Los nematodos entomopatógenos (Rhabditida: Steinernematidae y Heterorhabditidae) para el control del gusano cabezudo, *Capnodis tenebrionis* (Coleoptera: Buprestidae)

Tesis Doctoral
por

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Barcelona, 30 de Enero de 2009

A mi padre,
que siempre está conmigo

Agradecimientos

Al fin llega el día de escribir los agradecimientos, fuerte indicador de que la tesis, y con ello una nueva etapa, se termina. Llegado este momento, uno mira hacia atrás para recordar las experiencias personales que hicieron posible, ayudaron y apoyaron este proyecto. Muchas son las personas que me han acompañado en este largo e intenso viaje, y que han contribuido, en alguna medida, a que este proyecto se hiciera realidad. Aunque unas breves líneas no pueden dar cuenta de mi profunda gratitud y de los recuerdos imborrables que guardo, no por ello las obviaré.

Sin duda alguna, mi principal agradecimiento va dirigido a mi Director de tesis, Fernando García del Pino, mi tutor y amigo, por quien siento un profundo cariño y admiración. A él le agradezco, entre otras cosas, sus valiosos consejos, su constante paciencia, su gran generosidad y su plena disposición para dedicar parte de su tiempo en momentos de inseguridad y dispersión (que han sido muchos). Es un placer poder compartir y disfrutar de tu entusiasmo por la investigación.

Quisiera recordar a Dr. Antonio Garrido, del Instituto Valenciano de Investigaciones Agrarias (IVIA), un experto en la plaga de *Capnodis*. Me recibió en su despacho un día de Enero hace ya muchos años y después de plantearle la idea de la tesis me animó enérgicamente a llevarla a cabo.

Mi agradecimiento al Servei de Protecció dels Vegetals de la Generalitat de Cataluña, especialmente a Ricard Sorribes, que siempre ha estado dispuesto a ayudarme en todas mis peticiones y a Delfí Reinoso.

También quiero agradecer a los responsables y técnicos ADVs que me han ayudado en todo este tiempo, buscándome campos que muestrear, entre ellos Jordi Mateu, Ismael Balart, Joaquín García y Ramón Torà. Un agradecimiento muy especial a Andreu Vila, que siempre me recibía con una sonrisa y me acompañaba por los montes del Baix LLobregat, y a Anna Cáceres, ADV del Vallés Occidental, por contactar conmigo y ofrecerme un campo para hacer mis ensayos.

Gracias a Antonio Soler Montoya, del Servicio de Sanidad Vegetal de la Región de Murcia por guiarnos y ayudarnos en los muestreos de suelos durante los abrasadores días que estuvimos con él, por intercambiar experiencias y mandarme un poquito de su plaga de *Capnodis* de vez en cuando.

No me puedo olvidar de todos los agricultores que me han permitido, algunos sin saberlo, que agujereara por todos los rincones de sus campos. Gracias también a los que nos han dejado, con alegría y expectación, correr por sus campos con cazamariposas a la captura de los *Capnodis* adultos.

Quiero dar las gracias a Marià Vilajeliu, de la Fundació Mas Badia, por su ayuda en localizar zonas de muestreo y a la Cooperativa Frutícola del Empordà, de Sant Pere Pescador, en especial a Anna Cerdà, que me dejaran muestrear en sus campos.

Un agradecimiento especialmente a Xavier García, agricultor polifacético de Ullastrell, que me dejó sus cerezos para los ensayos, me ayudó cuanto pudo y del que no oí ningún improperio cuando vio su campo devastado.

Agradezco profundamente la ayuda de mis “alumnos colaboradores”, que se dejaron liar para recoger *Capnodis* sufriendo el calor de las mañanas veraniegas a cambio de un simple refresco y unas risas. Entre ellos Clara, Gemma, Toni, Berni, Patri, María, Gloria,…

Quiero agradecer a la empresa Agromillora Catalana por cederme plántulas de su producción para poder realizar los ensayos de laboratorio, especialmente a Jordi Monés y a Mª Angela Mestres por estar siempre dispuestos a ayudarme.

También quiero dar las gracias a la empresa e-nema GMBH (Alemania) que produjo y formuló la cepa de nematodos entomopatógenos utilizada en el ensayo de campo. Gracias a todo el equipo.

Gracias Dr. Ralf-Udo Ehlers por acogerme con tanto cariño en su laboratorio de Biotecnología y Control Biológico en Raisdorf, Alemania (Christian-Albrechts-University) durante mi estancia COST. Gracias a Johanna por enseñarme las técnicas de producción de los nematodos entomopatógenos. No me quiero olvidar de los compañeros que tuve, en especial Sibbylle, Alper, Olaf y por supuesto Mayra, que me acogió en su casa y a la que me liga una enorme amistad.

Quiero agradecer al Servei de microscopía electrónica de la UAB su disponibilidad para ayudarme con mis problemas con el microscopio electrónico.

Gracias a los ecólogos, que directa o indirectamente me han ayudado con los problemas en estadística.

Me gustaría agradecer también muy especialmente a mis compañeros de Unidad la ayuda que me han dado durante todo el camino. En especial a Alejandro, el primer nematólogo de la saga, por ser como es. A Maite por su sincera amistad y ser mi conciencia en todo momento. A Anna por tener siempre una sonrisa pase lo que pase. A Tomás por sus instructivas charlas en los cafés. A Francesc, Roger, Silvia, Paco, Marta, Queta y a todos los compañeros que han pasado por aquí o que acaban de llegar, especialmente a Laia que empieza una nueva etapa con nosotros.

A Pilar, Mª Carmen y Manolo por ayudarme en muchas pequeñas cosas.

A aquellos con los que he compartido algunas etapas desde que empecé en la UAB, mi formación o desarrollo de la tesis, que son muchos. Entre ellos Juan Carlos Balasch, con quien empecé el doctorado y con el que tengo una vida paralela, Joan Ruiz que me ha solucionado innumerables problemas con la microbiología, Sergi, con quien me encanta pelear y Joan Torrens, que me ha enseñado que la física también tiene su punto.

No quiero olvidar a mis amigos, que han aguantado estoicamente, algunos durante muchos años y otros no tanto, mis rollos sobre el “maravilloso” mundo de los nematodos entomopatógenos y mis explicaciones detalladas especialmente durante las comidas. Especialmente a Sonia, Pep, Xavi, Ona y Fernando.

A Sito, que es la persona que más ha vivido de cerca mi evolución personal y profesional durantes estos años y que me ha apoyado muchísimo para poder sacar este trabajo adelante.

A mi padre, Charles, por sus ánimos y sus inventos, estarías orgullosos de mi!. A mi madre, por animarme. Por ser la persona más valiente y fuerte del mundo. A mis hermanos por estar ahí. A Mark, por formar parte del equipo de nematodos con sus correcciones. Al resto de mi familia, por interesarse por mis avances. Y, por supuesto, a mis fans más incondicionales, Iñaki, David, Thomas y Miriam

A todos los que puedan sentir que forman parte de esto, y que no he nombrado por mi mala cabeza. Gracias.

Abstract

The Mediterranean flat-headed rootborer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae), is an economically important pest of stone fruit and seed fruit in Mediterranean areas. This report summarizes the studies carried out to prove the efficacy of entomopathogenic nematodes to control *C. tenebrionis:*

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae were isolated from stone-fruit orchards in two Mediterranean regions of Spain. A total of 630 soil samples (210 sites) from Catalonia and 90 soil samples (30 sites) from Murcia were evaluated resulting in 5.2% and 20% of the soils testing positive for nematodes, respectively. Ten steinernematid isolates and three heterorhabditid isolates were recovered using the *Galleria mellonella* baiting method. Based on morphometric data, molecular data and cross-breeding experiments the nematode species were identified as *Steinernema feltiae* and *Heterorhabditis bacteriophora*.

The efficacy of five entomopathogenic nematode strains of the families Steinernematidae and Heterorhabditidae was tested against the neonate larvae of *Capnodis tenebrionis*. The nematode strains screened included two of *Steinernema carpocapsae* (Exhibit and M137), and one each of *S. feltiae* (S6), *S. arenarium* (S2), and *Heterorhabditis bacteriophora* (P4). Exposure of neonate larvae of *C. tenebrionis* to 10 and 150 infective juveniles (IJs) per larva (equivalent to 3 and 48 IJs/cm2 respectively) in test tubes with sterile sand resulted in mortality between 60–91% and 96–100%, respectively. At a concentration of 150 IJs/larva, all of the nematode strains were highly virulent. Both *S. carpocapsae* strains (Exhibit and M137) caused infection and mortality to larvae more quickly than the other strains. However, at a lower concentration assay (10 IJs/larva), *S. arenarium* was the most virulent strain. The penetration rate as an indicator of entomopathogenic nematode infection was also evaluated. The highest value was recorded for *S. arenarium* (36%), followed by *H. bacteriophora* (30.6%), *S. feltiae* (23.1%), and *S. carpocapsae* (20.7%).

The virulence of 14 *Steinernema feltiae* isolates, one *S. carpocapsae* and three *Heterorhabditis bacteriophora* isolates was compared in the laboratory against larvae, pupae and adults of *Capnodis tenebrionis*. Larval mortality ranged from 50 to 100% and pupae mortality from 0 to 70%. Adults were exposed to different nematode concentrations, 50 and 100 infective juveniles/cm^2. *Steinernema feltiae* caused the highest mortality at the two doses tested, reaching 66.7% and 100%, respectively. Significant differences in mortality between *C. tenebrionis* males and

females were observed. The differences in virulence observed at each stage of *C. tenebrionis* vary within a species and can be higher than differences among species. The results of this study suggest that some of the isolate strains have potential for improved biological control of *C. tenebrionis*.

Environmental tolerance to heat, desiccation and hypoxia, the effect of temperature on infectivity and reproduction and nematode migration in sand columns were compared among isolates and one *S. carpocapsae* strain. Results showed differences among species and a great variability within species. Beneficial traits for each strain were added up to identify a superior candidate to control Mediterranean flat-headed rootborer, *Capnodis tenebrionis.* When all analyzed factors were considered, three *S. feltiae* isolates (Bpa, Sor and M116) obtained the best scores, and when hypoxia was removed, two of the strains (Bpa and Sor) continued ranking superior to other strains.

The susceptibility of larvae of *Capnodis tenebrionis* to 13 isolates of entomopathogenic nematodes was examined using GF-677$^{@}$ potted trees (peach x almond hybrid) as the host plant. The nematode strains tested included nine *Steinernema feltiae*, one *S. affine*, one *S. carpocapsae* and two *Heterorhabditis bacteriophora*. Nematodes showed the ability to locate and kill larvae of *C. tenebrionis* just after they enter into the roots of the tree. *S. feltiae* strains provided an efficacy ranging from 79.68% to 88.24%. *H. bacteriophora* strains resulted in control of 71.66–76.47%. *S. carpocapsae* (B14) and *S. affine* (Gspe3) caused lower control of *C. tenebrionis* larvae (62.03% and 34.76%, respectively). The influence of foraging strategy and the use of native nematodes to control *C. tenebrionis* larvae inside the roots is discussed.

The potential control of the entomopathogenic nematode *Steinernema feltiae* (Filipjev) (strain Bpa), isolated from a dead *C. tenebrionis* larva, was tested in a cherry tree orchard in Ullastrell, Barcelona (Spain). Nematode infective juveniles (IJs) were applied by drench and injection. In both the treatments, a rate of 1 million IJs was applied per tree every week during 4 or 8 weeks, with a total dose of 4 x 10^6 IJs/tree and 8 x 10^6 IJs/tree. Number, stage and localization of insects in each tree trunk were recorded. In both the experiments, *S. feltiae* significantly reduced the population of *C. tenebrionis* providing control ranging from 88.3% to 97%. No significant differences were recorded between the different treatments. Persistence of nematodes was recorded until 6 weeks after application. Results

indicate that the application of *S. feltiae* (Bpa) provides adequate control of *C. tenebrionis* in cherry trees.

Índice

"I think I could, if I only knew how to begin. For, you see, so many out-of-the-way things had happened lately that Alice had begun to think that very few things indeed were really impossible".

Lewis Carroll. *Alice's Adventures in Wonderland.*

1. Introducción

I.1. El gusano cabezudo, *Capnodis tenebrionis*

El gusano cabezudo, *Capnodis tenebrionis* L. (Coleoptera: Buprestidae) constituye una importante plaga de árboles frutales de hueso del género *Prunus* sp. (cerezo, melocotonero, albaricoquero, nectarina, almendro y ciruelo) y excepcionalmente de pepita (peral y manzano) desde mitad del siglo XVIII (Obenberger, 1926). Esta plaga causa pérdidas económicas importantes en viveros y en cultivos comerciales debido principalmente a las larvas, que se alimentan de las raíces y el tronco, causando la muerte de los árboles más jóvenes o debilitando progresivamente los árboles adultos hasta su muerte (Martin, 1951; Chrestian, 1955; Garrido, 1984; Ferrero, 1987; Hmimina *et al.*, 1988). Una sola larva es suficiente para matar un árbol joven, y unas pocas pueden causar la muerte de un árbol adulto en uno o dos años (Ben-Yehuda *et al.*, 2000). Debido a los daños que causa esta plaga en los cultivos, la Unión Europea, desde 1993, ha incluido este coleóptero en la lista de organismos nocivos que perjudican la calidad de los materiales de multiplicación de frutales de hueso (Dir. Com. 93/48/EEC).

Capnodis tenebrionis ha sido encontrado principalmente en el sur de Europa y en zonas Mediterráneas (figura I.1), si bien hay señales de su presencia en áreas de la Europa continental (Baviera, Ucrania o Francia) (Balachoswsky, 1962). Diversos autores han indicado la presencia de este insecto en España (Garrido, 1984; Sánchez-Capuchino *et al.*, 1987; Domínguez, 1976), Portugal (Cobos, 1986), Italia

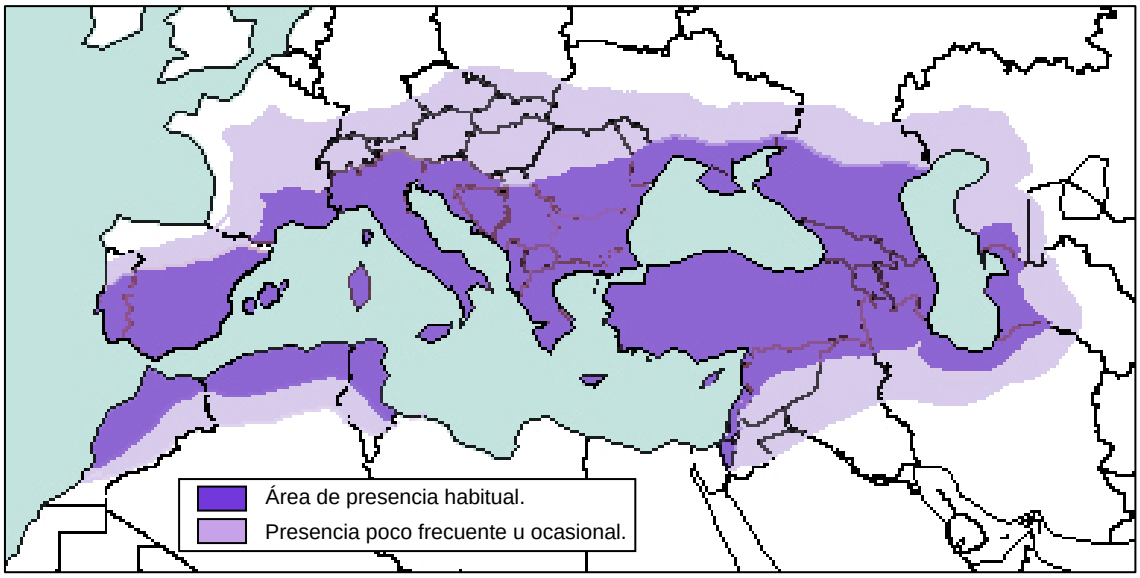


Figura I.1. Distribución de *Capnodis tenebrionis* a nivel mundial (Dibujo actualizado a partir de Malagón (1989)).

(Viggiani, 1991; Laccone, 1998), Francia (Besson, 1951), Marruecos (Perret y Berger, 1949; Chrestian, 1955; Hmimina *et al.*, 1988; Mahhou y Dennis, 1992), Israel (Ben-Yehuda y Mendel, 1997), Argelia (Gairaud y Besson, 1950a; Martin, 1951), Turquía (Tezcan, 1995; Cinar *et al.*, 2004), Líbano, Irán y Túnez (Balachoswsky, 1962).

La primera cita de *C. tenebrionis* en España fue realizada en 1893 por Ascarate, en el término municipal de Jerez (Cádiz) quien lo nombraba como perjudicial en los árboles de hueso y pepita. Actualmente, este bupréstido se encuentra ampliamente distribuido en la Península Ibérica, principalmente en plantaciones de frutales de hueso de secano (Garrido, 1984). Ha sido citado como plaga importante en Cataluña, Mallorca, Murcia (Del Cañizo, 1950), la Comunidad Valenciana (Garrido, 1984; Garrido y Del Busto, 1986), Andalucía (Cabezuelo *et al.*, 1986b), y en algunas provincias del interior de la Península como Cáceres (Del Cañizo, 1950).

I.1.1. Biología

Los adultos son de color negro mate y tienen un tamaño de 12 a 27 mm. Poseen una cabeza ancha con ojos oscuros, un pronoto compacto, más ancho que largo, con unos relieves simétricos lisos con fuerte puntuación (figura I.2), constituyendo

Figura I.2. *Adulto de Capnodis tenebrionis.*

un carácter diferenciador de la especie (Radjabi, 1973). Bajo los élitros, coriáceos y

muy duros, se encuentran las alas membranosas bien desarrolladas. En general las hembras son más grandes que los machos, aunque las oscilaciones de tamaño dentro de cada sexo hacen imposible distinguir entre sexos sólo por esta característica (García *et al.*, 1996). El dimorfismo sexual se observa en la forma del último segmento abdominal, trapezoidal en los machos y acampanada en las hembras (Malagón, 1989).

Los adultos son activos durante los meses cálidos. En primavera, cuando la temperatura en las horas centrales del día alcanza los 20-22 ºC se inicia la salida de imagos que emergen de las cámaras pupales de los árboles infectados. Los adultos comienzan a alimentarse activamente para alcanzar la madurez sexual. Su alimentación se basa en las partes tiernas de la planta, como son el córtex de yemas y ramas jóvenes (Garrido, 1984) y los peciolos de las hojas. Tienen preferencia por los árboles débiles y con poco vigor. El acoplamiento se produce generalmente a una temperatura de 25-26 ºC. La puesta comienza cuando las temperaturas máximas superan los 30ºC, abarcando un periodo desde mayo hasta finales de septiembre (Malagón, 1989). Las hembras depositan los huevos en el suelo, normalmente en grietas o debajo de piedras, en un radio de 50 cm alrededor del tronco del árbol, pudiendo depositar los huevos aisladamente o en grupos de hasta 23 huevos (Feron, 1949). Los huevos son depositados a una profundidad media de 7 mm, coincidiendo con la longitud del oviscapto de la hembra (Guessous, 1950; Reichart, 1967; Garrido, 1984). La puesta viene determinada por la temperatura y la precipitación. La temperatura óptima de oviposición se sitúa entre 28 y 32 ºC, y al aumentar ésta desciende la puesta por agotamiento de la hembra. La lluvia afecta negativamente a la puesta, observándose que la hembra realiza la oviposición siempre en terreno seco (Rivnay, 1946; Garrido *et al.*, 1987; Malagón, 1989). La fecundidad de *C. tenebrionis* según la bibliografía es muy variable, pudiendo llegar a 1000 huevos por hembra (Rivnay, 1944 y 1946) aunque la mayoría de autores sitúan la fecundidad media de este insecto alrededor de 200 a 400 huevos por hembra (Gairaud y Besson , 1950a; Guessous, 1950; Chrestian, 1955; Garrido et a., 1987; Malagón, 1989). Entre los meses de julio y agosto es cuando emerge el mayor número de adultos de las cámaras ninfales, produciéndose el máximo poblacional, al sumarse estos individuos a los hibernantes de la generación anterior. Así pues, se pueden encontrar individuos de dos generaciones, que se diferencian fácilmente por el desgaste de las mandíbulas (Reichart, 1967). Con el descenso de las temperaturas y la reducción de horas de sol los adultos, tras acumular sustancias de reserva, comienzan a buscar refugio bajo tierra, piedras o entre la hojarasca para pasar el invierno.

Los huevos de *C. tenebrionis* son blanquecinos y de forma ovalada, con un tamaño medio de 1,5 x 1,0 mm (Bonnemaison, 1964). Los huevos están recubiertos inicialmente de un líquido pegajoso que se endurece rápidamente, con lo que se recubren de partículas de suelo o de corteza, adquiriendo una forma irregular y pasan totalmente desapercibidos en el campo (figura I.3). El tiempo de desarrollo de los huevos depende principalmente de la temperatura, siendo de 35 a 40 días a 19-20 ºC y de 10 días a 30 ºC (Malagón, 1989).

Una vez ocurre la eclosión del huevo la larva neonata se desplaza por el suelo hacia la planta hospedadora por trofotropismo, atracción que varía según la especie de árbol frutal y que en suelos secos se une a un higrotropismo positivo (Rivnay, 1945; Chrestian, 1955). Las larvas neonatas poseen un tamaño de 3 a 4 mm y se caracterizan por tener pinceles de pelos laterales y caudales que les permiten avanzar por el suelo hacia la planta huésped (figura I.4). Esta estructura desaparece cuando realizan la primera muda, a los 6-15 días, después de haber penetrado en la planta. Las larvas neonatas, al igual que las de últimos estadios, son de color cremoso, tienen una cabeza pequeña, un protórax muy desarrollado y

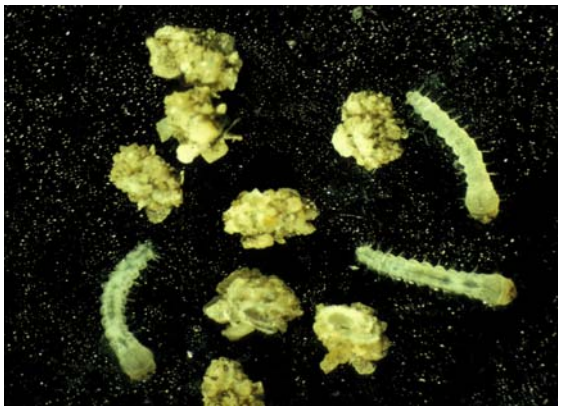


Figura I.3. Huevos recubiertos de partículas de suelo y larvas neonatas de *Capnodis tenebrionis*.

Figura I.4. Larva neonata de *Capnodis tenebrionis*.

un abdomen largo, formado por 10 segmentos subrectangulares. En el interior de la planta las larvas se alimentan principalmente de la capa interna de la corteza y el cambium. La larva mantiene una posición del abdomen característica, en forma de J o U, para facilitar el esfuerzo que realizan al alimentarse de las capas leñosas de la planta (figura I.5). Con el aumento de tamaño de la larva la galería se ensancha, penetrando en el xilema, y va alimentándose rodeando la raíz o el cuello del árbol. La duración del estadio larvario varía, de 1 a 2 años, según factores bióticos y

abióticos, como la temperatura ambiente y la especie de árbol atacada. La larva alcanza 70–75 mm de longitud al final del desarrollo, pero antes de transformarse

Figura I.5. Larva de último estadio de *Capnodis tenebrionis* dentro del tronco, con la posición típica del abdomen en forma de U.

en adulto pasa a estado preninfal en el cual sufre una reducción de longitud de hasta 20–30 mm al contraer los segmentos abdominales, deja de alimentarse y construye un capullo fibroso, denominado cámara ninfal, donde completa la metamorfosis. Esta cámara está formada por fibras de madera desmenuzada y un orificio de eclosión cerrado, fácil de ser perforado por el adulto. La cámara ninfal se encuentra situada generalmente en el cuello del árbol. La preninfa se transforma primero en una ninfa blanca (figura I.6) que esboza perfectamente la forma adulta, posteriormente va adquiriendo un color negro, dando lugar a la ninfa negra (figura I.7). El estado ninfal tiene una duración de 30 a 40 días. Al final de la metamorfosis aparece el adulto, que permanece durante 1 o 2 semanas en la cámara ninfal endureciendo sus tegumentos antes de su salida al exterior. El periodo de emergencia ocurre desde finales de julio a mediados de septiembre. No se conoce exactamente si las hembras de la nueva generación realizan la oviposición en el mismo periodo vegetativo de su emergencia, ya que existen, en la bibliografía, resultados totalmente contradictorios (Malagón, 1989).

En la zona mediterránea el ciclo biológico de *C. tenebrionis* se completa en 15–16 meses (2 periodos vegetativos) en el caso de los huevos puestos a principio de temporada, entre mediados de mayo y finales de julio, y en 25-26 meses (3 periodos vegetativos) en los huevos puestos entre mediados de agosto y

septiembre. Los huevos puestos entre finales de julio y primeros de agosto pueden

Figura I.6. Ninfa blanca de *Capnodis tenebrionis* en la cámara pupal.

Figura I.7. Ninfa negra de *Capnodis tenebrionis* en la cámara pupal.

completar el ciclo en uno u otro periodo (Malagón, 1989). La hibernación se produce en estado adulto, en estado de larva dentro de las galerías de la planta huésped, o bien en las cámaras ninfales, como preadultos o ninfas (Balachowsky, 1962).

A pesar de que se han realizado múltiples estudios sobre la biología del gusano cabezudo desde hace mucho tiempo, todavía existen aspectos que siguen despertando interés, como son los estudios sobre: las interacciones inter- e intra-específicas en *C. tenebrionis* (Bari *et al.*, 2002); la actividad y comportamiento de vuelo de los adultos (Bonsignore y Bellamy, 2007); la morfología de la larva neonata y el efecto de la humedad en la eclosión del huevo (Marannino y De Lillo, 2007); y la distribución de adultos en los cultivos (Bonsignore *et al.*, 2008).

I.1.2. Daños y control

Los ataques a los cultivos producidos por *C. tenebrionis* se manifiestan en la caída al suelo de hojas sanas, producida por los individuos adultos. También se observan árboles con ramas decaídas y secas (figuras I.8 y I.9), así como la aparición de gomosis en el tronco y la muerte más o menos repentina del árbol como consecuencia del ataque de las larvas. Las condiciones generales de cultivo influyen

decisivamente en la gravedad del ataque de *C. tenebrionis*; así los árboles con condiciones deficientes (con carencias nutritivas, déficit o exceso de riego) son mucho más atacados que los sanos y vigorosos (Malagón, 1989).

Figuras I.8 y I.9. Árboles de un cultivo de cerezo atacados por *Capnodis tenebrionis.*

Desde el inicio de la lucha contra *C. tenebrionis,* los métodos culturales han sido muy utilizados. La irrigación ha sido siempre el método más recomendado ya que está ampliamente constatado que el mayor ataque de esta plaga ocurre en condiciones de sequía. Schaefer (1949) y Del Cañizo (1950) ya señalaban la utilización de una malla metálica ajustada al tronco de los árboles y al suelo circundante para impedir la oviposición y evitar la emergencia de los adultos en los árboles infestados. Así mismo, Garrido y del Busto (1986) recomendaban el uso de plásticos con la misma función. También se ha practicado la recogida de adultos sobre árboles, aunque es una operación costosa y de limitada eficacia. Como medida complementaria, se ha recomendado la destrucción de los árboles atacados, que actúan como refugio de larvas y pupas, quemando las raíces y la parte basal del tronco hasta una altura de 30 cm (Garrido, 1984). La utilización de variedades resistentes también ha sido un método utilizado, así algunos autores observaron que el almendro amargo era el menos sensible de las especies usadas como portainjerto de los frutales de hueso (Rivnay, 1945; Guessous, 1949; Perret y Berger, 1949; Lozzia, 1950; Del Cañizo, 1950; Martin, 1951; Chrestian, 1955; Got, 1963; Forte, 1977; Sánchez-Capuchino *et al.*, 1987). Diversos autores (D'hallewin *et al.*, 1990; Malagón y Garrido, 1990; Mulas, 1994; Dicenta *et al.*, 1998; Ben-Yehuda *et al.*, 2001) han demostrado la sensibilidad de diferentes pies portainjerto frente al ataque de *C. tenebrionis*, y han estudiado la relación entre el nivel de compuestos cianógenos, como amigdalina y prunasina, que contienen las especies

de *Prunus* en las raíces, y la resistencia a las larvas de *C. tenebrionis.* También se ha estudiado la relación entre la cantidad de glicósidos cianogénicos de un árbol con la selección de huésped por los adultos para la alimentación y la puesta (Malagón y Garrido, 1990; Mendel *et al.*, 2003).

Los métodos químicos utilizados para controlar la plaga han ido encaminados tradicionalmente al control de los adultos cuando están alimentándose sobre la planta (Garrido *et al.*, 1990; Ben-Yehuda y Mendel, 1997; Colasurdo *et al.*, 1997; Ben-Yehuda *et al.*, 2000), y también contra las larvas neonatas cuando todavía están en el suelo (Sekkat *et al.*, 1997; Ben-Yehuda *et al.*, 2000; Sanna-Passino y Delrio, 2001). El uso de plaguicidas contra *C. tenebrionis* comenzó con el empleo de compuestos arsenicales, el fluosilicato de bario (Pussard, 1935) y la criolita (Rivnay, 1947) en aplicaciones aéreas para eliminar los adultos y el uso de fumigantes en aplicaciones de suelo, como naftaleno y paradiclorobenceno (Del Guercio, 1931; Rekk, 1932; Gairaud y Besson, 1950b; Del Cañizo, 1950) o de dicloroetano y dibromoetano (Rivnay, 1947; Del Cañizo, 1950) contra las larvas neonatas antes de entrar en las raíces. Después de 1940, con la aparición de los insecticidas de síntesis se generalizó el uso de insecticidas clorados, organofosforados y carbamatos, usados en aplicaciones aéreas contra los adultos (Rivnay, 1947, 1951; Feron, 1950; Delmas y Thermes, 1953; Chrestian, 1955; Kaitazov, 1958) o contra las larvas neonatas en el suelo (Schaefer, 1949; Gairaud y Besson, 1950b; Rivnay, 1951; Feron, 1952; Chrestian, 1955; Kaitazov, 1958; Saliba, 1963; Alavidze, 1965). También se recomendó el uso de una mezcla de tres insecticidas, triclorfón+paratión+carbaril, contra adultos (Alavidze, 1965). A finales de los años 80 y durante los 90, con más conocimientos sobre la biología de la plaga, se realizaron estudios para conocer el efecto de algunos insecticidas por contacto o ingestión sobre *C. tenebrionis* (Cabezuelo *et al.*, 1986a; Garrido y Del Busto, 1986; Malagón, 1989; Garrido *et al.*, 1990). Esto permitió tener un listado de productos más o menos eficaces contra *C. tenebrionis*, así como el momento óptimo de aplicación. Hasta finales de los 90 el plaguicida más utilizado fue el lindano, hasta que se prohibió su uso como agente fitosanitario en agricultura. En la actualidad existen en España cuatro formulados registrados para la lucha contra *C. tenebrionis*: Imidacloprid 20% (SL) P/V, del cual hay 34 productos comerciales; Clorpirifos 75% (WG) P/P que se aplica pulverizando los árboles y del cual hay un solo producto comercial registrado; Clorpirifos 25% (CS) P/V que se aplica por pulverización y se encuentran dos productos comerciales; y, Clorpirifos 25% (WP) P/P con 7 productos registrados.

Debido a la complejidad del control químico de *C. tenebrionis,* a que los plaguicidas químicos no son capaces de penetrar en el tronco y raíces para eliminar las larvas que se desarrollan dentro *y* a su impacto negativo sobre el medio ambiente, en estos últimos años se están buscando estrategias alternativas para controlar *C. tenebrionis* (Ben-Yehuda *et al.*, 2000). Se han iniciado estudios de laboratorio para evaluar la eficacia de ciertos hongos entomopatógenos en el control del gusano cabezudo. Marannino *et al.* (2006) utilizaron diversos aislados de *Beauveria bassiana* y *Metarhizium anisopliae* contra larvas neonatas de *C. tenebrionis* y obtuvieron una mortalidad de hasta el 100% y 94,5% respectivamente, demostrando que las larvas neonatas son susceptibles a los hongos entomopatógenos. Recientemente, los mismos autores han realizado un ensayo similar con adultos, mostrando que también éstos son susceptibles a las dos especies de hongos. Proponen utilizar unas bandas de fibras impregnadas con conídios de *M. anisopliae*, colocadas alrededor del tronco de los árboles, para que los insectos al pasar por ellas entren en contacto con los hongos (Marannino *et al.*, 2008).

I.1.3. *Capnodis tenebrionis* y los nematodos entomopatógenos

La susceptibilidad natural de las larvas de *C. tenebrionis* a los nematodos entomopatógenos se conoce desde hace algunos años debido al encuentro en el campo de larvas parasitadas por nematodos de las especies *Steinernema feltiae* (García-del-Pino, 1994) y *S. carpocapsae* (Santos Lobatón *et al.*, 1998). También se han realizado algunos estudios para evaluar la susceptibilidad de las larvas de *C. tenebrionis* en el laboratorio. García-del-Pino (1994) realizó ensayos previos con larvas neonatas y aislados de diferentes especies de nematodos y obtuvo una elevada mortalidad. Marannino *et al.* (2003) obtuvieron resultados similares con con *S. carpocapsae* y *H. bacteriophora.* En el presente trabajo también se muestra un estudio sobre la susceptibilidad de larvas neonatas ante diferentes especies de nematodos entomopatógenos. Existen también ensayos previos al presentado en este trabajo mostrando la susceptibilidad de las larvas de último estadio a un aislado del nematodo entomopatógeno *S. feltiae* (García-del-Pino, 1994) y a *S. carpocapsae* (Santos Lobatón *et al.*, 1998), y estudios iniciales de campo para evaluar su posible utilización comercial (Martínez *et al.*, 2008).

I.2. Los nematodos entomopatógenos

Dentro de grupo de los nematodos se conocen más de 30 familias que poseen relaciones con los insectos (Nickle, 1972; Poinar, 1975, 1990; Maggenti, 1981; Kaya y Stock, 1997) que van desde la foresis hasta el parasitismo o la patogénesis. Las especies que tienen alguna relación de parasitismo con los insectos pertenecen a 23 familias de nematodos. De estas, siete familias han sido estudiadas por su potencialidad para el control biológico de insectos: Mermithidae, Allantonematidae, Neotylenchidae, Sphaerulariidae, Rhabditidae, Steinernematidae y Heterorhabditidae, siendo las dos últimas familias, por su relación de patogénesis con los insectos, las que han sido más estudiadas como posibles agentes de control biológico de plagas de insectos (Alatorre-Rosas y Kaya, 1990; Peters, 1996; Lacey *et al.*, 2001). Su situación dentro del phylum Nematoda se muestra en la tabla I.1., según la clasificación propuesta por Stock y Hunt (2005), basada en De Ley y Blaxter (2002) y Siddiqi (2000).

Tabla I.1. Grupos con potencial de control biológico de insectos del phylum Nematoda (Stock y Hunt, 2005).

PHYLUM NEMATODA (Lankester, 2977) Rudolphi, 1808

CLASE CHROMADOREA Inglis, 1983
Subclase Chromadoria Pearse, 1942
ORDEN RHABDITIDA Chitwood, 1993
Suborden Tylenchina Thorne, 1949
Infraorden Panagrolaimomorpha De Ley y Blaxter, 2002
Superfamilia Strongyloidoidea Chitwood y McIntosh, 1934
Familia Steinernematidae Chitwood y Chitwood, 1937
Superfamilia Aphelenchoidea Fuchs, 1937
Familia Aphelenchidae Fuchs, 1937
Infraorden Tylenchomorpha De Ley y Blaxter, 2002
Superfamilia Sphaerularoidea Lubbock, 1861
Familia Allantonematidae Pereira, 1931
Familia Neotylenchidae Thorne, 1941
Familia Spaerulariidae Lubbock, 1861
Suborden Rhabditina Chitwood, 1933
Infraorden Rhabditomorpha De Ley y Blaxter, 2002
Superfamilia Rhaditoidea Örley, 1880
Familia Rhabditidae Örley, 1880

Superfamilia Strongyloidea Baird, 1853

Familia Heterorhabditidae Poinar 1975

Infraorden Diplogasteromorpha De Ley y Blaxter, 2002

Superfamilia Diplogasteroidea Micoletzky, 1922

Familia Diplogasteridae Micoletzky, 1922

CLASE ENOPLEA Inglis, 1983

Subclase Dorylaimia Inglis, 1983

ORDEN DORYLAIMIDA Pearse, 1942

Suborden Dorylaimiaa Pearse, 1942

Superfamilia Dorylaimoidea de Man, 1876

Familia Dorylaimidae de Man, 1876

Suborden Nygolaimia Thorne, 1935

Superfamilia Nygolaimoidea Thorne, 1935

Familia Nygolaimidae Thorne, 1935

ORDEN MONONCHIDA Jairajpuri, 1968

Superfamilia Mononchoidea Chitwood, 1937

Familia Mononchidae Chitwood, 1937

ORDEN MERMITHIDA Hyman, 1951

Suborden Mermithina, Andrássy, 1974.

Superfamilia Mermithoidea Braun, 1883

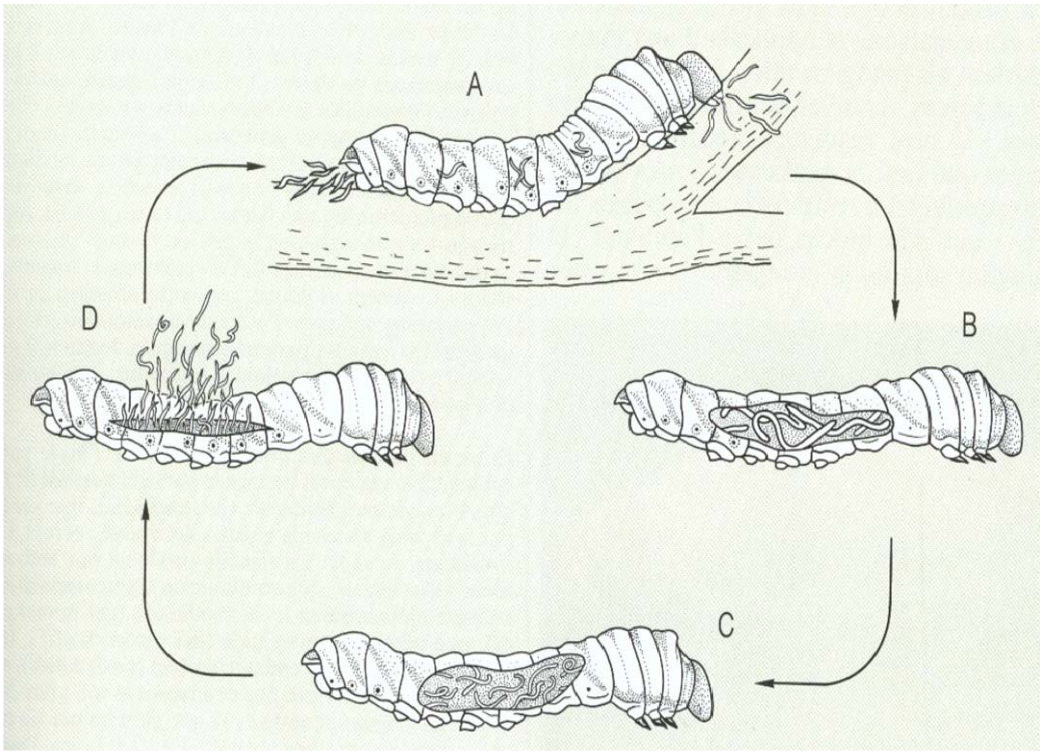
Familia Mermithidae Braun, 1883

I.2.1. Biología

Los nematodos entomopatógenos de las familias Steinernematidae y Heterorhabditidae son parásitos obligados de un gran número de especies de insectos (Poinar, 1979). Se caracterizan por vivir asociados en mutualismo con bacterias de la familia Enterobacteriaceae, del género *Xenorhabdus* y *Photorhabdus* respectivamente, que llevan en su intestino (Kaya y Gaugler, 1993; Boemare *et al.*, 1993).

El ciclo de vida de las dos familias de nematodos entomopatógenos es similar. El único estadio que vive fuera del insecto es el tercer estado juvenil, forma infectiva juvenil o larva “dauer”. Durante este periodo tiene la boca y el ano cerrados y no se

alimenta. El tercer estadio juvenil es el responsable de la localización e infección de los insectos hospedadores (Kaya y Gaugler, 1993). Las formas infectivas juveniles llevan células de la bacteria simbionte en su interior, formando lo que se conoce como complejo nematodo-bacteria. Las larvas "dauer" del género *Steinernema* llevan las células de la bacteria simbionte en una vesícula intestinal especializada (Bird y Akhurst, 1983; Forst y Clarke, 2002), mientras que en el género *Heterorhabditis* se encuentran en todo el intestino (Boemare *et al.*, 1996; Forst y Clarke, 2002; Ciche y Ensign, 2003). Después de localizar a su hospedador, las formas infectivas juveniles penetran en él a través de aberturas naturales (boca, ano y espiráculos) (figura I.10). Algunas especies pueden también penetrar a través de la cutícula (Peters y Ehlers, 1994), que en el caso de los



Figuras I.10. Ciclo de los nematodos entomopatógenos. **A:** Localización y penetración de las formas juveniles infectivas en el hospedador; **B:** Primera generación de adultos; **C:** Sucesión de generaciones; **D:** Salida de las nuevas formas infectivas.(

heterorhabdítidos es posible gracias a la posesión de un diente cuticular en la zona anterior (Bedding y Molyneux, 1982). Algunos steinernemátidos son capaces de penetrar por las zonas intertegumentales (carentes de epicutícula) del insecto entrando directamente al hemocele (Peters y Ehlers, 1994). Las formas infectivas juveniles que entran por el sistema traqueal o por el digestivo, deben atravesar sus paredes para llegar al hemocele. Si la pared es frágil, como la de las traqueolas,

ésta puede ser perforada fácilmente por presión mecánica. Las paredes del intestino, protegido en parte por la membrana peritrófica, son atravesadas por los heterorhabdítidos con la ayuda de su diente proximal. La secreción de enzimas proteolíticos también está implicada en la penetración de la membrana peritrófica por las formas infectivas, al menos en los steinernemátidos como se ha evidenciado en algunos trabajos (Abu Hatab *et al.*, 1995; Simoes, 1998; Dowds y Peters, 2002). Cuando las larvas "dauer" llegan al hemocele liberan la bacteria simbionte. *Xenorhabdus* es liberada de los nematodos steinernemátidos por defecación (Wouts, 1991; Martens *et al.,* 2003), mientras que *Photorhabdus* sale a través de la boca de los heterorhabdítidos (Ciche y Ensign, 2003). La bacteria se multiplica rápidamente, libera endo- y exotoxinas, causando la muerte del hospedador por septicemia en 24-72 h (Poinar, 1990). También los nematodos producen sustancias tóxicas contra el insecto. Así, *Steinernema* spp. inducen toxicogénesis (Boemare *et al.,* 1982, 1983) y producen un factor inmunodepresivo que actúa contra los péptidos antimicrobianos generados por los insectos (Götz *et al.*, 1981); en cambio, no se conoce nada a este respecto en *Heterorhabditis* (Forst y Clarke, 2002). La bacteria permite el crecimiento y reproducción del nematodo al suministrarle alimento y produciendo agentes antimicrobianos que inhiben el crecimiento de otros organismos, incluyendo bacterias, levaduras y hongos, y elementos que protegen a los nematodos de insectos saprófagos (Koppenhöfer, 2007). Los nematodos se alimentan de la bacteria, se desarrollan y dan lugar a una o más generaciones, dependiendo del tamaño del hospedador. Cuando se agotan los recursos aparecen las nuevas formas infectivas que, tras incorporar la bacteria simbionte en su tubo digestivo, emergen del cadáver del insecto (Adams y Nguyen, 2002) para buscar un nuevo hospedador (Kaya y Gaugler, 1993).

La infección por nematodos entomopatógenos causa un cambio de color en los cadáveres producido por la bacteria asociada. Generalmente los insectos parasitados por steinernemátidos adquieren una coloración marronosa, que va desde el ocre al casi negro, mientras que los parasitados por heterorhabdítidos toman un color rojizo, naranja, amarillo o verde. En estos últimos además se observa la emisión de bioluminiscencia producida por la bacteria simbionte, del género *Photorhabdus.* Esta bioluminiscencia es debida a un enzima típicamente bacteriano, la luciferasa, que produce una luz azul-verdosa (490 nm) (Poinar *et al.,* 1980). Sin embargo, el color de la cutícula del insecto es rojizo, debido presumiblemente al pigmento rojo producido por las bacterias que se acumula en esta, dando esta coloración. Los genes responsables de la luminiscencia de *Photorhabdus* son los genes lux, que han sido clonados, secuenciados y expresados

en *Escherichia coli* por muchos equipos de investigadores (Cochrum *et al.,* 1990; Frackman *et al,* 1990; Frackman y Nealson, 1990; Johnston *et al.*, 1990; Szittner y Meighen, 1990; Xi *et al.*, 1991; Meighen y Szittner, 1992). Al comparar estos genes con los de bacterias luminiscentes marinas se ha visto que el orden de los genes de los operones lux es similar en todas las bacterias luminiscentes y que las secuencias de los genes luxAB son similares a las de otras bacterias luminiscentes, lo que sugiere que estos genes lux tienen un origen evolutivo similar. Por otro lado, esta bioluminiscencia producida por *Photorhabdus* en los insectos parasitados por heterorhabdítidos parece impedir la actividad saprófita de otros invertebrados (Akhurst y Boemare, 1990).

La primera generación de adultos de los heterorhabdítidos está constituida por individuos hermafroditas que se autofecundan, y en las siguientes generaciones se producen machos, hembras y, a veces, hermafroditas (Dix *et al.*, 1992). En cambio, todas las generaciones que se suceden en los nematodos de la familia Steinernematidae son amfimícticas (Poinar, 1990) a excepción de una especie, *S. hermaprhoditum,* con individuos hermafroditas en la primera generación (Stock *et al.*, 2004). Las dos familias de nematodos entomopatógenos son ovíparas u ovovivíparas. Inicialmente los huevos se ponen en el medio, pero en muchos casos eclosionan dentro del útero de la hembra desarrollando formas juveniles que se alimentan de los tejidos maternos. Este proceso se conoce como endotoquia matricida (Wang y Bedding, 1996; Johnigk y Ehlers, 1999), y ocurre tanto en *Heterorhabditis* spp. como en *Steinernema* spp. La endotoquia matricida se ha observado principalmente en hembras hermafroditas y en hembras dioicas viejas (Johnigk y Ehlers, 1999) y parece ser una adaptación ecológica para asegurar la reproducción y la supervivencia de los nematodos entomopatógenos cuando el desarrollo de los nematodos se ve afectado por otros agentes biológicos o factores no biológicos, (Baliadi *et al.,* 2004). Este comportamiento también ha sido observado comúnmente en rhabdítidos de vida libre (Baliadi *et al.*, 2001) como respuesta a condiciones de stress y falta de alimento (Chen y Caswell-Chen, 2003).

I.2.2. Morfología y Clasificación

Las características generales de las dos familias de nematodos entomopatógenos, así como la clasificación en el momento de la presentación de este trabajo son las siguientes:

I.2.2.1. Familia Steinernematidae

Steiner (1923) describió por primera vez un ejemplar de steinernemátido, aislado en Alemania, y lo denominó *Aplectana kraussei*. Travassos (1927) renombró el género como *Steinernema,* proponiendo *S. kraussei* como especie tipo del género. En 1929 Steiner describió *Neoaplectana glaseri* y la designó como especie tipo del nuevo género *Neoaplectana.* Filipjev (1934) incluyó *Steinernema* y *Neoaplectana* en una nueva subfamilia Steinerneminae dentro de la familia Anguillulidae, orden Anguillulata (actualmente conocido como orden Rhabditida Chitwood, 1933). Chitwood y Chitwood, en 1937, elevaron la subfamilia Steinerneminae a la familia Steinernematidae. A pesar de que Filipjev, en 1934, sugiriera que *Steinernema* y *Neoaplectana* podían ser cogenéricos, el trabajo de Wouts *et al.* (1982) concluyó que *Neoaplectana* era sinónimo de *Steinernema*, dejando la familia con un solo género. Posteriormente Nguyen y Smart (1994) describieron un segundo género de la familia, llamado *Neosteinernema* asociado a termitas.

I.2.2.1.a. Género *Steinernema*

Hembras: Tamaño variable. Poseen una cutícula lisa o anillada con ausencia de campos laterales. Con poro excretor evidente. La región anterior es redondeada y posee 6 labios parcial o totalmente fusionados, con una papila labial en cada labio y a veces con otras estructuras papilares presentes. Poseen 4 papilas cefálicas. Anfidios presentes, de pequeño tamaño. Estoma colapsado formando un anillo que parece dos grandes puntos esclerotizados en vista lateral. Otras partes del estoma forman un canal asimétrico con un estrechamiento en la parte anterior. Esófago rhabditoide con el metacorpus ligeramente hinchado, istmo estrecho rodeado por el anillo nervioso, y un bulbo basal alargado con una reducida válvula cardiaca. Válvula esofágica-intestinal normalmente pronunciada. Sistema reproductor didélfico, anfidélfico, retroflexo con la vulva localizada en la mitad del cuerpo, a veces sobre una protuberancia, con o sin epitigma. Las hembras son ovovivíparas u ovíparas con juveniles desarrollados en estado infectivo antes de emerger del cuerpo de la hembra.

Machos: De tamaño más pequeño que las hembras. Extremo anterior normalmente con 6 papilas labiales, 4 papilas cefálicas y alargadas y generalmente con disco perioral. Esófago similar al de las hembras. Testículo único, retroflexo y espículas pares y separadas. Gubernáculo largo, a veces tanto como la espícula.

Bursa ausente. Punta de la cola redondeada, con o sin mucrón. Una papila simple y 10 a 14 pares de papilas genitales presentes junto con 7 a 10 pares de papilas precloacales.

Juveniles infectivos: Son el tercer estado juvenil. Estoma colapsado. Cuerpo alargado, a menudo manteniendo la cutícula del segundo estado juvenil. Campos laterales presentes, con 4 a 9 surcos y de 3 a 8 protuberancias lisas. Boca y ano cerrados, y esófago e intestino reducidos. Poro excretor visible, anterior al anillo nervioso. Fasmidios situados en la zona media del extremo caudal, pudiendo ser prominentes, poco o no observables. Cola conoide o filiforme.

I.2.2.1.b. Género *Neosteinernema*

Hembras: Con fasmidios prominentes situados en una protuberancia localizados en la mitad posterior de la cola. Longitud de la cola superior al ancho en el nivel del ano. Ovovivíparas, desarrollándose los huevos en formas infectivas antes de emerger de la hembra.

Machos: De tamaño más pequeño que las hembras. Parte posterior del cuerpo con una papila ventral y 13-14 pares de papilas genitales, 8 de ellas preanales. Fasmidios prominentes. Punta de la cola digitada, con la espícula en forma de pie con un abultamiento en la parte dorsal. Gubernáculo al menos tan largo como la espícula.

Juveniles infectivos: Con la parte anterior del cuerpo ligeramente engrosada. Fasmidios alargados. Cola alargada o filiforme, tan larga como el esófago, y normalmente curvada al final.

I.2.2.1.c. Especies descritas

La taxonomía y las especies de los géneros *Steinernema* y *Neosteinernema* descritas hasta el momento de esta revisión bibliográfica, basándonos en la revisión de Stock y Hunt (2005) y Nguyen y Hunt (2007), se muestran a continuación, ordenadas alfabéticamente junto con los autores, el año de la descripción y las sinonimias en el caso de que existan.

Filum: Nematoda (Lankester, 1977) Rudolphi, 1808

Clase: Chromadorea Inglis, 1983

Orden: Rhabditida (Oerly, 1880) Chitwood, 1933

Suborden: Tylenchina Thorne, 1949

Superfamilia: Strongyloidoidea Chitwood y McIntosh, 1934

Familia: Steinernematidae (Filipjev, 1934) Chitwood y Chitwood, 1937

Género *Steinernema* Travassos, 1927

= *Steineria* Travassos, 1927, nec *Steineria* Micoletzky, 1922

= *Neoaplectana* Steiner, 1929

= *Patanodontus* de Villalobos & Camino, 1997

Especie tipo: *Steinernema kraussei* (Steiner, 1923) Travassos, 1927

= *Aplectana krauss*ei Steiner, 1923

= *Steineria kraussei* (Steiner, 1923) Travassos, 1927

= *Oxysomatium kraussei* (Steiner, 1923) Skrjabin, Shikhobalova & Mozgovoi, 1951

Otras especies:

S. abbasi Elawad, Ahmad & Reid, 1997

= *S. thermophilum* Sudershan & Singh, 2000 n. syn.

S. aciari Qiu, Yan, Zhou, Nguyen & Pang, 2005

S. affine (Bovien, 1937) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Neoaplectana affinis* Bovien, 1937

S. akhursti Qiu, Hu, Zhou, Nguyen & Pang, 2005

S. anatoliense Hazir, Stock & Keskin, 2003

S. apuliae Triggiani, Mrácek & Reid, 2004

S. *arenarium* (Artyukhovsky, 1967) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Neoaplectana arenaria* Artyukhovsky, 1967

= *Neoaplectana anomali* Kozodoi, 1984

= *Steinernema anomalae* (Kozodoi, 1984) Curran, 1989

S. ashiuense Phan, Takemoto & Futai, 2006

S. asiaticum Anis, Shashina, Reid & Rowe, 2002

S. backanense Phan, Spiridonov, Subbotin & Moens, 2006

S. beddingi Qiu, Hu, Zhou, Pang & Nguyen, 2005

S. bicornutum Tallosi, Peters & Ehlers, 1995

S. carpocapsae (Weiser, 1955) Wouts, Mrácek, Gerdin & Bedding,1982

= *Neoaplectana carpoca*psae Weiser, 1955

= *Neoaplectana feltiae sensu* Stanuszek, 1974, nec Filipjev, 1934

= *Neoaplectana feltiae pieridarum* Stanuszek, 1974

= *Steinernema feltiae pieridarum* (Stanuszek, 1974) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Steinernema pieridarum* (Stanuszek, 1974) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Neoaplectana carpocapsae pieridarum* Stanuszek, 1974

= *Neoaplectana dutkyi* Turco, Thames & Hopkins, 1971

= *Steinernema dutkyi* (Turco, Thames & Hopkins, 1971) Wouts, Mrácek, Gerdin & Bedding, 1982

S. caudatum Xu, Wang & Li, 1991

S. ceratophorum Jian, Reid & Hunt, 1997

S. cholashanense Nguyen, Puza & Mrácek, 2008.

S. colombiense López-Núñez, Plichta, Góngora-Botero, Carmenza & Stock, 2008

S. costaricense Uribe, Mora & Stock, 2007

S. cubanum Mrácek, Hernandez & Boemare, 1994

S. cumgarense Phan, Spiridonov, Subbotin & Moens, 2006

S. diaprepesi Nguyen & Duncan 2002

S. eapokense Phan, Spiridonov, Subbotin & Moens, 2006

S. feltiae (Filipjev, 1934) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Neoaplectana feltiae* Filipjev, 1934

= *Neoplectana bibionis* Bovien, 1937

= *Steinernema bibionis* (Bovien, 1937) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Neoaplectana leucaniae* Hoy, 1954

= *Steinernema leucaniae* (Hoy, 1954) Wouts, Mrácek, Gerdin & Bedding, 1982

S. glaseri (Steiner, 1929) Wouts, Mrácek, Gerdin & Bedding, 1982

= *Neoaplectana glaseri* Steiner, 1929

S. guangdongense Qiu, Fang, Zhou, Pang & Nguyen, 2004

S. hebeiense Chen , Li, Yan, Spiridonov & Moens, 2006

S. hermaphroditum Stock, Griffin & Chaerani, 2004

S. ichnusae Tarasco, Mráce, Nguyen & Triggiani, 2008

S. intermedium (Poinar, 1985) Mamiya, 1988

= *Neoaplectana intermedia* Poinar, 1985

S. jollieti Spiridonov Krasomil-Osterfeld, Moens, 2004

S. karii Waturu, Hunt & Reid, 1997

S. khoisanae Nguyen, Malan & Gozel, 2006

S, kraussei Torr, Heritage & Wilson, 2007

S. kushidai Mamiya, 1988

S. leizhouense Nguyen, Qiu, Zhou & Pang, 2006

S. litorale Yoshida, 2005

S. loci Phan, Nguyen & Moens, 2001

S. longicaudum Shen & Wang, 1992

= *S. serratum* Liu, 1992 (= *nomen nudum*)

S. monticolum Stock, Choo & Kaya, 1997

S. neocurtillae Nguyen & Smart, 1992

S. oregonense Liu & Berry, 1996

S. pakistanense Shahina, Anis, Reid, Rowe & Maqbool. 2001

S. puertoricense Romin & Figueroa, 1994

S. puntauvense Uribe, Mora & Stock, 2007

S. rarum (de Doucet, 1986) Mamiya, 1988

= *Neoaplectana rara* de Doucet, 1986

S. riobrave Cabanillas, Poinar & Raulston, 1994

S. ritteri de Doucet & Doucet, 1990

S. robustispiculum Phan, Subbotin, Waeyenberge & Moens, 2005

S. sangi Phan, Nguyen & Moens, 2001

S. sasonense Phan, Spiridonov, Subbotin & Moens, 2006

S. scapterisci Nguyen & Smart, 1990

= *Neoaplectana carpocapsae* 'Uruguay strain' of Nguyen & Smart, 1988

S. scarabei Stock & Koppenhöfer, 2003

S. siamkayai Stock, Somsook & Reid, 1998

S. sichuanense Mrácek, Nguyen, Taillier, Bomoemare & Chen, 2006

S. silvaticum Sturhan, Spiridonov & Mrácek, 2005

S. tami Luc, Nguyen, Reid & Spiridonov, 2000

S. texanum Nguyen, Stuart, Andalo, Gozel & Rogers, 2007

S. thanhi Phan, Nguyen & Moens, 2001

S. websteri Cutler & Stock, 2003

S. weiseri Mrácek, Sturhan & Reid, 2003

S. yirgalemense Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004

Species inquirendae:

Neoaplectana agriotos Veremchuk, 1969 (? = *S. carpocapsae*)

Neoaplectana belorussica Veremchuk, 1969 (? = S. carpocapsae)

*Neoaplectana bothynoder*i KirJanova & Putschkova, 1955 (? = *S. feltiae)*
Neoaplectana georgica Kakuliya & Veremchuk, 1965 (? = *S. feltiae*)
Neoaplectana janickii Weiser & Ki5hler, 1955
Neoaplectana kirjanovae Veremchuk, 1969 (? = *S. feltiae*)
Neoaplectana melolonthae Weiser, 1958
Neoaplectana menozzii Travassos, 1932 (? = *S. affine/feltiae)*
Neoaplectana semiothisae Veremchuk & Litvinchuk, 1971 (? = *S. carpocapsae)*
Neoaplectana tabanivora Rubstov & Polevik, 1979

Nomina nuda:
Neoaplectana brevilarvalis pieridarum Sandner & Stanuszek, 1972
Neoaplectana chresima Steiner in Glaser, McCoy & Girth, 1942 (? = *S.carpocapsae*)
Neoaplectana dutkii Welch, 1963 (syn. of N. dutkyi Turco *et al.* 1971 = *S. carpocapsae*)
Neoaplectana elateridicola Veremchuk, 1970 (? = S*. carpocapsa*e)
Neoaplectana titovi Veremchuk, 1966 (syn. of *N. elateridicola*)
Steinernema serratum Liu, 1992

Género *Neosteinernema* Nguyen & Smart, 1994

Especie tipo: *Neosteinernema longicurvicauda* Nguyen & Smart, 1994

I.2.2.2. Familia Heterorhabditidae

Esta familia fue descrita por Poinar en 1976, quien describió la especie tipo *Herorhabditis bacteriophora*. La familia consta de un único género.

I.2.2.2.a. Género *Heterorhabditis*

Hembras hermafroditas: Parte anterior del cuerpo truncada o ligeramente redondeada. Presentan 6 labios cónicos bien desarrollados y separados, cada uno con una papila terminal. A veces cada labio posee una o dos pequeñas estructuras en la base. Anfidios con abertura pequeña. Queilorhabdiones presentes, formando un anillo, que en vista lateral se muestra como dos grandes puntos esclerotizados

en la región anterior del estoma. Región posterior del estoma colapsada y cubierta por el esófago. Esófago sin metacorpus, con istmo delgado, en cuya parte media se encuentra el anillo nervioso. Poro excretor generalmente posterior a la base del esófago. Vulva en la parte media, con forma de hendidura, rodeada de anillos elípticos. Ovotestículos anfidélficos, retroflexos. Son ovíparas, volviéndose ovovivíparas al final de su vida. Cola acabada en punta, más larga que la anchura a nivel anal, presentando generalmente una protuberancia postanal.

Hembras anfimícticas: Con morfología similar, aunque generalmente de menor tamaño, a las hembras hermafroditas. Papilas labiales prominentes. Sistema reproductor anfidélfico, con ovarios opuestos retroflexos, con la vulva no funcional para la deposición de huevos pero si para el apareamiento. Los huevos son incubados en el interior de la hembra y las formas juveniles emergen por endotoquia matricida.

Machos: Únicamente se producen durante la generación anfimíctica. Presentan un único testículo retroflexo. Espículas pares, separadas y ligeramente curvadas ventralmente y con la cabeza corta. Gubernáculo largo y delgado, generalmente representa la mitad de la longitud de la espícula. Bursa abierta, con nueve pares de costillas.

Juveniles infectivos: Este tercer estadio infectivo juvenil generalmente se encuentra dentro de la cutícula del segundo estadio. La cutícula del segundo estadio es estriada, mientras que la del tercer estadio presenta un campo lateral muy aparente formado por dos bandas longitudinales. Región anterior con un prominente diente dorsal. Boca y ano cerrados. Esófago e intestino reducidos. Poro excretor situado en posición posterior al anillo nervioso. Las bacterias simbióticas se encuentran en el interior del intestino. Cola acabada en punta.

I.2.2.2.b. Especies descritas

La taxonomía y las especies del género *Heterorhabditis* descritas hasta el momento de esta revisión bibliográfica se muestran a continuación, ordenadas alfabéticamente junto con los autores, el año de la descripción y las sinonimias en el caso de que existan.

Filum: Nematoda Chitwood, 1950
Clase: Chromadorea Inglis, 1983

Orden: Rhabditida (Oerly, 1880) Chitwood, 1933

Suborden: Rhabditina Chitwood, 1933

Superfamilia: Strongyloidea Baird, 1853

Familia: Heterorhabditidae Poinar, 1976

Género *Heterorhabditis* Poinar, 1976

= *Chromonema* Khan, Brooks & Hirschmann, 1976

Especie tipo: *Heterorhabditis bacteriophora* Poinar, 1976

= *Chromonema heliothidis* Khan, Brooks & Hirschmann, 1976

= *Heterorhabditis heliothidis (*Khan, Brooks & Hirschmann, 1976) Poinar, Thomas & Hess, 1977

= *Heterorhabditis argentinensis* Stock, 1993

Otras especies:

H. amazonensis Andaló, Nguyen & Moino, 2007

H. bacteriophora Poinar, 1976

H. baujardi Phan, Subbotin, Nguyen & Moens, 2003

H. downesi Stock, Griffin & Burnell, 2002

H. georgiana Nguyen, Shapiro-Ilan, David & Mbata, 2008

H. floridensis Nguyen, Gozel, Koppenhöfer & Adams , 2006

H. indica Poinar, Karunaka & David, 1992

= *Heterorhabditis hawaiiensis* Gardner, Stock & Kaya, 1994

H. marelatus Liu and Berry, 1996

= *Heterorhabditis hepialius* Stock, Strong & Gardner, 1996

H. megidis Poinar, Jackson & Klein 1987

H. mexicana Nguyen, Shapiro-Ilan, Stuart, James, McCoy & Adams, 2004

H. safricana Malan, Nguyen, de Waal & Tiedt, 2008

H. taysearae Shamseldean, El-Sooud, Abd-Elgawad & Saleh, 1996

H. zealandica Poinar, 1990

= *Heterorhabditis heliothidis apud* Wouts, 1979

Species inquirendae:

Heterorhabditis brevicaudis Liu, 1994

Heterorhabditis egyptii Abd-Elgawaad & Ameen, 2005

Heterorhabditis hambletoni (Pereira, 1937) Poinar, 1976

= *Rhabditis hambletoni* Pereira, 1937

Heterorhabditis hoptha (Turco, 1970), Poinar, 1979
= *Neoaplectana hoptha* Turco, 1970
Heterorhabditis poinari Kakulia & Mikaia 1997

Nomina nuda:
Heterorhabditis downesi Hass, Downes & Griffin, 2001 *nec Heterorhabditis downesi* Stock, Griffin & Burnell, 2002

I.2.3. Utilización de los nematodos entomopatógenos en el control biológico

Los nematodos entomopatógenos controlan de forma efectiva una gran variedad de plagas de insectos que causan importantes daños económicos (Shapiro-Ilan, 2004; Grewal *et al.*, 2005). Poseen además unas características importantes para ser utilizados como agentes de control biológico: son seguros para el medio ambiente, fauna y flora (Poinar *et al.*, 1982; Akhurst y Smith, 2002; Ehlers, 2005), pueden producirse en grandes cantidades en medios artificiales (Friedman, 1990; Ehlers y Shapiro-Ilan, 2005), y son fáciles de aplicar mediante simples equipos de pulverización, de irrigación o inyección (Georgis, 1990). Hay una gran evidencia de la eficacia de los nematodos entomopatógenos en el control de insectos. La primera utilización de nematodos entomopatógenos se realizó a mediados de los años 30, utilizando *S. glaseri* para el control del coleóptero escarabeido *Popillia japonica*, en Nueva Jersey y Maryland (EE.UU.) (Glaser, 1932; Glaser y Farrell, 1935). Pero no fue hasta los años 70, cuando algunos pesticidas fueron prohibidos por la EPA (*Environmental Protection Agency*, de EE.UU.), que aumentó el interés por el estudio de los nematodos entomopatógenos como agentes de control biológico. Como el medio natural de los nematodos entomopatógenos es el suelo, hay un gran número de estudios sobre la utilización de los nematodos entomopatógenos contra plagas de insectos en las que al menos una fase de su ciclo ocurre en él (Grewal et al., 2005). Pero en la actualidad los nematodos entomopatógenos también se utilizan con éxito contra insectos que se desarrollan en otros hábitats, como las hojas de las plantas (Tomalak *et al.*, 2005), hábitats crípticos como el interior de raíces, ramas o troncos de plantas (Kaya, 1985; Kaya y Brown, 1986; Cossentine *et al.*, 1990), e incluso contra plagas del entorno humano y veterinario (Glazer *et al.*, 2005).

A pesar del gran número de especies de nematodos entomopatógenos que se conocen, la posibilidad de su comercialización se ha limitado a unas pocas especies (Grewal y Georgis, 1999; Shapiro-Ilan *et al.*, 2002). Actualmente, en Europa y Estados Unidos, se comercializan cinco especies de steinernemátidos (*S. feltiae, S. carpocapsae, S. riobrave, S. kraussei* y *S. scapterisci*) y dos de *heterorhabdítidos* (*H. bacteriophora* y *H. megidis*), siendo *S. feltiae, S. carpocapsae* y *H. bacteriophora* los más utilizados comercialmente.

La producción de nematodos se puede realizar *in vivo*, sobre larvas de insectos (Woodring y Kaya, 1988) o *in vitro*, en medios sólidos (Bedding, 1984) o líquidos. Actualmente, la producción de los nematodos a nivel comercial se realiza *in vitro*, desarrollando cultivos líquidos en grandes biorreactores que permiten producir volúmenes de hasta 80.000 litros (Ehlers, 2005).

Los nematodos entomopatógenos difieren en eficacia dependiendo, entre otros factores, de la especie o aislado y de la susceptibilidad del insecto hospedador (Hay y Richardson, 1995; Glazer *et al.*, 1999). Factores abióticos (como textura y humedad del suelo, temperatura, aireación, exudados de las raíces de las plantas, etc.) y bióticos (como densidad de hospedadores, tamaño del hospedador, competencia inter- e intra-específica, enemigos naturales, etc.) también afectan la eficacia de los nematodos entomopatógenos (Zervos *et al.*, 1991, Koppenhöfer *et al.*, 1995; Kaya y Koppenhöfer, 1996; Ebssa *et al.*, 2004). La capacidad de supervivencia en el medio externo es un aspecto íntimamente relacionado con la eficacia. Como ocurre en muchos nematodos parásitos, únicamente el estadio que vive fuera del insecto, la forma infectiva juvenil, tiene la capacidad de sobrevivir fuera de éste. Como ya se ha citado anteriormente, la larva "dauer" no se alimenta y por ello, depende de las reservas energéticas iniciales que disponga en su interior para encontrar un nuevo insecto que parasitar, principalmente lípidos, ácidos grasos y glicógeno (Glazer, 2002). La supervivencia de las formas infectivas está también determinada por la capacidad de superar condiciones de stress ambiental, que se exponen a continuación:

- La tolerancia a **bajas temperaturas** se realiza mediante dos estrategias: en el primer caso, los nematodos sobreviven tolerando la formación de hielo extracelular; en el segundo caso evitan la congelación, es decir, mantienen los fluidos corporales en fase líquida a temperaturas inferiores al punto de congelación gracias a la producción de crioprotectores naturales, como la trehalosa (Wharton y Block,

1993). Además, las formas infectivas son capaces de resistir temperaturas inferiores a -80ºC en procesos de criopreservación, pudiendo ser conservadas indefinidamente en nitrógeno líquido (Popiel y Vasquez, 1989; Curran *et al.*, 1992; SeungHwa *et al.*, 2000; Bai *et al*, 2004).

- La exposición a **altas temperaturas,** superiores a 32ºC afecta negativamente a la supervivencia de los nematodos. La producción de proteínas de choque térmico ("heat-shock proteins", HPS) tiene un papel importante en la supervivencia en estas condiciones (Glazer, 2002). Estas proteínas y los genes que las producen están muy conservados en los organismos (Schlesinger, 1990). Así, algunos genes involucrados en la producción de proteínas HPS han sido identificados en el nematodo *Caenorhabditis elegans* (Rhabditida: Rhabditidae) (Jones *et al.*, 1986). La producción de *hsp*70 ha sido también detectada en *H. bacteriophora* HP88 (Selvan *et al.*, 1996) siendo esta cepa, viable, activa y con capacidad de reproducción a temperaturas superiores a 32ºC (Selvan *et al.*, 1996). Además, Hashmi *et al.* (1997) encontraron que existían homologías entre los genes que codifican *hsp*70 en nematodos entomopatógenos y en *C. elegans.* Para mejorar la tolerancia de los nematodos a las altas temperaturas, desde hace algunos años, se utilizan tanto métodos de genética clásica como es la selección por entrecruzamiento, como de ingeniería genética para incluir o sobreexpresar las proteínas HPS y obtener nematodos entomopatógenos resistentes al calor (Hashmi *et al.*, 1995, 1998; Shapiro-Ilan *et al.*, 1997). Pero la principal vía de obtención de cepas de nematodos entomopatógenos adaptados al calor es la selección de cepas localizadas en regiones áridas (Glazer *et al.*, 1991, 1996; Amarasinghe *et al.*, 1994; Shamseldean y Abd-Elgawad, 1994; Iraki *et al.*, 2000).

- Los nematodos para poder moverse necesitan una película de agua a su alrededor (Norton, 1978), que puede verse alterada por la **desecación**, afectando en consecuencia a su supervivencia. Para evitar la desecación las formas infectivas de los nematodos entomopatógenos poseen una doble cutícula (Patel y Wright, 1998), o desarrollan estrategias para conseguir sobrevivir a estas condiciones, como son la agregación de los individuos en el suelo (Simons y Poinar, 1973), refugiarse en el interior de los insectos parasitados (Brown y Gaugler, 1995, 1997; Koppenhöfer *et al.,* 1997) o migrar en la columna vertical de suelo buscando condiciones más adecuadas (Glazer *et al.*, 1996; García-del-Pino y Palomo, 1996). Bajo condiciones de desecación lenta, algunas especies de nematodos entomopatógenos puede sobrevivir entrando en anhidrobiosis, un estado de quiescencia, fisiológicamente reversible, que resulta de la ausencia de agua (Glazer y Salame, 2000). Durante la

anhidrobiosis se acumulan polioles y otros azúcares, como glicerol y trehalosa, que protegen las membranas y las proteínas intercelulares durante la deshidratación (Glazer, 2002).

- Los nematodos entomopatógenos se encuentran en la película de agua superficial de los poros del suelo, con lo que la concentración iónica influye en el equilibrio hídrico y osmótico de las células de los nematodos (Wharton, 1986; Piggot *et al.*, 2000). Aunque estudios demuestran que los nematodos entomopatógenos son capaces de resistir condiciones de **stress osmótico** (Thurston *et al.*, 1994; Finnegan *et al.*, 1999), no se conocen cuales son exactamente los mecanismos de osmoregulación que utilizan.

- La **disponibilidad de oxígeno** puede ser un factor limitante para la supervivencia de las formas infectivas, sobre todo en suelos arcillosos, suelos saturados de agua o con elevados niveles de materia orgánica (Glazer, 2002).En algunas especies de nematodos de vida libre la reducción de la concentración de oxígeno induce un estado de dormición llamado anoxibiosis (Wharton, 1986), pero no se ha observado en steinernemátidos ni en heterorhabdítidos. Los estudios realizados por Qiu y Bedding (1999) sobre *S. carpocapsae* mostraron que al someter a las formas juveniles a condiciones anaeróbicas estas podían sobrevivir hasta una semana sin perder su actividad. Además observaron que el tiempo de supervivencia en condiciones anaeróbicas está fuertemente relacionado con la temperatura. A mayor temperatura aumenta la movilidad y con ella la tasa metabólica, que implica una mayor demanda de oxígeno, y por tanto, una rápida mortalidad.

- La **radiación ultravioleta** es otro factor a considerar ya que afecta a los nematodos cuando se encuentran en la superficie del suelo, reduciendo su persistencia (Gaugler y Boush, 1978; Gaugler *et al.,* 1992). También se ha demostrado que produce una rápida inactivación de las formas infectivas de los steinernemátidos y heterorhabdítidos. Gaugler *et al.* (1992) observaron que las formas infectivas de *S. carpocapsae* perdían significativamente la capacidad patógena después de 5 minutos de exposición a la radiación ultravioleta y después de 7 minutos eran incapaces de causar infecciones letales, mientras que en *H. bacteriophora* ocurría en 3 y 4 minutos respectivamente. Además los insectos parasitados por formas infectivas expuestas durante un periodo de tiempo más largo (8-10 minutos), adquirían un color negro y se descomponían. Debido a que la bacteria es la responsable del color del cadáver y de evitar la putrefacción, parece

ser que la irradiación ultravioleta reprime a la bacteria, permitiendo así el establecimiento de invasores secundarios en el cadáver (Gaugler *et al*., 1992).

- **Pesticidas:** tanto los steinernemátidos como los heterorhabdítidos son capaces de sobrevivir a la exposición de diferentes productos fitosanitarios (Hara y Kaya, 1982; Rovesti et al, 1989; Rovesti y Deseö, 1990; Palomo y García-del-Pino 2000; García-del-Pino y Jové, 2005). Sin embargo, las formas infectivas son muy susceptibles a nematicidas que se encuentran en los cultivos agrícolas (Rovesti y Deseö, 1990, 1991).

Los nematodos entomopatógenos tienen enemigos naturales, siendo susceptibles a la infección por microorganismos y a la depredación. Entre sus enemigos se encuentran protozoos, hongos nematófagos, invertebrados depredadores como turbelarios, tardígrados, oligoquetos, ácaros o insectos, e invertebrados carroñeros como hormigas o isópodos (Kaya, 2002).

El comportamiento de búsqueda del hospedador es también un aspecto relevante en la eficacia de los nematodos entomopatógenos. Así pues, las formas infectivas pueden presentar dos tipos de estrategias que influyen en la respuesta a los diferentes estímulos físicos o químicos que reciban (Lewis *et al.*, 2006). Los nematodos denominados navegantes ("cruisers"), buscan activamente a su huésped, mientras que los que muestran un comportamiento de emboscada ("ambushers") esperan a que el insecto se acerque. Entre estas dos estrategias existen nematodos con un comportamiento intermedio (Campbell *et al.*, 2003). Parece existir una relación entre el tamaño de las formas infectivas y su estrategia de búsqueda. Así, especies de gran tamaño, como *S. glaseri* o *S. kraussei* se catalogan como "cruisers", mientras que las más pequeñas, como *S. carpocapsae* son "ambushers". Los nematodos de tamaño intermedio, como *S. feltiae* presentan una actividad intermedia (Campbell *et al.*, 2003).

Una vez localizado el insecto existen barreras físicas que superar en las vías de entrada. La anchura de la boca y el ano puede ser un factor limitante para el paso de las formas infectivas (Eidt y Thurston, 1995), mientras que las mandíbulas pueden dañar a los nematodos cuando estos intentan atravesarlas (Gaugler y Molloy, 1981). La entrada por el ano evita estos problemas y suele ser la ruta principal en larvas de muchos dípteros (Renn, 1998). Sin embargo, las defecaciones frecuentes de muchas larvas de insectos pueden dificultar la entrada de los

nematodos por esta vía. La entrada por los espiráculos puede verse dificultada en muchas larvas de insectos que habitan en el suelo y presentan estructuras que los protegen e impiden el paso de los nematodos.

Finalmente, si el nematodo consigue entrar en el insecto, hay un último factor que puede determinar su eficacia. Es la respuesta del sistema inmune del hospedador, que desencadena mecanismos de acción celular y humoral y mecanismos de acción hemolítica, como fagocitosis, encapsulación y la producción de péptidos antimicrobianos (Dowds y Peters, 2002). La encapsulación ocurre cuando no existe un reconocimiento del nematodo por el sistema inmunitario del insecto, siendo atrapado en cápsulas celulares endurecidas por melanina. La encapsulación de nematodos entomopatógenos se ha observado en algunos ortópteros (Wang *et al.,* 1994), coleópteros (Pye y Burman, 1978; Jackson y Brooks, 1989; Thruston *et al.,* 1994; Wang *et al.*, 1994; Steiner, 1996), dictiópteros (Zervos y Webster, 1989), dípteros (Welch y Bronskill, 1962; Poinar y Leutennegger, 1971; Peters y Ehlers, 1997) y lepidópteros (Simoes, 1998), aunque dentro de estos órdenes de insectos hay muchas especies que no realizan la encapsulación de nematodos. Además, esta respuesta del insecto depende de la combinación nematodo-insecto. Por ejemplo, en *Acheta domesticus,* los nematodos *S. carpocapsae* y *H. bacteriophora* son encapsulados, mientras que *S. scapterisci* no lo es (Wang *et al.*, 1994). Esta última especie se encuentra naturalmente asociada con el ortóptero *Scapteriscus vicinus.* Así, diversos estudios sugieren que los nematodos no sufren la encapsulación en hospedadores similares a los que están asociados de forma natural (Dowds y Peters, 2002). La encapsulación es evitada por los nematodos de distintas formas: segregando lípidos en la superficie de las formas infectivas en el momento de la penetración, que hace que los nematodos no sean reconocidos como ajenos al insecto, y no sean encapsulados (Dunphy y Webster, 1987); la actividad de unas proteínas de membrana de los nematodos pueden interferir en la capacidad de melanización y en la habilidad fagocitaria de los hemocitos (Dowds y Peters, 2002); finalmente, la entrada de muchos nematodos en el hospedador ayuda a que algunos escapen de la encapsulación ya que el potencial de encapsulación del insecto es limitada.

Most professional gardeners would be rather surprised to hear that all these insect-pests are an essential part of the world of life; that their destruction would be disastrous; and that without them some of the most beautiful and enjoyable of the living things around us would be either seriously diminished in numbers or totally destroyed.

Alfred Russel Wallace. The World of Life.

II. Objetivos

Este trabajo tiene como principal objetivo el estudio de la viabilidad de los nematodos entomopatógenos para controlar los diferentes estadios de *Capnodis tenebrionis.* Para ello se proponen los siguientes objetivos concretos:

- Aislar poblaciones de nematodos entomopatógenos de campos en los que se desarrolla *C. tenebrionis,* mediante muestreos en suelos de cultivos de frutales de hueso situados en áreas susceptibles de ser atacadas por el gusano cabezudo en las regiones de Cataluña y Murcia.

- Caracterizar morfológica y ecológicamente las poblaciones encontradas con el fin de identificar las cepas y conocer las adaptaciones de cada una a diferentes condiciones ambientales (temperatura, desecación, hipoxia, migración vertical).

- Estudiar la virulencia de los nematodos entomopatógenos contra los diferentes estadios de *C. tenebrionis.* En concreto:

    - Evaluar la susceptibilidad de las larvas neonatas durante las primeras 24 h de vida, cuando todavía están fuera de la planta hospedadora.

    - Evaluar la capacidad de los nematodos entomopatógenos de localizar y parasitar las larvas de primer estadio poco después de que hayan penetrado en las raíces de la planta.

    - Evaluar la susceptibilidad a los nematodos entomopatógenos de los estadios que se desarrollan dentro de la planta, las larvas de último estadio y las pupas.

    - Evaluar la susceptibilidad de las formas que se alimentan en la zona aérea del árbol e hibernan en el suelo, los adultos.

- Seleccionar la cepa más adecuada contra *C. tenebrionis* y evaluar su capacidad de control de la plaga en un ensayo de campo en una producción comercial de cerezos.

Para conseguir estos objetivos se han realizado diferentes trabajos que se presentan en cinco publicaciones, dentro de los siguientes capítulos:

CAPÍTULO I:

Efficacy of entomopathogenic nematodes against neonate larvae of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) in laboratory trials. Publicado en *BioControl (2005) 50: 307–316.*

CAPÍTULO II:

Virulence of entomopathogenic nematodes to different stages of the flatheaded rootborer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae). Publicado en *Nematology. Aceptado el 07/08/2008.*

CAPÍTULO III:

Effectiveness of different species of entomopathogenic nematodes for biocontrol of the Mediterranean flatheaded rootborer, *Capnodis tenebrionis* (Linné) (Coleoptera: Buprestidae) in potted peach tree. Publicado en *Journal of Invertebrate Pathology (2008) 97: 128–133.*

CAPÍTULO IV:

Field efficacy of the entomopathogenic nematode *Steinernema feltiae* against the Mediterranean flat-headed rootborer *Capnodis tenebrionis*. Publicado en *Journal of Applied Entomology (2008) 132: 632–63.*

ANEXO:

Morphological and ecological characterization of entomopathogenic nematode strains isolated in stone-fruit orchard soils of Mediterranean areas. Enviado a *Journal of Invertebrate Pathology*.

Capítulo I

Efficacy of entomopathogenic nematodes against neonate larvae of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) in laboratory trials

BioControl (2005) 50: 307-316

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Efficacy of entomopathogenic nematodes against neonate larvae of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) in laboratory trials

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Received 16 February 2004; accepted in revised form 21 June 2004

Abstract. The efficacy of five entomopathogenic nematode strains of the families Steinernematidae and Heterorhabditidae was tested against the neonate larvae of *Capnodis tenebrionis*. The nematode strains screened included two of *Steinernema carpocapsae* (Exhibit and M137), and one each of *S. feltiae* (S6), *S. arenarium* (S2), and *Heterorhanditis bacteriophora* (P4). Exposure of neonate larvae of *Capnodis* to 10 and 150 infective juveniles (IJs) per larva (equivalent to 3 and 48 IJs/cm^2 respectively) in test tubes with sterile sand, resulted in mortality between 60–91% and 96–100%, respectively. At a concentration of 150 IJs/larva, all of the nematode strains were highly virulent. Both *S. carpocapsae* strains (Exhibit and M137) caused infection and mortality to larvae more quickly than the other strains. However, at a lower concentration assay (10 IJs/larva), *S. arenarium* was the most virulent strain. The penetration rate as an indicator of entomopathogenic nematode infection was also evaluated. The highest value was recorded for *S. arenarium* (36%), followed by *H. bacteriophora* (30.6%), *S. feltiae* (23.1%), and *S. carpocapsae* (20.7%).

Key words: biological control, Buprestidae, *Capnodis tenebrionis*, *Heterorhabditis bacteriophora*, infectivity, neonate larvae, penetration rate, *Steinernema arenarium*, *Steinernema carpocapsae*, *Steinernema feltiae*

Introduction

Capnodis tenebrionis L. (Coleoptera: Buprestidae) is one of the most important pests of cultivated stone fruits (cherry, apricot, nectarine, peach, almond, and plum) and exceptionally of seed fruits such as apple and pear. Damage caused by this insect has been reported mainly from Southern European and Mediterranean areas such as Spain (Garrido, 1984; Sanchez-Capuchino et al., 1987; Domínguez, 1989), Italy (Viggiani, 1991; Laccone, 1998), Morocco (Chrestian, 1955; Hmimina et al., 1988; Mahhou and Dennis, 1992), Israel

(Ben-Yehuda and Mendel, 1997), Turkey (Tezcan, 1995), Palestine (Rivnay, 1944) and Algeria (Martin, 1951).

Adult beetles feed on the cortex of twigs and young branches, causing defoliation in trees. Such damage is not serious in fruit-bearing orchards, but can become more serious in nursery and greenhouse trees. Females oviposit on the ground, under stones or in cracks of dry soil. The neonate larvae find their way toward tree trunks, penetrating quickly by burrowing into the roots and feeding on the root cortex. This is the real damage caused by the larvae; a few can lead to the death of an adult tree within 1 or 2 years.

Control recommendations for *C. tenebrionis* currently consist of insecticide applications directed against emerging adults on stems and foliage, to kill adults while feeding on twigs and small branches (Garrido et al., 1990; Ben-Yehuda and Mendel, 1997; Colasurdo et al., 1997; Ben-Yehuda et al., 2000). Control of larvae with chemical insecticides has also been used, mainly by applying the insecticides on the soil surface to target the neonate larvae when emerging from the egg and moving to the roots (Sekkat et al., 1997; Ben-Yehuda et al., 2000; Sanna-Passino and Delrio, 2001).

Due to the difficulty in controlling this insect with chemical insecticides, and the costly damages associated with it, the development of biological control alternatives is needed. The use of entomopathogenic nematodes is one of the potential alternatives.

Entomopathogenic nematodes (EPNs) (Steinernematidae and Heterorhabditidae) are lethal obligate parasites of a large number of insect species (Smart, 1995), and have been used efficiently against many soil-inhabiting and burrowing insects (Klein, 1990). However, only limited research has been conducted on the susceptibility of *C. tenebrionis* to entomopathogenic nematodes (García del Pino, 1994; Lobatón et al., 1998) and only preliminary data on the relative susceptibility of neonate larvae are available (García del Pino, 1994; Marannino et al., 2004). Thus, the objective of this study was to screen a limited number of strains of different EPN species at various concentrations to control the neonate larvae of *C. tenebrionis*.

Materials and methods

Nematode culture

Five different nematode populations were used: a Spanish strain (M137), a commercial strain (Exhibit) of *Steinernema carpocapsae*, and Spanish strains of *S. feltiae* (S6), *S. arenarium* (S2) and *Heterorhabditis bacteriophora* (P4) (Table 1). The nematodes were reared in last instar larvae of the wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleridae) following the rearing

Table 1. Entomopathogenic nematode strains tested

Species	Name of the strains	Origin of the strains
Steinernema carpocapsae	M137	Catalonia (Spain)
S. carpocapsae	Exhibit	USA
S. feltiae	S6	Catalonia (Spain)
S. arenarium	S2	Salamanca (Spain)
Heterorhabditis bacteriophora	P4	Catalonia (Spain)

protocol of Woodring and Kaya (1998). Infective juveniles (IJs) were stored in water or damp sponges at 5–8 °C until used in the experiments. The nematodes were allowed to acclimate at room temperature for at least 12 h and checked for viability before use in the bioassays.

Neonate larvae of Capnodis

The neonate larvae of *C. tenebrionis* were obtained from eggs cultured following the protocol developed by Garrido et al. (1987). The adults were collected from the field and raised in containers of 50 cm × 50 cm × 50 cm. Six adult couples were put in each container and fed with fresh tree branches. Oviposition occurred in dishes filled with sand, previously sifted to 0.8 mm, placed in each container. Eggs were collected by sifting the sand; and were placed in an incubation chamber at 26 ± 2 °C to hatch. Emerging neonate larvae were first individually examined under a binocular microscope and immediately used for susceptibility tests to entomopathogenic nematodes.

Assay arena

The experiments were done in test tubes (2.5 cm × 2.0 cm) with 2.5 cm^3 of sterile sand. In each test tube one neonate larva was placed at the bottom of the sand and 0.5 ml of aqueous nematode suspension was added to the surface. The experimental tubes were sealed with parafilm to avoid dehydration, and maintained in a climate chamber at 23 ± 2 °C in the dark.

Two assays were carried out using two nematode application rates: 10 and 150 IJs per larva (equivalent to 3 and 48 IJs/cm^2 respectively). The five nematode strains were tested in the assay of 150 IJs per larva, but only four strains: *S. carpocapsae* (M137), *S. feltiae* (S6), *S. arenarium* (S2) and *H. bacteriophora* (P4) were used in the assay of 10 IJs per larva. The nematode dosage was calculated per individual nematode in the first assay (10 IJs/larva) and by volumetric estimation in the second one (150 IJs/larva). Each dose rate of a given strain was tested with 24 replicates. Controls were identical to the treatments except that no IJs were added. To evaluate the efficacy of each

nematode strain, in the assay of 150 IJs per larva, insect mortality was checked daily looking for the larvae on the surface of the sand and inside the sand, and dead larvae were dissected to verify the presence of nematodes. In the assay of 10 IJs per larva the insects were extracted from the sand five days following application of the nematodes in order not to destroy the soil structure each day and not to interfere with the process of the nematodes searching for larvae. The presence of nematodes inside the larvae were checked to determine their penetration rate (the percentage of initial infective juvenile inoculum that invades an insect host) as an indicator of nematode infection.

Statistical analysis

Differences between the percentages of host mortality for each strain of nematode used in the two dose rates were analysed by the χ^2 test (CROSSTABS, SPSS-PC). For each nematode strain, differences between means of the variables analysed were contrasted by an analysis of the variance (ONEWAY, SPSS-PC) and means were separated using Duncan's multiple means comparison procedure. A significance level of $p < 0.05$ was used in all analyses.

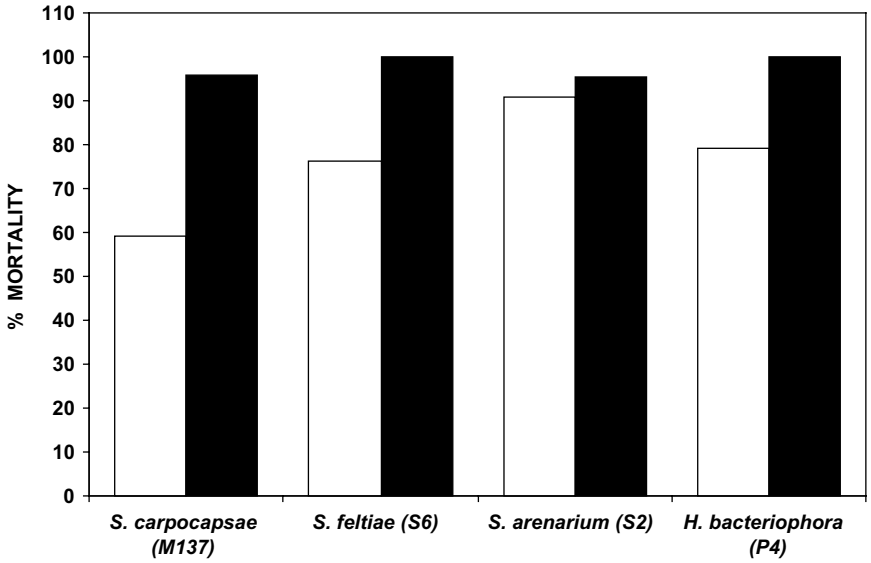

Figure 1. Mortality of neonate larvae of *Capnodis* five days following application of the nematode. (□) 10 IJs/larva (3 IJs/cm^2) and (■) 150 IJs/larva (48 IJs/cm^2).

Results

Neonate larvae of *C. tenebrionis* were susceptible to entomopathogenic nematodes. The infective juveniles of all assessed strains were able to penetrate and reproduce in the neonate larvae. The presence of infective juveniles inside the cephalic area of larvae observed within the first hours of application, indicates that the infective nematodes penetrate mainly through the mouth of neonate larvae.

At a concentration of 150 IJs/larva, no significant differences in larval mortality were observed between the EPN strains tested ($p > 0.05$). All nematode strains provided a high rate of parasitism, up to 95%, in five days (Figure 1). The mortality of untreated neonate larvae was 4.7% on the fifth day. However, the infection rate was different among the nematode species tested. The two *S. carpocapsae* strains (Exhibit and M137) caused infection and mortality to *Capnodis* larvae more quickly than the other nematodes (Figure 2). Two days after treatment, these nematodes caused 86% and 75% of insect mortality, respectively, whereas infection rates were 32% for *S. arenarium*, 12.5% for *H. bacteriophora*, and 4% for *S. feltiae*. Nevertheless, on the fourth day the infection rates were similar for all nematode strains.

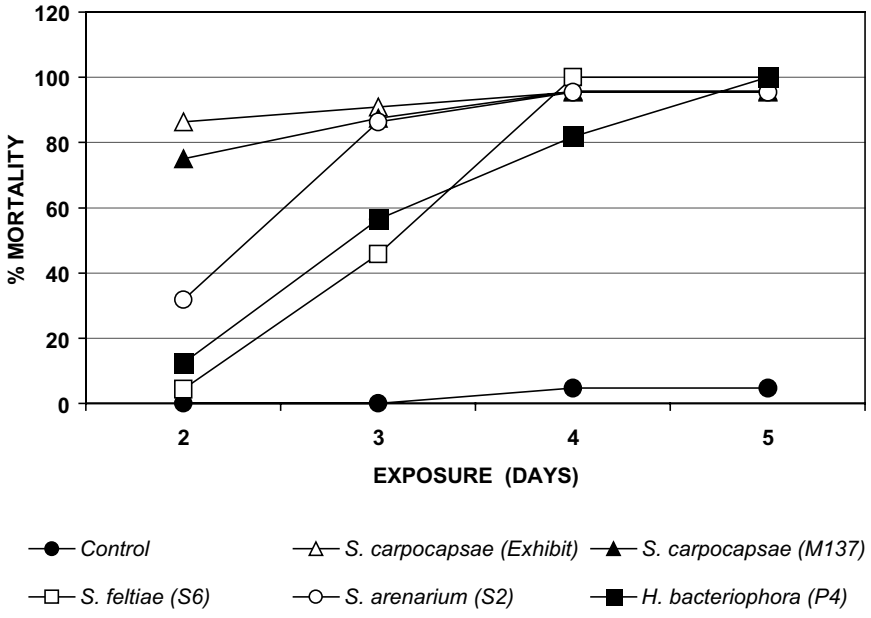

Figure 2. Evolution of infection of neonate larvae of *Capnodis* exposed to the nematode strains at 48 IJs/cm^2.

Table 2. Infection, penetration rate and sex ratio of the different strains tested at a dose of 10 IJs per neonate larva of *Capnodis* (equivalent to 3 IJs/cm^2)

Nematodes	Number IJs inoculum	Infection[a]	Mean penetration rate (%) (min–max)	Sex ratio (F: female; M: male)
S. carpocapsae (M137)	10	59.10% a	20.7 ± 13.8 (10–60)	51.85% F 48.15% M
S. feltiae (S6)	10	76.19% ab	23.1 ± 10.8 (10–50)	72.97% F 27.03% M
S. arenarium (S2)	10	90.91% b	36.0 ± 17.0 (20–90)	26.39% F 47.22% M 26.39% juv.
H. bacteriophora (P4)	10	79.19% ab	30.6 ± 19.8 (10–70)	100% H Hermafroditic

[a]Means with same letters are statistically similar ($p < 0.05$).

At a lower concentration assay (10 IJs/larva) significant differences in larval infection were observed between the nematode strains tested. The differences refer both to mortality and penetration rates (Table 2). Mortality of neonate larvae of *Capnodis* was higher in the *S. arenarium* treatment (90.91%) than in the other nematode strains (Figure 1). This level of control was significantly higher ($p < 0.05$) from *S. carpocapsae* (59.10%), and higher than *S. feltiae* (76.19%) and *H. bacteriophora* (76.19%) (Table 2).

Significant differences were observed among mean penetration rates of the four nematodes tested. The highest value was recorded for *S. arenarium* (36%) followed by *H. bacteriophora* (30.6%), *S. feltiae* (23.1%), and *S. carpocapsae* (20.7%) (Table 2).

The sex ratio of nematodes recovered from the neonate larvae of *Capnodis*, in the five nematode strains tested, changes according to the species. *S. carpocapsae* showed a sex ratio of nearly 50%. *S. feltiae* resulted in a majority of females (72.97%), whereas *S. arenarium* resulted in a majority of males (47.22%) but with a great proportion of juveniles (26.39%) at the end of the experiment.

Discussion

The results of our studies clearly demonstrate the susceptibility of neonate larvae of *C. tenebrionis* to infection by EPNs in soil their natural habitat. The *S. carpocapsae* (both commercial and autochthonous strains) nematode was the species which caused mortality of neonate larvae fastest at high density of infective juveniles (150 IJs per pot), where contact of the host and the

nematodes was ensured. In the low density infective juvenile experiments (10 IJs per pot), the probability of finding the insect decreased and nematodes were able to exhibit their foraging strategies. Under these conditions, *S. carpocapsae* was less effective, with a 59% mortality. Lewis et al. (1992) reported that this specie demonstrates a behaviour of 'ambushing', adopting a 'sit and wait' strategy in order to make contact with insects. This strategy implies that *S. carpocapsae* has a minor attraction to different insect excretions (Lewis et al., 1993) and a lower mobility in soil, where it prefers to stay near the surface to be close to its application place (Campbell and Gaugler, 1993). On the contrary, *S. arenarium,* with a foraging behaviour of 'cruiser' (actively searching for its host), was much more effective (91%) against neonate larvae of *Capnodis* in conditions where there was a low density of nematodes. *H. bacteriophora*, with a cruiser foraging behaviour, and *S. feltiae* with some characteristics of ambushing and some of cruiser foraging behaviour, resulted in a considerable efficacy (79.2% and 76.2%, respectively).

Hominick and Reid (1990) proposed the use of invasion efficiency as a direct measure of nematode infectivity. These authors assumed that the nematode with the greatest efficacy against a target insect would have the highest invasion efficiency. In this way, our results are in accordance with many other studies showing that the measurements of nematode invasion were in agreement with nematode insecticidal activity based on host mortality (Kondo and Ishibashi, 1986; Mannion and Jansson, 1993; Shannag et al., 1994). So, *S. arenarium* was the nematode with the greatest insecticidal activity (90.91%) against neonate larvae of *C. tenebrionis* and the highest penetration rate (36%), and *S. carpocapsae* was the nematode with the least insecticidal activity (59.10%) and lowest penetration rate (20.7%).

Caroli et al. (1996) showed that penetration rates differed among nematode species, host and substrate. The differences observed in the penetration rates of the nematode strains tested in neonate larvae of *Capnodis* may be related to the foraging behaviour of the different nematodes. Thus, species with a cruiser behaviour such as *S. arenarium* or *H. bacteriophora* provide a higher percentage of infective juveniles, infecting the host (invasion efficiency) by 36% and 31%, respectively. Species using an intermediate foraging strategy, such as *S. feltiae*, provide an invasion efficiency of 23%, while species with a clear ambushing behaviour, such as *S. carpocapsae*, provide a lower invasion efficiency, resulting in 21% of infective juveniles inoculated. Fan and Hominick (1991) and Epsky and Capinera (1993) reported that the invasion efficiency index is more sensitive than host mortality to determine nematode activity. These last authors also reported that for *S. carpocapsae* the invasion efficiency was positively related to increases of host exposure time and the number of hosts per arena, but negatively related to increases in substrate surface area per

host. This demonstrates, as we found, a relationship between the foraging strategies (ambushing or cruiser) of the nematodes and the invasion efficiency.

The sex ratio of the different nematode species observed inside the neonate larvae of *Capnodis* is similar to that Grewal et al. (1993) found in different *Steinernema* species developing on *Galleria mellonella* larvae, with a proportion of males ranging between 38% and 42%. The prevalence of females was reported by Gaugler et al. (1990), also in different strains of *S. carpocapsae* with 54% females, and by Bednarek et al. (1986) in a Polish strain of *S. carpocapsae* with 57% females. In our experiments, only *S. arenarium* provides a different sex proportion than the other species, with the number of males (64%) higher than females (36%). However, inside neonate larvae of *C. tenebrionis* parasitised by this specie, a high rate of juvenile forms was found (26.4%). This is because infective juveniles penetrate gradually, and by the fifth day of the assay they have not had enough time to develop into adults. When these infective juveniles eventually become adults, they are expected to develop as females. As Grewal et al. (1993) reported, in entomopathogenic nematodes (steinernematids) infective juveniles that develop into males disperse faster than females and colonise insects before them.

Capnodis' neonate larval stage and adult stage are the only ones that exist outside of tree trunks. Therefore, both stages have been considered as targets for different control agents (chemical pesticides and bio-insecticides). Sanna-Passino and Del Rio (2001) studied the application efficiency of different chemical insecticides for the control of neonate larvae of this insect in laboratory assays. These authors obtained a mortality of 67.63% for diazinon, 68.95% for carbaryl, 76.18% for isofenphos + phoxim, and 83.33% for chlorpyrifos, with a 31.04% mortality for the control. Present assays show that entomopathogenic nematodes cause a 60–91% mortality of the neonate larvae of *Capnodis*, at 10 IJs per larva (equivalent to a field application rate of 3 IJs/cm^2) and 96–100% mortality at 150 IJs per larva (equivalent to 48 IJs/cm^2), which is higher than the efficacy of chemical insecticides reported by Sanna-Passino and Del Rio (2001). Although it is necessary to compare the results with field assays, these results indicate that EPNs could be a more successful control agent for neonate larvae of *C. tenebrionis* than the chemical pesticides used at present.

Acknowledgement

We thank Mark Burch for the English correction of the manuscript.

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Capítulo II

Virulence of entomopathogenic nematodes to different stages of the flatheaded rootborer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae)

Nematology 8/09/2008

Nemo/2e[v.2006/11/21 12:15;] Prn:8/09/2008; 12:20 Tipas:?? F:nem2335.tex; /Diana p. 1(49-179)

Nematology, 2008, Vol. 00(0), 1-9

Virulence of entomopathogenic nematodes to different stages of the flatheaded root borer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae)

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Received: 5 June 2008; revised: 1 August 2008
Accepted for publication: 7 August 2008

Summary – The virulence of 14 *Steinernema feltiae* isolates, one *S. carpocapsae* and three *Heterorhabditis bacteriophora* isolates was compared in the laboratory against larvae, pupae and adults of the flatheaded root borer, *Capnodis tenebrionis*. Larval mortality ranged from 50 to 100% and pupae mortality from 0 to 70%. Adults were exposed to different nematode concentrations, 50 and 100 infective juveniles/cm^2. *Steinernema feltiae* caused the highest mortality at the two doses tested, reaching 66.7% and 100%, respectively. Significant differences in mortality between *C. tenebrionis* males and females were observed. The differences in virulence observed at each stage of *C. tenebrionis* vary within a species and can be higher than differences among species. The results of this study suggest that some of the isolate strains have potential for improved biological control of *C. tenebrionis*.

Keywords – biological control, *Heterorhabditis bacteriophora*, indigenous strains, larva, pupa, *Steinernema carpocapsae*, *Steinernema feltiae*, virulence.

Entomopathogenic nematodes (EPN) in the families Steinernematidae and Heterorhabditidae are obligate pathogens of a wide range of insects. These nematodes kill insects with the aid of mutualistic bacteria (*Photorhabdus* sp., *Xenorhabdus* sp.) that are carried in the nematode intestine (Poinar, 1990; Boemare, 2002). The non-feeding third-stage infective juveniles (IJ) penetrate into the haemocoel of host insects through natural openings, and in some species through the cuticle, and release the symbiotic bacteria. Toxins produced by the developing nematodes and bacteria kill the insect hosts within 2-3 days (Dowds & Peters, 2002), and the cadaver provides nutrition for one to three generations of nematodes (Poinar, 1990; Kaya & Gaugler, 1993). Once host resources are depleted, nematodes exit as IJ to repeat the cycle (Kaya & Gaugler, 1993). EPN are effective biocontrol agents of a variety of economically important insect pests (Klein, 1990; Shapiro-Ilan *et al*., 2002) and can provide effective biological control of some important soil insect pests and pests that occur in cryptic habitats (Georgis & Manweiler, 1994; Koppenhöfer, 2000). In fact, the ability of EPN to find hosts hidden in cryptic environments gives them an advantage over insecticides and other entomopathogens (Parsa *et al*., 2006). The selection of the best nematode species or strain for one target pest is important as there are large differences in the virulence of nematode species and strains against different species of pests (Grewal *et al*., 2005).

The flat-headed root borer, *Capnodis tenebrionis* L. (Coleoptera: Buprestidae), is an important pest of stone fruits (peach, cherry, apricot, almond, nectarine, plum) and seed fruit (apple, pear) in Southern European and Mediterranean areas (Morton & Garcia-del-Pino, 2008). These insects have a 1- or 2-year life-cycle. In Spain, adults emerge from tree trunks or soil in spring and summer, then feed on twigs and young branches (causing leaves to fall) and oviposit in soil near the trees at a distance up to 50 cm from the plant (Garrido, 1984; Malagón, 1989). Neonate larvae hatch, penetrate into the root of the tree and feed on the cortex (Malagón, 1989), progressing from small to large roots and trunk as larvae mature. Pupation occurs in the wood of the tree trunk (Mendel *et al*., 2003). Although adults cause damage to foliage, larvae are the real problem for plants killing

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one adult tree within 1 or 2 years (Ben-Yehuda *et al*., 2000). For many decades, chemical pesticides have been the most practical method used to control *C. tenebrionis*, focused on adults while they feed on trees (Garrido *et al*., 1990; Ben-Yehuda & Mendel, 1997; Colasurdo *et al*., 1997) and in neonate larvae when they are in the soil (Saba, 1979; Sekkat *et al*., 1997; Ben-Yehuda *et al*., 2000; Sanna-Passino & Delrio, 2001). Biological control with fungi (Marannino *et al*., 2006) and nematodes have been shown to be potential alternatives to chemical insecticides. Several species of EPN have been reported to infect *C. tenebrionis* larvae in the laboratory (García-del-Pino, 1994; Santos Lobatón *et al*., 1998; Maranino *et al*., 2003; García-del-Pino & Morton, 2005; Morton & García-del-Pino, 2008) and in the field (Martínez *et al*., 2008; Morton & García-del-Pino, 2008). Although the experiments have shown the effectiveness of EPN against *C. tenebrionis* larvae, there are no studies to assay the susceptibility of other stages or to select the appropriate species or strain.

The objective of the present work was to study the susceptibility of last instar larvae, pupae and adults of *C. tenebrionis* to different isolate EPN with the aim of selecting the most appropriate strains to control the different stages of *C. tenebrionis.* The knowledge obtained will allow the field control of *C. tenebrionis* to be improved.

Materials and methods

The virulence of fourteen *Steinernema feltiae* isolates, one *S. carpocapsae* and three *Heterorhabditis bacteriophora* isolates (Table 1) to *C. tenebrionis* was compared.

The nematodes of all species/strains were reared on last instar *Galleria mellonella* larvae according to Woodring and Kaya (1988). IJ were harvested from White (1927) traps and stored in tap water at 7°C for no longer than 2 weeks prior to the experiments. Before application, IJ of all nematode strains were acclimated at room temperature (21-23°C) for 12 h and their viability was checked under a stereomicroscope. All of the strains were checked for virulence by exposing ten *G. mellonella* larvae individually to 50 nematodes in Petri dishes at 25 ± 2°C. The experiment was repeated once.

Adults of *C. tenebrionis* were collected from an infested cherry tree orchard in La Beguda Baixa, Barcelona, Spain. In the laboratory, insects were maintained in cages at 25 ± 2°C and fed with fresh cherry tree branches. Before assay the sex of each insect was determined examining the last abdominal segment according to Garrido (1984). Larvae and pupae were collected from infected peach trees in fields in Els Guiamets (Tarragona), La Beguda Baixa and Ullastrell (Barcelona). Trees were pulled up and trunks and roots were dissected to find insects. Larvae and pupae were placed individually in Petri dishes with filter paper moistened with sterile tap water and maintained in coolers (18°C) during transit to the laboratory. For the assay only larvae of the two last stages and white pupae were selected.

Table 1. *Strains of* Steinernema feltiae*,* S. carpocapsae *and* Heterorhabditis bacteriophora *used in the susceptibility bioassays.*

Nematode species	Strain	Location	Orchard
S. feltiae	Bpa	Torrelles del Llobregat (Barcelona)	Cherry
S. feltiae	Bt2	Torrelles del Llobregat (Barcelona)	Cherry
S. feltiae	Bt4	Torrelles del Llobregat (Barcelona)	Cherry
S. feltiae	Bsor	Can Güell (Barcelona)	Cherry
S. feltiae	T91	Els Guiamets (Tarragona)	Peach
S. feltiae	T92	Falset (Tarragona)	Cherry
S. feltiae	Gsp	Sant Pere Pescador (Girona)	Peach
S. feltiae	L9	Almenar (Lleida)	Apple
S. feltiae	L11	Alfarràs (Lleida)	Pear
S. feltiae	L12	Alguaire (Lleida)	Apple
S. feltiae	M116	Casa del Francés (Murcia)	Cherry
S. feltiae	M117	Casa del Francés (Murcia)	Cherry
S. feltiae	M118	Pliego (Murcia)	Almond
S. feltiae	M123	Mula (Murcia)	Apricot
S. carpocapsae	B14	Barcelona (Barcelona)	Urban garden
H. bacteriophora	Gscl	L'Armentera (Girona)	Apple
H. bacteriophora	M110	Casablanca (Murcia)	Apricot
H. bacteriophora	M115	Calasparra (Murcia)	Cherry

A single insect was exposed to the IJ of each isolate in a sterile 9 cm diam. Petri dish containing 45 g sterile sand moistened with sterile distilled water (15%, w/w). IJ were transferred to Petri dishes in a volume of 500 μl of distilled water. Applied doses were 50 IJ/cm^2 in experiments with larvae, 100 IJ/cm^2 in experiments with pupae, and 50 and 100 IJ/cm^2 in experiments with adults. Control treatments received water only. Insects were held in an incubator at 25 ± 2°C. Petri dishes with larvae and pupae were maintained in the dark, while adults were stored with a 16 : 8 h L : D photoperiod. Insect mortality was recorded every 24 h. To confirm parasitism, dead larvae, pupae and/or adults were removed from the Petri dish, rinsed with distilled water to eliminate nematodes

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from their surface, transferred to Petri dishes filled with moist filter paper (Whatman No. 1) and dissected after 4 days. Twenty-four dishes were used in the assay with adults (12 males, 12 females) per treatment and nematode isolate, and the experiment was done twice. In the larvae and pupae assays, ten dishes for each treatment were used due to the difficulty to find enough appropriate individuals in the field.

Statistical analysis

Mortality data were presented in percentages and were analysed by the $\aleph^2$ test (SPSS 15.0 software). When mortality in the control exceeded 5%, data were corrected using Abbott's (1925) formula. A significance level of $P < 0.05$ was used in all analyses. The mortality percentages were statistically compared at 1, 3 and 5 days post exposure (PE) for larvae, at 4 and 6 days PE for pupae and at 5 and 8 days PE for adults.

Results

Virulence of the strains tested on *G. mellonella* larvae is presented in Figure 1. The majority of the strains caused 100% of larvae mortality, whereas two *S. feltiae* strains (L11, L12) only caused 75% mortality ($P < 0.05$). The susceptibility assays on larvae, pupae and adults of *C. tenebrionis* showed significant differences in percentage mortality among strains. Larvae of last instars of *C. tenebrionis* were susceptible to IJ of all tested isolates of *S. feltiae*, *H. bacteriophora* and to the isolate of *S. carpocapsae*. However, the mortality over time was different among the EPN strains tested (Fig. 2). Mortality caused by *S. feltiae* strains at 1 day PE ranged from 0 to 50%. *Steinernema feltiae* Bpa strain caused the highest mortality with significant differences ($P < 0.05$) from the rest of the strains (except T92, M116 and *S. carpocapsae*). All three strains of *H. bacteriophora* provided a low rate of mortality (10%). At 3 days PE, five *S. feltiae* strains reached 100% mortality, *Steinernema carpocapsae* caused 90% mortality and both species differed significantly with *H. bacteriophora* (40-60% mortality) ($P < 0.05$). At 5 days PE, seven of the 14 strains of *S. feltiae*, the three *H. bacteriophora* strains and *S. carpocapsae* strain caused 100% mortality of larvae. By contrast, *S. feltiae* isolates L11 and L12 did not reach 60% of larval mortality. There was no mortality in control treatments.

Pupae had a low susceptibility to nematodes (Fig. 3). Mortality of pupae at 4 days PE ranged from 0 to 30% for *S. feltiae isolates* with no significant differences between most of them and *S. carpocapsae* (10% mortality). Mortality caused by *H. bacteriophora* ranged from 30 to 50%. The mortality registered at 6 days PE for *S. feltiae* isolates only reached 40% (Bpa) and did not allow the separation of isolates with high pathogenicity. Mortality of pupae caused by *H. bacteriophora* ranged from 40 to 70% with Gscl strain as the highest virulent isolate, showing significant differences with all strains.

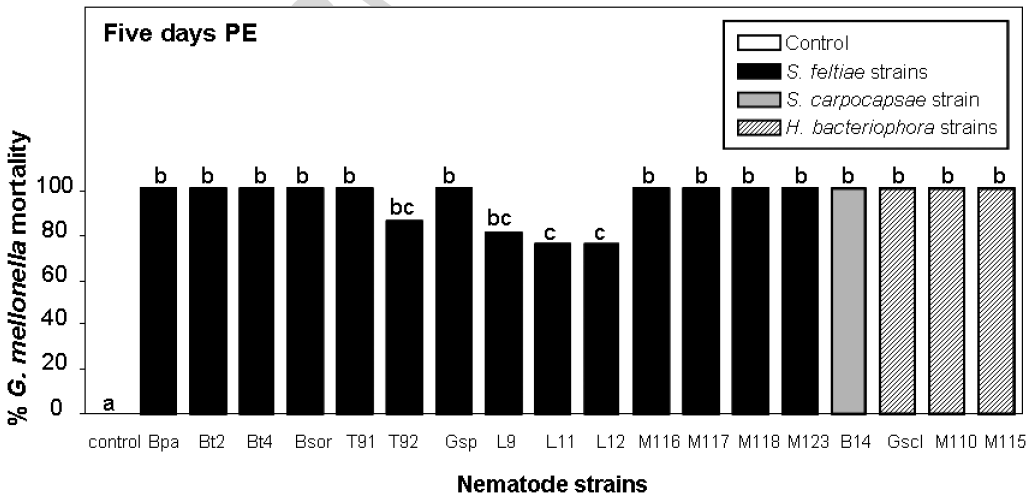

Fig. 1. *Mortality (%) of* Galleria mellonella *larvae in Petri dishes 5 days after exposure to 50 IJ of* Steinernema feltiae, S. carpocapsae *and* Heterorhabditis bacteriophora *isolates. Common letters above bars indicate no significant difference.*

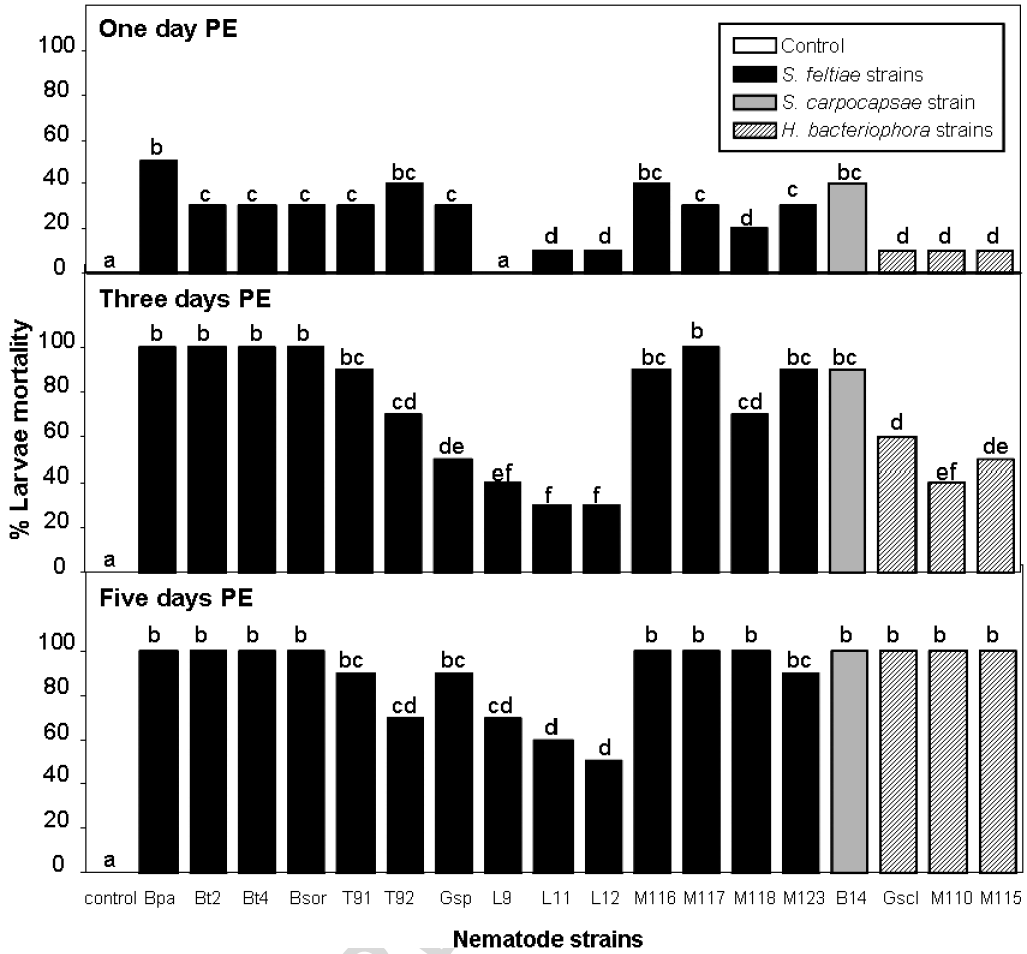

Fig. 2. *Mortality (%) of* Capnodis tenebrionis *larvae in Petri dishes 1, 3 and 5 days after exposure to 50 IJ/cm*^{2} *of* Steinernema feltiae*,* S. carpocapsae *and* Heterorhabditis bacteriophora *isolates. Common letters above bars indicate no significant difference.*

Steinernema carpocapsae provided a low control of pupae (20% mortality). There was no mortality in control treatments.

The mortality of adults registered with the dose of 50 IJ/cm^2 was lower than with 100 IJ/cm^2 (Fig. 4). At the dose of 50 IJ/cm^2, mortality at 5 days PE ranged from 0 to 55% for *S. feltiae* with significant differences among isolates. *Steinernema carpocapsae* (55% mortality) and all *S. feltiae* strains except L9 showed significant differences ($P < 0.05$) from *H. bacteriophora* isolates. At 8 days PE, mortality caused by *S. feltiae* reached 66.7% (Bpa, Bsor, T92), did not increase with treatments using *S. carpocapsae*, and in those treated with *H. bacteriophora* mortality ranged from 25 to 33%. At the dose of 100 IJ/cm^2 and at 5 days PE, adult mortality treated with *S. feltiae* ranged from 0% (M123, L9 y L11) to 87.5% (Bt4). *Heterorhabditis bacteriophora* caused low mortality (8.3%), whereas *S. carpocapsae* reached 62.5%. At 8 days PE the mortality distribution allowed isolates to be distinguished with high pathogenicity (that caused more than 50% mortality and differed from lower ones): eight *S. feltiae* strains (Bpa, Bt2, Bt4, Bsor, T91, T92, M116, M118) and *S. carpocapsae* B14; and isolates with low pathogenicity, causing up to 50% mortality: six *S. feltiae* (Gsp, L9, L11, L12, M117 and M123) and all *H. bacteriophora* (Gscl, M110, M115). Results obtained when comparing the two doses applied at 8 days PE showed significant differences ($P < 0.05$) for high pathogenicity isolates, except for Bsor and T92, and did not show significant differences ($P > 0.05$) for low pathogenicity isolates (except Gscl and M115). Mortality in control treatments was 8.3% for females and no mortality was recorded in males.

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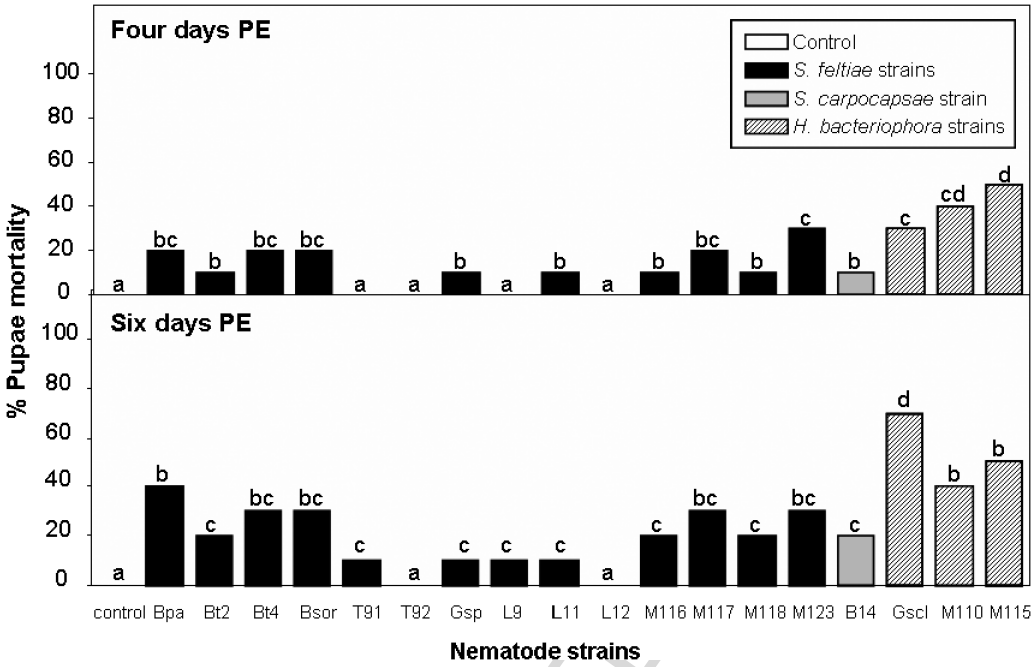

Fig. 3. *Mortality (%) of* Capnodis tenebrionis *pupae in Petri dishes 4 and 6 days after exposure to* Steinernema feltiae*,* S. carpocapsae *and* Heterorhabditis bacteriophora *isolates. Common letters above bars indicate no significant difference.*

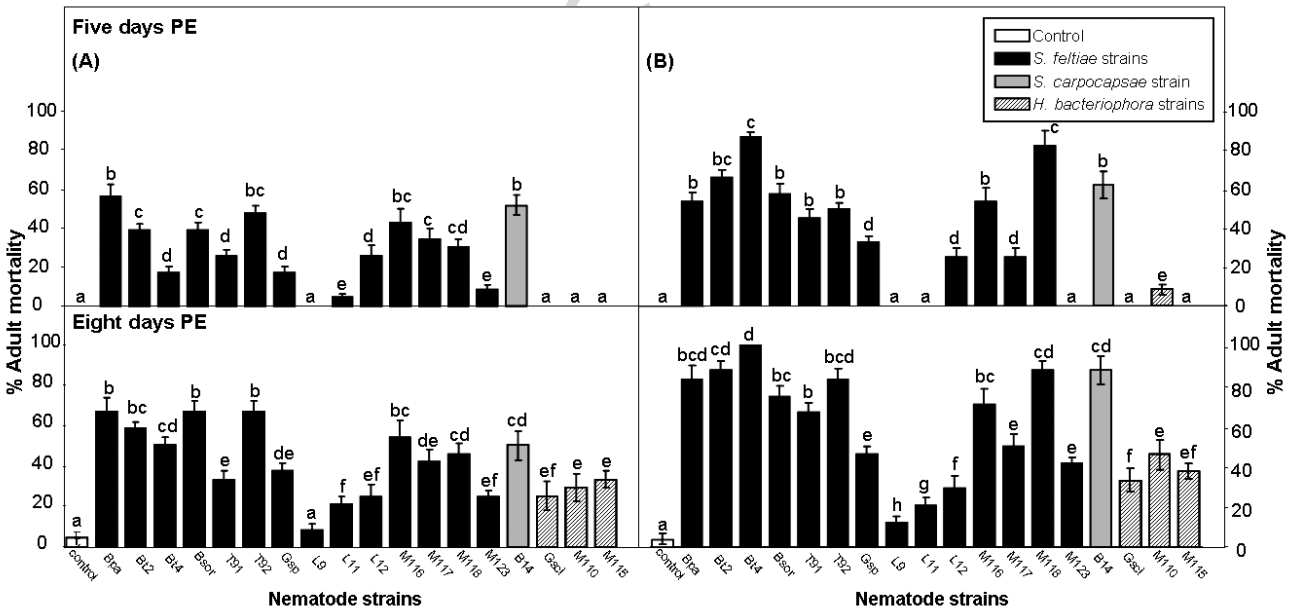

Fig. 4. *Mortality (%) of* Capnodis tenebrionis *adults in Petri dishes 5 and 8 days after exposure to (A) 50 IJ/cm*^{2} *and (B) 100 IJ/cm*^{2} *of* Steinernema feltiae*,* S. carpocapsae *and* Heterorhabditis bacteriophora *isolates. Common letters above bars indicate no significant difference.*

Table 2. *Mean percentage mortality (corrected with Abbott's formula) of males and females of* Capnodis tenebrionis *exposed to strains of entomopathogenic nematodes,* Steinernema feltiae*,* S. carpocapsae *and* Heterorhabditis bacteriophora*, with different concentrations of infective juveniles (IJ) in Petri dishes. For each dose, values in the same line followed by the same letter are not significantly different (*$P < 0.05$*).*

Strain	Dose of 50 IJ/cm^2		Dose of 100 IJ/cm^2	
	Males	Females	Males	Females
S. feltiae Bpa	83.3 a	45.5 b	91.7 a	72.7 a
S. feltiae Bt2	66.7 a	45.5 b	100 a	72.7 b
S. feltiae Bt4	58.3 a	36.4 b	100 a	100 a
S. feltiae Bsor	83.3 a	45.5 b	75 a	72.7 a
S. feltiae T91	66.7 a	0 b	66.7 a	63.6 a
S. feltiae T92	66.7 a	63.6 a	91.7 a	72.7 a
S. feltiae Gsp	41.7 a	27.3 b	50 a	36.4 b
S. feltiae L9	16.7 a	0 b	25 a	0 b
S. feltiae L11	33.3 a	0 b	33.3 a	0 b
S. feltiae L12	33.3 a	9.1 b	41.7 a	9.1 b
S. feltiae M116	66.7 a	36.4 b	100 a	36.4 b
S. feltiae M117	50 a	27.3 b	50 a	45.5 a
S. feltiae M118	50 a	36.4 b	91.7 a	81.8 a
S. feltiae M123	33.3 a	9.1 b	50 a	27.3 b
S. carpocapsae B14	58.3 a	36.4 b	91.7 a	81.8 a
H. bacteriophora Gscl	33.3 a	9.1 b	41.7 a	18.2 b
H. bacteriophora M110	50 a	0 b	66.7 a	18.2 b
H. bacteriophora M115	50 a	9.1 b	58.3 a	9.1 b

The effect of nematodes on the mortality of males and females for the two concentrations tested was also compared (Table 2). Using the dose of 50 IJ/cm^2, major mortality of males *vs* females occurred in all isolates, with significant differences except for T92. At 100 IJ/cm^2, differences among males and females were present in the three *H. bacteriophora* strains and in seven of the 14 *S. feltiae* isolates.

Discussion

This study documented that in the laboratory bioassays indigenous nematode isolates were able to infect and kill all stages of *C. tenebrionis*. Previous studies demonstrated that neonate larvae (García-del-Pino & Morton, 2005) and last instar larvae (García-del-Pino, 1994) of *C. tenebrionis* are susceptible to infection by EPN. The present study demonstrated that larvae of the two last stages, pupae and adults, are susceptible to nematode infection. Our results also indicate there are significant differences in virulence among isolates, allowing us to achieve a selection of the most virulent strains. Larvae of the last stages were highly susceptible to *S. feltiae*, *S. carpocapsae* and *H. bacteriophora*, resulting in 100% mortality with the three species, although some *S. feltiae* isolates worked faster than the other species. These results agree with those reported by García-del-Pino (1994) with *S. feltiae* and by Santos Lobatón *et al*. (1998) with *S. carpocapsae*. Similar results were observed in laboratory bioassays against neonate larvae. García-del-Pino and Morton (2005) tested nematode strains of *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* species, obtaining mortality above 95%. In the study carried out by Marannino *et al*. (2003), strains of *H. bacteriophora* and *S. carpocapsae* caused nearly 100% mortality of neonate larvae. Even though studies with other insects reported that first stages larvae are more susceptible than later stages (Glazer & Navon, 1990; Shapiro *et al*., 1999; Woon Lee *et al*., 2002; Grewal *et al*., 2004) and *vice versa* (Gaugler & Molloy, 1981; Fujiie *et al*., 1993; Smits *et al*., 1994), the results of this bioassay and other studies (García del Pino, 1994; Marannino *et al*., 2003; García-del-Pino & Morton, 2005) demonstrate that there are no differences in susceptibility among stages of *C. tenebrionis* larvae. The results obtained in this study with last instar larvae permit the rejection of *S. feltiae* isolates T92, L9, L11 and L12 because of their poor virulence. Moreover the assay on *G. mellonella* showed that these strains also caused lower mortality of larvae. Assuming that the infectivity test on *G. mellonella* is a quality control system of the IJ (Peters, 2005), the low virulence of these strains on *C. tenebrionis* and *G. mellonella* larvae seems not to be related to the susceptibility of the host but rather to the low infective quality of the strains.

There are no preliminary studies evaluating the susceptibility of pupae and adults of *C. tenebrionis* to EPN. In our study, pupae of *C. tenebrionis* were not especially susceptible to *S. feltiae* (up to 40% mortality) and *S. carpocapsae* (20% mortality), although *H. bacteriophora* (Gscl) reached 70% mortality. The result can be explained because in pupae the principal routes for nematodes to enter (spiracles, mouth and anus) are closed, obstructing the entrance of nematodes. However, *H. bacteriophora* is able to penetrate through the soft cuticle of the pupae of *C. tenebrionis* using their proximal tooth, as was reported by Bedding and Molyneux (1982) with other insects. Studies with EPN as insect control agents against adult insects have been done infrequently. The present survey with *C. tenebrionis* adults shows that they are less susceptible than larvae to EPN. The greater virulence of

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EPN has been reported in the immature stages of various other coleopteran, *e.g*., the fuller rose beetle *Asynonychus godmani* Crotch (Morse & Lindegren, 1996), the sweet potato weevil *Cylas formicarus* (F.) (Mannion & Jansson, 1992) and the West Indian sugarcane weevil *Metamasius hemipterus* (Oliver) (Giblin-Davis *et al*., 1996). The low mortality of *C. tenebrionis* caused by six strains of *S. feltiae* (L9, L11, L12, Gsp, M117, M123) and the three *H. bacteriophora* isolates (Gscl, M110, M115) justify discarding these strains for future control programmes of adults. Some of the discarded isolates were less successful against *G. mellonella*, but others (M117, M123, Gscl, M110, M115) caused 100% mortality. The low virulence on *C. tenebrionis* adults caused by all *H. bacteriophora* strains, with good results in the *G. mellonella* quality control test, could be related to their low capacity to infect adults of some coleopteran. Similar results were reported by Shapiro-Ilan (2001) in pecan weevil adults.

If studies about the susceptibility of adult insects to EPN are infrequent, studies about sex-related differences in susceptibility are rare. In some of these studies, males and females were equally susceptible to EPN (Renn, 1998; Van Sambeek & Wiesner, 1999; Buitenhuis & Shipp, 2005). However, Kotlarska-Mordzinska *et al*. (2000) indicated that males of the cockroach *Blatta orientalis* were more susceptible to *S. feltiae* and *H. bacteriophora* than females. Similar results were observed with the American cockroach *Periplaneta americana* infected by *S. carpocapsae* (Garcia-del-Pino, unpubl.). The present study is in accordance with these results, showing that males of the flat-headed root borer were significantly more susceptible to males than females. Although it is not evident why the sex-related differences in susceptibility occurs, factors such as the route of entry of nematodes into the insect (genital openings), insect behaviour (grooming activity), immune response or other physiological differences between males and females might be involved in the differences. Further research is required to validate predictions based on our results. The positive results of susceptibility of adults to EPN obtained in the laboratory suggest that some of the strains tested could be used to control adults in the field. One approach for controlling *C. tenebrionis* adults may be to apply EPN around each tree trunk. Thus, emerging adults would be infected when passing from the pupae camera through the soil. Other possibilities to control adults of *C. tenebrionis* in the field would be during the oviposition of the females or when adults take refuge in soil.

The results of this study show that there are strains or species with differences in virulence against each stage of *C. tenebrionis*. For example, the strain that caused the highest mortality in pupae, *H. bacteriophora* Gscl, was also effective against larvae but not against adults; however, a strain which provided greater control of larvae and adults, as with *S. feltiae* Bpa, did not show high efficacy against pupae. These findings enable us to select isolates with high pathogenicity in the laboratory against each insect stage, but future studies are necessary to characterise the isolates ecologically in order to select the most suitable strains to control *C. tenebrionis* in the field.

All nematodes used in this study (except *S. carpocapsae* B14) were found in orchards susceptible to flat-headed root borer attack. The presence of EPN in these orchards and the occurrence of *S. feltiae* and *S. carpocapsae* in *C. tenebrionis* larvae (García-del-Pino, 1994; Santos Lobatón *et al*., 1998; Morton & García-del-Pino, 2008) inside tree trunks suggest that EPN are natural enemies of this pest and could provide a natural biocontrol of *C. tenebrionis* in the field. In conclusion, the findings of the present study strongly suggest that EPN have a high potential for use as control agents for all stages of *C. tenebrionis* in stone fruit orchards.

Acknowledgement

We thank Mark Burch for the correction of the English of the manuscript.

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Capítulo III

Effectiveness of different species of entomopathogenic nematodes for biocontrol of the Mediterranean flatheaded rootborer, *Capnodis tenebrionis* (Linné) (Coleoptera: Buprestidae) in potted peach tree

Journal of Invertebrate Pathology (2008) 97: 128-133

Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 97 (2008) 128–133
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Effectiveness of different species of entomopathogenic nematodes for biocontrol of the Mediterranean flatheaded rootborer, *Capnodis tenebrionis* (Linné) (Coleoptera: Buprestidae) in potted peach tree

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Received 18 April 2007; accepted 5 September 2007
Available online 14 September 2007

Abstract

The susceptibility of larvae of the Mediterranean flatheaded rootborer (*Capnodis tenebrionis*) to 13 isolates of entomopathogenic nematodes was examined using GF-677® potted trees (peach × almond hybrid) as the host plant. The nematode strains tested included nine *Steinernema feltiae*, one *S. affine*, one *S. carpocapsae* and two *Heterorhabditis bacteriophora*. Nematodes showed the ability to locate and kill larvae of *C. tenebrionis* just after they enter into the roots of the tree. *S. feltiae* strains provided an efficacy ranging from 79.68% to 88.24%. *H. bacteriophora* strains resulted in control of 71.66–76.47%. *S. carpocapsae* (B14) and *S. affine* (Gspe3) caused lower control of *C. tenebrionis* larvae (62.03% and 34.76%, respectively). The influence of foraging strategy and the use of autochthonous nematodes to control *C. tenebrionis* larvae inside the roots is discussed.

Keywords: Biological control; *Capnodis tenebrionis*; *Steinernema feltiae*; *S. affine*; *S. carpocapsae*; *Heterorhabditis bacteriophora*

1. Introduction

The Mediterranean flatheaded rootborer, *Capnodis tenebrionis* (Linné) (Coleoptera: Buprestidae), is an economically important pest of cultivated stone and seed fruit. Damage caused by this insect has been reported mainly from Southern European and Mediterranean areas such as Spain (Garrido, 1984; Sánchez-Capuchino et al., 1987; Domínguez García-Tejero, 1989), Italy (Viggiani, 1991; Laccone, 1998), Morocco (Chrestian, 1955; Hmimina et al., 1988; Mahhou and Dennis, 1992), Israel (Ben-Yehuda and Mendel, 1997), Turkey (Tezcan, 1995; Cinar et al., 2004), Palestine (Rivnay, 1944) and Algeria (Martin, 1951). Larvae cause damage by feeding on the cortex which affects growth and may kill the tree, while adults feeding on twigs and young branches are particularly damaging to young trees and nurseries (Rivnay, 1946; Del Cañizo, 1950).

The biology of *C. tenebrionis* has been thoroughly studied (Garrido, 1984; Malagón, 1989). During the summer, eggs are laid on the ground, usually in cracks of dry soil or under stones within a distance of 50 cm around the plant. One female can lay more than 1000 eggs during her life. After the eggs hatch, neonate larvae are very active and move to the plant and penetrate into the root. Although they can survive up to 6 days outside the trunk, the penetration occurs mainly during the first 24 h (Balachowsky et al., 1962). During the first 1 or 2 years larva burrow into the root causing the real damage to the tree (Malagón, 1989). A few larvae can kill one adult tree within 1 or 2 years (Ben-Yehuda et al., 2000). Control of *C. tenebrionis* has been focused on using chemical insecticides against adults on stems and foliage (Garrido et al., 1990; Ben-Yehuda and Mendel, 1997; Colasurdo et al., 1997). Control of larvae with chemical pesticides has also been used when the

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0022-2011/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2007.09.003

Table 1
Entomopathogenic nematode strains tested and habitat of their isolation

Treatments	Name of the strains	Origin of the strains	Habitat
Steinernema feltiae	M116	Murcia (Spain)	Apricot orchard
S. feltiae	M117	Murcia (Spain)	Almond orchard with *C. tenebrionis*
S. feltiae	M118	Murcia (Spain)	Ecological Almond orchard
S. feltiae	M123	Murcia (Spain)	Apricot orchard
S. feltiae	Bpa	Catalonia (Spain)	*C. tenebrionis* larva parasited
S. feltiae	Bsor	Catalonia (Spain)	Cherry orchard with *C. tenebrionis*
S. feltiae	Bt2	Catalonia (Spain)	Cherry orchard with *C. tenebrionis*
S. feltiae	T91	Catalonia (Spain)	Cherry orchard
S. feltiae	T92	Catalonia (Spain)	Almond orchard
S. affine	Gspe3	Catalonia (Spain)	Apricot orchard
S. carpocapsae	B14	Catalonia (Spain)	Urban garden
Heterorhabditis bacteriophora	M110	Murcia (Spain)	Peach orchard
H. bacteriophora	Gscl3	Catalonia (Spain)	Apricot orchard

neonate larvae are in the soil moving to the roots (Saba, 1979; Sekkat et al., 1997; Ben-Yehuda et al., 2000; Sanna-Passino and Delrio, 2001). Because of the difficulty of controlling the larvae when penetrating into the roots, other control strategies, such as species of *Prunus* resistant to larvae of *C. tenebrionis*, have been considered (Malagón and Garrido, 1990; Mulas, 1994; Dicenta et al., 1998; Mendel et al., 2003). Increased efforts in recent years have been focused on biological control with entomopathogenic fungi (Marannino et al., 2006) and nematodes. Entomopathogenic nematodes (EPNs) (*Steinernema* spp. and *Heterorhabditis* spp.) and their symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) are obligate pathogens of insects in nature (Poinar, 1979). They are found in soils and persist as non-feeding infective juveniles, seeking out a potential insect host (Poinar, 1972). EPNs have been used efficiently against many soil-inhabiting and burrowing insects (Klein, 1990). A number of field trials have been conducted to evaluate the efficacy of EPNs for suppressing other wood-boring insects such as *Metamasius hemipterus* (L.) (Coleoptera: Curculionidae) (Giblin-Davis et al., 1996), *Synanthedon* spp. (Lepidoptera: Sesiidae) (Bedding and Miller, 1981; Miller and Bedding, 1982; Deseö et al., 1986; Kaya and Brown, 1986; Cossentine et al., 1990; Nachtigall and Dickler, 1992) or root-boring such as *Delia radicum* (L.) (Diptera: Anthomyiidae) (Willmott et al., 2002) and *Rhytidoderes plicatus* Oliv. (Coleoptera, Curculionidae) (Tarasco and Triggiani, 2002). Control of *C. tenebrionis* larvae with EPNs has also been tested under laboratory conditions (García del Pino, 1994; SantosLobatón et al., 1998; Marannino et al., 2003; García del Pino and Morton, 2005). The main objective of this study was to determine the efficacy of EPNs for control of *C. tenebrionis* larvae just after they enter into the roots of the tree, in potted trees under conditions mimicking those in the field.

2. Materials and methods

2.1. Nematode cultures

Thirteen Spanish isolates of EPNs (nine of *Steinernema feltiae* (Filipjev), one of *S. affine* (Bovien), one *S. carpocapsae* (Filipjev) and two of *Heterorhabditis bacteriophora* Poinar) were evaluated in this study (Table 1). All strains, except *S. carpocapsae* (B14) and *S. feltiae* (Bpa), were isolated from soils in orchards with presence of *C. tenebrionis*. *S. feltiae* (Bpa) was isolated from an infected larva of *C. tenebrionis* inside a cherry tree trunk. *Steinernema carpocapsae* (B14) was isolated from a soil of an urban garden. Nematodes were reared at 25 °C in last instar wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleridae), according to procedures described in Woodring and Kaya (1998). Infective juveniles (Ijs) were stored at 7 °C for 7–14 days before use. The nematodes in water suspensions were allowed to acclimate at ambient room temperature (21–23 °C) for 24 h prior to insect exposure.

2.2. Neonate larvae of C. tenebrionis

The neonate larvae of *C. tenebrionis* were obtained from eggs cultured following the protocol developed by Garrido et al. (1987). Adults of *C. tenebrionis* were obtained from an infested cherry tree orchard in La Beguda Baixa, Catalonia, Spain. Adults collected from the field were raised in cages of 50 cm × 50 cm × 50 cm. Fifteen females and 15 males were put together in each cage and fed every 2 days with fresh cherry tree branches. Oviposition occurred in dishes filled with sand, previously sifted to 0.8 mm, placed in each container. Eggs were collected every 3 days by sifting the sand, and were placed in an incubation chamber at 26 ± 2 °C to hatch. The eggs were observed every 24 h and emerging neonate larvae were individually examined under a binocular microscope and immediately used for the assay.

2.3. Bioassays

Experiments were carried out in potted trees to guarantee the location of the small larvae of *C. tenebrionis* inside galleries after treatments. Peach × almond hybrid tree (GF-677®) in 1000-ml plastic cups filled with 480 g of sandy soil (30% sand, 40% soil and 30% peat) were used in these experiments. Trees were 6–10 weeks old at the time of use and were kept without watering during 1 week before

use. Fifteen neonate larvae were placed in soil 1 cm deep and 4 cm from the base of the stem and left without watering during 5 days to allow larvae to enter into roots. After this time, trees were watered with 250 ml of tap water. A total of 3180 Infective juveniles (corresponding to a dose of 50 Ijs/cm^2) was applied in 10 ml of sterile tap water. Controls received water only. After nematode application, trees were watered every 2 days until the end of the experiment. Twenty days after the insect inoculation, the roots of each tree were washed and dissected. The percentage of the infested trees containing at least one live larva, the number of live larvae per tree, the cephalic capsule width and the length of each live larva were determined. There were 15 replications per treatment and the experiment was repeated three times. Potted trees were maintained in a greenhouse at 25 ± 5 °C during all the experiments.

2.4. Statistical analysis

Data for the number of live larvae per tree, and arcsine-transformed values of the percentage of trees containing at least one live larva were subjected to analysis of variance (ANOVA) (SPSS-PC v. 14.0). Significant differences between treatments were determined using Tukey's multiple range test at $P < 0.05$. Efficacy of nematodes in relation to the number of larvae per tree was calculated using Abbot's formula (1925).

3. Results

The mean number of *C. tenebrionis* larvae found in tree roots was 1.87 in control treatments compared to 0.30 (0.22–0.38) for *S. feltiae,* 0.49 (0.44–0.53) for *H. bacteriophora*, 0.71 for *S. carpocapsae* and 1.22 for *S. affine* (Table 2). Control differed significantly with all strains ($F = 26.569$; $P \leqslant 0.001$). Efficacy of nematodes ranged from 34.8% to 88.2% (Fig. 1). *S. feltiae* strains caused a high control of the insect ranging from 79.7% (T92 strain) to 88.2% (Bpa strain) but no significant differences were detected between the nine strains of *S. feltiae* ($F = 0.468$; $P > 0.05$). *H. bacteriophora* caused 71.7% (Gscl3 strain) to 76.5% (M110 strain) control of *C. tenebrionis* with no differences between strains and with *S. feltiae* strains ($P > 0.05$). *Steinernema carpocapsae* (B14) caused a 62.0% control and *S. affine* (Gspe3) caused the lowest control (34.8%), both significantly lower than the rest of the strains ($P < 0.05$).

Table 2
Mean (±SE) larvae per plant and percentage of infested plant produced by different species and strains of *S. feltiae, S. affine*, *S. carpocapsae* and *H. bacteriophora* against *C. tenebrionis* larvae after 20 days

Treatments	Larvae per plant	% Infested plants
Control	1.87 ± 0.76 a	95.6 a
S. feltiae M116	0.31 ± 0.47 b	31.1 b
S. feltiae M117	0.29 ± 0.51 b	26.7 b
S. feltiae M118	0.33 ± 0.60 b	26.7 b
S. feltiae M123	0.27 ± 0.54 b	22.2 b
S. feltiae Bpa	0.22 ± 0.42 b	22.2 b
S. feltiae Bsor	0.27 ± 0.50 b	24.4 b
S. feltiae Bt2	0.24 ± 0.43 b	24.4 b
S. feltiae L91	0.36 ± 0.53 b	33.3 b
S. feltiae L92	0.38 ± 0.58 b	33.3 b
S. affine Gspe3	1.22 ± 0.82 c	77.8 a
S. carpocapsae B14	0.71 ± 0.59 c	64.4 ac
H. bacteriophora M110	0.44 ± 0.59 b	40.0 bc
H. bacteriophora Gscl3	0.53 ± 0.66 b	44.4 bc

Same letters indicate no significant difference at $P > 0.05$ for a nematode species or strain

The percentage of infested trees containing at least one live larva of *C. tenebrionis* ranged from 95.6% (in control) to 22.2% with significant differences among trials (ANOVA: $F = 10.401$; $P \leqslant 0.001$). The lowest percentage of infested trees, ranging from 22.2% to 33.3%, was caused by *S. feltiae* strains, but no significant differences among strains were detected ($F = 0.437$; $P > 0.05$). Trees (40–44%) were infested with some live larvae following treatment with *H. bacteriophora*, with no differences between strains and with *S. feltiae* and *S. carpocapsae* strains ($P > 0.05$). *S. carpocapsae* (B14) resulted in 64.4% of trees being infested and did not significantly differ from *H. bacteriophora* strains, the *S. affine* strain and the control. Finally, *S. affine* (Gspe3) showed high infestation (77.8%) with significant differences from the rest of the strains ($P < 0.05$), except *S. carpocapsae* (B14), and it did not differ significantly from the control ($P > 0.05$).

During the experiment we observed that the holes made by larvae of *C. tenebrionis* leading to the tunnels were not sealed, and there was frass of the larvae throughout the galleries. We also observed that many trees treated with EPNs contained one or more galleries, probably made during the 5 days before nematode application, although no dead or live larvae were found. Only five dead larvae with nematodes inside were found in all the treatments (four in *S. feltiae* strains and one in a *H. bacteriophora* strain). The living larvae found in the tunnels of the roots measured 9.68 mm ± 0.32 mm length and 2.98 mm ± 0.03 mm cephalic capsule width. This size of larvae and the absence of the characteristic long setae of the first stage indicate that these larvae developed into second or third stage inside the gallery.

4. Discussion

Results of this study clearly demonstrate that EPNs are able to find and penetrate into the root of the tree, and to search for and kill the neonate *C. tenebrionis* larvae inside the gallery. The capacity of EPNs to locate *C. tenebrionis* larvae inside the roots of plants could be accomplished in a first stage by root exudates attraction, and then by host cues of the insect. Hui and Webster (2000) reported that nematodes may be attracted by gradients emanating from root exudates. This behaviour could explain the location of the entrance of the gallery by nematodes. Once the nematode is in the vicinity of the insect, the exudates of the host

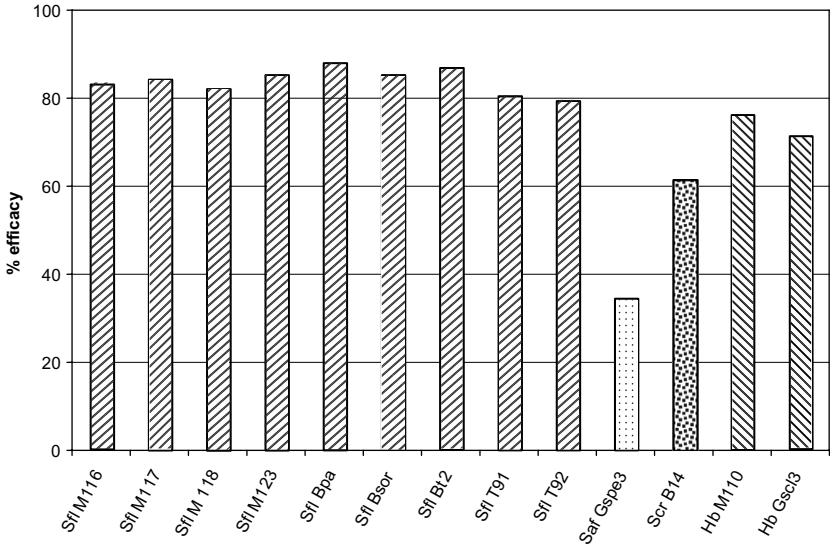

Fig. 1. Percentage of efficacy (Abbott's formula) of different species and strains of *S. feltiae*, *S. affine, S. carpocapsae* and *H. heterorhabditis* against *C. tenebrionis* larvae after 20 days. Sfl, *S. feltiae*; Saf, *S. affine*; Scr, *S. carpocapsae* and Hb, *H. bacteriophora*.

are more attractive for it, and nematodes enter into the gallery where they may locate and infect the larvae protected from hostile abiotic conditions (Arthurs et al., 2004). Nematode efficiency against *C. tenebrionis* larvae varies considerably with nematode species, but lower between different strains of the same species. From the results, it is apparent that *S. feltiae* and *H. bacteriophora* are more effective in controlling larvae of C. *tenebrionis*, inside their galleries, than *S. carpocapsae* and *S. affine*. But our experiments did not provide the appropriate conditions to distinguish any single *S. feltiae* or *H. bacteriophora* strain to be significantly superior to the others for controlling *C. tenebrionis* larvae. Larval survival in control treatment in this bioassay (12.5%) is in accordance with data obtained by Mendel et al. (2003), who recorded a survival ranging from 2.9% to 16.5% depending on *Prunus* rootstock. García del Pino and Morton (2005) reported that, under laboratory conditions, at a dosage of 48 Ijs/cm^2, strains of *S. feltiae, S. carpocapsae* and *H. bacteriophora* killed between 96% and 100% of the larval stage found in the soil, prior to entering the roots. Similar results were obtained by Marannino et al. (2003), who reported 100% mortality of *C. tenebrionis* neonate larvae with *S. carpocapsae* and *H. bacteriophora*, after applying 50 Ijs per larvae. Marannino et al. (2003) applied nematodes against *C. tenebrionis* neonate larvae in soil in the presence of plants just before root penetration of larvae, and obtained 100% control with *S. carpocapsae* and 98.9% with *H. bacteriophora*. Our experiment showed that when EPNs were applied after *C. tenebrionis* larvae penetrated into the galleries in the plant, larvae control up to 88.2% was obtained.

Steinernema carpocapsae (B14) and *S. affine* (Gspe3) showed an efficacy of 62% and 34.8%, respectively, but they did not reduce significantly the percentage of infested trees. However, all *S. feltiae* and *H. bacteriophora* strains provided a good control of the insect and reduced significantly the percentage of infested trees. These results may be related to the motile ability of different species to locate larvae of *C. tenebrionis* when they are inside the roots. The low efficacy of *S. carpocapsae* (62%), and the high percentage of infested trees (64.4%), could be explained by the ambushing behaviour of this species, staying near the surface close to its application place waiting for its host (Lewis et al., 1992; Campbell and Gaugler, 1993; Lewis, 2002). Poor migration of these nematodes could explain that only larvae in galleries near the application point were infected, with no search for the remote larvae. The low efficacy of *S. affine* could be related to the specificity of the host, as with parasite dipteran species in natural conditions (Peters, 1996). This species has been tested to control *D. radicum* with success (Willmott et al., 2002). However, *S. feltiae* and *H. bacteriophora* were significantly more effective against *C. tenebrionis* (88.2% and 76.5%, respectively) which have similar foraging strategies. *S. feltiae* displays an intermediate ambusher-cruiser foraging strategy (Lewis, 2002) and *H. bacteriophora* has the typical cruiser foraging strategy (Lewis et al., 1992). The high efficacy of *S. feltiae* and *H. bacteriophora* strains having the highest efficacy may be related to the origin of the nematodes. *Steinernema feltiae* (Bpa) was isolated from an infected larva of *C. tenebrionis* inside a cherry tree trunk, and the other strains were isolated from soils with the presence of *C. tenebrionis.* As Bedding et al. (1993) reported, improved efficacy can be achieved, in part, by using the most effective "wild type" nematode against a particular pest, with desirable traits physiologically adapted to the pest habitat. In this way Millar and Barbecheck (2001) suggested that endemic nematodes, adapted to the local habitat, are probably the best resource/strain to control the endemic insect pest.

Few nematode-infected larvae were recovered, but many tunnels made by larvae were found. This could be explained by the small size of the larva of *C. tenebri-*

onis that provides few nutrients for the nematode, allowing just one generation inside the larva. Probably, infective juvenile nematodes left the larvae, the cadavers decomposed during the 20 days of the assay, and we were not able to locate them among the frass inside the tunnels.

Although limited information on field efficacy of EPNs for the Mediterranean flatheaded rootborer (Barajas et al., 2002; Martínez de Altube et al., in press), the field presence of natural infections with EPNs of larvae of *C. tenebrionis* inside tree trunks and roots (García del Pino, 1994; SantosLobatón et al., 1998) shows a natural potential of this nematode to control such a pest. This and the results of this paper, indicate that EPNs can be an efficient biological agent of control of *C. tenebrionis* inside roots, but trials need to be conducted to assess its control ability in an open field situation.

Acknowledgments

We are grateful to Agromillora Catalana for supplying the potted trees, Antonio Soler for technical assistance and Mark Burch for language revision of the manuscript.

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Capítulo IV

Field efficacy of the entomopathogenic nematode *Steinernema feltiae* against the Mediterranean flat-headed rootborer *Capnodis tenebrionis*

Journal of Applied Entomology (2008) 132: 632-637

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Field efficacy of the entomopathogenic nematode *Steinernema feltiae* against the Mediterranean flat-headed rootborer *Capnodis tenebrionis*

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Keywords
biological control, Buprestidae, Coleoptera, field experiment, Rhabditida, Steinernematidae

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Received: January 14, 2008; accepted: April 22, 2008.

doi: 10.1111/j.1439-0418.2008.01300.x

Abstract

The Mediterranean flat-headed rootborer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae), is an economically important pest of stone fruit and seed fruit in Mediterranean areas. The potential control of the entomopathogenic nematode *Steinernema feltiae* (Filipjev) (strain Bpa), isolated from a dead *C. tenebrionis* larva, was tested in a cherry tree orchard in Ullastrell, Barcelona (Spain). Nematode infective juveniles (IJs) were applied by drench and injection. In both the treatments, a rate of 1 million IJs was applied per tree every week during 4 or 8 weeks, with a total dose of 4×10^6 IJs/tree and 8×10^6 IJs/tree. Number, stage and localization of insects in each tree trunk were recorded. In both the experiments, *S. feltiae* significantly reduced the population of *C. tenebrionis* providing control ranging from 88.3% to 97%. No significant differences were recorded between the different treatments. Persistence of nematodes was recorded until 6 weeks after application. Results indicate that the application of *S. feltiae* (Bpa) provides adequate control of *C. tenebrionis* in cherry trees.

Introduction

Capnodis tenebrionis (L.) (Coleoptera: Buprestidae), the Mediterranean flat-headed rootborer, is an important pest of stone fruit (cherry, apricot, peach, almond, nectarine and plum) and seed fruit (apple and pear). Damage caused by this insect has been reported mainly from Southern European and Mediterranean areas (Morton and García-del-Pino 2008). Adults of *C. tenebrionis* feed on twigs and young branches and may damage young trees in nurseries (Rivnay 1946; Del Cañizo 1950). Females lay eggs on the ground, usually in cracks of dry soil or under stones within a distance of 50 cm of the plant (Garrido 1984; Malagón 1989). When neonate larvae hatch, they move to the plant, penetrate into the root and start feeding. In 1 or 2 years, larvae burrow into the roots and thus cause the decline of the tree (Malagón 1989).

Control recommendations for *C. tenebrionis* currently consist of foliar applications of chemical insecticides against the adults (Garrido et al. 1990; Ben-Yehuda and Mendel 1997; Colasurdo et al. 1997) or soil application against neonate larvae before they penetrate into the roots (Saba 1979; Sekkat et al. 1997; Ben-Yehuda et al. 2000; Sanna-Passino and Delrio 2001). Because of the difficulty in controlling this insect with chemical insecticides when it is inside the root or trunk, latest studies have focused on the biological control with entomopathogenic fungi (Marannino et al. 2006) and nematodes (García-del-Pino and Morton 2005; Morton and García-del-Pino 2008).

Entomopathogenic nematodes are important biological control agents for a variety of economically important pests (Grewal et al. 2005). Nematodes can be mass-produced by *in vivo* or *in vitro* methods (Friedman 1990) and commercially applied as

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infective juveniles (IJs) in aqueous suspensions (Wright et al. 2005) by use of irrigation systems, sprayers or injection techniques (Grewal and Peters 2005). The efficacy of entomopathogenic nematodes has been investigated against several species of wood-boring insects such as *Metamasius hemipterus* (Coleoptera: Curculionidae) (Giblin-Davis et al. 1996) and *Synanthedon myopaeformis* (Lepidoptera: Sesiidae) (Nachtigall and Dickler 1992). Control of *C. tenebrionis* with entomopathogenic nematodes has also been tested under laboratory conditions against neonate larva (García-del-Pino 1994; Santos-Lobatón et al. 1998; Marannino et al. 2003; García-del-Pino and Morton 2005), and in potted trees after larvae had just entered into the roots (Morton and García-del-Pino 2008).

The main objectives of the research reported here were: (i) to examine the potential of a indigenous isolate of *S. feltiae* (strain Bpa) in controlling *C. tenebrionis* in the field and its persistence in the soil and (ii) to compare the efficacy of two different application methods, drench and injection.

Materials and Methods

The experiment was conducted in Ullastrell, Barcelona, in a commercial cherry orchard, attacked by *C. tenebrionis*. Trees had an average diameter of the trunk base of 23.2 cm and there was a distance between trees of 3 m. The soil analysis revealed a loam (22.8% clay, 44.6% silt and 32.6% sand) with a salinity of 0.19 dS/m and a pH of 8.5. The maximum temperature during the 3-month experimental period was 36.6°C and the minimum 6.3°C. Total rainfall of 64.4 mm was recorded. In pre-application sampling, a natural nematode population was not detected by baiting soil samples with wax moth larvae, *Galleria mellonella* (Lepidoptera: Galleridae) (Bedding and Akhurst 1975). Trees with symptoms of a *C. tenebrionis* infestation as reduced vigour or with some dried branches were selected for the experiments.

The nematode used in this experiment was *S. feltiae* (Bpa strain), isolated from a dead *C. tenebrionis* larva found inside a cherry tree trunk in an orchard located in Torrelles del Llobregat (Barcelona) (A. Morton and F. García-del-Pino, unpublished data). Nematodes were produced by e-nema GmbH (Raisdorf, Germany) in liquid culture according to Ehlers et al. (1998) and formulated with clay soil.

Two treatments were carried out. In the first treatment, entomopathogenic nematodes were applied through watering a ditch excavated 10 cm deep and 50 cm around the tree (drench). In the second treatment, nematodes were applied by injection, using an injector of 1.5 cm in diameter with four orifices of 2 mm in diameter at the end, which inserted the nematodes from soil surface and up to 50 cm deep. Each treatment applied 10^6 IJs/tree in 5 l of water. Nematode application started on June 1 and finished on August 3. Weekly treatments were done for 4 and 8 weeks which resulted in a total application rate of 4×10^6 and 8×10^6 IJs/tree, respectively. Control trees received an equal amount of water without nematodes. Each experimental unit comprised one tree with eight replicate trees per treatment and dose and eight control replicates. A totally randomized design was used. Before each application, nematodes were mixed with water and two samples were taken to check for IJ viability and dose. The nematodes in water suspensions were allowed to acclimate at ambient temperature for 1 h prior to the application. Treatments were performed during the sunset. During the experiment, the base of each tree was covered with a 1-m^2 piece of nylon cloth to control the emergence of adults. Every week, all trees were checked for adults emerging. Four weeks after the last application, trees were removed from the soil. Primary and secondary roots and trunk up to 40 cm above the soil surface were thoroughly dissected with a knife. The number of larvae, pupae and adults of *C. tenebrionis* was counted and their position recorded. The insects' positions were divided into four categories: adults emerged from the tree trunk; larvae and pupae inside the tree trunk above the soil surface; in the tree trunk under the soil surface and in the root system of the tree.

To determine the nematode persistence one soil core (3 cm in diameter and 20 cm deep) was taken randomly at a distance of 25 cm around each tree every week during 7 weeks after the last nematode application. The superficial 2 cm of dry soil were discarded and the rest of the core was divided into three 6-cm long sections. Each section was placed in a 9-cm diameter Petri dish. In the laboratory, the soil of each dish was baited with six *G. mellonella* larvae per sample. Water was added when necessary to provide moist conditions for IJ migration. Dishes were held at 23 ± 2°C and mortality of *G. mellonella* larvae was assessed after 7 days. Cadavers were dissected to confirm nematode infection. We assumed the number of infected larvae found by sampling was related to the number of nematodes that were present in the soil (Mràček 1982; Koppenhöfer et al. 1998).

Statistical analysis

Efficacy of nematodes relating to the number of surviving insects per tree was calculated using Abbott's formula (Abbott 1925). Data for the number of surviving insects per tree were subjected to Mann–Whitney non-parametric testing. To analyze the persistence of nematodes, percentage data of *G. mellonella* mortality were arcsine of square root transformed and subjected to analysis of variance. Means were compared using the Tukey's multiple range test. Significant differences between treatments were determined using a t-test. Differences among means were considered significant at $P < 0.05$. The statistical analysis was performed by using the program SPSS-PC 14.0. Data in text and figures are presented as non-transformed means with the standard errors of the means.

Results

The mean number of live insects of *C. tenebrionis* recorded in the two treatments is presented in table 1. In both the treatments, the majority of insects were in the larval stage. During the dissection of the trees, many galleries without insects in the tree trunk were observed but with moist excrements, signalling the past presence of *C. tenebrionis*. However, only 9 dead larvae were found inside treated tree trunks, in all 32 dissected trees. All dead larvae were mummified due to fungal attack and no nematodes were found inside the cadavers. No significant differences in reduction were observed between the two different doses, 4×10^6 IJs/tree and 8×10^6 IJs/tree in drench treatment (Mann–Whitney test, $P = 0.798$) and in injection treatment (Mann–Whitney test, $P = 0.721$) and between the different treatment techniques, drench and injection (Mann–Whitney test, $P = 0.959$ and $P = 1.0$, respectively). Both the treatments significantly reduced the number of live larvae and pupae relative to the untreated control (Mann–Whitney Test, $P < 0.001$) (table 1).

For control trees watered during 4 weeks, the mean numbers and positions of live insects found were: 2.25 emerged adults from the tree trunk, 10.75 larvae and pupae in the tree trunk above the soil surface, 7 in the tree trunk under the soil surface, and 7.75 in the tree's root system; and for control trees watered during 8 weeks, the numbers were 0.75, 6.5.5 and 7.25, respectively. The Abbott-corrected mortality of *C. tenebrionis* larvae and pupae for different sections of the plant was ranging from 88.3% to 97% (fig. 1). There were no significant differences between doses in each tree section and between treatments (Tukey's test, $P > 0.05$).

The vertical distribution of the nematodes in the soil during 7 weeks recorded as mean number of *G. mellonella* parasited is presented in fig. 2a for drench application and fig. 2b for injection. Nematodes were recovered during 6 weeks after the last application. During the first 2 weeks, both doses showed a higher presence of nematodes at the superficial layer (2–8 cm) in the drench compare to the injection treatment, with significant differences (*t*-test, $P < 0.01$). However, the presence of nematodes in the superficial layer decreased significantly in both the treatments by the third week (*t*-test, $P > 0.05$). At the intermediate (8–14 cm) and the deepest (14–20 cm) layers no significant differences between the first 4 weeks were found in both the treatments (Tukey's test, $P > 0.05$) and persistence declined faster by the fifth week (Tukey's test, $P < 0.05$).

Discussion

The results of this study indicate that *S. feltiae* is a valuable biological control agent to manage *C. tenebrionis* in the field. Applications of *S. feltiae* resulted

Table 1 Mean number of insects per plant (±SE) of *Capnodis tenebrionis* found after weekly applications of *Steinernema felti ae* by drench or injection and in water treated controls over a period of 4 or 8 weeks

	Treatment					
	Time (week)	Dose/tree	Larvae	Pupae	Adults	Total
Control	4	–	19.00 ± 13.29 a	4.00 ± 2.83 a	2.25 ± 1.26 a	25.25 ± 13.94 a
	8	–	10.75 ± 4.57 a	2.75 ± 2.06 a	0.75 ± 0.96 a	14.25 ± 3.77 a
Drench	4	4×10^6 IJs	1.00 ± 1.19 b	0.38 ± 1.06 b	0.13 ± 0.35 b	1.50 ± 1.93 b
	8	8×10^6 IJs	0.75 ± 1.16 b	0.13 ± 0.35 b	0.13 ± 0.35 b	1.00 ± 1.31 b
Injection	4	4×10^6 IJs	1.13 ± 1.25 b	0.00 ± 0.00 b	0.13 ± 0.35 b	1.25 ± 1.49 b
	8	8×10^6 IJs	1.00 ± 1.41 b	0.00 ± 0.00 b	0.00 ± 0.00 b	1.00 ± 1.41 b

Same letters in a column indicate no significant difference ($P > 0.05$).

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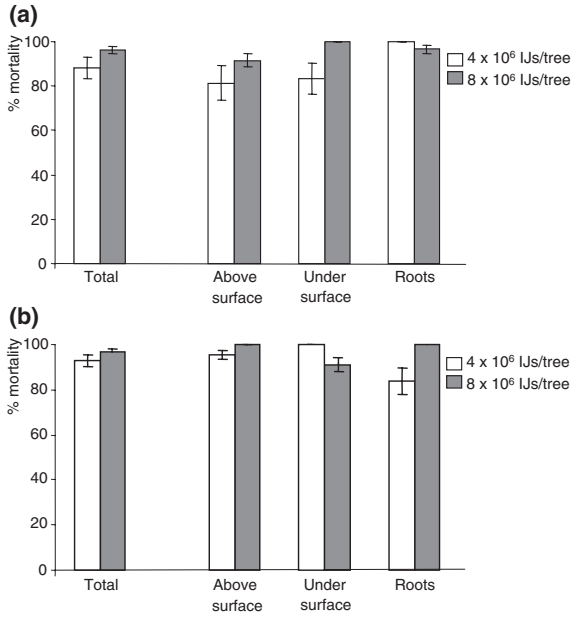

Fig. 1 Abbott-corrected mortality of *Capnodis tenebrionis* (larvae and pupae) collected from tree trunks (n = 8) above or below the soil surface and from the root system 4 weeks after (a) drench application or (b) soil injection of weekly treatments with 1×10^6 IJs/tree of *Steinernema feltiae* (strain Bpa) over a period of 4 or 8 weeks.

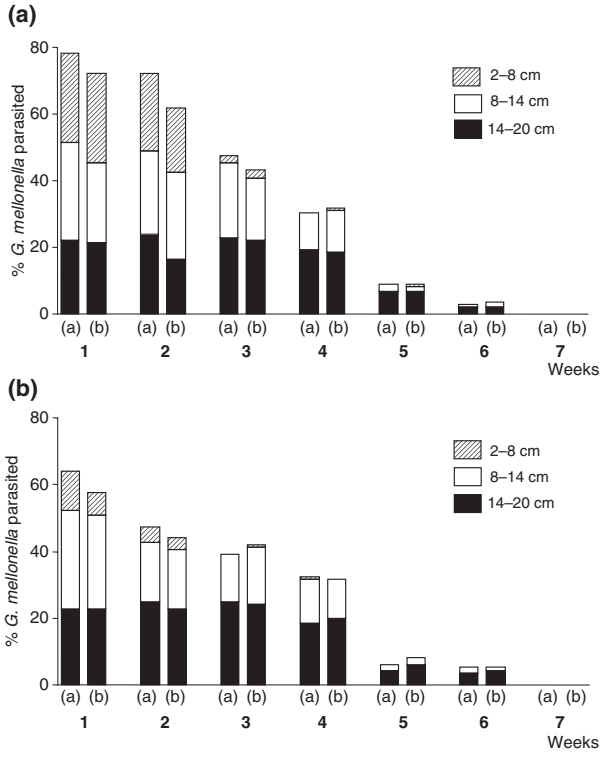

Fig. 2 Percentage of *Galleria mellonella* parasited by *Steinernema feltiae* (Bpa) 7 days after transfer into soil samples taken at three soil depths over a period of 7 weeks after the last application from trees (n = 8) treated by (a) drench or (b) injection with weekly sprays of 1×10^6 IJs/tree over a period of (i) 4 and (ii) 8 weeks.

in *C. tenebrionis* control ranging from 88.3% to 97%. Similar efficacies (80–95%) were obtained by Martínez de Altube et al. (2008) applying *Steinernema carpocapsae* together with Biorend R® (1.25% chitosan diluted in acetic acid). The presented results demonstrate that a high efficacy can be obtained with either formulation used for application of the nematodes. Efficacy of *S. feltiae* against *C. tenebrionis* obtained in this experiment confirms data obtained by Morton and García-del-Pino (2008) in pot experiments which reached 88.2% control with *S. feltiae* (Bpa) and 62% control with *S. carpocapsae* against first and second instars inside the galleries. The same authors reported 100% mortality of neonate larvae in laboratory assays in sand caused by *S. feltiae* and 96% by *S. carpocapsae* (García-del-Pino and Morton 2005). Results obtained with 4 or 8×10^6 IJs of *S. feltiae* per tree were not significantly different. Martínez de Altube et al. (2008) also did not record an increase of *C. tenebrionis* control when increasing a single dose from of 1×10^6 to 1.5×10^6 *S. carpocapsae* per tree. Other studies support the fact that from certain nematode concentrations, an increase in dosage does not result in a higher control (Loya and Hower 2003; Grewal et al. 2004).

There were no significant differences in efficacy between the two application methods, drench and injection. Martínez de Altube et al. (2008) also did not obtain differences between their treatments when applying *S. carpocapsae* mixed with Biorend R® by three application methods: drench, injection and drip irrigation. These results show that the application method has no effect on the capacity of nematodes to establish throughout the different soil layers, invade the galleries of the insect and provide high control. In this trial, when nematodes were applied by drench, a major number of *S. feltiae* were recovered from the superficial soil layer during the first 2 weeks. But from the first week and during the following 6 weeks, nematodes were also recovered at the deepest soil layer (14–20 cm deep), obtaining results comparable to the injection treatment. Because the drench application is an easier method than the injection, drench application is the recommended method for field use.

Persistence of nematodes might be important to obtain sustainable effects. Nematodes were found until 6 weeks after the last application. Favourable application conditions are usually obtained during spring and autumn when soil moisture is higher

than in summer. Egg laying of *C. tenebrionis* is during early summer. Thus, a treatment in spring will not protect the tree from neonate larvae attacking the trees in July and August. Because the limited persistence of up to 6 weeks a second treatment in autumn would be recommended.

During the dissection of the trees in our experiment, made 28 days after the last nematode application, we did not find any dead larva parasited by nematodes, but many moist and empty galleries of *C. tenebrionis* with frass were observed. Probably temperature and moisture inside the galleries provided optimal conditions for fast disintegration of the insect cadavers. Martínez de Altube et al. (2008) reported that nematode infested larvae were recorded even 170 days after the nematode application, suggesting that the conditions inside roots preserved cadavers, which did not decay due to limited moisture. Some studies confirm that for short periods with insufficient moisture, entomopathogenic nematodes remaining inside the host cadaver is an efficient mechanism for survival (Koppenhöfer et al. 1997; Puza and Mrácek 2007). But *C. tenebrionis* feeding galleries in the wood of living trees seem to produce enough moisture to allow IJs to exit the cadavers and search for other hosts, thus providing optimal conditions for the decomposition of the cadavers. A similar situation was observed by García-del-Pino and Morton (in press) with *Acrolepiopsis assectella* larvae (Lepidotera: Acrolepiidae) which feed on leeks, making galleries in leaves and bulbs. In this study, 9 days after nematode application, 30–44% of dead larvae were not found, probably due to decomposition of the insect killed by nematodes. Thus, the infected larvae detected by Martinez et al. (2008) might not have been infected by the applied nematodes, but by juveniles, which had reproduced in infected cadavers and then invaded surviving insects.

The persistence in soil and the efficacy obtained with the doses tested in the present study indicate that *S. feltiae* (Bpa strain) could contribute to control *C. tenebrionis* in the field. Determining an appropriate minimal concentration of entomopathogenic nematodes will now be the crucial step in order to develop a cost-effective strategy for *C. tenebrionis* control in cherry tree orchards.

Acknowledgements

Many thanks to e-nema GmbH for producing the nematodes, Xavier García to allow us to use his orchard, and Mark Burch for language revision of the manuscript.

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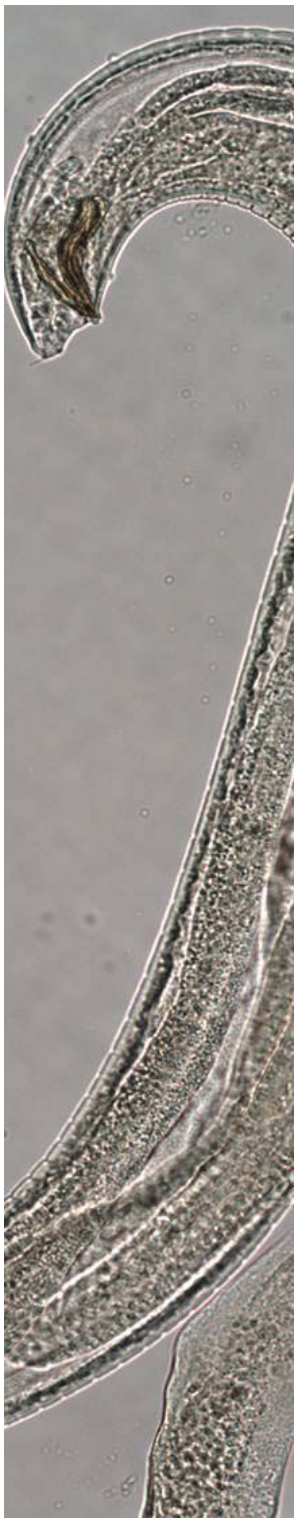


Anexo

Morphological and ecological characterization of entomopathogenic nematode strains isolated in stone-fruit orchard soils of Mediterranean Areas

Journal of Invertebrate Pathology (enviado)

Morphological and ecological characterization of entomopathogenic nematodes isolated in stone fruit orchard soils of Mediterranean areas.

Summary

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae were isolated from stone-fruit orchards in two Mediterranean regions of Spain. A total of 630 soil samples (210 sites) from Catalonia and 90 soil samples (30 sites) from Murcia were evaluated resulting in 5.2% and 20% of the soils testing positive for nematodes, respectively. Ten steinernematid isolates and three heterorhabditid isolates were recovered using the *Galleria mellonella* baiting method. Based on morphometric data, molecular data and cross-breeding experiments the nematode species were identified as *Steinernema feltiae* and *Heterorhabditis bacteriophora*. Environmental tolerance to heat, desiccation and hypoxia, the effect of temperature on infectivity and reproduction and nematode migration in sand columns were compared among isolates and one *S. carpocapsae* strain. Results showed differences among species and a great variability within species. Beneficial traits for each strain were added up to identify a superior candidate to control Mediterranean flat-headed rootborer, *Capnodis tenebrionis.* When all analyzed factors were considered, three *S. feltiae* isolates (Bpa, Sor and M116) obtained the best scores, and when hypoxia was removed, two of the strains (Bpa and Sor) continued ranking superior to other strains.

Keywords

Steinernema feltiae, Steinernema carpocapsae, Heterorhabditis bacteriophora, heat tolerance, desiccation, hypoxia, vertical migration.

Introduction

Entomopathogenic nematodes (EPNs) are biological control agents in the families Steinernematidae and Heterorhabditidae (Adams and Nguyen, 2002). The infective stage of EPNs is the unique free-living stage, the infective juvenile (IJ), which carries in its gut bacteria of the genus *Xenorhabdus* or *Photorhabdus* (Boemare, 2002). The IJs locate and enter the insect host through natural body openings such as the mouth, anus or spiracles (Forst and Clarke, 2002) or, in some cases, directly through the cuticle using a tooth (Kaya and Gaugler, 1993). Once inside the host, symbiotic bacteria are released into the insect haemocoel, causing septicaemia and death of the insect (Kaya and Gaugler, 1993). Nematodes feed on bacteria and disintegrate host tissues growing to adults, and producing two or three generations. When food resources in the host cadaver are exhausted, a new group of IJs is produced and emerges from the cadaver searching for new insect hosts. The infective juvenile is the only stage that prevails in the soil and is subject to environmental stresses.

Surveys of EPNs' isolation have been conducted in different countries in the south of Europe and Mediterranean areas such as Spain (Doucet and Gabarra, 1994; García-del-Pino and Palomo, 1996; Campos-Herrera *et al.*, 2007), Italy (Tarasco and Triggiani, 1997; Triggiani and Tarasco, 2000), France (Emelianoff *et al.*, 2008), Greece (Menti *et al*., 1997), Israel (Glazer *et al.*, 1991), Palestinian Territories (Iraki *et al.*, 2000), Turkey (Kepenekci, 2002; Susurluk *et al.*, 2001; Hazir *et al.*, 2003), Egypt (Shamseldean and Abd-Elgawad, 1994) or Syria (Canhilal *et al.*, 2006). However, there are no specific studies about the natural presence of EPNs in a particular kind of crop as in stone fruits (*Prunus* sp.). One of the most important pests of these crops in the Mediterranean countries is the Mediterranean flat-headed rootborer, *Capnodis tenebrionis* (Coleoptera: Buprestidae) (Morton and García-del-Pino, 2008a). Adults cause defoliation by feeding on twigs and young branches throughout the warm season (Garrido, 1984). Females oviposit on the ground of dry soil, near the trees, and the neonate larvae penetrate into the roots to grow by feeding on the root cortex. The presence of this pest is determined by high temperature and low humidity, with an optimal of 28-34ºC (Balachowsky *et al.*, 1962; Malagón, 1989) and soil humidity below 6% (Malagón, 1989). There are no surveys to assess the role of EPNs as natural controls of *C. tenebrionis*, despite strains of two species of EPNs (*S. feltiae* and *S. carpocapsae*) having been reported to occur naturally in flat-headed rootborer larvae (García-del-Pino, 1994; Morton and García-del-Pino, 2008a; Santos Lobatón *et al.*, 1998).

Studies of the isolation of new EPN species and strains focus on their taxonomic description, but this should be followed by an investigation of their ecological properties (Koppenhöfer and Kaya, 1999) to allow us to optimize the control potential of EPNs against a particular pest. There is variation in the survival and efficacy of infection

among strains within a species depending on abiotic and biotic factors. Differences in tolerance to environmental stress (heat, desiccation, UV and hypoxia) and fitness (virulence and reproductive potential) between strains have been observed using different populations of EPNs isolated from different localities (Gaugler *et al.*, 1989; Grewal *et al.*, 1994; Somasekhar *et al.*, 2002).

The objectives of the present work were to: (i) isolate native strains of EPNs in soils of orchards attacked by *C. tenebrionis*; and (ii) investigate the environmental tolerance of all isolates.

Materials and Methods

Soil Sampling

A total of 720 soil samples were collected at 210 sites in Cataluña and 30 sites in Murcia (Mediterranean regions of Spain). The collection sites were selected from stone-fruit orchards that were attacked by *C. tenebrionis*. The sample method involved collecting three samples of soil per site. Each sampling site was characterized by the type of crop, and latitude, longitude and altitude were all recorded with a GPS (Garmin®). Each soil sample (approximately 1 kg) was a composite of three subsamples collected at a depth of 2-20 cm and at a distance of 2 m from the other two subsamples, around a tree trunk. The three subsamples were pooled in a bucket and mixed gently, but thoroughly, by hand. Samples were placed in a polyethylene bag to prevent water loss, and transported under cool conditions to the laboratory. Each soil sample was sifted and any stones and plant residues were removed. Tap water was used to moisten dry soil if necessary.

In order to extract EPNs from soil samples the insect baiting technique (Bedding and Akhurst, 1975) was used. For each sample six Petri dishes (90 mm diameter) were filled up with soil and six *Galleria mellonella* L. (Lepidoptera: Galleridae) larvae were placed on the soil surface. Petri dishes were inverted, sealed and incubated at 23ºC ± 2ºC in the dark. Larvae were removed from the Petri dishes after seven days. When the result was negative for EPNs the assay was repeated once as above. Dead larvae from the baiting showing signs of infection were placed individually on modified White traps (Kaya and Stock, 1997) and held at 23ºC ± 2ºC. Infective juveniles were collected and stored at 7ºC. Some of the collected IJs were exposed to fresh *G. mellonella* larvae to confirm pathogenicity and to establish new cultures.

Soil samples containing EPNs were analyzed for soil type (USDA textural analysis), electrical conductivity (EC, as a proxy for salinity), organic matter and pH.

Identification of nematodes

Nematode isolates were identified by morphological criteria using a Zeiss Axio A-1 microscope equipped with differential interference contrast optics and a digital camera system, and scanning electron microscopy (SEM). Identification of nematodes was attempted by making mounts to 20 IJs and 20 first generation males for each isolate. Nematodes were killed, fixed in TAF, processed in glycerine and mounted as described in detail by Kaya and Stock (1997). Specimen measurements were made using DPxViewPro EE EF-Image Management software (DeltaPix) calibrated using a stage micrometer. The characters analyzed for identification were: total body length, maximum body width, distance from anterior end to excretory pore, distance from anterior end to nerve ring, distance from anterior end to base of oesophagus, tail length, body width at anus/cloaca and values of ratio a (total body length divided by maximum body width), b (total body length divided by oesophagus length), c (total body length divided by tail length), c' (tail length divided by anal/cloacal body width), and D (distance from anterior end to excretory pore divided by oesophagus length) (Adams and Nguyen, 2002; Hominick *et al.*, 1997). Within males, length of the spicules, length of the gubernaculum, length and width of the manubrium, length of the mucron, values of SW (length of spicules divided by anal body width) and GS (length of gubernaculum divided by length of spicules) were also examined. For IJs, values of H (hyaline portion in relation to length of tail) and E (distance from anterior end to excretory pore divided by tail length) were also considered. IJs of each isolate were processed following the method described by Hominick *et al.* (1997) to examine lateral fields by a scanning electronic microscopy (SEM).

In addition to morphological studies, one isolate (Bpa *Steinernema feltiae* strain) was selected for molecular characterization. This isolate was subjected to RFLP analysis as described by Reid and Hominick (1992) and compared to A1 and A2 RFLP variants of *S. feltiae* isolated in the UK (Reid *et al.*, 1997). The restriction enzymes used were Alu I, Dde I, Hae III, Hha I, Hinf I, Pst I, Rsa I, Sau3A I and Sau96 I.

For confirming the identity of the *Steinernema* isolates, cross-breeding experiments were carried out using the hanging blood drop technique (Kaya and Stock, 1997). The control treatments were also conducted in the same way except IJs from the same nematode population were used. Twenty replicates were made for each treatment. Slides were then incubated in Petri dishes lined with moist filter paper at 23ºC. The development and reproduction of the nematodes was observed every day. The experiment was repeated one time.

Ecological characterization

The nematodes used in these experiments were all the nematode strains isolated from the orchard soils and *C. tenebrionis* larva cadaver, and a *S. carpocapsae* strain (B14) isolated from an urban garden in Barcelona (Barcelona). The *S. carpocapsae* strain was evaluated because this species was isolated from a larva of *C. tenebrionis* by Santos Lobatón *et al.* (1998). All nematode strains were reared at 25ºC in last instar larvae of the greater wax moth, *G. mellonella*, according to procedures described by Woodring and Kaya (1998). IJs that emerged from cadavers were recovered using modified White traps (Kaya and Stock, 1997) and stored at 7ºC for 7-14 days before use. The nematodes in water suspensions were allowed to acclimate at room temperature (23ºC) for 12 h prior to use.

Heat tolerance

Petri dishes (50 mm diameter) were filled with 8 g of sterilized sand (15% water w/w). Twenty microliters of nematode inoculum containing 100 IJs was placed on the bottom. Dishes were sealed with Parafilm and incubated in the dark at 25, 30, 32, 35, 37, 40 and 42ºC during 2, 4, 6, 8, 10 and 12 h. There were 20 dishes per nematode strain, temperature and exposition time. After exposure, dishes were incubated at 25ºC during 24 h. Ten Petri dishes were transferred to Baermann funnels during 2 h and living nematodes were counted using a stereomicroscope. Nematodes were considered live if they were naturally moving or responded to probing with a fine needle. *Galleria mellonella* larvae were exposed individually in the other ten Petri dishes. Mortality of wax moth larvae was checked daily. Dead larvae were removed from Petri dishes, rinsed in distilled water and placed individually in dishes containing wet filter paper (Whatmann No. 1) for two days at 25ºC. Then each cadaver was dissected and adult nematodes were counted. The experiment was conducted twice.

Effect of Temperature on Infectivity

Wells (7.5 mm diameter) of tissue culture plates were filled with 0.5 g of sterilized sand and one *G. mellonella* (250-350 mg weight) was placed in each well. Plates had already equilibrated to the temperatures tested 30 minutes prior to the addition of nematodes. Sixty microliters of tap water containing 50 IJs were added per well. Plates were sealed with Parafilm and incubated in the dark at 5, 8, 10, 15, 20, 25, 28, 30, 32, 35 and 37ºC. The wells were checked every 24 h for *G. mellonella* mortality. Cadavers were removed from wells, and placed in Petri dishes filled with three filter papers (Whatmann No. 1) at 25ºC during three days, for larvae tested at 5-10ºC, two days for those tested at 15-20ºC and one day for larvae tested at 25-37ºC. After this period larvae were dissected and the number of nematodes established was counted. Each well was considered as one

replicate and there were 20 replicates per strain and temperature. The experiment was conducted twice.

Effect of Temperature on Reproduction

Last instar *G. mellonella* larvae (250-300 mg weight) were exposed individually to IJs in Petri dishes (50 mm diameter). Each plate was lined with 3 filter papers (Whatmann No. 1) and 50 IJs nematodes were added in 1 ml of tap water. The plates were sealed with Parafilm and incubated in the dark at 25ºC until death of the larvae. Cadavers were transferred to White traps and incubated at 5, 8, 10, 15, 20, 25, 28, 30 and 32ºC. The total number of IJs produced was counted. There were 10 replicates per strain and temperature and the experiment was repeated once.

Desiccation

Tolerance to desiccation was evaluated based on procedures described by Liu and Glazer (2000). Approximately 10,000 IJs were concentrated by vacuum filtration onto 50 mm diameter filter paper disks (Whatmann No. 1). Disks were placed on a cover of a Petri dish (50 mm diameter) and transferred to standard desiccators (Nalgene, Rochester, NY, USA) and exposed to different relative humidity (RH) levels generated using saturate salt solutions at 25ºC. Salt solutions used were K_2SO_4 for 97% RH, KNO_3 for 93% RH, $ZnSO_4.7H_20$ for 88% RH and KCl for 85% (Winston and Bates, 1960). After 72 h nematodes were rehydrated by immersion into Petri dishes (50 mm diameter) containing 10 ml of sterile tap water during 24 h. The number of dead and live IJs was counted under a stereomicroscope by taking three 50 µl samples for each plate and percentage survival was calculated. Nematode viability was determined by observing motility and they were considered dead if not responding to probing with a fine needle. There were four replications per strain and the experiment was repeated twice.

Hypoxia

Tolerance to hypoxia was evaluated based on procedures described by Somasekhar *et al.* (2002). Ten thousand IJs were held in Eppendorf tubes (1.5 ml capacity) with 1.5 ml of sterile tap water. Tubes were closed, placed horizontally in a shaker at 150 rpm and incubated in the dark at 25ºC. After 24, 48, 72 or 96 h nematodes were transferred to Petri dishes (50 mm diameter) containing 8.5 ml of sterile tap water and incubated at 25ºC during 24 h. The number of dead and live IJs was counted as above. Control treatment consisted of Petri dishes with 10 ml of water and IJs at the same concentration. The experiment was performed three times and there were 10 replications per strain and time.

Nematode migration in sand columns

PVC tubes having a 2.8 cm internal diameter and 20 cm height were used. A Petri dish lid of 5 cm diameter was put on the bottom of each tube with one *G. mellonella* immobilized by an inox mesh. Tubes were filled with 220 g of sterilized sand (particle size: 200-500 µm) that had been adjusted to 15% moisture (w/w). A 200 µm aliquot of sterile water containing approximately 1000 IJs was pipetted at the top of each tube and the top was sealed with a plastic lid to prevent desiccation. Control tubes were left without an insect. Tubes were kept vertically and incubated in the dark at 25ºC during 72h. After this period tubes were removed and larvae mortality was controlled. Live larvae were washed with sterile tap water and placed in Petri dishes at 25ºC during 48h to verify if they were infected. Sand columns were divided into four sections (50 mm), and each sample was placed in a beaker and washed three times with water to liberate the nematodes. The number of nematodes in the supernatant of each section was calculated by taking ten 1 ml samples and counted under a microscope. There were six sand columns per strain (five with one *Galleria* and the control). The experiment was replicated three times.

Qualitative analysis of beneficial traits among strains to control C. tenebrionis

Strains were compared based on procedures described by Shapiro-Ilan *et al.* (2003). Each strain was scored as 1 if performance did not differ significantly from the highest level for a trait, -1 if performance did not differ significantly from the lowest level for that trait, and 0 if performance was between the highest and lowest, or did not differ from either. The scores among traits were then added for each strain. For the traits comparison virulence to larvae, pupae and adults of *Capnodis tenebrionis* of a previous study (Morton and García-del-Pino, 2008b) were included.

Statistics

The variation in valuable morphometric variables according to Hominick *et al.* (1997), such as body length, distance from head to excretory pore, tail length, hyaline portion, cloacal body width, spicule length, gubernaculums length, SW, GS and D, of IJs and males of the *Steinernema* isolates, was examined through principal component analysis (PCA). Hierarchical cluster analysis was performed to explore the relation among different EPN strains, using squared Euclidean distance (SPSS 15.0 software).

Data on nematode establishment, reproduction, tolerance to desiccation, tolerance to hypoxia and migration in sand columns were subjected to analysis of variance (ANOVA) performed with the SPSS (15.0) computer package. The data obtained as percentages were arcsine transformed (arsine of square root) before they were subjected to the

analysis. When a significant F-value ($p < 0.05$) was found, differences between isolates were compared using Tukey's test mean separation test.

Results

Soil Sampling

EPNs were recovered from 11 (5.2%) of the 210 sites sampled in Catalonia and from 6 (20%) of the 30 sites sampled in Murcia. The characteristics of soils testing positive for nematodes are detailed in Table 1. *Steinernema* isolates were recovered from 14 locations, 10 in Catalonia and four in Murcia, and *Heterorhabditis* sp. isolates were found in three locations, one in Catalonia and two in Murcia. The steinernematid strain Bpa was isolated from an infected *C. tenebrionis* larva collected from inside a cherry tree trunk. The altitude of steinernematid soil samples varied from 2 to 693 m and for heterorhabditids from 5 to 374 m. Soil types were classified from sandy loam to silt loam. Steinernematid species were isolated from neutral (pH 6.7) to alkaline (pH 8.6) soils with organic matter content ranging from 0.84 to 3.21% and conductivity varying from 0.24 to 0.61. *Heterorhabditis* sp. species were found in alkaline (pH 8.3-8.5) soils with medium organic matter contents (2.15%-3.8%) and conductivity of 0.25 to 1.1.

Identification of strains

Morphological examination indicated that all *Steinernema* isolates recovered belong to *S. feltiae* (Tables 2 and 3). SEM examination of IJs showed eight ridges in lateral fields of all *Steinernema* isolates, characteristic of *S. feltiae.* Figure 1 shows two clusters, of males and IJs which present the relation, based on morphometric characters, among native *S. feltiae* isolates and *S. feltiae* according to Nguyen *et al.* (2006). Both IJs and males isolates were closer among them than with *S. feltiae* according to Nguyen *et al.* (2006). The molecular characterization of the Bpa isolate confirmed that it was *S. feltiae* (Filipjev). This strain was compared with the A1 RFLP variants of *S. feltiae* and revealed differences in the Hinf I and Rsa I digests, showing that Bpa belonged to the A2 RFLP variant (Figure 2). Cross-breeding experiments between each of the *Steinernema* isolates and Bpa strain resulted in fertile progeny thus confirming that all *Steinernema* isolates belong to *S. feltiae.* Morphometric identification of the three *Heterorhabditis* isolates (Tables 4 and 5) showed that they belong to *H. bacteriophora* (Poinar).

Table 1. Entomopathogenic nematode strains isolated from Catalonia and Murcia (Spain) showing the location and soil characteristics.

Location	GPS coordinates	Altitude (m)	Orchard	Soil type	pH	Conductivity (dS/m)	% OM	Strain
Torrelles del Llobregat (Barcelona)	N41º21.908' E001º58.753'	173	Cherry	Sandy loam	8.1	0.48	3.21	Bt2
Torrelles del Llobregat (Barcelona)	N41º21.774' E001º58.013'	263	Cherry	Sandy loam	7.9	0.32	2.43	Bt4
Can Güell (Barcelona)	N41º21.022' E001º58.001'	226	Cherry	Sandy loam	8.2	0.48	2.65	Bsor
Torrelles del Llobregat (Barcelona)	N41º21.578' E001º57.654'	200	Cherry	* From a parasited *C. tenebrionis* larva				Bpa
Els Guiamets (Tarragona)	N41º06.171' E000º44.692'	197	Peach	Silt loam	6.7	0.34	1.27	T91
Falset (Tarragona)	N41º09.248' E000º48.774'	300	Cherry	Silt loam	8.6	0.24	0.94	T92
Sant Pere Pescador (Girona)	N42º11.522' E003º05.585'	2	Peach	Silt loam	8.6	0.61	1.95	Gsp
L'Armentera (Girona)	N42º09.657' E003º35.586'	5	Apple	Sandy loam	8.5	0.25	2.47	Gscl
Almenar (Lleida)	N41º47.300' E000º35.539'	255	Apple	Silt loam	8.5	0.23	3.7	L9
Alfarràs (Lleida)	N41º49.426' E000º34.386'	271	Pear	Silt loam	8.7	0.24	1.25	L11
Alguaire (Lleida)	N41º44.142' E000º36.169'	256	Apple	Loam	8.2	0.25	0.84	L12
Casablanca (Murcia)	N38º17.660' W001º17.310'	394	Apricot	Loam	8.4	1.1	3.8	M110
Calasparra (Murcia)	N38º16.451' W001º35.113'	291	Cherry	Silt loam	8.3	0.59	2.15	M115
Casa del Francés (Murcia)	N37º56.824' W001º38.979'	632	Cherry	Loam	8.5	0.31	1.08	M116
Casa del Francés (Murcia)	N37º56.825' W001º38.969'	636	Cherry	Loam	8.5	0.26	1.23	M117
Pliego (Murcia)	N37º58.635' W001º30.865'	379	Apricot	Loam	8.5	0.27	1.56	M118
Mula (Murcia)	N38º06.763' W001º32.129'	496	Apricot	Loam	8.5	0.28	1.47	M123

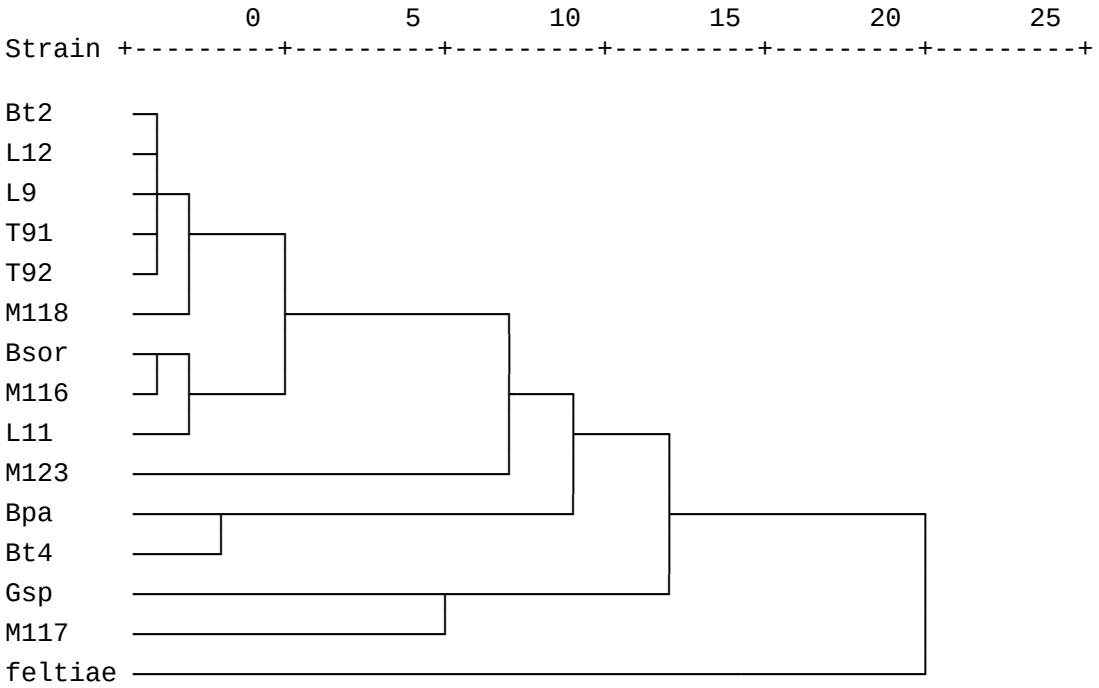
Table 2. Morphometric characters (in µm) of infective juveniles isolated from Catalonia and Murcia (Spain) comparable to *Steinernema feltiae*. Measurements in form: mean ± SD (range).

Character	**Bpa**	**Bt2**	**Bt4**	**Bsor**	**Gsp**	**L9**	**L11**	**L12**	**T91**	**T92**	**M116**	**M117**	**M118**	**M123**	***S. feltiae* (Nguyen *et al.*,2006)**
Total body length	946 ± 41 (873-1017)	834 ± 35 (769-883)	910 ± 47 (828-983)	845 ± 37 (752-924)	756 ± 41 (697-828)	840 ± 47 (744-913)	846 ± 37 (752-908)	839 ± 48 (746-912)	833 ± 36 (757-883)	869 ± 30 (819-922)	840 ± 42 (745-946)	787 ± 60 (662-930)	878 ± 25 (831-922)	872 ± 38 (789-939)	879 ± 49 (766-928)
Maximum body width	31± 7.5 (30-38)	28 ± 1.1 (26-30)	29 ± 1.8 (24-31)	27 ± 1.6 (24-31)	27 ± 1.5 (24-31)	29 ± 1.5 (26-31)	28 ± 1.5 (24-31)	29 ± 1.6 (26-31)	27 ± 1.3 (25-30)	28 ± 2 (26-34)	30 ± 1.7 (26-33)	26 ± 2.1 (23-31)	29 ± 1.6 (27-34)	30 ± 1.3 (28-31)	29 ± 1.9 (26-32)
Distance anterior end to excretory pore	64 ± 3.1 (58-70)	58 ± 3.7 (47-67)	62 ± 3 (57-67)	59 ± 2.3 (54-62)	57 ± 2.7 (52-61)	57 ± 4.2 (43-63)	61 ± 3.2 (54-66)	57 ± 4 (44-63)	58 ± 3.2 (54-67)	58 ± 2.6 (53-63)	61 ± 2.9 (56-66)	59 ± 3.6 (52-65)	59 ± 2.2 (55-63)	58 ± 3.3 (48-63)	63 ± 2.3 (58-67)
Distance anterior end to nerve ring	105 ± 5.5 (97-118)	95 ± 5.2 (84-104)	100 ± 4.4 (90-108)	93 ± 5.1 (80-100)	88 ± 5.7 (77-98)	94 ± 6.3 (77-104)	99 ± 4.3 (91-106)	94 ± 5.4 (80-101)	95 ± 4.7 (86-104)	95 ± 6.7 (86-113)	99 ± 4.2 (91-105)	95 ± 6.4 (83-105)	97 ± 5.2 (86-113)	102 ± 10 (87-138)	113 ± 5.1 (108-117)
Distance anterior end to esophagus base	133 ± 4.9 (126-143)	126 ± 5.6 (113-134)	131 ± 5.8 (120-139)	122 ± 6.7 (108-134)	114 ± 10 (79-127)	121 ± 7.1 (100-130)	129 ± 4.6 (120-137)	120 ± 6.1 (102-126)	126 ± 4 (119-134)	125 ± 8.1 (113-143)	126 ± 3.7 (118-132)	124 ± 7 (106-140)	127 ± 6.9 (113-143)	131 ± 11 (113-163)	136 ± 3.5 (130-143)
Tail length	80 ± 3.3 (73-86)	74 ± 4.1 (68-84)	80 ± 5.3 (69-85)	75 ± 3.9 (69-82)	68 ± 5.9 (54-78)	75 ± 4.6 (68-82)	74 ± 4.3 (69 ± 82)	75 ± 5.2 (67-82)	74 ± 3.3 (69-80)	74 ± 3.9 (68-81)	72 ± 4.7 (63-80)	75 ± 7.1 (61-91)	74 ± 3.9 (68-81)	79 ± 4.3 (70-86)	86 ± 2.6 (81-89)
Anal body width	18 ±1.1 (16-20)	28 ± 1.1 (26-30)	17 ± 1 (15-18)	17 ± 0.7 (15-18)	15 ± 1.2 (14-19)	17 ± 0.8 (15-18)	17 ± 1.1 (15-20)	16 ± 1 (15-18)	17 ± 0.8 (15-18)	16 ± 1.1 (14-18)	17 ± 0.8 (16-19)	16 ± 1.2 (13-18)	17 ± 0.9 (15-18)	17 ± 0.9 (15-19)	18 ± 0.8 (16-19)
Hyaline portion length	27 ± 2.7 (20-31)	26 ± 4.2 (17-33)	27 ± 3.3 (20-36)	28 ± 3.7 (21-35)	27 ± 4.4 (19-33)	26 ± 4.2 (19-35)	29 ± 3.8 (23-36)	26 ± 3.6 (19-31)	24 ± 3 (17-28)	26 ± 3.2 (20-31)	26 ± 4.8 (17-34)	33 ± 4.1 (25-41)	28 ± 3.4 (21-35)	29 ± 4.1 (21-35)	38 ± 3.2 (32-43)
Ratio a	29 ±1.6 (25-32)	30 ± 1.7 (27-34)	32 ± 1.4 (30-35)	31 ± 2 (26-34)	29 ± 4.7 (27-49)	29 ± 2.1 (25-33)	31 ± 1.7 (28-35)	29 ± 2.3 (26-33)	31 ± 2.2 (27-35)	31 ± 1.5 (7-33)	28 ± 1.5 (25-31)	30 ± 2 (26-33)	30 ± 1.4 (27-32)	30 ± 0.9 (28-32)	30 ± 1.9 (27-34)
Ratio b	7.1 ± 0.3 (6.5-7.9)	6.6 ± 0.4 (5.9-7.5)	6.9 ± 0.5 (6.2-8.2)	6.9 ± 0.4 (6.1-7.8)	6.7 ± 0.7 (6-8.8)	7 ± 0.4 (6.4-7.7)	6.5 ± 0.3 (5.9-7.1)	7 ± 0.4 (6.4-7.6)	6.6 ± 0.3 (5.9-7.2)	7 ± 0.4 (6.2-7.6)	6.7 ±0.3 (6-7.3)	6.4 ± 0.3 (5.6-6.7)	6.9 ± 0.3 (6.2-7.6)	6.7 ± 0.5 (5.4-7.8)	6.4 ± 0.3 (5.8-6.8)
Ratio c	12 ±0.5 (11-13)	11 ± 0.5 (10-12)	11 ± 0.6 (11-13)	11 ± 0.5 (11-12)	11 ± 0.7 (10-13)	11 ± 0.5 (10-12)	11 ± 0.6 (11-12)	11 ± 0.4 (10-12)	11 ± 0.4 (10-12)	12 ± 0.6 (11-13)	12 ± 0.6 (11-13)	11 ± 0.5 (9.7-12)	12 ± 0.6 (11-13)	11 ± 0.6 (10-13)	10 ± 0.5 (9.4-11)
Ratio c'	4.4 ± 0.3 (3.9-5.2)	4.5 ± 0.4 (3.8-5.5)	4.7 ± 0.3 (4.2-5.1)	4.5 ± 0.3 (4.1-5.2)	4.5 ± 0.4 (3.8-5.1)	4.5 ± 0.4 (3.8-5.3)	4.3 ± 0.3 (3.9-5.2)	4.6 ± 0.4 (3.8-5.3)	4.5 ± 0.3 (3.9-5.1)	4.6 ± 0.2 (4.2-5)	4.2 ± 0.3 (3.6-4.8)	4.8 ± 0.3 (4.2-5.6)	4.4 ± 0.3 (4-5.2)	4.6 ± 0.3 (4.1-5)	4.8 ± 0.2 (4.5-5.1)
D%	48 ± 2.8 (42-55)	46 ± 2.8 (40-53)	48 ± 3.1 (43-54)	48 ± 2.7 (44-56)	50 ± 5.4 (45-69)	47 ± 3.5 (36-52)	47 ± 1.6 (44-49)	47 ± 3.4 (36-53)	46 ± 2.7 (44-53)	47 ± 2.2 (44-52)	48 ± 2.2 (45-53)	48 ± 2.5 (43-54)	47 ± 1.8 (44-52)	44 ± 3.9 (36-52)	46 ± 1.4 (44-50)
H%	33 ± 3.5 (27-40)	35 ± 5.6 (24-46)	34 ± 4.2 (27-43)	37 ± 4.8 (28-48)	40 ± 7.4 (28-54)	35 ± 5.8 (27-45)	39 ± 4.7 (31-49)	34 ± 5 (28-44)	32 ± 4.1 (24-40)	35 ± 3.6 (29-41)	36 ± 6.3 (26-47)	44 ± 4.6 (36-53)	37 ± 4 (30-44)	36 ± 4.6 (27-44)	44 ± 4 (37-51)
E%	80 ±5 (72-92)	79 ± 6.1 (60-87)	78 ± 4.7 (69-87)	79 ± 4.8 (71-90)	83 ± 7.1 (76-104)	75 ± 5.1 (58-81)	83 ± 5.7 (71-93)	75 ± 6.8 (53-83)	79 ± 3.6 (73-86)	79 ± 5.2 (69-88)	84 ± 4.3 (77-93)	80 ± 6.5 (72-96)	80 ± 4.3 (71-88)	74 ± 4.8 (66-85)	74 ± 4 (67-81)
n	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

Table 3. Morphometric characters (in µm) of 1st generation males isolated from Catalonia and Murcia (Spain) comparable to *Steinernema feltiae*. Measurements in form: mean ± SD (range).

Character	**Bpa**	**Bt2**	**Bt4**	**Bsor**	**Gsp**	**L9**	**L11**	**L12**	**T91**	**T92**	**M116**	**M117**	**M118**	**M123**	***S. feltiae* (Nguyen *et al.*,2006)**
Total body length	946 ± 41 (873-1017)	834 ± 35 (769-883)	910 ± 47 (828-983)	845 ± 37 (752-924)	756 ± 41 (697-828)	840 ± 47 (744-913)	846 ± 37 (752-908)	839 ± 48 (746-912)	833 ± 36 (757-883)	869 ± 30 (819-922)	840 ± 42 (745-946)	787 ± 60 (662-930)	878 ± 25 (831-922)	872 ± 38 (789-939)	879 ± 49 (766-928)
Maximum body width	31± 7.5 (30-38)	28 ± 1.1 (26-30)	29 ± 1.8 (24-31)	27 ± 1.6 (24-31)	27 ± 1.5 (24-31)	29 ± 1.5 (26-31)	28 ± 1.5 (24-31)	29 ± 1.6 (26-31)	27 ± 1.3 (25-30)	28 ± 2 (26-34)	30 ± 1.7 (26-33)	26 ± 2.1 (23-31)	29 ± 1.6 (27-34)	30 ± 1.3 (28-31)	29 ± 1.9 (26-32)
Distance anterior end to excretory pore	64 ± 3.1 (58-70)	58 ± 3.7 (47-67)	62 ± 3 (57-67)	59 ± 2.3 (54-62)	57 ± 2.7 (52-61)	57 ± 4.2 (43-63)	61 ± 3.2 (54-66)	57 ± 4 (44-63)	58 ± 3.2 (54-67)	58 ± 2.6 (53-63)	61 ± 2.9 (56-66)	59 ± 3.6 (52-65)	59 ± 2.2 (55-63)	58 ± 3.3 (48-63)	63 ± 2.3 (58-67)
Distance anterior end to nerve ring	105 ± 5.5 (97-118)	95 ± 5.2 (84-104)	100 ± 4.4 (90-108)	93 ± 5.1 (80-100)	88 ± 5.7 (77-98)	94 ± 6.3 (77-104)	99 ± 4.3 (91-106)	94 ± 5.4 (80-101)	95 ± 4.7 (86-104)	95 ± 6.7 (86-113)	99 ± 4.2 (91-105)	95 ± 6.4 (83-105)	97 ± 5.2 (86-113)	102 ± 10 (87-138)	113 ± 5.1 (108-117)
Distance anterior end to esophagus base	133 ± 4.9 (126-143)	126 ± 5.6 (113-134)	131 ± 5.8 (120-139)	122 ± 6.7 (108-134)	114 ± 10 (79-127)	121 ± 7.1 (100-130)	129 ± 4.6 (120-137)	120 ± 6.1 (102-126)	126 ± 4 (119-134)	125 ± 8.1 (113-143)	126 ± 3.7 (118-132)	124 ± 7 (106-140)	127 ± 6.9 (113-143)	131 ± 11 (113-163)	136 ± 3.5 (130-143)
Tail length	80 ± 3.3 (73-86)	74 ± 4.1 (68-84)	80 ± 5.3 (69-85)	75 ± 3.9 (69-82)	68 ± 5.9 (54-78)	75 ± 4.6 (68-82)	74 ± 4.3 (69 ± 82)	75 ± 5.2 (67-82)	74 ± 3.3 (69-80)	74 ± 3.9 (68-81)	72 ± 4.7 (63-80)	75 ± 7.1 (61-91)	74 ± 3.9 (68-81)	79 ± 4.3 (70-86)	86 ± 2.6 (81-89)
Anal body width	18 ±1.1 (16-20)	28 ± 1.1 (26-30)	17 ± 1 (15-18)	17 ± 0.7 (15-18)	15 ± 1.2 (14-19)	17 ± 0.8 (15-18)	17 ± 1.1 (15-20)	16 ± 1 (15-18)	17 ± 0.8 (15-18)	16 ± 1.1 (14-18)	17 ± 0.8 (16-19)	16 ± 1.2 (13-18)	17 ± 0.9 (15-18)	17 ± 0.9 (15-19)	18 ± 0.8 (16-19)
Hyaline portion length	27 ± 2.7 (20-31)	26 ± 4.2 (17-33)	27 ± 3.3 (20-36)	28 ± 3.7 (21-35)	27 ± 4.4 (19-33)	26 ± 4.2 (19-35)	29 ± 3.8 (23-36)	26 ± 3.6 (19-31)	24 ± 3 (17-28)	26 ± 3.2 (20-31)	26 ± 4.8 (17-34)	33 ± 4.1 (25-41)	28 ± 3.4 (21-35)	29 ± 4.1 (21-35)	38 ± 3.2 (32-43)
Ratio a	29 ±1.6 (25-32)	30 ± 1.7 (27-34)	32 ± 1.4 (30-35)	31 ± 2 (26-34)	29 ± 4.7 (27-49)	29 ± 2.1 (25-33)	31 ± 1.7 (28-35)	29 ± 2.3 (26-33)	31 ± 2.2 (27-35)	31 ± 1.5 (7-33)	28 ± 1.5 (25-31)	30 ± 2 (26-33)	30 ± 1.4 (27-32)	30 ± 0.9 (28-32)	30 ± 1.9 (27-34)
Ratio b	7.1 ± 0.3 (6.5-7.9)	6.6 ± 0.4 (5.9-7.5)	6.9 ± 0.5 (6.2-8.2)	6.9 ± 0.4 (6.1-7.8)	6.7 ± 0.7 (6-8.8)	7 ± 0.4 (6.4-7.7)	6.5 ± 0.3 (5.9-7.1)	7 ± 0.4 (6.4-7.6)	6.6 ± 0.3 (5.9-7.2)	7 ± 0.4 (6.2-7.6)	6.7 ±0.3 (6-7.3)	6.4 ± 0.3 (5.6-6.7)	6.9 ± 0.3 (6.2-7.6)	6.7 ± 0.5 (5.4-7.8)	6.4 ± 0.3 (5.8-6.8)
Ratio c	12 ±0.5 (11-13)	11 ± 0.5 (10-12)	11 ± 0.6 (11-13)	11 ± 0.5 (11-12)	11 ± 0.7 (10-13)	11 ± 0.5 (10-12)	11 ± 0.6 (11-12)	11 ± 0.4 (10-12)	11 ± 0.4 (10-12)	12 ± 0.6 (11-13)	12 ± 0.6 (11-13)	11 ± 0.5 (9.7-12)	12 ± 0.6 (11-13)	11 ± 0.6 (10-13)	10 ± 0.5 (9.4-11)
Ratio c'	4.4 ± 0.3 (3.9-5.2)	4.5 ± 0.4 (3.8-5.5)	4.7 ± 0.3 (4.2-5.1)	4.5 ± 0.3 (4.1-5.2)	4.5 ± 0.4 (3.8-5.1)	4.5 ± 0.4 (3.8-5.3)	4.3 ± 0.3 (3.9-5.2)	4.6 ± 0.4 (3.8-5.3)	4.5 ± 0.3 (3.9-5.1)	4.6 ± 0.2 (4.2-5)	4.2 ± 0.3 (3.6-4.8)	4.8 ± 0.3 (4.2-5.6)	4.4 ± 0.3 (4-5.2)	4.6 ± 0.3 (4.1-5)	4.8 ± 0.2 (4.5-5.1)
D%	48 ± 2.8 (42-55)	46 ± 2.8 (40-53)	48 ± 3.1 (43-54)	48 ± 2.7 (44-56)	50 ± 5.4 (45-69)	47 ± 3.5 (36-52)	47 ± 1.6 (44-49)	47 ± 3.4 (36-53)	46 ± 2.7 (44-53)	47 ± 2.2 (44-52)	48 ± 2.2 (45-53)	48 ± 2.5 (43-54)	47 ± 1.8 (44-52)	44 ± 3.9 (36-52)	46 ± 1.4 (44-50)
H%	33 ± 3.5 (27-40)	35 ± 5.6 (24-46)	34 ± 4.2 (27-43)	37 ± 4.8 (28-48)	40 ± 7.4 (28-54)	35 ± 5.8 (27-45)	39 ± 4.7 (31-49)	34 ± 5 (28-44)	32 ± 4.1 (24-40)	35 ± 3.6 (29-41)	36 ± 6.3 (26-47)	44 ± 4.6 (36-53)	37 ± 4 (30-44)	36 ± 4.6 (27-44)	44 ± 4 (37-51)
E%	80 ±5 (72-92)	79 ± 6.1 (60-87)	78 ± 4.7 (69-87)	79 ± 4.8 (71-90)	83 ± 7.1 (76-104)	75 ± 5.1 (58-81)	83 ± 5.7 (71-93)	75 ± 6.8 (53-83)	79 ± 3.6 (73-86)	79 ± 5.2 (69-88)	84 ± 4.3 (77-93)	80 ± 6.5 (72-96)	80 ± 4.3 (71-88)	74 ± 4.8 (66-85)	74 ± 4 (67-81)
n	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

A



B

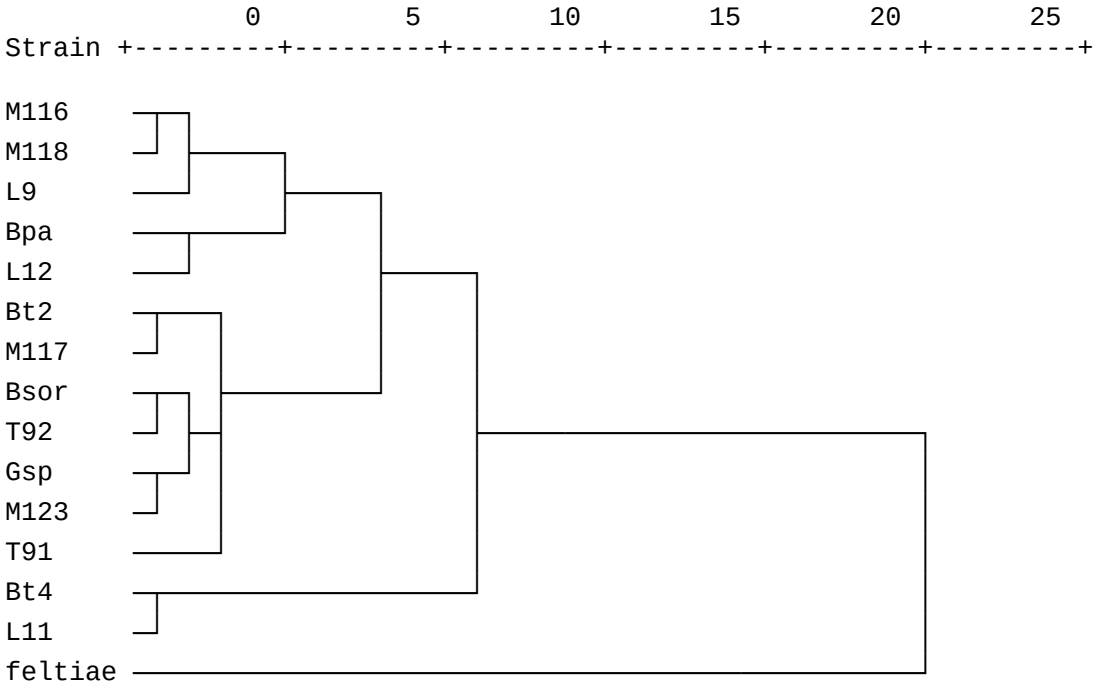


Figure 1. Cluster of relationship among isolates of *Steinernema feltiae* and compared to *S. feltiae* (Nguyen *et al.*, 2006). A: Infective juveniles; B: Males.

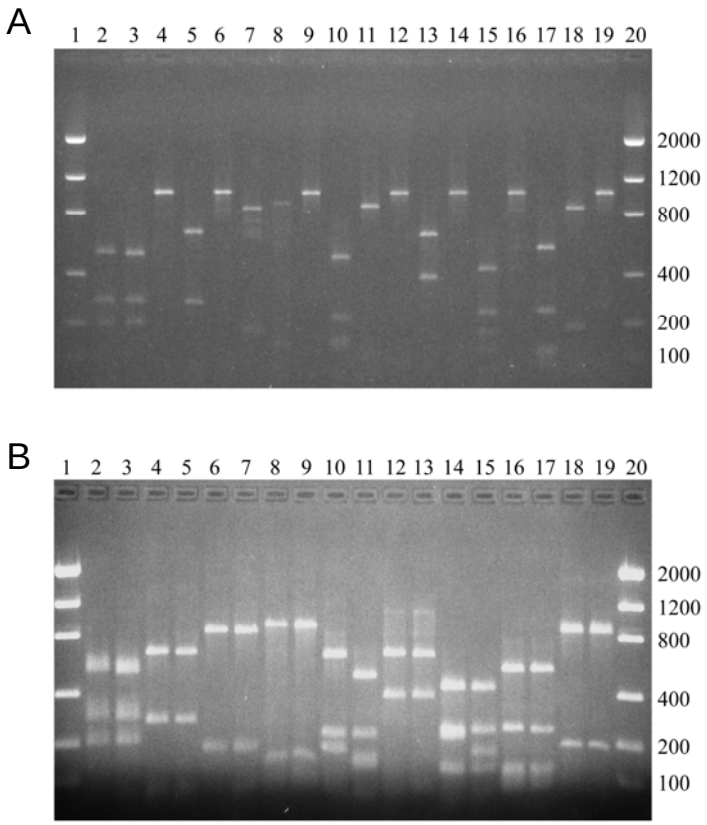


Figure 2. RFLP-PCR analysis of Bpa strain: (A) Gel lane 2 is a digest of Bpa with *Alu* I. Lanes 3-19 are individual digests with the following restriction enzymes: 3, *Alu* I; 4, *BstO* I; 5, *Dde* I; 6, *EcoR* I; 7, *Hae* III; 8, *Hha* I; 9, *Hind* III; 10, *Hinf* I; 11, *Hpa* II; 12, *Kpn* I; 13, *Pst* I; 14, *Pvu* II; 15, *Rsa* I; 16, *Sal* I; 17, *Sau*3A I; 18, *Sau*96 I*;* 19, *Xba* I. Lanes 1 and 20 are a molecular weight marker with band sizes shown in base pairs. (B) Comparison of RFLP profiles for *S. feltiae (*A1 RFLP type) and *Steinernema* sp. Bpa strain. Lane order: 1 and 20, molecular weight standard (bp); 2, *S. feltiae Alu* I; 3, Bpa *Alu* I; 4, *S. feltiae Dde* I *; 5, Bpa Dde* I*; 6, S. feltiae Hae* III; 7, Bpa *Hae* III; 8, *S. feltiae Hha* I; 9, Bpa *Hha* I; 10, *S. feltiae Hinf* I; 11, Bpa *Hinf* I; 12*, S. feltiae Pst* I *;* 13*, Bpa Pst* I *;* 14*,S. feltiae Rsa* I; 15, Bpa *Rsa* I; 16, *S. feltiae Sau*3A I; 17, Bpa *Sau*3A I; 18, *S. feltiae Sau*96 I; Bpa *Sau*96 I*.*

Table 4. Morphometric characters (in µm) of infective juveniles isolated from Catalonia and Murcia (Spain) comparable to *Heterorhabditis bacteriophora.* Measurements in form: mean ± SD (range).

Character	**Gscl**	**M110**	**M115**	***H. bacteriophora* (after Poinar 1975, 1990)**
Total body length	582 ± 20 (537-611)	590 ± 17 (560-622)	588 ± 32 (522-638)	588 (512-671)
Maximum body width	23 ± 1.2 (21-25)	23 ± 1.1 (20-24)	26 ± 2.3 (20-28)	23 (18-31)
Distance anterior end to excretory pore	107 ± 4.7 (101-116)	104 ± 6.4 (94-120)	106 ± 5.8 (88-113)	103 (87-110)
Distance anterior end to nerve ring	87 ± 3.9 (82-96)	87 ± 3.7 (79-93)	86 ± 3.3 (81-93)	86 (72-93)
Distance anterior end to esophagus base	123 ± 9.2 (89-132)	127 ± 4.7 (117-135)	129 ± 6.1 (117-139)	125 (100-139)
Tail length with sheath	98 ± 4.5 (91-107)	94 ± 3.8 (84-100)	93 ± 5.6 (82-101)	98 (83-112)
Anal body width	15 ± 0.8 (14-17)	15 ± 0.9 (13-16)	15 ± 0.8 (13-16)	-
Ratio a	25 ± 1.4 (23-28)	26 ± 1.5 (24-31)	23 ± 1.6 (21-28)	25 (17-30)
Ratio b	4.8 ± 0.4 (4.5-6.3)	4.6 ± 0.2 (4.3-5.2)	4.6 ± 0.3 (3.9-5.2)	4.5 (4-5.1)
Ratio c	9.7 ± 1.5 (5.7-12)	11 ± 0.6 (9.9-13)	10 ± 0.8 (9.1-12)	6.2 (5.5-7)
Ratio c’	3.7 ± 0.3 (3.1-4.2)	3.7 ± 0.2 (3.4-4.1)	3.8 ± 0.2 (3.3-4.2)	-
D%	84 ± 9.7 (69-113)	68 ± 1.9 (64-70)	67 ± 3.5 (59-75)	84 (76-92)
E%	109 ± 6.3 (97-121)	111 ± 7.4 (96-121)	114 ± 9.5 (88-135)	112 (103-130)
n	20	20	20	15

Table 5. Morphometric characters (in µm) of 2nd generation males isolated from Catalonia and Murcia (Spain) comparable to *Heterorhabditis bacteriophora*. Measurements in form: mean ± SD (range).

Character	**Gscl**	**M110**	**M115**	***H. bacteriophora* After Poinar (1975, 1990)**
Total body length	944 ± 53 (832-1056)	869 ± 61 (767-962)	836 ± 65 (726-958)	820 (780-960)
Maximum body width	52 ± 4 (46-58)	45 ± 2.2 (41-52)	45 ± 4.7 (36-53)	43 (38-46)
Distance anterior end to excretory pore	133 ± 7.6 (119-148)	125 ± 3.3 (120-132)	127 ± 6.2 (118-140)	121 (114-130)
Distance anterior end to nerve ring	79 ± 4.3 (70-85)	74 ± 6.1 (60-87)	75 ± 5.8 (64-84)	72 (65-81)
Distance anterior end to esophagus base	111 ± 3.2 (106-116)	101 ± 5.3 (91-114)	105 ± 5.1 (92-112)	103 (99-105)
Tail length	33 ± 2.6 (30-38)	27 ± 2.1 (24-33)	30 ± 3.6 (26-39)	-
Anal body width	22 ± 1.7 (19-25)	19 ± 1 (17-21)	19 ± 1.7 (16-22)	23 (22-25)
Spicule lenght	42 ± 2.5 (39-46)	42 ± 3.1 (35-47)	43 ± 3.4 (35-49)	40 (36-44)
Gubernaculum lenght	22 ± 1.7 (19-26)	21 ± 2.3 (16-25)	21 ± 1.7 (18-24)	20 (18-25)
Ratio a	18 ± 1.1 (16-21)	19 ± 1.4 (17-21)	19 ± 1.6 (15-22)	-
Ratio b	8.5 ± 0.5 (7.5-9.4)	8.6 ± 0.7 (7.4-9.6)	8 ± 0.7 (6.7-9.1)	-
Ratio c	29 ± 2.3 (25-32)	32 ± 2.5 (27-36)	28 ± 2.7 (23-34)	-
Ratio c’	1.5 ± 0.2 (1.3-1.9)	1.5 ± 0.1 (1.2-1.6)	1.6 ± 0.3 (1.2-2.4)	-
%D	119 ± 7 (108-132)	124 ± 6.5 (113-135)	122 ± 8.2 (110-142)	117
%SW	197 ± 23 (157-243)	224 ± 21 (178-253)	226 ± 31 (186-296)	174
%GS	52 ± 2.9 (46-56)	50 ± 4.9 (42-59)	49 ± 4.1 (42-58)	50
n	20	20	20	15

Heat tolerance

Infective juveniles were able of surviving at temperatures between 25 and 37ºC, but there were differences among strains and species. *Steinernema feltiae* and *S. carpocapsae* were recovered at temperatures ranging 25 to 35ºC while *H. bacteriophora* were recovered up to 37ºC at 2 h of exposure. The exposure at 40ºC resulted in 0% nematodes recovered in all of the strains.

After 4 h of exposure nematodes were recovered from 25 to 35ºC, except three strains of *S. feltiae* (L9, L11 and L12) that showed lesser tolerance to temperature (Figure 3).

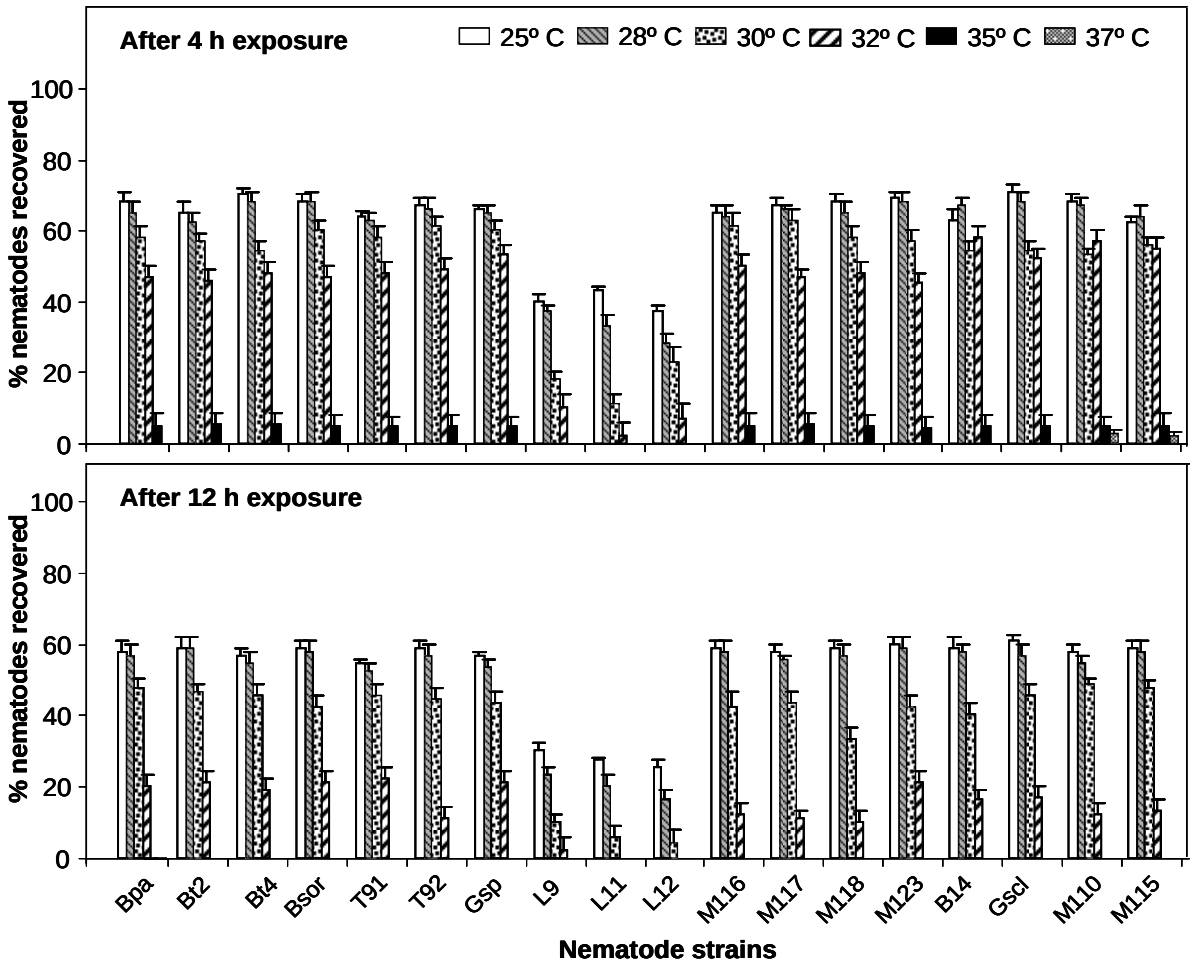


Figure 3. Percentage of nematodes of isolates of *Steinernema feltiae* (Bpa, Bt2, Bt4, Bsor, T91, T92, Gsp, L9, L11, L12, M116, M117, M118 and M123), *Steinernema carpocapsae* (B14) and *Heterorhabditis bacteriophora* (Gscl, M110 and M115), recovered after 4 and 12h of exposure to different temperatures.

When the exposure increased to 12h the survival temperature range decreased to 25-32ºC. There were no significant differences among species at the different exposure time.

Effect of Temperature on Infectivity

Temperature had an effect on the infectivity of the different nematode strains (Figure 4). There was no infection at 5ºC and at 37ºC during the period tested and all the *G. mellonella* larvae recovered from the Petri dishes were alive. Results showed great variability in the number of nematodes established in the insect larvae among strains within *S. feltiae* in all temperatures tested (Tukey test, $p < 0.05$) and establishment occurred between 8ºC and 30ºC. The number of nematodes that infected the insects increased with the temperature from 8ºC to 25ºC in all *S. feltiae* isolates. The maximum number of nematodes was recovered between 15ºC and 25ºC in eight of the 14 strains (Bpa, Bt2, Bt4, Bsor, Gsp, M116, M117 and M123), decreasing at 28ºC and at 30ºC (Tukey test, $p < 0.05$). Although no nematodes were found inside the insect at 32ºC and 35ºC many insect larvae recovered were dead. However, during the same period tested, larvae of the control (without nematodes) subjected to the same temperatures remained alive. Neither *S. carpocapsae* nor *H. bacteriophora* infected at 8 and 10ºC. The maximum infective capacity for *S. carpocapsae* B14 was recorded at 20-25ºC (Tukey test, $p \geq 0.05$) and nematodes were recovered up to 32ºC. Optimal temperature for infection of the three *H. bacteriophora* strains (Gscl, M110 and M115) was 25ºC. Although M110 and M115 strains were able to infect at 35ºC, Gscl and M110 strains showed the higher establishment at the optimal temperature of 25ºC.

Effect of Temperature on Reproduction

The time of the first emergence of IJs from the cadavers depended significantly on the temperature (Figure 5). Results also showed significant differences in the range of development temperatures among species (Tukey test, $p < 0.05$). The majority of *S. feltiae* strains were able to reproduce between 8ºC and 28ºC (except L11 and L12 strains), and there were significant differences among strains at each temperature tested. In all strains the lowest number of emerged IJs and the slowest emergence time occurred at 8ºC (Tukey test, $p \geq 0.05$) (Figure 6). The highest number of IJs was between 15ºC and 25ºC depending on the strains, but in all cases first emergence started before at 25ºC (Tukey test, $p < 0.05$). Except for three strains (L9, L11 and L12), *S. feltiae* showed faster emergence time than *S. carpocapsae* and *H. bacteriophora* between 15-20ºC (Tukey test, $p < 0.05$), whereas between 25-28ºC only differed with *H. bacteriophora*. For *S. carpocapsae* B14, the optimal temperature for development was 20ºC and nematodes emerged at the range of 15-30ºC. All three *H. bacteriophora* strains showed the greatest emergence of nematodes at 25ºC, with significant differences among strains (Tukey test, $p < 0.05$). Both *S. carpocapsae* and *H. bacteriophora* showed the fastest emergence between 25ºC and 30ºC (Tukey test, $p \geq 0.05$).

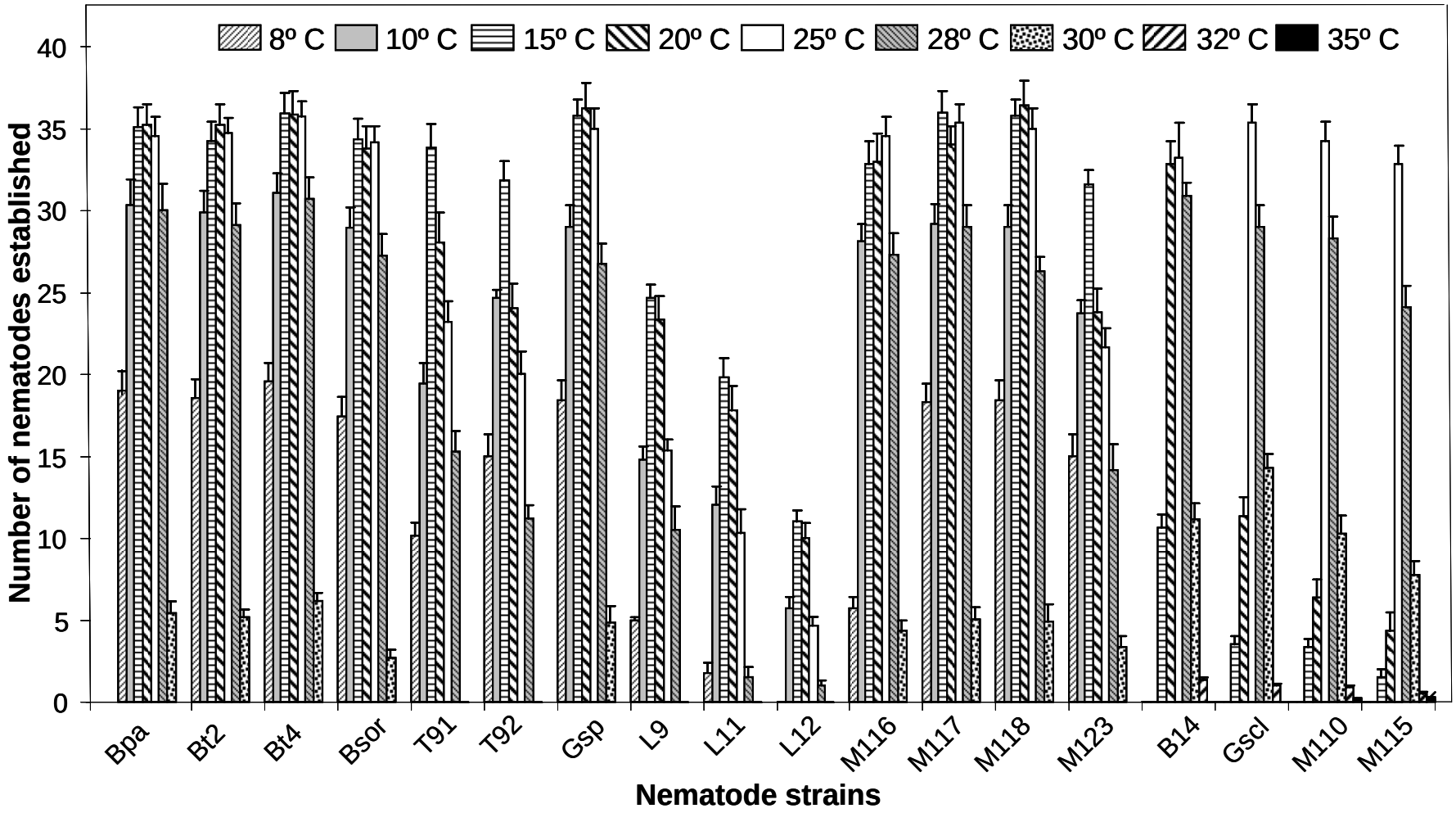


Figure 4. Number of nematodes of isolates of *Steinernema feltiae* (Bpa, Bt2, Bt4, Bsor, T91, T92, Gsp, L9, L11, L12, M116, M117, M118 and M123), *Steinernema carpocapsae* (B14) and *Heterorhabditis bacteriophora* (Gscl, M110 and M115), established in *Galleria mellonella* larvae at different temperatures.

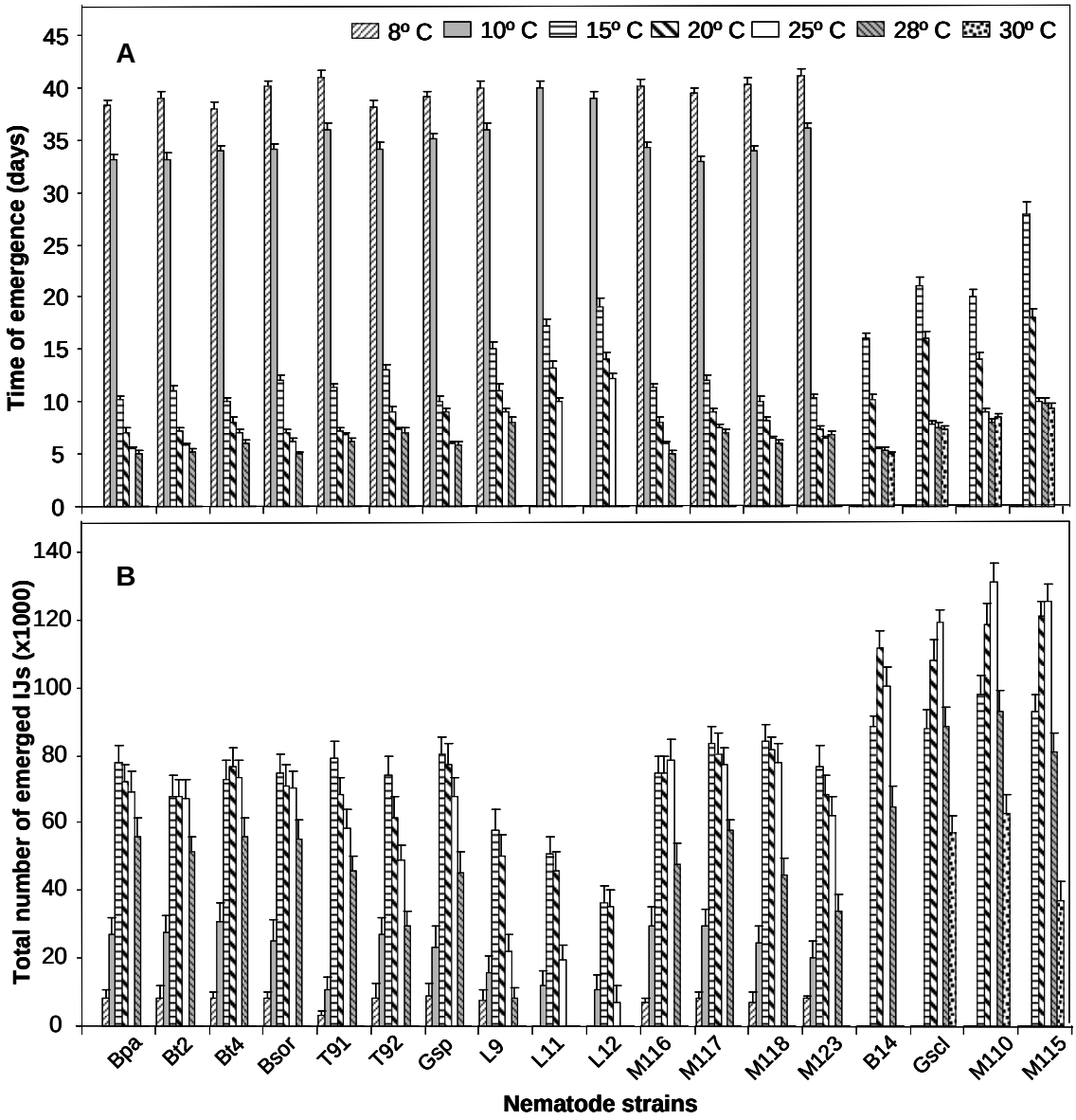


Figure 5. A- Time of emergence of first infective juvenile of isolates of *Steinernema feltiae* (Bpa, Bt2, Bt4, Bsor, T91, T92, Gsp, L9, L11, L12, M116, M117, M118 and M123), *Steinernema carpocapsae* (B14) and *Heterorhabditis bacteriophora* (GscI, M110 and M115) from *Galleria mellonella* cadaver at different temperatures. B-Total number of infective juveniles emerged per *G. mellonella* larvae.

Desiccation

The desiccation tolerances of the eighteen isolates varied among and within species in the different RHs tested (Figure 6). IJs from six strains of *S. feltiae* (Bpa, Bt4, Bsor, Gsp, M116 and M123) and the *S. carpocapsae* B14 were more tolerant when were exposed at 97% RH than the rest (Tukey test, $p < 0.05$) with a percentage of survival higher than 80%. The survival of *H. bacteriophora* strains at 97% RH ranged between 44 and 70%. Variation was also detected within *S. feltiae*, in strains with a poor ability to withstand desiccation as L9, L11 and L12 ($p < 0.05$). In the majority of the strains, the exposure at 93% and at 88% RH revealed a gradual reduction of the survival and the variability among strains was maintained. Exposing IJs at 85% RH resulted in a drastic reduction in survival of all strains tested, causing 100% mortality in eight isolates (six *S. feltiae* and two *H. bacteriophora*), and no more than 7.2% of survival in the rest of the strains. Nematodes used as the control treatments showed a percentage of survival higher than 95%, excepting three *S. feltiae* strains (L9, L11 and L12) which showed a percentage of survival between 76 and 80%.

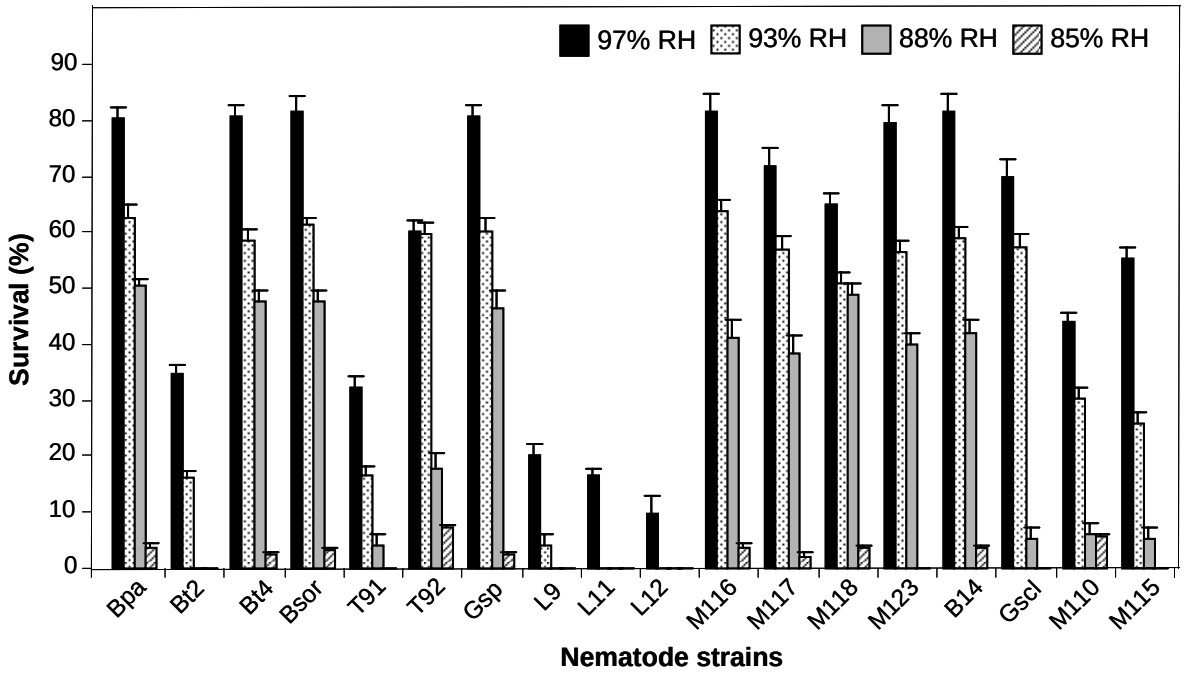


Figure 6. Mean survival (% ± SE) of infective juveniles of isolates of *Steinernema feltiae* (Bpa, Bt2, Bt4, Bsor, T91, T92, Gsp, L9, L11, L12, M116, M117, M118 and M123), *Steinernema carpocapsae* (B14) and *Heterorhabditis bacteriophora* (Gscl, M110 and M115) exposed to desiccation during 72 h.

Hypoxia

A gradual reduction of hypoxia tolerance, as indicated by IJ survival (Figure 7), was recorded during the four days of exposure. Survival of isolates differed significantly among those tested at different times of exposure. Survival recorded in all control treatments during the 96 h of the experiment was higher than 95%. After 24 h of exposure the nematode survival rate varied from 11.4% to 100%. The *S. feltiae Bsor* strain showed the highest tolerance and was significantly higher (Tukey test, $p < 0.05$) than the rest of the isolates. The lowest percentage of survival was observed in *S. feltiae* L11 (Tukey test, $p < 0.05$), and there was great variability in the tolerance to hypoxia within the species. Seven isolates also showed a high survival rate ranging between 83.2% and 90.8%, five *S. feltiae* strains (Bpa, Bt4, M116, M117 and M118) and two *H. bacteriophora* (M110 and M115). A moderate level of survival (60.1%) was recorded for the nematodes of *S. carpocapsae* B14. Survival of nematodes decreased with time in all of the isolates tested. After 96 h of exposure to hypoxia conditions, survival ranged from 0% (*S. feltiae* L12 and M123 strains) to 59.1% (*S. feltiae* M116). *Heterorhabditis bacteriophora* strains and *S. carpocapsae* B14 indicated low to moderate nematode survival (13.8 to 35.7% and 46.4% respectively).

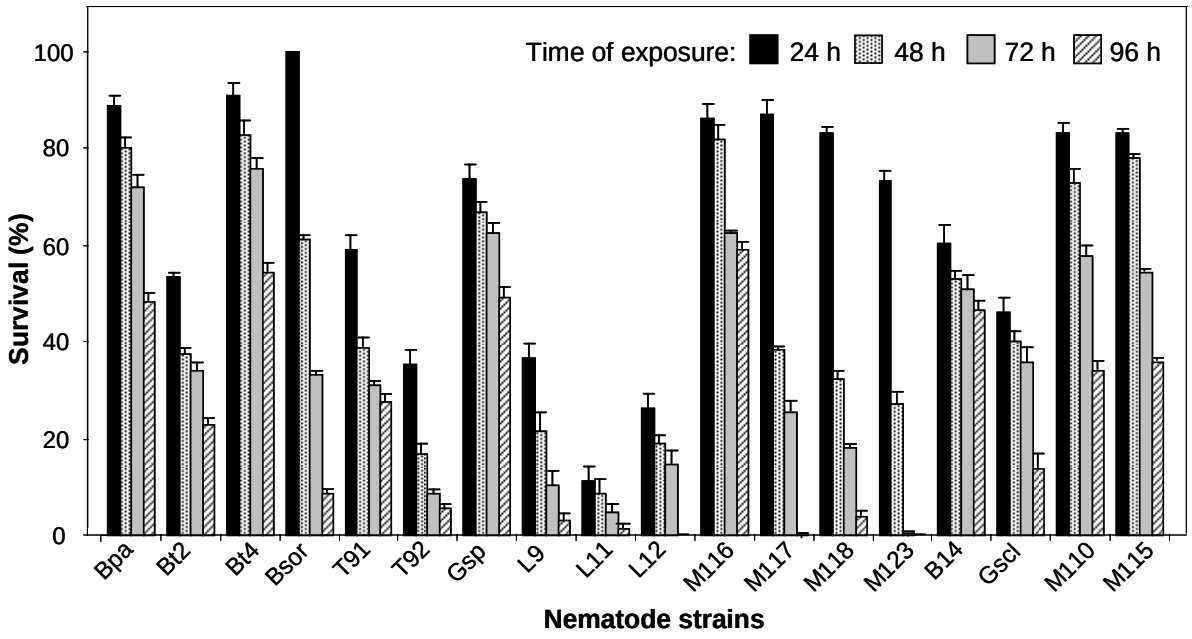


Figure 7. Mean survival (% ± SE) of infective juveniles of isolates of *Steinernema feltiae* (Bpa, Bt2, Bt4, Bsor, T91, T92, Gsp, L9, L11, L12, M116, M117, M118 and M123), *Steinernema carpocapsae* (B14) and *Heterorhabditis bacteriophora* (Gscl, M110 and M115) under hypoxia for 24, 48, 72 and 96 h.

Nematode migration in sand columns

All isolates moved from the surface where they were applied (0-5 cm), to the bottom section (15-20 cm) with and without a *G. mellonella* (Figures 8.A and 8.B). In the absence of *G. mellonella*, in general, more than 45% of the nematodes remained in the segment of application, but in all strains some nematodes were found also in the bottom section. *Steinernema carpocapsae* showed the highest percentage of nematodes

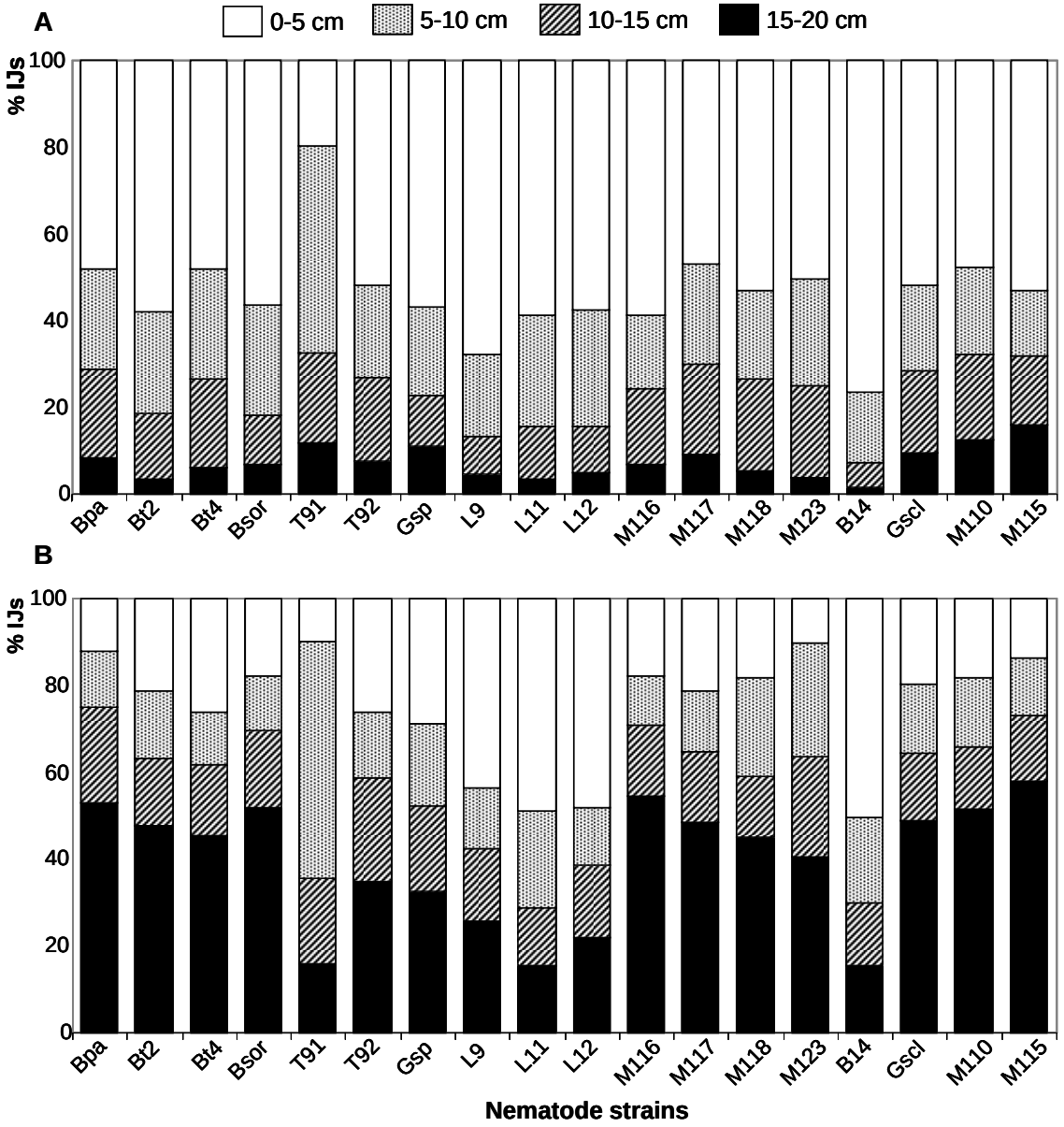


Figure 8. Percentage of nematodes detected in the absence (A) and in the presence (B) of a *G. mellonella* larva in the four sections of 20 cm sand columns of different strains of *Steinernema feltiae* (Bpa, Bt2, Bt4, Bsor, T91, T92, Gsp, L9, L11, L12, M116, M117, M118 and M123), *Steinernema carpocapsae* (B14) and *Heterorhabditis bacteriophora* (Gscl, M110 and M115).

recovered in the application section (0-5 cm), with significant differences in all strains (ANOVA, Tukey test, $p < 0.05$), except in *S. feltiae* L9 and with a small number of nematodes in the bottom section. The three strains of *H. bacteriophora* species moved similarly along the different sections, with 9.3-15% of nematodes found in the bottom section. *Steinernema feltiae* strains showed, in general, intermediate movement behaviour among the other two species. However there was variability in the vertical movement within the species. In *S. feltiae* T91 a small number of IJs remained in the top section while a high number migrated to the second section (5-10 cm), with significant differences with the rest of the strains (Tukey test, $p < 0.05$).

The presence of an insect in the sand columns had a significant effect on the majority of strains tested, increasing the movement of the nematodes to the bottom section. In general (except with *S. feltiae* T91) there was a greater number of nematodes on the bottom section in the sand columns with a *G. mellonella* than without it, showing significant differences between upper and bottom sections (T-test, $p < 0.05$). Strains within *S. feltiae* species showed high variability in migration. In three of them (L9, L11 and L12) and in *S. carpocapsae* high numbers of IJs (more than 43.5%) remained in the application section (Tukey test, $p < 0.05$). Three of the strains (T91, L11 and L12) differed from the rest of *S. feltiae* and *H. bacteriophora* strains (Tukey test, $p < 0.05$) because of the low percentage of nematodes on the bottom section, but not with *S. carpocapsae* B14, which also showed low migration capacity. All *H. bacteriophora* strains and eight *S. feltiae* strains resulted in a greater number of nematodes on the bottom section (40.5-58%), differing from the other isolates (Tukey test, $p < 0.05$). Mortality of *G. mellonella* larva was 100% in all isolates except in three *S. feltiae* strains (T91, L11 and L12) and in *S. carpocapsae* B14, reaching 70%.

Qualitative analysis of beneficial traits among strains to control C. tenebrionis

The traits chosen to select the best candidates to control *C. tenebrionis* in soil were as follows: virulence against larvae, pupae and adults; temperature tolerance to infection; temperature tolerance to reproduce; tolerance to desiccation at 88% RH; tolerance to hypoxia after 96 h and capacity of vertical migration with and without host. We assumed that all traits had equal importance in the control of *C. tenebrionis.* When comparing the addition of all beneficial traits among strains to select the most adequate isolates to control *C. tenebrionis,* the *S. feltiae* isolates Bpa, Sor and M116 obtained the highest scores. *Steinernema feltiae* Bt4 and Gsp were the second highest scoring, and the other two *S. feltiae* strains, L11 and L12, were the lowest (Table 6). When tolerance to hypoxia was excluded, *the S. feltiae* isolates Bpa and Sor continued scoring among the highest, *S. feltiae* Gsp and M116 strains scored the second, and the isolates L11 and L12 were among the lowest.

Table 6. Qualitative comparison of beneficial traits among strains for *Capnodis. tenebrionis* control.

Strain	**Virulence against larvae**	**Virulence against pupae**	**Virulence against adults**	**Temperature of infection**		**Reproductive capacity**		**Desiccation tolerance**	**Hypoxia tolerance**	**Vertical Migration**		**Total**
	5 DPE[(a)]	6 DPE[(a)]	8 DPE[(a)]	8-20 ºC	28-35ºC	8-20 ºC	25-30ºC	88 % RH	After 96 h	Without host	With host	
S. feltiae Bpa	1	0	1	1	1	1	0	1	0	0	1	7
S. feltiae Bt2	1	0	1	1	1	1	0	-1	0	-1	0	3
S. feltiae Bt4	1	0	0	1	1	1	0	1	1	0	0	6
S. feltiae Bsor	1	0	1	1	1	1	0	1	0	0	1	7
S. feltiae T91	1	0	0	0	0	0	0	0	0	1	-1	1
S. feltiae T92	-1	-1	1	0	-1	1	0	0	0	0	0	-1
S. feltiae Gsp	1	0	0	1	1	1	0	1	0	1	0	6
S. feltiae L9	-1	0	-1	0	-1	-1	-1	-1	0	0	0	-6
S. feltiae L11	-1	0	0	0	-1	-1	-1	-1	-1	-1	-1	-8
S. feltiae L12	-1	0	0	0	-1	-1	-1	-1	-1	-1	-1	-8
S. feltiae M116	1	0	1	0	1	1	0	1	1	0	1	7
S. feltiae M117	1	-1	0	1	1	1	0	1	-1	1	0	4
S. feltiae M118	1	0	0	1	1	1	0	1	0	-1	0	4
S. feltiae M123	1	0	0	0	0	1	0	1	-1	-2	0	0
S. carpocapsae B14	1	0	0	0	1	1	0	1	0	-1	-1	2
H. bacteriophora Gscl	1	1	0	-1	1	1	0	0	0	1	1	5
H. bacteriophora M110	1	0	0	-1	1	1	1	0	0	1	1	5
H. bacteriophora M115	1	0	0	-1	1	1	1	0	0	1	1	5

(a) Days post-exposition (DPE). Data were obtained from Morton and García-del-Pino (2008b).

Discussion

This study documents the occurrence of EPNs in stone fruit orchards in two Mediterranean regions of Spain. Moreover, it is the first report of isolation of EPNs from Murcia (Spain). The incidence of EPNs in the soils sampled was 5.2% in Catalonia, agreeing with data reported by Campos-Herrera *et al.* (2007), who recovered nematodes in 5.4% of the soil samples from La Rioja (north of Spain). The percentage of soils testing positive for nematodes in Murcia (a region with higher temperatures and low annual rainfall -less than 350 mm-) was 20%. This is consistent with the findings of García-del-Pino and Palomo (1996), who recovered EPNs in 28.2% of cultivated soils in Catalonia. This latter data however included orchards, vineyards, cereal and horticultural crops. Low rates of entomopathogenic nematode recovery have been reported in different regions around the world, and range from 2 to 30% (Mason *et al.*, 1996; Midituri *et al.*, 1997; Constant *et al.* 1998; Tangchitsomkid and Sontirat, 1998; Stock *et al.*, 1999; Griffin *et al.* 2000; Rosa *et al.* 2000; Lezama-Gutierrez *et al.* 2001; Hazir *et al.*, 2003; Canhilal *et al.* 2006). However, in a nearby region of Southern France, Emelianoff *et al.* (2008) isolated EPNs in 60% of soils but did not find EPNs in organic agricultural fields.

Hominick *et al.* (1996) reported that *S. feltiae* and *H. bacteriophora* are widely distributed throughout the world but steinernematids are generally recovered more often than heterorhabditids during non-targeted surveys (Hominick, 2002). In this study, the most common species was *S. feltiae*, which made up 82.3% of the nematode isolates versus 17.7% of *H. bacteriophora.* These results are similar to percentages of steinernematids and heterorhabditids reported by other studies in Spain (García-del-Pino and Palomo, 1996) and in different areas of the world (Mason *et al.*, 1996; Miduturi *et al.*, 1996; Stock *et al.*, 1999; Hazir *et al.*, 2003). However, Campos-Herrera *et al.* (2007) did not find steinernematids in La Rioja (Spain) and Emelianoff *et al.* (2008) found a higher proportion of heterorhabditids than steinernematids (72.7% and 27.3% respectively) in Southern France. *Steinernema feltiae* was isolated from soils with a range of soil textures, including sandy loam, loam and silt loam. It is apparent that soil texture is not significant in the habitat specificity of *S. feltiae*, as suggested by Sturhan (1999) in Steinernematidae. Although *Heterorhabditis* species have been found to be more common in sandy coastal soils (Griffin *et al.*, 1991; Hara *et al.* 1991; Liu and Berry, 1995), in the present study only one *H. bacteriophora* strain was isolated in sandy soil and near the sea (Gscl strain). However, results of other studies indicated that this species is not restricted to coastal strips (Stock *et al.*, 1996; Campos-Herrera *et al.*, 2007). The molecular characterization showed that *S. feltiae* Bpa belonged to the A2 RFLP variant, and to our knowledge until now the A2 RFLP variant of *S. feltiae* appears to be present only in the United Kingdom (Reid *et al.*, 1997). There is variability in the

morphometric characters of both IJs and males of *S. feltiae* isolates. Intraspecific morphological variation among strains was also observed in *Steinernema* species such as *S. glaseri* (Stock *et al.*, 1997) and *S. kraussei* (Stock *et al.*, 2000).

Environmental characterization of EPNs has been used to select the best candidates to control a pest and has reduced the number of strains that need to be tested in the field (Mannion and Jansson, 1992; Patterson Stark and Lacey, 1999; Shapiro-Ilan and McCoy, 2000; Shapiro-Ilan *et al.*, 2003). Although the aim of the present study was to identify a superior candidate to use against *C. tenebrionis*, the experiment did not demonstrate any single strain to be superior to the others for all traits tested. But the results showed differences among isolates in the response to the parameters tested. Other studies also show that different isolates of the same species of EPNs have different responses to various factors (Gaugler *et al.*, 1989; Griffin and Downes, 1991; Wright, 1992; Hazir *et al.*, 2001).

The thermal activity data obtained in this study show that isolates of *S. feltiae* are more adapted to cool temperatures than the *S. carpocapsae* and *H. bacteriophora* isolates, in agreement with other studies (Grewal *et al.*, 1994; Susurluk, 2008). The results also show that *S. feltiae* works well at warm temperatures, and can infect *G. mellonella* at a range between 8 to 30ºC (optimum 15-25ºC). Reproduction of this species was between 8 and 28ºC. Similar results were obtained by Hazir *et al.* (2001) with five different isolates of *S. feltiae.* Different studies classify *S. feltiae* as cold-adapted specie that infected their host from 8 to 28ºC and reproduced at a range between 8 and 25ºC (Grewal *et al.*, 1996; Hazir *et al.*, 2001). As Wright (1992) reported in his study of a *S. feltiae* isolate, the growth and development of *S. feltiae* strains at low temperatures would allow them to be used in controlling *C. tenebrionis* during winter. On the other hand, the warm-adaption of the majority of *S. feltiae* strains isolated in the present study could be related to their Mediterranean origin, as the original climatic locality of nematodes correlates with their temperature range (Kung *et al.*, 1991; Finnegan *et al.*, 1999). *Steinernema carpocapsae* and *H. bacteriophora* infected *G. mellonella* between 15 to 35ºC. Grewal *et al.* (1994) reported similar temperature activity ranges of 10-32ºC for *S. carpocapsae* and 10-35ºC for *H. bacteriophora*.

Tolerance to desiccation and hypoxia also varied significantly among the three species tested. Significant differences were observed even among the strains within each species. Our data indicate that *S. carpocapsae* and five *S. feltiae* strains are more adapted to humidity stress than *H. bacteriophora*. Both steinernematid species have been reported to be more desiccant tolerant than several other species (Glazer, 2002). The study of Shapiro-Ilan *et al.* (2005) reported no differences in survival of *H. bacteriophora*, *S. carpocapsae* and *S. feltiae* when they were exposed to 85% RH for 48h. Liu and Glazer (2000) showed that *H. bacteriophora* requires a more gradual and

longer period of adaptation than that of steinernematids. Preconditioning infective juveniles, for example to 97% RH, could improve survival levels (Womersley, 1990; Menti *et al.*, 1997). Low oxygen availability reduces the survival of EPNs (Glazer, 2002). The results of the present study showed high variability within *H. bacteriophora* and *S. feltiae.* The latter obtained the lowest and the highest percentage of survival after 24, 48, 72 and 96 h of exposure. Studies of other strains showed variability in mortality, such as 100% in *H. bacteriophora*, after 72 h of exposure (Shapiro-Ilan *et al.*, 2005), and 10-90% among different populations of *H. bacteriophora* after 96 h (Grewal *et al.*, 2002). In any case, hypoxia tolerance is not decisive since soils of orchards attacked by *C. tenebrionis* are not swamped or irrigated and the percentage of clay in soil is low.

Vertical movement is important to select adequate strains to reach the larvae of C. *tenebrionis* in the roots. The presence and absence of a host influence nematode movement in the soil (Molyneux, 1983). The three *H. bacteriophora* strains and most of *S. feltiae* isolates showed high migration capacity to the *G. mellonella* larva, and *S. carpocapsae* and some *S. feltiae* strains moved poorly across the 20 cm of soil, searching for the host. The vertical movement of the strains to the bottom of the cylinder also occurred without a *G. mellonella*, although most of the IJs remained in the two upper sections. Kaya (1990) studied vertical dispersal of different nematode species in different types of soil and reported dispersal distances in the presence of a host, in sandy soil, of 25 cm, 35 cm and 15 cm for *S. feltiae*, *H. bacteriophora* and *S. carpocapsae,* respectively. Susurluk (2008) found that *S. feltiae* possessed greater vertical dispersal ability than *H. bacteriophora.* Depending on the host seeking strategy, EPNs can be "cruisers", "ambushers" or "intermediates" (Kaya and Gaugler, 1993). *Heterorhabditis bacteriophora* is reported to be a cruiser (Lewis, 2002), *S. carpocapsae* as an ambusher and surface dweller (Campbell and Gaugler, 1993; Perez *et al.*, 2003), and *S. feltiae* is known as a species with intermediate foraging behaviour (Lewis, 2002). The strains tested in this study seem to match up to the previous classification even though some other studies with different EPN strains differed from it (Torr *et al.,* 2007). Differences between strains within *S. feltiae* species presented in this study could be due to genetic differences of species and strains that are also an important factor influencing nematode movement in soil (Glazer *et al.*, 1991).

In the qualitative analysis comparison of beneficial traits among strains, the traits were selected to be appropriate for an EPN strain to control *C. tenebrionis* in the soil (as larva or a hibernating adult) or in the roots (as mature larva, pupa or adult). The presence of the different stages of the insect in soil or in roots corroborates the importance of including into the analysis the virulence against larvae, pupae and adults. Desiccation tolerance is a determinant factor since most of the orchards with a presence of *C. tenebrionis* have poor or no irrigation. Furthermore, adults of *C. tenebrionis* usually

prefer dry soil to oviposite (Malagón, 1989; Garrido *et al.*, 1990) and to hibernate (Garrido, 1984). Capacities to infect and reproduce at high temperatures are important as they allow using nematodes during the warm months when the flatheaded rootborer larvae move through the soil and penetrate into roots. The capacity of nematodes to infect and reproduce at low temperatures would also allow EPNs to control this pest during the cold months, when larvae and pupae remain in roots and adults are hibernating in the soil. The results obtained in the analysis highlight three *S. feltiae* strains: Bpa, Bsor and M116, even though they did not show the best results in all of the assays. Both Bsor and M116 *S. feltiae* strains were isolated from soils without any irrigation. *Steinernema feltiae* Bpa was isolated from a *C. tenebrionis* larva inside a trunk which also was in an orchard without any irrigation. Apparently *S. feltiae* is more adapted to an environment where the flatheaded rootborer develops and any of the three strains may be an ideal candidate to control *C. tenebrionis* in the field.

Acknowledgements

We thank Antonio Soler Montoya (Servicio de Sanidad Vegetal, La Alberca, Murcia) and Andreu Vila (ADV de Fruita del Baix Llobregat, Barcelona) for assisting in the soil collection, and Mark Burch for the English correction of the manuscript.

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...if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable...we would find its mountains, hills, valleys, rivers, lakes, and oceans represented by a film of nematodes.

N.A. Cobb, 1914

III. Resumen de Resultados y Discusión

III.1. Aislamiento y caracterización morfológica de cepas de nematodos entomopatógenos en campos de frutales de hueso con presencia de *Capnodis tenebrionis*

El trabajo realizado (expuesto en el anexo) muestra la presencia de nematodos entomopatógenos en cultivos de frutales de hueso en dos regiones mediterráneas, así como el primer aislamiento realizado en la región de Murcia. La incidencia de nematodos entomopatógenos en las muestras de suelos fue del 5,2% (11 de 210 campos muestreados) en Cataluña, datos que se asemejan al 5,4 % de suelos positivos para nematodos obtenidos en suelos de La Rioja (Campos-Herrera, 2007) pero inferior al 28,2 % obtenido por García-del-Pino y Palomo (1996) en suelos de Cataluña. En la región de Murcia se encontraron nematodos en el 20 % (6 de 30 campos muestreados) de los campos, que concuerda con el porcentaje obtenido en suelos catalanes (García-del-Pino y Palomo, 1996). Sin embargo, tanto los datos obtenidos en Cataluña como en Murcia son inferiores al 60% de suelos con nematodos entomopatógenos encontrados en el sur de Francia (Emelianoff *et al.*, 2008). En general, el porcentaje de suelos positivos para nematodos entomopatógenos varía entre 2% y 30% en estudios realizados en distintas zonas del planeta como un 8,75% en Bélgica (Midituri *et al.*, 1997), un 13,8% en el Sur de Italia (Tarasco y Triggiani, 1997), un 2% en Turquía (Hazir *et al.*, 2003), un 20,3% en Indonesia (Griffin *et al.* 2000), o un 28,5% en el estudio conjunto de Arkansas, Georgia, Lousiana y Mississipi (EE.UU.) (Shapiro.Ilan *et al.*, 2003).

Se aislaron 14 cepas del género *Steinernema* sp. y tres del género *Heterorhabditis* sp. Todas las cepas fueron aisladas de muestras de suelos, a excepción de la denominada Bpa, que fue encontrada en el interior de una larva de *C. tenebrionis* dentro de un tronco de cerezo. *Steinernema feltiae* se aisló en el 82,3% de los suelos positivos para nematodos, frente a un 17,7% de *H. bacteriophora*. Aunque tanto *S. feltiae* como *H. bacteriophora* se encuentran ampliamente distribuidos por el mundo, los steinernemátidos se recogen más frecuentemente que los heterorhabdítidos (Hominick *et al.*, 1996), como se observa en previos estudios realizados en España (García-del-Pino y Palomo, 1996) y en otras áreas del mundo (Mason *et al.*, 1996; Midituri *et al.*, 1997; Stock *et al.*, 1999; Hazir *et al.*, 2003). En cambio, Campos-Herrera *et al.* (2007) no encontró steinernemátidos en La Rioja y Emelianoff *et al.* (2008) encontró un mayor proporción de heterorhabdítidos que steinernemátidos (72,7% y 27,3% respectivamente) en el sur de Francia.

Los suelos donde se aislaron los steinernemátidos se clasificaron desde neutros (pH 6,7) a alcalinos (pH 8,6), con un contenido de materia orgánica de 0,84-3,21% y una conductividad de 0,24-0,61dS/m. Los heterorhabdítidos fueron aislados de suelos alcalinos (pH 8,3-8,5) con un contenido medio de materia orgánica de 2,15-3,8% y una conductividad de 0,25-1,1dS/m. La textura de los suelos positivos a los nematodos entomopatógenos para ambos géneros varió entre franco-arenosa, franca y franco-limosa (según el triángulo textural de la USDA). El tipo de suelo parece no ser determinante para la presencia de *S. feltiae,* según los resultados obtenidos en este estudio y según sugiere Sturhan (1999) en los steinernematidos en general. En cambio, las especies de *Heterorhabditis* parecen ser más comunes en suelos arenosos de costas (Griffin *et al.*, 1991; Hara *et al.* 1991; Liu y Berry, 1995). En este trabajo únicamente se aisló una cepa de *H. bacteriophora* en este tipo de suelo. Así, estos resultados concuerdan más con los estudios que sugieren que esta especie no está restringida a la línea costera (Stock *et al.*, 1996; Campos-Herrera *et al.*, 2007).

La identificación morfológica con microscopía óptica y microscopía electrónica de barrido mostraron que todas las cepas de steinernemátidos pertenecían a la especie *S. feltiae*. Los datos morfométricos obtenidos de las formas infectivas y de los machos fueron comparados con los datos de Nguyen *et al.* (2006), observándose una gran similitud entre todas las cepas, como se observa en los cladogramas presentados en la figura III.1. Las medidas de los caracteres morfométricos de las formas infectivas y de los machos variaron dentro de la especie. Esta variación morfológica intraespecífica ha sido observada también en otras especies de *Steinernema*, como *S. glaseri* (Stock *et al.*, 1997) y *S. kraussei* (Stock *et al.*, 2000).

La caracterización molecular de la cepa Bpa confirmó que se correspondía a *S. feltiae,* concretamente a la variante A2 RFLP. Esta variante, ha sido citada únicamente en el Reino Unido (Reid *et al.*, 1997).

En los experimentos de entrecruzamiento entre *S. feltiae* Bpa y el resto de steinernemátidos se observó la aparición de una segunda generación de individuos, confirmando que todos pertenecían a la especie *S. feltiae.* Los datos morfométricos de las tres cepas de heterorhabdítidos mostraron que pertenecían a la especie *H. bacteriophora.*

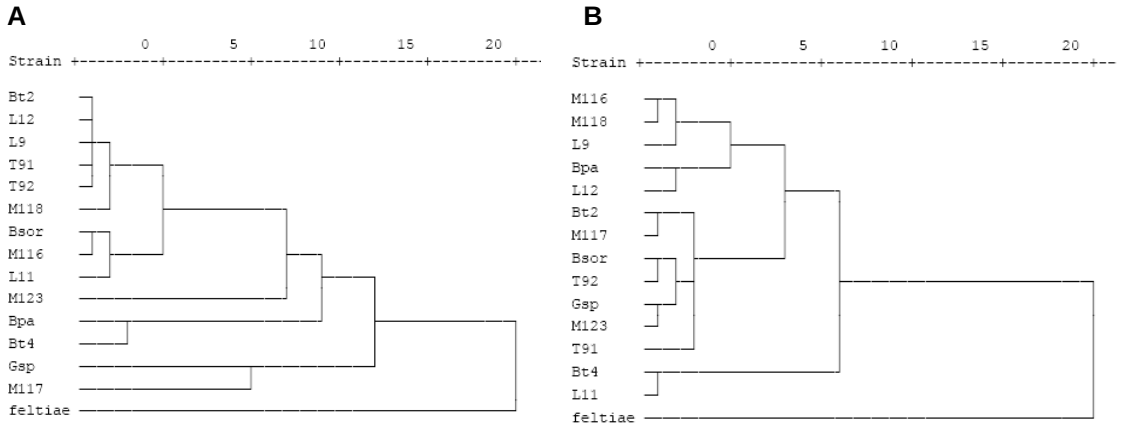

Figura III.1. Cladograma de la relación entre las cepas de *Steinernema feltiae* aisladas, comparándolas con *S. feltiae* (Nguyen *et al.*, 2006). **A**: Formas infectivas juveniles; **B**: Machos.

III.2. Eficacia de los nematodos entomopatógenos contra larvas neonatas de *Capnodis tenebrionis*

En el estudio sobre larvas neonatas de *C. tenebrionis,* los resultados, expuestos en el capítulo I, mostraron una gran susceptibilidad de las larvas a las diferentes especies de nematodos (figura III.2). Al aplicar una de concentración 150 formas infectivas por larva (FIs/larva) (equivalente a 48 FIs/cm^2), las cinco cepas utilizadas de nematodos (*S. feltiae* S6, *S. carpocapsae* Exhibit y M137, *S. arenarium* S2 y *H. bacteriophora* P4) obtuvieron más del 95% de mortalidad de las larvas tras 5 días de exposición, no observándose diferencias significativas entre cepas ($p > 0,05$). Cabe destacar que *S. carpocapsae* mostró una mayor rapidez en la infección, ya que a los dos días del ensayo habían causado una mortalidad de las larvas neonatas superior al 75%, mientras que el resto de especies no superaron el 35% de mortalidad (figura III.3).

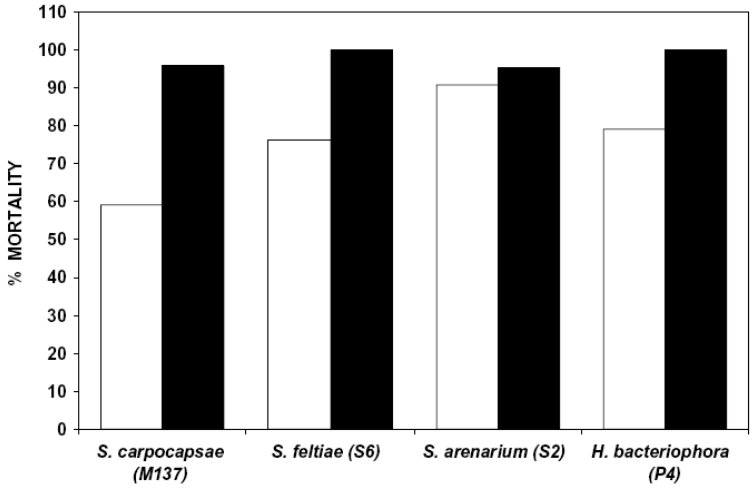

Figura III.2. Mortalidad de larvas neonatas de *Capnodis tenebrionis* después de cinco días de la aplicación de los nematodos. (□) 10 FIs/larva (= 3 FIs/cm^2); (■) 150 FIs/larva (= 48 FIs/cm^2).

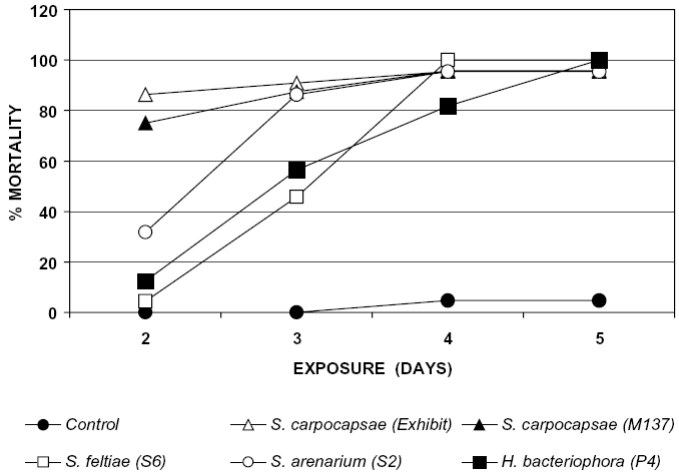

Figura III.3. Evolución de la infección de las larvas neonatas de *Capnodis tenebrionis* expuestas a las diferentes cepas de nematodos a 48 FIs/cm^2.

En un segundo ensayo se utilizaron cuatro de las cinco cepas de nematodos testadas anteriormente (*S. feltiae* S6, *S. carpocapsae* M137, *S. arenarium* S2 y *H. bacteriophora* P4) a una concentración de 10 FIs/larva (equivalente a 3 FIs/cm^2). La mortalidad de larvas neonatas alcanzó un 90,9% con *S. arenarium* S2 (figura III.2), siendo superior a la obtenida por *S. feltiae* S6 y *H. bacteriophora* P4 (76,19% y 79,19% de mortalidad respectivamente), y significativamente superior a la mortalidad causada por *S. carpocapsae* M137 (59,1% de mortalidad). En este ensayo, con una baja concentración de nematodos, es cuando la probabilidad de

encontrar al insecto se reduce, y las formas infectivas muestran su estrategia de búsqueda. La menor efectividad de *S. carpocapsae* en estas condiciones puede deberse a que tiene un comportamiento de emboscada ("ambushing"), adoptando la estrategia de esperar que el insecto se acerque (Lewis *et al.,* 1992). Esto implica también una menos atracción de esta especie por las excreciones de los insectos (Lewis *et al.*, 1993) y poca capacidad de movilidad en el suelo, prefiriendo permanecer cerca de la superficie, cerca del lugar de aplicación (Campbell y Gaugler, 1993). *Steinernema arenarium,* que resultó ser más efectivo, tiene un comportamiento de búsqueda activa del hospedador ("cruising"), así como *H. bacteriophora. Steinernema feltiae,* por su parte, tiene un comportamiento de búsqueda intermedia, como se observa también en los resultados obtenidos.

Al estudiar el rango de penetración de las distintas cepas (tabla III.1) se observó que el mayor porcentaje de formas juveniles encontradas en el interior de las larvas neonatas de *C. tenebrionis* respecto a las aplicadas en el ensayo correspondía

Tabla III.1. Infección, rango de penetración y ratio de sexos de las diferentes cepas testadas a una dosis de 10 FIs por larva neonata de *Capnodis tenebrionis* (equivalente a 3 FIs/cm^2).

Nematodes	Number IJs inoculum	Infection[a]	Mean penetration rate (%) (min–max)	Sex ratio (F: female; M: male)
S. carpocapsae (M137)	10	59.10% a	20.7 ± 13.8 (10–60)	51.85% F 48.15% M
S. feltiae (S6)	10	76.19% ab	23.1 ± 10.8 (10–50)	72.97% F 27.03% M
S. arenarium (S2)	10	90.91% b	36.0 ± 17.0 (20–90)	26.39% F 47.22% M 26.39% juv.
H. bacteriophora (P4)	10	79.19% ab	30.6 ± 19.8 (10–70)	100% H Hermafroditic

[a]Medias con la misma letra no difieren significativamente ($p < 0,05$).

también a *S. arenarium* S2 (36%) seguido de *H. bacteriophora* P4 (30,6%), *S. feltiae* S6 (23,1%) y *S. carpocapsae* M137 (20,7%). La eficacia de invasión puede ser utilizada como una medida directa que refleja la capacidad de infección de los nematodos (Hominick y Reid, 1990), así la cepa con mayor eficacia contra un insecto tendrá también la mayor eficiencia de invasión. Los resultados obtenidos en este trabajo concuerdan con otros estudios que muestran que existe esta relación (Kondo y Ishibashi, 1986; Mannion y Jansson, 1993; Shannag *et al.*, 1994). Además, Fan y Hominick (1991) y Epsky y Capinera (1993) concluyeron que, para determinar la actividad de los nematodos, el índice de invasión es mucho más

sensible que la mortalidad del hospedador. Estos últimos autores también mostraron en *S. carpocapsae* que la eficacia de invasión está positivamente relacionada con el aumento de tiempo de exposición y del número de huéspedes, pero negativamente relacionada con el aumento de la superficie del área por huésped. Esto demuestra una relación entre las estrategias de búsqueda de los nematodos y la eficacia de invasión.

La proporción de machos y hembras encontrados en las larvas neonatas varió según la especie (tabla III.1). Así, se observó una mayor penetración de hembras (72,97%) frente a machos en *S. feltiae*, un equilibrio entre sexos en *S. carpocapsae* y una mayoría de machos (47,22%) frente a hembras (26,39%) en *S. arenarium*, aunque en este último caso se contabilizó una gran proporción de formas infectivas (26,39%) al finalizar el ensayo. La prevalencia de hembras sobre los machos ya fue observado en diferentes cepas de *S. carpocapsae* por Gaugler *et al.* (1990) y Bednarek et al, (1986). El gran número de juveniles encontrados dentro del insecto parasitado por *S. arenarium* se debe a que las formas infectivas penetraron gradualmente, y al final del ensayo no habían tenido tiempo suficiente para desarrollarse en adultos. Cuando finalmente estos juveniles llegaran a adulto, posiblemente se desarrollarían en hembras, ya que según indican Grewal *et al.*, (1993) en los steinernemátidos las formas infectivas que se desarrollan en machos se dispersan y colonizan nuevos insectos más rápidamente, que las hembras.

Los estadios de larva neonata y adulto de *C. tenebrionis* son los únicos que viven fuera del tronco, siendo potencialmente más atacables por diferentes agentes de control, como insecticidas químicos o biológicos, que los demás estadios. Estudios realizados por Sanna-Passino y Del Rio (2001) con diferentes productos químicos contra larvas neonatas obtuvieron porcentajes de mortalidad no superiores al 83,3%. (67,63% con diazinon, 68,95% con carbaril, 76,18% con isofenphos+phoxim, y 83,33% con chlorpyrifos, con un 31,04% de mortalidad en el control). Estos resultados son inferiores a los obtenidos en el presente ensayo con nematodos entomopatógenos a las dosis de 3 y 48 FIs/cm^2 (60-91% y 96-100 % mortalidad respectivamente), mostrando que pueden ser más efectivos que los productos químicos que se utilizan actualmente.

III.3. Virulencia de los nematodos entomopatógenos frente a diferentes estadios de *Capnodis tenebrionis*

El ensayo de susceptibilidad de las 17 cepas de nematodos aisladas en campos de frutales de hueso, y una cepa de *S. carpocapsae,* se realizó sobre larvas de último estadio, pupas y adultos de *C. tenebrionis.* Los resultados se presentan en el capítulo II.

Para comprobar la virulencia de las cepas, en primer lugar se realizó un ensayo utilizando larvas de *G. mellonella* (figura III.4). Después de 5 días de exposición de las larvas a una dosis de 50 FIs/larva la mayoría de las cepas obtuvo un 100% de mortalidad, mientras que dos cepas de la especie *S. feltiae,* L11 y L12, sólo alcanzaron un 75% ($p < 0,05$). Este ensayo ha sido utilizado como sistema de control de calidad de la virulencia de las formas infectivas (Peters, 2005).

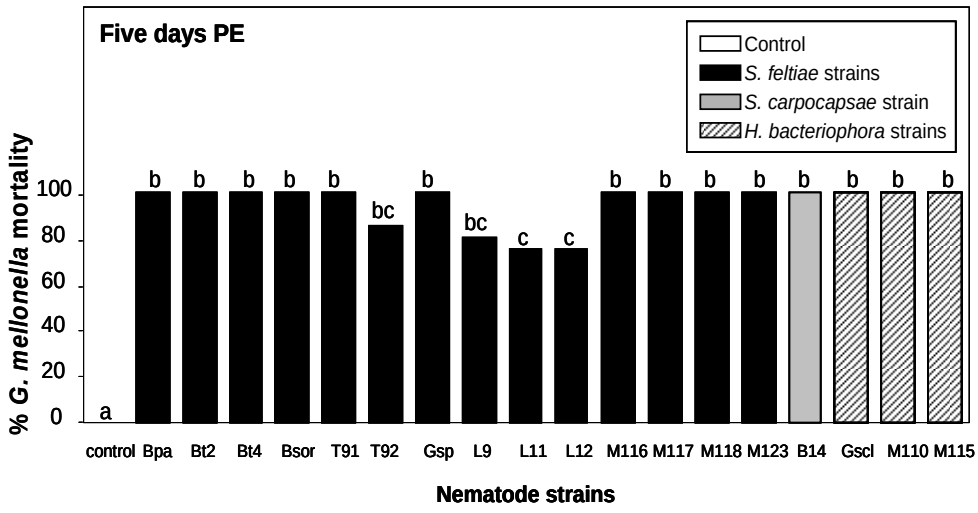

Figura III.4. Mortalidad (%) de larvas de *Galleria mellonella* después de cinco días de ser expuestas a la dosis de 50 FIs/larva de las cepas de *Steinernema feltiae, S. carpocapsae y Heterorhabditis bacteriophora.* Letras iguales sobre las columnas indican que los datos no difieren significativamente.

El ensayo sobre larvas de último estadio de *C. tenebrionis* mostró que eran muy susceptibles a los nematodos entomopatógenos a una concentración de 50 FIs/cm^2 (ver figura III.5). A los 5 días de ensayo, *S. carpocapsae* B14, las tres cepas de *H. bacteriophora* y siete de las cepas de *S. feltiae* obtuvieron una mortalidad del 100%. Hay que destacar, que la mayoría de las cepas de *S. feltiae* alcanzaron este valor al tercer día del ensayo, mientras que *S. carpocapsae* y especialmente las cepas de *H. bacteriophora* fueron más lentas en conseguir el 100% de mortalidad.

Los resultados de este trabajo son similares a los obtenidos por García-del-Pino (1994) con *S. feltiae* y por Santos Lobatón *et al.* (1998) con *S. carpocapsae*. Los bajos resultados obtenidos por algunas cepas de *S. feltiae* (T92, L9, L11 y L12) en *C. tenebrionis* concuerdan con los obtenidos en *G. mellonella*, mostrándonos que la baja virulencia de estas cepas no está relacionada con la susceptibilidad de *C. tenebrionis.*

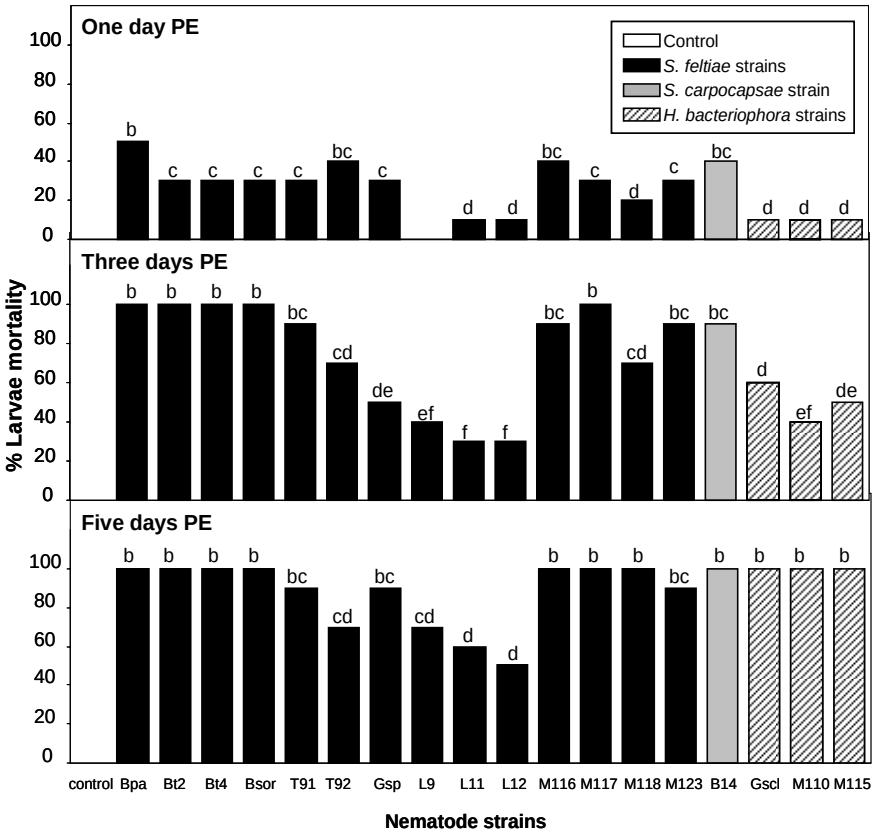

Figura III.5. Mortalidad (%) de larvas de *Capnodis tenebrionis* después de uno, tres y cinco días de ser expuestas a la dosis de 50 FIs/larva de las cepas de *Steinernema feltiae, S. carpocapsae y Heterorhabditis bacteriophora*. Letras iguales sobre las columnas indican que los datos no difieren significativamente.

La mortalidad de las pupas de *C. tenebrionis* resultó ser menor a la obtenida en las larvas aplicando una dosis de 100 FIs/cm^2 (figura III.6). La cepa que mayor mortalidad de pupas alcanzó tras 6 días de exposición a los nematodos fue *H. bacteriophora* Gscl, con un 70%. El resto de cepas obtuvo una mortalidad de pupas en un rango entre 0 y 50 %. Estos resultados pueden explicarse porque en las pupas las principales vías de acceso de los nematodos (boca, ano y espiráculos) están cerradas, impidiendo la entrada de los steinernemátidos. Sin embargo, *H. bacteriophora* es capaz de atravesar la cutícula de la pupa usando su diente proximal, como observaron Bedding y Molyneux (1982) en otros insectos.

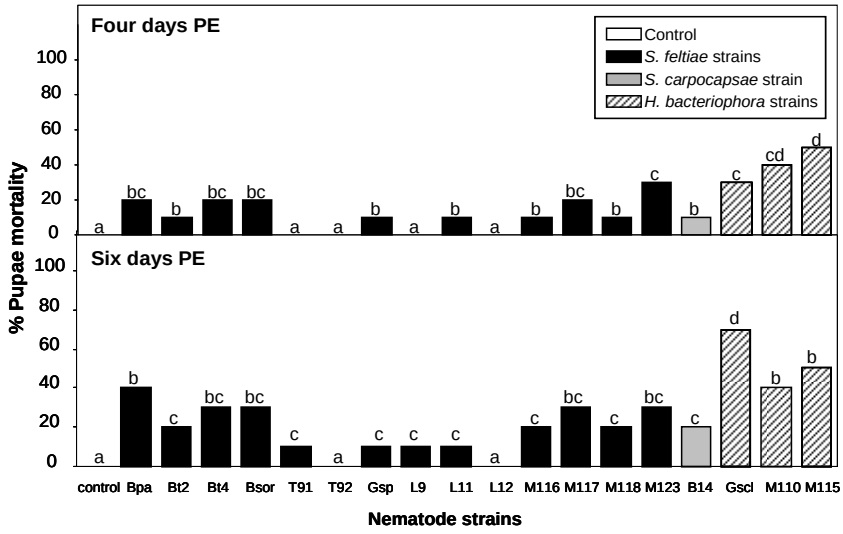

Figura III.6. Mortalidad (%) de pupas de *Capnodis tenebrionis* después de cuatro y seis días de ser expuestas a la dosis de 100 FIs/cm^2 de las cepas de *Steinernema feltiae, S. carpocapsae y Heterorhabditis bacteriophora*. Letras iguales sobre las columnas indican que los datos no difieren significativamente.

La mortalidad de los adultos de *C. tenebrionis* se evaluó a dos concentraciones de nematodos, 50 y 100 FIs/cm^2 (figura III.7). Los resultados mostraron un aumento de la mortalidad al aumentar la dosis aplicada, así como una gran variabilidad entre las distintas cepas. Así, al utilizar la dosis inferior, 8 días después del inicio del

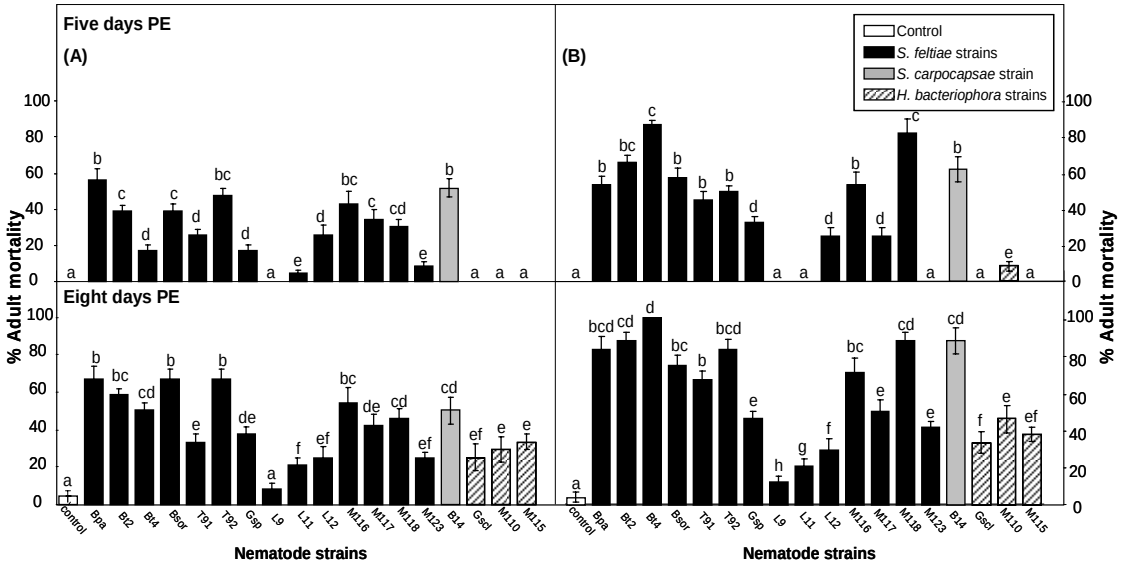

Figura III.7. Mortalidad (%) de adultos de *Capnodis tenebrionis* después de cinco y ocho días de ser expuestas a la dosis de (A) 50 FIs/cm^2 y (B) 100 FIs/cm^2 de las cepas de *Steinernema feltiae, S. carpocapsae y Heterorhabditis bacteriophora*. Letras iguales sobre las columnas indican que los datos no difieren significativamente.

ensayo, el porcentaje de mortalidad alcanzó el 66,7% en tres cepas de *S. feltiae* (Bpa, Bsor y T92), siendo inferior en el resto de las cepas. La exposición de los adultos a la dosis superior, de 100 FIs/cm^2, permitió diferenciar entre cepas con alta patogenicidad, es decir, aquellas cepas que causaron más de un 50% de mortalidad, que fueron *S. carpocapsae* y ocho *S. feltiae*, y cepas con baja patogenicidad (que causaron hasta un 50% de mortalidad) que resultaron ser seis *S. feltiae* y las tres *H. bacteriophora*. Los resultados de este trabajo muestran que los adultos son menos susceptibles a los nematodos entomopatógenos que las larvas. Estudios con otros coleópteros muestran que la mayor virulencia de los nematodos ocurre contra los estadios inmaduros del insecto, como *Asynonychus godmani* Crotch (Morse and Lindegren, 1996), *Cylas formicarus* (F.) (Mannion y Jansson, 1992) o *Metamasius hemipterus* (Oliver) (Giblin-Davis *et al.*, 1996).
La baja mortalidad de adultos producida por seis cepas de *S. feltiae* (L9, L11, L12, Gsp, M117 Y M123) y las tres de *H. bacteriophora* (Gscl, M110 y M115) permite descartarlas para futuros programas de control de adultos de *C. tenebrionis.* La poca virulencia *H. bacteriophora* frente a los adultos puede ser debida a una baja capacidad que tienen algunos nematodos de infectar adultos de ciertas especies de coleópteros (Shapiro-Ilan, 2001).

Al evaluar si existían diferencias en la susceptibilidad de los adultos entre sexos (tabla III.2), se observó que aplicando la concentración menor, de 50 FIs/cm^2, había una mayor susceptibilidad de los machos en todas menos una de las cepas testadas ($p < 0,05$). Al utilizar la concentración mayor (100 FIs/cm^2) las diferencias entre machos y hembras se mantuvieron en siete de las 14 cepas de *S. feltiae* y en las tres cepas de *H. bacteriophora.* Otros autores obtuvieron resultados similares usando *S. feltiae* y *H. bacteriophora* contra la cucaracha oriental, *Blatta* orientalis (Kotlarska-Mordzinska *et al.*, 2000), o *S. carpocapsae* contra la cucaracha americana *Periplaneta americana* (García-del-Pino, datos no publicados). En cambio hay otros estudios en que machos y hembras resultaron ser susceptibles por igual (Renn, 1998; Van Sambeek y Wiesner, 1999; Buitenhuis y Shipp, 2005). La diferencia en susceptibilidad entre sexos puede ser debida a diversos factores como la ruta de entrada de los nematodos en el insecto (aberturas genitales), el comportamiento del insecto (comportamiento diferente de acicalamiento), diferencias en la respuesta inmune u otras diferencias fisiológicas.

Tabla III.2. Porcentaje de mortalidad medio (corregido con la formula de Abbott) de machos y hembras de *Capnodis tenebrionis* expuestos a diferentes concentraciones de nematodos entomopatógenos. Para cada dosis, valores en la misma línea seguidos de la misma letra no son significativamente diferentes ($p<0,05$).

	Dose of 50 IJs/cm^2		**Dose of 100 IJs/cm^2**	
Strain	**Males**	**Females**	**Males**	**Females**
S. feltiae Bpa	83.3 a	45.5 b	91.7 a	72.7 a
S. feltiae Bt2	58.3 a	36.4 b	100 a	100 a
S. feltiae Bt4	66.7 a	45.5 b	100 a	72.7 b
S. feltiae Bsor	83.3 a	45.5 b	75 a	72.7 a
S. feltiae T91	66.7 a	63.6 a	91.7 a	72.7 a
S. feltiae T92	66.7 a	0 b	66.7 a	63.6 a
S. feltiae Gsp	66.7 a	36.4 b	100 a	36.4 b
S. feltiae L9	50 a	36.4 b	91.7 a	81.8 a
S. feltiae L11	50 a	27.3 b	50 a	45.5 a
S. feltiae L12	33.3 a	9.1 b	50 a	27.3 b
S. feltiae M116	33.3 a	9.1 b	41.7 a	18.2 b
S. feltiae M117	50 a	9.1 b	58.3 a	9.1 b
S. feltiae M118	50 a	0 b	66.7 a	18.2 b
S. feltiae M123	41.7 a	27.3 b	50 a	36.4 b
S. carpocapsae B14	16.7 a	0 b	25 a	0 b
H. bacteriophora Gscl	33.3 a	0 b	33.3 a	0 b
H. bacteriophora M110	33.3 a	9.1 b	41.7 a	9.1 b
H. bacteriophora M115	58.3 a	36.4 b	91.7 a	81.8 a

III.4. Caracterización ecológica de las cepas de nematodos entomopatógenos aisladas en campos de frutales de hueso con presencia de *Capnodis tenebrionis*

La caracterización ambiental de los nematodos ha sido utilizada para seleccionar los candidatos más adecuados para controlar una plaga determinada, reduciendo el número de cepas a testar en el campo (Mannion y Jansson, 1992; Patterson Stark y Lacey, 1999; Shapiro-Ilan y McCoy, 2000; Shapiro-Ilan *et al.*, 2003).

La caracterización de las cepas aisladas y una cepa de *S. carpocapsae* se centró en el estudio de la tolerancia de los nematodos a las altas temperaturas, el efecto de la temperatura en la capacidad de infección y reproducción, la tolerancia a la desecación y a la hipoxia y la capacidad de migración vertical en columnas de arena. Los resultados, que se muestran en el anexo, mostraron, en general, una gran variabilidad entre las distintas especies y cepas testadas. Estudios realizados por otros autores muestran que diferentes aislados de una misma especie presentan respuestas diferentes a varios factores (Gaugler *et al.*, 1989; Griffin y Downes, 1991; Wright, 1992; Hazir *et al.*, 2001).

En relación con la supervivencia de los nematodos a altas temperaturas (figura III.8) se observó que, después de 4h, la mayoría de cepas de *S. feltiae* (exceptuando L9, L11 y L12), *S. carpocapsae* y las tres cepas de *H. bacteriophora* eran capaces de sobrevivir a 35ºC, y únicamente *H. bacteriophora* M115 alcanzaba los 37ºC. Después de 12h de ensayo únicamente se recogieron nematodos vivos hasta una temperatura de 32ºC.

Al estudiar la temperatura a la cual los nematodos mantienen la capacidad de infectar al hospedador se observó una gran variabilidad entre cepas (figura III.9). En ninguna cepa testada se observó infección de *G. mellonella* a 5ºC ni a 37ºC. En las cepas de la especie *S. feltiae* el rango de temperatura de infección fue de 8 a 30ºC, aunque algunas cepas sólo consiguieron infectar hasta 28ºC (T91, L9, L11 y L12). En esta especie el número de nematodos encontrados dentro de *G. mellonella* aumentó con la temperatura hasta los 25ºC, siendo el rango de 15-25ºC donde se recogió el mayor número de nematodos por *G. mellonella.* Cabe destacar que a las temperaturas de 32ºC y 35ºC, se encontraron muchas larvas muertas del insecto aunque no se encontraron nematodos en su interior. El rango de infección de *S. carpocapsae* fue de 15-32ºC, con una máxima infección a 20-25ºC. *Heterorhabditis*

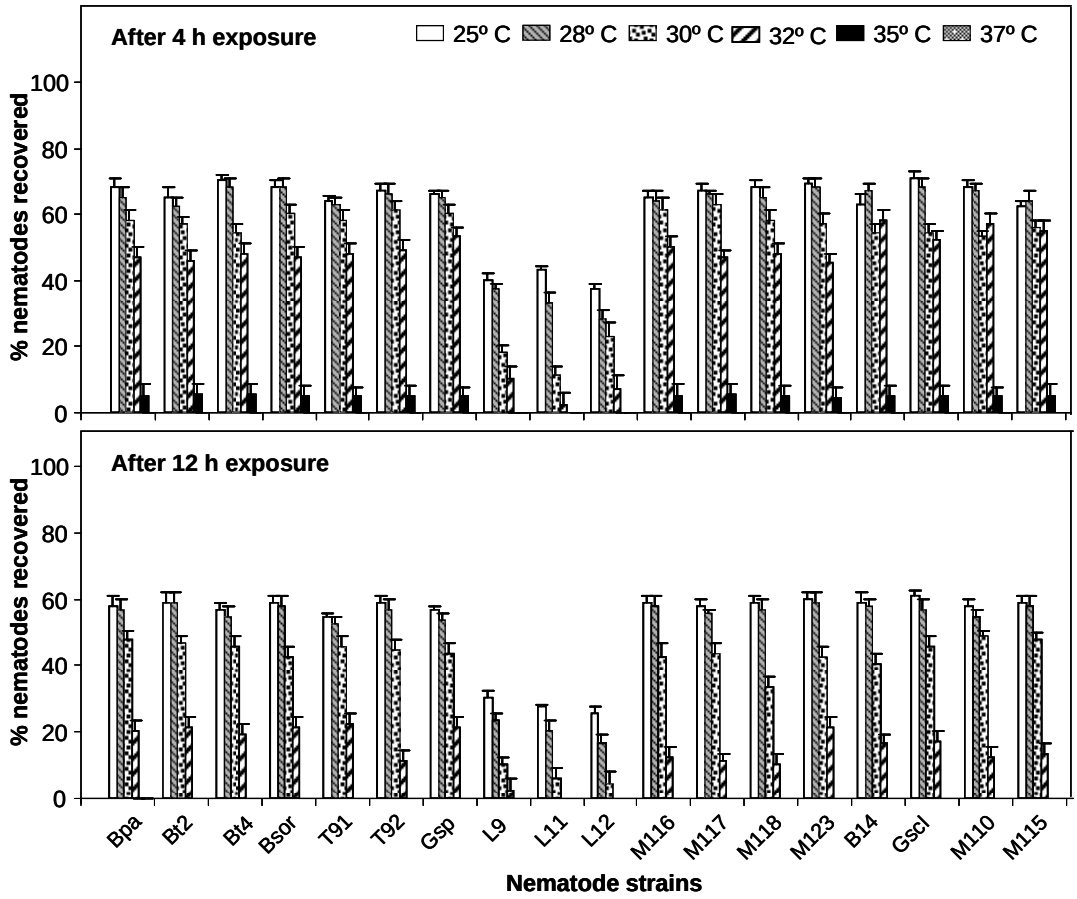

Figura III.8. Porcentaje de formas infectivas recuperadas después de 4 y 12 h de exposición a diferentes temperaturas.

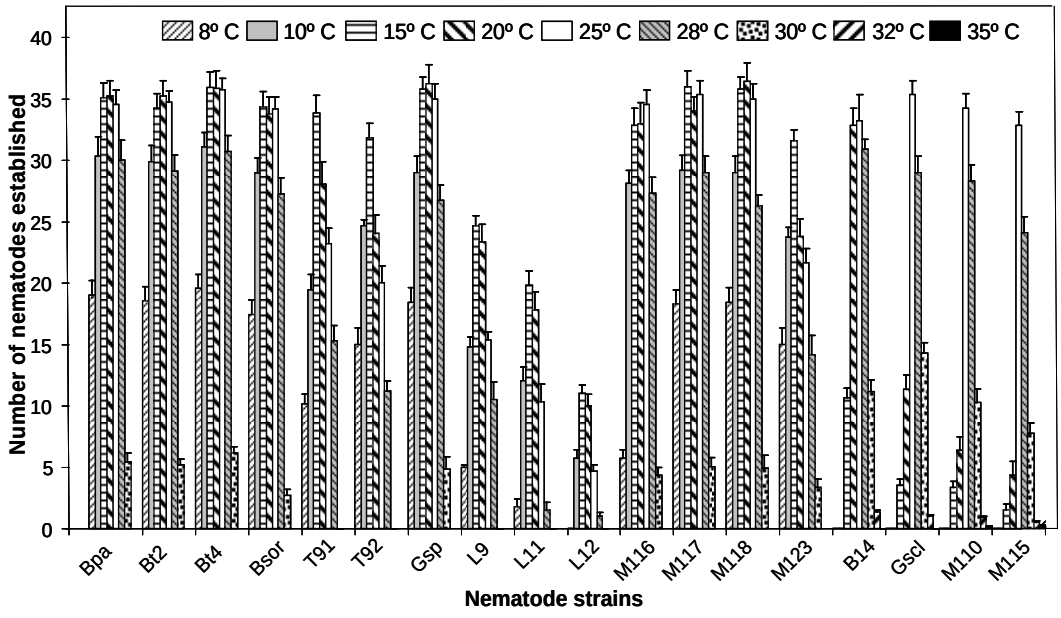

Figura III.9. Número de nematodos establecidos en larva de *Galleria mellonella* a diferentes temperaturas.

bacteriophora infectó entre 15 y 35ºC, con una temperatura óptima de infección a los 25ºC.

El tiempo de emergencia de los nematodos de los cadáveres y el número de formas infectivas producidas variaron también en función de la cepa y temperatura testadas (ver figura III.10). En *S. feltiae* las formas infectivas emergieron antes que en las otras especies a 15 y 20ºC. La temperatura óptima de reproducción de

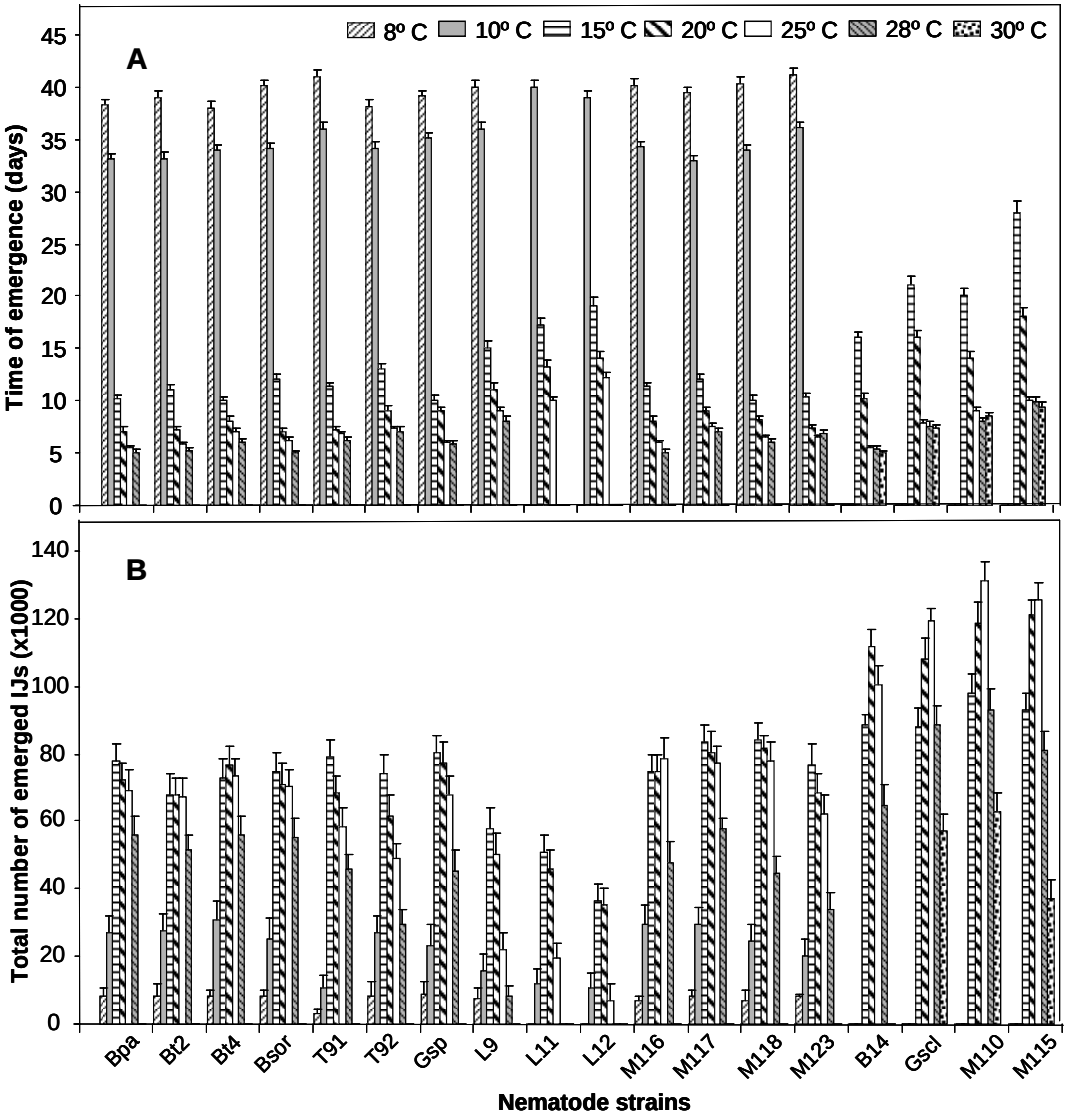

Figura III.10. A: Tiempo de emergencia de las primeras formas infectivas juveniles del cadáver de *Galleria mellonella*; B: Número total de formas infectivas emergidos de la larva de *G. mellonella*.

esta especie estuvo entre 15 y 25ºC dependiendo de la cepa testada. *Steinernema carpocapsae* se reprodujo dentro del rango de temperaturas de 15-28ºC, con un máximo número de juveniles emergidos a 20ºC. El valor óptimo de temperatura de

reproducción para las cepas de *H. bacteriophora* fue de 25ºC. Tanto para *S. carpocapsae* como para *H. bacteriophora* el inicio de emergencia más rápido de los nematodos resultó ser entre 25 y 30ºC

Los resultados de la capacidad infectiva de los nematodos a diferentes temperaturas muestran que las cepas de *S. feltiae* están más adaptadas a bajas temperaturas que las cepas de *S. carpocapsae* y *H. bacteriophora.* Los resultados también evidencian que *S. feltiae* actúa bien a temperaturas medias, pudiendo infectar *G. mellonella* entre 8 y 30ºC, y reproducirse entre 8 y 28ºC. Hazir *et al.* (2001) obtuvieron resultados similares estudiando cinco cepas de *S. feltiae.* Otros estudios clasifican *S. feltiae* como una especie adaptada al frío, que infecta en un rango de 8-28ºC y se reproduce entre 8 y 25ºC (Grewal *et al.*, 1996; Hazir *et al.*, 2001). Como Wright (1992) sugirió en su estudio con un aislado de *S. feltiae,* las cepas que muestran crecimiento y desarrollo a bajas temperaturas podrían ser usadas para controlar algunas plagas de insectos en invierno. Por otro lado, la adaptación de las cepas de *S. feltiae* de este estudio a altas temperaturas puede estar relacionada con su origen Mediterráneo, ya que hay estudios que evidencian que el origen de los nematodos está relacionado con el rango de temperatura en el cual se desarrollan (Kung *et al.*, 1991; Finnegan *et al.*, 1999).

La tolerancia a la desecación se estudió exponiendo los nematodos de las distintas cepas a humedades relativas de 85, 88, 93 y 97%. Los resultados mostraron una reducción en la supervivencia al disminuir la humedad relativa (HR) del 97 al 85% en todas las cepas, así como una gran variabilidad entre ellas (ver figura III.11). Las cepas más tolerantes al ser expuestas a un 97% HR resultaron ser seis *S. feltiae* (Bpa, Bt4, Bsor, Gsp, M116 y M123) y la cepa de *S. carpocapsae* B14. Al someter las cepas a un 85% HR se observó una drástica reducción en la supervivencia, que alcanzó el 100% en ocho cepas, seis *S. feltiae* (Bt2, T91, L9, L11, L12 y M123) y dos *H. bacteriophora* (Gscl y M115) y no más del 7,2% de supervivencia en el resto de las cepas testadas. Los resultados indican que *S. carpocapsae* B14 y cinco cepas de *S. feltiae* (Bpa, Bt4, Bsor, Gsp y M116) están mejor adaptadas a la disminución de la humedad que las cepas de *H. bacteriophora.* Glazer (2002) sugiere que estas dos especies de steinernemátidos son más tolerantes a la desecación que otras especies (Glazer, 2002). *Heterorhabditis bacteriophora,* en cambio, necesita un periodo gradual y más largo de adaptación a la desecación que los steinernemátidos (Liu y Glazer, 2000).

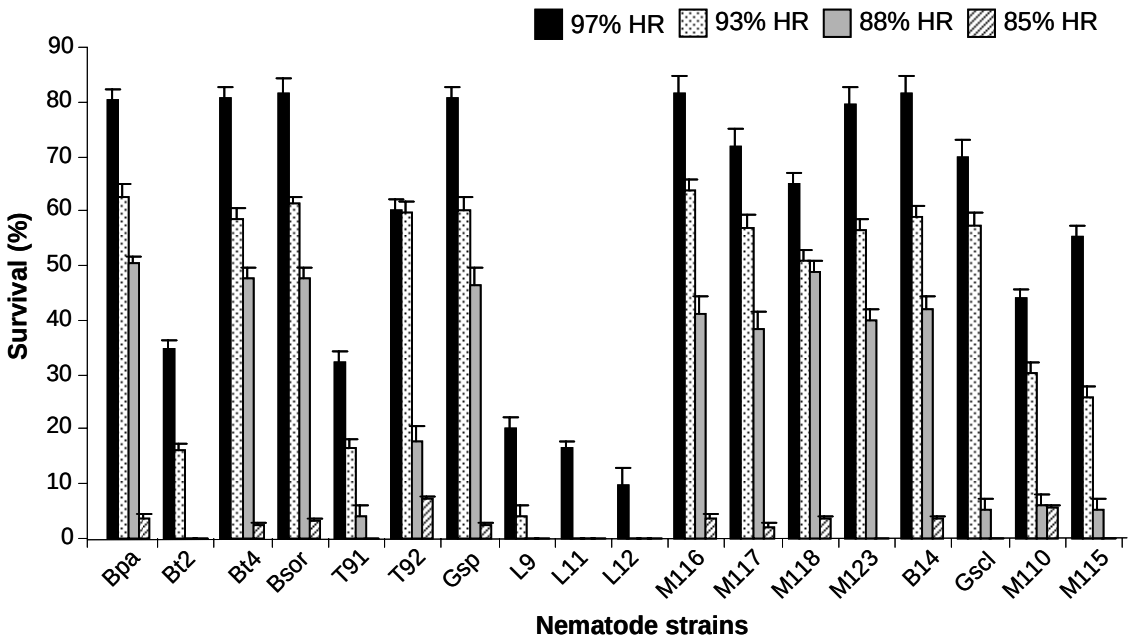

Figura III.11. Porcentaje de supervivencia (% ± SE) de las formas infectivas de diferentes cepas de *Steinernema feltiae, S. carpocapsae* y *Heterorhabditis bacteriophora* expuestos a humedades relativas (HR) de 97%, 93%, 88% y 85%.

La tolerancia a la hipoxia se evaluó manteniendo los nematodos en ausencia de oxígeno hasta cuatro días, observándose una disminución en la supervivencia de las formas infectivas durante este periodo (figura III.12). Después de 24h de exposición la supervivencia de las cepas dentro de cada especie varió ampliamente. Así, en las cepas de la especie *S. feltiae* el rango de supervivencia resultó ser de entre 100% (*S. feltiae* Sor) y 11,4% (*S. feltiae* L11). *Steinernema carpocapsae* sobrevivió moderadamente (60,1%), mientras que las cepas de *H. bacteriophora* lo hicieron entre un 45,9 y un 83,3%. La supervivencia de todas las cepas continuó reduciéndose y después de 96h de exposición a condiciones de hipoxia, se obtuvieron valores de supervivencia de 0 a 59,1% para las cepas de la especie *S. feltiae*, 46,5% para *S. carpocapsae* y entre 13,8 y 35,7% para las cepas de *H. bacteriophora.* La variabilidad entre cepas de la misma especie también la observaron Grewal *et al.* (2002) entre diferentes poblaciones de *H. bacteriophora,* obteniendo valores entre 10 y 90% de mortalidad después de 96 horas.

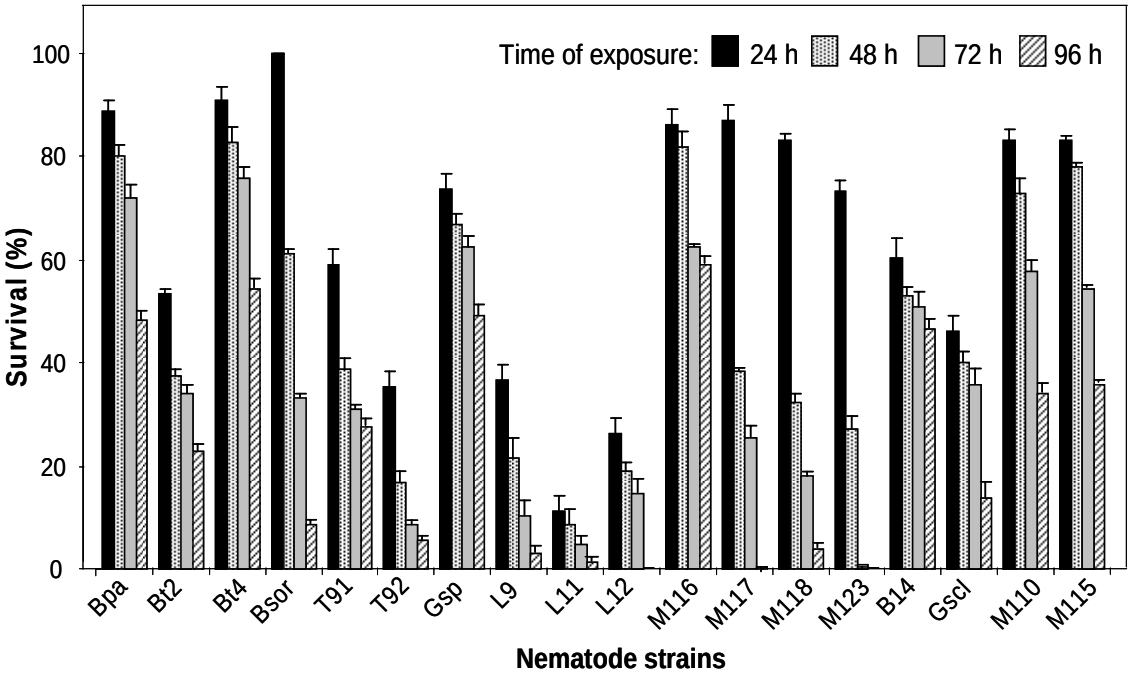

Figura III.12. Porcentaje de supervivencia (% ± SE) de las formas infectivas de las diferentes cepas de *Steinernema feltiae, S. carpocapsae* y *Heterorhabditis bacteriophora* expuestos a condiciones de hipoxia durante 24, 48, 72 y 96 h.

La capacidad de migración vertical a lo largo de una columna de arena de 20 cm se observó en todas las cepas ensayadas tanto en presencia como en ausencia de larva de *G. mellonella* (figura III.13). En el ensayo realizado sin *G. mellonella, S. carpocapsae* mostró una menor migración vertical con un mayor porcentaje de nematodos (76,35%) en la sección superior (0-5 cm) que el resto de aislados. Las tres cepas de *H. bacteriophora* tuvieron un comportamiento similar entre sí a lo largo de las distintas secciones de la columna vertical, con 9,3-15% de nematodos encontrados en la sección inferior (15-20 cm). La mayoría de cepas de *S. feltiae* tuvieron una capacidad de migración intermedia, aunque se observó variabilidad de comportamiento dentro de la especie. La presencia del insecto en la base de la columna de arena aumentó el movimiento vertical en la mayoría de las cepas, manteniéndose también la variedad de comportamientos. Algunas de las cepas mostraron poca capacidad de migración, como *S. feltiae* L9, L11, L12 y *S. carpocapsae* B14, en las que más de un 43,5% de formas infectivas se quedaron en la sección superior. En cambio, en ocho cepas de *S. feltiae* (Bpa, Bt2, Bt4, Bsor, M116, M117, M118 y M123) y en todas las de *H. bacteriophora* se encontró un alto porcentaje de nematodos en la sección inferior, entre 40,5 y 58%. La mortalidad de las larvas de *G. mellonella* fue de 100% para todas las cepas a excepción de las

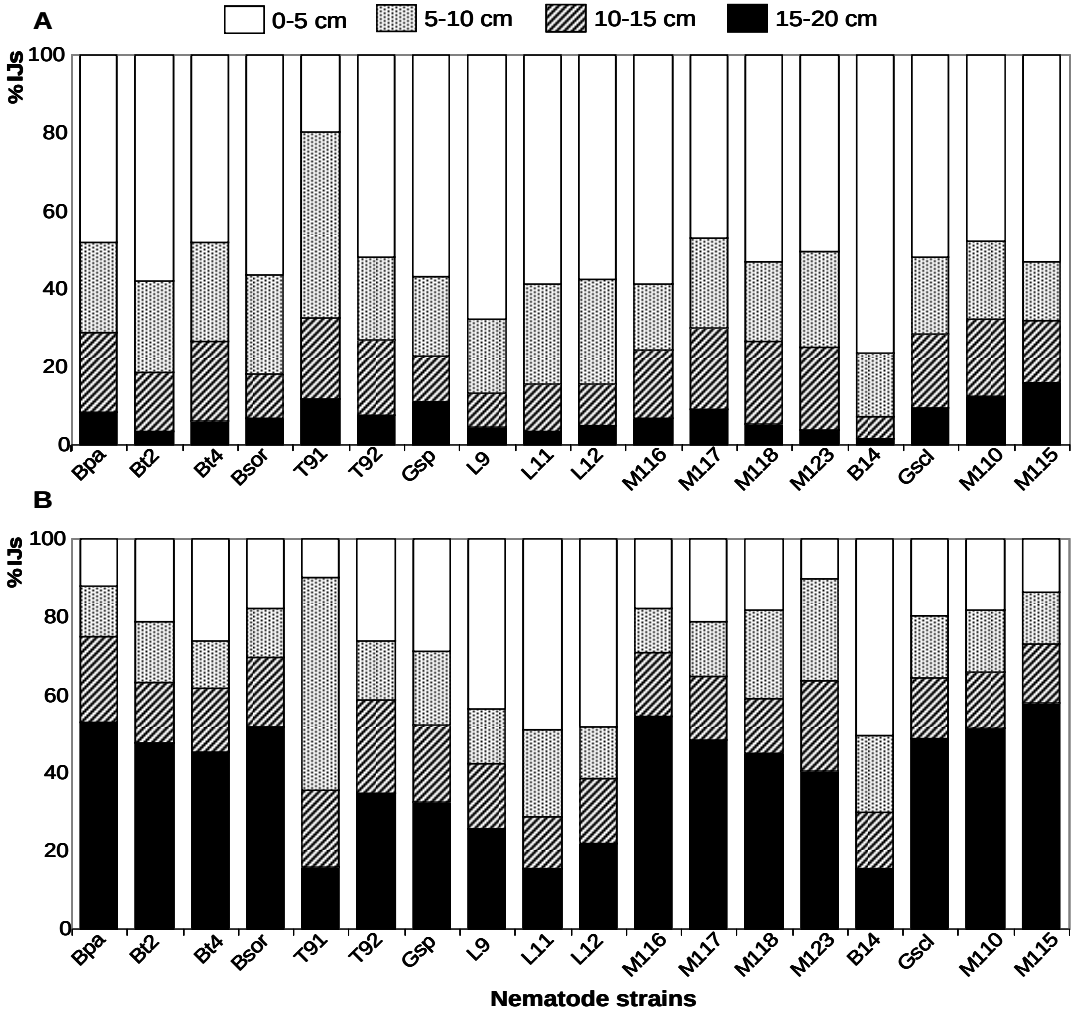

Figura III.13. Porcentaje de formas infectivas detectadas (A) en ausencia y (B) en presencia de larva de *Galleria mellonella* en las cuatro secciones de la columna vertical de arena, de las diferentes cepas de *Steinernema feltiae, S. carpocapsae* y *Heterorhabditis bacteriophora.*

cepas que ya demostraron un baja capacidad de migrar (*S. feltiae* T91, L11 y L12 y *S. carpocapsae* B14), en las que la mortalidad llegó únicamente al 70%. Kaya (1990) estudió la dispersión vertical de nematodos de las especies *S. feltiae, S. carpocapsae* y *H. bacteriophora* en presencia de un insecto y obtuvo migraciones de 25, 15 y 35 cm respectivamente. En cambio, Susurluk (2008) encontró que *S. feltiae* posee una mayor capacidad de migración que *H. bacteriophora.* Un factor importante implicado en la migración es la estrategia de búsqueda del hospedador. Como se ha comentado en la introducción, los nematodos pueden ser clasificados en navegantes ("cruisers"), con comportamiento de emboscada ("ambushers") e intermedios (Kaya y Gaugler, 1993). *Heterorhabditis bacteriophora* está clasificado como navegante (Lewis, 2002), *S. carpocapsae* muestra un comportamiento de

emboscada manteniéndose cerca de la superficie (Campbell y Gaugler, 1993; Perez *et al.*, 2003) y *S. feltiae* es conocida como intermedia (Lewis, 2002).

Con todos los resultados obtenidos en los ensayos de caracterización ecológica y virulencia contra los diferentes estadios de *C. tenebrionis,* se realizó una comparación entre cepas basada en los procedimientos descritos por Shapiro-Ilan *et al.* (2003). Los resultados obtenidos se muestran en la tabla III.3. La suma de los valores obtenidos para los distintas capacidades de los nematodos mostraron que las cepas, de la especie *S. feltiae,* Bpa, Sor y M116 obtenían las puntuaciones más altas, siendo, según este análisis cuantitativo, las más adecuadas para ser utilizadas en el control de *C. tenebrionis* en el campo. Tanto la cepa Bsor como la cepa M116 fueron aisladas de suelos sin ningún tipo de riego. *Steinernema feltiae* Bpa fue encontrada parasitando una larva de *C. tenebrionis* dentro de un tronco, también de un campo sin riego. Por los resultados obtenidos, *S. feltiae* parece estar más adaptada al ambiente donde se desarrolla el gusano cabezudo, y cualquiera de las tres cepas podrían ser buenas candidatas para controlar *C. tenebrionis* en el campo.

Tabla III.3. Análisis comparativo de las diferentes cepas de *Steinernema feltiae, S. carpocapsae* y *Heterorhabditis bacteriophora.*

Species	Strain	Virulence against larvae	Virulence against pupae	Virulence against adults	Temperature of infection		Reproductive capacity		Dessiccation tolerance	Hypoxia tolerance	Vertical Migration		Total
		5 DPE	6 DPE	8 DPE	8-20 °C	28-35°C	8-20 °C	25-30°C	88 % RH	After 96 h	Without host	With host	
S. feltiae	**Bpa**	1	0	1	1	1	1	0	1	0	0	1	**7**
	Bt2	1	0	1	1	1	1	0	-1	0	-1	0	**3**
	Bt4	1	0	0	1	1	1	0	1	1	0	0	**6**
	Bsor	1	0	1	1	1	1	0	1	0	0	1	**7**
	T91	1	0	0	0	0	0	0	0	0	1	-1	**1**
	T92	-1	-1	1	0	-1	1	0	0	0	0	0	**-1**
	Gsp	1	0	0	1	1	1	0	1	0	1	0	**6**
	L9	-1	0	-1	0	-1	-1	-1	-1	0	0	0	**-6**
	L11	-1	0	0	0	-1	-1	-1	-1	-1	-1	-1	**-8**
	L12	-1	0	0	0	-1	-1	-1	-1	-1	-1	-1	**-8**
	M116	1	0	1	0	1	1	0	1	1	0	1	**7**
	M117	1	-1	0	1	1	1	0	1	-1	1	0	**4**
	M118	1	0	0	1	1	1	0	1	0	-1	0	**4**
	M123	1	0	0	0	0	1	0	1	-1	-2	0	**0**
S. carpocapsae	**B14**	1	0	0	0	1	1	0	1	0	-1	-1	**2**
H. bacteriophora	**Gscl**	1	1	0	-1	1	1	0	0	0	1	1	**5**
	M110	1	0	0	-1	1	1	1	0	0	1	1	**5**
	M115	1	0	0	-1	1	1	1	0	0	1	1	**5**

III.5. Evaluación de la eficacia de diferentes especies de nematodos entomopatógenos para el control de *Capnodis tenebrionis* en plántulas

La eficacia de los nematodos entomopatógenos para el control de las larvas que han entrado en las raíces de plántulas se evaluó utilizando 13 de las cepas aisladas de campos de frutales de hueso y una cepa de *S. carpocapsae* a una concentración de 50 FIs/cm^2. Los resultados se presentan en el capítulo III. El número medio de larvas encontradas en el interior de las plántulas en la serie control (1,87±0,76) fue significativamente superior al encontrado en todas las series de plántulas tratadas con nematodos, siendo de 0,3 larvas de media para las tratadas *S. feltiae,* 0,49 para *H. bacteriophora*, 0,71 para *S. carpocapsae* y 1,22 para *S. affine* (tabla III.4). Los resultados del presente estudio demuestran claramente que los nematodos entomopatógenos son capaces de encontrar y penetrar en la raíz de la planta y de buscar y matar las larvas de *C. tenebrionis* dentro de su galería. La capacidad de los nematodos de localizar las larvas dentro de la planta pasa posiblemente por un primer estadio de atracción por los exudados de la raíz como sugieren Hui y Webster (2000), y una vez las formas infectivas están alrededor de las raíces, son atraídas ya por los exudados del insecto.

Tabla III.4. Número medio (±ES) de larvas de *Capnodis tenebrionis* por planta y porcentaje de plantas infectadas por diferentes cepas de nematodos entomopatógenos después de 20 días de su aplicación.

Treatments	Larvae per plant	% Infested plants
Control	1.87 ± 0.76 a	95.6 a
S. feltiae M116	0.31 ± 0.47 b	31.1 b
S. feltiae M117	0.29 ± 0.51 b	26.7 b
S. feltiae M118	0.33 ± 0.60 b	26.7 b
S. feltiae M123	0.27 ± 0.54 b	22.2 b
S. feltiae Bpa	0.22 ± 0.42 b	22.2 b
S. feltiae Bsor	0.27 ± 0.50 b	24.4 b
S. feltiae Bt2	0.24 ± 0.43 b	24.4 b
S. feltiae L91	0.36 ± 0.53 b	33.3 b
S. feltiae L92	0.38 ± 0.58 b	33.3 b
S. affine Gspe3	1.22 ± 0.82 c	77.8 a
S. carpocapsae B14	0.71 ± 0.59 c	64.4 ac
H. bacteriophora M110	0.44 ± 0.59 b	40.0 bc
H. bacteriophora Gscl3	0.53 ± 0.66 b	44.4 bc

Letras iguales indican que no existen diferencias significativas (p > 0,05).

El porcentaje de plántulas infectadas por larvas de *C. tenebrionis* en la serie control fue de un 95,6%, difiriendo significativamente con la mayoría de series tratadas con nematodos (a excepción de las tratadas con *S. carpocapsae*). El porcentaje de plántulas infectadas por larvas de *C. tenebrionis* tratadas con nematodos varió notablemente, en un rango de 22,2 a 77,8%, siendo las cepas de la especie *S. feltiae* las que obtuvieron los valores más bajos. El porcentaje de establecimiento de las larvas obtenido en el tratamiento control (12,5%) esta en el rango de los resultados obtenidos por Mendel *et al.* (2003) en el estudio encaminado a determinar la susceptibilidad de diferentes variedades de *Prunus* a las larvas de *C. tenebrionis*, con valores en un rango de 2,9-16,5% de establecimiento en las diferentes variedades de plantas.

Al aplicar la fórmula de Abbot (1925) para estudiar el porcentaje de eficacia de las distintas cepas de nematodos entomopatógenos (figura III.14) se observó que las cepas de la especie *S. feltiae* obtuvieron valores más altos, entre 79,7 y 88,2% de eficacia, pero no se pudo diferenciar significativamente entre las cepas de esta especie, ni con las de *H. bacteriophora* B14 que alcanzaron un porcentaje de

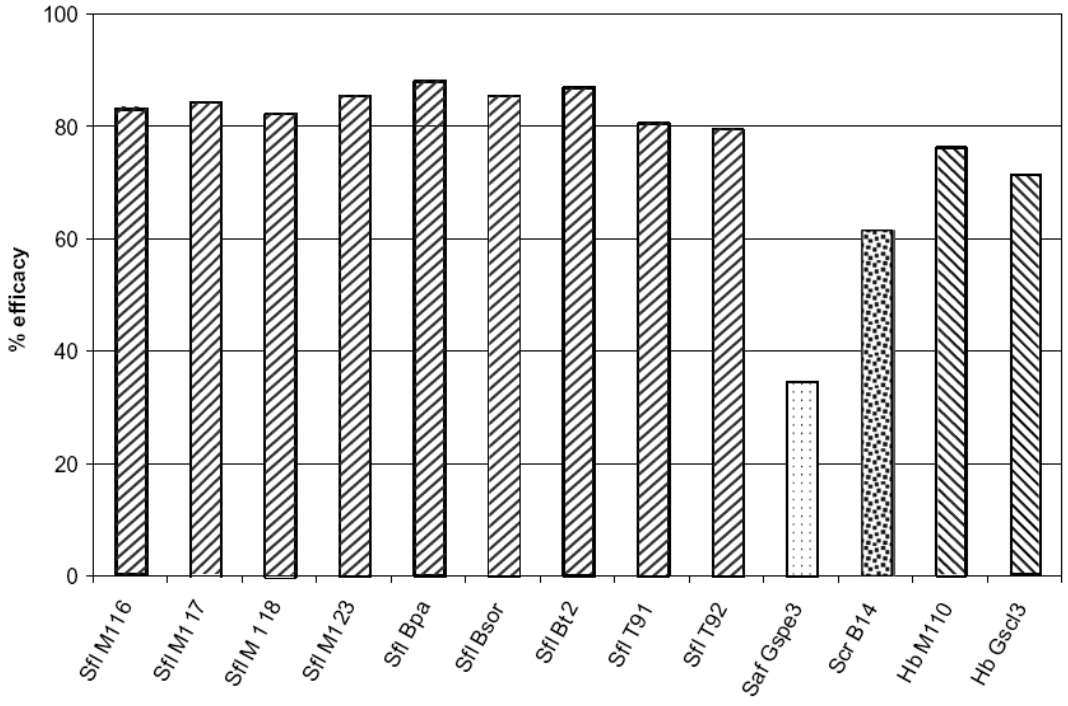

Figura III.14. Porcentaje de eficacia (según la fórmula de Abbott (1925)) de diferentes cepas de nematodos entomopatógenos contra larvas de *Capnodis tenebrionis* después de 20 días de su aplicación. Sfl: *Steinernema feltiae;* Saf: *S. affine;* Scr: *S. carpocapsae;* Hb: *Heterorhabditis bacteriophora.*

eficacia de 71,7 a 76,5%. *Steinernema carpocapsae* únicamente realizó un control del 62%. En esta especie, la baja eficacia obtenida y el alto porcentaje de plantas con larvas de *C. tenebrionis* encontrado pueden explicarse por el característico comportamiento de emboscada y que suelen permanecer en la superficie del suelo, cerca del punto de aplicación (Lewis *et al.*, 1992; Campbell y Gaugler, 1993; Lewis, 2002). La gran efectividad de *S. feltiae* y *H. bacteriophora* puede ser debida por un lado a su estrategia de búsqueda intermedia y de navegación, respectivamente; y por otro lado, el origen de las cepas, ya que fueron encontradas en campos afectados por *C. tenebrionis.* Diversos autores sugieren que los nematodos endémicos están más adaptados para controlar plagas del mismo hábitat (Bedding et al, 1993; Millar y Barbecheck, 2001).

Durante la realización del ensayo se observó que los agujeros realizados por las larvas neonatas al penetrar en las plántulas no estaban sellados, permitiendo así la entrada de los nematodos en busca del insecto. También se observó la presencia de galerías sin larvas pero con excrementos en plántulas tratadas con nematodos. Esto indicaría la entrada de un número mayor de larvas que las encontradas al diseccionar la planta, que pudieron descomponerse tras la infección por los nematodos. Las larvas vivas encontradas en el interior de las galerías fueron medidas, observando que habían conseguido desarrollarse hasta el segundo o tercer estadio larvario.

III.6. Evaluación de la eficacia del nematodo entomopatógeno *Steinernema feltiae* para el control de *Capnodis tenebrionis* en un ensayo de campo

La prueba de campo se realizó en un cultivo de cerezos atacado por *C. tenebrionis.* La cepa utilizada en este experimento fue *S. feltiae* Bpa que se aplicó con dos métodos diferentes, riego e inyección. La aplicación por ambos métodos se realizó durante 4 y 8 semanas, correspondiendo a dos dosis diferentes (4×10^6 y 8×10^6 FIs/árbol). El ensayo y los resultados se muestran en el capítulo IV. Tras la disección de la parte basal de los árboles se observó que tanto el tratamiento por riego como por inyección consiguieron reducir de forma significativa el número de larvas, pupas y adultos (tabla III.5). Por ejemplo, en las aplicaciones de 4 semanas el número de larvas encontradas en los árboles que formaban la serie control fue de 19 ± 13,29, mientras que fue de 1 ± 1,19 en el tratamiento con nematodos aplicados por riego y de 1,13 ± 1,25 en el tratamiento con nematodos aplicados

por inyección. Igual que en el ensayo con plántulas, se observaron numerosas galerías sin larva pero con excrementos, señal de la presencia anterior de larvas de *C. tenebrionis* que fueron muertas por los nematodos y posteriormente descompuestas, no pudiéndose encontrar los cadáveres de las mismas.

Tabla III.5. Número medio (±ES) de *Capnodis tenebrionis* por planta encontrados después de la aplicación durante 4 u 8 semanas de *Steinernema feltiae* por riego superficial o inyección y en los tratamientos control.

	Treatment					
	Time (week)	Dose/tree	Larvae	Pupae	Adults	Total
Control	4	–	19.00 ± 13.29 a	4.00 ± 2.83 a	2.25 ± 1.26 a	25.25 ± 13.94 a
	8	–	10.75 ± 4.57 a	2.75 ± 2.06 a	0.75 ± 0.96 a	14.25 ± 3.77 a
Drench	4	4×10^6 IJs	1.00 ± 1.19 b	0.38 ± 1.06 b	0.13 ± 0.35 b	1.50 ± 1.93 b
	8	8×10^6 IJs	0.75 ± 1.16 b	0.13 ± 0.35 b	0.13 ± 0.35 b	1.00 ± 1.31 b
Injection	4	4×10^6 IJs	1.13 ± 1.25 b	0.00 ± 0.00 b	0.13 ± 0.35 b	1.25 ± 1.49 b
	8	8×10^6 IJs	1.00 ± 1.41 b	0.00 ± 0.00 b	0.00 ± 0.00 b	1.00 ± 1.41 b

Letras iguales en una misma columna indican que no existen diferencias significativas ($p > 0,05$).

Se realizó una comparación entre tratamientos dividiendo la planta en tres zonas, raíces, tronco bajo la superficie y tronco por encima de la superficie (figura III.15). Los resultados, una vez aplicada la corrección de mortalidad de Abbott (1925), mostraron que no existían diferencias significativas para cada zona ni entre dosis ni tampoco entre tratamientos. Así, el control de *C. tenebrionis* resultó ser de entre el 88,3 y el 97%. Martínez *et al.* (2008) obtuvieron resultados similares (80-95%) aplicando *S. carpocapsae* junto con Biorend R® (formado por 1,25% de chitosan diluido en ácido acético). Por tanto, los resultados del presente trabajo indican que los nematodos entomopatógenos, aplicados sin chitosan obtienen también una alto control de *C. tenebrionis*. La eficacia obtenida en este ensayo de campo concuerda con los datos obtenidos en el ensayo sobre plántulas (capítulo III), con un control del 88,2% por *S. feltiae,* mientras que *S. carpocapsae* sólo alcanzó un control del 62%.

La evaluación de la persistencia de los nematodos en suelo se realizó durante 7 semanas a partir de la última aplicación (figura III.16), estudiando el porcentaje de larvas de *G. mellonella* parasitadas en tres secciones distintas de suelo. Durante las dos primeras semanas se obtuvo un mayor porcentaje de larvas parasitadas en la sección superior de suelo (2-8 cm) de los árboles tratados por riego que por inyección, que disminuyó de forma importante en la tercera semana. En las otras dos secciones (8-14 cm y 14-20 cm) no se detectaron diferencias significativas entre tratamientos durante las siete semanas. En ambos casos, el porcentaje de

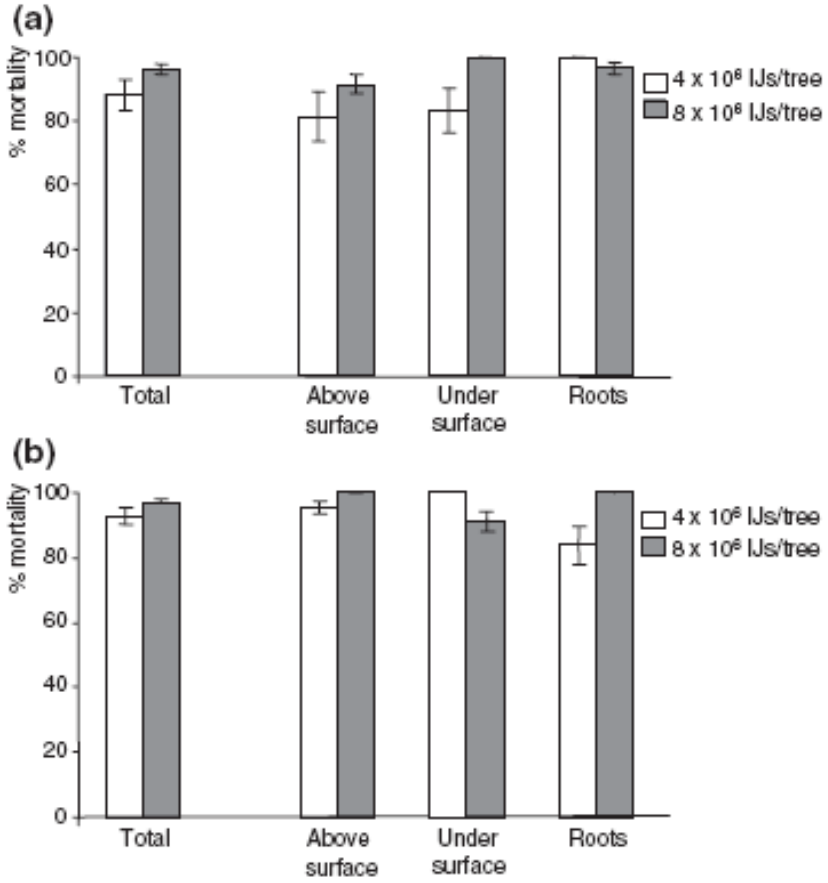

Figura III.15. Mortalidad (corregida con la fórmula de Abbot (1925)) de larvas y pupas de *Capnodis tenebrionis* de diferentes zonas del tronco y raíces 4 semanas después de la última aplicación por (a) riego superficial o (b) inyección, en dosis de 1x10^6 FIs/árbol durante un periodo de 4 u 8 semanas.

mortalidad de las larvas de *G. mellonella* se redujo de forma importante tras la cuarta semana. Después de 7 semanas no se observó ninguna larva parasitada en ninguno de los tratamientos. Aunque en las dos primeras semanas se observaron diferencias en la presencia de formas infectivas entre las distintas secciones de suelo, desde la primera semana se recogieron nematodos en la sección inferior (14-20 cm) tanto en los suelos tratados por riego como en los tratados por inyección.

Los resultados obtenidos con dosis de 4 y 8 x 10^6 formas infectivas por árbol de *S. feltiae* no mostraron diferencias significativas. Martínez *et al.* (2008) tampoco obtuvieron un aumento de control de *C. tenebrionis* al aumentar la dosis de 1 a 1,5 x 10^6 formas infectivas por árbol de *S. carpocapsae*. En este sentido, diversos estudios sugieren que a partir de ciertas concentraciones, un aumento de la dosis no implica un aumento del control (Loya y Hower 2003; Grewal *et al.* 2004). En este trabajo tampoco se obtuvieron diferencias de eficacia al utilizar distintos

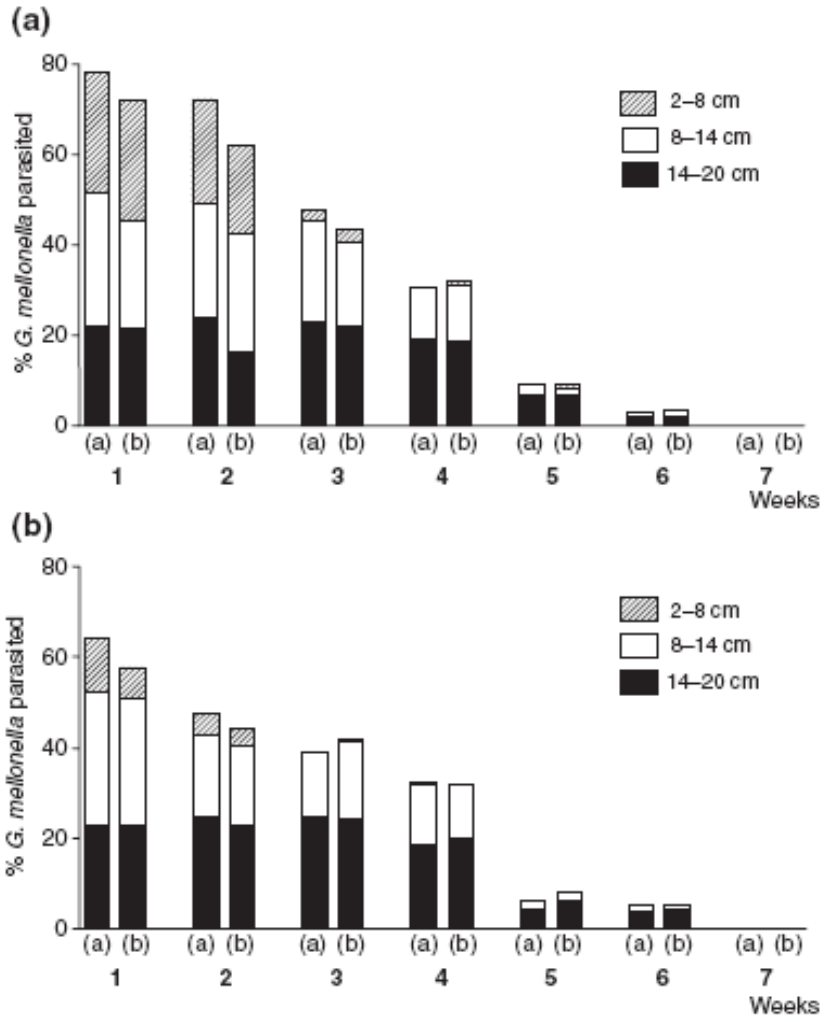

Figura III.16. Porcentaje de *Galleria mellonella* parasitadas por *Steinernema feltiae* (Bpa) durante las 7 semanas posteriores a la última aplicación por (a) riego superficial o (b) inyección, en dosis de $1x10^6$ FIs/árbol durante un periodo de 4 u 8 semanas.

métodos de aplicación. Martínez *et al.* (2008) utilizaron tres métodos de aplicación distintos, riego superficial, inyección y riego por goteo, y obtuvieron resultados similares en los tres tratamientos.

La persistencia en suelo y la eficacia obtenida con las distintas dosis testadas en el presente estudio indican que la cepa Bpa de *S. feltiae* pueden contribuir al control de *C. tenebrionis* en el campo. Los resultados de la distribución en suelo de los nematodos y la eficacia obtenida en los dos métodos de aplicación, riego e inyección, nos llevan a recomendar el riego superficial como el método más fácil y adecuado. Un paso crucial para desarrollar una estrategia económicamente efectiva para el control de *C. tenebrionis* en el campo es la determinación de la

concentración mínima apropiada de nematodos entomopatógenos a utilizar en cada aplicación.

Los resultados obtenidos en los distintos capítulos que se incluyen en este trabajo muestran como tanto las larvas neonatas en suelo, las larvas que han penetrado en las raíces, las pupas y los adultos de *C. tenebrionis* son susceptibles a los nematodos entomopatógenos. Los aislamientos de poblaciones nativas de campos de frutales de hueso nos permiten obtener nematodos entomopatógenos mejor adaptados a las características ambientales en que se desarrolla la plaga de *C. tenebrionis*. El estudio de las adaptaciones a distintos parámetros ambientales de las cepas aisladas nos muestra que existe una gran variabilidad entre ellas, pudiendo así seleccionar la cepa más adecuada para combatir ésta y otras plagas. En el caso de *C. tenebrionis,* hemos visto que su ciclo biológico ocurre en distintos sustratos: el suelo, el interior de raíces y tronco de los árboles y su parte aérea. Esto permite elegir la cepa más adecuada de nematodos entomopatógenos en cada uno de los momentos del ciclo de la plaga. El periodo de puesta de los huevos en suelo (de mayo a finales de septiembre) constituye un momento clave de la aplicación de los nematodos entomopatógenos. Debido a la gran susceptibilidad que tienen las larvas neonatas a los nematodos, la selección de la cepa a aplicar debería centrarse en la tolerancia a temperaturas medio-altas, y a la capacidad de buscar activamente al insecto, para alcanzar aquellas larvas que se dirigen hacia las raíces más profundas del árbol, así como para controlar las larvas que hubiesen podido penetrar en las raíces de los frutales. Los resultados de susceptibilidad de larvas neonatas realizado en el laboratorio sugieren que la dosis comercial de nematodos entomopatógenos que se utiliza en muchos cultivos (50 millones FIs/100m^2), sería suficiente también en este caso. La presencia de nematodos en suelo, observada en el ensayo de campo, de hasta 6 semanas sugiere realizar aplicaciones después de este periodo y hasta el fin de la época de puesta para mantener el número de nematodos entomopatógenos en suelo y conseguir controlar de forma adecuada las larvas neonatas. Esta aplicación debería realizarse en una franja de unos 50 cm alrededor de la base del tronco.

Las aplicaciones realizadas para combatir las larvas neonatas también servirían para combatir las larvas de últimos estadios y pupas que se encuentran en las galerías superiores, donde los productos químicos no pueden llegar.

Como el suelo es también el refugio de los adultos hibernantes de *C. tenebrionis*, sería adecuado realizar una aplicación durante los meses de invierno en las zonas donde se refugian estos adultos, con cepas más activas a bajas temperaturas como

han demostrado ser algunas cepas de *S. feltiae.* La baja eficacia de las cepas de *H. bacteriophora* permite descartar su uso en este caso. Los resultados obtenidos con las concentraciones testadas en el laboratorio (50 y 100 millones FIs/100m^2) indican que la dosis a aplicar contra los adultos debería ser superior a la que se utiliza comercialmente para obtener una mayor eficacia de los nematodos entomopatógenos. La aplicación de los nematodos entomopatógenos más adecuada sería por medio de riego superficial, ya que los resultados obtenidos muestran que son igual de eficaces que la aplicación por métodos de inyección, y, a diferencia de éstos, implican un menor esfuerzo de trabajo.

La eficacia de algunas cepas nativas de nematodos entomopatógenos en el control biológico del gusano cabezudo, *Capnodis tenebrionis*, obtenida en este estudio muestra que pueden ser utilizadas como productos comerciales en el control biológico de los diferentes estadios de esta plaga de los frutales.

It is an old maxim of mine that when you have excluded
the impossible, whatever remains, however improbable,
must be the truth.
Arthur Conan Doyle

IV. Conclusiones/Conclusions

1. En el muestreo realizado en Cataluña se han encontrado nematodos entomopatógenos en el 5,2% de los campos de cultivo muestreados atacados por *Capnodis tenebrionis*. Se han aislado e identificado 10 poblaciones nativas de nematodos entomopatógenos de *Steinernema feltiae* y una de *Heterorhabditis bacteriophora* procedentes de muestras de suelo, y una población de *Steinernema feltiae* aislada de una larva de *Capnodis tenebrionis* encontrada parasitando un árbol de un cultivo de cerezos. En el muestreo realizado en la región de Murcia se han encontrado nematodos entomopatógenos en el 20% de los campos muestreados. Se han aislado e identificado cuatro poblaciones de *Steinernema feltiae* y dos de *Heterorhabditis bacteriophora*, que constituyen las primeras citas de nematodos entomopatógenos aislados en la región de Murcia.

2. Los nematodos de la especie *Steinernema feltiae* han sido aislados en suelos neutros y alcalinos, con textura variable entre franco-arenosa y franco-limosa, siendo el tipo de suelo no determinante para la presencia de esta especie. Los nematodos de la especie *Heterorhabditis bacteriophora* han sido aislados en suelos más salinos, alcalinos, con textura variable entre franco-arenosa y franco-limosa.

3. Las cepas de nematodos entomopatógenos testadas son eficaces contra las larvas neonatas de *Capnodis tenebrionis.* Este estadio del coleóptero es el que se encuentra en suelo antes de penetrar en las raíces del árbol, así pues, la aplicación de nematodos entomopatógenos puede ser un primer método de control.

4. Tanto las larvas de último estadio como las pupas de *Capnodis tenebrionis,* estadios que se desarrollan dentro del árbol hospedador, son susceptibles a las cepas de nematodos entomopatógenos testadas en los ensayos de laboratorio. Una concentración de 50 FIs/cm^2 produce el 100% de mortalidad de las larvas por cepas de *Steinernema feltiae, Steinernema carpocapsae* y *Heterorhabditis bacteriophora.* Las pupas, menos susceptibles que las larvas, son más atacadas por *Heterorhabditis bacteriophora* que por los steinernemátidos, alcanzando el 70% de mortalidad al aplicar una concentración de 100 FIs/cm^2.

5. El estadio adulto de *Capnodis tenebrionis* ha mostrado ser susceptible a los nematodos entomopatógenos en los ensayos de laboratorio. Las especies *Steinernema feltiae* y *Steinernema carpocapsae* causan una mayor mortalidad

(hasta el 100%) que *Heterorhabditis bacteriophora* (menos del 50%), pudiendo descartar esta última para programas de control de adultos. Los machos de *Capnodis tenebrionis* han demostrado ser más susceptibles a la infección que las hembras.

6. Los estudios de caracterización ecológica de las distintas cepas de nematodos entomopatógenos aisladas muestran una gran variabilidad de adaptación a los diferentes factores analizados.

7. El comportamiento de los nematodos entomopatógenos al ser expuestos a diferentes temperaturas varía según la especie y la cepa estudiada. La mayoría cepas de *Steinernema feltiae* y *Steinernema carpocapsae* sobreviven como mínimo cuatro horas hasta 35ºC, mientras que *Heterorhabditis bacteriophora* alcanza 37ºC. Después de 12 horas de exposición ninguna cepa sobrevive a 35ºC. En *Steinernema feltiae* el rango de infección es de 8-30ºC y el de reproducción de 8-28ºC. *Steinernema carpocapsae* infecta entre 15 y 32ºC y se reproduce entre 15 y 30ºC y *Heterorhabditis bacteriophora* puede infectar entre 15 y 35ºC y reproducirse igual que la anterior. Estos valores indican que *Steinernema feltiae* está más adaptada a infectar insectos a bajas temperaturas que *Steinernema carpocapsae* y *Heterorhabditis bacteriophora*.

8. Los valores de tolerancia a la desecación de los nematodos entomopatógenos disminuyen de forma importante entre 97 y 85% HR, existiendo una gran variabilidad entre las cepas. *Steinernema feltiae* y *Steinernema carpocapsae* se muestran más adaptadas a la disminución de la humedad que *Heterorhabditis bacteriophora*, como también ha sido indicado por otros autores.

9. La capacidad de los nematodos entomopatógenos de tolerar la falta de oxígeno es muy variable según la cepa estudiada. Algunas cepas de las especies *Steinernema feltiae, Steinernema carpocapsae* y *H. bacteriohora,* han llegado a sobrevivir más de 96 horas en condiciones de hipoxia.

10. Las cepas de los nematodos entomopatógenos aislados han sido capaces de moverse hasta 20 cm de profundidad en un medio arenoso en presencia y ausencia de una larva de *Galleria mellonella*. La presencia de un insecto aumenta notablemente la migración vertical de los nematodos. *Steinernema feltiae* y *Heterorhabditis bacteriophora* muestran una mayor capacidad de migración en la columna de arena que *Steinernema carpocapsae*, en la que el

mayor porcentaje de individuos se quedan en la superficie de aplicación. El comportamiento de búsqueda de hospedador de las tres cepas de *Heterorhabditis bacteriophora,* de *Steinernema carpocapsae* y de la mayoría de las cepas de *Steinernema feltiae,* concuerda con la clasificación de navegantes ("cruisers"), de emboscada ("ambushers") e intermedias respectivamente, ya puesta de manifiesto por otros autores.

11. La realización de un análisis comparativo teniendo en cuenta los valores de la caracterización ecológica y de virulencia de las cepas muestran que tres cepas de *Steinernema feltiae* (Bpa, Bsor y M116) son las más adecuadas para utilizar en la lucha contra *Capnodis tenebrionis.*

12. El ensayo de susceptibilidad de larvas de *Capnodis tenebrionis* después de penetrar en la raíz de la planta muestra que los nematodos entomopatógenos son capaces de entrar por las galerías excavadas por las larvas, localizarlas y parasitarlas, lo que confirma la eficacia de los nematodos entomopatógenos para controlar los estadios larvarios del coleóptero que se desarrollan en el interior del árbol parasitado.

13. El ensayo de campo muestra que la cepa *Steinernema feltiae* Bpa es capaz de controlar *Capnodis tenebrionis* en un cultivo de cerezos comercial mediante técnicas aplicación por riego superficial o inyección. Los resultados similares obtenidos utilizando ambas técnicas nos llevan a proponer la aplicación de los nematodos por riego superficial al suponer un menor esfuerzo de trabajo.

14. El presente estudio ha mostrado la eficacia de algunas cepas nativas de nematodos entomopatógenos en el control biológico del gusano cabezudo, *Capnodis tenebrionis*, mostrando su potencialidad para ser utilizados como productos comerciales en el control biológico de los diferentes estadios de esta importante plaga de los frutales.

1. Entomopathogenic nematodes were recovered in 5.2% of the sampled orchards attacked by *Capnodis tenebrionis* in Cataluña. Ten entomopathogenic nematode strains of *Steinernema feltiae* and one of *Heterorhabditis bacteriophora* were isolated and identified from soil samples. One *Steinernema feltiae* was isolated from an infected *Capnodis tenebrionis* larva collected from inside a cherry tree trunk. Entomopathogenic nematodes were recovered in 20% of the orchards sampled in Murcia. Four *Steinernema feltiae* and two *Heterorhabditis bacteriophora* strains were isolated and identified and represent the first entomopathogenic nematode isolation described in the region of Murcia.

2. The *Steinernema feltiae* strains were isolated from neutral to alkaline soils and texture between sandy-loam and silt-loam, as the soil type is not decisive for the presence of this species. The *Heterorhabditis bacteriophora* strains were isolated from more saline soils, with alkalinity and texture between sandy-loam and silt-loam.

3. The entomopathogenic nematode strains tested were effective against neonate larvae of *Capnodis tenebrionis.* As this stage is found in soil before penetrating into roots, the application of entomopathogenic nematodes in soil could be a first method of control.

4. Last instar larvae and pupae of *Capnodis tenebrionis,* which develop inside the tree, were susceptible to the entomopathogenic nematode strains tested in the laboratory. A dose of 50 IJs/cm^2 resulted in 100% mortality of larvae by strains of *Steinernema feltiae, Steinernema carpocapsae* and *Heterorhabditis bacteriophora.* The pupae were less susceptible than larvae, and were more infected by *Heterorhabditis bacteriophora* (reaching 70% mortality at a dose of 100 IJs/cm^2) than the steinernematids.

5. Adults of *Capnodis tenebrionis* were susceptible to the entomopathogenic nematode strains tested in the laboratory. *Steinernema feltiae* and *Steinernema carpocapsae* caused major insect mortality (up to 100%) compared to *Heterorhabditis bacteriophora* (less than 50%), allowing us to discard the last species to control the adults. The adult males of *Capnodis tenebrionis* were more susceptible than females to entomopathogenic nematodes.

6. Environmental characterization of the entomopathogenic nematodes showed great variability among strains for all traits tested.

7. Temperature had an effect on the survival, infectivity and reproduction of the different nematode strains. The majority of *Steinernema feltiae* and *Steinernema carpocapsae* strains survived at least 4 h to 35ºC, while *Heterorhabditis bacteriophora* survived to 37ºC. After 12 h of exposure, no strain survived at 35ºC. *Steinernema feltiae* infected between 8 and 30ºC and reproduced between 8 and 28ºC. *Steinernema carpocapsae* was able to infect between 15 and 32ºC and to reproduce between 15 and 30ºC. *Heterorhabditis* bacteriophora infected between 15 and 35ºC and reproduced between 15 and 30ºC. These results showed *Steinernema feltiae* is better adapted to infect insects at low temperatures than *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*.

8. Tolerance of entomopathogenic nematodes to desiccation decreased drastically between 97 and 85% RH, with a wide variability among strains. *Steinernema feltiae* and *Steinernema carpocapsae* showed a greater adaptability to desiccation than *Heterorhabditis bacteriophora*.

9. Tolerance of entomopathogenic nematodes to hypoxia also showed a wide variability among strains. Some strains of *Steinernema. feltiae, S.carpocapsae* and *H. bacteriohora* survived more than 96 h under hypoxia conditions.

10. The entomopathogenic nematodes isolated were able to migrate up to 20 cm deep in sandy soil with and without a *Galleria mellonella* larva. The presence of an insect increased the vertical movement of the nematodes. *Steinernema feltiae* and *Heterorhabditis bacteriophora* showed a greater capacity to migrate in the sand column than *Steinernema carpocapsae,* with a larger percentage of infective juveniles remaining on the surface. The host seeking strategy of the three *Heterorhabditis bacteriophora* strains, the *Steinernema carpocapsae* strain and the majority of *Steinernema feltiae* strains match with the previous classification of cruisers, ambushers and intermediates, respectively, reported by other authors.

11. The qualitative analysis comparison, considering the ecological characterization and the virulence of the strains, showed that three *Steinernema feltiae* strains (Bpa, Bsor and M116) are ideal candidates to control *Capnodis tenebrionis.*

12. The assay of the *Capnodis tenebrionis*' larvae susceptibility showed that entomopathogenic nematodes are able to penetrate into larvae galleries, locate and kill them, and therefore confirms the efficacy of the entomopathogenic nematodes to control the larva stages inside the parasited tree.

13. The field assay showed that the *Steinernema feltiae* strain Bpa was able to control *Capnodis tenebrionis* in a commercial cherry orchard by drench and injection. The similar efficacy of the two application methods leads us to recommend the drench method as it requires less effort to apply.

14. The present study shows the efficacy of some native strains of entomopathogenic nematodes to control the flat-headed rootborer, *Capnodis tenebrionis,* and their potential to contribute, as a commercial product, to control the different stages of this important stone fruit pest.

Everything has been thought of before,
but the problem is to think of it again.

Johann Wolfgang Von Goethe

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