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CHEMICAL ANALYSIS OF SQUIRT-GUN DEFENSE IN *Bursera* AND COUNTERDEFENSE BY CHRYSOMELID BEETLES

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Abstract—The genus *Bursera* produces resin stored in canals in the leaf. When leaves are damaged, some, but not all, species release abundant resin. Species of *Blepharida* are specialized herbivores of *Bursera*, and they exhibit variation in their counterdefensive behavior. Species feeding on resin-releasing plants cut the leaf veins before feeding, which often makes them more prone to predation. They also adorn their backs with their feces and may regurgitate and release an anal secretion when attacked or disturbed by predators. Species that feed on *Bursera* species that release no fluids do not sever the leaf veins prior to feeding, and they do not carry their feces on their backs. Instead, they face their predators, raise their heads in a "boxing-like" display, and rapidly swing their abdomens from side to side. We performed a comparative chemical analysis of the compounds found in *Bursera schlechtendalii*, a species that releases abundant resins, and *B. biflora*, a species that does not. We also analyzed the frass, enteric discharges, and larvae of the two species of *Blepharida* that feed on each of these plants. The compounds found in the body, feces, and discharges of the *Blepharida* species that adorns itself with feces match the chemical mixture of its host plant, suggesting that this beetle species can compensate its higher risk of predation by using the compounds present in the plant for defense. The chemical mixture of *B. biflora* is more complex and does not match the compounds found in the body or frass of its beetle herbivore, suggesting that the defensive strategy of this insect is behavioral and does not rely on its host's constituents.

Key Words—*Bursera*, Burseraceae, *Blepharida*, Chrysomelidae, monoterpenes, chemical defense, fecal shield, insect defense.

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INTRODUCTION

The genus *Bursera* comprises about 100 plant species distributed from the southern United States to Peru (Becerra and Venable, 1999a). The New World members of *Blepharida* (Chrysomelidae: Alticinae) are specialized herbivores of *Bursera* (Becerra, 1997; Furth, 1998). The genus includes about 45 species, many of them monophagous. *Bursera* species produce an array of terpenes distributed in a reticulating network of resin canals in the cortex of the stems and throughout the leaves (Mooney and Emboden, 1968; Becerra and Venable, 1990). The resins have been shown to decrease *Blepharida* survival and growth rate (Becerra, 1994a). Plant chemistry has also had a strong impact on the macroevolution of this interaction as well. As they evolved, *Blepharida* species have shifted among members of *Bursera* that are chemically similar (Becerra, 1997; Becerra and Venable, 1999b).

Some *Bursera* species have added a mechanical defense to their chemical protection. When a leaf is damaged, there is an abundant release of resins, often as a squirt. Besides being repellent, the resins solidify and impede the movement of the insect's mandibles, and when abundant may completely entangle the insect. Thus, insects feeding on these *Bursera* have to overcome both physical and chemical challenges (Becerra and Venable, 1990; Becerra, 1994b). *Blepharida* species feeding on these plants have developed the ability to reduce their exposure to *Bursera's* resins by cutting the leaf veins before feeding on the leaves (Becerra, 1994b). These species typically have a characteristic defensive behavior against predators as well. Larvae festoon themselves with their own feces and may regurgitate or release an anal secretion when attacked or disturbed.

Larvae of *Blepharida* species feeding on *Bursera* species that release little or no fluids after damage do not sever the leaf veins before feeding on the leaves. These larvae also typically do not regurgitate, release an anal secretion, or carry their feces on their backs. Instead, they rear their heads up to face their insect predators in a "boxing-like" display and swing their abdomens rapidly and forcefully from side to side (Table 1).

There is variation in these plant and insect strategies. Besides squirting, in some species of *Bursera*, the resins rapidly bathe both surfaces of the leaf. Others release less resin that moves only a short distance from the cut. Some beetle species spend considerable time cutting veins while others only do a quick cut. Some carry feces on their back but do not regurgitate or discharge an anal secretion. Some species also carry big loads of feces that form a complete shield of their bodies, while others carry a minute amount on their heads only.

In this paper we report the results of chemical analyses of a *Bursera*–*Blepharida* interaction typical of the squirting strategy and an another interaction typical of the nonsquirting strategy. We ask: What are the primary chemical constituents in the leaves and the squirt of plants? Are they present and, if so,

TABLE 1. COMPARATIVE BIOLOGY OF DEFENSE AND COUNTERDEFENSE IN GENUS *Blepharida* AND ITS HOSTS[a]

Bursera host species	*Blepharida* species	Plant releases fluids when damaged	Larvae cut veins	Larvae with fecal shield	"Boxing-like" display[b]
B. attenuata	*B. alternata*	yes	yes	yes	no
B. grandifolia	*B. pallida*	yes	yes	yes	no
B. instabilis	*B. alternata*	yes	yes	yes	no
B. chaemapodicta	*B. alternata*	yes	yes	yes	no
B. crenata	*B. lineata*	yes	yes	yes	no
B. denticulata	*B. lineata*	yes	yes	yes	n/o
B. fagaroides	*B. multimaculata*	yes	yes	yes	no
B. fagaroides	*B. gabrielae*	yes	yes	yes	no
B. fragilis	*B. alternata*	yes	yes	yes	no
B. kerberi	*B. lineata*	yes	yes	yes	no
B. kerberi	*B. sparsa*	yes	yes	yes	no
B. morelensis	*B. verdea*	yes	yes	yes	yes
B. odorata	*B. atripennis*	no	no	yes	no
B. paradoxa	*B. gabrielae*	yes	yes	yes	no
B. schlechtendalii	*B. schlechtendalii*	yes	yes	yes	no
B. trifoliolata	*B. gabrielae*	yes	yes	yes	no
B. trimera	*B. lineata*	yes	yes	yes	no
B. aptera	*B. gabrielae*	yes	yes	yes	no
B. aptera	*B. multimaculata*	yes	yes	yes	no
B. discolor	*B. gabrielae*	no	no	yes	no
B. discolor	*B. multimaculata*	no	no	yes	no
B. fagaroides var. *fagaroides*	*B. multimaculata*	yes	yes	yes	no
B. fagaroides var. *purpusii*	*B. multimaculata*	yes	yes	yes	no
B. asplenifolia	*B. flavocostata*	no	no	no	yes
B. copallifera	*B. flavocostata*	no	no	no	yes
B. copallifera	*B. balyi*	yes	yes	yes	no
B. cuneata	*B.* unknown species	no	no	no	n/o
B. excelsa	*B. bryanti*	no	no	yes	no
B. hintoni	*B. flavocostata*	no	no	no	yes
B. sarukhanii	*B. flavocostata*	no	no	no	yes
B. velutina	*B. flavocostata*	yes	no	no	yes
B. biflora	*B. flavocostata*	no	no	no	yes
Rhus					
R. glabra	*B. rhois*[c]	no	no	yes	yes

[a]Data are based on personal observations made by J. Becerra and D. L. Venable during multiple visits to natural populations of each *Bursera* species over three to six years depending on the species. Fluid release was documented by cutting multiple leaves on multiple individuals with nail clippers. Vein cutting was directly observed in vivo.

[b]See text for description. This behavior was assayed by menacing the head of larvae with forceps; n/o, not observed.

[c]Vencl and Morton (1998).

are they modified in the insect body, feces, and regurgitate? The answers to these questions will allow us to determine whether the contrast in these *Blepharida* counterdefensive strategies is primarily one of chemistry or behavior.

METHODS AND MATERIALS

The Interacting Species

Squirting, Vein-cutting, Fecal Shield Strategy. Bursera schlechtendalii is native to the arid and semiarid regions of Central Mexico and Guatemala. Leaves of typical individuals release abundant resins when damaged, often in a syringe-like squirt. They may also release a flow of resin that can rapidly bathe both surfaces of the leaf. *Blepharida schlechtendalii* is a monophagous species that feeds on *Bursera schlechtendalii*. When climbing onto a new leaf, larvae position themselves along the midrib with their heads facing the petiole and repeatedly bite the midvein. This activity can last for as long as $1\frac{1}{2}$ hr, after which the flow of resins in the vein is interrupted (Becerra, 1994a). Larvae cover their backs with copious amounts of feces. We do not know the mechanism of fecal collection in this species, but we presume it is the same as that of other *Blepharida* species in which larvae have a dorsal anus and a neuromuscular propulsion system that directs feces forward over the larvae (Vencl and Morton, 1998). When approached by predators (ants, pentatomid bugs, or neuropteran larvae), they may regurgitate and release an anal secretion (Becerra and Venable, personal observation). The samples for this paper were collected at the Biological Reserve of the Jardín Botánico de Zapotitlán, in the Tehuacán Desert, near Zapotitlán, Puebla, Mexico.

Nonsquirting, No Vein-Cutting, No Fecal Shield Strategy. Bursera biflora is endemic to the Tehuacan Desert. Its leaves do not release fluids when damaged. *Blepharida flavocostata* attacks this and several other species of *Bursera* that release little or no fluid. Larvae do not sever veins on these nonsquirting hosts, and they discard their feces. When disturbed by predators, they do not regurgitate or release an anal secretion. Rather, the larvae stand up in a "boxing-like" display, and are often successful at warding off predators or knocking them off the branches with rapid movements of their abdomen. Samples of these plant and insect species were collected 20 km south of Tehuacán on the road to Zapotitlán, Puebla.

Chemical Analyses

Collected larvae, feces, and, for *Blepharida schlechtendalii*, regurgitate, and anal secretions were stored in ethyl acetate until analysis. The same solvent was used to store whole leaves, and, for *Bursera schlechtendalii*, resin squirt of a manually severed leaf. Gas chromatography–mass spectrometry (GC-MS) of the plant and insect extracts was performed on a Hewlett-Packard 5890 gas chromatograph linked to a Hewlett-Packard 5970B mass selective detector at 70 eV with a DB-5 column (J & W Scientific, Folsom, California) 25 m long × 0.32 mm ID and 0.52-μm film with helium carrier gas at a linear velocity of 28 cm/sec. The injector temperature was 250°C, detector 280°C, and the oven temperature programmed from 60°C to 230°C at 10°/min, then held at 230°C for 2 min.

All volatile compounds from *Blepharida schlechtendalii* and *Bursera schlechtendalii* extracts were identified by matching the obtained spectra with standard mass spectral libraries (NBS 75.K) and by comparing the mass spectra and retention times of authentic standards. Identification of compounds from *Blepharida flavocostata* and *Bursera biflora* extracts was performed by matching the obtained spectra with authentic standards, standard mass spectral libraries, and by interpreting the mass spectrum. Reference compounds were α-limonene and α-pinene from Eastman Organics, Rochester, New York; benzaldehyde from Mallinckrodt, St. Louis, Missouri; caryophyllene, from Givaudan Corp., Clifton, New Jersey; phytol, from Aldrich Chemical Corp. Milwaukee, Wisconsin; β-myrcene from oil of bay from Fritsche D&O Co. New York, New York; *p*-cymene from thyme oil supplied by Fritsche D&O Co.; β-phellandrene, a gift from Jorge E. Marcias-Samano of Simon Fraser University; nonane from a light petroleum hydrocarbon distillate; cryptone extracted from *Eucalyptus* sp. leaves from trees growing on the University of Arizona campus, Tucson, Arizona; cuminaldehyde from Givaudan Corp.; sabinene from Roth Chemicals, Karlsruhe, Germany, and 3-carene from Aldrich Chemical Co.

RESULTS

The volatile components of the resin of *Bursera schlechtendalii* are a mix of monoterpene hydrocarbons, primarily β-phellandrene and limonene (Figure 1). Extracts of whole *Blepharida* larvae feeding on the same *B. schlechtendalii* shrub contain all of the volatile terpenoid compounds identified from the plant (Table 2) plus benzaldehyde. The larval regurgitate, anal secretion, and fecal shield contain β-phellandrene, limonene, and sabinene in proportions similar to those found in the plant resin and extract of feeding larvae.

The chemical mixture found in leaves of *B. biflora* is less volatile, more diverse, and more complex than the one of *Bursera schlechtendalii* (Table 3). The primary compound found in the leaves of *B. biflora* is α-pinene, but there are also four sesquiterpenes, palmitic acid, phytol, and a number of unidentified compounds. The chemical analyses of the body and frass of *Blepharida flavocostata* reveals almost no match in constituents with respect to the ones found in *B. biflora*, indicating that this insect does not use this plant's volatile compounds for its own defense. The only compound found in both leaves and feces is palmitic acid, although there may be traces of caryophyllene and an unidentified diterpene (which are present in very small proportions in *B. flavocostata*) in *B. biflora* as well.

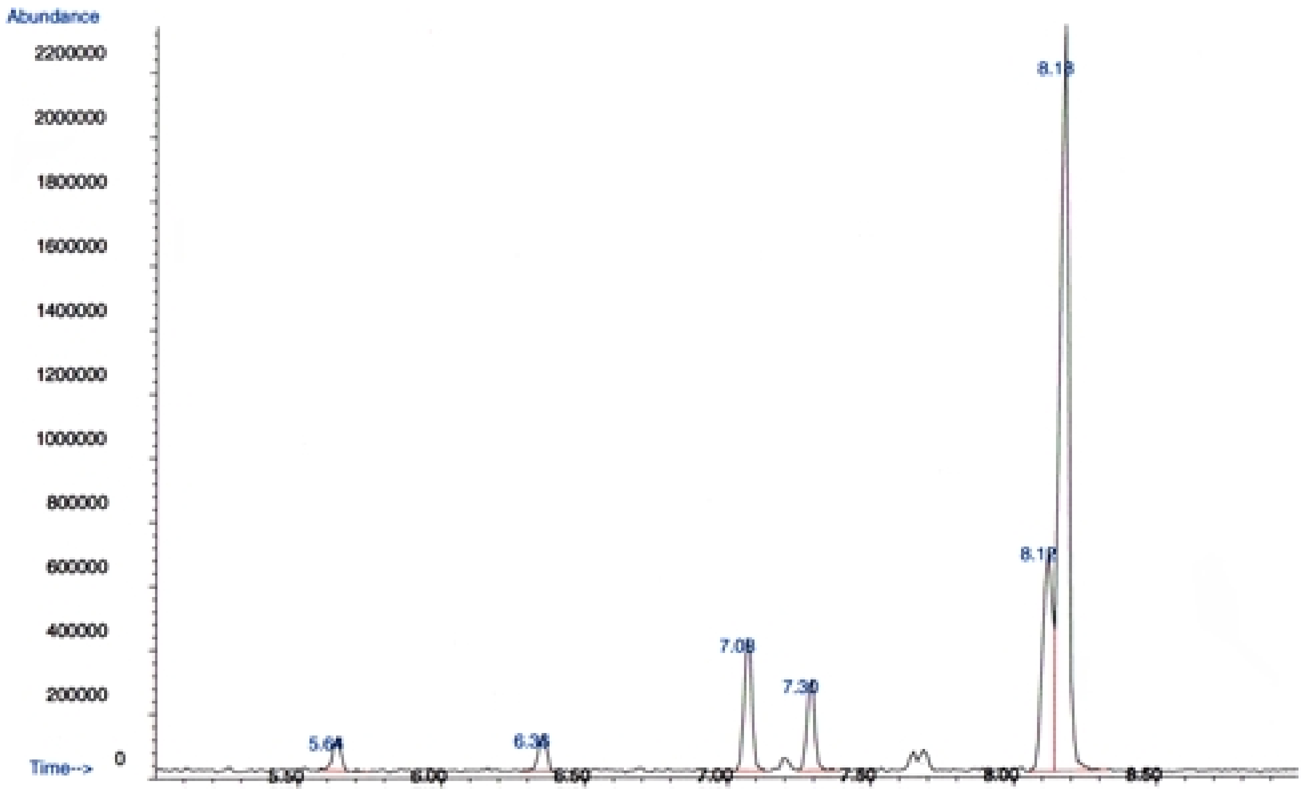

FIG. 1. Total ion chromatogram of the squirted resin of *Bursera schlechtendalii*. Retention time included on peaks corresponds to times and chemical identity given in Table 2.

TABLE 2. COMPOSITION OF ETHYL ACETATE EXTRACTS OF *Blepharida schlechtendalii* AND *Bursera schlechtendalii*

Retention (min)	Chemical	CAS#[a]	Amount (%)					
			Leaf	Resin	Larvae	Regurgitate	Anal secretion	Shield
5.64	nonane	111-84-2	3.4	2.4	3.1			
6.36	α-pinene	80-56-8	4.7	2.8	3.8			
6.91	benzaldehyde	100-52-7			3.9			
7.08	sabinene	3387-41-5	6.2	9.5	4.3	7.6	9.6	9.7
7.30	β-myrcene	123-35-3	8.3	6.3	3.1			
8.12	limonene	138-86-3	15.6	18.0	16.4	18.5	20.1	17.0
8.18	β-phellandrene	555-10-2	61.7	61.0	65.2	74.0	70.3	73.3

[a]Chemical Abstracts Service Registry Number.

TABLE 3. COMPOSITION OF ETHYL ACETATE EXTRACTS OF *Bursera biflora* AND *Blepharida flavocostata*

Retention (min)	Chemical	CAS#[a]	Identification method[b]	Amount (%) Leaf	Larvae	Feces
4.79	α-pinene	80-56-8	1	35.5		
5.20	benzaldehyde	100-52-7	1		4.8	
7.22	monacetin	106-61-6	2		4.0	
10.22	benzene acetaldehyde–α-ethylidene	4411-89-6	2		3.2	
12.57	caryophyllene	87-44-5	1			3.4
13.31			4			3.8
14.69	a sesquiterpene		3	7.4		2.2
15.00	octanoic acid	124-07-2	2			5.2
15.20			4		5.4	
15.45	a sesquiterpene		3	9.6		
15.65	a sesquiterpene		3	8.0		
16.05	a sesquiterpene		3	6.5		
16.47			4			3.0
16.57			4			5.0
16.60			4		6.7	
16.88			4		5.4	
17.56			4			3.4
17.97			4	4.9		
18.22			4	8.0		
18.35	a diterpene		3		9.7	2.7
18.83	palmitic acid	57-10-3	2	4.8	4.9	8.0
19.22	ethyl palmitate	628-97-7	2		12.7	6.2
20.15			4			19.6
20.50			4		11.1	22.1
20.59	phytol	150-86-7	1	6.3		
21.16	ethyl linoleate	544-35-4	2		5.0	5.4
21.27	ethyl oleate	111-62-6	2		19.3	
21.55	stearic acid	57-11-4	2		7.7	
21.63			2			4.1
21.90			2	9.1		6.0

[a]Chemical Abstracts Service Registry Number.
[b]Method of identification: 1, gas chromatographic retention coincidence and mass spectral matching with an authentic sample; 2, mass spectral matching with a library spectrum; 3, interpretation of the mass spectrum; 4, unidentified.

DISCUSSION

Blepharida schlechtendalii larvae are able to disarm the high-pressure resin defense of *Bursera schlechtendalli*, but it is apparent from the composition of the extract of whole larvae that the host defensive compounds are ingested. Analysis of the frass and anal secretions revealed that much of the monoterpene mix is excreted. We do not know whether this insect sequesters the dietary terpenoids in diverticular pouches for later use as a chemical deterrent as does the sawfly *Neodiprion sertifer* (Eisner et al., 1974), or if it simply expels available gut contents for defense. Other species of chrysomelid beetles have complex defensive behaviors, including the use of a fecal shield (Eisner et al., 1967) and de novo synthesis of defensive compounds (Pasteels et al., 1990).

Blepharida schlechtendalii has behavioral characteristics similar to other specialist herbivores feeding on plants with high concentrations of noxious chemicals. It has evolved a complex behavior to avoid the high terpenoid load in their diet, is partially resistant to the effects of the chemicals as evidenced by the high concentration in the gut, and uses the ingested plant resin for its own defense. We see no evidence of de novo synthesis of allomones or sequestration of certain components of the plant resin for use by *Blepharida schlechtendalii* in its defense against predators. The composition of the defensive secretions is similar to the terpenoid composition of the host plant resin.

Unlike the use of terpenoid compounds from *Busera schlechtendalii* by *Blepharida schlechtendalii*, *Blepharida flavocostata* does not sequester the major organosoluble volatile components from *Bursera biflora* in their feces. Perhaps the higher complexity of the plant's volatile mixture make it more difficult or more energetically expensive to use for antipredatory defense.

Becerra (1994a) has shown that the presence of resins under pressure increases the time it takes *Blepharida schlechtendalii* to process leaves. This slows larval growth rate and increases the risk of predation. Vein-cutting behavior increases the ability of the larvae to live on this plant by reducing their direct exposure to the resins. Our results indicate that resins have an indirect positive effect on larval survival through their incorporation into the larval protective arsenal. In contrast, *Blepharida flavocostata* does not obtain this indirect benefit from its heavier, more complex host chemistry, and it has developed an alternative behavioral defense.

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