



# Organic mulching modulated native populations of entomopathogenic nematode 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 (Flexas 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 (Pina-monti, 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  $\gamma$ -Proteobacteria, killing the arthropod by septicemia (Boemare, 2002; Stock, 2015). Diverse soil communities modulate the efficacy and persistence of EPNs in the agroecosystem through 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 *Acroboloides* 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 Csb 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 ostreatus* (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). Compositated samples, two per plot, comprised 12 single soil cores (2.5 cm  $\times$  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<sup>3</sup> 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

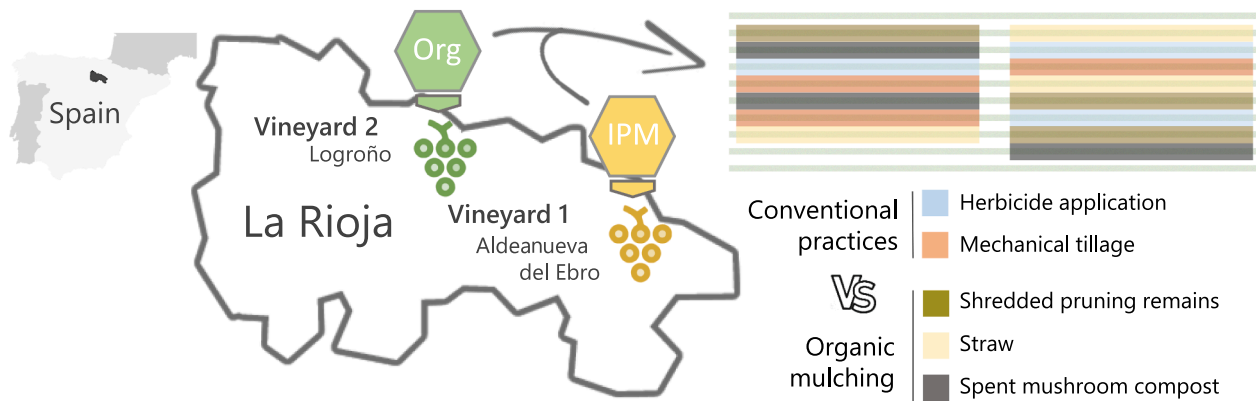


Fig. 1. Location of the experimental vineyards, handled for organic (Org) and integrated pest management (IPM), and experimental design of the evaluated treatments.

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<sub>4</sub>), 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 (nem-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.

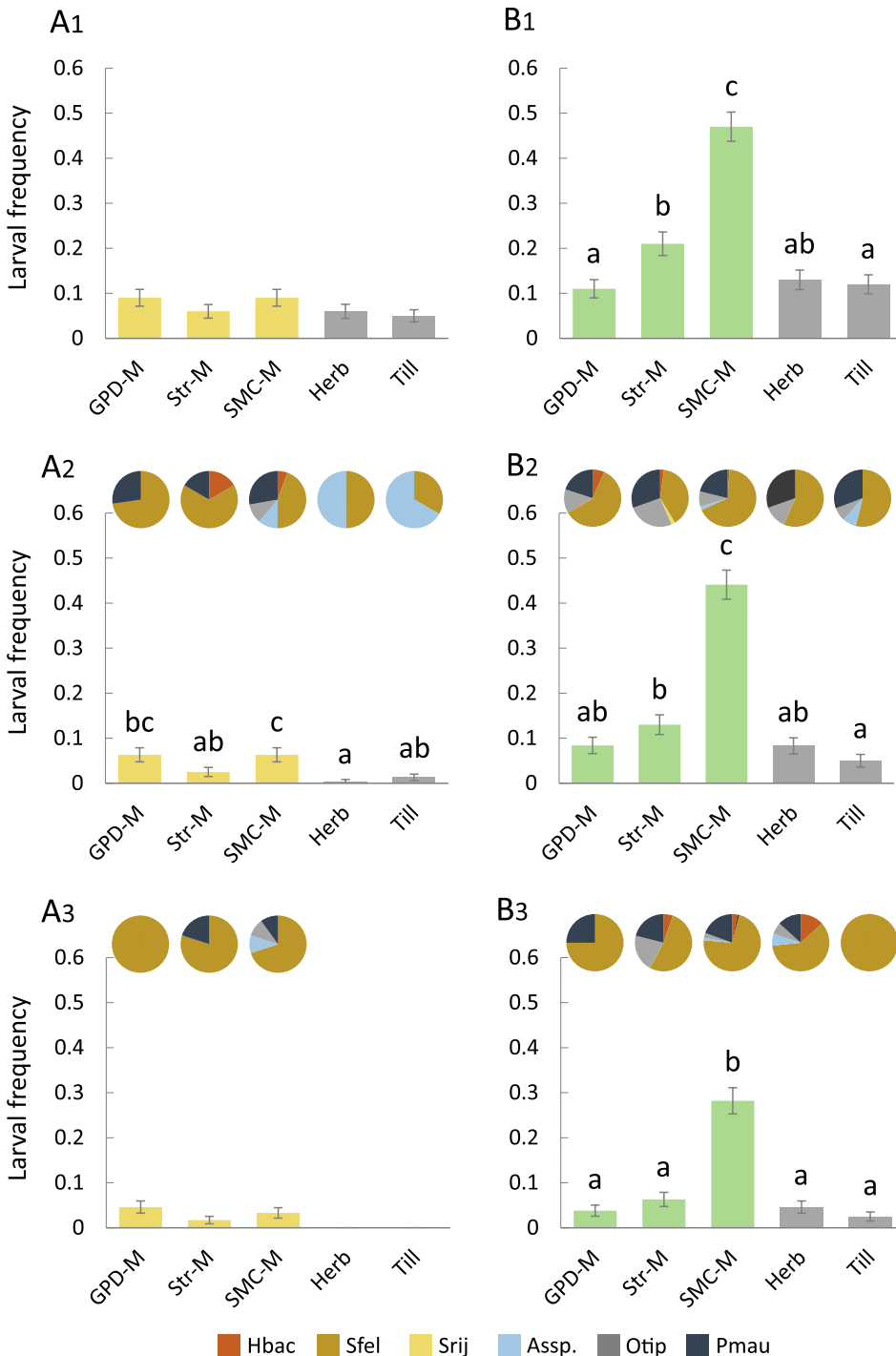
Type of organism / species	Population	GenBank ACNO ITS Region	Reference for primers and probes sequences
Entomopathogenic nematodes			
<i>Heterorhabditis bacteriophora</i>	(commercial)	KJ938576	Campos-Herrera et al. (2011b)
<i>Heterorhabditis indica</i>	Btw	KJ938571	Campos-Herrera et al. (2011a)
<i>Heterorhabditis megidis</i>	(commercial)	KJ938577	Campos-Herrera et al. (2011b)
<i>Steinernema affine</i>	CH	KJ938567	Torr et al. (2007)
<i>Steinernema carpocapsae</i>	DOK-83	KJ818295	Campos-Herrera et al. (2011b)
<i>Steinernema feltiae</i>	RS-5	KJ938569	Campos-Herrera et al. (2011b)
<i>Steinernema intermedium</i>	82	AF171290	Campos-Herrera et al. (2015b)
<i>Steinernema kraussei</i>	OS	KJ696686	Campos-Herrera et al. (2015b)
<i>Steinernema riojaense</i>	RM-30	MK503133	Blanco-Pérez et al. (2020)
<i>Steinernema sp. affine</i> -group	VO-53	MW480137	Blanco-Pérez et al. (2022)
Free-living nematodes			
<i>Acrobeloides</i> -group	RT1-R15C	JQ237849	Campos-Herrera et al. (2012)
<i>Oscheius tipulae</i>	MG68 P29	KJ938579	Campos-Herrera et al. (2015a)
<i>Oscheius onirici</i>	MG67 P20	KJ938578	Campos-Herrera et al. (2015a)
<i>Pristionchus maupasi</i>	AM-3	MG551681	Campos-Herrera et al. (2019)
Nematophagous fungi			
<i>Catenaria sp.</i>	1D	JN585805	Pathak et al. (2012)
<i>Arthrobotrys dactyloides</i>	H55	KJ938574	Pathak et al. (2012)
<i>Arthrobotrys musiformis</i>	11	KJ938572	Pathak et al. (2012)
<i>Arthrobotrys oligospora</i>	8	KJ938573	Pathak et al. (2012)
<i>Hirsutiella rhostiliensis</i>	2931	KM652168	Zhang et al. (2006)
<i>Purpureocillium lilacinum</i>	9357	KJ938575	Atkins et al. (2005)
Ectoparasitic bacteria			
<i>Paenibacillus nematophilus</i>	NEM2	AF480936	Campos-Herrera et al. (2011b)
<i>Paenibacillus sp.</i>	SdTc1FEE1	JF317562	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, ran 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<sub>i</sub>) by the highest (n<sub>max</sub>) according to 100 × n<sub>i</sub>/n<sub>max</sub> (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  $\pm$  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 (Assp.), *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  $\pm$  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<sub>1-3</sub>), but only compared to nem-act in the IPM vineyard except GPD-M (Fig. 2A<sub>2</sub>; 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<sub>1</sub>), nem-act than Till (Fig. 2B<sub>2</sub>), 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<sub>2</sub>). We found nematode emergences for the species *H. bacteriophora* (missed in Koch's postulate tests for IMP),

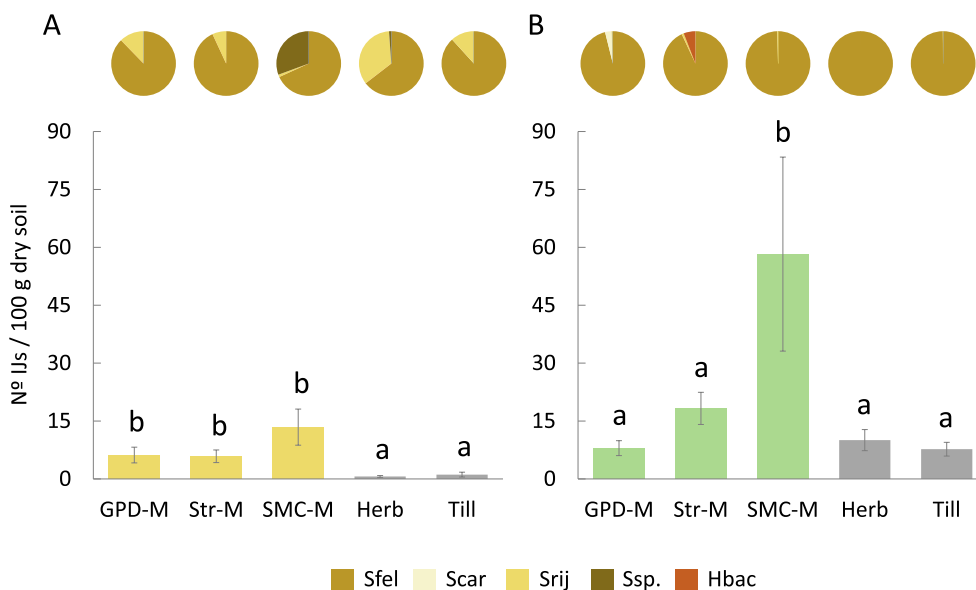
*S. feltiae* (the predominant EPN species), *S. riojaense* (only in a few numbers for Str-M in organic viticulture), *Acrobeloides*-group, *O. tipulae*, and *P. maupasi* (see Fig. S2-S4 for detailed statistics).

#### 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<sub>1</sub>, B<sub>1</sub>) 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<sub>1</sub>, B<sub>1</sub>). 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<sub>2</sub>, B<sub>2</sub>; Fig. S9). The only NF species occurring in all the treatments was *P. lilacinum*, in higher numbers for Herb than Till in IMP, 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  $\pm$  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 ( $x \pm$  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 ng/ $\mu$ l 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										
<i>Heterorhabditis bacteriophora</i>	0	0	0	0	0	0	1.08 $\pm$ 1.01	0	0	0
<i>Steinernema carpocapsae</i>	0	0	0	0	0	0.26 $\pm$ 0.26	0	0	0	0
<i>Steinernema feltiae</i>	5.43 $\pm$ 2.03 <b></b>	5.47 $\pm$ 1.56 <b></b>	9.15 $\pm$ 2.53 <b></b>	0.41 $\pm$ 0.23 <b>a</b>	0.99 $\pm$ 0.63 <b>a</b>	7.57 $\pm$ 1.99 <b>a</b>	17.01 $\pm$ 3.84 <b>a</b>	58.06 $\pm$ 25.16 <b></b>	10.06 $\pm$ 2.74 <b>a</b>	7.70 $\pm$ 1.78 <b>a</b>
<i>Steinernema riojaense</i>	0.76 $\pm$ 0.67	0.41 $\pm$ 0.28	0.16 $\pm$ 0.13	0.22 $\pm$ 0.13	0.13 $\pm$ 0.13	0.05 $\pm$ 0.05	1.20 $\pm$ 1.20	1.19 $\pm$ 1.14	0	0.01 $\pm$ 0.01
<i>Steinernema</i> sp. <i>affine</i> -group	0	0	4.11 $\pm$ 4.11	0.01 $\pm$ 0.01	0	0	0	0	0	0
FLN species										
<i>Acrobeloides</i> -group	1.5E-05 $\pm$ 4.6E-06 <b>bc</b>	7.9E-06 $\pm$ 2.3E-06 <b>ab</b>	2.2E-05 $\pm$ 2.5E-06 <b>c</b>	4.1E-06 $\pm$ 6.7E-07 <b>a</b>	4.9E-06 $\pm$ 1.1E-06 <b>ab</b>	1.5E-05 $\pm$ 2.0E-06 <b>c</b>	3.8E-06 $\pm$ 9.7E-07 <b>a</b>	3.2E-05 $\pm$ 3.1E-06 <b>c</b>	7.8E-06 $\pm$ 1.2E-06 <b>b</b>	5.1E-05 $\pm$ 1.0E-06 <b>ab</b>
<i>Oscheius onirici</i>	0	0	0	0	0	0	9.4E-02 $\pm$ 8.7E-02	0	0	0
<i>Oscheius tipulae</i>	0	5.7E-05 $\pm$ 5.7E-05	0	0	7.5E-06 $\pm$ 7.5E-06	8.9E-03 $\pm$ 8.3E-03	7.5E-03 $\pm$ 3.3E-03	0	8.4E-05 $\pm$ 8.4E-05	0
<i>Pristionchus maupasi</i>	0	2.4E-04 $\pm$ 2.4E-04	3.0E-05 $\pm$ 3.0E-05	7.2E-03 $\pm$ 7.2E-03	4.9E-03 $\pm$ 4.9E-03	2.0E-01 $\pm$ 1.0E-01	4.4E-01 $\pm$ 3.1E-01	1.8E-01 $\pm$ 1.0E-01	1.3E-01 $\pm$ 4.3E-02	5.4E-02 $\pm$ 1.6E-02
NF species										
<i>Arthrobotrys dactyloides</i>	0	3.3E-06 $\pm$ 3.3E-06	4.2E-05 $\pm$ 4.2E-05	0	0	2.9E-04 $\pm$ 2.3E-04	1.3E-04 $\pm$ 1.2E-04	0	0	0
<i>Arthrobotrys oligospora</i>	0	1.9E-03 $\pm$ 7.9E-04	0	0	4.0E-05 $\pm$ 4.0E-05	0	3.3E-03 $\pm$ 9.0E-04	6.6E-06 $\pm$ 6.6E-06	0	0
<i>Hirsutiella rhossiliensis</i>	0	0	0	0	0	0	2.0E-02 $\pm$ 1.3E-02	0	2.1E-02 $\pm$ 1.9E-02	0
<i>Purpureocillium lilacinum</i>	2.2E-04 $\pm$ 7.0E-05 <b>ab</b>	2.2E-03 $\pm$ 1.1E-03 <b>ab</b>	1.5E-04 $\pm$ 6.5E-05 <b>ab</b>	3.1E-03 $\pm$ 1.9E-03 <b></b>	8.3E-05 $\pm$ 3.5E-05 <b>a</b>	1.1E-03 $\pm$ 2.4E-04 <b></b>	1.3E-03 $\pm$ 3.0E-04 <b>ab</b>	5.8E-04 $\pm$ 1.0E-04 <b>ab</b>	6.2E-04 $\pm$ 1.1E-04 <b>ab</b>	5.0E-04 $\pm$ 8.3E-05 <b>a</b>

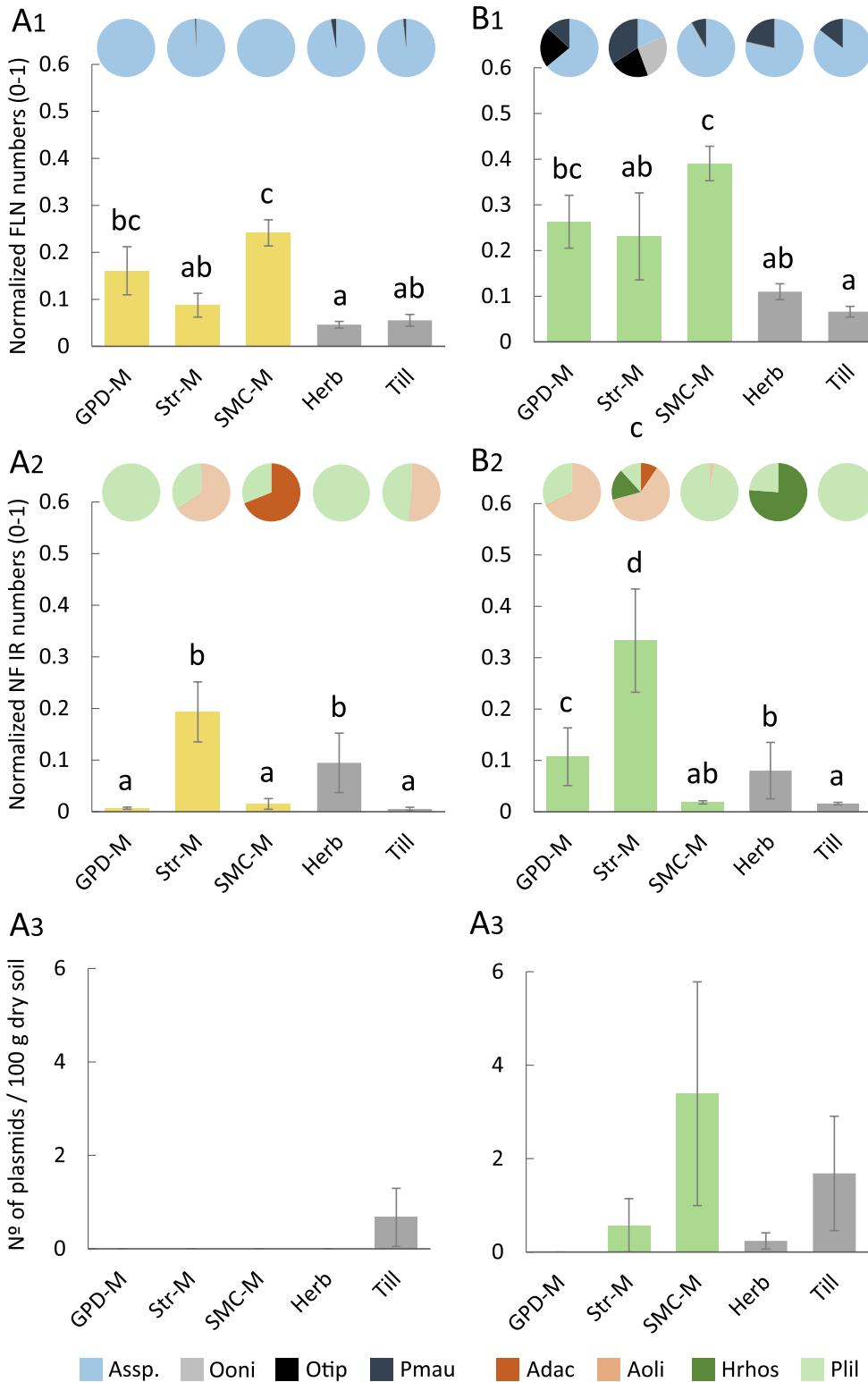
species identified were *A. dactyloides* (for organic mulches only), *A. oligospora*, and *H. rhossiliensis* (in organic farming only), without significant differences among treatments (Fig. 4A<sub>2</sub>,B<sub>2</sub>). Finally, we recorded low abundance for the EcPB *Paenibacillus* sp. (missed in IMP except for tilled soils) and no significant differences among treatments (Fig. 4A<sub>3</sub>,B<sub>3</sub>).

#### 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, preying 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  $\pm$  SE. Averages of the species identified are represented in pies: the FLNs *Acrobeloides*-group (Aspp.), *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). Moistened 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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