J Chem Ecol (2006) 32:2695–2708
DOI 10.1007/s10886-006-9193-x

Behaviorally Active Green Leaf Volatiles for Monitoring the Leaf Beetle, *Diorhabda elongata*, a Biocontrol Agent of Saltcedar, *Tamarix* spp.

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Received: 21 March 2006 / Revised: 9 June 2006 / Accepted: 18 August 2006 /
Published online: 28 November 2006

Abstract Biological activity and chemistry of host plant volatiles were investigated for *Diorhabda elongata*, Brullé (Coleoptera: Chrysomelidae), a biological control agent for the invasive tree, saltcedar (*Tamarix* spp., Tamaricaceae). Gas chromatographic–electroantennographic detection (GC-EAD) analysis of volatiles collected from adult *D. elongata* feeding on saltcedar foliage or from saltcedar foliage alone showed 15 antennally active compounds. These compounds were more abundant in collections from beetle-infested foliage. Antennally active compounds were identified by GC–mass spectrometry (MS) and confirmed with authentic standards. The emissions of the most abundant GC-EAD-active compounds, green leaf volatiles (GLV), were quantitated by GC-MS. A blend of four GLV compounds, mimicking the natural blend ratio, was highly attractive to male and female *D. elongata* in the field, and a combination of GLV and male-produced aggregation pheromone attracted significantly greater numbers of *D. elongata* than did either bait alone. A preliminary experiment with a blend of seven additional GC-EAD-active saltcedar volatiles did not show any behavioral activity. The combination of the pheromone and the green leaf

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odor blend could be a useful attractant in detecting the presence of the biocontrol agent, *D. elongata*, in stands of saltcedar newly colonized by the beetle.

Keywords Biological control · Chrysomelidae · Coleoptera · *Diorhabda elongata* · Electrophysiology · Field evaluation · Green leaf volatiles · Host-odor attractants · Saltcedar · *Tamarix ramosissima*

Introduction

The leaf beetle *Diorhabda elongata* Brullé (Coleoptera: Chrysomelidae) has been successfully introduced into the United States from China as a biocontrol agent for saltcedar (*Tamarix* spp., Tamaricaceae) (DeLoach et al., 2003, 2004). Saltcedars were originally imported from Eurasia as ornamentals and for erosion control of streambanks and river channels. However, these fast-growing shrubs or small trees are invasive and have become a major component of western riparian ecosystems (Friedman et al., 2005), causing serious environmental and economic damages (Zavaleta, 2000; Shafroth et al., 2005).

In an earlier study (Cossé et al., 2005), we identified the male-produced aggregation pheromone of *D. elongata* as a 1:1 blend of two seven-carbon compounds, (2*E*,4*Z*)-2,4-heptadienal (2*E*, 4*Z*-7:Ald) and (2*E*,4*Z*)-2,4-heptadien-1-ol (2*E*, 4*Z*-7:OH) and demonstrated attraction to the synthetic compounds in the field. The pheromone was identified in collections of volatiles from beetle-infested saltcedar by using electrophysiological analysis of these collections as a key tool. We noted that the antennae of male and female beetles responded to a number of compounds from the host foliage in addition to the pheromone components. Here, we report the identification and quantification of several saltcedar-emitted compounds and their electrophysiological and field activity for *D. elongata* adults.

Methods and Materials

Insects

Adult *D. elongata* used in this study were collected from a field population at Lovelock, NV, USA, or obtained from a colony maintained by one of us (DWB) at the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Exotic and Invasive Weed Research Unit, Albany, CA, USA. The beetles originated from sites near Fukang, in the Xinjiang province of northwestern China (DeLoach et al., 2003; Lewis et al., 2003). Upon arrival at the USDA-ARS National Center for Agriculture Utilization Research (NCAUR) in Peoria, IL, USA, adult beetles were kept at 25°C under a 17-hr light/7-hr dark photoperiod. *Tamarix ramosissima* Ledebour was grown year-round in a greenhouse facility at NCAUR to provide food for the beetles. The beetles were used for electrophysiological tests and also to create feeding damage on saltcedar foliage.

Collections of Volatiles

Two types of collection techniques for volatiles were used: solid-phase microextraction (SPME) and Super-Q/activated charcoal filters. The SPME collections allowed for rapid

detection of changes in saltcedar volatile profiles, whereas the Super-Q/charcoal collections served as the source of saltcedar volatiles for compound quantification and gas chromatographic–electroantennogram detection (GC-EAD) analysis.

Volatile saltcedar compounds, emitted during the first 30 min of experiments and directly after beetles were placed on the saltcedar foliage, were collected simultaneously on polydimethylsiloxane/divinylbenzene (65 μm) fibers of SPME needles (Supelco, Bellefonte, PA, USA) and on collection filters (Cossé et al., 2002) containing a mixture (40 mg, 1:1) of Super-Q porous polymer (80–100 mesh, Alltech, Deerfield, IL, USA) and activated charcoal (Darco G-60, EM Science, Cherry Hill, NJ, USA).

Collections of volatiles were made from saltcedar foliage (branch approximately 75 cm long), either infested with beetles (about 200, both sexes but predominately males) or without beetles. In either case, the foliage was placed in a vertical glass tube (5 cm inside diameter [i.d.]×65 cm) with the freshly cut end protruding from the lower opening into a beaker of water. A piece of fine mesh nylon cloth with a small central hole for the saltcedar stem was secured around the lower end of the tube to keep the beetles contained. In some cases, collections of volatiles were made specifically from the flowering parts on the foliage. The collections were made at 25°C under a 17-hr light/7-hr dark photoperiod.

Using several reducing glass adapters, the top of the tube (female 55/50 ground glass joint) was fitted with an adapter with a male 24/40 joint on one end and a threaded fitting with an air-tight "O" ring seal on the other (Ace Glass, Vineland, NJ, USA, #5028-30). The threaded airtight fitting held one leg of a T-shaped glass tube (0.5 cm outside diameter). A second leg received the SPME needle through a rubber septum, and the last leg was connected to the Super-Q/charcoal filter assembly, which was, in turn, connected to a vacuum line (for diagram, see Bartelt and Zilkowski, 1999).

With the vacuum line, air was drawn through the collection tube and past the SPME fiber at 25 ml/min during the SPME sampling. After 30 min, the SPME needle was removed and the flow rate adjusted to 1 l/min for the remainder of the 24-hr collection period. Foliage was replaced on a daily basis and any dead beetles in the collector were replaced with fresh ones. Collected volatiles were rinsed from the filters into vials using methylene chloride (400 μl) after every 24 hr. 1-Octanol (10 μl of a methylene chloride solution containing 250 ng/μl) was added to each vial as a quantitative internal standard.

It was confirmed that the 6-carbon and larger compounds did not break through the Super-Q/charcoal filters by placing a second filter in series and analyzing both separately. However, collection filters of Super-Q or 60–80 mesh-activated charcoal (GraphTrap-GB, Alltech) alone did not trap 6-carbon compounds quantitatively, and the very fine powdered Darco G-60 charcoal created excessive airflow resistance in the filters. The mixture of Super-Q and Darco G-60 charcoal resolved both problems.

Quantification

The amounts of (*E*)-2-hexenal (2*E*-6:Ald), (*Z*)-3-hexenal (3*Z*-6:Ald), (*Z*)-3-hexen-1-ol (3*Z*-6:OH), and (*Z*)-3-hexenyl acetate (3*Z*-6:OAc) in the collections of volatiles were quantified by coupled GC–mass spectrometry (GC-MS) using selected ion monitoring (SIM) mode. Two ions were monitored for each compound, *m/z 83* and 98 for 2*E*-6:Ald, *m/z 82* and 100 for 3*Z*-6:OH, *m/z 69* and 98 for 3*Z*-6:Ald, and *m/z 67* and 82 for 3*Z*-6:OAc. The selected ions for the internal standard, 1-octanol, were *m/z 70* and 84. Quantification was based on the italicized ions of the above-mentioned ion pairs, whereas the second ions served as qualifiers (proper ion ratios would support compound identity and purity in the GC peaks).

Serially diluted solutions of all the synthetic compounds and internal standard (0.01–30 ng/μl) were first analyzed by GC with flame-ionization detection (GC-FID) to verify proper concentrations and further analyzed by GC-MS in SIM mode to obtain linear calibration curves (log dose vs. log abundance). The linear calibration curves (average of three replicate injections for each concentration) served as the basis of the quantification. In SIM mode, compounds were still easily detectable at concentrations as low as 10 pg/μl.

Electrophysiology

GC-EAD analyses were made by methods and equipment generally described by Cossé and Bartelt (2000). GC-EAD connections were made by inserting a glass-pipette silver-grounding electrode into the back of an excised beetle head. A second glass-pipette silver-recording probe was placed in contact with the distal end of one antenna. Both pipettes were filled with Beadle–Ephrussi (Ephrussi and Beadle, 1936) saline.

Instrumentation

Volatile collections were analyzed by GC-FID and GC-MS. Samples were injected in splitless mode using Hewlett Packard 6890 (Palo Alto, CA, USA) instruments fitted with 30-m DB-1, DB-5, or DB-Wax capillary columns (0.25 mm i.d., with 1.0-, 0.25-, and 0.5-μm film thickness, respectively, J&W Scientific, Folsom, CA, USA). Temperature programs were from 40 to 280°C at 10°C per minute. Inlet temperatures were maintained at 280°C, and GC-EAD effluent interface from postcolumn splitter was kept at 280°C. For SPME analysis, the GC inlet port was fitted with an SPME-optimized glass liner (Supelco). Mass spectrometry was performed using a Hewlett Packard 5973 instrument (electron impact, 70 eV). The Wiley mass spectral library, with 275,821 spectra, was available on the MS data system (Wiley, 1995).

Chemicals

The two pheromone components, 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH, were synthesized according to Petroski (2003). The purities of the compounds were >95% by GC-FID; by GC-MS, impurities were other geometrical isomers. 3*Z*-6:Ald (50% in triacetin, Bedoukian, Danbury, CT, USA) was obtained in pure form by Kugelrohr distillation (room temperature, 40 mm Hg) and stored (−70°C) as a dilute solution in methylene chloride. *E*,*E*-Farnesene was obtained by gentle reflux (4 hr) of *E*,*E*-farnesol (1 g) with Dowex 50W-X4 cation exchange resin (2 g) in hexane (50 ml), followed by open column chromatography on silver nitrate-coated silica (25% $AgNO_3$, elution solvent 15% 1-hexene in hexane). All other compounds were purchased (Aldrich, Milwaukee, WI, USA) with purities >97%.

Field Lures

Two types of host odor lures were prepared for field testing. The first lure type consisted of four green leaf volatiles (GLV), 3*Z*-6:Ald (4 mg), 2*E*-6:Ald (4 mg), 3*Z*-6:OH (0.8 mg), and 3*Z*-6:OAc (0.6 mg), in mineral oil. The second lure type consisted of seven compounds, heptanal (0.1 mg), octanal (0.25 mg), nonanal (1 mg), *E*,*E*-2,4-heptadienal (0.2 mg), *E*,*E*-2,6-nonadienal, indole (2 mg), and *E*,*E*-farnesene (2 mg), in mineral oil. A bulk solution of the compounds needed for each lure type was prepared in mineral oil (enough of each for

50 lures, in a total volume of 25 ml). Then, aliquots (0.5 ml) of this mixture were placed in 1.5 ml polypropylene micro centrifuge tubes (Bio Plas Inc., San Rafael, CA, USA) together with a piece (1 cm long) of braided cotton roll (Richmond Dental, Charlotte, NC, USA). The emission rates of freshly prepared GLV lures were measured in an incubator, using the general reported methods and equipment reported earlier (Cossé et al., 2005). Specifically, volatiles were collected from lures at 25°C with a 150-ml/min airflow. Volatiles were collected periodically over 2 d, and the release rates of individual compounds were measured by GC-FID using 1-octanol as the quantitative internal standard.

Lures were stored at −20°C until needed. Prior to field deployment, the caps of the tubes were pierced with a single pinhole (1 mm) to allow compound release from the headspace.

Pheromone lures (Cossé et al., 2005) consisted of a 1:1 mixture of 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH (500 μg of each component), released in a nearly 1:1 ratio at 7 μg component^{-1} d^{-1}.

Field Study

Experiments were carried out between May and September 2004 in saltcedar stands along the Humboldt River (40°01′N, 118°31′W) 17 km southwest of Lovelock, NV, USA, where a population of *D. elongata* had been established (DeLoach et al., 2004).

Yellow sticky traps (15.5×30.5 cm, AgriSense, Pontypridd, UK) were attached to branches (oriented vertically) in the upper half of the trees, typically at a height of 2–4 m, and were placed in trees that were at least 10 m apart and were similar in size, foliage density, and in accessibility to beetles flying from downwind. There were always other saltcedar trees near the treatment trees and usually between them as well. The protective paper was removed only from one side of the trap, so that the sticky side was oriented downwind. The traps were set out in midafternoon, when flight activity usually began to intensify, and trap counts were made the following morning. Thus, each replicate lasted 1 d. A wire, wrapped around the bait tube, was used to secure the lure to a trap.

The field experiments were carried out in two types of saltcedar stands. Defoliated saltcedar consisted of saltcedar that had previously experienced complete defoliation and had little or no foliage present during the experiment. For the experiment beginning on May 6, 2004, the stand had experienced defoliation the previous season, and adult beetles had overwintered in the leaf litter beneath the stand. They were newly emerged from overwintering when the experiment was performed. Later in the season, newly defoliated saltcedar stands were the result primarily of heavy infestation with *D. elongata* larvae. The second stand type had little or no previous defoliation; adults were just beginning to move into these stands, whose trees had relatively lush green foliage during the tests.

Three sets of experiments were deployed. The first set was a preliminary assessment of the host odor lures. The two types of host odor lures (four-component and seven-component) were first combined on traps and compared to unbaited control traps in a paired design (N=12), followed by the pairwise comparison of only the four-component GLV lures and unbaited controls (N=6). In addition, there was one comparison of the seven-component lure vs. unbaited control.

The second experiment, deployed in both saltcedar stand types, was a more systematic pairwise comparison of the GLV lures and unbaited controls, throughout the 2004 season, starting on May 6, when the overwintered beetles were becoming active, and finishing on September 14, when all of the beetles had entered reproductive diapause (DeLoach et al., 2004). The first trapping day in both types of saltcedar stands had 18 replications per

treatment followed by six replications per treatment for all other days. Trapped beetles were sexed periodically (Cossé et al., 2005). Sweep net samples were taken on June 28 in both types of saltcedar stands and included both flying beetles and those present on foliage. Captured beetles were counted and sexed.

The third experiment measured the effects of combining pheromone and GLV lures and was set up on August 11, 2004, at least 200 m away from the nearest saltcedar trees. Traps used in this experiment were two-sided yellow sticky panel traps (15.2×30.5 cm, both sides exposed, Seabright Laboratories, Emeryville, CA, USA). Treatments were pheromone lure, GLV lure, pheromone and GLV lures combined, and unbaited control. A randomized complete block design was used (four traps per block), and there were four replicates of each treatment.

Statistical Analyses

Counts of *D. elongata* adults on traps were transformed using $\log(X+1)$ to stabilize variance. The data from the pairwise experiments were analyzed by paired *t*-tests, whereas data from the randomized complete block design were submitted to analysis of variance and means were compared using the least significant difference test.

Results

GC-EAD Analysis of Saltcedar Volatiles

GC-EAD comparisons of volatiles collected by Super-Q/charcoal from beetle-infested saltcedar foliage and foliage alone showed that male and female antennae responded to a variety of compounds (Fig. 1). Compounds that triggered male and female antennal responses were identified as 3*Z*-6:Ald, 2*E*-6:Ald, 3*Z*-6:OH, 2*E*-6:OH, 1-hexanol, heptanal, 3*Z*-6:OAc, octanal, (2*E*,4*E*)-2,4-heptadienal, benzyl alcohol, nonanal, (2*E*,6*Z*)-2,6-nonadienal, indole, and (*E*,*E*)-farnesene. By GC-MS, only trace amounts of the same compounds could be detected in collections of volatiles from uninfested saltcedar, and the antennal activity to these was in most cases inconclusive. More consistent GC-EAD activity from uninfested saltcedar could be obtained from collections of volatiles of crushed saltcedar foliage followed by sample concentration. Additional collections of volatiles from the flowering parts of saltcedar showed a relative abundance of benzyl alcohol, which was easily detected by the antennae of both sexes. Male and female antennae responded also to the two pheromone compounds, 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH, which were present in beetle-infested foliage collections. Identities of GC-EAD-active compounds were verified by GC-MS and GC-EAD analysis of authentic standards.

Collections of Volatiles by SPME

Thirty minutes after placing the beetles on saltcedar foliage, several GLV compounds were already easily collected by SPME and detected by GC-MS (Fig. 2A), whereas these compounds were almost absent in the SPME collections from uninfested saltcedar foliage (Fig. 2B). Compounds that were consistently more abundant in the beetle-infested SPME collections were 3*Z*-6:Ald, 2*E*-6:Ald, 3*Z*-6:OH, 3*Z*-6:OAc (coeluting with compound d in Fig. 2A), nonanal, and decanal. SPME collections from uninfested saltcedar showed only

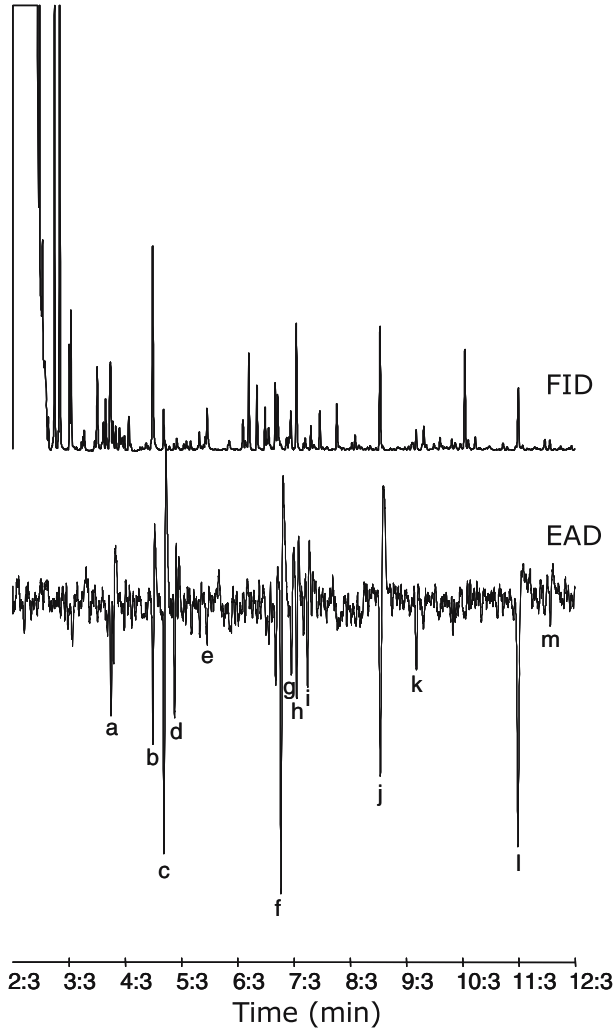

Fig. 1 Simultaneously recorded gas chromatogram with FID and EAD of female *D. elongata* antenna to volatiles collected from male and female *D. elongata* adults feeding on saltcedar. Letters denote antennal activity by: (*Z*)-3-hexenal (a), (*E*)-2-hexenal (b), (*Z*)-3-hexen-1-ol (c), (*E*)-2-hexen-1-ol (d), 1-hexanol (e), (*Z*)-3-hexenyl acetate (f), octanal (g), (2*E*,4*E*)-2,4-heptadienal (h), benzyl alcohol (i), nonanal (j), (2*E*,6*Z*)-2,6-nonadienal (k), indole (l), and (*E*,*E*)-farnesene (m)

trace amounts of 3*Z*-6:Ald, 2*E*-6:Ald, nonanal, and decanal. In addition, SPME collections from beetle-infested saltcedar also showed the presence of the two male-produced pheromone components, 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH. The most abundant and earliest detectable GC-EAD-active compounds in collections of volatiles from beetle-infested saltcedar were selected for further analysis.

Quantification of GLVs from Saltcedar

Quantification of four GLVs, 3*Z*-6:Ald, 2*E*-6:Ald, 3*Z*-6:OH, and 3*Z*-6:OAc, collected from saltcedar, with and without the feeding beetles, revealed that beetle-infested saltcedar foliage emitted about 50 times more of the four compounds than did uninfested foliage (Table 1). Nearly equivalent amounts of each of the four compounds were released by uninfested foliage, whereas proportionally greater amounts of 2*E*-6:Ald and lesser amounts

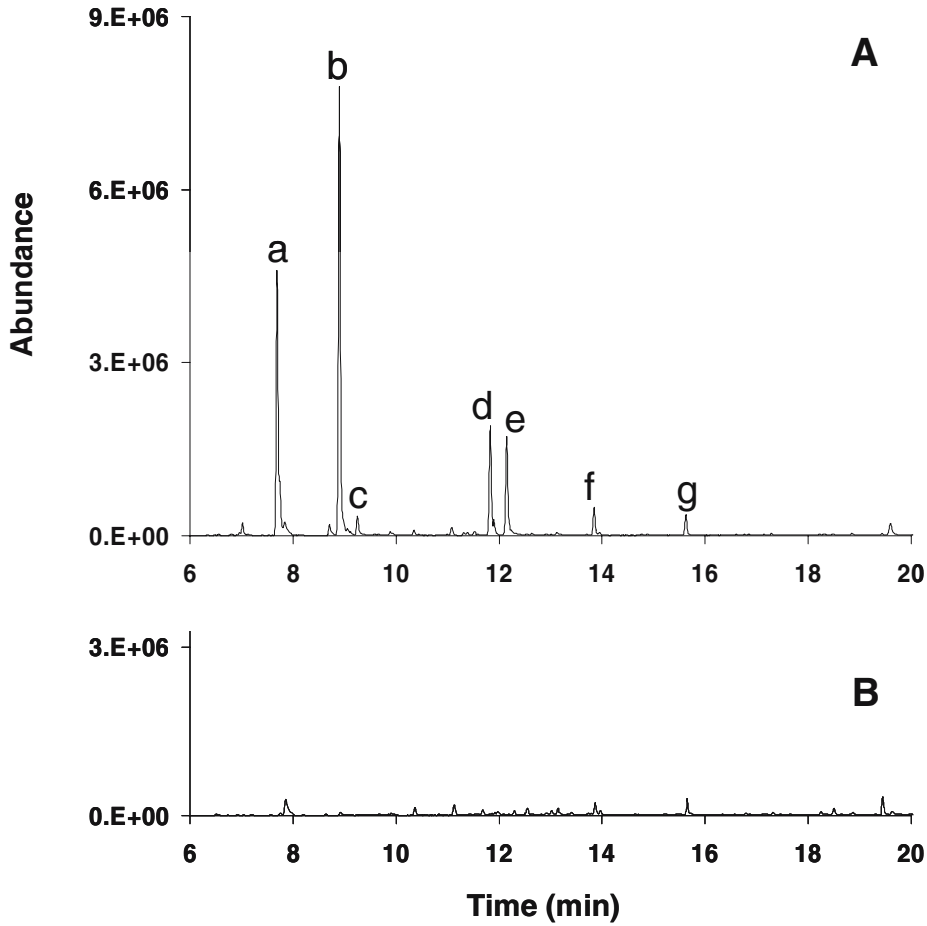

Fig. 2 GC-MS profiles of SPME-collected volatiles during the first 30 min of a 24-hr collection of volatiles from (A) saltcedar foliage infested with feeding adult *D. elongata* and (B) saltcedar foliage alone. Letters above the GC-MS peaks denote the presence of (*Z*)-3-hexenal (a), (*E*)-2-hexenal (b), (*Z*)-3-hexen-1-ol (c), (2*E*,4*Z*)-2,4-heptadienal (d), (2*E*,4*Z*)-2,4-heptadien-1-ol (e), nonanal (f), and decanal (g)

of 3*Z*-6:Ald and 3*Z*-6:OAc were released by infested foliage. Preliminary analysis of Super Q/charcoal collected saltcedar emissions (Cossé et al., 2005) showed that some of the less volatile compounds (2,6-nonadienal, indole, and *E*,*E*-farnesene) could only be detected towards the end of the 24-hr collection period.

Field Lures and Field Study

After 24 hr, the GLV field lures released approximately 30 μg/hr and in a ratio of nearly 32:3:1:1 (2*E*-6:Ald/3*Z*-6:OH/3*Z*-6:Ald/3*Z*-6:OAc; Table 2). Preliminary field trapping experiments captured 314±152 (Mean ± SD, N=12) beetles per trap per day in response to the two host odor baits combined, whereas the unbaited traps caught 32±60 (Mean ± SD, N=12) beetles per trap per day. The GLV lure alone caught 254±94 (Mean ± SD, N=6)

Table 1 Quantities of four GLVs released from saltcedar, *Tamarix ramosissima*, foliage infested with and without adult leaf beetles, *Diorhabda elongata*

Compound[a]	Foliage+200 Beetles		Foliage	
	Mean ± SD (μg/d) (*N*=8)	%	Mean ± SD (μg/d) (*N*=5)	%
3*Z*-6:Ald	1.26±0.57	4.7	0.13±0.02	24.3
2*E*-6:Ald	16.97±4.90	63.1	0.11±0.06	21.3
3*Z*-6:OH	6.17±3.71	23.0	0.13±0.07	24.6
3*Z*-6:OAc	2.48±1.02	9.2	0.15±0.08	29.8
Total	26.87±8.57		0.51±0.17	

[a] For compound abbreviations, see text.

beetles per trap per day, whereas the unbaited traps caught 12±24 (Mean ± SD, *N*=6) beetles per trap per day. A single pair of traps comparing the trap catch of the seven-compound host odor lure (16 beetles per trap per day) with that of the unbaited control (five beetles per trap per day) elicited a much lower response. Therefore, the GLV lure was selected for further field testing.

The second set of field trapping experiments demonstrated the attractiveness of the GLV lures to male and female *D. elongata* throughout the season and in the two types of saltcedar stands (Table 3). Significantly more adults were attracted to traps baited with GLV compared to the control traps during seven different periods in defoliated saltcedar stands. Similar results were obtained in saltcedar stands with abundant foliage during four trapping periods. In both types of saltcedar stands, the response ratio between GLV and control treatments was higher early in the season and slowly declined throughout the season (Table 3). Relatively high numbers of beetles were trapped on May 6 and August 12 in the defoliated saltcedar stands.

Trap counts showed a strong bias towards males in both baited and unbaited traps (Table 3). Similar male bias was found in aerial sweep net samples, collecting 38 males and nine females in areas with foliage and seven males and three females in defoliated areas. The observed sex ratio was consistent between the two saltcedar foliage categories (2×2 contingency table, X^2=0.58, 1 *df*, *P*=0.44; overall, 79% males). Sweep net samples of beetles from saltcedar trees showed a considerably more balanced sex ratio, with 43 males and 26 females from defoliated saltcedars (from 30 plants) and 29 males and 24 females from saltcedars with foliage (from 15 plants). Again, the sex ratio was not significantly different between the two saltcedar stand types (2×2 contingency table, X^2=0.72, 1 *df*, *P*=0.40; overall, 59% males). However, the proportion of males in the aerial sample was significantly higher than in the sample from the trees (after summing over stand type in the

Table 2 Quantities of four GLVs emitted from field lures for *Diorhabda elongata*

Hour	Total μg/hr	Mean ± SD (μg/hr) (*N*=3)							
		3*Z*-6:Ald	%	2*E*-6:Ald	%	3*Z*-6:OH	%	3*Z*-6:OAc	%
1	32.1	0.8±0.2	2.5	27.1±3.9	84.4	3.6±0.5	11.2	0.6±0.1	1.9
5	38.1	1.6±0.5	4.2	31.6±3.6	82.9	4.1±0.4	10.8	0.8±0.01	2.1
24	29.4	1.0±0.7	3.4	25.1±4.5	85.4	2.6±0.4	8.8	0.8±0.2	2.7
48	22.3	0.6±0.2	2.7	20.9±1.8	93.7	2.1±0.3	9.4	0.6±0.1	2.7

For compound abbreviations and applied dosages, see text.

Table 3 Response of *Diorhabda elongata* to sticky traps baited with four GLVs in defoliated and foliated saltcedar, *Tamarix ramosissima*, stands in Lovelock, NV, May–September 2004

Date	Response			GLV/Ctrl[a]	% Male Catch	
	Mean ± SD (Beetles per Trap per Day)					
	GLV	Ctrl	*t*		GLV	Ctrl
Defoliated Saltcedar						
6-May	293.9±135.4	22.1±50.8	12.14**	42.4	–	–
18-June	8.7±6.8	0.8±1.2	3.46*	11.2	–	–
28-June	13.7±13.9	1.0±1.3	4.87*	12.9	96.5	100
21-July	46.6±20.4	5.2±5.0	11.01**	12.1	95.8	73.7
23-July	20.8±12.8	2.7±2.8	7.59**	8.5	89.0	90.5
12-Aug.	156.6±45.3	30.4±15.9	8.76**	6.0	–	–
14-Sept.	60.8±75.5	1.0±0.9	7.99*	43.9	77.3	100
Saltcedar with Foliage						
18-June	34.7±43.2	1.0±2.4	3.54*	7.2	–	–
28-June	11.5±8.8	1.2±1.2	7.24**	10.1	96.6	100
6-July	19.4±20.0	4.1±5.2	3.81*	4.2	–	–
25-July	51.3±59.9	20.2±34.2	4.18*	4.0	92.1	65.4

For bait composition and emission rates, see Table 2.

[a] From the statistical analysis, ratios are based on log-transformed means.

*$P<0.01$.

**$P<0.001$.

above data sets, a 2×2 contingency table for sex vs. aerial/tree sampling gave $X^2=6.82$, 1 *df*, $P<0.01$).

In the third field experiment, adult *D. elongata* were attracted to traps baited with pheromone, GLV, or both, in numbers that were significantly higher than those found on the unbaited control traps (Table 4). There was no significant difference between the numbers of *D. elongata* attracted to traps baited with GLV lures compared to those attracted to traps baited with pheromone lures. The combination of GLV and pheromone lures attracted the highest number of beetles, with approximately six times more *D. elongata* than to GLV lures alone, and approximately four times more *D. elongata* than to pheromone lures alone (Table 4).

Table 4 Response of *Diorhabda elongata* to sticky traps baited with four GLVs (for bait composition and emission rates, see Table 2), a 1:1 mixture of 2*E*,4*Z*-7:ALD and 2*E*,4*Z*-7:OH (pheromone) (for bait composition and emission rates, see text), and a combination of GLV and pheromone in saltcedar, *Tamarix ramosissima*, stands in Lovelock, NV, August 12, 2004

Lure	Response Mean ± SD (beetles per trap per day)[a]
Pheromone+GLV	90.4a
Pheromone	23.7b
GLV	15.9b
Control	3.1c
F statistic	24.28 (*df*=3,12)
P	<0.001

[a] Means followed by the same letter do not differ by least significant difference test, $P>0.01$.

Discussion

In this study, we focused on possible biologically active saltcedar volatiles released during feeding by *D. elongata*. The experimental results demonstrated that at least 15 volatile compounds were antennally active to male and female *D. elongata*. Collections of volatiles from uninfested saltcedar showed relatively small amounts of GC-EAD-active material, but collections from beetle-infested foliage showed that the feeding activity of the beetles significantly increases the amounts of GC-EAD-active saltcedar volatiles released into the air. GLVs (Visser and Avé, 1978; Visser, 1986) especially were released in relative abundance and could be easily detected during the first 30 min after feeding began, whereas relatively lesser amounts could only be detected in the samples collected over a 24-hr period. Numerous studies have demonstrated the quantitative and qualitative differences between volatiles emitted by herbivore-infested plants compared to the volatiles emitted by uninfested plants (for reviews, see Dicke et al., 1998; Dicke and Vet, 1999; Dicke and van Loon, 2000), and several of these studies have shown the role of the identified GC-EAD-active compounds in tritrophic interactions (for reviews, see above). In this study, we focused only on GC-EAD-active material. Qualitative differences in these compounds were not noted between beetle-infested and noninfested plants, but the quantitative differences were dramatic.

The behavioral significance of the identified GLVs was demonstrated in the field. A blend of four of the most abundant, antennally active GLVs, released at compound ratios mimicking the beetle-infested plant release ratios, was highly attractive to male and female *D. elongata* in areas where most of the saltcedar stands were completely defoliated. Furthermore, this synthetic blend was still highly attractive to male (and, to a lesser extent, female) *D. elongata* in areas with an abundance of beetles and saltcedar foliage.

Further evidence for the importance of the GLV blend was demonstrated with the observed synergistic effect when pheromone and GLVs were combined (Table 4). It should be noted that this synergistic effect was demonstrated in previously uninfested saltcedar stands. As such, the combination of the pheromone and the green leaf odor blend could be a useful attractant in detecting the presence of *D. elongata* in newly colonized areas. This would be a useful tool in the saltcedar biocontrol program.

In beetle taxa with aggregation pheromones, pheromone emission occurs at the feeding/breeding site, and host-derived volatiles are potent synergists of the pheromones for some taxa (Borden, 1985; Bartelt, 1999). Only recently has the synergistic interaction of host compounds and pheromones been shown for chrysomelid beetles (Soroka et al., 2005; Tóth et al., 2005).

Field observation of *D. elongata* showed that the population density is not uniformly distributed among the saltcedar bushes. Especially in newly colonized stands of saltcedar, the presence of adult beetles is highly aggregated with, for example, a single plant containing hundreds of feeding beetles being surrounded by many other similar-looking plants without any beetles. In those instances where a small number of beetles landed and started feeding on a previously uninfested saltcedar bush, it was quickly (<30 min) followed by the arrival of numerous other beetles on the same saltcedar branch (personal observations by authors Cossé, Bartelt, and Bean). If the original group of pioneer beetles contained males, one could envision that the arrival of the additional beetles was caused by the release of the male-specific aggregation pheromone, the release of attractive host volatiles as a result of the feeding action, or both stimuli.

Our earlier study dealing with the identification of the male-specific aggregation pheromone of *D. elongata* showed that males feeding on saltcedar foliage released a 1:1 mixture of 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH, and that this mixture attracted significant numbers of males and females to traps placed in saltcedar stands in various stages of

defoliation (Cossé et al., 2005). In this study, the pheromone could be detected within the first 30 min after placing the beetles on the saltcedar foliage in the collector.

Certain chrysomelid beetles, such as *Diabrotica* spp. (for review, see Metcalf and Metcalf, 1992) and *Leptinotarsa decemlineata* (Dickens, 1999, 2000, 2006), are attracted to blends of some of the same GC-EAD-active compounds found in this study. For example, benzyl alcohol and indole, two volatile components associated with the blossoms of *Cucurbita* spp., were both antennally active and attractive to adult *Diabrotica undecimpunctata howardi* and *Diabrotica virgifera virgifera* in field studies (Andersen and Metcalf, 1986; Lampman et al., 1987). Antennae of *L. decemlineata* responded to several volatile potato foliage compounds, including 3*Z*-6:OH, 2*E*-6:OH, 3*Z*-6:OAc, nonanal, decanal, and indole (Dickens, 1999). However, Dickens (2000) showed that some blends containing the GLVs 3*Z*-6:OH and 2*E*-6:OH can also be unattractive or repellent for *L. decemlineata*. Also, cereal leaf beetles, *Oulema melanopus*, did not respond to a GC-EAD-active oat volatile, 3*Z*-6:OAc, in field tests (Rao et al., 2003). Behavioral activity to GLVs has also been demonstrated with other Coleoptera; for example, scarab beetles, *Melolontha melolontha* (Reinecke et al., 2005) and *Phyllopertha horticola* (Ruther and Mayer, 2005), are strongly attracted to single or blends of specific GLV compounds. In addition, GLVs significantly enhanced the responses of the smaller European elm bark beetle, *Scolytus multistriatus*, and boll weevil, *Anthonomus grandis*, to their respective pheromones (Dickens et al., 1990).

Future field tests with *D. elongata* will be necessary, using single components and different blends and blend ratios of saltcedar volatiles, to fully investigate the behavioral significance of all of the EAD-active compounds.

The field bioassays showed a male-biased sex ratio that was surprisingly strong on some of the trapping dates. It is possible that males were more attracted to the host plant odors, but with similar male sex ratios in unbaited control traps and aerial sweeps, a more likely explanation is that males are simply the more active fliers, especially because the beetle sex ratio on the foliage was far less male-biased. In previous field tests of the aggregation pheromone blend, only a slight male bias was noted in the captured beetles (Cossé et al., 2005). Again, it is possible that males are more strongly attracted to host plant odors than females and that this differential attraction is not as pronounced with aggregation pheromones. A more likely explanation is that the behavioral, physiological, and ecological context of these experiments is critical in the outcome, both in terms of attraction to the baits and the sex ratio of captured beetles. For instance, placement of traps outside of the areas where beetles are mating and ovipositing might bias the sex ratio toward males, if they are the primary colonizers. The physiological status of the beetles, whether it be reproductive, diapause, hungry, or fed, will also undoubtedly have a strong influence on response to behaviorally active compounds.

Future field studies might further investigate the hypothesis that male *D. elongata* are pioneers in the colonization of saltcedar and that successful establishment of new populations is being guided by both the release of the male-produced aggregation pheromone and some of the volatile compounds released by the host plants.

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