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Synthesis and secretion of volatile short-chain fatty acids in *Triatoma infestans* infected with *Beauveria bassiana*

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Abstract. Physically disturbed *Triatoma infestans* (Hemiptera: Reduviidae) adults, as well as adults of other Chagas' disease vectors, secrete a mix of volatile organic compounds (VOCs) with alarm and possible sexual and defence functions. The aim of the present research was to test whether infection with the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales: Clavicipitaceae) has an effect on VOC secretion in disturbed *T. infestans* and on the expression of two genes (*Ti-brnq* and *Ti-bckdc*) potentially involved in VOC biosynthesis. The volatiles released by insects at different time periods after fungal treatment were identified and their relative amounts measured. Isobutyric acid was the most abundant volatile found in both healthy and fungus-infected insects and underwent no significant relative changes through the infection process. The secretion of propionic acid, however, was significantly higher at 1–4 days post-infection (d.p. i.) compared with that in controls. A slight induction of both *Ti-brnq* and *Ti-bckdc* genes was found by real-time polymerase chain reaction at 4 d.p. i., with expression values reaching up to three-fold those in controls. The early stages of fungal infection seem to affect the composition of the alarm pheromone by changing the expression pattern of both genes analysed. These results help to elucidate the impact of fungal infections on the chemical ecology of triatomine bugs.

Key words. Chagas' disease vector, entomopathogenic fungi, gene expression, triatomine bugs.

Introduction

The triatomine bug *Triatoma infestans* (Klug) is the main vector of Chagas' disease in the southern region of South America. Failures of standard chemical control approaches attributable to pyrethroid resistance have been detected in Argentina and Bolivia (Mougabure-Cueto & Picollo, 2015). These findings emphasize a very strong need to develop innovative methods to control Chagas' disease vectors. Laboratory and field assays confirmed the efficacy of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. against *T. infestans* (Luz *et al*., 1998, 2004; Juárez *et al*., 2000; Lecuona *et al*., 2001; Pedrini *et al*., 2009; Forlani *et al*., 2015). Fungus-infected triatomines are able to transmit conidia to their congeners (a process known as fungal dissemination) and thereby contribute to overall mortality as a result of fungal infection (Forlani *et al*., 2011) and, in addition, infected insects are affected in their ability to oviposit and to reproduce (Forlani *et al*., 2015), as well as in their innate immunological responses (Lobo *et al*., 2015; Mannino *et al*., 2018).

Other strategies to control triatomine populations are focused on the exploitation of intraspecific chemical signals or so-called

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pheromones used by insects to communicate with their congeners. This method is already successfully employed against other pests and may also be applicable in the control of Chagas' disease vectors (Cruz-López *et al*., 2001). Chemical communication in triatomines is based on both contact and volatile signals. Epicuticular lipids of *T. infestans* are known to act as contact pheromones that mediate aggregation (Lorenzo-Figueiras *et al*., 2009) and recognition between adult mates (Cocchiararo-Bastias *et al*., 2011). In addition, cuticular signals have been previously reported to serve as chemical footprints that facilitate *T. infestans* aggregation (Lorenzo-Figueiras & Lazzari, 1998). The volatile organic compounds (VOCs) are secreted by two exocrine glands, namely the paired metasternal and Brindley's glands (Schofield & Upton, 1978). The metasternal gland is located in the thorax and has been associated with sexual communication (Crespo & Manrique, 2007; Pontes *et al*., 2008; Vitta *et al*., 2009; Zacharias *et al*., 2010; May-Concha *et al*., 2013, 2018); the abdominal Brindley's glands are involved in alarm and possible sexual and defence functions (Hack *et al*., 1980; Manrique *et al*., 2006; Crespo & Manrique, 2007; Palottini *et al*., 2014; Palottini & Manrique, 2016). The composition of Brindley's glands secretion includes a blend of straight and branched short-chain fatty acids, in addition to alcohols, esters and a ketone (Juárez & Brenner, 1981; Ward, 1981; Cruz-López *et al*., 1995; Rojas *et al*., 2002; Manrique *et al*., 2006; May-Concha *et al*., 2018) that are often referred to as 'alarm pheromone'.

Branched-chain short fatty acids are synthesized from the branched-chain amino acids in both insects (Halarnkar & Schooley, 1995) and plants (Van der Hoeven & Steffens, 2000). The short-chain fatty acids isobutyric, propionic and acetic are produced in mitochondria from isoleucine as part of the early steps in the biosynthesis of juvenile hormone or contact pheromones in several insect orders (Halarnkar & Schooley, 1995). In plants, the enzymes or enzyme complexes involved in branched-chain short fatty acids synthesis include aminotransferases, dehydratases and specific branched-chain amino acid transport systems and branched-chain alpha-keto acid dehydrogenase complex (Van der Hoeven & Steffens, 2000). To date, however, no information about insect genes involved in the synthesis of short branched-chain fatty acids is available.

In addition to alarm situations, other stress factors that potentially influence or determine volatile secretion by Brindley's glands are not well understood. For example, it is unknown whether a fungal infection would modify the profiles of VOCs during infection and consequently affect other nearby triatomine individuals. The aim of this research was to study the potential effect of *B. bassiana* infection on the synthesis and/or release of the volatile short-chain fatty acids that act as alarm pheromones in *T. infestans*. For this purpose, potential alterations attributable to fungal infection were analysed at two levels: (a) the mix of volatile compounds released by these insects, and (b) the patterns of expression of mitochondrial genes that are potentially involved in branched-chain amino acid uptake by mitochondria and the subsequent oxidative decarboxylation leading to the short-chain fatty acids.

Materials and methods

Fungal culture and preparation of conidial suspensions

Beauveria bassiana strain GHA (Laverlam International Corp., Butte, MT, U.S.A.) was grown on complete medium agar plates containing 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 0.7 g $NH_4NO_3 \cdot 7H_2O$, 10 g glucose, 5 g yeast extract and 15 g agar in 1000 mL of distilled water. Plates were incubated at 26 ± 1 °C for 12 days. Suspensions of conidia were prepared by rinsing fungal cultures in sterile distilled water and rubbing the sporulating surface with a bent needle. After debris had been filtered, the liquid was diluted in sterile distilled water containing 0.01% Tween 80. Conidial concentration was determined in a Neubauer chamber and the final concentrations were adjusted to the desired titres.

Insect rearing and preparation

All animal care and laboratory experimental protocols were approved by the Directive Board of the Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), La Plata, Argentina (Animal Welfare Assurance no. A5647–01) and carried out according to the American Veterinary Medical Association (AVMA) Animal Welfare Policies and AVMA Guidelines on Euthanasia (https//www.avma.org/KB/Policies/Documents/euthanasia.pdf).

Adult *T. infestans* were obtained from a colony reared at 30 °C, 50–60% relative humidity under an LD 12 : 12 h photoperiod and fed on chicken blood at the INIBIOLP. The colony is periodically renewed by incorporating field specimen-derived F1 insects, usually from Formosa Province, provided by the Servicio Nacional de Chagas, Cordoba, Argentina. For the tests, emergence of adults was observed daily; new adults were separated by sex and individually maintained in the same rearing conditions. Because the filling of Brindley's gland depends on several factors, such as whether the insect is reared in the laboratory or in a natural environment, adult development, the number and sequence of disturbance events and, more importantly, on feeding (Palottini *et al*., 2014), the adults used in the assays were aged 12–14 days and were used 7 days after their last pre-testing feeding. The latter condition and the care in handling insects (see below) were intended to ensure that only one discharge was produced by each insect throughout the post-infection trial.

Inoculation of fungi and bioassays

To avoid eventual disturbances that could potentially cause unwanted early gland discharges, individual adult *T. infestans* were first immobilized for 15 min in a refrigerator and were then carefully placed with a spoon in a Petri dish (100 × 20 mm) previously chilled in an ice-bath for 10 min. Ten adults were immobilized in this way on each dish. Semiautomatic micropipettes were used to administer two 10-μL droplets of the inoculum, one on the abdomen and the second on the thorax of adult insects; the conidial concentrations applied were either 1×10^6 or 1×10^8

conidia/mL for final dosages of 2×10^4 and 2×10^6 conidia per insect, respectively. Control insects were treated topically in the same manner with sterile distilled water containing 0.01% Tween 80. After treatments, insects were carefully returned to individual flasks and maintained without feeding in the rearing conditions described above. At different days post-infection (d.p. i.), different batches of adults were randomly separated for both VOCs collection and RNA extraction.

Sampling of VOCs by solid-phase micro-extraction

Volatile collections from both healthy and fungus-infected adults were performed with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre with a 50-μm film thickness (Supelco, Inc., Bellefonte, PA, U.S.A.). The fibre had been previously conditioned according to the manufacturer's instructions and was systematically reconditioned before each analysis. Volatile organic compounds were sampled from adults previously treated with either 1×10^8 conidia/mL or sterile distilled water containing 0.01% Tween 80 (controls). For each condition, one adult of known sex was gently placed in a 20-mL glass vial sealed with a Teflon cover with a rubber septum. The vial was vortexed for 10 s to elicit the release of volatiles, which were sampled immediately from the headspace corresponding to the gaseous phase in contact with the insect sample by solid-phase micro-extraction (SPME) (Arthur & Pawliszyn, 1990). Three independent replicates (each replicate from a different individual) were performed for each condition (fungus-treated and untreated males and females).Vials containing no insects were used as controls.

Analysis of VOCs by capillary gas chromatography coupled to mass spectrometry

Volatile organic compounds were identified using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Wilmington, DE, U.S.A.) equipped with an Omegawax 250 capillary column (30 m by 0.25 mm by 250 μm film; Supelco, Inc.) interfaced to an Agilent 5975C VL mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) operated at 70 eV. Samples were injected in splitless mode at 250 °C with helium as carrier gas. The column oven was programmed to 40 °C for 1 min, 12 °C/min to 260 °C, with a holding time of 10 min at the final temperature. The transfer line and quadrupole were held at 260 °C and 150 °C, respectively. Their mass fragmentation patterns were compared with commercial mass database data [National Institute of Standards and Technology (NIST05) and Adams (2007)], literature data (Juárez & Brenner, 1981; Cruz-López *et al*., 1995; Rojas *et al*., 2002; Manrique *et al*., 2006) and the Kovats retention index (KI) (Kovats, 1965). Peak areas of VOCs were calculated for each chromatogram and expressed as a percentage of the total peak area detected by the fibre after injection in the same equipment and chromatography conditions, but employing a flame ionization detector set at 280 °C.

Gene expression analysis

Total RNA was extracted from individual fungus-treated and control insects at 4 d.p. i. and 10 d.p. i. using the RNAeasy Mini Kit (Qiagen GmbH, Hilden, Germany) with an on-column DNA digestion step [DNAse I (Qiagen GmbH)]. For each treatment and control, RNA was extracted from at least three individuals. The RNA was quantified by a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, U.S.A.), and its integrity assessed on a 1% (w/v) agarose gel. Two-step real-time polymerase chain reaction (RT-PCR) was carried out with an iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Amplification was performed in an Mx3000P quantitative PCR System (Stratagene Corp., La Jolla, CA, U.S.A.) employing 20 ng reverse transcribed total RNA for each sample. The assays were performed in duplicate for each of the three independent biological replicates mentioned. The ΔΔCt method [threshold cycle (CT)] was used to quantify the data obtained from the RT-PCR system (Pfaffl *et al*., 2002), using actin as the housekeeping gene (Lobo *et al*., 2015). The primer sequences used were ACGCCAGCTGTACGCAGAATAG (Ti-brnq-f) and TCGCCCATCCTTTCCAGTTC (Ti-brnq-r), TGTTAATGCTGCCCGTTCACG (Ti-bckdc-f) and AGGTCGCCGCTAATTCTCACC (Ti-bckdc-r), and CACCCCAGCAATGTTATGTAG (Ti-actin-f) and ACCATCAGGAAGTTCGTAAG (Ti-actin-r). The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with three segment amplifications (30 s at 95 °C for denaturation, 30 s at 56 °C for annealing, and 30 s at 72 °C for DNA chain elongation). In order to confirm that only single products were amplified, a temperature-melting step was then performed. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The sequences corresponding to the putative branched-chain amino acid transport system (*Ti-brnq* gene) and the putative mitochondrial branched-chain alpha-keto acid dehydrogenase (*Ti-bckdc* gene) were obtained from an expression sequences tagged (EST) library synthesized from the cuticle of *T. infestans* nymphs (GenBank, BioProject PRJNA314811) (Calderón-Fernández *et al*., 2017). The sequences IDs are JAR98428 for *Ti-brnq* and JAR97322 for *Ti-bckdc*. The putative function of both genes was assigned after Gene Ontology (GO) analysis. The *Ti-brnq* gene encodes a carrier protein that may participate in the amino acid uptake by mitochondria, whereas the *Ti-bckdc* gene encodes the enzyme that catalyses the oxidative decarboxylation that results in the short-chain fatty acids.

Statistical analyses

Differences among the mean values obtained for each dose and time period were determined by analysis of variance (ANOVA), using the Student–Newman–Keuls post-test to separate treatment means ($P < 0.05$). Instat Version 3.10 (GraphPad Software, Inc., La Jolla, CA, U.S.A.) was used for all statistical analyses.

Table 1. Identification and relative composition (mean ± standard error of the mean percentage) of major volatile organic compounds released by Brindley glands of *Triatoma infestans* infected or not by *Beauveria bassiana*, at different periods post-infection.

			Relative composition	
Compound	KI	d.p. i.	Controls	Fungus-infected
Isobutyl propionate	1081	1–4	tr	tr
		5–10	tr	tr
		11–15	tr	tr
Isobutanol	1089	1–4	tr	tr
		5–10	tr	tr
		11–15	tr	tr
Isobutyl isobutyrate	1093	1–4	5.7 ± 2.6	2.1 ± 0.2
		5–10	5.0 ± 0.8	5.8 ± 2.4
		11–15	5.3 ± 1.3	5.4 ± 1.3
2/3-Methyl-1-butyl propionate mix	1193	1–4	tr	tr
		5–10	tr	tr
		11–15	tr	tr
3-Methyl-1-butyl-2-methyl propionate	1198	1–4	tr	tr
		5–10	tr	tr
		11–15	tr	tr
2-Methyl-1-butyl-2-methyl propionate	1201	1–4	tr	tr
		5–10	tr	tr
		11–15	tr	tr
2/3-Methyl-1-butanol mix	1206	1–4	10.4 ±3.1	7.2 ± 1.6
		5–10	9.9 ± 0.9	14.8 ± 5.1
		11–15	15.2 ± 2.6	13.7 ± 3.1
Propionic acid	1570	1–4	6.0 ± 1.7*	18.6 ± 5.8*
		5–10	6.0 ± 4.9	8.2 ± 3.8
		11–15	8.2 ± 3.8	5.3 ± 1.8
Isobutyric acid	1574	1–4	77.1 ± 5.2	70.8 ± 4.5
		5–10	78.1 ± 3.3	73.7 ± 7.0
		11–15	69.8 ± 4.4	74.1 ± 3.9
2-Methyl butanoic acid	1704	1–4	tr	tr
		5–10	tr	tr
		11–15	tr	tr

*Means in the same line were significantly different (ANOVA and Student–Newman–Keuls test at $P < 0.05$). d.p. i., days post-infection; KI, Kovats retention index; tr, traces (< 1% relative composition).

Results

Volatile organic compounds measurement

Table 1 lists the major compounds identified with corresponding KI values and relative amounts (percentages) in both healthy and fungus-infected insects. The major peak corresponded to isobutyric acid (KI 1574), but minor peaks were identified as propionic acid (KI 1570), 2- and 3-methyl-1-butanol mix (KI 1206), isobutyl isobutyrate (KI 1093), together with some trace quantities of 2- and 3-methyl-1 butyl 2-methyl propionate (KI 1198 and KI 1201, respectively), 2- and 3-methyl-1 butyl propionate mix (KI 1193), isobutyl propionate (KI 1081), and isobutanol (KI 1089). Isobutyric acid accounted for 70–78% of total volatiles in all samples, with no significant effect of the process of infection on its secretion ($F = 0.35$, d.f. = 5,53, $P = 0.9$). The only compound to present a significant change in its relative secretion throughout the infection process was propionic acid, which reached its highest values at 1–4 d.p. i. (18.6 ± 5.8%), and then distinctly decreased ($F = 2.8$, d.f. = 5,53, $P = 0.03$), with no difference with the control (8.2 ± 3.8% at 5–10 days d.p. i. and 5.3 ± 1.8% at 11–15 d.p. i.). Neither qualitative nor quantitative differences between sexes (data not shown) of fungus-treated adults and control individuals were found for any compound during the 2-week duration of these assays.

Gene expression analysis

With reference to short-chain fatty acids synthesis, the expression patterns of two insect genes (*Ti-brnq* and *Ti-bckdc*) potentially involved in this process were studied in *B. bassiana*-infected adults. Expression of the *Ti-brnq* gene was highest at 4 d.p. i. regardless of the conidial concentration: expression levels were 1.3 ± 0.5-fold and 3.0 ± 0.4-fold induction in individuals treated with 1×10^6 and 1×10^8 conidia/mL, respectively, compared with controls ($F = 11.3$, d.f. = 3,11, $P < 0.01$) (Fig. 1A). By contrast, no induction was observed for *Ti-bckdc* at 4 d.p. i. or for both genes at 10 d.p. i. (Fig. 1B). Both genes showed significant differences at 4–10 d.p. i. at the higher dose (1×10^8 conidia/mL), with values of 3.0 ± 0.4-fold and 1.1 ± 0.2-fold induction ($F = 11.3$, d.f. = 3,11, $P < 0.01$) and 2.5 ± 0.4-fold and 0.9 ± 0.1-fold induction compared with controls ($F = 5.1$, d.f. = 3,11, $P < 0.05$) for *Ti-brnq* and *Ti-bckdc* [Fig. 1(A) and (B), respectively].

Discussion

The composition of the Brindley's gland-emitted blend is known to be composed mostly of both straight and branched short-chain fatty acids (Schofield & Upton, 1978; Juárez & Brenner, 1981). The present work shows that although the *B. bassiana* infection did not influence the nature of the compounds comprising the volatile blend released by physically disturbed *T. infestans*, infected insects change their relative profile during the first days post-infection, probably by inducing expression of the two genes potentially involved in its biosynthesis.

The compounds identified here include most of those reported in a previous study with no fungus-infected *T. infestans* (Manrique *et al*., 2006); the reason why some compounds were not found in the present study may be because the earlier study used the same SPME technique but employed a different (CAR/PDMS) fibre. The main component of the Brindley's gland mix, isobutyric acid, has been shown to have a similar effect on insect behaviour as the total blends (Schofield, 1979; Ward, 1981), and the same as some doses of single compounds or binary mixtures (Palottini & Manrique, 2016). The current study found that fungal infection did not alter the relative secretion of isobutyric acid throughout the experimental period. However, little is known about the less prominent compounds of the blend; in this sense, propionic acid secretion was relatively higher after an incipient fungal infection (1–4 days).

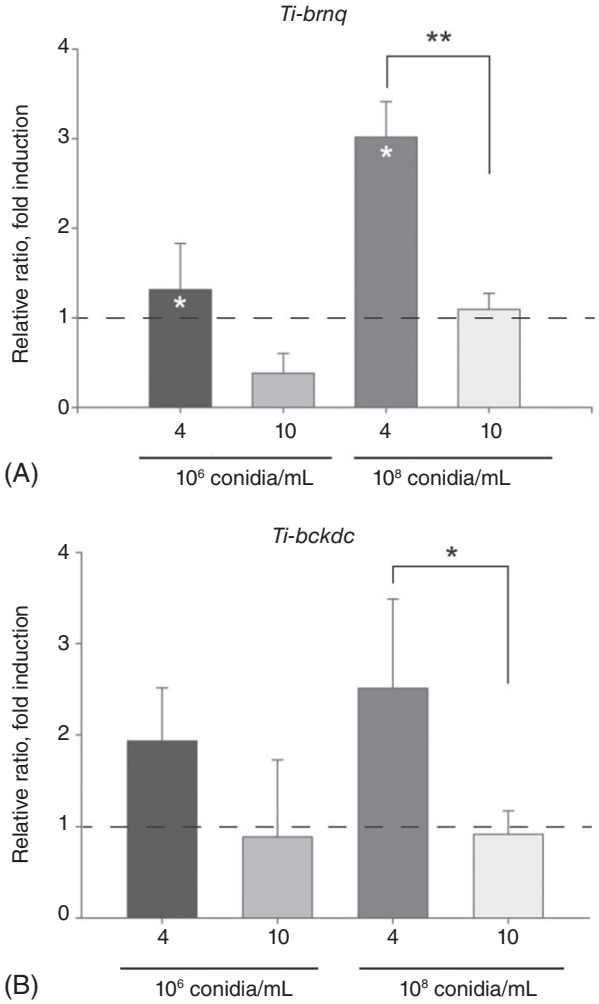

Fig. 1. Relative expression of (A) *Ti-brnq* and (B) *Ti-bckdc* genes at 4 and 10 days after topical treatment of *Triatoma infestans* adults with *Beauveria bassiana* conidial suspensions at 10^6 or 10^8 conidia/mL. Values are means of at least three replicates ± standard error of mean. The dashed line shows an expression ratio of 1, indicating that the target gene is expressed equally in control and fungus-infected insects. White asterisks within the bars indicate significant differences compared with controls. Black asterisks indicate significant differences in expression levels at 4 and 10 days post-infection in fungus-treated insects. $^*P < 0.05$; $^{**}P < 0.01$.

This compound is released by aphids and some coleopterans and is known to be repellent to grain storage weevils (Germinara *et al*., 2008). In triatomines, propionic acid was previously reported to be a part of their volatile blend (May-Concha *et al*., 2013, 2015, 2018).The role of some synthetic components forming the volatile blend was tested by Palottini & Manrique (2016) in order to detect any escape response of *T. infestans*; however, no activity was reported for propionic acid, suggesting that it has no effect as an alarm pheromone.

Although insects are able to produce and release chemicals in large quantities, those causing repellency behaviour in parasitic arthropods are usually present only in small quantities (Eisner & Grant, 1981; Berenbaum, 1995). In triatomines, isobutyric acid can be attractive or repellent depending on the dose (Guerenstein & Guerin, 2001), and plays multiple roles in the cognitive modulation of the insect's escape behaviour (Minoli *et al*., 2013) acting alone or in combination with other blend-included components (Palottini & Manrique, 2016). There is no information to date about the genes involved in the synthesis of straight and branched short-chain fatty acids in insects. Using parallel information available from plants and mammals (Van der Hoeven & Steffens, 2000), two sequences with high identity to branched-chain amino acid transport system (*brnq*) and branched-chain alpha-keto acid dehydrogenase (*bckdc*) genes were detected in an EST library from *T. infestans* cuticle (Calderón-Fernández *et al*., 2017). In an attempt to begin to understand their roles in the synthesis of volatile compounds, their expression levels were evaluated in infected individuals. *Ti-brnq* gene was more highly expressed in infected *T. infestans* adults than in control insects at 4 d.p. i. at the two conidial concentrations tested. At the beginning of a fungal infection, the insect may be affected by early infection events, such as attachment and adhesion of conidia to the cuticle; thus, a greater production of branched-chain short fatty acids by Brindley's glands may occur in response to this challenge. Nevertheless, the higher relative expression observed for the *Ti-bckdc* gene was not significant in fungus-infected triatomines. At 10 days after treatment, when infected individuals may be severely compromised, there was no induction of either of these genes in comparison with the control. At least two factors may contribute to an explanation of this observation: after the initial fungal challenge, insects may recover expression levels to normalize the synthesis of volatiles, correlating well with the levels detected by SPME at 10 days after treatment. However, it may be that individuals which, for some reason, survive 10 days after fungal inoculation may not have been seriously compromised by the fungal infection and thus may show expression levels of these genes similar to those found in healthy individuals.

The present research raises additional questions which the authors plan to address in future investigations. These include the issue of whether any intraspecific repellency that might be triggered by entomopathogenic fungi treatments may reduce the risk for transmission of the fungal disease to healthy insects that show aggregation behaviour and whether the behaviour of healthy individuals that are close to an infected individual may be influenced by the presence of the fungus in a mycoinsecticidal formulation.

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