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Sequestration of tropane alkaloids from *Brugmansia suaveolens* (Solanaceae) by the treehopper *Alchisme grossa* (Hemiptera: Membracidae)

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ABSTRACT

Treehoppers (Hemiptera: Membracidae) are sap-feeding insects distributed mainly in tropical regions. *Alchisme grossa* is a treehopper that has been reported in the Bolivian Yungas forests using mostly *Brugmansia suaveolens* (Solanaceae) as host-plant, where adult females oviposit and take care of their nymphs until they molt to adults. *Brugmansia* is a subtropical genus producing a variety of tropane alkaloids (TAs). We herein report the sequestration by adult males and females of *A. grossa* of TAs from *B. suaveolens*, examining separately the distinct body sections of insects. Purified extracts of *A. grossa* and *B. suaveolens* were analyzed by gas chromatography/mass spectrometry. TAs in *A. grossa* were the same as those in its host-plant; furthermore, they were equally distributed between sexes and they were differentially allocated within the body of adult individuals. An ecological role for sequestered TAs is discussed.

1. Introduction

Treehoppers (Hemiptera: Membracidae) are a diverse group (approximately 3300 spp.) of phytophagous insects distributed mainly in tropical regions of the world (Wood, 1993; Dietrich et al., 2001). They are sap feeders and their host-plants constitute a site for reproduction, oviposition and development. A conspicuous and morphologically variable pronotum constitutes a characteristic trait of treehoppers; this structure has been assigned a role in crypsis (Roy et al., 2007) and defense (Wood, 1974). Species range from monophagous to polyphagous (Dietrich and Deitz, 1991; Wood, 1993; Lin, 2006); diet breadth has been related to latitude, where tropical species tend to be oligophagous or polyphagous and temperate species tend to be monophagous, and to altitude, where higher elevations seemingly promote host specialization (Wood and Olmstead, 1984; Wood, 1984).

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The genus *Alchisme* thrives in humid montane and submontane ecosystems from northern Central America to the Brazilian shield and northern Chile, and shows preference for solanaceous host-plants (McKamey and Deitz, 1996; Godoy et al., 2006). *Alchisme grossa* is a subsocial species which displays diverse and complex maternal care traits such as egg and nymphal guarding, active defense against predators, and feeding facilitation (Godoy et al., 2006; Camacho et al., 2014; Torrico-Bazoberry et al., 2014). Adult females of *A. grossa* remain on the host-plant where they oviposit and take care of their nymphs until they molt to adults (Torrico-Bazoberry et al., 2014). In the summer (wet) season in the Yungas biogeographical region of Bolivia, *A. grossa* females oviposit mostly on young leaves of *Brugmansia suaveolens* (Solanaceae) (Torrico-Bazoberry et al., 2014), in spite of this being a habitat characterized as a site of high diversity of solanaceous species (Nee et al., 2007).

Host plant specialization is a common characteristic observed in phytophagous insects; the process is attributed in many cases to plant secondary metabolites which are a potential factor in the evolution of specific associations (Nishida, 2002; Schoonhoven et al., 2005). The Solanaceae is a large family comprising around 3500 species (D'Arcy, 1986), many of which contain alkaloids (Evans, 1986; Hawkes et al., 2000); in particular, *Brugmansia* produces tropane alkaloids (TAs) (Doncheva et al., 2006) which exhibit different levels of toxicity towards a wide variety of insects (Krug and Proksch, 1993; Detzel and Wink, 1993; Kitamura et al., 2004; Jolivet et al., 2012). In this study, we enquire about the sequestration of TAs from *B. suaveolens* in different body sections by *A. grossa* and their possible ecological role in the context of its pattern of specialization on this particular solanaceous species.

2. Materials and methods

2.1. *Alchisme grossa* and *B. suaveolens*

Periodic field observations showed that cohorts of *A. grossa* were synchronized, allowing collection of ten males and ten females, one from each of 20 different cohorts, within two days of having reached the adult stage. Immediately after collection, insects were taken to the laboratory, transferred to tomato plants (*Lycopersicum esculentum*), enclosed in tulle bags and allowed to feed for 24 h. The aim of this process was to allow insects to replace *B. suaveolens*-related contents in its gut by tomato-related contents (in terms of alkaloids, glycosteroidal alkaloids – Friedman, 2002); under these circumstances, TAs found in *A. grossa* should correspond only to compounds sequestered in the insect body.

Four groups of young leaves (ca. 20 g fr. wt each) were collected from different uncolonized individuals of *B. suaveolens*, carried to the laboratory, dried at 35 °C (Heraeus UT6 oven) for three days, ground in a laboratory mill and analyzed for TAs.

2.2. Extraction of alkaloids from leaves of *B. suaveolens*

Each pulverized sample (2 g) of *B. suaveolens* leaves was extracted with 40 ml CH_3OH and exposed to ultrasound in a bath (Power Sonic 405) at 25 °C for 30 min. The methanolic extract was filtered through a frit funnel and the resulting extract evaporated under reduced pressure on a rotatory evaporator (Büchi RE 111). The syrupy residue was dissolved in 3 ml 5% HCl. The acidic solution was washed with $CHCl_3$ (2 × 3 ml). The aqueous phase was adjusted to pH 10 with NH_4OH and was extracted twice with 3 ml $CHCl_3$; under these circumstances the aqueous extracts gave negative Dragendorff reaction. Finally, the organic extracts were dried with anhydrous Na_2SO_4, filtered through cotton wool placed at the tip of a Pasteur pipette and taken to dryness by means of a flow of nitrogen. Total alkaloids as free bases constituted on average of 2.1% dry weight of the plant extracts. The dry residues were disolved in 10 μl methanol before injecting 2 μl into the GC column.

2.3. Extraction of alkaloids from insects

Insects were sacrificed by freezing and then dried at 35 °C (Heraeus UT6 oven) for three days. Each adult was weighed (8.1 ± 1.2 mg dry weight; mean ± SD) and its body dissected under a stereoscopic magnifying lens (Olympus SZ61) into three sections: a) pronotum (18.7 ± 0.024% d.w.), b) head + thorax + abdomen (66.3 ± 0.083% d.w.), and c) wings + legs (15.0 ± 0.028% d.w.). Each section was ground by introducing it into a 1.8 ml stainless steel microvial with a polyethylene flange cap containing five 1.5-mm-diameter steel spheres and agitating it for 10 min in a bead beater (Mini-Beadbeater-96; Biospec Inc., Bartlesville, OK, USA). The pulverized sample was extracted with 2 ml CH_3OH and exposed to ultrasound in a bath (Power Sonic 405) at 25 °C for 30 min. The methanolic extract was not submitted to acid-base purification to avoid losses; it was filtered through cotton wool placed at the tip of a Pasteur pipette, collected in a 1.8 ml amber vial and dried under nitrogen flow. The dry residue was successively redissolved in small aliquots of methanol (ca. 50 μl) which were transferred to a 100 μl glass insert within an amber vial and evaporated to dryness by means of a nitrogen flow; this operation minimized the quantity of residue retained in the original vial walls. The dry residues were dissolved in 10 μl methanol before injecting 2 μl into the GC column.

2.4. Analysis of extracts by gas chromatography/mass spectrometry (GC/MS)

Purified extracts of *A. grossa* and *B. suaveolens* were analyzed by GC/MS (Shimadzu, GCMS-QS, 2010 Ultra), equipped with an Rtx-5MS Crossbond 5% diphenyl - 95% dimethyl polysiloxane capillary column (30 m length, 0.25 μm film thickness, 0.25 mm internal diameter). The GC was operated in the splitless injection mode; injection volume was 1 μl for plant extracts and 5 μl for insect extracts. The column temperature was held at 30 °C for 3 min, raised at 25°C/min to 230 °C, and maintained for 12 min at 230 °C. The carrier gas was helium at a flow rate of 1.3 ml/min. The mass spectrometer was used in the electron impact ionization mode (70 eV) with an emission current of 250 μA. The injection port, ion source and transfer line were kept at 250 °C.

2.5. Identification of alkaloids

Preliminary identification of alkaloids was achieved using the NIST 08 mass spectra library contained in the software GCMS Solution v. 2.61 (Shimadzu Corporation, Kyoto, Japan). Since this database contains few tropane alkaloids, the mass spectra and retention indexes of the chromatographic peaks were compared with those of TAs reported from *B. suaveolens* or other *Brugmansia* species. For mass spectral comparisons, similarity indexes were calculated based on the mean intensity (mean of four plant and 20 insect extracts) of the thirteen most abundant mass fragments using the algorithm in the Shimadzu software GCMS Solution v. 2.61. Retention indexes were compared with values from the literature or values calculated on the basis of retention times from the literature.

3. Results

Purified alkaloidal extracts from young leaves of *B. suaveolens* contained three alkaloids with retention times of 14.5, 15.5 and 18.5 min (Fig. 1). Their mass spectra (Table 1) with a base peak at *m/z* 94 corresponding to the *N*-methylpyridinium cation and a strong peak at *m/z* 42 best represented as $CH_3{-}N^+{\equiv}CH$ (Blossey et al., 1964), suggested they were tropane akaloids. The analysis of retention and similarity indexes identified them as 3-phenylacetoxy-6,7-epoxytropane, aposcopolamine and scopolamine, respectively (Fig. 2). The molecular peaks confirmed these assignments: $[M]^+$ at *m/z* 273 ($C_{17}H_{23}NO_3$) for 3-phenylacetoxy-6,7-epoxytropane (Vitale et al., 1995), at 285 ($C_{17}H_{21}NO_4$) for aposcopolamine, and at 303 ($C_{17}H_{21}NO_4$) for scopolamine.

Freshly emerged adults males and females of *A. grossa* contained the same three TAs identified in leaves of their host plant (3-phenylacetoxy-6,7-epoxytropane, aposcopolamine, and scopolamine). Total concentration of TAs did not differ between sexes (mean ± SD: $34.0 \pm 9.0 \times 10^6$ ions/mg dry wt for females and $26.7 \pm 12.3 \times 10^6$ ions/mg dry wt for males; one-way ANOVA: d.f. = 19, F = 2.321, P = 0.145). Global composition of alkaloids varied significantly between body sections (one-way ANOSIM: R = 0.207, P < 0.001); subsequent pairwise comparisons showed also significant differences between body sections (ANOSIM with 10,000 random permutations on Bray–Curtis similarity indices: pronotum vs. wings + legs, P = 0.006; pronotum vs. head + thorax + abdomen, P = 0.0009; wings + legs vs. head + thorax + abdomen, P = 0.021). Leaf extracts of *B. suaveolens* contained about 70 times more total TAs per mg of dry weight of tissue than insect extracts.

4. Discussion

GC–MS analysis of the alkaloidal fraction of *A. grossa* tissues revealed the presence of three TAs (3-phenylacetoxy-6,7-epoxytropane, aposcopolamine and scopolamine). While aposcopolamine may arise from pyrolysis of scopolamine inside the chromatographic column (Dräger, 2002), 3-phenylacetoxy-6,7-epoxytropane and scopolamine are potentially derived from their host, *B. suaveolens,* since leaf extracts of this plant also contained these compounds. The presence of TAs in both male and female adults of *A. grossa* is to be expected since the feeding behavior is similar in both sexes during a large part of

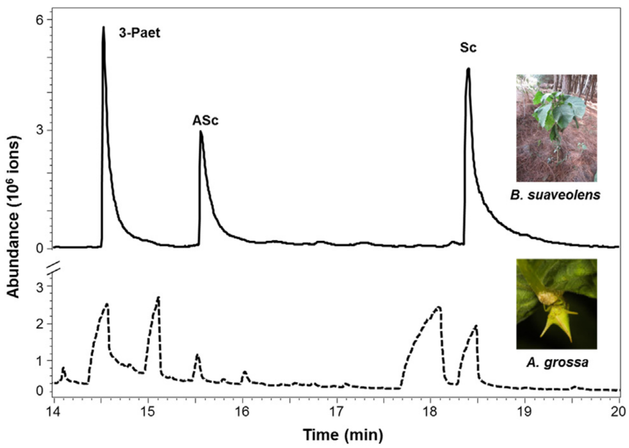

Fig. 1. Chromatogram of the alkaloid fraction of *B. suaveolens* young leaves (upper curve) and extracts of the pronotum of *A. grossa* (lower curve) showing tropane alkaloid peaks (see Fig. 2 for names and structures of alkaloids).

Table 1
Retention times (RT), retention indexes (RI) and mass spectra of alkaloids from leaf extracts of *B. suaveolens* (mean values of four extracts).

Compound	RT (min)	RI	M^+ (%)	Characteristic ions of mass spectra *m/z* (relative intensity, %)
1	14.5	2010	273 (21.0),	**94 (100)**, 138 (60.9), 91 (60.3), 42 (52.1), 108 (49.3), 154 (38.8), 136 (34.4), 97 (23.5), 81 (21.4), 41 (18.6), 65 (17.1), 57 (14.6)
2	15.5	2092	285 (26.3),	**94 (100)**, 103 (50.7), 138 (48.9), 42 (48.9), 108 (47.4), 154 (37.4), 136 (33.8), 77 (26.5), 97 (23.4), 81 (20.1), 41 (15.2), 110 (17.9)
3	18.5	2248	303 (16.6)	**94 (100)**, 138 (67.8), 108 (45.9), 42 (41), 154 (35.1), 136 (34.6), 97 (22.6), 81 (20.3), 103 (18.9), 137 (16.1), 120 (13.6), 110 (13.6)

Characteristic ions of mass spectra with parent peak in bold.

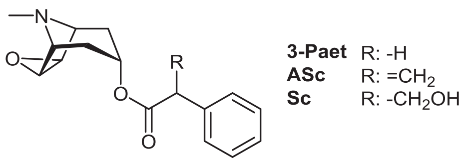

Fig. 2. Structures of tropane alkaloids found in *B. suaveolens* and *A. grossa*: 3-Paet: 3-phenylacetoxy-6,7-epoxytropane, ASc: aposcopolamine, Sc: scopolamine.

their lives and both sexes display fidelity to the host species where they were born (Camacho et al., 2014; Torrico-Bazoberry et al., 2014).

Different TAs show deleterious effects on a variety of phytophagous insects which feed on Solanaceae (Kitamura et al., 2004; Alves et al., 2007; Arab and Trigo, 2011; Arab et al., 2012; Jolivet et al., 2012); hence, a defense function may be proposed for TAs sequestered by *A. grossa*. If TAs are involved in defense, their similar concentration in both sexes may be related to a trade-off between two ways of being exposed to predators: while reproductive females of *A. grossa* could be more exposed to predators than males because they remain relatively immobile close to their offspring along their nymphal development (Torrico-Bazoberry et al., 2014), males could be more exposed to predators on account of their shorter dispersal ranges than females (Torrico-Bazoberry et al., 2014).

The overall effect of TAs on phytophagous insects may depend on their level of ecological specialization. For example, specialist insects such as adults of the butterfly *Placidula euryanassa* which feed on TA-containing *B. suaveolens* contain detectable amounts of TAs, proposed as chemical protection and host recognition cues (Freitas et al., 1996). In the case of other insects such as *Miraleria cymothoe* (Nymphalidae), which feeds on *B. suaveolens* without sequestering TAs, the role of compounds such as scopolamine in the plant has been suggested as feeding and oviposition attractants (Freitas et al., 1996; Kitamura et al., 2004; Alves et al., 2007; Arab et al., 2012).

Differences in concentrations of TAs between *A. grossa* body sections and *B. suaveolens* leaves may be related to differences in the concentration of compounds (e.g. alkaloids) between the phloem sap, where the insect feeds and the leaf tissue which was submitted to analysis.

We have shown that *A. grossa* sequesters alkaloids from its host-plant. The ecological roles associated with sequestration of TAs in this species remain unclear, although a defensive role may be suggested.

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