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Attraction of entomopathogenic nematodes to black truffle and its volatile organic compounds: A new approach for truffle beetle biocontrol

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ABSTRACT

The European truffle beetle, *Leiodes cinnamomeus*, is the most important pest in black truffle (*Tuber melanosporum*) plantations. Entomopathogenic nematodes (EPNs) are a promising biological control agents against *L. cinnamomeus*. EPNs may employ multiple sensory cues while seeking for hosts, such as volatile organic compounds (VOCs) and CO_2 gradients. We report for the first time the attraction of EPNs to truffle fruitbodies, and identified some VOCs potentially playing a key role in this interaction. We conducted olfactometer assays to investigate the attraction behavior of *Steinernema feltiae* and *Steinernema carpocapsae* towards both *T. melanosporum* fruitbodies and larvae of *L. cinnamomeus*. Subsequently, a chemotaxis assay using agar plates was performed to determine which of the 14 of the main VOCs emitted by the fruitbodies attracted *S. feltiae* at low (0.1 %) and high (mg/100 g truffle) concentrations. Both EPN species were attracted to mature fruitbodies of *T. melanosporum*, which may enhance the likelihood of encountering *L. cinnamomeus* during field applications. *L. cinnamomeus* larvae in the presence of truffles did not significantly affect the behavior of EPNs 24 h after application, underscoring the importance of the chemical compounds emitted by truffles themselves. Chemotaxis assays showed that four long-chain alcohol compounds emitted by *T. melanosporum* fruitbodies attracted *S. feltiae*, especially at low concentration, providing a first hint in the chemical ecology of a little-studied ecological system of great economical value. Further studies should be conducted to gain a finer understanding of the tritrophic interactions between *T. melanosporum*, EPNs, and *L. cinnamomeus*, as this knowledge may have practical implications for the efficacy of EPNs in the biological control of this pest.

1. Introduction

The black truffle, *Tuber melanosporum* Vittad., is a hypogeal fungus that grows belowground in ectomycorrhizal (ECM) association with oak trees (*Quercus* spp.) (Bonito et al., 2010). Primordium formation occurs from April to June. Subsequently, the immature fruitbodies continue to grow until December and ripen until March (Oliach et al., 2020). The black truffle fruit has gastronomic value and economic interest in some regions of Southern Europe, generating approximately €50 million annually (Oliach et al., 2020). Over the last years, this economic importance has led to the establishment of monocultures favoring the presence of fungivorous insect species, such as the European truffle beetle *Leiodes cinnamomeus* Panzer. This beetle is the most important pest in black truffle plantations (Martín-Santafé, 2020). Both adults and larvae feed on *T. melanosporum* fruiting bodies. While feeding, the insect burrows galleries in the fruiting body, reducing the quality of the truffle and its marketability. Alone, this insect causes up to 70 % of economic losses in certain plantations (Martín-Santafé, 2020).

Current control methods, which rely on frequent truffle harvests (Martín-Santafé et al., 2014) and the use of traps for mass capture of adults (Navarro-Llopis et al., 2021), are not efficient enough to reduce the population of *L. cinnamomeus,* therefore new sustainable strategies have been explored. Recently, the entomopathogenic nematodes (EPNs) *Steinernema feltiae* Filipje*, Steinernema carpocapsae* Weiser and *Heterorhabditis bacteriophora* Poinar have shown great potential as biological control agents against *L. cinnamomeus* larvae and adults under laboratory conditions (Fuentes-Boix et al., 2019; Julià et al., 2023a). Julià et al. (2023a) observed that *S. carpocapsae* induced 100 % mortality of adults at 20 °C, while *S. feltiae* caused 73 % mortality of mycophagous larvae at the same temperature. EPNs of the Steinernematidae and Heterorhabditidae families are obligate parasites of a wide range of insect species with great potential as biological control agents, presenting

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symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively (Lacey and Georgis, 2012; Shapiro-Ilan et al., 2017). EPN infective juveniles (IJs) locate their host insects by different cues (kairomones such as insect-produced odors and CO_2,) and enter via natural opening before releasing symbiotic bacteria in its hemolymph. The insect dies of septicemia within 24–72 h as the EPNs, feeding on bacteria, reproduce into the cadaver. As EPN population grows, resources and space become scarce, and new IJs emerge from the cadaver, ready to locate and infect new hosts (Grewal and Georgis, 1999).

Differential host-seeking behaviors are observed among EPNs: active cruisers, passive ambushers, or intermediates (Lewis, 2002; Campbell et al., 2003). However, alternate theories have challenged this classification and suggested that EPN species may show different behaviors depending on the substrate in which they forage (Wilson et al., 2012). There is yet a common agreement that EPNs may employ multiple sensory cues for their host-seeking behaviors (Zhang et al., 2021), such as insect-derived chemical signals for precise host localization (Campbell and Kaya, 2000), and CO_2 gradients and herbivore induced plant volatiles to locate potential host insects from a distance (Rasmann et al., 2005; Hallem et al., 2011; Dillman et al., 2012; Turlings et al., 2012). Recent studies suggest that EPNs may also have evolved to recognize and orient towards fungal chemicals, and even towards fungivore-induced fungal cues, enhancing the likelihood of encountering fungivorous insects (Wu et al., 2018; Wu and Duncan, 2020; Hummadi et al., 2021).

Volatile organic compounds (VOCs) emitted by truffle fruitbodies play a key role on their interactions with plants, insects, and mammals (Splivallo et al., 2011). These truffle VOCs consist of a blend of alcohols, aldehydes, aromatic and sulfuric compounds that vary during ripening (Tejedor-Calvo et al., 2021). Among them, dimethyl sulfide (DMS) has been used to mass trap *L. cinnamomeus* adults in an effort to manage this pest (Navarro-Llopis et al., 2021). Other VOCs have been detected from *T. melanosporum*, such as 1-octen-3-ol which characterized most of mature *T. melanosporum* fruitbodies (Caboni et al., 2020), and 1-Octanol and 3-Methylhexanol that were more frequently emitted by immature fruitbodies (Garcia-Barreda and Sánchez, unpublished results).

Whether EPNs respond to black truffle volatiles and if the presence of insects influences their behavior has yet been overlooked. Investigating the tritrophic relationship between EPNs, *T. melanosporum* and *L. cinnamomeus* could provide insights to better understand EPN foraging strategies and improve the biocontrol of this beetle. Therefore, the aims of our investigation were (1) to study the influence of mature black truffle fruitbodies and larvae of *L. cinnamomeus* on the behaviour of EPNs, and (2) to assess whether these nematodes are attracted by different VOCs emitted by *T. melanosporum*

2. Material and methods

2.1. Source of truffles, insects and nematodes

Mature black truffle fruitbodies (15–20 mm diameter) were collected from truffle plantations in Teruel, Spain, during January of 2023. They were maintained in closed boxes at 9 °C and covered with cellulose paper for a maximum of three weeks. To avoid elevated RH, cellulose paper was changed every two days until truffles were used.

Fungivorous larvae of *L. cinnamomeus*, with a body length of 0.5–0.9 cm (L2 and L3 instars), were collected from truffle plantations in Teruel, Spain, during January of 2023. They were maintained in boxes at 9 °C filled with soil from truffle plantations and small pieces of truffle fruitbodies as food source for a maximum of three weeks until being used.

Two EPN species were tested: *S. feltiae* TE15 strain, previously isolated in a truffle plantation (Julià et al., 2023b); and *S. carpocapsae* commercial strain (Capsanem® Koppert). Nematodes were reared at 25 °C in last instar larvae of the greater wax moth, *Galleria mellonella* (Linnaeus), according to Woodring and Kaya (1988). The emerged infective juveniles (IJs) were recovered using modified White traps (White, 1927) and stored at 9 °C for a maximum of two weeks. Before application, IJs were acclimatized at room temperature for 1 h and their viability was checked by observation of movement under a stereomicroscope.

2.2. Olfactometer assay

A belowground 6-arm olfactometer (Rasmann et al., 2005) was used to study the behavioral response of EPNs towards VOCs from *T. melanosporum* fruitbodies and larvae of *L. cinnamomeus* (Fig. 1). Briefly, the olfactometer consisted of a central glass arena connected to six pots equally distributed around it. Connecting arms consisted of a glass tube inserted in a Teflon connector. The one end of this connected was sealed with an ultra-fine mesh (2,300 mesh; Small Parts Inc.) to block nematodes in the arm and allowing further extraction and counting of EPNs. Details and quotes of the 6-arm belowground olfactometer are available in Rasmann et al. (2005).

For each assay, the olfactometer was filled with sterilized moist sand (10 % water, w/w) to approximately 5 cm from the rim of the pots. One glass pot contained a mature black truffle fruitbody, a second glass pot contained one mature black truffle fruitbody along with two larvae of *L. cinnamomeus*, and the third glass pot contained only two larvae of *L. cinnamomeus* (Fig. 1). Three additional pots, interspersed between treatment pots, were used as sand controls. Twenty-four hours after setting up the olfactometer, about 2000 IJs of *S. feltiae* or *S. carpocapsae* were released in the central arena of the olfactometer. One day after release, the sand from each detachable glass tube was transferred onto cotton filter disks 19 cm diameter (Hoeschele GmbH.) Subsequently, these disks were put into Baermann-funnel extractors, and nematode quantification was conducted on the following day. There were nine replicates for each EPN species.

2.3. Chemotaxis assay

A total of 14 VOCs emitted by black truffles, previously identified by Tejedor-Calvo et al. (2021), were tested at two concentrations. The high concentration was chosen to deliver to the assay plate (in 10 µL) the total amount (mg) emitted by 100 g truffle, reported by Tejedor-Calvo et al. (2021) and Garcia-Barreda and Sánchez (unpublished results) (Table 1). The low concentration for all VOCs was set at 0.1 % (in 10 µL). Synthetic VOCs were obtained from Sigma-Aldrich and their concentrations were adjusted in dimethyl sulfoxide (DMSO). The mixture was agitated in a shaker and immediately used in the bioassay.

The chemotaxis assay was based on the methodology developed by Ward (1973) and adapted by O'Halloran and Burnell (2003) and Laznik and Trdan (2016). *Steinernema feltiae* was the only species tested, based on the results obtained in the olfactometer assay. Petri dishes (9 cm diameter) containing 25 ml of 1.2 % technical agar were used (Fig. 2). All products were applied as a point source. For each assay, 10 µL of the test substance was applied to one side of the agar surface (treated area), while 10 µL of the DMSO only was spiked on the other side of the agar surface (control area). In the control treatment, 10 µL of DMSO were applied to the treated area and 10 µL of distilled water to the control area. A filter paper of 1 cm diameter was placed in the center of the agar surface. Subsequently, 50 µL of water containing 100 IJs of *S. feltiae* was applied on the filter paper (Fig. 2). Petri dishes were placed in a dark rearing chamber at 25 °C and the number of individuals in the treatment and control areas were counted after 4 h and 24 h with a binocular microscope. There were five replicates for each treatment and the experiments were conducted three times.

The specific chemotaxis index (CI) (Bargmann and Horvitz, 1991), which varied from 1.0 (perfect attraction) to − 1.0 (perfect repulsion), was calculated using the following formula:

$$CI = \frac{\text{Number of EPN in the treatment area} - \text{Number of EPN in the control area}}{\text{Total number of nematodes in the assay}}$$

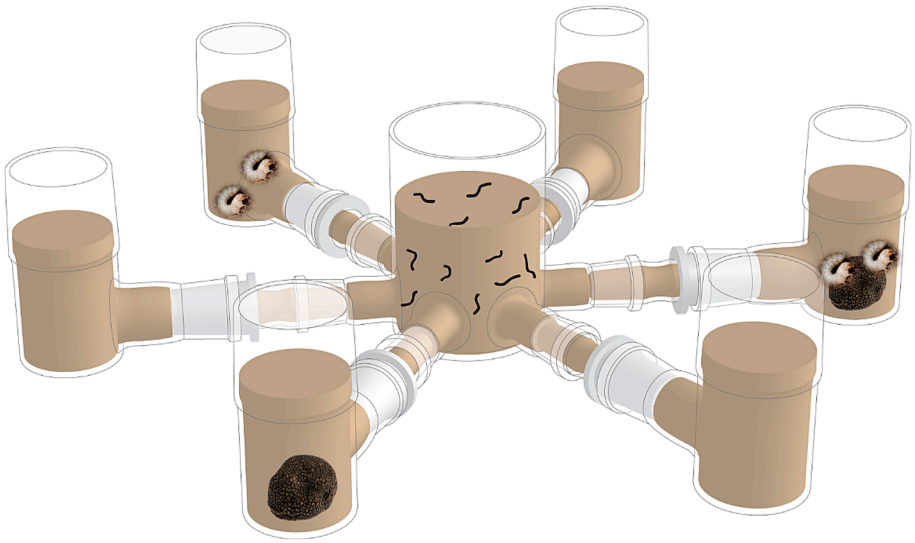

Fig. 1. Sketch of the belowground six-arm olfactometer in which nematode attraction to truffle fruitbodies and *L. cinnamomeus* larvae was tested (modified after Rasmann et al. 2005).

Table 1
List of volatile compounds (VOCs) and the high concentration used in the bioassay to give the amount of the volatile emitted by 100 g truffle (mg/100 g truffle), based on Tejedor-Calvo et al. (2021) and (2) Garcia-Barreda and Sánchez (unpublished results).

VOC	CAS n°	% Concentration (mg/100 g truffle)
Alcohol		
1-octen-3-ol	3391–86-4	25.78 (2.14) [(1)]
1-octanol	111–87-5	6.02 (0.5) [(2)]
2-methyl-1-butanol	137–32-6	25.28 (2.06) [(1)]
3-methyl-1-butanol	123–51-3	2.47 (0.2) [(2)]
3-methylhexanol	13231–81-7	9.90 (0.81) [(1)]
2-methylpropanol	78–83-1	34.91 (2.8) [(1)]
4-pentel-2-ol	625–31-0	83.63 (7) [(2)]
Aldehyde		
2-methylbutanal	96–17-3	100 (41.9) [(1)]
Hexanal	66–25-1	100 (9) [(2)]
Heptanal	111–71-7	10.63 (0.86) [(1)]
Sulfur-containing		
Dimethyl sulfide	75–18-3	15.36 (1.29) [(1)]
Dimethyl disulfide	624–92-0	41.49 (4.34) [(1)]
Ketone		
2,3-butanedione	431–03-8	11.92 (1.18) [(1)]
Aromatic compounds		
Anisole	100–66-3	10.05 (1) [(2)]

2.4. Statistical analysis

A Generalized Linear Model (GLM) with quasipoisson distribution and a log link function was used to test significant differences in the number of nematodes of *S. feltiae* and *S. carpocapsae* during the olfactometer assay. For the chemotaxis assay, a Linear Model (LM) or a Linear Mixed Model (LMM) (when the time factor was considered) were used to compare the response of *S. feltiae* to the tested VOCs under different exposure times and concentrations. For all models, main effects in the factorial design were analyzed for interactions, followed by a complete analysis of simple effects. Subsequently, Tukey's multiple range test was performed to compare differences among treatments. All data were analyzed using R software (version 4.3.1) (R Core Team, 2023). Any comparison was considered significant if the p-value was less than 0.05.

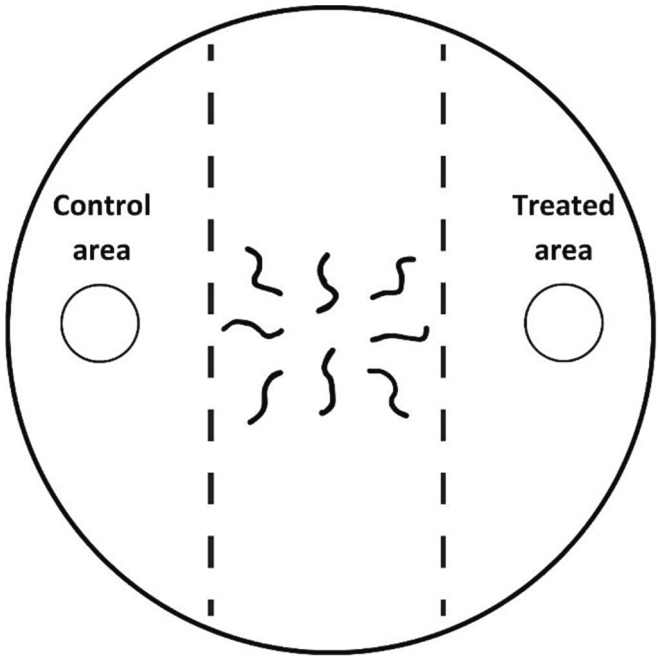

Fig. 2. Experimental arena (Petri dish) in which nematode attraction to different VOCs was tested.

3. Results

3.1. Olfactometer assay

When the main effects of the two factors (treatment and EPN species) were analyzed independently, both had a significant impact on the number of IJs ($\chi2 = 249.33$; df = 3, 68; $p < 0.001$ for treatment and $\chi2 = 245.21$; df = 1, 67; $p < 0.001$ for EPN species). However, their interaction did not show statistical significance ($\chi2 = 0.33$; df = 3, 64; $p = 0.983$).

The number of IJs that were attracted by *T. melanosporum* was higher than by the control, showing significant differences for both *S. feltiae* ($\chi2 = 218.26$; df = 3, 32; $p < 0.001$) and *S. carpocapsae* ($\chi2 = 31.40$; df = 3, 32; $p < 0.001$), regardless of the presence of *L. cinnamomeus* larvae

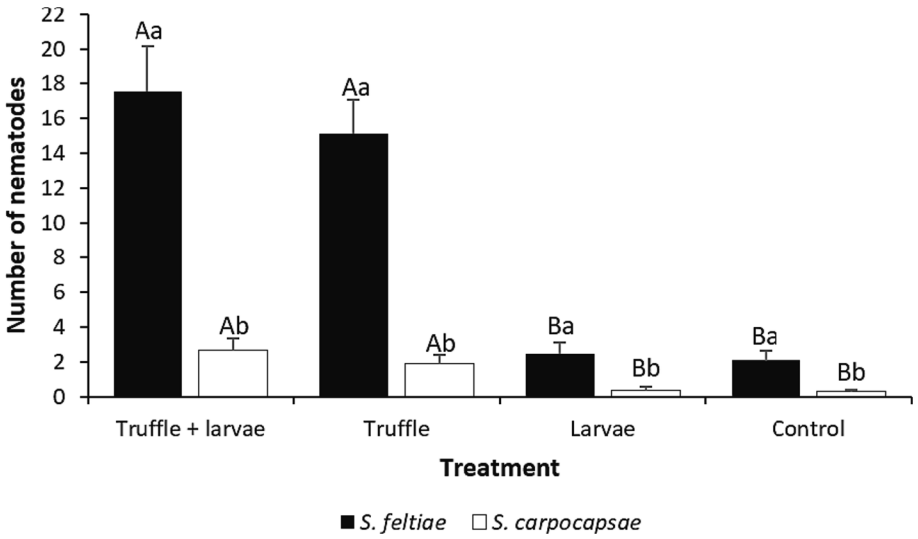

Fig. 3. Average number of nematodes (±SE) of *S. feltiae* and *S. carpocapsae* recovered from olfactometer arms 24 h after application. Uppercase letters indicate significant differences among treatments for each EPN species. Lowercase letters indicate significant differences between EPN species within each treatment. Data with the same letter are not significantly different ($p < 0.05$).

(Fig. 3). In fact, the number of nematodes in the treatment with only larvae was not significantly different from control (Fig. 3). Furthermore, there were also significant differences observed between both EPN species ($p < 0.01$). The number of *S. feltiae* IJs that moved to *T. melanosporum* fruitbodies, *L. cinnamomeus* larvae, and the control was higher than *S. carpocapsae* IJs (Fig. 3).

3.2. Chemotaxis assay

The analyses of the main effects showed that CI values were influenced by different factors (treatment and concentration) and their interactions (Table 2).

At low concentration, there were significant differences among VOCs after 4 h and 24 h ($F = 26.25$; $df = 14, 210$; $p < 0.001$ and $F = 26.07$; $df = 14, 210$; $p < 0.001$, respectively) (Table S1 and S2), with four alcohol compounds (1-octen-3-ol, 1-octanol, 3-methyl-1-butanol and 3-methylhexanol) being more attractive to *S. feltiae* than the control (CI = 0) (Fig. 4A and B). Compounds with the lowest CI were not significantly different from the control, indicating that none of them were repellent towards *S. feltiae*. When comparing both exposure times, only three VOCs (1-octen-3-ol, 1-octanol and Dimethyl disulfide) had a higher CI after 24 h compared to 4 h (Table 3).

At high concentration, there were also significant differences among VOCs after 4 h and 24 h ($F = 90.63$; $df = 13, 196$; $p < 0.001$ and $F = 120.97$; $df = 13, 196$; $p < 0.001$, respectively) (Table S1 and S2). The same alcohol compounds (1-octen-3-ol, 3-methyl-1-butanol and 3-methylhexanol), except for 1-octanol, were more attractive to *S. feltiae* than the control (Fig. 4A and B). In contrast, 2-methyl-1-butanol, 2-metilpropanol, 4-pentel-2-ol and 2,3-butanedione were repellent compared to the control, with a lower CI (Fig. 4A and B). When comparing both exposure times, the CI obtained by five VOCs (1-octen-3-ol, 2-methylpropanol, 4-pentel-2-ol, 2-methylbutanal and 2,3-butanedione) significantly differed between 4 and 24 h (Table 4). Moreover, Dimethyl disulfide was the only compound that paralyzed IJs after 4 h and 24 h at this concentration, so it is not present in Fig. 4A, B and Table 4.

Table 2
ANOVA results of the main effects and their interactions for the CI values.

Factor	F	df	P
Treatment	135.12	13	<0.001*
Concentration	350.25	1	<0.001*
Exposure time	1.27	1	0.268
Treatment x exposure time	5.94	13	<0.001*
Treatment x concentration	38.11	13	<0.001*
Concentration x exposure time	21.01	1	<0.001*
Treatment x concentration x exposure time	1.59	13	0.083

Residual df = 784.
*Indicates statistical significance.

4. Discussion

Our data show, for the first time, that *S. feltiae* and *S. carpocapsae* use VOCs cues (e.g. long-chain alcohols) emitted by black truffle fruitbodies. This suggests that EPN may use truffle-emitted blends of VOCs to enhance the likelihood of encountering fungivorous insects such as *L. cinnamomeus*. Various studies have reported that volatiles emitted by truffles mediate interactions with plants, insects, and mammals (Splivallo et al., 2011; Caboni et al., 2020; Garcia-Barreda et al., 2023) but this is the first evidence that truffle VOCs may play a role in the biological control of *L. cinnamomeus*, a major insect pest in truffle production.

EPNs were 8-fold more attracted to pots with *T. melanosporum* fruitbodies than to control pots, yet the presence of *L. cinnamomeus* larvae together with the fruitbodies only resulted in a marginal increase in *S. feltiae* and *S. carpocapsae* response. Scientific literature has widely reported that insect hosts and damaged plants can attract EPNs by emitting CO_2 and specific VOCs (Rasmann et al., 2005; Ali et al., 2012; Turlings et al., 2012). Moreover, recent studies have suggested that EPNs also have evolved to recognize and orient towards fungal chemicals, and even towards fungivore-induced fungal cues (Wu et al., 2018; Wu and Duncan, 2020; Hummadi et al., 2021). In fact, some evidence points towards a hierarchical order of EPN response to specific stimuli during foraging. It is believed that EPNs use CO_2 gradients and plant/fungus volatiles to locate insects from a distance, and insect-derived chemicals to accurately find a host (Turlings et al., 2012; Zhang et al., 2021). Therefore, our results suggest that chemical compounds emitted by truffles could be exploited by EPNs to locate insects from a distance.

The number of *S. feltiae* IJs that moved to *T. melanosporum* fruitbodies, *L. cinnamomeus* larvae, and control was significantly higher than *S. carpocapsae* IJs. Previous studies have observed that the behavioral response to these environmental cues may vary among EPN species or even strains (Hiltpold et al., 2010; Laznik and Trdan, 2016; Jagodič et al., 2017). It is generally accepted that *S. feltiae* evolved a more mobile foraging strategy compared to *S. carpocapsae*, which is mostly

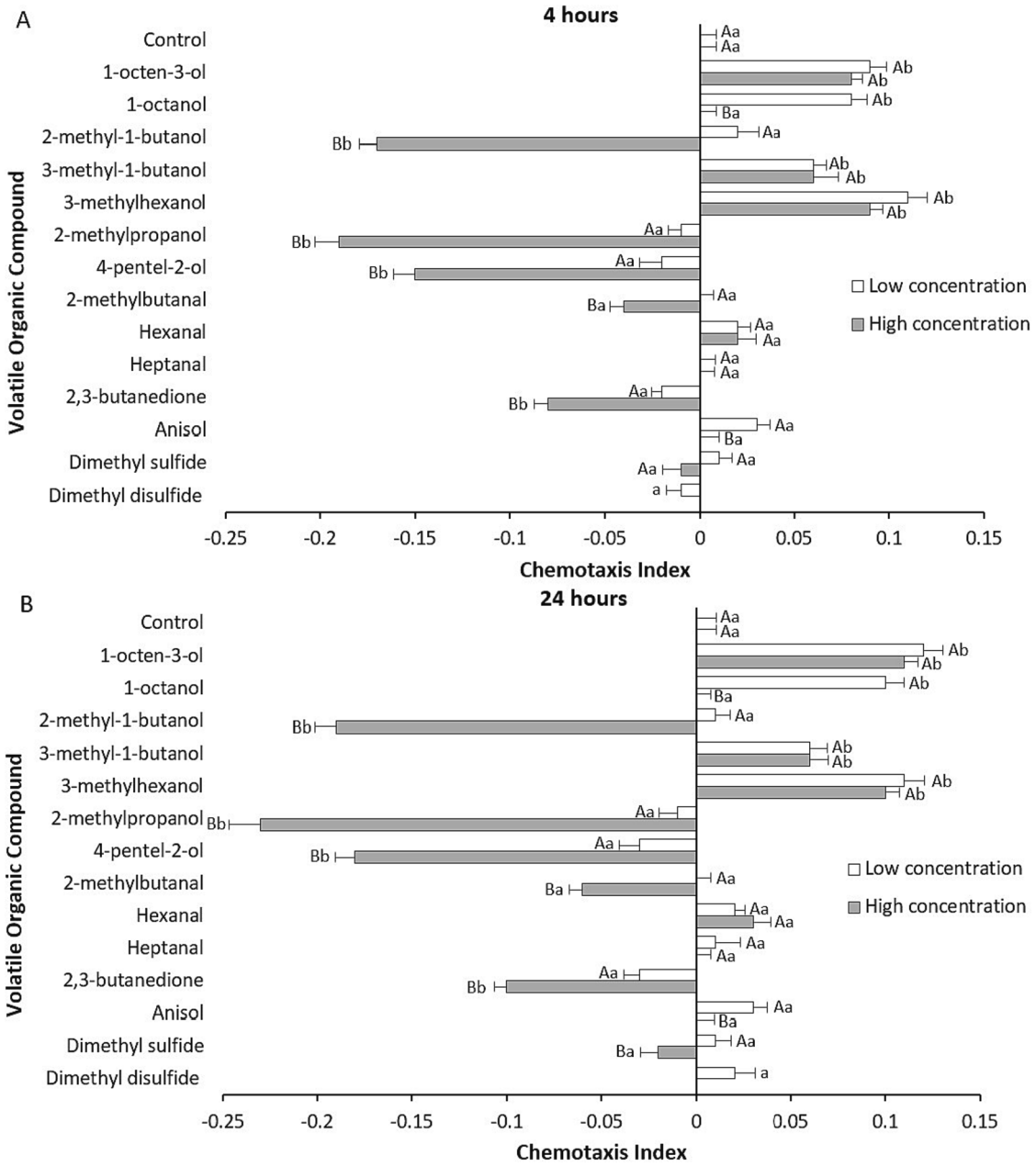

Fig. 4. Effect of different VOCs on the chemotactic response (CI ± SE) of *S. feltiae* at low and high concentration after (A) 4 h and (B) 24 h. Uppercase letters indicate significant differences between concentrations within the same VOCs. Lowercase letters indicate significant differences between the control and each VOC within the same concentration. Data with the same letter are not significantly different ($p < 0.05$).

considered as an ambusher nematode (Lewis, 2002; Lacey and Georgis, 2012), although several authors have challenged this classification (Hallem et al., 2011; Wilson et al., 2012). Wilson et al. (2012) suggested that *S. carpocapsae* prefers organic soils and so would be disadvantaged relative to *S. feltiae* in the sand medium used in the olfactometer of our study. It yet does not mean that *S. carpocapsae* would not move in the field as for example shown by Hiltpold and Hibbard (2018). This highlights the importance of behavioral ecology knowledge in the context of sustainable insect pest management with EPNs.

The results of our chemotaxis assay using *S. feltiae* varied depending on the VOCs tested, concentration, and several interactions among these factors, along with the exposure time. Although the low CI values (<0.2) obtained suggest that the attraction of IJs to VOCs should be considered as weak (Laznik and Trdan, 2016)., we also observed that IJs were more attracted to longer-chain alcohols such as 1-octen-3-ol, 1-octanol, 3-methyl-1-butanol, and 3-methylhexanol than to other VOCs and the control, especially at low concentration. Our results are consistent with those reported by O'Halloran and Burnell (2003), who observed that IJs of *H. bacteriophora* were highly attracted to long-chain alcohols compared to the control. Recently, 1-octen-3-ol was detected from the entomopathogenic fungus *Metarhizium brunneum* Petch, which attracted *H. bacteriophora* at low concentrations (Hummadi et al., 2021). Wu and Duncan (2020) also identified 1-octen-3-ol from the saprophytic fungus *Fusarium solani* Sacc., which was attractive for both fungivorous insects and *Steinernema diaprepesi* Nguyen & Duncan but repellent to non-fungivorous insects. In fact, they detected significantly less 1-octen-3-ol in *Fusarium oxysporum* Schlecht. than in *F. solani*, which may explain the lack of attraction of *S. diaprepesi* to *F. oxysporum* (Wu et al., 2018). Our data suggest that these long-chain alcohols may play a key role in the behavior of EPNs during their foraging in truffle soils. In fact,

Table 3
Effect of different VOCs on the chemotactic response (CI ± SE) of *S. feltiae* at low concentration after 4 and 24 h.

VOC	4 h	24 h	Statistical comparison
Alcohol			
1-octen-3-ol	0.09 ± 0.01a	0.12 ± 0.01b	F = 7.04; df = 1, 14; p = 0.018
1-octanol	0.08 ± 0.01a	0.10 ± 0.01b	F = 7.13; df = 1, 14; p = 0.018
2-methyl-1-butanol	0.02 ± 0.01a	0.01 ± 0.01a	F = 0.80; df = 1, 14; p = 0.380
3-methyl-1-butanol	0.06 ± 0.01a	0.06 ± 0.01a	F = 0.63; df = 1, 14; p = 0.441
3-methylhexanol	0.11 ± 0.01a	0.11 ± 0.01a	F = 0.71; df = 1, 14; p = 0.417
2-methylpropanol	−0.01 ± 0.01a	−0.01 ± 0.01a	F = 0.21; df = 1, 14; p = 0.654
4-pentel-2-ol	−0.02 ± 0.01a	−0.03 ± 0.01a	F = 1.69; df = 1, 14; p = 0.226
Aldehyde			
2-methylbutanal	0.00 ± 0.01a	0.00 ± 0.01a	F = 1.81; df = 1, 14; p = 0.198
Hexanal	0.02 ± 0.01a	0.02 ± 0.01a	F = 0.36; df = 1, 14; p = 0.554
Heptanal	0.00 ± 0.01a	0.01 ± 0.01a	F = 0.52; df = 1, 14; p = 0.483
Sulfur-containing			
Dimethyl sulfide	0.01 ± 0.01a	0.01 ± 0.01a	F = 0; df = 1, 14; p = 1
Dimethyl disulfide	−0.01 ± 0.01a	0.02 ± 0.01b	F = 6.79; df = 1, 14; p = 0.021
Ketone			
2,3-butanedione	−0.02 ± 0.01a	−0.03 ± 0.01a	F = 3.33; df = 1, 14; p = 0.091
Aromatic compound			
Anisole	0.03 ± 0.01a	0.03 ± 0.01a	F = 0; df = 1, 14; p = 1

Different letters indicate statistical significance between times for each row.

Table 4
Effect of different VOCs on the chemotactic response (CI ± SE) of *S. feltiae* at high concentration after 4 and 24 h.

VOC	4 h	24 h	Statistical comparison
Alcohol			
1-octen-3-ol	0.08 ± 0.01a	0.11 ± 0.01b	F = 23.5; df = 1, 14; p < 0.001
1-octanol	0.00 ± 0.01a	0.00 ± 0.01a	F = 0.13; df = 1, 14; p = 0.725
2-methyl-1-butanol	−0.17 ± 0.01a	−0.19 ± 0.01a	F = 3.34; df = 1, 14; p = 0.087
3-methyl-1-butanol	0.06 ± 0.01a	0.06 ± 0.01a	F = 0; df = 1, 14; p = 1
3-methylhexanol	0.09 ± 0.01a	0.10 ± 0.01a	F = 0.08; df = 1, 14; p = 0.786
2-methylpropanol	−0.19 ± 0.01a	−0.23 ± 0.02b	F = 17.42; df = 1, 14; p < 0.001
4-pentel-2-ol	−0.15 ± 0.01a	−0.18 ± 0.01b	F = 13.23; df = 1, 14; p < 0.01
Aldehyde			
2-methylbutanal	−0.04 ± 0.01a	−0.06 ± 0.01b	F = 10.27; df = 1, 14; p < 0.01
Hexanal	0.02 ± 0.01a	0.03 ± 0.01a	F = 0.20; df = 1, 14; p = 0.652
Heptanal	0.00 ± 0.01a	0.00 ± 0.01a	F = 0.48; df = 1, 14; p = 0.498
Sulfur-containing			
Dimethyl sulfide	−0.01 ± 0.01a	−0.02 ± 0.01a	F = 3.97; df = 1, 14; p = 0.064
Ketone			
2,3-butanedione	−0.08 ± 0.01a	−0.10 ± 0.01b	F = 13.65; df = 1, 14; p < 0.01
Aromatic compound			
Anisole	0.00 ± 0.01a	0.00 ± 0.01a	F = 0.06; df = 1, 14; p = 0.797

Different letters indicate statistical significance between times for each row.

EPNs may have evolved to exploit these VOCs as a means of encountering fungivorous insects.

We observed that the effect of these VOCs is concentration-dependent. On the one hand, 1-octen-3-ol, 3-methylhexanol and 3-methylbutanol were attractive towards *S. feltiae* at both concentrations. On the other hand, 2-methyl-1-butanol, 2-metilpropanol, 4-pentel-2-ol and 2,3-butanedione were repellent at high concentrations, but rather had a neutral impact at low concentration. Hummadi et al. (2021) showed that 1-octen-3-ol and 3-octanone caused mortality and did not attract IJs at high concentrations (100 %), while at low concentrations (1 % and 0.01 %) IJs were more attracted to this volatile with no significant mortality measured. Furthermore, the high concentrations used in our assays do not reflect natural conditions. These values were the total quantity extracted from 100 g of *T. melanosporum* fruitbodies (Tejedor-Calvo et al., 2021), but concentrations are much lower in soil due to their diffusion. It is important to consider that a Petri dish assay is suboptimal and prevents EPNs from exhibiting their natural behavior compared to using soil. Soil is a complex matrix, whose physical, biological, and chemical properties can considerably influence chemical emission, stability, diffusion, and degradation, thereby modulating EPN signal perception (Hiltpold and Turlings, 2008; Chiriboga et al., 2017; Som et al., 2017).

The blends of VOCs emitted by truffle fruitbodies vary during their ripening (Splivallo et al., 2011). Attractive compounds such as 1-octanol and 3-methylhexanol are relatively more frequent when *T. melanosporum* fruitbodies are immature (Jul-Sept) (Garcia-Barreda and Sánchez, unpublished results), while 1-octen-3-ol characterizes most of the mature fruitbodies (Caboni et al., 2020). Our olfactometer assays also demonstrated that EPNs are attracted to mature fruitbodies. Therefore, our results suggest that EPNs may be attracted to truffles throughout the entire ripening process. However, further studies should be conducted to assess the potential of *T. melanosporum* to attract EPNs throughout the ripening of the truffle fruitbodies.

5. Conclusions

This is the first demonstration that *S. feltiae* and *S. carpocapsae* are attracted towards mature fruitbodies of the black truffle *T. melanosporum,* which may enhance the likelihood of encountering *L. cinnamomeus* in the event of field application of EPNs. The presence of *L. cinnamomeus* larvae in the presence of truffle did not affect the behavior of EPNs 24 h after application, underlying the importance of chemical compounds emitted by the truffle itself (CO_2, VOCs). Chemotaxis assays highlighted the importance of long-chain alcohol compounds emitted by *T. melanosporum* fruitbodies in the attraction of EPNs, especially at low concentration, providing a first hint in the chemical ecology of a little-studied ecological system of great economical value. Further studies should be conducted to gain a better understanding of the tritrophic interactions between *T. melanosporum*, EPNs and *L. cinnamomeus* throughout the ripening of the truffle, as this knowledge may have practical implications for the efficacy of EPNs in the biological control of this pest.

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CRediT authorship contribution statement

Ivan Julià: Writing – review & editing, Supervision, Methodology, Investigation. **Ivan Hiltpold:** Writing – review & editing, Supervision, Methodology, Investigation. **Ana Morton:** Writing – review & editing, Supervision. **Fernando Garcia-del-Pino:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2024.108077.

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