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Plant volatiles are behavioral cues for adult females of the gall wasp *Antistrophus rufus*

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Summary. The purpose of this study was to identify plant volatiles that provide host location cues for adult females of the gall wasp *Antistrophus rufus* Gillette (Hymenoptera: Cynipidae). Larvae of this species inhabit flowering stems of the prairie perennial *Silphium laciniatum* L. (Asteraceae). Adult females responded to volatile compounds emitted by stems of *S. laciniatum* in field olfactometer bioassays. Plant volatiles were monoterpenes, including, in descending order of abundance, racemic α- and β-pinene (~50 % "+" enantiomer for both), (+)-limonene, (−)-camphene, and β-myrcene. In laboratory bioassays, females responded to aeration extracts of plant stems, the full blend of synthetic monoterpenes, and the four-component blend of α-pinene, β-pinene, (+)-limonene, and (−)-camphene. This monoterpene blend apparently serves as an olfactory cue for host plant location for female *A. rufus* and is the first such cue to be reported for a cynipid gall wasp. Species-specific ratios of plant monoterpenes may provide cues for gall wasp females to distinguish between plant species and choose appropriate hosts for oviposition. The olfactometer and bioassay techniques developed for this research may be useful for field bioassays of other types of minute arthropods.

Key words. Insecta – Hymenoptera – Cynipidae – Asteraceae – *Silphium laciniatum* – prairie – plant volatile – monoterpene – plant secondary compound

Introduction

Cynipid gall wasps (Hymenoptera: Cynipidae) are among the most diverse of gall-inducing arthropods (Abrahamson & Weis 1987; Ronquist & Liljeblad 2001). They are often ubiquitous and common insects, but nevertheless overlooked because of their minute size and endophytic niche. Little is known of their chemical ecology. The larvae may manipulate the physiology of their host plants, inducing the formation of nutritive tissues that are low in plant secondary compounds (Stone & Schönrogge 2003). Changes in host plant chemistry associated with galls provide cues for mate location by adult males (Tooker *et al*. 2002; Tooker & Hanks 2004c). The adult females apparently induce the formation of galls in plants by injecting compounds from the salivary glands, although the physiological mechanism of induction is unknown (Askew 1984; Stone *et al.* 2002). Plant tissues are vulnerable to gall induction for a limited time (Dreger-Jauffert & Shorthouse 1992; Stone *et al*. 2002), presumably necessitating efficient location of host plants by female gall wasps. The chemical basis of host plant location in cynipids apparently has yet to be investigated.

The purpose of our study was to identify plant volatiles that are cues for host location by adult females of the gall wasp *Antistrophus rufus* Gillette (Hymenoptera: Cynipidae). This species is a member of a complex of apparently monophagous species that inhabit the stems of herbaceous perennials in the genus *Silphium* (Asteraceae) that are endemic to tallgrass prairies of the Midwestern United States (for biology of *A. rufus*, see Tooker *et al.* 2002, 2004; Tooker & Hanks 2004a, b). Larvae of *A. rufus* feed during summer within galls in the pith and cambium of flowering stems of *Silphium laciniatum* L. The stems senesce in fall, and gall wasp larvae overwinter in the dead stems (Tooker & Hanks 2004b). Adult *A. rufus* (~3 mm long) emerge in spring, and females mate immediately upon emergence and disperse, primarily by walking and short, hopping flights (Tooker & Hanks 2004b). They encounter a diverse plant community, including other species of *Silphium*, but oviposit only in developing stems of *S. laciniatum*. We studied the chemical basis of host plant location in *A. rufus* by conducting Y-tube olfactometry bioassays to test the response of females to volatiles emitted by bolting stems of *S. laciniatum* in the field, we identified volatiles emitted by plants, and confirmed activity of synthetic blends of compounds with olfactometry bioassays in the laboratory. The olfactometer and bioassay techniques developed for this research may be useful for field bioassays of other types of minute arthropods.

Materials and methods

Olfactometry Methodology

Y-tube olfactometry is an effective bioassay technique for *Antistrophus* species because adults are relatively sedentary and

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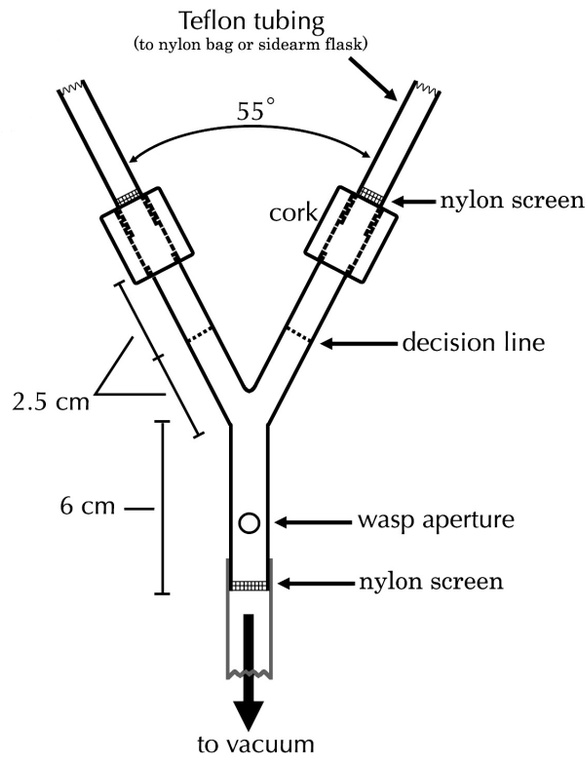

Fig. 1 Schematic of glass Y-tube olfactometer used to test response of adult gall wasps to volatiles of plants, aeration extracts, and synthetic compounds (see Materials and methods for definitions of terms)

respond to attractants by walking (Tooker *et al*. 2002; Tooker & Hanks 2004b, 2004c), obviating a larger wind tunnel to evaluate flight response. The minute size of adult gall wasps, however, necessitated a Y-tube apparatus of small dimensions. We created this apparatus by modifying a "U"-shaped glass tubing connector (no. 45030R-14, Kimble/Kontes, Vineland, NJ) to a "Y" shape by expanding the volume at the arm junction and sharpening the angle between the arms (Fig. 1). This modification greatly improved the linearity of airflow through the connector as indicated by vapor from dry ice. Wasps were introduced into the Y-tube through a ~4-mm diameter aperture in the main tube that was then sealed with a no. 3/0 cork. Air was drawn through the apparatus (~1.3 l/min) with a 1 hp vacuum cleaner on a variable power supply. In the field, the vacuum was run on a power inverter connected to a car battery.

Individual female wasps responded to an odor source by walking upwind 2.5 cm up an arm of the Y-tube and crossing the "decision line" (Fig. 1) and remaining beyond that line for at least 30 sec. Wasps not reaching a decision line within five minutes were recorded as "no response". We repeated this bioassay until there were at least 20 responding wasps, switching treatments between arms of the Y-tube every five trials to control for location effects, rinsing the tube with acetone, and allowing it to air dry. We compared percentages of wasps responding to treatments with the X^2 goodness-of-fit test (Sokal & Rohlf 1995).

We reared adult wasps for bioassays from *S. laciniatum* stems that were divided into ~20 cm sections and placed in screen rearing cages in an unheated outbuilding in Urbana, IL. Stems had been collected in March 2002 from unmanaged prairie sites in central Illinois (Ludlow Railroad Prairie, Ford Co., N 40° 24.33, W 88° 07.76, and Fithian Railroad Prairie, Vermilion Co., N 40° 06.78, W 87° 54.10). Adult wasps were held at 15 °C in cardboard containers with water and streaks of honey for food, and those used in bioassays were no more than five days old and were active under ambient laboratory conditions.

Bioassays of Plant Volatiles

To determine whether female wasps were attracted to volatile chemicals produced by host plants, we conducted olfactometry bioassays in a common garden plot. The plot had been established in Spring 2000 at the Landscape Horticulture Research Center (N 40° 05.43, W 88° 13.04) on the campus of the University of Illinois at Urbana-Champaign. We planted 30 bare root plants of *S. laciniatum* with two other species of *Silphium* (all plants 4-5 yrs old; Missouri Wildflower Nursery, Jefferson City, Missouri) one meter apart in a 30 × 3 array with position assigned randomly. We conducted bioassays on 28 and 29 May and 4 June 2002 from 10:00-14:00 hr. Weather conditions ranged from cloudy to partly sunny with air temperatures ~22 °C on all three days.

Plant volatiles were sampled by enclosing individual bolting stems of *S. laciniatum* (15-30 cm tall) in a nylon oven cooking bag (Reynolds®, Richmond, VA) supported by a cylindrical wire cage (~30 cm tall, 20 cm in diameter). The bag was connected to one arm of the Y-tube with Teflon® tubing and entering air was purified with activated charcoal. An empty oven bag with a charcoal scrubber and containing a wire cage and 5 ml of water was attached to the other arm of the Y-tube as a blank control. We used three different plants as sources of volatiles in these field bioassays.

We trapped volatile chemicals emitted by stems, during the same periods that we conducted bioassays, by drawing air (~1.3 l/min) from bagged stems through a 0.2 g column of the polymeric adsorbent SuperQ® (Alltech Industries, Deerfield, IL) for 15 min periods. We sampled volatiles from ten plants in the common garden, including the three that had been used in field bioassays (above). As a control, we also collected volatiles from empty oven bags (N = 3). We eluted the adsorbent with 2 ml of methylene chloride to yield aeration extracts for bioassays and analysis by coupled gas chromatography-mass spectrometry (see below).

We tested the response of female gall wasps to aeration extracts from five of the ten study plants in laboratory bioassays. The Y-tube olfactometer was modified by attaching two 25-ml glass sidearm flasks to the arms with Teflon® tubing. Air entering flasks was purified with activated charcoal. Emitters in the flasks were cotton wicks (Patterson Dental, St. Paul, MN) treated with 200 µl of extract or solvent as a control. Wicks were recharged every fifteen minutes, and solvent allowed to evaporate before bioassays. Both flasks also contained 200 µl of water to increase humidity. Laboratory bioassays were conducted in a windowless room under fluorescent lights in June and July 2002 and June 2003.

Identification of Volatile Attractants and Bioassays of Synthetic Blends

Volatile plant compounds in aeration extracts were identified by electron ionization-mass spectrometry with a Hewlett-Packard® 5973 mass spectrometer interfaced to a HP 6890 gas chromatograph equipped with HP-5MS (J&W Scientific, Folsom, CA) capillary column (30 m × 0.25 mm × 0.25 µm film thickness) in splitless mode with helium the carrier gas. The GC temperature was held at 38 °C for four minutes then ramped to 90 °C at 2 °C/min. Injector temperature was 250 °C and transfer line temperature was 280 °C. Plasticizer contaminants released by oven bags eluted well after plant volatiles. We confirmed identification of compounds by comparing retention times and spectra with those of synthetic standards (Sigma-Aldrich Chemical Corp., St. Louis, MO). Ratios of enantiomers [+:−] for α- and β-pinene in crude extracts (see Results) approximated ~50:50 (see Tooker *et al*. 2002), camphene was 100 % (−) enantiomer, and limonene was 100 % (+) enantiomer (J.F. Tooker, W.A. König, L.M. Hanks, unpublished data). Purity of standards: α-pinene (both enantiomers 98 %), (−)-camphene (80 %), α-pinene (both enantiomers >97 %), β-myrcene (90 %), (+)-limonene (97 %).

We tested the response of female wasps to synthetic blends of compounds in the Y-tube olfactometer by the same methods used

Table 1 Response of adult *Antistrophus rufus* females to volatiles of the host plant *Silphium laciniatum* and blends of synthetic standards (see text). See Materials and methods for definition of "Response". Significance levels of χ^2 test indicated by "ns" ($P > 0.05$), "*" ($P < 0.05$), and "**" ($P < 0.001$)

Source of volatiles	N	% Response to volatiles	% Response to control[a]	χ^2 statistic
Living plants[a]	20	85	15	9.8^{**}
Extract of plant volatile aeration	29	72.4	27.6	5.8^{*}
Full blend of synthetic compounds (α-pinene, (–)-camphene, β-pinene, β-myrcene, and (+)-limonene)	41	68.3	31.7	5.5^{*}
Full blend minus β-myrcene	20	90	10	12.8^{**}
Full blend minus β-myrcene and (–)-camphene	30	50	50	0^{ns}
Full blend minus β-myrcene and (+)-limonene	23	56.5	43.5	0.4^{ns}
Full blend minus β-myrcene and β-pinene	35	54.3	45.7	0.3^{ns}
Full blend minus β-myrcene and α-pinene	24	50	50	0^{ns}
Extract of plant volatile aeration[b]	43	76.7	23.3	12.3^{**}

[a]Control in bioassays of living plants was water, while controls in all other bioassays was pure solvent
[b]Bioassays of crude extract repeated during testing of synthetic blends to confirm that wasps were responsive

to bioassay aeration extracts (above). We adjusted the full blend of compounds and enantiomers, including racemic α-pinene, (–)-camphene, racemic β-pinene, β-myrcene, (+)-limonene (see Results) to produce ratios in headspace samples that approximated those of aeration extracts (quantified by sampling with SuperQ®). After confirming activity of the full blend (see Results), we identified active components by sequentially eliminating the least abundant compounds from the blends, starting with β-myrcene, and bioassaying each new blend until activity was lost. We then added the most recently omitted compound to the blend and proceeded with a subtractive scheme, individually eliminating each remaining compounds in random order and testing activity. We repeatedly confirmed that wasps were capable of responding to volatiles during these bioassays by testing their response to the aeration extract.

Results and discussion

Bioassays of Plant Volatiles

In field olfactometry bioassays, female gall wasps responded significantly to volatiles emanating from stems of host plants (Table 1). Most of the responding wasps walked directly up the appropriate arm of the Y-tube. In laboratory olfactometry bioassays, female *A. rufus* responded significantly to extracts of plant volatile aerations (Table 1).

Identification of Volatile Attractants and Bioassays of Synthetic Blends

The only compounds that were present consistently in extracts of plant aerations were monoterpenes, including racemic α-pinene, (–)-camphene, racemic β-pinene, β-myrcene, and (+)-limonene. The dominant compound was α-pinene, averaging of 0.13 ± 0.08 ng/µl (mean ± 1 SD N = 10 plants) in elutions of 15-min volatile collections. Amounts of β-pinene, (+)-limonene, (–)-camphene, and β-myrcene averaged 18 ± 6, 12 ± 11, 6 ± 5, and 5 ± 4 % of the α-pinene peak, respectively. None of these compounds were detected in aerations of empty oven bags.

Laboratory Bioassays

Wasps showed a significant response to the full synthetic blend of five monoterpenes, and there was no loss in activity when myrcene was subtracted (Table 1). Activity was lost when (–)-camphene was subtracted, however, and none of the three-component blends were active (Table 1). Although β-Myrcene had no activity in the full blend, the possibility remains that it might have contributed to activity when other components were eliminated. Nevertheless, the blend of four components showed activity similar to the plant extracts, supporting the notion that β-myrcene is not an important component.

The active monoterpene blend (racemic α-pinene, (–)-camphene, racemic β-pinene, and (+)-limonene) is apparently the first plant attractant to be identified for the family Cynipidae. Disparate taxa of insect herbivores, including bark beetles, dipteran leaf miners, and pyralid moths, respond to various combinations of the same monoterpenes (e.g. Byers *et al*. 1990; Ramachandran *et al*. 1990; Metcalf & Metcalf 1992; Zhao & Kang 2002). Male *A. rufus* use residues of α-pinene, camphene, β-pinene in dead stems of *S. laciniatum* as cues for mate location (Tooker *et al*. 2002; Tooker & Hanks 2004c). These monoterpenes are produced by many plant species (see Mabry & Gill 1979; Metcalf & Metcalf 1992), including other prairie plants (e.g. Mazza & Cottrell 1999), and their ubiquity would seem to confound location of host plants by *A. rufus*. The same monoterpenes, but in different ratios, are produced by *Silphium terebinthinaceum* Jacquin which co-occurs with *S. laciniatum*, but is not used as a host by *A. rufus* (Tooker *et al*. 2002, 2004). In preliminary field bioassays, female *A. rufus* did not respond to volatiles produced by *S. terebinthinaceum*, but there was a response by females of a congeneric gall wasp whose larvae inhabit stems of *S. terebinthinaceum*, *Antistrophus meganae* Tooker & Hanks (unpublished data). Species-specific ratios of plant monoterpenes may provide

cues for gall wasp females to distinguish between plant species and choose appropriate hosts for oviposition.

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