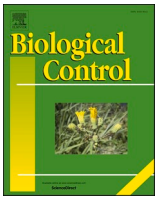
Biological Control 180 (2023) 105197

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon



The development of the truffle beetle *Leiodes cinnamomeus* at low temperature, a determining factor for the susceptibility of adults and larvae to entomopathogenic nematodes

Ivan Julià, Ana Morton, Fernando Garcia-del-Pino *

Departament de Biologia Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

HIGHLIGHTS

- Adults and larvae of *L. cinnamomeus* were susceptible to entomopathogenic nematodes.
- *S. carpocapsae* caused high mortality of adults at 10 °C with 6 h a day at 15 °C.
- Some populations of *S. feltiae* were virulent against mycophagous larvae at 10 °C.
- Larvae in summer diapause are susceptible to *H. bacteriophora* at 25 °C.
- There are short periods with optimal temperatures for EPN applications in autumn.

ARTICLE INFO

Keywords:
Entomopathogenic nematodes
Steinernema
Heterorhabditis
Low temperature
Leiodes cinnamomeus
Tuber melanosporum

ABSTRACT

The European truffle beetle *Leiodes cinnamomeus* (Panzer) (Coleoptera: Leiodidae) is the most important pest in black truffle (*Tuber melanosporum*) plantations. Adults and larvae feed on truffles during the cold months of autumn and winter, while during spring and summer larvae are in diapause. This study aims to test the susceptibility of *L. cinnamomeus* adults and larvae to different entomopathogenic nematode (EPN) species at various temperatures under laboratory conditions. Different populations of *Steinernema carpocapsae* and *Steinernema feltiae* were applied against adults and mycophagous larvae at 20 °C, 10 °C and 10–15 °C (10 °C during 18 h and 15 °C during 6 h a day), while *Heterorhabditis bacteriophora* was only applied against diapause larvae at 25 °C. *S. carpocapsae* caused 100% mortality of adults three days after application at 20 °C. At 10 °C, adults were not susceptible to any EPN species seven days after treatment, while at 10–15 °C *S. carpocapsae* was the most virulent species (76.6–96.6% mortality). In the case of larvae, all EPN species were infective at 20 °C (43.3–83.3% mortality), despite differences among some populations. At 10 °C, only two populations of *S. feltiae* caused higher mortality of larvae (50–53.3%) than control seven days after treatment. *H. bacteriophora* caused 100% mortality against diapause larvae five days after application at 25 °C. Soil temperature was measured in a truffle plantation for each hour every day from September 2021 to April 2022 at 20 cm depth. From September to mid-October it was registered a temperature above 15 °C for more than 20 h a day. Temperatures were generally below 10 °C from November to March. An appropriate timing of field applications should be considered due to the short periods of time when temperature is optimal for each EPN species tested.

1. Introduction

The black truffle or *Tuber melanosporum* Vittad. is a hypogeal fungus that establishes mutualistic relationships with different phanerogam species, mainly of the *Quercus* genus, that fructifies during autumn–-winter (Bonito et al., 2010). The black truffle fruit has gastronomic value and economic interest in some regions of Spain, Italy and France, where 40,000 ha of truffle plantations generates approximately €50 million per year (Oliach et al., 2020). All this leads to a monoculture situation that along with agricultural practices, such as irrigation, have favored the presence of some insect species, including the "truffle flies" of the genus *Suillia* sp (Diptera: Heleomyzidae) or the beetle *Leiodes cinnamomeus*

* Corresponding author.
E-mail address: Fernando.Garcia@uab.cat (F. Garcia-del-Pino).

https://doi.org/10.1016/j.biocontrol.2023.105197
Received 14 December 2022; Received in revised form 9 February 2023; Accepted 16 February 2023
Available online 18 February 2023

(Panzer) (Coleoptera: Leiodidae), becoming pests (Martín-Santafé, 2020).

The European truffle beetle, *L. cinnamomeus,* is the most important pest in black truffle plantations, being an univoltine species that is only distributed in Europe (Arzone, 1971; Martín-Santafé et al., 2014; Navarro-Llopis et al., 2021). In the province of Teruel (Spain), adults are observed from mid-September to mid-May and mycophagous larvae (L1 to L3) from October to March (Pérez-Andueza, 2015), during the coldest period of the year when temperature can reach 2 °C at 20 cm depth (unpublished results). L3 larvae enter diapause inside aestivation chambers, which last until the end of summer when several cohorts start to emerge as adults during autumn–winter (Pérez-Andueza, 2015). Larvae and adults are soil-dwelling stages and are distributed from 10 cm to 30 cm depth around the truffle fruits, except when adults rise to the soil surface to move to other truffles (Arzone, 1971). Both stages feed on *T. melanosporum* fruiting bodies, causing galleries which reduce quality and can generate up to 70% of economic losses in plantations (Barriuso et al., 2012).

Cultural practices, such as frequent recollections of truffles (Martín-Santafé et al., 2014) and the use of traps for mass capture of adults (Navarro-Llopis et al., 2021), are recommended in truffle plantations. However, these practices are not enough to reduce the population of *L. cinnamomeus* to acceptable levels. Limited research has been conducted on the biorational control of *L. cinnamomeus*, with mass trapping techniques based on adapted pitfall traps and the semiochemical dimethyl sulfide (DMS) as an attractant (Navarro-Llopis et al., 2021). The use of chemical products is not appropriate to control this insect because they may inhibit the growth of the mycelium (Trappe et al., 1984) and affect early stages of root colonization by *T. melanospotum* (Gómez-Molina et al., 2020). Moreover, there is a strict regulation of these chemical substances by the Administration, which tends towards their rational and sustainable use (Directive 2009/128/EC, 2009). Thus, alternative biological control methods are needed.

Entomopathogenic nematodes (EPNs) of the Steinernematidae and Heterorhabditidae families are obligate parasites of a wide range of insect species with great potential as biological control agents for different soil inhabiting insect pests on a variety of crops (Lacey and Georgis, 2012; Shapiro-Illan et al., 2017). These nematodes have proved to be successful against adults and larvae of different soil-dwelling coleoptera such as white grubs (Coleoptera: Scarabaeidae) (Patil et al., 2018; Benseddik et al., 2021), weevils (Coleoptera: Curculionidae) (Shapiro-Illan et al., 2003; Batalla-Carrera et al., 2016; Dlamini et al., 2019) and wireworms (Coleoptera: Elateridae) (Sandhi et al., 2020; Nikoukar et al., 2021). In field applications, the efficacy of EPNs could be affected by environmental factors (Shapiro-Ilan et al., 2006), such as low temperature, limiting the mobility and infectivity of some EPN species (Grewal et al., 1994). When adults and larvae of *L. cinnamomeus* feed on truffles (during autumn and winter), soil temperature in truffle plantations (2–20 °C) often drop below the acceptable threshold temperature for EPN activity (unpublished results).

At this moment, there are no relevant studies about the biological control of *L. cinnamomeus* and only preliminary data on the susceptibility of *L. cinnamomeus* to EPNs are available (Fuentes-Boix et al., 2019). Therefore, the objective of this research was to determine the susceptibility of adults and larvae of *L. cinnamomeus* to different EPN species exposed to different temperatures under laboratory conditions, with the aim to select the most appropriate population of EPNs and period of application against each stage of *L. cinnamomeus* in future field assays.

2. Material and methods

Source of insects and nematodes

Adults and larvae of *L. cinnamomeus* were collected from truffle plantations in Teruel, Spain, during October and December of 2021. Mycophagous larvae (which we will refer to as "larva" in the text) with a body length of 0.5–0.9 cm (L2 and L3 instars) were used. Both stages were maintained in boxes at 9 °C filled with soil from truffle plantations and small pieces of truffle fruit as food until being used. To obtain diapause larvae, L3 larvae were maintained in tubes (30 × 8 cm) filled with soil at 10 °C from February to April of 2022, while during May and June they were maintained at room temperature.

A total of eight EPN populations belonging to three different species were used: *Steinernema carpocapsae* (Weiser)*, Steinernema feltiae* (Filipjev) and *Heterorhabditis bacteriophora* (Poinar) (Table 1). Nematodes were reared at 25 °C in last instar larvae of the greater wax moth, *Galleria mellonella* (Linnaeus), according to the method of 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 3 h and their viability was checked by observation of movement under a stereomicroscope.

2.1. Insect susceptibility assays

The experiments were carried out in Petri dishes (5.5 cm diameter) filled with 15 g of sterile soil obtained from truffle plantations and moistened with sterile tap water (10%, w/w). One adult or larva was placed in each dish. *S. feltiae* and *S. carpocapsae* populations were used against adults and mycophagous larvae at three different temperatures: 20 °C, 10 °C and 10–15 °C (10 °C during 18 h and 15 °C during 6 h a day). *H. bacteriophora* was only tested against diapause larvae at 25 °C. All EPNs were used at a dose of 25 IJs/cm^2 (490 IJs/dish). The dishes were sealed with parafilm to avoid dehydration and maintained in a climate chamber at the temperature of each experiment. Control treatment received only sterile tap water. Insect mortality was checked every 24 h after application during three days for the treatments at 20 °C, for five days for those carried out at 25 °C and for seven days for those at 10 °C and 10–15 °C. Dead insects were individually placed in another Petri dish with two moistened filter paper disks without nematodes. 48 h after death, cadavers were dissected to confirm nematode infection. There were ten replicates for each treatment and the experiments were conducted three times.

2.2. Soil temperature analysis from truffle plantation

To evaluate the possible effect of the temperature tested against *L. cinnamomeus* for future field applications, soil temperature was measured for each hour every day from September 2021 to April 2022 at 20 cm depth in a truffle plantation located in Mora de Rubielos (Teruel, Spain). Mean temperature and the mean number of hours a day with $\geq$ 10 °C and $\geq$ 15 °C were calculated to select the best periods for potential EPN applications.

2.3. Statistical analysis

Generalized linear model (GLM), with binomial distribution and a logit link function, was used to test significant differences in mortality.

Table 1
Populations of *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* used in the susceptibility assays.

EPN species	Population	Habitat	Location
S. carpocapsae	B14	Urban garden	Barcelona
S. carpocapsae	e-nema	Commercial	
S. feltiae	TE15	Truffle plantation	Mora de Rubielos (Teruel)
S. feltiae	CT3	Truffle plantation	Naves (Lleida)
S. feltiae	e-nema	Commercial	
S. feltiae	Koppert	Commercial	
H. bacteriophora	CT47	Truffle plantation	Boixols (Lleida)
H. bacteriophora	Koppert	Commercial	

Main effects in the factorial design (EPN population and temperature) were analyzed for interactions and a complete analysis of simple effects was also conducted. Within each temperature, mortality caused by EPN populations were compared among them and to control. The impact of temperature on virulence within each EPN population was also analyzed. Subsequently Tukey's multiple range test was performed to compare differences among treatments. Lethal time 50 (LT_{50}) and corresponding 95% confidence intervals (CIs) were calculated for adult and larva treatments using probit analysis. All data were analyzed with the R software (version 4.1.0) (R Core Team, 2021). Any comparison was considered significant if p value was<0.05.

3. Results

3.1. Adult susceptibility assay

The interaction between EPN population and temperature factors on mortality of *L. cinnamomeus* adults was significant ($\chi^2 = 59.57$, df = 10, $p < 0.05$). When the main effects were analyzed independently, the EPN population had a significant impact on adult mortality ($\chi^2 = 85$, df = 5, $p < 0.05$). The effect of the temperature also had a significant impact on mortality of adults among the nematode treatments ($\chi^2 = 117.14$, df = 2, $p < 0.05$).

The results at 20 °C showed that all EPN populations, except *S. feltiae* CT3, caused higher mortality than control (13.3%) ($\chi^2 = 94.05$, df = 6, p < 0.05) three days after application, with significant differences between the species tested (Fig. 1). *S. carpocapsae* caused 100% adult mortality, being the most virulent species compared to *S. feltiae* (56.6–73.3%) ($p < 0.05$).

At 10 °C, there were no significant differences between treatments and control (3.3% mortality) ($\chi^2 = 8.65$, df = 6, p = 0.19) (Fig. 2) seven days after application. However, at 10–15 °C, only *S. carpocapsae* populations were significantly different from the other treatments ($\chi^2 = 108.17$, df = 6, $p < 0.05$) seven days after nematode application, with mortality of 96.6% for *S. carpocapsae* B14 and 76.6% for *S. carpocapsae* e-nema. *S. feltiae* populations were not significantly different from control (3.3% mortality), with mortality ranging from 10% to 30% (Fig. 2).

All EPNs were significantly more virulent at 20 °C (three days after application) than at 10 °C (seven days after application). Only *S. carpocapsae* populations were significantly more virulent at 10–15 °C (76.6–96.6% mortality) than at 10 °C (13.3% mortality) (Table 2).

Temperature also affected the rate of infection of both steinernematid species. At 20 °C, *S. carpocapsae* e-nema and B14 were the fastest in killing adults (LT_{50} = 27.8 h and 33.9 h, respectively), whereas *S. feltiae* CT3 was the slowest (LT_{50} = 89.8 h). Therefore, there were significant differences among EPN species (Table 3). At 10–15 °C, *S. carpocapsae* B14 and e-nema reached LT_{50} of 113.5 h ($\chi^2 = 9.98$, df = 4, p = 0.04) and 135.2 h ($\chi^2 = 3.96$, df = 4, p = 0.41), respectively (Table 3). LT_{50} data of *S. feltiae* populations are not shown because they were not significant due to the low mortality obtained.

3.2. Mycophagous larvae susceptibility assay

The interaction between EPN population and temperature factors was significant on mortality of *L. cinnamomeus* larvae ($\chi^2 = 28.01$, df = 10, $p < 0.05$). However, when the main effects were analyzed independently, the EPN population had not a significant impact on larvae mortality among nematode treatments ($\chi^2 = 6.35$, df = 5, p = 0.27), while the effect of temperature had a significant impact ($\chi^2 = 25.86$, df = 2, $p < 0.05$).

At 20 °C, all treatments were significantly more virulent than control (16.6% mortality) ($\chi^2 = 44.33$, df = 6, $p < 0.05$) (Fig. 3) three days after application. There were significant differences only between *S. carpocapsae* e-nema (83.3%) and two *S. feltiae* populations: *S. feltiae* e-nema (46.6%) and *S. feltiae* Koppert (43.3%) ($p < 0.05$).

At 10 °C, only *S. feltiae* TE15 (53.3%) and *S. feltiae* Koppert (50%) caused significantly higher mortality of larvae than control (10%) ($\chi^2 = 25.56$, df = 6, $p < 0.05$) (Fig. 4) seven days after application. At 10–15 °C, all treatments were significantly more virulent than control (16.6% mortality) ($\chi^2 = 22.91$, df = 6, $p < 0.05$), but there were no significant differences among EPNs (p greater than 0.05), with mortality ranging from 53.3 to 70% (Fig. 4).

Only *S. carpocapsae* caused significantly higher mortality of larvae at 20 °C (56.6–83.3%) three days after treatment than at 10 °C (17–20%) seven days after treatment. There were also significant differences in mortality between 10 °C and 10–15 °C with both *S. carpocapsae* populations, but not with *S. feltiae* (Table 4).

LT_{50} were significantly different among EPNs at 20 °C (Table 5), being *S. carpocapsae* e-nema and *S. feltiae* TE15 the fastest nematodes to kill *L. cinnamomeus* larvae (LT_{50} = 44.5 h and 48.1 h, respectively). At 10 °C, *S. feltiae* TE15 and *S. feltiae* Koppert registered LT_{50} of 151 h and 169 h, respectively. LT_{50} data of the other populations are not shown because they were not significant due to the low mortality obtained. At 10–15 °C, *S. feltiae* TE15 and *S. feltiae* Koppert were also the fastest nematodes to kill larvae (LT_{50} = 113.1 h and 101.5 h, respectively)

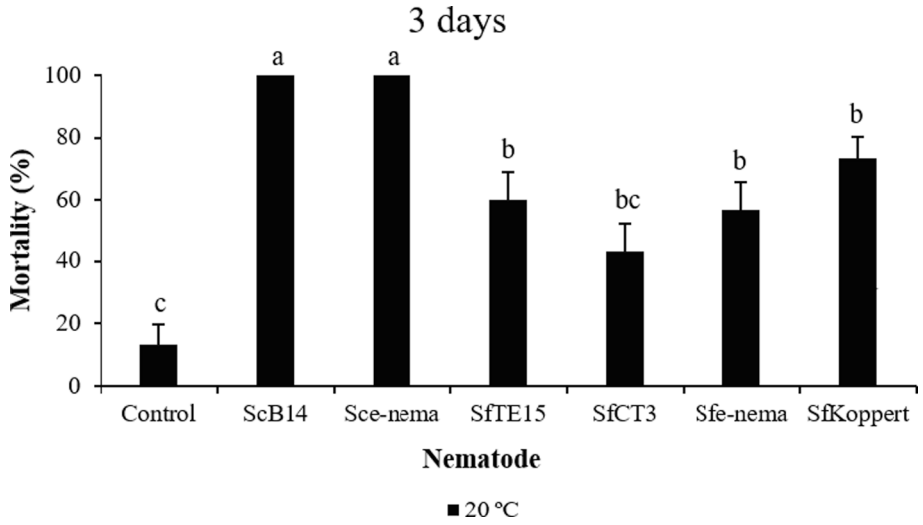


Fig. 1. Percentage of mortality (± SE) of *L. cinnamomeus* adults exposed to six different EPN populations and control at 20 ºC three days after treatment. Within each column, different letters indicate significant differences among treatments ($p < 0.05$). Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

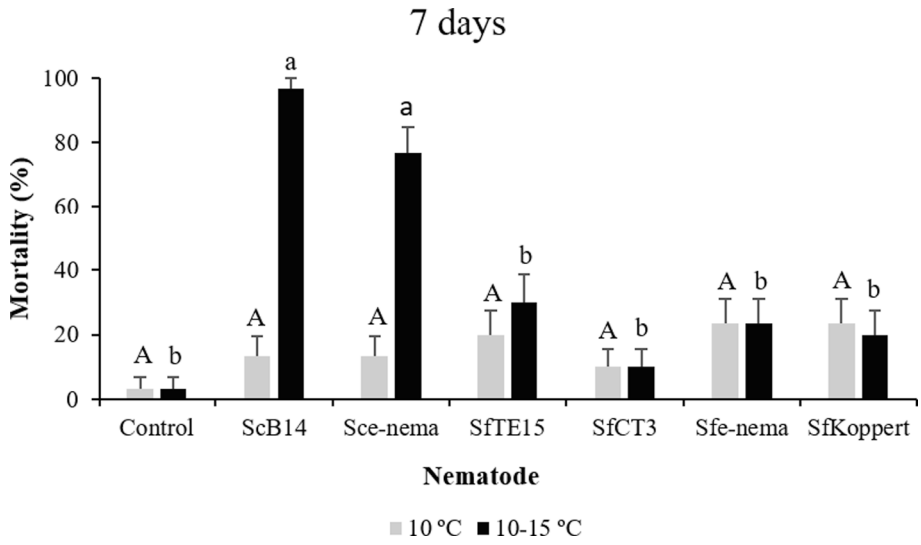


Fig. 2. Percentage of mortality (± SE) of *L. cinnamomeus* adults exposed to six different EPN populations and control at 10 ºC and at 10-15 ºC seven days after application. Within each column, uppercase letters indicate significant differences among treatments at 10 ºC ($p < 0.05$) and lowercase letters indicate significant differences among treatments at 10-15 ºC. Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

Table 2
Percentage of mortality (±SE) of *L. cinnamomeus* adults exposed to six different EPN populations at 20 °C three days after application, and at 10 °C and 10–15 °C seven days after application. Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

Treatment	20 °C	10 °C	10–15 °C	Statistical comparison
Sc B14 (%)	100 ± 0a	13.3 ± 6.3b	96.6 ± 3.3a	$\chi^2 = 77.62$, df = 2, p < 0.05
Sc e-nema (%)	100 ± 0a	13.3 ± 6.3c	76.6 ± 7.8b	$\chi^2 = 62.13$, df = 2, p < 0.05
Sf TE15 (%)	60 ± 9.1a	20 ± 7.4b	30 ± 8.5ab	$\chi^2 = 11.23$, df = 2, p < 0.05
Sf CT3 (%)	43.3 ± 8.7a	10 ± 5.5b	10 ± 5.5b	$\chi^2 = 12.71$, df = 2, p < 0.05
Sf e-nema (%)	56.6 ± 9.2a	23.3 ± 7.8b	23.3 ± 7.8b	$\chi^2 = 9.66$, df = 2, p < 0.05
Sf Koppert (%)	73.3 ± 6.9a	23.3 ± 7.8b	20 ± 7.4b	$\chi^2 = 22.87$, df = 2, p < 0.05

Different letters indicate statistical significance among temperatures for each row.

Table 3
Estimated exposure time to cause 50% mortality (LT_{50} in hours) of *L. cinnamomeus* adults by six EPN populations at 20 °C, with 95% confidence interval (CI). Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

Treatment	LT_{50} (CI 95%)	Pearson goodness
Sc B14	33.9 (30.0–38.4)a	$\chi^2 = 0.50$, df = 1, p = 0.97
Sc e-nema	27.8 (25.2–34.3)a	$\chi^2 = 0.26$, df = 1, p = 0.98
Sf TE15	62.7 (52.5–72.5)b	$\chi^2 = 3.12$, df = 5, p = 0.68
Sf CT3	89.8 (73.0–109)c	$\chi^2 = 0.68$, df = 3, p = 0.87
Sf e-nema	63.3 (53.9–72.2)b	$\chi^2 = 0.18$, df = 5, p = 0.99
Sf Koppert	58.9 (49.2–68.0)b	$\chi^2 = 4.53$, df = 5, p = 0.47

Values of Pearson goodness of fit χ^2, *df, p* are given (when p greater than 0.05, the model adequately fits the data). Different letters indicate statistical significance among treatments.

(Table 5).

3.3. Diapause larvae susceptibility assay

At 25 °C, *H. bacteriophora* CT47 and *H. bacteriophora* Koppert caused 100% mortality of diapause larvae five days after application, being significantly different from control (0% mortality) ($\chi^2 = 83.11$, df = 2, p < 0.05).

H. bacteriophora CT47, with a LT_{50} of 33.5 h (CI 95% = 28.2–38.5 h) ($\chi^2 = 0.45$, df = 1, p = 0.79), was significantly faster to kill 50% of diapause larvae than *H. bacteriophora* Koppert, with a LT_{50} of 42.2 h (CI 95% = 35.0–48.9 h) ($\chi^2 = 1.51$, df = 1, p = 0.68).

3.4. Soil temperature analysis from truffle plantation

The mean soil temperature and the mean number of hours a day at ≥ 10 °C and ≥ 15 °C during the period when *L. cinnamomeus* feeds on truffles in the plantation are given in Fig. 5. Temperature was above 15 °C for more than 20 h a day from September to mid-October. During these two months, temperature was above 10 °C 24 h a day, while in early November was<12 h a day. From late November to March temperature was generally below 10 °C. During April, temperature was above 10 °C for more than 12 h a day, but below 15 °C (Fig. 5).

4. Discussion

Our results indicate that EPNs were able to infect and kill adults and larvae of *L. cinnamomeus*, although there were differences of their susceptibility depending on the temperature and the EPN applied. *S. carpocapsae* was the most virulent (100% mortality) and fastest (LT_{50} = 27.8–33.9 h) EPN species against adults of *L. cinnamomeus* at 20 °C. In other studies, S. *carpocapsae* showed to be more virulent against adults of coleoptera than other nematodes when temperature is optimal for this species. Batalla-Carrera et al. (2016) reported that 3 days after the application of *S. carpocapsae*, adults of *Curculio nucum* (Linnaeus) caused mortality of 100% at 25 °C. Similar results were obtained by Shapiro-Illan et al. (2003) and Laznik et al. (2010) against *Curculio caryae* (Horn) and *Oulema melanopus* (Linnaeus), respectively.

Contrary to the differences observed between *S. carpocapsae* and *S. feltiae* against adults, mortality of larvae caused by *S. carpocapsae* populations (56.6–83.3%) were not significantly different from the two most virulent *S. feltiae* populations (50–73.3%) at 20 °C. Some studies did not find significant differences between EPN species against larvae of other coleoptera species. Batalla-Carrera et al. (2016) reported no differences between *S. feltiae* (50–65% mortality) and *S. carpocapsae* (55% mortality) against larvae of *C. nucum* at 25 °C. Likewise, Shapiro-Illan et al. (2000) did not observe differences between mortality caused by *S. carpocapsae* (18%) and *S. feltiae* (25–28%) against larvae of *Diaprepes abbreviatus* (Linnaeus) at 20 °C. In contrast, other studies found

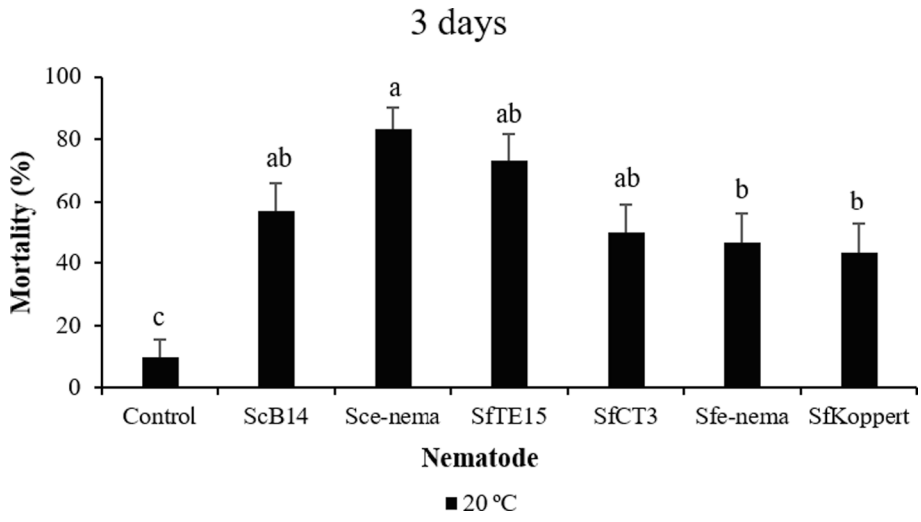


Fig. 3. Percentage of mortality (± SE) of *L. cinnamomeus* larvae exposed to six different EPN populations and control at 20 ºC three days after application. Within each column, different letters indicate significant differences among treatments ($p < 0.05$). Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

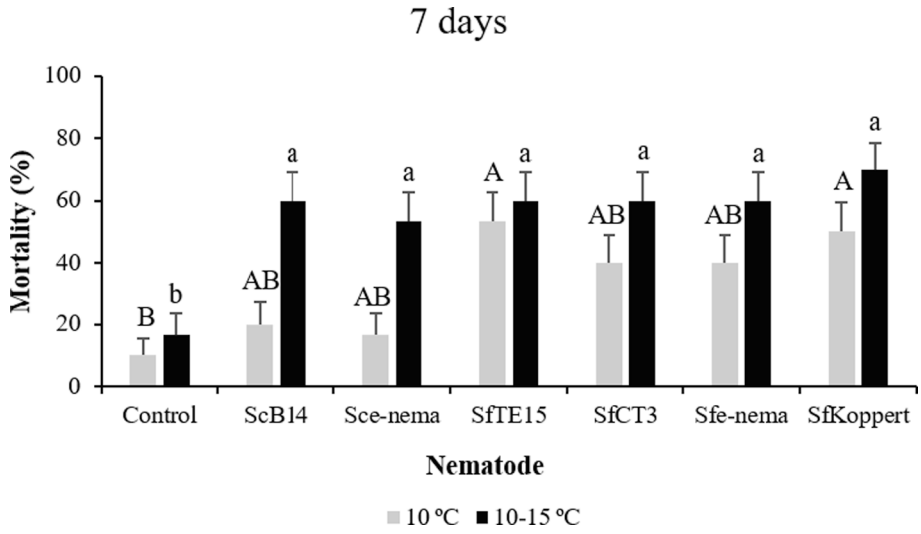


Fig. 4. Percentage of mortality (± SE) of *L. cinnamomeus* larvae exposed to six different EPN populations and control at 10 ºC and 10-15 ºC seven days after application. Within each column, uppercase letters indicate significant differences among treatments at 10 ºC ($p < 0.05$) and lowercase letters indicate significant differences among treatments at 10-15 ºC. Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

Table 4
Percentage of mortality (±SE) of *L. cinnamomeus* larvae exposed to six different EPN populations at 20 °C three days after application; and 10 °C and 10–15 °C seven days after application. Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

Treatment	20 °C	10 °C	10–15 °C	Statistical comparison
Sc B14(%)	56.6 ± 9.2a	20 ± 7.4b	60 ± 9.1a	$\chi^2 = 12.59$, df = 2, p < 0.05
Sc e-nema (%)	83.3 ± 6.9a	17 ± 6.9c	53.3 ± 9.2b	$\chi^2 = 29.19$, df = 2, p < 0.05
Sf TE15 (%)	73.3 ± 8.2a	53.3 ± 9.2a	60 ± 9.1a	$\chi^2 = 2.70$, df = 2, p = 0.26
Sf CT3 (%)	50 ± 9.3a	40 ± 9.1a	60 ± 9.1a	$\chi^2 = 2.41$, df = 2, p = 0.29
Sf e-nema (%)	46.6 ± 9.2a	40 ± 9.1a	60 ± 9.1a	$\chi^2 = 2.50$, df = 2, p = 0.28
Sf Koppert (%)	43.3 ± 9.2a	50 ± 9.3a	70 ± 8.5a	$\chi^2 = 4.76$, df = 2, p = 0.09

Different letters indicate statistical significance among temperatures for each row.

significant differences between EPN species. Nikoukar et al (2021) reported a significant higher mortality caused by S. feltiae (63%) against larvae of *Limonius californicus* (Mannerheim) than *S. carpocapsae* (30%) at 23 °C. Morton and Garcia-del-Pino (2016) observed that *S. carpocapsae* was significantly more virulent (68.9% mortality) against larvae of *Agriotes obscurus* (Linnaeus) than *S. feltiae* (8.9% mortality) at 23 °C. Thus, these variations in results among studies might be due to the different physical factors related with each coleoptera species (Bastidas et al., 2014; Sandhi et al., 2020).

One of the major obstacles to EPNs efficacy in field applications against *L. cinnamomeus* could be low temperature at which this insect develops during autumn and winter. Temperature is known to affect entomopathogenic nematode infectivity, virulence and reproductive capacity. Although most species are active at 20 to 30 °C, some of them are more tolerant to cold temperatures than others (Grewal et al., 1994). The adaptation of *S. feltiae* to low temperature has been reported by Hominick and Briscoe (1990) and Wright (1992), who observed that this species is more prevalent in cooler environments than *S. carpocapsae*. The optimal temperature range for *S. feltiae* to infect insects is usually 15–25 °C and 20–30 °C for *S. carpocapsae,* although

Table 5
Estimated exposure time to cause 50% mortality (LT_{50} in hours) of *L. cinnamomeus* larvae by six EPN populations at 20 °C and 10–15 °C, with 95% confidence interval (CI). Sf: *S. feltiae*; and Sc: *S. carpocapsae*.

Treatment	20 °C		10–15 °C	
	LT_{50} (CI 95%)	Pearson goodness	LT_{50} (CI 95%)	Pearson goodness
Sc B14	68.4 (52.2–85.8)b	$\chi^2 = 3.84$, df = 4, p = 0.42	153.2 (133.4–195.5c	$\chi^2 = 1.21$, df = 4, p = 0.87
Sc e-nema	44.5 (22.0–62.6)a	$\chi^2 = 11.5$, df = 4, p = 0.02	149.3 (130.9–187.8)c	$\chi^2 = 0.37$, df = 4, p = 0.98
Sf TE15	48.1 (23.1–69.6)a	$\chi^2 = 5.93$, df = 3, p = 0.11	113.1 (85.9–228.2)ab	$\chi^2 = 0.18$, df = 3, p = 0.98
Sf CT3	73.5 (47.0–97.2)bc	$\chi^2 = 0.34$, df = 2, p = 0.84	125.6 (101.6–188.0)b	$\chi^2 = 0.07$, df = 3, p = 0.99
Sf e-nema	83.6 (70.8–99.4)c	$\chi^2 = 1.06$, df = 4, p = 0.90	133.3 (106.4–209.7) bc	$\chi^2 = 0.59$, df = 4, p = 0.96
Sf Koppert	84.1 (56.5–107.6)c	$\chi^2 = 0.95$, df = 4, p = 0.91	101.5 (80.5–128.4)a	$\chi^2 = 0.83$, df = 4, p = 0.93

Values of Pearson goodness of fit χ^2, *df, p* are given (when p greater than 0.05, the model adequately fits the data). Different letters indicate statistical significance among treatments for each temperature.

both can infect at lower temperatures (8–10 °C and 12–15 °C, respectively) (Grewal et al., 1994; Shapiro-Ilan et al., 2006). Nevertheless, six hours a day at 15 °C were enough for *S. carpocapsae* to cause higher mortality rates of adults (76.6–96.6%) than *S. feltiae* (10–30%). In fact, only *S. carpocapsae* was significantly more virulent at 10–15 °C (76.6–96.6%) than at 10 °C (13.3%). These results agree with the results of Laznik et al. (2010), in which *S. carpocapsae* caused 72% and 100% mortality of adults of *O. melanopus* 48 h and 120 h after nematodes application at 15 °C, respectively. Our results also agree with those obtained by Grewal et al. (1994) and Radová and Trnková (2010), who reported a significant reduction of the infectivity of *S. carpocapsae* against larvae of *G. mellonella* and *Tenebrio molitor* (Linnaeus) at temperature below 15 °C. Although 15 °C is not the optimal temperature for *S. carpocapsae* to infect insects, its high virulence against adults of *L. cinnamomeus* indicates that it may be the best option against them. In truffle plantations in Teruel, these adults start to emerge on mid-September (Pérez-Andueza, 2015), when temperature data from the truffle plantation registered more than 20 h a day at ≥ 15 °C from September to mid-October. Therefore, *S. carpocapsae* should be applied during this period when temperature is above 15 °C.

In the larvae susceptibility assay, *S. feltiae* TE15 and *S. feltiae* Koppert were the only nematodes that caused significant higher mortality (50–53.3%) than control (10%) at 10 °C. When temperature was raised to 15 °C for 6 h a day, these two populations of *S. feltiae* caused the fastest death of larvae (LT_{50} = 113 h and 101 h, respectively). In fact, mortality caused by *S. feltiae* populations were not significantly different among the three temperatures tested, while *S. carpocapsae* populations were significantly less virulent at 10 °C than at 20 °C and 10–15 °C. *S. feltiae* is able to infect and cause mortality of insects at 8–10 °C, although the temperature is lower than its optimal range (15–25 °C) (Grewal et al., 1994). Shapiro-Illan et al. (2011) reported the virulence of *S. feltiae* against larvae of *Conotrachelus nenuphar* (Herbst) at low temperature. Twelve days after nematode application at 12 °C, *S. feltiae* caused significantly higher mortality of larvae (60%) than *S. carpocasae* (30%). Lacey et al. (2006) also found that *S. feltiae* and *S. carpocapsae* were both efficient against larvae of the codling moth *Cydia pomonella* (Linnaeus) at 20–25 °C, but when temperature fell below 15 °C, *S. feltiae* was significantly more active compared to *S. carpocapsae.* Therefore, our results suggest that for field applications, *S. feltiae* could be the best option to use against larvae of *L. cinnamomeus* in cold conditions, although temperature should not drop below 10 °C. Therefore, the optimal period to apply *S. feltiae* may be between early October and November, when larvae start feeding of *T. melanosporum*.

During spring and summer, when larvae of *L. cinnamomeus* are in diapause (Martín-Santafé, 2020), low temperature is not a limiting factor to apply EPNs. Our results showed that *H. bacteriophora* caused 100% mortality of these diapause larvae at 25 °C five days after application. Some other studies also reported high virulence of *H. bacteriophora* against larvae of other soil dwelling coleoptera species, such as *Popillia japonica* Newman (Paoli et al., 2017) and *Otiorhynchus sulcatus* Fabricius (Ansari and Butt, 2011). Therefore, *H. bacteriophora* could be a good option against diapause larvae of *L. cinnamomeus*.

The soil depth where the stages of the insect are present is another factor to consider before field application. There are different behaviors in relation to vertical migration depending on the EPN species. Within a favorable range of temperatures and adequate moisture, those EPNs with a mobile foraging strategy such as *S. feltiae* and *H. bacteriophora* could be considered for use in subterranean habitats, while those with a sit-and-wait foraging strategy, such as *S. carpocapsae*, could be most effective in soil surface habitats (Koppenhöfer et al., 1995; Lacey and Georgis, 2012). In our case, larvae and adults of *L. cinnamomeus* are soil dwelling stages that are found from 10 cm to 30 cm depth, depending on the presence of truffle fruits. However, adults also present activity on soil surface, being more mobile than larvae (Arzone, 1971). Therefore,

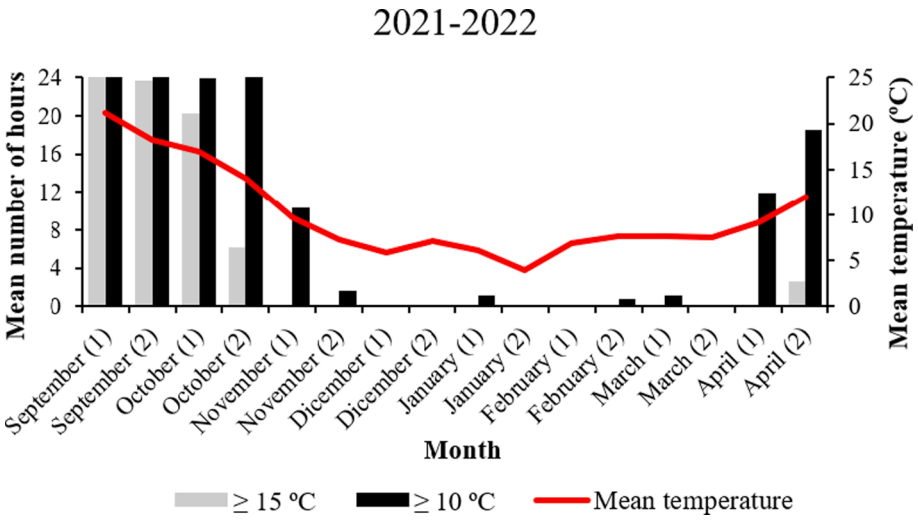


Fig. 5. Mean soil temperature and mean number of hours a day which the temperature registered was ≥ 10 ºC or ≥ 15 ºC in a truffle plantation at 20 cm depth (Teruel, Spain), during the period of September 2021 to April 2022. Each month is divided into the first half: 1st to 15th (1), and the second half: 16th to 31st (2).

the foraging strategy of EPNs and the results obtained in the susceptibility assay show *S. carpocapsae* as the best option against adults. For the treatment against larvae, *S. feltiae* would be the best option during autumn–winter, and *H. bacteriophora* would be the best choice against diapause larvae during spring-summer, due to the high mobility of both EPN species.

5. Conclusions

To conclude, our study showed that adults and larvae of *L. cinnamomeus* are susceptible to the EPN species tested. Even though adults are high susceptible to *S. carpocapsae* at 20 °C and 10–15 °C, the low mortality of adults at 10 °C suggests that field application of this nematode in Teruel (Spain) should be done during September and early October, when temperature is at least 6 h a day above 15 °C. In the case of mycophagous larvae, *S. feltiae* may be an optimal option at temperatures between 10 °C and 15 °C. However, it is necessary to prevent efficacy losses of this species due to temperatures below 10 °C from December to March. Application of *H. bacteriophora* against diapause larvae during spring and summer could be a complementary strategy to be considered due to the high mortality caused by this nematode. Therefore, a proper timing of EPN application should be considered in future field trials.

CRediT authorship contribution statement

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

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.

Acknowledgements

We would like to thank la Asociación de Truficultores de Teruel (ATRUTER), especially Julio Perales for providing soil temperature data of the truffle plantation and Rafael Doñate, for providing *L. cinnamomeus* adults and larvae for the assays. We also wish to thank Koppert and e-nema for providing commercial isolates of nematodes.

Funding.

This work was supported by Proyecto I + D + I CIBR – FITE 2021 (CITA) Gobierno de Aragón: Nuevas estrategias de control del escarabajo de la trufa en Teruel.

References

Ansari, M.A., Butt, T.M., 2011. Effect of potting media on the efficacy and dispersal of entomopathogenic nematodes for the control of black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Biol. Control 58 (3), 310–318. https://doi.org/10.1016/j.biocontrol.2011.05.016.

Arzone, A., 1971. Reperti ecologici ed etologici di *Leiodes cinnamomea* Panzer vivente Panzer su *Tuber melanosporum* Vittadini (Coleoptera Staphylinoidea). Ann. Fac. Sci. Agrar. Univ. Degli. Stud. Torino. 5, 317–357.

Barriuso, J., Martín-Santafé, M., Sánchez, S., Palazón, C., 2012. Plagas y enfermedades asociadas al cultivo de la trufa. In: Reyna, S. (Ed.), Truficultura, fundamentos y técnicas. Ediciones Mundi Prensa, Madrid, Spain, pp. 275–302.

Bastidas, B., Edgar, P., San Blas, E., 2014. Size does matter: The life cycle of Steinernema spp. in micro–insect hosts. J. Invertebr. Pathol. 121, 46–55. https://doi.org/10.1016/j.jip.2014.06.010.

Batalla-Carrera, L., Morton, A., Garcia-del-Pino, F., 2016. Virulence of entomopathogenic nematodes and their symbiotic bacteria against the hazelnut weevil *Curculio nucum*. J. Appl. Entomol. 140 (1–2), 115–123. https://doi.org/10.1111/jen.12265.

Benseddik, Y., Joutei, A.B., Laghfiri, M., Blenzar, A., Amiri, S., Ezrari, S., Saleh, A., Mokrini, F., Tahiri, A., Dababat, A.A., Lahlali, R., 2021. Efficacy assessment of entomopathogenic nematodes native to Morocco against the white grubs *Rhizotrogus obesus* Lucas and *Geotrogus olcesii* Fairmaire (Coleoptera: Scarabaeidae). Crop Protect. 143, 105534 https://doi.org/10.1016/j.cropro.2021.105534.

Bonito, G.M., Gryganskyi, A.P., Trappe, J.M., Vilgalys, R., 2010. A global meta-analysis of Tuber ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Mol. Ecol. 19 (22), 4994–5008. https://doi.org/10.1111/j.1365-294X.2010.04855.x.

Directive 2009/128/EC of the European Parliament and of the Council, 21 October 2009. Establishing a framework for Community action to achieve the sustainable use of pesticides, and its subsequent amendments. Official Journal of the European Union, L 309/71.

Dlamini, B.E., Malan, A.P., Addison, P., 2019. Control of the banded fruit weevil *Phlyctinus callosus* (Coleoptera: Curculionidae) using entomopathogenic nematodes. Aust. Entomol. 58, 687–695. https://doi.org/10.1111/aen.12386.

Fuentes-Boix, C., Martín-Santafé, M., Morton, A., Garcia-del-Pino, F., 2019. Susceptibilidad del escarabajo de la trufa, *Leiodes cinnamomeus*, a los nematodos entomopatógenos. In: Proceedings of XI Congreso Nacional de Entomología Aplicada, Madrid, Spain.

Gómez-Molina, E., Sánchez, S., Parladé, J., Cirujeda, A., Puig-Pey, M., Marco, P., Garcia-Barreda, S., 2020. Glyphosate treatments for weed control affect early stages of root colonization by *Tuber melanosporum* but not secondary colonization. Mycorrhiza 30 (6), 725–733. https://doi.org/10.1007/s00572-020-00990-8.

Grewal, P.S., Selvan, S., Gaugler, R., 1994. Nematodes: niche breadth for infection, establishment, and reproduction. J. Therm. Biol. 19, 245–253.

Homininck, W.M., Briscoe, B.R., 1990. Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in British soils. Parasitology 100 (2), 295–302. https://doi.org/10.1017/S0031182000061308.

Koppenhöfer, A.M., Kaya, H.K., Taormino, S.P., 1995. Infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae) at different soil depths and moistures. J. Invertebr. Pathol. 65 (2), 193–199. https://doi.org/10.1006/jipa.1995.1028.

Lacey, L.A., Arthurs, S.P., Unruh, T.R., Headrick, H., Fritts Jr, R., 2006. Entomopathogenic nematodes for control of codling moth (Lepidoptera: Tortricidae) in apple and pear orchards: effect of nematode species and seasonal temperatures, adjuvants, application equipment, and post-application irrigation. Biol. Control 37 (2), 214–223. https://doi.org/10.1016/j.biocontrol.2005.09.015.

Lacey, L.A., Georgis, R., 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. J. Nematol. 44 (2), 218–225.

Laznik, Ž., Tóth, T., Lakatos, T., Vidrih, M., Trdan, S., 2010. *Oulema melanopus* (L.) (Coleoptera: Chrysomelidae) adults are susceptible to entomopathogenic nematodes (Rhabditida) attack: results from a laboratory study. J. Plant. Dis. Prot. 117 (1), 30–32. https://doi.org/10.1007/BF03356330.

Martín-Santafé, M., 2020. Plagas y enfermedades asociadas a plantaciones truferas. Saragossa University). Saragossa University Research Repository. Doctoral dissertation,.

Martín-Santafé, M., Pérez-Fortea, V., Zuriaga, P., Barriuso-Vargas, J., 2014. Phytosanitary problems detected in black truffle cultivation. A review. For. Syst. 23 (2), 307–316. https://doi.org/10.5424/fs/2014232-04900.

Morton, A., Garcia-del-Pino, F., 2016. Laboratory and field evaluation of entomopathogenic nematodes for control of *Agriotes obscurus* (L.) (Coleoptera: Elateridae). J. Appl. Entomol. 141 (4), 241–246. https://doi.org/10.1111/jen.12343.

Navarro-Llopis, V., López, B., Primo, J., Martín-Santafé, M., Vacas, S., 2021. Control of *Leiodes cinnamomeus* (Coleoptera: Leiodidae) in cultivated black truffle orchards by kairomone- based mass trapping. J. Econ. Entomol. 114 (2), 801–810. https://doi.org/10.1093/jee/toaa317.

Nikoukar, A., Ensafi, P., Lewis, E.E., Crowder, D.W., Rashed, A., 2021. Efficacy of naturally occurring and commercial entomopathogenic nematodes against sugar beet wireworm (Coleoptera: Elateridae). J. Econ. Entomol. 114 (5), 2241–2244. https://doi.org/10.1093/jee/toab140.

Oliach, D., Morte, A., Sánchez, S., Navarro-Ródenas, A., Marco, P., Gutiérrez, A., Martín-Santafé, M., Fischer, C., Albisu, L.M., García-Barreda, S., Colina, C., 2020. Las trufas y las turmas. In: Sánchez-González, M., Calama, R., Bonet, J.A. (Eds.), Los productos forestales no madereros en España: del monte a la industria. Monografías INIA: Serie Forestal, 31. Spanish Ministry for Science and Innovation, Madrid, Spain, pp. 283–324.

Paoli, F., Marianelli, L., Torrini, G., Mazza, G., Benvenuti, C., Bosio, G., Roversi, P.F., 2017. Differential susceptibility of *Popillia japonica* 3rd instars to *Heterorhabditis bacteriophora* (Italian strain) at three different seasons. Biocontrol Sci. Technol. 27, 439–444. https://doi.org/10.1080/09583157.2017.1285866.

Patil, J., Rangasamy, V., Lakshmi, L., 2017. Efficacy of entomopathogenic Heterorhabditis and Steinernema nematodes against the white grub, *Leucopholis lepidophora* Blanchard (Coleoptera: Scarabaeidae). Crop Protect. 101, 84–89. https://doi.org/10.1016/j.cropro.2017.07.021.

Pérez-Andueza, G., 2015. Entomofauna asociada a la trufa negra (*Tuber melanosporum* Vittadini) cultivada en España Central: incidencia y valoración de las principales especies micófagas (Coleoptera, Leiodidae; Diptera, Heleomyzidae). Salamanca University). Salamanca University Research Repository. Doctoral dissertation,.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/.

Radová, S., Trnková, Z., 2010. Effect of soil temperature and moisture on the pathogenicity of two species of entomopathogenic nematodes (Rhabditida: Steinernematidae). J. Agrobiol. 27 (1), 1–7. https://doi.org/10.2478/s10146-009-0001-4.

Sandhi, R.K., Shapiro-Ilan, D., Sharma, A., Reddy, G.V., 2020. Efficacy of entomopathogenic nematodes against the sugarbeet wireworm, *Limonius californicus*

(Mannerheim) (Coleoptera: Elateridae). Biol. Control 143, 104190. https://doi.org/10.1016/j.biocontrol.2020.104190.
Shapiro-Ilan, D.I., Hazir, S., Glazer, I., 2017. Basic and applied research: entomopathogenic nematodes. In: Lacey, L. (Ed.), Microbial control of insect and mite pests, Academic Press, pp. 91–105. https://doi.org/10.1016/B978-0-12-803527-6.00006-8.
Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J., Fife, J.P., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol. Control 38 (1), 124–133. https://doi.org/10.1016/j.biocontrol.2005.09.005.
Shapiro-Ilan, D.I., Leskey, T.C., Wright, S.E., 2011. Virulence of entomopathogenic nematodes to plum curculio, *Conotrachelus nenuphar*: Effects of strain, temperature, and soil type. J. Nematol. 43 (3–4), 187–195.
Shapiro-Ilan, D.I., McCoy, C.W., 2000. Virulence of entomopathogenic nematodes to *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in the laboratory. J. Econ. Entomol. 93 (4), 1090–1095. https://doi.org/10.1603/0022-0493-93.4.1090.
Shapiro-Ilan, D.I., Stuart, R., McCoy, C.W., 2003. Comparison of beneficial traits among strains of the entomopathogenic nematode, *Steinernema carpocapsae*, for control of *Curculio caryae* (Coleoptera: Curculionidae). Biol. Control 28 (1), 129–136. https://doi.org/10.1016/S1049-9644(03)00030-6.
Trappe, J.M., Molina, R., Castellano, M., 1984. Reactions of mycorrhizal fungi and mycorrhiza formation to pesticides. Annu. Rev. Phytopathol. 22 (1), 331–359.
White, G.F., 1927. A method for obtaining infective nematode larvae from cultures. Science 66, 302–303.
Woodring, J.L., Kaya, H.K., 1988. Steinernematid and heterorhabditid nematodes: a handbook of techniques. South. Cooper. Bull. 331, 1–30.
Wright, P.J., 1992. Cool temperature reproduction of steinernematid and heterorhabditid nematodes. J. Invertebr. Pathol. 60 (2), 148–151. https://doi.org/10.1016/0022- 2011(92)90088-L.