Exposure to sugarcane borer-induced plant volatile (*E*)-caryophyllene enhances parasitoid recruitment

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Short title: (*E*)-caryophyllene enhances parasitoid recruitment

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Keywords: Sugarcane, tritrophic interaction, *Diatraea saccharalis*, *Cotesia flavipes*, HIPVs

Abstract

Natural enemy parasitoids locate herbivore-infested plants via detection of herbivore-induced plant volatiles (HIPVs) that are released in response to pest damage. Furthermore, synthetic HIPVs have been proposed as tools to enhance the biological control of crop pests. The sugarcane borer, *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae), is a key herbivore pest of sugarcane, *Saccharum* spp. (Andropogoneae), in the Americas. To manage *D. saccharalis* in Brazil, more than 3 million ha of sugarcane have been treated with the larval parasitoid, *Cotesia flavipes* (Hymenoptera: Braconidae). In this study, the role of sugarcane HIPVs as cues in the host-finding process of *C. flavipes* was investigated using a combination of dynamic headspace collection, chemical analysis and laboratory behaviour experiments. Comparison of volatile organic compounds (VOCs) collected from *D. saccharalis*-damaged and healthy sugarcane revealed very similar VOC profiles apart from significantly higher levels of the sesquiterpene (*E*)-caryophyllene released from damaged plants. Naïve female *C. flavipes* spent significantly more time in the olfactometer arm containing VOCs from *D. saccharalis*-damaged plants but showed no preference to VOCs from healthy plants. When (*E*)-caryophyllene was added to VOCs from healthy plants, parasitoids spent more time in the arm containing the combined treatment. Furthermore, in a dose-response experiment with synthetic (*E*)-caryophyllene, naïve parasitoids preferred the compound across a dose range of 3-300 ng, and experienced parasitoids (pre-exposed to (*E*)-caryophyllene) responded to doses of (*E*)-caryophyllene as low as 0.03 ng. These results suggest that *C. flavipes* can use (*E*)-caryophyllene as a cue to locate *D. saccharalis*-infested sugarcane plants. Moreover, experienced females appear to respond to lower doses than naïve females. These results potentially pave the way for increasing the efficiency of *C. flavipes* in biological control of *D. saccharalis*, the most important pest of sugarcane and maize crops in the Western Hemisphere, and also a major pest for rice and sorghum crops.

Introduction

Plants have evolved multiple ways to defend themselves against insect pests, including the recruitment of other organisms to reduce insect pressure ie. indirect defense (Heil, 2008). It has been well documented that plant volatiles released from herbivore-damaged plants are used by host-searching parasitoids as they predict host presence, but since volatiles have a plant origin, they are less reliable as a cue for the foraging of parasitoids than host cues (Vet et al, 1995: Turlings et al., 1990; Vet & Dicke, 1992). Insect attack triggers plant defense, leading to a systemic release of a blend of herbivore-induced plant volatiles (HIPVs) that makes plants attractive to natural enemies of the herbivores (Turlings & Erb, 2018). HIPVs have the potential to be exploited in biological control of agricultural insect pests, either as direct attractants for natural enemies, inducers of crop defence to increase their attractiveness to natural enemies, as targets for breeding or genetic engineering of crop plants, or as targets for companion cropping (Turlings and Erb, 2018). Attempts to use HIPVs, for natural enemy recruitment have been increasingly explored in recent years with some success eg companion cropping for management of pests on cereals (Pickett and Khan, 2016).

Sugarcane, *Saccharum officinarum L.* (Poaceae), is a global commercial agricultural crop, producing approximately 70% of the world's sugar, and is increasingly being used to generate a range of non-food products, particularly bioethanol and biomass electricity (Johnson & Seebaluck, 2013). Despite high yields, sugarcane production in Brazil is still impaired by a wide array of biotic and abiotic stresses, with insects being the main cause of economic loss (Silva et al., 2012). Of the insect pests that affect sugarcane crops, the sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Pyralidae), is the most important in Brazil and the rest of the Western Hemisphere. Damage caused by larvae may occur during all developmental stages of the plant, with young larvae feeding on leaves and mature larvae boring into the stalks. This direct damage facilitates the entrance of secondary phytopathogenic fungi such as *Colletotricum*

falcatrum and *Fusarium subglutinans* (Hughes et al., 1964). The combination of insect damage and pathogen infection causes significant losses in yield, quality and sugar content (Ogunwolu et al., 1991).

The stem-boring behaviour of *D. saccharalis* makes control of this pest difficult to accomplish as protection of sugarcane using synthetic pesticides is ineffective. Various other strategies, including manual removal, development of Bt sugarcane plants (Arencibia et al., 1999), and biological control (Parra, 2014) have been combined in integrated pest management strategies. The main biological control agent for *D. saccharalis* is the gregarious larval endoparasitoid and cenobiont wasp, *C. flavipes* (Cameron) (Hymenoptera: Braconidae) (Rossi, 2003), which is an endoparasitoid of lepidopterous stemborers in different Poaceae crops such as sugarcane, maize, sorghum and other perennial grasses (Nagarkatti & Nair, 1973). Females oviposit into the hemocoel of borers and alter host physiology with venom and polydnaviruses that reduce host immunity, thus allowing larval development (Scaglia et al., 2005). Deployment of *C. flavipes* has been the most efficient biological control strategy in Brazil, being applied across ca. 3.3 million ha of sugarcane (Parra, 2014). Release of *C. flavipes* has been shown to keep *D. saccharalis* infestation at a level of 2-3% (Botelho & Macedo, 2002; Rossi, 2003), which is assumed to be sufficient with respect to the economic damage threshold.

Female *C. flavipes* have been shown to exhibit a preference to odours of stemborer-infested plants of maize (Potting et al., 1995). Although the use of *C. flavipes* to control *D. saccharalis* in sugarcane is one of the most widely used biological control programs, little is known about the response of sugarcane during herbivory and the probable cues in the host-finding process of *C. flavipes*. In this study, we investigated the tritrophic interaction between sugarcane, *D. saccharalis* and *C. flavipes*, along with the composition and role of the blend of HIPVs in mediating *C. flavipes* behavior, with a view to understanding the role of sugarcane HIPVs in *D. saccharalis* biological control.

Materials and Methods

Plant and Insects. Sugarcane plants (cv. SP79-1011) were obtained from one-eyed seed sets by preparing cuttings of stalk containing one bud. The stalk cuttings were planted in 500 mL plastic pots containing a commercial planting mix (Bioplant, Bioplant Misturadora Agricola Ltda, Nova Ponte, MG, Brazil) and manually watered. Plants were further grown under natural light conditions, at 26±5 ºC, and 70±5 % relative humidity in a greenhouse until they were 45 days old and required for infestation / dynamic headspace collections (see below). *Diatraea saccharalis* larvae, *C. flavipes* parasitoids (adults and cocoons) and sugarcane cultures (for use as a seed to plant and carry out experiments) were obtained from the Biological Control Lab of the Sugar Industry Central Açucareira Santo Antonio S.A., Alagoas, Brazil. Young *D. saccharalis* larvae were individually reared in Petri-dishes on an artificial diet (Hensley & Hammond, 1968) and maintained at 26±5 ºC and 70±5 % relative humidity with a photophase of 12 h until required for infestation experiments (see below). *Cotesia flavipes* cocoons were transferred to 100 mL plastic pots and maintained at 26±5 ^{0}C, 70±5% relative humidity with a photophase of 12 h until adult emergence. Adults were sexed according to antennae size (females possessing antennae smaller than their body size) and females were used in olfactometer bioassays (see below).

Infestation of sugarcane plants. Sugarcane plants were infested with three second-instar *D. saccharalis* larvae (seven days post-hatch) that bored into the internode region of stems. Larvae were starved for 24 h prior to infestation to ensure complete boring into the base of the leaf sheath. Cylindrical plastic cages were used to confine the larvae and prevent them from escaping. Immediately after boring commenced, volatile organic compounds (VOCs) were collected by dynamic headspace collection (see below).

Dynamic Headspace Collection. Volatile organic compounds (VOCs) were collected from *D. saccharalis*-damaged and healthy sugarcane plants by dynamic headspace collection for a period of 48 h. The VOCs were trapped on either Tenax TA resin (60/80 mesh, 0.05 g, Supelco) or Porapak Q (80/100 mesh, 0.05 g, Supelco) as adsorbent. For Tenax TA collections, plants were placed in sealed glass chambers and charcoal-filtered air was admitted at the bottom of the vessel. The absorbent was contained in a glass GC inlet between glass-wool plugs and inserted in the top of the chamber, and air was drawn through the tube at a rate of 600 ml/min. VOCs were collected during three collections periods; 0-12 h, 12-24 h and 24-48 h. Once collections were complete, Tenax tubes were sealed in glass ampoules under nitrogen and stored at -20 ºC until required for GC-MS analysis. The total number of Tenax collections from *D. saccharalis*-damaged and healthy plants was 22 plants, ie. 11 damaged and 11 healthy plants. For Porapak Q collections, plants were placed in polyester bags as described by Stewart-Jones and Poppy, (2006), isolating the soil pot with aluminum foil. Air filtered through activated charcoal was pumped into the plastic bag at a flow of 600 ml/min per plant. Pyrex tubes with Porapak Q were inserted at top of the bag and the air was drawn at a flow rate of 400 ml/min for each plant. VOCs were collected continuously for 48 h. Once Porapak Q collections were complete, tubes containing trapped VOCs were desorbed using bidistilled HPLC grade hexane (500 µL) and eluted samples were stored at -20 ºC in tightly capped microvials until required for chemical analysis by GC-MS. Following GC-MS, individual samples were then pooled for quantification experiments and olfactometer bioassays. The total number of VOC collections from *D. saccharalis*-damaged and healthy plants was 12 each (three plants x four collections).

GC / GC-MS Analysis and (E)-Caryophyllene Quantification. VOCs collected from sugarcane plants and trapped onto Tenax TA were analyzed by gas chromatography (GC) using an Agilent 6890 GC instrument fitted with a non-polar HP-1 capillary column (J & W Scientific supplier,

50 m x 0.32 mm i.d. x 0.52 µm film thickness) and a flame ionization detector (FID). The carrier gas was hydrogen (flow 0.85 mL/min). VOCs were transferred onto the HP-1 column by inserting a Tenax tube into a programmable temperature vaporization (PTV) inlet (Anatune, Cambridge, UK) that was programmed to heat ballistically from 30ºC to 220ºC in 12 sec. The oven temperature programme was set to commence at 30ºC, maintained for 1 min, and then set to rise at 5ºC min^{-1} to 150ºC, then 10ºC min^{-1} to 230ºC and maintained for 20 min. VOCs collected from sugarcane plants and trapped onto Porapak Q were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GCMSQP 2010 Ultra instrument fitted with two columns of different polarities, Rtx-1 (60 m X 0.25 mm id x 0,25 µm film thickness) and DB-5 (J & W, 30 m x 0.25 mm id x 0,25 µm film thickness). Helium was used as the carrier gas (flow 1.2 mL/min). The samples were injected into a splitless injector at the temperature of 200 ºC. The oven temperature was set to commence at 50ºC and rise to 300ºC at a rate of 8ºC min^{-1}, with the injector and detector temperatures set at 250ºC. Tentative identification of (*E*)-caryophyllene and limonene in the Porapak Q VOC extracts by GC-MS analysis was confirmed by GC peak enhancement on two GC columns of differing polarity (Rtx-1 and DB-5), with the VOC extracts being co-injected with authentic standards (Sigma-Aldrich Co, St Louis, USA) that were diluted in HPLC grade bi-distilled hexane. The collected samples were analyzed individually by GC - MS and then pooled for quantification experiments and bioassays. For quantification of (*E*)-caryophyllene in the pooled Porapak Q sample, a calibration curve was prepared using an authentic standard diluted in HPLC grade bi-distilled hexane. GC Injections were carried out in triplicate using standard solutions of (*E*)-caryophyllene at the following concentrations: 0.27, 0.55, 1.09, 2.18 and 4.36 ng/µL. The mean peak areas corresponding to (*E)*-caryophyllene were plotted against the respective quantities. The data were adjusted by nonlinear regression to a straight-line equation and the angular coefficient and the point of intersection on the y axis were estimated, obtaining a linear correlation coefficient (r).

These parameters were used together with the line equation to calculate the concentration of (*E*)-caryophyllene in the pooled Porapak Q sample as 0.3 ng/µL.

Olfactometer Bioassays

Olfactometer apparatus*:* Behavioural assays with *C. flavipes* were performed using a Perspex four-arm olfactometer (Pettersson, 1970), lit from above by diffuse, uniform lighting and carried out at 26 ± 2ºC. The bottom of the olfactometer was lined with filter paper (Whatman no. 1). Air was drawn through the four arms toward the center at 300 ml.min^{-1}.

Olfactometry bioassays – general procedure*:* Single naïve or experienced female *C. flavipes* individuals were carefully introduced into the central chamber of the olfactometer with the help of a small brush. For each individually tested female, the time spent in each arm was recorded for a total time of 16 min using OLFA software (Exeter Software). During this time, the olfactometer was rotated 90 degrees clockwise at every 4 min to eliminate directional bias. This procedure was repeated 30 times for each sample tested. Thus, a total of 30 female parasitoids were individually assayed for each experiment. Parasitoids were only used once.

Preparation of experienced parasitoids for learning experiments*:* In order to allow naïve female *C. flavipes* to gain experience to *(E)*-caryophyllene, individual female *C. flavipes* were placed inside a 50-mL Becker cup together with a filter paper containing 1 µL of a 30 ng/µL *(E)*-caryophyllene solution in hexane (prepared as already described diluting appropriate amount with hexane). The Becker cup was then capped with aluminium foil for a total time of 1 minute, after which the experienced female parasitoid (EF) was then removed and introduced into the central chamber of an olfactometer. The olfactory response of EFs to 0.03, 0.3, and 3 ng of (*E*)-caryophyllene was then investigated, with each dose of compound being tested individually.

Odour treatments: The olfactory response of naïve female *C. flavipes* was investigated with the following samples: HIPV extract in hexane (HE), control plant extracts in hexane (CE), CE plus 3 ng (*E*)-caryophyllene in hexane (CE + (*E*)-caryophyllene); and 0.3 ng, 3 ng, 30, 300, and 3 µg (*E*)-caryophyllene, respectively. The response of experienced female parasitoids (EF) was investigated with 0.03 ng, 0.3 ng and 3 ng (*E*)-caryophyllene. All doses of (*E*)-caryophyllene tested were prepared by diluting (*E*)-caryophyllene in appropriate amounts of hexane, such that the following (*E*)-caryophyllene solutions were obtained: 0.003 ng/µL, 0.03 ng/µL, 0.3 ng/µL, 3 ng/µL, 30 ng/µL, and 300 ng/µL. Aliquots (10 µL) of these solutions were used to obtain the different final quantities of (*E*)-caryophyllene (0.03 – 3000 ng). An aliquot (10 µL) of each sample to be tested was added to a filter paper (0.5 x 1cm) and placed in one of the four arms, whereas an aliquot of bidistilled HPLC grade hexane (10 µL) was added to filter paper (0.5 x 1cm) and placed in the three other arms. Thus, for each experiment, one arm was used to release a treatment, while the other three arms were used as solvent controls. The solvent was allowed to evaporate from the filter paper for 30 s prior to starting experiments.

Statistical analysis. To test for differences in VOC quantities a Students t-test was used. To test for differences in parasitoid responses (the mean time spent in the treatment arms was compared with the mean time spent in the control arms) a Wilcoxon one-tailed rank sum test for two groups was used. Both tests were used with the significance level set at 0.01 ($p<0.01$). Statistical analyses were performed using GENES software package (Cruz, 2013).

Results

Sugarcane plants damaged by *D. saccharalis* larvae were found to release detectable levels of plant volatiles. GC analysis followed by quantification using an external standard method showed

that that the mean amount of (*E*)-caryophyllene released per damaged plant during 0-12 h, 12-24 h and 24-48 h collections was 7.50 ng/plant, 5.39 ng/plant and 7.64 ng/plant, respectively. (*E*)-Caryophyllene was not detected in healthy plants (Fig 1). Limonene (stereochemistry not determined) was detected only in healthy plants and not detected in *D. saccharalis*-damaged plants (Fig 2). There was no significant difference in the quantities of other volatiles between *D. saccharalis*-damaged plants and healthy plants and so further analysis of these volatiles was not warranted.

Bioassay data generated using olfactometer assays showed that naive females of *C. flavipes* spent significantly more time in the arm containing VOCs from *D. saccharalis*-damaged sugarcane (HE) compared to the control arms (Fig 3 experiment 1), but there was no difference in the time spent in the arm containing VOCs from healthy sugarcane (CE) and the control arms (Fig. 3 experiment 2). When VOCs from healthy plants were tested in combination with (*E*)-caryophyllene (CE + (*E*)-caryophyllene), *C. flavipes* spent significantly more time in the arm containing the combination than the control arms (Fig 3, experiment 3). Based on the concentration of (*E*)-caryophyllene present in the pooled Porapak Q VOC extract (Fig 2), ie. 0.3 ng/µL, a range of (*E*)-caryophyllene doses from 0.3 ng – 3 µg was assessed for behavioural activity (Fig 3, experiments 4-8). *Cotesia flavipes* spent significantly more time in the arm containing (*E*)-caryophyllene when doses of 3-300 ng were tested (Fig 3, experiments 5-7), but did not spend significantly more time in the arm containing doses of 0.3 ng and 3 µg (Fig 3, experiments 4 and 8). *Cotesia flavipes* (EF) that were pre-exposed to the odour of (*E*)-caryophyllene spent significantly more time in the arm containing the odour of (*E*)-caryophyllene when tested at a range of doses from 0.03 – 3 ng (Fig 3, experiments 9-11).

Discussion

Plants alter their metabolism following attack by herbivorous pests, releasing herbivore-induced plant volatiles (HIPVs) that participate in the recruitment of natural enemies that are able to exploit a diversity of foraging cues (Dicke & Baldwin, 2010). In this study, we investigated the composition of sugarcane volatiles in response to sugarcane borer, *D. saccharalis*, larvae damage and the action of sugarcane HIPVs in the recruitment of its natural enemy, *C. flavipes*. Even though these parasitic wasps have been inundatively released in sugarcane fields to control *D. saccharalis*, knowledge of specific chemical compounds involved in the recruitment of *C. flavipes* might be used to develop better strategies to control infestation by *D. saccharalis* on sugarcane plantations. Here, we have shown that emission of (*E*)-caryophyllene by sugarcane plants is induced by infestation with *D. saccharalis* larvae and that this sesquiterpene is able to attract wasps of *C. flavipes*. Odorants are emitted by other Poaceae species such as several wild maize relatives (Gouinguené et al., 2001), rice (Xiao et al., 2012) and Napier grass (Khan et al., 2007) in response to herbivore damage. It is known that (*E*)-caryophyllene is highly attractive to parasitic wasps and other natural enemies of caterpillars. It is worth noting that the level of (*E*)-caryophyllene emission by *D. saccharalis*-damaged sugarcane plants in the first 12 h after infestation was initiated was similar to the level released by plants 24 h and 48 h after the infestation began. Variation in the levels of limonene were also found between *D. saccharalis*-damaged and control sugarcane plants. Variation in limonene emission from herbivore-damaged plants has also been described for spider-mite infested tomatoes (Kant et al., 2004). Limonene has been associated with a low preference of *Helicoverpa zea* female moths for a wild tomato species (Paudel et al., 2019), and has been described as showing a repellent effect on various insect pests (Afifi et al., 2015; Zarrad et al., 2017; Schlaeger et al., 2018). The absence of limonene in *D. saccharalis*-damaged sugarcane plants in our study may result in reduced natural enemy repellency, however further studies are necessary to verify the effect of this volatile

compound on *C. flavipes* behaviour. Terpenoids such as (*E*)-caryophyllene and limonene are very often among the most reported bioactive compounds in plant-insect interactions (Aharoni et al., 2005) with high variability in their emission among different plant species and genotypes (Degenhardt et al., 2009). Terpenoids are a large and diverse class of compounds broadly found in plants and insects, and as secondary metabolites in plants they are frequently involved in environmental adaptation and stress tolerance. Biosynthesis of terpenoids *in planta* begins with the five-carbon precursor units isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). Based on the number of isoprene units, terpenes can be classified as monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes and higher terpenes. (*E*)-Caryophyllene is a sesquiterpene that is obtained from the cyclization of farnesyl pyrophosphate (FPP, C15), which is an intermediate in both the mevalonate and non-mevalonate (2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway - MEP/DOXP) pathways. The biosynthetic origin of (*E*)-caryophyllene in plants and the influence of insect herbivory in stimulating (*E*)-caryophyllene production leading to herbivore repulsion, natural enemy recruitment and plant-plant communication is well documented. (*E*)-Caryophyllene has been associated with inhibition of pathogen activity (Huang et al., 2012), interfering in the relationship of plants with herbivorous insects (Alquézar et al., 2017) and natural enemies (Büchel et al., 2011; Zhang et al., 2020), attracting pollinating insects (Zhang et al., 2016) and even recruiting entomopathogenic nematodes (Turlings et al., 2012; Chiriboga et al., 2018). Arabidopsis plants overexpressing (*E*)-caryophyllene production are repellent for *Diaphorina citri* (Alquézar et al., 2017). (*E*)-Caryophyllene shows potent contact toxicity and repellency against insect pests of stored products (Zhang et al., 2019), and is considered as safe for us in the control of stored product insects. Limonene has been shown to stimulate *in vitro* growth of the fungal pathogen *Penicillium digitatum* (Simas et al., 2017), but inhibit the conidial germination of *Colletotrichum lindemuthianum* at natural concentrations (Quintana-Rodriguez et al., 2015). Despite many

reports of antimicrobial activity for both compounds, further studies are required to investigate their effect on sugarcane pathogen development.

Parasitoids search for their hosts in a sequence involving host habitat location, host location, host acceptance and host suitability, utilising a wide diversity of foraging cues (Vinson, 1976). Plants provide the volatile chemical cues which helps *C. flavipes* to locate hosts (Potting et al., 1995; Gohole & Ngi-Song, 2001). Behavioural studies published by other groups have shown that (*E*)-caryophyllene can play an important role in the attraction of natural enemies both above and belowground. Aboveground, tests indicate that it is attractive to lacewings, *Chrysopa carnea*, in cotton fields (Flint et al., 1979) and to the egg parasitoid, *Annagrus nilaparvatae*, a natural enemy of *Nilapravata lugens*, an important rice pest (Xiao et al., 2012). Belowground, this compound has been described as an important signal to recruit enemies of the root-feeding pests in maize (Rasmann et al., 2005). In our work, the addition of (*E*)-caryophyllene to the VOCs collected from healthy sugarcane plants resulted in attraction of *C. flavipes* wasps, whereas VOCs collected from healthy plants were not significantly attractive (Fig. 3). Furthermore, naïve females of *C. flavipes* were significantly attracted to pure (*E*)-caryophyllene across a range of doses from 3 – 300 ng. Insects use volatiles for host location depending on their extreme ability to process the olfactory signals. The insect olfactory system has extremely high sensitivity to certain plant volatiles, with insect responses being elicited by doses that are far below the detection threshold of the GC flame ionization detector. Furthermore, insects display specificity towards volatiles, as they are able to discriminate between closely-related chemical structures (Pickett et al., 2012). However, insect behavioural responses to volatiles depends on the qualitative and quantitative differences. It has been frequently proposed that volatile blends elicit stronger behavioural responses than single odorants (Bruce & Pickett, 2011). Our results suggest that the presence of (*E*)-caryophyllene is sufficient for *C. flavipes* recruitment and that this compound can be utilised as a sustainable tool for pest control, as HIPVs can be used to recruit natural enemies to crops.

The use of kairomones to attract beneficial arthropods is an interesting area of research and a relatively new way of pest control, and the training of predatory wasps with HIPVs will enhance predator activity. However, arthropod attraction to chemical signals is a very complex process and field application of such signals has met with limited success (Kaplan, 2012; Gish et al., 2015; Stenberg et al., 2015). Based on our results, we believe that it may be possible to improve the success of chemical signals in recruiting beneficial insects in field crops. The response to an individual compound, such as (*E*)-caryophyllene, instead of an array of volatiles, could facilitate the development of tactics to enhance biological control of *D. saccharalis* populations.

Olfactory stimuli, including HIPVs, can be learned by parasitoids. Despite some parasitoids showing innate preference for HIPVs, many others require a previous oviposition experience to distinguish the odours of infested plants. Yet, even with the innate ability of the parasitoids to detect HIPVs, how can they benefit from adult learning, improving searching efficiency and success in foraging behaviour (Giunti et al., 2015)? For instance, responses of the parasitoid *C. plutellae*, an important natural enemy of the diamondback moth, *Plutella xylostella*, to volatile cues significantly increased after prior oviposition experience and contact with host-damaged leaves (Potting et al., 1999). Females of *C. marginimentris*, a generalist parasitoid, responded more significantly to odours of the plant-host complex after brief contact with host-damaged leaves contributing to female host searching efficiency (Turlings et al., 1989) . On the other hand, earlier studies have shown that neither previous contact with larval frass nor oviposition experience increased the responsiveness of naïve females of *C. flavipes* despite exhibiting a strong preference for odours of host-infested plants, suggesting that *C. flavipes* does not use learning (Potting et al., 1997). Based on these previous observations, we tested the hypothesis that previous experience of *C. flavipes* wasps with the HIPV (*E*)-caryophyllene would increase their attraction to VOCs. Furthermore, this behaviour could be extended to lower doses of (*E*)-

caryophyllene. Thus, naïve females of *C. flavipes* were pre-exposed to (*E*)-caryophyllene to gain experience and then, their attraction behaviour was reassessed at doses equal to or lower than the previously observed discrimination doses i.e. 3, 0.3, and 0.03 ng. Experienced wasps were able to orient towards lower (*E*)-caryophyllene doses, such as 0.3 and 0.03 ng, whereas as already described above, olfactometry experiments with naïve parasitoids demonstrated that the lowest dose capable of giving a statistically significant response was 3 ng, with the 0.3 ng dose not being significantly attractive (Fig. 3). It could be argued that for insects, information obtained through experience is critical for species survival. Learning, i.e. the acquisition and retention of neuronal representations of new information, plays an important role for essential life actions, such as feeding, aggression, interaction and sexual behaviour (Dukas, 2013). Giunti et al. (2015) suggested the employment of associative training to optimize parasitoid response to odours, using HIPVs to improve the foraging efficiency of parasitoids released in augmentation programs. Our results suggest that associative learning, ie. ie. the learning of reward-associated chemical cues, including long- and short-range olfactory cues originating from the plant-host complex or from the host itself, could be useful to improve the success of biological control of *D. saccharalis* using *C. flavipes* in sugarcane crops.

In conclusion, this study demonstrates that *C. flavipes* can use (*E*)-caryophyllene as a cue to locate *D. saccharalis*-infested sugarcane plants and suggests that exposure of *C. flavipes* to (*E*)-caryophyllene can increase their ability to detect this compound, resulting in their attraction towards lower doses. Most of the studies searching for beneficial effects of HIPVs use kairomones to lure beneficial arthropods and increase parasitism under natural conditions. However, under such conditions, plants attract and retain natural enemies offering shelter and food rewards (Heil, 2008). Therefore, the use of synthetic attractants could be translated as a false message, where attractants are not necessarily associated with the presence of hosts (pest

insects), leading to disruption of foraging and habituation of beneficials, where parasitoids start to respond less to chemical stimuli due to repeated exposure (Kaplan, 2012). Our results can be helpful to achieve biological control of *D. saccharalis* in a novel manner. Associative training could be employed in biological control programs to optimize parasitoid responses to (*E*)-caryophyllene (Giunti et al., 2015; Kruidhof et al., 2019). Thus, *C. flavipes* exposure to (*E*)-caryophyllene could be used to enhance host-seeking behaviour and target *D. saccharalis*. The potential application of (*E*)-caryophyllene in *D. saccharalis* management in agro-ecosystems is currently being examined in the field and will be reported at a later stage.

Acknowledgements

The authors would like to offer special thanks to Dr. Alessandro Riffel, who, although no longer with us, continues to inspire by his example and dedication to the students he served over the course of his career.

Funding

This study was supported by the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES), the National Institute of Science and Technology-Semiochemicals in Agriculture - INCT-Semioquímicos na Agricultura (CNPq Proc. 465511/2014-7 and Fapesp Proc. 2014/50871-0) and the National Council for Scientific and Technological Development (CNPq). Permission for collecting and use of insects and plants for research was obtained from The Genetic Patrimony Management Council - CGEN Access to Genetic Patrimony nº 010428 / 2012-7, National System of Management of Genetic Patrimony and Associated Traditional Knowledge (SisGen) n° ABE7A17. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom.

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Figure legends

Figure 1. Mean (ng ± SE) amount of (*E*)-caryophyllene produced per *D. saccharalis*-damaged sugarcane plant (black columns) and healthy (control) sugarcane plant (white columns) 0-12 h, 12-24 h and 24-48 h after damage was initiated. Volatiles were collected by headspace collection using Tenax TA as adsorbent. (*E*)-Caryophyllene was not detected in healthy plants. For (*E*)-caryophyllene quantification, a calibration curve was prepared using an authentic standard diluted in HPLC-bidistilled hexane grade. Data were analyzed by Students t test (* $P<0.01$).

Figure 2. Representative GC-MS analyses of volatile extracts collected from *D. saccharalis*-damaged sugarcane plants (A) and healthy (control) sugarcane plants (B). Volatiles were collected for a period of 0-48 h after *D. saccharalis* damage was initiated, using Porapak Q as adsorbent. (*E*)-Caryophyllene, which is produced only in *D. saccharalis*-damaged sugarcane plants, is labeled in graph A. X = Porapak Q contaminants.

Figure 3. Behavioral response of female *Cotesia flavipes* parasitoids in a four-arm olfactometer to VOCs collected for a period of 0-48 h and to (*E*)-caryophyllene. For experimets 1-3, CE = VOCs from healthy plants, HE = extracts from *Diatraea saccharalis*-damaged plants, CE + (*E*)-caryophyllene = VOCs from healthy plants plus 3 ng (*E*)-caryophyllene. For experiments 4-8, doses of 0.3 ng, 3 ng, 30 ng, 300 ng and 3 µg of (*E*)-caryophyllene were used with naïve parasitoids. For experiments 9-11, doses of 0.03 ng, 0.3 ng and 3 ng (*E*)-caryophyllene were used with experienced (pre-exposed to (*E*)-caryophyllene) parasitoids. The control in all experiments was hexane. Data were analyzed using GENES software (* $P<0.01$); NS = no significant response in preference between treatment and control.