pump was actuated by means of a 12-V DC gearmotor with a low power consumption (10 rpm, 40 mA; Japan Servo Co. Ltd., No. 4301). The motor armature is indirectly connected to a piston via two gears and a threaded-screw guide. The motor armature rotates gear 1 (2 cm OD, G1) at 10 rpm. The rotation of axial motion is translated from gear 1 to gear 2 (4.5 cm OD, G2) which is tightly fixed in the threaded-screw guide (25 cm long). This rotation is converted to linear motion through a piston soldered between two nuts attached along the guide (Figure 2). As the motor armature turns, the threaded guide slides, depending upon the direction of rotation, forward or backward and displaces the syringe piston causing fluid to move through the microtubing to the glass capillary tube. The flow rate of the fluid is determined by: (1) the speed of the motor, (2) the diameter of the syringe, and (3) the ratio of G1 : G2. In our set-up, (1) and (2) were constant; accordingly, the rate of the displaced fluid is determined by the ratio of G1 to G2 and is inversely proportional to the diameter of G2 and directly proportional to G1. The release rate of odorant was controlled by changing the concentration of the semiochemicals in solution or by changing the ratio of G1 to G2. In all experiments, the pump was set to deliver approximately 10 ± 3% μl/min by a gas-tight 1-ml syringe (Hamilton Bonaduz AG). Leakage and contamination are prevented by using tubing adapters (CMA/100) that connect the microtubing to the syringe tip and glass micropipet. One milliliter of semiochemicals when expelled at a rate of 10 μl/min typically took about 100 min.

Release of Semiochemicals in the Field. Test solutions of semiochemicals were prepared by dissolving the appropriate quantities of the synthetic semiochemicals in HPLC grade hexane. Solutions were pressed from a 1-ml syringe at 10 μl/min, through a microtube 1 m long, to the glass capillary tube. The glass micropipet was fixed and hung centered in one trap of a rotor trap pair (Figure 3). The traps in a pair were kept 6 m apart by two tubular-steel poles horizontally suspended by guy-wires from an upright center pole slowly rotated at 2 revolutions per hour (rph) by a 12-V regulated gearmotor (Byers et al., 1990, 1998). Each trap consisted of two panes of polycarbonate plastic (20 cm high × 32 cm wide) forming a cross-barrier trap. Wire from the cross-barrier suspended (15 cm below) a 32-cm-diam. plastic collecting funnel and bottle. The micropipet and piezoelectric vibrator, as well as the glass/plastic tubes with neat semiochemicals, were centered between the barrier trap and collecting funnel by a 1-mm wire (Figure 3).

Tests were performed to determine possible effects of hexane solvent, equality of trap pairs, and the relative attraction rates of beetles to components released by the sprayer versus the standard tube dispenser. (4*S*)-(−)-*cis*-Verbenol (99%, Borregaard) and grandisol, (1*R*,2*S*)-2-propenyl-1-methyl-cyclobutaneethanol (>98%, from G. Birgersson), both pheromone components of *P. bidentatus*, served as the attractive baits. In the standard dispensers, *cis*-verbenol

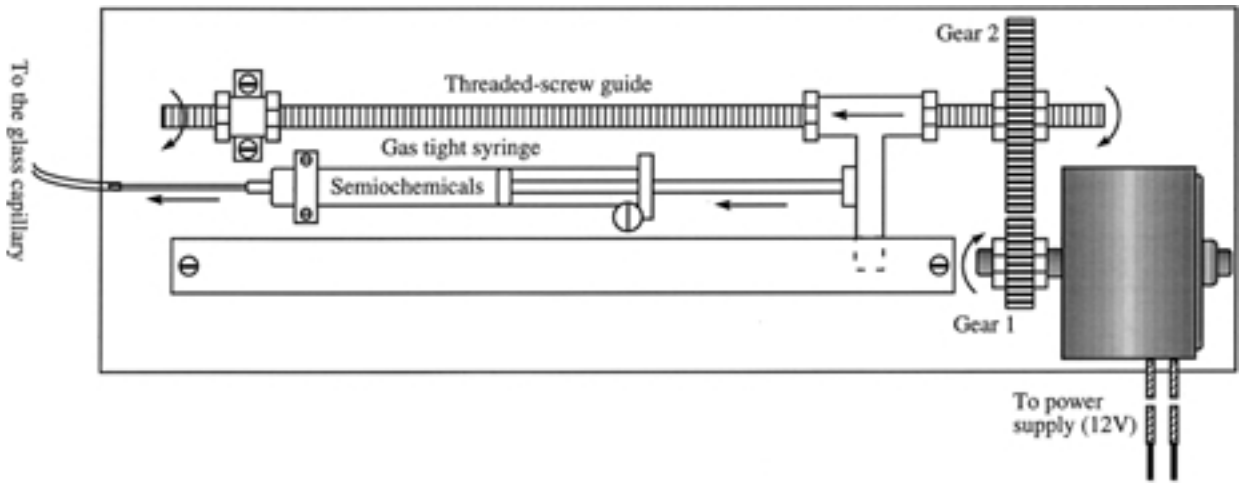

FIG. 2. Schematic diagram of a portable gear pump with low power consumption. The pump was used to deliver a specific amount of the aggregation pheromone of *P. bidentatus* or host Scotch pine monoterpenes via microtubing to a glass capillary tube fixed to a piezoelectric disk.

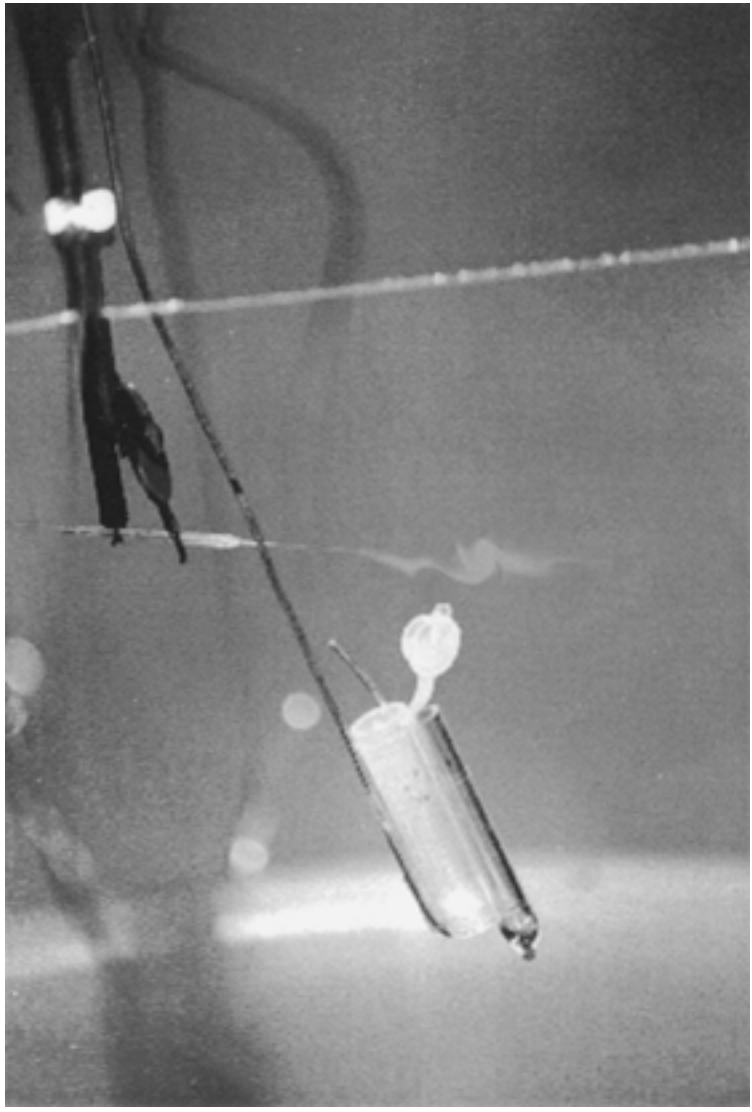

FIG. 3. Photograph of the active sprayer installed in a rotating trap for investigating the effect of Scotch pine monoterpenes on the attraction of *P. bidentatus* to its aggregation pheromone components in the field. (A) tubing, (B) capillary tube, (C) piezo disk, (D) aerosol of solvent containing semiochemicals, (E) standard polyethylene and glass dispenser tubes (F), and edge of plastic funnel in background.

was placed as a powder to cover the bottom of a 30-mm-long polyethylene tube (6 mm ID) while about 20 μl of grandisol was placed neat in the bottom of a 32-mm-long glass tube (3.5 mm ID). The release rates were estimated at 20°C to be 500 μg/day for *cis*-verbenol and 100 μg/day for grandisol. For the sprayer, the pheromone component test solution contained 17.5 ng each of grandisol and *cis*-verbenol per microliter hexane solvent. Since the pump and sprayer released about 10 μl/min, this was about 175 ng of each component per min or about half the rate for *cis*-verbenol and 2.5 times the rate for grandisol from the standard dispensers. However, in the trap catch comparison of the sprayer versus the standard dispensers, two such standard dispensers were used, so that the sprayer released 25% of the *cis*-verbenol and equivalent amounts of grandisol as the standard dispensers.

In the second series of tests to determine the effects of monoterpenes on attraction responses, only one tube for each pheromone component was used in each trap of the pair. One of these traps also used the sprayer to release a mixture of Scotch pine monoterpenes (−)-α-pinene ($[\alpha]_D^{20} = -50°$, >99.5% pure, Fluka), (+)-α-pinene ($[\alpha]_D^{22} = 46.5°$, >99%, Aldrich), (+)-3-carene ($[\alpha]_D^{20} = 17°$, >99%, Fluka), and terpinolene (>97.3%, Carl Roth). The concentrations of each monoterpene in the mixtures ranged in decadic steps, 0.01, 0.1, 1, and 10 μg/μl, again released at 10 μl solution/min (or 14.4 mg of each monoterpene/day) from the sprayer (Figure 4). These release rates are similar to what freshly cut Scotch pine logs (30 cm long × 15 cm diam.) emit at 0.01, 0.1, 1, and 10 log equivalents, respectively. The quantities of α-pinene and 3-carene from Scotch pine logs were mistakenly reported in Byers et al. (1985) as 13 or 14 μg/hr; they should have been micrograms per minute to give the measured amounts (20 mg/day). The monoterpenes also were tested for inhibition individually at the 10 log equivalent rate. Usually, one test was performed for a given semiochemical comparison, with a test usually conducted from 30 min to 1 hr or until the sprayer syringe was spent (ca. 100 min). Because of the continuous trap rotation, the population density of flying beetles is expected to be homogeneous for both treatments. Thus, the paired control and treatment were compared with a chi-square goodness of fit test to an expected catch if there were no differences based on the average for both traps.

RESULTS

The piezoelectric sprayer in one trap and the standard glass/plastic dispensers in the other trap of the rotating pair, releasing comparable amounts of pheromone components, caught similar numbers of *P. bidentatus* (male–female, 34 : 94 vs. 33 : 76, respectively, $P = 0.22$, chi square). The catching ability of both traps in the pair appeared balanced since placement of standard dispensers

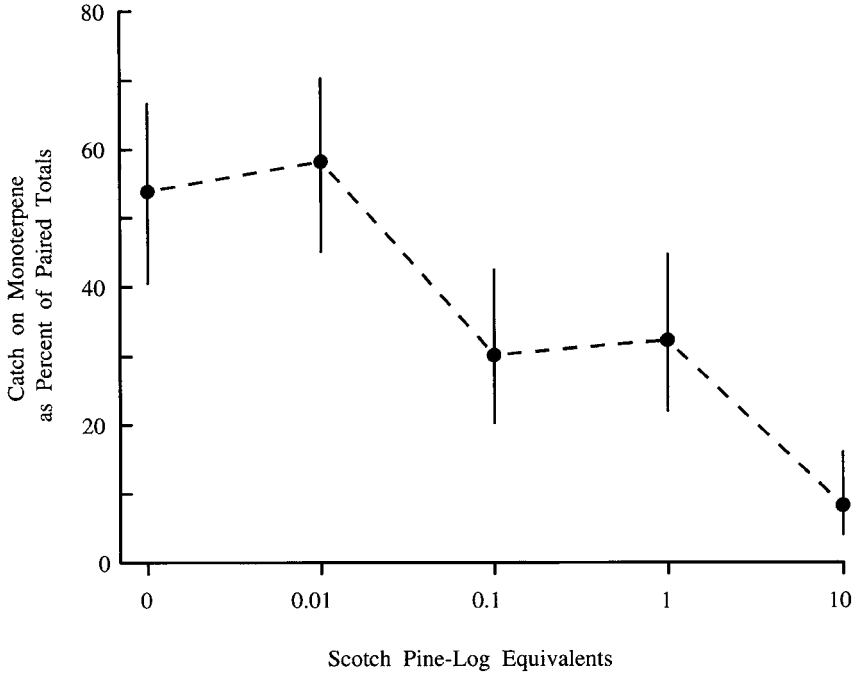

FIG. 4. Inhibition of *P. bidentatus* response to pheromone components (*cis*-verbenol and grandisol) by increasing release rates of a mixture of Scotch pine monoterpenes [(−)-α-pinene, (+)-α-pinene, (+)-3-carene, and terpinolene] each released at 10 μg/min in hexane with the piezoelectric sprayer (1.0 log-equivalent rate). The pheromone components were released from glass/plastic tubes in both traps of the pair rotated at 2 revolutions per hour. Error bars represent 95% binomial confidence limits for the monoterpene-releasing trap proportion based on the total paired catch of the rotor traps (chi square).

in both traps resulted in similar numbers caught (Figure 5; $P = 0.83$, not significantly different). A comparison of one unbaited trap and one with standard dispensers releasing aggregation pheromone showed that beetles oriented toward the pheromone trap with little interference by the unbaited trap (13 : 24 vs. 0; $P < 0.001$). Hexane atomized from the sprayer in one trap apparently had no effect on the response to pheromone from standard dispensers as the catches were similar (Figure 5; $P = 0.58$, not significantly different).

The sprayer was used to increase the release rate of a mixture of monoterpenes (+)- and (−)-α-pinene, (+)-3-carene, and terpinolene from 0.1 to 100 μg/min, which is equivalent to natural rates of release from Scotch pine logs from 0.01 to 10 log-equivalents, respectively. A significant decrease in attraction to aggregation pheromone components was found beginning at the 0.1 log-equiv-

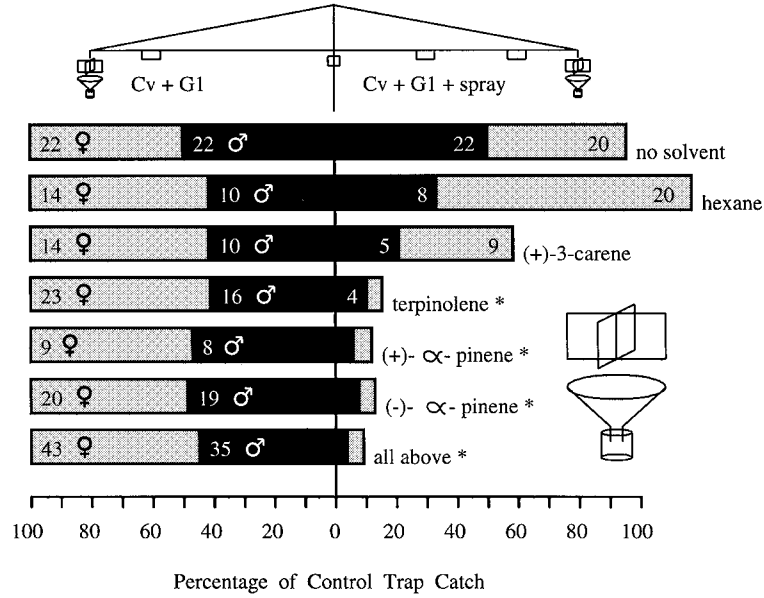

FIG. 5. Reduction of attraction of *P. bidentatus* to aggregation pheromone components (cV = *cis*-verbenol and G1 = grandisol) by host tree monoterpenes released in hexane by the piezoelectric sprayer in a trap rotating at 2 revolutions per hour. The pheromone components were released from glass/plastic tubes in both traps of the pair while the monoterpenes were released from the sprayer in one trap. Asterisks indicate a significant difference between trap baits of a pair at $P < 0.001$ (chi square). Release rates and details given in text.

alent, or 1 μg/min, release of each of the monoterpenes (Figure 4). Individual monoterpenes were also tested at 100 μg/min release (10 log-equivalents) to see if they inhibited attraction of *P. bidentatus* to the standard dispensers with aggregation pheromone (Figure 5). All of the tested monoterpenes reduced responses (Figure 5); however, the reduction by (+)-3-carene was not statistically significant ($P = 0.1$) with the numbers caught (Figure 5).

DISCUSSION

The piezoelectric sprayer dispensed aggregation pheromone components at a constant rate that attracted *P. bidentatus* males and females in numbers that were comparable to the same components released neat at equivalent rates. Although the rates were not identical, it would be expected that under uniform conditions the trap catches would not significantly differ unless releases from

the traps in a paired differed significantly (2–5 times). This is because significant differences in trap catches or behavioral responses are usually not observed unless there is a difference in release rates over an order of magnitude (Byers et al., 1988; El-Sayed, unpublished data), as is found in the release rates of monoterpenes over several orders of magnitude in our study (Figure 4).

Browne et al. (1974) devised a delivery system for releasing semiochemicals based on a spring-powered chart drive motor that depressed a plunger through a microliter syringe. The amount of material released could be changed by simply diluting the stock solutions. The major problem with this device, however, was that globules of solvent with semiochemicals might build up at the syringe tip. Furthermore, the solvent evaporates faster than the less volatile semiochemicals in a mixture, thereby concentrating them at different rates over time and causing increasing release rates of each during an experiment. Still another problem is that mixtures of semiochemicals compete for the vapor pressure, thus producing complicated effects on their release rates (Byers, 1988). Temperature and its potential changes during a field experiment will affect the volatilities of various semiochemicals and solvents differently, further confounding the even and precise release rates desired. These problems are avoided with the piezoelectric sprayer because each semiochemical is expressed to the atmosphere in the exact ratio of its concentration in solution.

Byers (1988) discussed dispenser technologies that use wicks, rubber septa, plastic bags, and "test-tubes." Rubber septa, zeolites, and other absorbent materials have release curves of semiochemicals that decline exponentially with time. Wicks have inexact surface areas, and there is the problem of differences in the elution of the solvent and the semiochemicals. Semipermeable plastic bags give constant rates that ought to vary with the dilution, but the release probably varies with the polarity of the solvent as well as the type of plastic used. The differences in elution of the solvent and semiochemicals would tend to change the ratios and release rates over time. Test-tube-type dispensers with dilutions of semiochemicals based on the diffusion–dilution method (Byers, 1988) give nearly constant rates for fairly long time periods, but eventually these tubes also show differences in elution of solvent and semiochemicals that result in a change in release rates of semiochemicals with time (usually an increase). With the piezoelectric sprayer, the ratio of semiochemical to solvent remains constant, and there is no difference in elution at the spray tip if the solvent is properly atomized. The piezoelectric sprayer can be made to dispense semiochemicals over longer periods by means of larger reservoirs and continuous pumping.

The release of either enantiomer of α-pinene, and terpinolene, alone or in combination at 100 μg/min, or 10-log equivalents, strongly inhibited attraction of *P. bidentatus* to its aggregation pheromone components, grandisol and *cis*-verbenol. Beetles could be seen in the evening orienting toward the aggregation pheromone but then becoming disoriented when within about 0.5–1 m from the

monoterpene release. It might be surprising that host Scotch pine monoterpenes are strongly inhibitory at rates similar to those from natural substrates. However, this bark beetle does not attack living trees that can produce significant amounts resin, or even healthy limbs, but rather colonizes diseased and dying limbs and small trees (Lekander et al., 1977). The avoidance of monoterpenes from resin exuding from Scotch pines would enable flying *P. bidentatus* to save time and energy while locating parts of the tree suitable for colonization. Some of the monoterpenes, e.g., α-pinene, are also found in Norway spruce, *Picea abies*, which is avoided in nature (Byers, unpublished). The bark beetle also avoids volatiles from Scotch pine needles or bark, Norway spruce needles or bark, and birch (*Betula pendula*) leaves or bark (Byers, unpublished).

Other bark beetles are known to avoid host monoterpenes. A plastic tube releasing GC effluents with monoterpenes from frass of male *I. paraconfusus* feeding in ponderosa pine caused walking females of this species to turn away from the effluents (Pitman et al., 1966). An attractive component (probably ipsenol), when eluting, caused a positive taxis toward the tube. The specific monoterpenes were not identified precisely but included one or more of the following: α-pinene, myrcene, β-pinene, 3-carene, and limonene (Pitman et al., 1966).

Bordasch and Berryman (1977) reported that the fir engraver, *Scolytus ventralis* LeC., was repelled by resin vapors, monoterpene fractions, and α-pinene from grand fir, *Abies grandis* (Dougl.) Lindl. On the other hand, Rudinsky et al. (1971) reported that the European spruce bark beetle, *Ips typographus* (L.), is attracted to α-pinene, β-pinene, and limonene (rates unknown) as compared to camphene (since there was no control). Later studies implied that the attraction by host monoterpenes must be rather weak since freshly cut Norway spruce did not attract *I. typographis* initially, but after storage some beetles were attracted (Lindelöw et al., 1992). In other field studies, traps with freshly cut logs or bark chips did not catch *I. typographus* (Byers, unpublished). In addition, volatiles (monoterpenes) from freshly cut host logs did not synergize attraction to pheromone components of *I. typographus* (Schlyter et al., 1987). In contrast, Reddemann and Schopf (1996) found that the attraction of *I. typographus* to aggregation pheromone is enhanced by large amounts (2 ml/dispenser) of (−)-α-pinene and (+)-limonene but decreased by (+)-α-pinene and (−)-β-pinene. (+)-α-Pinene reduced trap catch to only 6%. However, these results can be questioned if α-pinene oxidized to verbenone (an inhibitor) (Bakke, 1981) or to *cis*-verbenol (an aggregation pheromone component) (Bakke et al., 1977).

In the case of North American spruce beetles, *Dendroctonus rufipennis* (Kirby), Werner (1995) found that limonene, 4-allylanisole, myrcene, and β-phellandrene inhibited the response to frontalin, an aggregation pheromone component, while in the eastern larch beetle, *Dendroctonus simplex* LeC., myrcene and limonene inhibited response to seudenol, an aggregation pheromone compo-

nent. As mentioned earlier, many bark beetles are known to be attracted directly by monoterpenes (Byers et al., 1985; Byers, 1989, 1992; Phillips et al., 1988) or they synergize response to aggregation pheromones (Bedard et al., 1969; Werner, 1972; Rudinsky et al., 1972; Byers et al., 1988; Reddemann and Schopf, 1996). These diverse behaviors indicate a need for further research into the roles of host volatiles, especially monoterpenes, in the chemical ecology of bark beetles.

The piezoelectric sprayer should prove useful in many kinds of studies where precise quantities of semiochemicals need to be released in the lab or field. The piezoelectric sprayer can be constructed from a kit, including electronic components and the pump, obtainable from the first author.

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