

# Attraction of entomopathogenic nematodes to sugarcane root volatiles under herbivory by a sap-sucking insect

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**Abstract** Few systems have been described in which herbivore-induced root volatiles mediate attraction of entomopathogenic nematodes (EPNs), and they only concern root damage inflicted by chewing insects. EPNs, especially *Heterorhabditis indica* and *Steinernema carpocapsae*, are potential biological control agents of sugarcane spittlebug (*Mahanarva fimbriolata*) populations. Here, we investigated the response of these two species of EPNs to sugarcane root volatiles damaged by *M. fimbriolata* nymphs in a belowground six-arm olfactometer. We also examined changes on root volatile profile in response to herbivory of sugarcane spittlebug nymphs. Results showed that both EPN species did not discriminate between odors of undamaged sugarcane and moistened sand (blank). However, when EPNs were exposed to odors of spittlebug-damaged and undamaged sugarcane roots, both species significantly preferred odors of spittlebug-

damaged roots. Headspace collection followed by GC–MS analyses showed no qualitative difference (total of 11 compounds) between volatile profiles of spittlebug-damaged and undamaged sugarcane roots. In contrast to the previous studies involving feeding by root chewing insects, our root volatile analysis did not reveal any up-regulation resulting from sugarcane spittlebug damage, but the down-regulation of the terpenes dihydromyrcenol and  $\beta$ -isomethyl ionone when compared with the profile of undamaged sugarcane roots. Here, we propose alternative explanations for the EPN attraction to spittlebug-damaged roots as it is unlikely that reduced concentrations of the volatiles play a role in this interaction. Further studies are necessary to determine the key compounds of the root volatile emission to enhance biological control efficacy with EPNs against *M. fimbriolata* in sugarcane.

**Keywords** Host search · *Mahanarva fimbriolata* · Plant defenses · Soil insects · Spittlebug · Tritrophic interaction

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## Introduction

Plants defend themselves from herbivory by a broad spectrum of physical and chemical defenses that act directly on insect herbivores, or indirectly by emitting volatiles exploited by natural enemies as cues to locate their host/prey (Turlings et al. 1990; Dicke 1994; Turlings and Wäkers 2004). Attraction of parasitoids and predators to herbivore-induced plant volatiles (HIPVs) under attack of several feeding guilds of insect (e.g. chewing, sucking, mining, galling) has been intensively studied (Turlings et al. 1997; Van Poecke et al. 2001; Birkett et al. 2003; Zhu and Park 2005; Girling et al. 2006; Mumm and Hilker

2006; Tooker and Hanks 2006; De Boer et al. 2008). As a result, HIPVs have been regarded as a widespread defense mechanism in the plant kingdom, but their defensive role has been discussed as they also mediate interactions with other community members, attracting or repelling herbivore species (Bernasconi et al. 1998; Landolt et al. 1999; De Moraes et al. 2001; Carroll et al. 2006; Kaplan 2012).

Root tissue is also susceptible to herbivore attack by soil insects, which can compromise the uptake of nutrients and water. In a similar way to aboveground tritrophic interactions, roots under attack of soil herbivores emit a different volatile blend, which is exploited by soil-dwelling natural enemies, such as entomopathogenic nematodes (EPNs) (Rasmann et al. 2005), in host finding. These organisms penetrate into the insect, kill it with the aid of symbiotic bacteria, and feed on the proliferating bacteria as well as the decomposing insect cadaver (Strauch and Ehlers 1998).

In contrast to the vast knowledge on aboveground plant volatile-based interactions (reviewed by Mumm and Dicke 2010; Dicke and Baldwin 2010; Hare 2011), few systems have been described in which herbivore-induced root volatiles mediate the attraction of EPNs and little is known about the nature of these interactions. Recruitment of EPNs to plant root volatiles has been demonstrated in different plant species including crop plants, such as strawberry (Boff et al. 2002), corn (Rasmann et al. 2005), cabbage (Ferry et al. 2007), cotton (Rasmann and Turlings 2008) and citrus (Ali et al. 2010), and non-crop species, such as the conifer *Thuja occidentalis* L. (Van Tol et al. 2001) and common milkweed *Asclepias syriaca* L. (Rasmann et al. 2011). However, these studies focused on the root damage inflicted only by beetle larvae, which are chewing insects.

Physical injury inflicted by sap-sucking insects is almost imperceptible, unlike chewing insects that rapidly remove plant tissue and usually cause damage faster. Understanding of aboveground plant defenses indicates that plant recognition of herbivory is due not only to physical damage, but also to contact with insect-derived signals, the herbivore-associated molecular patterns (HAMPs) (Mithöfer and Boland 2008). As a result, sap sucking differently activates signal transduction pathways dependent on jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) compared to chewing insect damage (Walling 2000; Thaler et al. 2012; Lu et al. 2014). The balance of these three main phytohormones along with others will determine the expression of appropriate and specific defenses against herbivores (Erb et al. 2012b). Therefore, plant volatile composition is usually distinct under attack of chewing or sap-sucking insects (Leitner et al. 2005; Gosset et al. 2009), resulting in different responses of community members (Van Poecke et al. 2003).

So far, no studies have examined induced root response under attack by soil-dwelling insects that pierce and suck

phloem and xylem content, such as some species of aphids, leaf hoppers, stink bugs, and scales (Khan and Saxena 1984; Powel and Hardie 2002). Besides, according to the differences between the two plant parts (Erb et al. 2012a) and the fact that HIPV emission is tissue specific (Köllner et al. 2008), we cannot speculate about the mechanisms of recognition and response in roots based on the knowledge of aboveground-induced defenses.

Populations of the sugarcane spittlebug *Mahanarva fimbriolata* (Stål) (Hemiptera: Cercopidae) have greatly increased and become a serious pest in Brazilian sugarcane crops after prohibiting sugarcane harvest with the use of burning. Sugarcane spittlebug adults feed on and inject toxins into leaves, whereas nymphs live in the soil and specially suck the xylem content in the roots, blocking the transport of water and nutrients and causing a physiological disorder (Dinardo-Miranda et al. 2004; Garcia et al. 2007).

Since sugarcane is a perennial crop, not often perturbed, the use of EPNs to control the sugarcane spittlebug can be a potential management strategy (Southwood and Comins 1976). Particular strains of *Heterorhabditis indica* (Poinar, Jackson and Klein) (Rhabditida: Heterorhabditidae) and *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) have been selected as efficient biological control agents of sugarcane spittlebug populations (Leite et al. 2005). These species exhibit different foraging strategy: *H. indica* actively seeks out a host by crawling in the soil (cruiser), while *S. carpocapsae* is less active and usually waits for hosts passing by (ambusher) (Lewis 2002; Campbell et al. 2003; Lewis et al. 2006), although it can also behave like a cruiser (Wilson et al. 2012).

Here, we investigate the response of the two species of EPNs *H. indica* and *S. carpocapsae* of the same strain studied in Leite et al. (2005) to sugarcane root volatiles damaged by *M. fimbriolata* nymphs. We also examine changes in root volatile profile in response to sugarcane spittlebug herbivory. Knowledge on the cues used by herbivore natural enemies, such as EPNs, is of great relevance to develop integrated pest management techniques to enhance the biological control efficacy of soil-dwelling insects (Degenhardt et al. 2009; Hiltbold et al. 2010; Ali et al. 2012; Hiltbold and Turlings 2012).

## Materials and methods

### Plants

Sugarcane plants (*Saccharum officinarum* L. cultivar ‘SP 80-1842’) were grown in pots (200 ml) containing organic substrate (Golden-Mix<sup>®</sup>) and fertilizer (Osmocote<sup>®</sup>, 14-14-14 N-P-K) in an insect-free greenhouse from summer to autumn under natural light conditions (Piracicaba, SP,

Brazil). All plants used in bioassays had four opened leaves and were approximately 25 to 30 days old.

### Insects rearing

Nymphs of *M. fimbriolata* were collected in sugarcane crops in Piracicaba, SP, Brazil (22° 43' 14" to 22° 42' 01" S and 47° 38' 46" to 47° 36' 49" W) and reared on sugarcane plants under controlled conditions (25 ± 0.5 °C, 70 % RH, 14L:10D) (Garcia et al. 2007) for two to four generations. Briefly, *M. fimbriolata* adults were kept in cylindrical plastic cages containing sugarcane plants with wet cotton wool disks covering the soil, which served as a substrate for oviposition. After washing the cotton in running water over sieves, eggs were collected and maintained in Petri dishes on wet filter paper until hatching. Newly hatched nymphs were transferred to sugarcane roots and fed for 40 days until becoming adults.

### Nematodes

Populations of EPNs *H. indica* and *S. carpocapsae* were obtained from the Laboratory of Biological Control (Instituto Biológico, Campinas, SP, Brazil) and multiplied in the greater wax moth *Galleria mellonella* (L.) at 25 ± 0.5 °C, according to the method described in Kaya and Stock (1997). Infective juveniles (IJs) emerging from caterpillars in White traps (White 1927) were collected in distilled water and transferred to pots (1 L) at volumes just enough to cover the bottom (shallow). The IJs were then stored at 15 °C for 4–6 days prior to use.

### Olfactometer assays

Nematode preference of both *H. indica* and *S. carpocapsae* toward odors emitted by damaged sugarcane roots (five fourth-to-fifth-instar spittlebug nymphs feeding on roots for 24 h and then removed just before assays) and undamaged sugarcane roots was assessed in a six-arm olfactometer, consisting of a central glass chamber (10 cm diameter, 8 cm depth) with six arms connected to side chambers (5 cm depth, 3 cm diameter) (Rasmann et al. 2005). Four days before bioassays, sugarcane plants were transferred to the olfactometer side chambers, and the remaining space was filled with a mixture of sterile sand and rock (2:1) and moistened with 10 % water (dry sand:water; g/g). Each side chamber containing wet soil and plants was weighed before being transferred to the greenhouse. To maintain the moisture content at approximately 10 % during the 4 days before the bioassay, the chambers were weighed daily and an appropriate volume of water was added to return it to its initial weight (Santos et al. 2014). Positions of chambers containing damaged sugarcane roots were interchanged

with undamaged roots to avoid side bias. About 10,000 IJs were released into the central chamber, where they could freely choose among the chambers for over 24 h. After that, the olfactometer was disassembled, sand was collected from each detachable glass tube connecting the side chambers to the central arena, and nematodes were recovered in water by a modified Baermann funnel technique (Viglierchio and Schmitt 1983). After 24 h, 0.30 ml of water was collected to estimate the amounts of IJs using a McMaster counting slide. A total of eight replicates with more than 2000 recovered IJs were considered. Five and four replicates were discarded in bioassays with *S. carpocapsae* and *H. indica*, respectively.

### Headspace collection and analysis

Roots of nine undamaged and damaged sugarcane by *M. fimbriolata* nymphs were harvested and flash frozen in N<sub>2(1)</sub> and stored at −30 °C prior to headspace collection (Rasmann et al. 2005; Rasmann and Turlings 2008). To collect root volatiles, 2 g of frozen roots was ground into a powder in liquid nitrogen and transferred to 500-ml tightly closed chambers with two exits: one connected to a glass column filled with 50 mg of adsorbent polymer (Hayesep-Q, 80/100 Mesh, Alltech Assoc.) and the other to a charcoal filter. A vacuum pump was connected to the adsorbent polymer column, creating an air flow for 8 h. Subsequently, columns were eluted with 300 µl of dichloromethane and concentrated under clean nitrogen air flow to 50 µl. Each sample received 10 µL of nonyl acetate (internal standard solution at 100 ng/µL). A 2-µl aliquot of each sample was injected into a gas chromatograph coupled to mass spectrometer (GC–MS, Varian 4000) equipped with HP5-MS capillary column (JeW Scientific, Folsom, CA; 30 m × 0.25 mm × 0.25 µm), using helium as the carrier gas. The column temperature was held at 40 °C for 5 min, increased to 150 °C (5 °C/min) and held for one min, and then raised (5 °C/min) until reaching a final temperature of 250 °C. Compound identifications were acquired by comparing the obtained mass spectra retention times with those of the NIST 98 library and authentic standards (Sigma-Aldrich, St. Louis, MO, USA), when available, as well as the Kovats index (KI) using *n*-alkane (C<sub>7</sub>–C<sub>30</sub>) standards (Table S1). Quantification was estimated based on the peak area relative to the amount of internal standard and corrected by fresh root tissue used for the volatile collection.

### Statistical analysis

The normality and homogeneity of the number of nematodes recorded in the six-arm olfactometer assay and the relative amounts of each root volatile released by damaged

or undamaged sugarcane roots were analyzed by Shapiro–Wilk and Bartlett tests. We adopted a general log-linear model (glm) ( $P < 0.05$ ) and quasi-Poisson distribution for analyzing nematode choice assessed in olfactometer bioassay by one-way ANOVA. Relative amounts of volatiles were log-transformed, when required, to attend parametric assumptions. The composition of the volatile blend was analyzed by MANOVA ( $P < 0.05$ ) and individual compounds were analyzed using Student's test ( $P < 0.05$ ) when data were normal, whereas non-normal data were analyzed by Welch test ( $P < 0.05$ ). All analyses were performed using software package R 3.1.0 (R Development Core Team 2012).

## Results

Infective juveniles of both EPN species equally chose undamaged sugarcane roots and blank (only moistened sand) (Fig. 1, glm,  $N = 8$ ; *S. carpocapsae*:  $F_{(1, 14)} = 0.08$ ,  $P = 0.776$ ; *H. indica*:  $F_{(1, 14)} = 0.02$ ,  $P = 0.882$ ). However, they preferentially chose volatiles emitted by spittlebug-damaged over undamaged sugarcane roots (Fig. 1, glm,  $N = 8$ ; *S. carpocapsae*:  $F_{(1, 14)} = 6.29$ ,  $P < 0.05$ ; *H. indica*:  $F_{(1, 14)} = 9.21$ ,  $P < 0.01$ ).

Root volatile collection and analysis revealed that undamaged and spittlebug-damaged sugarcane roots emitted blends with similar composition (Fig. 2, MANOVA,  $N = 9$ ,  $P = 0.39$ ). Nevertheless, when compounds were examined individually, we found differences in amounts of dihydromyrcenol and  $\beta$ -isomethyl ionone, which were emitted at higher concentrations by undamaged sugarcane roots relative to spittlebug-damaged sugarcane root blend (Fig. 2, One-way ANOVA,  $N = 9$ ; dihydromyrcenol: Welch's test,  $t = 2.46$ ,  $P = 0.036$ ;  $\beta$ -isomethyl ionone: Student's test,  $t = 2.26$ ,  $P = 0.037$ ).

## Discussion

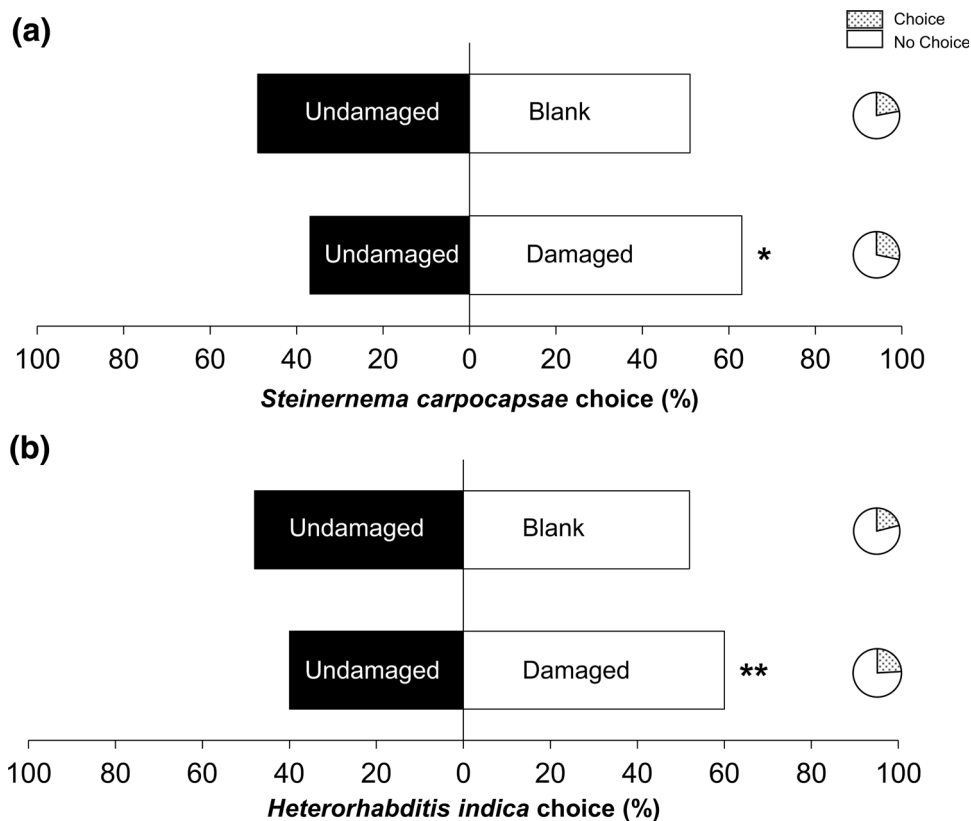
Our study confirms the previous findings showing that EPNs, irrespective of the foraging strategy (ambusher or cruiser), use herbivore-damaged root volatiles in host finding (Van Tol et al. 2001; Rasmann et al. 2005; Ali et al. 2010, 2011; Hiltbold et al. 2010). The most studied systems in the literature, corn and citrus, show that root feeding by beetle larvae mainly triggers the emission of terpenes, (*E*)- $\beta$ -caryophyllene, and pregeijerene, which mediate the attraction of EPNs (Rasmann et al. 2005; Ali et al. 2011, 2012). In contrast to these studies, our root volatile analysis did not reveal any up-regulation resulting from sugarcane spittlebug damage, but the down-regulation of the terpenes dihydromyrcenol and  $\beta$ -isomethyl

ionone when compared to the profile of undamaged sugarcane roots.

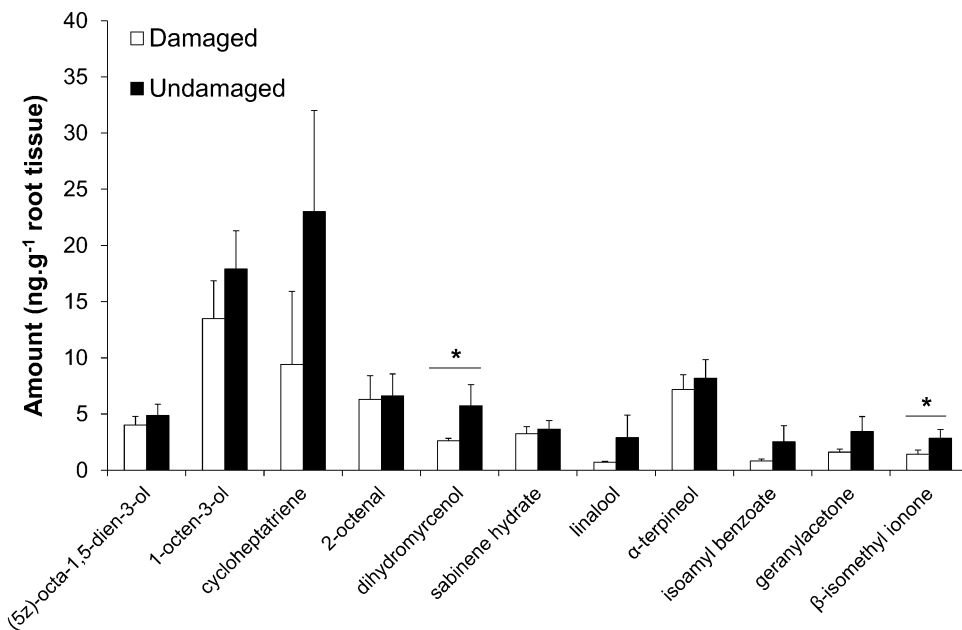
To collect sugarcane root volatiles, we performed a headspace collection with flash-frozen and ground root tissues, a method adapted from some classical studies in the literature (Rasmann et al. 2005; Rasmann and Turlings 2008). Ideally, root headspace collection would be performed without excising and grinding the roots and some researchers have developed such a method (Ali et al. 2011; Hiltbold et al. 2011; Eilers et al. 2015; Rostás et al. 2015). We tried collecting root headspace without grinding the tissue, but only collected detectable amounts in the GC–MS analysis using ground root tissue. Although our data did not likely reveal the exact belowground volatile emission in sugarcane since we collected volatiles from ruptured root cells, the method allowed us to compare the blend composition between the spittlebug-damaged and undamaged sugarcane roots. We found 11 volatile metabolites in the blends of both treatments. Besides dihydromyrcenol and  $\beta$ -isomethyl ionone, which were significantly reduced in the spittlebug-damaged sugarcane, we observed a trend of reduced levels of the terpenes linalool and geranylacetone, and the ester isoamyl benzoate in the spittlebug-damaged compared with undamaged roots (Fig. 2).

Feeding by hemipterans includes the secretion of salivary proteins that interact in the dynamics of induced plant defense (Sharma et al. 2014). Although attack by hemipterans on aerial plant parts often induces the emission of HIPVs (Du et al. 1998; Ninkovic et al. 2001; Birkett et al. 2003; Williams et al. 2005; Oluwafemi et al. 2011), some of the salivary components are involved in the strategies of manipulating plant defenses (Walling 2008; Felton et al. 2014). In some particular cases, feeding by sucking insects can either suppress or simply not elicit emission of HIPVs (Turlings et al. 1997; Schwartzberg et al. 2011). For example, the infestation of *Philaenus spumarius* (L.) (Hemiptera: Cercopidae), a spittlebug closely related to *M. fimbriolata*, does not elicit any volatile response in the late goldenrod (*Solidago altissima* L.) (Tooker et al. 2008), while herbivory by the tobacco budworm *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae) does. The underlying mechanism for not eliciting or suppressing induced volatile response, as in the example of the late goldenrod and our study system, can result from the activation of the SA-signaling pathway, which can interact negatively with the JA-signaling pathway (Zhang et al. 2009; Ali and Agrawal 2014). Despite the suppressing effect on JA-related defenses, activation of SA-signaling by sap-sucking herbivory can induce the release of specific herbivore-induced plant volatiles, which are exploited by natural enemies (Zhang et al. 2013).

**Fig. 1** Response of entomopathogenic nematodes to sugarcane root volatiles released by plants damaged by *Mahanarva fimbriolata*, undamaged, or only moistened sand (Blank) in a six-arm olfactometer. **a** *Steinernema carpocapsae*. **b** *Heterorhabditis indica*. Pie charts in the right side of the figure show proportions of responsive (choice) and non-responsive (no choice) nematodes in the assay. Asterisks indicate significant difference between treatments according to one-way ANOVA (quasi-Poisson glm, \* $P < 0.05$  and \*\* $P < 0.01$ )



**Fig. 2** Emissions of volatile compounds from sugarcane undamaged and damaged roots by *Mahanarva fimbriolata*. Bars represent mean amount  $\pm$ SE ( $N = 9$ ). Asterisks indicate significant difference between treatments according to Student's test ( $P < 0.05$ ) or Welch's test ( $P < 0.05$ )



Sugarcane spittlebug nymphs seem to be a prey that can be easily found by natural enemies as they stay in the same spot sucking the xylem and phloem content for 30–40 days (Garcia et al. 2007). One would expect that lower volatile emission caused by sugarcane spittlebug feeding on

sugarcane roots would be an adaptive strategy to becoming less detectable, therefore reducing chances of being found by natural enemies. Nonetheless, this hypothesis was not supported by our behavioral assays, which demonstrated that EPNs oriented themselves toward spittlebug-damaged



over undamaged roots, despite the reduced emission of some components (Figs. 1 and 2).

EPNs possibly do not exploit reduction of the two volatiles, especially because they did not closely evolve with sugarcane plants. Therefore, we propose two alternative explanations, not mutually exclusive: (i) EPNs are guided by increased concentrations of volatiles emitted from spittlebug-damaged sugarcane roots; however, up-regulation of specific compounds was not detected by the root volatile collection method adopted here; (ii) EPNs are attracted to spittlebug-damaged sugarcane roots because of a synergistic effect between root volatiles and increased carbon dioxide (CO<sub>2</sub>) concentration in a similar way to what has been shown for the attraction of *Heterorhabditis megidis* (Poinar, Jackson and Klein) to (*E*)- $\beta$ -caryophyllene and dimethyl disulfide (Turlings et al. 2012). Besides, we cannot discard the hypothesis that EPNs could have been attracted to residual odors left by spittlebug nymphs in damaged sugarcane roots, such as cues derived from the foam. Although we removed insects and foam, some could have been absorbed by the sand.

To the best of our knowledge, this is the first study that focused on belowground plant indirect defenses induced by sucking insects. Studying soil-dwelling insects is challenging and is one of the main reasons why belowground plant defenses have not been explored in detail as the aboveground environment. Unlike some EPN strains that do not respond to increased amounts of root volatiles (Anbesse and Ehlers 2013; Laznik and Trdan 2013), results show that our strains of *H. indica* and *S. carpocapsae* exploit changes on root volatile emission induced by spittlebug nymph feeding in host finding. However, it is unlikely that reduced concentrations of the terpenes dihydromyrcenol and  $\beta$ -isomethyl ionone in the blend potentially play a role on the discrimination of EPNs between spittlebug-damaged and undamaged sugarcane roots. As in any other study, it is possible that volatile compounds at undetectable amounts for the GC-MS analysis are exploited by EPNs. In contrast to previous studies involving feeding by root chewing insects (Rasmann et al. 2005; Ali et al. 2010), determining the key compound of the root volatile emission used as cues by EPNs to find a sucking insect, the sugarcane spittlebug, is a more complex task. As a result, further study is necessary before exploring root volatile emission to enhance biological control efficacy with EPNs against the spittlebug *M. fibriolata* in sugarcane.

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## Compliance with ethical standards

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**Conflict of interest** The authors declare that they have no conflict of interest.

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