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ORIGINAL CONTRIBUTION

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

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Abstract

The virulence of different 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 *Steinernema feltiae*, one *Steinernema 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 79% 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, *X. kozodoii* showed a LT50 of 22.7 h, and in the adults, it was 20.5 h. All nematodes species except *S. affine* tested against larvae showed great potential to control the insect, whereas *S. carpocapsae* was the most effective for controlling adults.

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 1 to 3 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 larvae of this insect in its cryptic habitat with chemical insecticides and the additional important environmental issues associated with their use for the control of the adults, the development of biological control alternatives is needed. Thus, entomopathogenic nematodes (EPNs) become potential biological control alternatives.

Entomopathogenic nematodes in the Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) families are lethal parasites of a large number of insect species (Dillon et al. 2006) and have been used efficiently against many soil-inhabiting and burrowing insects (Dillon et al. 2006; Georgis et al. 2006; Grewal and Peters 2005). Entomopathogenic nematodes have a symbiotic association with bacteria in the family Enterobacteriaceae. *Steinernema* spp. carry in a specialized intestinal lumen bacteria of the genus *Xenorhabdus*, and *Heterorhabditis* spp. carry throughout the intestine bacteria of the genus *Photorhabdus* (Fisher-Le Saux et al. 1999; Boemare 2002). The free-living stage, third-stage infective juveniles (IJs) actively search 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 and death of the insect usually within 24–48 h (Boemare and Akhurst 1988). The bacteria also release other virulence factors which cause immune system depression in the host and will prevent growth of non-symbiotic micro-organisms (Marokhazi et al. 2004). The developing nematodes feed upon the bacteria and liquefied host tissues, mate, and can produce one or more generations before food resources become scarce. Bacteria recolonize the nematodes (Martens and Goodrich-Blair 2005; Ciche et al. 2006), which emerge as IJs from the depleted insect cadaver in search of new hosts (Poinar 1990).

Nematodes have been applied against larvae of different curculionid 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), the pecan weevil, *C. caryae* (Horn) (Shapiro-Ilan 2001a,b), and *Rhytidoderes plicatus* (Oliv.) (Tarasco and Triggiani 2002). 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. 2007). However, adults' susceptibility to EPNs and the virulence of their symbiotic bacteria against larvae and adults of *C. nucum* have never been tested.

The objectives of this 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 symbiotic bacteria to the larvae and adults of this insect in order to select the most appropriate nematode–bacterium complex for the control of 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. All strains of *S. affine, S. feltiae*, *H. bacteriophora* and *Steinernema* sp. were isolated from soil samples from hazelnut orchards on Prades Mountain, Catalonia (NE Iberian Peninsula). *Steinernema carpocapsae* was isolated from a soil of an urban garden in Barcelona (E Catalonia). Nematodes were reared at 25°C on last instar of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae according to the method of Woodring and Kaya (1988). The IJs that emerged from cadavers were recovered using modified White traps (Kaya and Stock 1997). After storage at 7°C for a maximum of 2 weeks, they were allowed to acclimatize at room temperature before use and viability was checked by observation of movement under a stereomicroscope.

Infectivity test with nematodes

Larval assay

Last-instar larvae of *C. nucum* were field-collected from different hazelnut orchards located on Prades Mountain in the northeast portion of the Iberian Peninsula and stored in sterile soil at 10°C for 2 weeks to remove diseased individuals before being used. 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 diameter, 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 in a climate chamber in the dark at 23 ± 2°C 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 for 2 weeks and fed on apple for 1 week before being used in two different assays. The first, to assess the adults' susceptibility, was carried out in Petri dishes (5.5 cm diameter) 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 ± 2°C. 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 (five females and five 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 × 3 cm), were used as experimental units. A dose of 330 nematodes/adult was applied and a single adult weevil introduced into each Eppendorf tube. The tubes were placed in a climate chamber in the dark at 23 ± 2°C for 15, 30, 45, 60, 90, 120, 180 or 240 min. Afterwards, the adults were removed, rinsed with sterile tap water and individually placed in a Petri dish (5.5 cm diameter) with three dry paper discs for 24 h 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 7 days and verified by insect dissection.

Isolation and preparation of symbiotic bacteria for injection

All bacteria tested were isolated from *G. mellonella* larvae 30 h after EPN infection during septicaemia. 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 onto NBTA medium (Woodring and Kaya 1988; Boemare et al. 1997). The plates were sealed and incubated at 28 °C for 48 h. Colonies were cultured on Luria–Bertani broth (LB) with shaking (600 rpm) at 28°C for 20 h. After that, 100 μl from this culture was placed in 5 ml of fresh LB broth and incubated at 28°C to an exponential culture with an optical density (OD) of 0.8 at 600 nm (Jenway Colorimeter). This culture was washed with phosphate-buffered saline (PBS) and centrifuged three times to prevent bacterial aggregates. Bacteria were identified by molecular techniques by amplification of the 16S rDNA gene sequences as described by Tailliez et al. (2006).

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 before 20 μl of bacteria corresponding to 100 bacterial cells was injected into the dorsal part of the first segment of larvae using a Hamilton syringe. Bacterial concentrations were checked by plating serial dilutions onto GNO agar plates to check the number of injected bacteria. Insect mortality was assessed at regular point times after injection, to evaluate lethal time 50 (LT50) for each isolate. A total of 20 μl of sterile LB broth was injected into larvae as a control. After injection, the treated and control larvae were placed in plastic cages (2 × 2 cm) with a sterilized, moistened soil and incubated at 25 ± 2°C. Each treatment involved 15 larvae. Larval mortality was recorded every 2 h for a period of 65 h.

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. luminescens* (DG46) (table 1). The injection procedure and the concentration of the bacteria injected were the same as before except that the adults were injected in the left hind leg joint. Adults injected with bacteria were placed in plastic cages (2 × 2 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 2 h for a period of 65 h.

Statistical analysis

Analysis of 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

Table 1 Estimated exposure time of *Curculio nucum* larva to nine different strains of entomopathogenic nematode endosymbiotic bacteria to cause 50% larval mortality, LT_{50}, with 95% confidence interval (CI). Values of slope ± SE, and Pearson goodness of fit (χ^2, d.f., P) are also given (when $P > 0.05$, the model adequately fit the data). Different letters indicate statistical significance (Kaplan–Meier test, $P < 0.05$)

Bacterial strain	Slope ± SE	LT50 (CI 95%)	Pearson goodness of fit
Xenorhabdus kozodoii (D122)	15.374 ± 1.565	22.689 (21.964–23.406) a	9.427, 30, 1.000
Xenorhabdus bovienii (D114)	7.633 ± 0.694	25.933 (24.650–27.353) ab	34.802, 30, 0.250
Xenorhabdus nematophila (B14)	6.984 ± 0.582	25.954 (23.981–28.130) ab	57.170, 30, 0.002
X. bovienii (D245)	4.119 ± 0.327	27.424 (25.396–29.541) abc	25.878, 30, 0.681
X. bovienii (D113)	5.768 ± 0.393	34.505 (32.480–36.710) bc	27.048, 30, 0.621
Photorhabdus luminescens (DG46)	5.920 ± 0.407	35.375 (33.320–37.572) c	26.154, 30, 0.667
X. bovienii (D66)	6.405 ± 0.434	36.126 (34.087–38.285) c	25.423, 30, 0.704
X. bovienii (D108)	4.938 ± 0.357	38.374 (35.917–41.074) c	8.779, 30, 1.000
X. bovienii (D37)	2.336 ± 0.300	69.567 (58.744–89.435) d	5.760, 30, 1.000

strains were analysed with a generalized linear model (GLZ). LT_{50} slopes and 95% confidence intervals (CIs) for each bacteria isolate were analysed using PROBIT analysis, and the differences on the insect survival curves among bacterial strains were analysed using a Kaplan–Meier test. All these data were analysed using SPSS-PC v.19.0 (SPSS, 2011), and a level of significance of $P = 0.05$ was used for all tests.

Results

Infectivity test with nematodes

Larval assay

There were statistical differences in larval mortality after 14 days of exposure among the nematode strains tested (ANOVA: $F = 6.56, 8, 135, P < 0.05$) (fig. 1). All strains except *S. affine* caused more than 50% larval mortality with no significant differences between them. Mortality of larvae treated with *S. affine*, however, did not differ significantly from the mortality in the untreated control (10%).

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 (Student's t-test: $t = -0.993$; d.f. = 23; $P > 0.05$) or in terms of the time needed to infect the adults (Student's t-test: $t = -0.913$; d.f. = 46; $P > 0.05$). Consequently, data from males and females were combined in the subsequent data analyses. No mortality was recorded in the untreated control.

Differences in the virulence among nematode species were observed (ANOVA: $F = 45.46, 4, 19, P < 0.05$) (fig. 2). *Steinernema 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 test, $P > 0.05$).

In the time screening assay with *S. carpocapsae* (B14) on adults (fig. 3), 15 min was sufficient to infect 6.6–17% of the weevils, and adult mortality reached 100% in 120–240 min. No mortality was recorded in the untreated control. 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 61 h 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, $P < 0.05$). No mortality was recorded in the untreated control.

The time required to kill 50% (LT_{50}) of the *C. nucum* larvae ranged from 22.7 h with *X. kozodoii* belonging to *Steinernema* sp. (D122) to 69.6 h with *X. bovienii* belonging to *S. feltiae* (D37) (table 1).

Adult assay

All bacterial species injected caused 100% mortality of the hazelnut weevil after 69 h post-injection. No mortality was recorded in the untreated control. LT50 varied among strains ranging from 20.6 h due to *X. kozodoii* belonging to *Steinernema* sp. (D122) to 44.5 h caused by *X. bovienii* belonging to *S. feltiae* (D114) (table 2).

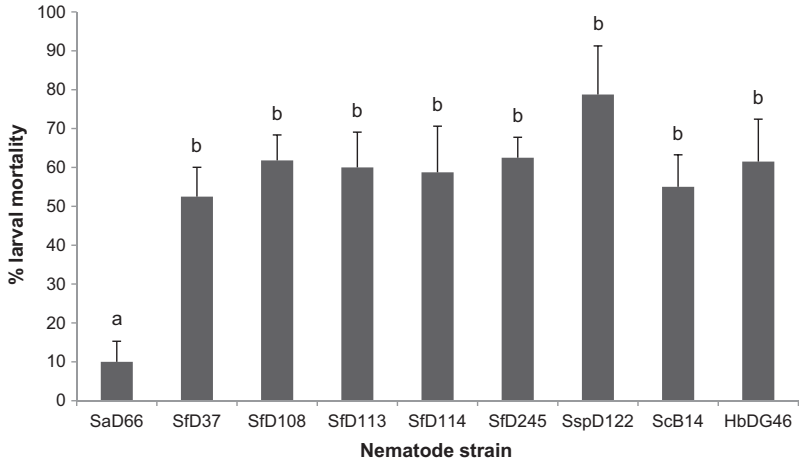

Fig. 1 Mortality of *Curculio nucum* larvae after exposure to nine entomopathogenic nematode strains observed 14 days after 50 IJs/cm^2 application. Different letters above bars (mean ± SEM) indicate statistically significant differences between means (Tukey's test, $P < 0.05$). Sa: *Steinernema affine*; Sf: *Steinernema feltiae*; Ssp: *Steinernema* sp; Sc: *Steinernema carpocapsae*; and Hb: *Heterorhabditis bacteriophora*.

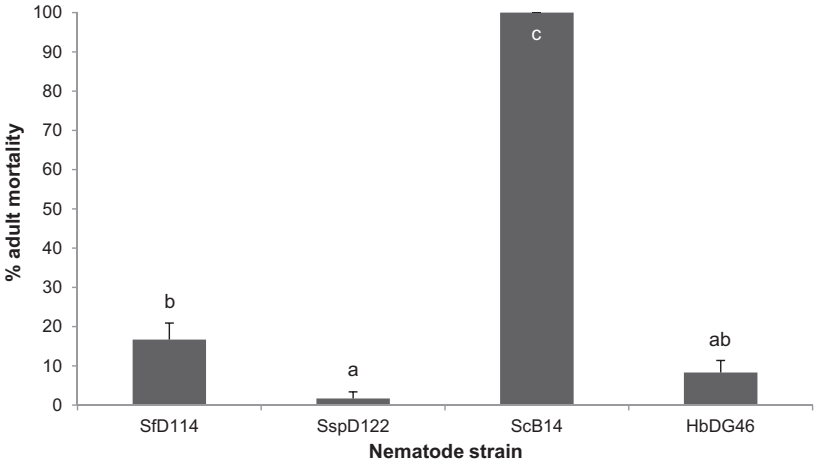

Fig. 2 Mortality of *Curculio nucum* adults after exposure to four entomopathogenic nematode strains observed 14 days after 50 IJs/cm^2 application. Different letters above bars (mean ± SEM) indicate statistically significant differences between means (Tukey's test, $P < 0.05$). Sf: *Steinernema feltiae*; Ssp: *Steinernema* sp; Sc: *Steinernema carpocapsae*; and Hb: *Heterorhabditis bacteriophora*.

Discussion

The results from the present study show that *C. nucum* larvae were susceptible to all 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 fit with those reported by Peters et al. (2007) 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 in *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) exposed to *S. affine*. The low efficacy of this nematode species has been related before to the host specificity because it has been found infecting mostly 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 that of larvae (except for *S. carpocapsae*). In other weevil species, adults were also less susceptible like for 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*

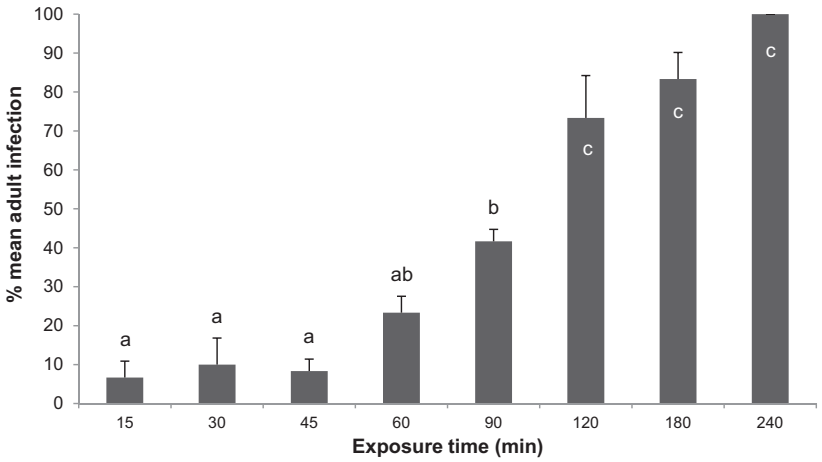

Fig. 3 Mortality of *Curculio nucum* adults exposed to 50 IJs/cm^2 of *Steinernema carpocapsae* for different time periods. Mortality was recorded 7 days after exposing the adults to the nematodes. Different letters above bars (mean ± SEM) indicate statistical significance (Tukey's test, $P < 0.05$).

Table 2 Estimated exposure time of *Curculio nucum* adult to four different strains of entomopathogenic nematode endosymbiotic bacteria to cause 50% adult mortality, LT_{50}, with 95% of confidence interval (CI). Values of slope ± SE, and Pearson goodness of fit (χ^2, d.f., P) are also given (when $P > 0.05$, the model adequately fit the data). Different letters indicate statistical significance (Kaplan–Meier test, $P < 0.05$)

Bacterial strain	Slope ± SE	LT50 (CI 95%)	Pearson goodness of fit
Xenorhabdus kozodoii (D122)	20.883 ± 2.901	20.576 (18.961–22.146) a	27.425, 10, 0.002
Photorhabdus luminescens (DG46)	13.383 ± 1.569	30.045 (28.650–31.538) b	10.551, 10, 0.394
Xenorhabdus nematophila (B14)	5.410 ± 0.686	41.049 (37.572–45.939) c	12.450, 10, 0.256
Xenorhabdus bovienii (D114)	3.327 ± 0.540	44.510 (38.711–55.228) c	12.495, 10, 0.253

(Herbst) (Shapiro-Ilan et al. 2002). The exception in hazelnut weevils, where adults are more susceptible to *S. carpocapsae* than larvae, has also been reported by other authors (Shapiro-Ilan 2001b; Shapiro-Ilan et al. 2003, 2005; Dillon et al. 2007) 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. Furthermore, *S. carpocapsae* has been reported to infect adults of other coleopteran species (Morris and Grewal 2011; Triggiani and Tarasco 2011; Morton and Garcia-del-Pino 2013) and in other insects such as cockroaches (Koehler et al. 1992; Maketon et al. 2010; Morton and Garcia-del-Pino 2013).

All the symbiotic bacterial strains proved to be highly pathogenic when injected into the haemocoel of larvae, reaching more than 93% mortality, and only *X. bovienii* belonging to *S. feltiae* (D37) showed lower pathogenicity (46.7%). Yeh and Alm (1992) also 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 nematode penetration into the haemocoel and/or release of the symbiotic bacteria are the limiting processes during infection of hazelnut weevils by *S. affine*. Therefore, this finding reasserts that *S. affine* is not a good candidate for controlling the larvae of *C. nucum*. The opposite situation was observed for *X. bovienii*, the symbiont of *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 bacterial dose injected (up to 100 cells) compared with the bacterial cell number (up to 250 cells) carried by one nematode (Snyder et al. 2007). 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 (except *S. carpocapsae*) suggests that nematodes have difficulties getting into the host and/or releasing symbiotic bacteria. Batalla-Carrera et al. (2014) observed differences in susceptibility of hazelnut weevil adults to *S. carpocapsae* strain B14 and *S. feltiae* strain D114 (92.5 and 2.5%, respectively), due to differences in the ability of the nematodes to penetrate the host. Same authors also reported any cellular immune response of *C. nucum* towards S. *feltiae* strain D114.

From our results, we rule out *S. affine* as a biocontrol agent for *C. nucum* larvae. *Steinernema feltiae* strains D37, D113, D114, D108 and D245, *Steinernema* sp. strain D122, *S. carpocapsae* strain B14 and *H. bacteriophora* strain DG46 have the 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, *S. carpocapsae* is the best nematode–bacterium complex to use against emerging adults due to its high virulence and rapid capacity for infection. The results of the present study show that some of the EPN strains tested could be used as biological control agents against underground stages of *C. nucum*, but field trials need to be conducted to assess its control ability under field conditions.

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