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TESIS DOCTORAL

Título

Effect of an alternative viticulture on native entomopathogenic nematodes and other organisms linked to their soil food web in DOCa Rioja vineyards

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Effect of an alternative viticulture on native entomopathogenic nematodes

and other organisms linked to their soil food web in DOCa Rioja vineyards

Efecto de una viticultura alternativa sobre poblaciones de nematodos entomopatógenos nativos

y organismos asociados a su red trófica en viñedos de la DOCa Rioja

Tesis Doctoral presentada por
Rubén Blanco Pérez
para optar al título de Doctor por
La Universidad de La Rioja

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DEPARTAMENTO DE AGRICULTURA Y ALIMENTACIÓN
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Programa de Doctorado Interuniversitario
en Enología, Viticultura y Sostenibilidad

Effect of an alternative viticulture on native entomopathogenic nematodes and other organisms linked to their soil food web in DOCa Rioja vineyards

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Por la presente declaran que:

La memoria titulada *Effect of an alternative viticulture on native entomopathogenic nematodes and other organisms linked to their soil food web in DOCa Rioja vineyards*, que presenta Rubén Blanco Pérez, Licenciado en Biología, ha sido realizada en la Universidad de La Rioja bajo su dirección y reúne las condiciones específicas para optar al grado de Doctor como compendio de publicaciones.

Lo que hacen constar en Logroño, a 5 de septiembre de 2022.

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Esta Tesis ha sido realizada en el Grupo de Investigación In-Vid del Departamento de Viticultura del Instituto de Ciencias de la Vid y del Vino (Gobierno de La Rioja, Consejo Superior de Investigaciones Científicas y Universidad de La Rioja), y apoyada la Ayuda predoctoral para la investigación en los Centros Tecnológicos, Centros de Investigación y Empresas de la Comunidad Autónoma de La Rioja, Resolución de concesión nº 1168/2018, de la Consejera de Desarrollo Económico e Innovación, el Proyecto Nacional AGL2014-53336R '*Efecto de la implantación de cubierta vegetal en viñedo sobre la comunidad de enemigos naturales de plagas de la vid*' (CUVEGENAT), el Contrato Ramón y Cajal RYC-2016-19939 (2018–2023), el Proyecto Regional I+D 2020 (P.R-05-20) '*Influencia de diferentes técnicas de manejo del suelo sobre la presencia y diversidad de organismos beneficiosos del suelo (nematodos entomopatógenos) y la asociación con sus enemigos naturales*', y la Ayuda IER 7/2021 '*Cuantificación molecular de nematodos entomopatógenos y micro/meso-fauna asociada en viñedos DOCa Rioja sujetos a distintos manejos agronómicos*'.

Durante la realización de la tesis doctoral se completaron estancias en el laboratorio LASBE del centro de investigación MeditBio de la Universidade do Algarve (Faro, Portugal) del 1 de agosto de 2017 al 20 de mayo de 2018, en el laboratorio de Ecología del Suelo del Departamento de Medio Ambiente y Agronomía del INIA (Madrid, España) del 14 al 18 de octubre de 2019 y en el laboratorio de Ecología Evolutiva de las Interacciones Planta-Herbívoro de la Misión Biológica de Galicia (CSIC) (Pontevedra, España) del 18 al 21 de octubre de 2021.

ARTÍCULOS INCLUIDOS EN LA TESIS

De acuerdo con la normativa vigente en la Universidad de La Rioja (Normativa para la defensa de tesis doctoral en la Universidad de La Rioja, aprobada por Consejo de Gobierno de 18 de marzo de 2022 y, específicamente con su Capítulo V), la tesis se presenta como compendio de publicaciones científicas. Las referencias completas de las mismas se listan a continuación en orden cronológico de publicación. Además, se presenta una copia de los artículos en el apartado ‘Compendio de publicaciones’.

La presente tesis se ha configurado a partir de tres artículos científicos, todos ellos publicados en revistas internacionales incluidas en los listados *Journal of Citation Reports-Science Edition* (JCR):

- **Blanco-Pérez, R.**, Sáenz-Romo, M.G., Vicente-Díez, I., Ibáñez-Pascual, S., Martínez- Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., 2020. *Impact of vineyard ground cover management on the occurrence and activity of entomopathogenic nematodes and associated soil organisms.* Agric. Ecosyst. Environ. 301, 107028. https://doi.org/10.1016/j.agee.2020.107028

  Área: *Agriculture/Multidisciplinary*; IF: **5,567** (2020), **Q1** (1/56)

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Para cada uno de estos estudios, el autor de esta tesis, bajo la supervisión de sus directores y en colaboración con el resto de coautores, ha desarrollado las siguientes funciones:

- ✓ Análisis del estado del arte.
- ✓ Planteamiento de objetivos y elección de los materiales y métodos.
- ✓ Recogida, procesamiento y análisis de muestras.
- ✓ Ejecución de los bioensayos y técnicas moleculares descritas en la metodología.
- ✓ Procesamiento de datos y análisis estadístico.
- ✓ Análisis de los resultados y discusión sobre los mismos.
- ✓ Redacción completa de los artículos.

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A mi padre,

que así lo quiso.

Cover:

Details of the lateral lines of a juvenile infective and the cephalic region of an adult specimen of the entomopathogenic nematode *Steinernema feltiae*. Photos provided with permission by Dra. Raquel Campos Herrera from Instituto de Ciencias de la Vid y del Vino, Spain. The photo of a grapevine leaf was downloaded from Pixabay.com and subsequently modified.

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Abstract

Entomopathogenic nematodes (EPNs) are biological control agents that often occur naturally in crop soils. The conventional agricultural practices of regular tillage and agrochemical applications predispose to soil biodiversity losses, compromising soil health and disrupting the natural balance of abiotic and biotic factors that might modulate EPN abundance and activity. The vineyard, which supports a relevant socio-economic sector worldwide, is one of the most intensively managed cropping systems. Therefore, approaches rather than reliance on mechanization and agrochemicals are needed to achieve more sustainable viticulture. We hypothesized that alternative strategies to tillage for soil management and the release of agrochemicals for pests, diseases, and weed management, such as cover cropping, mulching, and organic farming, would favor the native EPN community in vineyard soils. Therefore, our objective was to evaluate the impact of differentiated viticulture practices on native EPNs and other targeted organisms associated with their soil food web and how their assemblage might signal soil health in vineyards.

We implemented traditional and innovative methodologies to isolate and identify mesofauna to achieve this aim. Firstly, we estimated different soil activities, including those associated with EPNs, by baiting the soil samples with *Galleria mellonella* larvae. Besides, we used species-specific primers/probe qPCR sets to screen and quantify the occurrence and abundance of 10 EPN species and 12 organisms linked to their soil food web: four free-living nematodes (FLNs), six nematophagous fungi (NF), and two ectoparasitic bacteria (EcPB). Lastly, a third soil subsample set was employed to estimate the soil properties. Following this procedure, we performed three independent studies to evaluate the impact of different management practices on the EPN community and associated soils organisms in The Appellation of Origin (DOCa) Rioja vineyards (Northern Spain): *(i)* diverse cover crops (seeded with *Bromus catharticus*, flower-driven, and spontaneous) compared to regular tillage in an experimental vineyard, *(ii)* cover cropping and organic viticulture compared to regular tillage and Integrated Pest Management (IPM) in a survey comprising 80 vineyards, and *(iii)* various organic mulches (based on grape pruning debris, straw, and spent mushroom compost) compared to regular tillage and herbicide applications in an organic and IPM experimental vineyards.

We found seven EPN species and all the other screened species except the NF *Arthrobotrys musiformis* and the EcPB *Paenibacillus nematophilus*. The only EPNs reported in the three studies were *Heterorhabditis bacteriophora*, *Steinernema feltiae*, and the new EPN species *S. riojaense*, identified and isolated during the progress of this Thesis. Overall, EPN abundance and activity were higher for cover cropping and mulching than conventional soil management practices in both studies performed in experimental vineyards. However, the results obtained in the DOCa Rioja survey did not support this trend. It is possible that differential effects of diverse alternative strategies to regular tillage also affected the soil properties and, therefore, the EPN soil food web differentially. Indeed, we found lower numbers of potential enemies of EPNs, particularly NF, for spontaneous cover cropping and mulching based on spent mushroom compost, the treatments for which higher EPN activity rates and abundance were recorded.

On the other hand, in agreement with our hypothesis, organic viticulture enhanced the activity of native EPNs and the abundance and activity of the predominant EPN species, *S. feltiae*, in the DOCa Rioja survey. In addition, we obtained similar results for the organic vineyard in the mulching study. Organic viticulture also supported a higher FLN abundance and richness of the overall nematode species screened since the EPN species *Steinernema affine*, *S. carpocapsae*, and *S. kraussei*, as well as the FLN species *Oscheius onirici*, only occurred in organic vineyards.

Our results showed that organic viticulture and specific soil management practices that restrict or avoid regular tillage might support native EPNs in the vineyard, contributing to the maintenance of the ecosystem service these soil organisms offer as biological control agents. Moreover, these studies have illustrated how evaluating the EPN soil food web can signal soil health and the suitability of some viticulture practices over others. Applying innovative molecular tools and statistical analyses will improve understanding of the factors that determine the occurrence and distribution of EPNs in crop soils.

Resumen

Los nematodos entomopatógenos (NEPs) son agentes de control biológico comúnmente presentes en el suelo de los cultivos. Las prácticas agrícolas de laboreo convencional y aplicación de agroquímicos predisponen a la pérdida de biodiversidad del suelo, comprometiendo su salud y alterando el equilibrio natural de factores abióticos y bióticos que modulan la abundancia y actividad de los NEPs. El viñedo, soporte de un sector socioeconómico de gran relevancia mundial, es uno de los sistemas de cultivo más más se apoya en las prácticas del laboreo intensivo y la aplicación de productos agroquímicos. De este modo, con el fin de avanzar en su sostenibilidad, son necesarios nuevos enfoques en el manejo del viñedo. Nuestra hipótesis es que las estrategias alternativas al laboreo y a la liberación excesiva de agroquímicos en los manejos del suelo y de plagas, enfermedades y malas hierbas en vid, como son la implementación de cubiertas vegetales o acolchados y la agricultura ecológica, favorecerán la comunidad nativa de NEPs en el suelo del viñedo. De este modo, nuestro objetivo fue evaluar el impacto de diferentes prácticas vitivinícolas sobre las poblaciones nativas de NEPs y otros organismos específicos asociados a su red trófica del suelo y cómo sus interacciones podrían ser indicativo de la salud del suelo en los viñedos.

Para ello, combinamos metodologías tradicionales e innovadoras con el fin de aislar e identificar organismos de la mesofauna del suelo. En primer lugar, estimamos diferentes actividades del suelo, incluidas aquellas asociadas a los NEPs, empleando larvas de *Galleria mellonella* como cebos en las muestras de suelo usadas como trampas de insectos. Además, utilizamos cebadores y sondas específicos para identificar y cuantificar, mediante qPCR, la presencia y abundancia de 10 especies de NEPs y otros 12 organismos asociados a su red trófica: cuatro nematodos de vida libre (NVLs), seis hongos nematófagos (HNs) y dos bacterias ectoparásitas (BcPs). Por último, un tercer conjunto de submuestras de suelo fue utilizado para estimar sus propiedades físico-químicas. Siguiendo este procedimiento, realizamos tres estudios independientes para evaluar el impacto de diferentes prácticas de gestión sobre la comunidad de NEPs y organismos asociados en viñedos de la Denominación de Origen (DOCa) Rioja (Norte de España): *(i)* uso de diferentes cubiertas vegetales (sembradas con *Bromus catharticus*, mezcla de especies florícolas y vegetación espontánea) comparadas con la práctica de laboreo convencional en un viñedo experimental; *(ii)* cubiertas vegetales y viticultura ecológica comparadas con el laboreo convencional y el Manejo Integrado de Plagas (MIP) en 80 viñedos distribuidos

por toda la DOCa Rioja; y *(iii)* diferentes tipos de acolchados orgánicos (basados en restos de poda, paja y compost post-cosecha de champiñón) comparados con el laboreo convencional y aplicaciones de herbicidas en dos viñedos experimentales, uno manejado mediante MIP y otro en ecológico.

Identificamos siete especies de NEPs y la totalidad del resto de especies examinadas, excepto el HN *Arthrobotrys musiformis* y la BcP *Paenibacillus nematophilus*. Los únicos NEPs encontrados en los tres estudios fueron *Heterorhabditis bacteriophora*, *Steinernema feltiae* y la nueva especie *S. riojaense*, identificada y aislada durante el transcurso de estos estudios. En general, la abundancia y la actividad de los NEPs fueron mayores en cubiertas vegetales y acolchados que en las prácticas convencionales de manejo del suelo en los dos estudios realizados en viñedos experimentales, una tendencia que, sin embargo, no fue confirmada por el estudio regional de la DOCa Rioja. Es posible que el impacto diferencial que las diversas estrategias alternativas al laboreo convencional puedan ejercer sobre las propiedades del suelo también afecte de modo diferente a la red trófica de los NEPs. En efecto, encontramos un menor número de enemigos potenciales de los NEPs, en particular de HNs, en cubiertas vegetales espontáneas y en acolchados basados en sustrato de post-cosecha de champiñón, los tratamientos para los que se registraron mayores tasas de actividad y abundancia de NEPs.

En lo referente al manejo de plagas observamos, de acuerdo con nuestra hipótesis, que la viticultura ecológica promovió en el estudio regional DOCa Rioja la actividad de los NEPs, así como la abundancia y actividad de la especie de NEP predominante, *S. feltiae*. Además, obtuvimos resultados similares en el viñedo ecológico del estudio de acolchados orgánicos. La viticultura ecológica también favoreció una mayor abundancia de NVLs y la riqueza del conjunto de especies de nematodos analizadas, puesto que las especies de NEPs *Steinernema affine*, *S. carpocapsae* y *S. kraussei*, así como la especie de NVL *Oscheius onirici*, sólo fueron identificadas en los viñedos ecológicos.

Los resultados obtenidos en estos estudios indican que la viticultura ecológica y las prácticas específicas de gestión del suelo que sustituyan parcial o totalmente al laboreo convencional pueden potenciar la presencia y actividad de NEPs nativos en el viñedo, contribuyendo al mantenimiento del servicio ecosistémico que estos organismos del suelo ofrecen como agentes de control biológico. Además, estos estudios sirvieron de ejemplo de cómo la evaluación de la red trófica del suelo de los NEPs puede servir como indicativo de la salud del suelo y de la conveniencia de unas prácticas vitícolas sobre otras. En el futuro, técnicas moleculares y análisis estadísticos innovadores mejorarán nuestra comprensión de los factores que determinan la presencia y distribución de los NEPs en el suelo de los cultivos.

Introduction

Entomopathogenic nematodes

> *„In short, if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable […]"*
>
> Nathan Augustus Cobb
> in *Nematodes and their relationships*, 1914

Phylum Nematoda

Nematodes are the most abundant animals on Earth, successfully adapted to virtually every ecosystem (Cobb, 1914; Ettema, 1998; Bongers and Ferris, 1999; Bardgett and van der Putten, 2014). Although well known since antiquity, nematodes were formally recognized as a phylum relatively recently: firstly proposed by Nathan Cobb in 1919 under the name Nemates and elevated from the class level in 1932 with the current designation Nematoda (Cobb, 1919, 1932; Potts, 1932). Despite certain controversies, there is now consensus that nematodes are a sufficiently distinctive and well-defined group to be classified as a Phylum (De Ley and Blaxter, 2004; Hodda, 2022a). Among the attributes that define them, we can highlight that nematodes are triploblastic, bilaterally symmetrical, and nonsegmented animals of elongated, cylindrical shape. The body cavity is defined as pseudocoel, and the epidermis releases a thick and flexible collagenous cuticle that creates a hydroskeleton in combination with a layer of longitudinal muscle cells, which extend fibers into the nerve cells rather than vice versa. Several lateral lines and pores are present on the surface of the body. The circulatory and respiratory systems lack but not the digestive system, with a distinguishable mouth and anus. The nervous system is poorly developed, consisting of rudimentary cephalization, the circucumpharyngeal ring, and longitudinal nerve cords. The relatively distinct head is radially symmetrical with three to six lips and sensory papillae irradiating around the mouth. Although hermaphrodites are not uncommon, most nematode species have separate sexes and are dioecious, with larger females. The genital duct leads into the cloaca in males and the vulva in females (Figure 1). After the internal fertilization, the eggs are stored in the uterus until deposited on the exterior, although occasionally embryonic development begins inside the female. The four juvenile stages contain all the adult structures, except part of the reproductive system, and complete their growth after four cuticle sheds (Ruppert et al., 2004; Hickman et al., 2019).

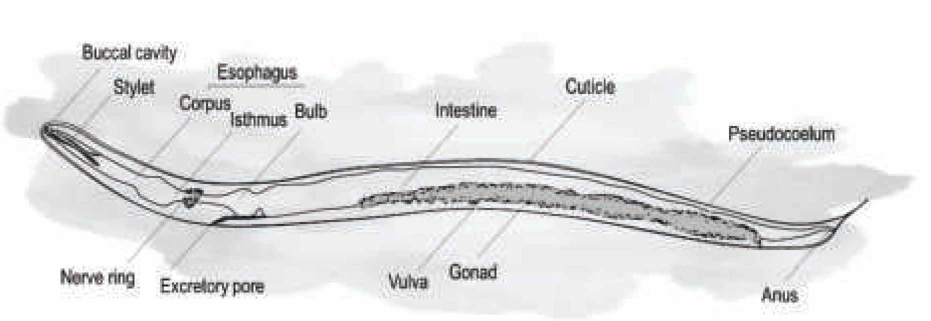


Figure 1. Simplified anatomy of a type specimen (adult female) of nematode.

Nematodes show exceptional morphological variability due to the multiple biotic and abiotic interactions they establish with the environment (Hodda, 2022b). Consequently, Nematoda is one of the phyla with the most described species (nearly 30,000), many substantially different from each other (Zhang, 2013). However, morphological identification of close related nematodes is often challenging due to their structural simplicity, small size, high abundance, and lack of expert knowledge for specific groups of interest (Floyd et al., 2002; Hodda, 2022a). In the last decades of the past century, numerous taxonomists trusted new molecular tools to identify and classify many organisms. Some of them pointed out incongruences between molecular analysis and comparative morphology data. For instance, a phylogenetic analysis using 53 small subunit ribosomal DNA sequences from different trophic groups concluded that the convergent morphological evolution of nematodes might be more extended than expected (Blaxter et al., 1998). Accordingly, the authors considered the clade Secernentea monophyletic but not Adenophorea, named Phasmidia and Aphasmidia, respectively, in an early classification that divided the phylum into these two classes (Chitwood and Chitwood, 1933). Subsequent studies incorporated the most relevant morphological attributes of the different groups of nematodes into evidence obtained through novel approaches, such as molecular phylogenetics, bioinformatics, and digital communication technologies, to move forward in the complex task of achieving an accurate phylogeny of nematodes (Floyd et al., 2002; Lambshead and Boucher, 2003; De Ley, 2006; Qing and Bert, 2019). Nowadays, according to morphological, molecular, and developmental evidence, the phylum Nematoda includes three classes: Chromadorea, Enoplea, and Dorylaimea (Hodda, 2022a) (Figure 2). Although a detailed linkage of these lineages is not yet determined, spermatogenesis and development provide evidence for the divergence of Chromadorea, the most diverse in morphological, genetic, and ecological range, from Enoplea and Dorylaimea, differing also by the pharyngeal structure and cuticle properties (Schierenberg and Lahl, 2004; Yushin and Malakhov, 2004; De Ley, 2006; Hodda, 2022a). Current progress in describing new nematodes, thanks, for example, to the increasing acknowledgment of widespread cryptic species (Derycke et al., 2012), is slowly advancing at a rate of 400 species per year, with estimates of total nematode species numbers between half and ten million species (Hodda, 2022b).

- Phylum **Nematoda** Cobb, 1932
  - Class **Enoplea** Inglis, 1983
    - Subclass **Enoplia** Pearse, 1942
      - (2 superorders, 6 orders, 8 suborders, 12 superfamilies, 21 families, 39 subfamilies, 145 genera, 1268 species)
    - Subclass **Oncholaimia** Hodda, 2007
      - (1 superorder, 1 order, 1 suborder, 1 superfamily, 3 families, 9 subfamilies, 56 genera, 534 species)
    - Subclass **Triplonchia** Hodda, 2007
      - (1 superorder, 2 orders, 3 suborders, 6 superfamilies, 11 families, 15 subfamilies, 42 genera, 495 species)
  - Class **Dorylaimea** Hodda, 2007
    - Subclass **Dorylaimia** Inglis, 1983
      - (1 superorder, 1 order, 2 suborders, 6 superfamilies, 18 families, 52 subfamilies, 269 genera, 3126 species)
    - Subclass **Bathyodontia** Hodda, 2007
      - (1 superorder, 3 orders, 4 suborders, 7 superfamilies, 12 families, 25 subfamilies, 174 genera, 1137 species)
    - Subclass **Trichocephalia** Hodda, 2007
      - (1 superorder, 4 orders, 4 suborders, 4 superfamilies, 10 families, 12 subfamilies, 56 genera, 654 species)
  - Class **Chromadorea** Inglis, 1983
    - Subclass **Chromadoria** Adamson, 1987
      - (1 superorder, 4 orders, 4 suborders, 5 superfamilies, 22 families, 36 subfamilies, 230 genera, 2163 species)
    - Subclass **Plectia** Hodda, 2007
      - (4 superorders, 11 orders, 27 suborders, 60 superfamilies, 178 families, 323 subfamilies, 2057 genera, 19154 species)

Figure 2. Summarized phylogeny of Nematoda according to Hodda (2022a).

Nematodes are ecologically and economically important, playing a significant function in processes that drive most ecosystem services on which human societies hang (Yeates et al., 2009; Hodda, 2021). As primary components of food webs, they accomplish vital ecological functions such as decomposition and carbon and nutrient cycling (Baldwin et al., 2000; Ferris, 2010; Nielsen et al., 2011). In soils, nematodes contribute to food web stability and the diversity of above and belowground plant-associated communities (De Deyn and Van Der Putten, 2005; Orgiazzi et al., 2016). Their ubiquity also makes them excellent bioindicators of soil environmental disturbance and biological control agents (BCAs) in pest and disease management programs (Ferris et al., 2001; Stuart et al., 2015). On the other hand, parasitic nematodes threaten plant, animal, and human health worldwide (Viglierchio, 1993). Plant-parasitic nematodes, for instance, cause considerable yield losses in crops, compromising the global food security (Bernard et al., 2017). Lastly, thanks to their structural simplicity and ecological relevance, nematode species (e.g., *Caenorhabditis elegans*, *Pristionchus pacificus*) have been extensively used as model organisms in experimental systems for diverse research fields (Brenner, 1974; Sommer and McGaughran, 2013).

The families Steinernematidae and Heterorhabditidae

According to Dillman et al. (2012a), a specific association with pathogenic bacteria to rapidly kill suitable hosts distinguish the species in Steinernematidae and Heterorhabditidae among the numerous insect-parasitic nematodes. This specific nematode parasitism has been named relatively recently in nematology as entomopathogeny (Spiridonov, 1981). Although an accurate definition of entomopathogenic nematode (EPN) is currently a matter of intense debate in the literature, there is agreement on the essential aspects that define these organisms. Both steinernematids and heterorhabditids are currently included in the superorder Rhabditica (Hodda, 2022a). Many nematodes of this clade successfully behave as parasites and extremophiles by developing a modified juvenile stage with a chemically impermeable cuticle explicitly adapted for long-term survival (De Ley, 2006). This dauer stage, named infective juvenile (IJ) for EPNs, persists in the soil until it locates and penetrates within suitable hosts (Figure 3). The IJs then release pathogenic bacteria they carry into the hemocoel. Bacteria proliferate exponentially, killing the host within 48–72 h of infection.

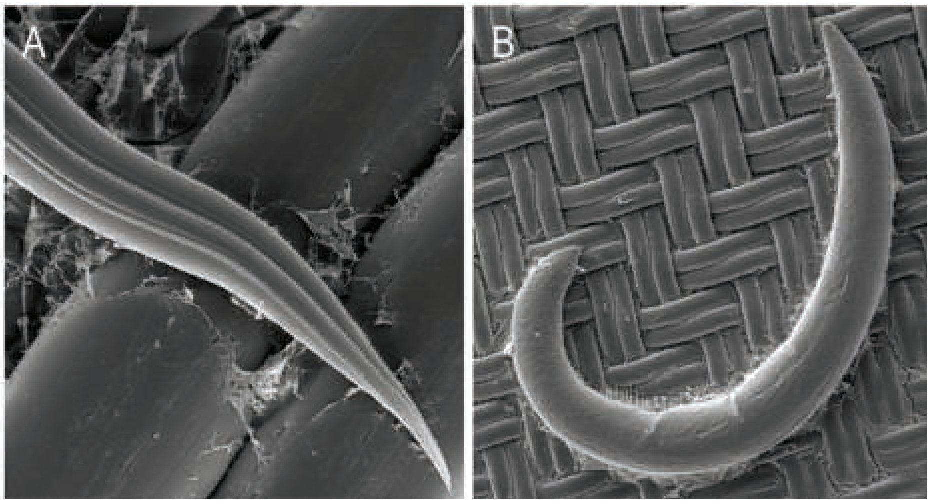


Figure 3. The entomopathogenic nematode *Steinernema carpocapsae*: (A) tail of an infective juveniles and (B) and an adult male. Photos provided with permission by Raquel Campos Herrera from Instituto de Ciencias de la Vid y del Vino, Spain.

Nematodes reproduce and complete several generations feeding on the cadaver and bacteria until sources deplete. Finally, a new generation of IJs reassociate some of the symbiont bacteria and leave the insect cadaver by thousands to start a new life cycle (Dillman et al., 2012a; Stock, 2015) (Figure 44).

Although this basic scheme is valid for all EPNs, steinernematids and heterorhabditids are phylogenetically distant, which share similarities due to convergent evolution (Blaxter et al., 1998). Shortly before, Poinar (1993) pointed in the same direction by suggesting that mutualistic relationships with Gram-negative bacteria began for nematodes in these two families independently about 350 million years ago. Based on Blaxter et al. (1998), steinernematids are closely related to free-living and insect associate nematodes in Panagrolaimoidea and vertebrate parasites in Strongyloididae. Indeed, according to Hodda (2022a), steinernematids no longer belong to the order Rhabtida but Panagrolaimida (Figure 55). On the other hand, heterorhabditids are most closely related to vertebrate parasites in Strongylidae (Blaxter et al., 1998). Accordingly, several attributes distinguish members of both genera. Many of these distinctions are outlined in the IJ stage for practical reasons. For example, the position of the excretory pore is anterior to the nerve ring in steinernematids and posterior in heterorhabditids, and the tail shape is conoid or filiform in steinernematids and

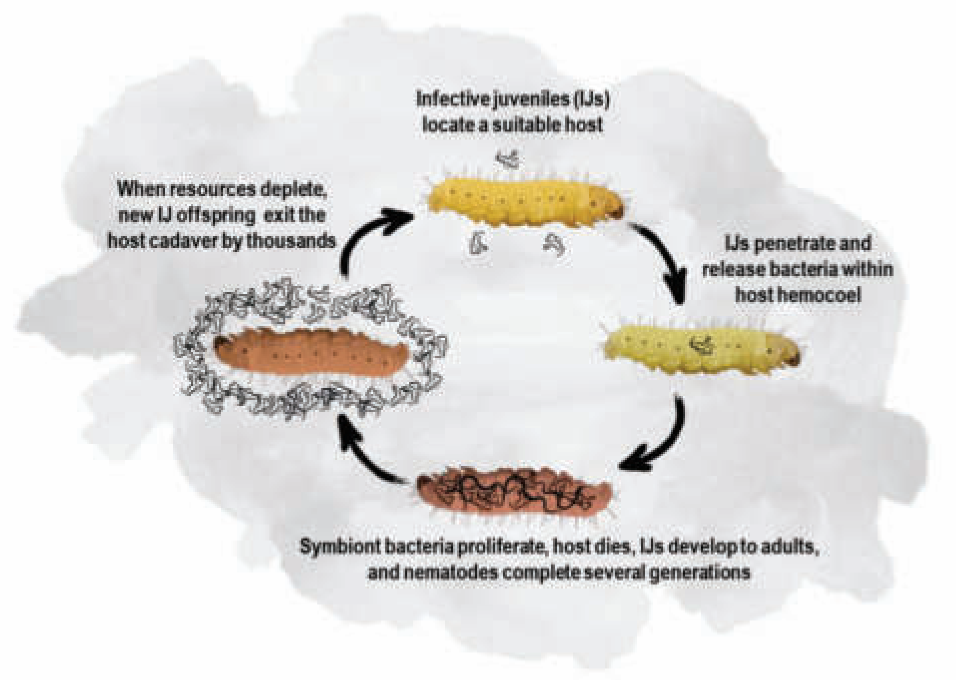


Figure 4. The life cycle of entomopathogenic nematodes.

pointed in heterorhabditids. Heterorhabditid IJs also exhibit a prominent dorsal tooth anteriorly in the head and a protective second-stage cuticle (Timper and Kaya, 1989; Adams and Nguyen, 2002). In addition, steinernematids are gonochoristic except for very few exceptions (Griffin et al., 2001; Stock et al., 2004), while heterorhabditids have a hermaphroditic first adult generation with similar morphology to females, followed by a gonochoristic second-generation (Stock, 2015). Even if some hermaphrodites deposit eggs, these usually develop inside in a process known as *endotokia matricida* that involves juvenile stages feeding on the adult intestine and symbiont bacteria (Ciche et al., 2008; Stock et al., 2012). This intrauterine development of juveniles is widespread in nematodes, but its incidence depends on the different feeding habitats of the species and environmental conditions (Baliadi et al., 2001). Thus, *endotokia matricida* also occurs in steinernematids when overcrowding and limited food resources make this strategy more effective than laying eggs by females (Bastidas et al., 2014).

- Subclass **Plectia** Hodda, 2007
  - Superorder **Rhabditica** Hodda, 2007
    - Order **Panagrolaimida** Hodda, 2007
      - Suborder **Panagrolaimina** Hodda, 2007
        - Superfamily **Steinernematoidea** Filipjev, 1934 (Hodda, 2007)
          - Family **Steinernematidae** Filipjev, 1934 (Chitwood & Chitwood, 1937)
            - Subfamily **Steinernematinae** Filipjev, 1934
              - Tribe **Steinernematini** Filipjev, 1934
                - Subtribe **Steinernematinii** Filipjev, 1934
                  - Genus **Steinernema** Travassos, 1927 (145 species)
      - Suborder **Cephalobina** Andrassy, 1974
        - Superfamily **Cephaloboidea** Filipjev, 1934
          - Family **Cephalobidae** Filipjev, 1934
            - Subfamily **Acrobelinae** Thorne, 1937
              - Tribe **Acrobelini** Thorne, 1937
                - Subtribe **Acrobelinii** Thorne, 1937
                  - Genus **Acrobeloides** Cobb, 1924 (26 species)
    - Order **Rhabditida** Chitwood, 1933
      - Suborder **Rhabditina** Chitwood, 1933
        - Superfamily **Rhabditoidea** Oerley, 1880 (Travassos, 1920)
          - Family **Rhabditidae** Oerley, 1880
            - Subfamily **Oryctonematinae** n. subfam.
              - Tribe **Oscheini** n. tribe
                - Subtribe **Oscheinii** n. subtribe
                  - Genus **Oscheius** Andrassy, 1976 (58 species)
        - Superfamily **Heterorhabditoidea** Poinar, 1975
          - Family **Heterorhabditidae** Poinar, 1975
            - Subfamily **Heterorhabditinae** Poinar, 1975
              - Tribe **Heterorhabditini** Poinar, 1975
                - Subtribe **Heterorhabditinii** Poinar, 1975
                  - Genus **Heterorhabditis** Poinar, 1976 (27 species)
    - Order **Diplogasterida** Inglis, 1983
      - Suborder **Diplogasterina** Paramonov, 1952
        - Superfamily **Diplogasteroidea** Micoletzky, 1922
          - Family **Diplogasteridae** Micoletzky, 1922
            - Subfamily **Diplogasterinae** Micoletzky, 1922
              - Tribe **Diplogasterini** Micoletzky, 1922
                - Subtribe **Diplogasterinii** Micoletzky, 1922
                  - Genus **Pristionchus** Kreis, 1932 (79 species)

Figure 5. Taxonomic classification of the entomopathogenic nematode genera *Steinernema* and *Heterorhabditis*, and the free-living nematode genera *Acrobeloides*, *Oscheius*, and *Pristionchus*, according to Hodda (2022a).

The symbiont bacteria

The relationships that EPNs establish with their symbiont bacteria, considered mutualistic and obligate under natural conditions (Stock, 2015), also differentiate these two nematode groups. Steinernematids and Heterorhabditids associate mutualistically with γ-proteobacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, both belonging to the order Enterobacterales and the family Morganellaceae (Boemare, 2002; Adeolu et al., 2016) (Figure 66). As these nematode-bacterium complexes, symbiotic associations of this kind have occurred multiple times for other members within the order Enterobacterales (Husník et al., 2011). However, *Xenorhabdus* and *Photorhabdus* provide their host more benefits than other bacterial symbionts (Russell, 2019), such as a source of nutrients, defense against competitors, and life-cycle signals (Maher et al., 2021). Indeed, a considerable loss of ancestral genes characterizes the species of both genera, particularly Xenorhabdus, probably due to a more refined adaptation to their respective ecological niches (Tobias et al., 2017). In the same direction, steinernematid IJs harbor symbiont bacteria in a specialized structure called the receptacle, formed by adapting the two most anterior intestinal cells (Flores-Lara et al., 2007; Snyder et al., 2007), while Photorhabdus adhere first to the esophagus–intestinal valve of IJs and then migrate to the intestinal lumen to proliferate (Ciche and Ensign, 2003; Stock, 2015). Although *Xenorhabdus* and *Photorhabdus* diverged more recently from a common ancestor than *Steinernema* and *Heterorhabditis* (Chaston et al., 2011), some phenotypic traits differentiate both genera: *Xenorhabdus* are catalase-negative while *Photorhabdus* are catalase-positive and the only known terrestrial bioluminescent bacteria (Koppenhöfer, 2007; Mulley et al., 2015).

The nematode-bacterium complexes produce large amounts of secondary metabolites of various chemical types (Tobias et al., 2017). These natural products are involved in diverse functions such as neutralizing the host immune system, supporting the development of nematodes, and shielding the insect cadaver from competitors thanks to their antibacterial, antifungal, and nematicidal activities (Crawford et al., 2012; Stock et al., 2017; Dreyer et al., 2018; Shi and Bode, 2018; Kusakabe et al., 2022). Moreover, symbiont bacteria also produce repellent compounds of an unknown nature until recent studies (Kajla et al., 2019; Jaffuel et al., 2022) to deter a broad range of scavengers, running from insects to birds and even fishes (Baur et al., 1998; Ramalingam et al., 2017, 2021).

The relationship between nematode and bacteria was considered, until relatively recently, species-specific (Adams et al., 2006). Indeed, the color of hosts colonized by EPNs, given by their symbiont bacteria, could serve as a distinct criterion for nematode species identification for both genera *Steinernema* and *Heterorhabditis* (Booysen et al., 2022) (Figure 77). If, as stated by the *Gause's Competitive Exclusion Principle*, species with identical niches cannot coexist indefinitely (Kneitel, 2008), it was supposed that each nematode species was associated with one bacterial species, a presumption that still appears to be mainly valid for the *Steinernema*-*Xenorhabdus* complex (Murfin et al., 2015). On the contrary, much evidence confirms that heterorhabditids, both isolated from

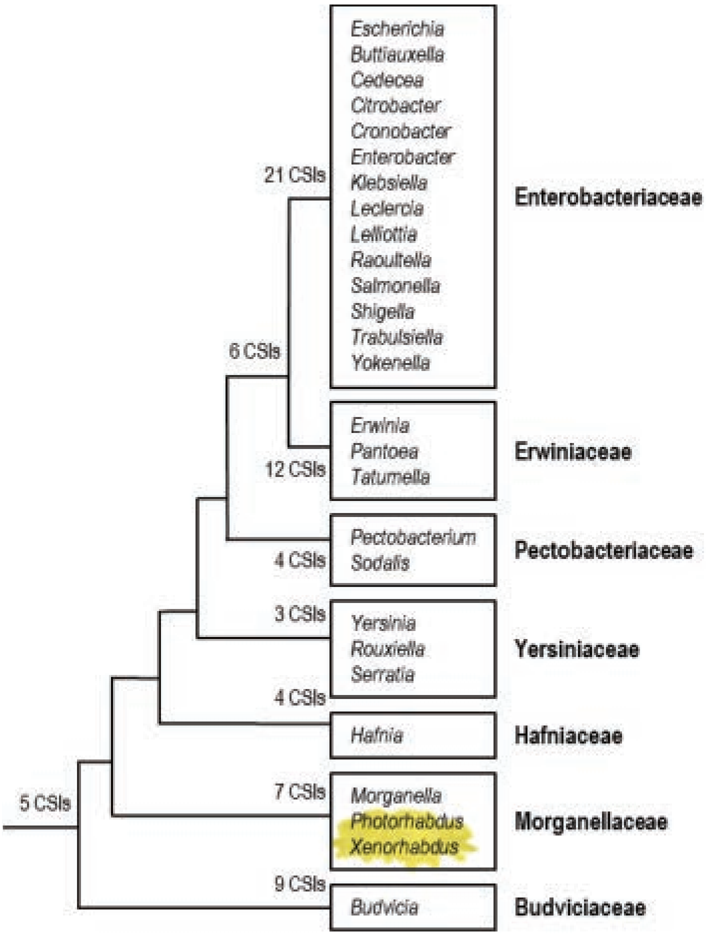


Figure 6. Summary diagram, modified from Adeolu et al. (2016), illustrating the distribution of conserved signature insertions/deletions (CSIs) for families (indicating some of the most representative genera of each family) within the order Enterobacteriales.

distant regions (Maneesakorn et al., 2011; Kazimierczak et al., 2017) or, although more rarely, from the same localities (Tóth and Lakatos, 2009; Maher et al., 2017; James et al., 2018), can associate different *Photorhabdus*. In a recent study, Maher et al. (2021) observed the co-existence of *Photorhabdus cinerea* and *P. temperata* in insect cadavers colonized by *Heterorhabditis downesi* collected in dune systems in Ireland. They suggested that different bacterial species could give differential benefits for a single niche under heterogeneous competitive conditions; in this case, *P. cinerea* providing better protection against desiccation and microbes, and *P. temperata* against scavengers that use their vision to forage for food.

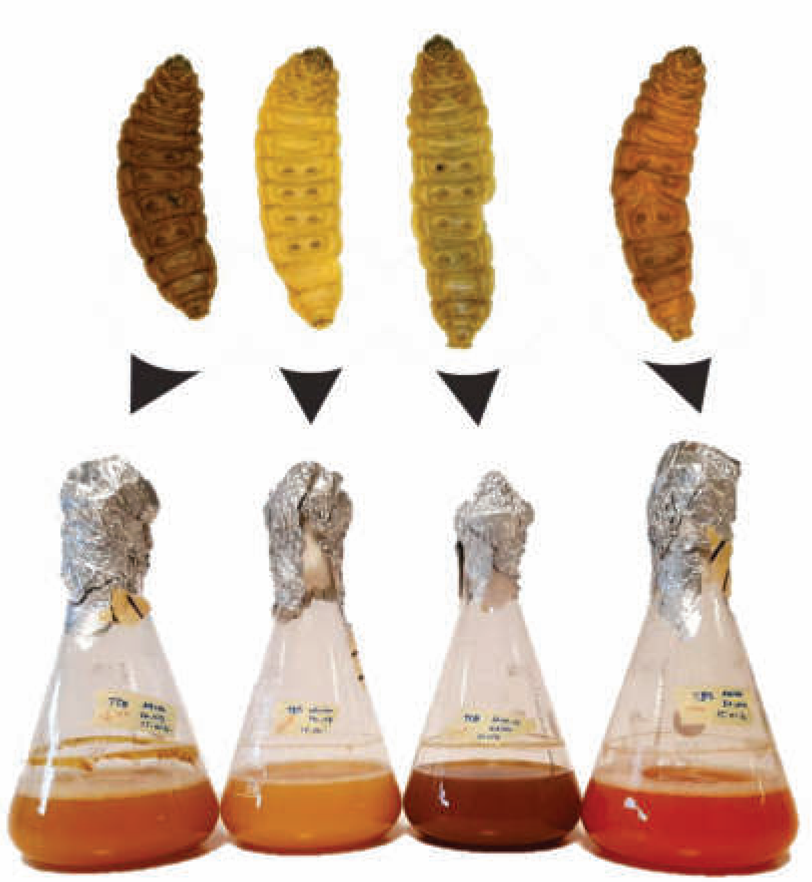


Figure 7. From left to right, distinctive coloration of *Galleria mellonella* larvae colonized by the entomopathogenic nematode espies *Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, and *Heterorhabditis bacteriophora*, and the culture of their symbiont bacteria *Xenorhabdus bovienii*, *X. nematophila*, *X. kozodoii*, and *Photorhabdus laumondii*. Photos by Rubén Blanco Pérez and Ignacio Vicente Díez from Instituto de Ciencias de la Vid y del Vino, Spain.

About habitat preference and abiotic drivers

As representative specimens of the phylum Nematoda, EPNs occur globally in soils of all continents except Antarctica, from coastal areas to mountains, from natural areas to agroecosystems (Mráček et al., 2005; Adams et al., 2006; Kergunteuil et al., 2016). Numerous surveys, achieved mainly in the last few decades, allowed researchers to find new EPN species and comprehend their distribution. Indeed, before the publication by Gaugler and Kaya (1990) of the book *Entomopathogenic Nematodes in Biological Control*, a milestone for entomopathogenic nematology, only 11 EPN species were described, 9 of them steinernematids (Hominick, 2002). Since then, these numbers have grown exponentially to over 100 species validated nowadays (Hunt, 2007; Nguyen et al., 2007; Bhat et al., 2020; Hodda, 2022a) (Figure 8). Some species (e.g., *Steinernema carpocapsae*, *S. feltiae*, *Heterorhabditis bacteriophora*, or *H. indica*) occur worldwide, and some others (e.g., *S. fabii* in South Africa, *S. huense* in Vietnam, or *S. ritteri* in Argentina) are characterized for their limited distribution (Hominick, 2002). Geostatistical techniques such as the *Ecological Niche Model* can today determine the potential distribution of specific EPN species in unexplored areas. For example, *H. indica* was found on the South Coast of the Iberian Peninsula (Campos-Herrera et al., 2016b; 2019a) after being predicted by Kour (2017).

EPN distributions show substantial seasonal, habitat, and geographical variability. Regional studies help to identify the environmental factors and soil properties that drive the occurrence of specific EPN species. Even if the IJ is a resistance stage, many abiotic factors can compromise their survival (Stuart et al., 2015). Firstly, nematodes inhabit water-filled pores with sufficient surface tension to allow movement (Wallace, 1971; Neher, 2010). X-ray computed tomography measurements recently ensured that the 20 to 220 µm size range of soil porosity is most suitable for these organisms (Schlüter et al., 2022). Still, IJs can overcome desiccation for 2-3 weeks by using various strategies, such as rolling or aggregating (Simons and Poinar, 1973; Glazer, 2002) or transitioning to quiescent or anhydrobiosis forms (Womersley, 1990).

The availability of habitable pores for nematodes depends on other factors like soil moisture and texture, formation of aggregates, or bioturbation by soil fauna and roots (Stuart et al., 2015). For example, many studies observed reduced EPN mobility and virulence in high clay soils (Kung et al., 1990a; Koppenhöfer and Fuzy, 2007; Ensafi et al., 2018; Lankin et al., 2020). Indeed, several surveys

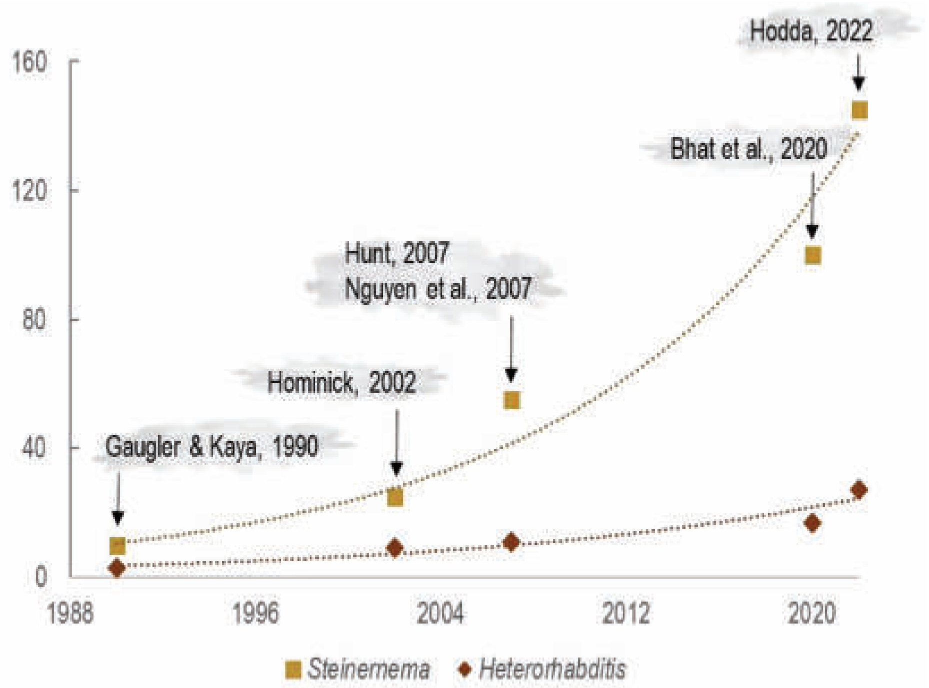


Figure 8. Exponential growth in the number of identified species of entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* over the last three decades.

(Campos-Herrera et al., 2013b, 2016a, 2019; Jaffuel et al., 2018) and greenhouse and manipulative field experiments (El-Borai et al., 2012; Duncan et al., 2013) highlighted the sand fraction as one of the main drivers of EPN natural occurrence. Compared with sandy soils, the low porosity of heavy soils prevents optimal aeration and mobility of soil organisms, including nematodes (Barbercheck, 1992), and the diffusion of specific volatile organic compounds (VOCs), such as those employed by plants as a cry-for-help signal that EPNs can recognize in the search for suitable hosts (Chiriboga et al., 2017; Som et al., 2017). The dimensions, shape, and composition of soil aggregates also define the proper formation of soil pores to provide optimal environments for edaphic organisms (Barbercheck, 1992; Portillo-Aguilar et al., 1999). Thus, most soil mesofauna inhabits pores among macroaggregates (approximately 0.1-2 µm in diameter), while microfauna occurs in micropores (with diameters less than 2 nm) comprised within the aggregates themselves (Elliott and Coleman, 1988; Schlüter et al., 2022). Oxygen content also decreases in-depth more sharply in clay or silty soils (Brady and Weil, 2013). Low oxygen concentrations reduce the ability of IJs to colonize and kill hosts (Kung et al., 1990b), with recovery times proportional to the period subjected to stress (Qiu and Bedding, 2000).

Suboptimal soil temperatures also restrict the EPN fitness, limiting the movement or penetration of IJs within the host or acceptable bacterial growth after colonization (Grewal et al., 2006). However, EPNs exhibit diverse mechanisms to persist in extreme temperatures, such as trehalose accumulation (Wharton and Surrey, 1994; Brown and Gaugler, 1997) or expression of *Heat-Shock Proteins* (Selvan et al., 1996), for cold or heat tolerance, respectively. Furthermore, soil pH could compromise the efficiency of EPNs as BCAs (Barbercheck, 1992), even though *in vitro* experiments have shown that EPNs, particularly steinernematids, survive a broad pH spectrum (Kung et al., 1990b; Khathwayo et al., 2021). However, the high presence of ions in the soil can activate cascade reactions with detrimental effects on EPNs. For instance, high contents of exchangeable H^+ in acid soils can cause the degradation of VOCs linked to plant-EPN communication (Som et al., 2017), or react with heavy metals, releasing possible toxic compounds for EPNs (Jaworska et al., 1996, 1997; Brady and Weil, 2013).

Soil food web and other biotic interactions

Nematodes may interact with myriad organisms, from micro and macrofauna to the root systems that connect them in the soil, arguably the most complex and diverse ecosystem on Earth (Wall and Moore, 1999; Cavigelli et al., 2012; Orgiazzi et al., 2016). IJs respond in various ways to the habitat, such as the *stimuli* from host and other soil-dwelling organisms that may be narrowly specific and presumably improve their chances of surviving in soils (Ishibashi and Kondo, 1990; Dillman et al., 2012b). EPN occurrence primarily depends on host availability (Mráček et al., 2005). Although laboratory studies suggested a broad host range for EPNs (Peters, 1996), it seems more restricted under natural conditions due to spatial or temporal distributions (Stuart et al., 2015). Beyond the pathogen-host relationship, *in vitro* experiments have proved that EPNs scavenger in insect cadavers (San-Blas and Gowen, 2008; Půža and Mráček, 2010) even in the presence of potential competitors (Blanco-Pérez et al., 2017). To what extent this behavior implies a biological significance under natural conditions is a question that will require further investigation. So far, there is only evidence that scavenging competition within the insect cadaver between specific EPN and free-living nematode (FLN) species could compromise the fitness of emerging IJs (Blanco-Pérez et al., 2019) (Figure 99).

Numerous studies have documented positive interactions of EPNs with other soil inhabitants in their environment, including other pathogens of insects. For example, synergistic effects have been reported for combined EPN applications with nuclear polyhedrosis virus (Narayanan and Gopalakrishnan, 2003) or diverse bacterial strains of *Bacillus thuringiensis* (Bacillales: Bacillaceae) (Kaya et al., 1995; Kaya and Koppenhöfer, 1996). Under specific circumstances, also entomopathogenic fungi can display additive and synergistic relationships with EPNs, as observed for the combinations of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) and *S. kraussei* (Ansari et al., 2010) and *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and different steinernematid and heterorhabditid species (Shaurub et al., 2016; Gulzar et al., 2021). Moreover, as shown by Bueno-Pallero et al. (2018) for co-formulations of *S. feltiae* IJs with *Arthrobotrys musiformis* (Orbiliales: Orbiliaceae) and *Purpureocillium lilacinum*

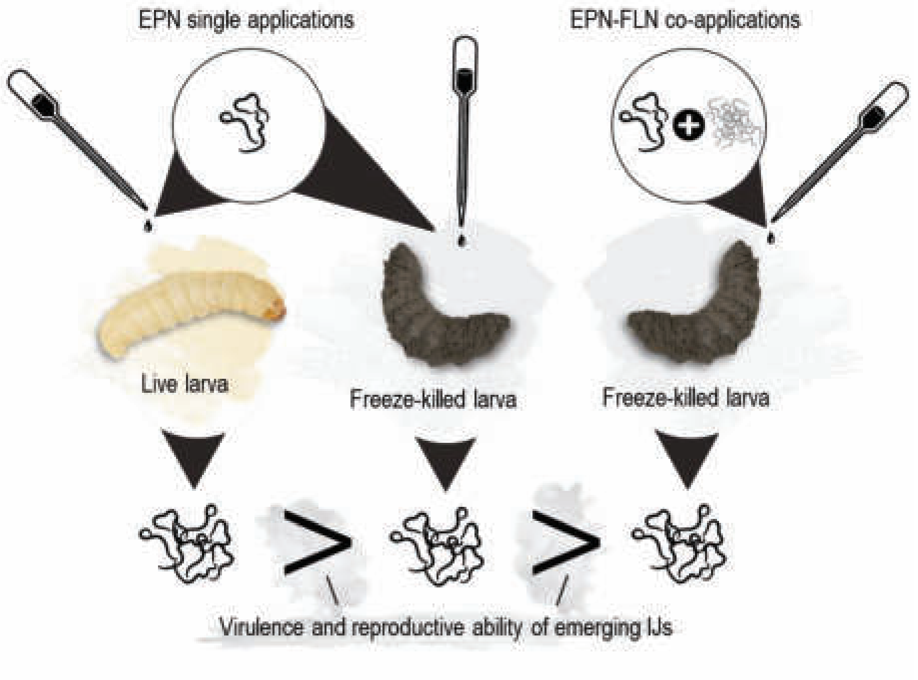


Figure 9. Schematic representation of the loss of fitness of infective juveniles (IJs) emerging from freeze-killed *Galleria mellonella* larvae, colonized by entomopathogenic nematodes (EPNs) alone or together with free-living nematodes (FLNs), compared to IJs emerging after completing the regular pathogenic cycle of EPNs (Blanco-Pérez et al., 2019).

(Hypocreales: Ophiocordycipitaceae), EPNs can establish beneficial associations even with nematophagous fungi (NF). Field applications mixing EPNs and parasitoids also reported synergistic effects, for example, *Steinernema scapterisci* and *Ormia depleta* (Diptera: Tachinidae) against the mole cricket *Scapteriscus* spp. (Orthoptera: Gryllotalpidae), or *S. carpocapsae* and *Mastrus ridibundus* and *Liotryphon caudatus* (Hymenoptera: Ichneumonidae) against the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) (Parkman and Frank, 2002; Lacey et al., 2003), respectively. On the other hand, earthworms and roots, for instance, can help the dispersion of EPNs by mixing the soil (Shapiro-Ilan et al., 1995; Shapiro-Ilan and Brown, 2013) and releasing chemical exudates that guide IJs to suitable hosts (Rasmann et al., 2005; Jaffuel et al., 2015; Willett et al., 2017).

Natural enemies and competitors

Although numerous in vitro experiments have revealed the potential of organisms of many kinds to compromise EPN fitness, no viruses or bacteria have been described as their competitors or natural enemies in complex soil environments (Kaya, 2002; Strong, 2002). However, laboratory experiments showed the establishment of bacteria attacked on the IJ surface concomitantly with the symbiont bacteria within the cadaver (Poinar, 1979; Molyneux et al., 1983; Bonifassi et al., 1999). Among them, ectoparasitic bacteria (EcPB) in the genus *Paenibacillus* (Bacillales: Paenibacillaceae) are particularly interesting because of the detrimental effect of endospores on IJs by adhering to the cuticle (Figure 1010), limiting their ability to migrate and hence, encounter hosts (El-Borai et al., 2005; Enright and Griffin, 2005). Even if *Paenibacillus* spp. seemingly showing low specificity to EPNs (Enright et al., 2003; Campos-Herrera et al., 2019a), there is evidence that these bacteria can modulate their abundance in nature. Thus, Campos-Herrera et al. (2019b) observed a higher frequency of bacterial endospores attached to the cuticle of *Steinernema diaprepesi* at alkaline sites in Florida and specific predator-prey dynamics between the two organisms.

On the other hand, it is well known that several invertebrates, including mites, collembolans, and tardigrades, can feed on EPNs (Poinar, 1979; Ishibashi et al., 1987; Read et al., 2006; Helmberger et al., 2018). These soil inhabitants have high rates of development, survival, dispersal, and reproduction (some of them, such as mesostigmatid mites, can do so by pathogenesis), are voracious feeders, and can show specificity towards nematodes (Stuart et al., 2015). All these properties define the effectiveness of a natural enemy (Pianka, 1999). Indeed, Walter et al. (1987) observed that mites develop and reproduce faster when feeding on nematodes than on arthropods. New molecular approaches for species identifications reported, for instance, an inverse correlation between mite and heterorhabditid abundances in natural conditions (Dritsoulas et al., 2021). However, some field studies provided contrasting results, not finding solid associations between microarthropod and EPN populations (Duncan et al., 2007; Jabbour and Barbercheck, 2011). Macroinvertebrates could also negatively affect the EPN community. Laboratory experiments have shown, for example, that the feeding and release of cutaneous excreta of earthworms can compromise the fitness of specific EPN species (Campos-Herrera et al., 2006; Chelkha et al., 2020, 2021).

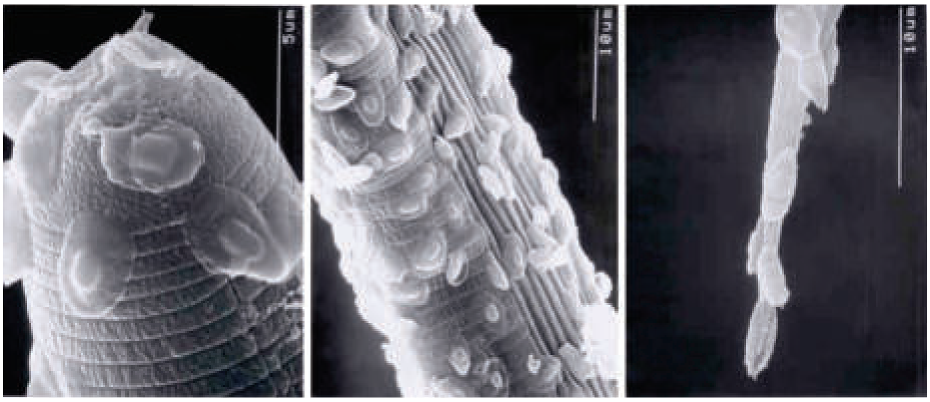


Figure 10. *Paenibacillus* sp. attached to the head, body, and tail (from left to right) of infective juveniles of *Steinernema diaprepesi*. Photo provided with permission by Dr. Fahiem El-Borai Kora from University of Florida, USA.

Certain fungal species deserve special attention in this respect. Found in all main taxonomic groups, NF survive as saprophytes in widespread soils but switch to parasitic stages in the presence of nematodes, developing specialized structures to prey on them (López-Llorca et al., 2007). Fungi of this type fall into three categories: nematode-trapping, endoparasitic, and egg/cyst-parasitic, which evolved independently as an adaptation to overcome competition for soil nutrients (Barron, 1992; Nordbring-Hertz et al., 2006). Trapping NF of the genus *Arthrobotrys* (Orbiliales: Orbiliaceae) are predators which form three-dimensional nets (e.g., *A. oligospora* and *A. musiformis*) or constricting rings (e.g., *A. dactyloides*) (Figure 11) to catch nematodes by adhesion or mechanically, respectively (Nordbring-Hertz et al., 2006). The endoparasites, which are more obligate parasites of nematodes than members of the other groups, produce

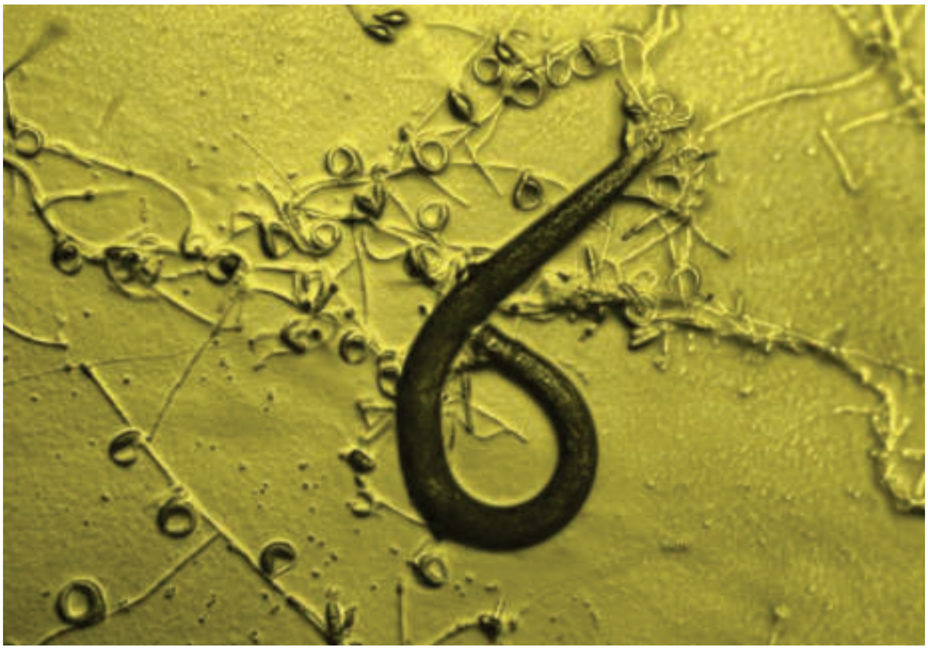


Figure 11. Constricting rings of *Arthrobotrys dactyloides* trapping an infective juvenile of *Steinernema diaprepesi*. Photo provided with permission by Dr. Larry W. Duncan from University of Florida, USA.

spores to infect them, for example, conidia by *Hirsutella* spp. (Globigerinida: Globorotaliidae) or zoospores by *Catenaria* spp. (Blastocladiales: Catenariaceae). (López-Llorca et al., 2007). Lastly, parasitic fungi of nonmotile nematode stages, like eggs or cysts, produce toxins and employ appressoria, a specialized flattened hypha used by many fungal pathogens to favor adhesion to hosts, before penetrating the eggshell. The species *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae), isolated from a wide range of habitats, can follow this strategy on nematode eggs and, occasionally, females of root-knot and cyst of plant-parasitic nematodes, so it has often been detected in the rhizosphere of many crops (Domsch et al., 1982; Money, 1998). Trapping-NF also appear frequently in the rhizosphere, perhaps due to root exudations linked to plant-parasitic nematode damages (Nordbring-Hertz et al., 2006). Even if correlations between NF and nematodes in field studies have been unclear (e.g., Persmark et al., 1996), there is evidence that high NF abundance could alter the EPN community in various crops (Campos-Herrera et al., 2015b; Pathak et al., 2017). EPN vulnerability to NF seems species-specific and environmental dependent, but the mechanisms behind their interactions are mainly unknown (Koppenhöfer et al., 1996; El-Borai et al., 2009; Bueno-Pallero et al., 2018). Plausibly, migration in the soil profile helps EPNs escape from NF, which would explain their absence in certain areas (Campos-Herrera et al., 2019b).

Among the most relevant antagonism effects for EPNs is competing for sources with entomopathogens of a different nature. Numerous studies approached the competition, understood as any mutually negative interaction not directly involving predation or parasitism (Wootton, 1994; Pianka, 1999), between EPNs and other BCAs. For example, co-infections of steinernematids and specific viruses (e.g., Nucleopolyhedrovirus, Granulovirus) adversely impact their offspring due to the disintegration of the insect carcass (Kaya and Brayton, 1978; Bednarek, 1986). In the same line, dual infections of EPNs and bacteria are infrequent and highly dependent on application timing, as an organism's colonization usually prevents the settlement within the host of others (Kaya and Burlando, 1989; Darissa and Iraki, 2013). Indirect antagonism of this kind also occurs for combined applications of EPNs and entomopathogenic fungi (EPF) since both BCAs produce specific natural products to prevent competition (Strasser et al., 2000; Tarasco et al., 2011; Lewis et al., 2015; Shi and Bode, 2018). In addition, several *in vitro* studies observed EPNs avoiding EPF-infected hosts and preferring other targets if available (Barbercheck and Kaya, 1991; Lezama-Gutiérrez et al., 1996). However, recent evidence suggested that the nature of the EPN-EPF interactions could be more species-specific (or even strain/population-specific) than previously thought. Thus, *Heterorhabditis heliothidis* and *S. carpocapsae* failed to reproduce (Barbercheck and Kaya, 1990), but not *S. feltiae* (Bueno-Pallero et al., 2018), in bioassays conducted with *G. mellonella* larvae previously inoculated with the EPF *B. bassiana*.

Nematode competition

Nematodes are essential components of soil food webs, so EPNs suffer, for instance, predation from some of them, such as species in the genera *Clarkus* (Mononchida: Mononchidae) and *Actinolaimus* (Dorylaimida: Actinolaimidae) (Ishibashi et al., 1987), but also exploitative competition by sharing resources with FLNs. For example, the presence of some FLN populations of the genera *Acrobeloides* (Panagrolaimida: Cephalobidae) and *Oscheius* (Rhabditida: Rhabditidae) within hosts caused a decrease in fitness for specific EPN species (Campos-Herrera et al., 2012a; Blanco-Pérez et al., 2019; Salari et al., 2021). Molecular analyses have also verified that several FLN species of these genera and *Pristionchus* (Diplogasterida: Diplogasteridae) (Figure 12) often co-emerged with

IJs from cadavers recovered from insect baits (Campos-Herrera et al., 2015b, 2019a; Jaffuel et al., 2016, 2018). However, since diverse FLNs might interact with EPNs in diverse ways, the complex connections they assemble in nature are challenging to interpret. Thus, Duncan et al. (2003) reported increased numbers of the FLN *Pellioditis* sp. (Rhabditida: Rhabditidae) emerging from *Diaprepes abbreviatus* (Coleoptera: Curculionidae) larvae infected by *Steinernema riobrave*, introduced in citrus groves of Florida as a BCA, but not from those colonized by native *S. diaprepesi* IJs.

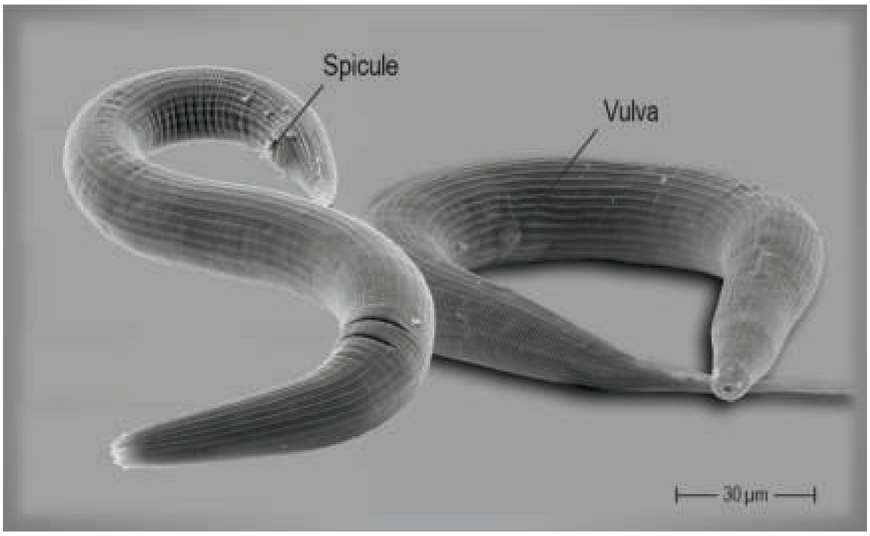


Figure 12. Male (left) and female (right) adults of the free-living nematode *Pristionchus pacificus*. Photos provided with permission by Dr. Ralf Sommer from the Max Planck Institute for Developmental Biology, Germany.

All these 'rhabditid' and diplogastrid FLN species (Figure 5) seemingly share an ancestral lineage with insect-parasitic nematodes, including EPNs (Blaxter et al., 1998). As mentioned above, this lineage of nematodes exhibits dauer stages that, according to the *concept of preadaptation*, can solve some of the problems that saprobic soil dwellers must face to become endoparasites (Sudhaus, 2008). This resistant, non-feeding third-stage juvenile is an adaptation of Chromadorea to terrestrial habitats that often became the phoretic stage in rhabditoid nematodes (Sudhaus, 2008; Blaxter and Koutsovoulos, 2014). Phoresy indeed allows them to colonize patchily distributed ephemeral habitats using insects for transportation (Félix et al., 2018). According to the *Dauer Hypothesis*, dauer stages can effectively adapt to being transported inside insect cavities (endo-phoresy) and then, step by step, switch to different host associations:

entoecy, necromeny, and, finally, entomopathogeny (Rogers and Sommerville, 1963; Sudhaus, 2008) (Figure 133). This hypothesis might explain the versatility of roles attributed to different 'rhabditid' and diplogastrid species. These nematodes have been described by establishing associations with insects in all the defined cathegories: (endo-) phoresy (e.g., *Oscheius dolichurus*), (facultative) necromeny (e.g., *Pristionchus maupasi*), and even pathogenicity (e.g., *Oscheius carolinensis*, *O. onirici*) (Herrmann et al., 2006; Kiontke and Sudhaus, 2006; Ye et al., 2010; Torrini et al., 2015).

There is also competition within the host cadaver among EPN species or populations. Bioassays with model insect larvae exposing different IJ species, or inoculating them into the hemocoel, showed that steinernematids appeared to show a competitive advantage over heterorhabditids (Alatorre-Rosas and Kaya, 1990; 1991). The authors supposed that even if dual infections eventually occurred, only one of the EPN species would have a chance of reproduction, presumably *Steinernema* spp. since *Xenorhabdus* spp. release natural products toxic to *Photorhabdus* spp. (Boemare, 2002). Similarly, it was widely accepted

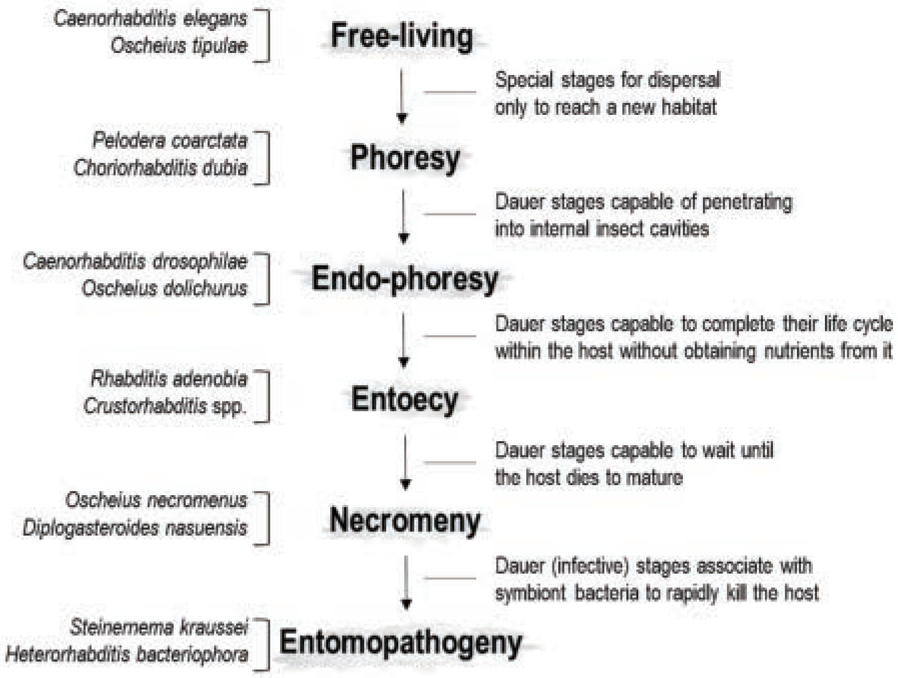
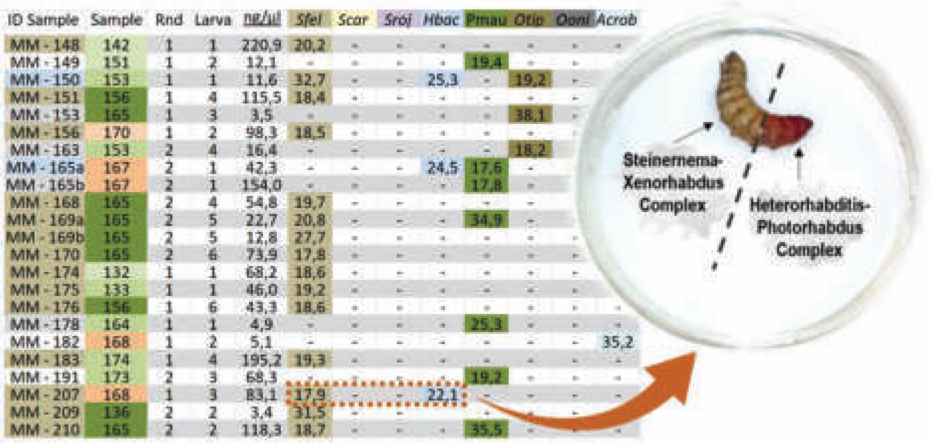


Figure 13. Evolution of nematode–host associations in rhabditoids according to the *Dauer Hypothesis*. Some of the most characteristic species for each adaptive step of their dauer juveniles are shown on the left. Simplified diagram adapted from Sudhaus (2008).

that always one *Steinernema* species predominates over another when co-infecting a host, presumably due to differences in specificity and growth capacity of its symbiotic bacteria (Koppenhöfer et al., 1995; Sicard et al., 2006; Půža and Mráček, 2009; Bashey et al., 2013). However, molecular analyses have recently probed that both FLNs and EPN species of diverse genera can successfully complete their development within the same host cadaver (Campos-Herrera et al., 2015a, 2015b, 2019a; Jaffuel et al., 2016, 2017) (Figure 144). Finally, male steinernematids have recently been observed to (at least) physically injuring males of their own species and males and females of other species when competing for hosts (O'Callaghan et al., 2014 ; Zenner et al., 2014), conceivably to mate females of better quality (Kapranas et al., 2020).



ID Sample	Sample	Rnd	Larva	ng/µl	*Sfel*	*Scor*	*Sroj*	*Hbac*	Pmau	*Otip*	Ooni	*Acrob*
MM - 148	142	1	1	220,9	20,2	-	-	-	-	-	-	-
MM - 149	151	1	2	12,1	-	-	-	-	19,4	-	-	
MM - 150	153	1	1	11,6	32,7	-	-	25,3	-	19,2	-	
MM - 151	156	1	4	115,5	18,4	-	-	-	-	-	-	
MM - 153	165	1	3	3,5	-	-	-	-	-	38,1	-	
MM - 156	170	1	2	98,3	18,5	-	-	-	-	-	-	
MM - 163	153	2	4	16,4	-	-	-	-	-	18,2		
MM - 165a	167	2	1	42,3	-	-	-	24,5	17,6	-		
MM - 165b	167	2	1	154,0	-	-	-	-	17,8	-		
MM - 168	165	2	4	54,8	19,7	-	-	-	-	-		
MM - 169a	165	2	5	22,7	20,8	-	-	-	34,9	-		
MM - 169b	165	2	5	12,8	27,7	-	-	-	-	-	-	
MM - 170	165	2	6	73,9	17,8	-	-	-	-	-	-	
MM - 174	132	1	1	68,2	18,6	-	-	-	-	-	-	
MM - 175	133	1	1	46,0	19,2	-	-	-	-	-	-	
MM - 176	156	1	6	43,3	18,6	-	-	-	-	-	-	-
MM - 178	164	1	1	4,9	-	-	-	-	25,3	-	-	-
MM - 182	168	1	2	5,1	-	-	-	-	-	-	-	35,2
MM - 183	174	1	4	195,2	19,3	-	-	-	-	-	-	-
MM - 191	173	2	3	68,3	-	-	-	-	19,2	-	-	-
MM - 207	168	1	3	83,1	17,9	-	-	22,1	-	-	-	-
MM - 209	136	2	2	3,4	31,5	-	-	-	-	-	-	-
MM - 210	165	2	2	118,3	18,7	-	-	-	35,5	-	-	-

Figure 14. Database table (left) representing Ct values obtained for qPCR analysis of nematode emergences from insect larvae used in Koch's postulates after insect baiting. Cases of co-emergence of nematodes of different natures were observed (e.g., large numbers of the free-living nematode *Oscheius tipulae* (Otip) co-emerged with some infective juveniles of the entomopathogenic nematodes (EPNs) *Heterorhabditis bacteriophora* (Hbac) and (in poor numbers) *Steinernema feltiae* (Sfel) from the larva MM-150), but also co-emergences of two EPN species, both with relatively high abundances (e.g., larva MM-207, framed in red). Larvae colonized by different EPN species may show the distinctive colorations of each nematode-bacterium complex at a time (right) (Partial data published in Blanco-Pérez et al., 2022a).

Soil health in vineyards

> *„C'est une triste chose de penser que la nature parle et que le genre humain ne l'écoute pas."*
>
> Victor Hugo

Facing soil conservation in agroecosystems

Decades of evidence have identified anthropogenic activities related to agriculture, climate change, and pollution as the main drivers of global environmental degradation and species extinction in above and belowground ecosystems (Veresoglou et al., 2015; Miličić et al., 2021). Specifically, agricultural soil biodiversity comprises an extraordinary abundance of microorganisms, mesofauna, and macroinvertebrates (Bardgett and van der Putten, 2014; Ramirez et al., 2015). This fact enables, under the influence of the abiotic soil environment, the self-sufficiency of the ecosystem functions of carbon transformation, nutrient cycling, soil structure keeping, and biological regulation of soil populations (Kibblewhite et al., 2008; Gunstone et al., 2021). As an illustration, recent studies showed the role of the nematode community, involved in primary roles in soil food webs, in controlling carbon fluxes in terrestrial ecosystems worldwide (Eisenhauer and Guerra, 2019; van den Hoogen et al., 2019) and how high biodiversity indexes of nematodes and bacteria, for instance, might positively influence ecosystem multifunctionality (Delgado-Baquerizo et al., 2020).

An integrated concept (rather than merely the sum of inputs from a set of individual constituents) of *soil health* arose in recent decades to referring the proper functioning of soil biota assemblage in an ecosystem that supports human land uses (Lehmann et al., 2020). In the view of Kibblewhite et al. (2008), a healthy agricultural soil must be able to sustain the production of goods adequate for human needs and, at the same time, conserve biodiversity and the quality of human life by providing essential ecosystem services to both present and future necessities. Conventional agricultural practices, such as primary tillage and chemical applications, maximize yields at the cost of unbalancing ecosystems and compromising the output of other environmental services (Leigh and Johnston, 1994; Altieri, 1999). Increased land conversion and agricultural intensification have indeed driven soil biodiversity losses to the extent of depleting approximately 60% of soil ecosystem services (Díaz et al., 2006; Veresoglou et al., 2015). Nowadays, agriculture is transitioning towards adopting new practices that reduce the use of agrochemicals and the intensity and depth of tillage and allow for improved fertilization and crop protection while diversifying crops through crop rotation (Christel et al., 2021). To face this challenge, farmers must introduce, among other things, alternative strategies in conservation biological control programs to favor biodiversity in soil crops (Altieri and Nicholls, 2004; Atwood et al., 2022).

Toward reduced tillage regimes

The introduction of mechanical tillage, one of the most significant steps in agricultural intensification, resulted in considerable human labor savings, improved the timing of other farming operations, and ensured a well-prepared seedbed (Kibblewhite et al., 2008). In contrast, powered tillage favors soil compaction and disruption of soil stratification and aggregate stability (Hendrix et al., 1986). As a result, the organic matter becomes available for microbial decomposition and the absence of macroaggregates, as explained above, compromises the proper settlement of mesofauna. Bacteria as primary decomposers imply an acceleration of nutrient cycling, increasing nitrogen mineralization and soil carbon losses, with decomposition and mineralization occurring in deeper areas and involving fewer trophic groups (House and Stinner, 1983; House and Parmelee, 1985; Hendrix et al., 1986). Conversely, the continuous deposition of organic matter stratifies the soil in no-tilled systems, where decomposition occurs mainly near the surface (Doran, 1980). More physically protected from microbiota than in tilled soils, crop residues are more affordable to fungi and mesofauna, which establish complex interactions in patterns similar to those found in forest ecosystems (House and Stinner, 1983; Kibblewhite et al., 2008) (Figure 155). Organisms' size and tillage intensity indeed negatively correlate in soil crops (Wardle, 1995).

In recent decades, agriculture has significantly returned to reduced tillage practices, especially in North and South America (Landers et al., 2003). Long-term studies have shown a progressive decline in soil carbon losses and an improvement of soil aggregate formation in crops managed with a reduced tillage regime, revealing the self-organizing capacity of agroecosystems (Six et al., 1999;Paustian et al., 2000). In addition, the raised activity of macrofaunal

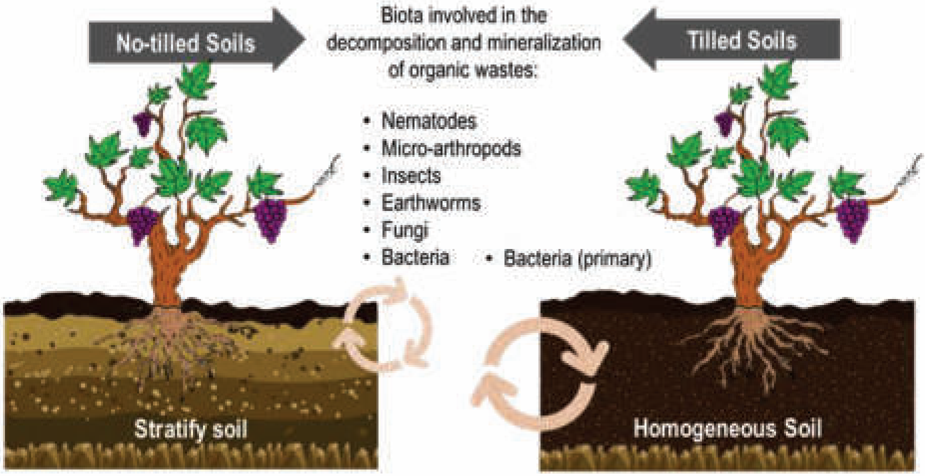


Figure 15. Main physicochemical and biological differences for nutrient cycling processes between tilled and no-tilled crop soils. Figure modified from House and Parmelee (1985), Soil and Tillage Research.

engineers (e.g., fungi, earthworms) appeared to support improving the water cycle functioning of no-tilled soils, such as reduced runoff and increased infiltration and storage (Kibblewhite et al., 2008). Alternative strategies to reduce tillage, like cover cropping or mulching, promote soil health by minimizing erosion, increasing organic matter content and water retention, and improving structural stability in soil crops (Santos et al., 2020), all improvements that, as discussed in detail, enhance biodiversity in agroecosystems.

Pest management and biodiversity

Pest management is a fundamental aspect of agriculture, but it has often focused on maximizing short-term benefits without considering long-term consequences for soil health (Atwood et al., 2022). This conventional agriculture, initiated during the industrial revolution at the end of the 19th century, still provides more than 95% of the world's agricultural yields (Christel et al., 2021). Habitat loss due to agricultural intensification and pollution, principally from the overuse of synthetic pesticides (e.g., insecticides, acaricides, herbicides, fungicides, bactericides) and fertilizers, has been identified as the major driving factor in soil biodiversity declines over the last decade (Sánchez-Bayo and Wyckhuys, 2019; FAO, 2020; Miličić et al., 2021). As a result, several governmental institutions have tried to control the use of agrochemicals through crop management programs, such as the *Integrated Pest Management* (IPM), enforced and regulated by the European Union (EC, 2009). While relying on synthetic organic pesticides, IPM aims to reduce their use by following a series of principles, ranging from preventive and control strategies to interventions, to, for instance, minimize the incidence of potential pests, diseases, and weeds below the *Economic Damage Threshold*, enhance soil health, and retract the development of chemical-resistant pest species (Barzman et al., 2015).

On the other hand, organic farming, initially introduced by Rudolf Steiner in 1924 in Germany to optimize natural resources in agriculture, drastically reduces agrochemical applications to the extent that copper and sulfur are the only natural chemicals allowed for crop protection and organic matter inputs for fertilization (Christel et al., 2021). The European Union regulates organic production and labeling through principles that promote environmental and animal care, biodiversity, and conservation of natural resources (Provost and Pedneault, 2016; EU, 2018). Indeed, a recent meta-analysis assessed that organic viticulture stimulates soil biodiversity over three times more than conventional practices (Karimi et al., 2020). In the last two decades, the area devoted to organic farming has increased nearly four times more in Europe (Figure 166) and fivefold worldwide (Willer et al., 2022), a fact that illustrates the expansion of this crop management type. The success of organic agriculture is thus essential for effectively implementing upcoming policies that claim to protect soil resources, such as those that comprise the European Union Soil Strategy for 2030, consisting of concrete actions to be activated in 2030 to achieve healthy soils by 2050 (EU, 2021).

Vine management

Viticulture and Enology are major socioeconomic sectors worldwide that provide more goods and services to people (e.g., enrich rural livelihoods) and nature (e.g., ensure functional ecosystems) than the production of grapes and wine (Hart et al., 2016; Santos et al., 2020). The world area under vines was estimated in 2020 at 7.3 million hectares, mostly distributed across arid and semi-arid ecosystems (Flexas et al., 2010; OIV, 2020). During the last decades, the rising environmental sensitivity of society, policies, and farmers (Nilsson et al., 2016) has led viticulture to evolve towards more sustainable practices. For example, the area devoted to organic viticulture, as for the other agricultural sectors, has increased fivefold over the over the current century, reaching more

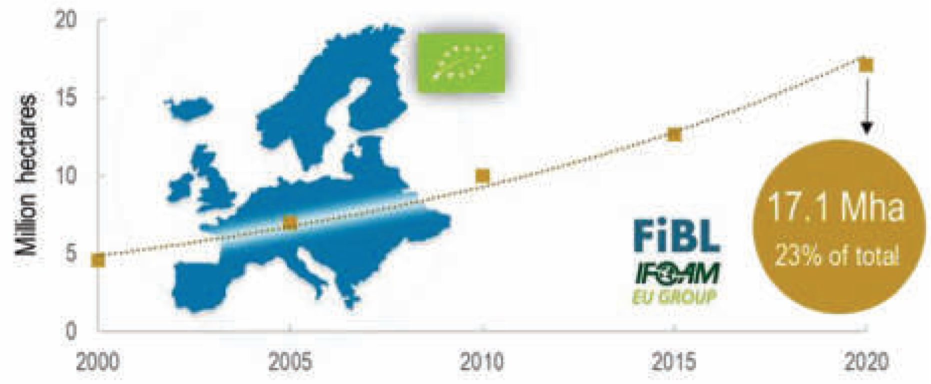


Figure 16. Growth trend of organic agricultural land in Europe during the 21st Century. Source: FiBL-IFOAM surveys 2001-2022 (Willer et al., 2022).

than 5% of the harvested grape area (FAOSTAT, 2018; Willer and Lernoud, 2019). In Europe, this rate rises to almost 10%, thanks to the significant investments in organic farming made by, for instance, the major producing countries: Spain, France, and Italy (Willer and Lernoud, 2019) (Figure 177). However, the generalized use of agrochemicals and tillage continues to make the vineyard one of the most intensively managed crops and erosion-prone land systems, also in Spain and neighboring countries (Nicholls et al., 2008; Rodrigo-Comino et al., 2018; Winter et al., 2018; Karimi et al., 2020).

Mulching and cover cropping in viticulture

For its preservation, grape growers must approach the vineyard as an ecosystem, considering its biodiversity when taking actions to optimize crop productivity (Thies and Tscharntke, 1999). Alternative strategies to tillage are particularly desirable in viticulture, even more so under the current context of climate change (Fraga and Santos, 2018; IPCC, 2018). For instance, shielding vineyard rows with cover crops or using mulches in the lines restrict damages resulting from elevated evaporation rates and soil erosion due to raindrop impact and water runoff (Pinamonti, 1998; Snapp et al., 2005; Dahiya et al., 2007). Besides reducing soil erosion and preserving soil moisture, these approaches provide other benefits in viticulture, such as supplying additional organic matter and nutrients, reducing weed invasion, and enhancing above and below-ground biota and crop plant health and yields (Rombough, 2002; Sanguankeo et al., 2009; Coll et al., 2011; Fredrikson et al., 2011; Pittelkow et al., 2014; Quintanilla-Tornel et al., 2016; Shapira et al., 2017). In addition to the advantages already

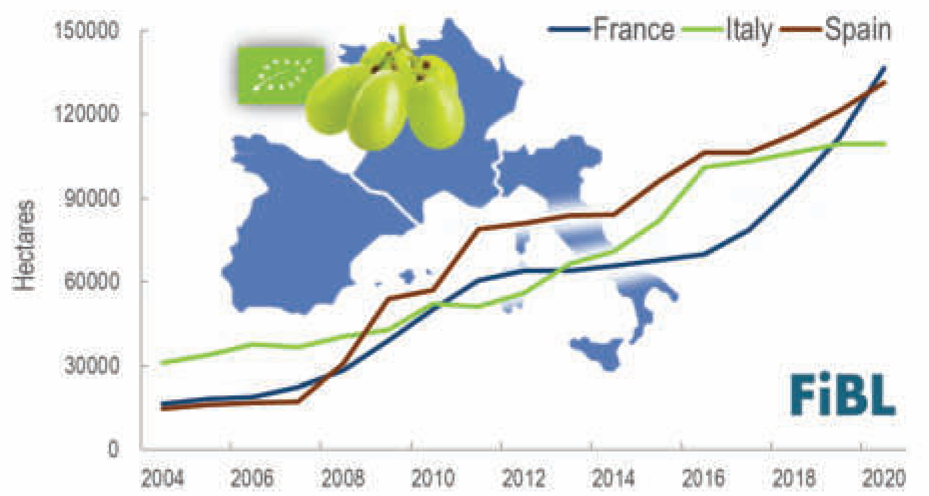


Figure 17. Growth of areas devoted to organic vineyards in the major producing European countries in recent years. Source: FiBL (Institute of Organic Agriculture) surveys (www.fibl.org).

detailed, there is evidence that organic amendments favored the abundance and diversity of beneficial organisms (e.g., earthworms, predatory arthropods, and parasitoids) and reduced the plant-parasitic nematode populations in the vineyard (Thomson and Hoffmann, 2007; Rahman et al., 2014). Indeed, according to Rombough (2002), mulches help restore the soil food web after the vine plantation. However, mulching can also cause certain disadvantages, such as excessive fungal growth in overly wet soils, which could lead to risks of acute fungal disease (Varga and Májer, 2004). In addition, urea and the products of the decomposition process of fresh manure amendments can reduce the availability of oxygen in the soil and be toxic to nematodes (Simpson, 1986; Kaplan and Noe, 1993). Proper mulch selection seems essential to avoid these types of problems. For example, the application of straw mulches helped control the wood-rotting fungus *Botrytis cinerea* (Helotiales: Sclerotiniaceae) in New Zealand vineyards (Mundy and Agnew, 2002; Jacometti et al., 2007). Another drawback of mulching is the cost of distributing and implementing amendments. To be efficient, growers must apply organic mulches in large quantities (at least 10 cm thick) and, depending on the materials, replace them every 2-3 years (Lanini et al., 2011). On the other hand, integrating mulching into circular economy programs at the local level that address the energy, organic farming, and integrated waste utilization approaches as self-sufficiently as possible can partially overcome these disadvantages, at least (Li et al., 2011; García Corral et al., 2022).

When sources such as water are not limited, the usefulness of cover crops in vineyards is more affordable. Their implementation significantly improves soil quality without negative impacts on the vine. For example, organic matter inputs due to cover cropping appear to increase the abundance of microbiota, nematodes, and earthworms (Pérès et al., 1998; Ingels et al., 2005; Coll et al., 2011). Their use is particularly convenient for weed control, specifically between rows, in organic viticulture since synthetic herbicides are prohibited (Pimentel et al., 1992; Sanguankeo et al., 2009). Another added benefit is that cover crops, depending on the plant species selected, can serve as habitats, refuges, alternative food sources, or attractants for BCAs. Thus, flowering cover crops, for instance, can enhance parasitism and predation of target pest species (Nicholls et al., 2000; Hanna et al., 2003; Sáenz-Romo et al., 2019). Lastly, among the main concerns of cover cropping in vineyards is the occurrence of fungal diseases, so, as in the case of mulching, a proper selection of plant varieties must be essential (Rombough, 2002; Pedneault and Provost, 2016).

Organic viticulture

Despite the correct implementation of IPM programs, most European farming soils retain pesticide residues, at least half with mixtures of several compounds of unknown combined effects (Silva et al., 2019). Faced with the urgent need to provide farmers with effective and non-polluting tools, the availability of permitted agrochemicals is decreasing every year, and vineyards also suffer from this current concern (Pertot et al., 2017; Campos-Herrera et al., 2021). As noted, organic pest management focuses primarily on preserving natural biodiversity and ecosystem balance with minimal intervention, e.g., enriching BCA occurrence to the detriment of pests and diseases, using economic and low-impact practices (Bruggisser et al., 2010). Although, in contrast to IPM, there has been an increase in new products compatible with organic farming, such conservation programs can be challenging when uncontrolled factors intensify pest and disease pressure (Provost and Pedneault, 2016). Fortunately, specific measures and tools are available for organic growers to constrain crop enemies when prevention methods become insufficient (Pertot et al., 2017). For example, periodic releases of BCAs in the vineyard achieve good results if, apart from other considerations, there is proper timing between the natural enemies and the target pests (Zehnder et al., 2007; Fiedler et al., 2008). Even with the strict limitations on chemical control in organic crop production, few non-synthetic compounds are allowed when other strategies have failed. Thus, biopesticides, defined by the European Union as biological control products and microbial biological control agents (EC, 2008), today represent only 5% of the total crop protection market, but their use is increasing rapidly every year (Olson, 2015 ; Damalas and Koutroubas, 2018). Finally, semiochemicals, such as sex pheromones, commonly implemented to control specific pests by mating disruption (Witzgall et al., 2010), have successfully controlled key pests in vineyards like the European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae) (Carlos et al., 2005).

The Appellation of Origin (DOCa) Rioja

Designated in 1925, the DOCa (from *Denominación de Origen Calificada*) Rioja is the oldest Appellation of Origin[1] in Spain ("CR Rioja wine," 2021), one of the world's leading wine-producing and exporting countries (OIV, 2020). The DOCa Rioja, located in the upper valley of the Ebro River (Northern Spain), comprises a wine-growing area of around 65,000 hectares for producing over 300 million liters of wine a year (Cabello et al., 2019). Is is spread over three Autonomous Communities of Spain: La Rioja, the Basque Country, and Navarra; in which three large zones are distinguished: Rioja Alta, Rioja Alavesa, and Rioja Oriental (Figure 18). A warm-summer Mediterranean climate with continental influence (classified as Csb by the Köppen-Geiger system) and haplocalcid semi-arid soils (e. g., low organic matter content, water deficiency, and accumulation of calcium carbonates) characterized this region (Soil Survey Staff, 2014). The benefits of the confluence of two opposing climates, Atlantic and Mediterranean, are excellent for vine growing with mild temperatures and annual rainfall slightly above 400 l/m^2 (Pérez-Marín, 2013).

Once the well-known vine pest of phylloxera *Daktulosphaira vitifoliae* (Homoptera: Phylloxeridae) is still successfully managed by using the appropriate rootstocks (Ollat et al., 2016), one of the pests that today mainly affect the vineyard in this region is *L. botrana*, also well-controlled with chemical applications and mating disruption employing sex pheromones (Palacios Ruiz et al., 1995). Maintaining low populations of this pest is also essential to control the

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[1] The International Organization of Vine and Wine (OIV) defines an *Appellation of Origin* as any denomination recognized and protected by the competent authorities, which serves to designate the particular quality of a wine or spirit as originating in an allocated geographical area, including the natural and human factors that are have given it its reputation (https://www.oiv.int/en/t/the-oiv-revises-its-definitions-of-gi-and-ao).

fungal disease of the gray mold of the clusters caused by *Botrytis cinerea* (Helotiales: Sclerotiniaceae), for which their larval stages are dispersal agents (Fermaud and Le Menn, 1989). Indeed, this and other diseases of fungal and oomycete causes, such as the downy mildew *Plasmopara viticola* (Peronosporales: Peronosporaceae) and the powdery mildew *Erysiphe necator* (Erysiphales: Erysiphaceae), are responsible for most of the phytosanitary treatments applied in vineyards (Pertot et al., 2017). The region's wine sector is also particularly concerned about the increasing impact of fungal wood diseases like the black rot of the vine *Phyllosticta ampelicida* (Botryosphaeriales: Phyllostictaceae) (Gramaje et al., 2018). Besides, mite pests, such as *Eotetranychus carpini* and *Tetranychus urticae* (Prostigmata: Tetranychidae), and the eriophyid *Calepitrimerus vitis* (Prostigmata: Eriophyidae), did not usually cause significant damage thanks to treatments based on sulfur powder (Pérez-Marín, 2013). Since 2001, national and regional polities have promoted sustainable programs for the region, starting with the implementation of the international sustainability principles proposed that year by the European Union (EC, 2001). Even so, vine management in the region implies generally conventional tillage and the widespread use of substantial amounts of pesticides, which are responsible for significant environmental impacts, including soil and water contamination (Pose-Juan et al., 2015; Herrero-Hernández et al., 2017).

Figure 18. Logotype and map representing the differentiated zones in the Appellation of Origin (DOCa) Rioja and their main edaphic and climatological characteristics.

Entomopathogenic nematodes and the vineyard

„Everything is connected to everything else."

Barry Commoner
in *The Closing Circle, Nature, Man & Technology*, 1979

Nematodes as biological control agents

The large availability of commercial products based on EPNs implemented into biological control programs against different pests in several crops (Lacey et al., 2015) illustrates their potential as BCAs. Most consist of IJ formulations on artificial substrates that aspire to improve storage periods (one to three months under refrigerated conditions) while minimizing negative impacts on their virulence (Hiltpold, 2015). Farmers can then apply them as water suspensions using, for instance, motorized tanks or irrigation lines (Shapiro-Ilan and Dolinski, 2015). An alternative approach is the release of EPN-infected insect larvae on the soil crops, which protects IJs from harassing conditions and increases their persistence (Gumus et al., 2015). Recent advances in specific formulations, adjuvants (e.g., antidesiccants, brighteners), and field application systems have begun targeting EPNs against aerial pests (Shapiro-Ilan and Dolinski, 2015; Nxitywa and Malan, 2021). The number of certified adjuvants in grapevines is limited today, but laboratory and greenhouse experiments have shown the great potential of this technology (Platt et al., 2019). Moreover, natural products produced by *Xenorhabdus* spp. and *Photorhabdus* spp. have great potential as biopesticides against numerous arthropod pests (e.g., *T. urticae* and *L. botrana*) and fungal diseases (e.g., *E. necator* and *B. cinerea*) of vineyards (Fang et al., 2011, 2014; Eroglu et al., 2019; Cevizci et al., 2020; Chacón-Orozco et al., 2020; Vicente-Díez et al., 2021a, 2021b). Exploring the compatibility of aerial applications based on the EPN-bacteria complex with authorized chemical compounds in viticulture will contribute to coordinating both strategies in the IPM of the vineyard (Gutiérrez et al., 2008; Cevizci et al., 2020; Özdemir et al., 2020).

Regardless of the great potential of native EPNs to control a wide variety of crop soil-dwelling pests, the vineyard remains under-researched as a target agroecosystem, perhaps because of the lack of severe problems besides those caused by the grape phylloxera *D. vitifoliae* or cutworms in the genus *Agrotis* (Lepidoptera: Noctuidae), among others, but also because of the real impact of specific pests or diseases is often hidden (Campos-Herrera et al., 2021; Marín et al., 2021). Thus, BCA preservation in vineyards must not be underestimated, especially under the changing climate. For example, the average temperature in the DOCa Rioja area has risen in the last decades between 0.9 and 1.2 °C, causing a shift towards warmer bioclimatic classes (Cabello et al., 2019). Such a new scenario may widely modify the distribution of species, so the spectrum of pests and diseases affecting vineyards could change, for example, being able to easily survive warmer winters (Schröter et al., 2005; Santos et al., 2020). Since similar concerns affect viticulture worldwide, several research lines, mainly through *in vitro* and semi-field experiments, are currently addressing the study of the efficacy of diverse BCAs against potential targets of agricultural value. Thus, laboratory studies targeting pests of local or regional relevance, such as the grape phylloxera *D. vitifoliae* on the East Coast of the USA (English-Loeb et al., 1999), and the vine mealybug *Planococcus ficus* (Hemiptera: Pseudococcidae) and the false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in South Africa (le Vieux and Malan, 2015; Steyn et al., 2021), obtained limited success. On the other hand, there are also some promising results, for example, for augmented EPN applications conducted in the States of Ohio and Georgia of the USA against the grape root borer *Vitacea polistimorfis* (Lepidoptera: Sessidae), that reached mortality rates of 55–74% (depending on the species) and IJ soil persistence from 1 year up to 21 months (Williams et al., 2010).

Entomopathogenic nematodes and soil health

As mentioned in the previous sections, nematodes are among the soil organisms that support most ecosystem functions (Delgado-Baquerizo et al., 2020) and, therefore, among those that suffer the most losses with soil degradation (Karimi et al., 2020). Thus, it is unsurprising that distinct trophic groups of nematodes are good bioindicators for assessing soil health (Ferris et al., 2001). Recent field studies have shown that adverse effects on soils resulting from cropping management may also negatively impact specific EPN populations, particularly in perennial crops (Campos-Herrera et al., 2008, 2014). Given that many factors affect their survival, there is increasing interest in identifying the best farming practices that favor optimal ecological scenarios to enhance their activity in soil crops (Campos-Herrera et al., 2021), including vineyards, where EPNs have often been found (Bélair et al., 2001; Mráček et al., 2005; Campos-Herrera et al., 2008).

Impact of soil management

There is evidence that reduced mechanization and the service of soil cover structures enhance the EPN community in crops by, as previously shown, improving soil health and supporting suitable host occurrence, but also by protecting IJs from the extreme conditions (e.g., suboptimal temperatures, UV light, desiccation) that they would suffer in bare soils (Stuart et al., 2015). For example, for EPN augmented applications in corn-soybean rotation crops, *S. carpocapsae* IJs persisted longer with crop residue implementations (Shapiro-Ilan et al., 1999). Similar results were observed for *H. bacteriophora* in no-tilled soils for different crops (Susurluk and Ehlers, 2008). Reduced or no-tillage also appears to favor EPN activity, although the findings depend on factors such as soil properties, type and quality of cover crops, and the EPN species considered (Brust, 1991; Hummel et al., 2002; Millar and Barbercheck, 2002). Thus, Marquez (2017) observed that black oat covers favored overall EPN activity in corn crops in Hawaii (USA), while those based on oilseed radish only the activity associated with heterorhabditids. Jaffuel et al. (2017) obtained comparable outcomes for EPN augmentation experiments in winter wheat crops in Switzerland, recording higher activity for *S. feltiae* in pea and mustard covers than in bare soils from samples collected after five months of applications, but not for *H. bacteriophora*. Overall, crop residues and composted organic amendments benefit EPN occurrence and activity, but also in a species-specific manner and depending on mulch types, among other factors (Ishibashi and Kondo, 1986; Lacey et al., 2006; Duncan et al., 2007; Campos-Herrera et al., 2015a; Khumalo et al., 2021; Renkema and Parent, 2021). For example, in vitro experiments found that IJs could move from soil into moist mulches, but their virulence varied depending on the substrate types and exposure temperature ranges (de Waal et al., 2011). In any case, how procedures of this kind affect the EPN community in viticulture is still not well known.

Impact of pest management

The effect of different management systems on EPN occurrence and activity in soil crops has not been thoroughly investigated, and findings have been inconclusive. For example, Ellers-Kirk et al. (2000) observed that inundative release of IJs of *Steinernema riobravis* in cucumber plantations in Pennsylvania (USA) better-controlled damages caused by the striped cucumber beetle *Acalymma vittatum* (Coleoptera: Chrysomelidae) for organic farming than IMP. Opposite, an extensive study involving three annual cropping systems (wheat, maize, and grass-clover ley) in Switzerland operated with different pest management practices (conventional, organic, and biodynamic, under differing fertilization and pesticide application regimens) resulted in no differences in the abundance of EPNs and soil organisms associated to their soil food web (Jaffuel et al., 2016). For perennial crops (vineyards and pear orchards) in La Rioja, Campos-Herrera et al. (2008, 2010) reported similar EPN activity for organic farming as in natural areas (Oaks) and much higher than for conventional agriculture and annual crops (onions, bell peppers, and wheat) independently of the pest management. Based on this and similar surveys (Campos-Herrera et al., 2013b, 2016a; Campos-Herrera, Palomare-Ruis, et al., 2022), a stable rhizosphere seems to favor the EPN community. Roots can, in fact, function as paths for EPNs in search of hosts (van Tol et al., 1998; Ennis et al., 2010) but can also emit volatiles when induced by insect pests that are attractive to EPNs (Rasmann et al., 2005; Ali et al., 2010; Hiltpold et al., 2011). That would contribute to explain why Campos-Herrera et al. (2008, 2010) found almost no sign of EPNs in annual crops regardless of the pest control implemented. Perennial crops such as the vineyard are thus much more suitable as a model agroecosystem for exploring the effect of agricultural management on the community of EPNs and associated soil organisms.

Objectives

„Ignoranti quem portum petat, nullus suus ventus est.“

Lucius Annaeus Seneca
in *Letters, 71, 3, c. 65 AD*

The main challenge of agriculture nowadays is to reconcile the production of goods that meet human demands with the conservation of ecosystem services and biodiversity on farmland (Altieri and Nicholls, 2004; Gunstone et al., 2021). As ambitious as necessary, this purpose is tightly linked to the notion of soil health (Kibblewhite et al., 2008). Maintaining biotic balances in crop soils is critical for preserving valuable beneficial organisms such as BCAs for conservation biological control programs (Lehmann et al., 2020). Therefore, it becomes necessary to evaluate farming management practices regarding their impact on soil biota to move towards more responsible agriculture. In this sense, the vineyard is a key agroecosystem due to its socioeconomic relevance (Santos et al., 2020) and because it is one of the most intensively managed agricultural sectors (Nicholls et al., 2008). In addition, being a perennial crop, the vineyard sustains a complex rhizosphere that allows stable relationships between soil organisms over time, including EPNs, whose foraging capacity and virulence are affected by root growth and structure and the emission of attractant volatiles (van Tol et al., 1998; Rasmann et al., 2005; Demarta et al., 2014). The results shown by Campos-Herrera et al. (2008, 2010), reporting lower activity values in conventional than organic vineyards, illustrate the suitability of this cropping system for assessing the impact of agricultural management on EPN activity since those differences were impossible to discern in annual crops of neighboring areas.

Optimization of molecular tools, such as the design of specific primers-probe sets for species identification through real-time qPCR, allowed in the last decade to quantify in field samples not only the EPN activity, as traditionally achieved with insect baits, but also their abundance (Torr et al., 2007; Campos-Herrera et al., 2011). Additionally, the study of the relationships between EPNs and other soil organisms addressed, until recently, through laboratory experiments can be more naturalized thanks to this methodology (Campos-Herrera et al., 2012a). Thus, this Thesis aimed to explore the impact of differentiated management in viticulture on the EPN community and other soil organisms associated with its food web and how their assemblage might inform the soil health in vineyards. The evaluation of the different agricultural practices was approached through three independent field studies:

- A field experiment to evaluate different cover crops (grass seeded, flower driven, and spontaneous) in an experimental DOCa Rioja vineyard (Logroño).
- A survey comprising 80 vineyards distributed throughout the DOCa Rioja area to evaluate the organic viticulture (*vs.* IMP) and cover cropping (*vs.* tillage).
- A field experiment to evaluate different organic mulches (consisting of grape pruning debris, straw, and spent mushroom compost) in two experimental DOCa Rioja vineyards handled for organic and integrated pest management (Logroño and Aldeanueva del Ebro, respectively).

This Thesis was based on the hypothesis that organic viticulture and alternative strategies to regular tillage (cover cropping and mulching) might enhance the abundance and activity of naturally occurring EPN species compared to conventional practices thanks, to a large extent, to more favorable biotic and abiotic conditions for their long-term persistence in crop soils. To this end, the specific objectives to accomplish were:

1. quantify abundance and activity of target EPN species,
2. quantify the abundance of target soil organisms associated with EPNs (FLNs, NF, and EcPB), and
3. contribute to discriminating the abiotic factors that drive their assemblage.

Material and Methods

> *„Conta ciò che si può contare, misura ciò che é misurabile e rendi misurabile ciò che non lo è.“*
>
> Galileo Galilei (1564-1642)

Experimental designs and soil sampling

Targeted native EPN species and other organisms linked to their soil food web were screened in three self-dependent studies conducted on DOCa Rioja vineyards (Figure 19). The study comparing the cover crops seeded with *Bromus catharticus* (Poaceae), flower-driven (commercial seed mixture), and spontaneous was evaluated seasonally (late spring and early autumn) for two consecutive years (2017 and 2018) in an experimental vineyard in Logroño owned by the Government of La Rioja (Figure 19 and Figure 20). The assessment of the organic mulches consisting of grape pruning debris, straw, and spent mushroom compost was only conducted seasonally (early and late spring and early autumn) during 2020 in two experimental vineyards belonging to the wineries Pernod Ricard (Logroño) and D. Mateos (Aldeanueva del Ebro) and handled for organic and IPM, respectively (Figure 19 andFigure 21). The experimental design for both studies was completely randomized divided into experimental units (plots). Lastly, the DOCa Rioja survey comprised 80 commercial vineyards sampled in autumn 2019. Each vineyard was assigned to one of the categories of two factors (soil and pest management), resulting in four possible combinations: IPM with tillage, IPM without tillage, organic farming with tillage, and organic farming without tillage (Figure 19). Throughout the studies, soil sampling was performed in spring and autumn, when EPN populations are assumed to be found in higher numbers at the soil surface in temperate climates (Akhurst and Bedding, 1986; Griffin et al., 1991; Efron et al., 2001). Indeed, temporal studies have shown that some EPN species migrate in the soil profile to avoid harassing conditions during summer and winter, such as extreme temperature or lack of moisture (García Del Pino and Palomo, 1997; Campos-Herrera et al., 2010).

Composited samples, two per plot (or vineyard in the DOCa Rioja survey), consisted of 12-20 single soil cores (2.5 cm Ø x 20-30 cm DP) randomly collected with auger soil samplers under the crop canopy of central vines to prevent border effects (Figure 22). Sampling a large number of soil cores per plot or vineyard may compensate for the patchy distribution of nematodes across spatial scales (Liu et al., 2019). Once collected, soil samples were mixed in individual plastic bags (~1800 cm^3 of soil) and stored in a chamber (4 °C in the dark) until processed (within 2–4 days). After being homogenized in the laboratory, subsamples of 200 g of fresh soil served to *(i)* isolate the nematofauna and other soil organisms through the sucrose centrifugation methodology, *(ii)* determine the soil activities by baiting insect larvae, and *(iii)* analyze the soil properties with the support of The Regional Laboratory of the Government of La Rioja (La Grajera, Logroño, Spain): soil texture (sand, silt, and clay percentages) (Bouyoucos, 1936), organic matter (Walkley and Black, 1934), pH (Millennia and Markewitz, 2004), electrical conductivity, macro-nutrients (NPK), oligo-nutrients (Mg, Ca, and SO_4), micro-nutrients (Fe, Mn, Zn, Cu, Al, and B), and other elements (Na and Pb) (Mehlich, 1978, 1984).

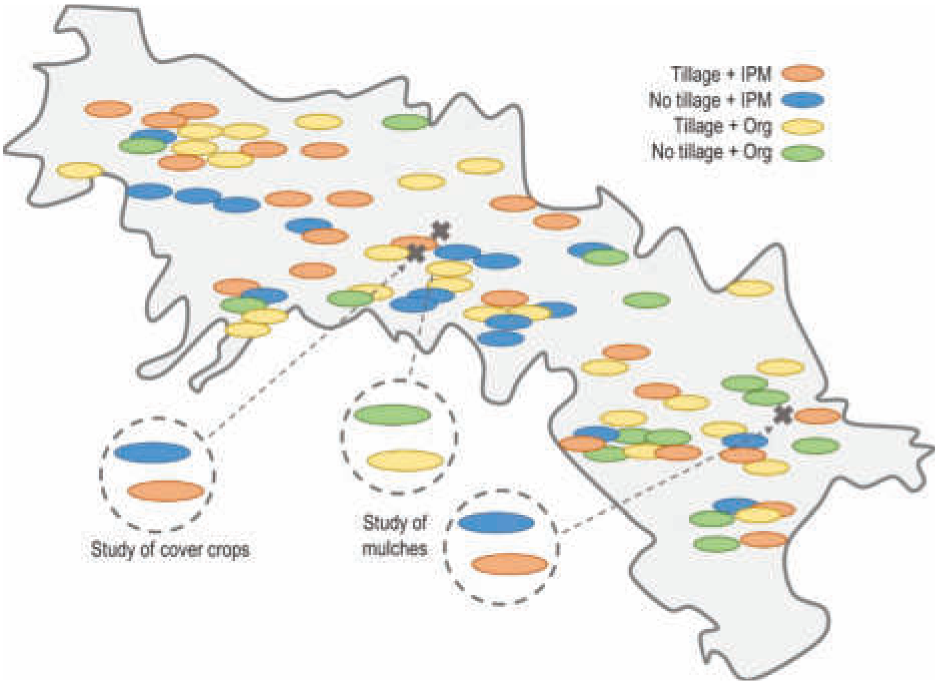

Figure 19. Distribution of the sampled vineyards managed with different agricultural practices: tillage *vs.* no-tillage and Integrated Pest Management (IMP) *vs.* organic farming (Org), for the DOCa Rioja survey and the cover cropping and mulching studies.

Figure 20. Treatments applied on inter-rows of an experimental vineyard to evaluate the impact of (A) regular tillage and the cover crops (B) grass-seeded, (C) flower-driven, and (D) spontaneous on native populations of entomopathogenic nematodes and other organisms linked to their soil food web. Photos by Rubén Blanco Pérez from Instituto de Ciencias de la Vid y del Vino, Spain.

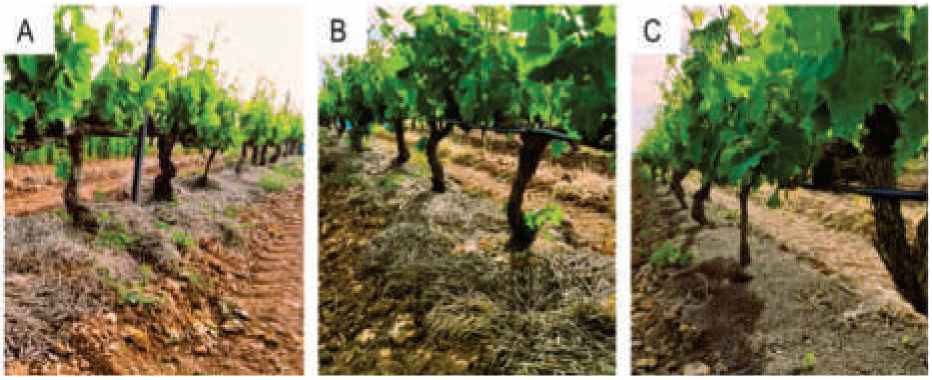

Figure 21. Differentiated organic mulches based on (A) grape pruning debris, (B) straw, and (C) spent mushroom compost on rows of two experimental vineyards to evaluate their impact on native populations of entomopathogenic nematodes and other organisms linked to their soil food web. Photos provided with permission by Andreu Mairata Pons from Instituto de Ciencias de la Vid y del Vino, Spain.

Figure 22. Soil sample collection under the vine canopy. Photos by Ignacio Vicente Díez and Rubén Blanco Pérez from Instituto de Ciencias de la Vid y del Vino, Spain.

Isolation and identification of soil mesofauna

For the studies that conform to this Thesis, we combine indirect and direct methodologies to isolate and identify soil organisms. Conventionally, most surveys in EPN biogeography studies have employed indirect methods based on soil bioassays using specific insects as baits (Figure 23), such as *G. mellonella*, which followed variants of the original procedure described by Bedding and Akhurst (1975). In addition, this procedure allows the estimation of soil activities, including those associated with EPN occurrence (Jaffuel et al., 2016). Briefly, we baited 200 g of fresh soil sample with 20 final instars of *G. mellonella* larvae in two independent rounds (10 larvae each) to optimize the detectability of nematode activity (Griffin, 2015). Larval mortality was assessed after four days of exposure (22–24 °C in the dark), indicating the total suppressive capacity of the soil for each sample. Dead larvae were recovered, placed in White traps (White, 1927), and monitored every 2-3 days for approximately one month to check for nematode emergencies, estimating the larval percentage that marked the soil activity associated with nematodes. Those emerging nematodes were used to perform Koch's postulates (Figure 24) for estimating entomopathogenic soil activity (Stock and Goodrich-Blair, 1997) and establishing laboratory cultures (Woodring and Kaya, 1988).

Figure 23. Baiting of *Galleria mellonella* larvae. Photo by Rubén Blanco Pérez from Instituto de Ciencias de la Vid y del Vino, Spain.

Consequently, insect baiting allowed us to isolate and identify a new EPN species: *Steinernema riojaense* (Půža et al., 2020; Figure 25) and to include an EPN population morphologically misidentified as *S. intermedium* by Valadas et al. (2014) within the species *S. affine*. Regarding the latter case, phylogenetic analysis based on the ITS rDNA regions conducted by Dr. Vladimir Půža (Czech Academy of Sciences, Czech Republic) suggested that our EPN population could be a new EPN species closely related to *S. affine* (named *Steinernema* sp. *affine*-group by Blanco-Pérez et al. (2022a, 2022b). Nonetheless, the variability of ITS sequences may occasionally be too high to reliably infer relationships among some steinernematid species (Nguyen et al., 2001; Nadler et al., 2006). Further analyses of the mitochondrial COI barcode region, also completedin collaboration with Dr. Vladimir Půza, strongly suggested that our isolate belongs to the EPN species *S. affine*. According to Dr. Vladimir Půza's ongoing studies, the Portuguese isolate of Valadas et al. (2014) and ours must typify a variant of this EPN species in the Iberian Peninsula that differs slightly from that predominant in Central Europe. Hence, according to this last molecular analysis, *S. intermedium*, topotype from the USA (Poinar, 1985), is still never found in Europe. This intraspecific variability might produce a low detection of our population using the primers/probe set that Torr et al. (2007) designed. A new molecular set is required to accurately quantify both *S. affine* populations in a single qPCR run.

Figure 24. Infective juveniles of the entomopathogenic nematode (EPN) *Steinernema feltiae* emerging from a baited *Galleria mellonella* larva (on the right) and new set of insect larvae infected with the EPN *Heterorhabditis bacteriophora* in a Koch's Postulates test (on the left). Photos by Rubén Blanco Pérez from Instituto de Ciencias de la Vid y del Vino, Spain.

For the molecular characterization of the ITS regions of both EPN populations (*S. riojaense* and *S. affine*), we employed the primers defined by Vrain et al. (1992) and provided by Biotools (B&M Labs S.A., Madrid, Spain). We followed the procedure described by Hominick et al. (1997) and modified by Campos-Herrera et al. (2015b), operating a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sequences obtained were assembled using specific forward and reverse primers (Geneious, R.5.6.5., Biomatters, Inc., New Zealand), compared to reported sequences of close related organisms using Blast (http://blast.ncbi.nlm.nih.gov), and submitted to Genbank (ITS GenBank accession numbers referred in Table 1 and primers/probe sets in Table 2).

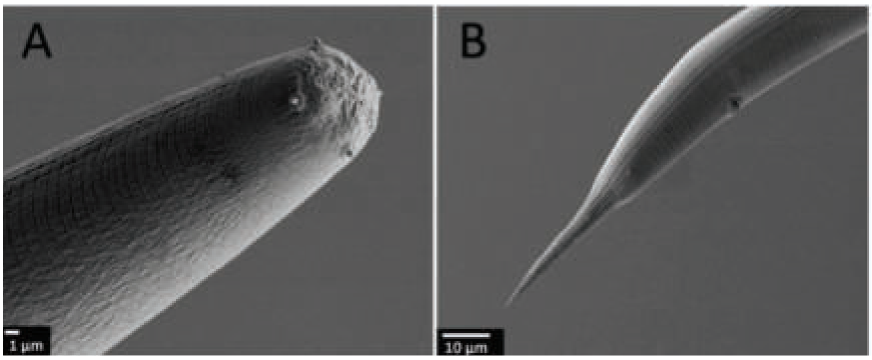

Figure 25. Scanning electron microscopy detailing the head (A) and tail (B) sections of an infective juvenile of *Steinernema riojaense* n. sp. Photos provided with permission by Dr. Vladimir Půza from the Czech Academy of Sciences, Czech Republic.

Table 1. Soil organisms evaluated.

Soil organisms	Population	GenBank AcNº ITS Region	Reference for primers/probes
Entomopathogenic nematodes			
Heterorhabditis bacteriophora	(comercial)	KJ938576	Campos-Herrera et al. (2011a)
Heterorhabditis indica	Btw	KJ938571	Campos-Herrera et al. (2011b)
Heterorhabditis megidis	(comercial)	KJ938577	Campos-Herrera et al. (2011a)
Steinernema affine	CH	KJ938567	Torr et al. (2007)
Steinernema affine	VO 53	MW480137	Blanco-Pérez et al. (2022b)
Steinernema arenarium	SA	KU194615	Campos-Herrera et al. (2019a)
Steinernema carpocapsae	DOK 83	KJ818295	Campos-Herrera et al. (2011a)
Steinernema feltiae	RS 5	KJ938569	Campos-Herrera et al. (2011a)
Steinernema intermedium	82	AF171290	Campos-Herrera et al. (2015b)
Steinernema kraussei	OS	KJ696686	Campos-Herrera et al. (2015c)
Steinernema riojaense	RM 30	MK503133	Blanco-Pérez et al. (2020)
Free-living nematodes			
Acrobeloides-group	RT1-R15C	JQ237849	Campos-Herrera et al. (2012a)
Oscheius onirici	MG 67	KJ938578	Campos-Herrera et al. (2015b)
Oscheius tipulae	MG 68	KJ938579	Campos-Herrera et al. (2015b)
Pristionchus maupasi	AM 3	MG551681	Campos-Herrera et al. (2019a)
Nematophagous fungi			
Arthrobotrys dactyloides	H55	KJ938574	Campos-Herrera et al. (2012a)
Arthrobotrys musiformis	11	KJ938572	Campos-Herrera et al. (2012a)
Arthrobotrys oligospora	8	KJ938573	Campos-Herrera et al. (2012a)
Catenaria sp.	1D	JN585805	Campos-Herrera et al. (2012a)
Hirsutella rhossiliensis	2931	KM652168	Zhang et al. (2006)
Purpureocillium lilacinum	9357	KJ938575	Atkins et al. (2005)
Ectoparasitic bacteria			
Paenibacillus nematophilus	NEM2	AF480936	Campos-Herrera et al. (2011a)
Paenibacillus sp.	SdTc1FEE1	JF317562	Campos-Herrera et al. (2011a)

Table 2. Primers/probe sets designed for the two new EPN isolates.

Species	Population	Sequence (5'-3')	Size (bp)	Reference
S. affine	VO 53	F: CGATTGCCTGTCATCAAATG R: AAGCAAAAGAGCACCCATCA P: AGGTGCCTGGAGCAGTTGTATGA	130	Blanco-Pérez et al. (2022b)
S. riojaense	RM 30	F: CGGCTAACTTGTGCGTTGAC R: CGACCCAAAAGGCACAAACG P: AGCTGTCGCGTATGTGCTGTTTCG	86	Blanco-Pérez et al. (2020)

Codes: F, forward; R, reverse; P, probe; bp, base pairs.

On the other hand, a drawback of the traditional baiting method is that it could strongly perturb estimations of EPN occurrence, for instance, by choosing inappropriate temperatures or unsuitable hosts to detect specific nematode species (Mráček et al., 2005). Advances in molecular tools allowed the characterization of soil organisms at the species level, including EPNs, solving some of the limitations of traditional methodologies, such as the extended period required for bioassays and morphological identification of species or the need for specialized expertise (Stock, 2015). Thus, several studies have revealed that qPCR analyses effectively screen for and quantify EPNs and other organisms related to their food wed in soils, even in low numbers (Campos-Herrera et al., 2012a, 2015b, 2019a; Pathak et al., 2012; Jaffuel et al., 2016, 2017, 2018). We selected sucrose centrifugation (Jenkins, 1964; Figure 26) for mesofauna extraction since it is a more efficient methodology than others, such as heptane flotation or Berlese funnels, particularly for nematofauna (Dritsoulas and Duncan, 2020).

We used commercial kits for DNA extraction procedures: the Speedtools Tissue Kit (Biotools, Madrid, Spain) for nematodes that emerged from insect baits and the DNeasy PowerSoil Kit (QIAGEN GmbH Co., Hilden, Germany) for soil organisms extracted through sucrose centrifugation. DNA samples were examined for quality and quantity in a Nanodrop system (Thermo Scientific 2000C spectrophotometer). Biotools also provided the species-specific primers/probe sets (Table 1) for identifying and quantifying the screened soil organisms in the qPCR analysis. The final concentrations of primers and probes were 400 and 200 nM, excluding *S. affine* VO 53 (100 and 40 nM). The qPCR conditions were: 2 min at 60 °C and 10 min at 95 °C, followed by selected cycles of 95 °C for 30 s and the adjusted temperature per organism for 1 min. We run 38 cycles for nematode species and 50 for NF and EcPB species in qPCR tests run on the Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems Corp. Waltham, MA, USA) for the cover crops study and a Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA) for the DOCa Rioja survey and the mulches study. Preliminary empirical optimization was necessary to determine the required conditions for the available equipment, reagents, and material. In all the preliminary checks and optimization protocols, DNA concentration was standardized to correspond to 30 IJs (for EPNs), 1 ng/µl of pure culture (for FLNs and NF), and 0.1 ng/ µl when plasmids were used (ITS rDNA sequence + pUC57 for the species *Steinernema poinari*, *S.*

Figure 26. Tubes with sieved soil samples ready for the sucrose centrifugation procedure. Photo by Rubén Blanco Pérez from Instituto de Ciencias de la Vid y del Vino, Spain.

phyllophagae, *S. intermedium*, *S. silvaticum*, *Acrobeloides*-group, *Oscheius* sp.3, and *Catenaria* sp.; and 16S rDNA + pUC57 or pDrive for EcPB (Table 3). The standard curves were obtained for the quantifications by 10-fold serial dilutions (n=5 points), starting with the highest concentration employed in the pre-screenings.

Table 3. Additional species of nematodes used for cross-amplification tests.

Soil organisms	Population	GenBank AcN° ITS Region
Entomopathogenic nematodes		
Heterorhabditis zealandica	Btw	GU174009
Steinernema bicornotum	D60 Pl	KJ938568
Steinernema glaseri	NC	GU173998
Steinernema glaseri-group	N7	MG711658
Steinernema intermedium-group	VAD 1067	KJ696684
Steinernema khuongi	ARC	GU174002
Steinernema phyllophagae	-	FJ410327
Steinernema phyllophagae-group	N10	MG711660
Steinernema poinari	1160	KF241754
Steinernema rarum	-	KJ938570
Steinernema riojbrave	Btw	GU174000
Steinernema silvaticum	IN30k3	KC631434
Steinernema weiseri	1117	KJ696686
Free-living nematodes		
Oscheius myriophilus	JU1386	KP792651
Oscheius sp.3	JU75	AJ297890
Pristionchus entomophagus	P0144	MG551683
Pristionchus lheritieri	P245	MG551684
Pristionchus pacificus	P2333	MG551685

Statistical analyses

We ran generalized mixed models (GLMMs), executed with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), testing the effect of differentiated farming practices of the three studies performed in DOCa Rioja vineyards on DNA quantifications and abundance/frequencies of occurrence of EPNs, FLNs, NF, EcPB, and soil activity measures. We adjusted all organism quantifications from DNA samples obtained by sucrose centrifugation to express the corresponding quantities per 100 g of dry soil. EPN and EcPB abundances were expressed as numbers of IJs and copies of plasmids per 100 g of dry soil, respectively, and numbers of FLNs and NF standardized to a 0–1 range by dividing all values recorded for a particular species (n_i) by the highest number (n_{max}) of that species, according to $100 \times n_i/n_{max}$ (Jongman et al., 1995). This standardization liberates the data set from variation caused by intrinsic differences, such as significant variations recorded for the copy numbers between the FLN and NF species evaluated. Since many NF species can survive in soil saprophytically (Nordbring-Hertz et al., 2006), we assumed most NF species isolated by sucrose centrifugation were within the infectious phase. Thus, NF numbers have been expressed as infection rate (IR), determined by dividing the DNA quantity of each NF species by the total amount of DNA (Campos-Herrera et al., 2012a).

In preliminary statistical analysis, variables expressed as a percentage and quantitative variables were arcsine and log (x + 1) transformed, respectively. For quantifying soil organisms and soil activities, we used a GLMM with a gamma distribution (log-link function) and a binomial distribution (logit-link function) for the frequencies of occurrence. We included as covariates in the GLMM tests those soil parameters for which we recorded significant differences among treatments, and then we removed all predictors that were not significant for preliminary statistical tests. We used the Least-Squares Means ± standard error of the mean (SEM) as descriptive statistics.

For the DOCa Rioja survey, we performed multivariate analyses of selected soil organisms and abiotic factors using CANOCO 5 (ter Braak and Šmilauer, 2002; Šmilauer and Lepš, 2014). Biotic and abiotic variables, chosen after avoiding strong co-linearities, were standardized by dividing by the highest values, ranking all values 0–1 (Šmilauer and Lepš, 2014). Since detrended canonical correspondence analysis indicated heterogeneous communities (values over 3.0 SD units), we run canonical correspondence analyses (constrained axes, interspecies correlations) with a Monte Carlo permutation (n=499) and automatic forward selection for the assignment of significant ($P<0.05$) abiotic factors (using the Bonferroni correction for P values). Results were visualized with bi–plot scaling (CANOCO 5). Finally, we employed Microsoft Excel 2016 (3D Maps) to visualize the sample sites and species distributions for this specific study.

Publications catalogue

(Section affected by confidentiality rights)

Agriculture, Ecosystems and Environment

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Journal of Invertebrate Pathology

Organic mulching modulated native populations of entomopathogenic nematode in vineyard soils differently depending on its potential to control outgrowth of their natural enemies

Rubén Blanco-Pérez[a], Ignacio Vicente-Díez[a], Alicia Pou[a], Ignacio Pérez-Moreno[b], Vicente Santiago Marco-Mancebón[b], Raquel Campos-Herrera[a,*]

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Publication 2

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Organic viticulture enhanced the activity of native entomopathogenic nematodes in DOCa Rioja soils (North of Spain)

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A R T I C L E I N F O

Keywords:
Beneficial soil organisms
Cover cropping
Free-living nematodes
Integrated pest management
Nematophagous fungi
Organic farming
Tillage

A B S T R A C T

Vineyards and their associated socio-economic activities are relevant sectors worldwide. Still, this agroecosystem is one of the most intensely managed crops and erosion-prone land areas. The conventional viticulture practices to control pests, diseases, and weeds, like tillage and agrochemical applications, accelerate the loss of soil biodiversity and compromise the presence of beneficial soil organisms such as the entomopathogenic nematodes (EPNs). Such human disturbances in the agroecosystems can strongly affect abiotic (e.g., soil texture and properties) and biotic factors (natural enemies and potential competitors) that modulate the EPN activity as biological control agents. For the first time in viticulture, this study aimed to investigate the impact of differentiating management on the EPN community and associated soil organisms and if their assemblage will provide indicators of better practices for sustainable farming. We hypothesized that organic pest management and alternative strategies to tillage might enhance the abundance and activity of the native EPN community in vineyard soils. In autumn 2019, we collected two composite soil samples from 80 vineyards distributed across the Guaranteed Designation of Origin (denominated DOCa) Rioja region. The sites belonged to one category of each of the two factors: pest management (integrated *vs.* organic, 40 plots each) and soil managing (tillage *vs.* cover cropping, 48 and 32 vineyards, respectively). Isolated through sucrose-gradient centrifugation and employing species-specific primers/probe qPCR sets, we investigated the presence of ten EPN species and associated soil organisms: four free-living nematodes (FLNs), six nematophagous fungi (NF), and two ectoparasitic bacteria (EcPB). Besides, we estimated the EPN activity using the traditional insect-bait method. We included in the analysis twenty soil variables to characterize the evaluated treatments and assess their impact on soil organism distribution. Our results provide evidence on the support of organic viticulture to beneficial soil organisms, notably the activity of native EPNs. We also reported a higher abundance of *S. feltiae* (the predominant steinernematid species in Europe) and FLNs for organic farming than IMP, while the presence of NF and EcPB resulted in unaffected. Contrarily, the soil management practices considered did not differ in their impact on EPNs or their natural enemies/competitors, even if contrasted for several soil properties. Future research may expand the screened soil-dwelling species using novel molecular technics to unravel their complex interactions and determine the best farming practices to preserve soil health.

1. Introduction

Viticulture and enology are relevant socioeconomic and cultural sectors worldwide that provide a broader range of services and goods to people and nature than the simple production of grapes and wine (Santos et al., 2020). The current viticulture has to afford appropriate yields while supporting ecosystem services such as regulating climate and hydrological services, preserving the habitat, or maintaining cultural heritage (Orgiazzi et al., 2016). Soils are possibly the most complex and biodiverse ecosystem on Earth (Orgiazzi et al., 2016). Soil biodiversity enables self-perpetuating of the ecosystem functions of carbon transformations, nutrient cycling, maintenance of soil structure, and the

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regulation of pests and diseases (Kibblewhite et al., 2008; Gunstone et al., 2021). Agricultural intensification accelerates the loss of soil biodiversity (Veresoglou et al., 2015), particularly in the last decade due to the overuse of chemical applications (FAO, 2020). Despite rising environmental awareness by farmers and society, which for instance drive in recent years the organic viticulture in the higher European wine-producing countries (FiBL, 2019), the vineyard is still one of the most intensely managed crops and erosion-prone land systems (Nicholls et al., 2008; Rodrigo-Comino et al., 2018; Winter et al., 2018; Karimi et al., 2020).

Located on both sides of the Ebro River, the Guaranteed Designation of Origin (denominated DOCa) Rioja (CR Rioja wine, 2021) is the oldest in Spain (1925), one of the principal vine-growing, wine-producing, and global exporter countries (OIV, 2020). Grapevine management in the region often involves conventional tillage and the widespread use of pesticides (herbicides, fungicides, insecticides, and acaricides) in substantial numbers, responsible for significant environmental impacts, including soil and water pollution (Pose-Juan et al., 2015; Herrero-Hernández et al., 2017). Integrated Pest Management (IPM) appeared as a viable solution to these problems. Strongly encouraged and regulated by the European Union (EC, 2009), IPM aims to keep the occurrence of potential pests, diseases, and weeds below the Economic Damage Threshold, following a series of principles ranging from preventive and monitoring strategies to intervention reducing as possible the use of pesticides (Barzman et al., 2015). Still, the wine sector and public opinion give today more attention to more sustainable practices (Aschemann-Witzel and Zielke, 2017). Organic viticulture, based in the European Union on the Regulation (EU) (2018)/848 containing the principles of organic production and labeling, is understood as an overall system that aims to reduce the use of approved pesticides by adopting alternative strategies that promote environmental care, biodiversity, preservation of natural resources, and high animal welfare standards (Provost and Pedneault, 2016; EU, 2018). Indeed, a recent meta-analysis estimated that organic viticulture promotes soil biodiversity over three times more than conventional practices (Karimi et al., 2020). Besides, soil management is also critical for soil and plant protection in crops. Traditional tillage negatively affects most soil microbes and their functioning and promotes the mineralization of organic matter and soil erosion, particularly in European vineyards, commonly placed on slopes (Santos et al., 2020). More sustainable solutions to reduce tilling, like cover cropping or mulching, decrease soil erosion and favor soil biodiversity by increasing organic matter content and porosity, structural stability, and water retention of soil crops (Santos et al., 2020).

Soil preservation brings several indirect benefits to farmer crops. Many soil invertebrates, like nematodes, mites, or different predators and parasitoids, play a role in controlling agricultural pests (Gunstone et al., 2021). The entomopathogenic nematodes (EPNs), well-known pathogens of soil inhabitant arthropods, are responsible for many ecological goods and services (Campos-Herrera et al., 2012; Lewis et al., 2015). Their non-feeding, infective juvenile (IJ) stages often occur in natural and agricultural soils where locate suitable hosts (Stock, 2015). Once penetrate within their hemocoel, IJs rapidly kill them (48–72 h after infection) with the aid of protein toxins and secondary metabolites produced by mutualistic enteric γ-Proteobacteria (Boemare, 2002; Bode, 2009; Dillman et al., 2012). Physical or chemical disturbance of soils resulting from human activities such as tillage and agrochemical applications can affect the occurrence and persistence of EPNs (Stuart et al., 2015). For example, a previous study conducted in La Rioja revealed a negative correlation between EPN activity and the intensity of agricultural management, with high activities in natural areas and perennial organic crops (vineyards included), low in conventional perennial crops, and rare in annual crops, regardless of pest control practices (Campos-Herrera et al., 2008). Similarly, recent investigations performed in an experimental DOCa Rioja vineyard concluded that turning conventional tillage to spontaneous cover cropping could favor the occurrence of above and below beneficial organisms, including EPNs (Sáenz-Romo et al., 2019; Blanco-Pérez et al., 2020). In addition to soil chemical and physical alterations, soil biota such as bacteria, fungi, or nematodes of other nature, also interact with EPNs and modulate their service as biological control agents (Helmberger et al., 2017). Nowadays, advances in molecular tools allow determining their relative importance within the EPN food web in an appropriate ecological context (Campos-Herrera et al., 2012). Diverse free-living nematode (FLN) and EPN species co-emerge from larvae used in insect baits (Duncan et al., 2003; Campos-Herrera et al., 2012, 2015a, 2019a; Jaffuel et al., 2016, 2018; Blanco-Pérez et al., 2020), a competition that plausibly reduces the virulence of emerging IJs (Blanco-Pérez et al., 2019). Also, the nematophagous fungi (NF), found in all main fungal taxonomic groups, are widespread in natural and agricultural soils, remaining as saprophytes until switching to their parasitic stage, developing specialized structures in the presence of nematodes (Nordbring-Hertz et al., 2006). The mechanisms behind these interactions seem to be environmental and species-specific dependent, but still mostly unknown (Koppenhöfer et al., 1996; El-Borai et al., 2009; Bueno-Pallero et al., 2018). Regional and temporal studies showed that the presence of particular NF species can contribute to the persistence of specific EPNs, although still poorly understood which are the scenarios that favor species-specific NF predation of this kind (Pathak et al., 2017; Campos-Herrera et al., 2019b). Besides, microorganisms such as ectoparasitic bacteria (EcPB) in the genus *Paenibacillus* can also compromise the fitness of some EPN species by reducing the motility and virulence of IJs (El-Borai et al., 2005; Enright and Griffin, 2005).

Despite the enormous potential of native EPNs in agroecosystems to control large host ranges, little is known about plausible soil-dwelling target pests in vineyards yet (Campos-Herrera et al., 2021; Marín et al., 2021). However, we should not underestimate the importance of conserving natural enemies of arthropod pests such as EPNs, particularly in the context of a changing climate that may vary the spectrum of pests and diseases affecting vineyards in warmer regions since, for example, they increasingly survive during warmer winters (Santos et al., 2020). Recognizing the factors that drive the activity and abundance of EPNs in the vineyard should allow the establishment of best practices to favor suitable ecological scenarios to enhance their functionality and long-term persistence in the agroecosystems. Additionally, soil nematode community members, involved in primary roles in soil food webs (Eisenhauer and Guerra, 2019), are good bio-indicators of soil health (Ferris et al., 2001). Moreover, recent studies provided evidence that linked high occurrences and activities of EPN species with sustainable agricultural practices (Campos-Herrera et al., 2008, 2014; Blanco-Pérez et al., 2020). However, limited to a few vineyards, these results can not define a whole wine-production area. Comprising 80 vineyards distributed throughout the DOCa Rioja region, our study aimed to explore the impact of differentiating management on the EPN community and associated soil organisms and if their assemblage will provide indicators of better practices for sustainable viticulture. We speculated that organic viticulture and the implementation of alternative strategies to regular tillage might enhance the abundance and the activity of naturally occurring EPN species compared to conventional practices. The main objectives of this study were to (i) quantify EPN abundance and activity, (ii) quantify the abundance of target soil organisms associated with EPNs (NF, FLNs, and EcPB), and (iii) contribute to discriminate the abiotic factors that drive them.

2. Material and methods

2.1. *Survey scheme, sampling methods, and soil properties analyses*

During early autumn 2019, we surveyed 80 vineyards distributed crosswise the DOCa Rioja region (Fig. 1; Supplementary data 1, Table S1), located in a warm-summer Mediterranean climate (classified as Csb by the Köppen-Geiger system) with a continental influence. We

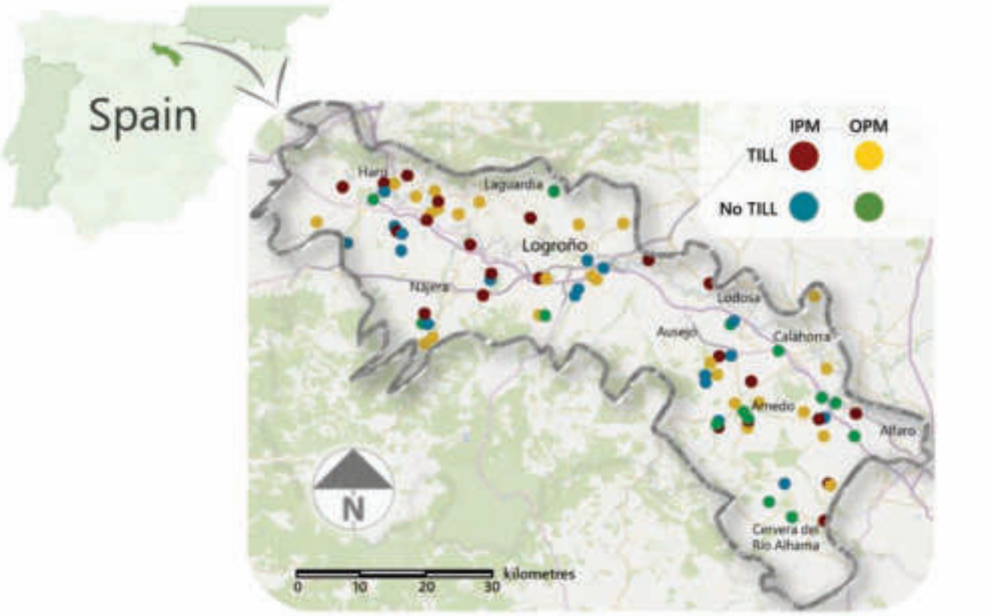

Fig. 1. Distribution of the sampled DOCa Rioja vineyards.

studied the effect of soil management (regular tillage *versus* cover cropping, 48 and 32 sites, respectively) and pest and diseases management (integrated *versus* organic, 40 locations each) on the EPN community and associated soil food web. Thus, each vineyard was characterized for one of the levels of the evaluated treatments, resulting in four possible combinations: tilled IMP, no-tilled IMP, tilled organic farming, and no-tilled organic farming (Table S1). Conventional soil management of DOCa Rioja vineyards consists of secondary tillage (not less than four times a year), mainly to provide weed control in inter-rows throughout the growing season during the maturation of the vine, generally using row crop cultivators (11 arms) with teeth that work at least 10–15 cm depth in the surface (Fernández Alcázar, 2011). We selected different types of cover cropping (a few of them combined with organic mulching) as alternative strategies to tillage that need no or minimum mechanization of soil crops (Table S1). The pest management of vineyards was adjusted to the current Spanish and regional regulations of sustainable use of agrochemical products (RD, 2012; Table S2) and organic farming (RD, 2014; DRioja, 2019), depending on the treatment.

We randomly collected two independent composited samples per vineyard, each consisting of 20 single soil cores (2.5 cm ø x −20 cm DP.), taken with auger soil samplers under the crop canopy of middle rows to avoid possible border effects. The 20 cores per sample were combined in individual plastic bags (−1800 cm^3 of soil) and stored in a chamber (4 °C in the dark) until processed (within 2–4 days). Following the protocol described by Campos-Herrera et al. (2019a), each sample was manually homogenized and divided into three subsamples of 200 g of fresh soil (Wiesel et al., 2015). Two of the subsamples were employed (i) to characterize the abundance of EPN community and associated organisms through sucrose-gradient centrifugation procedure and qPCR analysis, and (ii) determinate the soil suppressive capacity by using the traditional insect-bait method. The third soil set was dried at 40 °C for one week to measure the water content to express the number of identified organisms per 100 g of dry soil. Finally, we combined 100 g of oven-dried soil of both replicates of each vineyard to analyze the following soil properties (performed by Laboratorio Regional del Gobierno de La Rioja, La Grajera, Logroño, Spain): texture (sand, silt, and clay percentages) (Bouyoucos, 1936), pH (Millennia and Markewitz, 2004), electric conductivity, organic matter (Walkley and Black, 1934), macro-nutrients (P, K, and nitrates), oligo-nutrients (Mg, Ca, and SO_4), micro-nutrients (Fe, Mn, Zn, Cu, Al, and B), and other elements (Na and Pb) (Mehlich, 1978, 1984). Monthly precipitations in the DOCa Rioja region in 2019 were also recorded by the Agro-climatic Information Service in La Rioja (SIAR; Fig. S1).

2.2. Soil mesofauna isolation and estimation of soil activities against insect larvae

Following the procedure described by Blanco-Pérez et al. (2020), nematodes and other soil organisms were co-extracted through sucrose-gradient centrifugation (Jenkins, 1964) from sieved (aperture width of 25 µm) 200 g of fresh soil of each sample (Wiesel et al., 2015). Besides, we employed the traditional insect-bait method to estimate the soil activity ratios following a protocol adapted from Bedding and Akhurst (1975). Specifically, we calculated differentiated mortality percentages of final instars of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae (reared at ICVV): total mortality (total-act), mortality associated with nematode emergences (nem-act), and percentage of larvae for which Koch's postulates were confirmed (EPN-act). Additionally, we maintained some of the nematode emergences, from first and Koch's postulates test rounds (VO and VM aliquots, respectively), to establish laboratory cultures following the procedure described by Woodring and Kaya (1988). Both suspensions of soil organisms obtained through sucrose-gradient centrifugation and RO/RM aliquots were

stored at − 20 °C in the dark until used for DNA extraction procedures (see Blanco-Pérez et al., 2020 for a more detailed protocol).

2.3. Identification and quantification of soil organisms by real-time qPCR

For the DNA extraction procedure, we first mechanically disaggregated all samples with sterile blue pestles (15 seg) assembled to a Kontes™ Pellet Pestle™ motor (DWK Life Sciences GmbH, Mainz, Germany). We used the DNA extraction kits DNeasy PowerSoil Kit (QIAGEN GmbH Co., Hilden, Germany) and Speedtools tissue (Biotools, B&M Labs S.A., Madrid, Spain) for the samples obtained by sucrose-gradient centrifugation and RO/RM aliquots, respectively. DNA extractions were stored at − 20 °C until used. We screened for 22 soil organisms using species-specific primers and probes in qPCR tests (Table 1; Supplementary data 2, Table S3). Previous studies reported the presence in La Rioja of most of the evaluated species: the EPNs *Steinernema carpocapsae*, *S. feltiae*, *S. kraussei*, *S. riojaense*, *S. affine*, *Heterorhabditis bacteriophora*, and *H. indica*, the FLNs *Pristionchus maupasi* and *Acrobeloides*-group, the NF *Arthrobotrys oligospora*, *A. dactyloides*, *Purpureocillium lilacinum*, *Hirsutella rhossiliensis*, and *Catenaria* sp., and the EcPB *Paenibacillus* sp. (Campos-Herrera et al., 2007, 2008; Blanco-Pérez et al., 2020). Also, the EPN *S. intermedium*, the FLNs *Oscheius tipulae* and *O. onirici*, the NF *Arthrobotrys musiformis*, and the EcPB *Paenibacillus nematophilus* had been identified in the Iberian Peninsula (García del Pino, 2005; Campos-Herrera et al., 2011a, 2016, 2019a). On the other hand, there is still no record for *Heterorhabditis megidis* in Southwest Europe but Northern and Eastern European countries (Hominick, 2002). Besides, we designed a species-specific primers/probe set (Supplementary data 2) for the EPN species *Steinernema* sp. *affine*-group (recorded by Valadas et al., 2014 in continental Portugal, but misidentified as *S. intermedium* according to Dr. Vladimír Půža, Czech Academy of Sciences, Czech Republic), isolated from aliquots of insect-baits conducted in this study. Moreover, we included additional nematode species (13 EPNs and 5 FLNs) to validate the qPCR tools, avoiding cross-amplification and adapting previously published protocols to our experimental conditions (Table S4). Pure cultures of most evaluated soil organisms were preserved as described by Blanco-Pérez et al. (2020) (Supplementary data 2).

Final concentrations of species-specific primers and probes, synthesized by Biotools (Madrid, Spain), were 400 and 200 nM, respectively, except for *Steinernema* sp. *affine*-group, which was 100 and 40 nM. Probes were labeled at the 5' and 3' ends with the fluorogenic reporter dye FAM and the quencher BQH-1, respectively. The qPCR tests and quantifications were performed with the Quantimix Easy Probe mix (Biotools, Madrid, Spain) in Bio-Rad iCycler iQ® 96-well PCR plates, covered with Bio-Rad iCycler iQ® Optical tape, on the Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We run 38 cycles for nematodes species and 50 for NF and EcPB species. Before qPCR runs, all DNA samples were analyzed for quality and quantity in a Nanodrop system (Thermo Scientific 2000 C spectrophotometer) and adjusted to 1 ng/µl for all the organisms except NF, adjusted to 10 ng/µl. We optimized protocols and established positive controls described by Blanco-Pérez et al. (2020) (Supplementary data 2).

Table 1
Soil organisms tested.

Type of organism / species	Population	GenBank ACNO ITS Region	Reference for primers and probes sequences
Entomopathogenic nematodes			
Heterorhabditis bacteriophora	(commercial)	KJ938576	Campos-Herrera et al. (2011a)
Heterorhabditis indica	Btw	KJ938571	Campos-Herrera et al. (2011b)
Heterorhabditis megidis	(commercial)	KJ938577	Campos-Herrera et al. (2011b)
Steinernema affine	CH	KJ938567	Torr et al. (2007)
Steinernema carpocapsae	DOK-83	KJ818295	Campos-Herrera et al. (2011a)
Steinernema feltiae	RS-5	KJ938569	Campos-Herrera et al. (2011a)
Steinernema intermedium	82	AF171290	Campos-Herrera et al. (2013b)
Steinernema kraussei	OS	KJ696686	Campos-Herrera et al. (2013b)
Steinernema riojaense	RM-30	MK503133	Blanco-Pérez et al. (2020)
Steinernema sp. *affine*-group	VO-53	MW480137	(current study)
Free-living nematodes			
Acrobeloides-group	RT1-R15C	JQ237849	Campos-Herrera et al. (2012)
Oscheius tipulae	MG68 P29	KJ938579	Campos-Herrera et al. (2015a)
Oscheius onirici	MG67 P20	KJ938578	Campos-Herrera et al. (2015a)
Pristionchus maupasi	AM-3	MG551681	Campos-Herrera et al., 2019a
Nematophagous fungi			
Catenaria sp.	1D	JN585805	Pathak et al. (2012)
Arthrobotrys dactyloides	H55	KJ938574	Pathak et al. (2012)
Arthrobotrys musiformis	11	KJ938572	Pathak et al. (2012)
Arthrobotrys oligospora	8	KJ938573	Pathak et al. (2012)
Hirsutella rhossiliensis	2931	KM652168	Zhang et al. (2006)
Purpureocillium lilacinum	9357	KJ938575	Atkins et al. (2005)
Ectoparasitic bacteria			
Paenibacillus nematophilus	NEM2	AF480936	Campos-Herrera et al. (2011a)
Paenibacillus sp.	SdTc1FEE1	JF317562	Campos-Herrera et al. (2011a)

2.4. Statistical analyses

We ran generalized mixed models (GLMMs), executed with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), testing the effect of differentiated farming practices of DOCa Rioja vineyards, pest management (two levels: integrated *versus* organic), soil management (two levels: regular tillage *versus* cover cropping), and their interactions on DNA quantifications and abundance/frequencies of occurrence of EPNs, FLNs, NF, EcPB, and soil activity measures. The quantifications obtained by sucrose-gradient centrifugation were expressed per 100 g of dry soil: EPNs as number of IJs, EcPB as copies of plasmids, and FLNs and NF as standardized 0–1 range of the values recorded for each species (see Blanco-Pérez et al., 2020 for further details). The soil activities (total–act, nem-act, and EPN-act) recorded in the insect baits were represented as larval percentages and frequencies of occurrence. Before running the statistical analysis, quantitative variables were log(x + 1) transformed. We ran GLMMs with a gamma distribution (log-link function) for quantifying soil organisms and binomial distribution (logit-link function) for soil activities and frequencies of occurrence. We initially considered covariates in exploratory GLMM tests of all the abiotic variables that showed significant differences between treatments for general linear model (GLM) tests (Fig. 2; Supplementary data 3, Table S5). Subsequently, we included soil organic matter to test for differences in soil activities and quantifications of DNA, total FLNs, and specific target organisms. We used the Least-Squares Means ± standard error of the mean (SEM) as descriptive statistics. We employed Microsoft Excel 2016 (3D Maps) for visualizing the sample sites and species distributions.

For the multivariate analyses of selected soil organisms and abiotic factors, performed with CANOCO 5 (ter Braak and Šmilauer, 2002; Šmilauer and Lepš, 2014), we first chose some of the abiotic factors as

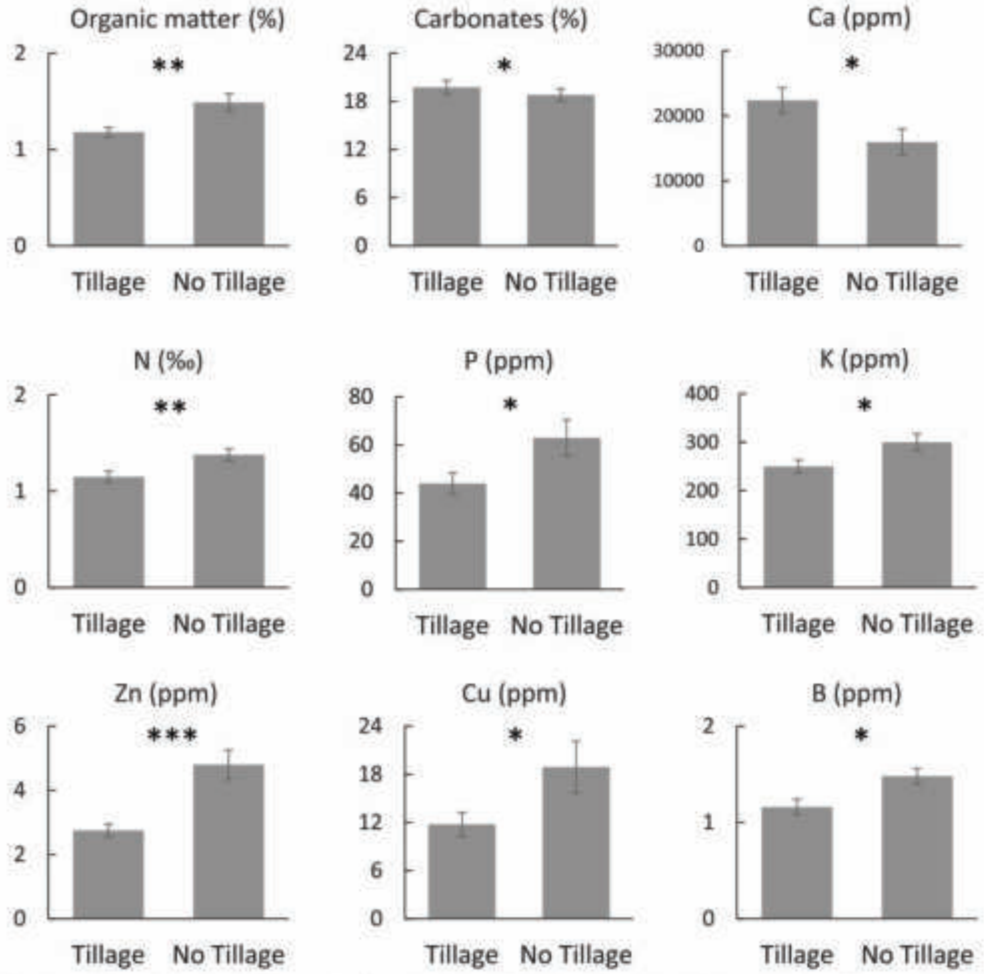

Fig. 2. General linear model testing for differences of soil properties between tilled and no-tilled soils. Asterisks indicate significant differences at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Values are least-square means±SE (see Table S5 for complete statistics).

exploratory predictors after avoiding strong co-linearities (Table S6). Then, both biotic and abiotic variables were standardized by dividing by the highest values, ranking all values 0–1 (Šmilauer and Lepš, 2014). Values over 3.0 maximum length (SD units) for detrended canonical correspondence analysis (DCCA) indicate heterogeneous communities, and canonical correspondence analysis (CCA, constrained axes) is recommended (ter Braak and Šmilauer, 2002). We run CCA (interspecies correlations) with a Monte Carlo permutation ($n = 499$) and automatic forward selection for the assignment of significant ($P < 0.05$) abiotic factors (using the Bonferroni correction for P values). The final results were visualized with bi–plot scaling (CANOCO 5).

3. Results

3.1. Soil properties and DNA quantification

We did not find differences for the evaluated abiotic factors between integrated and organic pest management but between regular tilling and cover cropping (Fig. 2; Supplementary data 3, Table S5). Overall, we recorded lower values for soil organic matter, N, P, K, Zn, Cu, and B in tilled soils but higher for carbonates (Fig. 2; Table S5). Among all these variables, only the soil organic matter was included in GLMMs testing the effect of our treatments on DNA quantifications and the abundances and occurrence of the target species *S. feltiae*, *O. tipulae*, *A. oligospora*, *H. rhossiliensis*, *Catenaria* sp., and *Paenibacillus* sp. Contrary, soil management did not affect the quantities of DNA obtained through sucrose-gradient centrifugation but pest management, higher in organic viticulture than IPM, although mediated by high values of soil organic matter (Fig. S2).

3.2. *Abundance and frequency of occurrence of the soil organisms*

We detected species of all the groups of soil organisms evaluated all across the DOCa Rioja region (Fig. 3), with no significant differences among treatments for the total EPN abundance and frequency of occurrence (Fig. 4A; Table 2). We detected four out of the ten EPN species screened: *S. feltiae*, *S. riojaense*, and *H. bacteriophora* recorded in all treatments, and *Steinernema* sp. *affine*-group only missed in tilled IMP vineyards (Fig. 4A; Table 2). Only for *S. feltiae* we reported statistical differences among treatments: higher abundance in organic viticulture and lightly higher frequency of occurrence ($P < 0.1$) in tilled soils, both mediated by soil organic content (Table 2; Table S7). Regarding FLNs, we reported higher abundance in organic vineyards than IPM, specifically for *Acrobeloides*-group and *O. tipulae*, both mediated by high values of soil organic matter (Table 2; Table S7), but not for soil management (Fig. 4B). In addition, we noticed the presence of the other two screened species, *O. onirici* and *P. maupasi*. The species *O. tipulae* was also detected in higher numbers in no-tilled soils, while *O. onirici* and *P. maupasi* were more abundant in bare soils (Table 2; Table S7). On the other hand, we detected five out of the six screened NF species: *A. dactyloides*, *A. oligospora*, *Catenaria* sp., *H. rhossiliensis*, and *P. lilacinum* (Fig. 4C). The viticulture practices evaluated did not affect the NF identified (Fig. 4C), except *A. dactyloides*, recorded in higher numbers and frequencies in organic viticulture, again mediated by high values of soil organic matter (Table 2; Table S7). Finally, we found one out of the two screened EcPB species, *Paenibacillus* sp., in higher abundance in organic vineyards than IPM, also mediated by high values of soil organic, while not for soil management (Fig. 4D; Table S7).

3.3. *Soil activities rates*

For all the soil activities evaluated, we recorded significantly higher values in organic viticulture than IPM, also registered in a higher frequency of organic vineyards for total-act and nem-act, but not for soil management (Fig. 5, Fig S4 and S5; Table S8). The same nematode species identified through sucrose gradient centrifugation emerged from insect baits plus the steinernematid species *S. carpocapsae* and *S. kraussei*. The species *S. feltiae*, *O. tipulae*, *P. maupasi*, and *Acrobeloides*-group occurred in all treatments except no-tilled IPM, for which no nematodes were found. The EPNs *Steinernema* sp. *affine*-group and *S. riojaense* happened in organic viticulture and *H. bacteriophora* in IPM. Finally, *S. kraussei* and *O. onirici* emerged from tilled IMP and *S. carpocapsae* from no-tilled organic vineyards only (Fig. 5A), none of them detected in the Koch`s postulates tests (Fig. 5B). Nevertheless, we recorded no significant differences among treatments for any nematode species identified, neither from VO nor VM aliquots.

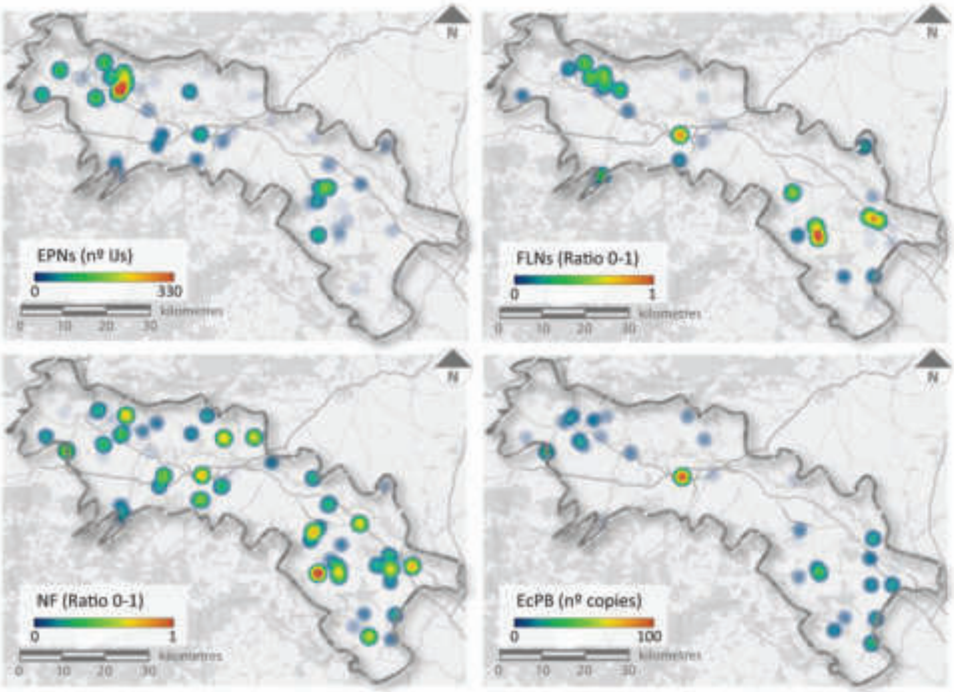

Fig. 3. Distribution in the DOCa Rioja region of the cumulative abundance of the soil organisms extracted through sucrose-gradient centrifugation: entomopathogenic nematodes (EPNs), free-living nematodes (FLNs), nematophagous fungi (NF), and ectoparasitic bacteria (EcPB).

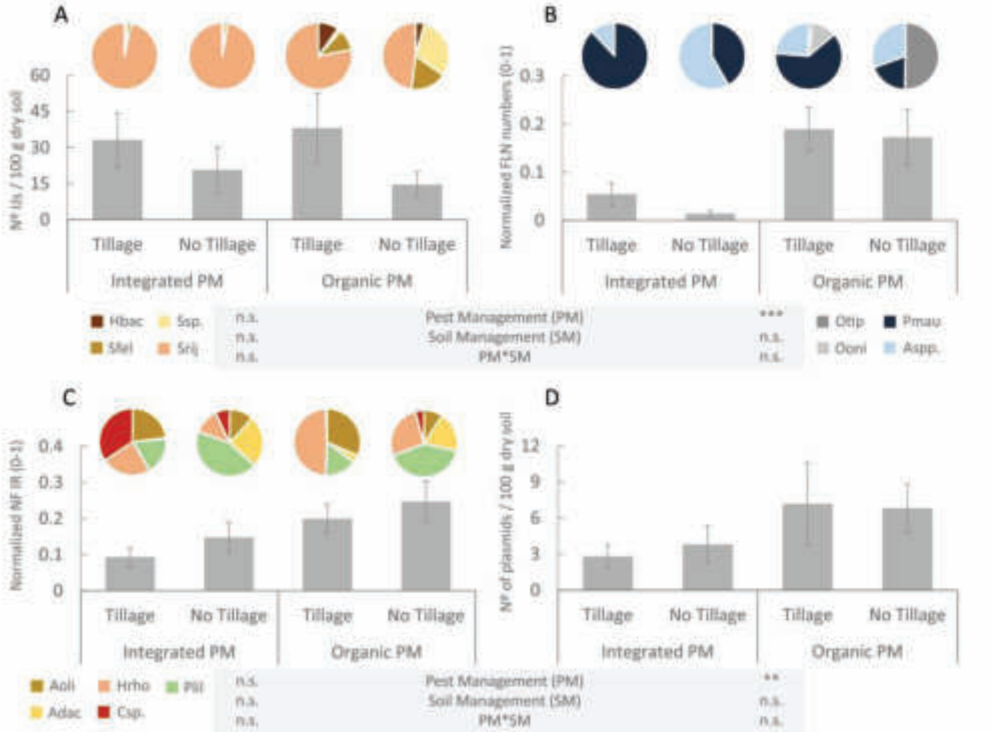

Fig. 4. Effect of different management practices in DOCa Rioja vineyards on abundance of (A) infective juveniles (IJ) of the entomopathogenic nematode species *Heterorhabditis bacteriophora* (Hbac), *Steinernema* sp. *affine*-group (Ssp.), *S. feltiae* (Sfel), and *S. riojaense* (Srij), (B) the free-living nematodes (FLNs) *Oscheius tipulae* (Otip), *O. onirici* (Ooni), *Pristionchus maupasi* (Pmau), and *Acrobeloides*-group (Aspp.), (C) nematophagous fungi (NF) infection ratio (IR) of the species *Arthrobotrys oligospora* (Aoli), *A. dactyloides* (Adac), *Purpureocillium lilacinum* (Plil), *Hirsutella rhossiliensis* (Hrhos), and *Catenaria* sp. (Csp.), and (D) the ectoparasitic bacterial species *Paenibacillus* sp. Asterisks indicate significant differences from generalized linear mixed model tests at $^{***}P < 0.001$ and $^{**}P < 0.01$ (n.s., not significant). Values are least-square means±SE. Soil species averages are represented in pies.

3.4. *Multivariate analysis of abiotic factors and target soil organisms*

Since the DCCA gradient was 3.1 SD units long, we performed CCA with the selected abiotic factors (Table S5) as exploratory variables, and the soil organisms isolated through sucrose-gradient centrifugation as response variables except for the NF species *Catenaria* sp. and the FLN species *O. tipulae* and *O. onirici*, present in less than 10% of the vineyards (Lepš and Hadincová, 1992). Four soil properties (pH and clay, organic matter, and Cu content) significantly ($P < 0.1$) contributed to explaining the soil composition and target species assemblage. The explained fitted variation of the two first axes reached 65%, Axis 1 primarily influenced by Cu and clay content, and Axis 2 by pH and soil organic matter (Fig. 6). Regarding the EPN species, *S. feltiae* was linked to high soil organic matter content and low pH and *S. riojaense* to high Cu and clay content, an opposite trend that observed for *Steinernema* sp. *affine*-group. The heterorhabditid *H. bacteriophora*, located near the origin axes, was unrelated to the predictor factors. The FLNs in the genus *Acrobeloides* correlated with low clay and organic matter contents and high pH, while the NF species *H. rhossiliensis* and *P. lilacinum* were located in the opposite quadrant. The NF species in the genus *Arthrobotrys* were associated with low organic matter content and high pH, particularly *A. dactyloides*. The FLN *P. maupasi* and the EcPB *Paenibacillus* sp. were widespread with no apparent association with the independent variables.

4. Discussion

4.1. *Impact of pest management*

The literature provides not many examples of the effect of different agronomic management systems on the EPN community. For instance, Ellers-Kirk et al. (2000) observed that the inundative release of *S. riobravis* IJs successfully controls *Acalymma vittatum* (Coleoptera: Chrysomelidae) populations in organic cucumber crops, in relative terms better than IMP. Contrary, no differences were found for the abundance and activity of EPNs and associated organisms in an ambitious study comprising four farming systems applied on three different crops (Jaffuel et al., 2016). However, and in agreement with our hypothesis, we observed higher soil activity rates for organic farming than IPM, also for those associated with nematode emergences, including EPNs. Conversely, we did not find significant differences for the total abundance of EPN species between the evaluated pest management treatments, probably due to the high numbers detected for *S. riojaense* also in IPM vineyards. Up to now, *S. riojaense*, the prevalent EPN species

Table 2

Descriptive (x̄ ±SEM) by treatments of abundances and frequencies of occurrence of the evaluated soil organisms. Asterisks indicate significant differences from generalized linear mixed models tests at *** $P<0.001$, ** $P<0.01$, and * $P<0.05$ (+ $P<0.1$; n.s., not significant). Abundances (per 100 g of dry soil) measured as infective juveniles for entomopathogenic nematodes (EPNs), and ng/µl of pure culture for free-living nematodes (FLNs), and nematophagous fungi (NF). Codes: PM, pest management; SM, soil management; IPM, integrated pest management; OPM, organic pest management; Till, tillage; OM, soil organic matter.

	Abundances								Frequencies of occurrence							
	Treatments				Factors and covariables				Treatments				Factors and covariables			
	IPM-Till	IPM-NoTill	OPM-Till	OPM-NoTill	PM	SM	PM*SM	OM (%)	IPM-Till	IPM-NoTill	OPM-Till	OPM-NoTill	PM	SM	PM*SM	OM (%)
EPN species																
Heterorhabditis bacteriophora	0.11 ±0.11	0.23 ±0.23	3.73 ±1.79	0.60 ±0.60	n.s.	n.s.	n.s.	-	0.02 ±0.02	0.03 ±0.03	0.15 ±0.05	0.04 ±0.04	n.s.	n.s.	n.s.	-
Steinernema feltiae	0.62 ±0.24	0.37 ±0.24	3.82 ±2.34	2.51 ±0.94	**	n.s.	n.s.	***	0.23 ±0.06	0.14 ±0.06	0.44 ±0.07	0.32 ±0.09	n.s.	+	n.s.	*
Steinernema riojaense	32.13 ±11.10	20.11 ±9.57	29.77 ±13.92	6.98 ±2.90	n.s.	n.s.	n.s.	-	0.30 ±0.07	0.39 ±0.08	0.40 ±0.07	0.32 ±0.09	n.s.	n.s.	n.s.	-
Steinernema sp. *affine*-group	0.33 ±0.24	0	0.77 ±0.39	4.49 ±4.25	n.s.	n.s.	n.s.	-	0.07 ±0.04	0	0.12 ±0.04	0.07 ±0.05	n.s.	n.s.	n.s.	-
FLN species																
Acrobeloides-group	6,5E-03 ±1,8E-03	8,2E-03 ±2,6E-03	4,5E-02 ±1,7E-02	5,3E-02 ±1,6E-02	*	n.s.	n.s.	**	0.82 ±0.06	0.83 ±0.06	0.98 ±0.02	0.96 ±0.04	n.s.	n.s.	n.s.	-
Oscheius tipulae	0	0	3,4E-03 ±2,4E-03	8,7E-02 ±4,3E-02	***	***	**	***	0	0	0.04 ±0.03	0.25 ±0.08	+	n.s.	n.s.	*
Oscheius onirici	0	0	2,3E-02 ±1,9E-02	0	n.s.	**	*	-	0	0	0.04 ±0.03	0	n.s.	n.s.	n.s.	-
Pristionchus maupasi	4,8E-02 ±2,4E-02	5,9E-03 ±5,9E-03	1,2E-01 ±3,4E-02	3,3E-02 ±2,3E-02	n.s.	*	n.s.	-	0.16 ±0.06	0.03 ±0.03	0.35 ±0.07	0.18 ±0.07	+	n.s.	n.s.	-
NF species																
Arthrobotrys dactyloides	4,1E-04 ±4,0E-04	3,7E-02 ±2,8E-02	7,4E-03 ±3,7E-03	4,8E-02 ±1,9E-02	n.s.	**	n.s.	**	0.05 ±0.03	0.17 ±0.06	0.10 ±0.04	0.29 ±0.09	+	**	n.s.	*
Arthrobotrys oligospora	2,1E-02 ±1,1E-02	1,7E-02 ±1,0E-02	6,3E-02 ±2,7E-02	2,2E-02 ±1,6E-02	n.s.	n.s.	n.s.	-	0.11 ±0.05	0.08 ±0.05	0.15 ±0.05	0.07 ±0.05	n.s.	n.s.	n.s.	-
Catenaria sp.	3,2E-02 ±2,4E-02	1,0E-02 ±1,0E-02	1,1E-03 ±1,1E-03	1,1E-02 ±1,0E-02	n.s.	n.s.	n.s.	***	0.07 ±0.04	0.03 ±0.03	0.02 ±0.02	0.07 ±0.05	n.s.	n.s.	n.s.	*
Hirsutella rhossiliensis	2,3E-02 ±8,3E-03	1,8E-02 ±1,7E-02	9,7E-02 ±3,5E-02	6,3E-02 ±4,1E-02	n.s.	n.s.	n.s.	-	0.20 ±0.06	0.14 ±0.06	0.23 ±0.06	0.14 ±0.07	n.s.	n.s.	n.s.	-
Purpureocillium lilacinum	1,7E-02 ±4,3E-03	6,5E-02 ±2,3E-02	3,1E-02 ±6,7E-03	1,0E-01 ±4,0E-02	n.s.	***	***	-	0.70 ±0.07	0.67 ±0.08	0.79 ±0.06	0.71 ±0.09	n.s.	n.s.	n.s.	-

in this study, has been only identified by Blanco-Pérez et al. (2020) in an experimental DOCa Rioja vineyard but in lower numbers. However, the high abundance recorded through sucrose-gradient centrifugation contrasted with the poor presence of *S. riojaense* obtained in insect baits and only from organic vineyards. Although the biology and ecology of this newly described species are still mostly unknown (Puza et al., 2020), this discrepancy might be due to the possible underperformance of *S. riojaense* for the host chosen in our bio-test (*G. mellonella* larvae). Indeed, Adams and Nguyen (2002) illustrated similar issues for EPNs of a narrow host range as *S. scarabaei* or *S. scapterisci*. Considering the rest of the identified EPN species, we only reported significant differences for higher abundance in organic viticulture of *S. feltiae*, the prevalent steinernematid species in Europe (Hominick, 2002; Bhat et al., 2020), and surveys completed in the Iberian Peninsula in the last decades (García del Pino and Palomo, 1996; Campos-Herrera et al., 2007, 2019a, 2019b; Valadas et al., 2014; Blanco-Pérez et al., 2020). Although these findings over mainland Spain and Portugal reported evidence of *S. feltiae* in a wide variety of habitats, studies performed in La Rioja found higher activity rates and abundance for this particular EPN species in less disturbed ecosystems like natural habitats, organic crops, and no-tilled vineyards (Campos-Herrera et al., 2007, 2010; Blanco-Pérez et al., 2020). The common practice of organic fertilizer applications in organic farming often enhances soil organic matter content (Leifeld and Fuhrer, 2010). Even if this assertion was not fully satisfied in our study, we established a correlation between organic matter and *S. feltiae* abundance in the overall soil community assemblage analysis (CCA). Linford's hypothesis proposed that organic matter inputs to soil generate rapid FLN population growth followed by their natural enemies (Linford, 1937; Cooke, 1962). Possibly, the higher soil organic matter contents that characterize organic vineyards favor the development of sustainable hosts for nematodes as *S. feltiae* to allow their long-term persistence. Other EPN species (*H. bacteriophora*, *Steinernema* sp. *affine*-group, *S. carpocapsae*, and *S. kraussei*) occurred at lower numbers, with no significant differences between the evaluated pest management practices. It is important to note that these three steinernematid species occurred only in organic viticulture, perhaps indicating the importance of this type of management to support the biodiversity within EPN communities.

The total abundance of our target FLN species was significantly higher in organic viticulture than IPM, particularly for bacterivores species in the genus *Oscheius* and *Acrobeloides* and, in agreement with Linford's hypothesis, enhanced by high soil organic matter contents. Bacterial feeder nematodes are good predictors of soil health in terms of soil structure, pH, and organic matter content (van den Hoogen et al., 2019). However, the few existing reports approaching the effects of organic farming on the nematode community suggested no clear

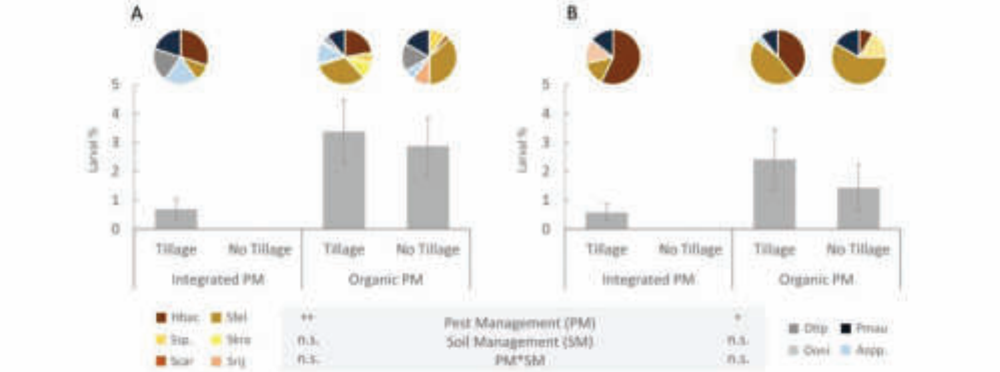

Fig. 5. Effect of different management practices in DOCa Rioja vineyards on soil activities measured as percentages of *Galleria mellonella* larvae (A) that showed nematode emergences and (B) positive for the Koch's postulates. Asterisks indicate significant differences from generalized linear mixed model tests at $^{**}P < 0.01$ and $^{*}P < 0.05$ (n.s., not significant). Values are least-square means ± SE (see Table S8 for complete statistics). Averages of the nematodes identified are represented in pies: the entomopathogenic nematode species *Heterorhabditis bacteriophora* (Hbac), *Steinernema* sp. affine-group (Ssp.), *S. carpocapsae* (Scar), *S. feltiae* (Sfel), *S. kraussei* (Skra), and *S. riojaense* (Srij); and the free-living nematode species *Oscheius tipulae* (Otip), *O. onirici* (Ooni), *Pristionchus maupasi* (Pmau), and *Acrobeloides*-group (Aspp.).

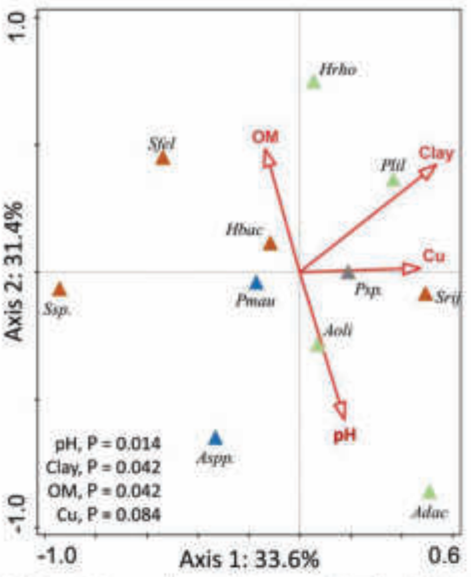

Fig. 6. Canonical correspondence analysis among significant ($P < 0.1$) abiotic factors (arrows) and soil organisms (triangles) isolated through sucrose gradient centrifugation from DOCa Rioja vineyards. Codes: entomopathogenic nematode species (orange triangles) *Heterorhabditis bacteriophora* (Hbac), *Steinernema* sp. *affine*-group (Ssp.), *S. feltiae* (Sfel), and *S. riojaense* (Srij); free-living nematode species (blue triangles) *Pristionchus maupasi* (Pmau) and *Acrobeloides*-group (Aspp.); nematophagous fungi species (green triangles) *Arthrobotrys oligospora* (Aoli), A. dactyloides (Adac), *Purpureocillium lilacinum* (Plil), and *Hirsutella rhossiliensis* (Hrhos), and the ectoparasitic bacterium (grey triangle) *Paenibacillus* sp. (Psp.); OM, soil organic matter content. The P values were adjusted with the Bonferroni correction.

benefits. For example, the study of soil fertility and soil food web structure in semiarid vineyards and olive orchards in South-Central Spain did not distinguish between conventional and organic management but between crop systems (Sánchez-Moreno et al., 2018). On the other hand, other studies reported higher nematode abundance in organic viticulture compared to conventional practices but only concerning specific trophic groups: fungal-feeding nematodes in Southern French vineyards (Coll et al., 2012), and omnivore-predator species occurring in inter-rows in Northern Israel (Schlüter et al., 2022). Anyhow, our study did not intend to characterize the free-living community but fit its co-occurrence within the EPN soil food web. Various studies have observed declines in the fitness of some EPN species in the presence of FLNs in the genus *Pellioditis* (Duncan et al., 2003), *Acrobeloides* (Campos-Herrera et al., 2012), and *Oscheius* (Blanco-Pérez et al., 2019). As highlighted by Blanco-Pérez et al. (2020), the relative abundance of diverse soil organisms associated with the EPN soil food web could partially explain the numbers we reported for EPN species. Our results showed that organic viticulture did not favor the proliferation of the NF species evaluated but the EcPB *Paenibacillus* sp. This bacterium, initially linked with *Steinernema diaprepesi* (Nguyen et al., 2007), has been detected in the Iberian Peninsula, putatively associated with other steinernematids close related to *S. diaprepesi* in the Clade V (Campos-Herrera et al., 2019a; Blanco-Pérez et al., 2020), especially *S. riojaense* (Pizano et al., 2020). Whether a possible association *S. riojaense*-*Paenibacillus* sp. limit the nematode movement is a matter of speculation that deserves further attention. In any case, it could explain, partially at least, the high abundance in the soil but the low activity of *S. riojaense* in organic vineyards. Overall, organic farming could enhance the occurrence and biodiversity in vine rows of certain soil mesofauna groups, nematofauna included, so probably EPNs have to deal with more potential competitors.

4.2. *Impact of soil management*

Although several studies revealed that inter-row tillage favors soil biota degradation in vineyards (Karimi et al., 2020), its impact on the nematofauna is still mostly unknown. Shapiro-Ilan et al. (1999) observed significantly higher persistence for the EPN *S. carpocapsae* in corn-soybean rotation ecosystems treated with crop residues based on soybean stubble than in bare soils. In the same direction, Susurluk and

Ehlers (2008) noted that no-tilled managements of different crops increase the persistence of *H. bacteriophora*, probably by maintaining host occurrence, improving soil health conditions, and reducing temperature fluctuations. Moreover, Blanco-Pérez et al. (2020) remarked a correlation between EPN abundance and activity and physical disturbance of inter-rows, recording higher EPN values for cover cropping than tilling, especially in the absence of mechanization in spontaneous cover crops. In disagreement with these results and our hypothesis, we observed that neither abundance nor activity of EPNs significantly varied among vineyards that differed in the inter-row soil management. Our observations also contrast with the results of several studies based on baiting methods and conducted in other crop systems, although with reservations. For example, no-tillage or strip-till significantly increased the infection of *G. mellonella* larvae by EPNs compared with traditional tillage in North Carolina, while other factors, such as soil properties, pesticide inputs, or type and quality of cover cropping, could similarly affect EPNs or the availability of target hosts (Brust, 1991; Hummel et al., 2002). Besides, Millar and Barbercheck (2002) suggested opposite sensitivities for different EPN species to the conditions created in tilled soils. Even if conventional tillage in viticulture rarely is intensive and does not directly affect rows (the sampling area in this study), we found several differences in the abiotic factors between till and no-till treatments that could modulate the occurrence of EPNs in a species-specific manner. For instance, metal ions like Zn and Cu, recorded at higher rates in our no-tilled vineyards, can exhibit very high toxicity on steinernematids but much less on heterorhabditids (Jaworska et al., 1996, 1997). This presumption seems to agree with our observations for *Steinernema* sp. *affine*-group and *S. feltiae*, both negatively affected by high Cu contents, while an opposite trend was noted for *S. riojaense*, closely associated with Cu in our CCA analysis. This example gives us an idea of the difficulty of determining the relative importance of different abiotic factors when evaluating their impact on the EPN community in complex soil environments.

In addition to soil properties, biotic factors can also affect the survival and reproduction of EPNs (Stuart et al., 2015). We found no differences between tilled and no-tilled vineyards for the total quantification of any other group of the soil organisms evaluated but for specific species. Alternative strategies to tillage and herbicide applications like cover cropping may enhance the nematode community by affecting trophic links associated with their soil food web (Sánchez-Moreno et al., 2015). However, in our study, no-tilled vineyards only appeared to favor the occurrence of the FLN *O. tipulae* and the NF *A. dactyloides* and *P. lilacinum*, while the nematode species *P. maupasi* and *O. onirici* were more abundant in tilled soils. The complex interactions that these soil organisms could establish with EPNs are not easy to interpret. For instance, the versatility of roles attributed to nematodes in the genus *Pristionchus* in natural conditions can serve as an illustration. The wide-spread hermaphroditic species *P. maupasi* is commonly present in rotting vegetal substrates (Félix et al., 2018), closely associated with scarabaeoid beetles in the genus *Melolontha* (Herrmann et al., 2006). The type of relationship with its hosts is controversial. According to Félix et al. (2018), *P. maupasi* displays a phoretic association with cockchafers to disperse the dauer juveniles (the stress-resistant, alternative third juvenile stage) and colonize new food sources. Certainly, Hong et al. (2008) concluded that *P. maupasi* is attracted to the most dispersive forms of cockchafers. But Herrmann et al. (2006) considered that the nematodes in the genus *Pristionchus* follow a necromeny behavior, an association much more specific with their hosts that is suggested, in evolutionary terms, as an intermediate step preceding true parasitism (Dillman et al., 2012). Although there is no evidence for considering *P. maupasi* a parasite of insects or a competitor of EPNs for hosts (Herrmann et al., 2006; Blanco-Pérez et al., 2019), the living and feeding habits of soil nematodes under natural conditions is still mostly unknown. Indeed, the specialized buccal cavity of *Pristionchus* and other diplogastrid nematodes allow them to feed on bacteria, fungi, and other nematodes (von Lieven, 2003), so they could also behave as predators if conditions require it. On the other hand, *in vitro* experiments showed that *O. onirici* could compromise *S. feltiae* virulence when acting as scavengers, a plausible strategy for EPNs to survive (Blanco-Pérez et al., 2019).

As mentioned, some FLN species can compete with EPNs for resources, but the nature of their interaction is still uncertain (Blanco-Pérez et al., 2017). We expected to notice a more straightforward relationship between NF and EPNs. Based on their observations, Blanco-Pérez et al. (2020) suggested that endoparasitic NF (*H. rhossiliensis* and *Catenaria* sp.) could be more efficient in killing EPNs than trapping NF (*Arthrobotrys* spp.). Since we obtained higher quantifications for *A. dactyloides* in no-tilled vineyards and no differences for *H. rhossiliensis* and *Catenaria* sp. between soil management treatments, our results did not ratify this premise. On the opposite, as also highlighted by Blanco-Pérez et al. (2020), the higher numbers reported for the eggs-trapping fungal species *P. lilacinum* on no-tilled vineyards could be due to an underestimation of the FLN abundance.

5. Conclusions

Our study supported the hypothesis that organic viticulture can favor the EPN activity and the abundance, at least, of the EPN *S. feltiae*, the predominant steinernematid species in Europe (Hominick, 2002; Bhat et al., 2020). In addition, we also observed evidence of higher quantities of FLNs and identified more nematode species (EPNs and FLNs) in organic agriculture. Nonetheless, we found no differences for the total EPN abundance between organic and integrated pest management, perhaps due to complex interactions among soil members assemblage to their soil food web. Conjointly, these results could indicate a higher resilience against pests and the healthiness of organic vineyard soils. Contrary, alternative strategies to tillage affected only a few of the evaluated soil species, without general distinctions on the EPN community or associated soil organisms. The vineyards selected for this study comprised, actually, multi-faceted management practices that could disturb soil biotic and abiotic characteristics differently. Since the nematode community could react to these singular disturbances even in a species-specific manner, our objective of establishing the causal effects of differentiated viticulture practices on the activity and abundance of native EPN species is considerably complex (Fiscus and Neher, 2002; Stuart et al., 2015). Proper viticulture practices will maximize profits and reduce potential problems (Provost and Pedneault, 2016), for instance, promoting optimal conditions for the occurrence of natural enemies of possible pests and pathogens in soil vineyards. Here we provide evidence on the support of organic viticulture to the activity of beneficial soil organisms, the EPNs. Future implementation of advanced molecular tools based on Next-Generation Sequencing (NGS) analysis (Geisen et al., 2018; Dritsoulas et al., 2020) will allow expanding the number of soil species investigated and hence, will contribute to a better understanding of the assemblages of EPNs and other soil organisms in natural conditions, and how abiotic factors affect them in the agroecosystems. These baseline data will provide objective assessments to add value to organic management strategies in sustainable viticulture.

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.2022.107931.

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Publication 3

Organic mulching modulated native populations of entomopathogenic nematodes in vineyard soils differently depending on its potential to control outgrowth of their natural enemies

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ABSTRACT

The entomopathogenic nematodes (EPNs) are biological control agents that are widespread in crop soils. However, traditional agricultural management practices such as cultivation and agrochemical usage can alter the soil balance that enables their occurrence and activity. Alternative strategies like mulching are commonly employed to prevent weed growth, enhance below-ground biodiversity by improving soil, organic matter content, fertility, and moisture. We hypothesized that organic mulches would favor biotic conditions for nematofauna development in crop soil, including EPNs, compared to herbicide application or tillage. Traditional (insect baits) and molecular (qPCR analysis) tools were used in this study to assess the abundance and activity of native EPNs, and the abundance of potential natural enemies, such as free-living nematode (FLN) competitors, nematophagous fungi (NF), and ectoparasitic bacteria, in soils managed with different organic mulches or traditional practices. As a model agroecosystem, we selected the vineyard, one of the most intensively managed crop systems. We compared mulches of grape pruning debris (GPD-M), straw (Str-M), and spent mushroom compost (SMC-M) in two commercial vineyards, which employed either integrated or organic pest and disease management. Following a completely randomized design, we retrieved two composite samples per plot (n = 3 per treatment in each vineyard) in April, June, and October 2020. Numbers of EPNs and selected members of their soil food web were higher in the organic than the integrated managed vineyard. Supporting our hypothesis, organic mulching overall favored nematode occurrence in both vineyards. We found higher NF abundance for Str-M, and GPD-M in the organic vineyard, which plausibly explained the lower EPN activity and occurrence compared to SMC-M in both vineyards. We conclude that the organic mulches can provide appropriate conditions for increasing nematofauna numbers but, depending on the mulch type, may also adversely affect EPNs by increasing their natural enemies. Our findings highlight the need to explore alternative farming practices to unravel complex biotic interactions that affect beneficial soil organisms in agroecosystems.

1. Introduction

Agricultural intensification accelerates the loss of soil biodiversity, arguably the most complex ecosystem on Earth (Orgiazzi et al., 2016). Consequently, alternative approaches to reliance on mechanization and agri-chemicals are required to achieve more sustainable farming (Veresoglou et al., 2015; FAO, 2020). Adding organic and inorganic amendments crops for weed control and preservation of soil moisture are among the oldest agricultural practices (Bednarek and Gaugler, 1997; Fredrikson et al., 2011; Pou et al., 2021). Additionally, mulching can enrich the soil with organic matter and nutrients, enhancing soil biota and crop plant health (Rombough, 2002; Pittelkow et al., 2014; Quintanilla-Tornel et al., 2016). Utilizing mulches made of by-products will also promote a circular economy. This state-of-the-art agriculture aligns with the current sustainability and territory conservation paradigm in a broad sense that concerns the environment, human health, and socioeconomic conditions (Orgiazzi et al., 2016; Santos et al., 2020).

The benefits of mulches are particularly interesting for the vineyard,

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one of the most intensively managed crop systems (Nicholls et al., 2008; Winter et al., 2018), predominantly placed on slopes in semi-arid regions (Peregrina et al., 2010; Santos et al., 2020). Their implementation shields the soil from extreme temperatures and other damaging weather events, which increase their frequency in the context of climate change (IPCC, 2018), as well as limiting the damage caused by high evaporation rates and soil erosion due to raindrop impact and water runoff (Pinamonti, 1998; Dahiya et al., 2007). However, mulching can produce a beneficial or detrimental impact on the soil biota. For example, it seems reasonable to expect higher fungal growth in moister soils, implying a risk of acute fungal disease incidences (Varga and Májer, 2004). Regardless, there is evidence of the beneficial effects of straw mulching against the wood-rotting fungus *Botrytis cynerea* (Helotiales: Sclerotiniaceae) in New Zealand vineyards (Mundy and Agnew, 2002; Jacometti et al., 2007). Studies also showed that organic amendments favored the abundance and diversity of beneficial organisms in viticulture, such as earthworms and predatory and parasitic arthropods (Thomson and Hoffmann, 2007), and induced a decrease in plant-parasitic nematode populations (Rahman et al., 2014). Hence, further insights into the effects of mulching on other beneficial soil organisms are needed to assess value of this practice.

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* (Rhabditida: Steinernematidae) and *Heterorhabditis* (Rhabditida: Heterorhabditidae) are well-known beneficial soil organisms that commonly occur in crop soils (Lewis et al., 2015). Their ability to rapidly kill (48–72 h after infection) a broad arthropod range makes them an excellent non-chemical alternative for managing insect pests (Kaya et al., 2006; Dillman et al., 2012; Dolinski et al., 2012). The non-feeding infective juvenile (IJ) stage penetrates the host hemocoel through natural openings and releases mutualistic enteric γ-Proteobacteria, killing the arthropod by septicemia (Boemare, 2002; Stock, 2015). Diverse soil communities modulate the efficacy and persistence of EPNs in the agroecosystem throught cooperative, antagonistic, or competitive relationships (Stuart et al., 2015; Helmberger et al., 2017). For example, diverse fungal species can develop specialized structures of different kinds to prey on nematodes (Nordbring-Hertz et al., 2006). According to several field studies, the high abundance of these nematophagous fungi (NF) could alter the EPN community in various crops, including vineyards, in a species-specific manner (Pathak et al., 2017; Campos-Herrera et al., 2015c; Blanco-Pérez et al., 2020, 2022). On the other hand, some free-living nematode (FLN) species appear to compete with EPNs for sources in the insect cadaver (Peters, 1996; Duncan et al., 2003; Campos-Herrera et al., 2015b), even reducing the IJ fitness of the subsequent offspring (Blanco-Pérez et al., 2019). Specialized bacteria also interact with EPNs, as is the case of ectoparasitic bacteria (EcPB) in the genus *Paenibacillus*, whose spore attachment to the IJ cuticle can reduce their motility (El-Borai et al., 2005; Enright and Griffin, 2005).

Diverse organic amendments affect EPNs differently in a species-specific manner. Several field studies reported that fresh manure and urea decrease the virulence and persistence of EPNs (Georgis et al., 1987; Mullens et al., 1987; Shapiro-Ilan et al., 1996, 1999a) since the decomposition process releases substances toxic to nematodes and reduces oxygen availability in the soil (Simpson, 1986; Kaplan and NOE, 1993). In contrast, despite few exceptions (Sweeney et al., 1998), crop residues and composted organic amendments of both animal and plant sources appear beneficial to the EPN community, possibly due to an increased abundance of soil-dwelling insects resulting from food and shelter provided by mulches (Ishibashi and Kondo, 1986; Shapiro-Ilan et al., 1999b; Lacey et al., 2006; Duncan et al., 2007; de Waal et al., 2011; Campos-Herrera et al., 2015a; Khumalo et al., 2021; Renkema and Parent, 2021). Beyond the evidence that organic amendments favor the presence of FLNs, especially *Acrobeloides* species (Jaffee et al., 1994; Duncan et al., 2007; Rahman et al., 2014; Campos-Herrera et al., 2015a; Quintanilla-Tornel et al., 2016), the impact of mulching on the EPN soil food web is still poorly understood.

This study evaluated the presence and activity of EPNs and some associated soil organisms in vineyards managed with different organic mulches and traditional practices. We (i) estimated soil activity rates linked with EPNs through the traditional insect baits, and (ii) quantified the abundance of EPN, FLN, NF, and EcPB target species *via* qPCR. We hypothesized that, compared to conventional soil management practices, organic mulching would enhance the EPN community in the vineyard. The organic mulches employed in this study consisted of straw and the by-products of grape pruning debris and spent mushroom compost, implemented in two vineyards which differed in edaphic properties and pest and disease management: conventional/integrated (IPM) vs. organic.

2. Material and methods

2.1. Treatments, experimental design, sampling, and abiotic factors

The native EPN community and associated soil food web were evaluated in 2020 in two experimental vineyards (var Tempranillo, spur-pruned on a bilateral Royat Cordon system) beneath the guaranteed designation of origin (denominated DOCa) Rioja (CR Rioja wine, 2021). The vineyards were located near Aldeanueva del Ebro and Logroño (North-Eastern of Spain) and handled according to the European Union and Spanish regulations for IPM (EC, 2009; RD, 2012) and organic farming (RD, 2014; EU, 2018), respectively (Fig. 1; Supplementary data 1, Table S1). This region is characterized by the warm-summer Mediterranean (with continental influence) climate (classified as Cab by the Köppen-Geiger system) and haplocalcid semi-arid soils (e.g., low organic matter content, water deficiency, and accumulation of calcium carbonates) (Soil Survey Staff, 2014). The treatments comprised differentiated organic mulches and conventional practices annually applied (in winter) on the crop rows since 2019. For mulches consisting of grape pruning debris (GPD-M), it was necessary to add wood of untreated vines from two additional rows to reach the quantities required. 'Sustratos de La Rioja SL' supplied the spent mushroom compost (SMC-M) amendment, consisting of a by-product made of straw amended with animal manure and urea used to grow the oyster mushroom *Pleurotus astreatus* (Agaricales: Pleurotaceae). Finally, the Government of La Rioja provided the straw (Str-M) mulches (physical and chemical properties of each mulch are detailed in Table S2). The amendments were deposited for an approximate dry matter rate of 14,000 Kg/ha in banks 60 cm wide (10–20 cm high after compaction). The conventional practices of herbicide applications (Herb), consisting of Terafit (25% p/p. Flazasulfuron) and glyphosate (100 l/ha), and secondary tillage (Till) using inter-row cultivators, the most common procedure in traditional viticulture in the region (Fernández Alcázar, 2011), were applied twice a year.

The experimental design in both experimental vineyards was a randomized complete block divided in experimental units (plots), each consisting of 30 vines (three plots per treatment, Fig. 1). Composited samples, two per plot, comprised 12 single soil cores (2.5 cm Ø x ~ 20 cm DP) randomly collected in April (22nd-24th), June (23rd-24th), and October (5th-6th) with auger soil samplers under the crop canopy of central vines to avoid possible border effects, mixed in separate plastic bags (~1800 cm^3 of soil), and stored in a chamber (4 °C in the dark) until processed (within 2–4 days). We avoided collecting mulch debris except for SMC-M, which consisted of a continuum with the underlying soil, because some EPN species, depending on differing foraging strategies, primarily occur near the surface. It was also confirmed that IJs could move from soil up to 10 cm into diverse mulch coatings to search for suitable hosts (de Waal et al., 2011). Thus, soil samples for this specific treatment could contain up to 30% of mulch substrate. After being homogenized in the laboratory, subsamples of 200 g of fresh soil were used to (i) isolate the nematofauna and other soil organisms through the sucrose centrifugation technique, (ii) determine the soil activities by baiting with insect larvae, and (iii) determine the soil properties. The remaining 200 g of fresh soil set was dried first at 40 °C

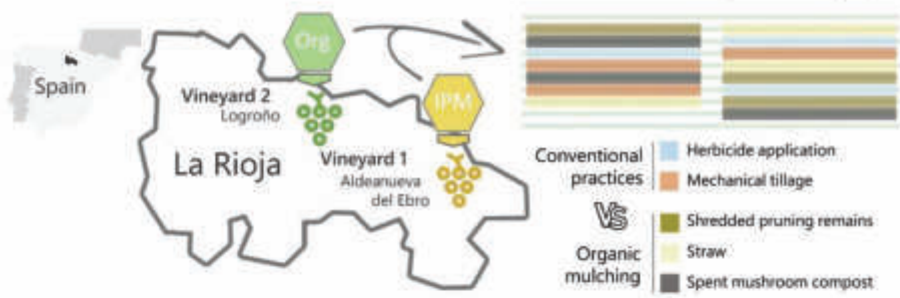


Fig. 1. Location of the experimental vineyards, handled for organic (Org) and integrated pest management (IMP), and experimental design of the evaluated treatments.

for one week to measure the water content, which is necessary to express the abundance of identified organisms per 100 g of dry soil (Wiesel et al., 2015; Campos-Herrera et al., 2019). The Regional Laboratory of the Government of La Rioja (La Grajera, Logroño, Spain) analyzed the following soil properties resulting from combining both replicates of each plot: pH (Millennia and Markewitz, 2004), electrical conductivity, organic matter (Walkley and Black, 1934), macro-nutrients (NPK), oligo-nutrients (Mg, Ca, and SO_4), micro-nutrients (Fe, Mn, Zn, Cu, Al, and B), and other elements (Na and Pb) (Mehlich, 1978; 1984). Soil texture (sand, silt, and clay percentages) (Bouyoucos, 1936) was analyzed only for the samples collected in April. Monthly precipitations of 2020 in the locations of both experimental vineyards, recorded by the Agro-climatic Information Service in La Rioja (SIAR), are also shown (Fig. S1).

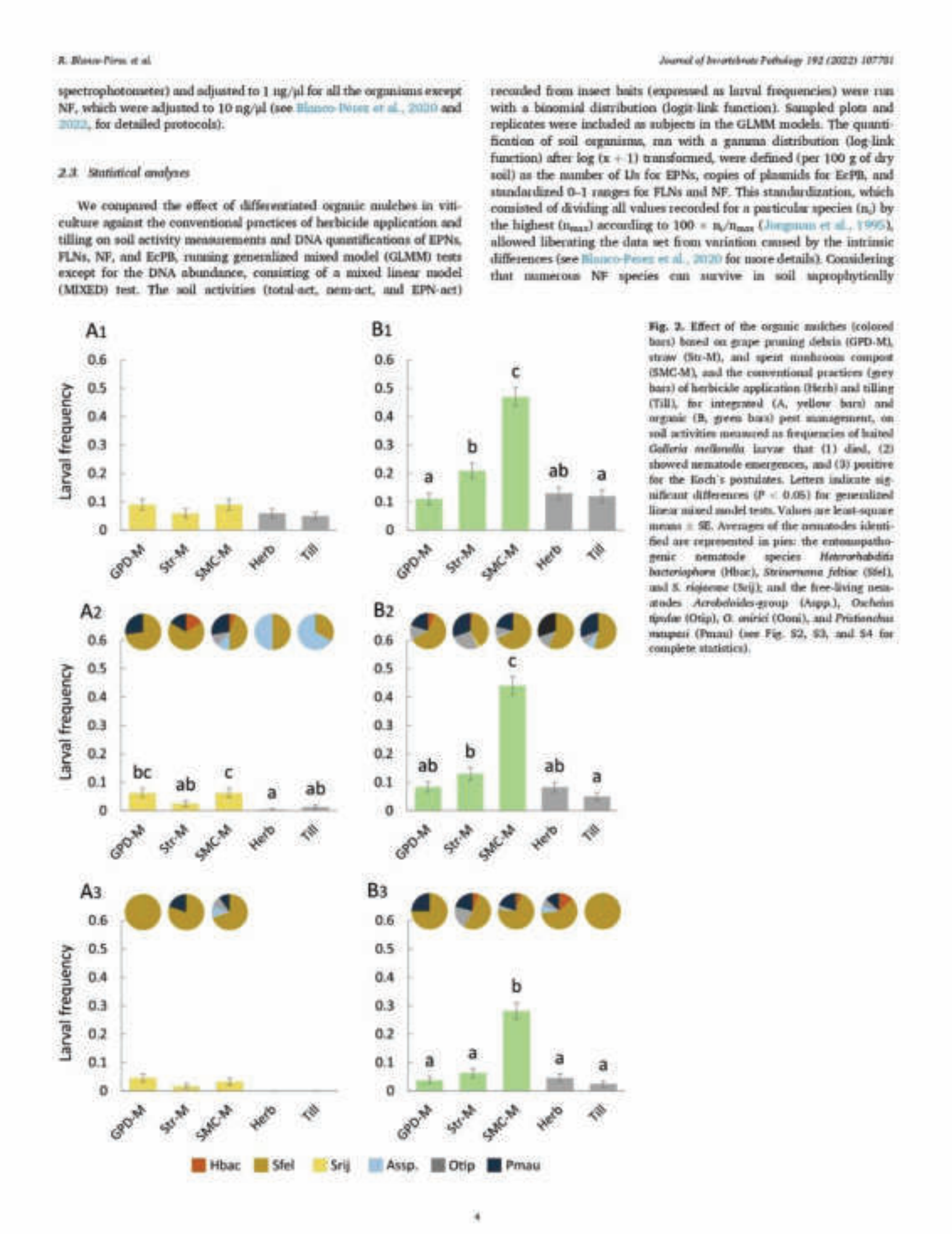
2.2. Accounting of soil activities and extraction and quantification of soil organisms

To explore the EPN soil food web assemblage, we isolated the nematodes and associated soil organisms from one subsample collection through sucrose centrifugation methodology (Jenkins, 1964). From the second, we calculated soil activity ratios (only from soil samples taken in June and September) using final instars of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae (reared at ICVV) baits, following a protocol adapted from Bedding and Akhurst (1975). As soil activity rates, we estimated differential larval percentages for total mortality (total-act), mortality associated with nematode emergences (nema-act), and confirmed Koch's postulates (EPN-act) (see Blanco-Pérez et al. 2020 for detailed protocols).

For the DNA extraction procedure, we used commercial kits: Speedtools tissue (Biotools, B&M Labs S.A., Madrid, Spain) for nematodes that emerged from insect baits and DNeasy PowerSoil Kit (QIAGEN GmbH Co., Hilden, Germany) for soil organisms extracted through sucrose-gradient centrifugation, all stored at −20 °C in the dark until used (see Blanco-Pérez et al., 2022 for a more detailed protocol). We screened in qPCR tests for 22 soil organisms using species-specific primers/probe sets (Table 1), synthesized by Biotools (Madrid, Spain), most of them previously identified in La Rioja vineyards (Blanco-Pérez et al., 2020, 2022). Some others, like the EPN *Steinernema intermedium* and the trapping NF *Arthrobotrys musiformis*, had been documented in the Iberian Peninsula (García del Pino, 2005; Campos-Herrera et al., 2011b, 2016, 2019). Final concentrations of species-specific primers and probes were 400 and 200 nM, respectively, excluding *Steinernema* sp. *affine*-group (100 and 40 nM). We run 38 cycles for nematodes species and 50 for NF and EcPB species for qPCR tests on the Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). DNA samples were examined for quality and quantity in a Nanodrop system (Thermo Scientific 2000C

Table 1
Soil organisms tested.

Type of organism / species	Population	GenBank ACNO ITS Region	Reference for primers and probes sequences
Entomopathogenic nematodes			
Heterorhabditis bacteriophora	(commercial)	KJ938576	Campos-Herrera et al. (2011b)
Heterorhabditis indica	Brw	KJ938571	Campos-Herrera et al. (2011a)
Heterorhabditis megidis	(commercial)	KJ938577	Campos-Herrera et al. (2011b)
Steinernema affine	CH	KJ938567	Torr et al. (2007)
Steinernema carpocapsae	DOK-83	KJ810295	Campos-Herrera et al. (2011b)
Steinernema feltiae	RS-5	KJ938569	Campos-Herrera et al. (2011b)
Steinernema intermedium	82	AF171290	Campos-Herrera et al. (2015b)
Steinernema kraussei	OS	KJ696686	Campos-Herrera et al. (2015b)
Steinernema riojanus	RM-30	MK503133	Blanco-Pérez et al. (2020)
Steinernema sp. *affine*-group	VO-53	MW480137	Blanco-Pérez et al. (2022)
Free-living nematodes			
Acrobeloides-group	RT1-R15C	JQ237849	Campos-Herrera et al. (2012)
Oscheius tipulae	MG68 P29	KJ938579	Campos-Herrera et al. (2015a)
Oscheius onirici	MG67 P20	KJ938578	Campos-Herrera et al. (2015a)
Pristionchus maupasi	AM-3	MG551681	Campos-Herrera et al. (2019)
Nematophagous fungi			
Catenaria sp.	1D	JN585805	Pathak et al. (2012)
Arthrobotrys dactyloides	H55	KJ938574	Pathak et al. (2012)
Arthrobotrys musiformis	11	KJ938572	Pathak et al. (2012)
Arthrobotrys oligospora	8	KJ938573	Pathak et al. (2012)
Hirsutella rhossiliensis	3931	KM652160	Zhang et al. (2006)
Purpureocillium lilacinum	9357	KJ938575	Atkins et al. (2005)
Ectoparasitic bacteria			
Paenibacillus nematophilus	NEM2	AF480936	Campos-Herrera et al. (2011b)
Paenibacillus sp.	SdTc1FEE1	JF817562	Campos-Herrera et al. (2011b)



spectrophotometer) and adjusted to 1 ng/µl for all the organisms except NF, which were adjusted to 10 ng/µl (see Blanco-Pérez et al., 2020 and 2022, for detailed protocols).

2.3. Statistical analyses

We compared the effect of differentiated organic mulches in viticulture against the conventional practices of herbicide application and tilling on soil activity measurements and DNA quantifications of EPNs, FLNs, NF, and EcPB, running generalized mixed model (GLMM) tests except for the DNA abundance, consisting of a mixed linear model (MIXED) test. The soil activities (total-act, nem-act, and EPN-act) recorded from insect baits (expressed as larval frequencies) were run with a binomial distribution (logit link function). Sampled plots and replicates were included as subjects in the GLMM models. The quantification of soil organisms, run with a gamma distribution (log link function) after $\log(x + 1)$ transformed, were defined (per 100 g of dry soil) as the number of IJs for EPNs, copies of plasmids for EcPB, and standardized 0–1 ranges for FLNs and NF. This standardization, which consisted of dividing all values recorded for a particular species (n_i) by the highest (n_{max}) according to $100 \times n_i/n_{max}$ (Jongman et al., 1995), allowed liberating the data set from variation caused by the intrinsic differences (see Blanco-Pérez et al., 2020 for more details). Considering that numerous NF species can survive in soil saprophytically

Fig. 2. Effect of the organic mulches (colored bars) based on grape pruning debris (GPD-M), straw (Str-M), and spent mushroom compost (SMC-M), and the conventional practices (grey bars) of herbicide application (Herb) and tilling (Till), for integrated (A, yellow bars) and organic (B, green bars) pest management, on soil activities measured as frequencies of baited *Galleria mellonella* larvae that (1) died, (2) showed nematode emergences, and (3) positive for the Koch's postulates. Letters indicate significant differences ($P < 0.05$) for generalized linear mixed model tests. Values are least-square means ± SE. Averages of the nematodes identified are represented in pies: the entomopathogenic nematode species *Heterorhabditis bacteriophora* (Hbac), *Steinernema feltiae* (Sfel), and *S. riojaense* (Srij); and the free-living nematodes *Acrobeloides*-group (Aspp.), *Oscheius tipulae* (Otip), *O. onirici* (Ooni), and *Pristionchus maupasi* (Pmau) (see Fig. S2, S3, and S4 for complete statistics).

(Nordbring-Hertz et al., 2006), we assumed that most NF species isolated by sucrose centrifugation were in the infectious phase (Pathak et al., 2012). Accordingly, NF numbers were expressed as infection rate (IR), determined by dividing the DNA abundance of each fungal species by the total amount of DNA (Campos-Herrera et al., 2012).

The two experimental vineyards comprising this study differed in their pest management (integrated and organic), soil properties (Table S3), and biotic factors (Table S4), so the recorded data from each of them were analyzed separately. Conversely, only a few differences among seasonal samplings were found (Table S5), so we included them as repeated measures in GLMM models. Soil factors displaying significant differences among treatments for general linear model (GLM) tests (Tables S6 and S7) were initially assessed as covariates in exploratory GLMM tests. Subsequently, we included electrical conductivity to test for differences in absolute EPN abundance in organic farming, clay and P contents in absolute NF abundance in IPM viticulture, and Fe content in absolute NF abundance in organic farming. We performed all the statistical analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), using the Least-Squares Means ± standard error of the mean (SEM) as descriptive statistics.

3. Results

3.1. Soil activities per treatments

Overall, the soil activities recorded for organic mulches were higher than for conventional practices, although not always significant (Fig. 2). The highest values were obtained for SMC-M, significantly higher than all the other treatments in the organic vineyard for the three soil activity rates (Fig. 2B$_{1-3}$), but only compared to nem-act in the IPM vineyard except GPD-M (Fig. 2A$_2$; Supplementary data 2, Fig. S2-S4). Similarly, in the organic vineyard, we recorded significantly higher frequencies for SMC-M of the EPN *S. feltiae*, both emerged from soil baits and Koch's postulate tests (Fig. S3 and S4), and the FLN *P. maupasi* for nem-act relative to all other treatments except Str-M (Fig. S3). Again for organic viticulture, Str-M registered significantly higher total-act than GPD-M and Till (Fig. 2B$_1$), nem-act than Till (Fig. 2B$_2$), and frequencies of *P. maupasi* reported in soil baits than GPD-M and Till (Fig. S3). Regarding GPD-M, the only difference obtained compared to the conventional practices was that the nem-act values were higher than those of Herb in IMP (Fig. 2A$_2$). We found nematode emergences for the species *H. bacteriophora* (missed in Koch's postulate tests for IMP), *S. feltiae* (the predominant EPN species), *S. riojaense* (only in a few numbers for Str-M in organic viticulture), *Acrobeloides*-group, *O. tipulae*, and *P. maupasi* (see Fig. S2-S4 for detailed statistics).

3.2. Abundance of soil organisms per treatments

More DNA was isolated from soil in the organic mulch plots than from conventional practice plots in both experimental vineyards (Fig. S5 and S6). In addition, we recorded higher DNA values for SMC than GPD and Str mulches and Herb than Till in organic viticulture (Fig. S5B and S6). The cumulative EPN and *S. feltiae* (the predominant species) abundances were significantly higher for organic mulching in the IPM vineyard (Fig. 3A; Table 2) but only for SMC-M (mediated by high values of electrical conductivity) in the organic vineyard (Fig. 3B; Table 2; Fig. S7). Additionally, we found the species *S. riojaense* (for all the treatments except Till in the organic vineyard), *H. bacteriophora* (only for Str-M in organic farming), *S. carpocapsae* (only for GPD-M in organic farming), and *Steinernema* sp. *affine*-group (for SMC-M and Herb in IPM), without significant differences among treatments for any of them (Fig. 3; Table 2).

On the whole, we recorded higher total FLN and *Acrobeloides*-group (identified in most of the soil samples) abundances for organic mulches than conventional practices in both vineyards, particularly for SMC-M, significantly different than all treatments except GPD-M (Fig. 4; Table 2; Fig. S8), Fig. S8 Furthermore, GPD-M showed higher absolute FLN abundance than Herb in IMP and Till in organic farming (Fig. 4A$_1$, B$_1$) and higher *Acrobeloides* spp. numbers than Herb and Till for both vineyards and Str-M and Till for organic viticulture, only (Table 2; Fig. S8). Conversely, *Acrobeloides* spp. abundance was significantly lower for Str-M than Herb in organic farming (Table 2; Fig. S8). The species *P. maupasi* was also well represented but absent in organic mulches of IMP, while the two species in the genus *Oscheius* occurred exclusively in organic mulches (Fig. 4A$_1$,B$_1$). No significant differences among treatments were found for any of them.

On the contrary, we did not observe clear patterns for the cumulative NF abundance. The treatment Str-M showed the highest NF numbers, significantly different from all the others except for Herb in IMP, followed by GPD-M, only in organic viticulture, and Herb, significantly higher than GPD-M and SMC-M in IPM, and Till in both vineyards (Fig. 4A$_2$,B$_2$; Fig. S9). The only NF species occurring in all the treatments was *P. lilacinum*, in higher numbers for Herb than Till in IPM, and GPD-M than SMC-M and Till in organic farming (Table 2; Fig. S9). The other NF

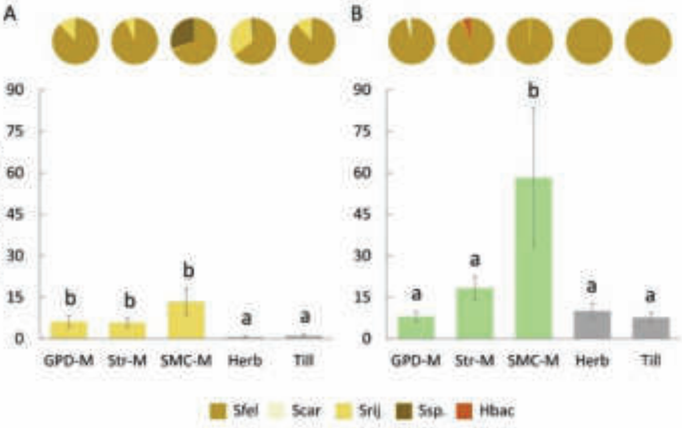


Fig. 3. Effect of the organic mulches (colored bars) based on grape pruning debris (GPD-M), straw (Str-M), and spent mushroom compost (SMC-M), and the conventional practices (grey bars) of herbicide application (Herb) and tilling (Till), for integrated (A, yellow bars) and organic (B, green bars) pest management, on the abundance of infective juveniles (IJs). Letters indicate significant differences ($P < 0.05$) for generalized linear mixed model tests. Values are least-square means ± SE. Averages of the entomopathogenic nematode species identified are represented in pies: *Steinernema feltiae* (Sfel), *S. carpocapsae* (Scar), *S. riojaense* (Srij), *Steinernema* sp. *affine*-group (Ssp.), and *Heterorhabditis bacteriophora* (Hbac) (see Fig. S7 for complete statistics).

Table 2
Descriptive (x ± SEM) of the abundance of the target species by treatments: the organic mulches based on grape pruning debris (GPD-M), straw (Str-M), and spent mushroom compost (SMC-M), and the conventional practices of herbicide applications (Herb) and tillage (Till). Bold letters indicate significant differences ($P < 0.05$) from generalized linear mixed model tests. Abundances (per 100 g of dry soil) measured as infective juveniles for entomopathogenic nematodes (EPNs) and ug/µl of pure culture for free-living nematodes (FLNs) and nematophagous fungi (NF).

	Vineyard 1 (Integrated Pest Management)					Vineyard 2 (Organic Pest Management)				
	GPD-M	Str-M	SMC-M	Herb	Till	GPD-M	Str-M	SMC-M	Herb	Till
EPN species										
Heterorhabditis bacteriophora	0	0	0	0	0	0	1.06 ± 1.01	0	0	0
Steinernema carpocapsae	0	0	0	0	0	0.26 ± 0.26	0	0	0	0
Steinernema feltiae	5.43 ± 2.03**b**	5.47 ± 1.56**b**	9.15 ± 2.53**b**	0.41 ± 0.23 **a**	0.99 ± 0.63 **a**	7.57 ± 1.99 **a**	17.01 ± 3.84 **a**	58.06 ± 25.16**b**	10.06 ± 2.74 **a**	7.70 ± 1.78 **a**
Steinernema riojaense	0.76 ± 0.67	0.41 ± 0.28	0.16 ± 0.13	0.22 ± 0.13	0.13 ± 0.13	0.05 ± 0.05	1.20 ± 1.20	1.19 ± 1.14	0	0.01 ± 0.01
Steinernema sp. *affine*-group	0	0	4.11 ± 4.11	0.01 ± 0.01	0	0	0	0	0	0
FLN species										
Acrobeloides-group	1.5E-05 ± 4.6E-06 **bc**	7.9E-06 ± 2.3E-06 **ab**	2.2E-05 ± 2.5E-06**c**	4.1E-06 ± 6.7E-07 **a**	4.9E-06 ± 1.1E-06 **ab**	1.5E-05 ± 2.0E-06**bc**	3.8E-06 ± 9.7E-07 **a**	3.2E-05 ± 3.1E-06**c**	7.0E-06 ± 1.2E-06**b**	5.1E-05 ± 1.0E-06 **ab**
Oscheius onirici	0	0	0	0	0	0	9.4E-02 ± 8.7E-02	0	0	0
Oscheius tipulae	0	5.7E-05 ± 5.7E-05	0	0	7.5E-06 ± 7.5E-06	8.9E-03 ± 8.3E-03	7.5E-03 ± 3.3E-03	0	8.4E-05 ± 8.4E-05	0
Pristionchus maupasi	0	2.4E-04 ± 2.4E-04	3.0E-05 ± 3.0E-05	7.2E-03 ± 7.2E-03	4.9E-03 ± 4.9E-03	2.0E-01 ± 1.0E-01	4.4E-01 ± 3.1E-01	1.8E-01 ± 1.0E-01	1.3E-01 ± 4.3E-02	5.4E-02 ± 1.6E-02
NF species										
Arthrobotrys dactyloides	0	3.3E-06 ± 3.3E-06	4.2E-05 ± 4.2E-05	0	0	2.9E-04 ± 2.3E-04	1.3E-04 ± 1.2E-04	0	0	0
Arthrobotrys oligospora	0	1.9E-03 ± 7.9E-04	0	0	4.0E-05 ± 4.0E-05	0	3.3E-03 ± 9.0E-04	6.6E-06 ± 6.6E-06	0	0
Hirsutella rhossiliensis	0	0	0	0	0	0	2.0E-02 ± 1.3E-02	0	2.1E-02 ± 1.9E-02	0
Purpureocillium lilacinum	2.2E-04 ± 7.0E-05 **ab**	2.2E-03 ± 1.1E-03 **ab**	1.5E-04 ± 6.5E-05 **ab**	3.1E-03 ± 1.9E-03**b**	8.3E-05 ± 3.5E-05 **a**	1.1E-03 ± 2.4E-04**b**	1.3E-03 ± 3.0E-04 **ab**	3.8E-04 ± 1.0E-04 **ab**	6.2E-04 ± 1.1E-04 **ab**	5.0E-04 ± 8.3E-05 **a**

species identified were *A. dactyloides* (for organic mulches only), *A. oligospora*, and *H. rhossiliensis* (in organic farming only), without significant differences among treatments (Fig. 4A_2,B_2). Finally, we recorded low abundance for the EcPB *Paenibacillus* sp. (missed in IMP except for tilled soils) and no significant differences among treatments (Fig. 4A_3,B_3).

4. Discussion

Consistent with various studies and our hypothesis, we recorded, excluding a few exceptions, higher EPN abundances and emergence rates from insect baits in plots handled with organic mulches than for the conventional practices of herbicide application and tilling. However, in agreement with previous findings reported by de Waal et al. (2011), we observed variable impact on EPNs presence depending on the type of mulch. Thus, while the amendment based on spent mushroom compost significantly increased EPN numbers in both experimental vineyards, the other two evaluated mulches (composed of grape pruning debris and straw) did not much differ from weed control conventional practices in organic viticulture. The prevalent steinernematid species in Europe (Hominick, 2002; Bhat et al., 2020), including the Iberian Peninsula (García del Pino, 2005; Valadas et al., 2014; Campos-Herrera et al., 2019), *S. feltiae*, mostly drove these results. Indeed, previous studies conducted in DOCa Rioja vineyards associated a high abundance and activity rates for this EPN species with less disturbed soils such as no-tilled organic crops (Campos-Herrera et al., 2007; Blanco-Pérez et al., 2020, 2022). The other EPN species we found (*Steinernema* sp. *affine*-group, *S. riojaense*, *S. carpocapsae*, and *H. bacteriophora*) were previously detected in the region in a study conducted on 80 vineyards throughout the DOCa Rioja by Blanco-Pérez et al. (2022). Compared to this study, we obtained similar or slightly lower EPN infection rates and quantifications except for SMC-M in the organic vineyard.

Our results support evidence for the potential of mulching to enhance the FLN community (Duncan et al., 2007), an effect that was particularly evident in our organic vineyard, which agreed with the observations reported by Blanco-Pérez et al. (2022). Nevertheless, as marked for EPNs, only SMC-M implied more pronounced increases in FLN abundances than conventional practices. In recent years, DNA analysis has verified that the FLN species here examined often co-emerged with EPNs from cadavers retrieved from baits (Campos-Herrera et al., 2012, 2015c, 2019; Jaffuel et al., 2016, 2018; Blanco-Pérez et al., 2020, 2022). Still, it is unknown to which extent the complex interactions established between these two groups of nematodes imply competitive relationships for resources in the insect cadaver. Nonetheless, diverse FLNs might interact with EPNs in many different ways, so the connections they will assemble in nature will not be easy to interpret. For example, specific populations in the genus *Acrobeloides*, the predominant FLNs in our study, have exhibited virulence against some insect pest larvae, such as *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in citrus grows in Florida (USA) and *Zeuzera pyrina* (Lepidoptera: Cossidae) in walnut trees in Iran, in laboratory and field experiments (Campos-Herrera et al., 2012; Salari et al., 2021). There is also evidence of entomopathogenic behavior by some *Oscheius* populations (Ye et al., 2010), including *O. onirici* (Torrini et al., 2015). Moreover, *in vitro* experiments suggested that the presence of this particular species may decrease the virulence of EPN offspring after scavenging within freeze-killed insect larvae, a plausible alternative strategy for EPNs to survive in soils (Blanco-Pérez et al., 2019). On the other hand, diplogastrid nematodes such as *Pristionchus* have specialized oral cavities that allow them to switch from feeding on bacteria to, for instance, predating on other nematodes (von Lieven, 2003). Field and laboratory experiments proved that competition from FLNs could displace augmented EPN

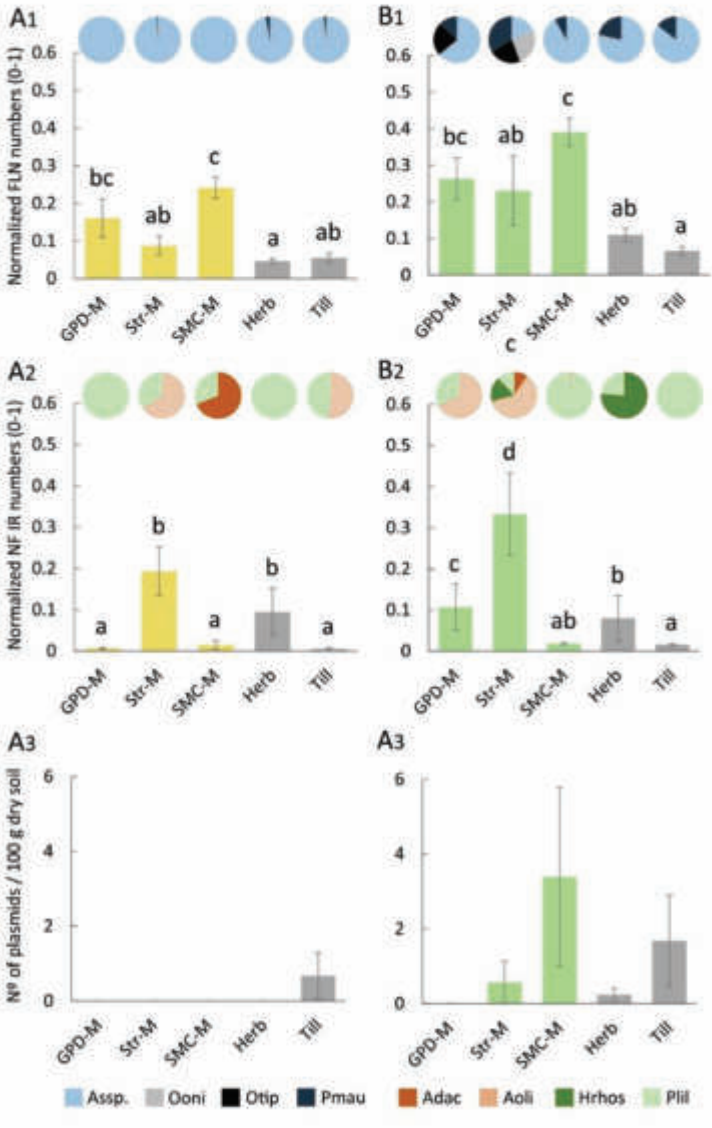


Fig. 4. Effect of the organic mulches (colored bars) based on grape pruning debris (GPD-M), straw (Str-M), and spent mushroom compost (SMC-M), and the conventional practices (grey bars) of herbicide application (Herb) and tilling (Till), for integrated (A, yellow bars) and organic (B, green bars) pest management, on the abundance of (1) free-living nematodes (FLNs), (2) nematophagous fungi (NF), and (3) ectoparasitic bacteria. Letters indicate significant differences ($P < 0.05$) for generalized linear mixed model tests. Values are least-square means ± SE. Averages of the species identified are represented in pies: the FLNs *Acrobeloides*-group (Assp.), *Oscheius tipulae* (Otip), *O. onirici* (Ooni), and *Pristionchus maupasi* (Pmau); and the NF *Arthrobotrys dactyloides* (Adac), *A. oligospora* (Aoli), *Hirsutella rhossiliensis* (Hrhos), and *Purpureocillium lilacinum* (Plil) (see Fig. S8 and S9 for complete statistics).

populations in citrus groves rather than endemics, also for mulching applications (Duncan et al., 2003, 2007). Our results showed that higher FLN numbers in mulched plots did not reduce EPN activity and occurrence. Indeed, previous field experiments found similar results on native EPN prevalence for composted chicken manure mulches added to citrus groves in Florida (USA), in which high FLN abundance did not interfere with their activity (Duncan et al., 2007) or reduce their occurrence (Campos-Herrera et al., 2015a).

Organic amendment applications in crops often result in a higher content of organic matter and water in the soil, enhancing soil biota (Pittelkow et al., 2014), nematofauna included. In this regard, it is worth noting that soil organic matter contents, higher for mulching than conventional practices and organic than IMP, positively correlated, as predicted by Linford's hypothesis (Linford, 1937; Cooke, 1962), to the absolute DNA abundance and nematode quantifications in our study. According to this premise, this increase in nematode populations should

induce a rapid proliferation of their natural enemies. In addition, high organic matter content is one of the main abiotic factors that favor NF occurrence in soils (Nordbring-Hertz et al., 2006; Pathak et al., 2017). While it is true that we obtained higher NF abundance in the organic than in the IMP vineyard, the higher values of organic matter content in the mulched plots did not always imply an increase of NF numbers, but it was dependent on the type of amendment applied, remarkably high for Str-M and low for SMC-M. In this line, some field and laboratory experiments observed no effect or slight suppression of NF occurrence or their parasitism against nematodes for different organic amendments (Jaffee, 2004; Duncan et al., 2007; Campos-Herrera et al., 2015a). Soil volumetric water content analysis confirmed that mulching favored soil water retention on our IMP vineyard but with minor annual fluctuations for straw mulches (Pou et al., 2021). Moisted soils for more extended periods could plausibly enhance fungal proliferation in this type of mulch. In contrast, despite the highest soil organic matter contents, NF incidence was minimal for SMC-M. The enzymatic activity of residual mycelia perhaps limited fungal proliferation. Indeed, under stress due, for example, to interspecific interactions with other fungi, it is known that *P. ostreatus* produces several secondary metabolites to suppress aerial mycelium expansion of a wide range of potential competitors (Rayner et al., 1994; Ocimati et al., 2021). Another reasonable explanation for our results is that SMC-M was somehow especially attractive to EPNs. For example, a recent study showed that octenol and other volatile organic compounds produced by filamentous fungi act as foraging cues used by fungivorous insects and EPNs (Wu and Duncan, 2020).

It is necessary to note that NF species, depending on environmental factors, can stay in soils saprophytically rather than in their infectious phase, so we should not assume that an increase in fungal growth will automatically translate to higher parasitism rates against nematodes (Jaffee, 2003, 2004). Fortunately, unlike traditional detection techniques based on soil baits, the sucrose centrifugation procedure enables the isolation of NF once they have developed specialized structures in the presence of nematodes or their eggs (Pathak et al., 2012). Thus, we could deduce that the high NF numbers quantified in the straw mulches directly relate to their preying activity against nematodes. However, to adequately explain their potential impact on EPNs, we should differentiate the NF types identified per treatment. We found fungal species belonging to the three main NF groups (Nordbring-Hertz et al., 2006): nematode-trapping fungi (*Arthrobotrys* spp.), endoparasites (*Catenaria* sp., and *H. rhossiliensis*), and eggs-parasitic fungi (*P. lilacinum*). The latter, *a priori* unrelated to EPNs, was predominant in this study and, like FLNs, more abundant in the organic vineyard than for IPM. Regarding other NF, it is revealing that most records of *Arthrobotrys* spp., particularly *A. oligospora*, occurred in the mulched plots with reduced EPN activity and abundance compared to the traditional practices: Str-M in both vineyards and GPD-M in the organic vineyard. Finally, the low endoparasitic NF numbers recorded in both vineyards are noteworthy, much more abundant than in a previous study comprising 80 DOCa Rioja vineyards that followed the same methodologies of isolation and quantification of soil organisms (Blanco-Pérez et al., 2022). Fungal species of this kind, more obligate parasites than other NF, produce fungal spores, in some cases mobile (zoospores), whose efficacy in infecting nematodes improved in soils with high water content (Nordbring-Hertz et al., 2006). Even if, according to this assumption, all the endoparasitic fungi were isolated wherein mulched plots, we found no evidence of *Catenaria* sp. and very few for *H. rhossiliensis*. On the other hand, nematode-trapping fungi seem to occur more often in the rhizosphere than other NF, perhaps due to root exudations linked to plant-parasitic nematode damages (Nordbring-Hertz et al., 2006). Finally, the EcPB *Paenibacillus* sp. numbers were lower than expected, particularly in the IPM vineyard, at least based on the results shown by Blanco-Pérez et al. (2022). Unfortunately, this does not allow us to draw any conclusions about the effect of mulching on the potential regulation of EPN populations by these bacteria.

5. Conclusions

In agreement with our hypothesis, different organic mulches affected the EPN soil food web in a specific manner in two experimental vineyards that differ in pest and disease management, integrated and organic. Our results were consistent with previous studies pointing out that overall organic mulching enhanced the nematode community, particularly in IPM plots, with poor baseline organic matter contents. The use of amendments will modify the soil's physicochemical properties, which, in turn, modulate biotic balances in the agroecosystem. For instance, we recorded higher soil organic matter in mulched plots than for the conventional practices of tilling or herbicide applications. Enriched soils imply higher occurrence and diversity of organisms such as nematodes, but their natural enemies also (Linford, 1937; Cooke, 1962). This study revealed that organic amendments of diverse nature could enhance or inhibit the development of fungal infective phases against nematodes. Thus, if mulching promotes excessive soil water content, as was possibly the case for the straw-based mulches in this study, is a conducive environment for fungal growth, and NF could efficiently restrict the abundance and activity of native EPN populations. Conversely, spent mushroom compost laid on vineyard plots provided optimal soil organic matter and water contents for nematode development but drastically declined NF-IR values. The absence of natural enemies, perhaps due to some antifungal activity exhibited by residual mycelia after oyster mushroom cultivation, could explain the remarkable raised EPN abundance and activity accounted for this mulch type, regardless of vineyard management. However, since mulching strongly affected the soil at abiotic and biotic levels, interpreting the results is complex. For instance, attractive volatiles for both IJs and insect larvae produced by these mycelia in SMC-M perhaps explained the high EPN occurrence and activity values recorded for this specific treatment. In any case, if NF populations modulated EPN community is still only a possibility, unconfirmed in some previous studies (Pathak et al., 2017). Mulching strongly affected many abiotic and biotic soil variables specifically, so it is unknown whether the relationships between NF and EPN activity were direct or independently caused by other variables that differed among treatments.

In short, organic mulches impacted the EPN food web differentially, favoring, in any case, the abundance of FLNs but enhancing or inhibiting, according to their typology, the occurrence of infective phases of NF . This study highlights the value of a comprehensive evaluation of alternative strategies to environmentally damaging viticultural practices. The motivation for this research arose from the need to protect native beneficial organisms in crop soils that serve as a natural and silent barrier against pests and diseases, preferably employing by-products from regional agro-industry to favor sustainable and circular processes. Moreover, our findings provide new insights into unraveling complex soil biotic interactions and identifying the abiotic factors that modulate them by combining traditional and molecular methodologies. Looking ahead, novel molecular tools such as Next-Generation Sequencing (NGS) analysis (Geisen et al., 2018; Dritsoulas et al., 2020) will contribute to advance in this line.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2022.107781.

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Results and Discussion

> *„Imaginada la hipótesis, menester es someterla a la sanción de la experiencia, para lo cual escogemos experimentos u observaciones precisas, completas y concluyentes."*
>
> Santiago Ramón y Cajal (1852-1934)

Soil organisms recorded

Entomopathogenic nematodes

The methodologies employed in this Thesis's studies allowed the identification of seven EPN species out of the ten initially screened (Table 4). The predominant species were *H. bacteriophora*, *S. feltiae*, and *S. riojaense*, all identified in high numbers through sucrose centrifugation and insect baiting procedures for all three studies and spread throughout the sampling area (Figure 27). The species *S. affine* (named *Steinernema* sp. *affine*-group in the studies published in 2022, see page 63 for more details) and *S. carpocapsae* also occurred in samples collected from all the experiments but at lower abundance and frequencies. Regarding the rest of the detected species, *H. indica* was prevalent in the experimental vineyard employed for evaluating the cover crops but lacking in the other studies, and *S. kraussei* was found emerging from very few baiting larvae in the DOCa Rioja survey (Figure 27).

Several studies have previously identified these EPN species in the Iberian Peninsula, with the exception of *S. riojaense*, isolated for the first time in these studies and recognized as a new species (Půža et al., 2020). The first EPNs reported in Spain were *H. bacteriophora* and *S. glaseri* (presumably misidentified with *S. arenarium* according to Sturhan and Mráček, 2003) in the locality of Cabrils in Catalonia (de Doucet and Gabarra, 1994). Nevertheless, García del Pino and Palomo (1996, 1997) were the pioneers in studying the EPNs in Spain. Using insect baiting as an isolation technique and covering the entire region of Catalonia, they found, in agreement with our results, *S. feltiae* to be the prevalent

Table 4. Target soil organisms screened and identified through sucrose centrifugation (Abundance) and insect baiting (Activity) for the studies that comprise this Thesis.

Type of organism /species	Publication 1		Publication 2		Publication3	
	Abundance	Activity	Abundance	Activity	Abundance	Activity
Entomopathogenic nematodes						
Heterorhabditis bacteriophora	√	√	√	√	√	√
Heterorhabditis indica	√	√	×	×	×	×
Heterorhabditis megidis	-	-	×	×	×	×
Steinernema affine	√	×	√*	√*	√*	×
Steinernema arenarium	×	×	-	-	-	-
Steinernema carpocapsae	×	√	×	√	√	×
Steinernema feltiae	√	√	√	√	√	√
Steinernema intermedium	-	-	×	×	×	×
Steinernema kraussei	×	×	×	√	×	×
Steinernema riojaense	√	√	√	√	√	√
Free-living nematodes						
Acrobeloides-group	√	√	√	√	√	√
Oscheius onirici	×	×	√	√	√	×
Oscheius tipulae	×	×	√	√	√	√
Pristionchus maupasi	√	√	√	√	√	√
Nematophagous fungi						
Arthrobotrys dactyloides	√	-	√	-	√	-
Arthrobotrys musiformis	×	-	×	-	×	-
Arthrobotrys oligospora	√	-	√	-	√	-
Catenaria sp.	√	-	√	-	×	-
Hirsutella rhossiliensis	√	-	√	-	√	-
Purpureocillium lilacinum	√	-	√	-	√	-
Ectoparasitic bacteria						
Paenibacillus nematophilus	×	-	×	-	×	-
Paenibacillus sp.	√	-	√	-	√	-

* Named *Steinernema* sp. *affine*-group

EPN species in the region, plus the additional occurrence of *H. bacteriophora*, *S. affine*, and *S. carpocapsae* (Figure 28). Other surveys employing a similar methodology, conducted in La Rioja (Campos-Herrera et al., 2007) and mainland Portugal (Valadas et al., 2014), also found *S. feltiae* in higher numbers and more widely distributed than other EPN species: *S. kraussei*, reported for both studies, *S. carpocapsae* in La Rioja, and *H. bacteriophora* and *S. affine* [2] in Portugal (Figure 28). Further studies conducted in other regions, such as Galicia and the Canary and Balearic Islands, have recognized other EPNs, probably in *the S. bicornutum* and *S. glaseri* groups (García del Pino, 2005). More recent surveys have addressed this issue through molecular techniques for species identification, such as those implemented in the current studies (Figure 28). Thus, Campos-Herrera et al.

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[2] Morphologically recognized as *S. intermedium* by Valadas et al. (2014) and named as *Steinernema* sp. *affine*-group in our studies, but belonging among *S. affine* populations according to Dr. Vladimir Půža (Czech Academy of Sciences, Czech Republic).

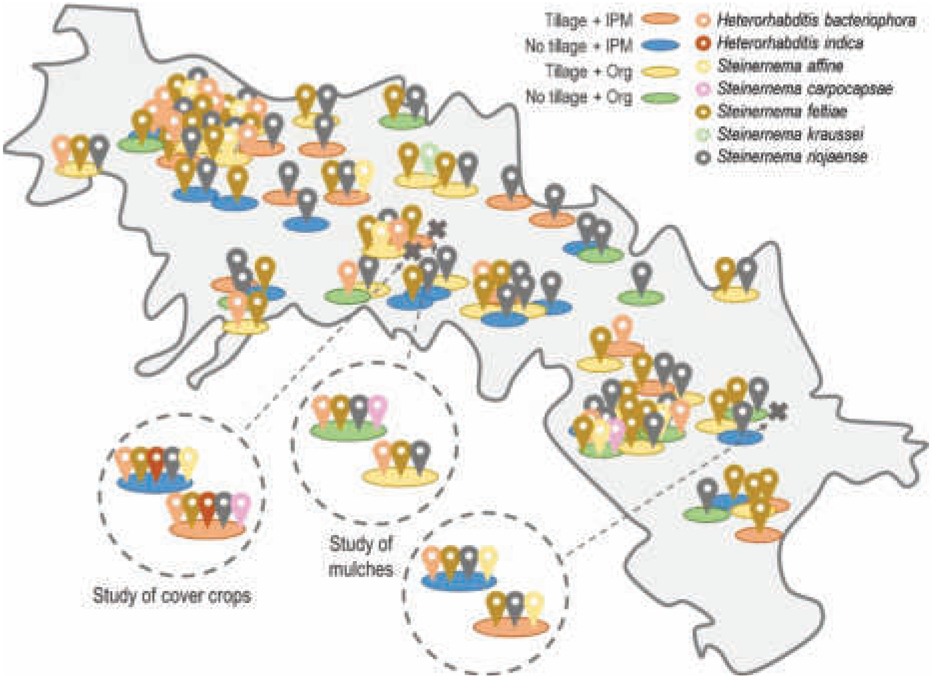

Figure 27. Species of entomopathogenic nematodes identified for the three studies that comprise this Thesis in vineyard soils managed with different agricultural practices: tillage *vs.* the use of alternative strategies such as cover cropping or mulching, and Integrated Pest Management (IMP) *vs.* organic farming (Org).

(2016b) confirmed the occurrence in the Iberian Peninsula of the EPN species *S. glaseri* and *H. indica*. The latter, subsequently detected by Campos-Herrera et al. (2019a) and in our cover crop study, had not been identified until then in such northern regions but predicted its occurrence in the Iberian Peninsula by estimating the ecological suitability of *H. indica* under a changing climatic scenario (Kour, 2017). However, even if Campos-Herrera et al. (2019a) observed a strong consilience of EPN detection using both nematode isolation techniques (bait and sucrose-centrifugation), all these evidence are based on molecular tools, so proofs of *H. indica* IJs emerging from baited larvae are needed to confirm definitively its natural occurrence in the Iberian Peninsula.

We obtained a considerable diversity of EPN species compared with the referred studies. For example, Campos-Herrera et al. (2007) reported the occurrence of only three EPN species (all steinernematids) in the same region under study, also identified in our samples. Undoubtedly, the molecular tools allowed us the recognition of additional species not yet described (as was the case

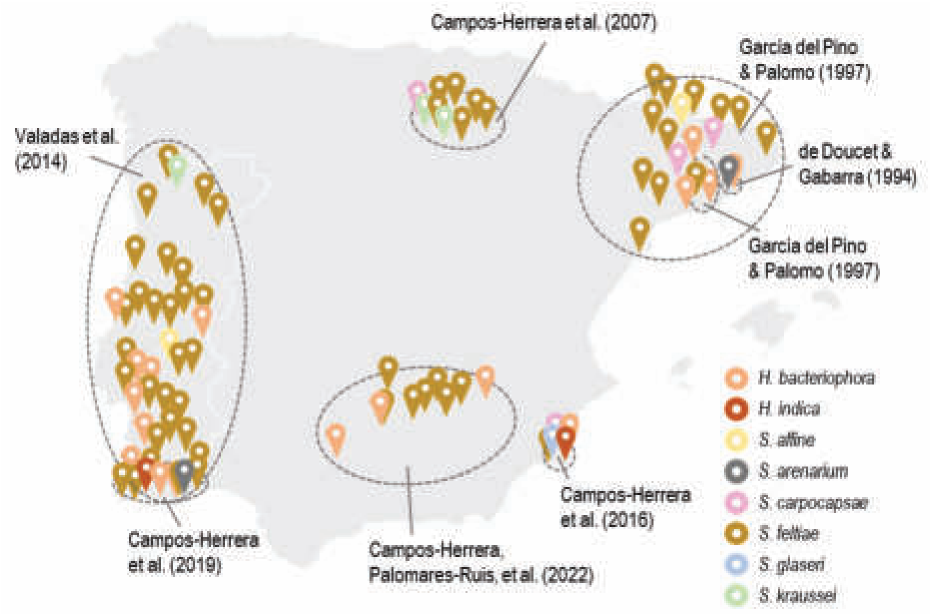

Figure 28. Species of entomopathogenic nematodes in the genera *Heterorhabditis* (H.) and *Steinernema* (S.) identified in some of the most representative studies performed in the Iberian Peninsula.

of *S. riojaense*) or found in low numbers. Besides, the combination of both methodologies seems to allow for a more refined screening of EPNs. Thus, a recent survey on olive groves in Southern Spain that employed systems for nematode extraction based on centrifugation techniques only found the common species of *S. feltiae* and *H. bacteriophora* (Campos-Herrera, Palomare-Ruis, et al., 2022). Regarding the frequency of EPN recovery from insect baits (expressed as percentages), our numbers (20-25%) were similar to those reported in surveys conducted in Catalonia, Galicia, and La Rioja (García del Pino and Palomo, 1996; García del Pino, 2005; Campos-Herrera et al., 2007). The other mentioned studies noted appreciably lower frequencies (de Doucet and Gabarra, 1994; García del Pino, 2005; Valadas et al., 2014) except for the values near 60% obtained in the Algarve (Southern Portugal) by Campos-Herrera et al. (2019a). Other studies of a similar type completed in European countries with Mediterranean influences also differed in the frequencies reported, for example, very low (~5%) in Greece (Menti et al., 1997), similar to our results (~15%) in Italy (Tarasco et al., 2015), or very high (~60%) in Southern France (Emelianoff et al., 2008). Lastly, the EPN abundance (4-60 IJs per 100g of dry soil) and recovery rates (40-70%) obtained in our studies were, depending on the treatments, comparable to those reported in the Algarve (Campos-Herrera et al., 2019a). Contrary, other surveys, such as those completed in polluted mining soils in Murcia (Southwestern Spain) and olive groves of Andalusia (Figure 28), registered abundance and frequencies of recovery lower than one IJs per 100g of dry soil and 25%, respectively (Campos-Herrera et al., 2016b; Campos-Herrera, Palomare-Ruis, et al., 2022).

Other targeted organisms

Both techniques of nematode extraction allowed the isolation and identification of all the FLNs screened (Table 4). Evidence of *Acrobeloide*s-group occurrence was found in almost all analyzed soil samples, but very few emerged from baited larvae, a pattern previously observed, for instance, in the Algarve (Campos-Herrera et al., 2019a). In contrast, the recovery rate was significantly lower (~60%) in polluted mining soils in Murcia (Campos-Herrera et al., 2016b). About the rest of the evaluated species, *O. tipulae* and *P. maupasi* occurred in all our experiments and Southern Iberian Peninsula (the Algarve and Andalusia), while *O. onirici*, usually found in lower numbers, was missed in our study of cover crops and Andalusia (Campos-Herrera et al., 2019a; Campos-Herrera, Palomare-Ruis, et al., 2022). Overall, total EPN findings correlated positively with FLN findings in all three studies, particularly *Acrobeloides* sp. and *P. maupasi* (Table 5).

Regarding the NF, we found all the screened species except *A. musiformis* (Table 4), the only one not detected in any of the mentioned studies conducted in the Iberian Peninsula. Recovery rates in our samples were up to 75% except for the cover crops study (20-45%), with *P. lilacinum* being the predominant species and *Catenaria* sp. lacking in the mulches study. Campos-Herrera et al. (2019a) obtained similar values in the Algarve but with an unclear prevalence of any NF species (apart from *Catenaria* sp., which occurred in low numbers). In contrast, the pattern observed for olive groves in Andalusia was quite different, with lower recovery frequencies (15-35%), *H. rhossiliensis* sharing predominance with *P. lilacinum*, and, most surprisingly, the absence of species in the genus *Arthrobotrys* (Campos-Herrera, Palomare-Ruis, et al., 2022). We found positive correlations between the abundance of trapping NF species in the genus *Arthrobotrys* and EPNs: *A. dactyloides* in the cover crops study and the DOCa Rioja survey (although with marginal significance for the latter) and *A. oligospora* in the mulches study (Table 5). FLNs in the *Acrobeloides*-group, widespread in all explored vineyards, also associated positively with *A. dactyloides* in the same studies as for EPNs, but *A. oligospora* did negatively in the mulches study (Table 6). Regarding endoparasitic NF, *Catenaria* sp. negatively correlated with nematode occurrence (although with marginal significance for *Acrobeloides* spp.) in the cover crop study (Table 5 and Table 6).

Table 5. The Spearman's rank-order correlation of total abundance of entomopathogenic nematodes and other soil organisms quantified in the three studies that comprise this Thesis. Significant differences (*P* < 0.05) are highlighted in bold. Codes: no data, n.d.

Type of organism /species	Publication 1		Publication 2		Publication3	
	Spearman's ρ	*P*	Spearman's ρ	*P*	Spearman's ρ	*P*
Free-living nematodes						
Acrobeloides-group	0,248	**0,001**	0,181	**0,022**	0,199	**0,007**
Oscheius onirici	n.d.	n.d.	0,007	0,925	0,085	0,189
Oscheius tipulae	n.d.	n.d.	-0,047	0,553	0,173	**0,020**
Pristionchus maupasi	0,203	**0,005**	0,166	**0,036**	0,207	**0,005**
Nematophagous fungi						
Arthrobotrys dactyloides	0,204	**0,005**	0,142	0,073	0,106	0,158
Arthrobotrys oligospora	-0,102	0,161	0,028	0,728	0,192	**0,010**
Catenaria sp.	-0,163	**0,024**	-0,103	0,196	n.d.	n.d.
Hirsutella rhossiliensis	0,065	0,371	0,139	0,080	0,062	0,407
Purpureocillium lilacinum	0,041	0,573	0,093	0,242	0,086	0,250
Ectoparasitic bacteria						
Paenibacillus sp.	0,136	0,060	0,055	0,489	0,092	0,220

Table 6. The Spearman's rank-order correlation of *Acrobeloides* spp. abundance and quantifications for fungal and bacterial species in the three studies that comprise this Thesis. Significant differences (*P* < 0.05) are highlighted in bold. Codes: no data, n.d.

Type of organism /species	Publication 1		Publication 2		Publication3	
	Spearman's ρ	*P*	Spearman's ρ	*P*	Spearman's ρ	*P*
Nematophagous fungi						
Arthrobotrys dactyloides	0,263	**<0,001**	0,207	**0,009**	-0,046	0,363
Arthrobotrys oligospora	-0,205	**0,004**	0,065	0,412	-0,145	0,053
Catenaria sp.	-0,136	0,060	-0,043	0,590	n.d.	n.d.
Hirsutella rhossiliensis	-0,002	0,974	0,096	0,228	-0,051	0,497
Purpureocillium lilacinum	0,069	0,339	0,052	0,510	-0,068	0,363
Ectoparasitic bacteria						
Paenibacillus sp.	0,178	**0,013**	0,293	**<0,001**	0,160	**0,032**

Finally, we found evidence of EcPB *Paenibacillus* sp. in vineyards of all three studies but not of *P. nematophilus* (**¡Error! No se encuentra el origen de la referencia.**). Interestingly, similar surveys conducted in Switzerland (Jaffuel et al., 2016, 2017, 2018) and Portugal (Campos-Herrera et al., 2019a) also failed to identify *P. nematophilus*, a bacterial species first described in Ireland (Enright and Griffin, 2005), but did document the occurrence in the Algarve of *Paenibacillus* sp., a species reported in Florida, USA (El-Borai et al., 2005). Campos-Herrera et al. (2019a) indeed found high EcPB abundance (7 plasmids per 100 g of dry soil) and recovery rates (50%), similar to those obtained for our cover crops study and DOCa Rioja survey, and much higher than the numbers found for the mulches study (less than one plasmid on average and frequencies above 10%). Our findings for *Paenibacillus* sp. positively correlated with Acrobeloides spp. abundance in all three studies (Table 6) but not with EPNs (only marginally in the cover crop study). As already discussed (page 37), *Paenibacillus* sp. generally shows low specificity for NEPs (Enright et al., 2003) except for specific hosts, as is the case of *Paenibacillus* sp. and *S. diaprepesi* in Florida (El-Borai et al., 2005). However, it is possible to detect appreciable numbers of EcPB in the digestive tract of FLNs since they presumably can actively acquire them (Campos-Herrera et al., 2012b).

Impact of viticulture practices

Soil management

As discussed, alternative strategies to tillage might stimulate trophic linkages associated with the nematode community, enhancing their presence and biodiversity in crop soils (Sánchez-Moreno et al., 2015). Based on this premise, we hypothesized that implementing cover crops and organic mulches that limit or prevent the practice of tillage in vineyards could favor the abundance and activity of native EPNs. As expected (Pittelkow et al., 2014), no-tilled soils in our studies overall contained higher organic matter contents than bare soils, which generally resulted in higher concentrations of mesofaunal DNA extracted through sucrose centrifugation (Figure 29). However, the analysis of our findings revealed contrasting results. Our assumption was partially satisfied for the studies focused on experimental vineyards, particularly for mulches based on spent mushroom compost and spontaneous cover crops implemented on rows and inter-rows, respectively (Figure 30). Thus, compared to regular tillage, most of the cover crop and mulch treatments appeared to favor the occurrence of the EPN species *H. bacteriophora* and *S. feltiae*, respectively. On the contrary, *S. feltiae*, which seems to be replaced by *H. bacteriophora* on no-tilled soils, occurred at lower numbers in all cover crop types evaluated (see Blanco-Pérez et al., 2020 and 2022a for further details).

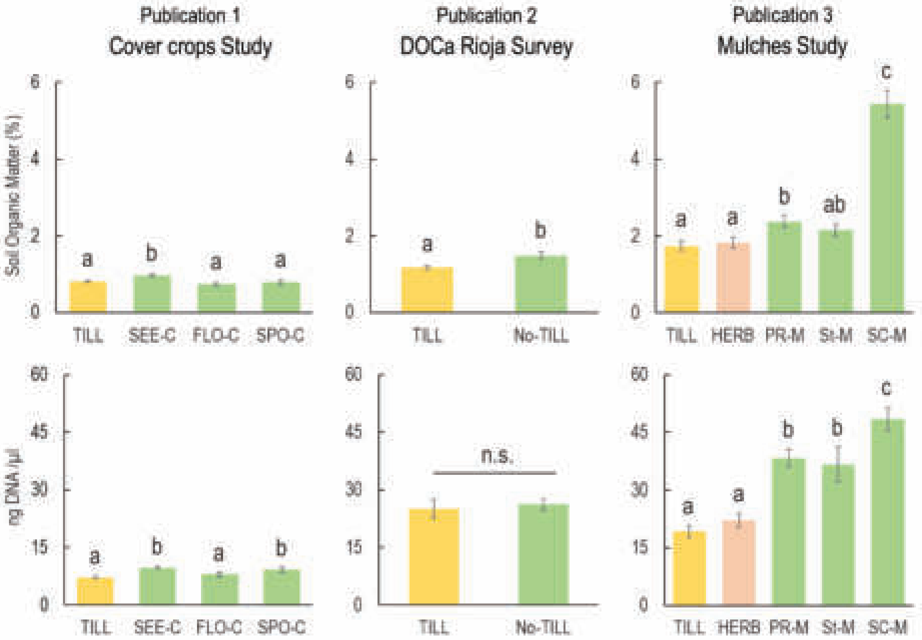

Figure 29. Quantifications for soil organic matter (graphs above) and DNA extracted through sucrose centrifugation (graphs below) for the three studies that comprise this Thesis. Letters indicate significant differences ($P < 0.05$) for general linear mixed model tests (n.s., no significant). Values are least-square means ± SE. Codes: tillage, TILL; no tillage, No-TILL; herbicide application, HERB; cover crops based on grass-seed, SEE-C; flower-driven, FLO-C; and spontaneous SPO; mulches based on grape pruning debris, PR-M; straw, St-M; and spent mushroom compost, SC-M.

Nevertheless, the DOCa Rioja survey did not validate this trend. Even if cover cropping promoted soil organic matter in the evaluated vineyards, we obtained higher values (no significant) for most of the biotic variables analyzed in the tilled soils (Figure 29, Figure 30, and Figure 31). These results disagree with those obtained in the other two studies and other research discussed in the introduction (see page 48). Perhaps experimental designs counting with replicates in space and time are more suitable for sampling organisms that, like EPNs, show a pronounced patchy distribution (Spiridonov et al., 2007). In addition, this study includes vineyards with multi-faceted management practices that could alter biotic and abiotic soil attributes differently, creating unique disturbances that may affect the nematofauna in a species-specific manner, including the EPNs (Fiscus and Neher, 2002; Stuart et al., 2015). Hence, several soil properties differed strongly between soil management practices in the DOCa Rioja survey. We recorded, for example, higher NPK values for cover cropping but

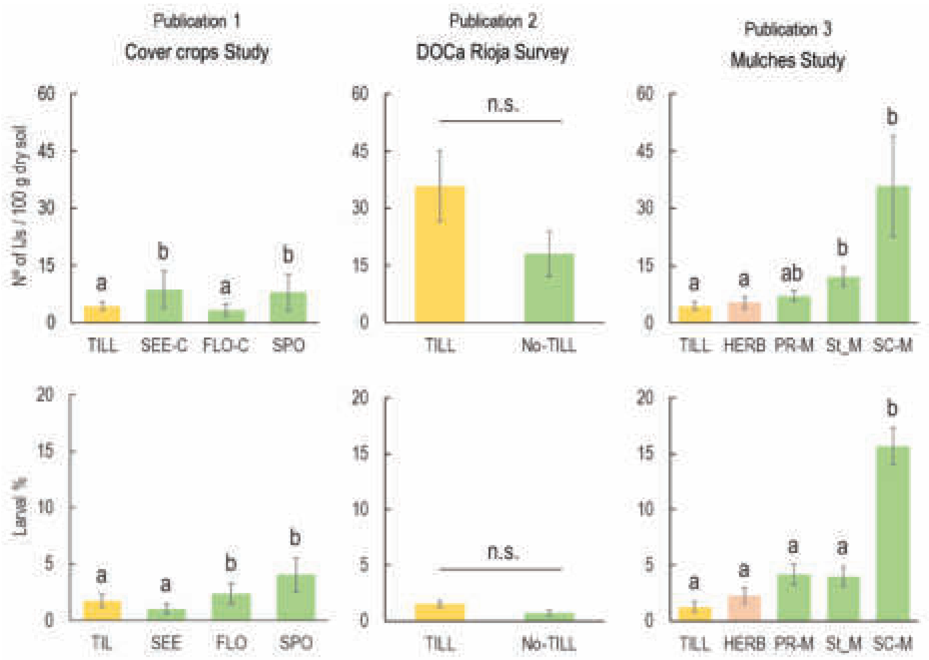

Figure 30. Abundance (graphs above) and activity (graphs below) of entomopathogenic nematodes for the three studies that comprise this Thesis, measured as the number of infective juveniles (IJs) extracted through sucrose centrifugation and percentage of larvae that showed nematode emergencies and were positive for the Koch's Postulates, respectively. Letters indicate significant differences ($P < 0.05$) for generalized linear mixed model tests (n.s., no significant). Values are least-square means ± SE. Codes: tillage, TILL; no tillage, No-TILL; herbicide application, HERB; cover crops based on grass-seed, SEE-C; flower-driven, FLO-C; and spontaneous SPO; mulches based on grape pruning debris, PR-M; straw, St-M; and spent mushroom compost, SC-M.

also increased contents of metal ions such as Zn and Cu, which can cause high toxicity on EPNs, notably in steinernematids (Jaworska et al., 1996, 1997). Indeed, the abundance we reported for *S. feltiae* and *S. affine* negatively correlated with high Cu contents in our soil community assemblage analysis (see Blanco-Pérez et al., 2022b for further details).

The difficulty of discerning the impact of different abiotic drivers on the EPN community in complex environments is amplified by their interactions with other soil organisms. Overall, we found a significantly higher abundance of FLNs in mulches based on grape pruning debris and spent mushroom compost, primarily due to nematodes in the *Acrobeloides*-group (the most widespread FLNs in our studies), and NF in grass-seeded cover crops and mulches based on straw (most notably) and grape pruning debris. In contrast, in the DOCa Rioja

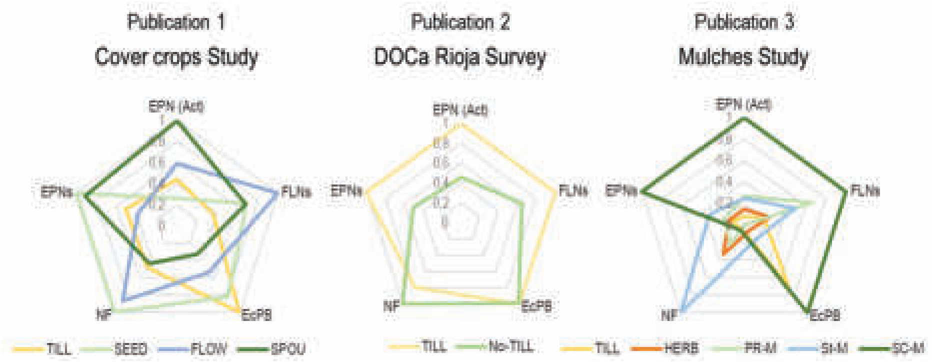

Figure 31. Proportional impact of soil management for the three studies that comprise this Thesis on the activity (Act) of the entomopathogenic nematodes (EPNs) and the abundance of these and other associated soil organisms: free-living nematodes (FLNs), nematophagous fungi (NF), and ectoparasitic bacteria (EcPB). Codes: tillage, TILL; no tillage, No-TILL; herbicide application, HERB; cover crops based on grass-seed, SEE-C; flower-driven, FLO-C; and spontaneous SPO; mulches based on grape pruning debris, PR-M; straw, St-M; and spent mushroom compost, SC-M.

survey, we did not find any significant differences in the abundance of any group of organisms examined (Figure 31), but only for specific species. Thus, the FLNs *P. maupasi* and *O. onirici* were more abundant in tilled soils, while no-tilled vineyards favored the occurrence of *O. tipulae* and the fungal species *A. dactyloides* and *P. lilacinum*. No species-specific differences were observed for FLNs in the cover crop and mulch studies, nor for endoparasitic NF (*Catenaria* sp. and *H. rhossiliensis*) and the EcPB *Paenibacillus* sp. for any of the three studies. On the other hand, the results obtained for the cover crops and mulches studies also support the possibility that tillage negatively affects the infective stage occurrence of the eggs-parasitic fungus *P. lilacinum*, a fungal species that, according to our observations, did not interact directly with IJs in soils (Table 5). It seems plausible that this may be due to an underestimation of FLN abundance, analysis beyond this Thesis' scope. However, in no-tilled soils from the DOCa Rioja study, we obtained, as noted above, larger values for *P. lilacinum* but not for the DNA extracted by sucrose centrifugation (Figure 29), a technique optimized for nematofauna extraction (Dritsoulas et al., 2021).

In summary, alternative strategies to regular tillage could increase native EPN populations in the vineyard but selectively, probably due in part to differential impacts on their soil food web. Approaches of this kind modify physicochemical properties in crop soils, enriching them and promoting biodiversity in the agroecosystem (Atwood et al., 2022). Thus, increased soil organic matter contents (Figure 29) may imply, for instance, a higher abundance and diversity of nematodes, although followed by subsequent increases in their natural enemies (Linford, 1937; Cooke, 1962). In the study of cover crops, we recorded, for example, higher organic matter and clay contents in grass-seeded cover crops, two of the key factors favoring NF occurrence (Nordbring-Hertz et al., 2006; Pathak et al., 2017). Indeed, we observed higher NF abundances, particularly for endoparasitic species, for this specific treatment that could explain the poor values reported for the EPN activity (Table 5); Figure 31). On the contrary, we obtained opposite numbers for spontaneous cover crops, with a significantly lower abundance of *A. oligospora* and a lack of endoparasitic NF. Based on these observations, we initially suggested that *H. rhossiliensis* and *Catenaria* sp. could inhibit the pathogenic capacity of EPNs more efficiently than other NF species, but the results obtained in the DOCa Rioja study did not support this premise.

In this regard, the study of organic mulches was even more illustrative. This study showed that different mulches could enhance or inhibit NF growth, for instance, by contributing to excessive soil water contents, which presumably limited the abundance and virulence of native EPNs in an environment otherwise favorable for their optimal establishment. All the analyzed organic mulches favored soil moisture but with minor annual oscillations in straw mulches (Pou et al., 2021), the treatment for which we found higher NF numbers in both experimental vineyards (Figure 31). Although to a lesser extent, mulches of grape pruning debris also promoted the proliferation of NF infective stages in organic viticulture. That could also explain the lack of statistical significance between this treatment and bare soils for both abundance and activity of EPNs in this specific vineyard. In contrast, we found low numbers of NF infective stages for mulches based on spent mushroom compost (Figure 31). Somehow, this treatment inhibited the development of, at least, this type of fungi despite creating *a priori* favorable conditions for it. This kind of organic mulch contains residual mycelia of *Pleurotus ostreatus* (Agaricales: Pleurotaceae) that can produce diverse secondary metabolites to prevent the fungal growth of a wide range of possible competitors (Rayner et al., 1994; Ocimati et al., 2021). The decline of natural enemies of nematodes could explain the exceptional EPN and FLN abundances obtained for this treatment in both experimental vineyards (Figure 30 andFigure 31). Moreover, a recent study revealed that volatile organic compounds such as octenol, produced by filamentous fungi like *P. ostreatus*, serve as foraging cues used by fungivorous insects and EPNs (Wu and Duncan, 2020). Thus, another plausible explanation for these findings is that mulches based on spent mushroom compost are specifically attractive to EPNs.

Besides, as previously mentioned, the FLN species examined in these studies were selected on the criteria of their ability to emerge from insect larvae previously colonized by EPNs and, therefore, on their ability to compete with them for food sources within the host cadaver. However, although we found a positive correlation between EPN abundance and most of the FLNs analyzed (Table 5), the specific interactions that these two groups of nematodes would establish in soils may be too diverse and complex to be easily interpretable, even more considering that their living and feeding habits under natural conditions are still mostly unknown. For instance, according to the *Dauer Hypothesis* (Rogers and Sommerville, 1963), rhabditid and diplogastrid nematodes can exhibit a remarkable versatility of functions, from bacteriophagy as free-living organisms in soils to pathogenesis (Figure 13). Moreover, specialized oral cavities of diplogastrids enable nematodes such as *Pristionchus* spp. to switch from feeding on bacteria to predating on other nematodes (von Lieven, 2003). However, we only reported significant differences for differentiated soil management practices in the abundance of FLNs in the DOCa Rioja survey, and the higher occurrences of the species *O. onirici* and *P. maupasi* in tilled soils do not justify the highly raised EPN abundance obtained for this particular treatment or the lower EPN activity than expected for untilled soils.

Pest management

We found higher biotic numbers for organic viticulture than for IMP, both for the abundance of all types of soil organisms screened and for the total quantification of DNA extracted by sucrose centrifugation (Figure 32Figure 33). These numbers should explain the higher soil activity rates we obtained, in agreement with our hypothesis, for organic farming than IPM, including those associated with EPN emergences (positive for Koch's Postulates) (Figure 34). In the organic vineyard employed for the mulches study, we also observed increased organic matter contents and EPN abundance not confirmed for the DOCa Rioja survey (Figure 32 andFigure 34). Perhaps this lack of significance in the EPN numbers reported for the 80 vineyards comprising this study was due to the remarkably patchy records obtained for *S. riojaense*, found in very high abundance regardless of farming management, but only in a limited number of

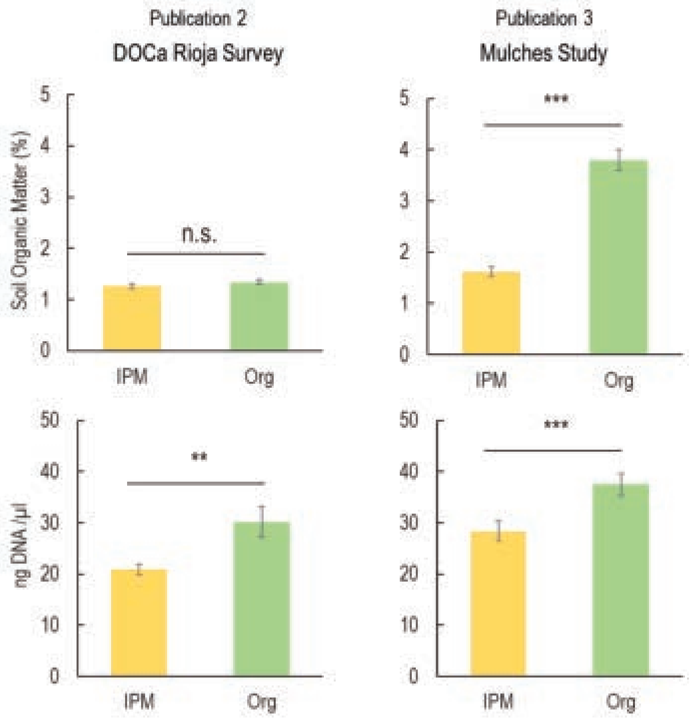

Figure 32. Quantifications for soil organic matter (graphs above) and DNA extracted through sucrose centrifugation (graphs below) for two of the studies that comprise this Thesis. Asterisks indicate significant differences from generalized linear mixed model tests at ***$P < 0.001$ and **$P < 0.01$ (n.s., not significant). Values are least-square means ± SE. Codes: IPM, Integrated Pest Management; Org, organic viticulture.

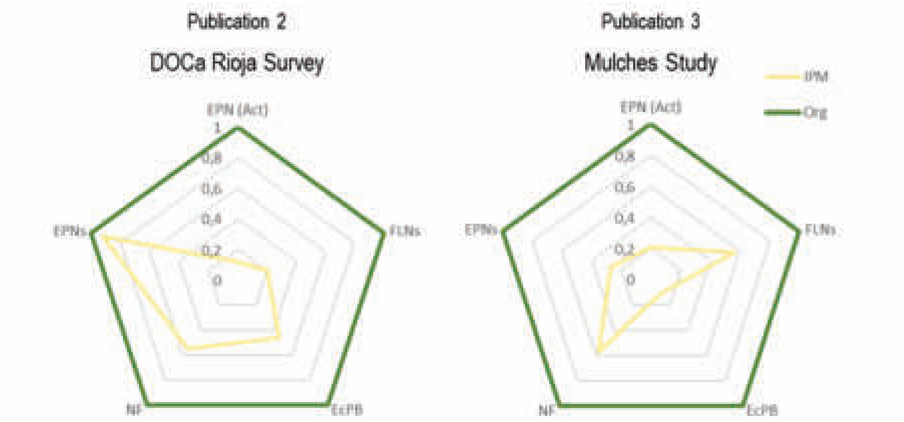

Figure 33. Proportional impact of pest management for two of the studies that comprise this Thesis on the activity (Act) of the entomopathogenic nematodes (EPNs) and the abundance of these and other associated soil organisms: free-living nematodes (FLNs), nematophagous fungi (NF), and ectoparasitic bacteria (EcPB). Codes: IPM, Integrated Pest Management; Org, organic viticulture.

vineyards. Conversely, organic viticulture enhanced the abundance of *S. feltiae* (Figure 33), the predominant species in the overall of our studies and those completed in the Iberian Peninsula and Europe (Hominick, 2002; Bhat et al., 2020). Moreover, in this survey, more EPN emergences from insect baits in soil samples collected from organic vineyards were identified for *S. feltiae* and not for *S. riojaense*, which showed meager activity rates relative to its abundance.

Organic viticulture also seemed to sustain a higher richness of nematode species. In the DOCa rioja survey, for example, the steinernematid species *S. affine*, *S. carpocapsae*, and *S. kraussei* only occurred in organic vineyards. In addition, we documented no evidence of *O. onirici* for IPM nor the experimental vineyard used in the mulches study, and the abundance of the rest of the screened FLNs was higher for organic farming in both studies (Figure 35). Bacterial feeder nematodes of this type are, in fact, good predictors of soil health in terms of, for instance, soil structure and organic matter contents (van den Hoogen et al., 2019). Plausibly linked to this raised presence of nematodes (Table 6), *Paenibacillus* sp. numbers were also significantly higher in organic than in IPM vineyards. Similar may occur for the NF absolute abundance, although no statistically significant differences were obtained for the DOCa Rioja survey (Figure 35). Regarding NF richness, we found the same fungal species for both treatments, except for the non-presence of *H. rhossiliensis* in the IPM vineyard of the mulches study.

Altogether, increased abundance and richness of potential natural enemies could partially explain the lack of significant differences in EPN numbers between IPM and organic farming in the DOCa Rioja survey. Beyond the evident adverse impact of increased NF abundance on EPN populations in crop soils, nematodes in the genera *Acrobeloides*, *Oscheius*, and *Pristionchus* could, as noted above, disrupt EPN fitness by establishing with them diverse and complex interactions that are challenging to predict. On the other hand, the EcPB *Paenibacillus* sp. could affect IJs in a species-specific manner. Indeed, El-Borai et al. (2005) associated this bacterial species with *S. diaprepesi*, an EPN species closely related to *S. riojaense* (Půža et al., 2020). Perhaps *Paenibacillus* sp. affected *S. riojaense* more than other EPN species in the organic vineyards of the DOCa Rioja study, limiting its abundance for organic viticulture and overall activity. However, given that the biology and ecology of this recently described species are still largely unknown (Půža et al., 2020), multiple reasons could be considered to explain the infrequent numbers we report for this particular EPN species in this study. For

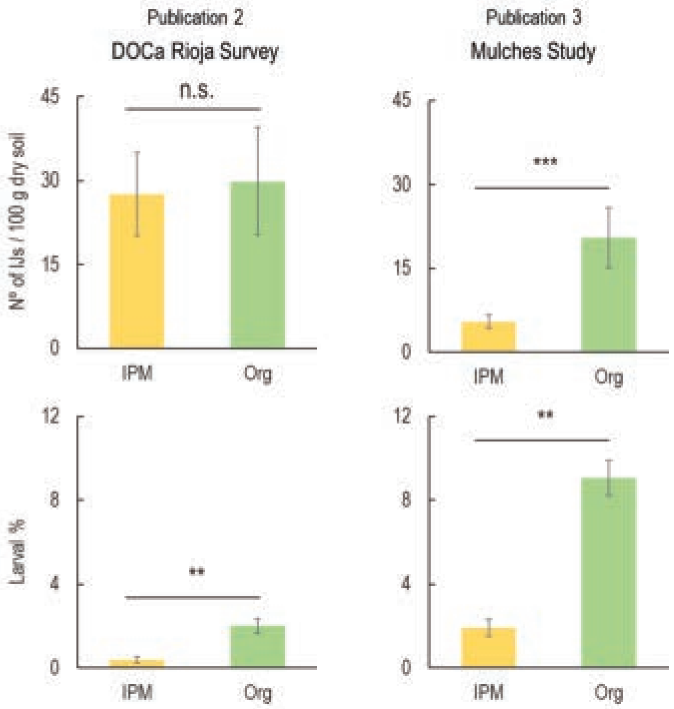

Figure 34. Abundance (graphs above) and activity (graphs below) of entomopathogenic nematodes for two of the studies that comprise this Thesis, measured as the number of infective juveniles (IJs) extracted through sucrose centrifugation and percentage of larvae that showed nematode emergencies and were positive for the Koch's Postulates, respectively. Asterisks indicate significant differences from generalized linear mixed model tests at ***$P < 0.001$ and **$P < 0.01$ (n.s., not significant). Values are least-square means ± SE. Codes: IPM, Integrated Pest Management; Org, organic viticulture.

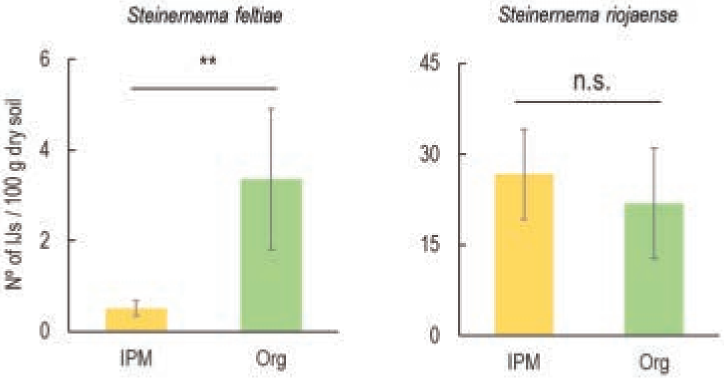

Figure 35. . Abundance of infective juveniles (IJs) of the two predominant species for the DOCa Rioja survey. Asterisks indicate significant differences from generalized linear mixed model tests at **$P < 0.01$ (n.s., not significant). Values are least-square means ± SE. Codes: IPM, Integrated Pest Management; Org, organic viticulture.

example, the meager activity rates recorded for *S. riojaense* compared to its high abundance could be due to the wrong host choice (*G. mellonella* larvae) in our bioassays (Adams and Nguyen, 2002). This illustration, for instance, accentuates the complexity of unraveling multiple biotic interactions under naturalized conditions.

Outlooks

Conserving soil biodiversity and understanding the natural interrelationships of soil organisms is a fundamental milestone in improving soil health (Lehmann et al., 2020; Gunstone et al., 2021). Indeed, irreparable soil losses have generated a global alarm that justifies and drives the European Union Soil Strategy 2030 (EU, 2021). The studies included in this Thesis have shown that diverse management practices in viticulture affect the EPN community and its soil food web differently and, hence, modulating the ecosystem service these BCAs provide. Molecular tools accurately characterized target species in cryptic environments. However, these results are difficult to interpret regarding possible spatial and temporal links that soil organisms may establish among them and the abiotic conditions in which they coexist. Approaches of this type must be addressed with multivariate statistical and geostatistical methods to assess rather than infer patterns (Campos-Herrera et al., 2013a). For example, *Spatial Analysis by Distance Indices* (SADIE) (Perry and Hewitt, 1991) was already successfully applied to characterize the spatial patterns of EPNs, FLNs, and NF in Florida citrus orchards (Campos-Herrera et al., 2011, 2012a), so it could similarly help to unravel their interactions in soil vineyards.

Furthermore, understanding BCA biogeography and identifying factors that drive their occurrence and abundance in crop soils, crucial for conservation biological control programs, require finer-scale taxonomic resolution than qPCR analysis provides (Dritsoulas et al., 2020). Although species-specific primer-probe sets have allowed advances in understanding EPN communities, particularly for sympatric distributions (Campos-Herrera et al., 2011b), this approach is limited to identifying target organisms only. Moreover, the specificity of qPCR analysis is occasionally excessive for communities with marked intraspecific variability. Certainly, closely related EPN species exhibit divergent phenotypes due to, for instance, habitat adaptation or host specificity (Lewis et al., 2006; El-Borai et al., 2016). Novel molecular tools, such as high-throughput sequencing (HTS), provide more accurate taxonomic resolution and recognize more rare taxa than morphological or qPCR analysis (Treonis et al., 2018). In addition, the rapidly decreasing cost of this procedure makes HTS a more affordable tool for measuring organisms in cryptic environments. For example, Geisen et al. (2018) obtained higher taxonomic resolution for native nematode populations at a lower cost than qPCR and morphological approaches.

Regarding EPNs, Dritsoulas et al. (2020) reanalyzed 56 samples out of 100 that comprised Campos-Herrera et al. (2019a)'s survey using universal primers targeting the ITS1 region for an HTS procedure, finding all the species also detected by qPCR plus *H. megidis*. Besides, the predominant EPN species, *S. feltiae*, was not only found at 55% more sites and in increased numbers, but HTS measurements indicate that it tended to be highly aggregated rather than random, as suggested by Campos-Herrera et al. (2019a). Based on these results, HTS analysis arises as a promising tool for studying EPN biogeography and their soil food web since HTS potentially reveals everything in a soil sample that universal primers can amplify, including unknown organisms and novel functions of soil communities (He et al., 2010). However, this technology still needs further optimization to improve its accuracy (Waeyenberge et al., 2019). Hence, future research will integrate such molecular tools with spatial-temporal dynamics and multitrophic interactions analyses to reveal the EPN biogeography and identify the best viticulture practices for their implementation in conservation biological control programs. Finally, choosing the vineyard as a model framework in this Thesis provides new lessons in these research areas that lay the groundwork for advanced studies in other cropping systems.

Conclusions

The main conclusions resulting from the studies presented in this Thesis were as follows:

1. Alternative strategies to tillage, such as cover cropping and mulching, can support native entomopathogenic nematodes (EPNs) in the vineyard but depending on typologies.
2. Comprehensive, no-tilled soils retained higher organic matter contents than tilled soils and favored the appearance of nematodes, although, according to *Linford's Hypothesis*, also that of their antagonisms, such as nematophagous fungi (NF) and free-living nematodes (FLNs).
3. The soil management practices of spontaneous cover cropping and mulching based on spent mushroom compost, which promoted EPN occurrence and activity, also maintained low numbers of potential enemies.
4. Organic viticulture enhanced FLN numbers, EPN activity, an overall nematode richness, and the occurrence of the predominant EPN species *Steinernema feltiae*.
5. Seven EPN species were reported in the DOCa Rioja vineyards, with *S. feltiae* and *Heterorhabditis bacteriophora* as the dominant of each genus.
6. The combination of molecular identification approaches and the traditional insect baiting enabled the isolation and the biogeographical study of the new EPN species *Steinernema riojaense*.
7. Our approach demonstrated that EPN soil food web characterization could signal soil health in perennial crops such as vineyards.
8. Overall, combining organic viticulture and specific soil management practices to replace regular tillage could enhance the ecosystem service that native EPNs perform as biological control agents in the vineyard.

Conclusiones

Las principales conclusiones resultantes de los estudios presentados en esta Tesis fueron las siguientes:

1. Tipologías específicas de cubiertas vegetales y acolchados orgánicos que reemplacen total o parcialmente la práctica del laboreo pueden favorecer la presencia y actividad de nematodos entomopatógenos (NEPs) nativos en el viñedo.

2. En líneas generales, los suelos no labrados mantuvieron mayores contenidos de materia orgánica que los suelos labrados y favorecieron la presencia de nematodos, pese a que, según predice la *Hipótesis de Linford*, también la de sus antagonistas, como los hongos nematófagos (HNs) y nematodos de vida libre (NVLs).

3. Las prácticas de manejo del suelo mediante cubierta vegetal espontánea y acolchados a base de compost de post-cosecha de champiñón, que potenciaron una mayor abundancia y actividad de NEPs, fueron también las que registraron menores números de sus enemigos naturales.

4. La viticultura ecológica favoreció la abundancia de NVLs, la actividad de los NEPs, la riqueza general de nematodos y la presencia de la especie predominante de NEPs, *Steinernema feltiae*.

5. Siete especies de NEPs fueron identificadas en los viñedos de la DOCa Rioja, con *S. feltiae* y *Heterorhabditis bacteriophora* como las especies dominantes para cada género.

6. La combinación de técnicas de identificación molecular y la tradicional trampa de insectos permitió el aislamiento y el estudio biogeográfico de la nueva especie de NEP *Steinernema riojaense*.

7. Estos estudios señalan que la caracterización de la red trófica de los NEPs en el suelo podría ser indicativo de la salud del suelo en cultivos perennes como el viñedo.

8. En general, la combinación de la viticultura ecológica con prácticas específicas de manejo del suelo que sustituyen el laboreo convencional pueden mejorar el servicio ecosistémico que los NEPs nativos realizan como agentes de control biológico en el viñedo.

Supplementary scientific activity

completed during the PhD progress

Publications

JCR Scientific publications

- Bueno-Pallero, F.A., **Blanco-Pérez, R.**, Dionísio, L., Campos-Herrera, R., 2018. *Simultaneous exposure of nematophagous fungi, entomopathogenic nematodes and entomopathogenic fungi can modulate belowground insect pest control.* J. Invertebr. Pathol. 154, 85-94. https://doi.org/10.1016/j.jip.2018.04.004

  Discipline: *Zoology*; IF: **2,101** (2018), **Q1** (25/170)

- Jaffuel, G., **Blanco-Pérez, R.**, Hug, A.S.., Chiriboga, X., Meuli, R.G., Mascher, F., Turlings, T.C.J., Campos-Herrera, R., 2018. *The evaluation of entomopathogenic nematode soil food web assemblages across Switzerland reveals major differences among agricultural, grassland and forest ecosystems*. Agric. Ecosyst. Environ. 262, 148-157. https://doi.org/10.1016/j.agee.2018.04.008

  Discipline: *Agriculture/Multidisciplinary*; IF: **3,954** (2018), **Q1** (2/56)

- Campos-Herrera, R., **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Duarte, A., Nolasco, G., Sommer, R.J., Rodríguez-Martín, J.A., 2019. *Vegetation drives assemblages of entomopathogenic nematodes and other soil organisms: Evidence from the Algarve, Portugal*. Soil Biol. Biochem. 128, 150-163. https://doi.org/10.1016/j.soilbio.2018.10.019

  Discipline: *Soil science*; IF: **5,795** (2019), **Q1** (2/34)

- **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Vicente-Díez, I., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., 2019. *Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode* Steinernema feltiae. J. Invertebr. Pathol. 164, 5-15. https://doi.org/10.1016/j.jip.2019.04.002

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- Chelkha, M., **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Amghar, S., El Harti, A., Campos-Herrera, R., 2020. *Cutaneous excreta of the earthworm* Eisenia fetida

(Haplotaxida: Lumbricidae) might hinder the biological control performance of entomopathogenic nematodes. Soil Biol. Biochem. 141, 107691. https://doi.org/10.1016/j.soilbio.2019.107691

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- Dritsoulas, A., Campos-Herrera, R., **Blanco-Pérez, R.**, Duncan, L.W., 2020. *Comparing high throughput sequencing and real time qPCR for characterizing entomopathogenic nematode biogeography*. Soil Biol. Biochem. 145, 107793. https://doi.org/10.1016/j.soilbio.2020.107793

  Discipline: *Soil biology*; IF: **7,609** (2020), **Q1** (1/37)

- Půža, V., Campos-Herrera, R., **Blanco-Pérez, R.**, Jakubíková, H., Vicente-Díez, I., Nermuť, J., 2020. Steinernema riojaense *n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from Spain*. Nematology 22, 825-841. https://doi.org/10.1163/15685411-00003343

  Discipline: *Zoology*; IF: **1,442** (2020), **Q3** (88/175)

- Bueno-Pallero, F.A., **Blanco-Pérez, R.**, Vicente-Díez, I., Rodríguez-Martín, J.A., Dionísio, L., Campos-Herrera, R., 2020. *Patterns of occurrence and activity of entomopathogenic fungi in the Algarve (Portugal) using different isolation methods*. Insects 11, 352. https://doi.org/10.3390/insects11060352

  Discipline: *Entomology*; IF: **2,769** (2020), **Q1** (18/102)

- Castruita-Esparza, G., Bueno-Pallero, F.A., **Blanco-Pérez, R.**, Dionísio, L., Aquino-Bolaños, T., Campos-Herrera, R., 2020. *Activity of* Steinernema colombiense *in plant-based oils*. J. Nematol. 52, e2020-72. https://doi.org/10.21307/jofnem-2020-072

  Discipline: *Zoology*; IF: **1,402** (2020), **Q3** (93/175)

- Vicente-Díez, I., **Blanco-Pérez, R.**, González-Trujillo, M.M., Pou, A., Campos-Herrera, R., 2021. *Insecticidal effect of entomopathogenic nematodes and the cell-free supernatant from their symbiotic bacteria against* Philaenus spumarius *(Hemiptera: Aphrophoridae) nymphs.* Insects 11, 352. https://doi.org/10.3390/insects12050448

  Discipline: *Entomology*; IF: **3,139** (2021), **Q1** (16/105)

- Chelkha, M., **Blanco-Pérez, R.**, Vicente-Díez, I., Bueno-Pallero, F.A., Amghar, S., El Harti, A., Campos-Herrera, R., 2021. *Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi.* J. Invertebr. Pathol. 184, 107620. https://doi.org/10.1016/j.jip.2021.107620

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- Campos-Herrera, R., Vicente-Díez, I., **Blanco-Pérez, R.**, Chelkha, M., González-Trujillo, M.M., Puelles, M., Čepulyte, R., Pou, A., 2021. *Positioning entomopathogenic nematodes for the future viticulture: exploring their use against biotic threats and as bioindicators of soil health.* Turk. J. Zool. 45, 335-346. https://doi.org/10.3906/zoo-2106-40

  Discipline: *Zoology*; IF: **0,932** (2021), **Q4** (148/174)

- Vicente-Díez, I., **Blanco-Pérez, R.**, Chelkha, M., Puelles, M., Pou, A., Campos-Herrera, R., 2021. *Exploring the use of entomopathogenic nematodes and the natural products derived from their symbiotic bacteria to control the grapevine moth,* Lobesia botrana *(Lepidoptera: Tortricidae).* Insects 12, 1033. https://doi.org/10.3390/insects12111033

  Discipline: *Entomology*; IF: **3,139** (2021), **Q1** (16/105)

- Campos-Herrera, R., Palomares-Ruis, J.E., **Blanco-Pérez, R.**, Rodríguez-Martín, J.A., Landa, B.B., Castillo, P., 2022. *Irrigation modulates entomopathogenic nematode community and its soil food web in olive groves under different agricultural managements*. Agric. Ecosyst. Environ. 337, 108070. https://doi.org/10.1016/j.agee.2022.108070

  Discipline: *Agriculture/Multidisciplinary*; IF: **6,576** (2021), **Q1** (5/59)

Outreach publications

- **Blanco-Pérez, R.**, Sáenz Romo, M.G., Vicente-Díez, I., Ibáñez-Pascual, S., Martínez-Villar, E., Pérez-Moreno, I., Marco-Mancebón, V.S., Campos-Herrera, R., 2018. *Cubiertas vegetales en viña y su impacto en la riqueza y actividad de los nematodos entomopatógenos.* Ae 34, 28-29.

- Vicente-Díez, I., Sáenz Romo, M.G., **Blanco-Pérez, R.**, Ibáñez-Pascual, S., Martínez-Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., 2019. *Impacto de la implantación de cubiertas vegetales en la presencia de agentes de control biológico en viñedos.* Viticultura 3555, 1844-1850.

- Campos-Herrera, R., **Blanco-Pérez, R.**, Vicente-Díez, I., 2020. *Nematodos entomopatógenos en el control biológico de ácaros e insectos.* Cuaderno de Canpo 63, 34-39.

- Chelkha, M., **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Vicente-Díez, I., Amghar, S., El Harti, A., Campos-Herrera, R., 2020. *Coexistencia de dos organismos beneficiosos del suelo: ¿Pueden las lombrices de tierra alterar la actividad* *beneficiosa de los nematodos entomopatógenos como agentes de control biológico?* Ae 39, 26-27.

- **Blanco-Pérez, R.**, Sáenz Romo, M.G., Vicente-Díez, I., Ibáñez-Pascual, S., Martínez-Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., 2021. *Cubiertas vegetales y conservación de poblaciones nativas de nematodos entomopatógenos en viña.* Cuaderno de Campo 65, 34-39.

Seminars and conference presentations

Seminars

- *Presencia y actividad de la comunidad de nematodos entomopatógenos en viñedos de la DOCa Rioja sujetos a diferentes manejos agronómicos.* Seminarios internos del ICVV. Instituto de Ciencias de la Vid y el Vino (ICVV). Febrary 25th 2020. Logroño, Spain.

- *Nematodos entomopatógenos como potenciales bio-indicadores en viñedos sujetos a diferentes manejos.* Seminario Internacional de Viticultura: Avances y Desafíos Coyunturales. Universidad Mayor en Santiago de Chile. September 1st 2021. Santiago de Chile, Chile.

Presentations at International Conferences

ESA International Branch and Virtual Symposium
April 16-18th 2018
Entomology Society of America (ESA)
USA (virtual)

- Castruita-Esparza, G., Bueno-Pallero, F.A., **Blanco-Pérez, R.**, Aquino-Bolaños, T., Campos-Herrera, R., *Exploring low-cost vegetable adjuvants for entomopathogenic nematode application: impact of temperature and combination with other entomopathogens.*

  Poster presentation

AMAS International Conference IV
May 9-11th 2018
American Moroccan Agricultural Sciences (AMAS)
Meknes, Morocco

- Chelkha, M., **Blanco-Pérez, R.**, Bueno-Pallero, F.A., El Harti, A., Amghar, S., Campos-Herrera, R., *Earthworm feeding activity and mucus secretion can decrease entomopathogenic nematodes activity as biological control agents.*

  Poster presentation

33rd ESN Symposium. 9-13 September 2018
September 9-13th 2018
European Society of Nematologists (ESN)
Ghent, Belgium

- **Blanco-Pérez, R.**, Sáenz Romo, M.G., Ibáñez-Pascual, S., Martínez-Villar, E., Pérez-Moreno, I., Marco-Mancebón, V.S., Campos-Herrera, R., To*ward conservation biological control in vineyards: how the implementation of different cover crops can affect the activity of native entomopathogenic nematodes?*

  Poster presentation

- Campos-Herrera, R., Palomares-Ruis, J.E., **Blanco-Pérez, R.**, Landa, B.B., Castillo, P., *Soil texture and olive cultivar determine natural occurrence and assemblage of entomopathogenic nematode in Southern Spain: consilience with main drivers for plant-parasitic nematode community.*

  Oral presentation

- Chelkha, M., **Blanco-Pérez, R.**, Bueno-Pallero, F.A., El Harti, A., Amghar, S., Campos-Herrera, R., *The presence of earthworm mucus secretion could altered entomopathogenic nematodes activity as biological control agents.*

  Poster presentation

International Congress on Gravevine and Wine Sciences
November 7-11th 2018
Institute of Grapevine and Wine Sciences (ICVV)
Logroño, Spain

- **Blanco-Pérez, R.**, Sáenz Romo, M.G., Ibáñez-Pascual, S., Vicente-Díez, I., Martínez-Villar, E., Pérez-Moreno, I., Marco-Mancebón, V.S., Campos-Herrera, R., *Entomopathogenic nematode natural distribution in vineyards managed with cover crops: impact on soil organisms' assemblage.*

  Poster presentation

52nd SIP Annual Meeting
July 28th – August 1st 2019
Society for Invertebrate Pathology (SIP)
Valencia, Spain

- **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Vicente-Díez, I., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., Steinernema feltiae *scavenging behavior: offspring fitness is modulated by various insect cadaver scenarios.*

  Poster presentation
  Honorable Mention in the Best Poster Presentation Contest

- Campos-Herrera, R., **Blanco-Pérez, R.**, Duncan, L.W., *Drivers of assemblages of entomopathogenic nematodes and other soil organisms from the same habitats on two continents: singularities or general trends?*

  Oral presentation

- Chelkha, M., **Blanco-Pérez, R.**, Bueno-Pallero, F.A., El Harti, A., Amghar, S., Campos-Herrera, R., *The presence of cutaneous excreta of earthworms and their feeding activity can decrease the biological control action by entomopathogenic nematodes and entomopathogenic fungi.*

  Poster presentation

11th Spider Mite Genome Meeting
November 4-6th 2019
Universidad de La Rioja and Western University
Logroño, Spain

- Campos-Herrera, R., Vicente-Díez, I., Moreira do Nascimento, J., Chelkha, M., **Blanco-Pérez, R.**, El Harti, A., Grbic, V., *Novel strategies for the management of* Tetranychus urticae *using beneficial soil organisms: direct and indirect measures concept.*

  Oral presentation

Virtual SON Conference
December 15-16th 2020
Society of Nematologists (SON)
USA (virtual)

- Vicente-Díez, I., **Blanco-Pérez, R.**, Chelkha, M., González-Trujillo, M.M., Pou, A., Campos-Herrera, R., *Enhancing organic viticulture: insecticidal effect of entomopathogenic nematodes and the cell-free supernatant from* Xenorhabdus *and* Photorhabdus *bacteria against* Philaenus spumarius *(Hemiptera: AphrophoProteobacteria: Xanthomonadaceae).*

Poster presentation

- Chelkha, M., **Blanco-Pérez, R.**, Vicente-Díez, I., González-Trujillo, M.M., Amghar, S., El Harti, A., Campos-Herrera, R., *Unraveling earthworm impact over entomopathogenic nematode infectivity: general trend or species-specific dependent?*

  Poster presentation

ESA International Branch and Virtual Symposium
April 26-28th 2021
Entomology Society of America (ESA)
USA (virtual)

- **Blanco-Pérez, R.**, Vicente-Díez, I., Marco-Mancebón, V.S., Pérez-Moreno, I., Pou, A., Campos-Herrera, R., *Impact of mulching on the activity of entomopathogenic nematode community in DOCa Rioja vineyards (Spain)*.

  Poster presentation

53nd SIP Annual Meeting
June 26th – July 2nd 2021
Society for Invertebrate Pathology (SIP)
France - México (virtual)

- **Blanco-Pérez, R.**, Vicente-Díez, I., Ramos de Ojer, J.L., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., *Impact of differentiated vineyard management on the activity of entomopathogenic nematodes in La Rioja (Spain)*.

  Poster presentation

- Campos-Herrera, R., **Blanco-Pérez, R.**, *The cost of fighting for surviving in a complex world: entomopathogenic nematodes as scavengers*.

  Oral presentation (invited speaker)

- Vicente-Díez, I., **Blanco-Pérez, R.**, Chelkha, M., Puelles, M., Pou, A., Campos-Herrera, R., Steinernema carpocapsae *and* Xenorhabdus nematophila *based products for the control of the grapevine moth and the grey mold in vineyards*.

  Poster presentation

- Chelkha, M., del Toro Hernández, M., Vicente-Díez, I., **Blanco-Pérez, R.**, Amghar, S., El Harti, A., Pou, A., Campos-Herrera, R., *Unraveling the effect of the presence of earthworms or their cutaneous excreta and entomopathogenic nematodes in the soil bacterial community, biocontrol capacity, and plant traits.*

  Poster presentation

- González-Trujillo, M.M., Čepulyte, R., Vicente-Díez, I., **Blanco-Pérez, R.**, Chelkha, M., Puelles, M., Gámez, A., Ramos-Sáez de Ojer, J.L., Campos-Herrera, R., *Screening of adjuvants to enhance the entomopathogenic nematode survival and adherence after aerial application on grapevine leaves.*

  Poster presentation

7th International Congress of Nematology
May 1-6th 2022
Society of Nematologists
Antibes Juan-Les-Pins, France

- **Blanco-Pérez, R.**, Vicente-Díez, I., Ramos de Ojer, J.L., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., *Impact of differentiated farming practices on the native entomopathogenic nematodes in DOCa Rioja vineyards (Northern Spain)*.

  Poster presentation

- Vicente-Díez, I., **Blanco-Pérez, R.**, Chelkha, Pou, A., Campos-Herrera, R., *Possibilities of using* Xenorhabdus nematophila *and* Photorhabdus laumondii *against Botrytis cinerea*.

  Poster presentation

Presentations at National Conferences

X Congreso Nacional de Entomología Aplicada,
XVI Jornadas Científicas de la SEEA
October 16-20th 2017
Sociedad Española de Entomología Aplicada (SEEA)
Logroño, Spain

- **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Campos-Herrera, R., *Entomopathogenic nematode scavenging and its implications for their long term persistence in soil.*

  Poster presentation

- Campos-Herrera, **Blanco-Pérez, R.**, Bueno-Pallero, F.A., Duarte, A., Nolasco, G., Rodríguez-Martín, J.A., *Entomopathogenic nematode soil food web assemblage in Algarve region: habitat preference and spatial patterns.*

  Oral presentation

- Bueno-Pallero, F.A., **Blanco-Pérez, R.**, Dionísio, L., Campos-Herrera, R., *To kill or to be killed: belowground interaction between soil beneficial organisms, implications in biological control.*

  Oral presentation

XIII Congreso SEAE de Agricultura Ecológica
November 14-17th 2018
Sociedad Española de Agricultura Ecológica (SEAE)
Logroño, Spain

- **Blanco-Pérez, R.**, Sáenz Romo, M.G., Vicente-Díez, I., Ibáñez-Pascual, S., Martínez-Villar, E., Pérez-Moreno, I., Marco-Mancebón, V.S., Campos-Herrera, R., *Impacto de la implementación de cubiertas vegetales en la distribución natural y actividad de los nematodos entomopatógenos.*

  Poster presentation

XI Congreso Nacional de Entomología Aplicada,
XVII Jornadas Científicas de la SEEA
November 4-8th 2019
Sociedad Española de Entomología Aplicada (SEEA)
Madrid, Spain

- Vicente-Díez, I., **Blanco-Pérez, R.**, Moreno, A., Fereres, A., Campos-Herrera, R., *Capacidad infectiva del nematodo entomopatógeno* Steinernema feltiae *(Filipjev) (Rhabditida: Steinernematidae) frente a ninfas de* Philaenus spumarius *(Linnaeus) (Hemiptera: Aphrophoridae), vector de* Xylella fastidiosa *Wells, Raju, Hung, Weisburg, & Beemer (g-Proteobacteria: Xanthomonadaceae) en Europa.*

  Poster presentation

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