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

# Control biològic del diabló de l'avellaner *Curculio nucum*, L. (Coleoptera: Curculionidae), mitjançant nematodes entomopatògens (Rhabditida: Steinernematidae i Heterorhabditidae)

Laia Batalla Carrera

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**Tesi doctoral dirigida per**

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**Universitat Autònoma de Barcelona**

**2013**

Aquest treball de Recerca - que forma part del programa de Doctorat en Biologia Animal de la Universitat Autònoma de Barcelona- ha estat desenvolupat en el marc del projecte "Control biològic del diablò de l'avellaner, Curculio nucum L. (Coleoptera, Curculionidae) mitjançant organismes entomopatògens (nematodes i fongs)" del Ministeri d'Educació i Ciència (AGL2006-03728/AGR).

A la meva família, a la meva gent, a la meva terra

A la sort que m'acompanya

Som la flor que naix de la llavor que vareu sembrar
Sou la llum que guia en l'obscuritat
Som les vostres veus i no ens faran callar
Perquè mai perdrem la nostra dignitat
Continuar la senda de la nostra essència i trobar
I trobar una resposta per demà
No ens podran guanyar mai, si ens donem la mà
I agafem l'herència que ens vareu deixar

Hui cantarem la vida d'un poble
Vida d'un poble que no vol morir
Vull donar-te les gràcies perquè vull
Perquè tu em fas sentir-me viu
Gràcies a tu que cantaves furtiu
Gràcies a tu que em donaves caliu!
Gràcies a tu que cantaves furtiu
Gràcies a tu hui sóc l'últim que riu!!!

(ASPENCAT-L'Herència)

# Agraïments

***I així pren tot el fruit que et pugui donar el camí que, poc a poc, escrius per a demà. Que demà mancarà el fruit de cada pas; per això, malgrat la boira, cal caminar.***

*(Lluís Llach - Que tinguem sort)*

Als que em coneixeu, és a dir, la majoria dels qui us esteu prenent la molèstia de llegir això, sabeu que podria escriure i escriure sense parar agraint per tot allò que m'envolta, i també sabeu, que el meu caparró de mustèlid farà que m'oblidi de gent a qui porto al cor, sovint res a vore amb el que tinc al cap… Començaria per la meva sort que sempre m'ha acompanyat a la vida i arribaria a aquelles persones, desconegudes, que et somriuen quan te'ls creues pel carrer… així que intentaré sintetitzar.

Vull agrair als meus directors Fernando i Ana, la paciència i la gran dedicació d'aquests anys. Per donar-me l'oportunitat de realitzar aquesta tesi doctoral, i tot el que d'ella n'ha derivat. Sento haver aprés molt durant aquests anys, i la recerca n'és només una part. Gràcies.

A tots aquells que en l'àmbit acadèmic han fet que aquesta Tesi sigui, avui, una realitat. A casa, al SSV, especialment a l'Anna Aymamí per la il·lusió de trobar sempre el diabló que s'amaga a la caixa màgica i al Sergi Santamaria per l'ajuda amb els fongs. A França, a tota la gent de l'INRA, en especial a Sylvie Pagès, per introduir-me al món dels bacteris. Semblava impossible treballar amb alguna cosa més petita que un nematode, però... A l'altra banda de l'oceà, al David i al Mike pels consells, l'ajuda i la confiança.

A tota la gent que en algun moment us heu deixat enredar per baixar (o pujar) a Prades, ja sigui a la primavera a buscar adults, amb un fred que pela, com a l'estiu, arrastrant borrasses, amb la xafogor característica de Prades! Moltes gràcies Oscar, Marcos, Eva, Jose, Mayra, Cristina, Dani, Berni, Maria B, Anna, Francesc. A la meva tieta, i els meus pares per venir, en ple agost, a posar larves als tubs.

Al Frede, qui a part d'ajudar-me en totes les tasques de camp, incloses les llargues hores de persistència, m'ha regalat la seva companyia i amistat durant aquests anys ensenyant-me tantes coses. Per descobrir-me els racons més bonics de Prades, les abelles, els bolets, com es fa el vi i iniciar-me en el món de l'agricultura. A la Julia, l'Oscar i el David per l'interès i l'amabilitat.

Als companys i companyes de la Unitat de Zoologia, en especial a la Nuria (plega-tin), la Cecília, la Carolina i la Laia (aranya) amb qui hem compartit aquest viatge com només es pot fer amb aquelles que estan passant per la mateixa experiència. Al Lector per aguantar les meves impertinències estoicament i pels cafès (descafeïnats) del matí, que sempre són més bons amb tu al costat. A l'Emili i l'Anna per ser com són. A l'Anna...per tantes coses i tants moments que no sabria ni per on començar. Moltes gràcies.

Al CREAF en general, per tota l'ajuda i… per la trampa de llum, i la sorra puticlubera, i l'aigua mQ, i els garbells, i la clau de la tanca del Titllar (que prometo tornar algun dia)…i a la gent amb qui he tingut la sort de compartir aquests anys les hores de dinar i les mil aventures que allà s'han covat. Per la festa dels 30, les bodes, les arrossades, calçotades, diamantades, però sobre tot, per un dia-a-dia que em desperta sempre un somriure. Al Jordi, pels trajectes en ferrocarril més emocionants. Al Josep per encertar sempre el moment concret amb la paraula ideal, a l'Albert per no deixar mai de sorprendrem amb tot el que et passa, però sobre tot, per voler-ho compartir amb mi. Al Vicens per deixar-se molestar. A la Mireia Bartrons i l'aire nou que va dur a la gespa, que el reubicarem, segur. I

com no, a les gripaues, que cada dia en som més! mariab (barrachina), mariad (bledi-meri), marta (martacoooll), montse (montxi/xispimor), Rebeca (Rebetxins/clon!), Txell (coushi) Virgina, Mireia, Helena, Lucia per les explosions de rialles i els bon moments. Per fer que res sembli impossible!

A la Maria, per descobrir-me un món. Per totes les aventures que transgradeixen l'espai-temps (visca els neutrins!), per una llibreta que porto al cor, per les sorpreses a la porta pel matí! Per explicar-me mil històries i no retreure'm mai que no les sàpiga. Per voler-ho intentar. Per fer-me tenir ganes de millorar cada dia.

A la Marilins, per ser-hi. Per compartir. Per no jutjar. Per aguantar les atabalamentes que últimament han estat unes quantes...per els caps de setmana a Viladrau amb la taula parada durant hores i hores... per l'energia amb què vius!

A la Rebeca, per estar sempre apunt pel què calgui. Per les diamantades romàntiques. Per tantes aventures. I a la Txell per la força que transmets. A la Montse per estar tan disponible! Al Javi i la Burguipedia per la paciència amb tres lloques com nosaltres. A vosaltres, per aquestes últimes setmanes plenes d'activitats banyades amb mimos i alegria.

Als Biohooligans!! Per ser així. Per la capacitat de riure de tot i retornar el sentit a les coses importants en moments de fluixera. Pels plans, independentment que es cumpleixin o no (perímetre d'Amèrica?) que acostumen a ser tan surrealistes com les formes de morir i fotos que envia l'Oleguer. A la Marta Coll, per cuidar-me i escolar-me tantíssim i potser sense saber-ho haver-se convertit en un gran pilar. Merci. A la Patrim-patram, per tot. Per les excursions, les birres, els ping-pongs, les xerrades, per venir-me a buscar a l'Autònoma encara que acabis d'entrar per la Meridiana.

Gràcies a la penya espardenya, al Coro, al Charlie, al Niño & Co per descobrir-me la millor manera de passar un cap de setmana i ajudar-me a desconnectar. Per tenir la paciència d'aguantar la meva hiperactivitat de bon matí i la meva por a dalt del cim. Per no deixar de portar-me d'excursió per molt que plori cada vegada que arribem a un pendent!

Als tombs per gràcia, que m'han decobert perquè també s'anomenen vols, i és que el temps passa volant quan passeges pel barri. Al Marc, per fer que defensar la Tesi sembli fàcil comparat amb convèncer-te, i pels ànims d'aquest darrers dies.

A Belis, por estos cuatro años de convivencia. Por las pompas de jabón en el salón, los picnics en la plaza, las palomitas en la cama. Por estar siempre dispuesta a escuchar el relato de un mal día. Por hacerme siempre reír y por la paciencia de estos últimos días.

A la meva família que sempre m'ha donat l'amor i suport que es necessita per ser feliç. Que m'estima de manera incondicional i ha fet possible que avui posi aquest punt i final. Gràcies.

# Abstract/Resum

***I, a vegades, contra tot pronòstic una gran bestiesa capgira allò que crèiem lògic, tot fent evident, que per un moment, ens en sortim.***

*(Manel – Ens en sortim)*

The Hazelnut weevil, *Curculio nucum* (L.) (Coleoptera: Curculionidae), is a key pest of hazelnuts causing an important economical impact on crop yield. This report summarizes the studies developed to prove the efficacy of entomopathogenic nematodes to control *C. nucum*.

Entomopathogenic nematodes in the Steinernematidae and Heterorhabditidae families are insect lethal parasites with an endosymbiotic bacteria associated. From a total of 295 soil samples taken from hazelnut areas in the NE of the Iberian Peninsula, 11 (3.73%) were positive to entomopathogenic nematodes. Ten steinernematids and one heterorhabditid were molecular identified as two *S. affine*, five *S. feltiae,* two *S. intermedium,* one *Steinernema* sp. and one *H. bacteriophora*. Also, entomopathogenic fungi were recovered in 133 (45.08%) of the total samples, being *Metarhizium anisopliae* (36.09%) and *Beauveria bassiana* (42.8%) the most frequently isolated.

The virulence of eight of these nematode species and strains, together with a *S. carpocapsae* isolated from an urban garden in Barcelona, was tested against larvae of *C. nucum*. Larval exposure to entomopathogenic nematodes resulted in mortality between 10% caused by *S. affine* and 78.75% by *Steinernema* sp. with significant differences. The virulence of the endosymbiotic bacteria was 100% mortality of larvae except for the *X. bovienii* belonging to *S. affine* (93.3%) and *X. bovienii* belonging to *S. feltiae* (46.7%). The susceptibility of the adult weevils to four nematodes species: *S. feltiae, Steinernema* sp., *S. carpocapsae* and one *H. bacteriophora* was tested. The mean mortality percentages caused by *Steinernema* sp., *S. feltiae* and *H. bacteriophora* were 1.7%, 16.7% and 8.3% respectively, while *S. carpocapsae* proved to be the most virulent nematode, causing 100% insect mortality. The adult exposure to *S. carpocapsae* over different periods of time revealed that 15 minutes were long enough to infect 6.6-17% of the weevils, and adult mortality reached 100% in 120 to 240 minutes of exposure.

To assess the observed differences in susceptibility in relation to the insect stage of development and the nematode species used, penetration into the larva and adult of *C. nucum*, routes of entrance to the insect and insect immune response were evaluated. Results revealed larvae to be more susceptible to *S. feltiae* than to *S. carpocapsae* and adults were highly susceptible to *S. carpocapsae*. The anus was determined as the main entry route of *S. carpocapsae* and *S. feltiae* in adult insects and larvae. The penetration rate was found to be related to the nematode virulence. Our findings suggest rejecting the immune system as being responsible for the different susceptibility between larvae and adults.

The virulence of six entomopathogenic fungi against larvae of *C. nucum* was also tested. The highest larvae mortality (reaching 80%) was due to *M. anisopliae.* When entomopathogenic fungi

were applied in combination with entomopathogenic nematodes no antagonistic or synergistic effects were observed.

Finally, short-term field persistence and the vertical distribution were assessed showing that nematodes can persist nine weeks after application and that nematodes can reach depths of 40 cm. The entomopathogenic nematodes can effectively reduce *C. nucum* populations in field. Efficient biocontrol requires a combined strategy. A summer application using *S. feltiae*, *Steinernema* sp. or *H. bacteriophora* to control the larvae when they are burying into the ground and a spring application with same nematodes species to reduce the overwintering larvae population. An additional spring application with *S. carpocapsae* would effectively control the overwintering and the emerging adults.

El diabló de l'avellana, *Curculio nucum* (L.) (Coleoptera: Curculionidae) és una plaga clau de l'avellaner, responsable de pèrdues important en la collita. Aquest treball pretén estudiar l'efectivitat dels nematodes entomopatògens per controlar *C. nucum*.

Els nematodes entomopatògens pertanyen a les famílies Steinernematidae i Heterorhabditidae. Són paràsits d'insectes que porten associat un bacteri simbiòtic. D'un total de 295 mostres agafades en sòl d'avellaners al NE de la Península Ibérica, en 11 (3,73%) es van aïllar nematodes. Deu steinernemàtids i un heterorhabdítid van ser molecularment identificats com dos *S. affine*, cinc *S. feltiae,* dos *S. intermedium,* un *Steinernema* sp. i un *H. bacteriophora*. També es van aïllar fongs entomopatògens en 133 (45,08%) de les mostres, apareixent *Metarhizium anisopliae* (36,09%) i *Beauveria bassiana* (42,8%) com les espècies més freqüents.

La virulència de vuit d'aquestes espècies de nematodes juntament amb una *S. carpocapsae*, aïllada en un jardí urbà de Barcelona, contra larves de *C. nucum* va ser avaluada. La mortalitat de larves deguda als nematodes varià del 10% causat per *S. affine* i el 78,75% per *Steinernema* sp. amb diferències significatives. La virulència del bacteri simbiont fou del 100% de mortalitat de larves a excepció de *X. bovienii* bacteri simbiont de *S. affine* (93,3%) i *X. bovienii* de *S. feltiae* (46,7%). La susceptibilitat de l'adult a quatre espècies de nematodes: *S. feltiae, Steinernema* sp., *S. carpocapsae* i *H. bacteriophora* també va ser avaluada. Els percentatges de mortalitat observats per *Steinernema* sp., *S. feltiae* i *H. bacteriophora* foren 1,7%, 16,7% i 8,3% respectivament, mentre que *S. carpocapsae* va demostrar ser el nematode més virulent, causant el 100% de mortalitat en l'adult. L'exposició d'adults a *S. carpocapsae* durant diferents períodes de temps va revelar que 15 minuts són suficients per matar 6,6-17% dels diablons i que el 100% de mortalitat es dóna a partir dels 120-240 minuts d'exposició.

Per tal d'estudiar les diferències de susceptibilitat observades entre la larva i l'adult de *C. nucum* i les diferents espècies de nematodes entomopatògens utilitzats, s'avaluaren la penetració, les vies d'entrada i la resposta immunològica en la larva i l'adult de *C. nucum*. Els resultats indicaren que la larva és més susceptible a *S. feltiae* que a *S. carpocapsae* mentre que els adults foren molt susceptibles a *S. carpocapsae*. Es va determinar l'anus com a via preferent d'entrada en la larva i l'adult, tant per *S. feltiae* com per *S. carpocapsae.* La capacitat de penetració s'observà estar en relació amb la virulència del nematode. Basat en les nostres observacions la resposta immunològica queda descartada com a causa principal per explicar les diferències observades en la susceptibilitat als nematodes entomopatògens de larva i adult.

La virulència de sis fongs entomopatògens contra la larva de *C. nucum* també fou testada. La major mortalitat de larves (80%) fou causada per *M. anisopliae.* Quan es van aplicar fongs i nematodes entomopatògens de manera combinada no s'observaren efectes antagònics o sinèrgics.

Finalment, la persistència i la distribució vertical van ser avaluades en assajos de camp, mostrant que els nematodes foren capaços de persistir un mínim de 9 setmanes després de l'aplicació i que assoliren profunditats de fins a 40 cm. Els nematodes entomopatògens pogueren reduir de forma efectiva la població de *C. nucum* al camp. Un control eficaç requereix la combinació de diverses estratègies. Una aplicació d'estiu, utilitzant *S. feltiae*, *Steinernema* sp. o *H. bacteriophora* per controlar les larves quan s'enterrin al sòl, i una aplicació de primavera amb les mateixes espècies de nematodes per reduir la població d'insectes hivernants. Una aplicació addicional de primavera amb *S. carpocapsae* actuarà controlant de manera efectiva tant els adults hivernants com els que emergeixin del terra.

# Índex

***I la pluja es va assecar, i els núvols es van obrir, i en un instant, explosiu,***
***el món es va enfonsar més enllà de l'horitzó.***

*(Roger Mas – I la pluja es va assecar)*

# Introducció

***Pare, si no hi ha pins no es fan pinyons ni cucs, ni ocells.***
***Pare, on no hi ha flors no es fan abelles,***
***cera, ni mel..***

*(Joan Manel Serrat – Pare)*

## Les Muntanyes de Prades i l'agrosistema de l'avellaner

Les Muntanyes de Prades, anomenades així des de l'edat mitjana (Planas, 2001), formen part del sistema litoral de Catalunya, a la província de Tarragona. L'orientació d'aquesta serralada és NE-SW on dominen els altiplans, les moles i les cingleres. Amb una extensió de 260 $Km^2$, el massís de Prades separa el Priorat del Camp de Tarragona. El clima predominant és mediterrani amb estius calorosos i secs i hiverns relativament suaus. No obstant, a les parts més altes el clima és més continental, és a dir, més fred i humit (Domingo i Duran, 1998). La pluviometria és superior a la part nord del massís, amb uns valors mitjans de 600 mm i inferior al sector meridional, amb mitjanes de 500-560 mm. Quan a la temperatura, l'oscil·lació és molt gran, registrant mínims absoluts de -8$^oC$ a màxims de 38$^oC$ (Domingo i Duran, 1998). Els recursos econòmics, basats en l'agricultura de muntanya, són pocs i la demografia molt baixa; menys de 10 habitants per quilòmetre quadrat (Planas, 2001). El cultiu de l'avellana té una llarga tradició a Tarragona. Els primers documents que hi fan referència al terme d'Alforja daten de l'any 1287 (Tasias, 1975). El 1862, en una assemblea del Institut Agrícola Català Sant Isidre feta a Reus, es va recomanar que es canviés la vinya per l'avellaner per combatre la crisi provocada pel fong anomenat oïdi (Canela et al., 2004). Amb l'aparició de la fil·loxera (s. XIX) es va acabar imposant per força aquest canvi, donant així lloc a l'àrea contínua més extensa d'aquest cultiu a tot Europa (Anguera, 1996). L'avellaner és un arbre exigent amb aigua, fet que explica la presència d'aquest conreu a la muntanya, on el clima és més humit que a la plana on es requereixen notables aportacions hídriques en forma de reg (Girona, 1995). La impossibilitat de satisfer tota la demanda hídrica, juntament amb la dificultat de mecanització en bona part de l'àrea conreada degut a la forta pendent dels terrenys on es troben assentats els conreus, té una incidència negativa en el rendiment del cultiu (Reguant, 1996). A aquests problemes estructurals del cultiu de l'avellaner es suma la pressió dels mercats i l'elevada producció per part de països com Turquia que fan necessari explorar nous camins per comercialitzar aquest producte (Ismet et al., 1991). És per això que l'administració ha desenvolupat diverses línies d'ajuts per a la producció d'avellanes dins del marc del "Programa zonal de l'avellaner" establert per l'Ordre de 17 de novembre de 1995 (DOGC nº 2134 de 29/11/95). En els darrers anys ha potenciat la producció integrada de l'avellana, desenvolupant normes tècniques de producció integrada (Resolución ARP/2164/2005 de 12 de julio. DOGC nº 4427 de 15/07/2005) que malauradament no s'han traduït en un increment del valor de l'avellana.

L'alta qualitat del producte representa una opció necessària per a atendre el repte d'un mercat fortament competitiu que compta amb un consumidor cada cop més exigent (Reguant, 1996). Així mateix, per tal de fer front a la pressió de diversos factors de cost (baix rendiment, cost de mà d'obra, orografia que impedeix la mecanització, cost de l'aigua) cal cercar els marges de rendibilitat suficients, oferint una qualitat del producte que marqui la diferència (Monfort, 1992). La producció ecològica de l'avellana, pràctica encara molt minoritària, pretén donar resposta a la creixent

demanda de productes d'aquestes característiques que hi ha actualment. D'aquesta manera es donaria viabilitat al cultiu de l'avellaner de muntanya, es mantindria la xarxa social i econòmica que genera la seva producció i l'interès ecològic de les Muntanyes de Prades.

Un dels motius que impedeixen l'expansió del cultiu ecològic de l'avellana és el conjunt de plagues que l'ataquen, arribant a produir pèrdues de fins al 80% en alguns casos (AliNiazee, 1998). A Catalunya, àcars com *Phytoptus avellanae*, *Eotetranychus carpini* o *Tetranychus urticae*; lepidòpters com *Zeuzera pyrina*, *Archips rosana* o *A. xylosteana*; homòpters com *Myzocallis corylii*, *Corylobium avellanae*, o *Eulecanium caryli*, heteròpters com *Pantilius tunicatus* o *Nezara viridula*; o coleòpters com *Cerambyx cerdo* o *Melolontha*, poden provocar pèrdues en el cultiu de l'avellaner, fent necessari l'ús de plaguicides pel seu control (Barrios, 1997). Per alguna d'aquestes plagues no s'ha trobat agent de control compatible amb la normativa de producció ecològica. Tal és el cas de *Curculio nucum*, considerat plaga clau de l'avellaner, per a la qual no es disposa de control efectiu (Barrios, 1997; DAR, 2012).

### *Curculio nucum*, el Diabló de l'Avellaner

El diabló de l'avellaner, *C. nucum* L. (Coleoptera: Curculionidae), abans *Balaninus nucum* L., és l'insecte plaga amb major impacte econòmic en el cultiu de l'avellaner avui en dia (AliNiazee, 1998). La seva distribució s'estén per tota l'Europa temperada i septentrional. A la Península Ibèrica s'ha descrit a Astúries, Galícia i Catalunya (AliNiazee, 1998). A escala local, la distribució de *C. nucum* queda parcialment determinada per les diferents varietats d'avellaners que existeixen i l'altitud a la que aquestes són plantades (Tuncer i Ecevit, 1997).

**Figura 1.** Adult del Diabló, *C. nucum* fent la picada alimentícia.

## Biologia i ecologia

Tot i la importància de la plaga, són pocs els estudis detallats de la seva biologia i ecologia. Els adults mesuren uns 6,5 mm (sense comptar el rostre) són de color marró-verdós, recoberts de pubescència (Akça i Tuncer, 2005) (figura 1). Com tots els curculiònids, el tret més característic és el rostre que presenta l'adult, un terç més llarg en femelles que en mascles, tret que ajuda a determinar el sexe dels individus. Les larves passen per quatre estadis, mesuren entre 4 i 8 mm, són gruixudes, corbades i de color blanquinós, posseeixen una càpsula cefàlica ben diferenciada de color marró i un aparell bucal mastegador amb mandíbules molt esclerotitzades (Akça i Tuncer, 2005) (figura 2).

**Figura 2.** Tercer estadi larvari de *C. nucum* alimentant-se de l'avellana.

L'adult emergeix del sòl a finals d'abril i durant els mesos de maig i juny s'alimenta del fruit immadur, fulles tendres i flors del l'avellaner (Akça i Tuncer, 2005). En aquest període també s'ha observat *C. nucum* alimentant-se d'arbres fruiters com nogueres, pereres, cirerers i presseguers (AliNiazee, 1998). Passades de 4 a 8 setmanes, quan els adults maduren sexualment es dóna l'aparellament (Bel-Venner et al., 2009). L'ovoposició té lloc des de finals de maig a principis de juliol. La femella perfora l'avellana amb el rostre i seguidament introdueix un únic ou mitjançant l'ovopositor (Akça i Tuncer, 2005). Cada femella pot pondre entre 25 i 30 ous. L'ou eclosiona als 8-10 dies de la posta, donant lloc a una larva que es desenvoluparà dins l'avellana de la qual s'alimenta durant el següent mes (Akça i Tuncer, 2005) (figura 2). Generalment, les avellanes afectades cauen de manera prematura al terra. A començaments d'agost, la larva ha completat el seu desenvolupament, fa un orifici circular a la closca de l'avellana, anomenat xiulet (figura 3), pel que sortirà per tal d'enterrar-se a una profunditat d'entre 10-40 cm, depenent de les característiques del sòl. És aquí on tindrà lloc una prolongada diapausa en forma de larva (Akça i Tuncer, 2005; Bel-Venner et al., 2009). El diabló hiberna al terra en forma de larva o d'adult

(Coutin, 1992; AliNiazee, 1998, Akça i Tuncer, 2005; Bel-Venner et al., 2009) i emergeix en forma d'adult a principis de maig. Tot i que la majoria d'individus emergeixen la primavera següent, un cert nombre pot romandre enterrat durant de dos a cinc anys (Akça i Tuncer, 2005; Bel-Venner et al., 2009).

**Figura 3.** Quart estadi larvari de *C. nucum* sortint de l'avellana pel xiulet, orifici que fa amb les mandíbules.

### Danys i control

Distingim dos tipus de danys produïts per aquest insecte. El primer, però menys important, és degut a la picada alimentària dels adults sobre el fruit immadur, arribant també a ferir el mànec del floc d'avellanes, pecíol i fulles (Paparatti, 1990; Pucci, 1992) (figura 1). L'arbre pot avortar els fruits depredats. Tanmateix, aquest fet es considera que té poca importància econòmica. Els danys més importants són els causats la larva en alimentar-se del fruit de manera que el destrueix (figura 2).

El control cultural que tradicionalment s'utilitzava contra aquesta plaga consistia en sacsejar les branques a la matinada i recollir en borrasses les avellanes atacades i els insectes, per després cremar-los, sent aquest mètode poc eficaç (Barrios, 1997; Barrios et al., 2011). Actualment, el control cultural aposta per plantar varietats primerenques d'avellaner que endureixen ràpidament el fruit de manera que, quan la femella emergeix del sòl, la closca de l'avellana és massa dura per poder-la perforar amb el rostre i dipositar-hi l'ou (Chambers et al., 2011). No obstant, el mètode més estès per combatre aquesta plaga des dels anys 40 és el químic. Es dirigeix contra l'adult en el període en què aquest s'està alimentant o aparellant damunt la planta (AliNiazee, 1998). Entre 1940 i 1945 es feia servir el diclorodifeniltricloroetà (DDT) del grup dels organoclorats, fins que va ser substituït per insecticides de la família dels carbamats, que esdevingué clau en la lluita contra el diabló (Barrios et al., 2011). Com a conseqüència de la lluita química va aparèixer una nova plaga secundària, el badoc (*P. avellanae*), un àcar que afecta les gemmes vegetatives de l'avellaner

(Barrios et al., 2011). Durant els anys 60 va sortir al mercat un nou producte l'endosulfan, que controlava les dues plagues (el diabló i el badoc), seguit de metiocarb (Couturié, 2008). També s'utilitzà monocrotofos, que més tard es comprovà que eliminava els ocells insectívors controladors de la *Z. pyrina*, que conseqüentment va esdevenir una altra plaga de l'avellaner (Barrios et al., 2011). Durant els anys 1960-1970 s'institucionalitzà el calendari de tractaments i fou aleshores quan la problemàtica de les plagues secundàries va començar a sortir a la llum, encapçalada pel badoc, l'aranya roja (*T. urticae*) i seguida per la del borró sec (*Cryptosporium corily*) (Barrios et al., 2011). Així doncs, l'ús de plaguicides en general, i contra *C. nucum* en particular, s'ha vist reduït en els darrers anys. Mentre l'any 2002 encara s'empraven endosulfan, clorpirifos, metomilo, carbaril, benfuracarb, bifentrin (segons les normes tècniques de producció integrada de fruits secs, Decreto 241/2002, DOGC nº3744 de 21/10/2002), actualment el clorpirifos al 3% és l'únic plaguicida permès (MAAM, 2010). Degut a les restriccions i la dificultat de controlar *C. nucum* amb plaguicides químics cal trobar nous mètodes de control.

### *C. nucum* i el Control biològic

El control biològic és l'ús dels anomenats enemics naturals (predadors, paràsits i patògens) per controlar una determinada plaga. Darrerament es tendeix a conservar i augmentar la fauna autòctona amb potencial d'agent de biocontrol (Van Driesche i Bellows, 1996). En el cas del diabló de l'avellaner, el control biològic mitjançant fauna auxiliar és molt limitat. No hi ha evidències de l'ús efectiu de parasitoids ni depredadors contra aquest insecte (Paparatti i Speranza, 2005). Pel que fa a la utilització de virus i bactèries, s'ha observat una mortalitat del 90% utilitzant el Virus de la Poliedrosis Nuclear (VPN, baculoviridae) (Mustafa et al., 1999) i un 45% de mortalitat utilitzant la soca de *Bacillus thuringiensis* serovar kurstaki (H3abc) (Bn1) (Kati et al., 2007). No obstant, tot i els bons resultats que s'ha obtingut, el fet que tots dos agents actuin per ingestió i el poc temps que l'insecte queda a l'abast d'aquests, dificulta molt l'ús de virus i bacteris a gran escala i els fa poc efectius com a agents de control d'aquesta plaga al camp. És per això que calen nous agents de control biològic compatibles amb l'agricultura ecològica, com podrien ser els agents entomopatògens, fongs i nematodes, paràsits de gran nombre d'espècies d'insectes (Georgis et al., 2006; Grewal i Peters, 2005; Khachatourians i Sohail, 2008).

Els fongs entomopatògens, pertanyen majoritàriament a les divisions Ascomycota i Zygomycota, es troben àmpliament distribuïts arreu del món (Meyling i Eilenbergi, 2005) i s'ha comprovat el seu potencial com agents de control biològic (Charnley i Collins, 2007). La seva virulència contra diverses espècies d'insectes ha estat testada (Khachatourians i Sohail, 2008; de La Rosa et al., 2000; Amer et al., 2008), essent *B. bassiana* i *M. anisopliae* els més utilitzats (Petlamul i Prasertsan, 2012) i que actualment es poden trobar al mercat en productes comercials (Ferron et al. 1991; Ekesi et al. 2001). Els fongs entomopatògens s'han aïllat en diferents hàbitats, un d'ells, les plantacions d'avellaners (Keller et al., 2003; Sevim et al., 2010). Paparatti i Speranza (2005) van avaluar

l'efectivitat d'una soca comercial de *B. bassiana* contra la larva de *C. nucum* en tractaments al camp, obtenint un 99,5% de mortalitat en front a un 63,5% de mortalitat observada en el control.

## Els nematodes entomopatògens i el seu bacteri simbiont

Dins del grup dels nematodes es coneixen més de 30 famílies que tenen algun tipus de relació amb insectes (Nickle, 1972; Poinar, 1975, 1990; Maggenti, 1981; Kaya i Stock, 1997). Dins d'aquestes, hi ha set famílies amb capacitat de parasitar insectes entre les que destaquen Heterorhabditidae i Steinernematidae pel seu potencial com agents de control biològic d'insectes (Alatorre-Rosas i Kaya, 1990; Peters, 1996; Lacey et al., 2001) (taula 1).

**Taula 1.** Situació de les famílies Steinernema i Heterorhabditis segons Stock i Hunt (2005)

FILUM NEMATODA (Lankester 1977) Rudolphi 1808
Classe Chromadorea Inglis 1983
Subclasse Chromadoria Pearse 1942
Ordre Rhabditida Chitwood 1993
Subordre Tylenchina Thorne 1949
Infraordre Panagrolaimomorpha De Ley i Blaxter 2002
Superfamília Strongyloidea Chitwood i McIntosh 1934
**Família Steinernematidae Chitwood i Chitwood 1937**
Gènere *Steinernema* Travassos 1927
Gènere *Neosteinernema* Nguyen i Smart 1994
Subordre Rhabditina Chitwood 1933 (Oerly, 1880)
Infraordre Rhabditomorpha De Ley i Blaxter 2002
Superfamília Strongyloidea Baird 1853
**Família Heterorhabditidae Poinar 1975**
Gènere *Heterorhabditis* Poinar 1976

Dins la família Heterorhabditidae trobem un únic gènere *Heterorhabditis* Poinar, 1975 amb més de 14 espècies (Stock et al., 2009). La família Steinernematidae avarca dos gèneres: *Steinernema* Travassos, 1927 i *Neosteinernema* Nguyen i Smart, 1994. El primer, amb una única espècie representant *N. longicurvicauda* i amb 66 espècies (Lee, 2009; Shapiro-Ilan et al., 2012). Malgrat *Steinernema* i *Heterorhabditis* no pertanyen a famílies filogenèticament properes (Blaxter et al. 1998) presenten característiques comunes com a conseqüència d'una convergència evolutiva (Poinar, 1993; De Ley i Blaxter 2002) (taula 2). Es creu que *Steinernema* prové d'un ancestre terrestre, proto-rhabditonema, mentre que el d'*Heterorhabditis* el trobaríem en nematodes Rhabditids marins com *Pellioditis marina* (Poinar 1993; Blaxter et al., 1998).

**Taula 2.** Característiques diferencials de les famílies Steinernematidae i Heterorhabditidae (Forst i Clarke, 2002).

| Tret fenotípic | *Steinernema* | *Heterorhabditis* |
|---|---|---|
| Primera generació d'adults | Amfimíctiques | Hermafrodites |
| Localització del bacteri | En una vesícula intestinal específica | Part distal de l'intestí |
| Relació filogenètica | Rhabditida(Strongyloididae) i Rhabditida (Panagrolainidae) | Rhabditida(Strongyloididae) i Stongylida |
| Retenció de bacteri en fase II | Si | No |
| Formes infectives | Sense dent cuticular<br>Porus excretor per damunt de l'anell nerviós<br>6-8 línies laterals | Amb dent cuticular<br>Porus excretor per sota de l'anell nerviós<br>2 línies laterals |
| Primera generació de mascles | Sense bursa<br>10 o 11 parells més una imparell papil·les genital | Amb bursa<br>9 parells papil·les genital o número inferior |

Les dues famílies s'han descrit com patògens obligats d'un ampli ventall d'insectes (Georgis et al., 2006). Presenten un únic estadi de vida lliure, anomenat juvenil infectiu, que no s'alimenta de tal manera que utilitza les reserves emmagatzemades fins que troba un hoste (Poinar, 1990) i el parasita mitjançant la interacció mutualista de bacteris dels generes *Xenorhabdus* sp. o *Photorhabdus* sp. (Poinar, 1979). No obstant, darrerament s'ha posat en dubte el concepte de parasitisme obligat per descriure la relació entre els nematodes entomopatògens i l'insecte (Griffin, 2012), ja que s'ha vist que les formes infectives també poden penetrar en cadàvers morts per altres causes com pesticides, virus o congelació (San Blas i Gowen, 2008; Puza i Mráček, 2010). San Blas i Gowen (2008) recomanaren que es consideri els nematodes entomopatògens com a carronyaires facultatius en comptes de paràsits obligats.

Els gèneres *Xenorhabdus* i *Photorhabdus* es troben en associació simbiòtica amb els nematodes *Steinernema* i *Heterorhabditis*, respectivament (Forst et al., 1997). *P. asymbiotica* i un altre espècie no identificada de *Photorhabdus* ,que s'han descrit com a paràsit oportunista d'humans, en són l'excepció (Farmer et al., 1989; Peel et al.,1999). *Xenorhabdus* i *Photorhabdus* pertanyen a la subdivisió gamma dels Proteobacteris, família dels Enterobacteris. Són bacils Gram negatius que posseeixen l'antigen ECA (*Enterobacterial Common Antigen*) característic d'aquesta família de bacteris (Ramia et al., 1982). Tanmateix, fenotípicament (Holt et al., 1994) i genotípicament (Brenner i Farmer, 2005) presenten diferències amb la resta de representants de la família Enterobactericeae (taula 3). També s'observen caràcters diferents entre els dos gèneres; *Photorhabdus* presenta bioluminescència i és catalasa positiu, mentre que *Xenorhabdus* no (Forst et al., 1997). La presència de bioluminescència en *Photorhabdus* es relaciona amb l'origen marí d'aquest bacteri (Dunlap i Kita-Tsukamoto, 2006). Al igual que s'ha vist en els nematodes entomopatògens, estudis genètics i fisiològics suggereixen que *Xenorhabdus* i *Photorhabdus* no

tenen un origen comú, però en canvi, han convergit en l'estratègia d'establir una associació simbiòtica amb nematodes entomopatògens (Goodrich-Blair i Clarke, 2007;.Griffin et al., 2001; Poinar, 1993; Chaston et al., 2011).

**Taula 3.** Característiques principals de *Xenorhabdus* i *Photorhabdus* (Boemare, 2002).

| | *Xenorhabdus* | *Photorhabdus* |
|---|---|---|
| Bioluminescència | - | + |
| Catalasa | - | + |
| Hemòlisis | - | d |
| Activitat ureasa | - | d |
| Producció $H_2S$ | - | - |
| Reducció nitrats | - | - |
| Manosa | + | + |
| Pigments | - | Anthraquinones |
| Antibiòtics | Xenocoumacins, Xenorhabdins | Hidroxistilbenes |
| Proteïnes cristall | 26 KDa, 22 KDa | 11.6 KDa, 11.3 KDa |

+ 90-100% de les soques són positives; d 26-75% són positives; - 0-10% són positives

Tant *Xenorhabdus* com *Photorhabdus*, presenten una conversió fenotípica que diferencia entre l'anomenada fase I i la fase II del bacteri. Aquesta conversió s'atribueix a inversions en el material genòmic que afecta la morfologia, motricitat i activitat enzimàtica entre d'altres (Boemare, 2002; Givaudan et al., 1995; Smigielski et al., 1994). Diverses característiques presents en la fase I com la bioluminescència, motilitat o la producció d'enzims extracel·lulars, en la fase II no existeixen o es manifesten més atenuadament (Akhurst, 1980; Boemare et al., 1997) (taula 4). Mentre que la fase I és transportada de manera natural pels formes infectives (Boemare 2002; Smigielski et al., 1994), la fase II només s'ha aïllat al laboratori i no és capaç de sustentar el creixement i reproducció dels nematodes (Akhurst, 1980; Forst i Clarke, 2002). S'ha suggerit que la funció d'aquesta fase II podria estar relacionada amb un possible estat de resistència d'aquests bacteris per sobreviure fora del nematode (Smiglielski et al., 1994).

**Taula 4.** Caracterització general de les fases de variació descrites per *Xenorhabdus* i *Photorhabdus* (Forst i Clarke, 2002)

| | *Xenorhabdus* | | *Photorhabdus* | |
|---|---|---|---|---|
| Característiques | **Fase I** | **Fase II** | **Fase I** | **Fase II** |
| Hàbitat natural | Nematode | Cultius estacionaris | Nematode | Cultius estacionaris |
| Producció d'antibiòtics | Si | No | Si | No |
| Absorció de pigments | Si | No | Si | No |
| Bioluminescència | No | No | Si | <Fase I |
| Activitat lipolítica | Si | >Fase I | Si | <Fase I |
| Activitat proteasa | Si | <Fase I | Si | <Fase I |
| Inclusions proteiques | Si | No | Si | No |
| Mobilitat | Si | No | - | - |
| Pigmentació en agar nutritiu | Si | Canvia | Si | >Fase I; canvia |
| Canvi de fase | Si | Si (reversible) | Si | No |

## Complex nematode-bacteri

*Xenorhabdus* i *Photorhabdus* són filogenèticament més propers entre si que els nematodes que els allotgen (Chaston et al., 2011), fet que suggereix que tant *Xenorhabdus* com *Photorhabdus* van divergir del seu ancestre comú més recentment (Chaston et al., 2011). Aquest ancestre comú, potser va ser capaç de colonitzar *Steinernema* i *Heterorhabditis*, i l'associació amb l'espècie de nematode podria haver donat lloc a l'especiació de *Xenorhabdus* i *Photorhabdus* (Chaston et al., 2011). Diverses evidències suporten aquesta hipòtesi, tanmateix encara no s'ha pogut afirmar de manera concloent (Lee, 2009).

El número de bacteris que cada nematode porta associats varia entre 50 i 200 (Goetsch et al., 2006) depenent de l'espècie de nematode (Sicard et al., 2003). *Heterorhabditis* els porta allotjats a l'intestí (Endo i Nickle, 1991; Ciche i Ensign, 2003) i els allibera a l'interior de l'insecte hoste regurgitats per la boca (Bird i Akhurst, 1983), mentre que *Steinernema* els transporta en una vesícula específica (Bird i Akhurst, 1983; Martens et al., 2003) (figura 4) i els allibera per l'anus (Boemare et al., 1996). Els nematodes allotgen una única espècie de bacteri al seu interior, són monoaxènics (Bonifassi et al., 1999), mentre que una determinada espècie de bacteri pot ser albergat per més d'una espècie de nematode (Boemare, 2002) (taula 5); és el cas de *X. bovienii* que es troba en simbiosi amb diverses espècies de steinernematids (Adams et al., 2006). La relació nematode-bacteri queda assegurada gràcies als components antimicrobians que produeixen els bacteris endosimbiòtics, que inhibeixen el creixement de qualsevol altre microorganisme perpetuant, d'aquesta manera l'associació entre el nematode i la soca determinada de bacteri (Boemare et al., 1993; Hu et al., 1998; Maxwell et al., 1994; Thaler et al., 1997).

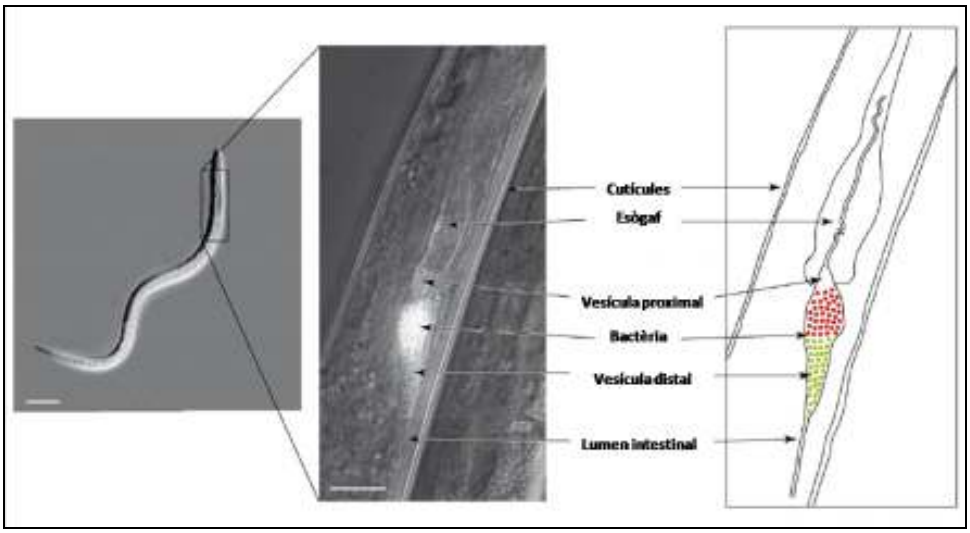


**Figura 4.** Detall i esquema d'un juvenil infectiu transportant *Xenorhandus* a la vesícula d'Akhurst (Goodrich-Blair, 2007).

En aquesta relació mutualista, el nematode transporta el bacteri i li permet l'entrada dins l'insecte hoste, el qual serveix de font de nutrients pel bacteri. El bacteri al seu torn allibera

components químics (SDF, *Scavengers Deterrent Factor*) que dissuadeixen els organismes carronyaires d'alimentar-se d'insectes parasitats per nematodes entomopatògens (Gulcu et al., 2012). Nematodes i bacteris treballen junts, alliberant toxines, per tal de vèncer la resposta immunològica de l'insecte hoste, de manera que el bacteri pugui proliferar i el nematode créixer i reproduir-se (Kim et al., 2005; Park et al., 2003; Park i Forst, 2006; Shrestha et al., 2007; Koppenhöfer, 2007).

**Taula 5.** Relació nematodes entomopatògens i bacteri simbiont (Adams et al. 2006).

| Complexe *Steinernema- Xenorhabdus* | |
|---|---|
| *S. carpocapsae* | *X. nematophila* |
| *S. affine, S. feltiae, S. intermidium, S. kraussei* | *X. bovienii* |
| *S. glaseri, S. cubanum* | *X. poinarii* |
| *S. longicaudum* | *X. beddingii* |
| *S. kushidai* | *X. japonica* |
| *S. bicornotum* | *X. budapestensis* |
| *S. serratum* | *X. ehlersii* |
| *S. scaperisci* | *X. innexi* |
| *S. rarum* | *X. szentirmaii* |
| Complexe *Heterorhabditis-Photorhabdus* | |
| *H. bacteriophora* Brecon | *P. luminiscens* subsp. *luminiscens* |
| *H. indica* | *P. luminiscens* subsp. a*khurstii* |
| *H. bacteriophora* HP88 | *P. luminiscens* subsp. *laumondii* |
| *H. bacteriophora* (Turquia) | *P. luminiscens* subsp. *kayaii* |
| *H. bacteriophora* (Turquia) | *P. luminiscens* subsp. *thracensis* |
| *H. zealandica, H. bacteriophora* NC1, *H. megidis* (soqu | *P. temperata* |
| Neàrtiques) | |
| *H. megidis* (soques Paleàrtiques) | *P. temperata* subsp. *temperata* |

Aquesta associació nematode-bacteri que tradicionalment s'ha descrit com a mutualista, s'ha vist que és complexa, ja que la relació que es dóna entre els dos organismes té un alt cost pel nematode en la seva fase de vida lliure, formes infectives, i no és fins que el nematode entra dins l'insecte hoste que comença a rebre els beneficis d'aquesta interacció (Emelianoff et al., 2007). En aquest sentit, Sicard et al (2003) va demostrar com la *fitness* dels nematodes dins de l'insecte hoste era major en presència de bacteri simbiont que en la seva absència. Per altra banda, Mitani et al (2004) ha posat de manifest una major mortalitat de les formes infectives que carreguen bacteri que d'aquelles sense bacteri després d'un perllongat emmagatzemament a 25$^{o}$C. És sabut que *Xenorhabdus* es multiplica lentament dins la vesícula de les formes infectives (Martens et al., 2003; Sicard et al., 2003) i per tal de multiplicar-se, els bacteris necessiten nutrients que, en aquesta situació, únicament poden obtenir de les reserves del juvenil infectiu. En aquest sentit, s'especula que aquelles formes infectives que porten una major quantitat de bacteri al seu interior perden més reserves energètiques sustentant-los (Emelianoff et al., 2007). Això suggereix que mentre que el bacteri és sempre beneficiós pels nematodes quan aquests es troben parasitant un insecte, podrien suposar un cost per a les formes infectives (Emelianoff et al., 2007).

## Biologia i ecologia dels nematodes entomopatògens

Tots els estadis del cicle dels nematodes entomopatògens són endoparàsits, a excepció de, com s'ha mencionat, les formes infectives les quals presenten una doble cutícula amb anus i boca tancats, i que per tant, no s'alimenten (Poinar, 1990). Aquestes formes infectives són les responsables de la infecció, penetrant a l'insecte hoste a través de les obertures naturals (boca, anus i/o espiracles) o, en alguns casos, travessant la cutícula fins arribar a l'hemocel (Peters i Ehlers, 1994; Dowds i Peters, 2002), on després de desfer-se de la doble cutícula, alliberen el bacteri que ràpidament es començarà a multiplicar. Les toxines produïdes principalment pels bacteris, però també pels nematodes, maten l'insecte en 24-72 hores (Dows i Peters, 2002). El nematode s'alimenta del cultiu format pels teixits degradats de l'insecte i el bacteri i es desenvolupa, arribant-se a produir d'una a tres generacions de nematodes adults, en funció de la quantitat de nutrients disponibles (Poinar, 1990; Kaya i Gaugler, 1993) (figura 5).

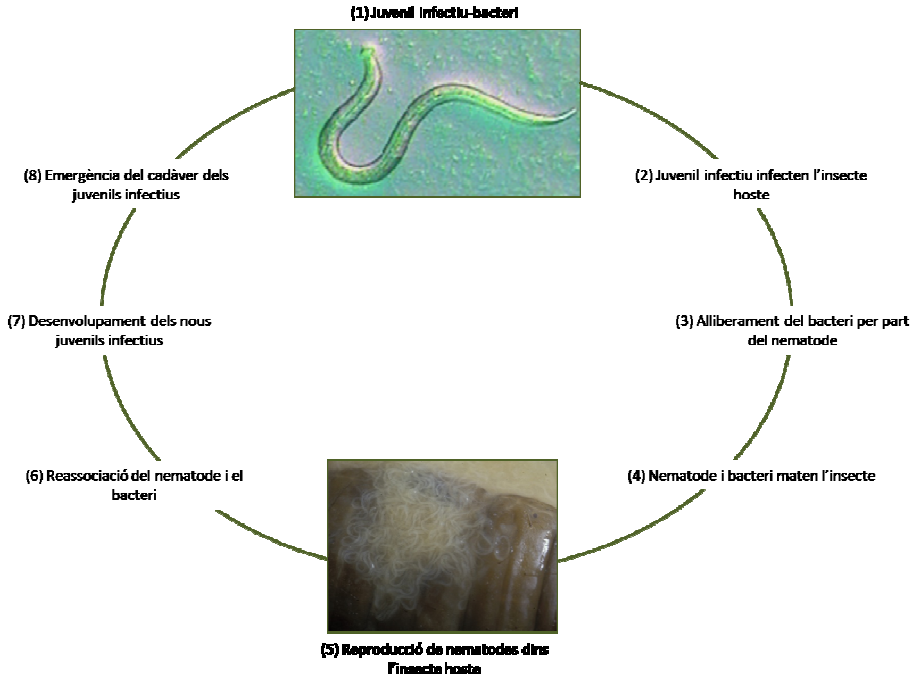


**Figura 5.** Cicle biològic dels nematode entomopatògens i el seu bacteri simbiont.

La primera generació d'adults en els heterorhabditids és hermafrodita que per autofecundació donen lloc a una segona generació amfimíctica, i en algun cas noves formes hermafrodites (Dix et al., 1992). En el cas de la gran majoria de steinernematids totes les generacions són amfimíctiques (Poinar, 1990). Les dues famílies presenten oviparisme o ovoviviparisme i dipositen els ous en l'interior de l'insecte. Sovint l'eclosió dels ous té lloc quan aquests encara són dins l'úter matern, situació que porta al desenvolupament de les formes infectives que s'alimenten dels teixits materns. Aquest procés es coneix amb el nom d'endotòquia matricida (Wang i Bedding, 1996; Johnigk i Ehlers, 1999) i es dóna tant en heterorhabditids com en steinernematids. És una característica que sembla respondre a una adaptació ecològica quan els nutrients escassegen per

assegurar la reproducció i la supervivència dels nematodes entomopatògens (Johnigk i Ehlers, 1999). Quan es consumeixen els recursos alimentaris del cadàver de l'insecte, els nematodes es transformen en noves formes juvenils infectives de manera sincronitzada gràcies a l'acció de feromones Ascarosides (Kaplan et al., 2012; Noguez et al., 2012). Les formes infectives retenen novament el bacteri simbiont, fet que en el cas d'*Heterorhabditis* s'ha descrit que succeeix dins l'úter del nematode (Ciche et al., 2008). Les formes infectives amb el bacteri simbiont associat abandonen el cadàver de l'insecte per buscar un nou hoste que parasitar (Martens et al., 2003).

Els nematodes entomopatògens perceben senyals, senyals químiques, tèrmiques i mecàniques de l'ambient i dels insectes que hi habiten. Les fan servir per definir la trajectòria i localitzar l'hoste (Lewis et al., 1993; Rasmann et al., 2005; Lei et al., 1992; Van Tol et al., 2001). Algunes d'aquestes senyals que s'han descrit són el $CO_2$ emès pels insectes, que activa el nematode per tal de trobar la font emissora (Lewis et al., 1993; O'Halloran i Burnell, 2003; Hallem et al., 2011), els excrements dels insectes (Grewal et al., 1993; Schmidt i All, 1979) i la temperatura (Byers i Poinar, 1982). Però els nematodes entomopatògens, no només perceben senyals provinents d'hostes potencials directament, sinó que també reben senyals de plantes que són atacades per herbívors com son kairomones o sinomones (Turling et al., 1990; Heil, 2008; Dicke i Baldwin; 2010; Ennis et al., 2010). En aquest sentit, s'ha vist que arrels de cítrics atacades per insectes com *Diaprepes abbrebiatus* emeten sinomones com a mecanisme de defensa indirecte ja que atrau a nematodes entomopatògens.

S'ha vist que els nematodes poden infectar hostes que ja han estat parasitat prèviament per nematodes. Això té uns costos i uns beneficis associats (Grewal et al., 1993; 1996). Els nematodes entomopatògens reben senyals químics que els indiquen si l'hoste està parasitat (l'insecte estarà debilitat i el nematode tindrà més fàcil accés) (Lewis et al., 2006) i si el nematode que l'ha parasitat és de la mateixa espècie (assegurant-se trobar un co-específic amb qui reproduir-se) (Lewis et al., 2006).

Les formes infectives presenten dos tipus d'estratègies que influeixen en la resposta als diferents senyals físics i/o químics que reben (Lewis et al., 2006). Els nematodes anomenats navegants (*cruiser*), rastregen activament el seu entorn en busca d'un hoste i s'aturen poc mentre que aquells nematodes que mostren un comportament d'emboscada (*ambusher*) esperen el pas d'un hoste potencial (Campbell et al., 2003; Lewis et al., 2006). Existeix una relació entre la mida de les formes infectives i l'estratègia de cerca. Espècies grans, com *S. glaseri* o *S. kraussei* es consideren navegants, mentre que espècies petites com *S. carpocapsae* es consideren d'emboscada (Campbell i Gaugler, 1997; Campbell et al., 2003). Existeix, no obstant, una gradació entre una i altra estratègia com seria el cas de *S. feltiae* (Campbell et al., 2003; Morton and Garcia-del-Pino, 2009). En alguns casos el tipus d'estratègia determina el tipus d'organismes que trobaran. Sovint, nematodes que adopten una estratègia navegant arribaran a insectes sedentaris o de

moviment reduït, mentre que els que desenvolupen una estratègia d'emboscada seran més eficients contra insectes més actius (Lewis et al., 2006). Tanmateix, també s'ha demostrat que espècies típicament d'emboscada com *S. carpocapsae* poden infectar larves i pupes d'insectes que viuen immòbils enterrades a 40 cm de profunditat, com és el cas de *Hylobius abietis* (Dillon et al., 2006).

L'aproximació i el reconeixement de l'hoste van seguits de la penetració del nematode a l'hemocel d'aquest. El comportament dels insectes ha evolucionat amb estratègies evasives amb que poden dificultar el parasitisme. (Cabanillas, 2002; Sicard et al., 2004; Mankowski et al., 2005; Klein et al., 2007). Així, un primer obstacle amb que es troben els nematodes és els moviments violents i d'autoneteja que efectuen molts insectes per repel·lir els nematodes adherits a la cutícula (Gaugler et al., 1994; Koppenhöffer et al., 2000; Koppenhöffer et al., 2007; Mankowski et al., 2005). A més, cada una de les possibles vies d'entrada a l'interior de l'insecte (boca, anus, espiracles i tegument) poden presentar barreres que els nematodes hauran de superar per tal de penetrar a l'interior de l'insecte. Els nematodes que intenten entrar per la boca poden morir esclafats per les mandíbules de l'insecte (Gaugler i Molloy, 1981) i aquells que atempten per l'anus poden ser repel·lits com a conseqüència d'una constant defecació (Bedding i Molyneux, 1982). La pròpia mida de l'obertura pot representar un repte per a la parasitació (Edit i Thurson, 1995). Els nematodes que aconsegueixen penetrar a l'interior de l'insecte via intestinal, hauran de sobreviure als fluids intestinals de l'insecte, que s'ha comprovat que poden matar el 40% dels nematodes que inicialment penetren l'hoste (Wang et al., 1995), i travessar la membrana peritròfica que actua com a defensa morfològica (Forschler i Gardner, 1991). La penetració a través dels espiracles es veu dificultada per estructures d'exclusió, com els espiracles cribiformes de molts escarabèids, o una mida de llum inferior al diàmetre dels nematodes (Koppenhöfer et al., 2007).

Un cop el nematode es troba a l'interior de l'hemocel de l'insecte encara ha de superar la resposta immunològica d'aquest (Castillo et al., 2011). El sistema immunològic dels insectes es basa en una resposta humoral i una resposta cel·lular que actuen conjuntament i sistemàtica contra els agents entomopatògens (Strand, 2008) (figura 6). La resposta humoral es basa en pèptids antimicrobians, radicals d'oxigen, formacions de melanina i coagulants que actuen contra els diferents patògens (Lemaitre i Hoffmann, 2007; Feldhaar i Gross, 2008). La resposta cel·lular es dóna per l'acció d'unes cèl·lules, que es troben circulant a l'hemolimfa de l'insecte, anomenades hemòcits. Els hemòcits són els responsables de processos de defensa com la fagocitosi, la formació de nòduls o l'encapsulació (Strand, 2008).

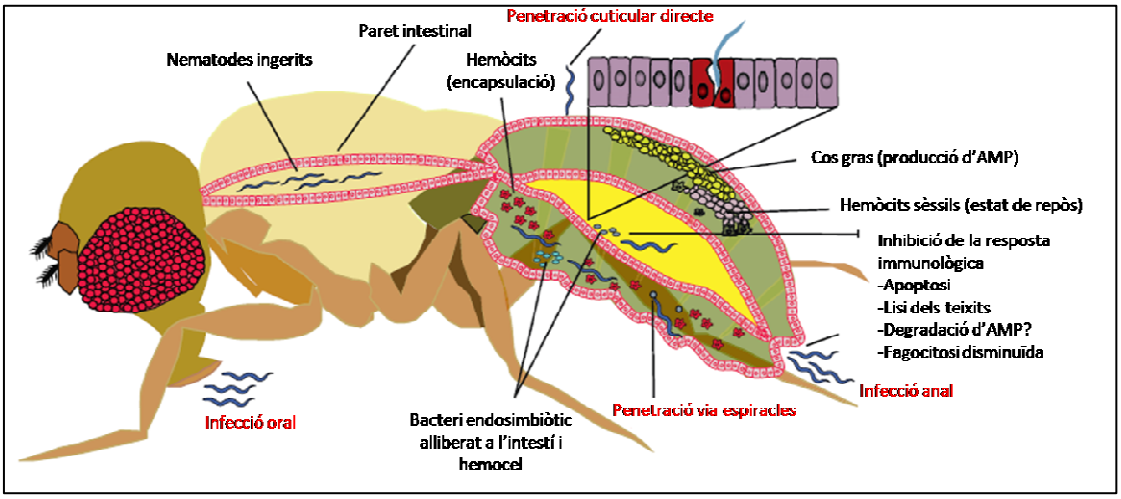


**Figura 6.** Diagrama on es mostren les vies d'entrada preferents dels nematodes entomopatògens a l'interior de l'insecte i les principals respostes immunològiques que desencadenen (Castillo et al., 2011).

Els insectes poden reaccionar a la infecció de nematodes des de desencadenant una forta resposta cel·lular (encapsulació efectiva) fins a la manca de resposta immunològica (Ebrahimi et al., 2011). Així, l'encapsulació efectiva per la qual moren diverses espècies de nematodes s'observa en diversos ordres d'insectes com per exemple els lepidòpters i coleòpters (Armer et al., 2004; Steiner, 1996; Thurston et al., 1994; Wang et al., 1994; 95). No obstant, s'ha observat com *S. glaseri* i *H. bacteriophora*, escapen a l'acció dels hemòcits després d'haver estat encapsulats pel sistema immunològic de *Popillia japonica* (Wang et al., 1994; 1995). En l'altre extrem, veiem com *S. carpocapsae* és capaç de suprimir la resposta cel·lular d'insectes com *Galleria mellonella* o *Malacosoma disstria* (Walter et al., 2008).

La resposta immunològica variarà en funció de diversos factors ambientals, com la temperatura, el fotoperíode o la humitat relativa, així com també en funció de factors relatius a l'insecte com és la nutrició (Bedding et al., 1983). L'espècie o soca de nematode i bacteri també tenen un paper important com a factor desencadenant (Moret, 2006; Li et al., 2007). En aquest sentit, s'ha observat com determinats nematodes no són encapsulats per aquells hostes als quals parasiten més freqüentment. És el cas de *S. glaseri,* que sovint ha estat aïllat en poblacions naturals de *P. japonica,* i que no és encapsulat per aquest hoste, mentre que *S. carpocapsae* i *H. bacteriophora* reben una forta resposta immunològica del mateix insecte (Lewis et al., 2006).

## Distribució i hàbitat dels nematodes entomopatògens

Els nematodes entomopatògens es troben distribuïts per tots els continents del món excepte l'Antàrtic (Griffin et al., 1990; Hominick, 2002; Adams et al., 2006; Stock et al., 2008). Existeixen espècies de nematodes endèmiques com és el cas de *S. cubanum* o *S. puertoricensis*, mentre que d'altres són ubiqüistes, com per exemple *S. carpocapsae*, *S. feltiae* o *H. bacteriophora* (Mráček et

al., 2005). A la Península Ibèrica s'ha aïllat set espècies de nematodes entomopatògens, la majoria dels quals són steinernematids (taula 6). Els nematodes han estat aïllats d'un elevat nombre d'hàbitats diferents, com prats, sòls agrícoles, boscos i selves (García-del-Pino i Palomo, 1996; Sturhan i Liskova, 1999; Stock et al., 1999; Emelianoff et al., 2008) i estan adaptats a la majoria de condicions climàtiques, havent-se trobat des de en hàbitats com platges a estatges alpins (Steiner, 1996). Aquestes adaptacions s'observen a nivell d'espècie i respecte un determinat hàbitat. Tal és el cas de *S. feltiae* i *S. affine* que es troben habitualment en zones obertes com prats o camps de cultiu (Sturhan i Lišková, 1999), mentre que *S. kraussei* s'ha trobat principalment en boscos (Mráĉek et al., 1999; Sturhan, 1995). El tipus de sòl i la seva textura són factors que determinen la distribució dels nematodes i la seva capacitat de dispersió i persistència, essent els substrats lleugers com sorra o terra rica en matèria orgànica aquells que permeten una major prevalença dels nematodes (Georgis i Poinar, 1983; Kung et al., 1990; Miduturi et al., 1996). També el pH té un impacte en la diversitat dels nematodes entomopatògens, de manera que s'ha vist que diferents espècies de nematodes seleccionen un rang de pH determinat. *S. kraussei* s'ha trobat majoritàriament en pH àcids, mentre que *S. affine* selecciona pH neutres i *S. intermedium* i *S. feltiae* eviten els pH extrems (Steiner, 1996).

**Taula 6.** Espècies de nematodes entomopatògens aïllats a la Península Ibèrica

| **Espècie** | **Localitat** | **Referència** |
|---|---|---|
| *S. feltiae* (Filipjev, 1934) Wouts, Mráĉek, Gerdin and Bedding, 1982 | Catalunya | García-del-Pino i Palomo, 1996 |
| | La Rioja | Campos-Herrera et al., 2007 |
| | Murcia | Morton i García-del-Pino, 2008 |
| | Portugal | Valdas et al., 2013 |
| *H. bacteriophora* Poinar, 1976 | Catalunya | García-del-Pino i Palomo, 1996 |
| | Murcia | Morton i García-del-Pino, 2008 |
| | Portugal | Valdas et al., 2013 |
| *S. carpocapsae* (Weiser, 1955) Wouts, Mracek, Gerdin i Bedding, 1982 | Catalunya | García-del-Pino i Palomo, 1996 |
| | La Rioja | Campos-Herrera et al., 2007 |
| *S. affine* (Bovien, 1937) Wouts, Mráĉek, Gerdin i Bedding, 1982 | Catalunya | García-del-Pino i Palomo, 1996 |
| | | Morton i García-del-Pino, 2008 |
| *S. intermedium* (Poinar, 1985) Mamiya, 1988 | Catalunya | García-del-Pino, 2002 |
| | Portugal | Valdas et al., 2013 |
| *S. kraussei* (Steiner, 1923) Travassos, 1927 | Catalunya | García-del-Pino, 2002 |
| | La Rioja | Campos-Herrera et al., 2007 |
| | Portugal | Valdas et al., 2013 |
| *S. glaseri* (Steiner, 1929) Wouts, Mráĉek, Gerdin and Bedding, 1982***** | Sevilla | De Doucet i Gabarra, 1994 |

***** Identificada posteriorment com a *S. arenarium* (Artyukhovsky, 1967) Wouts, Mracek, Gerdin and Bedding, 1982 (García-del-Pino, 2002)

La preferència d'hàbitat dels nematodes entomopatògens està relacionada amb l'estabilitat del propi hàbitat, les condicions del sòl i la presència d'hostes potencials (Millar i Barbercheck, 2002; Mráĉek, 2005). La capacitat de supervivència en el medi ve determinada per la capacitat de superar les condicions d'estrès ambiental com poden ser les temperatures extremes, la dessecació, l'estrès

osmòtic, la disponibilitat d'oxigen i la radiació ultraviolada (Glazer, 2002; Solomon et al., 2000; Piggot et al., 2000; Gaugler et al., 1992). Així com també els enemics naturals amb els que cohabiten (Georgis i Gaugler, 1991; Kaya i Gaugler, 1993; Kaya, 2002) com poden ser fongs nematòfags (Jaffe i Muldon, 1995) i alguns depredadors invertebrats: àcars *Sancassania* sp. o *Alycus roseus* (Epsky et al., 1988; Karagoz et al., 2007), tardígrads com *Macrobiotus richtersi* (Ishibashi et al., 1987), col·lèmbols com *Hypogastura scotti* (Epsky et al., 1988) i altres nematodes depredadors com *Clarkus* sp. (Mononchida: Mononchidae) (Epsky et al., 1988).

L'aïllament i identificació de poblacions autòctones de nematodes entomopatògens és considerada una part essencial per encaminar el seu ús com agents de control biològic ja que les soques autòctones tenen trets fisiològics que les permeten adaptar-se millor a les condicions ecològiques locals i per tant, poden ser més efectives (Millar i Barbercheck, 2001; Campos-Herrera i Gutiérrez, 2009). Aquestes soques autòctones de nematodes entomopatògens poden arribar a ser nous agents de control biològic (Grewal et al., 2002; Lewis et al., 2006).

## Els nematodes entomopatògens com a agents de biocontrol

L'objectiu del control biològic en l'agricultura ecològica és maximitzar l'efectivitat dels enemics naturals per tal de regular de forma natural la població de la plaga (DeBach i Rosen, 1991; Norris et al., 2003). Els nematodes entomopatògens representen uns dels agents de control de plagues compatibles amb l'agricultura ecològica amb més projecció (Shapiro-Ilan et al., 2002 a, b; Nguyen et al., 2006). Són capaços de controlar de forma efectiva una gran varietat de plagues d'insectes que provoquen grans pèrdues econòmiques (Shapiro-Ilan, 2004; Grewal et al., 2005a), com noctuids, escarabèids, curculiònids i elatèrids entre d'altres (Grewal et al., 2005a; Klein, 1990; Shapiro-Ilan et al., 2002). La capacitat de buscar activament l'insecte plaga, la rapidesa amb que el maten i l'elevat potencial reproductiu els converteix en agents de control biològic ideals (Shapiro-Ilan et al., 2012), especialment tenint en compte que el 90% dels insectes plaga passen algun estadis de desenvolupament enterrats (Akhurst, 1986). El nombre d'espècies de nematodes que s'ha aïllat darrerament ha incrementat considerablement (Poinar, 1990; Adams i Nguyen, 2002; Lewis i Clarke, 2012). Des del 1923, data en què s'aïllà el primer nematode entomopatogen, *Aplectana kraussei* (ara *S. kraussei*) (Steiner, 1923; Poinar, 1990), s'ha descrit més de 85 espècies noves (Nguyen i Buss, 2011; Lewis i Clarke, 2012) i és d'esperar que el nombre segueixi incrementant (Shapiro-Ilan et al., 2012). Actualment s'ha avaluat l'eficàcia d'una petita part de les espècies/soques descobertes (Shapiro-Ilan et al., 2012), i només es comercialitzen unes poques espècies (*S. feltiae*, *S. carpocapsae*, *S. riobrave*, *S. kraussei*, *S. scapterisci*, *H. bacteriophora* i *H. megidis*) (Shapiro-Ilan et al. 2002a). La majoria d'aplicacions de nematodes s'han dirigit contra insectes que habiten al sòl, o que hi passen alguna fase del seu desenvolupament. Alguns dels exemples en que els nematodes s'han utilitzat eficaçment el trobem en escarabeids i curculiònids. Els escarabeids són plaga important de prats i pastures als EEUU i s'ha demostrat la seva

susceptibilitat als nematodes (Grewal et al., 2005b; Klein et al., 2007). Actualment, *H. bacteriophora* i *H. zealandica* es comercialitzen pel control de Escarabeids (Grewal et al., 2005b). El control de curculiònids en plantacions de fruiters, plantes ornamentals, prats i pastures amb nematodes també s'ha dut a terme, amb bons resultats (Kakouli-Duarte et al., 1997). Aquests autors, van provar *S. carpocapsae* contra *Otiorhynchus sulcatus* en una plantació de maduixes obtenint una reducció 49,5% en una aplicació d'estiu i del 65% en una aplicació de primavera. Un altre curculiònid que provoca grans pèrdues en cultius de cítrics als EEUU és *D. abbreviatus* pel qual fa més de 20 anys que es comercialitza *H. indica* i *S. riobrave.* També s'ha demostrat d'eficiència de *S. feltiae* i *Heterorbaditis* spp. pel control de fòrids i esciàrids, dípters que malmeten les produccions de xampinyons, com *Lycoriella auripilla*, *L. mali*, *L. solani*, *B. coprophila* i *B. difformis* (Scheepmaker et al., 1998a, 1998b; Jagdale et al., 2004; 2007; Jess et al., 2005; Tomalak et al., 2005; Grewal, 2007). Més recentment, els nematodes s'han emprat per controlar plagues defoliadores i similars que actuen sobre les parts aèries de les plantes (Gaugler, 1988; 1993). El major factor limitant per aquest ús és la ràpida dessecació de les formes infectives, tot i que l'aplicació amb adjuvants n'ha incrementat l'efectivitat (Lacey i Georgis, 2012). *S. carpocapsae* és l'espècie de nematode que s'ha utilitzat més freqüentment pel control de plagues en les parts aèries de les plantes, amb resultats variables (Lacey i Georgis, 2012). L'efectivitat dels nematodes augmenta quan són aplicats contra insectes minadors, ja que les galeries que produeixen serveixen de refugi als nematodes, permetent les condicions necessàries per a trobar i infectar l'insecte (Begley, 1990; Klein, 1990; Williams and Walters, 1999; Tomalak et al., 2005; Batalla-Carrera et al., 2010). L'ús combinat dels nematode entomopatògens amb altres agents de control biològic pot suposar un augment de l'efectivitat. La combinació dels nematodes amb *B. thuringiensis* o els fongs entomopatògens ha demostrat actuar de forma sinèrgica contra diverses plagues. En aquest sentit, Baur et al (1998) va comprovar la superior eficiència en combinar *S. carpocapsae* i *B. thuringiensis* contra *Plutella xylostella* en creixens, i de manera similar, Ansari et al (2008) van observar com l'aplicació de *M. anisopliae* seguida de *H. bacteriophora, S. feltiae* o *S. kraussei* va donar com a resultat un 100% de control de la larva de *O. sulcatus*. A part de la combinació d'agents de control, si les espècies i soques ja existents o les que es descobreixin a partir d'ara no aconseguissin els nivells de control biològic desitjat, la millora genètica, ja sigui mitjançant mètodes moleculars (transgènics), no moleculars (selecció i hibridació) o la combinació d'ambdós podria resultar en soques de nematodes millor adaptades a les condicions particulars d'un determinat hàbitat i amb una elevada virulència (Mukuka et al., 2010).

**Taula 7.** Comparació dels diferents mètodes de procucció dels nematodes entomopatògens (Shapiro-Ilan et al., 2012)

| | Mètode de producció | | |
|---|---|---|---|
| **Factor** | **In Vivo** | **In Vitro-Sòlid** | **In Vitro- Líquid** |
| Cost Inicial | Baix | Mig | Alt |
| Coneixements tècnics | Mínim | Mig | Ampli |
| Economia d'escala | Baix | Mig | Alt |
| Qualitat del producte | Elevada | Difícil | Difícil |
| Esforç de treball | Alt | Mig | Baix |

Els nematodes es poden produir *in vivo* o *in vitro* (en cultius sòlids o líquids) (Shapiro-Ilan et al., 2012). Es considera la producció de nematodes *in vitro* en medi líquid, el mètode més eficient i conseqüentment és el que actualment s'utilitza per produir els productes comercials que surten al mercat (Georgis et al., 1995; Shapiro-Ilan et al., 2012). Tanmateix, cada un dels mètodes de producció té avantatges i desavantatges en relació al cost de producció, coneixements tècnics, economia d'escala i qualitat del producte (taula 7). Aquests factors poden ser millorats per tal d'incrementar l'eficàcia dels nematodes entomopatògens (Shapiro et al., 2012). A la millora dels sistemes de producció la segueixen els diferents tipus de formulats i les alternatives d'aplicació (Grewal, 2002). La possibilitat de produir-los en medis artificials en grans quantitats, la seguretat del seu ús (tant per la flora i la fauna, com pel medi ambient), i la facilitat d'aplicació, simplifiquen l'entrada d'aquest sistema a l'agricultura (Griffin, 2012; Shapiro-Ilan et al., 2012). El fet que els nematodes entomopatògens siguin compatibles amb la major part dels mètodes d'aplicació de plaguicides ja existents i per tant, el fet que el seu ús no impliqui noves necessitats instrumentals, ha sigut cabdal per a la seva bona acollida i la voluntat de desenvolupar-los (Lacey i Georgis, 2012; Shapiro-Ilan, 2012).

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# Objectives/Objectius

***No es senzill saber cap on has de marxar, pren la direcció del teu cor. Mai no es massa tard per tornar a començar, per sortir a buscar el teu tresor.***

***(Sopa de Cabra –Camins)***

The main goal of this Thesis is to study the viability of native entomopathogenic nematodes to control *Curculio nucum,* an insect pest damaging the hazelnut. In order to achieve this goal, the following specific objectives were defined:

To isolate entomopathogenic nematodes and fungi native to hazelnut orchards where *C. nucum* is present and causes damage.

To molecularly identify the isolated nematode species and strains and the associated endosymbiotic bacteria.

To evaluate the virulence of the isolated nematode strains and their symbiotic bacteria against the larvae and adults of *C. nucum*.

To determine the possible causes (nematodes entry route, penetration rate and immune response) that are responsible for the virulence of different entomopathogenic nematodes against the larvae and adults of *C. nucum*.

To assess the immune response of larvae and adults of *C. nucum* against the entomopathogenic nematodes.

To evaluate the virulence of entomopathogenic fungi and to estimate the combined use of entomopathogenic nematodes and fungi against the larva of *C. nucum*.

To study the field persistence and the vertical migration of the entomopathogenic nematodes in organic hazelnut orchards.

To assess the field efficacy of entomopathogenic nematodes to control *C. nucum* in organic hazelnut orchards and to identify the optimal time for their application.

In order to accomplish these objectives, the following chapters were developed:

**Chapter 1.**

Isolation and molecular identification of entomopathogenic nematodes and their symbiotic bacteria from hazelnut growing areas.

**Chapter 2.**

Virulence of native entomopathogenic nematodes and their symbiotic bacteria against larvae and adults of the hazelnut weevil *Curculio nucum.*

**Chapter 3.**

Different susceptibility of the hazelnut weevil stages to Steinernematidae: entry routes versus host immune response.

**Chapter 4.**

Isolation and virulence of entomopathogenic fungi against hazelnut weevil larvae and their combination with entomopathogenic nematodes.

**Chapter 5.**

Field efficacy and short-term persistence evaluation of entomopathogenic nematodes against the Hazelnut weevil, *Curculio nucum.*

Aquest treball té com objectiu central l'estudi de la viabilitat de nematodes entomopatògens autòctons per controlar *Curculio nucum,* insecte plaga de l'avellaner. Per tal d'aconseguir-ho, s'han proposat els següents objectius concrets:

Aïllar poblacions autòctones de nematodes i fongs entomopatògens mitjançant el mostreig de sòls en àrees d'avellaners i està present *C. nucum* a Catalunya.

Identificar, amb tècniques moleculars, les espècies de nematodes entomopatògens aïllades i els bacteris endosimbiòtics que els nematodes porten associat.

Avaluar la virulència de les soques aïllades de nematodes entomopatògens i el seu bacteri simbiont contra la larva i l'adult de *C. nucum*.

Estudiar les possibles causes (vies d'entrada, taxa de penetració i resposta immunològica) que determinen la diferent virulència dels nematodes entomopatògens sobre larves i adults de *C. nucum*.

Estudiar la resposta immunològica de la larva i l'adult de *C. nucum* envers els nematodes entomopatògens.

Avaluar la susceptibilitat de la larva i l'adult de *C. nucum* als fongs entomopatògens i la virulència de la combinació de nematodes i fongs entomopatògens contra la larva de *C. nucum*.

Estudiar la persistència i la migració vertical dels nematodes entomopatògens després de la seva aplicació en camps d'avellaners de producció ecològica.

Avaluar l'eficàcia en proves de camp dels nematodes entomopatògens per controlar *C. nucum* en cultius ecològics d'avellaner i identificar el moment òptim per a la seva aplicació.

Per tal d'assolir aquests objectius s'ha desenvolupat una sèrie de treballs que es presenten en els següents cinc capítols:

**Capítol 1.**

Isolation and molecular identification of entomopathogenic nematodes and their symbiotic bacteria from hazelnut growing areas.

**Capítol 2.**

Virulence of native entomopathogenic nematodes and their symbiotic bacteria against larvae and adults of the hazelnut weevil *Curculio nucum.*

**Capítol 3.**

Different susceptibility of the hazelnut weevil stages to Steinernematidae: entry routes versus host immune response.

**Capítol 4.**

Isolation and virulence of entomopathogenic fungi against hazelnut weevil larvae and its combination with entomopathogenic nematodes.

**Capítol 5.**

Field efficacy and short-term persistence evaluation of entomopathogenic nematodes against the Hazelnut weevil, *Curculio nucum.*

# Capítol I

**Isolation and molecular identification of entomopathogenic nematodes and their symbiotic bacteria from hazelnut growing areas**

*(Enviat a Nematropica)*

**Isolation and molecular identification of entomopathogenic nematodes and their symbiotic bacteria from hazelnut growing areas**

Isolation and identification entomopathogenic nematodes

**Abstract**

Entomopathogenic nematodes (EPNs) in the Steinernematidae and Heterorhabditidae families were isolated from hazelnut growing areas in the northeast of the Iberian Peninsula. Of a total of 295 samples, 11 (3.73%) tested positive for the presence of EPNs. Nematode species were found mainly in wild hazelnut growing areas and there were none isolated in conventionally managed hazelnut orchards. Soil pH characteristics and altitude had no effect on the occurrence of nematodes. Sequencing and characterization of the internal transcribed spacer (ITS) region was used to identify all nematode isolates to the species level. Ten different *Steinernema* strains were isolated (two *S. affine*, two *S. intermedium*, five *S. feltiae* and one *Steinernema* sp., in the *glaseri* group), and one *Heterorhabditis* (*H. bacteriophora*). The 16S rDNA gene was amplified to identify the symbiotic bacteria of each strain, showing two different groups of *Xenorhabdus* including *X. bovienii* symbiont of different strains of *S. feltiae, S. affine* and *S. intermedium* and *X. kozodoii* symbiotic of *Steinernema* sp. *Photorhabdus luminiscens* strain was found associated with *H. bacteriophora.*

*Keywords*: altitude, habitat, *Heterorhabditis,* isolation, ITS, molecular identification, pH, *Photorhabdus*, *Steinernema*, *Xenorhabdus*

**Introduction**

Entomopathogenic nematodes (EPNs) of the families *Steinernematidae* and *heterorhabditidae*, with their associated symbiotic bacteria, are widely distributed in soils throughout the world (Hominick et al., 1996; Hominick, 2002; Adams et al., 2006; Stock et al., 2008) and have been recovered from a large range of ecosystems such as sandy beaches, meadows, non-agricultural fields, organic vines, oak woodlands, and forests (Sturhan and Liskova, 1999; Stock et al., 1999; Emelianoff et al., 2008).
Nguyen et al (2010) reported 61 species of *Steinernema* and 24 species of *Heterorhabditis* and a total of 21 *Xenorhabdus* spp. in association with *Steinernema* and 3 *Photorhabdus* spp. associated with *Heterorhabditis* have been cited by Tailliez et al (2010).
They are lethal parasites of insects and particularly suited for controlling soil dwelling pests (Klein, 1990). Infective juvenile nematodes, the only free-living stage, penetrate the insects and kill them with the help of symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus* (Boemare et al., 1997; Boemare, 2002). Nematodes feed and develop inside the insect host completing from 2 to 3

generations until the food source becomes scarce and the new infective juveniles exit the cadaver and seek a new host (Poinar, 1990).

EPNs have emerged as important biological control agents for their ability to cause rapid death, to reach insects in cryptic habitats, their high reproductive ability and persistence (Georgis and Manweiler, 1994; Koppenhöfer, 2000; Parsa et al., 2006). Nematodes are easily mass-produced and are safe for humans and other vertebrates (Shapiro-Ilan et al., 2012). They can be easily incorporated into Integrated Pest Management (IPM) programs since they can be applied in standard spray equipment and are compatible with many agrochemicals (Koppenhöfer and Grewal, 2005).

Hazelnut orchards are economically important crops in the northeast of the Iberian Peninsula (FAO, 2009). In these orchards the hazelnut weevil (HW), *Curculio nucum* L. (Coleoptera: Curculionidae) is the major pest, causing up to 80% yield loss in unprotected orchards (AliNiazee, 1998). Chemical pesticides are the most commonly used method for controlling *C. nucum*, focused only on the emerging adult (Akça and Tuncer, 2005). Due to the difficulty in controlling this insect with chemical insecticides and, moreover, the crucial environmental issues associated with their use, there is a need to develop biological control alternatives. EPNs become potential biological control alternatives against this insect. The susceptibility of *C. nucum* larvae to EPNs has been tested in previous studies, proving that some commercial nematodes are capable of infecting larvae in the laboratory (Blum et al 2009) and of significantly reducing (41% to 75%) *C. nucum* population in field (Peters et al. 2009; Kuske et al. 2005). Different studies have shown that native EPNs from the pest area might improve biological control strategy. Native EPNs might be adapted to the abiotic conditions of the pest area (i.e. soil type, humidity, temperature and pH) which influence nematode establishment and persistence in soil (Grewal et al., 1994; Kung et al., 1990a; Kung et al., 1990b). For this reason, many surveys have been conducted in numerous regions in order to find new, more efficient nematode species and strains (Hominick et al., 1996; Kaya et al., 2006). Therefore the objective of this study was to isolate and identify EPNs and their associated bacteria from hazelnut growing areas with presence of *C. nucum* and to show how the types of habitat, altitude and soil pH affect the occurrence of nematodes.

**Material and Methods**

*Nematode isolation*

A total of 295 soil samples were collected from different locations distributed throughout Catalonia (NE Iberian Peninsula) during 2007. The survey was carried out in hazelnut areas with the presence of *C. nucum* distributed as follows: 21% conventional management hazelnut plantation, 48% integrated management, 3.4% organic farming, 17% abandoned hazelnut plantations and 10% wild hazelnuts. The locations and altitudes of the sampled soils were recorded using global positioning

system equipment (Garmin®) and the cropping system was noted. Soil pH was measured by the potential difference between electrodes immersed in standard and test solutions (potentiometric method). Soil samples were collected with a hand trowel (cleaned after each sample) to a depth of 20 cm. Each soil sample was a composite of four subsamples collected at a distance of 2 m from the other subsamples, around the hazelnut tree. The four subsamples were pooled in a bucket and mixed gently. Approximately 1 kg of sampled soil was placed in a polyethylene bag to prevent water loss, and transported to the laboratory under cool conditions. Each soil sample was sifted and any stones and plant residues were removed. Soil samples were baited following the "Galleria bait method" (Bedding and Akhurst, 1975).

The relationships between the occurrence of EPNs and sample characteristics (altitude, pH and cropping system) were analyzed with a Generalized Lineal Model (GLZ) using SPSS-PC v. 19. Variables were categorized into groups as follows: altitude: 1: 25-350 m, 2: 351-678 m, 3: 679-980 m, 4: 981-1331 m; and pH: acid: <7.0, neutral: 7.01–7.50, and alkaline: >7.60.

*Molecular identification of nematode species and their symbiotic bacteria*

Nucleic acid preparations used for polymerase chain reaction (PCR) amplifications of *Steinernema* spp. were extracted from one first-generation female reared in *Galleria mellonella* L. (Lepidoptera: Pyralidae). Samples were incubated at 56$^{o}$ C overnight in ATL buffer and proteinase K to digest. Nucleic acids were extracted from the digestion supernatant using Qiagen's DNeasy tissue kit (QIAGEN Inc., Santa Clarita, CA). PCR was used to amplify a region within the 5'-end of the ITS region of the ribosomal DNA. The primers used in this study were reported by Vrain et al. (1992): 18S-F 5'-TTGATTACGTCCCTGCCCTTT-3' and 26S-R: 5'-TTTCACTCGCCGTTACTAAGG-3'. All PCR reactions were conducted in a PTC-100 Thermal Cycler (MJ Research, Inc., Waltham, MA) with the cycling profile suggested by Nguyen et al. (2004). The same procedure was carried out in the molecular analysis of *Heterorhabditids* but different primers were used to amplify the complete ITS region: TW81-F = 5'-GTTTCCGTAGGTGAACCTGC-3' and AB28-R = 5'-ATATGCTTAAGTTCAGCGGGT-3' (Hominick et al. 1997). One microliter of each PCR amplification was used for agarose gel electrophoresis (1X agarose in TAE buffer) to confirm product size and yield. PCR products were purified with a QIAquick PCR purification kit (QIAGEN Inc., Santa Clarita, CA). Purified DNA was sequenced directly using an ABI PRISM ™ Dye Terminator Cycling Sequencing Ready Reaction Kit (Perkin-Elmer Corp., Foster City, CA). The primers used for sequencing were 18S and 26S for steinernematids and TW81 and AB28 for heterorhabditids described earlier. Two internal primers were used to obtain a complete sequence of both strands of the PCR products. Internal primers for *Steinernema* were KN58-F 5'-GTATGTTTGGTTGAAGGTC-3' and KNRV-R 5'CACGCTCATACAACTGCTC-3' (Nguyen et al., 2001) and for *Heterorhabditis* were H58P-F 5'-ACGAATTGCAGACGCTTAG-3' and H58R-R 5'GTGCGTTCAAAACTTCACC-3') (Nguyen et al., 2004). For molecular identification of the symbiotic bacteria, 500 sterile IJs were used to extract the bacteria. These IJs were crushed in 1 ml

of sterile PBS buffer without $Mg^{2+}$ and $Ca^{2+}$ salts and 100µl of the suspension was streaked onto an NBTA medium. Petri dishes were incubated at 28°C for 48h. Colony forming units (CFUs) were taken for molecular analyses. DNA extraction was performed with the nucleic acid extraction kit Isoquick (ORCA Research, Inc., Bothell, Wash.) according to the rapid DNA extraction protocol of the manufacturer. The primers used were 63B-F: 5'-GAAGAGTTTGATCATGGCTC-3' and 153R-R: 5'-AAGGAGGTGATCCAGCCGCA-3' to amplify the 16S rDNA gene sequences as described by Tailliez et al (2006). PCR products were visualized on ethidium bromide stained agarose-gel electrophoresis and purified using Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan). Purified PCR products were sequenced by Millegene, Toulouse, France.

*Molecular data analysis*

The resulting sequences were compared to a library of more than different EPN species from Genbank. Similarity studies with already described species were conducted using the Poisson correction model. Multiple alignments of the gene sequences were obtained using Clustal X ver. 1.83 software (Thompson et al., 1997) and phylogenetic and molecular analyses based on an Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm using MEGA software (Tamura et al., 2007). The reliability of the trees was assessed by bootstrap analysis (Felsentein, 1985) with 1000 replications. For the nematode and bacterial phylogenetic analyses, we chose the species *Panagrellus redivivus* (Panagrolaimidae) and *Escherichia coli* (Enterobacteriaceae), respectively, as outgroups.

## Results

*Nematode isolation*

Of a total of 295 soil samples, 11 (3.73%) were positive for EPNs. The isolation characteristics (location, altitude, cropping system and pH) for each EPN strain isolated are detailed in Table 1.

**Table 1.** Geographical location of soil sampling sites (habitat type, altitude (m) and soil pH) in hazelnut areas.

| Sample | Locality | Longitude | Latitude | Habitat | Altitude | pH | Nematode sp | Bacteria sp |
|---|---|---|---|---|---|---|---|---|
| D3 | Capafons | 339875,9756 | 4575959,188 | Organic | 590±5 | 6.74 | *S. affine* | *X. bovienii* |
| D37 | Constantí | 348181,3770 | 4561289,1740 | Integrated | 111±6 | 7,34 | *S. feltiae* | *X. bovienii* |
| D66 | Aleixar | 338702,0245 | 4565395,8175 | Abandoned | 462±5 | 5,22 | *S. affine* | *X. bovienii* |
| D102 | Vilanova S | 449033,3461 | 4640738,517 | Wild | 536±8 | 5.8 | *S. intermedium* | *X. bovienii* |
| D104 | Espinelves | 453657,3547 | 4638714,483 | Wild | 962±13 | 5.4 | *S. intermedium* | *X. bovienii* |
| D108 | Rupit i Pruit | 452899,9863 | 4648881,9885 | Wild | 511±12 | 7,07 | *S. feltiae* | *X. bovienii* |
| D113 | Sta. Maria | 451849,3000 | 4656451,8615 | Wild | 911±5 | 7,12 | *S. feltiae* | *X. bovienii* |
| D114 | Sta. Maria | 449151,7147 | 4654336,3067 | Wild | 680±9 | 6,83 | *S. feltiae* | *X. bovienii* |
| D122 | Prades | 331433,8859 | 4577665,4180 | Integrated | 959±6 | 4,8 | *Steinernema* sp. | *X. kozodoii* |
| D245 | Sta. Pau | 447925,9576 | 4669557,3650 | Wild | 1166±6 | 7,34 | *S. feltiae* | *X. bovienii* |
| DG46 | Anglès | 469769,0000 | 4648003,0000 | Integrated | 269±6 | 5,14 | *H. bacteriophora* | *P. luminiscens* |

Nematode occurrence varied significantly among the different habitats surveyed (GLZ: $\chi^2$=12.75; df= 4; P<0.05). The highest occurrence of EPNs was 20% positive samples in wild hazelnut areas, followed by 9.1% in organic orchards, 2.17% in integrated management, and 1.52% in abandoned hazelnut orchards. There were no nematodes isolated from conventionally managed hazelnut orchards. Most of the isolated nematodes were found at an altitude of over 500 m except for *H. bacteriophora* (269±6 m) and one isolate of *S. feltiae* (111±6 m ) but this factor had no effect on the occurrence of nematodes (GLZ: $\chi^2$=7.33; df= 4; P>0.05). EPNs were isolated from soils with pH acid (4.8), neutral and alkaline (up to 7.3) with no significant differences (GLZ: $\chi^2$=4.40; df= 2; P>0.05).

*Molecular identification of nematode species and their symbiotic bacteria*

*Identification of nematode species*

Ten of the isolates were steinernematids (90.9%) and one was heterorhabditid (9.1%). Based on the distance matrix the nematode isolates were identified as two *S. affine*, five *S. feltiae*, two *S. intermedium*, one *H. bacteriophora* and one as yet undescribed *Steinernema* sp. which, through the analysis of the ITS rDNA region, based on UPGMA, was situated in the *glaseri* group (Fig. 1). Pairwise distances showed >99% similarity of isolates D3 and D66 to *S. affine* AY230159, >93% of isolates D102 and D104 to *S. intermedium* AF122016, >97% of isolates D37, D108, D113, D114 and D245 to *S. feltiae* HM461997, and 100% of isolate DG46 to *H. bacteriophora* FJ346826. The similarity between isolate D122 and *Steinernema* sp. AY171285 was 92.73%.

*Identification of bacterial symbiont isolates*

The UPGMA tree based on 16s rRNA showed that the *Xenorhabdus* isolates studied fell into two different groups including *X. bovienii* symbiont of different strains of *S. feltiae*, *S. intermedium* and *S. affine* and *X. kozodoii* symbiont of *Steinernema* sp. The only *Photorhabdus* strain found, *P. luminiscens*, was associated with *H. bacteriophora.* The phylogenetic tree was well supported by bootstrap values (Fig. 2). Comparing *X. bovienii* EU190978 with the isolated strains, pairwise distances showed a similarity of 99.7-100% to bacterial strains belonging to *S. feltiae,* 99.8% to those belonging to *S. affine* and 99.7% to *S. intermedium* strains.

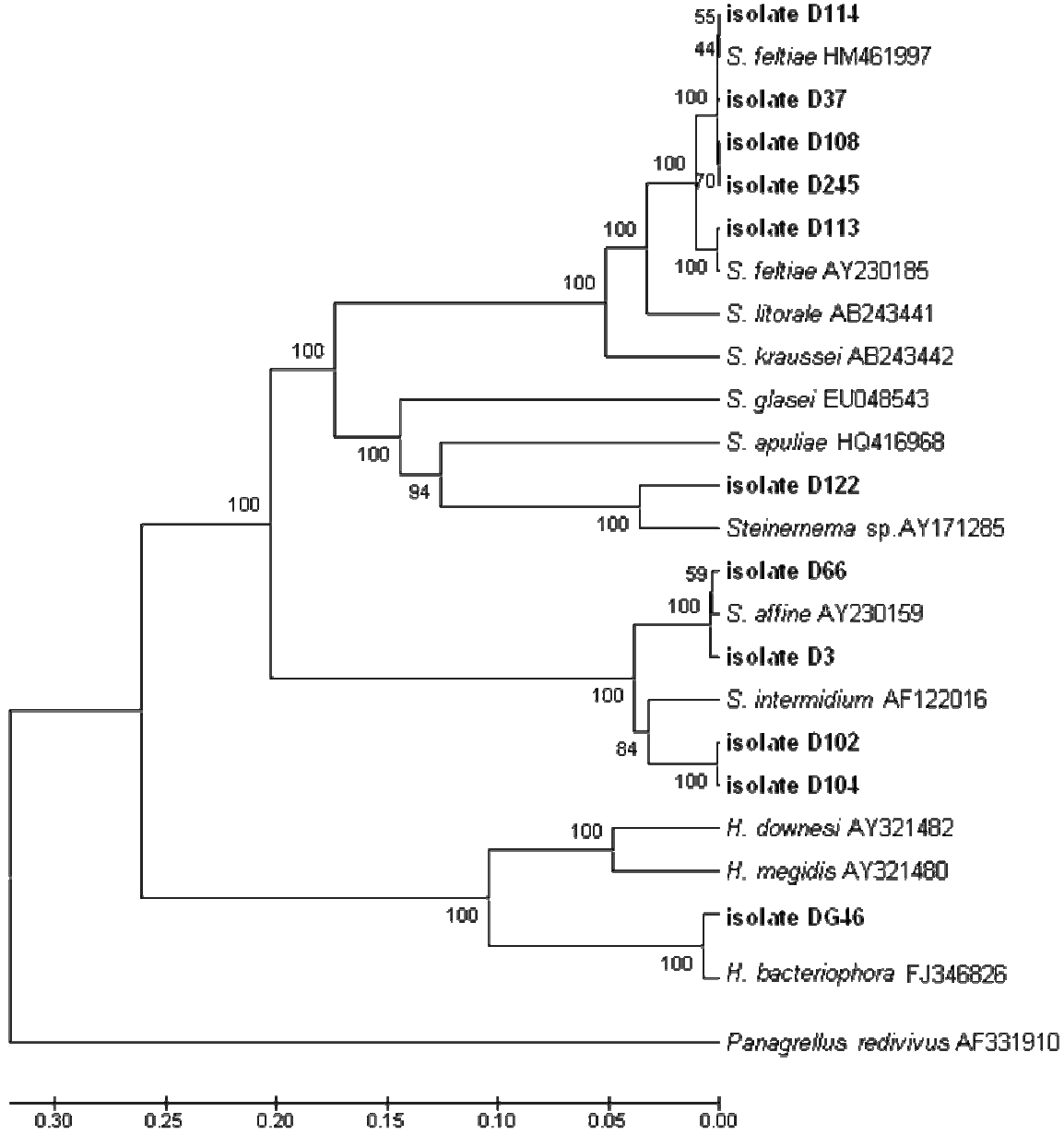


**Figure 1.** Phylogenetic relationship of the nematode isolates found during the survey based on UPGMA method analysis of the ITS rDNA regions. Numbers indicated at the nodes represents bootstrap proportion values (1000 replicates). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Nematodes isolates in this study are in bold and numbers after each species indicate the GenBank Accession numbers.

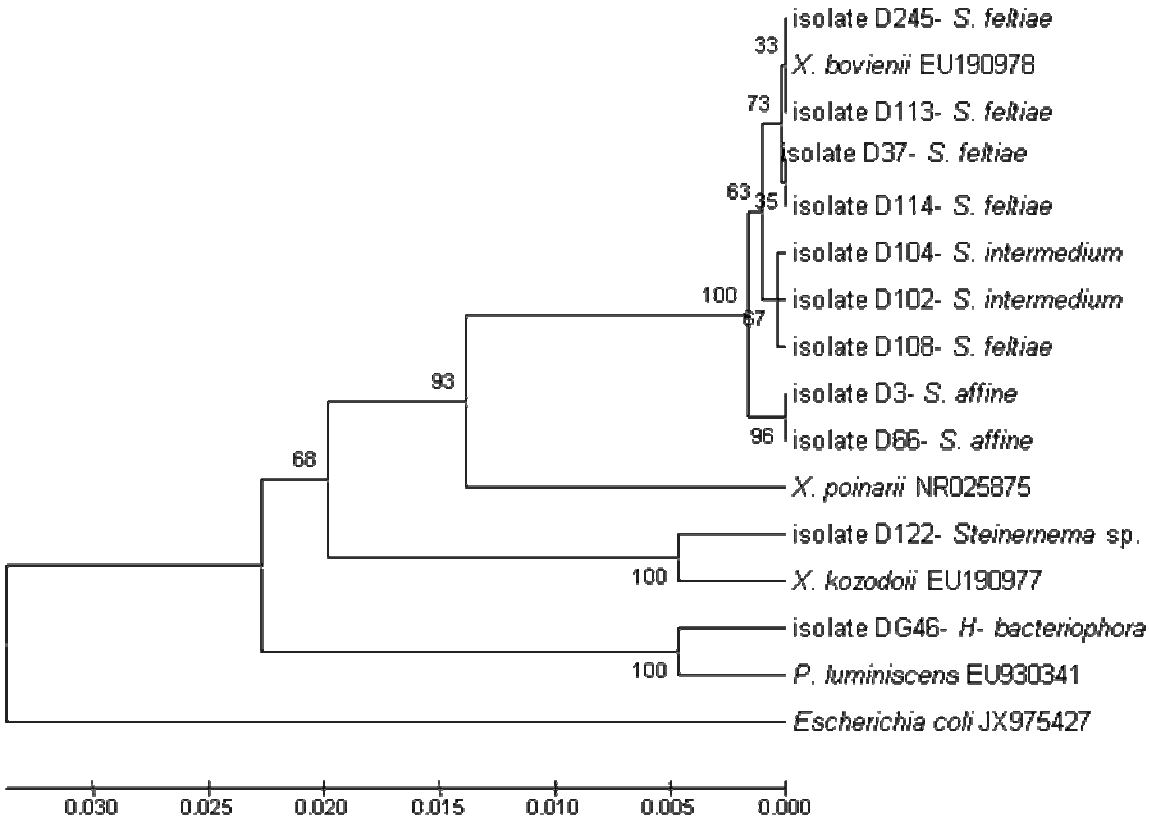


**Figure 2.** Phylogenetic relationship of the endosymbiotic bacteria strains based on UPGMA method analysis of the 16s-rRNA regions. Numbers indicated at the nodes represents bootstrap proportion values (1000 replicates). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Next to each bacterial strain is written the nematode species associated. Numbers after each species indicate the GenBank Accession numbers.

**Discussion**

The results of the present study showed a 3.73% occurrence of EPNs in hazelnut growing areas in the NE Iberian Peninsula. Previous surveys on croplands, woodlands and pastures in the same region reported a higher presence (23.3%) of nematodes (Garcia-del-Pino and Palomo, 1996). However, the percentages of soils testing positive for nematodes in our study coincide with those of Morton and Garcia-del-Pino (2009) who recovered 5.2% nematodes in stone-fruit orchards in the same region and with Campos-Herrera et al. (2007) who found nematodes in 5.4% of the soil samples in the northern Iberian Peninsula.

The percentage of EPNs recovered in our surveys falls within the range of occurrence observed in other surveys in the Mediterranean basin such as those of Jordan (Stock et al., 2008) where 0.9% of the soil samples were positive for EPNs; Turkey (Hazir et al., 2003) where 2% of entomopathogenic nematodes were recovered; Syria where 2.37% of the samples were positive (Canhilal et al., 2006);

and Italy where 8.5% of the total samples collected presented nematodes (Triggiani and Tarasco, 2000). Nevertheless, Emelianoff et al. (2008) observed 60% occurrence in Hérault and Gard (southern France).

The identification of nematodes from the positive samples showed a higher presence of *Steinernema* species (in 90.9% of the samples) than *Heterorhabditis* species (9.1%), according to Hominick (2002) who stated that steinernematids are generally recovered more often than heterorhabditids. Other studies in the Iberian Peninsula have also found *Steinernema* to be more frequently isolated than *Heterorhabditis* (Garcia-del-Pino and Palomo, 1996; Campos-Herrera et al., 2007; Morton and Garcia-del-Pino, 2009). The same occurrence has been reported in other regions around the world (Mason et al., 1996; Midituri et al., 1997; Stock et al., 1999; Hazir et al., 2003). Nevertheless, Emelianoff et al. (2008) found more *Heterorhabditis* than *Steinernema* species in the south of France. Different studies reported *Heterorhabditis* species to be more common in sandy coastal soils (Hara et al., 1991; Liu and Berry, 1995; Griffin et al., 2000; Rosa et al., 2000). However, other authors have suggested that heterorhabditids are not restricted to coastal strips (Stock et al., 1996; Stock et al. 2005; Malan et al. 2011).

In our study the most common species isolated was *S. feltiae*, which accounts for 46% of all isolated EPNs. Other studies on the Iberian Peninsula have reported the same predominance of *S. feltiae* (Garcia-del-Pino and Palomo, 1996; Morton and Garcia-del-Pino, 2009). This species was isolated from wild hazelnut growing areas (80%) or from integrated managed hazelnut plantations (20%). Similarly, Susurluk et al. (2007) found 45.5% of the total *S. feltiae* isolates in woodlands, 31.8% in orchards and 22.7% in cave and wetlands. The habitat preference reported by Hominick et al. (1996) for *S. feltiae* was fields and verges, but Sturhan (1999) reported *S. feltiae* also occurring in woodlands, thus supporting our findings and confirming the ubiquity of this species. The two strains of *S. affine* were isolated from an organic managed hazelnut plantation and from an abandoned plantation. *S. intermedium* strains were isolates from wild hazelnut trees growing in the forest, which is consistent with Sturhan's (1999) findings in Germany confirming *S. intermedium* as a natural forest inhabiting species (Hominick et al. 1996). *H. bacteriophora* has no habitat preference, since it was recorded from croplands, woodlands, pastures, orchards and native vegetation (Rosa et al. 2000). In our study, the only *H. bacteriophora* was isolated from an integrated managed hazelnut orchard.

During our survey, the absence of EPNs in conventional managed hazelnut orchards could be due to intensive use of broad-spectrum chemical pesticides that reduce the potential nematode host populations necessary for nematode establishment and persistence (Strong, 2002). The higher occurrence of EPNs in wild hazelnut areas than in managed hazelnut fields surveyed could be related to the lower human impact in these areas. In this way, some authors suggest that agricultural practices could affect EPN occurrence (Barbercheck, 1992; Hummel et al., 2002;

Campos–Herrera et al., 2008). Similarly, other studies reported a higher presence of nematodes in natural habitats such as forests where human impact is low (Stock et al. 1999, 2008; Sturhan and Lišková, 1999; Stock et al. 2005).

Nematodes were isolated within a pH range of 4.8 (*Steinernema* sp.) to 7.3 (*S. feltiae*) with no differences between nematodes species or strains. This pH range tallies with what has been observed by other authors (Steiner, 1996; Nyasani et al. 2008; Morton and Garcia-del-Pino, 2009) confirming that pH values of the soil do not have any effect on their occurrence (Koppenhöfer and Kaya, 2001).

The altitude range of nematode occurrence in our survey (111-1166 m) had no effect on EPN presence, which is consistent with the findings of Mráček et al. (2005). EPNs have been isolated from an altitude of 2000 to 2500 m (Steiner, 1996; Khatri-Chhetri et al., 2010) down to sea level (Rosa et al. 2000) suggesting that nematodes are well adapted to a wide range of altitudes (Steiner, 1996).

The use of native EPNs can provide effective pest control, preventing possible environmental issues (Lewis et al., 1998; Stuart et al., 2006). Isolation of indigenous EPNs is essential in order to provide a pool of potential biological control agents for pest-control purposes and to gain a better understanding of nematode habitat characteristics. Therefore, the EPNs isolated in this study could be good candidates for controlling insect pests in the hazelnut areas where these nematodes were found. Further research should be conducted in order to assess the virulence of these nematode strains against hazelnut pests, in particular against *C. nucum*, the most harmful insect to this crop.

**Acknowledgments**

This research was supported by a Spanish Ministerio de Educación y Ciencia Project (AGL2006-037228/AGR). Special recognition to Sylvie Pagès and Patrick Tailliez for all guidance on bacterial assays, without their help this study would not have been possible. We are also thankful to Anna Aymamí, Gonçal Barrios, Jordi Mateu, from the Servei de Sanitat Vegetal (Generalitat de Catalunya) for all their effort and help and to Helena Sanchez and Sergi Santamaria for the technical assistance. We are especially grateful to all the farmers who collaborate allowing us access to their fields to carry on with the present survey.

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# Capítol II

**Virulence of native entomopathogenic nematodes and their symbiotic bacteria against larvae and adults of the hazelnut weevil *Curculio nucum***

*(Acceptat a Biocontrol)*

## Virulence of native entomopathogenic nematodes and their symbiotic bacteria against larvae and adults of the hazelnut weevil *Curculio nucum*

**Abstract**

The virulence of native entomopathogenic nematode strains of the families Steinernematidae and Heterorhabditidae, isolates from Catalonia (NE Iberian Peninsula), and their symbiotic bacteria was assessed with regard to the larvae and adults of the hazelnut weevil, *Curculio nucum* L. (Coleoptera: Curculionidae). The nematode strains screened included one *Steinernema affine*, five *S. feltiae*, one *S. carpocapsae*, one *Steinernema* sp. (a new species not yet described), and one *Heterorhabditis bacteriophora*. The pathogenicity of all the strains of nematodes was tested on larvae and only four of them on adults of the hazelnut weevil. Larval mortality ranged from 10% with *S. affine* to 78.8% with *Steinernema* sp. Adult mortality was higher in *S. carpocapsae*, achieving 100% adult weevil mortality. The pathogenicity of the symbiotic bacteria *Xenorhabdus bovienii, X. kozodoii, X. nematophila* and *Photorhabdus luminescens* was studied in larvae and adults of *C. nucum*. In the larvae one of the fastest bacteria was *X. kozodoii* and in the adults it was the fastest of all. All nematodes species apart from *S. affine* tested against larvae showed great potential to control the insect, whereas *S. carpocapsae* was the most effective for controlling adults. Therefore, a strategy combining the use of different nematodes species might be the best approach to successfully controlling this pest.

*Key words:* biological control, entomopathogenic nematodes, *Xenorhabdus, Photorhabdus, Curculio nucum, Curculionidae*

### Introduction

The hazelnut weevil, *Curculio nucum* L. (Coleoptera: Curculionidae) is a key pest in hazelnut orchards throughout Europe (AliNiazee 1998). This weevil has a life cycle ranging from one to three years (Bel-Venner et al. 2009). Adults emerge from the soil and lay their eggs inside the hazelnut fruits. Larval stages develop inside the nut, and last instar larvae burrow into the ground and make a nymphosis chamber where they overwinter as larvae or adults (Bel-Venner et al. 2009). Current control recommendations for the hazelnut weevil are focused on chemical pesticide applications (Akça and Tuncer 2005). Due to the difficulty in controlling this insect in its cryptic habitat with chemical insecticides and the additional important environmental issues associated with their use, the development of biological control alternatives is needed. Thus, entomopathogenic nematodes (EPNs) become potential biological control alternatives.

EPNs in the Steinernematidae and Heterorhabditidae (Order Rhabditida) families are lethal parasites of a large number of insect species (Smart 1995), and have been used efficiently against

many soil-inhabiting and burrowing insects (Klein 1990). EPNs have a symbiotic association with bacteria in the family Enterobacteriaceae. *Steinernema* spp. carry in their gut symbiotic bacteria of the genus *Xenorhabdus* and *Heterorhabditis* spp. carry symbiotic bacteria of the genus *Photorhabdus* (Boemare 2002; Fisher Le-Saux et al. 1999). As infective juveniles (IJs), which is to say in the free-living stage, they actively look for hosts and penetrate through their natural openings (anus, mouth and spiracles). Once inside, the nematodes release bacteria (up to 250 cells) (Snyder et al. 2007) which rapidly multiply and overwhelm the hosts' defences by means of toxins (Waterfield et al. 2001) causing septicaemia, usually within 24 to 48 h (Boemare and Akhurst 1988). The bacteria also release other virulence factors which cause immune system depression in the host and will prevent other bacterial growth (Marokhazi et al. 2004). The developing nematodes feed upon the bacteria and liquefied host tissues, mate, and can produce two or more generations before food resources become scarce. Bacteria re-colonize the nematodes, which emerge as IJs from the depleted insect cadaver in search of new hosts (Poinar 1990).

EPNs are potential biological control agents of soil insect pests (Georgis et al. 2006; Grewal and Peters 2005) and the selection of the best nematode species or strains for a target pest is important in achieving control success. Consequently, the discovery of endemic EPNs can be beneficial in terms of finding strains or species that are well adapted to the local environment and specific target insects. Laboratory trials for screening the virulence of native EPN species and strains and their symbiotic bacteria are crucial in selecting the best nematode-bacterium complex for each pest. This means that choosing the best nematode-bacterium complex can improve biological control strategy (Duncan and McCoy 1996; Koppenhofër and Fuzy 2003; Shapiro-Ilan et al. 2002).

Nematodes have been applied against larvae of different curculionidae species, such as the black vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Kepenekci et al. 2004), the chestnut weevil, *C. elephas* (Gyllenhal) (Karagoz et al. 2009), the citrus root weevil, *Diaprepes* spp. (Shapiro-Ilan and McCoy 2000) and the pecan weevil, *C. caryae* (Horn) (Shapiro-Ilan 2001a, 2001b). The virulence of EPNs against *C. nucum* larvae has been tested in previous studies, proving that some commercial nematodes are capable of infecting larvae in the laboratory (Blum et al. 2009) and of significantly reducing *C. nucum* population in field (Kuske et al. 2005; Peters et al. 2009). However, adults' susceptibility to EPNs and the virulence of their symbiotic bacteria against larvae and adults of *C. nucum* has never been tested.

The objectives of the present work were (i) to study the susceptibility of larvae and adults of the hazelnut weevil to several native isolates of EPNs and (ii) to determine the virulence of the nematode's symbiotic bacteria to the larvae and adults of this insect in order to select the most appropriate nematode-bacterium complex in order to control the different stages of *C. nucum*. The knowledge obtained will enable the field control of *C. nucum* to be improved.

## Material and Methods

*Source of nematodes*

Different entomopathogenic nematode strains were used: *S. affine* strain D66; *S. feltiae* strains D37, D108, D113, D114 and D245; an undescribed *Steinernema* sp. strain D122 (*glaseri* group); *S. carpocapsae* strain B14 and *H. bacteriophora* strain DG46 (table 1). All strains of *S. affine, S. feltiae*, and *H. bacteriophora* and the *Steinernema* sp. were isolated from soil samples from hazelnut orchards on Prades Mountain, Catalonia (NE Iberian Peninsula). *S. carpocapsae* was isolated from a soil of an urban garden in Barcelona (E Catalonia). Nematodes were reared at 25ºC on last instar of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae according to the method of Woodring and Kaya (1998). The IJs that emerged from cadavers were recovered using modified White traps (Kaya and Stock 1997). After storage at 7ºC for a maximum of two weeks, they were allowed to acclimatise at room temperature before use and viability was checked by observation of movement under a stereomicroscope.

Table 1. Entomopathogenic nematodes and their associated bacteria used in this study.

| Associated nematode species | Strain | Bacterial species |
|---|---|---|
| *S. feltiae* | D37 | *X. bovienii* |
| *S. affine* | D66 | *X. bovienii* |
| *S. feltiae* | D108 | *X. bovienii* |
| *S. feltiae* | D113 | *X. bovienii* |
| *S. feltiae* | D114 | *X. bovienii* |
| *S. feltiae* | D245 | *X. bovienii* |
| *Steinernema* sp | D122 | *X. kozodoii* |
| *S. carpocapsae* | B14 | *X. nematophilus* |
| *H. bacteriophora* | DG46 | *P.luminiscens* |

*Infectivity test with nematodes*

*Larval assay*

Last instar larvae of *C. nucum* were obtained from different hazelnut orchards, located on Prades Mountain and stored in sterile soil at $10^{O}$C. Susceptibility of hazelnut weevil larvae was tested with nine strains of entomopathogenic nematodes: *S. affine* (D66), *S. feltiae* (D37, D108, D113, D114 and D245), *Steinernema* sp. (D122), *S. carpocapsae* (B14) and *H. bacteriophora* (DG46). Experimental

units consisted of plastic cups (3.5 cm diam., 5 cm deep) filled with 45 g of sterile sand. A dose of 50 IJs/cm$^2$ (481 nematodes/larvae) was applied onto the soil surface of each cup in 0.5 ml of water to adjust the moisture to 10% w/w. Afterwards, a single larva per cup was placed on the soil surface and covered with parafilm to avoid dehydration. Control treatments received water only. Cups were incubated at 23 ± 2ºC for 14 days when larval mortality was determined. Dead larvae were dissected and nematode infection confirmed. There were four replicates of 10 cups per treatment (strain) and the experiment was conducted twice with each nematode strain.

*Adult assays*

Adults were collected from several abandoned or organically managed hazelnut orchards on Prades Mountain. The weevils were kept at room temperature and fed on apple for one week before being used in two different assays. The first, to assess the adults' susceptibility, was carried out in Petri dishes (5.5 cm diam.) filled with 23 g of sterile sand moistened with sterile tap water (10% w/w). A dose of 50 IJs/cm$^2$ (981 IJs/adult) of *S. feltiae* (D114), *S. carpocapsae* (B14) *Steinernema* sp. (D122) and *H. bacteriophora* (DG46) was applied. Adult sex was determined and a single individual was placed in each Petri dish together with a 1 cm$^2$ of apple as the food and moisture source. The dishes were sealed with parafilm to prevent dehydration and kept in a climate chamber in the dark at 23± 2ºC. Untreated controls were identical to the treatments except that no IJs were added. Dead adults were dissected to ensure nematode infection. There were three replicates of 10 Petri-dishes per treatment (5 females and 5 males) and the experiment was repeated twice. Adult mortality was determined 14 days after EPN application.

The second assay, to establish the minimum time needed for EPNs to invade the host (screening time assay), was carried out with *S. carpocapsae* (B14), the most virulent species against the adult weevil. Eppendorf tubes, each wrapped with a piece of filter-paper (2.2 x 3 cm), were used as experimental units. A dose of 50 IJs/cm$^2$ (330 nematodes/adult) was applied and a single adult weevil introduced into each Eppendorf tube. The tubes were placed in a climate chamber (23± 2ºC) for 15, 30, 45, 60, 90, 120, 180 or 240 minutes. Afterwards, the adults were removed, rinsed with sterile tap water and individually placed in a Petri dish (5.5 diam.) with three dry paper-discs for 24h to ensure no living nematodes remain on the surface of the insect. Then, the paper discs were moistened with sterile water. There were 30 replicates (15 females and 15 males) for each time interval, and the experiment was conducted twice. Nematode infectivity was determined by adult mortality after seven days and verified by insect dissection.

*Isolation and inoculums preparation of symbiotic bacteria*

All bacteria tested were isolated from *G. mellonella* larvae 30h after EPN infection. Larvae were surface sterilized in 70% alcohol and the haemolymph was obtained from the first prothoracic leg using a sterile needle. A 1 µl drop was then streaked on to NBTA medium (Woodring and Kaya

1988). The plates were sealed and incubated in the dark at 28 ºC for 48 h. Two to three colonies were taken and placed on Luria Bertani medium (LB) kept in agitation (600 rpm) at 28ºC for 20 h. After that, 100 µl from this culture was placed in 900 µl of fresh LB medium and incubated as before for 3 to 4 hours. This culture was washed with Phosphate Buffered Saline (PBS) and centrifuged 3 times in order to prevent bacterial aggregates.

*Infectivity test with symbiotic bacteria*

*Larval assay*

The last instar larvae of the hazelnut weevil, *C. nucum*, were surface sterilized with 70% alcohol and rinsed in sterile distilled water. The larvae were then injected into the dorsal part of the first segment, under aseptic conditions, with 20 µl of the bacterial strains in LB medium solution containing up to 30 bacterial cells/larvae. The bacterial strains used are all listed in table 1. The same volume of sterile LB medium without bacteria was injected into larvae as a control mechanism. After injection, the treated and control larvae were placed in plastic cages (2x2 cm) with a sterilized, moistened soil and incubated at 25±2 ºC. Each treatment involved 15 larvae. Larval mortality was recorded every 2h. Lethal Time 50 ($LT_{50}$) was calculated for each isolate.

*Adult assay*

Four bacterial species were chosen to assess the virulence towards adults of *C. nucum*: *X. bovienii* (D114), *X. nematophila* (B14), *X. kozodoii* (D122) and *P. luminiscens* (DG46) (table 1). The injecting procedure was the same as before except that the adults were injected in the left hind leg joint. A volume of 20 µl of bacterial solution of LB medium containing up to 30 bacterial cells was injected and adults were placed in plastic cages (2x2 cm) with filter paper and 1 $cm^2$ piece of apple as the food and moisture source. Control was identical to treatments but LB medium with no bacterial cells being injected. Each treatment involved 20 adults that were kept at room temperature and checked for mortality every 2h.

*Statistical analysis*

Analysis of the Variance (ANOVA) and Tukey's multiple range test were used to test for differences among nematode treatments in all susceptibility assays. Mortality data were arcsine transformed for larvae and square rooted for adults before analysis. A t-test was used to determine whether there were differences in susceptibility to EPNs between insect sexes. Screening time assay was analysed with Pearson correlation and a General Linear Model (GLM) test was performed to discern between mortalities caused at each period of time. Differences in larval mortality due to bacterial strains were analysed with a Generalized Linear Model (GLZ). All these data were analysed using SPSS-PC v.19.0 and a level of significance of p=0.05 was used for all tests. $LT_{50}$ slopes and 95% confidence intervals (CI) for each bacteria isolate were analyzed using Polo Pc statistical software

(LeOra, 1987) and the differences on the insect survival curves among bacterial strains was analysed using a Kaplan-Meier test.

**Results**

*Infectivity test with nematodes*

*Larval assay*

The results revealed that the last instar larvae of *C. nucum* were susceptible to the nine nematodes strains tested (fig. 1). The response of the hazelnut weevil larvae to all EPN strains was variable but, in all cases except *S. affine* (D66), mortality exceeded 50%. There were statistical differences in larval mortality after 14 days of exposure among the nematode strains tested (ANOVA: $F=6.56, 8$, $P<0.05$). All strains belonging to *S. feltiae* reached similar mortality rates (ranging from 52.5% to 65%), showing no statistical differences either among strains, or with regard to the other nematode species tested: *S. carpocapsae* (B14), *Steinernema* sp. (D122) and *H. bacteriophora* (DG46).

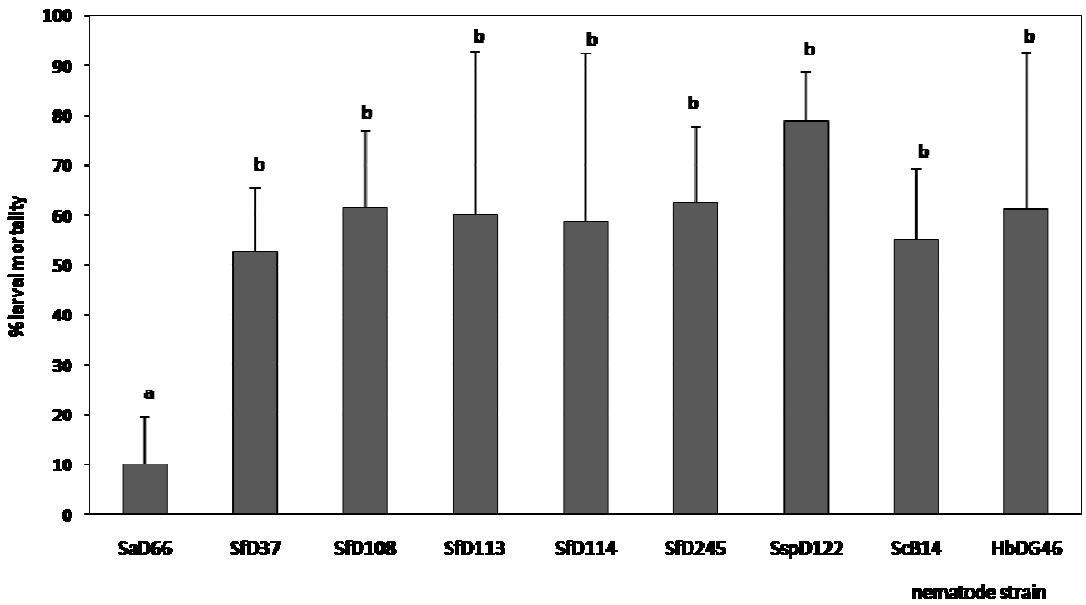


**Figure 1.** Mean percentage mortality larvae of *C. nucum* exposed to nine different EPN strains observed 14 days after 50 IJs/ $cm^2$ application. Different letters above bars (mean ± SD) indicate statistical significance (Tukey's test).

*Adult assays*

All nematode strains tested were able to infest the adult of the hazelnut weevil (fig. 2). Differences between males and females were not observed either in nematode infection (t-student: t=-0.993; *df* = 23; P>0.05) or in terms of the time needed to infect the adults (t-student: t=-0.913; *df* = 46; P<0.05). Consequently data from males and females were combined in the subsequent data analyses.

Differences in the virulence among nematode species were observed (ANOVA: $F=45.46, 4$, $P<0.05$). *S. carpocapsae* (B14) proved to be the most virulent nematode, causing 100% insect mortality,

while the mean mortality percentages caused by *Steinernema* sp. (D122), *S. feltiae* (D114) and *H. bacteriophora* (DG46) were 1.7%, 16.7% and 8.3% respectively with no significant differences among these species (Tukey's P>0.05).

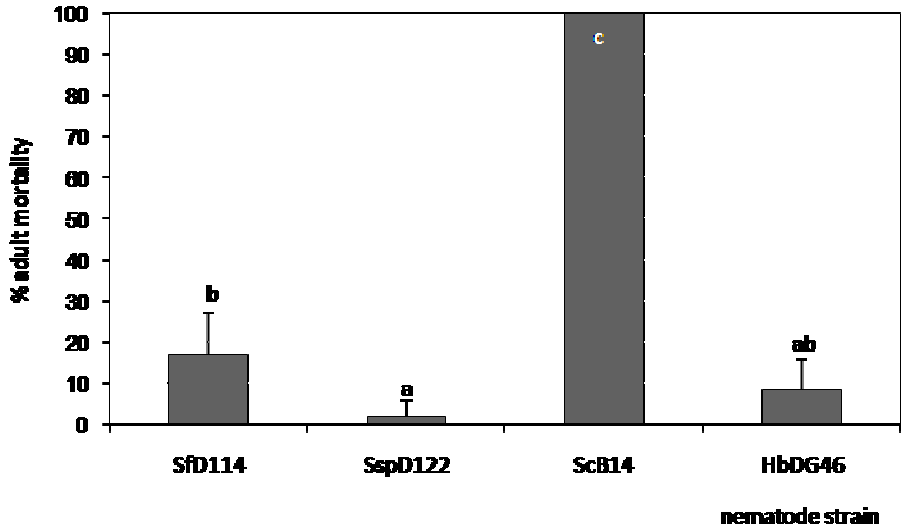


**Figure 2.** Mean percentage mortality of adults of *C. nucum* exposed to D114, DG46 and *S. carpocapsae* at dose of 50 IJs/cm$^2$ observed 14 days after EPNs application. Different letters above bars (mean ± SD) indicate statistical significance (Tukey's test).

In the time screening assay with *S. carpocapsae* (B14) on adults (fig. 3), 15 minutes were long enough to infect 6.6-17% of the weevils, and adult mortality reached 100% in 120 to 240 minutes. There was a positive correlation (r=0.904, P<0.01) between the exposure time and the mortality caused by *S. carpocapsae*.

*Infectivity test with symbiotic bacteria*

*Larval assay*

The results of bacterial virulence showed that after 61h post-injection all strains tested killed 100% of larvae except for the *X. bovienii* belonging to *S. affine* (D66) and *X. bovienii* belonging to *S. feltiae* (D37), reaching 93.3% and 46.7% respectively (GLZ: $\chi^2$=19.44, 8, P<0.05).

The time required to kill 50% ($LT_{50}$) of the *C. nucum* larvae ranged from 22.7h with *X. kozodoii* belonging to *Steinernema* sp. (D122) to 69.6h with *X. bovienii* belonging to *S. feltiae* (D37) (table 2).

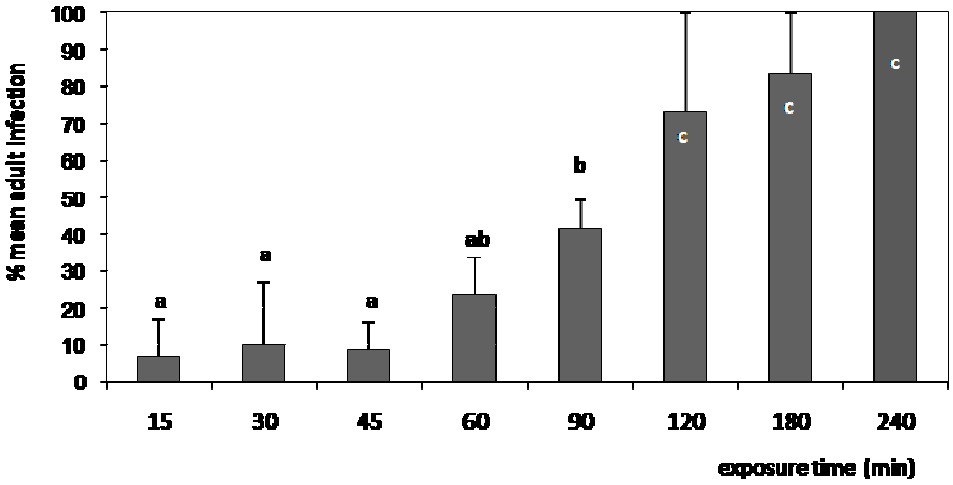


**Figure 3.** Mean percentage ± SE mortality of *C. nucum* adults caused by different time exposure to 50 IJs/ $cm^2$ of *S. carpocapsae*. All mortality was recorded after 7 days incubation. Different letters above bars indicate statistical significance (Tukey's test).

*Adult assay*

All bacterial species injected caused 100% mortality of the hazelnut weevil after 69h post-injection. LT50 varied among strains ranging from 20.6 due to *X. kozodoii* belonging to *Steinernema* sp. (D122) to 41.9 h caused by *X. bovienii* belonging to *S. feltiae* (D114) (table 3).

**Table 2.** Estimated exposure time of *C. nucum* larva to nine different strains of entomopathogenic nematode endosymbiotic bacteria to cause 50% larval mortality, $LT_{50}$, at 95% of confidence interval (CI). Values of slope±SD, and goodness of fitness are also given. Different letters indicate statistical significance (Kaplan-Meier test).

| Bacterial strain | Slope +/-SE | TL50 (IC 95%) | Goodness of fit |
|---|---|---|---|
| *X. kozodoii* (D122) | 15.37±1.565 | 22.689 (21.694-23.406) a | 9.427, 30, 0.028 |
| *X. bovienii* (D114) | 7.633±0.694 | 25.933 (24.496-27.545) ab | 34.802, 30, 0.276 |
| *X. nematophila* (B14) | 6.984±0.582 | 25.954 (23.981-28.130) ab | 57.170, 30, 0.038 |
| *X. bovienii* (D245) | 4.119±0.327 | 27.424 (25.396-29.541) abc | 25.878, 30, 0.017 |
| *X. bovienii* (D113) | 5.768 ±0.393 | 34.505 (32.480-36.709) bc | 27.04, 30, 0.126 |
| *P. luminiscens* (DG46) | 5.780±0.389 | 35.057 (32.999-37.262) c | 26.931, 30, 0.013 |
| *X. bovienii* (D66) | 3.894±0.233 | 36.126 (34.087-38.285) c | 25.42, 30, 0.177 |
| *X. bovienii* (D108) | 4.938±0.357 | 38.374 (35.917-41.074) c | 8.779, 30, 0.014 |
| *X. bovienii* (D37) | 2,336±0,299 | 69.562 (58.740-89.430) d | 5.760, 30, 0.063 |

**Table 3.** Estimated exposure time of *C. nucum* adult to four different strains of entomopathogenic nematode endosymbiotic bacteria to cause 50% adult mortality, $LT_{50}$ , at 95% of confidence interval (CI). Values of slope±SD, and goodness of fitness are also given. Different letters indicate statistical significance (Kaplan-Meier test).

| Bacterial strain | Slope +/-SE | TL50 (IC 95%) | Goodness of fit |
|---|---|---|---|
| *X. kozodoii* (D122) | 20.883 2.902 | 20.576 (19.338-21.788) a | 27.425, 14, 0.174 |
| *X. bovienii* (D114) | 3.327 0.540 | 41.901 (36.345-49.106) c | 25.586, 14, 0.091 |
| *X. nematophila* (B14) | 5.741 0.515 | 40.319 (37.360-43.560) c | 19.083, 14, 0.050 |
| *P. luminiscens* (DG46) | 13.383 1.569 | 30.045 (28.409-31.818) b | 10.551, 14, 0.477 |

**Discussion**

The results from the present study show that *C. nucum* larvae were susceptible to all native EPN strains tested. Larval mortality ranged from 10% for *S. affine* (D66) up to more than 78.75% of the other species and strains tested. These results coincide with those reported by Peters et al. (2009) in laboratory bioassays, who obtained 23% mortality of *C. nucum* larvae with *H. bacteriophora* and 76% with *S. carpocapsae*. The low virulence of *S. affine* against larvae of *C. nucum* obtained in this study has also been reported in other coleopterans by different authors. Morton and Garcia-del-Pino (2008) obtained 34.76% larval mortality in *Capnodis tenebrionis* L. (Coleoptera: Buprestidae) compared with 80% achieved by different *S. feltiae* strains tested. In the same way, Triggiani and Tarasco (2011) reported a 20% larval mortality when exposed *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) to *S. affine*. The low efficacy of this nematode species could be related to the host specificity, which has been found in parasitizing dipteran species under natural conditions (Peters 1996). Nevertheless, other studies showed poor virulence of *S. affine* against mushroom flies (Scheepmaker et al. 1998). These results suggest that the efficacy of this nematode is not related to the specificity for dipterans and *S. affine* proves to be an inadequate biocontrol agent.

Data from adult mortality showed low virulence in *S. feltiae*, *Steinernema* sp. and *H. bacteriophora* but very high virulence in *S. carpocapsae*, evidencing that the susceptibility of *C. nucum* adults was lower than of larvae (except for *S. carpocapsae*). In other weevil species, adults were also less susceptible than larvae, as was the case of the sweetpotato weevil *Cylas formicarius,* (F.) (Mannion and Jansson 1992), the West Indian sugarcane weevil *Metamasius hemipterus* (Oliver) (Giblin-Davis et al. 1996), the pecan weevil *C. caryae* (Shapiro-Ilan 2001b) and the plum curculio *Conotrachelus nenuphar* (Shapiro-Ilan et al. 2002). The higher virulence of *S. carpocapsae* against adult weevils observed in the present work has also been reported by other authors (Dillon et al. 2007; Shapiro-Ilan 2001b, Shapiro-Ilan et al. 2005) tallying with our results. The potential of *S. carpocapsae* to

control the adult of *C. nucum* is supported by the short time needed by this species to cause weevil mortality, as observed in our time screening assay.

All the symbiotic bacterial strains proved to be highly pathogenic when injected into the hemocoel of larvae, reaching more than 93% mortality and only *X. bovienii* belonging to *S. feltiae* (D37) showed lower pathogenicity (46.7%). Like our own our research, Yeh and Alm (1992) obtained up to 96% of mortality when testing different *Xenorhabdus* species on *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae), and Ansari et al. (2003) recorded 99.7% mortality when 25 bacterial cells of *X. poinarii* were injected into *Hoplia philanthus* (Füessly) (Coleoptera: Scarabaeidae). In our assay, the endosymbiotic bacterial strain *X. bovienii* belonging to the nematode *S. affine* was as pathogenic as other *X. bovienii* strains when it was injected into the *C. nucum* larvae. In contrast, the nematode-bacterium complex was less virulent than the other strains tested. This fact suggests that the nematode may have difficulties penetrating into the host and/or releasing its symbiotic bacteria. Therefore, this finding confirms that *S. affine* is a poor nematode species for controlling the larvae of *C. nucum*. The opposite situation is described by *X. bovienii* belonging to *S. feltiae* (D37). This bacterium showed lower virulence than the other bacteria strains tested while the nematode *S. feltiae* (D37) caused larval mortality that was similar to the other *S. feltiae* strains evaluated. One possible explanation for this particular case could be related to the low bacteria dose injected (up to 30 cells) compared with the bacterial cells number (up to 250 cells) carried by one nematode (Snyder et al. 2007), suggesting that a single nematode is enough to kill a larva of *C. nucum*. This finding and the fact that more than one nematode infected each larva, could explain the equal virulence of the *X. bovienii-S. feltiae* (D37) complex to the other *S. feltiae* strains tested.

The high adult mortality observed when *Xenorhabdus* and *Photorhabdus* were injected in contrast to the low susceptibility obtained with the EPNs (excepting *S. carpocapsae*) suggest that nematodes have difficulties getting into the host and/or releasing symbiotic bacteria (Armer et al. 2004).

In conclusion, this study indicates that efficient biocontrol of the hazelnut weevil requires a strategy combining different EPN species. From our results we rule out *S. affine* as a biocontrol agent for *C. nucum* larvae. *S. feltiae* strains D37, D113, D114, D108 and D245, *Steinernema* sp. strain D122, *S. carpocapsae* strain B14 and *H. bacteriophora* strain DG46 have potential against the larvae although further research focused on hybridization and genetic selection of EPNs could improve the biocontrol of *C. nucum* larvae by combining the best nematode species with the most virulent compatible symbiotic bacteria. For adult weevils we conclude that *S. carpocapsae* is the best nematode-bacterium complex to use against emerging adults due to its high virulence and rapid capacity for infection. Moreover, *S. carpocapsae* could be effective against overwintering adults (Bel-Venner et al. 2009), becoming a promising nematode for controlling all underground stages of *C. nucum*.

**Acknowledgement**

This research was supported by a Spanish Ministerio de Educación y Ciencia Project (AGL2006-037228/AGR). Many thanks to Gonçal Barrios, Jordi Mateu, Anna Aymamí from the Servei de Sanitat Vegetal (Generalitat de Catalunya) and Frederic Mallol (ADV Associació pel Desenvolupament Rural de les Muntanyes de Prades) for all their effort and help, and to Helena Sanchez for the technical assistance. We are especially grateful to Patrick Talliez and Sylvie Pagès for their guidance and collaboration in bacteria assays.

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# Capítol III

**Different susceptibility of the hazelnut weevil stages to Steinernematidae: entry routes versus host immune response**

**Different susceptibility of the hazelnut weevil stages to Steinernematidae: entry routes versus host immune response**

**Abstract**

We investigated the basis for differential susceptibility of the hazelnut weevil, *Curculio nucum* L. (Coleoptera, Curculionidae) to entomopathogenic nematodes. Specifically, we investigated the main route of entry of the nematodes, *Steinernema carpocapsae* and *S. feltiae,* into larvae and adult insects, as well as host immune response. The main entry route of *S. carpocapsae* and *S. feltiae* in adult insects and larvae was the anus. Larvae were more susceptible to *S. feltiae* than *S. carpocapsae* and adults were highly susceptible to *S. carpocapsae*. The penetration rate was related to the nematode virulence; nematodes with higher virulence exhibited a higher penetration rate. There were no signs of cellular immune response in larvae of the hazelnut weevil towards *S. carpocapsae* or *S. feltiae*. We observed hemocytes as a cellular immune response towards *S. carpocapsae*in only a few adult insects and no response was observed towards *S. feltiae*. We conclude that the differences in susceptibility of hazelnut weevil larvae and adults to the *S. carpocapsae* and *S. feltiae* are due to the different ability of these two nematodes to penetrate the host, and not to host immune mechanisms developed by the insect.

*Key words: Steinernema carpocapsae*, *S. feltiae*, entry routes, penetration rate, immune response, Curculionidae, susceptibility

**Introduction**

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are obligate parasites of a large number of insect species (Kaya and Gaugler, 1993) and are used in biological pest control (Parkman and Smart, 1996). These nematodes have a mutualistic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus. Xenorhabdus* is associated with *Steinernema* and localize to specific vesicle of the infective juveniles (IJs). *Photorhabdus* is associated with *Heterorhabditis* and is carried in the intestine (Bird and Akhurst, 1983; Silva et al., 2002). The IJ is a third larval stage of nematode which is ensheathed in the cuticle of the second stage, allowing it to survive for long periods of time before locating an insect host (Kung et al., 1990). Nematodes locate their potential host by following insect cues (Lewis et al., 2006). After IJs have located an insect, they infect the host through orifices such as the anus, mouth and spiracles or they sometimes penetrate the cuticle. Once IJs enter the host, they shed their outer cuticle (Sicard et al., 2004) and begin ingesting hemolymph, which triggers the release of symbionts by defecation or regurgitation (Martens and Goodrich-Blair, 2005; Martens et al., 2004).

The insect immune system consists of humoral and cellular components that interact to protect against a diversity of entomopathogenic agents (Uvell and Engström, 2007; Lemaitre and

Hoffmann, 2007). Hemocytes play a key role in defense against nematodes by mounting a defense response called encapsulation, which involves the binding of hemocytes to the surface of the nematodes to form an overlapping sheath of cells (Strand, 2008). Capsules thereafter sometimes melanize owing to activation of the phenoloxidase cascade (Cerenius et al., 2010). However, the symbionts associated with *Steinernema* and *Heterorhabditis* nematodes produce molecules that often disable hemocyte function and capsule formation (Dunphy and Webster, 1984; Castillo et al., 2011). Symbionts also produce antibiotics, which inhibit the growth of other bacteria and fungi (Li et al., 1995) and proteases, which digest host tissues (Nealsonet al., 1990). The developing nematodes then consume the bacteria and liquefied host tissues (Kondo and Ishibashi, 1988). When food is depleted, new IJs exit the host cadaver to search for new hosts (Grewal and Georgis, 1999). In addition to the immune system, insects have also evolved behavioral, morphological, and physiological barriers to nematode infection (Sicard et al., 2004). Grooming behaviors including rubbing and using the mandibles to scrape the cuticle can remove nematodes attached to the surface of the insect (Gaugler et al., 1994; Koppenhöfer et al., 2000). The morphology of orifices restricts the entry of nematodes into some insects (Ishibashi and Kondo, 1990).

EPNs are one of the most effective biological control agents of soil insect pests (Grewal and Peters 2005; Georgis et al., 2006) and consequently their virulence has been tested in the laboratory against many different insect species (Grewal et al., 2005). It is widely reported that different stages of an insect species respond differently to EPNs (Grewal et al. 2005; Lewis et al., 2006; Shapiro-Ilan, 2001a, 2001b). Our preliminary laboratory studies indicated that EPNs kill the hazelnut weevil (HW), *Curculio nucum* (L.) (Coleoptera: Curculionidae) (Batalla-Carrera et al., *unpublished data*) but efficacy varied with nematode species and weevil developmental stage. The aim of this study was to elaborate on these observations by assessing the basis for differential susceptibility of HW adults and larvae to the nematodes *S. carpocapsae* and *S. feltiae*. We suggest that these differences might be related to the penetration ability of the nematodes and differences in the immune response of weevil larvae and adults.

**Material and Methods**

*Source of insect and nematodes*

Larvae and adults of HW were field-collected from hazelnut orchards in the NE of the Iberian Peninsula. Insects were maintained in boxes filled with autoclaved soil at 25°C for 2 weeks to remove diseased individuals before being used. *S. carpocapsae* and *S. feltiae* were reared at 25ºC in last instar *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) according to the method of Woodring and Kaya (1998). IJs that emerged from cadavers were recovered using modified White traps (Kaya and Stock, 1997). After storage at 7ºC for a maximum of two weeks, the nematodes were

acclimatised at room temperature for 3 hrs before use and viability was checked by observation of movement under a stereomicroscope.

*Routes of entry used by nematodes*

Nematode routes of entry into HW larvae and adults were assessed as follows. To determine whether nematodes preferentially enter HW larvae through the anus or mouth, larvae were ligated between the sixth and seventh abdominal segment using a cotton thread, which prevented IJs entering from the anterior of the larva from migrating to the posterior and vice versa. Larvae were then individually exposed for 48 h to 2716 IJs (100 IJs/cm$^2$) of *S. carpocapsae* or *S. feltiae* in 5.5 cm Petri disheslined with two moistened filter paper discs. After the exposure period, all larvae were rinsed with sterile tap water followed by dissection in physiological saline to determine the number of nematodes present in the anterior and posterior domains. If no nematodes were found, we further confirmed the absence of endosymbiotic bacteria by culturing a drop of hemolymph in NBTA medium (NA + 0.00 25% bromothymol blue + 0.004% triphenyltetrazoliumchloride) (Akhurst, 1980). We assumed that all nematodes in the anterior hemocoel penetrated via mouth or one of the spiracles in the anterior portion of the larval while any nematodes in the posterior hemocoel penetrated through the anus or spiracles in the posterior portion of the larva. Controls consisted of ligated larvae with no IJs added. A total of 15 larvae per treatment were examined and each treatment was replicated 5 times using different batches of nematodes. Entry into adults was using 1 ml Eppendorf tubes filled with sand. We pierced the cap of each tube and threaded the weevil's rostrum through the opening and then glued it in place using SuperGlue3 (Loctite, Henkel adhesives SL). For half of the samples the beetles were on the outside of the cap with the rostrum pointing to the inside, while for the other half the beetles were placed abdomen first into the tube with the rostrum oriented to the outside. We exposed only the mouth to nematodes by using beetles with the rostrum pointing to the inside of the tube. Nematodes were then added to the sand in the tube and then the tube was closed which put the rostrum in contact with the sand. Reciprocally, we exposed only the anus and spiracles to nematodes using beetles inserted in the tube where with the rostrum pointed outward. Again, nematodes were added to the sand followed by closure of the tube. Nematodes were applied in tubes at a dose of 157 IJs (200 IJs/cm$^2$) of *S. carpocapsae* or *S. feltiae*. We used two control treatments: (1) to measure whether gluing affected weevil survival, glued weevils were kept in same conditions as above but no IJs were added to the sand, and (2) to compare mortality when all orifices were exposed to nematodes, weevils were placed completely inside the Eppendorf tubes surrounded by sand with the same dose of IJs. Adult weevils were exposed to nematodes for a period of 3 h. Weevils still glued to the Eppendorf caps were then rinsed and placed in Petri dishes with filter paper. Mortality was assessed daily and dead weevils were dissected to determine the presence of nematodes. A total of 30 adults (15 males and 15 females) per treatment were assayed and the experiment was repeated twice. Finally, scanning

electron micrographs (SEM) studies were conducted on the mouth, anus and spiracles of larval and adult stage HW to determine the ultrastructure of the main entry routesused by the nematodes. In brief, samples were not treated but fresh exanimate using a Zeiss Evo ® MA 10.

*Host immune response*

We assessed whether *S. carpocapsae* and *S. feltiae* IJs trigger an encapsulation response in HW larvae or adults by conducting laboratory assays. In brief, individual last-instar HW were exposed for 12 h to a low 50 IJs (4.4 IJs/$cm^2$) or high 500 IJs (44.1 IJs/$cm^2$) dose of *S. carpocapsae* and *S. feltiae* by filling cups with 45 g of soil at field capacity (12 % w/w). After the exposure period, each larva was rinsed with sterile tap water to remove any external IJs and replaced in a new cup containing soil without nematodes. For each nematode and concentration five individuals were randomly chosen (dead or alive) and dissected daily during the subsequent 4 days. For each dissected larva the following data were recorded: number of nematodes inside, whether the nematodes were dead or alive, whether the nematodes were encapsulated by hemocytes, and whether any deposition of melanin was visible on the surface of nematodes. Assays with each nematode species and dose were repeated twice. Adults were similarly examined with the exception that assays were conducted in 5.5 cm Petri dishes with 23 g soil at field capacity. After 12 h, cohorts of 5 adults were dissected daily for 4 days with same data collected as described for the larvae. Assays with each nematode species and dose were also repeated twice.

*Statistical analysis*

To determine the effect of nematode treatment and dose on larval and adult HW mortality independent crosstabs test were used. Differences in penetration rate between species were analyzed using a Mann-Whitney (M-W) non-parametric test. To compare insect mortality on partially exposed adults and larvae to nematodes, crosstabs test was used and M-W was employed to differentiate the mean number of nematodes in each section of the insect. For all tests a level of significance of $P<0.05$ was used. All data were analysed using SPSS-PC v.19.0.

**Results**

*Routes of entry used by nematodes*

In the absence of nematode infection there was no mortality of ligated HW larvae over the duration of our assays. We thus concluded that ligation was a useful approach for assessing whether *S. carpocapsae* and *S. feltiae* preferentially enter the anterior or posterior of HW larvae. We visually observed *S. carpocapsae* and *S. feltiae* IJs around both the head and anus of HW larvae in our assay areas. However, our dissection results strongly indicated a much higher proportion of ligated HW larvae had *S. carpocapsae* in the posterior hemocoel (91.7%) than in the anterior hemocoel (16.7%) ($\chi 2=14.22$, 1, $P<0.05$). The mean number of nematodes was also higher in the

posterior (5.45 nematodes) than anterior (3.50 nematodes) (M-W test P<0.05). In two individuals, we observed nematodes in both the posterior and anterior. . In no case did we detect symbiotic bacteria in the anterior or posterior hemocoel if no nematodes were present in the same domain.

Our dissection results indicated that a higher proportion of ligated HW larvae contained *S. feltiae* IJs in their posterior hemocoel (97%) than in their anterior hemocoel (44%) ($\chi$2=73.1, 1, P<0.05). The mean number of nematodes was also higher in the posterior (8.97 nematodes) than in the anterior (2.07 nematodes) (M-W test P<0.05). In 11 of these larvae, nematodes were found simultaneously in the anterior and posterior portions of the insect. Similar to *S. carpocapsae*, no symbiotic bacteria were detected in the anterior or posterior hemocoel if no *S. feltiae* were present in the same domain.

Our assays with adults showed that gluing the rostrum to the cap of Eppendorf tubes had no adverse effect on mortality relative to unglued controls (X2=something; p>0.1). Our results also showed that adult mortality approached 90% when beetles were fully exposed to *S. carpocapsae* reached. Exposure of the anus plus spiracles to *S. carpocapsae* resulted in 55% mortality, while exposure of only the rostrum resulted 15% mortality ($\chi$2=7.033, 1, P<0.05). When using *S. feltiae,* weevil mortality was 20% in adults that were fully exposed to nematodes. In those adults with the anus + spiracles exposed, mortality was also 20%, whereas no mortality occurred when only the rostrum was exposed to *S. feltiae* ($\chi$2=4.44, 1, P<0.05).

Examination of larvae by SEM showed the head capsule and mandibles of HW larvae are heavily schlerotized. Spiracles were biforous (having two small accessory chambers adjacent to the margin) on larvae with a peritreme surrounded by cuticular wrinkles. The anal orifice was simple with no external protective structures. Adult openings showed a mouth with a long-snout and small, saw-like terminal teeth. Spiracles presented swollen peritreme and spiral ridges of taenidia composed of many inwardly curved small spines. The anal orifice was Y shaped and lacked any apparent protective structures (fig. 1).

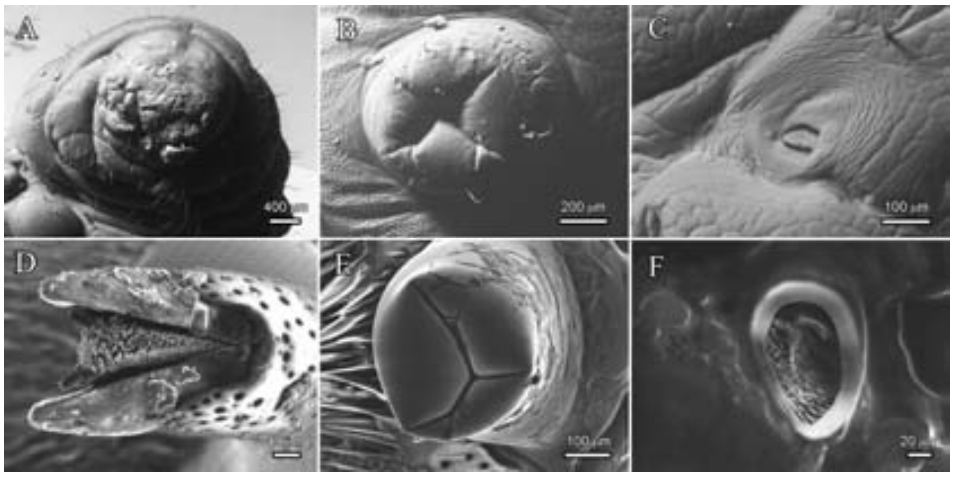


**Figure 1.** SEM micrographs of (A) HW larval cephalic capsule, (B) HW larval anus, (C) HW larval spiracle, (D) HW adult mouth, (E) HW adult anus, (F) HW adult spiracle.

*Host immune response*

Larval mortality using a low or high dose of *S. carpocapsae* IJs was 5%, which was significantly lower than the mortality associated with a low and high dose of *S. feltiae* (15% and 20% respectively) ($\chi$2=11.12, 1, P<0.05; $\chi$2=13.09, 1, P<0.05. Differences in larval mortality due to dose were not detected in either nematode species ($\chi^2$=5.92, 1, P>0.05; $\chi^2$=5.02, 1, P>0.05). The nematode penetration rate into larvae of HW after 12 h exposure was low for both nematode species tested. The *S. carpocapsae* penetration rate at the low dose was 0.05% (0.025 nematodes/larva) and at high dose was 0.31% (1.55 nematodes/larva). The penetration of *S. feltiae* was 2.6% (1.28 nematodes/larva) and 1.4% (7.13 nematodes/larva) at the low and high dose, respectively. There was a significant dose effect for *S. carpocapsae* (M-W test, P<0.001) but not for *S. feltiae* (M-W test, P=0.516). The *S. feltiae* penetration rate was higher than *S. carpocapsae* at low dose (M-W test, P<0.05) but not at the high dose (M-W test, P=0.444). No nematodes collected from the hemocoel of larvae were either encapsulated or melanized. (in the methods you talked about determining whether the nematodes you found from your dissections were alive or dead. You need to report this here).

In the case of adults, *S. carpocapsae* caused much higher mortality than *S. feltiae* ($\chi^2$=108.49, 1, P<0.05). The low dose of *S. carpocapsae* caused 77.5% adult mortality and the high dose caused 92.5%. In contrast, *S. feltiae* did not kill any weevils at the low dose and caused only 2.5% adult mortality at the high dose. Mortality rates were unaffected by dose for either *S. carpocapsae* ($\chi^2$=3.11, 1, P=0.08) or *S. feltiae* ($\chi^2$=1.01, 1, P=0.314). *S. carpocapsae* penetration rates into adult weevils was 0.51% (2.53 nematodes/adult) at the low dose and 3.9% at the high dose (19.58 nematodes/adult). Penetration of *S. feltiae* was 0% and 0.02% (0.1 nematodes/adult) at the low and high dose, respectively (M-W test, P<0.001). There was a significant effect of dose on the penetration rate for *S. carpocapsae* (M-W test, P<0.001) but not for *S. feltiae* (M-W test, P=0.056). The penetration rate was also higher for *S. carpocapsae* than *S. feltiae* at both doses tested (M-W test, P<0.05). Inspection of *S. carpocapsae* from the hemocoel of dissected adults showed that almost no nematodes were encapsulated or melanized. Only one encapsulated nematode was observed from a total of ten adults dissected at 24 h post-infection. This nematode was also still alive as evidenced by its ability to move inside the capsule. After 72h, two adult insects out of ten contained encapsulated nematodes. One of these insects had a single living nematode surrounded by hemocytes, and the other had two living nematodes with only one of them surrounded by hemocytes. No encapsulated or melanized nematodes were identified from dissected HW adults exposed to *S. feltiae*.

Comparing the susceptibility of larva and adult stage HW to EPNs, mortality ($\chi^2$=1.54, 1, P<0.05) and penetration rate (M-W test, P<0.05) was higher in larvae than adults for *S. feltiae*. In contrast, morality ($\chi^2$=104.76, 1, P<0.001) and penetration rate was higher in adults than larvae for *S. carpocapsae* (M-W test, P<0.05).

**Discussion**

Differences in the susceptibility of larvae and adults to EPNs have been reported in studies with several species of insects (McGraw and Koppenhöfer, 2008; Shapiro-Ilan et al., 2002; Shapiro-Ilan et al., 2005). Overall, larvae tend to be more susceptible than adults (Boivin and Bélair, 1989; McGraw and Koppenhöfer, 2008; Morton and Garcia-del-Pino, 2009), although higher rates of adult susceptibility are also known (Laznik et al., 2010; Loya and Hower, 2003; Schroeder et al., 1994; Shapiro-Ilan 2001a, b; Shapiro-Ilan et al., 2005). Collectively, these findings suggest the relationship between stage and susceptibility to EPNs is species specific. In the case of *C.nucum*, previous findings obtained by Batalla-Carrera et al. (*unpublished data*) showed that larvae were more susceptible to EPN infection than adults. Studies by Shapiro-Ilan (2001a; 2001b) with the pecan weevil, *Curculio caryae* (Horn) (Coleoptare: Curculionidae) similarly showed that larvae were equally susceptible to *S. feltiae* and *S. carpocapsae* but adults were more susceptible to *S. carpocapsae* than *S. feltiae.*

Differential susceptibility among larval and adult stages of insects might be related to differences in the infection process among nematodes. First, differential attraction of EPNs to volatile cues emanating from the different host stages could be a factor (Lewis et al., 2006). However, due to the experimental design in this study, in which insects were confined in a very small arena, it is doubtful that host attraction had an impact on the observed differences in HW susceptibility.

Differential host susceptibility can also vary with IJ entry strategy (Cabanillas, 2002) or host behavior (Bedding and Moulyneux, 1982; Koppenhöfer et al., 2000). Our results clearly show that the anus is the main entrance for *S. carpocapsae* and *S. feltiae* into HW larvae. The anus is also the primary route of nematode entry for other species including *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) (LeBeck et al., 1993) and *Musca domestica* (L.) (Diptera: Muscidae) (Renn, 1998). In contrast, Koppenhöfer et al. (2007) observed equal or higher penetration through the mouth than the anus when exposing four different species of white grub larvae (Scarabeidae) to *S. glaseri* and *S. scarabaei*. The size of natural openings in HW larvae does not seem to be a prime determinant for the preference we observed since neither the mouth nor anus have morphological features that would impede nematode penetration. Thus, HW behavior or differences between the mouth and anus in the cues used by nematodes to locate HW may account for the penetration preferences we identified. Nematodes attached to the cuticle may be eliminated by insect grooming behavior (Gaugler et al., 1994; Koppenhöffer et al., 2000; Koppenhöffer et al., 2007; Mankowski et al., 2005). Nematodes can also be destroyed by insects' mandibles when IJs try to enter through the mouth (Gaugler and Molloy, 1981). We observed nematodes triggered noticeable grooming behaviour in HW larvae. Furthermore, the mandibles of HW larvae have hard sclerotized structures, which could crush nematodes entering the mouth. In contrast, we did not observe evasive behaviors like defecation by HW larvae to reduce the anal route of entry as has been observed in some insects

(Bedding and Molyneux, 1982). Our results also suggest that HW larval gut fluids are not detrimental to nematodes as has been described for some other hosts (Wang et al., 1995).

Our results cannot rule out spiracles as a possible route of penetration for nematodes into the HW larvae. However, since the same numbers of spiracles were present in the anterior and posterior portions of the ligated insects we exposed it is unlikely that spiracles account for the strong tendency for nematodes to infect the posterior hemocoel. Thus, we conclude the anus is the main opening used by nematodes to enter HW larvae. Furthermore, the ultrastructure of the larval spiracles showed that they are protected with sieve plates, which allow the free exchange of oxygen and $CO_2$ but exclude invading nematodes. Similar ultrastructures have been reported on the larva of *Phyllophaga hirticula* (Knoch) (Coleoptera: Scarabaeidae) to avoid EPN infection (Forschler and Gardner, 1991). We also rule out direct penetration of steinernematids through the integument because this entry route has rarely been observed in insects with an epicuticle (Peters and Ehlers, 1994).

In adults, the higher penetration observed through anus could be related to orifice size because the anus is bigger than the mouth. Direct observations showed that adult weevils display evasive behavior towards nematodes; mobility increased when nematodes were present. However, this avoiding behavior does not explain the differences observed on the entry route. In the case of adults we also rule out the spiracles and direct penetration through the integument as entry routes because adults have spiracles firmly enclosed by the elytra, which provide difficult access for nematodes. This fact was also observed in other Curculionidae as *Cosmopolites sordidus* (Padilla, 2003).

Hominick and Reid (1990) reported that IJ penetration rate could be a good indicator of the nematode virulence. Our results demonstrate how penetration into HW depends more on life stage. Our results also indicate that increasing nematode dosage does not necessarily result in higher mortality. Similar results have been obtained by other authors who confirmed the lack of a correlation between penetration rate and host mortality (Epsky and Capinera, 1994; Gaugler et al., 1990; Morton and Garcia-del-Pino, 2007).

Finally, our results show that HW larvae and adults do not usually encapsulate or melanized *S. carpocapsae* or *S. feltiae* that enter the hemocoel.. The lack of response could be due to a depression of hemocyte activity caused by the founding population of EPNs (Silva et al., 2002). Moreover, the observation of hemocytes inside dead adult insects imply that when the nematode penetrated the insect immune system was triggered, but the response came too late to interfere with the lethal action of symbiotic bacteria. Thus, the period from nematode invasion to bacterial release is crucial for counteracting the immune response (Dowds and Peters, 2002). As many factors may be involved in the differences between insect stages in susceptibility to EPN, further studies are needed to deepen our knowledge of this subject and to allow us to optimize the

efficiency of insect control efforts, e.g., by selecting the appropriate nematode species against each insect stage.

**Acknowledgement**

This research was supported by a Spanish Ministerio de Educación y Ciencia Project (AGL2006-037228/AGR). Many thanks to Gonçal Barrios, JordiMateu, Anna Aymamí from the Servei de Sanitat Vegetal (Generalitat de Catalunya) and especially to Frederic Mallol (ADVAssociaciópelDesenvolupament Rural de les Muntanyes de Prades) for all their effort and help.

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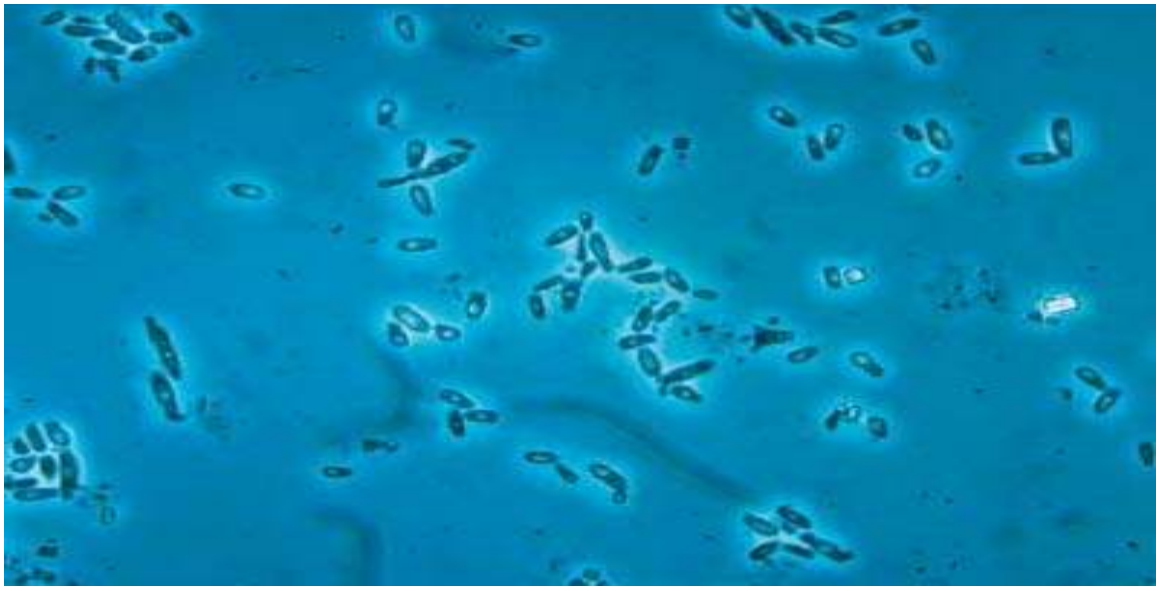
# Capítol IV



**Isolation and virulence of entomopathogenic fungi against hazelnut weevil larvae and its combination with entomopathogenic nematodes**

*Biocontrol Science and Technology (2012) 23, 101-125*

# Isolation and virulence of entomopathogenic fungi against hazelnut weevil larvae and its combination with entomopathogenic nematodes

Isolation, virulence and combined use

**Abstract**

An approach to ensure effective pest biocontrol would be to select native isolates of Biological Control Agents (BCAs). A survey to isolate entomopathogenic fungi from a hazelnut growing area has been carried out. Entomopathogenic fungi were recovered from 133 of the 295 soil samples. The main species isolated were *Metarhizium anisopliae* (36.09%) and *Beauveria bassiana* (42.8%). With regard to controlling abiotic factors, altitude had an effect on the distribution of *B. bassiana*, but not on *M. anisopliae*. Cropping system did not have an effect on the occurrence of these entomopathogenic fungi, while pH appeared as a predictive variable for both. In addiction we tested the virulence of six of these isolates: three *M. anisopliae* and three of *B. bassiana* against larvae of *Curculio nucum* L. The highest larvae mortality (reaching 80%) was due to *M. anisopliae* (strain 34) when applied in simultaneous combination with four entomopathogenic nematode species: *Steinernema carpocapsae* (strain B14), *S. feltiae* (strain D114), *Steinernema.* sp. (strain D122) and *Heterorhabditis bacteriophora* (strain DG46). The effect of nematodes was greater and no antagonistic or synergistic effects were observed.

*Key words: Metarhizium*, *Beauveria*, *Steinernema*, *Heterorhabditis*, synergism, laboratory

## Introduction

The hazelnut weevil, *Curculio nucum* L. (Coleoptera: Curculionidae) is a key pest of hazelnut orchards throughout Europe (AliNiazee, 1998).This weevil has a cycle of life ranging from 1 to 3 years (Bel-Venner et al. 2009). Adults emerge from soil in late April and lay their eggs inside the hazelnut fruits. Last instar larvae drop to the ground where they burrow and make a nimphatic camera where they will overwinter (Akça and Tuncer, 2005).

Traditionally, control of this weevil in Catalonia (NE Iberian Peninsula) has involved the application of chemical treatments; nowadays an organophosphate insecticide (Clorpyrifos 3%) is the normally used pesticide to control the hazelnut weevil (MAAM, 2012). Chemical treatments have resulted in adverse effects on the environment and non-target organisms in the past; therefore other mechanisms of pest control are required. Consequently, other biological agents such as bacteria, viruses, nematodes and fungi for control of insect pests are required. These biological agents including entomopathogenic fungi can provide an alternative and more environmentally friendly approach to the control of pests. Entomopathogenic fungi are versatile biological control agents, due to their wide host range that often results in natural epizootics. An excellent feature of these

fungi is that infectivity is by contact (Nadeau et al. 1996). Entomopathogenic fungi belonging to the order Hypocreales (Ascomycota) that inhabit the soil such as *Metarhizium anisopliae* and *Beauveria bassiana* l are considered promising biological control agents (St. Leger et al. 1996). Several studies have documented that entomopathogenic fungi *B. bassiana* and *M. anisopliae* can be used with success against insect pests and some products are commercially available (Ferron et al. 1991; Ekesi et al. 2001). More recently, a commercial product made of *B. bassiana* was tested by Paparati and Speranza (2005) against *C. nucum* demonstrating the feasibility of using fungi to control the weevil.

The soil environment is an important reservoir for soil-inhabiting entomopathogenic fungi (Sevim et al. 2010). Bidochka et al (2001), has shown that the occurrence of genetic groupings in *B. bassiana* and *M. anisopliae* is influenced by habitat type. Although it remains unclear how this genetic variation is related to virulence, several authors have reported that isolates of entomopathogenic fungi are generally more pathogenic to the species of insect from which they are isolated (Poprawski et al. 1985; Maniania 1992). Bruck (2005) suggested that success in application of Biological Control Agents (BCAs) in nature has close relationship with their related biological systems especially with their ecological niche. Additionally, abiotic factors have been proved to affect the ability and speed by which entomopathogenic fungi can infect and colonize host insects (Benz 1987). For all these reasons, it makes sense to believe that native isolates would be the best approach for hazelnut weevil control.

The integrated use of different biological control agents has increased in pest control programs over last few decades. The combined used of entomopathogenic fungi and nematodes is considered an interesting approach in pest control. Different studies have proved a synergistic effect in the use of both entomopathogenic agents against many insects pest such as *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) or *Hoplia philanthus* (Füessly) (Scarabaeidae: Melolonthinae) (Ansari et al. 2004, 2010). Although entomopathogenic fungi and nematodes have been studied separately to control *C. nucum* (Blum et al. 2009; Paparatti and Speranza 2005) with variable results, the potential for achieving a higher rate of pest control when combining these agents to control *C. nucum* has not been studied yet. Since all three stages of *C. nucum* occur in the soil, achieving infection with entomopathogenic agents becomes feasible. Nevertheless, pupation is an ephemeral stage that happens inside a cocoon and young adults remain in it, therefore the use of entomopathogenic fungi and nematodes should be orientated to control the larvae. For these reasons, the three main objectives in this study were first, to isolate native entomopathogenic fungi, second, to test the virulence of six strains against larvae of the hazelnut weevil and third, to assess the effect of combine application of fungi and nematodes on *C. nucum* larvae.

**Material and Methods**

*Fungal isolation*

Soil samples were collected from different sampling sites in a hazelnut-growing area of Catalonia, in the Iberian Peninsula, at 38 different locations, during the period from February to July 2007. The locations and altitudes of the sampled soils were recorded using global positioning system (GPS) equipment and the cropping system was noted. Soil pH was by measured by the potential difference between electrodes immersed in standard and test solutions (potentiometric method). From a total of 295 soil samples 21.36% belonged to conventional management hazelnut plantation, 48.14% to integrated management, 3.39% to organic farming, 16.95% to abandoned fields and 10.17% to wild hazelnuts. Soil samples were collected with a hand trowel (sterilized with 70% ethanol after each sample) to a depth of about 20 cm after removing surface litter and immediately placed in plastic bags, sealed and transported to the laboratory. Soil samples were baited following the ''Galleria bait method'' (Zimmermann, 1986) with *Galleria mellonella* L. (Lepidoptera: Galleridae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). The entomopathogenic fungi isolated were identified according to Samson et al (1988) and Humber (1997) based on morphological characteristics in the culture. Morphological characteristics of the isolates were studied by phenotypic methods and were maintained in pure cultures on SDAY (Sabouraud Dextrose Agar with Yeast extract). To obtain feasible conidia to use on the virulence assay all fungi were cultured on Sabouraud Dextrose Agar (SDA) (10% neopeptone, 40% dextrose, 15% agar, pH 5.6 ± 0.2) in Petri dishes and incubated in the dark at 25 ± 1 ºC for 15 days for complete sporulation, and stored at 4 ºC. A mixture of conidia and hyphae was harvested by flooding the dishes with sterile distilled water containing 0.1% aqueous (w/v) Tween 80 and agitating with a glass rod. Conidia were separated from hyphae and substrate and immediately used for testing.

*Virulence test*

Last instar larvae of *C. nucum* were obtained from different hazelnut orchards, from Prades Mountain (S Catalonia), by knocking hazelnuts off the tree and immediately taking them to the laboratory. Once there were placed inside a mesh container, so that when larvae naturally emerged from the hazelnut they were collected and stored in sterile soil at 25ºC.

Virulence toward hazelnut weevil larvae was tested using those strains that had caused higher mortality when baiting the soil samples. Six different entomopathogenic fungi were tested: three *M. anisopliae* (strains 27, 59 and 72) and three *B. bassiana* (29, 30 and 34).

Experimental units consisted of plastic cups (3.5 cm i.d. 5 cm deep) filled with 45 g of sterile sand with a 10% moisture content w/w. A dose of $9 \cdot 10^4$ conidia/$cm^2$ was applied to the soil surface and after inoculation a single larvae was placed on the sand surface. Cups were sealed with parafilm to avoid dehydration and maintained in a climate chamber at 24± 2ºC in the dark. Untreated control

was identical to the treatments except that no conidia were added. There were four replicates of 10 cups per treatment. Larval mortality was determined 14 days after entomopathogenic fungi application. The experiment was repeated twice with each entomopathogenic fungi isolate.

*Entomopathogenic agents' combination assay*

For the fungi and nematode combination assay, *M. anisopliae* strain (34) was used together with different entomopathogenic nematode strains: *S. feltiae* (D114), *Steinernema* sp. (D122), *S. carpocapsae* (B14) and *H. bacteriophora* (DG46). All strains were isolated from soil samples from hazelnut orchards in Catalonia with the exception of *S. carpocapsae* that was isolated from the soil of an urban garden in Barcelona.

Combination assay was based on the same method described for virulence test. Pathogens were each applied at the same time onto the soil surface of each cup in 0.5 ml of water to adjust the moisture to 10% w/w. After 24 hours a single larva per cup was placed on the soil surface and it was covered with parafilm to avoid dehydration. Cups were incubated at 24 ± 2 ºC for 14 days and larval mortality was assessed 14 days.

Four different combinations were assessed: (1) *S. carpocapsae* (B14) with *M. anisopliae* (34), (2) *S. feltiae* (D114) with *M. anisopliae* (34), (3) *Steinernema sp.* (D122) with *M. anisopliae* (*34*), and (4) *H. bacteriophora* (DG46) with *M. anisopliae* (34). The experiments were arranged in completely randomized designs with three replicates of 10 cups for each treatment. All experiments were repeated twice.

*Statistical analysis*

The relationships between the occurrence of entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) and sample characteristics (altitude, pH and cropping system) were analysed with a Generalized Lineal Model (GLZ). Variables were categorized into groups as follows: altitude: 1: 25-350m, 2: 351-678m, 3: 678-980m, 4: 981-1331m; and pH: acid: <7, neutral: 7.1–7.5, and alkaline: >7.6.

Data of virulence of entomopathogenic fungi on larvae was subjected to General Lineal Model (GLM) followed by a Tukey's multiple range test to discriminate means using SPSS-PC v17.0. Additive, antagonistic or synergistic interactions between agents when combining treatments were determined using a $x^2$ test. The expected additive proportional mortality (Me) for the nematode-fungus combinations was calculated by Me = Mn + Mf (1-Mn), where Mn and Mf are respectively the observed proportional mortalities caused by nematodes and fungus alone. Mnf is the observed mortality for the nematode-fungus combinations. Thus, the results from a $x^2$ test, $x^2$ = (Mnf-Me)$^2$/Me, were compared with the $x^2$ table value for 1 df. If the calculated $x^2$ value exceeded the table value, a non additive effect between the two entomopathogenic agents was suspected. If the difference Mnf-Me had a positive value, a significant interaction was considered synergistic. If the

difference had a negative value, a significant interaction was considered antagonistic. Differences among means in all experiments were considered significant at $P < 0.05$. Means ± SE are presented.

**Results**

*Fungal isolation*

Entomopathogenic fungi were recovered from 133 of the 295 soil samples (45.08%) collected from the hazelnut growing areas surveyed. Locations, altitude, cropping system, and pH for each isolate are given in Table 1. The isolates were found belonging to 5 different entomopathogenic fungi genera: *Beauveria, Metarhizium, Aspergillus, Fusarium,* and *Paecilomyces*. The most abundant were the *B. bassiana* (57 isolates), and *M. anisopliae* (48 isolates). In addition, another 17 samples presented both fungi together. While *Aspergillus* and *Fusarium* were always isolated alone, *Paecilomyces* was found together with *B. bassiana* in all cases. The percentage of fungi in conventional management soil was 37.88%, integrated management soil 45.65%, organic farming soil 45.65%, abandoned fields soil 53.06%, and wild hazelnut soil 27.67%. Regarding the effect of the characteristics of the soil where fungi were isolated, from the contingency table results (Table 2) we can see in the first place, that there is an effect of the altitude on *B. bassiana* (showing a preference for altitudes below 350m), secondly that different crop systems do not have an effect on the occurrence of entomopathogenic fungi, and finally that pH, ranging from 5.32 to 7.19, has an effect on both species, *M. anisopliae* and *B. bassiana* (fig. 1).

**Table 2.** Maximum likelihood analysis of variance from Generalized lineal models analyses for the effects of altitude, cropping system and pH on the occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from Catalonia (NE Iberian Peninsula)

| | Altitude | | | pH | | | Cropping system | | |
|---|---|---|---|---|---|---|---|---|---|
| | DF | F | P | DF | F | P | DF | F | P |
| *M. anisopliae* | 3 | 1.751 | 0.156 | 2 | 3.038 | 0.049 | 5 | 0.613 | 0.689 |
| *B. bassiana* | 3 | 2.804 | 0.04 | 2 | 3.217 | 0.041 | 5 | 1.336 | 0.248 |

*Virulence test*

All entomopathogenic fungi isolates caused significantly higher mortality of larvae of *C. nucum* when compared with the control treatment. (GLM: $F=8.464$; df= 6, 42 $P<0.05$). The results showed varying levels of virulence: mortality caused by *M. anisopliae* ranged from 52.5- 80% and from 40-52% for *B. bassiana*. The most pathogenic isolate was *M. anisopliae* (34), killing the 80% of the larvae. Overall, strains of *M. anisopliae* showed an equal or superior virulence compared with all *B. bassiana* strains tested (fig. 2).

*Entomopathogenic agents' combination assay*

The interaction between nematodes and *M. anisopliae* (34) caused mortalities ranging from 78.3 to 80% of the larvae. There were no differences between the four distinct combinations tested in the present study (GLM: F=0.574; df= 8, 46 P>0.05) (fig. 3). Entomopathogenic agent causing of mortality was recorded and results show a higher larvae mortality due to entomopathogenic nematodes than entomopathogenic fungi. Both entomopathogenic agents were not found together in a dead larva. Nematodes were found in 56.6% of the total number of larvae for *S. feltiae* (D114), 51.6% for *Steinernema* sp. (D122), 50% for *S. carpocapsae* and 55% for *H. bacteriophora*. The number of dead larvae caused by *M. anisopliae* was from 16.6% to 26.6%. Despite the difference in mortalities caused by one or other agent, results did not show any antagonistic or synergistic relationship.

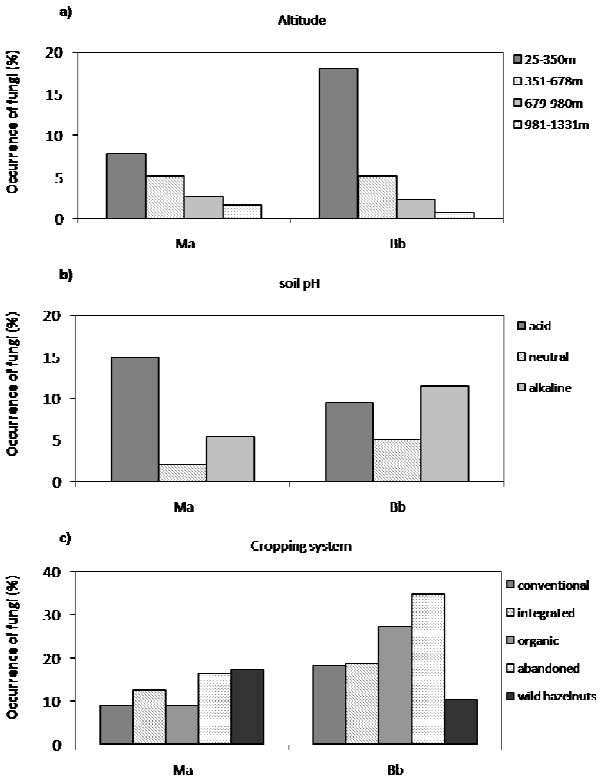



**Figure 1.** Effect of altitude (A), cropping system (B) and soil pH (C) on the occurrence of entomopathogenic fungi in hazelnut growing soils. Data are relative frequencies of isolation of each entomopathogenic species from 295 soil samples. Soil and geographic variables were categorized into groups indicated in the corresponding plot legend. Bars represent mean percentages (±SE) 14 days after inoculation.

## Discussion

From the isolations made we have obtained general information about the diversity and abundance of entomopathogenic fungi in hazelnut orchards in Catalonia. We isolated entomopathogenic fungi in 133 of the 295 soil samples (45.08%) belonging to 5 different genera:

*Beauveria, Metarhizium, Aspergillus, Fusarium,* and *Paecilomyces*. Despite we found higher diversity, the actual number of isolates we found is slightly lower than that recorded by Quesada et al (2007) (71.7% entomopathogenic fungi isolates) when surveying different geographical sites distributed throughout the continental area of Iberian Peninsula and the Canary and the Balearic Archipelagos. However these authors monitored a wider range of habitats than in the present study. In our survey in hazelnut orchards, the main occurrence was accounted for by 42.86% isolates of *Beauveria bassiana*, 36.09% of *Metarhizium anisopliae* and both species were found together in a further 12.78% of cases. If we look at the entomopathogenic fungi frequencies' that Quesada et al (2007) found in cultivated habitats (field crops and fruit crops sub-habitat) we see similar results with *B. bassiana* (30.6% and 40.5% in each sub-habitat respectively), but much lower percentages with *M*. anisopliae (14.1% and 2.41%, respectively). The fruit crop sub-habitat surveyed by Quesada et al (2007) did not include any hazelnut plantations, which could be part of the explanation for the differences observed. In support of this, the study carried out by Sevim et al (2010) in hazelnut growing areas in Turkey reported *M. anisopliae* as the most common entomopathogenic fungus isolate followed by *B*. *bassiana* (54.83% and 20.96% respectively). Nevertheless different results have been reported regarding the association of these fungi genera with cultivated habitats. While Quesada et al (2007) observed a stronger association of *M. anisopliae* with cultivated habitats, Meyling and Eilenberg (2006) showed in Denmark that *B. bassiana* was the most common fungus in agricultural fields; they recorded *M. anisopliae* as rarely found in agricultural soils. In the present work, we found both fungi equally and no effect of the cropping system on the occurrence. Similar results were obtained by Beavers et al (1982), however Klingen et al (2002) reported more common occurrence of entomopathogenic fungi in organically managed arable fields compared with conventionally managed. These authors explain their results as an effect of reduction of suitable hosts due to use of synthetic insecticides. A high population of insects would enhance the preservation of fungi by providing them with abundant hosts. From our results presented here, we could hypothesize a slight trend of higher occurrence of entomopathogenic fungi in integrated and organic farming systems which would match their statement, although no statistically difference was found.

Some studies have suggested that both *B. bassiana* and *M. anisopliae* have the potential to engage in fungus–plant interactions (Saikkonen et al. 1998; Arnold and Lewis 2005). Likewise, it has recently been proved that hazelnut shrubs are a suitable host for establishing a fungus-plant interaction (Bonfante 2010). This association could provide the fungus with nutrient when there is a lack of insect hosts to parasite. Therefore hazelnut shrub communities could have an effect on elevating fungi occurrence than the cropping system itself. But this entire hypothesis requires further research.

Most of the entomopathogenic fungi isolates were found in samples taken between 25-350m. Altitude does have an effect on the occurrence of *B. bassiana* but not on *M. anisopliae*. Quesada et al (2007) reported a similar effect of altitude on the distribution of all fungal species harboured, *B. bassiana* being more frequently found from 400-1000m and *M. anisopliae* from 50-400m. Vänninen (1996) also reported geographical location as the strongest factor in determining the occurrence of entomopathogenic fungi in Finland, while other authors such as Rath et al (1995) demonstrated that altitude had minor or no effect on the distribution of strains of *M*. *anisopliae* isolated from Tasmanian soils.

In our study pH appear to have an effect on the occurrence of both, *M. anisopliae* and *B. bassiana.* We found *M. anisopliae* mainly in acidic soils and *B. bassiana* in alkaline soils which is in accord with results obtained by Quesada et al (2007) and Padmavathi et al (2003). Issaly et al (2005) reported *M. anisopliae* better adapted than *B. bassiana* to acidic soils. However, different authors have showed distinct results on the effect of pH on the survival, ecological distribution and virulence of entomopathogenic fungi, showing a wide range of tolerance for both B. bassiana and *M. anisopliae* (Sanzhimitupova 1980; Galani 1988; Milner 1989; Rath et al. 1992; Hallsworth and Magan 1996; Shimazu and Sato 1996)

The results of virulence tests suggest that all entomopathogenic fungi strains tested were capable of causing mortality and forming feasible conidia on the cadavers of *C. nucum* larvae. However, differences in the larvae mortality were observed among strains, which also have been reported on Plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae) and Lesser mealworm *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) larvae by Alston et al (2005) and Gindin et al (2009) respectively. In our assays *M. anisopliae* (34) caused the highest mortality (80%) and overall, *M. anisopliae* strains showed equal or superior virulence compared with *B. bassiana*. Our results are in accord with the pattern found by Shapiro-Ilan et al (2009) when testing *M. anisopliae* on *Curculio caryae* (Horn) (Coleoptera: Curculionidae). Nevertheless, many studies have reported a higher efficacy when using *B. bassiana* to control different insects such as in the cases of *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) (Reithinger et al. 1997) or *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) (Rohde et al. 2006). Focusing on *C. nucum*, Paparatti and Speranza (1999; 2005) obtained a 35% efficacy against the hazelnut weevil and chestnut weevil, *C. elephans* L. (Coleoptera, Curculionidae) when they tested a commercial product of *B. bassiana* on field applications. In our study *C. nucum* larvae mortality by *B. bassiana* ranged from 40% to 52%. This could be highlighting the importance of the use of native species, as these isolates might be better adapted or prepared to infect a particular host that cohabit in the same location. Regarding the use of *M. anisopliae* against *C. nucum*, we are not aware of any study published.

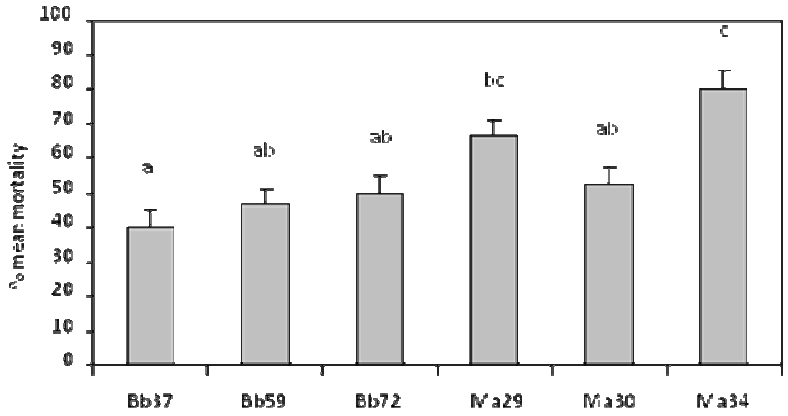



**Figure 2.** Percentage larvae *Curculio nucum* mortality and mycosis following applications of *Metarhizium anisopliae* (Ma) or *Beauveria bassiana* (Bb) strains in the laboratory. Bars represent mean percentages (±SE) 14 days after inoculation. Different letters above bars indicate statistically significant differences.

In the present work, we also provide results from combining the most virulent fungus isolate, *M. anisopliae* (34), with four native species of entomopathogenic nematode: *S. carpocapsae* (B14), *S. feltiae* (D114), *Steinernema* sp (D122) and *H. bacteriophora* (DG46). To our knowledge this is the first report that evaluates the combined effect of entomopathogenic nematodes and fungi on *C. nucum*. Nevertheless, there are several reports on combinations response when applying entomopathogenic nematodes together with other entomopathogenic agents on other insect hosts (Koppenhöfer and Kaya 1997; Koppenhöfer et al.1999; Thurston et al. 1993; Shapiro et al. 2004; Ansari et al. 2010). Our results did not show either synergistic or antagonistic effect when combining entomopathogenic fungi and entomopathogenic nematodes on the *C. nucum* larvae mortality. Shapiro et al (2004) tested various entomopathogenic agents together against *C. caryae* larvae and reported antagonistic effect for some of the combinations in the laboratory. However, these authors did not find synergistic or antagonistic effect when combining *H. indica* and *S. carpocapsae* at a rate of 40 IJs/cm$^2$ with *M. anisopliae* and *B. bassiana* ($8 \cdot 10^4$ conidia/cm$^2$), respectively, which is consistent with data presented here.

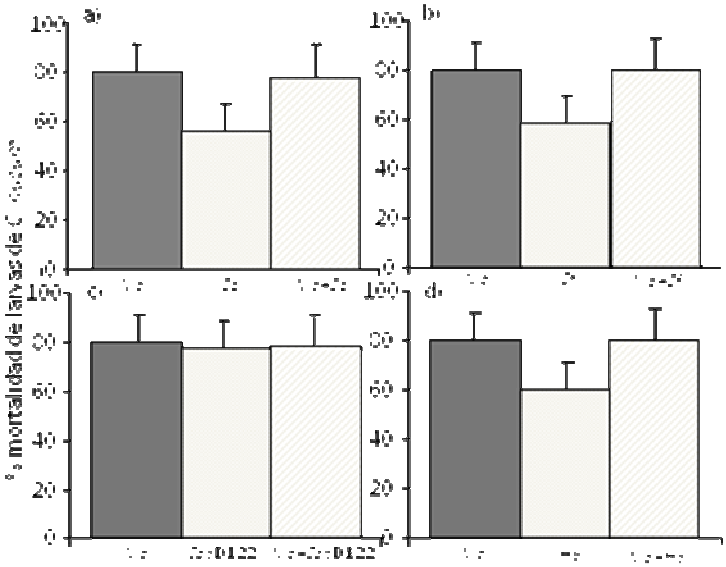


**Figure 3.** Percentage mortality caused by entomopathogenic agents used alone and in combination for the control of *Curculio nucum* larvae. Treatments consisted of: *Metarhizium anisopliae* strain 34 (Ma), *Steinernema feltiae* strain D144 (Sf), *Steinernema* sp. strain D122 (SspD122), *S. carpocapsae* strain B14 (Sc), *Heterorhabditis bacteriophora* strain DG46 (Hb), and the combinations of fungus and nematodes of: *M. anisopliae* 34 with S. feltiae D114 (Ma-Sf), *M. anisopliae* 34 with *Steinernema sp* D122 (Ma-SspD122), *M. anisopliae* 34 with *S. carpocapsae* B14 (Ma-Sc) and *M. anisopliae* 34 with *H. bacteriophora* DG46 (Ma-Hb). Bars represent mean percentages (±SE) 14 days after inoculation.

Similar findings to our results were also obtained by Ansari et al (2004) when applying nematodes (*H. megidis* or *S. glaseri*) and *M. anisopliae* simultaneously to third-instar of *H. philanthus* in a laboratory experiment. However Ansari et al. 2004 recorded synergistic effect when nematodes were applied at least 1 week after the *M. anisopliae*. It has been described that stressed insects are more susceptible to entomopathogenic nematodes (Brown et al. 2006). There are different causes of stress, such as temperature extremes, insecticides, or metal cations that have been proved to cause a greater effect on the host mortality by nematodes (Kaya and Gaugler 1993; Brown et al. 2006). Perhaps previous exposure to another pathogen compromises host defences, thus permitting a more efficient nematode infection. Since there are many reports confirming the slow action of *M. anisopliae* against other soil inhabiting larvae, such as *H. philanthus, Adoryphorus couloni* (Burmeister) (Coleoptera: Scarabaeidae) or *Lepidiota negatora* (Coleoptera: Scarabaeidae) (Ansari et al. 2004; Rath et al. 1995; Samson et al. 1997), the observed lack of effect when combining EPNs and EPFs in the present paper might change if *C. nucum* larvae were exposed to entomopathogenic fungi previously to the application of entomopathogenic nematodes. This might both, enhance the stressing factor that the fungi could produce into the larvae, and minimize the possible inhibitory effect of the fungal toxins on the nematodes in order to achieve a synergistic effect.

Mortality observed on *C. nucum* during our assay was caused by fungi (22.5%) or by nematodes (53.3%) but it was never recorded by both agents in the same larvae. Similar results were obtained

by Sankar et al (2009) who combined *H. indica* and *M. anisopliae* on *G. mellonella* and obtained higher mortality due to the nematode (76%) compared with the fungi (24%). It has been described how symbiotic nematode bacteria can inhibit fungi development and how the fungi, in turn, can produce toxins that impair bacterial growth (Barbercheck and Kaya 1990; Ansari et al. 2004; Tarasco et al. 2011). In their studies on *G. mellonella* on the competition for insect's haemocoel between *B. bassiana* and *S. ichnusae*, Tarasco et al (2011) reported no effect of dual infection in causing the insect mortality. However, they found a strong competition between both entomopathogens in the larvae haemocoel. The decrease observed on the mortality, from when *M. anisopliae* was used alone (80%) compared with the mortality reached when combined with entomopathogenic nematodes (22.5%), it seems clear that nematode are interfering on the fungi infecting process. This is consistent with the theory that fungal antibiotic toxin effect occur more slowly than the release of antifungal compounds by the nematode symbiotic bacteria. Thus observed larval mortality being predominantly due to nematode activity may reflect the relative rates at which the bio-processes proceed. In conclusion, we can state that entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* inhabit hazelnut growing areas, and that altitude and pH seems to have an effect on the occurrence of the fungi. Isolated entomopathogenic fungi have been proven capable of killing *C. nucum* larvae, and *M. anisopliae* (34) appears to be the most virulent strain. The combination of *M. anisopliae* (34) with *S. carpocapsae* (B14), S. *feltiae* (D114), *Steinernema* sp. (D122) and *H. bacteriophora* (DG46) are not likely to improve suppression of *C. nucum* larvae beyond what is expected from single application of either pathogen.

**Acknowledgement**

This research was supported by a Spanish Ministerio de Educación y Ciencia Project (AGL2006-037228/AGR). Many thanks to Gonçal Barrios, Jordi Mateu, Anna Aymamí from the Servei de Sanitat Vegetal (Generalitat de Catalunya) and Frederic Mallol (ADV Associació pel Desenvolupament Rural de les Muntanyes de Prades) for all their effort and help, and to Helena Sanchez and Romina Torre for the technical assistance. We thank Rob Nunny for language revision of the manuscript.

**Table 1.** Geographical location of soil sampling sites, altitude, cropping system, and soils pH

| Sample | Locality | Longitude | Latitude | Cropping system | Altitude (m) | pH | Species isolated | species isolated 2 |
|---|---|---|---|---|---|---|---|---|
| **D1** | Alcover | 350482,81 | 4571432,1 | Integrated | 154 | 7,5 | *B. bassiana* | |
| **D2** | Capafons | 339878,02 | 4576051,7 | Organic | 590 | 6,78 | *B. bassiana* | |
| **D3** | Capafons | 339875,98 | 4575959,2 | Organic | 590 | 6,74 | | |
| **D4** | Capafons | 339702,98 | 4575814,9 | Organic | 596 | 6,78 | | |
| **D5** | Capafons | 339284,58 | 4576040,1 | Organic | 596 | 8,01 | | |
| **D6** | Capafons | 335377,66 | 4574347,3 | Organic | 717 | 7,56 | | |
| **D7** | Prades | 331960,67 | 4574299,1 | Organic | 946 | 6,47 | | |
| **D8** | Alcover | 346530,4 | 4569098,7 | Integrated | 234 | 7,6 | *B. bassiana* | |
| **D9** | Alcover | 346685,15 | 4568928,8 | Integrated | 225 | 7,6 | | |
| **D10** | Alcover | 346636,49 | 4569161,2 | Integrated | 230 | 7,09 | | |
| **D11** | Alcover | 347180,56 | 4566795,7 | Integrated | 208 | 7,3 | *B. bassiana* | |
| **D12** | Alcover | 347634,88 | 4567026,8 | Organic | 179 | 7,7 | *B. bassiana* | |
| **D13** | Alcover | 347736,65 | 4566993,9 | Organic | 172 | 7,7 | *B. bassiana* | |
| **D14** | Alcover | 347670,82 | 4566853,3 | Integrated | 184 | 7 | | |
| **D15** | Alcover | 348057,96 | 4568998,7 | Integrated | 193 | 6,53 | | |
| **D16** | Alcover | 348411,57 | 4566523,2 | Organic | 160 | 8 | *B. bassiana* | *M. anisopliae* |
| **D17** | Alcover | 348992,46 | 4567134,3 | Integrated | 151 | 7,5 | | |
| **D18** | Milà, el | 350267,54 | 4567533,7 | Integrated | 153 | 7,6 | | |
| **D19** | Alcover | 350287,62 | 4571334,3 | Integrated | 155 | 8,1 | *Paecilomyces* | *B. bassiana* |
| **D20** | Alcover | 350215,07 | 4571428,4 | Abandoned | 158 | 7,14 | *B. bassiana* | |
| **D21** | Alcover | 350119,98 | 4571217,4 | Abandoned | 156 | 7,24 | *B. bassiana* | |
| **D22** | Alcover | 342950,79 | 4570881,3 | Abandoned | 358 | 6,34 | *B. bassiana* | |
| **D23** | Alcover | 343955,86 | 4571075,6 | Integrated | 308 | 6,76 | *Fusarium* | |
| **D24** | Alcover | 343054,74 | 4570413,2 | Integrated | 410 | 7,7 | | |
| **D25** | Alcover | 344524,03 | 4571085,1 | Integrated | 297 | 7,45 | | |
| **D26** | Aixàviga-Montral | 339969,12 | 4571526,4 | Integrated | 744 | 8,1 | *B. bassiana* | |
| **D27** | Aixàviga-Montral | 339803,6 | 4571517,7 | Integrated | 741 | 8,1 | | |
| **D28** | Aixàviga-Montral | 339728,29 | 4571374,4 | Integrated | 746 | 8,3 | *Fusarium* | |
| **D29** | Aixàviga-Montral | 340370,92 | 4571381,8 | Integrated | 744 | 8,3 | | |
| **D30** | Aixàviga-Montral | 339865,79 | 4571590,4 | Integrated | 751 | 8,1 | | |
| **D31** | Aixàviga-Montral | 339680,91 | 4573131 | Integrated | 775 | 6,6 | | |
| **D32** | Aixàviga-Montral | 339892,09 | 4572996,7 | Integrated | 756 | 6,6 | | |
| **D33** | Albiol | 342322,61 | 4568429,7 | Integrated | 404 | 8,1 | *M. anisopliae* | |
| **D34** | Constantí | 348540,02 | 4559253,8 | Integrated | 109 | 8,3 | *B. bassiana* | |
| **D35** | Constantí | 348300 | 4560941,2 | Integrated | 103 | 8,3 | *B. bassiana* | |
| **D36** | Constantí | 348511,92 | 4561044,7 | Integrated | 111 | 7,55 | | |
| **D37** | Constantí | 348181,38 | 4561289,2 | Integrated | 111 | 7,34 | *B. bassiana* | |
| **D38** | Constantí | 346360,61 | 4561157,6 | Integrated | 120 | 7,63 | *B. bassiana* | *M. anisopliae* |
| **D39** | Constantí | 346174,57 | 4561288 | Integrated | 130 | 7,67 | | |
| **D40** | Perafort | 347565,06 | 4562912,5 | Integrated | 132 | 6,8 | *Paecilomyces* | *B. bassiana* |
| **D41** | Perafort | 347271,3 | 4563017,4 | Integrated | 139 | 7,83 | | |
| **D42** | Perafort | 347926,52 | 4562590,3 | Abandoned | 125 | 7,56 | *B. bassiana* | |
| **D43** | Constantí | 348017,81 | 4562273,7 | Integrated | 126 | 7,98 | *B. bassiana* | |
| **D44** | Vilallonga del Camp | 348350,98 | 4563726 | Integrated | 121 | 5,99 | *B. bassiana* | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **D45** | Vilallonga del Camp | 348596,93 | 4563452,5 | Integrated | 125 | 6,5 | *M. anisopliae* | *B. bassiana* |
| **D46** | Vilallonga del Camp | 349742,72 | 4563879,3 | Integrated | 109 | 5,32 | *M. anisopliae* | |
| **Sample** | **Locality** | **Longitude** | **Latitude** | **Cropping system** | **Altitude (m)** | **pH** | **Species isolated** | **species isolated 2** |
| | | | | | | | | |
| **D47** | Pobla de Mafumet | 349543,73 | 4561007,9 | Integrated | 99 | 7,7 | *B. bassiana* | |
| **D48** | Pobla de Mafumet | 349673,09 | 4562085,1 | Integrated | 97 | 7,24 | *B. bassiana* | *Paecilomyces* |
| **D49** | Morell | 352570,49 | 4564537,2 | Abandoned | 77 | 7,9 | *M. anisopliae* | |
| **D50** | Morell | 352348,47 | 4563699,5 | Integrated | 78 | 7,82 | *B. bassiana* | |
| **D51** | Morell | 352462,62 | 4563351,6 | Abandoned | 73 | 7,95 | | |
| **D52** | Constantí | 352533,85 | 4558232 | Integrated | 34 | 7,94 | | |
| **D53** | Constantí | 352238,52 | 4558271,8 | Abandoned | 33 | 7,87 | | |
| **D54** | Constantí | 352749,29 | 4558971,1 | Integrated | 38 | 8,92 | *M. anisopliae* | |
| **D55** | Constantí | 351506,22 | 4559866,3 | Abandoned | 54 | 7,95 | *M. anisopliae* | *B. bassiana* |
| **D56** | Constantí | 352654,11 | 4558991,6 | Integrated | 49 | 8,03 | *B. bassiana* | *M. anisopliae* |
| **D57** | Tarragona (la Canonja) | 347367,06 | 4555336,2 | Conventional | 41 | 8,17 | *B. bassiana* | |
| **D58** | Tarragona (la Canonja) | 347465,27 | 4554340,7 | Integrated | 25 | 8 | *M. anisopliae* | |
| **D59** | Reus | 338220,96 | 4561250,3 | Integrated | 225 | 7,93 | | |
| **D60** | Aleixar | 336495,09 | 4563494,9 | Integrated | 260 | 6,97 | | |
| **D61** | Aleixar | 337177 | 4562205,4 | Integrated | 213 | 6,83 | | |
| **D62** | Aleixar | 336229,81 | 4563926,8 | Integrated | 268 | 7,39 | | |
| **D63** | Aleixar | 337781,67 | 4564552,3 | Abandoned | 397 | 4,79 | | |
| **D64** | Aleixar | 338625,92 | 4565746,2 | Conventional | 492 | 4,26 | | |
| **D65** | Aleixar | 338906,96 | 4565079,6 | Conventional | 499 | 4,36 | | |
| **D66** | Aleixar | 338702,02 | 4565395,8 | Abandoned | 462 | 5,22 | | |
| **D67** | Aleixar | 337615,35 | 4566496,8 | Abandoned | 424 | 4,39 | | |
| **D68** | Aleixar | 337128,85 | 4565785,6 | Integrated | 375 | 5 | | |
| **D69** | Aleixar | 338654,34 | 4566714,4 | Integrated | 442 | 6,21 | | |
| **D70** | Aleixar | 334677,27 | 4565948,8 | Integrated | 357 | 5,84 | | |
| **D71** | Aleixar | 334337,11 | 4567175,4 | Integrated | 463 | 4,1 | *Fusarium* | |
| **D72** | Vilaplana | 335305,42 | 4567869,3 | Integrated | 429 | 4,7 | *B. bassiana* | |
| **D73** | Aleixar | 338764,49 | 4567375,3 | Conventional | 540 | 4,24 | *M. anisopliae* | |
| **D74** | Vilaplana | 335827 | 4568070,4 | Integrated | 463 | 4,89 | | |
| **D75** | Alforja | 330077,9 | 4565616,6 | Integrated | 413 | 7,4 | | |
| **D76** | Alforja | 328553,9 | 4563921,3 | Integrated | 508 | 5,28 | | |
| **D77** | Alforja | 328934,36 | 4564939,9 | Abandoned | 441 | 5,26 | *B. bassiana* | |
| **D78** | Alforja | 330445,5 | 4565796,2 | Integrated | 397 | 8 | *B. bassiana* | |
| **D79** | Riudecols | 329845,42 | 4560848,6 | Integrated | 378 | 6,49 | *M. anisopliae* | |
| **D80** | Riudecols | 329911,09 | 4561066,1 | Integrated | 385 | 6,25 | | |
| **D81** | Riudecols | 329616,13 | 4561609,8 | Abandoned | 406 | 7,42 | | |
| **D82** | Riudecols | 330367,75 | 4562456,3 | Conventional | 425 | 6,45 | *Aspergillus* | |
| **D83** | Riudecols | 329698,92 | 4562262,1 | Conventional | 418 | 4,99 | | |
| **D84** | Riudecols | 329960,54 | 4562286,9 | Abandoned | 429 | 5,08 | | |
| **D85** | Riudecols | 328555,68 | 4559832,7 | Conventional | 371 | 7,74 | | |
| **D86** | Riudecols | 331603,45 | 4559443,9 | Conventional | 262 | 5,79 | | |
| **D87** | Riudecols | 331726,59 | 4559323,8 | Integrated | 250 | 5,24 | | |
| **D88** | Marçà | 316357,28 | 4557111,5 | Integrated | 301 | 7,9 | | |
| **D89** | Marçà | 316838,01 | 4557025,4 | Integrated | 316 | 7,82 | | |
| **D90** | Marçà | 317050,43 | 4557217,6 | Integrated | 331 | 7,87 | *B. bassiana* | |
| **D91** | Marçà | 314504,66 | 4555535,1 | Integrated | 252 | 7,91 | | |
| **D92** | Marçà | 314505,44 | 4555843,7 | Abandoned | 242 | 8,15 | *B. bassiana* | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **D93** | Falset | 314555,75 | 4558925,3 | Abandoned | 273 | 7,94 | | |
| **D94** | Falset | 314867,35 | 4558982,2 | Abandoned | 272 | 7,75 | *B. bassiana* | |
| **D95** | Falset | 317286,33 | 4558254,7 | Integrated | 334 | 7,67 | *B. bassiana* | |
| **Sample** | **Locality** | **Longitude** | **Latitude** | **Cropping system** | **Altitude (m)** | **pH** | **Species isolated** | **species isolated 2** |
| **D96** | Falset | 317429 | 4558550,5 | Organic | 332 | 7,53 | | |
| **D97** | Falset | 317912,02 | 4559868,4 | Integrated | 340 | 5,32 | *M. anisopliae* | *B. bassiana* |
| **D98** | Falset | 318493,4 | 4558209,2 | Conventional | 378 | 7,63 | | |
| **D99** | Falset | 315857,86 | 4558583,7 | Conventional | 311 | 7,84 | | |
| **D100** | Falset | 316510,81 | 4558672,3 | Conventional | 315 | 7,12 | | |
| **D101** | St. Martí d'Osormort | 448260,21 | 4639593,7 | wild | 543 | 5,95 | *M. anisopliae* | |
| **D102** | Vilanova de Sau | 449033,35 | 4640738,5 | wild | 536 | 5,8 | | |
| **D103** | Vilanova de Sau | 452609,66 | 4639862,5 | wild | 867 | 6,05 | *B. bassiana* | |
| **D104** | Espinelves | 453657,35 | 4638714,5 | wild | 962 | 5,14 | *B. bassiana* | |
| **D105** | Vilanova de Sau | 452298,34 | 4636382,5 | wild | 730 | 5,65 | | |
| **D106** | Vilanova de Sau | 448874,2 | 4644228 | wild | 621 | 5,37 | | |
| **D107** | Vilanova de Sau | 449836,08 | 4645775,6 | wild | 511 | 4,72 | *M. anisopliae* | |
| **D108** | Rupit i Pruit | 452899,99 | 4648882 | wild | 511 | 7,07 | | |
| **D109** | Rupit i Pruit | 455864,91 | 4652027,4 | wild | 750 | 7,07 | | |
| **D110** | Rupit i Pruit | 456080,63 | 4652325,2 | wild | 818 | 5,07 | | |
| **D111** | Rupit i Pruit | 456057,1 | 4654095,7 | wild | 867 | 7,09 | | |
| **D112** | Stª Mª de Corcó | 452961,63 | 4655735 | wild | 988 | 6,83 | *M. anisopliae* | |
| **D113** | Stª Mª de Corcó | 451849,3 | 4656451,9 | wild | 911 | 7,12 | *M. anisopliae* | |
| **D114** | Stª Mª de Corcó | 449151,71 | 4654336,3 | wild | 680 | 6,83 | | |
| **D115** | Stª Mª de Corcó | 449554,62 | 4653124,4 | wild | 794 | 7,19 | | |
| **D116** | Prades | 330892,06 | 4576770,8 | Integrated | 931 | 4,77 | | |
| **D117** | Prades | 330849,26 | 4576932,3 | Integrated | 940 | 4,38 | | |
| **D118** | Prades | 330949,02 | 4577019,4 | Integrated | 938 | 5,76 | *B. bassiana* | |
| **D119** | Prades | 331033,22 | 4576841,6 | Integrated | 947 | 4,56 | *B. bassiana* | |
| **D120** | Prades | 331158,23 | 4576912,8 | Integrated | 955 | 6,35 | | |
| **D121** | Prades | 331323,85 | 4576331,9 | Integrated | 917 | 5,54 | | |
| **D122** | Prades | 331433,89 | 4577665,4 | Integrated | 959 | 4,8 | | |
| **D123** | Prades | 331408,63 | 4576780,4 | Abandoned | 935 | 6,14 | | |
| **D124** | Prades | 332478,5 | 4577175,3 | Integrated | 983 | 4,79 | | |
| **D125** | Prades | 332198,16 | 4577425,5 | Integrated | 991 | 4,67 | *M. anisopliae* | |
| **D126** | Prades | 332895,53 | 4577104 | Integrated | 985 | 6,23 | *M. anisopliae* | |
| **D127** | Prades | 332895,53 | 4577104 | Integrated | 983 | 4,91 | | |
| **D128** | Prades | 333197,39 | 4577587,7 | Integrated | 1013 | 5,17 | *M. anisopliae* | |
| **D129** | Prades | 333406,6 | 4577783,4 | Integrated | 1025 | 4,99 | *M. anisopliae* | |
| **D130** | Prades | 331735,27 | 4576421,1 | Integrated | 942 | 5,51 | *M. anisopliae* | |
| **D131** | Prades | 331775,37 | 4576142,4 | Integrated | 919 | 4,17 | | |
| **D132** | Prades | 331712,34 | 4576335,2 | Integrated | 923 | 4,57 | | |
| **D133** | Prades | 331601,85 | 4576785,2 | Integrated | 931 | 6,07 | | |
| **D134** | Prades | 331415,74 | 4576783,4 | Integrated | 933 | 5,25 | | |
| **D135** | Prades | 331632,84 | 4576716,6 | Integrated | 920 | 5,67 | *M. anisopliae* | |
| **D136** | Prades | 331584,86 | 4577254,6 | Integrated | 919 | 5,3 | | |
| **D137** | Vilanova de Prades | 326479,83 | 4579035,1 | Integrated | 690 | 7,74 | | |

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|---|---|---|---|---|---|---|---|---|
| **D138** | Vilanova de Prades | 326687,16 | 4578952,9 | Integrated | 696 | 7,87 | | |
| **D139** | Prades | 331907,7 | 4577635,9 | Integrated | 955 | 6,19 | | |
| **D140** | Prades | 330785,34 | 4576884,4 | Integrated | 926 | 5,16 | | |
| **Sample** | **Locality** | **Longitude** | **Latitude** | **Cropping system** | **Altitude (m)** | **pH** | **Species isolated** | **species isolated 2** |
| **D141** | Prades | 330696,56 | 4568375,9 | Integrated | 924 | 5,37 | | |
| **D142** | Riudoms | 335384,05 | 4556491,2 | Conventional | 140 | 7,98 | *B. bassiana* | *M. anisopliae* |
| **D143** | Riudoms | 335259,87 | 4556052,8 | Conventional | 137 | 7,57 | *M. anisopliae* | |
| **D144** | Riudoms | 335183,71 | 4555881,8 | Conventional | 135 | 7,3 | *B. bassiana* | |
| **D145** | Riudoms | 335415,03 | 4556003,1 | Conventional | 136 | 7,71 | *Aspergillus* | |
| **D146** | Riudoms | 335284,2 | 4556200,4 | Conventional | 140 | 6,84 | | |
| **D147** | Riudoms | 335735,49 | 4555835,3 | Conventional | 128 | 7,58 | | |
| **D148** | Riudoms | 336226,51 | 4556824 | Conventional | 136 | 7,82 | | |
| **D149** | Riudoms | 335846,26 | 4557026,9 | Conventional | 149 | 7,57 | | |
| **D150** | Riudoms | 335621,5 | 4557195,5 | Conventional | 157 | 7,53 | | |
| **D151** | Riudoms | 335411,74 | 4557515 | Conventional | 158 | 7,85 | | |
| **D152** | Riudoms | 335172,14 | 4557850,5 | Conventional | 165 | 7,82 | | |
| **D153** | Riudoms | 335757,24 | 4558253,9 | Conventional | 172 | 7,87 | | |
| **D154** | Riudoms | 335501,33 | 4557556,2 | Conventional | 178 | 7,88 | | |
| **D155** | Alforja | 333021,56 | 4563333,1 | Conventional | 290 | 7,76 | *M. anisopliae* | |
| **D156** | Alforja | 332782,5 | 4563477,5 | Abandoned | 294 | 5,87 | *M. anisopliae* | |
| **D157** | Alforja | 332700,61 | 4563056,6 | Conventional | 298 | 5,22 | | |
| **D158** | Alforja | 332903,92 | 4563079,7 | Conventional | 308 | 7,23 | *B. bassiana* | |
| **D159** | Alforja | 332547,6 | 4564309,7 | Abandoned | 303 | 7,22 | | |
| **D160** | Alforja | 332352,1 | 4564262,2 | Conventional | 313 | 7,08 | | |
| **D161** | Alforja | 332332,2 | 4563555,7 | Conventional | 309 | 7,4 | | |
| **D162** | Alforja | 331944,69 | 4564940,8 | Conventional | 354 | 7,82 | | |
| **D163** | Alforja | 329950,06 | 4565030,2 | Conventional | 374 | 6,98 | *B. bassiana* | |
| **D164** | Alforja | 329890,53 | 4565275,4 | Conventional | 382 | 6,5 | *M. anisopliae* | |
| **D165** | Alforja | 330277,65 | 4565093,5 | Conventional | 382 | 6,94 | | |
| **D166** | Alforja | 330167,28 | 4564654,9 | Conventional | 408 | 4,78 | *M. anisopliae* | *B. bassiana* |
| **D167** | Alforja | 330425,3 | 4565429,6 | Conventional | 388 | 6,91 | | |
| **D168** | Alforja | 333208,9 | 4564593,8 | Conventional | 352 | 7,89 | | |
| **D169** | Botarell | 334066,55 | 4558560,6 | Conventional | 181 | 7,57 | *B. bassiana* | |
| **D170** | Botarell | 334108,61 | 4558460,9 | Conventional | 192 | 5,42 | *B. bassiana* | *Paecilomyces* |
| **D171** | Botarell | 334479,4 | 4558153,1 | Conventional | 183 | 7,8 | | |
| **D172** | Botarell | 334426,77 | 4557583,5 | Abandoned | 189 | 7,3 | *B. bassiana* | |
| **D173** | Botarell | 333825,66 | 4558016,8 | Abandoned | 186 | 7,7 | *B. bassiana* | |
| **D174** | Botarell | 332916,02 | 4557911 | Conventional | 193 | 7,2 | | |
| **D175** | Botarell | 334599,81 | 4557909,8 | Conventional | 170 | 6,98 | *B. bassiana* | |
| **D176** | Botarell | 334083,54 | 4557668,5 | Abandoned | 163 | 7,68 | *B. bassiana* | |
| **D177** | Botarell | 334008,56 | 4557343,1 | Conventional | 166 | 7,75 | | |
| **D178** | Botarell | 334585,86 | 4556367,4 | Conventional | 152 | 7,57 | | |
| | | | | | | | | |
| **D179** | Botarell | 334316,03 | 4556503 | Conventional | 153 | 7,55 | *B. bassiana* | |
| **D180** | Botarell | 333793,09 | 4556585,9 | Conventional | 163 | 7,6 | | |
| **D181** | Botarell | 334544,36 | 4556800,3 | Conventional | 160 | 7,22 | *B. bassiana* | |
| **D182** | Riudoms | 335278,38 | 4556564,5 | Conventional | 150 | 7,5 | *B. bassiana* | *M. anisopliae* |
| **D183** | Riudoms | 335060,24 | 4557334,7 | Conventional | 158 | 7,07 | | |
| **D184** | Riudoms | 334593,69 | 4557539,6 | Conventional | 165 | 7,74 | *B. bassiana* | *M. anisopliae* |
| **D185** | Riudoms | 335885 | 4556572,5 | Conventional | 150 | 6,8 | *B. bassiana* | |
| **D186** | Riudoms | 335557,31 | 4556104,7 | Conventional | 144 | 7,79 | *B. bassiana* | *M. anisopliae* |
| **D187** | Riudoms | 335834,99 | 4556215,8 | Conventional | 142 | 6,46 | *B. bassiana* | *M. anisopliae* |
| **D188** | Riudoms | 336818,15 | 4556369,6 | Conventional | 121 | 7,26 | *M. anisopliae* | |

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|---|---|---|---|---|---|---|---|---|
| **D189** | Riudoms | 337909,8 | 4559418,4 | Conventional | 178 | 7,95 | | |
| **D190** | Riudoms | 336721,85 | 4560509,3 | Conventional | 186 | 7,23 | *B. bassiana* | |
| **D191** | Riudoms | 336952,04 | 4560170,9 | Conventional | 178 | 6,78 | | |
| **Sample** | **Locality** | **Longitude** | **Latitude** | **Cropping system** | **Altitude (m)** | **pH** | **Species isolated** | **species isolated 2** |
| **D192** | Riudoms | 337096,14 | 4560568,8 | Conventional | 187 | 8,43 | *B. bassiana* | |
| **D193** | Riudoms | 335813,49 | 4559511,5 | Conventional | 168 | 7,43 | *B. bassiana* | *M. anisopliae* |
| **D194** | Fatarella | 288097 | 4559105,5 | Integrated | 485 | 6,78 | *B. bassiana* | *M. anisopliae* |
| **D195** | Fatarella | 287949,13 | 4558835,1 | Integrated | 525 | 7,23 | *M. anisopliae* | |
| **D196** | Vilalba | 286850,78 | 4559410,3 | Intergada | 504 | 6,79 | | |
| **D197** | Fatarella | 288266,51 | 4558353,6 | Integrated | 537 | 7,56 | *B. bassiana* | |
| **D198** | Corbera d'Ebre | 288222,16 | 4557067,7 | Abandoned | 501 | 6,36 | *B. bassiana* | |
| **D199** | Corbera d'Ebre | 287876,18 | 4555840,1 | Integrated | 401 | 7,41 | *M. anisopliae* | |
| **D200** | Corbera d'Ebre | 287934,11 | 4555745,7 | Integrated | 386 | 7,34 | *B. bassiana* | |
| **D201** | Corbera d'Ebre | 287532,94 | 4556235,8 | Integrated | 408 | 6,07 | *M. anisopliae* | |
| **D202** | Corbera d'Ebre | 286845,38 | 4557067,6 | Integrated | 511 | 6,87 | *M. anisopliae* | |
| **D203** | Corbera d'Ebre | 286880,86 | 4556924,7 | Abandoned | 511 | 7,56 | *B. bassiana* | |
| **D204** | Vilalba | 286089 | 4557537,3 | Integrated | 517 | 8,02 | | |
| **D205** | Corbera d'Ebre | 286596,88 | 4556550,2 | Integrated | 497 | 7,99 | *B. bassiana* | |
| **D206** | Vilalba | 286535,84 | 4558215,7 | Integrated | 508 | 8,05 | *M. anisopliae* | |
| **D207** | Vilalba | 286369,3 | 4558822,5 | Abandoned | 487 | 7,77 | *B. bassiana* | |
| **D208** | Vilalba | 285946,81 | 4559294,8 | Integrated | 496 | 8 | *M. anisopliae* | |
| **D209** | Vilalba | 286148,27 | 4559798,2 | Integrated | 499 | 7,86 | *B. bassiana* | |
| **D210** | Vilalba | 286495,48 | 4559788 | Abandoned | 476 | 7,7 | | |
| **D211** | Prades | 332472,83 | 4576527,4 | Abandoned | 960 | 5,97 | | |
| **D212** | Prades | 332426,53 | 4576636,4 | wild | 980 | 6,6 | *Monilia* | |
| **D213** | Prades | 325219,6 | 4576821,9 | Abandoned | 960 | 5,99 | | |
| **D214** | Prades | 331281,8 | 4576727,8 | Abandoned | 931 | 6,2 | *M. anisopliae* | |
| **D215** | Prades | 331114,51 | 4576833,5 | Abandoned | 927 | 6,95 | | |
| **D216** | Prades | 331173,51 | 4576770,5 | Abandoned | 932 | 5,93 | *M. anisopliae* | |
| **D217** | Prades | 330815,24 | 4576769,6 | Abandoned | 939 | 5,5 | | |
| **D218** | Prades | 330350,01 | 4576768 | Abandoned | 942 | 6,19 | | |
| **D219** | Prades | 330320,81 | 4577012,5 | Abandoned | 959 | 5,21 | | |
| **D220** | Prades | 330188,04 | 4577203,8 | Abandoned | 966 | 4,92 | | |
| **D221** | Prades | 329898,12 | 4576149,2 | Abandoned | 978 | 4,96 | *M. anisopliae* | |
| **D222** | Prades | 329755,76 | 4576328,4 | Abandoned | 990 | 5,42 | *B. bassiana* | |
| **D223** | Prades | 329769,12 | 4576201,5 | Abandoned | 983 | 5,7 | | |
| **D224** | Prades | 331674,78 | 4575015,4 | Abandoned | 946 | 6,14 | | |
| **D225** | Prades | 331484,88 | 4575454,9 | Abandoned | 934 | 5,53 | *M. anisopliae* | *Monilia* |
| **D226** | Prades | 331920,85 | 4575191,7 | Abandoned | 936 | 7,47 | | |
| **D227** | Prades | 332085,65 | 4574672,6 | Abandoned | 946 | 7,71 | | |
| **D228** | Prades | 331829,15 | 4576764,5 | Abandoned | 955 | 6,36 | | |
| **D229** | Prades | 332905,67 | 4577140,7 | Abandoned | 984 | 5,68 | *B. bassiana* | |
| **D230** | Prades | 331949,58 | 4577036,3 | Abandoned | 955 | 5,43 | *M. anisopliae* | |
| **D231** | Prades | 331227,08 | 4576874,1 | Abandoned | 922 | 5,94 | *B. bassiana* | |
| **D232** | Prades | 330162,93 | 4576331,2 | Abandoned | 917 | 5,85 | | |
| **D233** | Vidrà | 439972,88 | 4665962,6 | wild | 828 | 7,54 | | |
| **D234** | Vidrà | 439433,59 | 4665714,2 | wild | 904 | 7,46 | | |
| **D235** | Vidrà | 442940,01 | 4664127,3 | wild | 971 | 7,32 | *B. bassiana* | |
| **D236** | Vidrà | 444517,03 | 4665243,7 | wild | 982 | 7,52 | | |
| **D237** | Vidrà | 443800,65 | 4665514,6 | wild | 960 | 7,48 | | |

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|---|---|---|---|---|---|---|---|---|
| **D238** | Vidrà | 444460 | 4666166,4 | wild | 1002 | 6,72 | | |
| **D239** | Vidrà | 445587,27 | 4667379 | wild | 1202 | 7,22 | | |
| **D240** | Riudaura | 445634,61 | 4667859,8 | wild | 1304 | 6,76 | | |
| **Sample** | **Locality** | **Longitude** | **Latitude** | **Cropping system** | **Altitude (m)** | **pH** | **Species isolated** | **species isolated 2** |
| **D241** | Riudaura | 446041,15 | 4667912,2 | wild | 1331 | 6,66 | | |
| **D242** | Santa Pau | 446963,24 | 4669373,3 | wild | 1231 | 7,19 | | |
| **D243** | Santa Pau | 447925,96 | 4669557,4 | wild | 1166 | 7,34 | | |
| **D244** | Vidrà | 447708,93 | 4666955,9 | wild | 408 | 7,23 | | |
| **D245** | Santa Pau | 441647,16 | 4666863 | wild | 603 | 7,56 | *M. anisopliae* | |
| **DG1** | Brunyola | 473747 | 4640092 | Integrated | 179 | 4,94 | *M. anisopliae* | |
| **DG2** | Brunyola | 473386 | 4639824 | Integrated | 177 | 4,8 | *M. anisopliae* | |
| **DG3** | Brunyola | 473344 | 4639768 | Integrated | 183 | 4,87 | *M. anisopliae* | |
| **DG4** | Brunyola | 472381 | 4640069 | Integrated | 225 | 4,47 | *M. anisopliae* | |
| **DG5** | Brunyola | 472338 | 4639493 | Conventional | 206 | 5,05 | | |
| **DG6** | Brunyola | 472387 | 4639467 | Integrated | 207 | 6,11 | *M.anisopliae* | |
| **DG7** | Brunyola | 471652 | 4640221 | Integrated | 236 | 6,29 | *M.anisopliae* | |
| **DG8** | Brunyola | 472980 | 4641551 | Integrated | 210 | 4,58 | | |
| **DG9** | Brunyola | 472970 | 4641573 | Integrated | 209 | 5,41 | | |
| **DG10** | Brunyola | 473334 | 4641751 | Integrated | 200 | 5,42 | | |
| **DG11** | Brunyola | 474004 | 4642316 | Conventional | 165 | 5,38 | *M. anisopliae* | |
| **DG12** | Brunyola | 474038 | 4642314 | Integrated | 165 | 4,68 | | |
| **DG13** | Vilobí d'Onyar | 475226 | 4641167 | Integrated | 151 | 5,54 | | |
| **DG15** | Vilobí d'Onyar | 476003 | 4641180 | Integrated | 145 | 4,73 | *B. bassiana* | |
| **DG16** | Brunyola | 476367 | 4640007 | Integrated | 164 | 7,99 | | |
| **DG17** | Brunyola | 476485 | 4640246 | Integrated | 164 | 6,38 | | |
| **DG18** | Vilobí d'Onyar | 477337 | 4640266 | Integrated | 131 | 5,87 | | |
| **DG19** | Vilobí d'Onyar | 477474 | 4640344 | Integrated | 134 | 5,85 | | |
| **DG20** | Vilobí d'Onyar | 477292 | 4640429 | Integrated | 140 | 5,35 | | |
| **DG21** | Vilobí d'Onyar | 477484 | 4640606 | Integrated | 143 | 6,57 | | |
| **DG22** | Bescanó | 478083 | 4641939 | Integrated | 200 | 6,15 | | |
| **DG23** | Bescanó | 477777 | 4643095 | Integrated | 196 | 5,4 | | |
| **DG24** | Bescanó | 477719 | 4642328 | Organic | 187 | 6,68 | *M. anisopliae* | |
| **DG25** | Vilobí d'Onyar | 477695 | 4640573 | Integrated | 169 | 6,4 | | |
| **DG26** | Vilobí d'Onyar | 477095 | 4638084 | Conventional | 142 | 7,9 | | |
| **DG27** | Vilobí d'Onyar | 477016 | 4638098 | Conventional | 136 | 7,18 | | |
| **DG28** | Vilobí d'Onyar | 477019 | 4638054 | Integrated | 134 | 6,38 | | |
| **DG29** | Vilobí d'Onyar | 477054 | 4637834 | Integrated | 121 | 5,36 | *M. anisopliae* | |
| **DG30** | Brunyola | 474197 | 4639966 | Abandoned | 174 | 4,67 | *M. anisopliae* | |
| **DG31** | Brunyola | 471559 | 4642469 | Conventional | 224 | 4,6 | | |
| **DG32** | Brunyola | 471535 | 4641656 | Conventional | 237 | 4,8 | | |
| **DG33** | Brunyola | 471777 | 4642968 | Conventional | 200 | 5,13 | | |
| **DG34** | Brunyola | 471902 | 4643036 | Conventional | 184 | 5,26 | | |
| **DG36** | Brunyola | 472728 | 4642904 | Integrated | 214 | 5,47 | *B. bassiana* | |
| **DG37** | Brunyola | 472299 | 4642512 | Integrated | 211 | 5,15 | | |
| **DG38** | Brunyola | 472269 | 4642391 | Integrated | 210 | 4,8 | *M. anisopliae* | |
| **DG39** | Brunyola | 472394 | 4642154 | Integrated | 217 | 4,74 | *B. bassiana* | |
| **DG40** | Brunyola | 472738 | 4642000 | Integrated | 188 | 4,58 | | |
| **DG41** | Brunyola | 473401 | 4642165 | Integrated | 184 | 5,4 | | |

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|---|---|---|---|---|---|---|---|---|
| **DG42** | Brunyola | 473346 | 4642108 | Integrated | 180 | 6,03 | *M. anisopliae* | |
| **DG43** | Brunyola | 473346 | 4642108 | Integrated | 180 | 5,32 | *B. bassiana* | *M. anisopliae* |
| **DG44** | Brunyola | 472241 | 4642136 | Integrated | 220 | 4,65 | *M. anisopliae* | |
| **Sample** | **Locality** | **Longitude** | **Latitude** | **Cropping system** | **Altitude (m)** | **pH** | **Species isolated** | **species isolated 2** |
| **DG45** | Brunyola | 472273 | 4642137 | Integrated | 225 | 5,35 | *M. anisopliae* | |
| **DG46** | Anglès | 469769 | 4648003 | Integrated | 269 | 5,14 | *M. anisopliae* | *B. bassiana* |
| **DG47** | Anglès | 469840 | 4648188 | Integrated | 306 | 6,88 | | |
| **DG48** | Brunyola | 470998 | 4642114 | Integrated | 225 | 4,38 | *M. anisopliae* | |
| **DG49** | Brunyola | 471133 | 4641632 | Integrated | 247 | 4,59 | *B. bassiana* | *M. anisopliae* |
| **DG50** | Brunyola | 471130 | 4641604 | Integrated | 249 | 5,17 | | |

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# Capítol V

**Field efficacy and short-term persistence evaluation of entomopathogenic nematodes against the Hazelnut weevil, *Curculio nucum***

*(Enviat a Spanish Journal of Agricultural Research)*

# Field efficacy and short-term persistence evaluation of entomopathogenic nematodes against the Hazelnut weevil, *Curculio nucum*

Efficacy and persistence of entomopathogenic nematodes against Hazelnut weevil


## Abstract

The hazelnut weevil, *Curculio nucum* L. (Coleoptera: Curculionidae) is a pest affecting hazelnut orchards in Europe, with an important economical repercussion. Its potential control, short-term field persistence and the vertical distribution of native entomopathogenic nematode strains were tested in Muntanyes de Prades, Tarragona (NE Iberian Peninsula) over two consecutive years. *Steinernema feltiae* strain D114, *Steinernema* sp. strain D122 and *Heterorhabditis bacteriophora* strain DG46 were used in summer and spring applications at a dosage of $5 \cdot 10^5$ IJs/$m^2$. The three nematode species reduced the hazelnut weevil population without significant differences in efficacy or between the two applications. Persistence evaluation was carried out during 9 weeks for *S. feltiae* (D114), *Steinernema* sp. (D122) and *H. bacteriophora* (DG46) and showed all species capable of lasting for this period. Nematodes and larval vertical distribution was assessed. Most of the hazelnut weevil stayed within the first 25 cm although some were found as deep as 40 cm. Entomopathogenic nematodes were found along all 40 cm depth. This study proves the suitability of entomopathogenic nematodes to control the hazelnut weevil.


*Keywords:* hazelnut orchard, biological control, *Steinernema*, *Heterorhabditis*, vertical distribution, spring application

## Introduction

The hazelnut weevil (HW), *Curculio nucum* L. (Coleoptera, Curculionidae) is a major pest of hazelnut orchards. In the Mediterranean region adults emerge from the soil in April, and feed during May-June on the immature fruits. Oviposition take place from June to July in the hazelnut fruit and the larvae develop inside the nuts. At the beginning of August the larvae emerge from the nuts and burrow into the ground, where this insect spends a wintering diapause (Akça & Tuncer, 2005). The weevil life cycle can last for 2 years, including overwintering larval and adult stages (AliNiazee, 1998; Bel-Venner et al., 2009; Coutin, 1992).

Spain, in the Iberian Peninsula, is the eighth world hazelnut producer, with 15,100T during 2010 (FAO, 2010). 95% of the hazelnut growing area is in the North East of the Iberian Peninsula, in Catalonia (FAO, 2009). HW may cause up to 80% yield loss in unprotected orchards (AliNiazee, 1998).Current control relies on chemical insecticides and due to the cryptic habitat of larvae,

chemical control is directed only against emerging adults, limiting its success (Akça & Tuncer, 2005). Due to the difficulty of controlling this insect with chemical insecticides and the important environmental issues associated with this procedure, alternative control methods are needed.

Entomopathogenic nematodes are important biological control agents for a variety of economically important pests (Grewal et al., 2005) and particularly suited to controlling soil pests (Klein, 1990). They have potential for use in augmentative and/or inundative biological control (Parkman & Smart, 1996), they can be mass produced in vitro (Ehlers, 2001) and they have a high control potential when applied to control weevils (Curculionidae) in nurseries (van Tol & Raupp, 2006), tuber crops (Bélair et al., 2003) and forestry (Torr et al., 2007). The virulence of EPNs against HW larvae has been tested in previous studies proving that some commercial nematodes are capable of infecting larvae in the laboratory (Blum et al., 2009) and significantly reducing HW population in the field (Kuske et al., 2005; Peters et al., 2009).

Concurrence of the biology and ecology of nematodes and the target pest are basic for a successful application (Hazir et al., 2003). Native EPNs might be better adapted to the abiotic conditions of a certain locality, and thus extend their persistence, which is an important characteristic for their wider use. Different abiotic factors (i.e. soil type, humidity, temperature and pH) influence the establishment and persistence of the nematodes in soil (Grewal et al., 1994; Kung et al., 1990a; Kung et al., 1990b). But biotic factors (i.e. alternative host availability) also have an effect on the different persistence of nematode species and strains (Strong, 2002).

The main objectives of the research reported here were: (i) to determine the potential of EPN strains isolated in hazelnut orchards to control HW under field conditions using two different application strategies: one as barrier strategy, directed against the larvae when they bury themselves in the ground and the other against the overwintering stages and (ii) to evaluate the nematodes' vertical distribution and persistence under field conditions in order to determine optimal application strategy.

**Material and Methods**

Experiments were conducted in 2 organic managed hazelnut orchards (Hortals and Mallola) located in Muntanyes de Prades, Catalonia (NE Iberian Peninsula), an area naturally attacked by HW. Prior to all experiments soil samples were taken to confirm no presence of EPNs. Soil analysis for both fields was conducted (Table 1) and data of soil temperature and moisture during the study were recorded. Nematodes used for these experiments were *S. feltiae* strain D114, *S. carpocapsae* strain B14, *Steinernema* sp. strain D122 (glasseri group) and *H. bacteriophora* strain DG46. All strains were isolated from hazelnut orchards soil. Nematodes were cultured on last instar of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae according to the method of Woodring & Kaya (1998) and stored in

tap water at 7$^{o}$C for no longer than 2 weeks prior to the experiments. Before application, the Infective juveniles (IJs) viability was checked under a stereomicroscope.

Table 1. Chemical characteristics and granulometric analysis of soil samples from hazelnut orchards.

| | Orchard1:Hortals | Orchard2:Mallola |
|---|---|---|
| Humidity | 1.4 % | 1.1 % |
| pH | 7.9 | 8.1 |
| conductivity | 0.23 dS/m | 0.24 dS/m |
| Organic mater | 2.90 % | 2.96 % |
| Sand (0.05<D<0.2 mm) | 20.8 % | 22.7 % |
| Sand (0.2<D<2 mm) | 9.5 % | 18.7 % |
| Silt (0.02<D<0.05 mm) | 21.3 % | 20.4 % |
| Silt (0.002<D<0.02 mm) | 23.2 % | 19.3 % |
| Clay (D<0.002 mm) | 25.2 % | 18.9 % |
| Carbonate | 5 % | 12 % |

*Nematode field efficacy*

The experiment was conducted in 2009 and 2010. The experimental units, plots, were plastic tubes (12 cm diameter, 40 cm length) with an open bottom for water drainage and a trap top with a mesh tightly attached to collect possible emerging HW adults. Plots were installed under the canopy of shrubs within 2 m of the trunk and local soil was transferred into pots and left to settle for several months. Two different trials were conducted to test the suitability of controlling HW with EPNs.

*Summer application*

This trial was designed to determine the effectiveness of the application of EPNs, as a possible barrier strategy, to attack the insect when the larvae are burying themselves in the soil.

Three different treatments, corresponding to three different EPN species were used to assess its efficacy: *S. feltiae* (D114), *Steinernema* sp. (D122) and *H. bacteriophora* (DG46). Nematodes were applied at a dose of $5{\cdot}10^5$ IJs/m$^2$ (5,655 IJs/plot) in 10 ml of sterile tap water per plot during the last week of August. Application was at dusk to reduce the adverse effects of high temperatures and UV. One day after nematode application 15 last instar larvae were placed on the soil surface and allowed to naturally burrow into the soil. Controls received only water. There were 10 replications per treatment and the trial was repeated over two consecutive years.

*Spring application*

The spring application would have the aim of determining whether nematodes are capable to seek the overwintering HW when they are buried in the soil. During the last week of August 2010 last instar larvae were placed on the surface of each plot and waited until they had buried themselves. Seven months later (last week of March) nematodes were applied with the same methodology as the spring application.

In both applications, seven months later the nematode treatment plots were taken to the laboratory to determine larval vertical distribution and the nematodes presence and distribution. Presence of nematodes was evaluated by the *Galleria* baiting method according to Bedding & Akhurst (1975).

*Nematode persistence*

Persistence was assessed in two different fields and each one comprised five randomized plots ($1m^2$) per nematode species. The nematode species *S. feltiae* (D114) and *H. bacteriophora* (DG46) were applied at the end of April 2009 and 2010 and *Steinernema* sp. (D122) at the end of April 2010 and beginning of May 2011. Nematodes were applied in a concentration of $5 \cdot 10^5$ IJs/$m^2$ in 8 liters of water and administered by watering each plot. Each plot was treated only once. Nematode persistence was investigated over the spring and summer by taking one 25cm depth soil sample per plot with a drill. Each sample was divided in 5 subsamples corresponding to different depths (0-5cm; 5-10cm; 10-15cm; 15-20cm and 20-25cm) and then placed independently in a 90 cm diam. Petri dish. The persistence (indicated by number of positive samples) was determined using the *Galleria* baiting method as before. Persistence was assessed once a week in 2009, and every two weeks for the next years, up to a period of maximum 9 weeks.

*Statistical analysis*

Efficacy of nematodes relating to the number of surviving insects found in the non-treated plots was calculated using Abbott's formula (Abbott, 1925). Generalized Linear Model (GLZ) was used to test differences in efficacy between nematodes treatments within each year and between summer and spring applications. Presence of nematodes on the efficacy plots (percentage of the positive samples for presence of nematodes) in summer and spring application was subjected to GLZ. In all GLZ analysis pairwise comparisons were adjusted using Sequential Sidak.

Differences in persistence of nematodes between fields for each nematode species was estimated using a GLZ analysis. To evaluate the effect of each sample year on the persistence of nematode species a chi-square test was developed. Based on these results, for each year data was pulled together for further analysis. To assess differences between strains on the persistence over time and the vertical distribution of nematodes in soil, a GLZ analysis followed by Sequential Sidak comparison was used.

A level of significance of p<0.05 was used for all tests. The statistical analysis was performed using the programme SPSS-PC 19.0 (SPSS Inc., 2007).

**Results**

*Nematode field efficacy*

The efficacy of the three different nematode treatments in both summer and spring applications is presented in Figure 1. In summer application the efficacy recorded was 34.0%, 44.3% and 51.5% for *S. feltiae* (D114), *Steinernema* sp. (D122) and *H. bacteriophora* (DG46), respectively in 2010. In 2011 the efficacy was 32.2% in plots treated with *S. feltiae* (D114) and 60.5% treated with *Steinernema* sp. (D122) and *H. bacteriophora* (DG46). No differences were found between different nematode species treatments (GLZ: $\chi$2=3.55, 2, P>0.05).

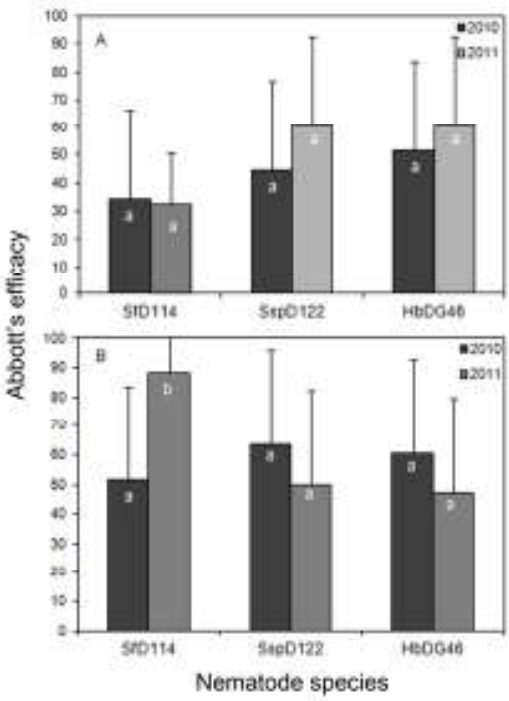


**Figure 1.** Abbott's efficacy in (A) summer application and (B) spring application using three different entomopathogenic nematodes, SfD114: *S. feltiae* (D114), SspD122: *Steinernema* sp. (D122) and HbDG46: *H. bacteriophora* (DG46), against the Hazelnut weevil, *Curculio nucum*.

In the spring application a similar pattern was observed in 2010. The treatments showed efficacies of 51.5%, 63.6% and 60.6% for *S. feltiae* (D114), *Steinernema* sp. (D122) and *H. bacteriophora* (DG46), respectively without significant differences between nematode treatments (GLZ: $\chi$2=2.33, 2, P>0.05). In 2011, *S. feltiae* (D114) achieved 88.2% efficacy, significantly higher (GLZ: $\chi^2$5.171, 1, P=0.023) than the 50.0% accounted by *Steinernema* sp. (D122) and 47.1% by *H. bacteriophora* (DG46) (Figure 1).

Comparing the efficacy of summer and spring applications there were no differences in 2010 (GLZ: $\chi^2$=3.13, 1, P>0.05) and a marginal difference in 2011 (GLZ: $\chi^2$=3.84, 1, P=0.05). Both treatments showed similar efficacy when pulling together the two assessed years (GLZ: $\chi^2$=1.56, 1, P>0.05).

After 7 months of nematodes application, all nematode species were present in all depths, although 50% of them were found in the first 20cm. The HW distribution found in the control plots showed that nearly 90% of found larvae were located within the first 20 cm of the soil (16.51% at 5cm, 35.92% at 10cm, 28.03% at 15cm, 7.64% at 20cm, 3.96% at 25cm, 0.81% at 30 cm, 6.33% at 35cm and 0.81% at 40cm).

*Nematode persistence*

There were no differences in the persistence of nematodes species between the two fields assessed (GLZ: $\chi^2$=0.010, 1, P>0.05) thus data was pulled together to develop further statistic analysis. Persistence data over the time (Figure 2) revealed a no strict linear relationship between the presence of nematodes and time after application.

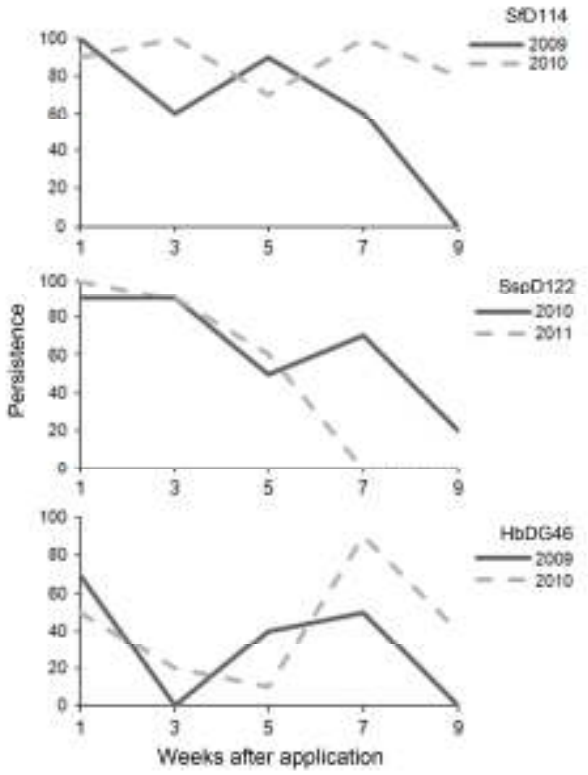


**Figure 2.** Percentage of soil samples containing entomopathogenic nematodes released on spring on the field over 9 weeks. The nematodes used were SfD114: *S. feltiae* (D114), SspD122: *Steinernema* sp. (D122) and HbDG46: *H. bacteriophora* (DG46).

*S. feltiae* (D114) was present at a high rate during the nine surveyed weeks in 2009, increasing 3 and 7 weeks after application. Fluctuations were also observed in 2010 but no positive samples were

found after nine weeks. *Steinernema* sp. (D122) presented oscillations and was still present nine weeks after application in 2010 but not in 2011. The number of *H. bacteriophora* (DG46) positive samples dropped 3 weeks after application in 2009 and then increased again at the 7th week after application when the positive samples decreased again down to 0 at the end of the sampling season. A similar pattern was observed in 2010 with decreasing numbers during 5 weeks after application and increasing again to high rates 7 weeks after application. At the end of each sampling period, the total number of samples with presence of nematodes showed significant differences between strains (GLZ: $\chi^2$=62.44, 2, P<0.05). *S. feltiae* (D114) and *Steinernema* sp. (D122) were more abundant than *H. bacteriophora* (DG46) (75%, 57% and 37% respectively).

**Table 2.** Pearson coefficient for the effects of the sampled year on the vertical distribution of the entomopathogenic nematodes SfD114: *S. feltiae* (D114), SspD122: *Steinernema* sp. (D122) and HbDG46: *H. bacteriophora* (DG46).

| | 5cm | 10cm | 15cm | 20cm |
|---|---|---|---|---|
| **SfD114** | $\chi^2$=1.058, 1 , p>0.05 | $\chi^2$=0.292, 1, p>0.05 | $\chi^2$=0.182, 1, p>0.05 | $\chi^2$=1.267, 1, p>0.05 |
| **SspD122** | $\chi^2$=1.232, 1 , p>0.05 | $\chi^2$=1.309, 1 , p>0.05 | $\chi^2$=0.052, 1 , p>0.05 | $\chi^2$=2.450, 1 , p>0.05 |
| **HbDG46** | $\chi^2$=1.350, 1 , p>0.05 | $\chi^2$=0.119, 1 , p>0.05 | $\chi^2$=0.790, 1 , p>0.05 | $\chi^2$=0.078, 1 , p>0.05 |

Distribution of nematodes in soil showed no differences between years for any of the strains (Table 2). Nematodes were more abundant on the surface than deep into the soil column (Figure 3). More than 50% of the positive samples were found in the first 10 cm of soil in all species and decreased towards a depth of 20 cm. *H. bacteriophora* (DG46) was the most abundant in the first 5 cm of the soil, statistically different than *S. feltiae* (D114) and *Steinernema* sp. (D122) (GLZ: $\chi^2$=11.44, 2, P<0.05). The distribution through the soil column didn't show differences at 10 cm (GLZ: $\chi^2$=0.208, 2, P>0.05). *S. feltiae* (D114) and *Steinernema* sp. (D122) were more abundant at 15 and 20 cm than *H. bacteriophora* (DG46) (GLZ: $\chi^2$=14.256, 2, P<0.05 and $\chi^2$=10.697, 2, P<0.05).

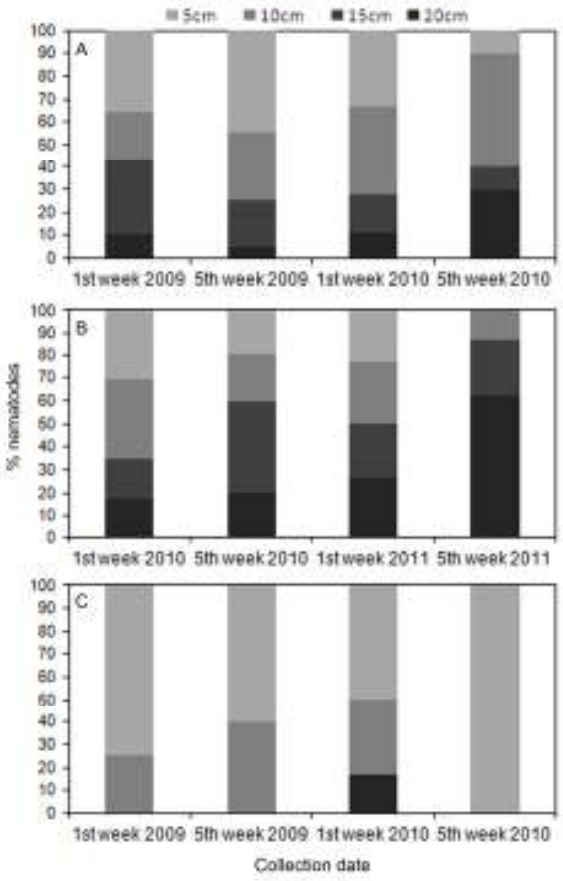


**Figure 3.** Percentage of nematodes detected in four sections of 20 cm soil samples of different species (A) SfD114: *S. feltiae* (D114), (B) SspD122: *Steinernema* sp. (D122) and (C) HbDG46: *H. bacteriophora* (DG46) during the first and the fifth week each sampled year.

**Discussion**

The results of the field experiment showed that the nematodes tested were capable of finding and parasiting HW in field conditions. The reduction of the HW population by the three strains is consistent with the virulence observed for the same strains in previous laboratory assays (58.6-70.6%) (Batalla-Carrera et al., *unpublished data*). Our results agree with Kuske et al. (2005) who obtained a field efficacy from 43.3% to 75.5% using commercial strains of *S. feltiae* and *H. bacteriophora* respectively and Peters et al. (2009) who observed an insect mortality ranging from 41% to 75% using the same nematode species. But in contrast to our results both authors found significant differences between the nematode species that they tested.

Our results did not reveal differences between the two strategies tested to control HW. All nematodes showed the capacity of controlling larvae both in spring, when HW is overwintering, and during the summer when they are buried in the soil. Moreover, the results of HW and nematodes distribution demonstrate the capacity of nematodes to find and invade overwintering HW at any depth. Peters et al. (2009) applied nematodes in August and based on their results recommended as the best approach to control this pest by hitting the larvae when they are in the top soil layer in order to optimize the efficacy of the nematodes. Nevertheless, these authors used irrigation during their experiments which could have improved nematode success. Based on our results we recommend applying the nematodes in spring to avoid the use of irrigation and minimize the negative factors for the survival of nematodes. Moreover, if we release nematodes in spring, when soil temperatures and moisture levels are optimal for nematode survival, we would ensure longer persistence of nematodes.

Different studies have shown the influence of abiotic factors on the nematodes persistence in the soil (Grewal et al., 1994; Kung et al., 1990a; Kung et al., 1990b). In our study, the locality where experiments were developed has Mediterranean climate characteristics: dry and hot summer, cold winter and rainfall condensed in spring and autumn. Nematode persistence was assessed from April to July when temperature starts to rise. The agrometeorological data of the study area during 2010, 2011 and 2012 showed an increment of soil temperatures from 14$^{o}$C in April, 18$^{o}$C in May, 21$^{o}$C in June and 24$^{o}$C in July and moisture of 23.73cbar in April, 15.5cbar in May and 32.65 in July. Data of temperature and moisture during our experiment would not seem to imply any limiting factors for short-term persistence of the nematodes tested. Nematodes were present up to nine weeks after application drawing a fluctuating pattern. Since persistence studies of EPNs cannot distinguish between the recovery of a released population and the recovery of offspring, it could be possible that the fluctuations in nematode presence were closely related to the insect population dynamics. Fenton et al. (2002) data also showed oscillating trends in nematode abundance throughout their experiment, suggesting that there were substantial levels of nematode recycling. In hazelnut orchards, over 200 species of insects and mites have been identified associated with hazelnuts

(AliNiazee, 1998) and some of them might be soil-dwelling insects. This established insect population could easily work as a potential nematode reservoir keeping base levels of EPNs in the soil. Although abiotic factors are essential for the EPN's establishment and short-term persistence, these factors could have a lesser effect on the longer persistence. The major factor on long-term persistence of EPNs might be the presence of host insects providing a basis for the nematodes population (Strong, 2002). This would also explain the nematode presence observed by the efficacy experiments 7 months after the nematode's application as well as the longer persistence of EPNs reported by Susurluk & Ehlers (2008) who found nematodes in different crops two years after application.

Regarding the vertical distribution most of the nematodes were found within the first 10 cm depth. In our study *H. bacteriophora* (DG46) was mainly found in the surface, while *S. feltiae* (D114) and *Steinernema* sp. (D122) presented a more uniform distribution. The vertical distribution of nematodes is often justified by their different foraging strategies (Campbell & Gaugler 1993; Campbell et al., 2003; Spiridonov et al., 2007*).* A gradient between ambusher and cruiser has been recognized for entomopathogenic nematodes (Campbell et al., 2003). Foraging strategy was considered a species characteristic (Campbell & Gaugler, 1997), but nowadays many authors have proved that nematode's behaviour and virulence go down to the strain level. Morton & García-del-Pino (2009) proved that often intra-specific differences are as important as inter-specific. When testing the tolerance and foraging behavior of different strains of *S. feltiae* towards different abiotic factors, these authors obtained different strain behaviors and virulence within the same species. While *H. bacteriophora* has been frequently described as an active cruiser nematode and has been isolated from deeper soil layers, in our field study it was mainly found in the first 5 cm. Susurluk (2009) also found *H. bacteriophora* at 10-15 cm depths during his study in fallow, evidencing the intra-specific character of the foraging behavior.

This study confirms that entomopathogenic nematodes can effectively reduce HW populations in field and suggests that a spring application could be an alternative to summer application in order to minimize negative abiotic factors and improve the nematode persistence. Future research focused on hybridization and genetic selection of EPNs could improve the biocontrol of *C. nucum* by enhancing the foraging efficiency, persistence and virulence against this insect.

**Acknowledgement**

This research was supported by a Spanish Ministerio de Educación y Ciencia Project (AGL2006-037228/AGR). Many thanks to Anna Aymamí, Gonçal Barrios and Jordi Mateu from the Servei de Sanitat Vegetal (Generalitat de Catalunya) for all the assistance. We are especially thankful to Frederic Mallol (ADV Associació pel Desenvolupament Rural de les Muntanyes de Prades) for all his effort, dedication and long hours of drilling in the field. We are also grateful to meteoprades.cat for

sharing with us all agronometeorogic data, particularly to Eduard and Romà Ogaya for atmospheric data. And finally, we thank Sharon Robinson for the language revision of the manuscript.

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# Conclusions

***No deixis mai de bategar els sentiment de llibertat, no deixis mai d'anar endavant, perquè tot el poble avançarà.***

*(Cesk Freixas – La princesa de la revolta)*

1. Entomopathogenic nematodes were recovered in 3.73% of the sampled hazelnut growing areas in Catalonia. Ten entomopathogenic nematode strains of *Steinernema* and one of *Heterorhabditis* were isolated from the soil samples.

2. The main occurrence of entomopathogenic nematodes was in wild hazelnuts area (20%), followed by organic orchards (9.1%), integrated managed (2.2%) and abandoned fields (1.5%). The altitude and pH did not affect the presence of the entomopathogenic nematodes.

3. The molecular analysis revealed that the isolated entomopathogenic nematode strains belong to *S. affine*, *S. feltiae*, *S. intermedium*, and *H. bacteriophora*. A new species of *Steinernema* belonging to the *glaseri* group was also isolated.

4. The symbiotic bacteria of the isolated entomopathogenic nematodes were identified as nine *Xenorhabdus bovienii, associated to S. affine, S. intermedium* and *S. feltiae*, one *X. kozodoii* associated to the new *Steinernema* sp. and one *Photorhabdus luminiscens* associated to the nematode *H. bacteriophora*.

5. Last instar larvae of *C. nucum*, which overwinter in the ground, were susceptible to all of the native entomopathogenic nematodes tested in the laboratory. All nematodes caused similar larval mortality (52.2%-78.8%) except *S. affine* which caused the lowest mortality (less than 10%).

6. Bacteria strains caused 100% larvae mortality except *X. bovienii* (93.3%) belonging to *S. affine* and *X. bovienii* (46.7%) that belonging to *S. feltiae*.

7. *S. carpocapsae* was very effective against the adult of *C. nucum* achieving 100% weevil mortality and emerging as the best entomopathogenic nematode species to control the adult stage of *C. nucum*.

8. Bacterial strains caused 100% adult mortality, therefore the low adult mortalities caused by some of the entomopathogenic nematodes species are due to the nematodes ability to infect the host and not to the symbiotic bacteria virulence.

9. The main route of penetration used by *S. carpocapsae* and *S. feltiae* to get into the hemolymph of the larva and adult of *C. nucum* was the anus followed by the mouth. The

spiracles are not a preferential route for entomopathogenic nematodes to penetrate into the *C. nucum*.

10. The host immune response against *S. carpocapsae* and *S. feltiae* was not observed in larvae of *C. nucum* and only a few *S. carpocapsae* infective juveniles were observed surrounded by hemocytes in adult weevils. The observation of hemocytes inside dead insects implies that nematodes triggered the immune system, but the response comes too late to interfere with the lethal action of symbiotic bacteria.

11. Five different entomopathogenic fungi genera were isolated: *Beauveria*, *Metarhizium*, *Aspergillus*, *Fusarium*, and *Paecilomyces*. The most abundant were the *B. bassiana* (57 isolates), and *M. anisopliae* (48 isolates).

12. Entomopathogenic fungi were virulent against *C. nucum* larvae. The larval mortality caused by the combination of entomopathogenic nematodes and fungi did not increase the mortality obtained by the application of either pathogen alone.

13. Entomopathogenic nematodes can persist in the field for a duration of nine weeks and most of the nematodes were found within the first 10 cm depth although some reached 40 cm depth. The vertical distribution of nematodes ensures the encounter with the overwintering stages of *C. nucum*.

14. The entomopathogenic nematodes, *S. feltiae*, *Steinernema* sp. and *H. bacteriophora*, can effectively reduce *C. nucum* populations in field. They are especially suitable for controlling the larvae as they bury, as well as while they are overwintering in the ground.

15. Efficient biocontrol of *C. nucum* with entomopathogenic nematodes requires a combined strategy. A summer application using *S. feltiae*, *Steinernema* sp. or *H. bacteriophora* would be the best approach to control the larvae when they are burying into the ground. A spring application with same nematodes species could decrease the overwintering larvae population and an additional spring application with *S. carpocapsae* would effectively control the overwintering and the emerging adults.

1. Es va aïllar nematodes entomopatògens en un 3.73% de les mostres agafades en àrees on creixen avellaners i es van identificar com deu soques pertanyents a *Steinernema* i una a *Heterorhabditis*.

2. La major incidència de nematodes entomopatògens es donà en avellanoses salvatges (20%), seguida d'explotacions ecològiques (9.1%), integrades (2.2%) i camps d'avellaners abandonats (1.5%). L'altitud i el pH no va afectar a la presència dels nematodes entomopatògens aïllats.

3. L'anàlisi molecular va revelar que les soques aïllades de nematodes entomopatògens corresponien a *S. affine*, *S. feltiae*, *S. intermedium*, i *H. bacteriophora*. Addicionalment, es va aïllar una nova espècie de *Steinernema* identificada dins del grup *glaseri*.

4. El bacteri endosimbiòtic dels nematodes entomopatògens van ser identificats com nou *Xenorhabdus bovienii* associats a *S. affine, S. intermedium* i *S. feltiae*, un *X. kozodoii* associat a la nova espècie *Steinernema* sp. i un *Photorhabdus luminiscens* associat al nematode *H. bacteriophora*.

5. Les larves de *C. nucum*, que hibernen enterrades al sòl, resultaren susceptibles a les soques aïllades de nematodes entomopatògens testades al laboratori. Tots els nematodes causaren una mortalitat similar (52.2%-78.8%) excepte *S. affine* que va provocar la mortalitat més baixa (10%).

6. Les soques de bacteri van provocar un 100% de mortalitat en larves a excepció de *X. bovienii* associada a *S. affine* (93.3%) i *X. bovienii* associada a *S. feltiae* (46.7%).

7. *S. carpocapsae* fou molt efectiva contra l'adult, provocant el 100% de mortalitat i esdevenint l'espècie de nematode entomopatogen idònia pel control de l'adult de *C. nucum*.

8. Les soques de bacteri causaren el 100% de mortalitat en adults, posant de manifest que la baixa mortalitat d'adults obtinguda amb altres espècies de nematodes entomopatògens és degut a l'habilitat del nematode i no a la virulència del bacteri.

9. La principal via de penetració de *S. carpocapsae* i *S. feltiae* a l'hemolimfa de la larva i adult de *C. nucum* és l'anus.

10. La resposta immunològica de *C. nucum* contra *S. carpocapsae* i *S. feltiae* no fou observada en la larva i només s'observà alguna forma infectiva de *S. carpocapsae* envoltada d'hemòcits. L'observació d'aquests hemòcits dins de l'adult mort implica que els nematodes activen el sistema immunològic de l'hoste, però que la resposta arriba massa tard per frenar l'acció letal del bacteri simbiont.

11. Es va aïllar fongs entomopatògens de cinc gèneres diferents: *Beauveria*, *Metarhizium*, *Aspergillus*, *Fusarium* i *Paecilomyces*. Els més abundants van ser *B. bassiana* (57 aïllats) i *M. anisopliae* (48 aïllats).

12. Els fongs entomopatògens van demostrar la seva virulència envers la larva de *C. nucum*. La mortalitat de larves causada per la combinació de nematodes i fongs entomopatògens no va reduir *C. nucum* més que cada un dels agents per separat.

13. Els nematodes entomopatògens van persistir durant nou setmanes i la majoria van es localitzaren en els primers 10 cm de sòl, tot i que van ser trobats fins a 40 cm. La distribució vertical dels nematodes assegura l'encontre d'aquests amb les fases hibernant de *C. nucum*.

14. Els nematodes entomopatògens, *S. feltiae*, *Steinernema* sp. i *H. bacteriophora* poden reduir la població de *C. nucum* de manera efectiva. Seran especialment eficients tant contra la larva quan aquesta s'està enterrant com contra la larva que hiberna enterrada al sòl.

15. Un control efectiu de *C. nucum* amb nematodes entomopatògens requereix una combinació d'estratègies. Una aplicació d'estiu utilitzant les espècies *S. feltiae*, *Steinernema* sp. o *H. bacteriophora* seria la millor per tal de controlar les larves quan aquestes s'enterren al sòl. Una aplicació de primavera amb les mateixes espècies podria reduir les larves hivernants, mentre que una aplicació addicional de primavera utilitzant *S. carpocapsae* reduiria de manera efectiva els adults hivernants i els que emergeixin del sòl.