



Irrigation modulates entomopathogenic nematode community and its soil food web in olive groves under different agricultural managements

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ABSTRACT

Entomopathogenic nematodes (EPNs) are biological control agents distributed worldwide in natural and agricultural soils. Recent evidence supports the hypothesis that stable rhizospheres of perennial crops can favor the occurrence of EPNs. However, different agricultural management practices can modulate their natural distribution in agroecosystems, hence should be considered in conservation biological control programs to support as high as possible native EPN populations. Cultivated olive (*Olea europaea* subsp. *europaea*), originally from the Mediterranean Basin, is a key perennial crop susceptible to a diverse range of arthropod pests that account for their life with at least one soil inhabitant stage. In this study, we investigated, for the first time, the occurrence of EPNs and associated soil organisms in olive groves managed with different practices. We surveyed 89 commercial olive groves located in Andalusia (Southern Spain) and differently handled for pest management (conventional vs. organic), soil management (tilling vs. cover cropping), and irrigation regime (rain feed vs. drip-irrigation). By using species-specific primers-probe sets and quantitative real-time PCR approaches, we screened the occurrence of nine species of EPNs, five free-living nematodes (FLNs), and six nematophagous fungi (NF). We found EPNs in 23.6% of the sites, identifying only two EPN species: *Steinernema feltiae* (15.7%) and *Heterorhabditis bacteriophora* (7.9%). Pest and soil management did not affect EPN abundance, but drip irrigation increased their occurrence ($P < 0.025$). On the contrary, we recorded lower FLN numbers in irrigated groves ($P = 0.003$), particularly for *Oscheius tipulae* and *Pristionchus maupasi*. Regarding NF, both organic management ($P < 0.001$) and drip irrigation ($P < 0.035$) enhanced their abundance. Four abiotic explanatory variables (extractable P, clay content, elevation, and precipitation) explained 85.6% of the EPN, FLN, and NF species variation in multivariate analysis. Overall, drip irrigation in the olive groves enhanced the EPN community but also NF growth, natural enemies of nematodes. Additional studies are required to determine the best combination of alternative strategies that will enhance a balanced soil biota in olive groves in Mediterranean regions and other areas where this crops is cultivated that enable conservation ecosystem services, such as those provided by the EPNs.

1. Introduction

Soil is a non-renewable asset of high natural and socio-economic value, composed of a mixture of mineral material and organic matter, pores with a variable amount of air and water, and a myriad of organisms (Wall, 2012). The organisms that comprise soil ecosystems, possibly the most complex on Earth, establish dynamic interactions of different nature among themselves, from detrimental (competitive,

predatory, parasitic) to beneficial (phoresy, commensalism, symbiosis), that depend on the species, time and space (Orgiazzi et al., 2016). Human activities such as agriculture often alter the balance of these interactions, compromising various valuable ecosystem services mediated by soil biota (Bardgett and Van Der Putten, 2014; Delgado-Baquerizo et al., 2016). In the current sustainable development paradigm, preserving soil health and quality (e.g., structure, biodiversity, and functions) is a milestone for preventing losses in agricultural

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yield and promoting crop quality. Hence, understanding how traditional agricultural practices (tillage, pest management, irrigation) affect soil biodiversity and their functions will contribute to achieving sustainable farming by improving resilient and beneficial biota in soil crops.

Entomopathogenic nematodes (EPNs) are members of the soil biota and provide the biocontrol of arthropod pests, a critical ecosystem service in agriculture. Their infective juvenile (IJ) stages naturally occur in soils where arthropods can cohabit and serve as hosts, are being distributed from the sea level areas to high up in the mountains, from natural to agricultural ecosystems, even highly polluted soils (Adams et al., 2006; Stuart et al., 2015; Campos-Herrera et al., 2016a; Ker-gunteuil et al., 2016). The IJs actively search for hosts in the soil profile, and once located, they penetrate by natural openings or injuries, reach the hemocoel, and release the bacterial symbiont. The concomitant activity of nematode and bacteria causes the death of the insect within 24–48 h (Griffin, 2015; Lu et al., 2017). EPNs complete various generations if food is available to finally emerge from the cadaver carcass in the form of IJs to start their life cycle over again (Dillman et al., 2012; Stock, 2015).

The study of EPNs in the context of Agroecology and Applied Soil Ecology is increasing in the last decades (Campos, Herrera et al., 2012a; Helmberger et al., 2017). Several studies illustrate how changes in soil properties affect the EPN community and associated organisms such as nematophagous fungi (NF) and free-living nematodes (FLNs), their natural enemies and potential competitors (Campos, Herrera et al., 2013, 2016b, 2019a, 2019b; Pathak et al., 2017; Dristoulas et al., 2021). For example, recent evidence suggests that agroecosystems with a stable rhizosphere, like the perennial crops of citrus groves and grapevine, might be more conducive to EPN occurrence and activity (Blanco-Pérez et al., 2022a; Campos-Herrera et al., 2019a; Blanco-Pérez et al., 2022b). The management practices of these farming systems also modulate the abiotic sphere (soil texture, pH, moisture level, nutrients availability) and, in turn, the EPN and associated organisms assemblage (Blanco-Pérez et al., 2020, 2022a; Campos-Herrera et al., 2014; Blanco-Pérez et al., 2022b). Thus, unraveling how agricultural management alters the EPN community in biotic and abiotic interphases is crucial for advancing the use of these organisms in conservation biological control programs in target crop systems.

The olive *Olea europaea* subsp. *europaea* is a widespread crop in the Mediterranean and Middle Eastern areas, with high socio-economic relevance. Spain's current olive grove surface is over 2.6 million ha, representing around 50% in the EU and 21% globally, generating 58% of the olive production in the EU and 34% globally (FAO, 2020). Andalusia (Southern Spain) accumulates most olive groves in the country, often cultivated under rain-fed production and monoculture systems. As in other crops, olive cultivation often is managed using conventional tillage and application of pesticides following the integrated pest management (IPM) frame. The existing movement towards sustainable agriculture also promotes adopting alternative agricultural practices, such as cover cropping and organic farming, following the European Union on the Regulation (EU) (2018)/848, to manage pests, diseases, and weeds. Overall, the area of olive groves dedicated to organic production has doubled in the last decade, particularly in Spain, the third organic olive producer country in the World with 222,723.00 ha (FiBL, 2020).

Recent studies have shown that alternative strategies of this kind can contribute positively to the soil biodiversity in olive groves, although with some exceptions. For example, García et al. (2009) showed that organic farming increased the soil enzyme activity in a restricted set of olive groves, but the presence of nematode bioindicators of soil health was only higher in one of the three evaluated groves. Similarly, Montes-Borrego et al. (2013) revealed relevant differences in soil properties and microbial diversity between organic and conventional systems. However, Glisakis et al. (2016) suggested that other factors such as temperature, humidity, and tilling drove soil arthropod biodiversity rather than pest management practices. Tillage for controlling weed

growth, often used in olive groves, can also reduce soil biodiversity (Lisek and Sas, 2015; Rodríguez Pleguezuelo et al., 2018). For example, Palomares-Rius et al. (2015) showed that no-till organic olive groves favored plant-parasitic nematodes (PPN) diversity and abundance in Andalusia, while soil properties and the olive cultivar drove the structure and diversity of this specific nematode community. However, PPNs represent a small fraction of the whole nematode soil diversity. Additional studies are required to understand the crucial drivers of soil biodiversity in the olive sphere, necessary for enhancing ecosystem services that sustain olive production with low external inputs, such as conservation biological control.

Olive fruit and trees can be affected by several pests, some of them limiting the quantity and quality of oil production in many areas of the world (Therios, 2009; Alvarado et al., 2017). However, few studies have evaluated the impact of olive grove management on ecosystem services such as biocontrol programs. Cárdenas et al. (2015) observed that the organic olive groves registered a higher abundance and diversity of spiders as natural enemies. Similarly, Picchi et al. (2017) showed that the overwintering pupae of the fruit fly *Bactrocera oleae* (Diptera: Tephritidae) were more consumed by natural predators in organic than in conventional olive groves. Regarding EPNs, there is still no information about their natural occurrence in organic or integrated pest-managed olive groves or how biotic and abiotic factors can modulate their natural enemies' populations in this agroecosystem. However, they have potential against various insects as target. The most important pest is the olive fruit fly *B. oleae* that affects the olive fruit by causing it to fall prematurely or by reducing its weight and quality (Therios, 2009; Alvarado et al., 2017). This pest passes the winter period in the soil and can be potentially attacked by EPN in soil. Indeed, various studies showed that EPN can be efficient biocontrols of this pests under controlled conditions as well field trials (Sirjani et al., 2009; Torrini et al., 2017, 2020). In addition, some secondary olive pests that reside in the soil such as weevils (*Otiorrhynchus cribricollis*, Coleoptera: Curculionidae) or white grubs (*Melolontha* spp., Coleoptera: Scarabaeoidea) are often group of insects susceptible to the EPN attack (Lacey et al., 2015). Additionally, there are a plethora of insects that inhabit the olive groves, often associated with weeds, that can serve as host for EPN for certain part of the year. In this study, we investigated the impact of olive grove management on the occurrence of native EPNs and associated soil organisms by taking advantage of the DNA samples extracted from soil nematode populations in a study conducted by Palomares-Rius et al. (2015) on PPNs. Based on results from recent similar studies on other perennial crop systems (grapevine), our hypotheses are: (i) organic farming might sustain a higher EPN occurrence (Blanco-Pérez et al., 2022a), and (ii) the presence of cover crops might modulate the EPN soil food web if homogeneous conditions are available (Blanco-Pérez et al., 2020). This study aimed to investigate, for the first time, the impact of agricultural management practices on the EPN natural distribution and the assemblage of associated soil organisms in olive groves to disentangle the biotic and abiotic factors that can drive their presence and activity. The specific objectives were to (1) evaluate the presence and abundance of EPNs in olive groves subjected to diverse agricultural management practices, (2) determine the presence and abundance of associated soil organisms (FLNs and NF), and (3) discriminate the abiotic explanatory variables that may describe their community assemblage.

2. Material and methods

2.1. Survey design, sampling methods, and characterization of abiotic properties

Eighty-nine commercial olive groves were surveyed in Andalusia (Southern Spain) from May to July 2009 (Fig. 1). Montes-Borrego et al. (2014) and Palomares-Rius et al. (2015) provided the details of specific geographical locations and general descriptions of the sampled groves

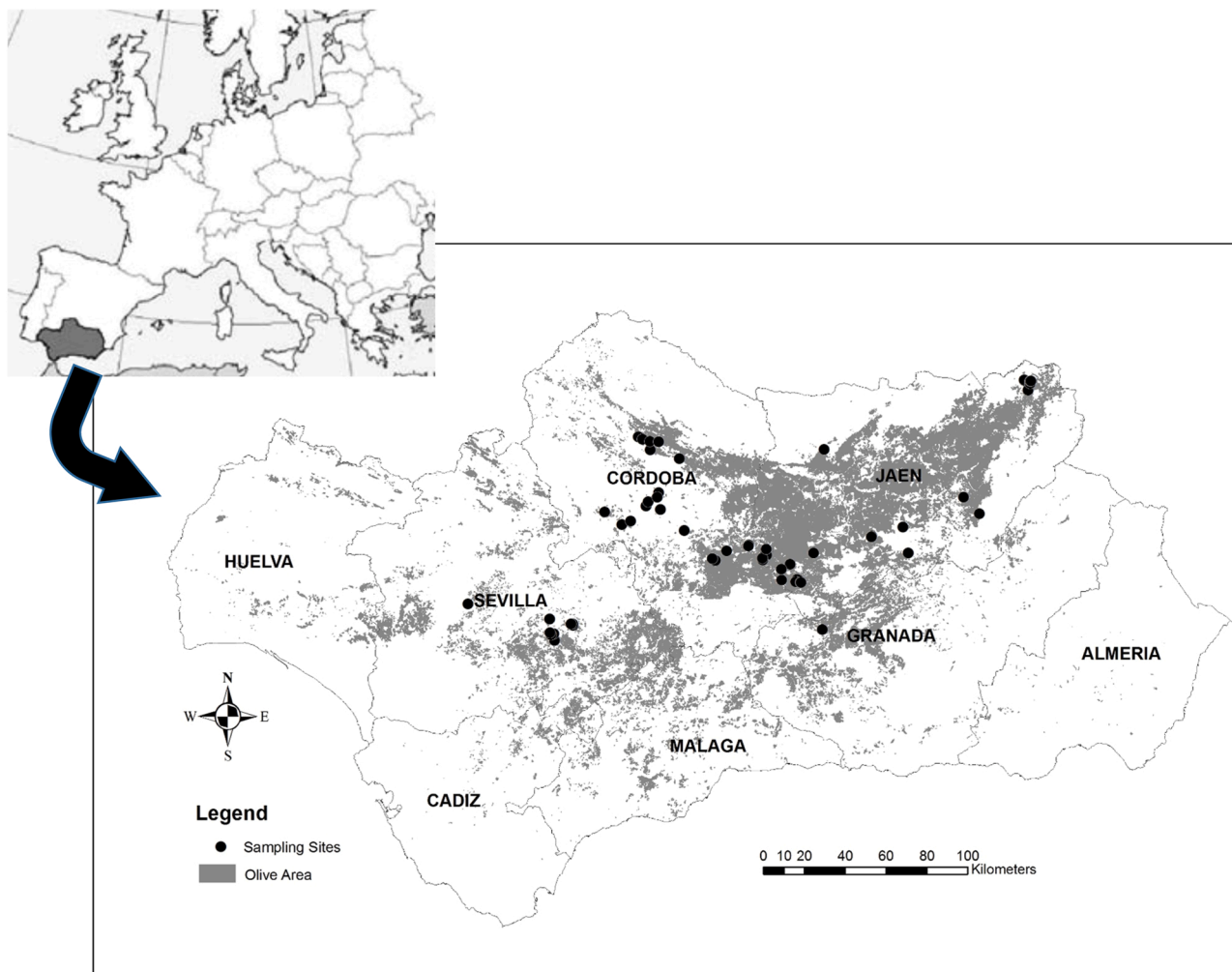


Fig. 1. Distribution of the sampling sites in the olive production areas in Andalusia (Southern Spain).

(soil properties, agronomic and climatic characteristics). In this study, we evaluated the impact of agronomic management on the EPN soil food web, considering the variables (i) type of pest management (organic vs. conventional), (ii) soil management (tilling vs. cover cropping), and their interaction. The organic farms sampled were all accredited through the Andalusian Committee of Organic Farming (CAAE, Junta de Andalucía). The orchards sampled were selected for being under organic production at least for 5 years at sampling time. The groves under conventional farming follow the corresponding national and regional rules. In any case, the use of active ingredients was strongly regulated and those were all approved to be used in olive crops by the Spanish Ministry of Agriculture (<https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro-productos/>). In addition, we explored the effect of the presence/absence of an irrigation system. For the sampling, briefly, a composite sample of the rhizosphere of eight olive trees randomly selected was obtained at each site by sampling to a depth of 40 cm, ensuring the close vicinity of active olive roots (Palomares-Rius et al., 2015). About first top 5 cm of soil in the sampling was discarded (Palomares-Rius et al., 2015). Samples were taken before irrigation to prevent differences in soil water content (Montes-Borrego et al., 2013). Sites with herbaceous plants in the surrounding area were avoided (Palomares-Rius et al., 2015). Samples were transported in coolers to the laboratory and stored at 5 °C in the dark until processed.

In the laboratory, each sample was thoroughly mixed. The nematode community and associated soil organisms were extracted from soil subsamples of 500 cm³ through the magnesium sulfate centrifugal-

flotation method (Coolen, 1979; Palomares-Rius et al., 2015). In the suspension, nematodes and other minor amount of soil organisms (endomycorrhizal survival spores, mites, springtails and collembolan individuals), big and easy to pick microorganisms, were removed from the suspension using forceps. Hence, mainly nematodes and intimately associated organisms in the solution were concentrated in an Eppendorf tube and stored at – 20 °C until DNA processing. Another soil subsample set was air-dried, sieved (2–5 mm mesh size), and processed to analyze the soil properties: sand, silt, and clay content, pH, cation exchange capacity (CEC), soil organic matter (SOM), organic carbon (C) and nitrogen content (N), extractable phosphorous (P) and exchangeable potassium (K) (Official Agroalimentary Laboratory of Córdoba, Spain) (Montes-Borrego et al., 2013). The environmental variables (elevation, average rainfall, and average temperature) were retrieved as described by Aranda et al. (2011) and Montes-Borrego et al. (2013).

2.2. Identification and quantification of target organisms by real time qPCR

We employed the DNA extraction kit PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc, Carlsbad, CA, USA). DNA samples were analyzed for quality and quantity using a Nanodrop system (Thermo Scientific 2000C spectrophotometer) and stored at – 20 °C until qPCR analysis (see Blanco-Pérez et al., 2022a for a more detailed protocol). Briefly, by using species-specific primers/probe sets, we screened for the occurrence and abundance of 20 soil species (Table 1), comprising 9 EPNs, 5 FLNs, and 6 NF (Atkins et al., 2005; Zhang et al., 2006; Torr

Table 1
Species and sources of the soil organisms screened in this study.

Type of organism/ species	Population	Material used/Unit of measurements	GenBank accession number ITS region	Reference for primers and probe sequence / employed for cross-check
Nematodes: Entomopathogenic nematodes				
<i>Heterorhabditis bacteriophora</i>	Commercial	Infective juvenile (IJs) / no. IJs	KJ938576	Campos-Herrera et al. (2011a)
<i>Heterorhabditis indica</i>	Btw	IJs / no. IJs	KJ938571	Campos-Herrera et al. (2011b)
<i>Steinernema affine</i>	CH	IJs / no. IJs	KJ938567	Torr et al. (2007)
<i>Steinernema arenarium</i>	SA	IJs / no. IJs	KU194615	Campos-Herrera et al. (2019a)
<i>Steinernema carpocapsae</i>	DOK-83	IJs / no. IJs	KJ818295	Campos-Herrera et al. (2011a)
<i>Steinernema feltiae</i>	RS-5	IJs / no. IJs	KJ938569	Campos-Herrera et al. (2011a)
<i>Steinernema glaseri</i>	NC	IJs / no. IJs	GU173998	Campos-Herrera et al. (2011b)
<i>Steinernema intermedium</i>	82 from USA	ITS rDNA sequence + pUC57 / pg DNA	AF171290	Campos-Herrera et al. (2015a)
<i>Steinernema intermedium</i> -group	VAD-1067	IJs / no. IJs	KJ696684	Campos-Herrera et al. (2015a)
Nematodes: Free-living nematodes				
<i>Acrobeloides</i> -group	RT1-R15C	18 S rDNA sequence + pUC57 / pg DNA	JQ237849	Campos-Herrera et al. (2012b)
<i>Oscheius tipulae</i>	MG68 P29	Nematodes/ ng DNA	KJ938579	Campos-Herrera et al. (2015b)
<i>Oscheius onirici</i>	MG67 P20	Nematodes/ ng DNA	KJ938578	Campos-Herrera et al. (2015b)
<i>Pristionchus maupasi</i>	AM-3	Nematodes/ ng DNA	MG551681 ^a	Campos-Herrera et al. (2019a)
<i>Pristionchus pacificus</i>	P2333	Nematodes/ ng DNA	MG551685 ^a	Campos-Herrera et al. (2019a)
Nematophagous fungi				
<i>Catenaria</i> sp.	1D	ITS rDNA sequence + pUC57 / pg DNA	JN585805	Pathak et al. (2012)
<i>Arthrobotrys dactyloides</i>	H55	Pure culture / pg of DNA	KJ938574	Pathak et al. (2012)
<i>Arthrobotrys musiformis</i>	11	Pure culture / pg of DNA	KJ938572	Pathak et al. (2012)
<i>Arthrobotrys oligospora</i>	8	Pure culture / pg of DNA	KJ938573	Pathak et al. (2012)
<i>Hirsutiella rhossiliensis</i>	2931	Pure culture / pg of DNA	–	Zhang et al. (2006)
<i>Purpureocillium lilacinum</i>	9357	Pure culture / pg of DNA	KJ938575	Atkins et al. (2005)

et al., 2007; Campos-Herrera et al., 2011a, 2011b, 2012b, 2015a, 2015b, 2019a; Pathak et al., 2012). Except for the EPNs *Heterorhabditis megidis*, *Steinernema intermedium*, and *S. intermedium*-group, all were recently reported in the Iberian Peninsula in surveys that employed a matching methodology with the current study (Campos-Herrera et al., 2019a; Campos-Herrera et al., 2016a; Blanco-Pérez et al., 2020, 2022a). We screened for fungal species belonging to the three NF major classes (Nordbring-Hertz et al., 2006): nematode-trapping fungi (*Arthrobotrys dactyloides*, *A. musiformis*, and *A. oligospora*), endoparasites (*Hirsutiella rhossiliensis* and *Catenaria* sp.), and eggs-parasitic fungi (*Purpureocillium lilacinum*). All the organisms were maintained in laboratory cultures to provide the corresponding positive controls as described by Campos-Herrera et al. (2019a), except when alive material was not available and a plasmid containing the complete published ITS region was used (Table 1).

We used species-specific primers and TaqMan® probe (5' fluorogenic reporter dye FAM; 3' end quencher BQH-1) sets, synthesized by Biosearch Technologies (supplied by Biotools, Spain), for the qPCR analysis (see Campos-Herrera et al., 2019a for further details). The standard curves included serial dilutions (5 points) of pure DNA culture of a known quantity. The unknown DNA samples were 10-fold diluted before qPCR analysis, performed on the iCycler iQ (Bio-Rad) real-time qPCR equipment, with the Quantimix Easy Probe mix (Biotools, Spain) as a buffer, the iCycler iQ® PCR as plates, and iCycler iQ® as optical tape (Bio-Rad). The species were identified and quantified in independent runs, with the positive (standard curve) and negative (no sample) controls in all plates, each sample per duplicate, following MIQE procedure and quality standard (Bustin et al., 2009). Details of the primers/probe concentrations, final volumes, temperatures, and cycles are shown in Campos-Herrera et al. (2019a).

2.3. Statistical analyses

We ran generalized linear models (GLMz), performed with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), testing the effect of the following factors on the abundance and frequency of occurrence of EPNs and associated soil organisms (FLNs and NF). First, we evaluated the

agronomic management impact on: (1) Pest Management (PM): organic (n = 42) vs. conventional (n = 47); (2) Soil Management (SM): use of cover cropping (n = 43) vs. tilling (n = 46); and their interaction (PM * SM). In addition, we assessed for the effect of the presence (n = 43) or absence (n = 46) of an irrigation system in the evaluated olive groves. Quantifications of soil organisms were expressed (per 500 cm³ of fresh soil) as the abundance of IJs (for EPNs) and standardized 0–1 range of the values recorded for the FLN and NF species. NF abundances were transformed as infection rate (IR), estimated by dividing the corresponding DNA quantity of each NF species by the total amount of DNA per sample (Campos-Herrera et al., 2012b). Tests for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test) were performed, and variables were transformed when homoscedasticity was violated. Overall, we ran GLMz tests with a gamma distribution (log-link function) for quantitative variables, previously transformed by log₁₀ (x + 1), and a binomial distribution (logit-link function) for the frequencies of occurrence (see Blanco-Pérez et al., 2020 for more details). We also looked for statistical differences in general linear model tests between treatments for the abiotic variables. We used the Least-Squares Means ± standard error of the mean (SEM) as descriptive statistics and employed ArcGIS 10 for visualizing the sample locations and species distributions.

To account for the general assemblage of the screened species and abiotic factors, we ran a multivariate analysis using the software CANOCO 5 (ter Braak and Šmilauer, 2002; Šmilauer and Lepš, 2014). First, we established the abiotic variables selected through Pearson Correlation as exploratory predictors to avoid strong co-linearity. Then, both biotic and abiotic variables were standardized by dividing by the highest values, ranking all between 0 and 1 (Šmilauer and Lepš, 2014). We obtained values over 3.0 maximum length (SD units) for detrended canonical correspondence analysis (DCCA), indicating heterogeneous communities, so canonical correspondence analysis (CCA, constrained axes) was recommended (ter Braak and Šmilauer, 2002). We ran a CCA (interspecies correlations) with Monte Carlo permutation (n = 499) and automatic forward selection, presenting the bi-plot scaling graphs as described by Campos-Herrera et al. (2019a).

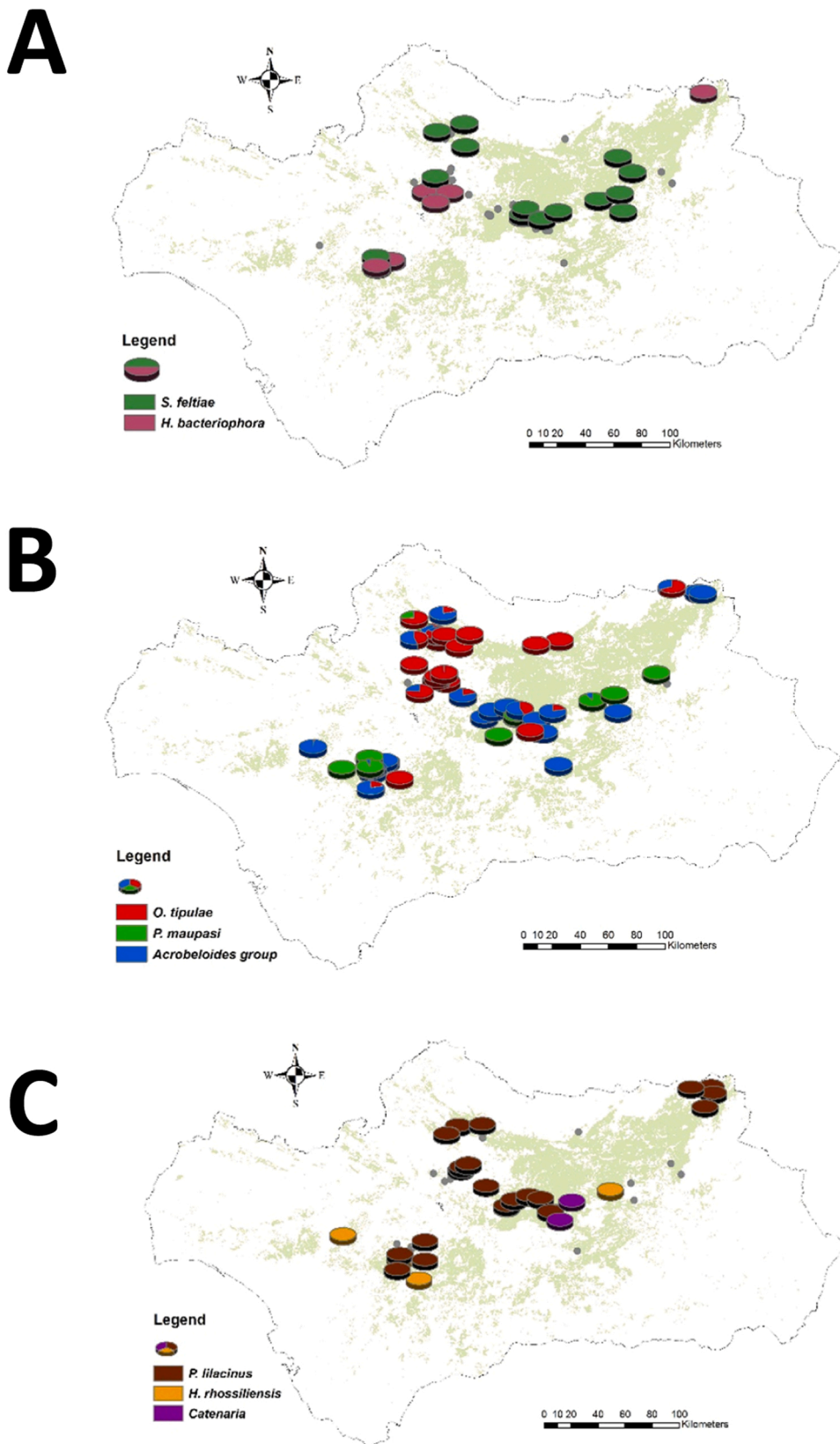


Fig. 2. Geographical distribution of the three trophic groups evaluated. **A.** Entomopathogenic nematodes (EPNs): *Steinernema feltiae* and *Heterorhabditis bacteriophora*. **B.** Free-living nematodes (FLNs): *Oscheius tipulae*, *Pristionchus maupasi*, and *Acrobelloides*-group. **C.** Nematophagous fungi (NF): *Purpureocillium lilacinum*, *Hirsutella rhossiliensis*, and *Catenaria* sp. In each map, the circles correspond to the relative presence of each of the species described. Sampling groves with no detection are presented as small grey circles.

3. Results

3.1. Natural occurrence of entomopathogenic nematodes and associated soil organisms

Overall, 23.6% of the olive groves registered a presence of EPNs, widespread in the study area (Fig. 2A). Only *Steinernema feltiae*, the

predominant species in this survey (15.7% sites), and *Heterorhabditis bacteriophora* (7.96% sites) were detected out of the nine EPN species screened. Co-occurrence of both species in the same olive grove was not detected. FLNs were found in 52% of the olive groves, *Oscheius tipulae* in 29% sites, *Pristionchus maupasi* in 12% sites, and *Acrobeloides*-group in 33% sites (Fig. 2B). The FLNs *O. tipulae* and *Acrobeloides* spp. co-existed in 12 localities, while the three were sympatric in three olive groves.

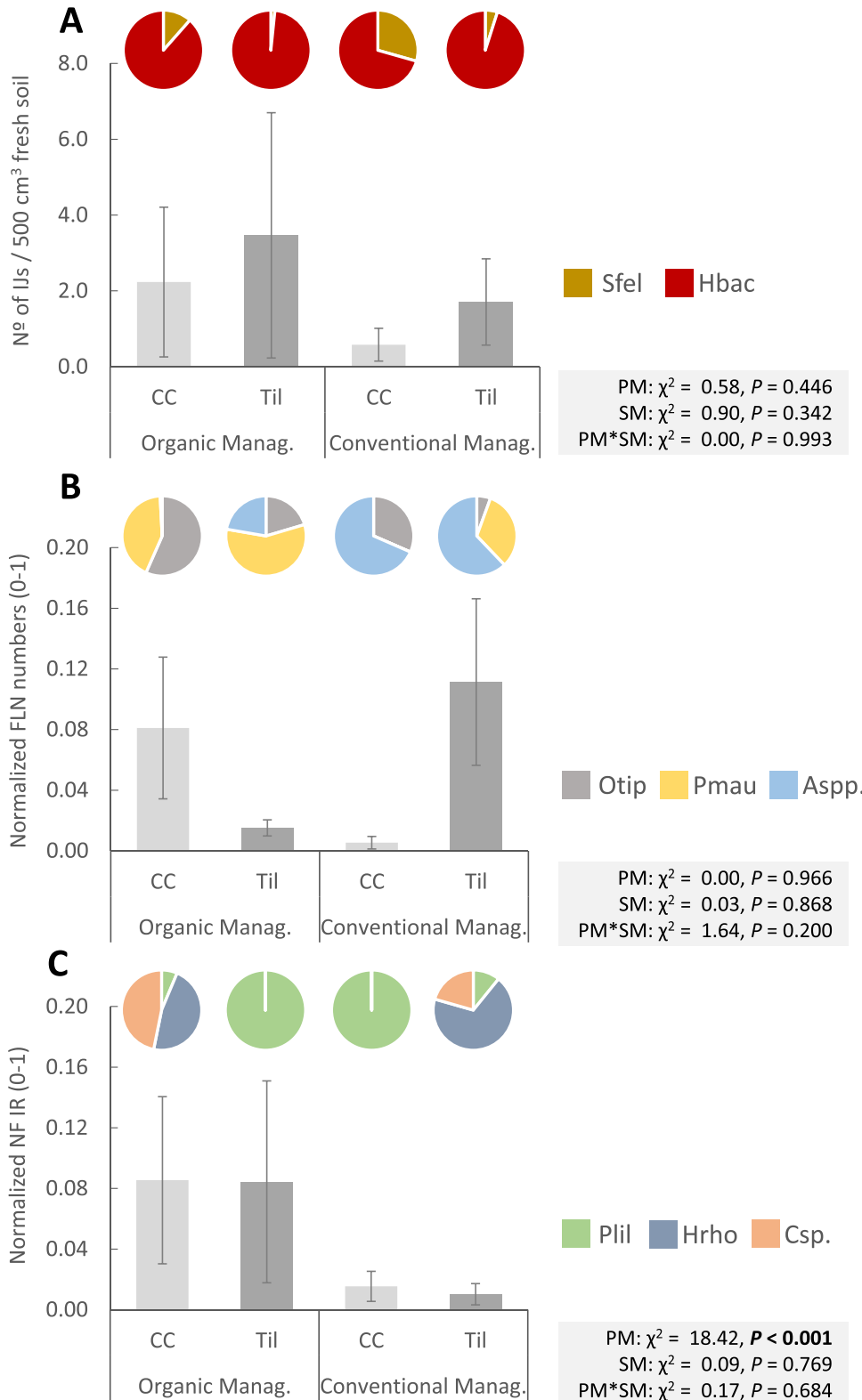


Fig. 3. Effect of pest (PM: organic vs. conventional) and soil (SM: cover cropping –CC– vs. tilling –Til–) management in olive groves on the abundance of (A) infective juveniles (IJs) of the entomopathogenic nematodes *Heterorhabditis bacteriophora* (Hbac) and *Steinernema feltiae* (Sfel), (B) the free-living nematodes (FLNs) *Oscheius tipulae* (Otip), *Pristionchus maupasi* (Pmau), and *Acrobeloides*-group (Aspp), and (C) the infection ratio (IR) of the nematophagous fungi (NF) *Purpureocillium lilacinum* (Plil), *Hirsutiella rhossiliensis* (Hrho), and *Catenaria* sp. (Csp.). Linear generalized mixed models (GLMz) testing within pair-treatment comparisons of each trophic group per PM and SM treatments. Significant differences ($P < 0.05$) are highlighted in bold. Values are least-square means \pm SE. Soil species averages are represented in pies.

Finally, NF were detected in 26% of olive groves (Fig. 2C), with *P. lilacinum* being the predominant species (21% sites). No sympatric distribution was observed among the three NF species.

3.2. Effect of agronomic practices in the natural occurrence of entomopathogenic nematodes and associated soil organisms

For total accounts of the different organisms investigated, neither occurrence nor abundance of nematodes but NF-IR were affected by any of the agricultural practices evaluated (Fig. 3; Supplementary material Fig. S1). Specifically, we found significantly higher NF abundance in organic olive groves than in conventional farming (Fig. 3C), although not for the frequency of NF occurrence (Fig. S1C). By species, we recorded higher levels for organic than IPM for the FLN *O. tipulae* and the NF-IR *H. rhossiliensis* and *P. lilacinum* (Fig. 3; Table 2). The endoparasitic fungi *H. rhossiliensis* was the only screened species significantly more abundant in cover cropped groves than bare soils, despite the marginal significance ($P = 0.052$) obtained for *O. tipulae* (Fig. 3; Table 2). Numbers for the nematode *P. maupasi* and the fungi *H. rhossiliensis* and *P. lilacinum* were affected by the PM*SM interaction (Table 2). Finally, none of the management treatments affected the abundance of the two EPN species identified and the FLN *Acrobeloides*-group, except for the marginally ($P = 0.077$) higher numbers reported in tilled soils for *H. bacteriophora*, nor the frequency of occurrence of any of the evaluated species (Table 2).

3.3. Effect of irrigation in the natural occurrence of entomopathogenic nematodes and associated soil organisms

Drip-irrigated soils overall contained significantly higher abundances of EPNs and NF but lower than in non-irrigated olive groves for FLNs (Fig. 4). However, we found no differences in occurrence frequencies for these trophic groups (Supplementary material Fig. S2). Specifically, the EPN *H. bacteriophora* and the NF *P. lilacinum* were more abundant in irrigated soils, the opposite that observed for the FLNs *O. tipulae* and *P. maupasi* and the NF *H. rhossiliensis* (Fig. 4; Table 3). The abundances of the rest soil organisms (*S. feltiae*, *Acrobeloides* spp., and

Table 2

Linear generalized mixed models (GLMz) testing within pair-treatment comparisons of pest (PM) and soil management (SM) for the abundance and frequency of occurrence in olive grove soils of the screened soil organisms. Significant differences ($P < 0.05$) are highlighted in bold (no significant denoted as n.s.).

Soil organisms	Abundance			Frequency of occurrence		
	PM (χ^2 , P)	SM (χ^2 , P)	PM vs SM (χ^2 , P)	PM (χ^2 , P)	SM (χ^2 , P)	PM vs SM (χ^2 , P)
Entomopathogenic nematodes						
<i>Steinernema feltiae</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Heterorhabditis bacteriophora</i>	n.s.	3.12, 0.077	n.s.	n.s.	n.s.	n.s.
Free-living nematodes						
<i>Oscheius tipulae</i>	4.51, 0.034	3.78, 0.052	6.34, 0.012	n.s.	n.s.	n.s.
<i>Pristionchus maupasi</i>	n.s.	n.s.	10.75, 0.001	n.s.	n.s.	n.s.
<i>Acrobeloides</i> -group	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Nematophagous fungi						
<i>Purpureocillium lilacinum</i>	5.59, 0.018	n.s.	16.29, < 0.001	n.s.	n.s.	n.s.
<i>Hirsutiella rhossiliensis</i>	3.88, 0.049	3.88, 0.049	25.28, < 0.001	n.s.	n.s.	n.s.
<i>Catenaria</i> spp.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Catenaria sp.) were unaffected by the presence or the absence of irrigation, nor the frequency of occurrence of any of the evaluated species except *P. maupasi*, more often found in non-irrigated soils (Fig S2; Table 3).

3.4. Abiotic factors and multivariate assemblage

Soils managed with different practices slightly differed for the analyzed abiotic factors. The proportion of irrigated olive groves was significantly higher for those driven with cover crops than tillage ($P = 0.008$) and for the interaction of management practices (PM*SM) evaluated ($P = 0.042$; Supplementary material Table S1). None of the climatic factors were affected, but a few of the soil physico-chemical variables: organic matter and N contents were significantly higher in organic olive groves ($P < 0.01$) and extractable P for cover cropping ($P = 0.002$; Table S1). We obtained similar results for irrigation considered a treatment. Climatic variables were not different, but few soil properties, precisely organic matter, C, and N contents, higher in non-irrigated groves, and P and K in irrigated groves ($P < 0.02$; Supplementary material Table S2).

In the multivariate analysis, the preliminary screening by DCCA gradient was 3.63 SD units long, so a CCA was selected to assess the species community assemblage. A total of seven abiotic factors (explanatory variables) and eight identified organisms were included in the analysis (Supplementary material Table S3). After the forward selection procedure, four variables (clay content, elevation, extractable P, and rain) significantly contributed to describing the assemblage of abiotic factors and selected species (Fig. 5). The CCA explained 85.6% of the fitted variation. Specifically, axis 1 explained 49.2% of the variability and was primarily influenced by the extractable P, while axis 2 contributed to explaining 36.4% and was mainly influenced by clay content, elevation, and mean annual rainfall. The NF *Catenaria* sp. and *H. rhossiliensis* were mainly associated with the extractable P. In axis 2, we observed two differentiated groups: *S. feltiae*, *P. maupasi*, and *P. lilacinum*, more present in groves with high clay content, elevation, and rainfall, and *H. bacteriophora* and *O. tipulae* showing an opposite association (Fig. 5). Finally, the FLN *Acrobeloides*-group was not linked with the selected abiotic variables.

4. Discussion

Quantification and isolation of EPNs in regional surveys depend on many variables, including the detection method (molecular vs. traditional approaches), temperature and hosts used as insect baits, and sampling effort and schedule (Campos-Herrera and Lacey, 2018). Not many studies have addressed the characterization of the EPN community in Spain or nearby countries through a similar methodology, i.e., by employing the sucrose gradient centrifugation for the isolation of mesofauna and species identification via qPCR analysis. Compared to them, our results for the overall EPN prevalence in olive groves of Andalusia (23.6% positive sites) were higher than those obtained in mining polluted areas of Southeastern Spain as well as natural and agricultural sites all across Switzerland (<15%) (Campos-Herrera et al., 2016a; Jaffuel et al., 2018), but remarkably lower than the numbers reported for natural and agricultural areas in Southern Portugal and vineyards in Northern Spain (>40%) (Blanco-Pérez et al., 2022a; Campos-Herrera et al., 2019a). Considering other surveys performed in West Europe but based on insect baits, we assume that our results for EPN occurrence are among the average. Thus, EPN findings were similar in uncultivated and farming areas (20–25%) of Catalonia and La Rioja, Northern Spain (García del Pino and Palomo, 1996; Campos-Herrera et al., 2007), lower in soil samples collected throughout continental Portugal and Italy (<16%) (Valadas et al., 2014; Tarasco et al., 2015), and higher in natural and agricultural areas in Southern France and grassland from the Swiss Alps (>60%) (Emelianoff et al., 2008; Kergunteuil et al., 2016).

Although our EPN numbers were, to some extent, as expected, we

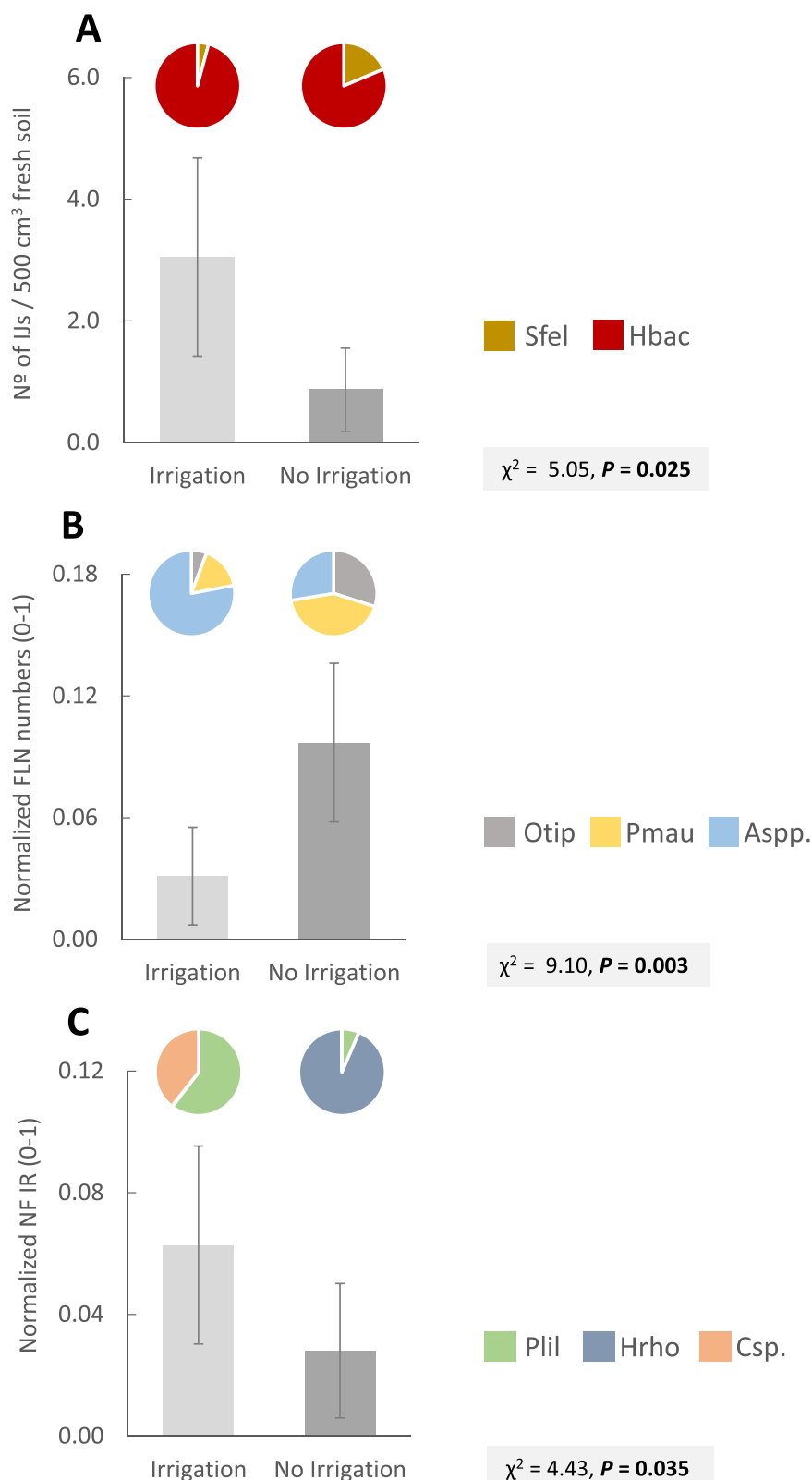


Fig. 4. Effect of irrigation in olive groves on the abundance of (A) infective juveniles (IJs) of the entomopathogenic nematodes *Heterorhabditis bacteriophora* (Hbac) and *Steinernema feltiae* (Sfel), (B) the free-living nematodes (FLNs) *Oscheius tipulae* (Otip), *Pristionchus maupasi* (Pmau), and *Acroboides*-group (Aspp), and (C) the infection ratio (IR) of the nematophagous fungi (NF) *Purpureocillium lilacinum* (Plil), *Hirsutella rhossiliensis* (Hrho), and *Catenaria* sp. (Csp.). Linear generalized mixed models (GLMz) testing within pair-treatment comparisons (irrigation vs. absence of irrigation) of each trophic group. Significant differences ($P < 0.05$) are highlighted in bold. Values are least-square means \pm SE. Species averages are represented in pies.

only identified two species: *S. feltiae* and *H. bacteriophora*, the predominant species of each genus in the Iberian Peninsula (Blanco-Pérez et al., 2022a; Campos-Herrera et al., 2007, 2014, 2019a; García del Pino and Palomo, 1996, 1997). However, we are aware that one of the possible limitations of the current study might be that we only searched for those

species that we expect to find through previous survey records (Homnick, 2002; Campos-Herrera et al., 2016a; Valadas et al., 2014). Thus, unpredictable or undescribed species, detection of which could increase the overall EPN numbers described in olive groves, could be lacking in this survey. For example, *Steinernema riojaense*, the most abundant EPN

Table 3

Linear generalized mixed models (GLMz) testing within pair-treatment comparisons of irrigation system implemented in the orchard (presence or absence) for the abundance and frequency of occurrence in olive grove soils of the screened soil organisms. Significant differences ($P < 0.05$) are highlighted in bold (no significant denoted as n.s.).

Species	Abundance (χ^2 , P)	Frequency of occurrence (χ^2 , P)
Entomopathogenic nematodes		
<i>S. feltiae</i>	n.s.	n.s.
<i>H. bacteriophora</i>	10.06, 0.002	n.s.
Free-living nematodes		
<i>O. tipulae</i>	9.60, 0.002	n.s.
<i>P. maupasi</i>	14.44, < 0.001	3.90, 0.048
Acrobelloides-group	n.s.	n.s.
Nematophagous fungi		
<i>P. lilacinum</i>	51.95, < 0.001	n.s.
<i>H. rhossiliensis</i>	34.57, < 0.001	n.s.
<i>Catenaria</i> spp.	n.s.	n.s.

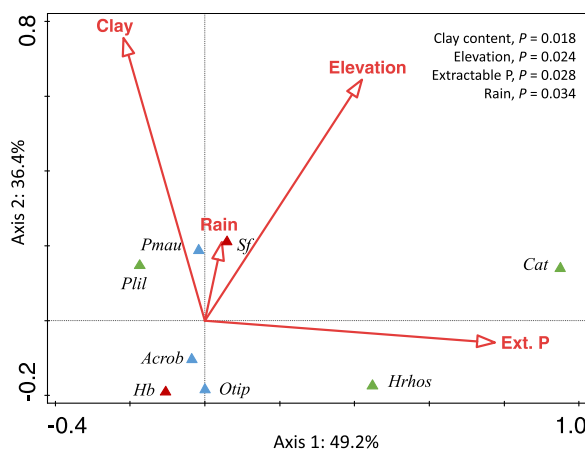


Fig. 5. Canonical correspondence analysis of the abundance of soil organisms and explicative abiotic variables in olive groves of Andalusia (Southern Spain). Abiotic factors are represented as arrows: clay content, elevation, average annual rain, and extractable P. Biotic factors (species abundance) are represented as triangles: entomopathogenic nematodes (red triangles), *Steinernema feltiae* (Sf), and *Heterorhabditis bacteriophora* (Hb); free-living nematodes (blue triangles), *Acrobelloides*-group (Acrob), *Oscheius tipulae* (Otip), and *Pristionchus maupasi* (Pmau); and nematophagous fungi (green triangles), *Hirsutiella rhossiliensis* (Hrhos), *Purpureocillium lilacinus* (Plil), and *Catenaria* sp. (Cat).

species for a survey conducted on vineyards of Northern Spain (Blanco-Pérez et al., 2022a), was isolated, identified, and described for the first time only two years earlier (Blanco-Pérez et al., 2020; Püza et al., 2020). The same could ensue in the olive groves of Southern Spain. Therefore, we consider that further studies combining traditional and molecular methods are needed to reveal other plausible naturally occurring EPN species.

Contrary to our hypothesis, the natural occurrence of EPNs and FLNs was not affected by the management practices evaluated, and for NF, only conventional reduced their presence. The relatively low nematode abundances obtained were possibly insufficient to establish statistical differences among treatments. Jaffuel et al. (2016), for instance, reported even lower numbers in a long-term field trial of annual crops in Switzerland involving organic, conventional, and biodynamic production systems, probably due to the limited stability of the rhizosphere of the analyzed soils. According to this, agricultural practices contributed less to the EPN soil food web modulation than the annual crop rotation. Similarly, Campos-Herrera et al. (2010) recorded lower EPN activity rates in annual than perennial crops in Northern Spain, with no differences between conventional and more sustainable environmental

practices, while differences were observed when perennial crops were compared. A recent study in vineyards in the same region (La Rioja) also showed high EPN abundance, more increased for the species *S. feltiae* and FLNs associated with the EPN community in organic than IPM (Blanco-Pérez et al., 2022a). Hence, low EPN number detected in the olive agroecosystems might have limited the observation of crop management effect and further studies are recommended. It is possible that the low number of soil organisms recovered in our study could be related to the poor soil moisture that characterizes regions of Southern Spain. Although Sánchez-Moreno et al. (2018) distinguished between vineyards and olive groves in terms of soil fertility and soil food web structure using FLNs as a model framework, they also found low nematode numbers in semiarid areas of South-Central Spain and no differences between conventional and organic management. Analysis of other soil organisms in the same olive orchards employed in our study revealed no differences in the abundance of culturable heterotrophic bacteria and arbuscular mycorrhizal fungi among grove management systems (Montes-Borrego et al., 2013, 2014). Conversely, Palomares-Rius et al. (2015) found higher numbers and diversity of PPN species for organic than conventional management, particularly in no-tilled soils, possibly because olive trees serve as hosts to numerous PPN species (Castillo et al., 2010; Ali et al., 2014). In addition, many nematodes exhibit a wide range of morphologies that allow them to rapidly adapt to changing environments to feed in other host or food resources (Bird et al., 2014). For example, PPNs were the only trophic nematode group to react to altered precipitation in low moisture content soils in New Mexico (USA) desertified areas (Vandegheuchte et al., 2015). Palomares-Rius et al. (2015) suggested that regular food sources during the crop season and different soil niches generated in perennial crops probably explained the higher PPN abundance and diversity observed for cover-cropper organic orchards than conventional farming.

If soil moisture content was indeed a critical factor in explaining the low EPN numbers recorded in this study, it is revealing that we found significantly higher EPN abundance in drip-irrigated than rain-fed olive groves. Overall, nematodes are considered aquatic animals which only inhabit soil pores filled with water (Neher, 2010), with an appropriate surface film to allow tension to support their locomotion (Wallace, 1971). Besides, a continuum of water input may enhance the formation of microhabitats that benefit the development of immature stages of insects that are suitable hosts for EPNs. Thus, available moisture levels in the groves during periods of special drought conditions must be critical for the long-term persistence of EPNs, which agreed with previous regional surveys that highlighted the relevance of soil water contents for the EPN community (Campos, Herrera et al., 2013, 2016b, 2019a).

Surprisingly, drip-irrigation limited FLN occurrence in olive groves, specifically of the species *O. onirici* and *P. maupasi*. It is plausible that specific root exudates, litter inputs, or differentiated agricultural practices could alter the soil biota assemblage and behavior (Sánchez-Moreno et al., 2018; Yang et al., 2016). We obtained higher organic matter, N, and NPK values in irrigated soils than for the rain-fed regime. Tenuta and Ferris (2004) suggested that excess nitrogenous solutions in soils can reach toxic levels for bacterivorous nematodes, as is the case of the two mentioned FLNs. Moreover, we also found large NF-IR in irrigated groves, particularly for the endoparasitic *H. rhossiliensis* and the egg-parasitic *P. lilacinum* fungal species. Zoospores and other structures that require moisture for their optimal functioning supported the reproduction of these specific NF groups (Pathak et al., 2017). Accordingly, constant water supply in the olive groves may initially favor the nematode occurrence, followed by the population growth of their natural enemies such as NF, in our case, fungal species that can affect most of the screened FLNs. We could not find a relationship between NF-IR and numbers of detected PPNs in the studied samples by Palomares-Rius et al. (2015) (data not shown), as just a few in some samples were found positive for specific NF species. Additionally, other nematode species (not the ones studied in this

article) could be present and they were not quantified in Palomares-Rius et al. (2015). *Purpureocillium lilacinum* has been successfully applied to control important PPNs as *Meloidogyne* spp. (Parajuli et al., 2014) and some strains (i.e. strain 251) are used commercially in many parts of the world (Atkins et al., 2005). *Hirsutella rhossiliensis* has been associated with the nematode number reduction of *Criconeoides xenoplax* and *Heterodera* spp. (Stirling, 2014). In this respect, *H. rhossiliensis* and *Catenaria* sp. are more difficult to be used as an active inundate biocontrol agents and they must be maintained in a conservation approach by adapting soil management. A good soil management (reduced tillage, increase in organic matter content, rational cover crop use,...) could help to increase the presence of these NF and help to balance soil levels of PPN (Stirling, 2014), including in olive ecosystems. Hence, further studies are required to investigate the best practices that ensure the natural presence of EPN, limit the PPN prevalence and ensure the action of NF in suitable levels.

The multivariate linkages between abiotic factors and selected organisms associated with the EPN were consistent with previous studies, where clay content was a key explanatory variable in citrus groves, annual crops, grasslands, and natural areas in subtropical, temperate, and Mediterranean regions (Campos, Herrera et al., 2013, 2016b, 2019a; Jaffuel et al., 2018). Soil texture is a key soil property affecting EPN movement, infectivity, and overall survival, linked to the soil water content (Stuart et al., 2015). In agreement with previous reports, *H. bacteriophora* was associated with soils with low clay content (Hominick, 2002), although studies in temperate regions showed its presence in heavy soils (Jaffuel et al., 2018). Elevation resulted in an explanatory variable that negatively affected all the considered organisms except *S. feltiae*, prevalent in olive groves at slightly higher altitudes. Previous studies finding EPN species associated with high elevation areas, such as *S. poinari* and *S. krausseii* in temperate Swiss soils (Jaffuel et al., 2018), suggested habitat modulations that alter, for instance, the occurrence of susceptible hosts and natural enemies of EPNs.

Regarding FLNs, none of the explanatory variables strongly impacted the investigated species in our CCA analysis. The FLN *O. tipulae* was associated with *H. bacteriophora* assemblage and low clay content and elevation. However, in previous studies, this species remained unrelated to the abiotic factors considered (Jaffuel et al., 2018; Campos-Herrera et al., 2019a). The FLN *P. maupasi* showed an opposite trend than noted by Campos-Herrera et al. (2019a), mostly occurring in soils with high clay content. Finally, the NF *H. rhossiliensis* and *Catenaria* sp. were linked to high P contents, agreeing with Pathak et al. (2017) for the last species. However, previous studies proposed that high P levels can negatively affect the chytrid community composition and diversity (Letcher et al., 2004). The knowledge of how abiotic conditions modulate NF occurrence and activity in natural and agricultural areas is still limited, so further regional studies will be required to expand our understanding of the ensemble of these organisms in the soil food web and their impact on beneficial organisms for crops.

5. Conclusion

In this study, we have shown, for the first time, the occurrence of native EPNs and associated soil organisms in olive groves managed through diverse practices. Recent studies demonstrated that EPNs biological control agents are a real option in controlling key pests such as *B. oleae* in olive groves (Torrini et al., 2017, 2020). In the current global context of sustainable agriculture, it is essential, more than ever, to promote alternative strategies to support their natural occurrence and long-term persistence, including in the olive groves. We expected to find a high abundance of nematodes in an agroecosystem with a stable rhizosphere that characterizes perennial crops like olive groves (Campos-Herrera et al., 2019a). This presumption was only partially fulfilled for EPNs recovered in drip-irrigated soils. Water restriction in rain-fed groves (expected in Southern Europe and in other Mediterranean climate regions where olive is cultivated) was plausibly a primary driver

for limiting the abundance of nematodes occurring naturally in soils, at least those species screened in this survey. Indeed, we did not find differences in EPN abundance among the evaluated agricultural practices (pest and soil management) but for the irrigation regime. However, complex biotic interactions established in the soil may modulate the most suitable traits of individual inhabitants. Thus, higher soil moisture increased NF populations in our samples, which should cause a decline in specific nematode populations. Additional studies are required to determine the best combination of alternative strategies (e.g., particular types of mulches or cover crops) that will enhance the development of rich and equilibrated soil biota in olive groves (Calabrese et al., 2015) thereby promoting conservation ecosystem services such as provided by the EPNs. This study illustrates the value of such approaches that can be extended to other regions where olives are cultivated, from Italy and Greece in South Europe and Mediterranean associated regions (Turkey, Morocco, etc.), as well as other key areas in Australia, South Africa, Chile, Argentina and USA where this crop has commercial relevance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2022.108070](https://doi.org/10.1016/j.agee.2022.108070).

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