**Impact of vineyard ground cover management on the occurrence and activity of
entomopathogenic nematodes and associated soil organisms**

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17 **Abstract**

18 Viticulture is a valuable sector worldwide with an extraordinary socio-economic impact in
19 Spain. Numerous pests and diseases threaten vineyards, and their management primarily relies on
20 the use of conventional agrochemicals. The current paradigm of sustainability pursues the
21 implementation of ecologically sound strategies in vineyard ecosystems. The use of cover crops
22 is arising as an alternative with numerous benefits, including favoring above-belowground
23 biodiversity and the presence of beneficial soil organisms such as the entomopathogenic
24 nematodes (EPNs). We hypothesized that the use of specific cover crops in vineyards might
25 enhance the natural occurrence and activity of EPNs by modulating the assemblage with
26 associated organisms. We performed the experiments in an ongoing experimental vineyard (*Vitis*
27 *vinifera* var Tempranillo, clon RJ-26, rootstock ‘110-Richter’) located in Logroño (Spain), drove
28 with different soil management systems (three replicates each): conventional tillage practice and
29 the cover crops (i) seeded with *Bromus catharticus* (Poaceae), (ii) flower-driven, and (iii)
30 spontaneous. We took four soil composite samples per plot (n=48 per sampling time) late spring
31 and early autumn in two consecutive years (2017 and 2018). By using species-specific
32 primers/probes qPCR sets, we screened for the presence and abundance of eight EPNs species
33 and 12 related soil organisms: six nematophagous fungi, four free-living nematodes, and two
34 ectoparasitic bacteria. Additionally, we assessed the EPN activity by the traditional insect-bait
35 method. Overall, we recorded higher EPN numbers or activity rates on cover crops than on bare
36 soils. However, some of the results were divergent among no-till treatments. We observed not
37 only higher EPN abundance and activity on spontaneous covers but lower numbers of antagonistic
38 organisms, particularly endoparasitic nematophagous fungi. Thus, according to our results, the
39 use of spontaneous covers could be the most promising strategy to support the conservation
40 biological control service provided by the naturally occurring EPN species in vineyards, plus with
41 a low cost for the sector.

42

43 Keywords: Agroecology; beneficial soil organisms; cover crop; free-living nematodes;
44 nematophagous fungi; sustainable viticulture; tillage.

45 **1. Introduction**

46

47 The wine sector represents a relevant socioeconomic asset worldwide. Spain is among the
48 top five grape-producing countries (FAOSTAT, 2018), has the largest area of vineyards in the
49 world (almost one million hectares), and with France and Italy, is the world leader for wine
50 exports (OIV, 2018). Since conventional viticulture production practices, such as the widespread
51 use of agrochemicals and soil tillage, make the vineyard one of the most intensive management
52 systems (Nicholls et al., 2008), defining sustainable strategies is critical for the sector within the
53 current context of environmental protection. Indeed, areas devoted to organic grape production
54 significantly increased during the last two decades, particularly in Spain, Italy (each with more
55 than 100'000 hectares) and France (with over 78'000 hectares), the countries that cover the largest
56 organic grape areas (FiBL, 2019; Provost and Pedneault, 2016).

57 Sustainable viticulture considers the vineyard as an ecosystem where every resource is
58 optimized to maintain rich biodiversity and decrease pest and disease pressures (Thies and
59 Tschardtke, 1999). Consequently, viticulture must face the current challenge of reducing
60 agrochemicals by providing alternative management strategies that respect the environment.
61 When sources like water are not limited, the use of cover crops in vineyards is arising as an
62 alternative with numerous benefits, including reducing soil erosion, providing additional organic
63 matter, improving soil structure, and favoring above-belowground biodiversity (Coll et al., 2011;
64 McGourty and Reganold, 2005; Sáenz-Romo et al., 2019a; Shapira et al., 2017; Wheaton et al.,
65 2008). Continuous no-tillage practices stratify the soil, concentrating organic matter, nutrients,
66 and microbial activity near the soil surface (Doran, 1980). Thus, crop residue decomposition
67 occurs mostly through the activity of a diverse soil biota community in which complex
68 interactions are established (House and Stinner, 1983). Conversely, organic matter, nutrients, and
69 biodiversity decrease in conventionally tilled soils because decomposition and mineralization
70 process occurs faster and over a deeper area of the soil, involving fewer types of organisms,
71 primarily microbiota (House and Stinner, 1983).

72 Favored by high biodiversity rates, conservation biological control plays a key role in

integrated and organic pest management programs (Altieri, 1999; Altieri and Nicholls, 2004). Among the soil organisms responsible for ecological goods and services, the entomopathogenic nematodes (EPNs) are well recognized as biological control agents and excellent models in ecological studies (Campos-Herrera et al., 2012; Lewis et al., 2015; Stuart et al., 2015). Nematodes in the families Steinernematidae and Heterorhabditidae show pathogenicity against arthropods in symbiosis with enteric γ -Proteobacteria species in the genera *Xenorhabdus* and *Photorhabdus*, respectively (Boemare, 2002). EPNs occur in soils of natural and agricultural areas worldwide in their non-feeding stage, called infective juvenile (IJ), capable of locating suitable hosts (Stock, 2015). Once inside, the symbiotic nematode-bacterium complex overcomes the host's immune response resulting in death by septicemia within 48–72 hours of infection (Dillman et al., 2012). But first, IJs must cope with soil and environmental conditions (i.e. limited water content, extreme temperatures, etc.), overcome competitors for hosts and avoid predators and other soil organisms that could compromise their reproductive success (Lewis et al., 2015; Stuart et al., 2015). Regarding specific biotic interactions, several surveys have revealed, through traditional and molecular methods, the co-emergence of IJs and free-living nematodes (FLNs) naturally occurring in soils (Liang et al., 2005) from insect larvae colonized by EPNs (Campos-Herrera et al., 2012; 2015; 2019; Duncan et al., 2003; Jaffuel et al., 2016; 2018). Aside from competitive interactions that could occur between these two groups of nematodes, recent *in vitro* experiments suggest that IJ pathogenicity could decrease when competing with FLNs for nutrient sources inside the insect cadaver (Blanco-Pérez et al., 2019). On the other hand, nematophagous fungi (NF), found in all major fungal taxonomic groups, are also widespread in natural and agricultural soils, where mainly survive as saprophytes until they switch to the parasitic stage in the presence of nematodes, developing traps or mature spores (Nordbring-Hertz et al., 2006). Thus, NF are natural enemies of EPNs, but the mechanisms behind their interactions are not well known except that EPN susceptibility to NF seems to be environmental and species-specific dependent (Bueno-Pallero et al., 2018; El-Borai et al., 2009; Koppenhöfer et al., 1996). Finally, other soil organisms such as ectoparasitic bacteria (EcPB), can alter the fitness of some EPN species by reducing their motility and pathogenicity (El-Borai et al., 2005; Enright and Griffin,

101 2005).

102 Due to their notable value as biological control agents against a wide range of soil-borne
103 insect pests, there is increasing interest in identifying the factors that define EPN population
104 dynamics in agroecosystems (Griffin, 2015; Lewis et al., 2015). Numerous studies link the use of
105 cover crops and the nematode community with beneficial actions, for example, to evaluate soil
106 health by using soil nematodes as bio-indicators (Bongers and Ferris, 1999; DuPont et al., 2009;
107 Ferris et al., 2012; Ito et al., 2015), or to enhance the control of plant-parasitic nematodes
108 (Hiltpold, 2015). In this regard, there is evidence of high EPN occurrence and activity in perennial
109 crops, including vineyards, would be linked to soil health (Campos-Herrera et al., 2008; 2014).
110 However, few studies address the relationship of cover crops presence with EPN occurrence.
111 Susurluk and Ehlers (2008) suggested that no-till ground cover managements increase the
112 persistence of the EPN species *Heterorhabditis bacteriophora* by supporting alternative hosts,
113 improving soil health conditions, and reducing temperature fluctuations. However, recent studies
114 advise that case-by-case investigations might be necessary to confirm a positive impact of cover
115 crops on EPN populations. For example, black oat covers favored EPN activity in corn crops
116 compared to conventional tillage practice, while the implementation of oil radish covers only
117 implied higher records of heterorhabditid IJs without affecting the global EPN activity (Marquez,
118 2017). Similarly, for EPN augmentation experiments in winter wheat crops, *Steinernema feltiae*
119 activity remained significantly higher in pea and mustard covers than in bare soils five months
120 after IJ application but not its abundance. Conversely, no differences were observed when *H.*
121 *bacteriophora* was applied, nor for the abundance of EPN natural enemies and antagonists such
122 as NF and FLN species (Jaffuel et al., 2017).

123 A previous study performed in the same experimental Denominación de Origen Calificada
124 (DOCa) Rioja (<https://www.riojawine.com/es-en/>) vineyard, where we carried the current
125 experiments, concluded that cover crops could promote beneficial above-ground entomofauna,
126 effective in conservation biological control programs (Sáenz-Romo et al., 2019a; 2019b). Our
127 study aimed to explore the impact of the implementation of different cover crops (grass-seeded,
128 flower-driven, and spontaneous) on the presence and activity of EPN populations, and to identify

the biotic and abiotic factors that could modulate them under those different ecological scenarios. We speculated that the use of cover crops might enhance the abundance and the activity of naturally occurring EPN species compared to conventional tillage practice. Thus, the main objectives of this study were (i) to quantify EPN abundance and activity, and (ii) to quantify the abundance of target soil organisms associated with EPNs, like natural enemies and potential competitors or antagonisms (NF, FLNs, and EcPB), on soils managed with different practices.

2. Material and Methods

2.1. Field experiment characteristics and design, sampling method, and soil properties analyses

The EPN activity and natural occurrence was evaluated for two consecutive years (2017 and 2018) on *Vitis vinifera* var Tempranillo (clon RJ-26, rootstock '110Richter'), in an experimental vineyard located in La Grajera (Logroño, Spain, 42°26'N, 2°30'W, 455 masl, ~8,5% slope), and belonging to the Government of La Rioja. The vineyard training systems were bush (goblet) and double Guyot (trunk height ~40 cm, 1.15 m between vines / 2.90 m between rows), set in a plot characterized by loam and sandy-loam soils with low content of soil organic matter (0.88% on average in 2016; data reported by Sáenz-Romo et al., 2019a). The region is located in a warm-summer Mediterranean climate (classified as Csb by the Köppen-Geiger system), with continental influence, characterized by average annual data of 13.9 °C, 67% relative humidity, and 405 mm of total precipitations (Spanish Meteorological Agency, AEMET; years 1981-2010). Monthly climatological data for the two sampling years were recorded (Agro-climatic Information Service in La Rioja, SIAR; Supplementary data 1, Table S1).

The experiment compared the use of three different cover crops (seeded with *Bromus catharticus* (Poaceae), flower-driven, and spontaneous) versus a regular tillage practice. Settled in 1995, the vineyard was fully managed by tilling until the cover crops were implemented in 2016. The selected covers were selected to study the impact of the use of different cover crops on the abundance and biodiversity of above-ground insect communities (Sáenz-Romo et al., 2019a). The grass/graminaceous *Bromus catharticus* Vahl cv. Samson was chosen for grass-seeded

covers, a species adapted to drought and the basic pH levels registered in the experimental vineyard, but also easy to implement and develop due to its high self-seeding capacity (Ibáñez-Pascual, 2014). After removing spontaneous vegetation by using an agriculture rotary tiller (AGRIC® GLF-70-BM, 5–10 cm depth), *B. catharticus* was mechanically seeded 1-2 cm depth with a mechanical seed drill (AGRIC® KSA/HER-70) twice a year (early autumn and late winter). The flower-driven covers were manually seeded once a year (early spring, 20 kg/ha) without any previous inter-row management to disturb as little as possible soil structure. The seed commercial mixture (Deco Vignes Anuelles, Nova Flore) was selected for its spread over time/gradually flowering. The spontaneous covers were designated as areas without any tillage or seeds application, allowing the natural bank of seeds to growth. The weeds community composition of flower-driven and spontaneous covers were estimated in mid-May of each year (see Sáenz-Romo et al., 2019a). No herbicides were applied during conducting the study, but acaricides and fungicides to control *Eotetranychus carpini* (Acari: Tetranychidae), and fungal diseases such as powdery (Oidium) and downy mildew, respectively (see Sáenz-Romo et al., 2019b for further information). The insect pest *Lobesia botrana* (Lepidoptera: Tortricidae) was controlled by a mating disruption technique applied throughout the sampling area (Palacios Ruiz et al., 1995). Sáenz-Romo et al. (2019a) described more details of the experimental design, treatments and aboveground fauna.

Treatments were placed on the experimental vineyard following a completely randomized design, three plots of 1200-2100 m² (~360 vine stocks) per treatment (Fig. 1). To ensure balanced spatial distribution, we divided each plot in two areas and we collected two samples in each subarea (one pair's row / inter-row per subarea). The samples were taken in the middle rows to avoid as much as possible the possible border effect. Therefore, we obtain four samples per plot and a total of 48 samples per sampling time (early autumn and late winter 2017 and 2018). Each sample was composed of 12 single soil cores (2.5 cm ø x 20 cm DP., ca. ~1200 cm³), pooled in individual plastic bags. The samples were stored at 4 °C in the dark until processed (within 2–4 days). In the laboratory, each sample was manually homogenized and divided in three soil subsamples of 200 g (fresh weight). Following the protocol described by Campos-Herrera et al.

185 (2019), two of the subsamples were used (i) to characterize EPNs and associated organisms
186 through sucrose-gradient centrifugation technique and qPCR identification, and (ii) to determine
187 the soil suppressive capacity by using the traditional insect-bait method (see detailed descriptions
188 below). To express the number of organisms identified per 100 g of dry soil, the third set of
189 aliquots was dried at 40 °C for one week to measure the water content. Thereafter, 200 g of oven-
190 dried soil, result of combining the samples from each subareas (n = 24 per sampling time), were
191 used for the analyses of soil properties: pH (Millennia and Markewitz, 2004), electric
192 conductivity, organic matter (Walkley and Black, 1934), and micro-nutrient elements (P, K, Mg,
193 Ca, Zn, Mn, Fe, and Cu) (Mehlich 3 acid extraction/ICAP for elements; Mehlich, 1978; 1984)
194 (Supplementary data 1, Table S2). The soil texture (sand, silt and clay percentages) (Bouyoucos,
195 1936) was analyzed only once (Supplementary data 1, Table S3). All soil analyses were
196 performed by Laboratorio Regional del Gobierno de La Rioja, La Grajera (Logroño, Spain).

197

198 2.2. Characterization of entomopathogenic nematodes and associated organisms, and estimation 199 of soil activities against insect larvae

200 Following Campos-Herrera et al. (2019), nematodes and other organisms were co-extracted
201 from 200 g fresh soil samples (Wiesel et al., 2015) by sucrose-gradient centrifugation (Jenkins,
202 1964), retrieving the nematode community in 50 ml Falcon tubes (Becton Dickinson Labware,
203 USA). After allowed all the organisms be placed in the bottom of the tubes (4 °C overnight), each
204 bottom content (organisms and some rest of soil) was transferred to 1.5 ml Eppendorf tubes by
205 successive centrifuge-evacuation steps. The final concentrated material was stored at -20 °C until
206 DNA extraction procedures.

207 Following the protocol described by Bedding and Akhurst (1975) with few modifications
208 (see Campos-Herrera et al., 2019), each sample of the second set of 200 g fresh soil was baited
209 with 20 final instar of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae (reared at ICSVV) in
210 two independent rounds (n = 10 larvae per round) to allow nematodes to become active (Griffin,
211 2015). After four days of exposure (22–24 °C in the dark), larval mortality was assessed and dead

larvae recovered, rinsed with tap water, and individually placed in White traps (White, 1927). Live larvae were additionally incubated for 24 h to record possible late mortality. The percentage of dead larvae marked the total suppressive capacity of the soil for each sample (hereafter Total-act). Dead larvae were checked every 2–3 days under the stereoscope for about a month to verify nematode occurrence. The percentage of larvae that recorded any nematode emergency indicated the soil activity associated with nematodes (hereafter Nem-act). Emerging nematodes were recovered in tap water (hereafter RO aliquots) and stored (14 °C in the dark). Their entomopathogenic activity was tested by performing the Koch's postulates (Stock and Goodrich-Blair, 1997). Briefly, two Petri dishes lined with filter paper and three *G. mellonella* larvae were inoculated with 0.5 ml of RO aliquots. This procedure was repeated when not nematode progeny was reported to avoid false negatives. The emerging nematodes were recovered in tap water (hereafter RM aliquots) and stored at 14 °C in the dark. The percentage of larvae that recorded positive Koch's postulates indicated the soil entomopathogenic activity (hereafter EPN-act). Besides, 1 ml of concentrated nematode suspension of both RO and RM aliquots was saved (-20 °C) for later analysis. Besides, we recovered and maintained (Woodring and Kaya, 1988) some of the emerging nematodes to establish laboratory cultures.

228

229 *2.3. Identification and quantification of entomopathogenic nematodes and associated organisms* 230 *by real time qPCR*

Before DNA extraction, all samples were mechanically disaggregated (15 sec) by using sterile blue pestles assembled to a pellet mixer (VWR International, UK). Then, we used the PowerSoilR DNA Isolation Kit (MOBIO Laboratories, Inc, Carlsbad, CA, USA) for the aliquots obtained by sucrose-gradient centrifugation, and the Speedtools tissue DNA extraction kit (Biotools, Spain) for the RO/RM aliquots. Thereafter, DNA extractions were analyzed for quality and quantity using 1 µl per duplicate in a Nanodrop system (Thermo Scientific 2000C spectrophotometer) and then stored at -20 °C until qPCR analysis.

We screened for the occurrence and abundance of 20 soil organism species by using species-specific primers/probe (Table 1), comprising 8 EPNs, 4 FLNs, 6 NF and 2 EcPB.

Specifically, soil baits in La Rioja have previously reported three of the EPN species under study: *Steinernema carpocapsae*, *S. feltiae*, and *S. kraussei* (Campos-Herrera et al., 2007; 2008). The EPN species *Steinernema affine*, *S. arenarium*-like, *Heterorhabditis indica*, and *H. bacteriophora* have been also reported in the Iberian Peninsula (Campos-Herrera et al., 2016; 2019; Doucet and Gabarra, 1994; García del Pino and Palomo, 1996; Valadas et al., 2014). We made an additional screening for a new EPN species, *Steinernema riojaense* (Půža et al., 2020), identified from aliquots of the insect-baits conducted in this study, after designing a species-specific primers/probe set (Supplementary material 2). The FLNs screened in this study, *Oscheius tipulae*, *O. onirici*, *Pristionchus maupasi*, and *Acrobelloides* spp. (Campos-Herrera et al., 2012; 2015), were also previously recorded in the Iberian Peninsula, specifically in the Algarve Region, in South Portugal (Campos-Herrera et al., 2019). Moreover, other nematode species (15 EPNs and 9 FLNs) were included in this study to validate the qPCR tools, avoiding cross-amplification and adapting previously published protocols to our experimental conditions (Supplementary data 2, Tables S4 and S5). We maintained pure cultures of most nematode species, EPNs reproduced in *G. mellonella* larvae (Woodring and Kaya, 1988), and FLNs in 1% nutrient agar (NA; Difco, MD, USA) feeding on associated bacteria (R.J. Sommer and R. Campos-Herrera reference laboratory populations). A plasmid containing the complete published ITS region was used when living material was not available (Supplementary data 2). EPN and FLN populations were maintained in tap water and Buffer M9, respectively, and stored at 14 °C in dark conditions.

We screened for fungal species belonging to the three main groups of NF (Nordbring-Hertz et al., 2006): nematode-trapping fungi (*Arthrobotrys dactyloides*, *A. musiformis*, and *A. oligospora*), endoparasites (*Hirsutella rhossiliensis* and *Catenaria* sp.), and eggs-parasitic fungi (*Purpureocillium lilacinum*). These species were also previously recorded in Iberian Peninsula (Campos-Herrera et al., 2016; 2019). We maintained pure cultures of all fungi in Corn Meal Agar (Fluka Analytical, Sigma-Aldrich, CO, USA) (Pathak et al., 2012) except *Catenaria* sp., for which we used as positive control/standard curve a plasmid containing the complete published ITS region (Table 1). Finally, we screened for two bacterial species in the genus *Paenibacillus*: *P. nematophilus* and *Paenibacillus* sp. (Campos-Herrera et al., 2011a; 2019). Because no living

268 material was available, we used a plasmid containing a fragment of the 16S rDNA region (Table
269 1).

270 Biosearch Technologies (supplied by Biotools, Spain) synthesized species-specific primers
271 and TaqMan® probes. The probes were labeled at the 5' end with the fluorogenic reporter dye
272 FAM and the 3' end with the quencher BQH-1. We established positive controls by the
273 combination of three independent DNA extractions, using Power SoilR DNA Isolation Kit for
274 each pure culture or QIAprep Spin Miniprep for plasmid. In all the preliminary checks and
275 optimization protocols, DNA concentration was standardized to correspond to 30 IJs (for EPNs),
276 1 ng/μl of pure culture (for FLNs and NF), and 0.1 ng/μl when plasmids were used
277 (Supplementary material 2). For the quantifications, the standard curves were obtained by 10-fold
278 serial dilutions (n = 5 points), starting with the highest concentration employed in the pre-
279 screenings. Real-time qPCR tests and quantifications were performed with the Quantimix Easy
280 Probe mix (Biotools, Spain) in Applied Biosystems® MicroAmp™ Optical 96-Well reaction
281 plates on the Applied Biosystems® 7500 Real-Time PCR System. We run 38 cycles for
282 nematodes species and 50 cycles for NF and EcPB species at 60° C annealing temperature (Table
283 1; see Supplementary material 2 for more details). The DNA samples obtained by sucrose-
284 gradient centrifugation were adjusted to 10 ng/μl before qPCR tests, employing a correction factor
285 to transform qPCR data to original quantities per sample. Similarly, the quantification of the DNA
286 samples obtained from the RO/RM aliquots was estimated by using a dilution between 0.5 and 1
287 ng/μl, recording the threshold cycle (Ct) values for estimation of the relative abundance of
288 nematodes emerged per insect larva.

289

290 *2.4. Statistical analyses*

291 We ran generalized linear mixed models (GLMMs) testing the effect of the use of cover
292 crops pair-treatment compared with regular tillage (fixed factor) on numbers and frequencies of
293 occurrence of EPNs, FLNs, NF, EcPB, and soil activity measures, performed with SPSS 25.0
294 (SPSS Statistics, SPSS Inc., Chicago, IL, USA). Sampling area into plots (pair's rows / inter-
295 rows) was included as subjects of the models since no significant differences for any of the

variables under study were observed (data not shown). In each model, we also accounted for yearly and seasonally repeated measures as random factors.

We adjusted all organism quantifications from DNA samples obtained by sucrose-gradient centrifugation to express the corresponding quantities per 100 g of dry soil. Thus, EPN and EcPB abundances were expressed as number of IJs and copies of plasmids per 100 g of dry soil, respectively. Numbers of FLNs and NF were standardized to a 0–1 range by dividing all values recorded for a particular species (n_i) by the largest number ($n_{\max}$) of that species, according to $100 * n_i/n_{\max}$ (Jongman et al., 1995). By this standardization, the data set is liberated from variation caused by the intrinsic differences such as large variations recorded for the copy numbers between the FLN and NF species analyzed. Since many NF species can survive in soil saprophytically (Nordbring-Hertz et al., 2006), we can considerate that most of the NF species isolated by sucrose-gradient centrifugation were in the infectious phase. Thus, NF numbers were 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., 2012). Finally, we evaluated the soil activities and nematode occurrence in the RO/RM aliquots as larval percentages recorded in insect-baits.

Prior statistical analysis, variables expressed as a percentage and quantitative variables were arcsine and $\log(x+1)$ transformed, respectively. For the quantification of soil organisms and soil activities, we used a GLMM with a gamma distribution (log-link function), and for the frequencies of occurrence a binomial distribution (logit-link function). Besides, we looked for significant differences among treatments in the abiotic soil parameters analyzed. For the soil texture (sand, silt and clay percentages), we run one-way ANOVA and Tukey's HSD test SPSS 25.0 (Table S3), and for the rest of soil properties, we used a linear mixed models (LMM) in SPSS 25.0 within pair-treatment comparisons of soil tillage management and each cover crop treatment (Fig. 2; Table S2). We initially included as covariates in the GLMM all soil parameters for which we recorded significant differences, and then we removed all predictors that were not significant from those statistical models. Thus, for the pair-treatment comparisons between tillage and grass-seeded cover crop treatments, we included soil organic matter to test for differences of total NF numbers. We used least square means $\pm$ SEM as descriptive statistics.

324

325 **3. Results**

326

327 *3.1. Soil properties analysis*

328 The experimental field was homogeneous for most of the soil properties (Supplementary
329 data 1, Tables S1 and S2), except for few variables in specific treatments when compared with
330 the tillage management. In the case of the grass-seeded cover, only soil organic matter (Fig. 2)
331 and Mg content (Table S1) resulted higher than in the tillage plots. In the flower-driven cover, the
332 water content and electro conductivity (Table S2) registered lower values than in the tillage
333 treatment. Finally, the spontaneous cover only had a significantly superior value of Zn content
334 than the control (Table S2). Among all these variables, only the soil organic matter (Fig. 2) were
335 included in the NF presence pair-treatment comparisons between tillage and grass-seeded cover
336 crop treatments.

337

338 *3.2. Numbers and frequency of occurrence of the soil organisms identified by sucrose-gradient* 339 *centrifugation*

340 The total IJ numbers on grass-seeded and spontaneous cover crops were significantly
341 higher than recorded on tillage management (Fig. 3A; Supplementary data 3, Table S6). However,
342 no differences for flower-driven cover crops were observed, nor for the frequency of EPN
343 occurrence among treatments (Fig. 3B). We detected five out of the eight EPN species screened:
344 the heterorhabditids *H. bacteriophora* and *H. indica*, and the steinernematids *S. feltiae*, *S. affine*,
345 and the new species *S. riojaense* (Fig. 3; Table 2). The only EPN species detected for all
346 treatments were *H. indica* and *S. feltiae*. While no difference was observed for *H. indica* among
347 treatments, compared to tilled soils *S. feltiae* was found in lower numbers on grass-seeded and
348 spontaneous covers, and higher numbers on flower-driven covers (Table 2). However, the
349 frequency of *S. feltiae* occurrence was similar for all treatments except grass-seeded covers, that
350 it was significantly lower than tillage (Table 2). The presence of *H. bacteriophora* explained the
351 differences for EPN numbers among treatments, higher on grass-seeded and spontaneous covers

352 compared to tilled soils, and absent on flower-driven covers, while no significant differences were
353 observed among treatments for its frequency of occurrence (Table 2). *S. riojaense* was detected on
354 all cover crops but not on tilled soils, and *S. affine* only on grass-seeded covers and in very low
355 numbers (without significant differences among treatments for any of the two species; Table 2).

356 Overall, FLNs were more abundant on cover crops than tilled soils, both in numbers and
357 frequency of occurrence (Fig. 4), but with no significant differences. We detected two out of the
358 four FLNs screened: *P. maupasi* and *Acrobelloides* spp., both present on all treatments but with
359 no significant differences even if proportional *P. maupasi* numbers were higher on tillage and
360 grass-seeded treatments (Fig. 4A; Table 2). The NF IR was only significantly higher on grass-
361 seeded covers than tilled soils, both for the frequency of occurrence and numbers (Fig. 5; Table
362 S6), in the latter case mediated by the higher percentages of soil organic matter found for grass-
363 seeded covers (Fig. 2; Table S6). We detected five out of the six NF species screened: *A.*
364 *oligospora*, *A. dactyloides*, *H. rhossiliensis*, *Catenaria* sp., and *P. lilacinum*. The NF *A.*
365 *oligospora* was the only species identified on all treatments. No records of *A. dactyloides* and *H.*
366 *rhossiliensis* were observed on grass-seeded and flower-driven covers, respectively. *Catenaria*
367 sp. was the less abundant NF, only identified on grass-seeded and flower-driven covers (Fig. 5).
368 However, the only significant difference was recorded for the frequency of occurrence of *P.*
369 *lilacinum*, absent on bare soils (Table 2). Finally, we only detected one out of the two EcPB
370 screened, *Paenibacillus* sp., in higher numbers and frequency of occurrence in cover crops than
371 tilled soils but with no significant differences (Fig. 6).

372

373 3.3. Soil activities recorded in insect-baits

374 Compared to tillage treatment, we recorded significant differences for the EPN-act
375 registered for flower-driven and spontaneous covers (Fig 7A; Table S6), while no differences for
376 the frequencies of occurrence of any soil activity tested (total activity, Nem-Act nor EPN-act)
377 were obtained (Fig 7B; Supplementary material 3, Fig. S1). We identified the same nematode
378 species from the RO/RM aliquots than through sucrose centrifugation technic except for *S.*
379 *carpocapsae*, registered instead of *S. affine*, also in very low numbers. On the contrary, the

nematode species *H. bacteriophora*, *S. feltiae*, *S. riojaense*, and *Acrobeloides* spp. were the nematode species identified for all treatments. However, we did not observe any significant differences for the Nem-act nor EPN-act among treatments for any nematode species (Fig 7; Fig. S1).

4. Discussion

According to our hypothesis, we observed evidence of some positive effects on EPN populations resulting from the use of cover crops, although with reservations. The spontaneous cover was the only no-till treatment that recorded both numbers and activity of EPNs significantly higher than conventional soil management (Fig. 3 and 7). Conversely, grass-seeded and flower-driven covers showed opposite patterns with each other. We found high EPN abundance but low activity on grass-seeded cover crops, while high pathogenicity rates but low EPN numbers characterized flower-driven covers. Compared to other surveys performed by insect baiting in the Iberian Peninsula, we recorded similar frequencies of EPN occurrence (or slightly higher) than registered before in La Rioja and Catalonia regions (Campos-Herrera et al., 2007; García del Pino and Palomo, 1996). However, Valadas et al. (2014) and Campos-Herrera et al. (2019) reported completely different numbers for two surveys conducted in continental Portugal, approximately three times lower (~ 7%) and three times higher (~ 60%), respectively. Regarding the EPN abundance, we recorded lower numbers than Campos-Herrera et al. (2019) did in the Algarve region (South Portugal) using the same methodology, specifically for citrus and oak groves (~ 25 IJs / 100 g of dry soil).

The abundance and frequency of occurrence of all soil organisms evaluated were not affected by the sampling location within the vineyard (row / inter-row), suggesting that attending to the soil microhabitat, the plots comprise a homogeneous set. The prevalent EPN species in Europe *S. feltiae* and *H. bacteriophora* (Hominick, 2002) were the most commonly found in our study. In particular, previously mentioned findings in the Iberian Peninsula reported more evidence of *S. feltiae* activity, in a wide variety of habitats (Campos-Herrera et al., 2007; García

del Pino and Palomo, 1996; Valadas et al., 2014), and more numerous (Campos-Herrera et al., 2019), than other EPN species. Overall, we also found *S. feltiae* in higher frequencies of occurrence, but, except for flower-driven covers, it seems to be replaced by *H. bacteriophora* on cover crop treatments. Bare soils easily suffer abrupt temperature changes that affect various EPN biological processes such as IJ infectiveness, development to adults within the host, and productiveness, and thus, could reduce the persistence of sensitive EPN species during the spring and autumn seasons. According to Grewal et al. (1994), *S. feltiae*, with thermal niche breadths between 8-25°C, tolerates low temperatures much better than *H. bacteriophora*, that establish and reproduce in *G. mellonella* larvae up to 14°C. Hence, the use of cover crops could favor cold-sensitive EPN species as *H. bacteriophora*. The new EPN species identified in this study, *S. riojaense*, was also more abundant on no-till soils, particularly on flower-driven and spontaneous covers, the managements that less disturb the soil structure. However, additional studies to verify the ecological characteristics of this new EPN species are needed. On the other hand, it is worth noting the presence of *H. indica* in our samples, recently identified for the first time in the Iberian Peninsula (Campos-Herrera et al., 2016; 2019), and now reported in a high-latitude region that still within the marginal ecoclimatic suitability for *H. indica* predicted by Kour (2017).

The numbers reported for other organisms linked to EPN soil food web may explain, partially at least, our observations. In particular, spontaneous and grass-seeded covers, similar for the abundance of IJs, remarkably differ for the frequency of *G. mellonella* larvae killed by EPNs after baiting, probably due to the differences in the abundance of EPN antagonist organisms observed between treatments. Indeed, spontaneous covers registered, despite the lack of significant differences, the lowest numbers of NF, FLN, and EcPB species among the no-till treatments, while just the opposite trend characterized grass-seeded covers. It is likely that not only abundance but also the proportions of the identified NF species could be one of the keys to explaining these observations. We reported significantly higher presence of NF-IR on grass-seeded covers, the treatment that recorded the highest content in organic matter and the lowest sand percentages, two of the key factors that favor NF occurrence on soils (Nordbring-Hertz et al., 2006; Pathak et al., 2017). Endoparasitic fungi (*H. rhossilliensis* and *Catenaria* sp.) were well

436 represented on grass-seeded covers and bare soils, but almost absent on spontaneous and flower-
437 driven covers, the treatments with higher evidence of EPN activity, and for which nematode-
438 trapping fungi in the genus *Arthrobotrys* were the predominant NF species. Altogether, the results
439 obtained in this study suggest the possibility that the endoparasitic NF species identified were
440 more efficient in killing EPNs than the trapping NF species. Timper and Kaya (1989) pointed out
441 the protective role of the second-stage cuticle of heterorhabditid IJs (third-stages) against
442 endoparasitic NF by preventing the penetration of adhering fungal spores within the nematode
443 body cavity. Consequently, Timper et al. (1991) confirmed that *H. rhossiliensis* infects
444 ensheathed *H. bacteriophora* IJs less efficiently than steinernematid IJs, a supposal that support
445 our observations of both the large numbers recorded for this EPN species and the low presence
446 of steinernematids on grass-seeded covers. However, *in vitro* experiments reported that two
447 endoparasitic NF species *Catenaria* sp. *Myzocyttium* sp. exposed to ensheathed IJs did not reduce
448 the prevalence of *H. indica* but *H. zealandica* (El-Borai et al., 2009), and propose that the NF
449 efficacy in killing EPNs could be more species-specific dependent. For example, large and highly
450 motile IJs will encounter more *H. rhossiliensis* conidia than less motile nematodes (Timper et al.,
451 1991). Similarly, big nematodes might able to escape more readily from adhesive or constricting
452 hyphal structures than small nematodes will do (El-Borai et al., 2009). In agreement with these
453 observations, we reported higher occurrence and numbers of *S. riojaense*, characterized by large
454 IJs (up to 1000 µm), on flower-driven and spontaneous covers, where trapping-nematode fungi
455 were the predominant NF species, than on grass-seeded covers or bare soils, more abundant in
456 endoparasitic NF species.

457 Various studies have addressed the competitive relationships among EPN and FLN species
458 (Blanco-Pérez et al., 2019; Campos-Herrera et al., 2012; 2015b; Duncan et al., 2003), but the
459 nature of possible occurring interaction between these two groups of nematodes seems to be much
460 more uncertain. For example, Blanco-Pérez et al. (2017) suggest that, in particular cases, the
461 presence of bacteriophagous nematodes within the insect's cadaver could assist EPN
462 development. Of the two identified FLN species in this study, *P. maupasi* co-emerged more
463 frequently with EPNs from insect larvae baited on soil samples collected in the Algarve region

(Campos-Herrera et al., 2019). We found *P. maupasi* in higher and lower numbers on grass-seeded and spontaneous covers, respectively, similarly than observed for the other EPN antagonism organisms tested (NF and EcPB). However, *in vitro* experiments conducted by Blanco-Pérez et al. (2019) discarded any pathogenic capacity of a Portuguese strain of *P. maupasi* against *G. mellonella* larvae, and concluded that IJ infectiveness and reproduction for the EPN species *S. feltiae* were not affected by the presence of this particular FLN strain even in large numbers. Besides, in our insect-baits, we did not report many emergences for *P. maupasi*, but nematodes in the *Acrobeloides*-group. Surprisingly, we did not found evidence for the presence of nematodes in the genus *Oscheius*, commonly existing in soils (Félix et al., 2001), so it seems plausible that other FLN species may occupy their ecological niche. The remarkable abundance of the eggs-trapping fungal species *P. lilacinum* on no-till soils point in this direction and suggests that we may be underestimating the total number of FLNs that these treatments would contain. Expanding the molecular qPCR tools for new FLN species associated with EPNs, such as *Caenorhabditis elegans* (R. Blanco-Pérez, personal observation), or using Next-generation sequencing (NGS) analysis to expand the number of species explored (Dritsoulas et al., 2020; Geisen et al., 2018), might provide additional insight on the interactions between these two groups of nematodes in nature.

481

482 5. Conclusion

483

484 Pest prevention is one of the main aims of current agriculture. Natural enemies occurring
485 in crop soils allow for protection against pests and pathogens if conditions favor their survival.
486 Overall, our results confirm a tendency of the positive impact of the implementation of specific
487 cover crops on natural occurrence and activity of EPNs, which should be an indicator of more
488 high-grade soil health. But the proper selection of alternative management strategies in
489 agroecology is essential to maximize benefits and reduce potential problems (Provost and
490 Pedneault, 2016), and viticulture is not an exception. For example, we observed that the use of
491 grass-seeded covers seems to enhance IJ abundance, but also endoparasitic NF proliferation that

might reduce their activity, perhaps by accumulating more organic matter than other no-till managements. Conversely, the spontaneous cover showed an equilibrium in the abundance and activity of the EPN species naturally occurring, while maintaining low levels of abundance for antagonistic organisms. Besides, the evaluation of above-ground arthropods in the same experimental vineyard revealed that several predator taxa, including ants, ground beetles, earwigs, and vespid wasps, were also significantly better represented on spontaneous cover crops (Sáenz-Romo et al., 2019b). Remarkably, this particular treatment should not contribute to increasing viticulture costs. These promising results can contribute to expanding the use of strategies that enhance the occurrence of natural enemies of arthropods potentially damaging to vineyards at a reasonable cost for the farmers. Thus, future studies to confirm this general trend and unravel the factors associated with the beneficial effect of cover crop implementations in vineyards will be needed.

Acknowledgements

The authors thank Dr. Xoaquín Moreira (Misión Miológica de Galicia, CSIC, Spain) and Laura Barrios (Área de Informática Científica, CSIC, Spain) for their help with the statistical analysis, and members of Vitigen Lab (ICVV) for kindly sharing their equipment. This study was grant supported by the Ministry of Economy and Competitiveness (AGL2014-53336R). The pre-doctoral contracts CAR-2018 (Department of Economic Development and Innovation of the Government of La Rioja) and FPI-UR 2015 (University of La Rioja) support RBP and MGS, respectively. IVD awarded the ADER I+D+i (2019) fellowship by the Rioja Agency of Economic Development (La Rioja, Spain). The Government of Spain currently supports RCH with a Ramón y Cajal contract award (RYC-2016-19939).

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795

796 **Figure legends**

797

798 **Figure 1.** Experimental design of the experimental vineyard performed in 2017 and 2018. The
799 experiment was settle on the vine variety Tempranillo (clon RJ-26, rootstock ‘110-
800 Richter’). The plots consisted of four to seven plant rows (1.15 m between strains / 2.90
801 m between rows). Different treatments (n = 3) are indicated with different colours: tillage
802 (TIL) and three different cover crops, seeded (SEE) with *Bromus perenne* (Poaceae),
803 flower-driven (FLO), and spontaneous (SPO).

804

805 **Figure 2.** Linear mixed models testing for differences of the percentage soil organic matter (OM)
806 between till (TIL) and no-till soils but the implementation of the cover crops grass-seeded
807 (SEE), flower-driven (FLO), and spontaneous (SPO). Asterisks indicate significant
808 differences within pair-treatment comparisons at *** $P < 0.001$, and n.s., not significant.
809 Values are least-square means $\pm$ SE.

810

811 **Figure 3.** Effect of the use of the cover crops grass-seeded (SEE), flower-driven (FLO), and
812 spontaneous (SPO), on (A) number of infective juveniles (IJs) and (B) frequency of
813 occurrence of the entomopathogenic nematode (EPN) species identified: *Steinernema*
814 *feltiae* (Sfel), *S. affine* (Saff), *S. riojaense* (Rroj), *Heterorhabditis bacteriophora* (Hbac),
815 and *H. indica* (Hind). Asterisks indicate significant differences from generalized linear
816 mixed models testing within pair-treatment comparisons of tillage management (TIL) and
817 each cover crop at * $P < 0.05$ and n.s., not significant. Values are least-square means $\pm$
818 SE. EPN species averages are represented in pies (see Table 2 for statistics).

819

820 **Fig. 4.** Effect of the use of the cover crops grass-seeded (SEE), flower-driven (FLO), and
821 spontaneous (SPO), on (A) quantification and (B) frequency of occurrence of the free-
822 living nematodes (FLNs) identified: *Pristionchus maupasi* (Pmau) and *Acrobelloides* ssp.
823 (Assp.). Not significant (n.s.) differences were found from generalized linear mixed

models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop. Values are least-square means $\pm$ SE. FLN species averages are represented in pies.

Fig. 5. Effect of the use of the cover crops grass-seeded (SEE), flower-driven (FLO), and spontaneous (SPO), on (A) quantification and (B) frequency of occurrence of nematophagous fungi (NF) infection ratio (IR) for the identified species: *Arthrobotrys oligospora* (Aoli), *A. dactyloides* (Adac), *Purpureocillium lilacinum* (Plil), *Hirsutella rhossiliensis* (Hrhos), and *Catenaria* ssp. (Cssp.). Asterisks indicate significant differences from generalized linear mixed models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop at *** $P < 0.001$, ** $P < 0.01$, and n.s., not significant. Values are least-square means $\pm$ SE. NF species averages are represented in pies (see Table 2 for statistics).

Fig. 6. Effect of the use of the cover crops grass-seeded (SEE), flower-driven (FLO), and spontaneous (SPO), on (A) number of plasmids and (B) frequency of occurrence of the ectoparasitic bacterial species *Paenibacillus* sp. (Psp.). Not significant (n.s.) differences were found from generalized linear mixed models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop. Values are least-square means $\pm$ SE.

Fig. 7. Effect of the use of the cover crops grass-seeded (SEE), flower-driven (FLO), and spontaneous (SPO), on entomopathogenic nematode (EPN) activity (A) measured as the percentage of *G. mellonella* larvae that recorded positive Koch's postulates nematode emergence, and (B) the frequency of occurrence of EPN activity. Asterisks indicate significant differences from generalized linear mixed models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop at *** $P < 0.001$, ** $P < 0.01$, and n.s., not significant. Values are least-square means $\pm$ SE. Averages of the nematode species identified are represented in pies: *Steinernema feltiae* (Sfel), *S.*

850 *riojaense* (Rroj), *S. carpocapsae* (Scar), *Heterorhabditis bacteriophora* (Hbac), *H. indica*
851 (Hind), *Pristionchus maupasi* (Pmau), and *Acrobeloides* ssp. (unk., unknown species).

FLO-3 6 rows / 1210 m ²	TIL-3 6 rows / 1330 m ²	SPO-3 6 rows / 1410 m ²
SPO-2 7 rows / 1350 m ²	SEE-3 7 rows / 1490 m ²	FLO-2 7 rows / 1450 m ²
TIL-2 6 rows / 2100 m ²		SPO-1 6 rows / 2050 m ²
SEE-2 6 rows / 2020 m ²		TIL-1 6 rows / 1820 m ²
FLO-1 4 rows / 1740 m ²		
SEE-1 7 rows / 1570 m ²

A **B**

A

B

A

B

1 **Table 1.** Soil organisms tested and qPCR running conditions.

Type of organism / species	Population	GenBank ACNO ITS Region	Primers / Probes final concentration (nM)	Reference for primers and probes sequences
Entomopathogenic nematodes				
<i>Heterorhabditis bacteriophora</i>	(commercial)	KJ938576	400 / 200	Campos-Herrera et al. (2011b)
<i>Heterorhabditis indica</i>	Btw	KJ938571	400 / 200	Campos-Herrera et al. (2011a)
<i>Steinernema affine</i>	CH	KJ938567	600 / 300	Torr et al. (2007)
<i>Steinernema arenarium</i>	SA	KU194615	600 / 300	Campos-Herrera et al. (2019)
<i>Steinernema carpocapsae</i>	DOK-83	KJ818295	400 / 200	Campos-Herrera et al. (2011b)
<i>Steinernema feltiae</i>	RS-5	KJ938569	600 / 300	Campos-Herrera et al. (2011b)
<i>Steinernema kraussei</i>	OS	KJ696686	266 / 150	Campos-Herrera et al. (2015b)
<i>Steinernema riojaense</i>	RM-30	MK503133	600 / 300	Current study
Free-living nematodes				
<i>Acrobeloides</i> -group	RT1-R15C	IQ237849	400 / 200	Campos-Herrera et al. (2012)
<i>Oscheius tipulae</i>	MG68 P29	KJ938579	400 / 200	Campos-Herrera et al. (2015a)
<i>Oscheius onirici</i>	MG67 P20	KJ938578	400 / 200	Campos-Herrera et al. (2015a)
<i>Pristionchus maupasi</i>	AM-3	MG551681	400 / 200	Campos-Herrera et al. (2019)
Nematophagous fungi				
<i>Catenaria</i> sp.	1D	JN585805	400 / 200	Pathak et al (2012)
<i>Arthrobotrys dactyloides</i>	H55	KJ938574	400 / 200	Pathak et al (2012)
<i>Arthrobotrys musiformis</i>	11	KJ938572	400 / 200	Pathak et al (2012)
<i>Arthrobotrys oligospora</i>	8	KJ938573	400 / 200	Pathak et al (2012)
<i>Hirsutella rhossiliensis</i>	2931	-	400 / 200	Zhang et al. (2006)
<i>Purpureocillium lilacinum</i>	9357	KJ938575	400 / 200	Atkins et al. (2005)
Ectoparasitic bacteria				
<i>Paenibacillus nematophilus</i>	NEM2	AF480936	400 / 200	Campos-Herrera et al. (2011b)
<i>Paenibacillus</i> sp.	SdTc1FEE1	JF317562	400 / 200	Campos-Herrera et al. (2011b)

Table 2

Effect of the implementation of the cover crops grass-seeded (SEE), flower-driven (FLO), and spontaneous (SPO), on quantification and frequency of occurrence of the isolated species by sucrose-gradient centrifugation of entomopathogenic nematodes (EPNs) and the associated organisms free-living nematodes (FLNs) and nematophagous fungi (NF). Results from generalized linear mixed models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop. Asterisks indicate significant at *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s., not significant, and n.d., no data.

Dependent variable	Quantification			Frequency of occurrence		
	TIL vs SEE (χ^2 , P)	TIL vs FLO (χ^2 , P)	TIL vs SPO (χ^2 , P)	TIL vs SEE (χ^2 , P)	TIL vs FLO (χ^2 , P)	TIL vs SPO (χ^2 , P)
Total EPNs – n° IJs						
<i>S. feltiae</i>	13.16 ***	4.17 *	6.72 ***	1.78 *	n.s.	n.s.
<i>S. riojaense</i>	n.d.	n.s.	n.s.	n.d.	n.s.	n.s.
<i>S. affine</i>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>H. bacteriophora</i>	123.40 ***	n.d.	47.02 ***	n.s.	n.d.	n.s.
<i>H. indica</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Total FLNs – ratio 0-1						
<i>P. maupasi</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Acrobelloides</i> ssp.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Total NF – IR ratio 0-1						
<i>A. oligospora</i>	n.s.	n.s.	48.53 ***	n.s.	n.s.	n.s.
<i>A. dactyloides</i>	33.32 ***	n.s.	n.d.	n.s.	n.s.	n.s.
<i>P. lilacinum</i>	n.s.	n.s.	n.s.	2.71 *	2.08 *	2.30 *
<i>H. rhossiliensis</i>	n.d.	n.d.	n.d.	n.d.	n.d.	n.s.
<i>Catenaria</i> ssp.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Supplementary Data

Entomopathogenic nematode natural distribution in vineyards managed with cover crops: impact on activity and associated soil organisms

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Contents:**Supplementary data 1.** Climatological data and soil parameters.

- **Table S1.** Monthly climatological data for the two years of experimentation (2017 and 2018).
- **Table S2.** Soil properties.
- **Table S3.** Soil texture.

Supplementary data 2. Development, optimization and implementation of the qPCR toolkit to characterize the entomopathogenic nematodes and associated soil organisms.

- **Table S4.** Soil organisms searched on this study, and used on *S. riojaense* cross-amplification test.
- **Table S5.** Additional species of nematodes which cross-amplification for *S. riojaense* was tested.

Supplementary data 3. Results

- **Table S6.** Statistics of the soil organism species isolated by sucrose-gradient centrifugation.
- **Figure S1.** Total and nematode activity recorded from insect-baits.

Table S1. Monthly climatological data for the two years of experimentation (2017 and 2018). Sampling seasons are marked in gray (Agro-climatic Information Service in La Rioja, SIAR).

Year	Month	Air Temperature (°C)				Soil Temperature (°C)				P (l/m ²)
		max .	avg.	max. avg.	min. avg.	min.	max .	avg.	min.	
2017	Jan	14,3	5,1	8,8	2,0	-4,5	8,8	4,1	1,2	37,4
	Feb	18,7	8,3	13,3	3,9	0,9	11,5	7,3	3,7	28,9
	Mar	27,2	11,0	17,4	5,8	0,7	17,2	10,9	5,7	32,1
	Apr	27,2	12,8	19,6	6,6	-0,3	20,9	14,7	8,7	5,7
	May	32,9	17,4	24,2	11,4	2,4	28,3	18,9	9,0	74,7
	Jun	36,2	21,3	28,4	15,8	9,9	31,3	23,5	14,8	42,6
	Jul	36,5	22,1	29,6	16,1	11,8	33,5	25,6	16,4	15,8
	Ago	36,0	21,6	28,8	15,9	9,9	33,8	26,0	18,8	36,4
	Sep	30,4	17,1	23,4	11,8	6,4	27,7	20,0	14,3	5,7
	Oct	27,1	15,6	21,9	10,3	4,3	23,9	16,6	10,2	21,4
	Nov	19,5	8,8	13,5	4,8	-0,2	15,5	9,1	3,6	18,2
	Dec	16,6	5,9	9,6	3,0	-1,1	9,2	5,4	2,2	58,6
2018	Jan	17,7	6,9	10,4	3,5	-1,8	11,0	5,9	2,2	115,3
	Feb	16,9	4,8	8,2	1,7	-3,8	11,4	4,9	1,6	35,8
	Mar	21,3	8,2	13,0	4,2	0,9	13,2	7,7	2,1	52,3
	Apr	23,9	12,0	17,3	7,5	1,9	19,2	12,5	6,5	86,1
	May	25,8	14,4	19,9	9,8	2,8	20,3	15,6	9,9	65,6
	Jun	33,6	19,1	25,2	13,9	10,3	28,3	20,4	14,7	39,4
	Jul	33,9	22,0	28,9	16,7	13,8	27,4	23,0	19,2	117,8
	Ago	37,0	22,5	30,0	16,4	11,4	30,4	25,7	20,9	0,0
	Sep	32,9	20,1	27,3	14,5	9,9	28,5	21,9	17,1	45,5
	Oct	26,1	13,7	19,5	9,3	0,5	21,5	15,6	7,1	28,5

Table S2. Descriptive by treatments of the soil properties analyzed (Mean $\pm$ SEM), and linear mixed models testing within pair-treatment comparisons of soil tillage management and the implementation of the cover crops grass-seeded (SEE), flower-driven (FLO), and spontaneous (SPO). Probability level, P, designed as $P < 0.001$, ***, $P < 0.05$ *, not significant, n.s.. Codes of soil characteristics: WC, water content; EC, electro conductivity; and OM, soil organic matter.

	Treatment descriptive				Pair-treatments comparisons		
	Tillage (TIL)	Seeded (SEE)	Flower-driven (FLO)	Spontaneous (SPO)	TIL vs. SEE	TIL vs. FLO	TIL vs. SPO
General properties							
WC (%)	6.29 ± 0.39	5.90 ± 0.4	5.32 ± 0.39	5.38 ± 0.39	n.s.	F _{1,38} =6.32 *	n.s.
pH	8.15 ± 0.02	8.13 ± 0.01	8.20 ± 0.02	8.18 ± 0.03	n.s.	n.s.	n.s.
Fertility							
EC (μS/cm)	175.8 ± 5.2	183.5 ± 4.8	162.0 ± 5.4	170.5 ± 5.9	n.s.	F _{1,27} =6.13 *	n.s.
OM (%)	0.81 ± 0.02	0.97 ± 0.03	0.74 ± 0.04	0.79 ± 0.05	F _{1,45} =22.23 ***	n.s.	n.s.
P (mg/kg)	38.53 ± 1.86	37.16 ± 1.37	39.57 ± 1.57	40.14 ± 3.12	n.s.	n.s.	n.s.
K (mg/kg)	186.8 ± 10.0	203.0 ± 10.1	181.8 ± 8.6	212.6 ± 9.3	n.s.	n.s.	n.s.
Mg (mg/kg)	290.4 ± 7.1	306.0 ± 7.2	282.3 ± 8.2	312.9 ± 12.0	F _{1,40} =4.27 *	n.s.	n.s.
Ca (mg/kg)	2.6E4 ± 9.4E2	2.5E4 ± 8.8E2	2.6E4 ± 6.3E2	2.7E4 ± 10.0E2	n.s.	n.s.	n.s.
Zn (mg/kg)	1.58 ± 0.06	1.67 ± 0.06	1.62 ± 0.06	1.84 ± 0.11	n.s.	n.s.	F _{1,44} =4.93 *
Mn (mg/kg)	78.38 ± 5.28	76.22 ± 2.80	66.90 ± 4.07	81.65 ± 2.71	n.s.	n.s.	n.s.
Fe (mg/kg)	57.56 ± 4.57	51.65 ± 3.40	51.00 ± 2.85	63.41 ± 3.06	n.s.	n.s.	n.s.
Cu (mg/kg)	4.11 ± 0.24	4.37 ± 0.26	3.54 ± 0.24	3.54 ± 0.27	n.s.	n.s.	n.s.

Table S3. Soil texture in terms of percentage of sand, silt and clay (Mean $\pm$ SEM) and statistical analysis among crop managements (one-way ANOVA).

Soil texture	Crop management			Statistics
	Tillage	Grass-seeded	Flower-driven	Spontaneous
Sand (%)	47.20 $\pm$ 4.42	46.27 $\pm$ 5.78	56.27 $\pm$ 8.59	51.87 $\pm$ 4.44
Silt (%)	36.83 $\pm$ 3.12	37.00 $\pm$ 4.26	29.13 $\pm$ 6.95	33.83 $\pm$ 3.41
Clay (%)	15.93 $\pm$ 1.28	18.17 $\pm$ 0.32	14.63 $\pm$ 1.82	14.37 $\pm$ 1.04
				$F_{3,8}$ (P)
				0.58 (0.643)
				0.62 (0.623)
				1.96 (0.198)

Supplementary data 2 Development, optimization and implementation of the qPCR toolkit to characterize the entomopathogenic nematodes and associated soil organisms.

To perform a comprehensive characterization of the categorical variables that drive the presence, abundance and activity of the entomopathogenic nematodes (EPNs), we developed, optimized and implemented the qPCR toolkit for nematodes (free-living nematodes –FLNs- included), nematophagous fungi (NF) and ectoparasitic bacteria (EcPB). Herein, we detail all the steps involved, main results and discussion of the advantages and limitations.

1. Molecular characterization of the ITS regions of *S. riojaensis*

The ITS region was amplified using the primers described by Hominick et al (1997). For ensuring optimal amplification in conventional PCR, the DNA sample was previously diluted to a range between 0.5-1 ng/ µl. The reaction was performed in a final volume of 20 µl, and included 1 µl DNA template, 200 nM dNTP mix (Promega), 1x PCR buffer (5X ColorlessGoTaq® Reaction buffer, Promega), 400 nM of each primer (Biosearch Technologies, supplied by Biotools, Spain) with 0.68 U GoTaq® G2 DNA Polymerase (Promega). Amplification procedure was performed as described Hominick et al. (1997), employing the Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR product was verified for expected size by visualization after electrophoresis in 2% agarose gel in TAE (pH 8.3 ± 0.1) (Fisher Bioreagents, Ltd) run along the reference BenchTop 100 bp DNA ladder (Promega). The band of interest was purified by QIAquick Gel Extraction (Qiagen®), cloned by pGEM®-T Easy Vector System I (Promega) kit and transformation of the JM109 High-Efficiency Competent Cells (Promega). After plating in selective LB agar (ampicillin and IPTG/X-Gal, ChromoMax™, Fisher Scientific), selected colonies were cultured overnight in selective LB (with ampicillin). Finally, the plasmid containing the insert was extracted (QIAprep Spin Miniprep, Qiagen®), verified the occurrence of the expected insert in a TAE 0.8% agarose gel, sequenced at Macrogen (Macrogen Europe Laboratory, Inc.). The sequence obtained was assembled (forward and reverse sequences) (Geneious, R.5.6.5., Biomatters, Inc.), compared to reported sequences using Blast (<http://blast.ncbi.nlm.nih.gov>) and submitted to Genbank. The new sequence generated for the target species was identified as a new steinernematid species (ITS GenBank accession number referred in Table S2).

2. Protocols for qPCR tool kits adaptation

A total of 20 species-specific primers and probe combinations were employed for evaluating the EPN soil food assemblage, comprising 8 EPN, 4 FLN, 6 NF, and 2 EPB species (Table S2), including *de novo* designed for this study (*Steinernema riojaense*) The rest of the primers/probe combinations were optimized from the original description to adapt our conditions (Atkins et al., 2005; Zhang et al., 2006; Torr et al., 2007; Campos-Herrera et al., 2011a, 2011b, 2012, 2015a, 2015b, 2019; Pathak et al., 2012). We followed Campos-Herrera et al. (2011a, 2015b) for both adapting protocols from the published molecular tools, and designing/optimizing the new systems. Briefly, sequences of the target species were retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/Genbank/>). In addition, we included the ITS

sequence produced in this study (Table S2). Independently, for each species, multiple alignments of the close related sequences (Goujon et al., 2010) allowed to select areas of variability in the ITS region for designing primers and probes (Primer-Blast, Rozen and Skaletsky, 2000; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Species-specific primers and TaqMan® probe were synthesized by Biosearch Technologies (supplied by Biotools, Spain). The probe was labeled at the 5' end with the fluorogenic reporter dye FAM and the 3' end with the quencher BQH-1. Positive controls were established by the combination of three independent DNA extractions using Power SoilR DNA Isolation Kit for each pure cultures or QIAprep Spin Miniprep for plasmid. In all the preliminary checks and optimization protocols, DNA concentration was standardized to correspond to 30 IU (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 *S. poinari*, *S. phyllophagae*, *S. silvaticum*, *Acrobeloides*-group, *Oscheius* sp.3, and *Catenaria* sp.; and 16S rDNA + pUC57 or pDrive for EPB. For the quantifications, the standard curves were obtained by 10-fold serial dilutions ($n = 5$ points), starting with the highest concentration employed in the pre-screenings.

Real-time PCR test and quantifications were performed with Quantimix Easy Probe mix (Biotools, Spain) in Applied Biosystems® MicroAmp™ Optical 96-Well reaction plates covered with Applied Biosystems® MicroAmp™ adhesive Film (by Thermo Fisher Scientific) on the Applied Biosystems® 7500 Real-Time PCR System. In each run, 1 μl of sample/positive control/negative control (mQ water) was tested in a final volume of 20 μl, with two technical repetitions per sample. 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 organisms for 1 min. The final number of cycles were 38 for nematodes species and 50 for NF and EcPB species. As highlighted by Campos-Herrera et al. (2015b, 2019), preliminary empirical optimization was necessary to determine the required conditions for the available equipment, reagents and material. Primers/probe final concentrations were 200/400 nM respectively, and annealing temperature was 60 °C. With few exceptions, all the molecular sets (EPN, FLN, NF and bacteria) achieved qPCR efficiency ranging 95-105% with $R^2 \sim 0.99$ (Table S2). For the evaluation of the specificity of *S. riojaensis* primers/probe set, and avoid possible cross-amplifications, it was tested on 22 EPN and 9 FLN species (Tables S2 and S3). Overall, none of the primers/probe combinations provided unspecific amplifications with non-target species.

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Table S4. Soil organisms under study used on *Steinernema riojaense* cross-amplification test.

Type of organism / species	Population	GenBank ACNO ITS Region	Standard curve	R ² , Eff. (%)	Ct value (per)	Reference for primers and probes sequences
Entomopathogenic nematodes						
<i>Heterorhabditis bacteriophora</i>	(commercial)	KJ938576	y = 27.715-3.434x	1.000, 96	22.7 (30 IIs)	Campos-Herrera et al. (2011a)
<i>Heterorhabditis indica</i>	Btw	KJ938571	y = 27.702-3.287x	0.996, 101	23.2 (30 IIs)	Campos-Herrera et al. (2011b)
<i>Steinernema affine</i>	CH	KJ938567	y = 25.727-3.331x	0.999, 100	20.8 (30 IIs)	Torr et al. (2007)
<i>Steinernema arenarium</i>	SA	KU194615	-	-	-	Campos-Herrera et al. (2019)
<i>Steinernema carpocapsae</i>	DOK-83	KJ818295	-	-	-	Campos-Herrera et al. (2011a)
<i>Steinernema feltiae</i>	RS-5	KJ938569	y = 27.076-3.427x	0.997, 96	21.9 (30 IIs)	Campos-Herrera et al. (2011a)
<i>Steinernema kraussei</i>	OS	KJ696686	y = 26.133-3.376x	0.998, 98	21.4 (30 IIs)	Campos-Herrera et al. (2015a)
<i>Steinernema riojaense</i>	RM-30	MK503133	y = 27.887-3.341x	0.999, 99	23.0 (30 IIs)	Current study
Free-living nematodes						
<i>Acroboloides</i> -group	RT1-R15C	JQ237849	y = 10.934-3.182x	0.999, 106	14.0 (0.1 ng)	Campos-Herrera et al. (2012)
<i>Oscheius tipulae</i>	MG68 P29	KJ938579	y = 17.083-3.478x	0.999, 106	17.2 (1 ng)	Campos-Herrera et al. (2015b)
<i>Oscheius onirici</i>	MG67 P20	KJ938578	-	-	-	Campos-Herrera et al. (2015b)
<i>Pristionchus maupasi</i>	AM-3	MG551681	y = 18.616-3.187x	0.998, 106	21.7 (1 ng)	Campos-Herrera et al. (2019)
Nematophagous fungi						
<i>Catenaria</i> sp.	ID	JN585805	y = 10.146-3.217x	0.997, 105	13.4 (0.1 ng)	Pathak et al. (2012)
<i>Arthrobotrys dactyloides</i>	H55	KJ938574	y = 22.930-3.148x	1.000, 108	26.1 (1 ng)	Pathak et al. (2012)
<i>Arthrobotrys musiformis</i>	11	KJ938572	y = 22.955-3.324x	0.998, 100	22.8 (1 ng)	Pathak et al. (2012)
<i>Arthrobotrys oligospora</i>	8	KJ938573	y = 23.056-3.147x	0.998, 108	23.0 (1 ng)	Pathak et al. (2012)
<i>Hirsutella rhossiliensis</i>	2931	-	y = 23.925-3.043x	0.993, 113	27.0 (0.1 ng)	Zhang et al. (2006)
<i>Purpureocillium lilacinum</i>	9357	KJ938575	y = 19.489-3.453x	0.998, 95	19.4 (1 ng)	Atkins et al. (2005)
Ectoparasitic bacteria						
<i>Paenibacillus nematophilus</i>	NEM2	AF480936	-	-	-	Campos-Herrera et al. (2011a)
<i>Paenibacillus</i> sp.	SdTc1FEE1	JF317562	y = 11.029-3.216x	0.996, 105	17.4 (0.1 ng)	Campos-Herrera et al. (2011a)

Table S5. Additional species of nematodes which cross-amplification for *S. riojaense* was tested.

Type of organism / species	Population	Unit of measurements	GenBank ACNO ITS region
Entomopathogenic nematodes			
<i>Heterorhabditis megidis</i>	(commercial)	No. infective juveniles	KJ938577
<i>Heterorhabditis zealandica</i>	Btw	No. infective juveniles	GU174009
<i>Steinernema bicornotum</i>	D60 Pl	No. infective juveniles	KJ938568
<i>Steinernema glaseri</i>	NC	No. infective juveniles	GU173998
<i>Steinernema intermedium</i>	82	ng DNA	AF171290
<i>Steinernema intermedium</i> -group	VAD-1067	No. infective juveniles	KJ696684
<i>Steinernema khuongi</i>	ARC	No. infective juveniles	GU174002
<i>Steinernema phyllophagae</i>	-	ng DNA	FJ410327
<i>Steinernema poinari</i>	1160	ng DNA	KF241754
<i>Steinernema rarum</i>	-	No. infective juveniles	KJ938570
<i>Steinernema riobrave</i>	Btw	No. infective juveniles	GU174000
<i>Steinernema silvaticum</i>	IN30k3	ng DNA	KC631434
<i>Steinernema weiseri</i>	1117	No. infective juveniles	KJ696686
<i>Steinernema</i> sp. <i>glaseri</i> -group	N7	No. infective juveniles	MG711658
<i>Steinernema</i> sp. <i>phyllophagae</i> -group	N10	No. infective juveniles	MG711660
Free-living nematodes			
<i>Oscheius myriophilus</i>	JU1386	ng DNA	KP792651
<i>Oscheius</i> sp. 3	JU75	ng of DNA	AJ297890
<i>Pristionchus pacificus</i>	P2333	ng DNA	MG551685
<i>Pristionchus entomophagus</i>	P0144	ng DNA	MG551683
<i>Pristionchus lheritieri</i>	P245	ng DNA	MG551684

Table S6. Effect of the use of the cover crops seeded (SEE), flower-driven (FLO), and spontaneous (SPO) on numbers of the identified species of entomopathogenic nematodes (EPNs) and associated organisms (free-living nematodes (FLNs), nematophagous fungi (NF), and the ectoparasitic bacteria (EcPB) species *Paenibacillus* sp.) by sucrose-gradient centrifugation, and soil activities recorded in the insect-baits. Results from generalized linear mixed models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop at ***P < 0.001, **P < 0.01, *P < 0.05 and n.s., not significant.

Dependent variable	Factor and covariates	Quantification			Frequency of occurrence		
		TIL vs SEE (χ^2 , P)	TIL vs FLO (χ^2 , P)	TIL vs SPO (χ^2 , P)	TIL vs SEE (χ^2 , P)	TIL vs FLO (χ^2 , P)	TIL vs SPO (χ^2 , P)
Total EPNs – n° infective juveniles	Treatment	0.66 *	n.s.	1.41 *	n.s.	n.s.	n.s.
Total FLNs – ratio 0-1	Treatment	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Total NF – IR ratio 0-1	Treatment	13.43 ***	n.s.	n.s.	3.37 ***	n.s.	n.s.
	OM	15.34 ***	-	-	-	-	-
	Treatment * OM	13.68 ***	-	-	-	-	-
EcPB – n° plasmids	Treatment	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Total activity – larvae %	Treatment	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Nematode activity – larvae %	Treatment	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
EPN activity – larvae %	Treatment	n.s.	2.75 **	1.62 ***	n.s.	n.s.	n.s.

Figure S1. Effect of the use of the cover crops grass-seeded (SEE), flower-driven (FLO), and spontaneous (SPO), on the total soil activity, measured as (A) the percentage of dead insect larvae and (B) its frequency of occurrence, and on the nematode activity measured as (C) the percentage of insect larvae that recorded nematode emergence and (D) its frequency of occurrence. No significant differences from generalized linear mixed models testing within pair-treatment comparisons of tillage management (TIL) and each cover crop were obtained. Values are least-square means $\pm$ SE. Averages of the nematode species identified are represented in pies: *Steinernema feltiae* (Sfel), *S. riojaense* (Sroj), *S. carpocapsae* (Scar), *Heterorhabditis bacteriophora* (Hbac), *H. indica* (Hind), *Pristionchus maupasi* (Pmau), and *Acroboloides* ssp. (unk., unknown species).

1 **Research Highlights**

2

- 3 • Cover crops might enhance entomopathogenic nematodes (EPN) presence in vineyards
4 • More EPNs and higher activity observed in spontaneous cover (SPO) than tilled (TIL)
5 • Lower presence of EPN natural enemies in SPO than TIL treatments
6 • The cover crops seeded (SEE) and flower-driven (FLO) produced alteration on EPN assemblage
7 • SPO covers could be a promising strategy to support EPN in Mediterranean vineyard