Article published in Journal of Invertebrate Pathology Volume 207, November 2024, 108231

To be a good killer: Evaluation of morphometry and nematodes-bacteria complex effect on entomopathogenic nematodes virulence against wireworms.

Andrea Chacon-Hurtado[1], Fanny Ruhland[1], Salimata Drabo[1], Thibaut Smeets[1], Brice Checconi[1], Raquel Campos-Herrera[2] & François J. Verheggen[1]*.

[1]Chemical and Behavioral Ecology, Gembloux Agro-Bio Tech, University of Liege, Avenue de la Faculté d'Agronomie 2B, 5030, Gembloux, Belgium.

[2] Departamento de Viticultura, Instituto de Ciencias de la Vid y del Vino (CSIC, Universidad de La Rioja, Gobierno de La Rioja), Finca La Grajera, Ctra. de Burgos Km. 6 (LO-20 - salida 13), 26007 Logroño, Spain.

* Corresponding author :

François Verheggen
2B Avenue de la faculté d'Agronomie, 5030 Gembloux, Belgium
+3281622662 (tel)
fverheggen@uliege.be

Highlights

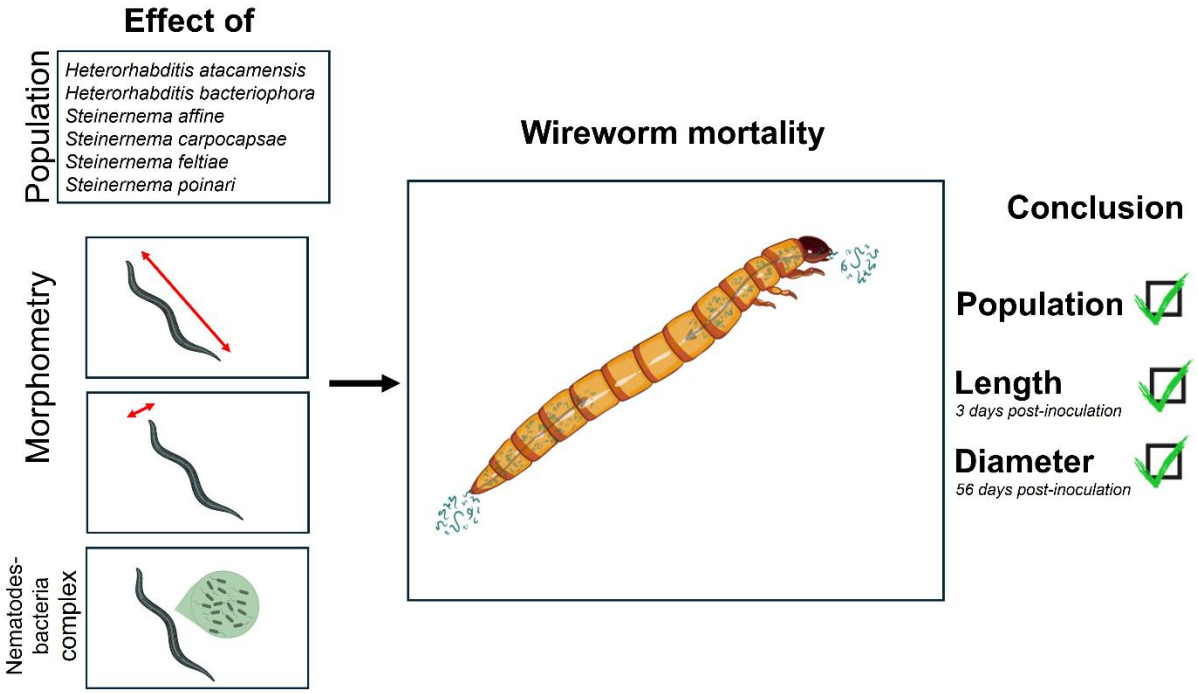
- The virulence of 16 entomopathogenic nematodes (EPN) populations was evaluated against a soil-dwelling insect (*Agriotes* spp).
- Six EPN populations demonstrated notable efficacy.
- Smaller-diameter nematodes induced higher mortality after 56 days post-inoculation.
- Nematodes length affects virulence at three days post-inoculation but not at 56 days post-inoculation.
- Bacteria biochemical profiles did not reflect the differences in virulence of nematodes-bacteria complex against wireworms.

Abstract

Entomopathogenic nematodes (EPNs) have emerged as a promising tool for controlling soil-dwelling crop pests. However, their efficacy varies according to EPN populations and targeted hosts. Wireworms are polyphagous insects causing significant crop losses, especially since the ban on pesticides previously used for their control. They are highly resistant to EPN populations and require high number of infective juveniles (IJs) to achieve optimal mortality rates. In this research, we collected and compared the virulence of 16 EPN populations, of foreign origin, purchased or collected from wireworms infested fields. Then, we have tested two hypotheses: (i) smaller nematodes induce heightened mortality rates against wireworms; (ii) virulence levels can be linked to nematodes-bacteria complex. Mortality rates scaled from three to 43% after 56 days of continuous exposure across the 16 tested EPN populations (*Heterorhabditis* spp. and *Steinernema* spp.). Morphometric analysis of IJs revealed both intra- and interspecific variations in length and diameter among populations. Interestingly, while EPN length influence mortality at three days post-inoculation. We found leaner IJs (< 25µm) to induce higher mortality rates at 56 days post-inoculation. To better determine the structure and dimensions of the primary entry routes utilized by EPNs, we provide optical microscope micrographs of wireworm *Agriotes* spp. spiracle, anal sclerotized coating anus and anal muscles. Symbiotic bacteria of each EPN population were identified, and a biochemical characterization was performed using Analytical Profile Index tests. The symbiotic bacteria belong to the species

Photorhabdus antumapuensis, *P. laumondii* subsp. *laumondii*, *P. thracensis*, *Xenorhabdus bovienii* and *X. nematophila*. Bacteria biochemical profiles did not reflect the differences in virulence of nematodes-bacteria complex against wireworms. These findings highlight the importance of considering EPN morphometry and intraspecific variability in designing applications to control wireworms.

Graphical abstract



Keywords

Elateridae, Click beetle, Insect pathogens, Biocontrol, Crop Protection.

1. Introduction

Entomopathogenic nematodes (EPNs) in the families Heterorhabditidae and Steinernematidae (Nematoda: Rhabditida and Panagrolamida, respectively) (Hodda, 2022) are efficient at controlling several crop pests, especially soil-dwelling arthropods (Campos-Herrera et al., 2012). Their free-living juveniles (dauer stage) infect and kill the insect host in cooperation

with symbiotic enteric bacteria, *Photorhabdus* spp. and *Xenorhabdus* spp. (Boemare, et al., 1993). In the soil, their foraging strategies rely on a diversity of cues (Grewal et al., 1994; Dillman et al., 2012), some being released by their insect hosts (CO_2) as well as by plants under insect infestations (volatile organic compounds) (Rasmann et al., 2005; Turlings et al. 2012; Laznik & Trdan, 2016; Machado and Reuss, 2022). Once a suitable insect is located, the infective juveniles (IJ) use natural openings or break integument to penetrate inside the hemocoel of the host and release their symbiotic bacteria stocked in the anterior portion of the intestine (for *Heterorhabditis* spp.) (Ciche and Ensign, 2003) or in the bilobed vesicle (for *Steinernema* spp.) (Bird and Akhurst, 1983). These bacteria then secrete toxins that induce rapid insect death within 48 to 72 hours, provide a suitable nutrient medium for nematodes growth and reproduction, and inhibit competing organisms by producing antimicrobial, antifungal and nematicidal metabolites (Wang et al., 2011, Tobias et al., 2017; Blanco Perez et al., 2019). Nematodes undergo multiple generations within the host, and when the food is depleted and excreta products excessive, IJs are formed, re-establish symbiosis and emerge to seek new hosts (Stock et al., 2019).

Wireworms, the soil-dwelling larvae of click beetles (Coleoptera: Elateridae) are ubiquitous polyphagous pests. The most damaging species for agricultural sector in Europe include *Agriotes lineatus*, *A. obscurus* and *A. sputator* (Parker & Howard 2001; Vernon & van Herk, 2022). They may be found in various cultivated and non-cultivated areas where they feed on the roots and collars of young seedlings and the underground organs of older plants. Their subterranean habits and extended development cycle, covering 2 to 5 years (Miles, 1942; Furlan, 2004), make them particularly challenging to manage. Until recently, wireworm populations were controlled through a combination of insecticides (Furlan and Toffanin 1998). Nevertheless, the gradual prohibition of these molecules (Curia Europa, 2023), has led to a notable upsurge in wireworm-related damages over the past 15 years (Le Cointe et al., 2023).

Diverse strategies are being tested and implemented, including intercropping, crop rotation, tillage, resistant varieties seeding, and adult trapping (Parker and Howard 2001; Furlan and Toth, 2007; Johnson et al., 2008; La Forgia et al., 2020; Thibord and Larroude, 2023; Chacon Hurtado et al., 2023). Despite some successes in monitoring, these alternatives are only partially efficient (Barsics et al., 2013; La Forgia and Verheggen 2019).

Since EPNs are well-known biocontrol agents, they have been tested against wireworms, but these insects are particularly resistant to EPNs (Campos-Herrera & Gutiérrez, 2009; Williams et al., 2022). They have a sclerotized, wear-resistant integument, and thick intersegmental membranes, along with a dense brush of branched hairs located in the preoral cavity that prevents EPNs larger than 18 µm in diameter to enter (Eidt & Thurston, 1995). Also, their rectal muscles are likely capable of completely closing the anus (Eidt & Thurston, 1995). Coupled with the cellular and humoral responses that can offset the effect of the bacteria (Li et al., 2007; Rahatkhah et al., 2015), these various physical barriers must be considered when assessing the efficacy of EPN populations against wireworms (Grewal et al., 2005).

In this study, we evaluated the efficacy of different EPN populations from several origins to infected and kill wireworms. Also, we assessed whether this efficacy is related to EPN morphometry and/or their association with specific symbiotic bacteria (Campos-Herrera and Gutierrez, 2009; Lacey and Georgis, 2012; Williams et al., 2022).

2. Materials and Methods

2.1. Insect and nematodes collection, maintenance, and rearing

Agriotes spp. larvae (Coleoptera: Elateridae) were collected from two locations in Belgium: Gembloux and Gedinne (Figure 1 [7 and 11 sites], Supplementary Material). The collection sites were chosen based on their organic cultivation history of at least two years. In

both collection sites, a mix of two species was observed and confirmed by molecular analyses: *Agriotes obscurus* and *A. lineatus* (GenBank PP892771 and PQ066666, Supplementary Material). Larvae were separated by weight and up to 10 individuals were placed in rearing boxes (500 ml – 11 × 11 × 6 cm) to avoid cannibalism. They were maintained in a potting soil (La Plaine Chassart brand; dry matter: 26%, organic matter 20%, pH (water) 5-6.5, Electrical Conductivity (EC) 400-600) - vermiculite mixture (v:v, 1:1) at 65% Water Holding Capacity (WHC) at control room temperature (20 ± 1°C) and fed germinating organic barley (culture of Gembloux AgroBio-Tech) *ad libitum* renovated every three weeks. The stage L11 (14-16 mm) (Sufyan et al., 2014) larvae were selected for the nematode screening.

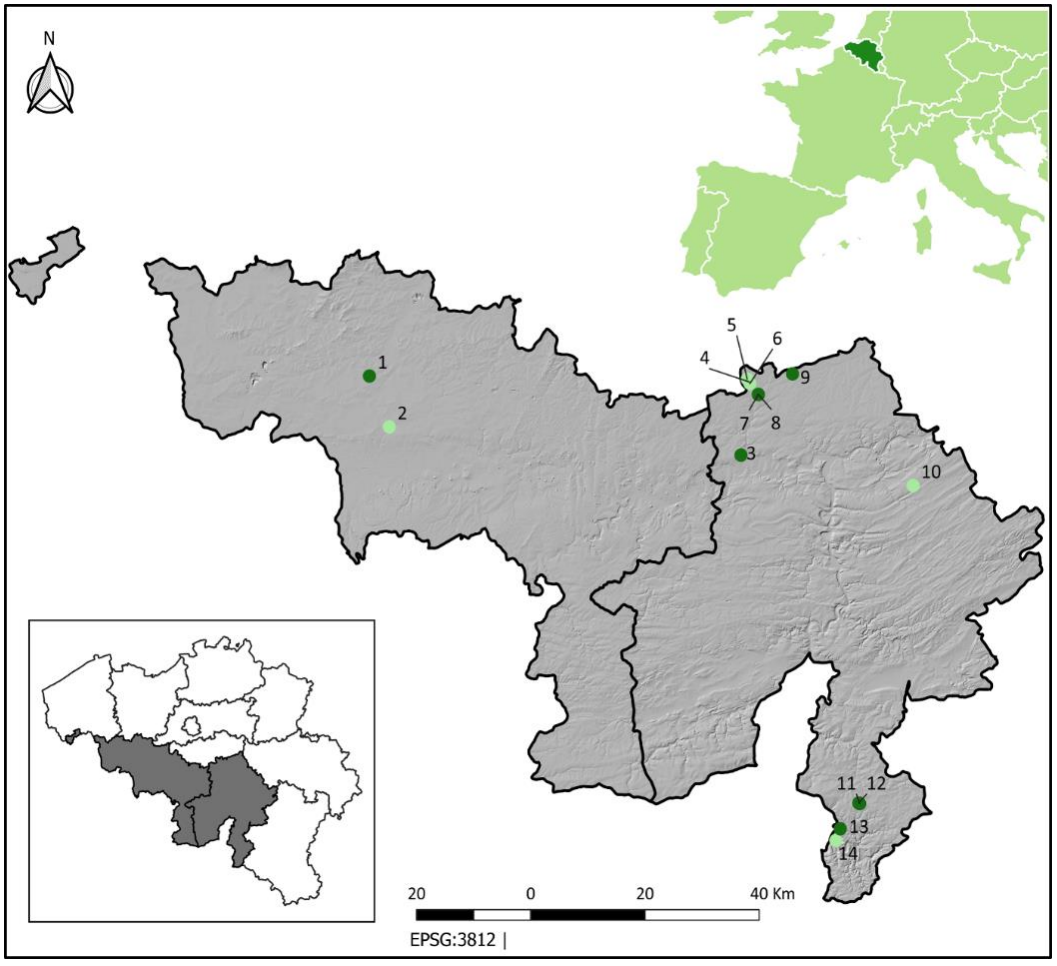


Figure 1. Distribution of the sampling sites of wireworms and EPN along Wallonia region (Belgium) dark green dots: sites with presence of EPNs, light green dots: sites without presence of EPNs. Code of reference and land use in agreement with Table S1. EPSG: Projected coordinate system Lambert 2008.

Galleria mellonella larvae (Lepidoptera: Pyralidae) were reared under laboratory conditions according to the protocol of Vicente-Díez et al. (2021a). Last larval stage was used for all experiments.

Native populations of EPNs were isolated during the spring and autumn of 2022 from agriculture, forests, grasslands, and horticulture sites (with and without treatment of phytosanitary products) in the Wallonia region (Belgium) (Figure 1) where wireworm presence was previously documented (Supplementary Material). The populations used in the screening were selected based on species diversity, soil usage, and agricultural conditions of the collection sites (conventional or organic) (Supplementary Material). Foreign populations of EPNs were provided by the Department of Animal Biology, Plant Biology, and Ecology, Faculty of Biosciences (Autonomous University of Barcelona, Spain), Institute of Grapevine and Wine Sciences – (ICVV, Government of La Rioja, CSIC, and University of La Rioja, Logroño, Spain) and Department of Plant Protection, Faculty of Agricultural Sciences (University of Chile, Santiago, Chile). These populations were collected in Spain for *Steinernema carpocapsae* B14 and *Heterorhabditis bacteriophora* 0943 (horticultural field, personal communication), in Portugal for *S. feltiae* AM25 (citrus field) (Campos-Herrera et al., 2019), in Chile for *Heterorhabditis atacamensis* UCH-31936, UCH-33043 (walnut orchard and grapevine field) (Castaneda-Alvarez et al., 2022) and *Heterorhabditis bacteriophora*. UCH-32913 (grapevine field, personal communication). Populations Sc B14 and Sf AM25 were used previously in wireworms screening (Morton and Garcia-del-Pino, 2017; La Forgia et al., 2021). Commercial EPN populations were used for comparison: *H. bacteriophora* and *S. carpocapsae* from e-nema GmbH and *S. feltiae* from insectosphere (Table 1). All EPNs were multiplied every four months on *G. mellonella* and maintained in ventilated culture flasks (250 mL / 75 cm^2; Falcon®) stored at 14°C, at a concentration of 5 IJs/µl.

2.2. Screening of the entomopathogenic nematode activity

A total of 16 populations from six different species of EPNs were tested (Table 1). The experimental unit was a plastic microcosm (6.6 cm^3) filled with 10 g of autoclaved sand (Cobo garden, Belgium), and dried at 80°C for 48 h. Each microcosm was moistened to 90% (WHC) using a solution of IJs and tap water, achieving a 250 IJs/cm^3 concentration (Campos-Herrera & Gutiérrez, 2009). EPNs were reared in *G. mellonella* and used 24 h after emergence. A single wireworm was then placed in each microcosm (n = 15 per population). A positive control was established by substituting the wireworm with a *G. mellonella* (n = 5 per population). Negative controls were conducted by introducing wireworm (n = 10) into microcosms containing only tap water. Wireworms were not fed, considering that they can live without food for one year (Furlan, 2004). Microcosms were stored in control room temperature at 21±1°C in dark. Two independent trials, one week apart, were conducted with new and fresh material in each time. Larval mortality (wireworms and *G. mellonella*) was assessed 24 h, 48 h, and 72 h post-inoculation. Subsequently, mortality was monitored weekly for up to 56 days post-inoculation. Each week, microcosms were weighed, and the WHC was adjusted to 90%. To confirm that the observed mortality was attributable to EPNs, each dead larvae was rinsed with tap water and then individually placed in White traps (White, 1927). They were monitored until the emergence of new IJs or dissected if melanization occurred and the IJs were unable to emerge (Li et al., 2007; La Forgia et al., 2021). At 56 days post-inoculation, alive wireworms were removed from the microcosm and a *G. mellonella* was introduced to assess the virulence of the EPNs.

2.3. Characterization of entomopathogenic nematode morphometry

For each EPN population, recently emerged IJs from five *G. mellonella* were transferred to an Eppendorf tube containing Ringer's solution (NaCI 7.5 g, KCl 0.35 g, $CaCl_2$ 0.21 g, 100

ml distilled water) according to the protocol of Hominick et al. (1996). After a 30 minutes incubation period at 21±1°C (when the IJs became immobile) they were examined and photographed using a microscope (Axioskop 2 MOT) coupled with a camera (AxioCam). Body length and diameter were measured using Inkscape software (version 1.3) on 40 individuals per population randomly chosen.

2.4. Measurement of wireworm openings

Micrographs of the spiracles and anus of L11 stage wireworms (n=10) were captured using an optical microscope. These images were used to determine the structure and dimensions of the primary entry routes utilized by EPNs. Freshly dissected individuals were examined without treatment, following the same procedures used for EPN morphometry measurements.

2.5. Entomopathogenic nematode bacteria isolation and molecular identification

Bacterial isolation was conducted modifying the protocols of Boemare and Akhurst (1988) and Vicente-Díez et al. (2021b). We started from 1 mL of suspension containing around 1000 IJs of each EPN population. After centrifugation (1890.515g for 120s) the supernatant was discarded, and the IJs were cleaned by immersion in 1% NaClO for two minutes. Once again, a centrifugation was performed (1890.515g for 120s), NaClO was carefully removed by pipetting, preserving the EPNs at the bottom of the Eppendorf tube. Subsequently, 1 mL of distilled water was added, followed by centrifugation (1890.515g for 120s), and the supernatant was discarded. This last procedure was carried out twice. The IJs were further disaggregated in 500 µl of Tryptone Soy Broth (TSB, PanReac AppliChem) using the ProCulture™ Cordless Homogenizer Unit (Bel-Art™). Subsequently, 50 µl of shredded was diluted with 950 µl of TSB. A total of 100 µl of each sample was inoculated onto Petri dishes (3 replicates per sample) containing NBTA culture medium [Nutrient agar N1 (VWR chemicals), bromothymol blue (VWR Chemicals) and 2,3,5-triphenyltetrazolium chloride (Merck)]. Petri dishes were then

stored for 48 h at 21±1°C at darkness. Finally, for each sample (n = 3 per population), a single bacterial colony presenting the morphology associated with *Photorhabdus* (Enright et al., 2003) and *Xenorhabdus* (Boemare and Akhurst, 1988; Givaudan et al. 1995) species (colorant absorption capacity, rounded …) were isolated and sub-cultured in NBTA medium (stored for 48 h at 21±1°C at darkness). One colony was selected for DNA extraction using NucleoSpin Tissue kit (Macherey Nagel™). After extraction and amplification, the DNA concentration of each sample was measured using a Nanodrop™ 2000/2000c spectrophotometer. The 16S region was amplified using the primers EB27 forward (5′-AGAGTTTGATCCTGGCTCAG-3') and UN 1492 reverse (5'-GGTTACCTTGTTACGACTT-3') (Enright et al., 2003; Campos-Herrera, et al., 2011) under the following conditions: 60s at 98°C, 10s at 98°C, 30s at 63°C, 45s at 72°C, and 120s at 72°C, with a total of 30 cycles. The species of each sample was then identified by sequence alignment using the BLAST tool on the National Library of Medicine (NIH) website and submitted to Genbank (Table 1).

2.6. Biochemical characterization of the entomopathogenic nematode symbiotic bacteria

All bacteria were cultured in PCA (Plate Count Agar) standard medium (Biokar) to avoid potential metabolic interferences associated with the culture medium. Forty-one biochemical characterizations through microtesting were conducted using the Analytical Profile Index (API) 20E and 20NE galleries (Biomerieux SA, France). Following manufactured protocols. Results were read after an incubation period during 24 hours for the 20E gallery and 48 hours for the 20NE gallery. Coloration gram tests were performed using 77730 Gram Staining Kit (Merck).

2.7. Statistical analysis

All statistical analyses were conducted using R Studio software (version 4.2.3) with the following packages: survminer, survival, lme4, car, dplyr, dunn.test, coxphf, rcompanion,

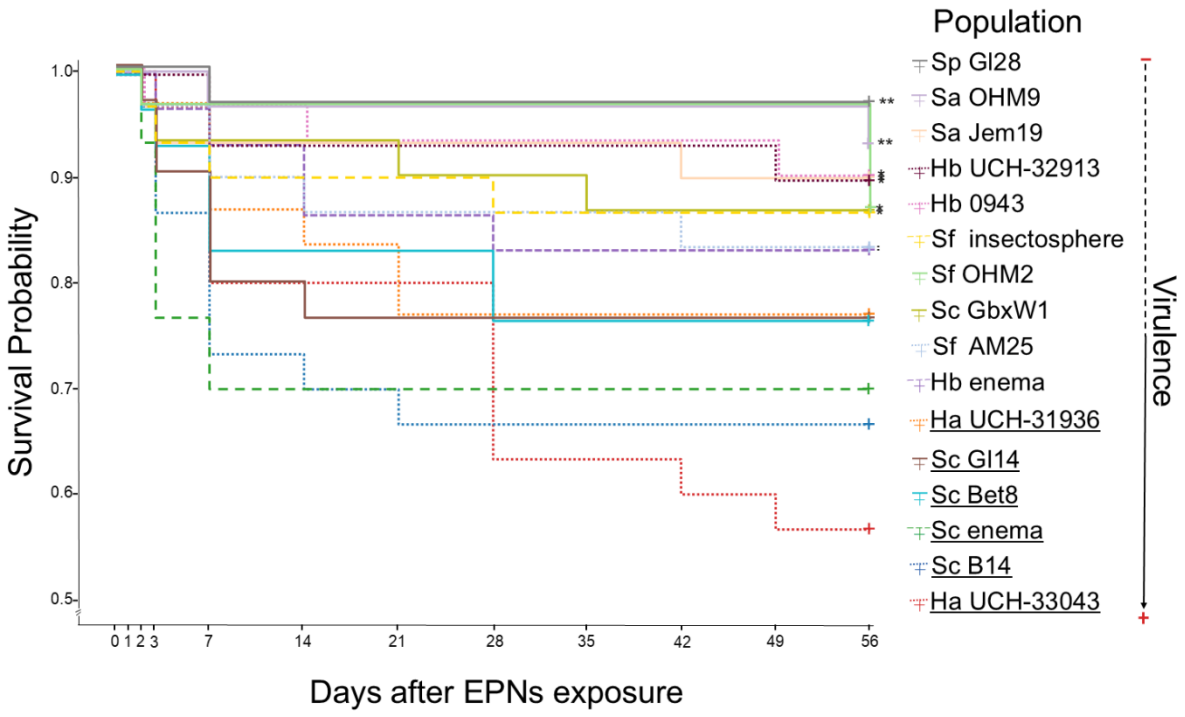
multcomp, and ggplot2. Regarding the screening, a χ^2 test with Yates' continuity correction was employed to identify differences between the two temporal replications. To identify a group of the most virulent populations, Cox regressions were applied, excluding the negative control (0% of natural mortality) and using the most virulent population, "Ha UCH-33043," as a reference. The impact of the population factor on wireworms' survival probability was assessed by comparing the distribution of residuals from the null model (intersect only) and the model containing the factor of interest (population or group) with a χ^2 distribution. Subsequently, Kaplan-Meier survival curves were generated, and a Log-Rank Mantel-Cox test was employed to determine whether the progression of survival probability, with the population factor, exhibited similar trends. To evaluate differences in length and diameter among different populations, an analysis of variance (ANOVA) was performed. Data normality was assessed using a Shapiro test, and homogeneity of variances was tested with a Bartlett test. If conditions allowed, a Kruskal-Wallis test was performed, followed by a post hoc Dunn test with Bonferroni correction. Two general linear mixed models were initially fitted with the mortality rate at three days (time required of EPNs to kill the host) and 56 days as the response variable. These models included EPN length and EPN diameter as explanatory factors, with EPN and bacteria species as random factors. Since the random factors had no significant effect and there variances were null, the models were simplified. Consequently, two general linear models (binomial family) were fitted with the mortality rate at three days and 56 days as the response variable, and EPN length and EPN diameter as explanatory factors.

3. Results

3.1. Screening of the entomopathogenic nematode activity

The χ^2 test did not reveal significant differences between the two trials ($\chi^2 = 2.021$, df =1, $P = 0.155$). Every EPN population induced wireworm mortality (from 3 to 43% after 56

248 days post-inoculation), while no mortality was observed in the negative control (Figure 2).
249 Mortality rates varied across EPN populations (log-rank test χ^2 = 35.5, df =15, *P* = 0.002). Ha
250 UCH-33043 (43% of mortality) were significantly different from 10 populations: Sa OHM9 (*P*
251 = 0.008), Sp Gl28 (*P* = 0.009), Sf insectosphere (*P* = 0.031), Hb UCH-32913 (*P* = 0.014), Hb
252 0943 (*P* = 0.015), Sa Jem19 (*P* = 0.016), Sf OHM2 (*P* = 0.025), Sc GbxW1 (*P* = 0.030), and
253 nearly significantly different from Hb enema (*P* = 0.053) and Sf AM25 (*P* = 0.054). Results
254 showed six EPN populations that can be grouped as "virulent populations": Ha UCH-33043,
255 Sc B14 (*P* = 0.633), Sc enema (*P* = 0.537), Sc Gl14 (*P* = 0.213), Sc Bet8 (*P* = 0.173), Ha UCH-
256 31936 (*P* = 0.169), (Figure 2). The EPN population displaying the fastest mortality rate in
257 wireworms was Sc enema, with 23.3% induced mortality after 72h post-inoculation. In
258 assessing EPN survival within the microcosms, *G. mellonella*, introduced at 56 days, displayed
259 a 100% mortality rate for EPN treatments and 0% in the control group.



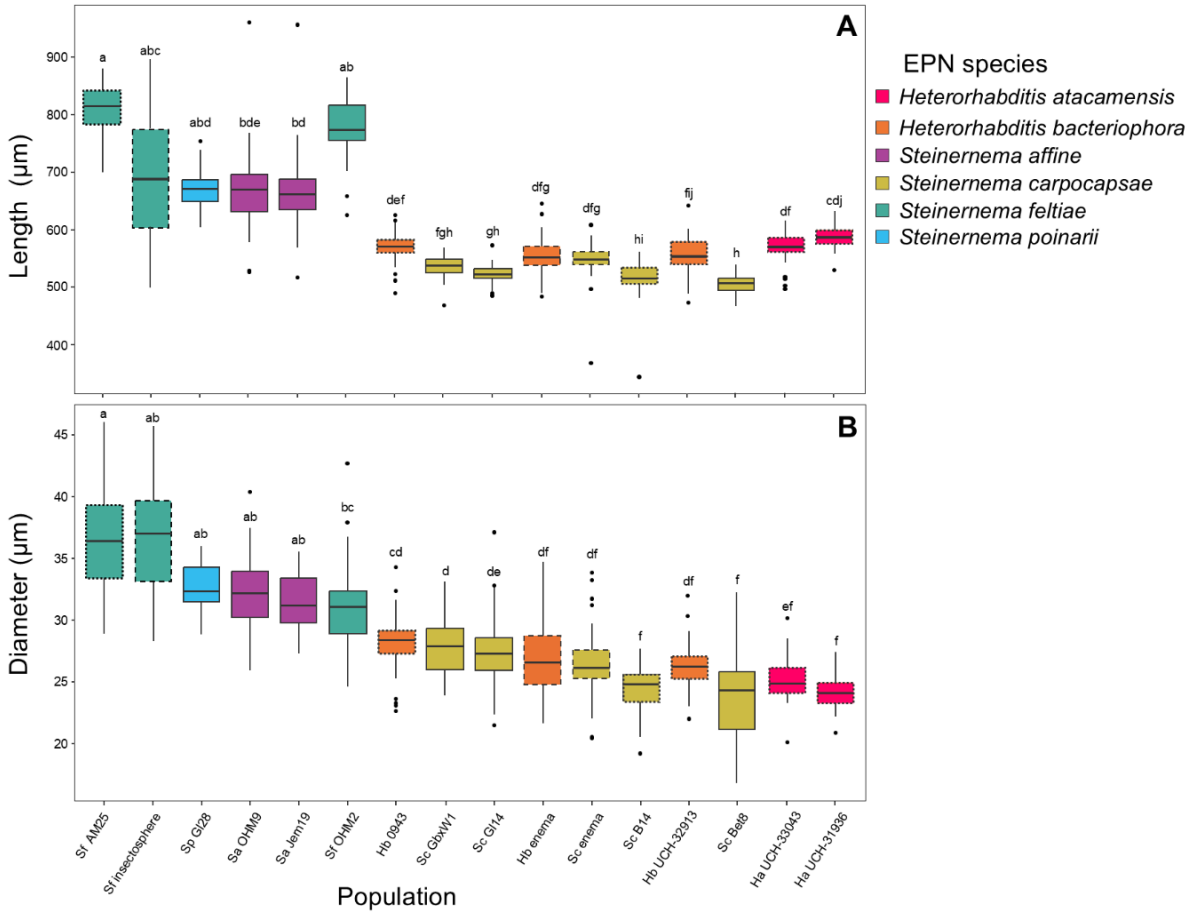
260 **Figure 2**. Wireworm infection by various entomopathogenic nematode populations:
261 from commercial sources (- - -), foreign (⋯) and native (—) populations. Ha (*Heterorhabditis*
262 *atacamensis*), Hb (*Heterorhabditis bacteriophora*), Sa (*Steinernema affine*), Sc (*Steinernema*
263 *carpocapsae*), Sf (*Steinernema feltiae*), Sp (*Steinernema poinari*). Differences in mortality rate
264 (with Ha UCH-33043 as reference) are designated by asterisks (** = p<0.01; * = p<0.05; · =

p<0.1) and were determined by Pairwise comparisons using the Log-Rank test. Underlined populations refer to the most virulent group.

3.2. Characterization of entomopathogenic nematode morphometry

Significant differences were observed in the EPNs length across populations (Kruskal-Wallis $\chi^2 = 486.65$, df = 14, $P < 0.001$; Figure 3A). Sf AM25 stood out as the longest (809.1 ± 6.4 µm), while Ha UCH-33043 (568.7 ± 4.12 µm) and Ha UCH-31936 (586.2 ± 3.13 µm) were the shortest. As for EPN diameters, significant differences were also observed across the populations (Kruskal-Wallis $\chi^2 = 87.24$, df = 14, $P < 0.001$), with Sf insectosphere having the largest diameter (37.1 ± 0.7 µm). Sc Bet8 (24.2 ± 0.5 µm), and Ha UCH-31936 (24.3 ± 0.2 µm) exhibited the smallest diameters (Figure 3B).

The length of EPNs influenced significantly wireworms' mortality after three days post-inoculation (Z-value = -1.967, df = 15, $P = 0.049$) but not at 56 days post-inoculation (Z-value = 0.608, df = 15, $P = 0.543$). Concerning the diameter, our results didn't found any effect at three days post-inoculation (Z-value = 1.049, df = 15, $P = 0.294$) but revealed a significant negative effect after 56 days post-inoculation (Z-value = -2.357, df = 15, $P = 0.018$) with leaner EPNs associated with higher mortality rate.



283 **Figure 3.** Length (A) and diameter (B) of different EPN populations (n = 40 individuals per
284 population). Letters assigned denote significant differences between populations, determined
285 by Dunn-Bonferroni post hoc analysis. Populations from commercial sources (- - -), foreign
286 (⋯) and native (—) populations.

287

288 *3.3. Measurement of wireworm openings*

289 Wireworms possess eight lateral spiracles, which measure up to 805.7 µm in length and 85.7
290 µm^2 when opened (Figure 4A). The anus can be opened (Figure 4C) or completely closed
291 (Figure 4D). It measured up to 240 µm in length. When fully opened, it can reach a maximal
292 average area of 33733 µm^2, which corresponds to the air inside the chitinous ring surrounding
293 the anal muscle (Supplementary material).

294

295

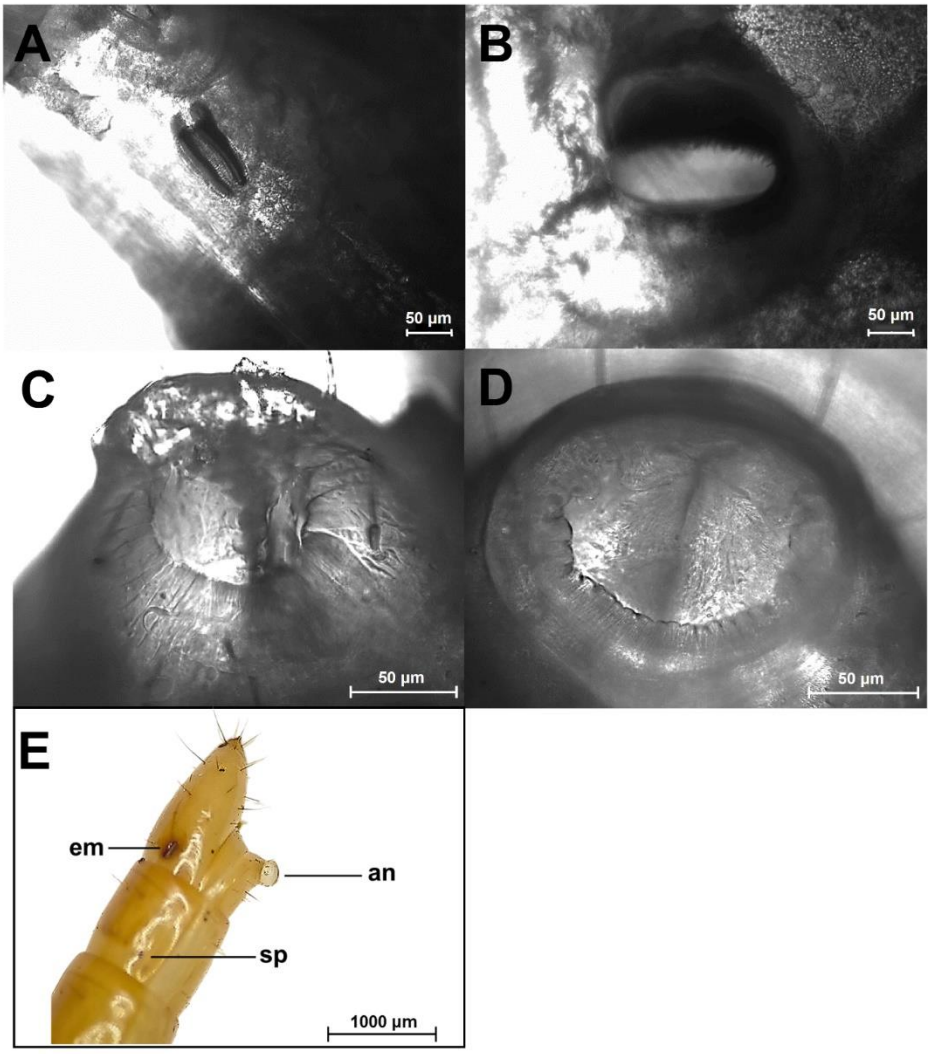


Figure 4. Optical microscope micrographs of wireworm Agriotes spp. (A) spiracle, (B) "eye-like" impression, (C) anal sclerotized coating (D) anus and anal muscles, (E) Stereo microscope image. Side view of posterior part (eighth, ninth and tenth segments) of wireworm: em, "eye like" impression; an, anus; sp, spiracle.

3.4. Entomopathogenic nematode bacteria isolation and molecular identification

The phenotypic characteristics of the bacterial isolates were consistent with Phase I, indicative of the direct isolation of symbiotic bacteria from IJs. These isolates presented convex, circular colonies and exhibited positive dye absorption on NBTA plates (Akhurst, 1983; Akhurst et al., 1992; Campos-Herrera et al., 2009). A total of five species of symbiotic bacteria were identified across our 16 population, with a successfully amplified 1500 bp rDNA fragment for each isolate: populations of *H. atacamensis* were associated with *Photorhabdus*

antumapuensis (Castaneda-Alvarez et al., 2022); *H. bacteriophora* (Poinar, 1976) was associated with two symbiotic bacteria: *P. laumondii subsp. laumondii* (Machado et al., 2018) and *Photorhabdus thracensis* (Machado et al. 2018). *S. carpocapsae* (Weiser, 1955) were associated with *Xenorhabdus nematophila* (Poinar and Thomas 1965; Thomas and Poinar 1980); *S. affine* (Bovien, 1937), *S. feltiae* (Filipjev, 1934), and *S. poinari* (Mrácek, Puza & Nermut, 2014), were associated with *Xenorhabdus bovienii* (Akhurst 1983; Akhurst and Boemare 1993) (Table 1).

T**able 1**. Populations of entomopathogenic nematode (EPN) of the genera Heterorhabditis and Steinernema and their symbiont bacteria, tested against wireworms of the genus Agriotes spp.

EPN species	**Geographic origine**	**Population**	**ITS-sequence** (GenBank accession number)	**Symbiont bacteria**	**16S-sequence** (GenBank accession number)
Steinernema affine	Belgium	Sa Jem19	PP375812	*Xenorhabdus bovienii*	PP209855
	Belgium	Sa OHM9	PP375815	*Xenorhabdus bovienii*	PP209857
Steinernema carpocapsae	Belgium	Sc Bet8	PP375816	*Xenorhabdus nematophila*	PP209849
	Belgium	Sc GbxW1	PP375813	*Xenorhabdus nematophila*	PP209853
	Belgium	Sc Gl14	PP375817	*Xenorhabdus nematophila*	PP209859
Steinernema feltiae	Belgium	Sf OHM2	PP375814	*Xenorhabdus bovienii*	PP209851
Steinernema poinari	Belgium	Sp Gl28	PP375818	*Xenorhabdus bovienii*	PP209860
Heterorhabditis atacamensis	Chile	Ha UCH-31936	MZ676563	*Photorhabdus antumapuensis*	MZ676562
	Chile	Ha UCH-33043	PQ436959	*Photorhabdus antumapuensis*	PQ198615
Heterorhabditis bacteriophora	Spain	Hb 0943	PQ199402	*Photorhabdus thracensis*	PP401697

	Chile	Hb UCH-32913	PQ436960	*Photorhabdus thracensis*	OR140086
Steinernema carpocapsae	Spain	Sc B14	PQ199401	*Xenorhabdus nematophila*	PP401689
Steinernema feltiae	Portugal	Sf AM25	MG551674	*Xenorhabdus bovienii*	PP401693
Heterorhabditis bacteriophora	Commercial	Hb enema	PQ199404	*Photorhabdus laumondii subsp. laumondii*	PP401695
Steinernema carpocapsae	Commercial	Sc enema	PQ199403	*Xenorhabdus nematophila*	PP401691
Steinernema feltiae	Commercial	Sf Insectos-phere	PQ199505	*Xenorhabdus bovienii*	PP401687

332

333 *3.5. Biochemical characterization of the entomopathogenic nematode symbiotic bacteria*

334 The 16 bacterial isolates under study (Table 2) revealed similar trends, with most results
335 indicating negative outcomes for enzyme reactions and carbon source fermentation and
336 assimilation, aligning with characteristics typical of *Xenorhabdus* isolates, as described by
337 Campos-Herrera et al., (2009). The bacterial cells displayed Gram-negative staining. No
338 oxidase activity or carbon source fermentation was observed. Gelatin hydrolysis was only
339 detected in *Photorhabdus* isolates. Positive L-tryptophane hydrolysis was observed in isolates
340 *P. thracensis* 0943 and *X. bovienii* Jem19. D-glucose and D-mannose assimilation was positive
341 for isolates *Photorhabdus* UCH-33043, UCH-913 and *X. nematophila* B14, Gl14, enema.
342 Additionally, D-glucose assimilation was positive for *Photorhabdus* UCH-936 and enema. All
343 isolates of *Photorhabdus* and isolates of *X. nematophila* B14, Gl14, enema, *X. bovienii* AM25,

344 Jem19, OHM9, exhibited assimilation of N-acetyl-glucosamine. Malic acid assimilation was
345 positive for isolate *Photorhabdus* UCH-33043.

Table 2 Phenotypic characterization of the bacteria isolates of *Heterorabditis* and *Steirnernema* populations tested again wireworms**.** [a]Type of reaction: positive (+), negative (-).

Bacteria isolates	*Photorhabdus*					*Xenorhabdus bovienii*						*Xenorhabdus nematophila*				
Strain	antumapuensis		*laumondii*	*thracensis*		AM25	Gl28	Insectosphere	Jem19	OHM2	OHM9	Bet8	B14	GbxW1	Gl14	enema
	UCH33043	UCH936	enema	0943	UCH 913											
Reactions/Enzyme[a]																
2-nitrophenyl-ßd-galactopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-nitrophenyl-βD-galactopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin ferric citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
L-arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-ornithine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-tryptophane	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Potassium nitrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium pyruvate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium thiosulfate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trisodium citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbon source fermentation [a]																
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbon source assimilation																
D-glucose	+	+	+	-	+	-	-	-	-	-	-	-	+	-	+	+
L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-mannose	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	+
D-mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-glucosamir	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+
D-maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium gluconat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Capric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adipic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malic acid	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trisodium citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenylacetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4. Discussion

While previous studies have examined nematodes virulence against wireworms, our unique approach focuses on exploring inherent factors within the nematode that could influence its virulence. We tested populations from various biogeographic sites and commercial origins inferring that different populations of the same nematode species may exhibit variations in traits associated with infection dynamics, such as mortality and penetration percentage (Campos-Herrera & Gutiérrez, 2014). We found six populations (native, foreign, and commercial) demonstrating to be virulent against wireworms. Prior studies evaluating virulence against wireworms have reported mortality rates reaching 50% for Sf AM25 and 75% for Sc B14 populations (La Forgia et al., 2021; Morton & Garcia-del-Pino, 2017). In contrast, our study only reached a mortality rate of 43% for the Ha UCH-33043 population under continuous exposure to EPNs over 56 days. This suggests that the chosen inoculation protocol (quantity of EPN, application method, duration of exposition ...) may significantly influence the observed mortality. Additionally, variations in susceptibility or resistance to EPN infection among different wireworm populations could also account for these differing results.

In the current screening, inter- and intraspecific morphometric differences were found across EPN populations. Stock et al. (2000), previously identified EPN morphological differences according to their biogeographic origin. These differences could reflect a morphological adaptation from the entomopathogen to hosts' physical barriers. In wireworms the pre-ecdysis phase makes up most of their larval development (Furlan, 2004). Consequently, nematodes attached to the wireworm's cuticle may be eliminated promptly during ecdysis.

Wireworms have a highly chitinous exoskeleton acting as a strong physical barrier. *Heterorhabditis* spp. infective juveniles possess a dorsal tooth that assist in penetrating the intersegmental regions (Kaya and Gaugler, 1993). However, in our study, we were unable to

demonstrate an advantage of this structure at the time of penetrating the wireworm. Of the five tested populations of *Heterorhabditis spp.*, only two were classified within the group of "most virulent". These nematodes have difficulty penetrating the intersegmental region as well as other natural openings. *Agriotes* spp. present a big "eye-like" structure in the ninth abdominal segment. However, this structure is not an entry hole but rather an aposematic tegument coloration that serves as a deterrent to predators (Skelhorn et al., 2016). Wireworms' mouth may not be a natural opening targeted by IJs either. Indeed, wireworms possess an oral filter that can exclude IJs which have diameter of 18 µm or greater (Eidt & Thurston, 1995). Examination of larvae showed that the head capsule and mandibles are heavily sclerotized structures (Furlan et al., 2021; Supplementary material), which could crush nematodes attempting to enter the mouth (Gaugler and Molloy, 1981). Wireworms possess eight lateral spiracles, the size of which could allow EPN entrance. However, these primary orifices are closed most of the time. The secondary orifices through which oxygen diffuses are densely covered with branched hairs and, in some Elateridae species, they are further protected by a thick septum (Snodgrass 1935; Lanchester 1939; Eidt, 1958). These closed structures hinder the access of IJs (Eidt et al., 1995). Other studies have shown that the anus serves as the primary entrance for nematodes into insects (Batalla-Carrera et al., 2014). In the case of wireworms, the anus is not just a posterior opening of the digestive tract but also functions as a locomotive organ fitted with strong muscles that act like a vacuum cup, pushing the larva forward or assisting in its withdrawal (Lanchester, 1939). It can measure up to 240 µm in length when opened (Supplementary material). However, due to its structure, wireworms can close it completely, preventing the entry of microorganisms. These results suggest that leaner juveniles may hold a competitive advantage in swiftly penetrating wireworms' physical barriers, a crucial initial step in the successful infection. *Agriotes* wireworms can be found as deep as 60cm in the soil (Furlan, 2004), *Heterorhabditis* spp. adopts a cruiser foraging strategy, they can move

through the soil using its pores (Kaya and Gaugler, 1997), making these EPNs promising candidates for controlling wireworms.

In this study, all EPN populations were cultivated on *G. mellonella* larvae, which are known for their minimal physical barriers, particularly their large natural orifices. Considering the characteristics of wireworms and the potential impact of orifice width on penetration success and mortality rates, cultivating EPN populations directly on wireworms could induce natural selection favoring leaner infective juveniles. Studies suggest that morphometric variability may also arise from interactions with the host (Poinar, 1992); therefore, cultivating the most virulent species in the target host could enhance selectivity and, thereby, population effectiveness.

We identified a diversity of entomopathogenic nematodes-bacteria complex, comprising six nematode species and four bacteria species, exhibiting differences in their success in killing wireworms. Although the isolated effect of bacterial virulence was not investigated here, their biochemical characterization does not reflect the variability observed in wireworms' mortality rates. Certain enzymatic reactions could play a role in the establishment of infection in the host (Massaoud et al., 2010). Additionally, it has been noted that biochemical profile varies among different bacteria strains of *Photorhabdus* spp. and *Xenorhabdus* spp. (Machado et al., 2018; Castaneda-Alvarez et al., 2022). Here, we highlighted the assimilation by symbiotic bacteria of N-acetyl-glucosamine, a principal component of chitin (Son et al., 2024). Initially expected for enteric bacteria responsible for degrading the insect exoskeleton, this may be considered in future experiments that would assess bacteria's ability to assimilate this monosaccharide. *Photorhabdus spp.* and *Xenorhabdus spp.* bacteria produce distinct immunosuppressants, antibiotics, and insecticides (Goodrich-Blair and Clarke, 2007; Bode, 2009). Further investigation into the nematode-bacteria complex is essential, particularly concerning the protein toxins (secondary metabolites) these bacteria produce and their role in

any process as the mortality rate of hosts. Analyzing bacterial viability and growth rates within the host may uncover significant differences that influence virulence outcomes. This information could enhance our understanding of the mechanisms driving host-pathogen interactions in this system.

REFERENCES

Akhurst, R. J. (1983). Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. *International Journal of Systematic and Evolutionary Microbiology*, 33(1), 38-45. doi.org/10.1099/00207713-33-1-38

Akhurst, R. J., Smigielski, A. J., Mari, J., Boemare, N., & Mourant, R. G. (1992). Restriction analysis of phase variation in *Xenorhabdus* spp. (Enterobacteriaceae), entomopathogenic bacteria associated with nematodes. *Systematic and applied microbiology*, 15(3), 469-473. doi.org/10.1016/S0723-2020(11)80224-9b

Batalla-Carrera, L., Morton, A., Shapiro-Ilan, D., Strand, M. R., & García-del-Pino, F. (2014). Infectivity of Steinernema carpocapsae and S. feltiae to larvae and adults of the hazelnut weevil, Curculio nucum: differential virulence and entry routes. Journal of nematology, 46(3), 281. PMC4176411

Barsics, F., Haubruge, E., & Verheggen, F. (2013). Management: An Overview of the Existing Methods, with Particular Regards to *Agriotes* spp. (Coleoptera: Elateridae). *Insects*, 4(1), 117-152. doi:10.3390/insects4010117

Bird, A.F., Akhurst, R.J., 1983. The nature of the intestinal vesicle in nematodes of the family Steinernematidae. Int. J. Parasitol. 13, 599–606.

Blanco-Pérez, R.; Bueno-Pallero, F.Á.; Vicente-Díez, I.; Marco-Mancebón, V.S.; Pérez-Moreno, I.; Campos-Herrera, R. Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode Steinernema feltiae. J. Invertebr. Pathol. 2019, 164, 5–15.

Bode, H. B. (2009). Entomopathogenic bacteria as a source of secondary metabolites. Current opinion in chemical biology, 13(2), 224-230. doi.org/10.1016/j.cbpa.2009.02.037

Boemare, N. E., & Akhurst, R. J. (1988). Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). *Microbiology*, 134(3), 751-761. doi.org/10.1099/00221287-134-3-751

Boemare, N. E., Akhurst, R. J., & Mourant, R. G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology, 43(2), 249-255. doi.org/10.1099/00207713-43-2-249

Campbell, J. F., & Gaugler, R. R. (1997). Inter-specific variation in entomopathogenic nematode foraging strategy: dichotomy or variation along a continuum?. Fundamental and Applied Nematology, 20(4), 393-398. ISSN 1164-5571

Campos-Herrera, R., & Gutiérrez, C. (2009). Screening Spanish isolates of steinernematid nematodes for use as biological control agents through laboratory and greenhouse microcosm studies. *Journal of Invertebrate Pathology*, 100(2), 100–105. doi.org/10.1016/j.jip.2008.11.009

Campos-Herrera, R., & Gutierrez, C. (2014). *Steinernema feltiae* intraspecific variability: infection dynamics and sex-ratio. *Journal of Nematology*, 46(1), 35. PMCID: PMC3957570

Campos-Herrera, R., Barbercheck, M., Hoy, C. W., & Stock, S. P. (2012). Entomopathogenic Nematodes as a Model System for Advancing the Frontiers of Ecology. *Journal of Nematology*, 44(2), 162. PMCID: PMC3578465

Campos-Herrera, R., Blanco-Pérez, R., Bueno-Pallero, F. Á., Duarte, A., Nolasco, G., Sommer, R. J., & Rodríguez Martín, J. A. (2019). Vegetation drives assemblages of entomopathogenic nematodes and other soil organisms: Evidence from the Algarve, Portugal. Soil *Biology and Biochemistry*, 128, 150–163. doi.org/10.1016/j.soilbio.2018.10.019

Campos-Herrera, R., El-Borai, F. E., Stuart, R. J., Graham, J. H., & Duncan, L. W. (2011). Entomopathogenic nematodes, phoretic *Paenibacillus* spp., and the use of real time quantitative PCR to explore soil food webs in Florida citrus groves. *Journal of Invertebrate Pathology*, 108(1), 30–39. doi.org/10.1016/j.jip.2011.06.005

Campos-Herrera, R., Tailliez, P., Pagès, S., Ginibre, N., Gutiérrez, C., & Boemare, N. E. (2009). Characterization of *Xenorhabdus* isolates from La Rioja (Northern Spain) and virulence with and without their symbiotic entomopathogenic nematodes (Nematoda: Steinernematidae). *Journal of Invertebrate Pathology*, 102(2), 173–181. doi.org/10.1016/j.jip.2009.08.007

Castaneda-Alvarez, C., Machado, R. A., Morales-Montero, P., Boss, A., Muller, A., Prodan, S., & Aballay, E. (2022). Photorhabdus antumapuensis sp. nov., a novel symbiotic bacterial species associated with Heterorhabditis atacamensis entomopathogenic nematodes. International Journal of Systematic and Evolutionary Microbiology, 72(10), 005525. doi.org/10.1099/ijsem.0.005525

Chacon Hurtado, J. A., Ruhland, F., Boullis, A., & Verheggen, F. (2023). Potato cultivar susceptibility to wireworms: feeding behaviour, fitness and semiochemicals-based host selection. *Entomologia Generalis*, 43(6). DOI: 10.1127/entomologia/2023/2168

Ciche, T.A., Ensign, J.C., (2003). For the insect pathogen, *Photorhabdus luminescens*, which end of a nematode is out? Appl. Environ. Microbiol. 69, 1890–1897. doi.org/10.1128/AEM.69.4.1890-1897.2003

Curia Europa (2023). Available at:

https://curia.europa.eu/juris/document/document.jsf?text=&docid=269405&pageIndex=0&doclang=FR&mode=req&dir=&occ=first&part=1&cid=1486798 (Accessed: 15 January 2024).

Dillman, A. R., Guillermin, M. L., Lee, J. H., Kim, B., Sternberg, P. W., & Hallem, E. A. (2012). Olfaction shapes host–parasite interactions in parasitic nematodes. Proceedings of the National Academy of Sciences, 109(35), E2324-E2333. doi.org/10.1073/pnas.121143610

Eidt, D. C. (1958). Anatomy and histology of the full-grown larva of Ctenicera aeripennis destructor (Brown) (Coleoptera: Elateridae). Canadian Journal of Zoology, 36(3), 317-361. doi.org/10.1139/z58-030

Eidt, D. C., & Thurston, G. S. (1995). Physical deterrents to infection by entomopathogenic nematodes in wireworms (Coleoptera: Elateridae) and other soil insects. *The Canadian Entomologist*, 127(3), 423-429. doi:10.4039/Ent127423-3

Enright, M. E., McInerney, J. O., & Griffin, C. T. (2003). Characterization of endospore-forming bacteria associated with entomopathogenic nematodes, *Heterorhabditis* spp., and description of *Paenibacillus nematophilus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 53(2), 435–441. doi.org/10.1099/ijs.0.02344-0

Furlan, L. (2004). The biology of Agriotes sordidus Illiger (Col., Elateridae). Journal of Applied Entomology, 128(9-10), 696-706. doi.org/10.1111/j.1439-0418.2004.00914.x

Furlan, L., & Toffanin, F. (1998). Effectiveness of new insecticides used as seed dressing (imidacloprid and fipronil) against wireworms in a controlled environment.

Furlan, L., & Toth, M. (2007). Management and biological control of wireworm populations in Europe: current possibilities and future perspectives. IOBC WPRS BULLETIN, 30(7), 11-16.

Furlan, L., Benvegnù, I., Bilò, M. F., Lehmhus, J., & Ruzzier, E. (2021). Species identification of wireworms (Agriotes spp.; coleoptera: Elateridae) of agricultural importance in Europe: A new "horizontal identification table". Insects, 12(6), 534. doi.org/10.3390/insects12060534

Gaugler, R., & Molloy, D. (1981). Instar susceptibility of Simulium vittatum (Diptera: Simuliidae) to the entomogenous nematode Neoaplectana carpocapsae. Journal of Nematology, 13(1), 1. PMC2618041

Givaudan, A., Baghdiguian, S., Lanois, A., & Boemare, N. (1995). Swarming and Swimming Changes Concomitant with Phase Variation in *Xenorhabdus nematophilus*. In *Applied and environmental microbiology*, 61(4), 1408-1413. doi.org/10.1128/aem.61.4.1408-1413.1995

Goodrich-Blair, H., & Clarke, D. J. (2007). Mutualism and pathogenesis in Xenorhabdus and Photorhabdus: two roads to the same destination. Molecular microbiology, 64(2), 260-268. doi.org/10.1111/j.1365-2958.2007.05671.x

Grewal, P. S., Ehlers, R. U., & Shapiro-Ilan, D. I. (Eds.). (2005). Nematodes as biocontrol agents. *Cabi publishing*.

Grewal, P. S., Lewis, E. E., Gaugler, R., & Campbell, J. F. (1994). Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. Parasitology, 108(2), 207-215. doi:10.1017/S003118200006830X

Hodda, M. (2022). Phylum Nematoda: a classification, catalogue and index of valid genera, with a census of valid species. Zootaxa, 5114(1), 1-289. doi.org/10.11646/zootaxa.5114.1.1

Hominick, W. M., Reid, A. P., Bohan, D. A., & Briscoe, B. R. (1996). Entomopathogenic nematodes: biodiversity, geographical distribution and the convention on biological diversity. Biocontrol Science and Technology, 6(3), 317-332. doi. org/ 10. 1007/ s0044 20050 108

Johnson, S. N., Anderson, E. A., Dawson, G., & Griffiths, D. W. (2008). Varietal susceptibility of potatoes to wireworm herbivory. Agricultural and Forest Entomology, 10(2), 167-174. doi.org/10.1111/j.1461-9563.2008.00372.x

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. Annual review of entomology, 38(1), 181-206. doi.org/10.1146/annurev.en.38.010193.001145

La Forgia, D., & Verheggen, F. (2019). Biological alternatives to pesticides to control wireworms (Coleoptera: Elateridae). Agri Gene, 11, 100080. doi.org/10.1016/j.aggene.2018.100080

La Forgia, D., Bruno, P., Campos-herrera, R., Turlings, T., & Verheggen, F. (2021). The lure of hidden death: development of an attract-and-kill strategy against wireworms combining semiochemicals and entomopathogenic nematodes. Turkish Journal of Zoology, 45(8), 347-355. doi.org/10.3906/zoo-2106-38

La Forgia, D., Thibord, J. B., Larroudé, P., Francis, F., Lognay, G., & Verheggen, F. (2020). Linking variety-dependent root volatile organic compounds in maize with differential infestation by wireworms. Journal of pest science, 93, 605-614. doi.org/10.1007/s10340-019-01190-w

Lacey, L. A., & Georgis, R. (2012). Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. Journal of nematology, 44(2), 218. PMID: 23482993; PMCID: PMC3578470

Lanchester, H. P. (1939). The external anatomy of the larva of the Pacific Coast wireworm. USDA Technical Bulletin

Laznik, Ž., & Trdan, S. (2016). Attraction behaviors of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to synthetic volatiles emitted by insect damaged potato tubers. *Journal of Chemical Ecology*, 42, 314-322. DOI:10.1007/s10886-016-0686-y

Le Cointe, R., Plantegenest, M., & Poggi, S. (2023). Wireworm management in conservation agriculture. *Arthropod-Plant Interactions*, 1-7. doi.org/10.1007/s11829-023-09966-9

Li, X. Y., Cowles, R. S., Cowles, E. A., Gaugler, R., & Cox-Foster, D. L. (2007). Relationship between the successful infection by entomopathogenic nematodes and the host immune

response. International journal for parasitology, 37(3-4), 365-374. doi.org/10.1016/j.ijpara.2006.08.009

Machado, R. A., & Von Reuss, S. H. (2022). Chemical ecology of nematodes. Chimia, 76(11), 945-945. doi.org/10.2533/chimia.2022.945

Machado, R. A., Wüthrich, D., Kuhnert, P., Arce, C. C., Thönen, L., Ruiz, C., ... & Erb, M. (2018). Whole-genome-based revisit of *Photorhabdus* phylogeny: proposal for the elevation of most *Photorhabdus* subspecies to the species level and description of one novel species *Photorhabdus bodei* sp. nov., and one novel subspecies *Photorhabdus laumondii* subsp. clarkei subsp. nov. International journal of systematic and evolutionary microbiology, 68(8), 2664-2681. doi.org/10.1099/ijsem.0.002820

Massaoud, M. K., Marokházi, J., Fodor, A., & Venekei, I. (2010). Proteolytic enzyme production by strains of the insect pathogen *Xenorhabdus* and characterization of an early-log-phase-secreted protease as a potential virulence factor. Applied and environmental microbiology, 76(20), 6901-6909. doi.org/10.1128/AEM.01567-10

Miles, H. W. (1942). Wireworms and agriculture, with special reference to *Agriotes obscurus* L. doi.org/ 10.1111/j.1744-7348.1942.tb07585.x

Morton, A., & Garcia-del-Pino, F. (2017). Laboratory and field evaluation of entomopathogenic nematodes for control of *Agriotes obscurus* (L.) (Coleoptera: Elateridae). *Journal of Applied Entomology*, 141(4), 241–246. doi.org/10.1111/jen.12343

Parker, W. E., & Howard, J. J. (2001). The biology and management of wireworms (*Agriotes* spp.) on potato with particular reference to the UK. *Agricultural and Forest Entomology*, 3(2), 85-98. doi.org/10.1046/j.1461-9563.2001.00094.x

Poinar Jr, G. O. (1992). Steinernema feltiae (Steinernematidae: Rhabditida) parasitizing adult fungus gnats (Mycetophilidae: Diptera) in California. *Fundamental and applied nematology*, 15(5), 427-430.

Rahatkhah, Z., Karimi, J., Ghadamyari, M., & Brivio, M. F. (2015). Immune defenses of *Agriotes lineatus* larvae against entomopathogenic nematodes. *BioControl*, 60, 641-653. doi.org/10.1007/s10526-015-9678-z

Rasmann, S., Köllner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J., & Turlings, T. C. J. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, *434*(7034), 732-737. doi.org/10.1038/nature03451

Skelhorn, J., Holmes, G. G., Hossie, T. J., & Sherratt, T. N. (2016). Eyespots. Current Biology, 26(2), R52-R54

Snodgrass, R.E. (1935). Principles of Insect Morphology. *McGraw-Hill*, New York. 667.

Son, D. J., Kim, G. G., Choo, H. Y., Chung, N. J., & Choo, Y. M. (2024). Functional Comparison of Three Chitinases from Symbiotic Bacteria of Entomopathogenic Nematodes. *Toxins*, 16(1), 26. doi.org/10.3390/toxins16010026

623 Stock, S. P., Mrácek, Z., & Webster, J. (2000). Morphological variation between allopatric
624 populations of *Steinernema kraussei* (Steiner, 1923) (Rhabditida: Steinernematidae).
625 *Nematology*, 2(2), 143-152. doi.org/10.1163/156854100509033

626 Stock, S. P. (2019). Partners in crime: symbiont-assisted resource acquisition in Steinernema
627 entomopathogenic nematodes. *Current opinion in insect science*, 32, 22-27.
628 doi.org/10.1016/j.cois.2018.10.006

629 Sufyan, M., Neuhoff, D., & Furlan, L. (2014). Larval development of Agriotes obscurus under
630 laboratory and semi-natural conditions. Bull. Insectol. 2014, 67, 227–235

631 Thibord, J. B., & Larroude, P. (2023). Baiting to protect maize against wireworms. Arthropod-
632 Plant Interactions, 17(4), 507-515. doi.org/10.1007/s11829-023-09971-y

633 Tobias, N. J., Wolff, H., Djahanschiri, B., Grundmann, F., Kronenwerth, M., Shi, Y. M., ... &
634 Bode, H. B. (2017). Natural product diversity associated with the nematode symbionts
635 Photorhabdus and Xenorhabdus. Nature microbiology, 2(12), 1676-1685.

636 Turlings, T. C., Hiltpold, I., & Rasmann, S. (2012). The importance of root-produced volatiles
637 as foraging cues for entomopathogenic nematodes. *Plant and Soil*, 358, 51-60.
638 doi.org/10.1007/s11104-012-1295-3

639 Vernon, B., & van Herk, W. (2022). Wireworms as pests of potato. *Insect Pests of Potato*.
640 *Academic press*, 103-148. doi.org/10.1016/b978-0-12-821237-0.00020-2

641 Vicente-Díez, I., Blanco-Pérez, R., Chelkha, M., Puelles, M., Pou, A., & Campos-Herrera, R.
642 (2021a). Exploring the use of entomopathogenic nematodes and the natural products
643 derived from their symbiotic bacteria to control the grapevine moth, *Lobesia botrana*
644 (Lepidoptera: Tortricidae). Insects, 12(11), 1033. doi.org/10.3390/insects12111033

645 Vicente-Díez, I., Blanco-Pérez, R., González-Trujillo, M. D. M., Pou, A., & Campos-Herrera,
646 R. (2021b). Insecticidal effect of entomopathogenic nematodes and the cell-free
647 supernatant from their symbiotic bacteria against *Philaenus spumarius* (Hemiptera:
648 Aphrophoridae) nymphs. *Insects*, *12*(5). doi.org/10.3390/insects12050448

649 Wang, Y., Fang, X., Cheng, Y., & Zhang, X. (2011). Manipulation of pH shift to enhance the
650 growth and antibiotic activity of *Xenorhabdus nematophila*. BioMed Research
651 International, 2011. doi.org/10.1155/2011/672369

652 White, G. F. (1927). A method for obtaining infective nematode larvae from cultures. *Science*,
653 66(1709), 302-303. DOI: 10.1126/science.66.1709.302.b

654 Williams, L., Cherry, R., & Shapiro-Ilan, D. (2022). Effect of host size on susceptibility of
655 Melanotus communis (Coleoptera: Elateridae) wireworms to entomopathogens. Journal of
656 Nematology, 54(1). doi: 10.2478/jofnem-2022-0033

657

658

659

AKNOWLEDGEMENTS

The authors thank Dr Patrick Fickers and Sebastien Steels for the guidance and laboratory access for molecular identification (laboratory of Microbial Processes and Interactions, Gembloux Agro-Bio Tech). Dr. Philippe Maesen and Virginie Byttebier to advice in the realization of biochemical test (Chemistry for Sustainable Food and Environmental Systems). Dr. Fernando García del Pino (Autonomous University of Barcelona) and Dr. Carlos Castaneda-Alvarez (University of Chile) for providing EPN populations. Dr Carlos Molina for providing e-nema populations. Julie Bonnet for her hard work during field collection, rearing and bioassays conduction. Dr. Yves Brostaux for his valuable advice on statistical analyses. The anonymous reviewers for their helpful feedback. This work was supported by Erasmus mundus grants: Brice Checconi (IN221021) and Salimata Drabo (FAMIENS01). Andrea Chacon Hurtado was awarded an FRIA grant (Fund for research training in industry and agriculture, FRIA/FC-2576 FC46977) to achieve a PhD thesis. Fanny Ruhland is supported by Attrack-and-kill Wallonia project (D65-1401/S1, Belgium). Raquel Campos-Herrera collaborated under the frame of the Grant PID2022-136487OB-I00 funded by MCIN/AEI/ 10.13039/501100011033 and, by "ERDF A way of making Europe".

Supplementary material

Entomopathogenic nematodes resource

We followed the protocols described by Campos-Herrera et al. (2019) and Blanco-Pérez et al. (2020) to isolate soil nematodes and confirm they were entomopathogens. Native populations of EPNs were isolated during the spring and autumn of 2022 from agriculture, forests, grasslands, and horticulture sites (with and without treatment of phytosanitary products) in the Wallonia region (Belgium) (Figure1) where wireworm presence was previously documented. For each site, composite samples (n = 2) were collected, each comprising 20 soil

cores (diameter: 2.5 cm; depth: 20 cm; over 120 meters). In the laboratory, composite samples were mixed, homogenized, and humidified at 80% Water Holding Capacity (WHC) manually and divided into three 200 g (fresh weight) soil subsamples individually placed into a plastic container of 200 mL with a perforated lid. Each subsample was baited with *G. mellonella* larvae (n = 5). This procedure was repeated after one week in a second round of nematode isolation. Plastic containers were stored in the dark at room temperature (21 ± 1°C). After five days of exposure, dead larvae were collected, rinsed with tap water, and individually placed in White traps (White, 1927). Alive larvae were also introduced into White traps and observed for 48h to document any subsequent mortality. If IJs emerged from the cadaver, their entomopathogenic activity was confirm with a Koch's postulate where five additional *G. mellonella* larvae were inoculated with 250 ± 50 IJs. In case of a positive Koch's postulate, the IJs emerging from cadavers were harvested and stored in tap water in culture flasks ventilated (250 mL / 75 cm²; Falcon®) at 14°C, at a concentration of 2 IJs/µl.

All the EPNs, both from our collection and those provided by other laboratories or commercial resources, were reared every four months. For this, a solution of 250 ± 50 IJs was inoculated on filter paper in a 55 mm diameter Petri dish, to which five *G. mellonella* larvae were added (this procedure was performed twice per EPN population). After 48-72 hours, larval

mortality was recorded. Subsequently, the dead larvae were placed following a star pattern (the heads facing the center of the Petri dish), and the 55 mm Petri dish was floating inside a larger. Petri dish (90mm) containing 5 mL of tap water. This facilitates the migration of IJs towards the water upon emergence. Emergence was monitored every three to four days under a stereoscope. Once the IJs emerged, the two replicates were mixed and collected in culture flasks ventilated (250 mL / 75 cm²; Falcon®) and stored at 14°C, at a concentration of 2 IJs/µl.

Molecular identification of the isolates

The identification process followed a protocol adapted from Campos-Herrera et al. (2011). We transferred 1 mL of suspension containing around 1000 IJs of each EPN population in an Eppendorf tube and mechanically disaggregated (20s) using sterile pestles assembled to a ProCulture™ Cordless Homogenizer Unit (Bel-Art™). For each population, DNA was extracted following NucleoSpin Tissue kit (Macherey Nagel™). After the extraction and amplification steps, the DNA concentration of each sample was measured using a Nanodrop™ 2000/2000c spectrophotometer. Then, we used high-quality DNA for further amplification by polymerase chain reaction (PCR), using as amplification primers: forward 18S (5′-TTGATTACGTCCCTGCCCTTT-3′) and reverse 26S (5′-TTTCACTCGCCGTTACTAAGG-3′) (Vrain et al., 1992 ; Hominick et al., 1996) for analysis of the ITS region, then forward D2F (5′-CCTTAGTAACGGCGAGTGAAA-3′) and reverse 536(5′-CAGCTATCCTGAGGAAAC-3′) (Nguyen and Hunt, 2007) for analysis of the D2D3 region enabling species confirmation. We employed Taq polymerase Q5®. The PCR on a FastGene® gradient thermocycler. PCR conditions for the ITS region were: 60s at 98°C, 15s at 98°C, 30s at 58°C, 60s at 72°C and 120s at 72°C. The PCR conditions for the D2D3 region were: 30s at 98°C, 10s at 98°C, 30s at 57°C, 60s at 72°C and 120s at 72°C. These conditions were carried out over a total of 30 cycles. All PCR products generated were checked in a 2% Tris-acetate-EDTA (TAE) (pH 8.3±0.1; Fisher Thermo fisher scientific) agarose gel (Nippon genetics) and visualized in the UVP (VWR GenoSmart). Samples were purified by Gel and PCR Clean up kit (Macherey Nagel™). The amplified fragments were sent for sequencing to Eurofins Genomics laboratory. The obtained sequences were aligned using the BLAST tool on the National Library of Medicine (NIH) website and submitted to Genbank.

733 Table S1.

734 Sites sampled in the Wallonia region, Belgium.

Code	Site	GPS coordonates	Land use	Codification	EPNs species isolated
1	Ormeignies	50.35255N 3.44159E	Horticultural/ Organic	OHM	*Steinernema carpocapsae* (Weiser, 1955) *Steinernema affine* (Bovien, 1937) *Steinernema feltiae* (Filipjev, 1934)
2	Sirault	50.30466N 3.47163E	Corn, wheat/ Organic	Sir	NO
3	Jemeppe-sur-Sambre	50.28152N 4.39210E	Corn/ Organic	Jem	*Steinernema carpocapsae* (Weiser, 1955) *Steinernema affine* (Bovien, 1937) *Steinernema feltiae* (Filipjev, 1934)
4	Ernage	50.34578N 4.40358E	Sugar beet/ Pesticide	Bet	*Steinernema carpocapsae* (Weiser, 1955)
5	Ernage	50.35132N 4.40195E	Corn growing/ Pesticide	MaER	NO
6	Ernage	50.34491N 4.40432E	Potato/ Pesticide	PdER	NO
7	Gembloux	50.33485N 4.41584E	Vegetable gardening / Organic	GbxW	*Steinernema carpocapsae* (Weiser, 1955)
8	Gembloux	50.33485N 4.41584E	Grassland	GbxP	*Steinernema carpocapsae* (Weiser, 1955)
9	Grand-Leez	50.594595N 4.784013E	Forest	Gl	*Steinernema carpocapsae* (Weiser, 1955) *Steinernema feltiae* (Filipjev, 1934) *Steinernema poinari* (Mrácek, Puza & Nermut, 2014)
10	Gesves	50.25212N 5.04466E	Potato / (CuSO4)	Gev	NO

11	Ardennes - Gedinne	49.56158N 4.56314E	Wheat/ Organic	Arc	*Steinernema carpocapsae* (Weiser, 1955) *Steinernema affine* (Bovien, 1937)
12	Ardennes - Gedinne	49.56191N 4.56237E	Grassland	Arp	*Steinernema feltiae* (Filipjev, 1934)
13	Ardennes - Herisson	49.535836N 4.533970E	Corn / Organic	Herma	*Steinernema carpocapsae* (Weiser, 1955)
14	Ardennes - Vresse sur semois	49.881757N 4.883395E	Forest	Arf	NO

735

736 *Molecular identification of wireworms*

737 The identification process followed a protocol adapted from Staudacher et al., 2011 with
738 any modifications. We transferred one wireworm in an Eppendorf tube and mechanically
739 disaggregated (1 min) using sterile pestles assembled to a ProCulture™ Cordless Homogenizer
740 Unit (Bel-Art™). 200 µl of sample was used to DNA extraction. DNA was extracted following
741 NucleoSpin Tissue kit (Macherey Nagel™). After the extraction and amplification steps, the
742 DNA concentration of each sample was measured using a Nanodrop™ 2000/2000c
743 spectrophotometer. Then, we used high-quality DNA for further amplification by polymerase
744 chain reaction (PCR), using as amplification primers: forward LCO1490 (5′-
745 GGTCAACAAATCATAAAGATATTGG-3′) and reverse HC02198 (5′-
746 TAAACTTCAGGGTGACCAAAAAATCA-3′) for analysis of the mitochondrial cytochrome
747 *c* oxidase subunit I (COI) gene (approx. 660bp) (Vrijenhoek, 1994). We employed Taq
748 polymerase Q5®. The PCR on a FastGene® gradient thermocycler. PCR conditions for the COI
749 region were: 60s at 98°C, 10s at 98°C, 30s at 52°C, 20s at 72°C by 5 cycles, then 15s at 98°C,
750 30s at 64°C, 45s at 72°C by 35 cycles with a final step of 60s at 72°C. All PCR products
751 generated were checked in a 2% Tris-acetate-EDTA (TAE) (pH 8.3±0.1; Fisher Thermo fisher
752 scientific) agarose gel (Nippon genetics) and visualized in the UVP (VWR GenoSmart).

Samples were purified by Gel and PCR Clean up kit (Macherey Nagel™). The amplified fragments were sent for sequencing to Eurofins Genomics laboratory. The obtained sequences were aligned using the BLAST tool on the National Library of Medicine (NIH) website and submitted to Genbank.

Measurement of wireworm's openings

A total of 10 wireworms in the larval stage L11 were dissected, examined, and photographed using a microscope (Axioskop 2 MOT) equipped with a camera (AxioCam). The openings (spiracle, anus) and eye like impression were measured using Inkscape software (version 1.3). The measure of anus open correspond to inside area of sclerotized coating.

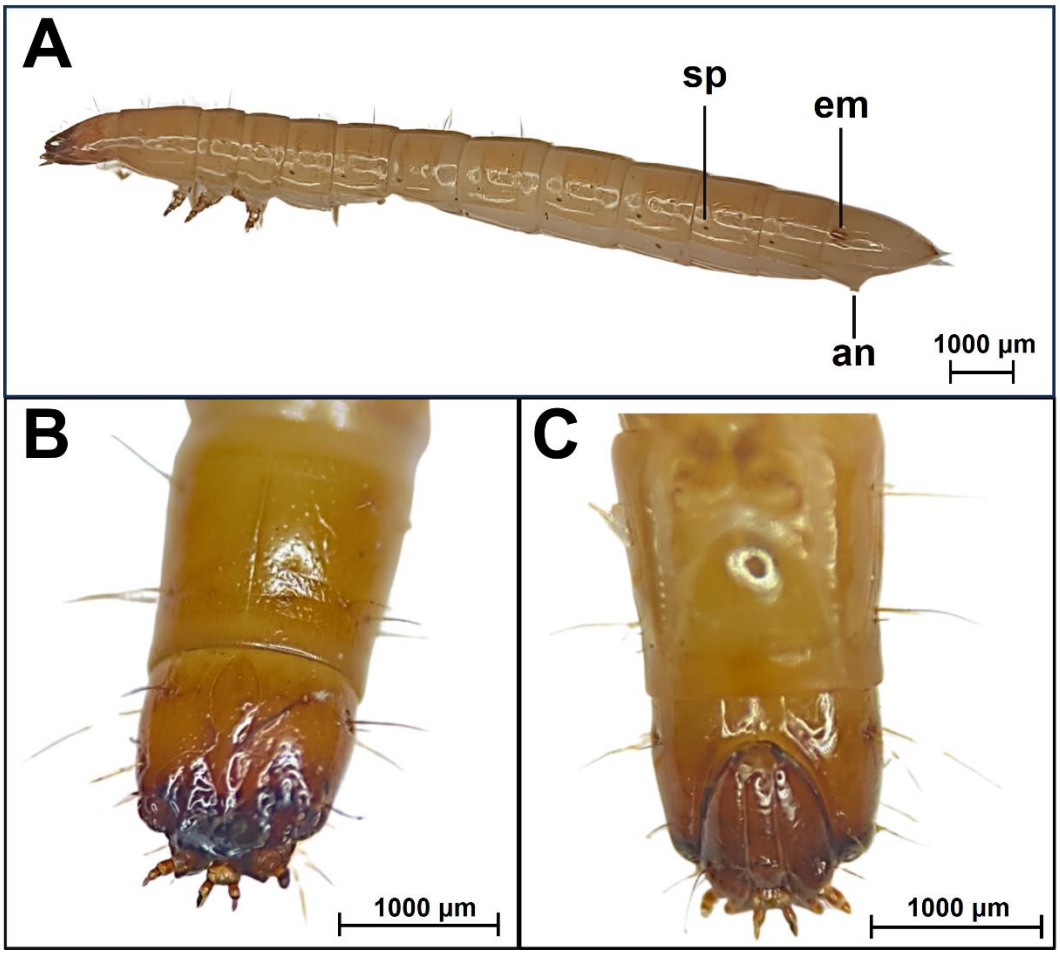


Figure S1. Stereo microscope image of wireworms *Agriotes* spp.: (A) Abdominal segments (dorso-lateral view): sp, spiracle; em, "eye-like" impression; an, anus. (B) Head Capsule (dorsal view); (C) Head capsule (ventral view).

Table S1. Measurements (mean ± SEM) of the longest length (µm) and area of openings (µm²) of different parts of the body of wireworms (n = 10; larvae mean length = 13.5 ± 0.3). Spiracles were measured from the first to the eighth abdominal segment.

		Length (µm)	Opening area (µm²)
Spiracles	1	635.1 ± 75.8	84.4 ± 4.6
	2	676.1 ± 66.2	77.0 ± 2.6
	3	628.1 ± 66.2	81.0 ±3.5
	4	706.3 ± 91.7	80.2 ± 3.5
	5	620.2 ± 45.5	79.2 ± 2.8
	6	694.5 ± 76.6	78.9 ± 2.8
	7	703.5 ± 80.7	85.7 ± 5.7
	8	805.7 ± 98.8	83.4 ± 3.6
Anus		296.4 ± 59.8	15.7 ± 1.6

References

Blanco-Pérez, R., Sáenz-Romo, M. G., Vicente-Díez, I., Ibáñez-Pascual, S., Martínez-Villar, E., Marco-Mancebón, V. S., Pérez-Moreno, Campos-Herrera, R. (2020). Impact of vineyard ground cover management on the occurrence and activity of entomopathogenic nematodes and associated soil organisms. *Agriculture, Ecosystems and Environment*, 301(May), 107028. doi.org/10.1016/j.agee.2020.107028

Campos-Herrera, R., Blanco-Pérez, R., Bueno-Pallero, F. Á., Duarte, A., Nolasco, G., Sommer, R. J., & Rodríguez Martín, J. A. (2019). Vegetation drives assemblages of entomopathogenic nematodes and other soil organisms: Evidence from the Algarve, Portugal. Soil *Biology and Biochemistry*, 128, 150–163. doi.org/10.1016/j.soilbio.2018.10.019

Campos-Herrera, R., El-Borai, F. E., Stuart, R. J., Graham, J. H., & Duncan, L. W. (2011). Entomopathogenic nematodes, phoretic *Paenibacillus* spp., and the use of real time quantitative PCR to explore soil food webs in Florida citrus groves. *Journal of Invertebrate Pathology*, 108(1), 30–39. doi.org/10.1016/j.jip.2011.06.005

785 Staudacher, K., Pitterl, P., Furlan, L., Cate, P. C., & Traugott, M. (2011). PCR-based species
786 identification of Agriotes larvae. Bulletin of entomological research, 101(2), 201-210.
787 doi:10.1017/S0007485310000337

788 Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase
789 subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol, 3(5), 294-9.

790 White, G. F. (1927). A method for obtaining infective nematode larvae from cultures. *Science*,
791 66(1709), 302-303. DOI: 10.1126/science.66.1709.302.b

792

793