

Research Paper

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Comparative virulence of the fungus *Lecanicillium muscarium* and two entomopathogenic nematodes against adult truffle beetles (*Leiodes cinnamomeus*) at low temperatures

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Abstract

The truffle beetle, *Leiodes cinnamomeus*, is the most important pest in black truffle (*Tuber melanosporum*) plantations during autumn and winter. Entomopathogenic nematodes (EPNs) are promising biological control agents against *L. cinnamomeus*, while entomopathogenic fungi (EPF) are also commonly used in Integrated Pest Management (IPM) programmes to enhance the control of insect pests. Our aims were to test the virulence of a cold-adapted commercial strain of the EPF *Lecanicillium muscarium* Ve6 and two EPN species, *Steinernema carpocapsae* and *Steinernema feltiae*, against adults of *L. cinnamomeus* at 15 °C and 10 °C, and to assess the effect of their combined application. Two susceptibility assays were performed using different treatments: (1) immersion of adults in a fungal suspension and (2) direct soil treatment of the EPF. Mortality of *L. cinnamomeus* was recorded 7 days post-treatment at 15 °C and 15 days post-treatment at 10 °C. *Lecanicillium muscarium* did not cause significant mortality of *L. cinnamomeus*. *Steinernema feltiae* did not cause significant mortality at 15 °C after 7 days but resulted in a statistically significant low mortality (33.3%) at 10 °C after 15 days compared to the control. *Steinernema carpocapsae* caused the highest mortality rates (63.3–100%) at both temperatures, suggesting it would be the best option to control adults of *L. cinnamomeus* in field applications. The addition of *L. muscarium*, did not enhance pest suppression compared to EPNs alone. To optimise IPM strategies for *L. cinnamomeus* control, further research is recommended to explore potential synergistic effects of combining EPNs with other cold-adapted EPF.

Introduction

The black truffle or *Tuber melanosporum* Vittad., 1831 (Pezizales: Tuberaceae) is a hypogaeal fungus with high gastronomic value and economic interest that establishes mutualistic relationships with different phanerogam species, mainly of the *Quercus* genus. The primordium formation occurs from April to June, while immature fruit bodies continue to grow until December and ripen until March (Bonito *et al.*, 2010). In truffle plantations, especially in southern Europe, the truffle beetle, *Leiodes cinnamomeus* (Panzer, 1793) (Coleoptera: Leiodidae), is considered the most important pest (Arzone, 1971; Martín-Santafé *et al.*, 2014). This beetle has a life cycle that coincides with the fructification period of *T. melanosporum*. Adults are active from mid-September to mid-May, whereas mycophagous larvae (L1–L3) occur from October to March (Pérez-Andueza, 2015). This period coincides with the coldest months of the year, when soil temperatures at 20 cm depth can reach 2 °C (Julià *et al.*, 2023a). In fact, it has been previously observed that adults exhibit nearly 100% mortality after three days at a constant temperature of 20 °C, whereas most adults and larvae survive for at least two weeks at 10 °C, highlighting their adaptation to low temperatures (Julià *et al.*, 2023a). Subsequently, L3 larvae leave the truffle and enter diapause within earthen aestivation chambers, which persist until the end of summer when several cohorts pupate and emerge as adults during autumn-winter (Pérez-Andueza, 2015). Both adult and mycophagous larval stages cause galleries within truffle fruit bodies, which reduce their quality and can lead to economic losses of up to 70% in plantations (Barriuso *et al.*, 2012).

Current control methods, relying on frequent truffle harvesting (Martín-santafé *et al.*, 2014) and mass trapping of adult beetles (Navarro-Llopis *et al.*, 2021), have proven insufficient to

effectively reduce *L. cinnamomeus* populations. The use of chemical products is not appropriate for controlling this insect because they can inhibit the growth of *T. melanosporum* mycelium (Trappe *et al.*, 1984) and negatively affect early stages of root colonisation by the fungus (Gómez-Molina *et al.*, 2020). Thus, alternative biological control methods have been explored.

Recent studies have demonstrated the potential of entomopathogenic nematodes (EPNs) as biological control agents (BCAs) against both larval and adult stages of *L. cinnamomeus* under laboratory conditions (Julià *et al.*, 2023a). These nematodes, belonging to the Steinernematidae and Heterorhabditidae families, are obligate parasites of a wide range of insect species (Shapiro-Ilan *et al.*, 2017; Tarasco *et al.*, 2023). Infective juveniles (IJs) locate their host insects using various cues and enter the host through natural openings before releasing symbiotic bacteria into the insect's haemolymph. The insect dies within 24–72 h, and the EPNs reproduce within the insect cadaver, feeding on the symbiotic bacteria. These bacteria prevent other bacteria and fungi from colonising the host while providing nutrients to the nematodes (Koppenhöfer, 2007). As the EPN population grows within the cadaver, resource limitations trigger the development of new IJs that emerge and search for new insect hosts (Grewal and Georgis, 1999). These nematodes have proven to be successful against adults and larvae of different soil-dwelling insects (Koppenhöfer *et al.*, 2020). However, their efficacy can be affected by several factors. Intrinsic factors such as specific species or population, and environmental factors such as temperature, can influence their success (Shapiro-Ilan *et al.*, 2006). For example, low temperatures can hinder the mobility and infectivity of certain nematode species (Grewal *et al.*, 1994). In the case of *L. cinnamomeus*, *Steinernema carpocapsae* (Weiser, 1955) (Panagrolaimida: Steinernematidae) and *Steinernema feltiae* (Filipjev, 1934) (Panagrolaimida: Steinernematidae) have shown significant virulence, causing 100% and 73.3% mortality, respectively, in adult beetles at 20 °C (Julià *et al.*, 2023a). However, their efficacy is temperature-dependent. While *S. carpocapsae* caused significant mortality (96 %) under a fluctuating temperature regime (10 °C for 18 hours and 15 °C for 6 hours) 7 days after treatment, neither species exhibited significant virulence at a constant 10 °C after 7 days (Julià *et al.*, 2023a). These findings highlight the importance of considering temperature regimes when evaluating the potential of EPNs for biological control.

Other BCAs commonly used alongside EPNs in Integrated Pest Management (IPM) programmes are entomopathogenic fungi (EPF) (Půža and Tarasco, 2023). EPF exhibit a broad host range, frequently leading to natural epizootics, particularly in soil environments. These fungi target insects by adhering conidia to the insect cuticle. Under favourable temperature and humidity conditions, conidia germinate forming hyphae that penetrate and colonise the insect's body. The insect dies after a few days (4–7 days), primarily due to fungal toxins. Sporulation then occurs on or within the cadaver, producing new infective spores (Quesada-Moraga *et al.*, 2020). As with EPNs, the success of EPF depends on selecting strains adapted to the environmental conditions of the target ecosystem (Fernandes *et al.*, 2008). While most EPF exhibit optimal growth between 25 and 35 °C (Půža and Tarasco, 2023), the commercial *Lecanicillium muscarium* (Hypocreales: Cordycipitaceae) strain Ve6 (Mycotal) presents a conidial germination of 20.6% at 10 °C and 98% at 15 °C, and is commonly used in Europe and India against whiteflies (Hemiptera) and thrips (Thysanoptera) (Reddy, 2020). Although there are few studies

on the virulence of *L. muscarium* against insect hosts beyond whiteflies and thrips, some studies have reported its potential to cause mycosis in adult curculionid beetles at 20–25 °C (Kope *et al.*, 2006, 2008; Ak, 2019; Mitina *et al.*, 2020; Altinok *et al.*, 2023).

The integrated use of different BCAs has increased in pest control programmes over the last few decades. Both EPNs and EPF are considered safe for non-target organisms and the environment (Půža and Tarasco, 2023). Studies on their compatibility have revealed different types of interactions, including additive, synergistic, and antagonistic effects (Půža and Tarasco, 2023). However, no research to date has explored the potential application of EPF, either alone or in combination with EPNs, for the control of *L. cinnamomeus*. While all life stages of this beetle occur in the soil, adults are more mobile and often emerge to move between truffles (Arzone, 1971). Hence, an integrated EPF and EPN control strategy for *L. cinnamomeus* should focus on controlling the adult beetles.

This study was designed to analyse whether an EPF treatment could complement nematode activity under temperatures lower than 20 °C, where nematode virulence is reduced against *L. cinnamomeus* (Julià *et al.*, 2023a). Specifically, the aims were to evaluate the virulence of one EPF species, *L. muscarium* strain Ve6, and two EPN species, *S. carpocapsae* and *S. feltiae*, against adults of *L. cinnamomeus* at 15 °C and 10 °C, and to assess the effect of their combined application. We hypothesised that the combination of cold-adapted EPF and EPNs would enhance the mortality of *L. cinnamomeus* adults at 15 °C and 10 °C compared to individual treatments.

Material and methods

Source of EPF, EPNs, and insects

Lecanicillium muscarium strain Ve6 (Mycotal, Koppert) was the selected EPF species for this experiment. This commercial strain, originally isolated from the whitefly genus *Trialeurodes* (Hemiptera) in the United Kingdom, is recommended for low-temperature applications against various Hemipteran pests. Conidia were stored at 4 °C for a maximum of two weeks. Before application, a conidial suspension was spread onto Malt extract agar (3.5%) plates and incubated in the dark at 25 °C for 24 hours. Germination was assessed by examining 100 conidia per plate under a microscope. A conidium was considered germinated if it had developed a germ tube at least half the width of the conidium (Wyss *et al.*, 2001).

Two commercial strains belonging to two different EPN species were used: *Steinernema feltiae* (Entonem® Koppert) and *Steinernema carpocapsae* (Capsanem® Koppert). Nematodes were reared at 25 °C in last-instar larvae of the greater wax moth, *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) (Woodring and Kaya, 1988). IJs emerging from cadavers were recovered using modified White traps (White, 1927) and stored at 9 °C for no longer than two weeks. Prior to application, IJs were acclimatised at room temperature for 2 h, and their viability was checked under a stereomicroscope.

Adults of *L. cinnamomeus* were collected from black truffle plantations in Teruel, Spain, during November and December 2024. They were maintained in plastic boxes filled with soil from truffle plantations and provided with pieces of truffle fruit bodies as a food source. They were kept at 9 °C until use in the experiments.

Susceptibility assay using adult submersion in the EPF and soil treatment of the EPNs

The experimental unit consisted of a Petri dish (5.5 cm diameter) filled with 20 g of sterile soil collected from truffle plantations and moistened with sterile tap water (10% w/w). A single adult of *L. cinnamomeus* and a small piece of black truffle fruitbody as a food resource were placed in the Petri dish. The virulence of the EPF species, *L. muscarium*, and one EPN species, *S. carpocapsae*, was tested against *L. cinnamomeus* adults. *S. carpocapsae* was used because of its high virulence against adults at 15 °C, as previously reported by Julià *et al.* (2023a). The experiments were conducted at 15 °C and 10 °C, as well as the effect of their combination. Susceptibility at a higher temperature (20 °C) could not be assessed, as this temperature lies outside the natural thermal range of *L. cinnamomeus* adults, resulting in high background mortality within three days. There were two doses of *L. muscarium*: 6×10^6 and 1×10^7 conidia/mL. Adult beetles were submerged in the conidial suspension for 15 seconds and then transferred to the Petri dishes (Kope *et al.*, 2006). *S. carpocapsae* IJs were applied directly to Petri dishes at two doses: 10 (196 IJs/dish) and 25 IJs/cm² (490 IJs/dish). Dishes were placed in boxes with controlled humidity and temperature within a climate chamber.

Insect mortality was recorded daily for the initial four days, followed by assessments every 48 hours until 7 days post-treatment at 15 °C and 15 days post-treatment at 10 °C. These different observation periods were established because control mortality at 15 °C increased significantly after the first week, whereas adults maintained at 10 °C showed higher survival rates over a longer period. There were 15 replicates per treatment, and the experiment was conducted twice, resulting in a total of 30 biological replicates per treatment.

Dead insects from each treatment were individually transferred to a new Petri dish containing two moistened filter paper disks and incubated at 22 °C. For EPF treatments, insects were checked 14 days after death, and the presence of fungal growth was evaluated. In the case of EPN treatments, cadavers were dissected four days after death to check the presence of nematode adults.

Susceptibility assay using soil treatment of the EPF and EPNs

Since *L. cinnamomeus* was not susceptible to *L. muscarium* infection in the previous assay, we conducted an experiment under more field-like conditions to determine whether the fungus could interact with EPNs and enhance their infectivity. Therefore, while *S. carpocapsae* was retained as a positive control due to its demonstrated efficacy against adult beetles, we also included *S. feltiae*, which has previously shown low virulence against adults (Julià *et al.*, 2023a). This allowed us to evaluate whether the presence of the fungus could enhance the virulence of *S. feltiae* through a synergistic interaction.

The experimental unit and temperature conditions were the same as in the assay above. *L. muscarium* was applied at a dose of 6×10^6 viable conidia/dish and thoroughly mixed with the soil. *S. carpocapsae* or *S. feltiae* were applied at a dose of 25 IJs/cm² (490 IJs/dish). Control and incubation conditions, as well as the mortality recording schedule (7 days for 15 °C and 15 days for 10 °C), remained consistent with the previous assay. There were 15 replicates per treatment, and the experiment was conducted twice, resulting in a total of 30 biological replicates per treatment.

Infection of *L. cinnamomeus* adults by BCAs was determined using the same method as previously described.

Statistical analysis

A generalised linear model (GLM), with binomial distribution and logit link function, was used to test significant differences in mortality on the final day of the assay. Subsequently, Tukey's multiple range test was performed to compare differences among treatments. Lethal time 50 (LT₅₀) and corresponding 95% confidence intervals (CIs) were also calculated using probit analysis.

A chi-square (χ^2) test was used to analyse the interaction between the EPF and EPNs. Before analysis, all mortality data were corrected for control mortality (Abbott, 1925). The method of determining the type of interaction (synergistic, additive, or antagonistic) was based on Finney (1964). The expected additive proportional mortality M_E for the EPN-EPF combinations was calculated using the formula $M_E = M_N + M_F (1 - M_N)$, where M_N and M_F represent the observed proportional mortalities caused by individual treatments of EPN and EPF, respectively. Subsequently, a chi-square test was carried out using the formula $\chi^2 = (M_{NF} - M_E)^2 / M_E$, where M_{NF} represents the observed mortality for the EPN-EPF combination. The calculated value from the chi-square test was then compared with the chi-square table value for one degree of freedom ($\chi^2_{1, 0.05} = 3.84$). If the calculated χ^2 value exceeded the table value, a non-additive interaction between the two entomopathogenic agents was suspected (Finney, 1964). A significant interaction was considered synergistic when the difference $M_{NF} - M_E$ was positive, and antagonistic if the difference was negative.

All data were analysed using R software (version 4.3.1) (R Core Team, 2023). All comparisons were considered significant if the p-value was less than 0.05.

Results

In the subsequent assays, the conidial viability of *L. muscarium* was assessed at 85%, indicating a high proportion of viable and potentially infective conidia.

Susceptibility assay using adult submersion in the EPF and soil treatment of the EPNs

At 15 °C, treatments with *L. muscarium* alone resulted in 13.4–26.7% mortality after 7 days of exposure, which did not differ significantly from the control (33.4%) ($p > 0.05$), even at the higher fungal dose (fig. 1). In contrast, all *S. carpocapsae* treatments, regardless of dose, resulted in 100% mortality ($\chi^2 = 153.25$, $df = 6$, $p < 0.001$). No significant dose-dependent effect was observed regarding the speed of kill between the highest (LT₅₀ = 2.2 days) and lowest (LT₅₀ = 2.8 days) doses (table 1).

At 10 °C, treatments with *L. muscarium* alone resulted in 16.7–30% mortality, which was not significantly different from the control (23.4%) ($p > 0.05$) after 15 days of exposure, even at the higher fungal dose (fig. 2). In contrast, *S. carpocapsae* treatments at both doses (80–96.7% mortality) were significantly more effective than the control and *L. muscarium* alone ($\chi^2 = 92.79$, $df = 6$, $p < 0.001$). Additionally, a significant dose-dependent effect was observed for *S. carpocapsae*, with the higher dose (LT₅₀ = 6.2 days) resulting in faster mortality than the lower dose (LT₅₀ = 9.5 days) (table 1).

In treatments combining both BCAs, no synergistic effect was observed. All interactions between *L. muscarium* and *S. carpocapsae* at both temperatures were classified as additive (Supplementary table S1).

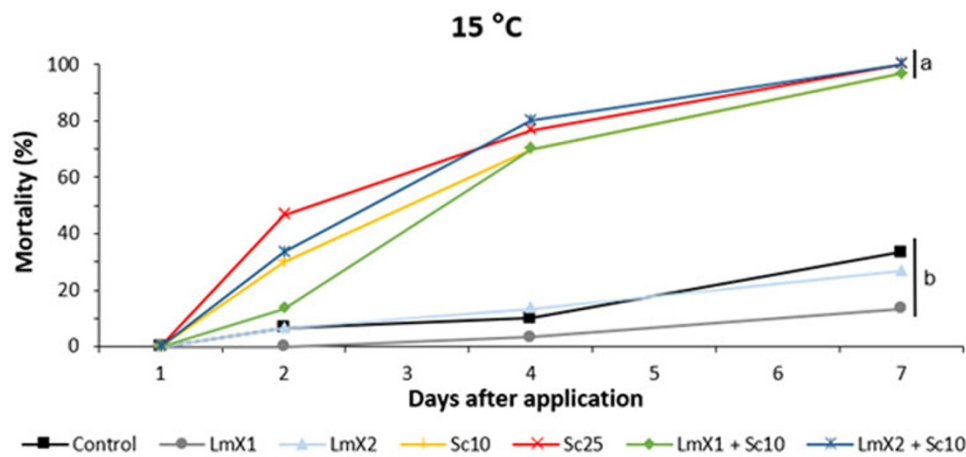


Figure 1. Susceptibility of *Leiodes cinnamomeus* adults exposed to *Lecanicillium muscarium* by submersion in conidial suspension and to *Steinernema carpocapsae* through soil treatment, applied alone or in combination, and to the control at 15 °C for 7 days. Different letters indicate significant differences among treatments ($p < 0.05$) at the end of the experiment. LmX1 (*L. muscarium* at 6×10^6 conidia/mL); LmX2 (*L. muscarium* at 1×10^7 conidia/mL); Sc10 (*S. carpocapsae* at 10 IJs/cm²); Sc25 (*S. carpocapsae* at 25 IJs/cm²).

Table 1. Estimated exposure time to cause 50% mortality (LT_{50} in days) of *Leiodes cinnamomeus* adults by the four treatments at 15 °C and 10 °C, with 95% confidence interval (CI). Sc10: *Steinernema carpocapsae* at 10 IJs/cm²; Sc25: *S. carpocapsae* at 25 IJs/cm²; LmX1: *Lecanicillium muscarium* at 6×10^6 conidia/mL; LmX2: *L. muscarium* at 1×10^7 conidia/mL.

Treatment	15 °C		10 °C	
	LT_{50} (CI 95%)	Pearson goodness	LT_{50} (CI 95%)	Pearson goodness
Sc10	2.8 (2.2–3.3) ^a	$\chi^2 = 0.51$, df = 1, $p = 0.47$	9.5 (6.2–15.5) ^{bc}	$\chi^2 = 10.1$, df = 4, $p = 0.08$
Sc25	2.2 (1.6–2.7) ^a	$\chi^2 = 2.05$, df = 1, $p = 0.15$	6.2 (5.3–7.0) ^a	$\chi^2 = 1.12$, df = 3, $p = 0.77$
LmX1 + ScX1	3.2 (2.7–3.7) ^a	$\chi^2 = 0.00$, df = 1, $p = 0.98$	10.8 (9.7–12.2) ^c	$\chi^2 = 2.26$, df = 3, $p = 0.52$
LmX2 + ScX1	2.5 (2.0–2.9) ^a	$\chi^2 = 0.82$, df = 1, $p = 0.36$	8.5 (7.3–9.9) ^b	$\chi^2 = 0.88$, df = 4, $p = 0.93$

Note: Values of Pearson’s goodness-of-fit χ^2 , df, and p are given (when $p > 0.05$, the model adequately fits the data). Different superscript letters indicate statistical significance among treatments. LT_{50} of *L. muscarium* treatments applied alone (LmX1 and LmX2) were not calculated due to the low mortality obtained.

No fungal mycelium growth was observed on cadavers two weeks after death, indicating a lack of fungal establishment within the beetles.

Susceptibility assay using soil treatment of the EPF and EPNs

The susceptibility assay revealed different effects of the BCAs on adult *L. cinnamomeus* survival at the two tested temperatures (15 °C and 10 °C).

At 15 °C, neither *L. muscarium* treatment alone (20% mortality) nor *S. feltiae* treatments alone or combined (23.3–30% mortality) were significantly virulent compared to the control (10%) after 7 days of exposure ($p > 0.05$) (fig. 3). In contrast, all *S. carpocapsae* treatments (100% mortality), whether applied alone or in combination with *L. muscarium*, showed significant differences compared to the control, *S. feltiae* and *L. muscarium* treatments (GLM: $\chi^2 = 130.20$, df = 5, $p < 0.001$).

In line with previous results, at 10 °C, *L. muscarium* treatment alone resulted in only 13.3% mortality, which was not significantly different from the control (0%) ($p > 0.05$) after 15 days (fig. 4). In contrast, mortality caused by *S. carpocapsae* (63.3%) and *S. feltiae* (33.3%) treatments, both alone and in combination with the fungus, was significantly different from the control (GLM: $\chi^2 = 53.02$, df = 5, $p < 0.001$). No significant differences in adult mortality were observed between *S. carpocapsae* and *S. feltiae* treatments

($p > 0.05$) at the end of the assay (fig. 2). However, *S. carpocapsae* ($LT_{50} = 12$ days) exhibited a significantly shorter time to kill than *S. feltiae* ($LT_{50} = 22.7$ days) (table 2).

Despite the significant virulence of *S. feltiae* when combined with *L. muscarium* at 10 °C, no synergistic effect was detected, with interactions being mainly additive between *L. muscarium* and *S. feltiae* or *S. carpocapsae* at both temperatures (Supplementary table S2). However, an antagonistic interaction was detected for *S. feltiae* combined with the fungus when applied at 15 °C.

No fungal mycelium growth was detected on cadavers even two weeks after death, indicating a lack of fungal establishment. In the case of EPNs, although *S. carpocapsae* caused 100% mortality 8 days after treatment, dissection of *L. cinnamomeus* cadavers 4 days later revealed that nematodes developed into adults (males and females) in only 14% of the beetles.

Discussion

One of the main challenges in applying BCAs, including EPF and EPNs, against *L. cinnamomeus* is the low temperature regime prevalent during autumn and winter, which coincides with the beetle’s active period (Julià *et al.*, 2023a). The successful implementation of EPF requires selecting strains tolerant of the target ecosystem’s temperature range (Fernandes *et al.*, 2008). In our study, the *L. muscarium* Ve6 strain used was specifically chosen

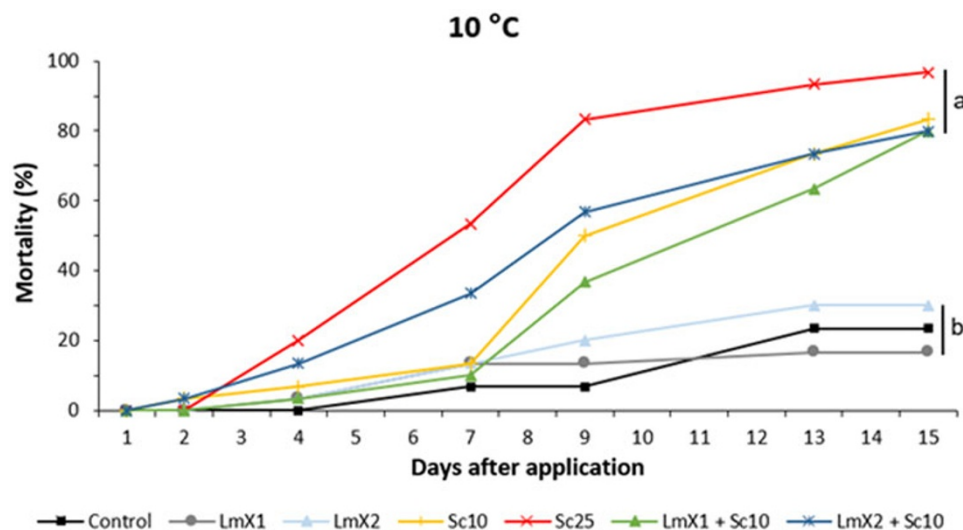


Figure 2. Susceptibility of *Leiodes cinnamomeus* adults exposed to *Lecanicillium muscarium* by submersion in conidial suspension and to *Steinernema carpocapsae* through soil treatment, applied alone or in combination, and to the control at 10 °C for 15 days. Different letters indicate significant differences among treatments ($p < 0.05$) at the end of the experiment. LmX1 (*L. muscarium* at 6×10^6 conidia/mL); LmX2 (*L. muscarium* at 1×10^7 conidia/mL); Sc10 (*S. carpocapsae* at 10 IJs/cm²); Sc25 (*S. carpocapsae* at 25 IJs/cm²).

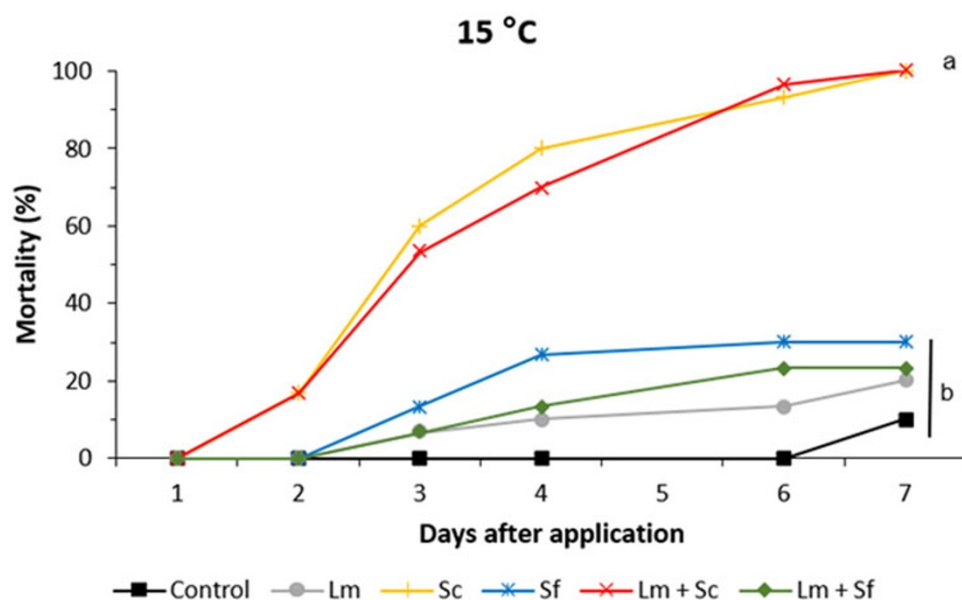


Figure 3. Susceptibility of *Leiodes cinnamomeus* adults exposed via soil treatment to *Lecanicillium muscarium* (Lm), *Steinernema carpocapsae* (Sc) and *Steinernema feltiae* (Sf), alone or in combination, and to the control at 15 °C for 7 days. Different letters indicate significant differences among treatments ($p < 0.05$) at the end of the experiment.

for its reported low-temperature adaptability, presenting a conidial germination of 20.6% at 10 °C and 98% at 15 °C (Reddy, 2020). However, we observed that *L. cinnamomeus* adults were not significantly susceptible to this EPF at either 10 °C or 15 °C, regardless of whether the EPF was applied directly to the soil or via adult submersion, with no evidence of mycelial growth detected in the cadavers. Moreover, susceptibility at 20 °C could not be assessed, as this temperature lies outside the natural thermal range of *L. cinnamomeus* adults, resulting in high background mortality within three days. Although *L. muscarium* is mostly used against whiteflies and thrips (Reddy, 2020), previous studies have reported the potential of this species to also cause mycosis in adult curculionid beetles at 20–25 °C (Ak, 2019; Mitina *et al.*, 2020). Altinok *et al.* (2023)

reported a significant effect of the *L. muscarium* Ve6 strain, causing 30% mortality in adult *Sitophilus granarius* (Linnaeus, 1758) (Coleoptera: Curculionidae) at 25 °C, 10 days after treatment, compared to 1.3% mortality in the control group. Therefore, the lack of efficacy in our study may be attributed to the low susceptibility of *L. cinnamomeus* adults to this EPF, possibly in combination with the low temperatures used in the experiment, which may further hinder the mycosis.

Temperature is also a key factor influencing infectivity, virulence, and reproductive capacity of EPNs (Grewal *et al.*, 1994). While *S. feltiae* typically performs best at 15–25 °C and *S. carpocapsae* at 20–30 °C, both species can infect hosts at lower temperatures (8–10 °C and 12–15 °C, respectively) (Grewal *et al.*, 1994;

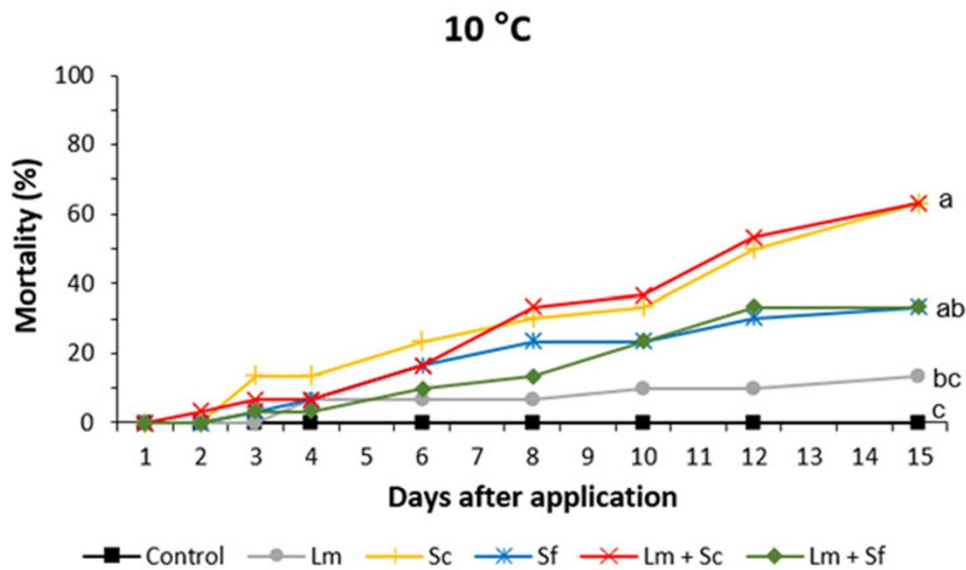


Figure 4. Susceptibility of *Leiodes cinnamomeus* adults exposed via soil treatment to *Lecanicillium muscarium* (Lm), *Steinernema carpocapsae* (Sc) and *Steinernema feltiae* (Sf), alone or in combination, and to the control at 10 °C for 15 days. Different letters indicate significant differences among treatments ($p < 0.05$) at the end of the experiment.

Table 2. Estimated exposure time to cause 50% mortality (LT_{50} in days) of *Leiodes cinnamomeus* adults by the four treatments at 15 °C and 10 °C, with 95% confidence interval (CI). Sc: *Steinernema carpocapsae*; Sf: *Steinernema feltiae*; and Lm: *Lecanicillium muscarium*

Treatment	15 °C		10 °C	
	LT_{50} (CI 95%)	Pearson goodness	LT_{50} (CI 95%)	Pearson goodness
Sc	2.9 (2.5–3.2) ^a	$\chi^2 = 1.81$, df = 3, $p = 0.61$	12.0 (10.0–15.6) ^a	$\chi^2 = 1.30$, df = 4, $p = 0.94$
Sf	11.0 (6.1–15.3) ^b	$\chi^2 = 0.61$, df = 1, $p = 0.43$	22.7 (15.0–71.7) ^b	$\chi^2 = 0.81$, df = 4, $p = 0.94$
Lm + Sc	3.0 (2.6–3.3) ^a	$\chi^2 = 1.46$, df = 3, $p = 0.69$	12.7 (10.1–19.7) ^a	$\chi^2 = 2.23$, df = 5, $p = 0.69$
Lm + Sf	11.6 (6.8–15.8) ^b	$\chi^2 = 0.03$, df = 1, $p = 0.85$	22.8 (15.8–75.3) ^b	$\chi^2 = 0.39$, df = 4, $p = 0.98$

Note: Values of Pearson’s goodness-of-fit χ^2 , df, and p are given (when $p > 0.05$, the model adequately fits the data). Different superscript letters indicate statistical significance among treatments. LT_{50} of *L. muscarium* treatment applied alone (Lm) was not calculated due to the low mortality obtained.

Shapiro-Ilan *et al.*, 2006). Our results support this, demonstrating that both *S. carpocapsae* (63.3%) and *S. feltiae* (33.3%) could kill adults of *L. cinnamomeus* at 10 °C after 15 days of treatment compared to the control (0%), with *S. carpocapsae* exhibiting a faster kill rate. Furthermore, *S. carpocapsae* treatments caused mortalities between 96 and 100% at 15 °C within 7 days even at the lowest dose (10 IJs/cm²), while *S. feltiae* did not cause significant virulence compared to the control at this temperature. The observed difference in virulence of *S. feltiae* at 15 °C and 10 °C might be attributed to the unequal exposure times (7 days at 15 °C versus 15 days at 10 °C), which resulted from the higher mortality of *L. cinnamomeus* in untreated control dishes at 15 °C. Our results extend the observations of Julià *et al.* (2023a), who reported *S. carpocapsae* as a more virulent species (76–96% mortality) than *S. feltiae* (10–30% mortality) after 7 days at a fluctuating temperature regime (10 °C for 18 hours and 15 °C for 6 hours). However, in that study, none of the EPN species caused significant adult mortality at 10 °C (10–23%). Our findings confirm that *L. cinnamomeus* adults are susceptible to these EPNs, particularly to *S. carpocapsae*, even at 10 °C, provided there is sufficient exposure time.

Although *S. carpocapsae* caused 100% mortality at 15 °C in our study, only 14% of cadavers contained adult nematodes. This low recovery rate may be due to the presence of phoretic or

necromenic nematodes, such as *Pristionchus* spp., which are frequently found on *L. cinnamomeus* adults (Julià *et al.*, 2023b). These phoretic nematodes, which sometimes can exceed 100 individuals per beetle (unpublished data), may compete with EPNs for cadaver resources, reducing EPN development (Duncan *et al.*, 2003; Campos-Herrera *et al.*, 2012). Furthermore, the relatively small size of *L. cinnamomeus* adults (3.5–5 mm) might limit the available space and nutrients, favouring competition with saprophytic or phoretic species. Julià *et al.* (2020) observed a similarly low proportion (14.50%) of adults of *S. carpocapsae* developing within the even smaller nymphs (1.3–2.3 mm) of *Corythucha ciliata* (Say, 1832) (Hemiptera: Tingidae). Another factor that may have contributed to the low development of EPNs could be an inadequate medium for EPNs to develop inside the insect host after infection. Griffin (2012) proposed that species like *S. carpocapsae*, which do not require symbiotic bacteria to kill the host, may present a low quantity of bacteria to enhance IJs’ survival at the expense of a lower reproduction rate within the host. Thus, the potential competition between EPNs and the phoretic/necromenic nematodes for the host resources, the relatively small size of *L. cinnamomeus* adults, and other factors related to the medium inside the insect host may all contribute to the low development of IJs into adult stages.

The combined application of EPNs and EPF has been reported to produce additive or synergistic effects, whereas antagonistic interactions have been observed less frequently (Půža and Tarasco, 2023). Synergistic effects may occur because EPF weaken the host insect through tissue colonisation and immune suppression, thereby increasing its susceptibility to nematode infection (Půža and Tarasco, 2023). In our study, however, no synergistic effects were observed. Most interactions were additive, with one case of antagonism (*S. feltiae* at 15 °C). This lack of synergism is likely explained by the high susceptibility of *L. cinnamomeus* adults to EPNs and the near-zero virulence of the EPF strain used. This finding aligns with other studies reporting no synergistic effects when one of the agents is significantly more dominant than the other (Batalla-Carrera *et al.*, 2013; McNamara *et al.*, 2018; Uzun Yiğit *et al.*, 2022). Antagonistic effects, even when the EPF alone is ineffective, have also been documented in other Coleoptera (Shapiro-Ilan *et al.*, 2004), suggesting that the presence of fungal conidia might sometimes interfere with nematode host-finding or penetration.

To conclude, our study demonstrates significant differences in virulence among the biological control agents tested. *Lecanicillium muscarium* did not cause significant mortality in *L. cinnamomeus* adults at either 10 °C or 15 °C, regardless of the treatment method (via adult submersion or directly to the soil) or dose. This lack of efficacy may be attributed to the low susceptibility of *L. cinnamomeus* adults to this EPF. In contrast, both *S. feltiae* and *S. carpocapsae* demonstrated virulence against *L. cinnamomeus* adults at 10 °C after 15 days of exposure. *Steinernema carpocapsae* consistently caused the highest mortality rates (63.3–100%) at both temperatures, even at the lowest dose tested, highlighting its potential as a promising candidate for field application in truffle orchards during the cold season. Our findings also indicate that combining *L. muscarium* with either *S. feltiae* or *S. carpocapsae* does not enhance the suppression of *L. cinnamomeus* adults compared to EPNs applied alone. Further research should focus on identifying other cold-adapted EPF strains with greater virulence and better compatibility with EPNs to enhance IPM programmes for this pest.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0007485326101357>.

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Competing interests. The authors declare no conflicts of interest.

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