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

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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, ranging from 32% to 88% efficacy, 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.

Additional key words: 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 (Coutin, 1992; AliNiazee, 1998; Bel-Venner *et al*., 2009).

Spain, in the Iberian Peninsula, is the eighth world hazelnut producer, with 15,100 t during 2010 (FAO, 2010). More than 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 (EPNs) 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),

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Abbreviations used: EPNs (entomopathogenic nematodes); GLZ (generalized linear model); HW (hazelnut weevil); IJ (infective juvenile).

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 (Kung *et al*., 1990a,b; Grewal *et al*., 1994). 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 to: (i) 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) evaluate the nematodes' vertical distribution and persistence under field conditions in order to determine optimal application strategy.

Material and methods

Experiments were conducted in two 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°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

	Orchard 1: Hortals	**Orchard 2: Mallola**
Humidity (%)	1.4	1.1
pH	7.9	8.1
Conductivity (dS m^{-1})	0.23	0.24
Organic mater (%)	2.90	2.96
Sand ($0.0\,5 < D < 0.2$ mm) (%)	20.8	22.7
Sand ($0.2 \leq D < 2$ mm) (%)	9.5	18.7
Silt ($0.02 < D < 0.05$ mm) (%)	21.3	20.4
Silt ($0.002 < D \leq 0.02$ mm) (%)	23.2	19.3
Clay ($D < 0.002$ mm) (%)	25.2	18.9
Carbonate (%)	5	12

D: diameter.

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 2 m distance from the trunk and local soil was transferred into pots and left to settle for nine 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 $plot^{-1}$) 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 replica-

tions 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 (1 m^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 L 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 25 cm depth soil sample per plot with a drill. Each sample was divided in 5 subsamples corresponding to different depths (0-5 cm; 5-10 cm; 10-15 cm; 15-20 cm and 20-25 cm) 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. Each Petri dish with dead *G. mellonella* larvae was counted as a positive sample. 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 sampled year on the persistence of nematode species a chi-square test was developed. Based on these results, for each year data were 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, 2007).

Results

Nematode field efficacy

The efficacy of the three different nematode treatments in both summer and spring applications is presented in Fig. 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: $\chi^2 = 3.55, 2, p > 0.05$).

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) (Fig. 1).

Comparing the efficacy of summer and spring applications there were no differences in 2010 (GLZ:

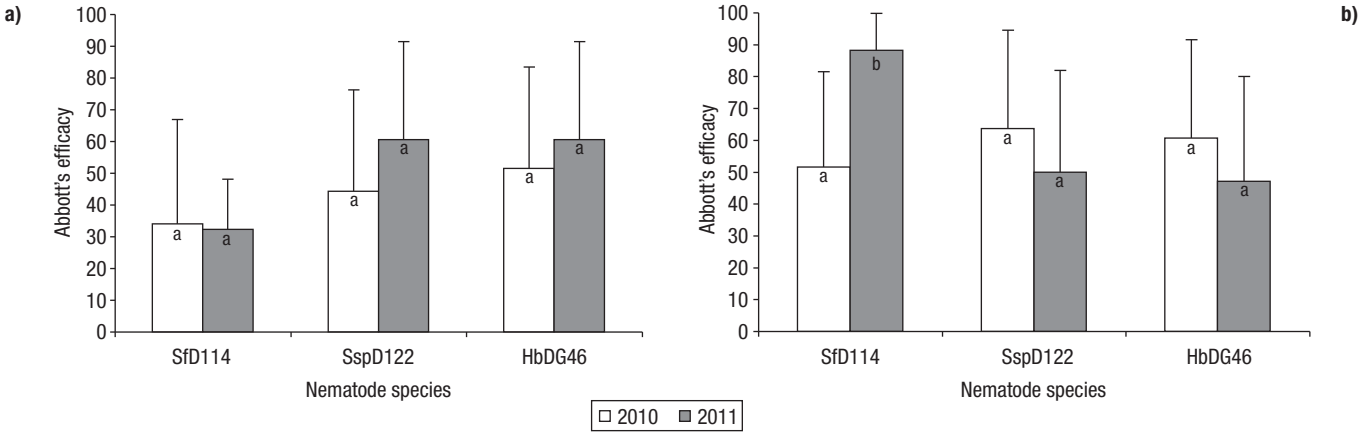

Figure 1. Abbott's efficacy in (a) summer application and (b) spring application using three different entomopathogenic nematodes, SfD114: *Steinernema feltiae* (D114), SspD122: *Steinernema* sp. (D122) and HbDG46: *Heterorhabditis bacteriophora* (DG46), against the hazelnut weevil, *Curculio nucum.*

$\chi^2 = 3.13$, 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 20 cm. 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 5 cm, 35.92% at 10 cm, 28.03% at 15 cm, 7.64% at 20 cm, 3.96% at 25 cm, 0.81% at 30 cm, 6.33% at 35 cm and 0.81% at 40 cm).

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 were pulled together to develop further statistic analysis. Persistence data over the time (Fig. 2) revealed a no strict linear relationship between the presence of nematodes and time after application.

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 9 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).

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 (Fig. 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 did not 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, $p < 0.05$).

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

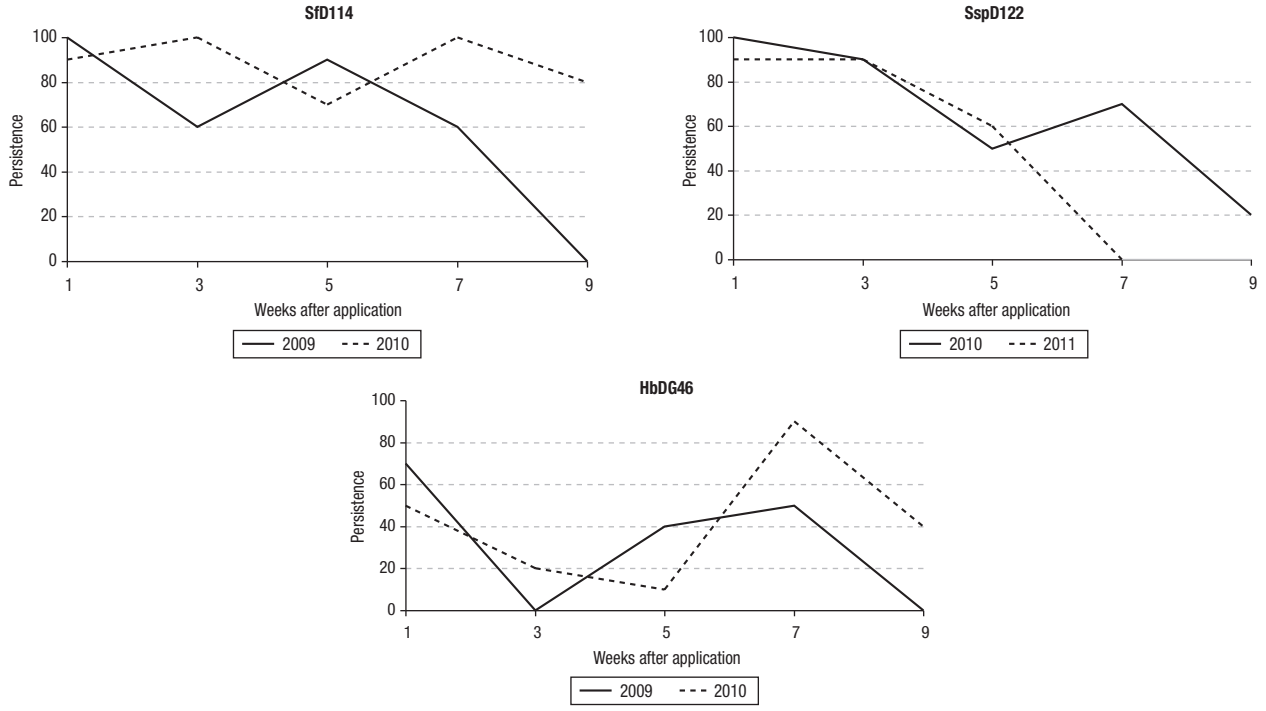

Figure 2. Percentage of soil samples containing entomopathogenic nematodes released on spring on the field over 9 weeks. The nematodes used were SfD114: *Steinernema feltiae* (D114), SspD122: *Steinernema* sp. (D122) and HbDG46: *Heterorhabditis bacteriophora* (DG46).

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, 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.

Table 2. Pearson coefficient for the effects of the sampled year on the vertical distribution of the entomopathogenic nematodes to different depths (0-5, 5-10, 10-15 and 15-20 cm)

Nematodes[1]	0-5 cm	5-10 cm	10-15 cm	15-20 cm
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, $p > 0.05$

[1] SfD114: *Steinernema feltiae* (D114). SspD122: *Steinernema* sp. (D122). HbDG46: *Heterorhabditis bacteriophora* (DG46).

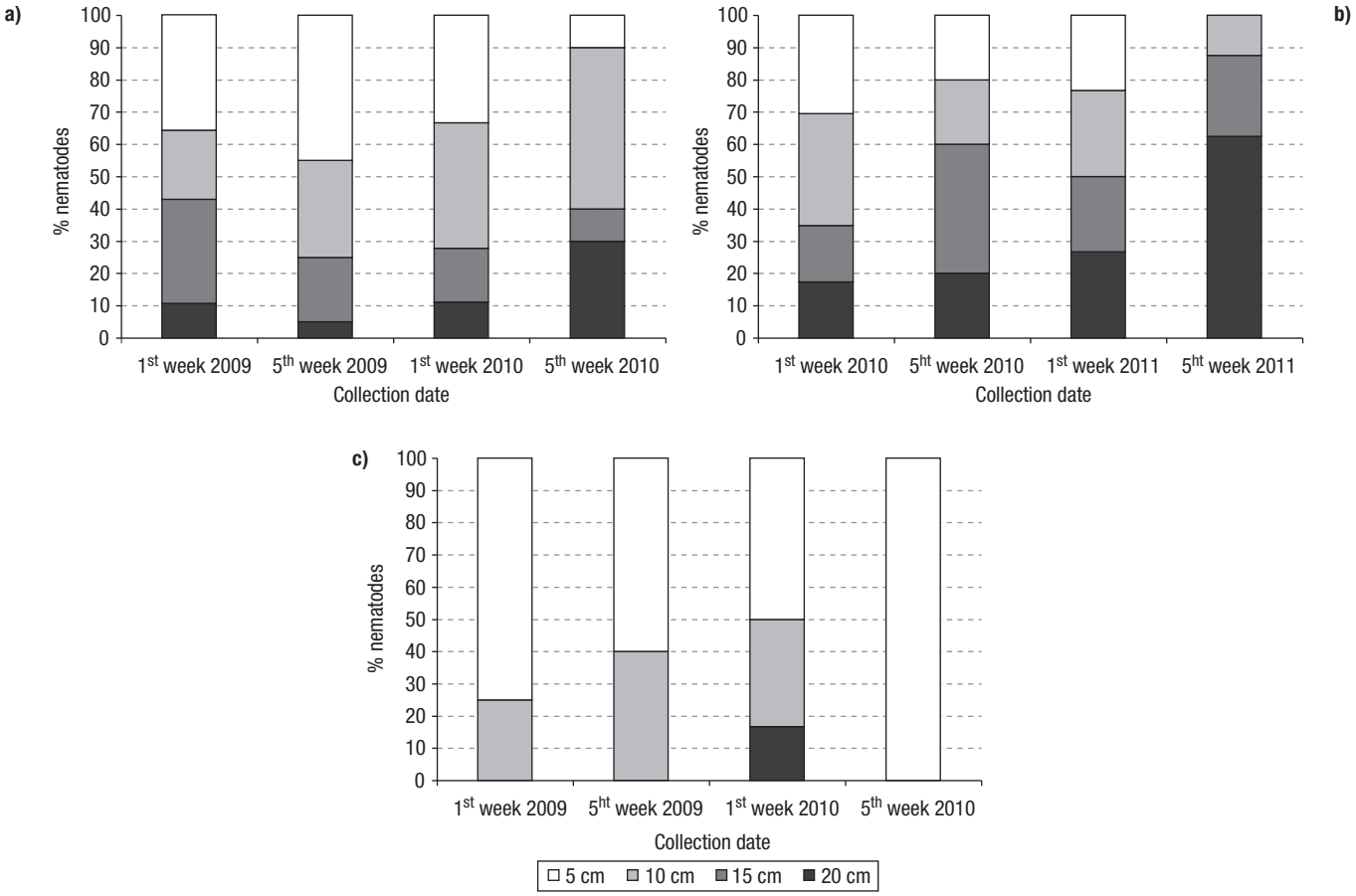

Figure 3. Percentage of nematodes detected in four sections of 20 cm soil samples of different species (a) SfD114: *Steinernema feltiae* (D114), (b) SspD122: *Steinernema* sp. (D122) and (c) HbDG46: *Heterorhabditis bacteriophora* (DG46) during the first and the fifth week of each sampled year.

Different studies have shown the influence of abiotic factors on the nematodes persistence in the soil (Kung *et al*., 1990a,b; Grewal *et al*., 1994; Glazer, 2001). 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°C in April, 18°C in May, 21°C in June and 24°C in July and moisture of 23.73 cbar in April, 15.5 cbar in May and 32.65 cbar 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 9 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 (Wilson *et al*., 2012). 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.

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