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ORIGINAL PAPER

Virola sebifera Aubl. (Myristicaceae) leaf chemical composition and implications on leaf-cutter ant foraging choice

Amanda Aparecida Carlos[1] · Karla da Silva Malaquias[2] · Rafael Camargo Consolmagno[3] · André Lucio Franceschini Sarria[2] · João Batista Fernandes[2] · Odair Correa Bueno[1]

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Abstract Leaf-cutter ant plant material choice is essential for colony maintenance and growth. Plant material is used as a substrate for cultivating symbiotic fungus, and the ants' preference for particular leaves, tends to be determined by vegetal age-related physicochemical factors. The plant species *Virola sebifera* Aubl. (Myristicaceae), for example, shows a large number of leaf surface trichomes. Although non-glandular, *V. sebifera* trichomes may gradually retain an increasing amount of chemical compounds over the lifetime of the leaf. Thus, the present study aims to investigate the role of plant chemical compounds on *Atta sexdens rubropilosa* preference for *V. sebifera* leaves of different ages. For this purpose, the chemical composition of trichomes on young and senescent leaves was analyzed, and ants' preference tested. The chemical compositions differ between *V. sebifera* young and senescent leaves, with triacontane (C30) predominance in young leaves and tetratriacontane (C34) predominance in senescent leaves. Ants' preference choice was tested by randomly offering leaves of different ages to *A. sexdens rubropilosa* workers, following six different treatments: (1) young leaf fragments; (2) young leaf fragments with few trichomes removed; (3) loose trichomes from young leaves; (4) senescent leaf fragments; (5) senescent leaf fragments with few trichomes removed, and (6) loose trichomes from senescent leaves. Ants' preference was observed for young leaves fragments with a few trichomes removed and also for young leaves loose trichomes. Ants' preference might be due to specific volatile compounds (GLV) preset in *V. sebifera* young leaves. Results suggest occurrence of ants' selectivity resulting from changes on trichomes chemical composition between *V. sebifera* leaves different age stages.

Keywords Ants preference · Trichomes · Substrate selection · Substrate chemical variation · Plant selection

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✉ Amanda Aparecida Carlos
amandacarlos@yahoo.com.br

1 Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Avenida 24A, 1515, Bela Vista, Rio Claro, SP 13506-900, Brazil

2 Centro de Ciências Exatas e de Tecnologia, Departamento de Química, Laboratório de Química de Produtos Naturais, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, Caixa Postal 676, São Carlos, SP 13565-905, Brazil

3 Departamento de Botânica, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Avenida 24A, 1515, Bela Vista, Rio Claro, SP 13506-900, Brazil

Introduction

Leaf-cutter ants colonies rely on obligatory mutualism to grow fungus over plant material, as symbiotic fungus is the major food source for immature ant larvae. However, for adult workers ants, the amount of energy and nutrients derived from fungus as a food source is very low (Bass and Cherrett 1995; Silva et al. 2003). Only 5% of an adults' energy requirement is derived from symbiotic fungus ingestion. Adult ants, therefore, feed directly on plant sap while cutting out and shredding leaves (Quinlan and Cherrett 1979; Forti and Andrade 1999), or they ingest fluids or tissues from a variety of plant structures (e.g. food bodies and seed elaiosomes; Bueno et al. 2008).

Hence, selection of appropriate plant material by leaf-cutter ants is essential for colony maintenance and growth, since plant fragments are used as a substrate for cultivating symbiotic fungus. Furthermore, the fact that ants choose a variety of plant species or vegetative structures while foraging may guarantee that potential toxic substances do not overload either ants or their symbiotic fungus (Howard et al. 1988). Therefore, plant selection by foraging leaf-cutter ants is a complex process that involves different factors (Verza et al. 2007). The foraging pattern of the genus *Atta* does not appear to be random, as it is not based on spatial distribution nor on plant material abundance, but rather on plants' attractiveness and repellency. Hence, the ants' preference for particular vegetal material comprises a wide range of characteristics in which chemical composition and leaf content are determinant factors (Howard 1987; Howard et al. 1988; Salatino et al. 1998).

Further research is still needed to determine which factors specifically cause a plant to be chosen by leaf-cutter ants. Although many authors suggest quality and physical characteristics of leaves to be determinant factors (Rockwood 1976; Stradling 1978), others indicate that a plant's chemical compounds may be attractive, inhibitory, or even repellent, (Littledyke and Cherrett 1978; Carlos et al. 2010; Heil 2014). However, researchers are unanimous in highlight the importance of chemical and physical plant features act as the first line of defense against herbivory (Crawley 1983; Marquis et al. 2012). For example, the vegetal species *Qualea multifora* shows three different and effective defense mechanisms against herbivory during its development: (1) higher number of trichomes during early leaf development; (2) extrafloral nectary activity during mid-phase leaf development; and (3) increase in leaf toughness at advanced developmental stages (Calixto et al. 2015).

Conversely, some specific volatile compounds (GLV) may act as attractive factors for herbivorous species (Heil 2014). Attraction of leaf-cutter ants may be seen as a co-evolutionary relationship as it is also known that plant phenology can be directly influenced by plant-herbivorous interactions (e.g. some species of Malpighiaceae family have considerably reduced leaf areas caused by ants herbivory, although no damage is done to the plants' reproductive structures; Vilela et al. 2014). As postulated from Verza and colleagues (2007), leaf-cutting ants' plant material choice is primarily associated to chemical characteristics of the vegetal substrate. Thus, only after the choice is made, physical factors may play secondary role on leaf-cutting ants' choice. Furthermore, plant age-related factors, such as compound production, reduced water content, increased hardness and leaf nutritional state, are involved in ant selectivity (Stradling 1978; Waller 1986). Howard (1987) described the existence of a positive correlation between quantitative variations in nutrient and water content and ant-workers' foraging choice of substrate. According to this author, plants' secondary compounds production would be an additional variable to contribute to the ants' choice (Howard 1987). Rockwood (1976) emphasizes the importance of chemical compounds on leaf-cutter ants' choice, but adds that seasonal interference on composition of compound and on variation of its quantity should also be taken into consideration.

Many plant species gradually retain an increasing amount of chemical compounds over its lifetime. Compounds found in trichomes are metabolics synthetized within plant cells that may or may not have attached ions directly absorbed from the environment. It is also known that leaf trichomes gradually change the amount and type of chemical compounds they retain as leaves age. For example, the species *Virola sebifera* Aubl. (Myristicaceae), from Brazilian Cerrado, shows a large number of non-glandular trichomes on leaf surfaces. Increase in chemical compounds retention during *V. sebifera* the leaf lifecycle may be one of the physical features that change considerably in the process of ageing. This indicates that trichome quantity and leaf age could influence cutting-leaf ants preference for *V. sebifera* leaves. The present study aims to investigate the role of plant chemical compounds on *Atta sexdens rubropilosa* preference for *V. sebifera* in relation to different trichome quantities and leaf ages. Hypotheses tested consisted of checking if (1) presence or low quantity of trichomes and (2) leaves of different ages influenced the foraging preferences of *A. sexdens rubropilosa* workers. It is expected that the amount of trichomes should be inversely proportional to workers' loading frequency of leaves, as a reduced number of trichomes should stimulate ants' choice; and that ants would show preference for young leaves rather than old ones due to high concentrations of chemical compounds in aged leaves.

Materials and methods

Bioassay of retained chemical compounds on *Virola sebifera* Aubl. (Myristicaceae) foliar surface

Young and senescent *V. sebifera* leaves were collected in the Cerrado area of the Federal University of São Carlos (UFSCar, São Carlos, Brazil). Leaves were collected from trees with a mean size of 3 m height for both chemical analyzes bioassays and behavioral tests. Young leaves were defined as those 16–18 cm in size, with vivid green color, low leaf stiffness and arranged in the most apical portion of branches. Senescent leaves were defined as those 18–25 cm in size, with loss of coloration, greater leaf stiffness and from basal parts of branches. The material, separated by foliar ontogeny, was individually packed in plastic bags (50 × 70 cm) and immediately transported to the laboratory. Plant species and collected samples were identified by Dr. Leonardo Biral dos Santos, Msc Marcelo Monge Egeã and Msc Rafael Camargo Consolmagno. A voucher specimen (HRCB 57764) was deposited at Herbário Rioclarense (HRCB), Bioscience Institute of São Paulo State University "Júlio de Mesquita

Filho" (UNESP, Rio Claro, São Paulo). Trichomes from the foliar abaxial face (Fig. 1) were removed by carefully scraping the fresh leaf surface with a blade under a stereomicroscope (Zeiss, Model Stemi 2000), avoiding leaf damage. Some photos of fresh material were also taken using an inverted microscope (Zeiss Model Primovert). A portion of the freshly removed trichomes was sampled for chemical compound identification and quantification.

Chemical analyses were conducted at the Natural Product Chemistry Laboratory of UFSCar Chemistry Department (São Carlos, Brazil). A preliminary test series exploring solvents of different polarities was carried out to choose the most appropriate solvent for extraction. In this test, dichloromethane was found to be the most efficient, and was used in the subsequent analysis. For extraction, 500 mg trichome samples from young leaves and 500 mg trichome samples from senescent leaves were separately mixed with 5 mL dichloromethane, and each sample was immersed for 30 s in an ultrasonic bath (Model Bl4). Obtained extracts were analyzed by gas chromatography combined with mass spectrometry (GC-MS; Shimadzu, Model TQ8040) to determine all compounds present. Ramp rates were 100 °C for 2 min, 8 °C min^{-1}; 200 °C for 5 min, 8 °C min^{-1}; and 300 °C for 15 min. Injector and detector temperatures were 320 and 100 °C, respectively (McCaffery and Wilson 1990). Compounds were identified by comparison to standard compounds and ions diagnostic described in literature.

Trichomes morphology analysis of *Virola sebifera* Aubl (Myristicaceae) young and senescent leaves

Young and senescent leaf fragments were observed under a scanning electron microscope. Fresh leaf fragments (1 cm × 1 cm) from leaves of both ages were dehydrated and placed on steel stubs to reach the critical point at which to be coated in gold. Subsequently, each stub sustaining a golden-coated sample was examined and photographed on a scanning electron microscope (Hitachi Model TM 3000).

Behavioral bioassays

Behavioral bioassays were conducted at the Center for Social Insect Studies (CEIS in the Portuguese acronym), Bioscience Institute of São Paulo State University "Júlio de Mesquita Filho" (UNESP, Rio Claro-SP). *Atta sexdens rubropilosa* queens were collected during nuptial flight in Edmundo Navarro de Andrade State Forest, municipality of Rio Claro-SP (22°25′S, 47°33′W). Queen ants were kept in the laboratory (air conditioned room 25 °C ± 2 °C and 70% relative humidity) inside plastic containers (200 mL) with 1 cm plaster (gypsum) in the bottom for maintaining moisture. Ant colonies started to develop by oviposition of fertilized queens. When colonies increased in size, they were transferred to larger plastic pots (800 mL), also with 1 cm bottom plaster layers. Each plastic container was interconnected to two other gypsum-free containers (foraging chamber and depleted material, 500 mL each) by plastic tubes. For colony maintenance and growth, leaves of *Acalypha wilkesiana* var. *marginata* Muell. Arg., *Syzygium jambolanum* Lam. e *Mangifera indica* L. were provided daily, *ad libitum*. Colonies that reached 800 mL volume of symbiont fungus content were used for preference tests.

Behavioral bioassays were performed using a larger foraging arena connected as replacement for preceding ones (length 28.5 × width 24 × height 5 cm). Exposure to new larger foraging arena was made 1 day before behavioral bioassays were conducted so ants could be familiarized to this new environment. Larger foraging arenas were chosen so there was a greater spacing between all substrates to be tested, facilitating visualization of ant

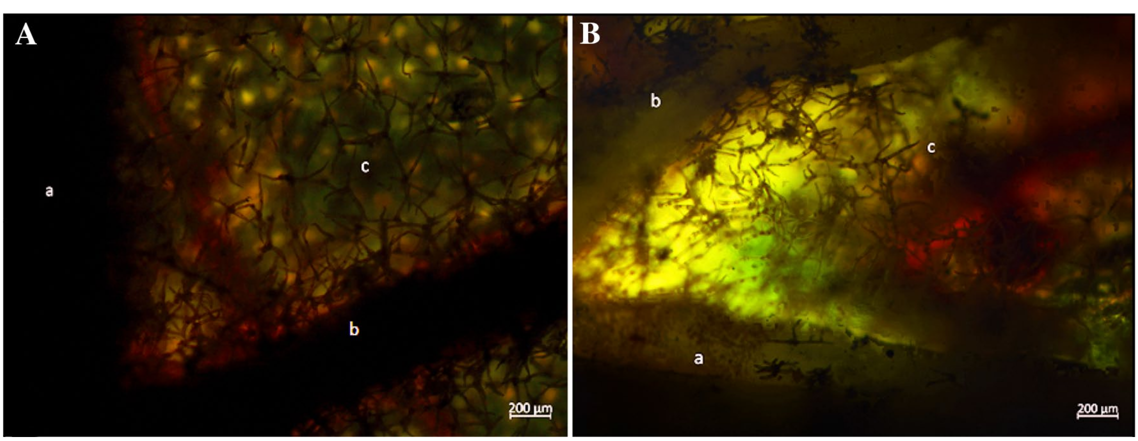

Fig. 1 Stereomicroscope images of *Virola sebifera* Aubl (Myristicaceae) leaf abaxial faces. **A** Young leaf, **B** senescent leaf. *a* Main leaf vein, *b* secondary leaf vein, and *c* leaf blade

workers' interest in foliar fragments and trichomes. Preference test were performed by suppressing the supply of plant substrate leaves for 24 h before bioassay. Four different colonies (repetitions) of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) were used for the preference choice test. The test was carried out as proposed by Forti et al. (1993), after 24 h of plant substrate privation.

Behavioral bioassays of preference choice consisted in randomly offering in foraging arenas six treatments of different types of *Virola sebifera* leaves fragments (five leaf fragments 1.0 × 1.0 cm each, cut from different leaves) and a quantity of trichomes (2 grams) as substrates per each one of the four colonies. Treatments consisted of (1) young leaf fragments; (2) young leaf fragments with few trichomes removed; (3) loose trichomes from young leaves; (4) senescent leaf fragments; (5) senescent leaf fragments with few trichomes removed, and (6) loose trichomes from senescent leaves; 2 g of trichomes from senescent leaves. For treatments 2 and 5, trichomes were gently scraped off with a blade under a stereo microscope, avoiding as much mechanical damage to the leaf as possible (Fig. 2). The number of leaf fragments carried by *A. sexdens rubropilosa* workers was determined after 60 min (treatments 1, 2, 4 and 5). For evaluating interest in trichomes, the presence or absence of ant workers on the offered trichome samples (treatments 3 and 6) was registered every 15 min, totaling 60 min of experimental time. Afterthe experiment was conducted, all treatments were left in the foraging arena for 24 h so the total number of *V. sebifera* leaf fragments and trichomes carried by each colony could be determined.

Statistical analysis

Behavioral bioassays data were analyzed by Kruskal–Wallis analysis ($p \leq 0.05$). Student–Newman–Keuls test and descriptive statistic (presence and absence) were used on statistical analysis a posteriori (Bioestat 2011).

Results

Trichomes morphology analysis

Dendroid trichomes were observed as described by Barthlott et al. (2017). Trichome structures are present on both abaxial and adaxial sides of leaf blades, on both young and senescent leaves. However, the abaxial face shows fewer trichomes in the foliar limbus of both young and senescent leaves. (Fig. 3).

Trichomes present in young leaves were more preserved and uncontaminated, while those present in senescent leaves were mostly brittle (Fig. 4). In addition, a high concentration of organic and inorganic material, such as fungal hyphae, was obsserved adhering to trichomes in senescent leaves (Fig. 4).

Chemical analysis

The chromatographic method for the determination of the compounds found in trichomes was validated. Linearity and sensitivity were verified and the coefficient of correlation was sufficient for quantification of the compounds (R^2: 0.99). Tables 1 and 2 show the compounds found, which included hydrocarbons, fatty acids and ketones.

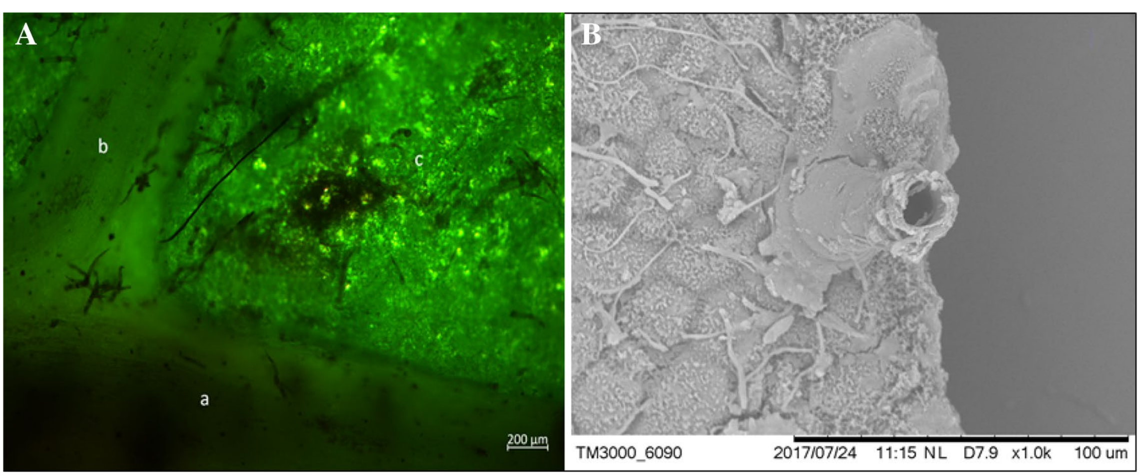

Fig. 2 Scanning electron microscope images of *Virola sebifera* Aubl. (Myristicaceae) leaf blade abaxial face. **A** Young leaf abaxial face showing few trichomes after scraping, **B** remaining base of a removed trichome, in detail

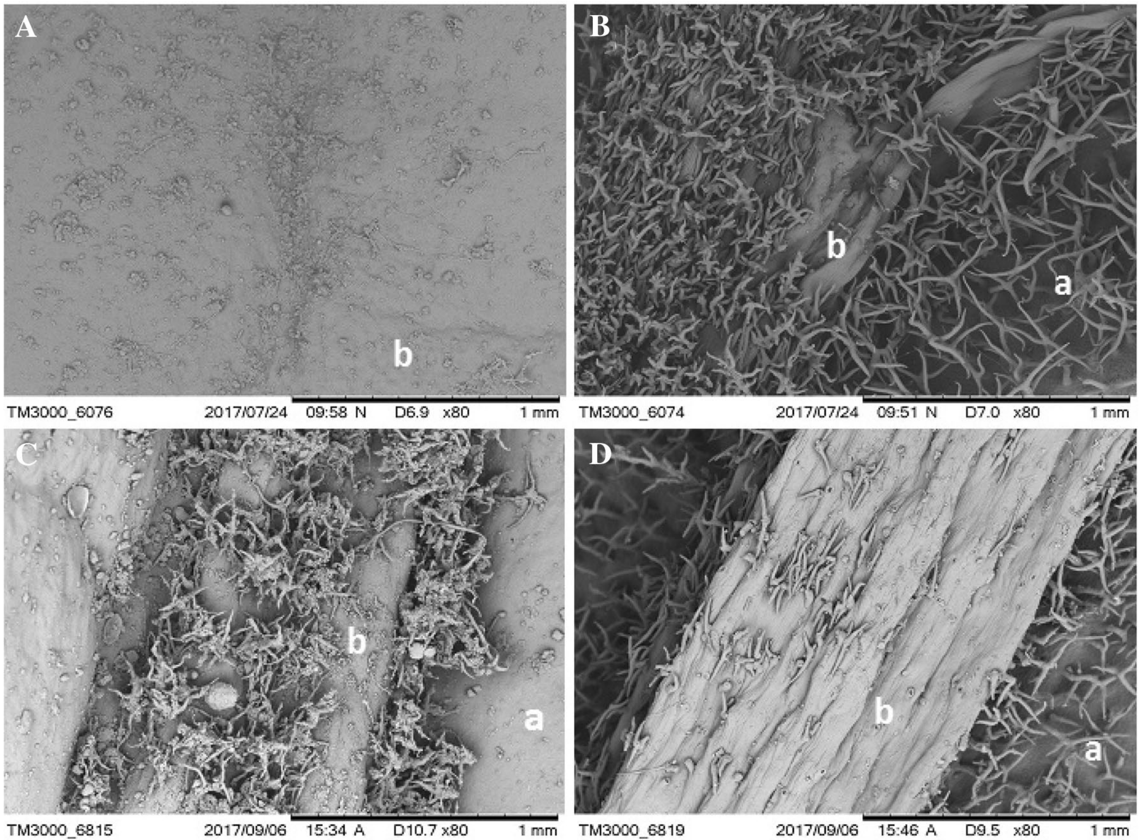

Fig. 3 Scanning electron microscope images of *Virola sebifera* Aubl (Myristicaceae) leaf. **A** Young leaf adaxial face, **B** young leaf abaxial face, **C** senescent leaf adaxial face, **D** senescent leaf abaxialface. *a* main leaf vein, *b* leaf blade

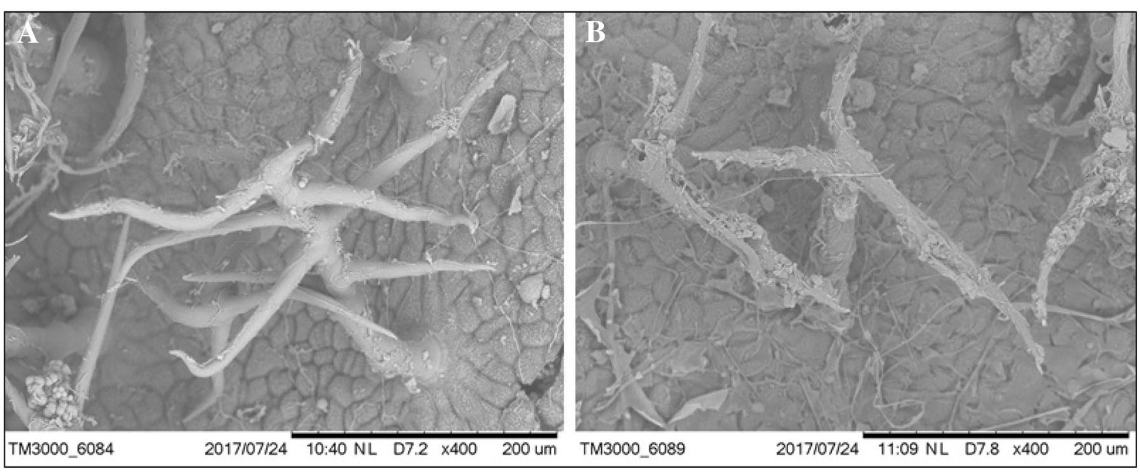

Fig. 4 Scanning electron microscope images of *Virola sebifera* Aubl. (Myristicaceae). **A** Young leaf trichomes, **B** senescent leaf trichomes

Behavioral analysis

With respect to leaf fragments and trichomes carried to the colonies, *Atta sexdens rubropilosa* workers carried preferentially young leaf fragments with few trichomes (Treatment 2) within 60 min, and no worker carried senescent leaf fragments (Treatment 4) ($H = 8.50$; $p = 0.0367$) (Fig. 5).

Evaluation of ant workers' interest in trichomes over the period of 60 min in the four repetitions (colonies) showed that ants explored young trichomes more frequently than

Table 1 Compounds found in the extract of young leaf trichomes

N	RT	Compound	m/z	Concentration (ppm)
1	20.063	Hexadecanoic acid	60, 73, 129, 256	10.66
2	21.989	Heptadecanoic acid	60, 73, 129	1.67
3	24.883	Octacosane (C28)	57, 71, 85	1.21
4	26.450	Phytosteroid	–	16.70
5	27.920	Triacontane	57, 71, 85	20.30
6	28.081	2-Pentacosanone	135, 146, 366	4.54
7	29.479	Dotriacontane (C32)	57, 71, 85	8.45

N number of the compound obtained, *RT* retention time (in minute), *m/z* M stands for mass and Z stands for charge number of ions

Table 2 Compounds found in the extract of senescent leaf trichomes

N	RT	Compound	m/z	Concentration (ppm)
1	17.790	o-Toluenesulfonamide	90, 106, 171	1.66
2	18.394	*p*-Toluenesulfonamide	91, 155, 171	2.76
3	21.401	3.3.5-Trimethylcyclohexyl salicylate	69, 138, 262	1.06
4	22.250	Di-octyl ether	71, 113, 143	3.29
5	27.297	Phytosteroid	–	3.68
6	28.297	Triacontine	57, 71,85	2.13
7	30.249	Dotriacontane	57, 71,85	12.02
8	32.069	Tetratriacontane	57, 71,85	15.64
9	32.260	2-Hexacosanone	135, 146, 365	1.69
10	33.828	Hexatriacontane	57, 71,85	7.96
11	36.143	1-Heptacosanol	97, 125, 139	5.45

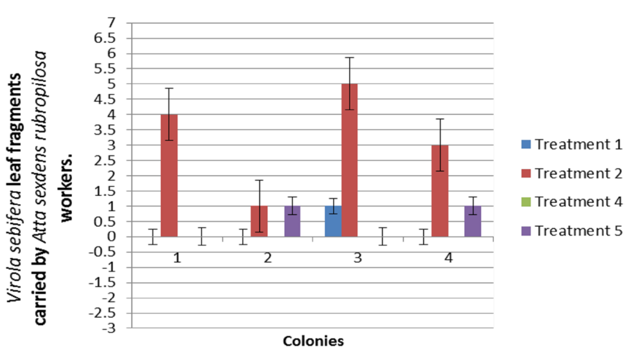

Fig. 5 Percentage of different *Virola sebifera* leaf fragments carried by *Atta sexdens rubropilosa* workers. Treatments: 1—young leaf fragments; 2—young leaf fragments with few trichomes; 4—senescent leaf fragments; 5—senescent leaf fragments with few trichomes. Tukey test (p = 0.05). Treatments 3 and 6 were not evaluated because of the difficulty in quantifying the small-sized leaf structures (trichomes)

Table 3 Presence (+) and absence (−) of *Atta sexdens rubropilosa* workers exploring *Virola sebifera* trichomes

Colony	Trichomes	Time (min)			
		0	15	30	45
1	J	+	+	+	+
	S	−	−	−	−
2	J	+	+	+	+
	S	−	−	+	+
3	J	+	+	+	+
	S	−	−	+	+
4	J	+	+	+	−
	S	−	−	−	−

Trichomes removed from young leaves (Treatment 3) J, Trichomes removed from senescent leaves (Treatment 6) S

old trichomes, irrespective of their position in the foraging arena (Table 3).

After 24 h, senescent leaves with few trichomes remained in the foraging arena. The other leaf fragments were carried and incorporated to symbiotic fungi. Trichomes were still left in the foraging arena. However, due to the small size and color of the structures, it was not possible to visualize their presence or absence on the symbiont under a stereo microscope.

Discussion

In this study, it could be observed that *Atta sexdens rubropilosa* workers prefer to carry young *V. sebifera* leaves with few trichomes and showed interest only in young trichomes, ignoring the trichomes of senescent leaves.

The slow speed of carrying the samples may be explained by the conditions of the colonies. Although the colonies had been fasted for 24 h, all had substrate incorporated into the symbiotic fungus from previous feeding. However, characteristics such as fungal volume and estimated number of ants were the same for all colonies. Thus, although the colonies used had been standardized and kept under laboratory conditions, each possessed its own individual characteristics. This obviously influenced the carrying of leaves, as observed in field colonies, which can also vary in activity, being more or less active.

Differences were observed in the compounds present between trichomes of different ages as well as in compounds adhered to them, which could have explained whether the samples were carried to colonies. Analysis of the superfície foliar identified hentriacontane (C31) as the predominant compound in both young and senescent leaves. When comparing ants' loading of these leaves, there was no preference for workers of *A. sexdens rubropilosa*, both were loaded and incorporated into the symbiotic fungus (Carlos 2013). In that study, triacontane (C30) was the major chemical compound in young leaves and dotriacontane (C32) in senescent leaves. In contrast, in the present study, a higher concentration of triacontane (C30) was detected in the trichomes of young leaves and of tetratriacontane (C34) in those of senescent leaves.

In plants, the biosynthesis of fatty acids involves a complex enzymatic system in which hydrocarbons are reduced and oxidized, resulting in saturated or unsaturated short-, medium- and long-chain fatty acids. Chemical analysis of the trichomes revealed the presence of these intermediates, with the detection of alcohols, aldehydes and ketones (Tables 2, 3). Substances such as lignans, sesamin, hinokinin and kusunokinin have been detected in crude methanol extracts of *V. sebifera*, in addition to flavonoids in ethyl acetate extracts. The methanol extracts exhibited insecticidal activity against *A. sexdens rubropilosa* (Bicalho et al. 2012). In general, trichomes are described as structures with negative effects, which release exudates that negatively affect insects when they come in contact with the plant (Kogan 1976), thus reducing or impairing the consumption of leaves (Schillinger and Gallum 1968; Singh et al. 1971) by physically damaging insects (Pillemer and Tingey 1978).

For leaf-cutting ants, thick trichomes impair the cutting of leaves (Oliveira et al. 2002). In the present study, workers carried a significant number of young leaves without trichomes. At the beginning, workers whose bodies were impregnated with these trichomes exhibited an a priori cleaning behavior, but accepted these structures over time and apparently did not bother to carry them intentionally, or when they were exploring other samples. On experiments carried by Oliveira et al. (2002), no selectivity was observed in a preference study of *Atta bisphaerica* testing plants of eight grass species and one legume species. The authors explained this finding by the absence of trichomes or, when present, that they were very thin. As mentioned earlier, trichomes are thin structures in *Virola sebifera* and, according to these authors, therefore, probably acting as a physical barrier but not influencing plant choice negatively.

However, both the chemical profile and worker behavior suggest that trichomes contribute to attractiveness of plants, regardless of the trichome morphology. The bodies of workers were impregnated with the trichomes of young leaves, but they avoided trichomes collected from senescent leaves, which are morphologically similar. However, senescent leaf trichomes are more damaged than those of young leaves, and the presence of adhered materials is much higher, including fungi hyphae, which could negatively affect both the ant and the fungus that it grows (Fig. 4). Thus, the structural characteristics of both types of trichomes were similar, while the chemical compounds were different, possibly influencing the foraging of these structures by workers.

In addition, worker ants preferentially carried young leaves with few trichomes, possibly to avoid overloading the ants and/or symbionts with a given chemical compound. Another explanation would be the release of compounds that render these leaves more attractive when compared to other treatments. In the first case, the chemical compounds identified as hexadecanoic and heptadecanoic acids are toxic when present in excess, especially to ants (Levin 1987).

However, this could be a very complex response, with workers first evaluating the quality and ideal dosage of compounds on a given substrate. The second, and more likely, hypothesis is that when the trichomes were extracted, even with the greatest possible care, the leaves under this stress released volatile substances of young plants that were perceptible at low concentrations and that could be attractive compounds for the workers. It has already been described that these compounds may indicate the presence of a plant being damaged by herbivores and therefore can also be used by herbivores themselves to locate suitable hosts (Heil 2014). In addition, trichomes collected from senescent leaves contained chemical compounds such as dioctyl ether, 2-hexacosanone and 1-heptacosanol. These compounds result from reactions with hydrocarbons and may be unattractive for workers, with the result that the trichomes are therefore not carried.

In this respect, the presence of some chemical compounds may render the plants attractive to leaf-cutting ants so that they are carried, as observed for leaf fragments with few trichomes, and may cause the exploration of trichomes present on young leaves. It should be noted that this study did not characterize the volatile compounds released by the plants suffering mechanical damage (standardized cutting of leaves offered to the ants) and after the stress caused by removal of the trichomes. These factors probably exerted an important influence on the choice of foraged material.

In conclusion, this study shows how chemical compounds are involved in the selection of plant material, since morphologically similar, but chemically different, trichomes elicit different behavioral responses during foraging of these structures by workers. This occurs even in non-glandular trichomes, such as those of *V. sebifera*, since these structures allow the retention of many substances that adhere to them, whether they originate from the plant itself or from the environment in which it is found. We suggest that a set of factors, i.e., the leaf as a whole including nutrients, volatile compounds and attractive hydrocarbons, define whether or not a plant material is cut and carried by leaf-cutting ants.

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