NEMATODES IN PHYTOBIOMES

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NEMATODES IN PHYTOBIOMES

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Table of Contents

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Novel Strategies for Soil-Borne Diseases: Exploiting the Microbiome and Volatile-Based Mechanisms Toward Controlling *Meloidogyne*-Based Disease Complexes

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Under more intensified cropping conditions agriculture will face increasing incidences of soil-borne plant pests and pathogens, leading to increasingly higher yield losses world-wide. Soil-borne disease complexes, in particular, are especially difficult to control. In order to better understand soil-borne *Meloidogyne*-based disease complexes, we studied the volatile-based control mechanism of associated bacteria as well as the rhizospheric microbiome on Ugandan tomato plants presenting different levels of root-galling damage, using a multiphasic approach. The experimental design was based on representative samplings of healthy and infected tomato plants from two field locations in Uganda, to establish species collections and DNA libraries. Root galling symptoms on tomato resulted from a multispecies infection of root-knot nematodes (*Meloidogyne* spp.). Results revealed that 16.5% of the bacterial strain collection produced nematicidal volatile organic compounds (nVOC) active against *Meloidogyne*. Using SPME GC-MS, diverse VOC were identified, including sulfuric compounds, alkenes and one pyrazine. Around 28% of the bacterial strains were also antagonistic toward at least one fungal pathogen of the disease complex. However, antagonistic interactions appear highly specific. Nematicidal antagonists included *Pseudomonas*, *Comamonas*, and *Variovorax* and fungicidal antagonists belonged to *Bacillus*, which interestingly, were primarily recovered from healthy roots, while nematode antagonists were prominent in the rhizosphere and roots of diseased roots. In summary, all antagonists comprised up to 6.4% of the tomato root microbiota. In general, the microbiota of healthy and diseased root endospheres differed significantly in alpha and quantitative beta diversity indices. Bacteria-derived volatiles appear to provide a remarkable, yet wholly unexploited, potential to control *Meloidogyne*-based soil-borne disease complexes. The highly specific observed antagonism indicates that a combination of volatiles or VOC-producing bacteria are necessary to counter the range of pathogens involved in such complexes.

Keywords: root-knot nematodes, tomato microbiome, biocontrol, antagonists, *Pseudomonas*, *Comamonas*, *Variovorax*, *Bacillus*

INTRODUCTION

Agriculture causes long-lasting anthropogenic environmental impacts as it replaces natural vegetation, alters biogeochemical cycles, and decreases biodiversity; this defined a new human-dominated geological epoch, the Anthropocene. The soil disturbance by the conversion of land to agriculture has resulted in species extinctions 100–1,000 times higher than natural rates, and likely constitutes the beginning of the sixth mass extinction in Earth's history (Lewis and Maslin, 2015). However, much less is known about the loss of microbial diversity (Berg et al., 2017) due to human activity than the loss of macroscopic diversity. In particular, bacteria occupy important niches and roles, linking plant microbial diversity and ecosystem functioning, such as productivity, host plant fitness and resilience (Bais et al., 2004; Tilman et al., 2012; Vandenkoornhuyse et al., 2015; Laforest-Lapointe et al., 2017). Among the most striking direct consequences of agricultural intensification is the elevated presence and impact of soil-borne pests and pathogens (Mendes et al., 2012). Soil-borne diseases are often "microbiome diseases"; they signify the result of a loss of microbial diversity and dysbiosis in soil and consequently in the rhizosphere and endosphere of plants (van Elsas et al., 2012). Once established, bacteria, fungi, and nematode pathogens accumulate, often as a synergistic combination, leading to high yield losses, which prove difficult to control (Oerke, 2006; Bennett et al., 2012). The soil environment is a complex arena, the biological permutations of which are little understood (Bais et al., 2004), especially in areas such as Africa, where assessments and understanding of the microflora and microbiota remain negligible.

Root-knot nematodes Göldi 1892 (RKN, genus *Meloidogyne*) infect over 5500 host plants, including plant species from nearly every extant known plant family (Trudgill and Blok, 2001). Species of *Meloidogyne* can be extremely polyphagous, are mainly parthenogenetic, and are highly adapted obligate sedentary plant parasites. They are regarded as the most economically important plant-pathogenic nematode group worldwide (Jones et al., 2013) and in the tropics, are viewed as the most significant biotic threat to crop production (Karssen et al., 2013; Coyne et al., 2018). Host roots are infected by freshly hatched, motile second stage juveniles (J2), which, upon establishing a feeding site behind the root tip, become sedentary, feeding from cells which it modifies to provide a constant supply of nutrients. Infection by RKN often leads to typical symptoms of root damage and gall formation, with above-ground symptoms of stunting, wilting, leaf chlorosis, reduced yield, which are symptomatic of water or nutrient scarcity (Karssen et al., 2013). Nematode-infected plants tend to be more susceptible to other diseases (Coyne et al., 2018). These unspecific above-ground symptoms, however, lead to an excess overuse of fertilizers and ineffective treatment with pesticides (Karungi et al., 2011). Excessive and frequent pesticide applications, in combination with inappropriate handling, increase the risks to human health as well as to water resources and the ecological system (Coyne et al., 2018). The impact of RKN on the host is significantly exaggerated through secondary pathogen infections, such as root rot pathogens, bacterial and fungal wilts, e.g., *Fusarium oxysporum* and *Verticillium dahliae* (Back et al., 2002; Karssen et al., 2013). These plant pathogens do not necessarily need the presence of RKN to successfully infect their hosts, but since RKN may act as casual agents, the disease can be seen as *Meloidogyne*-based disease complex. The interactions between RKN, secondary pathogens, host plant and plant-associated microorganisms lead to the resulting effects on plant health (Karssen et al., 2013). The management of RKN would therefore benefit from a more holistic approach, taking into consideration the management of soil-borne microbial complexes. A deep understanding of the plant-associated microbiomes would be beneficial, given that a proportion of microorganisms are antagonistic toward soil-borne pests and pathogens (Berg et al., 2002). One recently discovered indirect mode of plant disease prevention in bacteria is the production of volatile organic compounds (VOC) (Effmert et al., 2012; Cernava et al., 2015). VOC are semiochemicals that act as "long-range" allelochemicals in soil, which can have growth-promoting or -inhibiting effects on other microorganisms (Effmert et al., 2012) and plants (Ryu et al., 2003). The fact that VOC can have communicational, controlling or inhibitory effects that act inter- and/or intra-specifically, make them a highly interesting field of study for biological control (Effmert et al., 2012; Torto et al., 2018). However, despite their potential, VOC have received only limited attention and are yet to be fully exploited for biocontrol strategies. Our hypothesis was that RKN can be negatively affected and controlled by bacteria-derived VOC in the microbiome.

We selected tomato as a model plant, given its susceptibility to RKN and soil-borne disease complexes, using two RKN-infected field sites in Uganda. Tomatoes in Uganda are a key source of income and food security for smallholders (Ssekyewa, 2006) who often own less than 2 ha of land (Karungi et al., 2011). A major challenge in controlling the RKN-disease complex is the need for simultaneous control of all involved pathogens. Novel mechanisms are urgently needed to address these soil-borne challenges, especially in Africa where the need to sustainably intensify cropping production systems is critical (Bennett et al., 2012; Vanlauwe et al., 2014). Our current study focused on three main objectives: (i) identify the *Meloidogyne* species present, (ii) screen bacterial strains capable of producing nematicidal VOC or fungicidal metabolites, (iii) analyze the microbiome shift in the root endosphere due to RKN activity. Results of this study will create a better understanding of the soil-pathogen-host interactions in the RKN-disease complex, which will be translated to developing novel control strategies.

MATERIALS AND METHODS

Sampling Design

Three bulk samples, each comprising ten roots of fruit-bearing tomato plants (*Solanum lycopersicum* L.) with adhering soil, were collected from two field sites in Uganda in April 2017. Gall formation was categorized according to a root galling index (RGI) from 1 (no visible galling damage) to 5 (severe/lethal damage) (Coyne et al., 2007); two plants were selected for each RGI score from each site during uprooting. Sampling site

"Luwero" (0°39′20″ N, 32°24′38″ E, 1187 m) was a rural farmer's open field with 1-year-old virgin soil. Tomato cv. "Rio Grande," received unknown application levels of pesticides but included generic fungicides, mainly mancozeb-based and the insecticides cypermethrin and chlorpyriphos. Sampling site "Namulonge" was at the IITA research station (0°31′46″ N, 32°36′45″ E, 1170 m) and consisted of a RKN-infected sandy soil within a concrete tomato outdoor bed with no direct connection to surrounding soils. Tomato cv. "Moneymaker" received no pesticide applications. A 300 g soil sample from each sample site was assessed for pH, nutrient (K, P, Mg, organic matter) content and soil type by "AGROLAB Agrar und Umwelt GmbH" (Sarstedt, Germany) to compare soil composition between the two sampling sites.

Bacterial Strains and Isolation of Total Community DNA

Samples were recovered from different microhabitats associated with RKN infection: bulk soil constituting the native bacterial community in the field, rhizosphere representing root-associated bacteria, galled and non-galled rhizoendosphere. Bacterial suspensions were recovered using 0.9% NaCl from a 5 g sub-sample of the bulk soil (soil between tomato plants, three samples/site, $n = 6$), from rhizosphere (root adhering soil, one/plant, $n = 20$) and surface sterilized sections of roots from both galled (RE-D, $n = 16$) and non-galled (RE-H, $n = 17$) roots. Suspensions were used for DNA extractions for both amplicon analysis and isolation of bacterial strains (for details, see **Supplementary Material** – Additional Methods). Suspensions were plated onto NA plates (nutrient terestingly agar; Sifin GmbH, Berlin, Germany); in total 260 strains were isolated and screened for nematicidal (see section "Screening for nVOC-Producing Strains") and fungicidal properties (see section "Bacterial Antagonistic Activity Against Fungal Pathogens"). Extraction of the DNA pellet was conducted using "FastDNA Spin Kit for soil" (MP Biomedical, Eschwege, GER). PCR-products were cleaned with GENECLEAN Turbo^{TM} Kit (MP Biomedicals, Eschwege, GER) following the manufacturer's instructions for genomic DNA. 16S rRNA gene amplifications were carried out in 3 × 30 µl reactions with the Illumina barcode universal bacterial primer set 515f-806r (Caporaso et al., 2011) and PNA Mix (Lundberg et al., 2013) to remove plastid DNA. PCR products of barcoded samples were pooled to equimolarity; sequencing was carried out by Eurofins MWG Operon (Ebersberg, GER[1]) with an Illumina HiSeq 2500 system (for details, see **Supplementary Material** – Additional Methods).

Identification of Nematodes and Inocula Production

Randomly selected females with eggs were dissected from diseased roots. Perineal patterns of females were used for morphological diagnosis; body content of the same crushed females were used for molecular identification using the molecular key method of Adam et al. (2007). Furthermore, the region for NAD dehydrogenase subunit 5 was amplified for genetic determination using the body content of individual females (Janssen et al., 2016). Data were combined to determine species identification. Eggs were used to re-infect tomato seedlings in the fourth-true-leaf stadium to establish pure cultures. J2 of identified pure cultures were partially used in further experiments. For extracting J2, roots were rinsed free of adhering soil. Diseased root sections were chopped coarsely, placed in 1.2% NaOCl solution and blended with a hand blender for 3 min. The suspension was rinsed with tap water on nested 100–25 µm sieves. Eggs were caught on 25 µm sieve and collected into a beaker, which was aerated for 10 days to allow hatching. The J2 suspensions were placed on a Baermann funnel filter for 24 h at room temperature (**Supplementary Figure S1A**). The resulting ∼30 ml J2 suspension was stored horizontally in 50 ml Sarstedt tubes at 6°C until use.

Screening for nVOC-Producing Strains

We used a variation of the two clamp VOC assay (TCVA, Cernava et al., 2015): Bacterial strains were streaked on 12-well plates containing NA and incubated at 30°C for 24 h. Each plate had a blank well, containing NA only. Plates were inversed onto another 12-well plate containing ∼100 J2 of *M. incognita* (provided from Julius Kühn-Institut, Münster, Germany) on 2%-tap water agar. A silicon foil with a 5 mm hole between the two opposing chambers separated the two 12-well plates. The two plates were clamped together to provide airtight test conditions (**Supplementary Figure S1B**) and then maintained for 24 h at room temperature. Dead J2 were assigned dead if the body was straight and did not react when prodded with a dissection needle. Percentage J2 mortality was calculated after correcting for the blank value of the corresponding plate blank. Bacterial strains were categorized according to their activity: non-active (<10% mortality), slightly active (>10–80%), active (>80–95%), and highly active (>95%). Distributions of the number of bacterial strains within the categories were compared between sampling sites, microhabitat (healthy/diseased root, rhizosphere) and RGI. The experiment was repeated two more times for those samples demonstrating >80% nematicidal activity. The strains showing consistent nematicidal activity were identified by sequencing of 16S rRNA gene.

Bacterial Antagonistic Activity Against Fungal Pathogens

All 260 isolated bacterial strains were tested for their antagonistic activity against the fungal pathogens *Botrytis cinerea*, *F. oxysporum*, *Fusarium verticillioides*, *Sclerotium rolfsii*, and *V. dahliae* (provided by Institute of Environmental Biotechnology Graz) in dual cultures on Waxman agar in three replicates. Antifungal activity was categorized according to: 0 (fungi overgrow bacterial colony), +1 (hyphae reach bacteria, but do not overgrow), +2 (lateral inhibition zone <0.5 cm), and +3 (lateral inhibition zone >0.5 cm). The mean category across the three repeat assessments was calculated. Bacterial strains showing a strong antifungal effect (category +3) were compared

[1]http://www.eurofinsgenomics.eu/

using VENN[2]. DNA of strains with a mean antifungal activity of +3 against at least four pathogens (n = 23, see also **Figure 2A**) was extracted. A BOX-PCR was carried out to identify clones, resulting in five genotypes. 16S rRNA gene of the resulting five genotypes was amplified and sequenced for identification.

SPME GC-MS of nVOC and Nematicidal Effect of Single Compounds

Nematicidal volatile organic compounds were identified using an adapted version of the method from Verginer et al. (2010) (for details, see **Supplementary Material** – Additional Methods). A total of nine compounds (purity >98%) partially found in the GC-MS samples were tested against *M. javanica* J2 in a chambered Petri dish, namely decene (10en), undecene (11en), undecane-2-on (11on), dodecene (12en), 2-methoxy-3-methyl pyrazine (2M3MP), 2,5-dimethyl pyrazine (25DP), 5-isobutyl-2,3-dimethyl pyrazine (5I23DP), 2-ethyl-3-methyl pyrazine (2E3MP), and 2-isobutyl-3-methoxy pyrazine (2I3MP) (all Sigma-Aldrich, Darmstadt, Germany). 2M3MP was used as a substitute for 3-methoxy-2,5-dimethyl pyrazine, which was consistently detected in nVOC-volatilome of *Pseudomonas koreensis* T3GI1 (see also **Table 2**). The compound 2-undecanone was used as a positive control, due to its known nematicidal effects on *M. incognita* (Huang et al., 2010) and other nematodes (Gu et al., 2007). On one side of the Petri dish, a 500 µl suspension of *M. javanica* (∼250 J2) was placed on 8 ml of 2%-tap water agar. On the opposite side, three concentrations (1, 5, and 20 µl) of a single compound were placed on a microscopic slide, which prevented interactions of the compound with the Petri dish plastic. A Petri dish with 20 µl distilled water represented the control. Plates were maintained at room temperature for 24 h and the experiment repeated a further two times and the blank-corrected mortality rate calculated for each compound.

Amplicon Analysis and Statistics

Pre-processing of the reads obtained by the sequencing company of Eurofins MWG Operon was carried out using QIIME 2 (2017.12 release) and QIIME 1 (Caporaso et al., 2010) following the protocol of Schwendner et al. (2017). Demultiplexing, denoising (400 bp length, including phiX and chimera filtering) and generation of ribosomal sequence variants (RSVs) was carried out with the DADA2 algorithm (Callahan et al., 2016). RSVs were summarized in a feature table. The taxonomic analysis was based on a customized naïve-bayes classifier trained on 16S rRNA gene features clustered at 97% similarities within the Silva128 database release and trimmed to a length of 400 bp. Reads for mitochondria and chloroplasts were filtered using QIIME 2 before analysis. The feature table was rarefied to 6,890 reads for core metrics analysis. Alpha diversity indices were analyzed using Pairwise Kruskal–Wallis test and beta diversity indices with PERMANOVA. Bray–Curtis dissimilarity and unweighted UniFrac dissimilarity between habitats were visualized using EMPEROR[3]. One rhizosphere sample (T4R) was removed due to poor quality. Differential abundances were subjected to ANCOM and Gneiss test, implemented in QIIME 2. Bacterial network of the core microbiome of each habitat (>1% relative abundance within habitat) was visualized using Cytoscape 3.3.0 (Shannon et al., 2003) on order level. The core microbiomes were defined with an occurence of >75% throughout the replicates for each habitat. Mean relative abundance of fungal and nematode antagonists found in this study was calculated for each data set. Bacterial abundances on family level were compared between healthy and diseased root samples to visualize bacterial community shift due to RKN infection.

[2]http://bioinformatics.psb.ugent.be/webtools/Venn/

[3]https://view.qiime2.org

RESULTS

Meloidogyne Were the Causal Agents of the Disease Complex in Tomato

The combination of identification approaches (SCAR-primer molecular key, morphological examination, NAD5 sequences) identified 10 adult females as *M. incognita* and two as *M. incognita* sensu lato. in Luwero (site 1). Just *M. incognita* was reliably identified from Luwero. In samples from Namulonge (site 2), three females were identified as *M. incognita*, six as *M. javanica* and five as *M. incognita* s. lat. Thus, a multispecies infection was confirmed in Namulonge (see **Supplementary Material** – Additional Results, **Supplementary Figures S2, S3**).

Bacterial Strains With Antagonistic Activity Toward *Meloidogyne*

Representative bacterial strains from each of the two sampling sites caused similar mean nematode mortality rates through nVOC (32.1 ± 25.4% in Luwero, 30.9 ± 28.1% in Namulonge). Most strains showed no effect (mortality <10%) or only slight nematicidal effects. In total, 43 strains were categorized as active (80–95% mortality; n = 20) or highly active (>95% mortality; n = 23). When comparing the overall nematicidal activity of bacteria from plants of different RGI, a trend was apparent for higher mortality rates of strains from highly diseased plants. Most active and highly active strains (n = 43) were recovered from diseased rhizoendosphere (RE-D) samples, independent of site (**Figure 1**). Of these 43 strains, six demonstrated repeated nematicidal activity (>80% mean mortality). Five out of these six highly active strains were bacteria isolated from RE-D. Moreover, three of these strains were collected from the same tomato plant (T13), a heavily galled (RGI = 4.5) plant from Namulonge. Just one strain was isolated from healthy, non-galled rhizoendosphere (RE-H) from Luwero. Sequences of the 16S rRNA genes identified the nematicidal bacteria as *Pseudomonas koreensis*, *Comamonas sediminis*, *Variovorax paradoxus*, *P. soli* and two strains of *P. monteilii*. Strains with high nematicidal activity showed no high antifungal activity and *vice versa* (**Table 1**).

The volatilomes of the nVOC-producing strains mainly consisted of alkenes, sulfuric compounds, alcohols, ketones, and aldehydes. Additionally, one pyrazine (3-methoxy-2,5-dimethyl pyrazine) was consistently detected in *P. koreensis* T3GI1, the strain with the highest nematicidal effect (**Table 2**).

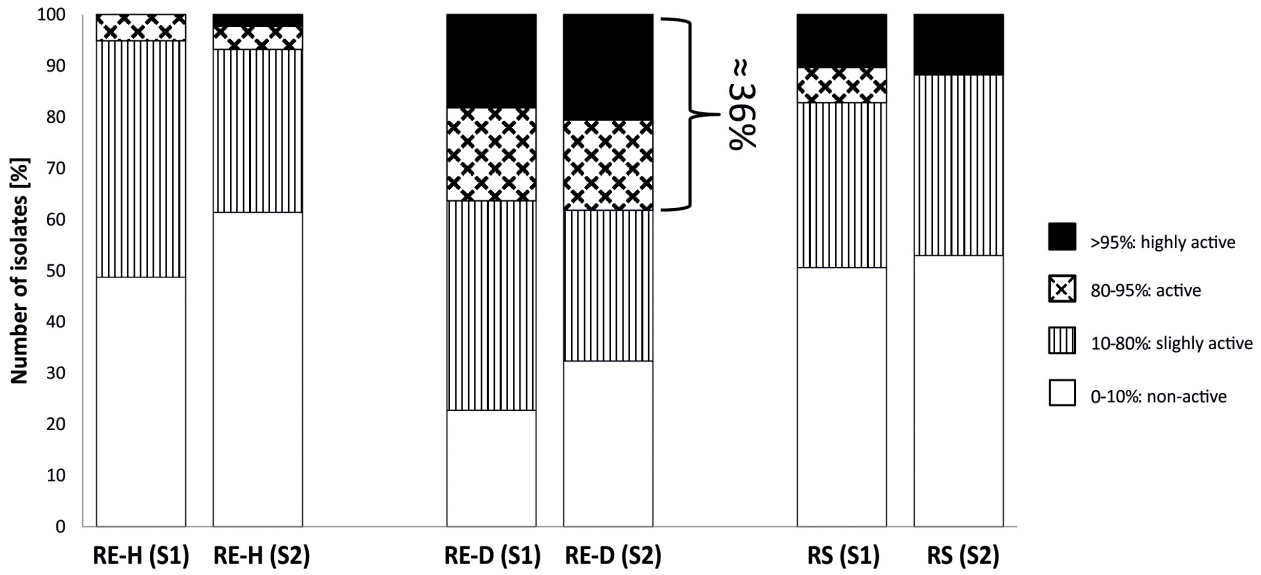

FIGURE 1 | Proportion of nematicidal active bacterial strains. Proportion of nematicidal active strains is highest in diseased root endosphere, independent of site. RE-H, rhizoendosphere healthy; RE-D, rhizoendosphere Meloidogyne-diseased; R, rhizosphere; S1, Site 1, Luwero; S2, Site 2, Namulonge; Pattern refer to the activity category; in RE-D, around 36% of bacterial strains are categorized active or very active (>80% mortality) in nVOC-production.

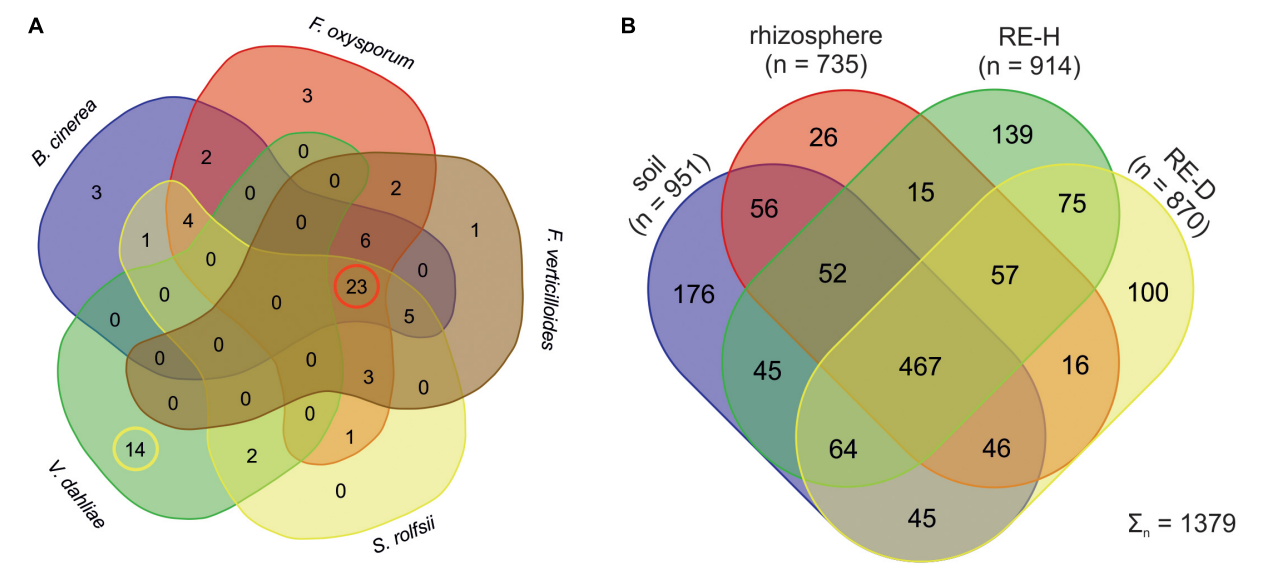

FIGURE 2 | (A) Venn diagram of bacterial strains showing high antagonism against fungal pathogens. Strains that showed high antagonism against *Botrytis cinerea*, *Fusarium oxysporum*, *F. verticillioides*, and *Sclerotium rolfsii* did not show high effects against *Verticillium dahliae*. Red circle: active strains against four fungal pathogens. Yellow circle: *Verticillium* antagonists. **(B)** Venn Diagram displaying the number of taxa on genus level of bacterial strains. Rhizosphere sample have the least microhabitat-specific taxa. Endosphere samples have more specific than shared taxa.

1-undecene was one of the main components of the volatilome of *C. sediminis*, *P. monteilii*, and *P. soli*. Except for *P. monteilii* T8GH4, dimethyl disulfide was the main component of the volatilome but strangely, was also found in the blank (**Table 2**). When comparing the overall number of mass spectra ion counts, the total ionic counts of bacterial volatilomes were up to sevenfold higher (in *P. soli* T13GI4) than in the blank (**Table 2**). When individually assessing 2-undecanone, several alkenes and pyrazine derivates against J2, just three compounds, 2-undecanone (11on), 2-methoxy-3-methyl pyrazine (2m3mp), and 2-ethyl-3-methyl pyrazine (2e3mp), gave significantly higher mortality rates than the blank mortality after 1-day incubation using quantities of 5 and 20 µl. Only 11on and 2m3mp gave significantly higher mortality rates than the blank when using 1 µl of compound (**Supplementary Figure S4**). At the highest concentration, live J2 were mainly found surrounded

TABLE 1 | Identification of bacterial strains with antagonistic properties against either root-knot nematodes or fungal pathogens.

Strain ID	Best hit (ref_seq)	Ident.	NCBI Acc. No.	Identified as	Mortality	Antifungal category				
						B. c.	*F. o.*	*F. v.*	*S. r.*	*V. d.*
T3GI1	*Pseudomonas koreensis*	99%	NR_025228.1	*P. koreensis* T3GI1	97.84%	1	2	2	0	1
T1GI1	*Variovorax paradoxus*	99%	NR_113736.1	*V. paradoxus* T1GI1	96.19%	0	1	1	0	1
T13GI2	*Comamonas sediminis*	99%	NR_149789.1	*C. sediminis* T13GI2	93.77%	1	1	2	1	0
T8GH4	*P. monteilii*	100%	NR_114224.1	*P. monteilii* T8GH4	87.66%	0	1	0	0	0
T13GI4	*P. soli*	100%	NR_134794.1	*P. soli* T13GI4	86.68%	2	1	2	2	2
T13GI6b	*P. monteilii*	100%	NR_114224.1	*P. monteilii* T13GI6b	83.54%	0	1	2	0	1
T3R11	*Bacillus velezensis*	98%	NR_075005.2	*B.* cf. *velezensis* T3R11	1.9%	3	3	3	3	1
T17GI1	*Bacillus velezensis*	97%	NR_075005.2	*B.* cf. *velezensis* T17GI1	36.56%	3	3	3	3	1
T17GI2	*B. amyloliquefaciens*	99%	NR_117946.1	*B. amyloliquefaciens* T17GI2	3.47%	3	3	3	3	2
T7GH4a	*B. methylotrophicus*	99%	NR_116240.1	*B. methylotrophicus* T7GH4a	0%	3	3	3	3	0
T21GH5	*B. velezensis*	97%	NR_075005.2	*B.* cf. *velezensis* T17GI1	0%	3	3	3	3	1
T1R1	–	–	–	T1R1	6.52%	2	2	2	2	3
T1R12	–	–	–	T1R12	4.76%	2	2	2	0	3
T1R14	–	–	–	T1R14	4.11%	2	2	2	2	3
T2R18b	–	–	–	T2R18b	0%	2	2	2	2	3
T14R6	–	–	–	T14R6	3.07	2	2	2	1	3

Mortality: mean mortality of Meloidogyne incognita juveniles due to bacterial volatiles. Antifungal category: mean values for antagonism against Botrytis cinerea (B. c.), Fusarium oxysporum (F. o.), F. verticillioides (F. v.), Sclerotium rolfsii (S. r.), and Verticillium dahliae (V. d.), where 0, no antagonism; 1, hyphae do not overgrow colony; 2, inhibition zone <5 mm; and 3 = inhibition zone >5 mm. Values for nematode antagonists and fungal antagonists with five examples of V. dahliae-antagonistic strains.

by or within a mass of dead J2 individuals. Three more compounds showed nematicidal effects at 20 µl following incubation for 4 days, namely 2-isobutyl-3-methyl pyrazine (67.8%), 5-isobutyl-2,3-dimethyl pyrazine (44%), and 2-ethyl-3-methyl pyrazine (50.2%).

Bacterial Strains With Antagonistic Activity Toward Fungal Pathogens

Of 260 bacterial strains 72 showed a high antagonistic effect on at least one fungal pathogen tested *Botrytis cinerea*, *F. oxysporum*, *F. verticillioides*, *S. rolfsii*, and *V. dahliae* (**Figure 2A**). Strains showing a fungal antagonistic effect were mostly isolated from RE-H (n = 30 strains) rather than from RE-D (n = 14 strains) samples. Antagonists of *V. dahliae* were recovered only from the root adhering soil, or "rhizosphere." Further analysis was carried out only with strains showing antagonism toward at least four different plant pathogenic fungi (n = 23, **Figure 2A**), thus *Verticillium* antagonists were excluded. Five separate genotypes were revealed by analyzing the BOX-PCR patterns of the 23 strains that complied with this requirement. Sequencing the 16S rRNA gene of these strains identified them all as members of the *Bacillus amyloliquefaciens* complex, namely one strain of *B. amyloliquefaciens* (T17GI2), one strain of *B. methylotrophicus* (T7GH4), and three strains of *B. velezensis* (T3R11, T17GI1, T21GH5). None of these strains showed high antagonistic activity against *V. dahliae* (**Table 1**).

Deciphering the Tomato Microbiome and Surrounding Soil Microbiome

Physical soil composition was comparable at both sampling sites; soil type was loamy sand (**Table 3**). Amplicon libraries of different tomato microhabitats (rhizosphere; RE-H: rhizoendosphere healthy; RE-D: rhizoendosphere diseased) as well as from surrounding soil were analyzed. When taxa were compared at the genera level between all four microhabitats, a total of 1,379 taxa were found with 467 taxa present in all microhabitats. The rhizosphere presented the smallest number of microhabitat-specific taxa, while RE-H and RE-D had more microhabitat-specific than shared taxa (**Figure 2B**). When visualizing beta diversity with a PCoA-plot of Bray–Curtis dissimilarities, samples from the same sampling site did not cluster. Axis 1 (16.45% variation explained) showed no two-dimensional clustering of the samples in combination with axis 2 (12.96%) or axis 3 (8.11%), but samples clearly cluster habitat-specific with axis 2 and 3 (**Supplementary Figures S5A–C**). The combination of all three axes 1–3 shows a clear clustering of soil, rhizosphere and RE-D, while RE-H samples overlap with rhizosphere and RE-D (**Figure 3A**).

Shannon alpha diversity of the four microhabitats showed a high alpha diversity in soil and a decline in alpha diversity from rhizosphere to RE-H to RE-D (**Figure 3B**). Pairwise Kruskal–Wallis test for alpha diversity indices showed highly significant ($p < 0.01$) differences in alpha diversity between all microhabitats in most cases. Only rhizosphere and RE-H samples failed to differ significantly in all tested alpha diversity indices (**Figure 3B** and **Table 4**). Pairwise PERMANOVA test of beta diversity indices significantly differed between all microhabitats, except qualitative indices (Jaccard and unweighted UniFrac distance) in RE-H and RE-D samples (**Table 4**).

Amplicon analysis of the soil samples revealed a total of 763 taxa at the genus level in Luwero (site 1) and 782 in Namulonge (site 2), with 594 taxa shared between sites. The most abundant classes in soil were Alphaproteobacteria

TABLE 2 | SPME GC-MS: volatilome composition of nematicidal VOC-producing bacterial strains associated with root-knot nematode infection in Ugandan tomato.

Compounds	RI measured	RI NIST (mainlib)	NIST match	*P. koreensis* T3GI1	*V. paradoxus* T1GI1	*C. sediminis* T13GI2	*P. monteilii* T8GH4	*P. soli* T13GI4	*P. monteilii* T13GI6b	blank (NA)
Alkenes										
1-nonene	889	892	868	–	–	0.68	1.96	–	–	–
1-decene	989	993	933	–	–	0.78	0.94	–	–	–
1-undecene	1091	1093	932	1.2	–	47.12	68.43	17.83	28.45	–
E-1,4-undecadiene	1081	–	–	–	–	–	0.99	–	–	–
E-3-undecene	1085	1085	884	–	0.3	–	–	–	–	–
1-dodecene	1186	1193	913	–	–	0.38	0.6	–	–	–
1-tridecene	1286	1293	919	–	–	–	0.04	–	–	–
1,12-tridecadiene	1272	1279	871	–	–	–	0.08	–	–	–
Sulfuric compounds										
Methanethiol	–	464	976	4.45	5.39	2.03	1.03	2.27	1.13	–
Dimethyl sulfide	520	515	745	1.73	–	0.55	–	1.01	1.64	–
Dimethyl disulfide	733	740	968	70.43	81.69	44.44	14.19	73.6	39.01	25.38
Dimethyl trisulfide	959	972	926	0.62	2.88	–	–	–	6.58[a]	16.37
S-methyl propanethioate	784	785	781	–	1.44	–	2.25	–	–	–
2,3-dimercaptopropan-1-ol	835	–	680	1	–	–	–	–	–	–
2-me-2-methylthiobutan	836	847	755	–	–	–	0.17	–	–	–
Methyl thiolacetate	698	701	877	–	1.2	–	5.45	–	–	–
S-methyl 3-methyl-butanethioate	931	938	727	–	–	–	0.13	–	–	–
S-methyl ester octanethioic acid	1291	1293	809	–	–	–	0.16	–	–	–
Oxygen-containing compounds										
CO_2	–	–	999	2.92	2.66	1.83	1.43	2.54	3	–
Acetone	502	503	741	0.84	1.7	1	0.5	1.09	1.32	0.79
2-butanone	601	601	820	1.56	1.5	1.19	0.29	1.25	3.31	3.89
2-methyl butanal	656	659	838	–	–	–	–	–	2.03	7.82
3-methyl butanal	646	649	949	–	–	–	–	–	3.6	13.82
2-methyl butanol	728	736	739	–	0.27	–	–	–	–	–
3-methyl butanol	725	734	799	–	0.96	–	0.25	–	–	–
2,3-epoxybutane	510		602	–	–	–	–	0.17[a]	–	–
Benzaldehyde	951	958		–	–	–	–	–	9.93[a]	31.93
2-undecanone	1288	1291	949	–	–	–	1.11	–	–	–
3-methoxy-2,5-dimethyl pyrazine	1046	1054		14.95	–	–	–	–	–	–
Others										
Methyl (Z)-N-hydroxybenzene-carboximidate	899		813	0.15	–	–	–	–	–	–
Unidentified substance (Rt = 1.581)				0.16	–	–	–	–	–	–
Unidentified substance (Rt = 12.277)				–	–	–	–	0.24	–	–
(Total ion counts)/(total ion counts blank)				5.59	6.02	5.17	2.95	7.38	1.85	1

Values of compounds are percentage of area of ionic counts. RI, retention index for non-isothermal GC; NA, nutrition broth agar. For identification references, see ***Supplementary Table S1****; for mass spectra, see* ***Supplementary Table S2****.* [a]*Compound was not found in all repeats.*

(13.7%), Planctomycetacia (7.2%), and Bacilli (6.2%) (**Figure 4**). Caulobacterales, Cytophagales, Rhodocyclales, and Rhodospirillales are shared between soil and rhizosphere samples (**Figure 5**).

All plant-associated samples were dominated by Gammaproteobacteria. The classes most abundant in the rhizosphere were Gamma- (47.4%), Alpha- (19.8%), and Betaproteobacteria (11.6%) (**Figure 5**). The most abundant bacterial genera in the rhizosphere were *Pseudomonas* spp. (25.5%), unidentified Enterobacteriaceae species (17.2%) and *Sphingobium* spp. (8.2%). Endosphere samples mainly consisted of orders of the core microbiome (**Figure 5**).

Differential abundance analysis using Gneiss proved that the "habitat" category is the main driver of variance within our

TABLE 3 | Physical soil parameters at the two sampling sites.

Sampling site	Soil type	pH	K (mg/kg)	P (mg/kg)	Mg (mg/kg)	Org. matter (%)
Luwero	S'l	5.3	142	40	250	3.9
Namulonge	S'l	5.6	213	31	196	3.2

The two soils mainly differ in potassium concentration. S'l: loamy sand.

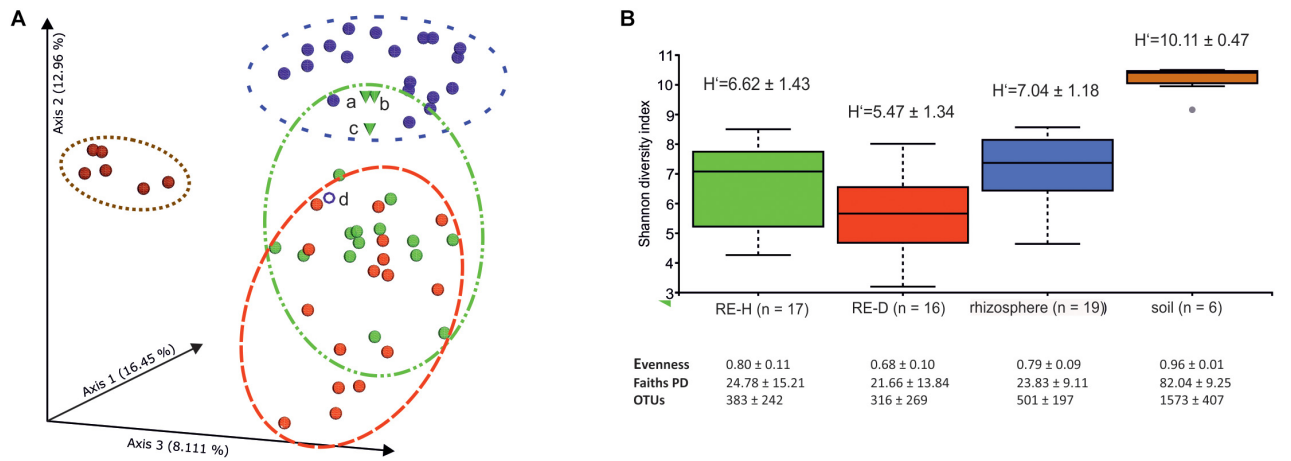

FIGURE 3 | (A) PCoA plot of Bray–Curtis dissimilarity between microhabitats. PCoA-plot shows clear clustering in soil (brown) in rhizosphere (blue) while healthy rhizoendosphere (green) sampling points (a-c) overlap with *Meloidogyne*-diseased rhizoendosphere (red) and rhizosphere; sample point d (rhizosphere) does not cluster with other rhizosphere samples and was removed in statistical analysis. **(B)** Barplots of Shannon diversity of microhabitats. Alpha diversity is highest in soil and lowest in diseased rhizoendosphere (galls). Alpha diversity index values are given.

TABLE 4 | *P*-values of diversity indices reveal significant microhabitat-specific bacterial community differences in root-knot nematode-diseased tomato roots.

	Alpha diversity indices				Beta diversity indices			
	Shannon	**Observed OTUs**	**Faith's PD**	**Evenness**	**Jaccard**	**Bray–Curtis**	**Unweighted Unifrac**	**Weighted UniFrac**
RE-H/RE-D	*0.028*	0.505	0.746	***0.002***	0.061	*0.022*	0.396	*0.013*
RE-H/R	0.350	0.084	0.788	0.579	***0.001***	***0.001***	***0.006***	***0.003***
RE-H/S	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***0.001***	***0.001***	***0.001***	***0.001***
RE-D/R	***0.002***	***0.004***	0.289	***0.001***	***0.001***	***0.001***	***0.001***	***0.001***
RE-D/S	***<0.001***	***0.001***	***<0.001***	***<0.001***	***0.001***	***0.001***	***0.001***	***0.001***
R/S	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***0.001***	***0.001***	***0.001***	***0.001***

P-values for Kruskal–Wallis and PERMANOVA results of alpha and beta diversity indices comparing microhabitats, significant (<0.05) p-values formatted italic, highly significant (p < 0.01) values formatted bold. RE-H, healthy rhizoendosphere; RE-D, Meloidogyne-diseased rhizoendosphere; R, rhizosphere; S, bulk soil.

dataset, changes of the microbiome composition through higher RGI were not significant. Enterobacteriaceae, Burkholderiaceae, Pasteuriaceae, and Rhizobiaceae were identified as the main drivers of microbiome changes in rhizoendosphere (**Supplementary Figures S6A–D**). When comparing core taxa (present in >75% of the samples) on family level, these families showed a strong abundance shift (**Figure 6**).

Linking the Microbiome With Antagonistic Strains

Antagonists differ in their overall abundance between microhabitats. *Bacillus* spp. had the highest mean abundance in RE-H (4.1%), *Variovorax* spp. in RE-D (1%), *Comamonas* spp. (0.3%), and *Pseudomonas* spp. (22%) across the 19 rhizosphere samples. Taxa showing >98% identity with 16S rRNA sequences of isolated antagonists were represented by 6% of the root endosphere microbiome (**Table 5**). Focusing on the three most abundant antagonistic genera (*Pseudomonas*, *Pasteuria*, *Bacillus*), no direct correlation between their abundance and RGI was detected (**Figure 7**). *Pseudomonas* was especially abundant in diseased plants with a RGI of 5, whereas *Pasteuria* showed highest abundance in moderately diseased (RGI = 3 ± 1) plants (**Figure 7**).

DISCUSSION

Bacteria and nVOC

In our comprehensive study of soil-borne diseases of tomato from Uganda, we discovered novel principles, which help to explain the disease complex and offer new potential strategies as to how to suppress them. Diseased tomatoes suffered from a multispecies infection of *Meloidogyne*. We clearly identified two species (*M. incognita*, *M. javanica*) but results indicate that more species are involved (Adam et al., 2007; Janssen et al., 2016).

Our novel VOC-based screening method toward RKN resulted in the identification of several nematicidal antagonists, namely *Comamonas sediminis*, *P. koreensis*, *P. monteilii*, *P. soli*, and *Variovorax paradoxus*. *P. koreensis* and *P. monteilii* have known potential for biocontrol against oomycetes (Hultberg et al., 2010a,b) and fungi in general through VOC, respectively (Dharni et al., 2014). In contrast, *P. soli* and *C. sediminis* have not previously been identified as potential biocontrol

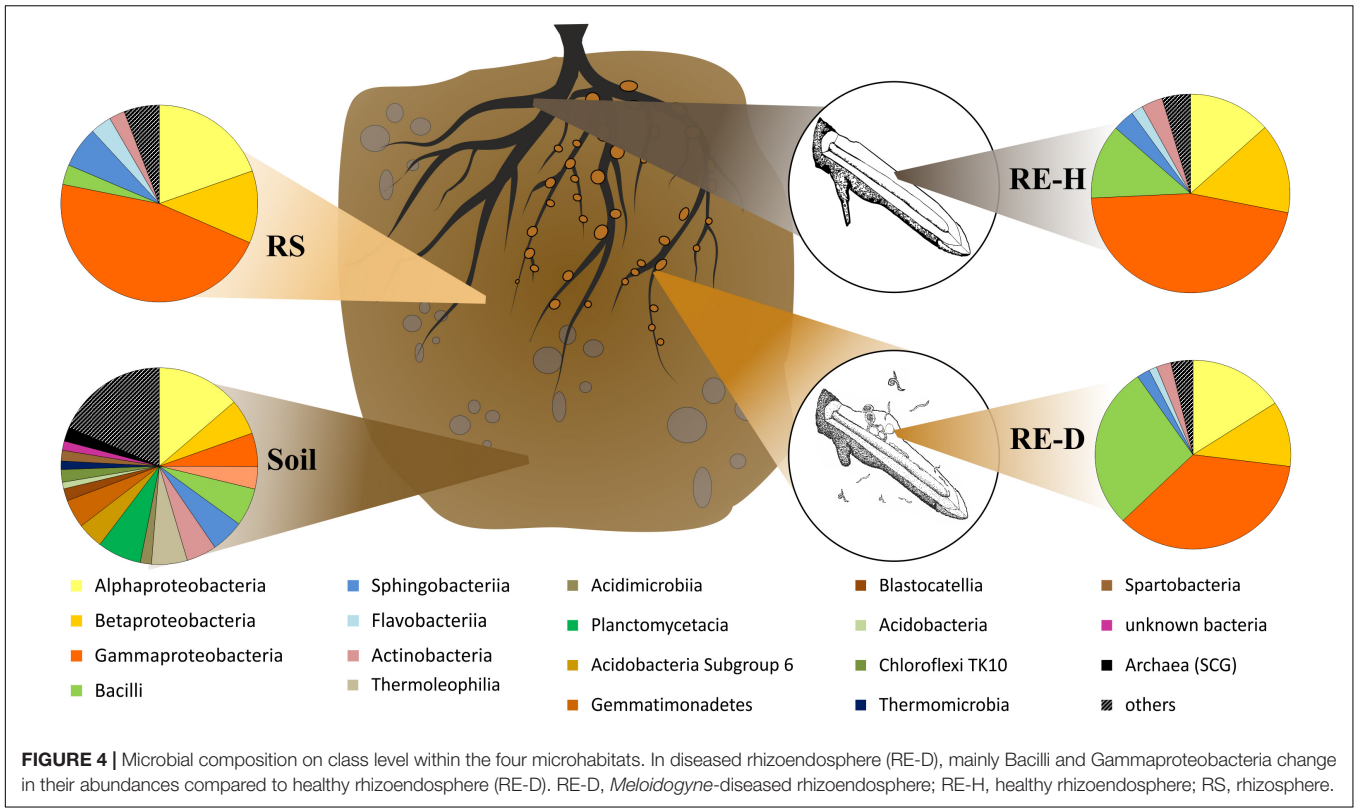

FIGURE 4 | Microbial composition on class level within the four microhabitats. In diseased rhizoendosphere (RE-D), mainly Bacilli and Gammaproteobacteria change in their abundances compared to healthy rhizoendosphere (RE-D). RE-D, *Meloidogyne*-diseased rhizoendosphere; RE-H, healthy rhizoendosphere; RS, rhizosphere.

candidates. However, the closely related *C. acidovorans* has shown antagonistic effects toward plant pathogens (El-Banna, 2007; Liu et al., 2007). *Variovorax paradoxus* has great potential for bioremediation, biotechnology (Satola et al., 2013), and plant-protection (Chen et al., 2013). *Variovorax* spp. and *Comamonas* spp. occurred in relatively low abundance in endosphere samples (**Table 5**). The question remains as to whether they can become sufficiently abundant to affect RKN through their nematicidal nVOC and support the plant indirectly, or if they inherit direct plant-promoting traits.

A considerable aspect of the cultivable bacteria isolated in our study demonstrated their negative impact on RKN by producing nVOC. VOC produced by the bacterial community – among other factors – may contribute to the overall suppressiveness toward RKN of different soils. This may also explain the efficacy of RKN management methods that promote bacterial growth and diversity, e.g., the use of soil amendments (Viaene et al., 2013). We identified six nVOC-producing strains and 72 potential fungal antagonists. However, our tests did not result in any single strain that controlled both RKN and phytopathogenic fungi to any great extent. Furthermore, individual antagonists tended to attain high abundances in different, separate microhabitats. Fungal antagonists were mainly isolated from, and abundant in, RE-H, as supported by the amplicon data, indicating an important role of these strains for host-plant protection. The strong antagonistic effects on fungal pathogens by members of the *B. amyloliquefaciens* complex is well-known (Chowdhury et al., 2015). The strains found in our study did not effectively inhibit growth of *V. dahliae* in dual cultures, although *in vitro* antagonism toward *V. dahliae* was been reported for other *Bacillus* strains (Danielsson et al., 2007). The antimicrobial activity of their metabolites is well-studied, showing that induced systemic resistance (ISR) and antimicrobial metabolite production are their main mechanism of plant protection (Chowdhury et al., 2015). Our findings exclude nVOC of *Bacillus* strains as a controlling component for RKN, though they may have a repellent effect *in vitro*. We question that *Bacillus* spp. in the root endosphere are enriched from surrounding soil, because *Bacillus* spp. are a part of the core microbiome of the tomato seeds itself (Bergna et al., 2018). In this case, susceptibility of tomatoes toward diseases may also be a consequence of declining bacterial diversity through breeding practices or the inability to establish native antagonists.

One component of our most effective nVOC-producing strain was a pyrazine, which have known antimicrobial effects (Haidar et al., 2016; Kusstatscher et al., 2017), we therefore focused on different pyrazines in the single compound test. We found that the effect of pyrazines on RKN appears dependent on their functional groups. Although all pyrazines were not as effective as 2-undecanone, they may enhance the nematicidal effect of the total volatilome of *P. koreensis* T3GI1. Alkenes were consistently found in high concentrations in the nVOC spectra but they showed no nematicidal effect. This may be a result of their hydrophobicity, as J2 were protected by a thicker water layer in the single compound test, than in the modified TCVA-screening. Therefore, hydrophobic compounds would be less able to affect the nematode cuticle. We also found several sulfuric compounds with strong odors (e.g., dimethyl sulfide, octanethioic acid

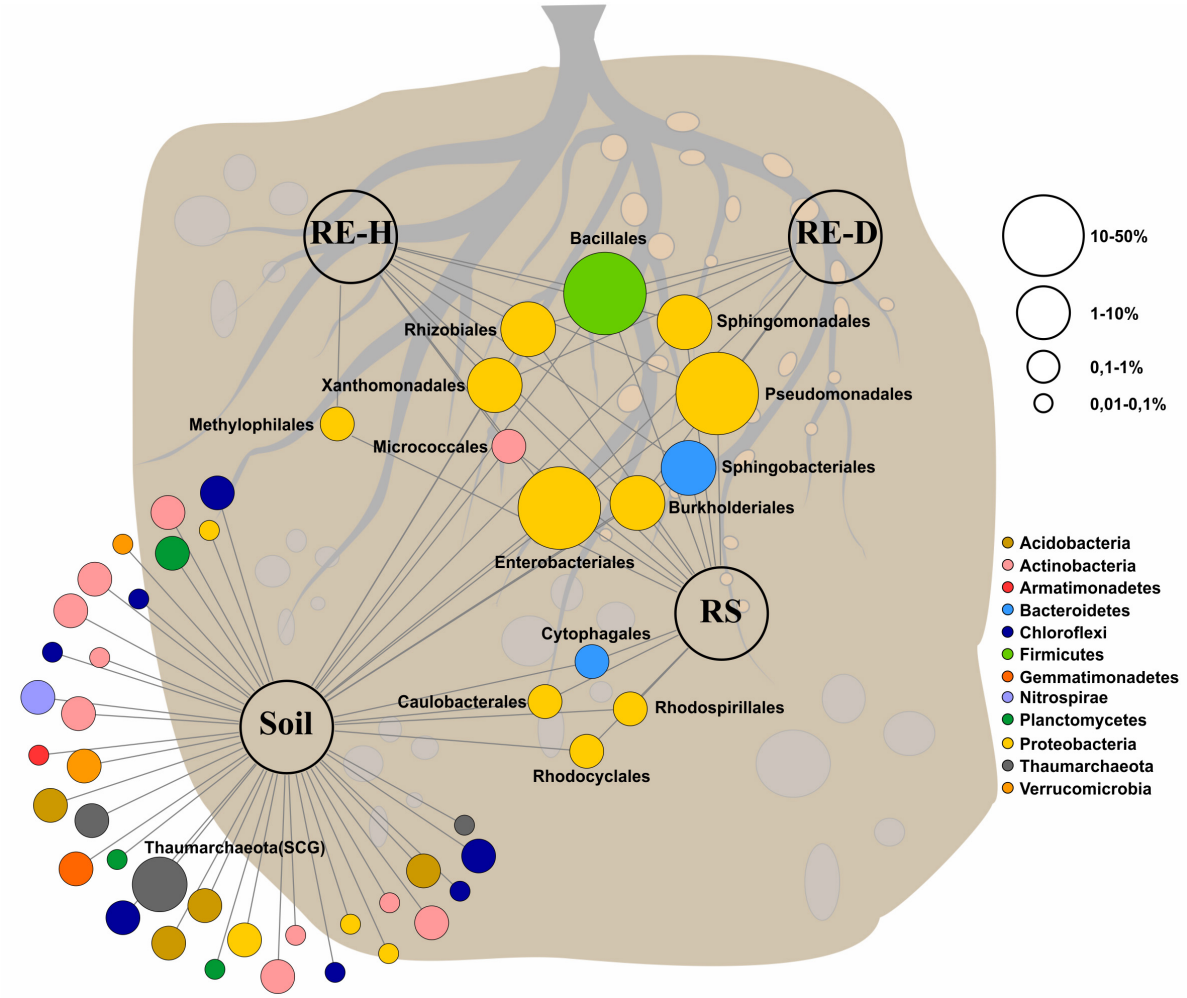

FIGURE 5 | Feature network of core taxa (≥80% of samples) at class level (>1% relative abundance) found within the four microhabitats. Bacterial community in soil is very diverse, all endosphere taxa belong to the core microbiome, Methylophilales are <1% abundant in diseased endosphere. Core microbiome covers around 90% of all abundant orders except for soil (soil: 68.9%; rhizosphere: 92.7%; healthy rhizoendosphere/RE-H: 91.27%; *Meloidogyne*-diseased rhizoendosphere/RE-D: 91.24%).

S-methylester). Nematicidal properties may mostly arise from dimethyl disulfide, which is an effective nematicide (Gómez-Tenorio et al., 2015) and was the main volatilome component in all strains except *P. monteilii* T8GH4 in our assessment. Sulfur is generally regarded to be in low concentrations in tropical soils (Blair et al., 1980). As elemental sulfur is known to reduce RKN densities in non-sterile soil (Rumiani et al., 2016), VOC of the microbial sulfur metabolism may enhance RKN control. Although our study revealed the potential of VOC in suppressing multispecies plant diseases, in order to be able to translate this concept into plant protection a greater understanding and more research is needed.

The Microbiome of *Meloidogyne* Disease Complexes

Our study provides the first in-depth analysis of the influence of RKN on the bacterial community under field conditions. Results of Tian et al. (2015), which studied microbiomes of RKN-diseased tomatoes under controlled, greenhouse conditions differ heavily from our study in terms of the dominating taxa, microbiome composition, and microbiome shift due to RKN infection. These differences are not entirely surprising, however, given that our study was performed on plants removed from fields following natural infection by RKN, compared with the controlled pot environment and artificial inoculation of RKN by Tian et al. (2015). Some of this variance may also be due to cultivar differences (Rybakova et al., 2017) but which is not expected to be apparent at this taxonomic level. Infection with nematodes was correlated with a strong bacterial community shift in tomato roots, with a microbiome from healthy plants differing from infected roots, even though this was not necessarily dependent upon the RGI. Regarding the beta diversity, only quantitative indices revealed significant differences between RE-D and RE-H. Thus, nematode feeding site (NFS) induction

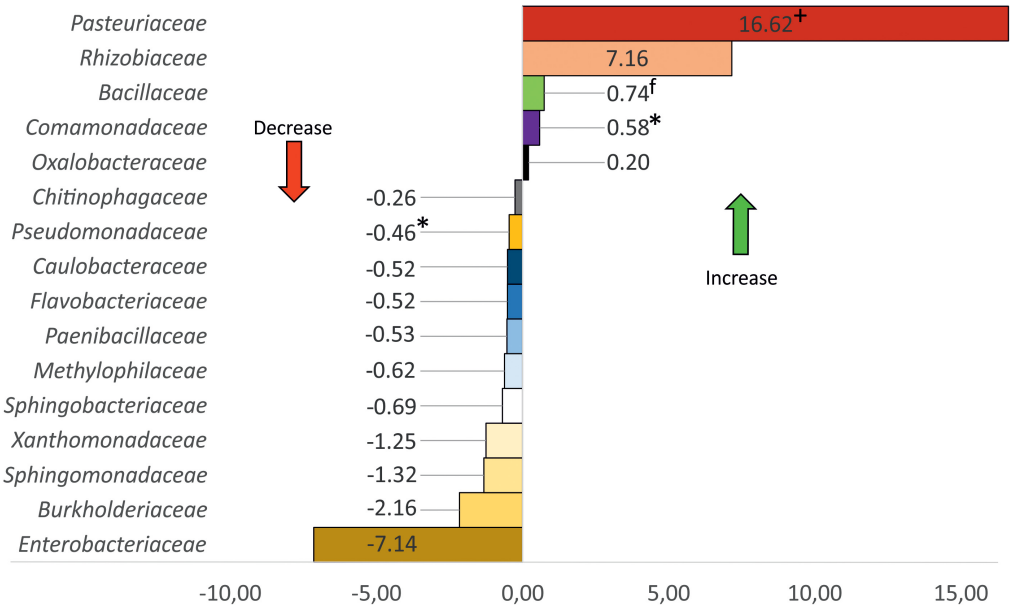

FIGURE 6 | Changes of relative abundance of bacteria families due to root-knot nematode (*Meloidogyne* spp.) infection. Only families with >1% relative abundance shown. +, obligate nematode parasites; f, fungi antagonists; *, nematode antagonists found in this study.

TABLE 5 | Relative abundance of taxa most similar to 16S rRNA sequences of isolated bacterial antagonists in different microhabitats.

Relative abundance of antagonists	Ident. (%)	n(taxa)	Soil	Rhizosphere	RE-H	RE-D
Best hit *Pseudomonas koreensis* T3GI1	98.97	7	0	0.176	0.189	0.000
Pseudomonas putida-group	>98	18	0.331	23.295	5.515	5.338
Best hit *P. monteilii* T8GH4	98.97		0	0.335	0.131	0.084
Best hit *P. soli* T13GI4	98.63		0.031	1.691	0.069	0
Best hit *P. monteilii* TT13GI6b	98.97		0.044	2.828	0.185	0
Variovorax	>98	5	0.052	0.677	0.538	1.060
Best hit *V. paradoxus* T1GI1	98.97		0.008	0.084	0.046	0
Comamonas	>98	4	0	0.138	0.033	0.017
Best hit *C. sediminis* T13GI2	98.97		0.025	0	0.004	0
nVOC antagonists total	>98		0.382	23.972	6.053	6.398
Pasteuria	>98	7	0.002	0.118	3.115	17.948
Best hit *P. penetrans* (AF077672.1)	98.97		0	0	0.136	1.104
Bacillus amyloliquefaciens-group	>98	6	0.008	0.141	0.286	0.128
Best hit *B. amyloliquefaciens* s. lat.	99.32		0	0	0.007	0

n(taxa) refers to the number of taxa with >98% identic 16S rRNA sequences. RE-H, rhizoendosphere healthy; RE-D, Meloidogyne-diseased rhizoendosphere.

would appear to have a greater impact on the abundance of bacterial taxa that are present and highly abundant in both RE-D and RE-H (**Figure 6**), rather than the microhabitat-specific colonization pattern of low-abundant taxa (**Figure 2B**). A higher diversity of endophytes due to RKN infection (Tian et al., 2015) could not be confirmed; cultivable bacteria and habitat-specific taxa were more abundant in RE-H than in RE-D. Further, RE-H and rhizosphere did not significantly differ in alpha diversity indices. This appears to be extraordinary: the rhizosphere is regarded as a biodiversity hotspot and root endosphere diversity in tomato was found to be lower than in rhizosphere. Nevertheless, both rhizosphere and to a lesser extent root endosphere diversity are influenced by the surrounding soil (Bergna et al., 2018). Since tomato are not indigenous to Uganda, the rhizoendophytic alpha diversity may be raised because of the combination of tomato core microbiota and an uptake of native soil bacteria. Interestingly, alpha diversity in RE-D is significantly lowered compared to RE-H despite comparable numbers of species. Therefore, RKN may favor roots with lower microbiome diversity for NFS selection. Diversity of endophytic microbiota is regarded as a key factor for plant health (Berg et al., 2017) but RGI and Shannon diversity do not clearly correlate with healthy and diseased roots (data not shown). Furthermore, RE-D microbiomes of moderately damaged plants

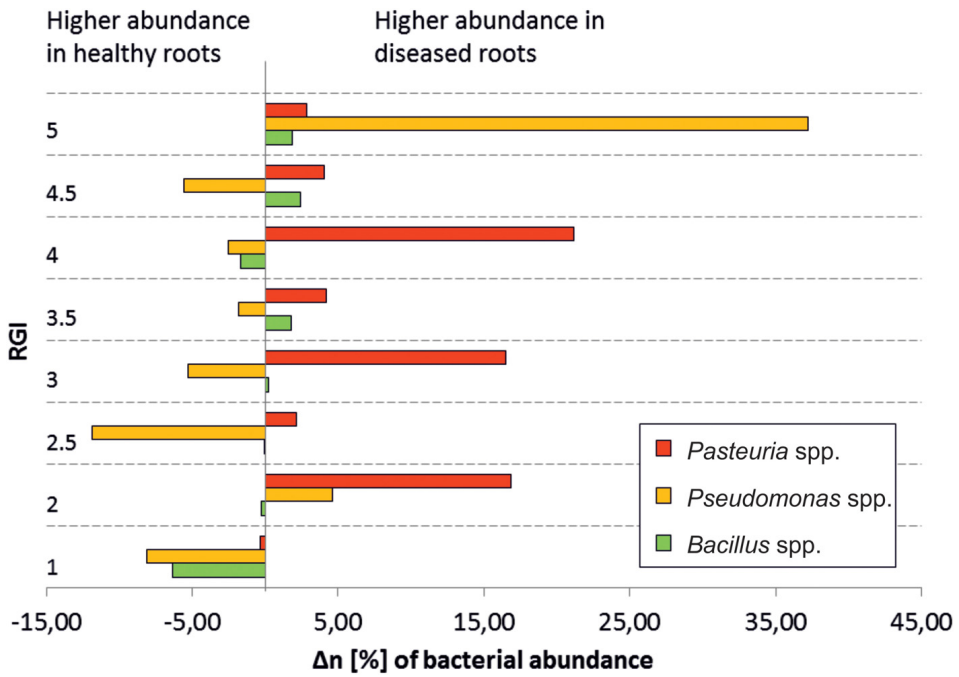

FIGURE 7 | Abundance of selected nematode-antagonistic genera in relation to severity of *Meloidogyne*-infection. ΔrA, difference of relative abundances, = abundance (diseased root) – abundance (healthy root); RGI, root galling index; 1, symptomless; 5, severely damaged roots; antagonistic genera are generally higher in healthy roots, except for obligate nematode parasites *Pasteuria* spp. The high abundance of *Pseudomonas* in the RG5 plant may be due to saprophytic species, which already degrade the plant material.

(RGI 2–3.5) show a more asymmetric composition with fewer but more dominant taxa than the severely damaged plants (RGI = 4.5–5). This may be due to delayed establishment of slower growing saprophytic or commensal species, although, Tian et al. (2015) also found a specific enrichment of some bacterial groups in the NFS, which indicated specific association of these groups with the NFS and nematode pathogenesis. Our results indicate that the changed physiological conditions of plant cells at the NFS is responsible for microbiome changes. The microbial community within the NFS is influenced by microbes that are able to adhere to the nematode cuticle (Elhady et al., 2017). This is most obvious when looking at the abundance of obligate parasites of plant pathogenic nematodes, such as *Pasteuria* spp. within RE-D samples (six samples with >20% relative abundance). Their abundance did not correlate with RGI (**Figure 7**), maybe because it is dependent on a successful transportation adhered to the cuticle of RKN to the NFS. The increase of Rhizobiaceae in RKN galls seems to be a constant effect (Hallmann et al., 2001; Tian et al., 2015). There are three possible explanations: (i) Rhizobiaceae have a preference to attach to the nematode surface during soil migration, as reported for *Neorhizobium* (Elhady et al., 2017); (ii) it is a side effect of NFS induction, since RKN manipulate the gene expression of plant hormones and nodulation factors (Gheysen and Fenoll, 2002; Jones et al., 2013); (iii) it contributes to a defense reaction of the host plant, since Rhizobiaceae are known to closely interact with plants. The latter two hypotheses are connected to the regulation of plant flavonoids, which have several important functions in the plant-nematode interaction (Hutangura et al., 1999; Weston and Mathesius, 2013) and there is evidence that RKN counteract the effects of *Rhizobium*, since RKN reduce nodulation in legumes (Kimenju et al., 1999). Enterobacteriaceae and Burkholderiaceae were the families with the highest negative shift in abundance in RE-D. Whether there was a lower abundance at the NFS beforehand, or this was lowered following RKN infection remains unclear. However, Enterobacteriaceae and Burkholderiaceae appear to be less competitive following the physiological and physical changes that occur as galls develop in the roots. Due to the high abundance of Enterobacteriaceae (31.8%) and Burkholderiaceae (5.3%) in RE-H their abundance shift is most obvious. Still, when examining the abundance changes separately, the percentage change in abundances is higher in low-abundant taxa, such as Caulobacterales or Methylophilales (**Supplementary Table S3**).

Managing the RKN disease complexes through biocontrol requires a detailed knowledge on the antagonists and their effects. Since antagonistic strains of bacteria are here shown to prefer different microhabitats, they would likely affect RKN at different stages of their life cycle, which would indicate the need for a more holistic consortia of biological control agents. Further, some bacterial strains are known to impact on multiple targets, such as both fungi and RKN (Adam et al., 2014). As we did not observe this in the current study, we hypothesize that the fungal antagonists in our study would affect RKN with alternative mode of action than with nVOC.

CONCLUSION

Our results indicate that VOC and plant-associated microbial diversity offers promise for RKN-defense management. Based on our data, we suggest three methods for RKN-control: (i) application of a consortia containing bacterial, fungi and nematode antagonists; (ii) application of sulfur-containing fertilizers to enhance sulfur-containing VOC in the rhizosphere for J2-reduction; (iii) screening and application of Rhizobiaceae-strains that produce nematicidal metabolites to take advantage of their increased abundance in galls. The combination of different management methods can lead to synergistic beneficial effects in tropical climates (Viaene et al., 2013). Implementing environmentally sensitive biocontrol strategies in agricultural programs, especially on smallholder farms, is an alternative to the harmful and often unspecific toxic biocides, toward preserving the stability and diversity of macro- and microhabitats. It would also help alleviate agriculture-related health issues, hunger and social conflicts while simultaneously providing economic and nutritional needs of the local people.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available in the European Nucleotide Archive (ENA) under project no. PRJEB28436 under the accession numbers ERS2856266–ERS2856324. Reference sequences of *Meloidogyne incognita* (KJ476151.1), *M. javanica* (KP202352.1), *M. arenaria* (KP202350.1), *M. ethiopica* (KU372360), and *M. chitwoodi* (KJ476150.1) are publicly available at the NCBI database (https://www.ncbi.nlm.nih.gov/) under the corresponding accession numbers.

AUTHOR CONTRIBUTIONS

DC, GB, and AW designed the study. AW, JT, DC, RG, and GB performed the sample process. AW and JT analyzed the data. AW and GB wrote the manuscript. All authors improved and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01296/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
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Pochonia chlamydosporia Induces Plant-Dependent Systemic Resistance to *Meloidogyne incognita*

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Meloidogyne spp. are the most damaging plant parasitic nematodes for horticultural crops worldwide. *Pochonia chlamydosporia* is a fungal egg parasite of root-knot and cyst nematodes able to colonize the roots of several plant species and shown to induce plant defense mechanisms in fungal-plant interaction studies, and local resistance in fungal-nematode-plant interactions. This work demonstrates the differential ability of two out of five *P. chlamydosporia* isolates, M10.43.21 and M10.55.6, to induce systemic resistance against *M. incognita* in tomato but not in cucumber in split-root experiments. The M10.43.21 isolate reduced infection (32–43%), reproduction (44–59%), and female fecundity (14.7–27.6%), while the isolate M10.55.6 only reduced consistently nematode reproduction (35–47.5%) in the two experiments carried out. The isolate M10.43.21 induced the expression of the salicylic acid pathway (*PR-1* gene) in tomato roots 7 days after being inoculated with the fungal isolate and just after nematode inoculation, and at 7 and 42 days after nematode inoculation too. The jasmonate signaling pathway (*Lox D* gene) was also upregulated at 7 days after nematode inoculation. Thus, some isolates of *P. chlamydosporia* can induce systemic resistance against root-knot nematodes but this is plant species dependent.

Keywords: *Cucumis sativus*, induced resistance, root endophytes, root-knot nematodes, *Solanum lycopersicum*

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INTRODUCTION

The root-knot nematodes (RKN), *Meloidogyne* spp., are obligate parasites of plants. The genus comprises more than 100 species, but only four of them are considered the most damaging plant parasitic nematodes due to its wide range of plant hosts, worldwide distribution, and high reproductive capacity (Jones et al., 2013). The RKN infective juveniles (J2) enter the root near the elongation zone and migrate intercellularly to establish a permanent feeding site into the vascular cylinder, inducing the formation of giant cells and root galls by affecting cell wall architecture, plant development, defenses, and metabolism (Shukla et al., 2018). Once the infection occurs, J2 become sedentary, and molt three times to achieve the mature adult female stage. The most frequent and damaging tropical species, *M. arenaria*, *M. incognita*, and *M. javanica*, reproduce parthenogenetically.

The female lays a large number of eggs in a gelatinous matrix, the egg mass, located on the surface or within the galled roots.

The damage potential of some *Meloidogyne* species has been summarized (Greco and Di Vito, 2009; Wesemael et al., 2011). *M. arenaria*, *M. incognita* and *M. javanica* are responsible for the majority of vegetable yield losses caused by plant parasitic nematodes (Sikora and Fernández, 2005). Among vegetables, those belonging to the solanaceae and cucurbitaceae families are commonly included in rotation schemes because they are economically important for growers. The estimation of maximum crop yield losses caused by the nematode in field and plastic greenhouse cultivation varies according to the plant germplasm, environmental conditions and agronomic practices. For instances, maximum yield losses from 62 to 100% have been reported in susceptible tomato cultivars (Seid et al., 2015; Giné and Sorribas, 2017), 30 to 60% in aubergine (Sikora and Fernández, 2005), 50% in cantaloupe (Sikora and Fernández, 2005), 37 to 50% in watermelon (Sikora and Fernández, 2005; López-Gómez et al., 2014), and 88% in non-grafted or grafted cucumber on *Cucurbita* hybrid rootstocks (Giné et al., 2014, 2017). Control of RKN is conducted mainly with fumigant and non-fumigant nematicides (Djian-Caporalino, 2012; Talavera et al., 2012). However, due to environmental and toxicological concerns, some legislative regulations, such as the European Directive 2009/128/EC, aim to reduce the use of pesticides by promoting alternative methods such as biological control and plant resistance.

Several nematode antagonists belonging to different taxonomic groups have been described (Stirling, 2014). They can act in three ways: (1) directly parasitizing several or specific RKN development stages, such as *Pasteuria penetrans* (Davies et al., 2011); (2) indirectly by repelling, immobilizing and/or killing them by means of metabolites, and/or inducing plant response, such as *Fusarium oxysporum* strain Fo162 (Dababat and Sikora, 2007a,b); or (3) both directly and indirectly, such as *Trichoderma atroviride* strain T11 or *Trichoderma harzianum* strain T-78 (de Medeiros et al., 2017; Martínez-Medina et al., 2017). *Pochonia chlamydosporia* (syn. *Metacordyceps chlamydosporia*) is a fungal antagonist of RKN and cyst nematodes that acts directly by parasitizing eggs, and could also acts indirectly. This fungal species colonizes endophytically the root of several plants, including barley (Maciá-Vicente et al., 2009), tomato (Bordallo et al., 2002), potato (Manzanilla-López et al., 2011), or *Arabidopsis* (Zavala-Gonzalez et al., 2017), inducing plant defense mechanisms, such as the formation of papillae (Bordallo et al., 2002) and the modulation of miRNA in tomato (Pentimone et al., 2018) or plant defense genes related to salicylic acid and jasmonic acid pathways in barley and *Arabidopsis* (Larriba et al., 2015; Zavala-Gonzalez et al., 2017). It is assumed that some of these defense mechanisms could suppress root infection, development and/or reproduction of RKN (Escudero and Lopez-Llorca, 2012), but as far we know, only one study has proven the induction of local resistance (de Medeiros et al., 2015), and none to elucidate the capability of this fungal species to induce systemic resistance. As *P. chlamydosporia* parasitizes RKN eggs, a split-root system is required to determine the capability of this nematophagous fungus to induce plant resistance avoiding the direct interaction with the nematode. Therefore, in the present study, the capability of five *P. chlamydosporia* isolates to induce plant resistance against *M. incognita* was assessed in a split-root system. To assess whether the response was plant dependent, tomato and cucumber were used as representatives of solanaceous and cucurbit crops frequently including in rotation schemes.

MATERIALS AND METHODS

Plant Material, Nematode, and Fungi

Tomato cv. Durinta and cucumber cv. Dasher II were used in this study. For all the experiments, seeds were surface-sterilized in a 50% sterilized bleach solution (35 g L^{-1} active chlorine) for 2 min, washed three times in sterilized distilled water for 10 s each, sown in a tray containing sterile vermiculite, and maintained in a growth chamber at 25°C ± 2°C with a 16 h:8 h (light:dark) photoperiod.

Five *P. chlamydosporia* isolates M10.41.42, M10.43.21, M10.51.3, M10.55.6, and M10.62.2 were used. The fungal isolates were obtained from horticultural commercial growing sites in northeastern Spain from RKN eggs (Giné et al., 2012) and maintained as single-spore isolates at the Universitat Politècnica de Catalunya. Fungal chlamydospores were produced in barley seeds following the procedure of Becerra and collaborators (Becerra Lopez-Lavalle et al., 2012) with some modifications. Briefly, for each isolate, three 200 g batches of barley seeds were soaked for 18 h and each batch sterilized in an Erlenmeyer flask at 121°C for 22 min over two consecutive days, then were incubated at 25°C ± 2°C in the dark. Afterward, 10 5-mm plugs from the edge of each *P. chlamydosporia* isolate grown in CMA were added to each Erlenmeyer flask and they were shaken at 5-day intervals to homogenize fungal growth. After a month, the number of chlamydospores produced on barley was determined following the procedure of Kerry and Bourne (2002). Three 10-seed subsamples per Erlenmeyer were plated onto CMA and incubated at 25 ± 2°C in the dark for 2 weeks to assess putative contaminations prior to being used. The viability of the chlamydospores was assessed as in Escudero et al. (2017).

J2 of the isolate Agropolis of *Meloidogyne incognita* were used as inoculum. Eggs were extracted from tomato roots by blender maceration in a 5% commercial bleach (40 g L^{-1} NaOCl) solution for 5 min (Hussey and Barker, 1973). The egg suspension was passed through a 74-µm aperture sieve to remove root debris, and eggs were collected on a 25-µm sieve and placed on Baermann trays (Whitehead and Hemming, 1965) at 25 ± 2°C. Nematodes were collected daily using a 25-µm sieve for 7 days and stored at 9°C until their use.

Induction of Systemic Plant Resistance by *P. chlamydosporia* Isolates Against Root-Knot Nematodes

Tomato and cucumber were grown in a split-root system as described in previous studies (de Medeiros et al., 2017; Martínez-Medina et al., 2017). In this system, the root is divided into

two halves transplanted in two adjacent pots: the inducer, inoculated with the antagonist, and the responder, inoculated with the nematode. Briefly, the main root of 5-day-old seedlings was excised and plantlets were individually transplanted in seedling trays containing sterile vermiculite and maintained under the same conditions for 2 weeks for cucumber, and 3 weeks for tomato plants. Afterward, plantlets were transferred to the split-root system by splitting roots into two halves planted in two adjacent 200 cm^3 pots filled with sterilized sand. Four treatments were assessed for each fungal isolate: Fungi-RKN and None-RKN, to assess the capability of each fungal isolate to induce plant response against RKN, and Fungi-None and None-None, to assess the effect of each fungal isolate on plant growth. Each treatment was replicated 10 times, and the experiment was conducted two times. The inducer part of the root of the treatments containing Fungi was inoculated with 10^5 viable chlamydospores of *P. chlamydosporia* just before transplanting. One week later, the responder part of the root of the treatments containing RKN was inoculated at a rate of 1 J2 per cm^3 of soil. The treatment None-None, with no inoculation with either fungi or RKN received the same volume of water. The plants were maintained in a growth chamber in the same conditions described previously in a completely randomized design for 40 days. The plants were irrigated as needed and fertilized with Hoagland solution twice per week. Soil temperatures were recorded daily at 30-min intervals with a PT100 probe (Campbell Scientific Ltd) placed in the pots at a depth of 4 cm. At the end of the experiments, both inducer and responder root fresh weight and the shoot dry weight of each single plant were measured. Roots from the RKN-inoculated responder were immersed in a 0.01% erioglaucine solution for 45 min to stain the egg masses (Omwega et al., 1988) before counting them. Afterward, the eggs were extracted from the roots as in Hussey and Barker (1973)'s method and counted. The number of egg masses was considered as the infective capability of the nematode because it indicates the number of J2 able to penetrate, to infect the root, and to develop into egg-laying females. The number of eggs was considered the reproductive capability of the nematode, and the female fecundity was calculated as the number of eggs per egg mass.

The tomato and cucumber root colonization by each fungal isolates was estimated by quantifying the fungal DNA by qPCR at the end of the second experiment. The inducer part of the root was washed three times in sterilized distilled water for 10 s each and then blotted onto sterile paper. Per each fungal isolate three biological replicates were assessed. Each biological replicate consisted of the inducer part of the roots from three plants pooled together. The DNA was extracted from each biological replicate following the Lopez-Llorca et al. (2010)'s procedure. qPCR reactions were performed using the FastStart Universal SYBR Green Master (Roche) mix in a final volume of 25 µl containing 50 ng of total DNA and 0.3 µM of each primer (5′ to 3′ direction) VCP1-1F (CGCTGGCTCTCTC ACTAAGG) and VCP1-2R (TGCCAGTGTCAAGGACGTAG) (Escudero and Lopez-Llorca, 2012). Negative controls containing sterile water instead of DNA were included. Reactions were performed in duplicate in a Stratagene Mx3005P thermocycler (Agilent Technologies) using the following thermal cycling conditions: initial denaturation step at 95°C for 2 min, then 40 cycles at 95°C for 30 s, and 62°C for 30 s. Genomic DNA dilutions of the fungal isolate M10.43.21 were used to define a calibration curve from 5 pg to 50 ng. After each run, the specificity of the PCR amplicons was verified by melting curve analysis and agarose gel electrophoresis. The fungal DNA biomass of each isolate was referred to the total DNA biomass (50 ng) and expressed as a proportion.

Dynamic Regulation of the Jasmonic and Salicylic Acid Pathways by *P. chlamydospora* and *M. incognita*

Tomato seeds were sterilized as previously described. Three-week-old tomato seedlings were transferred to 200 cm^3 pots with sterilized sand and maintained in a growth chamber as previously described. The fungal isolate M10.43.21 was selected for this experiment because it reduced nematode infectivity and reproduction in the split-root system experiments. The experiment consisted of two treatments: non-inoculated and co-inoculated to determine the expression of genes related to the salicylic acid and jasmonic acid pathways. In the co-inoculated treatment, the soil was inoculated with 10^5 viable chlamydospores just before transplanting and with 1 J2 of *M. incognita* per cm^3 1 week after transplanting. Each treatment was replicated 40 times. An additional treatment only inoculated with the nematode was included to determine the effect of the fungal isolate on nematode reproduction. The fungus and nematode inoculation procedure was as previously stated.

The expression of the pathogenesis-related protein 1 (*PR-1*) gene and the lipoxygenase (*Lox D*) gene the from the salicylic acid and jasmonic acid pathways, respectively, was evaluated at three time points: just after nematode inoculation, that is, at 0 days after nematode inoculation (dani), at 7 dani, and at 42 dani. At each assessment time, roots were washed three times with sterile distilled water, placed onto sterilized filter paper, frozen in liquid nitrogen and stored at −80°C until being used. At 7 dani, the J2 were stained inside roots with acid Fuchsin following the Byrd et al. (1983) procedure to confirm that nematode had penetrated and infected. At the end of the experiment (42 dani), the number of eggs per plant from three plants for each treatment was determined by extracting them as described previously.

Total RNA from roots was isolated using the PureLink RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Afterward, the DNA-free kit (Invitrogen) was used to remove the remaining DNA from the sample. Total RNA integrity and quantity of the samples were assessed by means of agarose gel, NanoDrop 1000 Spectrophotometer (Thermo Scientific) and Qubit RNA BR assay kit (Thermo Fisher Scientific). To assure that the sample was DNA free, a PCR was carried out. Then, the RNA was retro-transcribed with the SuperScript II (Invitrogen) according to manufacturer's instructions. The relative gene expression was estimated with the ΔΔCt methodology (Livak and Schmittgen, 2001), using the ubiquitin (*UBI*) gene as a reference gene (Song et al., 2015).

Primers used in the RT-qPCR were (5′ to 3′ direction): LeUbi3-F (TCCATCTCGTGCTCCGTCT), LeUbi3-R (GAACCTTTCCA GTGTCATCAACC) Song et al. (2015), LoxD-F (GACTGGTCCA AGTTCACGATCC), LoxD-R (ATGTGCTGCCAATATAAAT GGTTCC) Fujimoto et al. (2011), LEPR1F (GCAACAC TCTGGTGGACCTT), and LEPR1R (ATGGACGTTGTCCTC TCCAG) Gayoso et al. (2007). qPCR reactions were performed in a final volume of 20 µl with 1 µl of cDNA, 0.3 mM of the corresponding primers, and 1X Fast SYBR Green Master Mix (Applied Biosystems). The qPCR was performed in a 7900HT Fast Real Time PCR System thermocycler (Applied Biosystems) using: 20 s at 95°C followed by 40 cycles of 30 s at 95°C and 60 s at 60°C (Gayoso et al., 2007). The specificity of PCR amplicons was verified as described previously. Reactions were performed with three biological replicates per treatment. Each biological replicate consisted of the roots from three plants pooled together. Two technical replicates per biological replicate were assessed.

Statistical Analysis

Statistical analyses were performed using the JMP software v8 (SAS institute Inc., Cary, NC, USA). Both data normality and homogeneity of variances were assessed. When confirmed, a paired comparison using the Student's *t*-test was done. Otherwise, paired comparison was done using the non-parametric Wilcoxon test, or multiple comparison using the Kruskal-Wallis test and groups separated by Dunn's test ($p \leq 0.05$). The repetitions of the split-root experiments for each crop were compared using the non-parametric Wilcoxon test, and considered as one experiment when no differences ($p \leq 0.05$) were found.

TABLE 1 | Capability of *P. chlamydosporia* isolates to induce systemic resistance in tomato cv. Durinta or cucumber cv. Dasher II against *Meloidogyne incognita* in two split root experiments.

Crop-experiment	Fungal isolate	Egg masses per plant	Eggs (×10^3) per plant	Eggs per egg mass
Tomato – 1	M10.41.42	110 ± 14	56.6 ± 8.3	517 ± 41
	M10.43.21	67 ± 16*	29.5 ± 7.3*	445 ± 41*
	M10.51.3	91 ± 5	45.1 ± 3.2*	500 ± 27*
	M10.55.6	82 ± 9*	38.0 ± 6.1*	506 ± 102
	M10.62.2	103 ± 8	44.3 ± 2.4*	434 ± 16*
	Non-inoculated	118 ± 5	72.4 ± 3.3	615 ± 31
Tomato – 2	M10.41.42	71 ± 4	62.1 ± 4.4	876 ± 37
	M10.43.21	57 ± 7*	44.4 ± 5.3*	812 ± 68*
	M10.51.3	65 ± 7	58.9 ± 8.1	881 ± 40
	M10.55.6	62 ± 9	51.3 ± 7.6*	831 ± 28*
	M10.62.2	68 ± 5	60.4 ± 3.8	897 ± 38
	Non-inoculated	84 ± 7	79.3 ± 7.1	952 ± 41
Cucumber – 1 and 2	M10.41.42	52 ± 6	24.9 ± 4.3	459 ± 42
	M10.43.21	52 ± 5	24.7 ± 3.0	464 ± 22
	M10.51.3	54 ± 6	26.1 ± 3.6	466 ± 28
	M10.55.6	53 ± 7	25.4 ± 5.2	463 ± 40
	M10.62.2	51 ± 6	24.8 ± 4.4	458 ± 39
	Non-inoculated	46 ± 6	20.5 ± 4.2	422 ± 45

The inducer part of the root was inoculated with 10^5 chlamydospores of the fungus just before transplanting and the responder part of the root was inoculated with 200 nematode juveniles a week later. The parameters assessed in the responder part of the root at the end of the experiments, 40 days after being inoculated with the nematode, were: the number of egg masses per plant (infectivity), the number of eggs per plant (reproduction), and the number of eggs per egg mass (fecundity).
*Data of each tomato experiment are mean ± SE of 10 replicates. Data of the cucumber experiments 1 and 2 are mean ± SE of 20 replicates because no differences ($p < 0.05$) were found between experiments and data were considered as a single experiment. Data within the same column per crop and experiment followed by *differ ($p < 0.05$) from the non-inoculated treatment according to the non-parametric Wilcoxon test.*

RESULTS

Induction of Systemic Plant Resistance by *P. chlamydosporia* Isolates Against *M. incognita*

The split-root system experiments with tomato differed ($p < 0.05$) between them and were treated separately. But no differences were found between the two split-root experiments with cucumber and thus we considered them as replicates of a single experiment. Both tomato and cucumber fresh root weight of the two halves of the split-root system of the None-None treatment did not differ ($p < 0.05$) (data not shown), showing that the split-root system did not influence root development. Shoot dry biomass did not differ in any fungal isolate-plant species combination ($p < 0.05$) (data not shown).

Two of the five *P. chlamyodosporia* isolates induced resistance in tomato plants in both experiments, but none of them did in cucumber (**Table 1**). The fungal isolate M10.43.21 reduced both the number of egg masses per plant (32–43%), the number of eggs per plant (44–59%), and the female fecundity (14.7–27.6%), while the isolate M10.55.6 reduced the number of eggs per plant in both experiments (35–47.5%) but the number of egg masses or the female fecundity in only one.

P. chlamydosporia isolates differed in the level of root colonization estimated by qPCR irrespective of the plant species (**Figure 1**). The standard curves for qPCR obtained by representing the cycle thresholds (Ct) against the log of 10-fold serial dilution of DNA from isolate M10.43.21 were accurate and reproducible to estimate the DNA concentration of the different treatments (tomato = $-3.66x + 19.36$; $R^2 = 0.9736$ and cucumber $y = -3.4937x + 21.29$; $R^2 = 0.9947$). Regarding tomato, isolate M10.43.21 was the best root colonizer followed by M10.55.6 (**Figure 1A**). In relation to cucumber, isolate M10.55.6 was the best root colonizer followed by M10.43.21 (**Figure 1B**). The fungus was not detected in the inducer part of the root from treatments None-None and None-RKN.

Dynamic Regulation of the Jasmonic and Salicylic Acid Pathways by *P. chlamydosporia* and *M. incognita* in Tomato

Changes in the expression of genes *PR-1* and *Lox D* from the salicylic acid and jasmonic acid pathways at 0, 7, and 42 dani are shown in **Figure 2**. The expression of the *PR-1* gene in roots inoculated with the fungal isolate M10.43.21 was upregulated at 0, 7, and 42 dani compared to the non-inoculated plants (**Figures 2A–C**). Regarding the jasmonic acid pathway (**Figures 2D–F**), the gene *Lox D* was only upregulated at 7 dani.

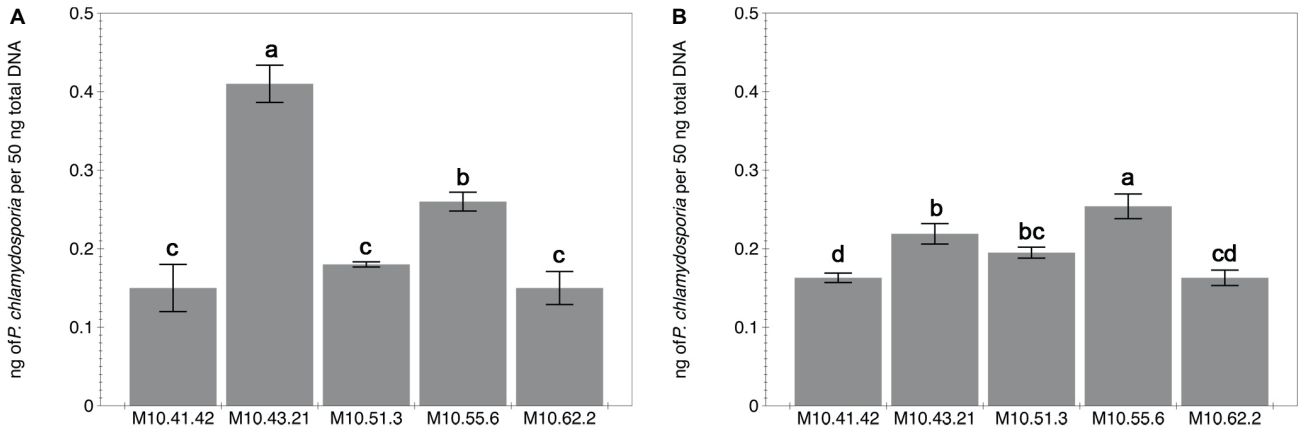

FIGURE 1 | Capability of five *P. chlamydosporia* isolates to colonize roots of tomato cv. Durinta **(A)** and cucumber cv. Dasher II **(B)**. The root colonization is expressed as the proportion of fungal DNA biomass (isolates) per 50 ng of the total DNA biomass extracted from the inducer part of the root of the split-root experiment 2. Each value is mean ± SE of three biological samples with two technical replicates each. Different letters indicate statistical differences between isolates ($p < 0.05$) according to the Dunn's test.

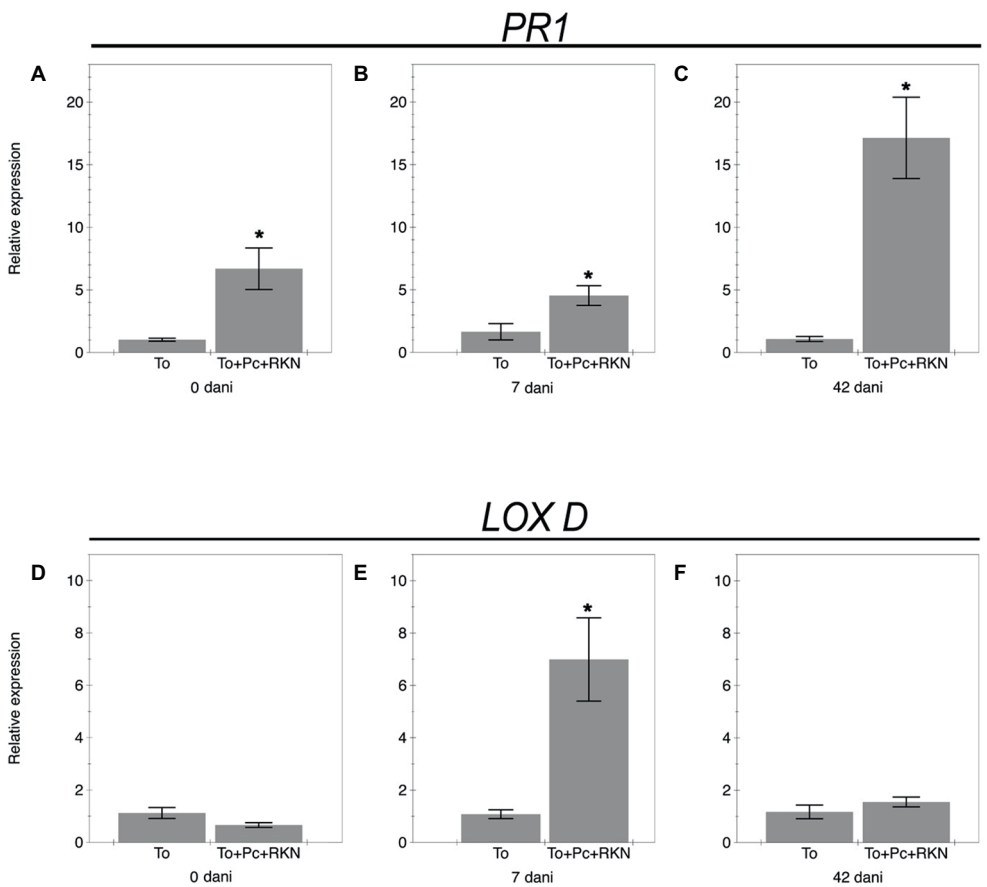

FIGURE 2 | Relative expression of genes related to the salicylic acid and jasmonic acid pathways. The expression of genes *Pr1* **(A–C)** and *Lox D* **(D–F)** in roots of the tomato cv. Durinta non inoculated (To) or inoculated with 10^5 chlamydospores of *P. chlamydosporia* isolate M10.43.21 just before transplanting and with 200 J2 of *M. incognita* per plant a week after transplanting (To+Pc + RKN) at three time points: just after nematode inoculation (0 dani), at 7 dani, and at 42 dani. Each value is mean ± SE of three biological samples with two technical replicates each. Asterisks indicate significant differences ($p < 0.05$) according to the non-parametric Wilcoxon test.

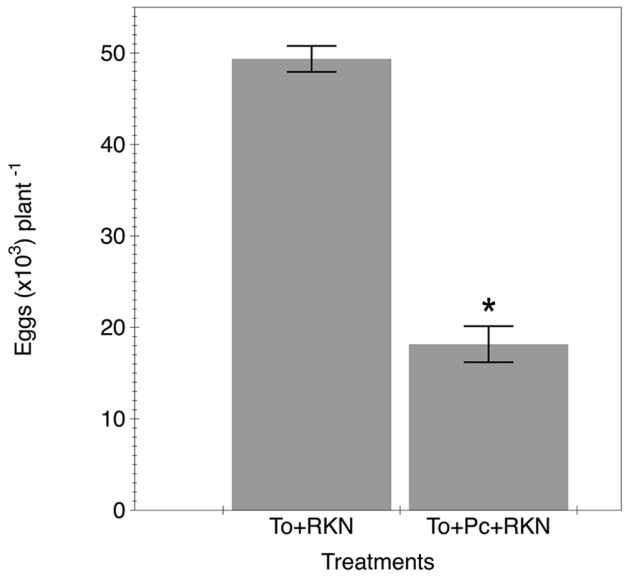

FIGURE 3 | Effect of primed tomato plants by *P. chamydosporia* on *M. incognita* reproduction. Number of eggs produced in the tomato cv. Durinta after 42 days of being inoculated with 200 J2 of *M. incognita* per plant a week after transplanting (To + RKN) or inoculated with 10^5 chlamydospores of *P. chlamydosporia* isolate M10.43.21 just before transplanting and with the nematode at the same rate and time mentioned before (To + Pc + RKN). Each value is mean ± SE of three replications. Asterisk indicates significant differences ($p < 0.05$) according to the non-parametric Wilcoxon test.

The nematode reproduction in plants co-inoculated with the fungal isolate was suppressed by 60% compared to plants only inoculated with the nematode (**Figure 3**).

DISCUSSION

The results of this study provide evidence for the ability of some *P. chlamydosporia* isolates to induce systemically resistance against *M. incognita*, and that this induction is dependent on the plant species. The isolate M10.43.21 showed the most consistent response in both split-root experiments with tomato, and was the reason for selecting it to determine the hormone modulation in this plant species. The mechanisms responsible for the endophyte-induced resistance are unclear (Schouten, 2016). Both salicylic acid- and jasmonic acid-dependent signaling pathways have been proposed as responsible for the systemically induced resistance to *Meloidogyne* spp. in tomato in split-root experiments (Selim, 2010; de Medeiros et al., 2017; Martínez-Medina et al., 2017). Martínez-Medina et al. (2017) reported that *Trichoderma harzianum* T-78 induced the upregulation of genes related to salicylic acid at early stage of nematode infection, whereas those related to jasmonic acid were upregulated from 3 to 21 days after nematode inoculation. In our study, the *P. chlamydosporia* isolate primed salicylic acid from the first assessment time (7 days after fungal inoculation and just after nematode inoculation) until the end of the experiment (42 dani). This effect could be responsible for the suppression of nematode infection at early stages, as well as the infection by the J2 produced by the primary inoculum that were able to overcome the plant defense mechanisms. In addition, the upregulation of the *Lox D* gene, related to jasmonic acid, at 7 dani could affect nematode reproduction and fecundity. Thus, the induction of the salicylic acid and jasmonic acid signaling pathways by the fungal isolate M10.43.21 in tomato counteract the suppression of these phytohormones by the nematode described in the susceptible tomato-nematode interaction (Shukla et al., 2018). The three-phase model proposed to explain the induced protection to RKN by *Trichoderma harzianum* T-78 in tomato consisting of salicylic acid induction suppressing RKN infection followed by jasmonic acid induction suppressing RKN reproduction and fecundity and finally salicylic acid induction affecting root infection by the next J2 generation (Martínez-Medina et al., 2017) is valid for *P. chlamydosporia*. Other local plant defense mechanisms induced by *P. chlamydosporia* against RKN have been reported, including the increase of the peroxidases (*POX*) and poliphenoloxidases (*PPO*) enzymes activity at root nematode invasion stage (24–96 h after nematode inoculation) (de Medeiros et al., 2015). However, considering that *P. chlamydosporia* does not extensively colonize the root (Maciá-Vicente et al., 2009; Escudero and Lopez-Llorca, 2012), even being improved by chitosan irrigation (Escudero et al., 2017), the effect of local defense mechanisms alone may be insufficient to achieve significant nematode suppression.

Not all *P. chlamydosporia* isolates induced systemic resistance in tomato. The variability of this attribute among fungal isolates can be added to other observations previously reported, such as the production of chlamydospores, root colonization, plant growth promotion, or egg parasitism (Kerry and Bourne, 2002; Zavala-Gonzalez et al., 2015). In fact, egg parasitism can even differ between single-fungal spore isolates originating from the same field population (Giné et al., 2016). The frequency of occurrence of *P. chlamydosporia* in horticultural production sites under integrated and organic standards has increased since the 1990s in northeastern Spain (Giné et al., 2012), showing that this fungal species is adapted to environmental characteristics and agronomic practices. Field populations of *P. chlamydosporia* can contain individuals representing a diversity of functions that are highly beneficial to plants, such as plant growth promoters which enhance plant tolerance; inducers of plant defense mechanisms suppressing infection, development and reproduction of RKN; efficient egg parasites suppressing the RKN inoculum; or saprophytic behavior contributing to the organic matter cycle and plant nutrition. A given proportion of *P. chlamydosporia* representing some or all of these functions could be present in a given soil and adapted to the plant species involved in the rotation scheme and contributing to their health status. It seems that most of these functions are not interlinked. In fact, none of the five fungal isolates assessed in our study was a plant growth promoter. Thus, molecular tools must be developed to facilitate knowledge of the functional composition of the fungal field population in a given soil.

P. chlamydosporia has been reported as the main biotic factor responsible for soil suppressiveness to RKN in horticultural crops (Giné et al., 2016). In soils with low antagonistic potential, the use of fungal isolates with both direct and indirect action mechanisms could suppress RKN. Indeed, primed plants along

with egg parasitism will protect against infection and reproduction of RKN and decrease the inoculum viability. The combination of the two modes of action will result in a decrease of the nematode population growth rate, and consequently lower crop yield losses. Alternatively, combining the use of *P. chlamydosporia* with plant defense activators can produce a similar effect. Vieira Dos Santos et al. (2014) found a reduction in RKN reproduction when *P. chlamydosporia* was combined with the application of cis-jasmone, as well as an increase in fungal egg parasitism.

In conclusion, this study proves that some *P. chlamydosporia* isolates induce systemic resistance to *M. incognita* in tomato but none of them in cucumber. Thus, this response is plant species dependent. In future studies, the interaction between *P. chlamydosporia* isolates and selected economically important crops should be characterized to elucidate the mechanisms and genes involved in inducing plant resistance in order to maximize the efficacy of control.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

FS and NE conceived, designed, supervised the experiments, the data collection, and analyses. ZG performed the experiments, analyzed the data, and wrote the draft of the manuscript. NE and ES performed the gene expression analyses. TG provided reagents, materials, and advice. NE, ES, TG, and FS reviewed and wrote the final draft of the manuscript.

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Bacterial Metabolites Produced Under Iron Limitation Kill Pinewood Nematode and Attract *Caenorhabditis elegans*

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Pine Wilt Disease (PWD) is caused by *Bursaphelenchus xylophilus*, the pinewood nematode, and affects several species of pine trees worldwide. The ecosystem of the *Pinus pinaster* trees was investigated as a source of bacteria producing metabolites affecting this ecosystem: *P. pinaster* trees as target-plant, nematode as disease effector and its insect-vector as shuttle. For example, metals and metal-carrying compounds contribute to the complex tree-ecosystems. This work aimed to detect novel secondary metabolites like metallophores and related molecules produced under iron limitation by PWD-associated bacteria and to test their activity on nematodes. After screening 357 bacterial strains from Portugal and United States, two promising metallophore-producing strains *Erwinia* sp. A41C3 and *Rouxiella* sp. Arv20#4.1 were chosen and investigated in more detail. The genomes of these strains were sequenced, analyzed, and used to detect genetic potential for secondary metabolite production. A combinatorial approach of liquid chromatography-coupled tandem mass spectrometry (LC-MS) linked to molecular networking was used to describe these compounds. Two major metabolites were detected by HPLC analyses and described. One HPLC fraction of strain Arv20#4.1 showed to be a hydroxamate-type siderophore with higher affinity for chelation of Cu. The HPLC fraction of strain A41C3 with highest metal affinity showed to be a catecholate-type siderophore with higher affinity for chelation of Fe. LC-MS allowed the identification of several desferrioxamines from strain Arv20#4.1, in special desferrioxamine E, but no hit was obtained in case of strain A41C3 which might indicate that it is something new. Bacteria and their culture supernatants showed ability to attract *C. elegans*. HPLC fractions of those supernatant-extracts

of *Erwinia* strain A41C3, enriched with secondary metabolites such as siderophores, were able to kill pinewood nematode. These results suggest that metabolites secreted under iron limitation have potential to biocontrol *B. xylophilus* and for management of Pine Wilt Disease.

Keywords: metallophores, siderophore, nematode, *Erwinia*, *Rouxiella*, *Caenorhabditis elegans*, *Bursaphelenchus xylophilus*, secondary metabolites

INTRODUCTION

The pine wilt disease (PWD) is one of the most devastating diseases of forests pine trees in the world, with tremendous ecological, environmental, and economic damage. Native to North America (United States and Canada), it has spread to Asia and Europe (Proença et al., 2017b). The only known causal agent is the nematode *Bursaphelenchus xylophilus*, the pinewood nematode (PWN; Nickle et al., 1981). PWN is transferred when an insect vector feeds on young shoots of pine trees and, if the tree is susceptible, develops the disease and starts the PWN-insect life cycle (Proença et al., 2017b). The ecosystem in which the PWD is developed is rather complex and comprises next to above mentioned tree-nematode-insect also adjacent microorganisms. Respectively, the endophytic microbiome of pine trees was recently characterized (Proença et al., 2017a; Alves et al., 2018), and the bacteria carried by PWN (Proença et al., 2010, 2014; Vicente et al., 2012) and insect vector were studied (Alves et al., 2016, 2018). Especially, the microbiome of the nematode seems directly linked to the tree-wilting (Alves et al., 2018). Further, the tree and nematode microbiome differ significant with respect to genus/species level. In addition, some of the relevant and more tree-related bacteria are endophytic opportunistic, living in soil and may enter the tree through the roots (Proença et al., 2017a). They are believed to be plant growth promoting rhizobacteria (PGPR) or do at least affect and thus interact with plants. They might not only promote the growth of the trees but also can control plant pests (Niranjan Raj et al., 2005). PGPR produce various compounds as for example small organic acids, phytohormones, antibiotics and siderophores among others. Metabolites like organic acids and siderophores interfere with metal or metalloid abundance and mobility in rhizosphere and surrounding environment. Especially the availability of metals, like iron (Fe^{3+} or Fe^{2+}), was determined to be crucial within plant-microbe-nematode interactions (Ramamoorthy et al., 2001). It was reported that under iron limitation not lipopolysaccharides but siderophores as pseudobactin induce systemic resistance towards plant pests (Saikia et al., 2005; Romera et al., 2019). This response seems to be complementary to other defense mechanisms in dependence on the environmental conditions.

Metals are essential for life, acting as cofactors of enzymes and participating in central biological processes, as for example oxygen binding, activation and transport, photosynthesis, and within electron transport chains (Fones and Preston, 2013). The oxidation state of each metal is dependent on the environmental conditions that in turn results in its bioavailability for living organisms. Iron and copper are important metals for biological processes and its availability in soil influences plant growth as well as microorganisms (beneficial and pathogenic) (Złoch et al., 2016). Thus the redox environment and availability of certain metals and metalloids defines the ecosystem, especially in the rhizosphere and its vicinity (Zobel et al., 2005; McNear, 2013). In order to acquire certain metals and metalloids, organisms like microbes (e.g., the PGPR) and plants secrete metallophores which support the mobilization of poorly soluble metals (in case of Fe^{3+} designated as siderophores) (Hider and Kong, 2010).

Metallophores have distinct biochemical characteristics and are divided into various families, i.e., hydroxamates, catecholates, carboxylates, and mixed types (Hider and Kong, 2010; Saha et al., 2013). Different types of siderophores have different metal binding capabilities and are often not restricted to iron but can scavenge different metals like copper, zinc, vanadium, gallium, and cadmium from the environment (Mehnert et al., 2017; Wiche et al., 2017; Schwabe et al., 2018). There are several biotechnological applications of siderophores, for example, enhancing of plant growth, acting as biological control agents (BCA), to perform bioremediation of environments contaminated with metals, to recover rare earth elements, and to act as antibiotic carriers or inhibitors of metalloenzymes (Ahmed and Holmström, 2014; Kurth et al., 2016). Plants are not able to assimilate iron in its predominant form in soil, ferric ion or Fe^{3+}, and produce phytosiderophores that mobilize Fe^{3+} and are being taken up as Fe^{3+}-complexes (Ahmed and Holmström, 2014). Bacterial siderophores have been shown to be important in this interplay, increasing uptake of iron into tissues of the model plant *Arabidopsis thaliana* and improved plant growth (Vansuyt et al., 2007). On the other hand, bacterial siderophores are suggested to be pathogenicity factors because they compete for iron, limiting iron uptake by the plant (Gamalero and Glick, 2011; Wilson et al., 2016).

Overall it can be stated that metal limitation is stress for all the organisms of such an ecosystem described herein and the response siderophore production, and secretion can have various outcomes for the individuals. And as described above this can also affect the PWD-causing nematode and needs, therefore, to be investigated. The effects of siderophores towards the model nematode *Caenorhabditis elegans* is controversial. It was shown that bacterial siderophores from pseudomonads chelate iron and lead to death of *C. elegans* (Kirienko et al., 2013; Kang et al., 2018). However, siderophores like enterobactin caused an increase in iron levels in mitochondria, promoting the growth of *C. elegans* (Qi and Han, 2018).

Considering the large interest in the metabolic arsenal of environmental bacteria with biotechnological potential, the present work focused on the siderophores produced by wood

colonizing and/or endophytic bacteria from the rhizosphere, where a complex community comprising plants and nematodes among others, interacts. Therefore, the present work aims to identify the siderophores produced by endophytic bacteria isolated associated with *Pinus* trees, in order to understand the role of bacterial siderophores using the model nematode *C. elegans* and verify their nematicidal activity towards the PWN. Two strains producing distinct siderophore patterns were chosen as model systems to investigate siderophore production, metal binding properties, and the biochemical properties of the siderophores, as well as their pathogenicity against nematodes.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions and Biochemical Characterization

Two hundred and seventy-one bacterial strains isolated as wood colonizers/endophytes of *P. pinaster* trees from Portugal (Proença et al., 2017a), 48 bacterial strains carried by PWN from Portugal (Proença et al., 2010), and 38 bacterial strains carried by PWN from United States (Proença et al., 2014) were evaluated for their production of siderophores on Iron-CAS agar plates (Schwyn and Neilands, 1987). The screening for bacterial endophytes and bacteria carried by PWN nematode from Portugal were re-evaluated in this study alongside with bacterial strains carried by PWN from United States for a confident result. The two best bacterial siderophores-producers candidates, with highest orange halo (positive for siderophore secretion) and lack of information of siderophores-producing species, strains Arv20#4.1 and A41C3, were selected for further production and characterization.

These two strains were grown in tubes with 5 ml of M9 media, supplemented with glucose (4% w/v), succinic acid (4 g/l), and Mg/Ca/B1/Goodie Mix (Esuola et al., 2016) for seven days. To investigate siderophore production and bacterial growth, the supernatant of each strain was obtained at days 0, 3, 5, and 7 and the OD_{600} was determined at the same time points.

The phenotypes of strains Arv20#4.1 and A41C3 were analyzed focusing on the solubilization of phosphate and zinc, production of siderophores, and hydrolyzing activities including protease (casein), lipase (Tween 20, 40, 60 and 80), cellulase, and chitinase activities (Naveed et al., 2014). The ability to oxidize different carbon sources was assessed using Biolog GN2 MicroPlates incubated at 30°C. The results were recorded daily for up to 72 h using a MicroPlate reader (Tecan Infinite M200).

Phylogenetic Analysis

The strains Arv20#4.1 and A41C3 were grown on R2A agar media at 30°C for 48 h and its genomic DNA was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions.

These strains were phylogenetically identified by 16S rRNA gene sequencing using primers 27F and 1525R as previously described (Rainey et al., 1992). All obtained sequences from bacterial isolates were compared with sequences available in the EMBL/GenBank database using BLASTN network services (Altschul et al., 1997) and with sequences available on the Eztaxon-e server[1] (Kim et al., 2012). Sequences were aligned within the SINA alignment service (Pruesse et al., 2012). Sequences were also checked for chimeric artifacts by using Mallard software (Ashelford et al., 2006). Sequences were included in 16S rRNA-based Living Tree Project (LTP) release 115 database[2] by parsimony implemented in the ARB software package version 5.5 (Ludwig et al., 2004). Phylogenetic dendrograms of sequences from this study and closest reference sequences were constructed by the Randomized Axelerated Maximum Likelihood (RAxML) method with GTRGAMMA model (Stamatakis, 2006) included in the ARB software (Ludwig et al., 2004).

Genome Sequencing, Annotation and Siderophore Mining

The draft genome sequencing of strains Arv20#4.1 and A41C3 was generated on an Illumina platform (MiSeq) as described previously (Retamal-Morales et al., 2018). To this end standard Illumina protocols and kits were employed. The extracted genomic DNA (see above) was used to prepare a genomic library using the NexteraXT DNA library kit. A quality check was performed using an Agilent Bioanalyzer 2100. The paired-end libraries obtained were sequenced employing v3-v4 chemistry on the MiSeq platform. The reads obtained were analyzed by applying FastQC, TrimGalore including the Phred20 quality filter, and SPAdes 3.5 for read assembly by using the GALAXY webtool[3]. The quality of the assembled genomes was accessed by means of Quast (Gurevich et al., 2013). The assembled genomes were annotated by using automatic pipelines of NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP[4]), RAST (Aziz et al., 2008) and JGI servers (Huntemann et al., 2015; Chen et al., 2016, 2017).

Both annotated draft genomes were subjected to secondary metabolite gene cluster analysis by using the platform antiSMASH 3.0 (Weber et al., 2015), with specific interest for siderophores. Therefore, a BLAST analysis was performed for all protein sequences codified by each gene from siderophore gene cluster by using NCBI (Altschul et al., 1997) and UniProtKB/Swissprot and Uni-ProtKB/TrEMBL (Schneider et al., 2004) databases.

Determination of Siderophore Production

Supernatants obtained from cultures of strains Arv20#4.1 (bacterium carried by PWN from United States) and A41C3 (endophytic bacterium of *P. pinaster* from Portugal) were assessed for the siderophore activities by a modified CAS assay and a modified Csáky test (Esuola et al., 2016; Mehnert et al., 2017). EDTA and desferrioxamine B (DFOB) were used as positive controls. The modified CAS assay was used to determine

[1] http://eztaxon-e.ezbiocloud.net/

[2] http://www.arb-silva.de/projects/living-tree/

[3] https://usegalaxy.eu

[4] http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html

Fe, Ga, Cu, V, and Al chelating compounds in comparison to the same control reagents as for the Csáky test (Mehnert et al., 2017). Fe-, Ga-, Al-CAS solutions were prepared as follows (100 ml): 1.5 ml of 1 mM $FeCl_3 \times 6H_2O$ (in 10 mM HCl) or 1.5 ml of 1 mM $Ga_2(SO_4)_3$ or 1.5 ml of 1 mM $AlCl_3 \times 6 H_2O$ was added to 7.5 ml of 2 mM aqueous CAS solution. Six ml of a 10 mM hexadecyltrimethylammonium bromide (CTAB) solution was added slowly. Subsequently MES buffer was added (10.67 g MES in 80 ml H_2O with a pH of 5.6 by adjusting with 50% KOH). The mixture obtained was filled to a final volume of 100 ml with H_2O. Cu-CAS solution was prepared as follows (100 ml): 2 ml of 10 mM $CuCl_2$, 10.5 ml of 2 mM aqueous CAS solution, 2.5 ml of ddH_2O and 85 ml of MES buffer at pH 5.7. V-CAS solution was prepared as follows (100 ml): 1.5 ml of 5 mM VCl_3, 7.5 ml of 2 mM aqueous CAS solution, 6 ml of 10 mM CTAB and 85 ml of MES buffer at pH 4.1. Supernatants were mixed with Fe-CAS, Al-CAS, or V-CAS solution in a ratio 1:1 or were mixed with Ga-CAS or Cu-CAS solutions in a ratio 1:5. 33 mM of EDTA (final concentration) was used as positive control and ddH_2O was used as negative control. Absorbance was measured at 630 nm for Fe, Ga, and Al assays, at 590 nm for V assays, and at 582 nm for Cu assays, after 4 h of incubation (except 6 h for Al assay).

Catecholate siderophore production was estimated using the colorimetric Arnow assay (Arnow, 1937) with following modifications: 150 µl of supernatant, 150 µl of 0.5 N HCl, 150 µl of nitrite-molybdate (10 g sodium nitrite and 10 g sodium molybdate per 100 ml) and 150 µl of 1 N NaOH were added in this order. The absorbance was measured at 510 nm. The standard curve was based on 3,4-dihydroxybenzoic acid (DHBA) (Sigma).

Hydroxamate siderophore production was estimated using the colorimetric Atkin assay (Atkin et al., 1970). Briefly, 104 µl of supernatant was added to 52.5 µl of 2 N perchloric acid. From this mix, 47.5 µl were added to 47.5 µl of 10% sodium acetate. Then, 47.5 µl of 1% sulfanilic acid and 19 µl of 0.5% I_2 and incubated at room temperature (RT) for 15 min. 19 µl of 0.1 N sodium thiosulfate and 19 µl of 0.6% α-naphthylamine were added consequently and incubated at RT for 45 min. The absorbance was measured at 562 nm. The standard curve was based on NH_2OH (Sigma).

Enrichment of Siderophores by HPLC

The strains Arv20#4.1 and A41C3 were selected to be grown in high volumes, 1 l of M9 medium mention above for each strain and supernatant was harvested by centrifugation at 10,000 rpm at 4°C for 1 h. The pH of each supernatant was measured. The siderophores in the supernatants of each strain were obtained by ion exchange-based solid phase extraction as follows: Amberlite XAD-4 (4 g/l) with Amberlite XAD-16 (4 g/l) activated with dH_2O, followed by methanol and washed with dH_2O, and incubate at 4°C for 3 days. Then, the supernatant was discarded and beads with siderophores were washed 3 times with 30 ml of dH_2O, followed by shaking 3 times with 20 ml methanol at 65 rpm at room temperature. These volumes of methanol were evaporated in an evaporator system at low pressure to a final volume of 5 ml.

The siderophores were enriched by HPLC to a C18-RP column (1 ml; Thermo, Auqastar) equilibrated in 28% of methanol (buffer A) and 72% of ddH_2O with 0.1% of TFA (buffer B) (Anke et al., 2017; Schwabe et al., 2018). The column elution was performed as described and after 8 min a linear gradient from 28 to 95% buffer A was performed to 12 min, buffer A concentration kept constant for 3 min before switching back to initial conditions (total time of elution 15 min at 1 ml/min flow rate). The eluted molecules were detected at 217 nm and 430 nm. The resulting 30 fractions were characterized for their Fe, Ga, Cu, V, and Al-CAS activities and hydroxamate and catecholate siderophores were quantified as mentioned in previous section.

LC-MS/MS Analysis

Fractions from HPLC profiling with a significant CAS activity were analyzed by LC-MS/MS and fragment spectra were investigated by molecular networking. Samples were dried under reduced pressure and reconstituted in 1 ml of MS-grade methanol. To achieve a better solubility these samples were sonicated for 5 min. Mass spectrometric analysis was performed similarly as described earlier (Senges et al., 2018). Liquid chromatography (LC) was performed on a nanoACQUITY-UPLC system (Waters, Milford, MA, United States) with a Mixer Assy (Waters, zirc bead, inner cross section 1 mm, length 50 mm) and an AcquityUPLC HSS T3 column (Waters, pore size 100 Å, particle size 1.8 µm, inner cross section dimension 1 mm, length 100 mm). 4.7 µl of each sample was injected and separated by means of a H_2O/acetonitril (ACN) gradient with 0.1% formic acid (FA) and a flow rate of 25 µl min^{-1} (**Table 1**).

Subsequently, the mass spectrometry (MS) was performed with a Synapt G2-S $HDMS^E$ system (Waters) with an ESI source operated in positive and negative mode and a TOF detector. Masses in the range of 50 to 3000 m/z were detected with 0.5 s per scan and leucine enkephalin injected as reference mass every 30 s. Mass spectrometry settings: lockspray capillary current 2.5 kV, capillary current 2.5 kV, cone voltage 30 V, source temperature 120°C, cone gas flow 60 l/h, flushing gas flow 550 l/h, with a temperature of 150°C. Fragmentation in MS/MS mode was conducted by collision induced diffraction with argon and a collision energy ramp of 10–25 V. Fragmentation was started when the intensity of a mass signal exceeded 6000 counts/s, finished after 6 s at max, and stopped prematurely if the intensity

TABLE 1 | Gradient used for LC-MS/MS analysis.

Time [min]	% ACN with 0.1% FA
0.0	5.0
2.5	5.0
21.0	99.5
23.0	99.5
28.5	5.0
30.0	5.0

dropped below 6000 counts/s. Spectra were processed with MassLynx V4.1 SCN932 (Waters).

Using Proteowizard (ver. 3.0.9490) waters.raw-files were converted to.mzXML, with 32-bit binary encoding precision and peak picking. Spectra were uploaded to the UCSD GNPS FTP-server [ucsd.gnps.edu] and investigated using the METBOLOMICS-SNETS1 workflow with following parameters: parent mass tolerance 2 Da, ion tolerance 0.5 Da, minimal pairs cos 0.7, network topK 10, maximum connected component size 100, minimum matched peaks 6, minimum cluster size 2, run MSCluster. Data were visualized with Cytoscape (ver. 3.4.0). Network nodes corresponding to solvent background were excluded. Annotation of fragment spectra was aided by *in silico* fragmentation with MetFrag.

Nematode Strains and Growth Conditions

The nematode wild type *Caenorhabditis elegans* N2, obtained from Caenorhabditis Genetic Center, Minneapolis, MN (United States) were propagated at 19.5°C on nematode growth agar medium (NGM) with *Escherichia coli* OP50 lawn (Brenner, 1974). After 3 days, nematodes were harvested by rinsing the plates with sterile M9 broth medium, transferred to 15 ml tubes in a total of 2 ml. It was added a fresh solution containing 0.5 ml of 2N NaOH and 1 ml of 3% sodium hypochlorite, followed by vortexing every 2 min with final duration of 10 min. The nematodes and eggs were centrifuged 1,300 g x 30 s at 22°C. Supernatant was removed and the nematodes and eggs were washed 2 times with sterile water. The eggs were transferred into new NGM plates and incubated at 19.5°C during 18 h - 24 h. The pinewood nematode *B. xylophilus* PtAS18, was maintained on agar plates containing *Botrytis cinerea* Pers. at 25°C (Fonseca et al., 2012).

C. elegans-Bacterial Strains/Supernatants Attraction Assay

Chemotaxis assays were performed according to Akduman et al. (2018) with some modifications. Briefly, 10 µl of bacterial suspensions obtained after 24 h of growth in M9 broth media supplemented with iron, at 30°C at 130 rpm, were placed 0.5 cm away from the edge of a 6 cm NGM agar plate. Ten µl of *E. coli* OP50 were added on the opposing side. Idealizing an equilateral triangle, 10 µl containing approximately 30 J3 stage nematodes were placed on the remaining vertex, with all spots at the same distance.

In the same line, 10 µl of bacterial supernatants, obtained from bacterial growth as mentioned above, was used to perform the chemotaxis attraction. Moreover, the bacterial strains were also grown in M9 broth medium without supplementation of iron, and also its suspensions and its supernatants were tested in chemotaxis attraction of *C. elegans* N2.

Different combinations were performed to understand the attraction of the nematodes by different bacteria, its supernatants and if the influence of the presence of iron in M9 broth media also affects their attraction. M9 broth media with or without iron supplementation was also tested in chemotaxis assays. After 3h the number of nematodes were recorded in each test spot. All the assays were performed in triplicate.

Nematodes Survival Assays

The bacterial strains and their supernatants mentioned above were used to evaluate their effect on *C. elegans* N2 viability. The nematodes were observed and scored at 24 h, 48 h, and 72 h for with a dissecting microscope. On the other hand, the nematicidal activity of bacterial strains, its supernatants and HPLC fractions were evaluated towards the *B. xylophilus* PtAS18 (mixed life stages: J4 and adults) and towards *C. elegans* N2 (mixed life stages: L4 and adults) according to Paiva et al. (2013) with little modifications. Briefly, the bacterial suspensions obtained were centrifuged (20 min, 4°C, 13,000 rpm), in order to drastically reduce the number of cells, without removing any growth product. To test the nematicidal activity, 500 µl of each bacterial supernatant, with less than 0.06 O.D.600 nm, were incubated with 30 disinfected nematodes, for 48 h at 25°C. The assays were performed in triplicate. Nematodes were disinfected by sequential washes in sodium hypochlorite 0.1% (one wash, 1 min at 4°C) and in 1 ml sterilized water (two washes, 3 min at 4°C), followed by centrifugation. Last water (100 µl) was inoculated on R2A for control of the disinfection efficiency. The number of dead nematodes was assessed under the stereoscopic microscope. Nematodes were considered dead when linearized and not able to recover after being transferred to water. The controls, nematodes in CAA, were incubated under the same conditions.

Statistical Analysis

Chi-squared test was used to evaluate the significance of the differences in the chemotaxis of *C. elegans* with bacterial and supernatant samples. All differences were considered to be statistically significant for $p < 0.05$.

The significance of the differences, observed between the different nematicidal treatments towards *B. xylophilus* PtAS18 by using bacterial supernatants or HPLC fractions, was evaluated with two-way analysis of variance (ANOVA), using Bonferroni post-tests. The statistical analysis was performed with GraphPadPrism v5.0 for windows, GraphPad software, San Diego, CA, United States. All differences were considered to be statistically significant for $p < 0.05$.

Accession Numbers

The Whole Genome Shotgun project of strain Arv20#4.1 and A41C3 have been deposited at ENA under the accession numbers RQVU00000000 and RQVV00000000, respectively. The 16S rRNA gene sequences of the isolates, have been previously deposited in the NCBI GenBank database under the accession numbers according KF214948 (Proença et al., 2014) and KJ654833 (Proença et al., 2017a). Accession links for metabolomic data sets are provided at: positive mode (http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=bbd4f668b6224bd381617001be917381); negative mode (http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e3009a90a0954791ae9b747e727c6515).

RESULTS

Siderophore Screening, Bacterial Phylogeny and Biochemical Characterization

In this study, 357 bacterial strains were tested and belonging to 52 genera according to 16S rRNA gene analysis (Proença et al., 2010, 2014, 2017a). Numerous bacterial strains were positive on CAS agar medium that serves to detect siderophores: 103 endophytic strains (38.0%), 39 strains carried by *B. xylophilus* from Portugal (81.2%) and 20 strains carried by *B. xylophilus* from United States (52.6%) (**Supplementary Figure S1**). Among those, two isolates showed a distinct siderophore production on CAS agar plates and have been chosen for further study: strains Arv20#4.1 and A41C3.

According to neighbor-joining and maximum-likelihood phylogenetic trees, the closest relatives to strain Arv20#4.1 were *Rouxiella chamberiensis* (99.6% sequence similarity), *R. silvae* (99.6%), and *R. badensis* (99.6%). *Erwinia endophytica* (98.1%), *Pantoea dispersa* (97.7%), and *E. psidii* (97.6%) were the closest relatives to strain A41C3. Thus, the best two bacterial isolates with higher biotechnological potential analyzed in this study belong to the family *Enterobateriaceae*, and were *Erwinia* sp. A41C3 (endophyte from Portuguese pines) and *Rouxiella* sp. Arv20#4.1 (bacterium isolated as carried by PWN from United States) (**Supplementary Figure S2**).

Biochemical characteristics of strains Arv20#4.1 and A41C3 are summarized in **Table 2**. Both strains were able to solubilize phosphate and zinc, produce siderophores, degrade Tween 60 and carboxymethyl cellulose (CMC), and produced catalases; but were negative for protease production, chitin, Tween 40 and Tween 80 degradation. Both strains oxidized 43 carbon sources in GN2 MicroPlate (and 6 more carbon sources were weakly oxidized). Only the strain A41C3 was able to oxidize m-inositol, β-hydroxybutyric acid, α-keto butyric acid, α-keto glutaric acid, sebatic acid, succinamic acid, L-pyroglutamic acid, γ-amino butyric acid in GN2 MicroPlates (**Table 2**).

TABLE 2 | Differential characteristics between strains *Rouxiella* sp. Arv20#4.1 and *Erwinia* sp. A41C3.

Characteristic	Arv20#4.1	A41C3
Phosphate solubilization	W	+
Siderophores	+++	++
Tween 20	++	−
CMC degradation	+	−
Catalase	+	++
BIOLOG		
Tween 80	W	+
M-Inositol	−	+
D-Sorbitol	+	W
Turanose	W	+
Pyruvic Acid Methyl Ester	−	W
Cis-Aconitic acid	W	+
Formic acid	+	W
D-Glucosaminic acid	W	+
α-Hydroxybutyric acid	W	+
β-Hydroxybutyric acid	−	+
p-Hydroxy Phenylacetic acid	−	W
α-Keto butyric acid	−	+
α-Keto glutaric acid	−	+
α-Keto valeric acid	−	W
Malonic acid	W	+
Quinic acid	W	+
Sebatic acid	−	+
Bromosuccinic acid	W	+
Succinamic acid	−	+
L-Alaninamide	−	W
D-Alanine	+	W
L-Glutamic acid	W	+
Glycyl-L-aspartic acid	W	+
L-Proline	W	+
L-Pyroglutamic acid	−	+
L-Threonine	W	+
γ-Amino butyric acid	−	+
Urocanic acid	W	+

Both strains were positive on solubilization of zinc, degradation of Tween 60; and negative on production of proteases, degradation of Tween 40, Tween 80 and chitin. Both strains oxidized Tween 40, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, m-inositol, α-D-lactose, lactulose, D-maltose, D-mannitol, D-mannose, D-melibiose, b-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, pyruvic acid methyl ester, succinic acid mono-methyl-ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebatic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, L-hydroxyproline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ-amino butyric acid, urocanic acid, inosine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L,α-glycerol phosphate, α-D-glucose-1-phosphate, D-glucose-6-phosphate. +, Positive; W, weakly positive; −, negative.

Genome Analysis Showed (Novel) Siderophore Gene Clusters

The draft genome sequence of strains Arv20#4.1 contained 29 contigs, totaling 5.37 Mbp in size with a mapped coverage of 547-fold of the genome. The G + C content was 53.0 mol%. The genome encoded 4,936 putative coding sequences (CDSs) (**Table 3**). The draft genome sequence of strain Arv20#4.1 contained loci for three ribosomal RNAs and 72 tRNAs (**Table 3**).

In case of strain A41C3, the draft genome sequence contained 61 contigs, totaling 4.22 Mbp in size with a mapped coverage of 792-fold of the genome. The G + C content was 51.6 mol%. The genome encoded 3,953 putative CDSs (**Table 3**). The draft genome sequence of strain A41C3 contained loci for seven ribosomal RNAs and 57 tRNAs (**Table 3**). A general overview of the biological subsystems obtained using RAST for both strains is summarized in **Table 4**.

By using the webtool antiSMASH, it was possible to identify the gene cluster for secondary metabolite, especially siderophore, production close to desferrioxamine, in particular the operon that is composed by decarboxylase, monooxygenase, and acetyltransferase in strain Arv20#4.1 (**Table 5** and **Supplementary Figure S3A**). For strain A41C3, it was possible

TABLE 3 | General characteristics of the genome sequences of the strains *Rouxiella* sp. Arv20#4.1 and *Erwinia* sp. A41C3.

Characteristic	Arv20-4-2	A41C3
Sequence size	5,371,123	4,227,107
GC content (%)	53.0	51.6
N50	431,840	138,450
L50	4	8
Number of contigs (with PEGs)[a]	29	61
Number of Subsystems[a]	401	359
Number of RNAs[a]	74	64
Genome coverage[b]	547x	792x
Genes (total)[b]	5,021	4,023
CDSs (total)[b]	4,936	3,953
Genes (coding)[b]	4,867	3,765
CDSs (with protein)[b]	4,867	3,765
Genes (RNA)[b]	85	70
rRNAs (5S, 16S, 23S)[b]	1, 1, 1	5, 1, 1
Complete rRNAs[b]	1, 1, 1	5, 1, 1
tRNAs[b]	72	57
ncRNAs[b]	10	6
Pseudogenes (total)[b]	69	188
CDSs (without protein)[b]	69	188
Pseudogenes (ambiguous residues)[b]	0	0
Pseudogenes (frameshifted)[b]	25	86
Pseudogenes (incomplete)[b]	36	126
Pseudogenes (internal stop)[b]	20	36
Pseudogenes (multiple problems)[b]	11	54
CRISPR Arrays[b]	0	2

[a]*Data from RAST annotation;* [b]*Data from PGAP from NCBI.*

TABLE 4 | Biological subsystem distribution of annotated genes in strains *Rouxiella* sp. Arv20#4.1 and *Erwinia* sp. A41C3.

Code	Description	Arv20#4.2	A41C3
A	Cofactors, vitamins, prosthetic groups, pigments	188	167
B	Cell wall and capsule	70	53
C	Virulence, disease and defense	61	42
D	Potassium metabolism	19	11
E	Miscellaneous	23	15
F	Phages, prophages, transposable elements, plasmids	79	12
G	Membrane transport	138	125
I	RNA metabolism	54	58
J	Nucleosides and nucleotides	101	96
K	Protein metabolism	221	202
L	Cell division and cell cycle	8	8
M	Motility and chemotaxis	14	0
N	Regulation and cell signaling	76	64
O	Secondary metabolism	5	4
P	DNA metabolism	78	84
Q	Fatty acids, lipids, and isoprenoids	71	55
R	Nitrogen metabolism	25	22
S	Dormancy and sporulation	3	2
T	Respiration	106	92
U	Stress response	96	73
V	Metabolism of aromatic compounds	14	12
W	Amino acids and derivatives	401	357
X	Sulfur metabolism	27	37
Y	Phosphorus metabolism	37	33
Z	Carbohydrates	382	243

to identify multiple genes in the gene cluster for siderophore production (**Table 6** and **Supplementary Figure S3B**). However, it was not possible to predict which siderophore is produced. There are several putative siderophore biosynthesis clusters identified by antiSMASH that could be attributed to strain A41C3 like as turnerbactin, griseobactin among others.

Production and Biochemical Characterization of Siderophores

Both bacterial strains reached maximum of optical density after 3 days of incubation in 10 ml tubes containing 5 ml of M9 modified medium. Siderophore production by both strains depended on days of cultivation, but both supernatants showed activity for hydroxamate and catecholate-type siderophores (**Figure 1**). Strain Arv20#4.1 produced mainly hydroxamate-type siderophores (25 μM of NH_2OH equivalents) while strain A41C3 produced mainly catecholate-type siderophores (70 μM of DHBA equivalents) and showed more affinity to copper or to iron, respectively.

After three days, the growth medium of strain Arv20#4.1 had a pH of 9 while, that of A41C3 a pH of 5. At this moment siderophore comprising supernatants were harvested and processed by ion exchange material to obtain an enriched siderophore pool for each strain. After concentration by evaporation, siderophores were enriched by HPLC and 30 fractions per strain were obtained (**Figure 2**). Both strains showed ability to produce hydroxamate and catecholate-type siderophores but with different concentrations. The best metal affinity was identified in an HPLC fraction (1.25) of supernatant of strain Arv20#4.1 for a hydroxamate-type siderophore (92 μM of NH_2OH equivalents) with higher affinity for Cu (**Figure 2B**). On the other hand, the best supernatant HPLC fraction (3.26) of strain A41C3 contained a catecholate-type siderophore (309 μM of DHBA equivalents) with higher affinity for Fe (**Figure 2D**).

Siderophore Identification by Mass Spectrometry

The collected compounds in HPLC fractionations were characterized by using LC-MS. Fragment spectra were analyzed by molecular networking, which allows sorting of spectra according to their similarity that is ultimately based on the structural similarity of molecules analyzed (Wang et al., 2016). Guided by the genome-based biosynthetic potential mass spectrometric data were compared with literature and databases to identify the enriched molecules (Senges et al., 2018).

As indicated by its desferrioxamine biosynthetic gene cluster, Arv20#4.1 produces a set of desferrioxamines (**Figure 3A**). These were detected in both, positive and negative mode from the LC-MS procedure (**Supplementary Figure S4**). As the eponymous compound for this molecular subnetwork the hydroxamate siderophore desferrioxamine E was identified

TABLE 5 | Secondary metabolite clusters identified in strain *Rouxiella* sp. Arv20#4.1 with antiSMASH3.0.

Cluster	Type	Start	Stop	Similarity to known cluster	MIBiG BGC-ID
1	Hserlactone	87033	107683	–	–
2	Cf_fatty_acid	446284	467504	–	–
3	Arylpolyene	787129	821369	APE Ec biosynthetic gene cluster (68% of genes show similarity)	BGC0000836_c1
4	Cf_putative	25500	34695	Emulsan biosynthetic gene cluster (9% of genes show similarity)	BGC0000760_c1
5	Cf_fatty_acid-Cf_saccharide	24604	60942	Lipopolysaccharide biosynthetic gene cluster (18% of genes show similarity)	BGC0000776_c1
6	Cf_putative	27900	36897	–	–
7	Cf_putative	89005	95978	–	–
8	Cf_fatty_acid	165825	186739	–	–
9	Cf_saccharide	485089	506842	S-layer glycan biosynthetic gene cluster (20% of genes show similarity)	BGC0000794_c1
10	Cf_fatty_acid	532454	553725	–	–
11	Cf_putative	586070	593059	Polysaccharide B biosynthetic gene cluster (6% of genes show similarity)	BGC0001411_c1
12	Cf_putative	712377	716502	–	–
13	Cf_putative	103014	112356	–	–
14	Cf_putative	283336	295617	–	–
15	Siderophore	270474	282858	Desferrioxamine B biosynthetic gene cluster (60% of genes show similarity)	BGC0000941_c1
16	Cf_putative	164758	170600	–	–
17	Cf_saccharide	97264	131452	Capsular polysaccharide biosynthetic gene cluster (33% of genes show similarity)	BGC0000731_c1

NRPS, Non-ribosomal peptide synthetase cluster; Cf, ClusterFinder.

TABLE 6 | Secondary metabolite clusters identified in strain *Erwinia* sp. A41C3 with antiSMASH3.0.

Cluster	Type	Start	Stop	Similarity to known cluster	MIBiG BGC-ID
1	Cf_putative	284735	289620	Polysaccharide B biosynthetic gene cluster (6% of genes show similarity)	BGC0001411_c1
2	Cf_putative	15697	24996	–	–
3	Cf_putative	68401	78206	–	–
4	Thiopeptide	210237	236598	O-antigen biosynthetic gene cluster (14% of genes show similarity)	BGC0000781_c1
5	Cf_fatty_acid	347964	368917	Svaricin biosynthetic gene cluster (6% of genes show similarity)	BGC0001382_c1
6	Cf_putative	72078	80802	–	–
7	Cf_fatty_acid	1	11819	–	–
8	Cf_putative	18987	28258	Emulsan biosynthetic gene cluster (9% of genes show similarity)	BGC0000760_c1
9	Cf_fatty_acid	1	7355	–	–
10	Cf_fatty_acid -Ladderane	35473	78034	–	–
11	Cf_fatty_acid	41264	62484	–	–
12	Cf_saccharide	70023	92437	–	–
13	Cf_saccharide	57635	93206	Lipopolysaccharide biosynthetic gene cluster (60% of genes show similarity)	BGC0000779_c1
14	Hserlactone	73942	94574	–	–
15	Cf_saccharide	30591	54347	–	–
16	Cf_saccharide	103519	141097	Stewartan biosynthetic gene cluster (85% of genes show similarity)	BGC0000763_c1
17	Cf_saccharide-NRPS	131937	194079	Turnerbactin biosynthetic gene cluster (30% of genes show similarity)	BGC0000451_c1
18	NRPS	271826	315746	–	–
19	Cf_putative	129079	138254	–	–
20	Cf_putative	93961	107030	–	–
21	Cf_putative	143921	152024	–	–
22	Butyrolactone	192718	198822	–	–

NRPS, Non-ribosomal peptide synthetase cluster; Cf, ClusterFinder.

by comparison with reference spectra described in Senges et al. (2018) and through manual mapping of the fragment spectrum onto the molecular structure (**Figure 3B**). As sortation of fragment spectra by molecular networking allows sortation into structurally related families, further compounds in the desferrioxamine subnetwork are likely desferrioxamine E analog. This is supported by five further desferrioxamine-like siderophores, identified by their complete masses (**Supplementary Table S1**).

Of the 421 and 1,034 nodes in the molecular network of A41C3, originating from positive and negative mode respectively, none could be identified. One fragment spectrum showed similarity to a database entry of a desferrioxamine, but its characteristics (parent and fragment masses) were unlike those

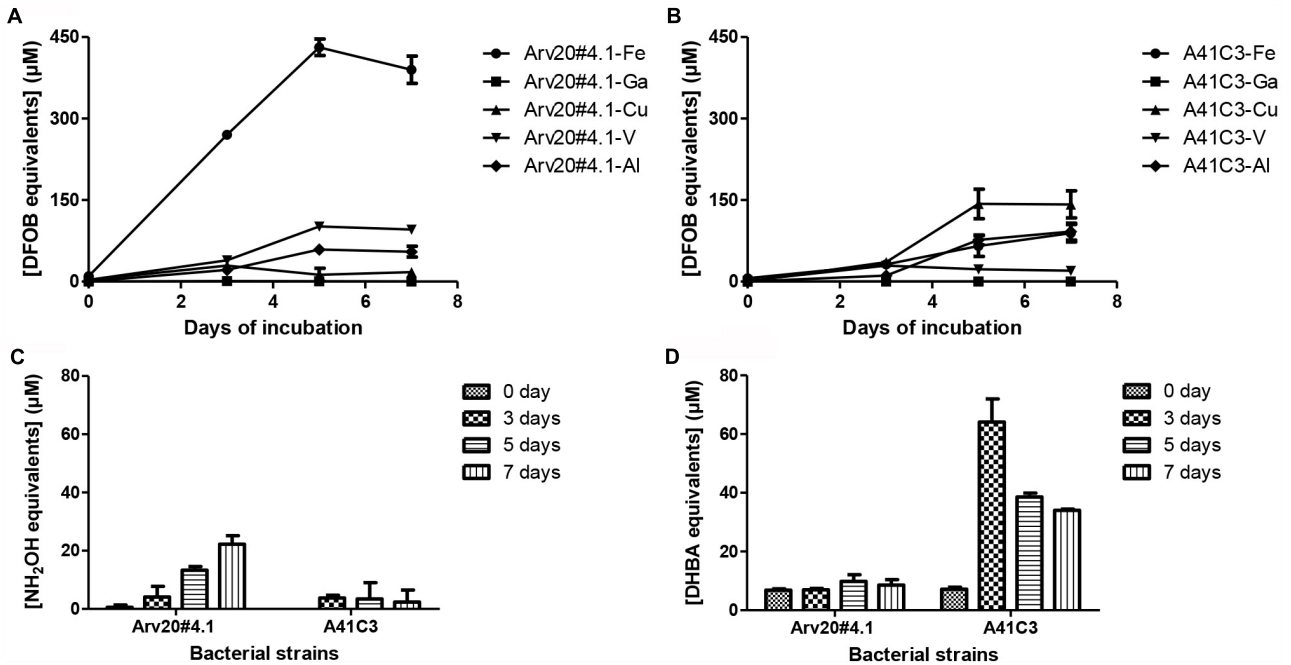


FIGURE 1 | Bacterial production and characterization of siderophores with different metal ion affinities. The two bacterial strains produced siderophores with different metal binding affinities, Arv20#4.1 siderophores showed higher affinity to iron **(A)** and A41C3 siderophores to Cu **(B)**. The production of siderophores is dependent on the growth of the strains. The strain Arv20#4.1 predominantly produced hydroxamate-type siderophores **(C)**, strain A41C3 catecholate-type siderophores **(D)**.

of typical desferrioxamines. Iron chelating and biological activity are likely to originate from different, yet unknown compounds. Respectively, an overview on siderophore activity of fractions with corresponding signals in mass spectrometry are provided in **Figure 2** and **Supplementary Table S2**.

C. elegans Attracted by Siderophore-Producing Bacteria

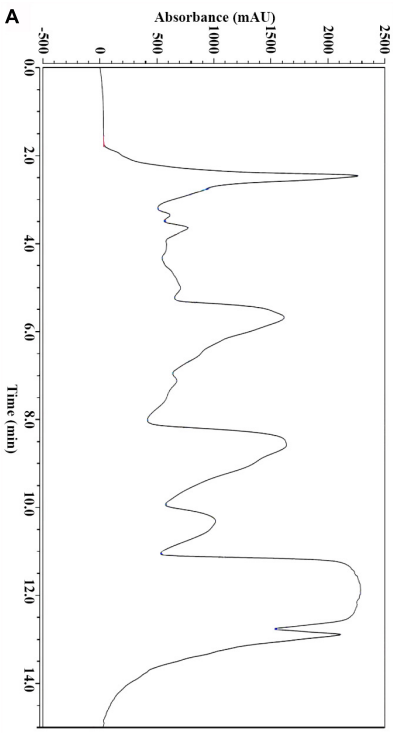
Both bacteria, after 24 h growth, showed higher OD_{600} in the M9 growth media supplemented with iron than in low-iron medium. This indicates iron limitation creates stress and should induce a metabolic response as shown above for siderophore production. Strain Arv20#4.1 reached an OD_{600} of 2.06 when growth medium was supplemented with iron and 0.84 when growth medium was deprived of iron. In the same way, strain A41C3 showed OD_{600} of 2.19 when growth medium was supplemented with iron and 0.54 when growth medium was deprived of iron.

C. elegans N2 was attracted by strains Arv20#4.1 and A41C3 and by their supernatants (**Figure 4**). The M9 broth media supplemented or not supplemented with iron did not attracted nematodes by itself. The *E. coli* OP50 was used as control and nematodes showed preference for their regular food source in comparison with both strains but without statistical significance. The nematodes were significantly attracted by strain A41C3 (+ Fe) compared to Arv20#4.1 (+ Fe), but no significant attraction of nematodes was observed when both strains were grown in M9 broth medium without iron supplementation. These differences might be due to differences in the metabolites produced and maximum cell density reached (OD_{600}). The supernatant of Arv20#4.1 with iron in the growth medium showed significant attraction of nematodes when compared with same strain supernatant without iron. The same was observed when supernatant of Arv20#4.1 (+ Fe) compared with supernatant of A41C3 supplemented with iron (**Figure 4**). On the other hand, supernatant of Arv20#4.1 without iron showed significant attraction of nematodes when compared with supernatant of A41C3 without supplementation of iron (**Figure 4**).

Nematicidal Activity

The herein investigated bacteria and their supernatants did not show nematicidal activity against *C. elegans* N2 evaluated at 24 h, 48h, and 72h incubation.

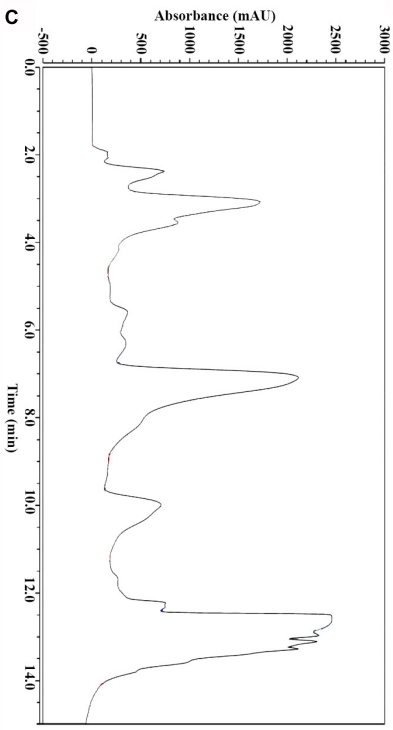
In the same line, the bacterial supernatants and fractions of both strains containing siderophores were tested against *B. xylophilus*. It was possible to observe significant nematicidal activity for HPLC fractions of the samples A-G against *B. xylophilus* PtAS18 (**Figure 5**). The sample G, containing HPLC fractions 3.25 + 3.26 + 3.27 + 3.28, showed the strongest nematicidal activity that includes combination of HPLC fractions 25, 26, 27, 28 (equal ratio) of the strain A41C3. The compounds present in these HPLC fractions, with nematicidal activity, showed similarity to a number of database entries with respect to the MS analysis. However, an identification was impossible so far. Therefore, the amounts were too little to obtain a relevant fragmentation pattern or even NMR data after iron removing.



B

Fraction (t-min)	Fe*	Ga*	V*	Cu*	Al*	Hydroxamate#	Catecholate$
1.1 (0.0)	3.2	0.3	35.2	8.2	-	-	-
1.2 (0.5)	-	0.5	37.3	9.5	-	-	-
1.3 (1.0)	2.2	0.5	38.8	9.2	-	-	-
1.4 (1.5)	-	0.5	41.0	10.1	-	-	-
1.5 (2.0)	-	1.2	-	14.0	-	-	-
1.6 (2.5)	-	0.5	32.2	12.7	-	-	-
1.7 (3.0)	5.0	1.6	45.5	10.3	-	-	-
1.8 (3.5)	2.9	1.4	46.1	7.2	-	-	-
1.9 (4.0)	3.7	2.0	45.9	7.1	-	6.1	5.8
1.10 (4.5)	8.4	4.4	56.9	9.4	-	12.1	6.3
1.11 (5.0)	12.3	6.3	58.5	9.9	-	8.0	6.5
1.12 (5.5)	57.3	26.5	64.7	16.0	-	11.4	6.5
1.13 (6.0)	67.9	20.1	63.1	16.5	-	17.2	6.4
1.14 (6.5)	35.8	11.3	60.7	9.0	-	12.4	6.2
1.15 (7.0)	24.1	8.8	60.0	9.4	-	9.6	6.0
1.16 (7.5)	16.4	8.6	62.7	13.5	-	6.7	5.8
1.17 (8.0)	15.9	13.3	14.1	14.1	-	6.2	6.0
1.18 (8.5)	51.4	40.8	29.9	29.9	-	9.1	6.0
1.19 (9.0)	56.6	34.9	23.5	23.5	-	22.7	6.0
1.20 (9.5)	36.7	18.6	14.5	14.5	-	26.5	6.1
1.21 (10.0)	19.6	18.6	15.3	15.3	-	11.3	6.0
1.22 (10.5)	24.2	27.8	15.5	15.5	-	8.9	6.0
1.23 (11.0)	38.9	52.8	38.5	38.5	-	12.7	5.9
1.24 (11.5)	290.5	89.8	535.3	535.3	13.7	67.2	5.9
1.25 (12.0)	246.9	88.2	355.3	355.3	8.1	92.0	6.1
1.26 (12.5)	171.4	70.4	40.4	40.4	-	18.4	6.5
1.27 (13.0)	24.7	7.6	5.9	5.9	-	-	7.0
1.28 (13.5)	1.3	0.9	2.0	2.0	-	-	-
1.29 (14.0)	1.1	0.4	1.4	1.4	-	-	-
1.30 (14.5)	-	-	-	-	-	-	-

*[DFOB equivalents] (μM); #[NH_2OH equivalents] (μM); $[DHBA equivalents] (μM); -, negative.



D

Fraction (t-min)	Fe*	Ga*	V*	Cu*	Al*	Hydroxamate#	Catecholate$
3.1 (0.0)	-	-	32.5	3.6	-	-	-
3.2 (0.5)	-	-	34.8	6.4	-	-	-
3.3 (1.0)	-	-	34.3	3.1	-	-	-
3.4 (1.5)	-	0.1	34.8	8.5	-	-	-
3.5 (2.0)	-	2.0	-	13.0	-	-	-
3.6 (2.5)	-	0.8	31.5	8.7	-	-	-
3.7 (3.0)	1.4	6.7	44.4	13.7	-	-	54.4
3.8 (3.5)	-	1.3	40.7	6.2	-	-	43.9
3.9 (4.0)	-	0.4	37.7	4.5	-	-	-
3.10 (4.5)	-	0.4	37.5	3.5	-	-	12.3
3.11 (5.0)	-	0.6	37.9	6.1	-	-	123.7
3.12 (5.5)	1.8	0.5	39.3	6.2	-	-	42.9
3.13 (6.0)	1.9	0.4	40.2	6.3	-	-	7.8
3.14 (6.5)	6.5	2.5	46.8	5.9	-	-	12.7
3.15 (7.0)	165.2	15.9	45.7	9.7	-	3.4	123.7
3.16 (7.5)	69.2	4.7	48.0	8.2	-	-	42.9
3.17 (8.0)	35.9	2.1	47.7	3.3	-	-	24.9
3.18 (8.5)	7.5	1.0	47.0	2.5	-	-	12.7
3.19 (9.0)	0.7	0.8	45.0	4.4	-	-	-
3.20 (9.5)	-	0.6	38.9	6.0	-	-	-
3.21 (10.0)	-	0.3	35.5	6.7	-	-	-
3.22 (10.5)	-	0.4	34.4	4.9	-	-	-
3.23 (11.0)	-	0.5	36.5	6.6	-	-	-
3.24 (11.5)	5.4	0.6	43.5	6.1	-	-	-
3.25 (12.0)	40.5	7.4	30.1	10.7	5.7	-	26.0
3.26 (12.5)	96.7	62.0	16.6	29.6	-	18.6	309.2
3.27 (13.0)	176.1	12.7	11.7	10.4	12.2	7.3	150.6
3.28 (13.5)	65.6	2.9	-	3.8	-	-	35.6
3.29 (14.0)	-	0.6	-	-	-	-	-
3.30 (14.5)	-	-	-	-	-	-	-

*[DFOB equivalents] (μM); #[NH_2OH equivalents] (μM); $[DHBA equivalents] (μM); -, negative.

FIGURE 2 | Purification and characterization of the siderophores in supernatant of strains Arv20#4.1 **(A,B)** and A41C3 **(C,D)**. **(A,C)** The siderophores were enriched by HPLC using a C18-RP column (1 ml; Thermo, Auqastar) equilibrated in 28% methanol (buffer **A**) and 72% ddH_2O with 0.1% TFA (buffer **B**). The elution from the column was performed as described and after 8 min, a 4-min linear gradient 28–95% buffer **A** followed by 3 min 95% buffer **A** was applied before going back to initial conditions (total time of elution 15 min at 1 ml/min flow rate). **(B,D)** Characterization of 30 fractions for Fe, Ga, Cu, V, and Al-CAS activities and quantitation of hydroxamate and catecholate-type siderophores. The best supernatant HPLC fraction of strain Arv20#4.1 containing hydroxamate-type siderophores (92 μM of NH_2OH equivalents) most actively chelated Cu **(B)**. The best supernatant HPLC fraction of strain A41C3 contains catecholate-type siderophores (309 μM of DHBA equivalents) with higher affinity for Fe **(D)**.

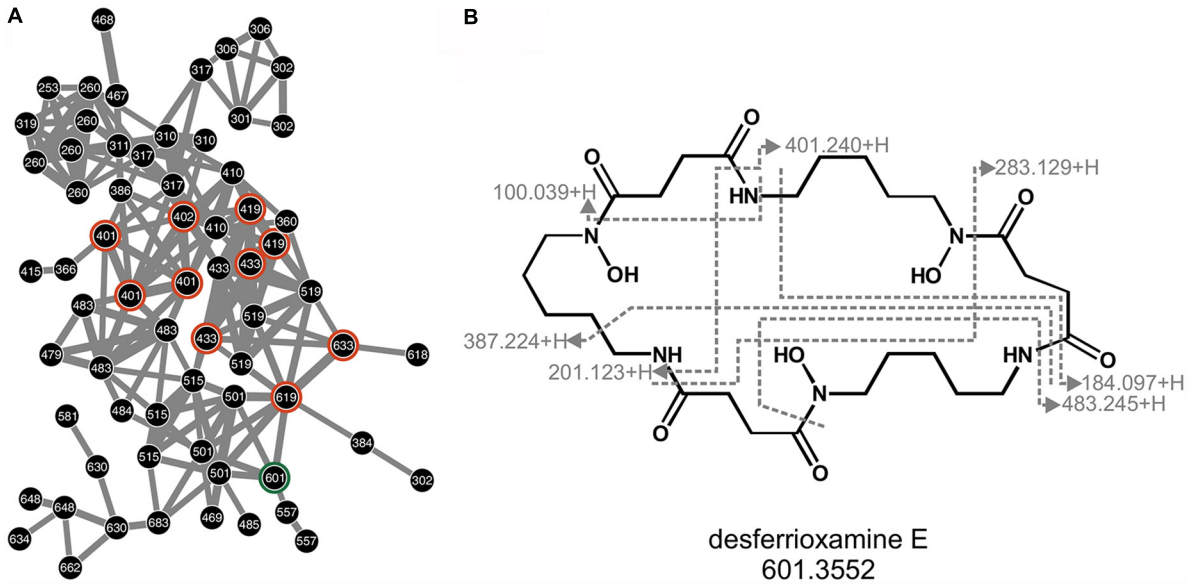


FIGURE 3 | Desferrioxamines of strain Arv20#4.1. Subnetwork of desferrioxamines as produced by Arv20#4.1 and detected in positive mode **(A)**. Nodes are labeled with corresponding m/z values. Highlighted in orange are nodes which resemble identified desferrioxamines. As this network is not dereplicated, some desferrioxamines are represented by several nodes. Marked in green is desferrioxamine E, for which the corresponding fragment spectrum was mapped onto the molecular structure **(B)**. Given are the m/z of fragments as measured in positive mode.

DISCUSSION

Bacteria are important in the ecosystem of *P. pinaster* trees as they are involved in key activities and interactions such as production of proteases, lipases, cellulases, secretion of metabolites for signaling and auxins (Proença et al., 2010, 2017a,b; Vicente et al., 2012). They are further involved in solubilization of phosphate and zinc and mobilization of metals and metalloids by the production of siderophores (Proença et al., 2010, 2017a,b). Among those activities, (secondary) metabolite production and release by microbes represents an important trigger in the ecosystems. This is often a response towards a limitation of resources within such an ecosystem. Here, we focused especially on metallophores produced under metal (iron) limitation and known to have species overlapping activities (Hider and Kong, 2010; Wiche et al., 2017; Senges et al., 2018). Metallophores are known to be part of the arsenal of plant-growth promoting (rhizo)bacteria and to interact with other microorganisms, nematodes, higher animals, and plants within the ecosystem (Mercado-Blanco et al., 2018). Metabolites like siderophores play important roles in ecosystems to chelate metallic micronutrients essential for microorganisms and plants. However, siderophores have been recognized to have a key role in virulence of pathogenic organisms (Soutar and Stavrinides, 2018) and thus its screening is important.

Species belonging to the family *Enterobacteriaceae* are known to grow in soil next to plants, to engage in plant-microbe interactions, and thus are part of a complex network in the rhizosphere. Strains of the genus *Rouxiella* have been isolated from peat bog soil (Le Fléche-Matéos et al., 2017) and are recognized to produce biosurfactants (Kügler et al., 2015). Strains belonging to both genera *Erwinia* and *Pantoea* have been demonstrated to produce hydroxamate siderophore desferrioxamine E among others that supports the iron acquisition by the strains. This ability favors host colonization and it is recognized as a factor of pathogenicity (Dellagi et al., 2009).

Here, we found that the endophytic *Erwinia* strain A41C3 produces a catecholate-type siderophore based on analytical methods and bioinformatics genome annotation pipeline. The latter hints towards turnerbactin but this was not verified by LC-MS/MS and thus, these compounds remain enigmatic and await structural identification. *Erwinia* species are known to produce achromobactin or aerobactin (Soutar and Stavrinides, 2018), two hydroxamate-type siderophores, that have not yet been described to produce catecholate-type metallophores like turnerbactin-related compounds. This will be investigated in more detail in the near future.

In this study, we found that PWN-carried *Rouxiella* strain Arv20#4.1 produces desferrioxamine-like compounds based on analytical methods and also predicted from the genome (**Figure 3** and **Supplementary Figure S2**). This was verified by LC-MS/MS confirming the identification of desferrioxamine-like structures, with desferrioxamine E (DFOE) as major metabolite. This is the first time that siderophore production was described in more detail by a strain of the genus *Rouxiella* and the respective siderophore was identified as DFOE. The HPLC-enriched fraction of DFOE and related molecules (**Figure 2**) showed affinity to various metals and metalloids as described previously for other desferrioxamines obtained from soil bacteria (Mehnert et al., 2017; Wiche et al., 2017; Schwabe et al., 2018).

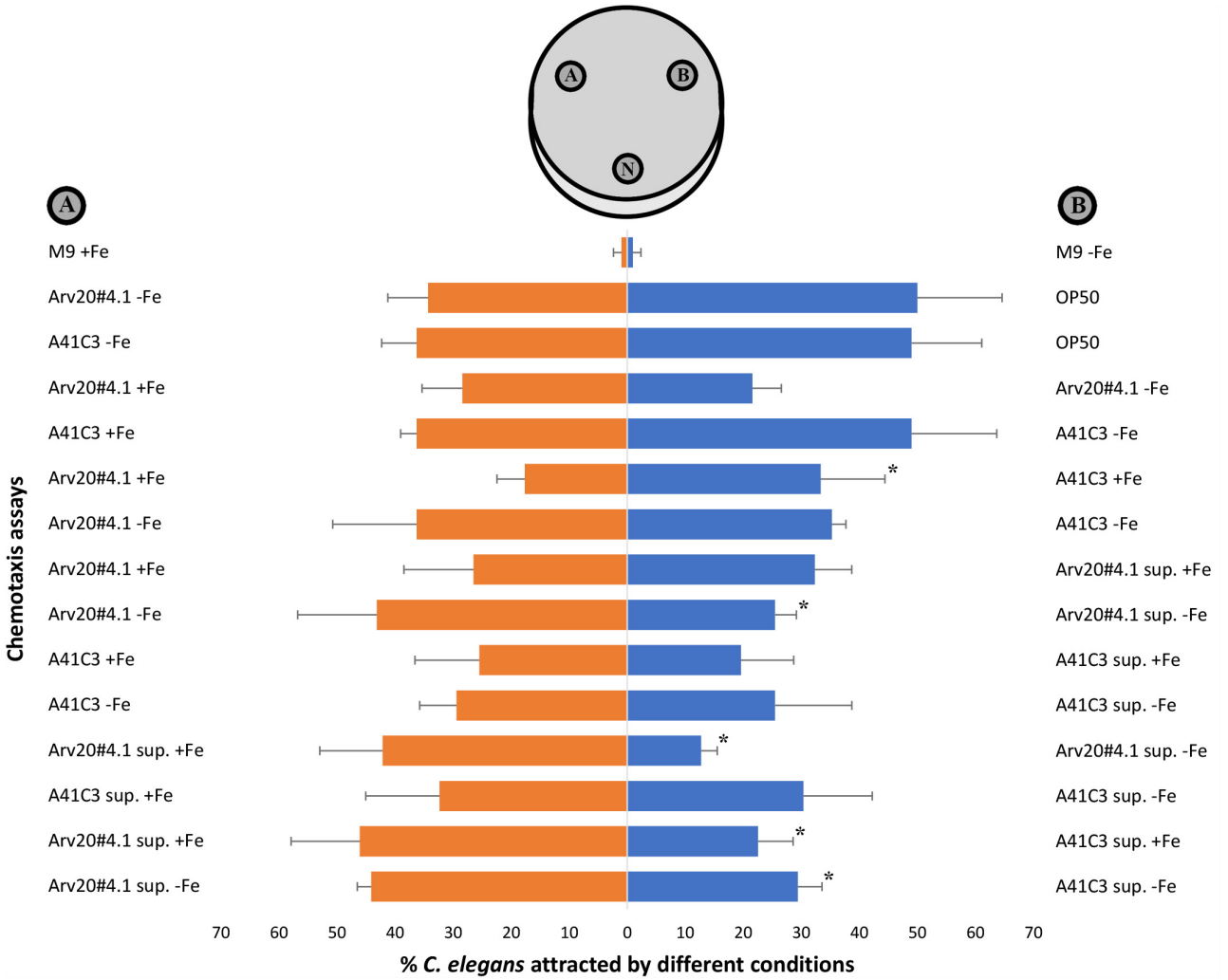


FIGURE 4 | Attraction of *C. elegans* N2 by strains Arv20#4.1 and A41C3 and by their supernatants. The nematodes were significantly attracted by strain A41C3 (+Fe) instead Arv20#4.1 (+Fe) that was not significantly attracted in assay of both bacterial strains without iron supplementation in M9 broth media. The supernatant of Arv20#4.1 (+ Fe) showed significant attraction of nematodes when compared with supernatant of Arv20#4.1 (–Fe) or in the presence of supernatant of A41C3 (+ Fe). On the other hand, supernatant of Arv20#4.1 (–Fe) showed significant attraction of *C. elegans* N2 when compared with supernatant of A41C3 (–Fe). M9 broth media supplemented or not with iron did not attracted nematodes by itself. The *E. coli* OP50 was used as control and nematodes showed preference for their regular food source in comparison with both strains but without statistical significance. N, application spot of nematodes; **(A,B)**, application spots of strains, supernatants or M9 medium. *Indicate significantly different percentages of attraction ($p < 0.05$) between treatments. sup., abbreviation of supernatant. +Fe, growth medium supplemented with iron. –Fe, growth medium without supplementation of iron.

As described above both strains, A41C3 and Arv20#4.1, produced different siderophores which have different properties. As various metals and metalloids may play a role in the rhizosphere ecosystem it is of interest to verify the capability of the siderophores to bind various types of ions (Mehnert et al., 2017; Wiche et al., 2017; Schwabe et al., 2018). Therefore, we decided to choose a selection of ions which can form a CAS-complex and thus allow to show and to discriminate affinity of siderophores for Fe, Ga, Cu, V, and Al (**Figure 2**). The siderophore of strain Arv20#4.1 is a hydroxamate-type siderophore and has a high affinity for Cu. The one of strain A41C3 is a catecholate-type siderophore and has a high affinity for Fe.

Caenorhabditis elegans N2 were attracted by both strains and by their supernatants. This attraction of this nematode through different bacterial species has been recognized in several studies with other bacterial strains (Choi et al., 2016). No nematicidal activity against *C. elegans* was demonstrated by either species, by bacterial supernatants, or by HPLC fractions containing siderophores. Monitoring food behavior of *C. elegans* is a simple approach to identify potential bacterial virulence (Lewenza et al., 2014). Considering that the biological role of siderophores is to ensure the acquisition of iron necessary for metabolism, their production is expected to be lower in bacteria growing in iron-supplemented media. This was the case, however, the nematode behavior was similar in both supernatants. *C. elegans* preferred

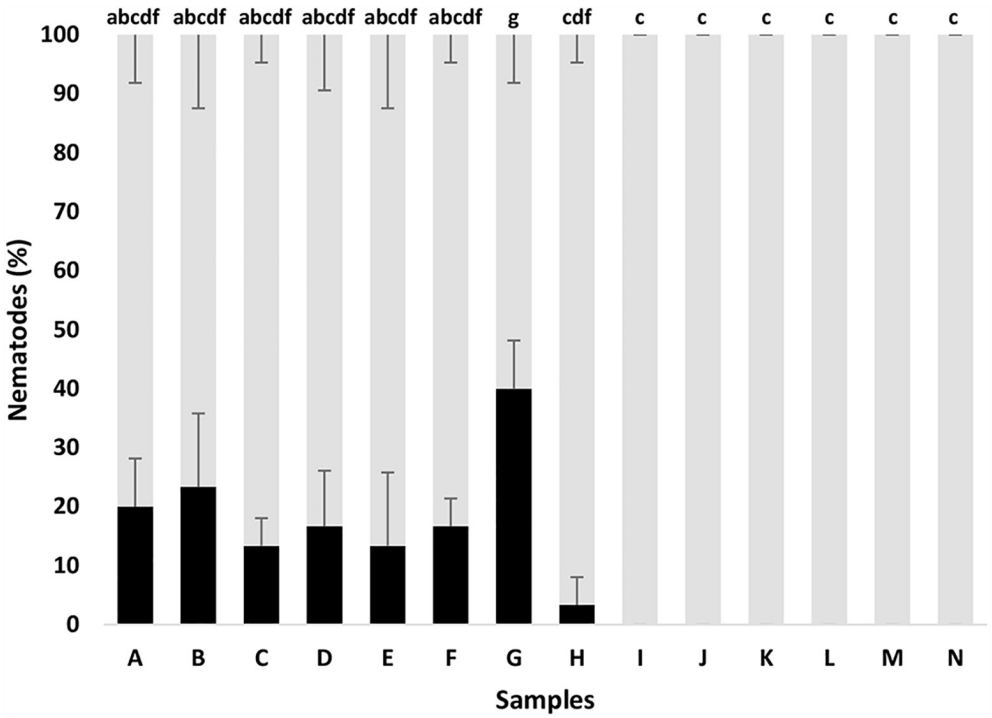


FIGURE 5 | *Bursaphelenchus xylophilus* dead (black) and alive (gray) after different *in vitro* treatments with bacterial supernatant and fractions containing siderophores, after 24 h incubation. Fractions, mentioned in **Figure 2**, with siderophores resuspended in 0.1 M NaCl. (A) HPLC fractions 1.12 + 1.13; (B) 1.17 + 1.18 + 1.19; (C) HPLC fractions 1.21 + 1.22; (D) HPLC fractions 1.23 + 1.24 + 1.25 + 1.26; (E) HPLC fractions 3.14 + 3.15 + 3.16; (F) HPLC fractions (3.20 + 3.21); (G) HPLC fractions 3.25 + 3.26 + 3.27 + 3.28; (H) NaCl 0.1 M; (I) Arv20#4.1 supernatant (growth in CAA medium); (J) A41C3 supernatant (growth in CAA medium); (K) Arv20#4.1 supernatant (growth in M9 medium); (L) A41C3 supernatant (growth in M9 medium); (M) CAA medium; and (N) M9 medium. Different letters above the bars indicate significantly different percentages of mortality ($p < 0.05$) between treatments.

bacterial supernatants to bacterial cells, independently of the bacterial growth conditions. This behavior is in congruence with the fact that bacterial supernatants and siderophores do not kill *C. elegans* and may be used by the nematode as food sources. The type of siderophores, and probably the concentration, might be crucial for nematicidal activity as it was demonstrated in pathogenicity of *P. aeruginosa* by its siderophore pyoverdin (Kirienko et al., 2013). On the other hand, the nematode behavior seems to indicate that *Rouxiella* sp. Arv20#4.1 is more virulent than *Erwinia* sp. A41C3, using virulence factors different from siderophores. Both bacterial species are *gamma-proteobacteria* reported to be pathogenic (Paiva et al., 2013).

The effect of enriched siderophore fractions on *B. xylophilus* behavior was evaluated for the first time. The isolate Arv20#4.1 originating from PWN did produce mainly desferrioxamines which had no effect on *B. xylophilus*. Thus it might be reasoned that desferrioxamines interfere with the mobility of metals but do not affect other organisms of this ecosystem to a large extent. However, this nematode showed to be highly susceptible to supernatants containing siderophores and other metabolites produced by the endophytic *Erwinia* sp. A41C3 under iron limitation. Here, the highest nematicidal activity was determined from the fractions comprising highest siderophore titer, respectively. This seems to be a clear correlation and needs to be elucidated with pure compounds in more detail. Siderophores produced by entomopathogenic bacteria (Hirschmann et al., 2017), endophytic plant growth-promoting bacteria (Tashi-Oshnoei et al., 2017), and rhizospheric bacteria (Khan et al., 2016) have been related with the pathogenic activity of these bacteria against insects, plant pathogenic bacteria and root-knot nematode, respectively. The role of this *Erwinia* strain when part of the endophytic bacterial community needs to be explored. In spite of that, their metabolites produced under iron limitation should be investigated further as they might prove useful in biocontrol *B. xylophilus* and management of Pine Wilt Disease.

DATA AVAILABILITY STATEMENT

The Whole Genome Shotgun project of strains Arv20#4.1 and A41C3 has been deposited at ENA under the accession numbers RQVU00000000 and RQVV00000000, respectively. The 16S rRNA gene sequences of the isolates have been previously deposited in the NCBI GenBank database under the accession numbers KF214948 (Proença et al., 2014) and KJ654833 (Proença et al., 2017a). Accession links for metabolomic data sets are provided at: positive mode (http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=bbd4f668b6224bd381617001be917381); negative

mode (http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e3009a90a0954791ae9b747e727c6515).

AUTHOR CONTRIBUTIONS

DP, PM, and DT designed the experiments and wrote the manuscript. DP did all the experiments. TH did the genome sequencing and analysis. CS performed the mass spectrometry analysis. DT and PM contributed with reagents and laboratory conditions. JB provided input to the manuscript. All authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02166/full#supplementary-material

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COP9 Signalosome CSN4 and CSN5 Subunits Are Involved in Jasmonate-Dependent Defense Against Root-Knot Nematode in Tomato

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COP9 signalosome (CSN) is an evolutionarily conserved regulatory component of the ubiquitin/proteasome system that plays crucial roles in plant growth and stress tolerance; however, the mechanism of COP9-mediated resistance to root-knot nematodes (RKNs, e.g. *Meloidogyne incognita*) is not fully understood in plants. In the present study, we found that RKN infection in the roots rapidly increases the transcript levels of CSN subunits 4 and 5 (*CSN4* and *CSN5*) and their protein accumulation in tomato (*Solanum lycopersicum*) plants. Suppression of *CSN4* or *CSN5* expression resulted in significantly increased number of egg masses and aggravated RKN-induced lipid peroxidation of cellular membrane but inhibited RKN-induced accumulation of CSN4 or CSN5 protein in tomato roots. Importantly, the RKN-induced accumulation of jasmonic acid (JA) and JA-isoleucine (JA-Ile), as well as the transcript levels of JA-related biosynthetic and signaling genes were compromised by *CSN4* or *CSN5* gene silencing. Moreover, protein–protein interaction assays demonstrated that CSN4 and CSN5B interact with the jasmonate ZIM domain 2 (JAZ2), which is the signaling component of the JA pathway. Silencing of *CSN4* or *CSN5* also compromises RKN-induced *JAZ2* expression. Together, our findings indicate that CSN4 and CSN5 play critical roles in JA-dependent basal defense against RKN.

Keywords: basal defense, COP9 signalosome subunit 4 (CSN4), CSN5, jasmonic acid, root knot-nematode, tomato

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INTRODUCTION

Plant parasitic nematodes attack majority of agricultural crops, causing an annual loss of approximately 157 billion USD (Abad et al., 2008; Holbein et al., 2016). Root-knot nematodes (RKNs, *Meloidogyne* spp.) such as *Meloidogyne arenaria*, *Meloidogyne javanica*, *Meloidogyne incognita*, and *Meloidogyne hapla* are among the most economically important sedentary endoparasitic nematodes (Jones et al., 2013). In RKN life cycle, infective juveniles (J2s) hatch from eggs in soil. Infective J2s penetrate the host roots and then migrate towards the plant vascular system, where the nematode provokes the generation of giant cells leading to the formation of a gall. This feeding site is the nutrient source for the RKN. The egg masses (EMs) are formed within the gall tissue or on the surface of root galls (Williamson and Kumar, 2006). To overcome RKN infection, plant defense responses are triggered by activation of pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and

resistance (R)-protein-activated effector-triggered immunity (ETI) (Zhou et al., 2018). PTI is a basal defense response that resists most non-adapted pathogens, leading to basal immunity in plants during pathogen infection (Couto and Zipfel, 2016). ETI is a second immune response that is classically associated with the recognition of pathogen-secreted effectors (Cui et al., 2015). In solanaceous crops, several specific *R*-genes, such as *Me* genes in pepper (*Capsicum annuum*) and *Mi* genes in tomato (*Solanum lycopersicum*), have been identified and successfully used to limit the establishment and spread of RKNs (Milligan et al., 1998; Djian-Caporalino et al., 2007). The *R*-genes encode structurally similar nucleotide-binding site, leucine-rich repeat (NBS-LRR) proteins which can recognize RKN-derived molecules and induce hypersensitive response (HR) through their anti-RKN complexes with chaperones such as Hsp90 and Sgt1 in RKN-resistant plants (Takahashi et al., 2003; Shirasu, 2009).

Phytohormones salicylic acid (SA) and jasmonic acid (JA) play critical roles in the plant defense system, and their signaling pathways often interact in an antagonistic manner in plant immunity (Berens et al., 2017). The application of SA or its analogs can activate a strong defense against RKNs in tomato and rice (*Oryza sativa*) plants (Nahar et al., 2011; Molinari et al., 2014). In contrast, overexpression of bacterial salicylate hydroxylase *NahG*, which can catalyze the degradation of SA, affects neither basal defenses nor *Mi-1* resistance to RKNs in tomato (Bhattarai et al., 2008), showing the ambiguous function of SA in plant RKN defense. The JA-dependent signaling pathway plays pivotal roles in both ETI and PTI against RKNs in plants (Nahar et al., 2011; Manosalva et al., 2015). The application of JA or its methyl ester (MeJA) enhances RKN resistance in *Arabidopsis*, tomato, and rice (Nahar et al., 2011; Zhou et al., 2015; Gleason et al., 2016). Similarly, the reproduction of RKNs is reduced by overexpression of tomato *Prosystemin* gene, which can constitutively induce the JA-dependent signaling pathway, but is increased in the tomato JA-biosynthetic mutant *suppressor of prosystemin-mediated responses 2* (*spr2*) (Sun et al., 2010). Furthermore, proteinase inhibitors (PIs) are involved in JA-induced resistance against RKN infection (Fujimoto et al., 2011). Silencing of *PI1* compromises JA-induced basal defense and increases RKN reproduction in tomato (Zhou et al., 2015).

The COP9 signalosome (CSN) is an evolutionarily conserved protein complex which regulates the ubiquitin/proteasome system (UPS) that specifically guides ubiquitylated proteins to the 26S proteasome for degradation in plants, animals, and fungi (Schwechheimer, 2004). Previously identified CSNs have eight subunits, CSN1 to CSN8, according to their electrophoretic mobility (Wei et al., 1994; Wei et al., 2008; Stratmann and Gusmaroli, 2012). Six subunits (CSN1–CSN4, CSN7, and CSN8) contain a PCI (proteasome, CSN, and eukaryotic initiation factor 3, eIF3) domain, and two (CSN5 and CSN6) contain a MPN (Mpr1p-Pad1p-N-terminal) domain (Kotiguda et al., 2012). Recently, a subunit, CSN acidic protein (CSNAP) without PCI or MPN domain, has been identified in humans (Rozen et al., 2015). CSN subunits can act independently as free forms or combine with other CSN subunits to form CSN complexes (Dubiel et al., 2015). In *Arabidopsis*, loss of function of either subunit destabilizes the CSN complex, and all *csn* mutants exhibit lethal phenotype after germination (Wei et al., 1994; Gusmaroli et al., 2007).

The CSN regulates multiple phytohormone signaling pathways through interacting with SCF-type E3 ubiquitin ligases such as SCF^{TIR1} in auxin, SCF^{COI1} in jasmonate, and SCF^{SLY1} in gibberellic acid signaling in *Arabidopsis* (Schwechheimer et al., 2001; Feng et al., 2003; Dohmann et al., 2010). Moreover, silencing of *CSN5* reduces JA biosynthesis, and its signaling response comes along with reduced resistance against herbivorous *Manduca sexta* larvae and the necrotrophic fungal pathogen *Botrytis cinerea* in tomato plants (Hind et al., 2011). These studies indicate that the CSN complex orchestrates both JA biosynthesis and signaling responses. CSN subunits also play critical roles in phytohormone regulation and plant defense response. For instance, the geminiviral C2 protein interacts with CSN5 and alters phytohormonal pathways which are regulated by CUL1-based SCF ubiquitin E3 ligases in *Arabidopsis* (Lozano-Durán et al., 2011). CSN5A interacts with JAZ1, and it is targeted by more than 30 virulence effectors from various pathogens (Mukhtar et al., 2011). However, a CSN5-like gene negatively regulates wheat (*Triticum aestivum* L.) leaf rust resistance (Zhang et al., 2017a). Recently, Bournaud et al. (2018) found that soybean (*Glycine max*) CSN5 interacts with the *M. incognita Passe-Muraille* (*MiPM*) gene which encodes a cell-penetrating protein. However, the precise role of CSN in plant defense against RKN infection and the relationship between CSN and JA signaling pathway in tomato RKN resistance remain unclear.

In the present study, we have found that there are 10 COP9 subunits (including CSN1, CSN2A, CSN2B, CSN3, CSN4, CSN5A, CSN5B, CSN6, CSN7, and CSN8) in tomato plants and show that CSN4 and CSN5 play a critical role in tomato basal defense against *M. incognita* infection. The expression of *CSN4* and *CSN5* genes and the accumulation of their proteins were induced by RKN infection in tomato plants. Silencing of *CSN4* or *CSN5A* aggravated RKN infection and damaged the cell membrane in tomato roots. The accumulation of JA and JA-isoleucine (JA-Ile) and the transcript levels of JA biosynthetic and signaling genes were attenuated by the reduced expression of *CSN4* and *CSN5*. Moreover, using yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC), we demonstrated that CSN4 and CSN5B could both interact with jasmonate ZIM domain 2 (JAZ2), which is the signaling module of the coronatine-insensitive protein 1 (COI1)-JAZ co-receptor for JA-Ile (Thines et al., 2007; Fonseca et al., 2009). Taken together, the results indicate that CSN4 and CSN5 are involved in tomato RKN resistance by activating JA biosynthesis and signaling pathway.

MATERIALS AND METHODS

Plant Materials and Virus-Induced Gene Silencing

A susceptible tomato (*S. lycopersicum*) cultivar Ailsa Craig was used in all experiments. Germinated seeds were grown in 100-cm^3 plastic pots filled with steam-sterilized river sand and watered daily with Hoagland's nutrient solution in the growth

room. The growth conditions were as follows: 23/20°C day/night temperature, a 14-h photoperiod and 600 μmol m^{-2} s^{-1} photosynthetic photon flux density.

Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) constructs used for silencing the tomato *CSN* genes were generated by cloning specific cDNA fragments, which were amplified using specific primers as shown in **Supplemental Table S1**. The PCR products were digested by specific restriction enzymes and ligated into the same restriction sites in the TRV-based VIGS vectors TRV-*RNA2* (TRV2). The specific restriction sites of each vector are shown in **Supplemental Table S1**. TRV-*CSN4* contains 276 base pairs (bp) and TRV-*CSN5* contains 367 bp. The resulting plasmids were transformed in *Agrobacterium tumefaciens* strain GV3101. A mix of the TRV-based VIGS vectors TRV-*RNA1* (TRV1) and gene-targeted TRV2 in a 1:1 ratio was co-infiltrated into germinating seeds by vacuum infiltration. The infiltrated seeds were sown, and the seedlings were grown in a growth room at 22°C for 4 weeks before they were used for experiments (Zhou et al., 2018). The transcript levels of silenced genes were measured by quantitative real-time PCR (qRT-PCR) using the primers shown in **Supplemental Table S2**.

RKN Infection Assays

The seedlings were used for RKN infection at the four-leaf stage. The RKN *M. incognita* line, race 1, was cultured on Ailsa Craig plants in a greenhouse at 22–26°C. Nematode eggs were extracted from infected roots by processing in 0.52% NaOCl in a blender for 2 min at 12,000 rpm (Zhou et al., 2015). The eggs and root debris were passed through 0.15-mm pore sieves (100 mesh), and eggs were collected on 0.025-mm pore sieves (500 mesh). J2s were obtained by hatching the eggs in a modified Baermann funnel. The mesh sieves were linked with two layers of paper towels which were set in petri dishes. The dishes were incubated at 27°C, and J2 hatchlings were collected after 48 h (Martinez de Ilarduya et al., 2001). The tomato seedlings were inoculated with 500 J2s or mock inoculated with water over the surface of the sand around the primary roots. In each experiment, more than 30 plants of each genotype were infected with RKNs. Four weeks after inoculation, the EMs were evaluated by staining with 3.5% acid fuchsin (Zhou et al., 2015). The roots were placed in acidified glycerin and photographed.

RNA Isolation and qRT-PCR

Total RNA was extracted from 100 mg of root tissue using an RNA extraction kit (Tiangen, Shanghai, China) and reverse transcribed using a ReverTra Ace qRT-PCR kit (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. The qRT-PCR was performed using the LightCycler 480 RT PCR system (Roche, Basel, Switzerland), as described earlier (Wang et al., 2019). The PCR program was performed using pre-denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and then a final extension at 72°C for 5 min. Relative gene expression was calculated as described previously (Livak and Schmittgen, 2001). Three biological replicates were analyzed. The primers specific for target genes and the internal control *ACTIN* gene are presented in **Supplemental Table S2**.

Protein Extraction and Western Blotting

For protein extraction, root tissue (0.3 g) was ground in liquid nitrogen and homogenized in an extraction buffer (100 mM of Tris-HCl, pH 8.0, 10 mM of NaCl, 1 mM of EDTA, 1% Triton X-100, 1 mM of phenylmethylsulfonyl fluoride, and 0.2% β-mercaptoethanol). Protein concentration was measured by a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). For western blotting, 30-μg total proteins were loaded and separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in a Tris-buffered saline (TBS) buffer (20 mM of Tris, pH 7.5, 150 mM of NaCl, and 0.1% Tween 20) with 5% skim milk powder at room temperature and then incubated overnight in TBS buffer with 1% bovine serum albumin (BSA) containing a rabbit anti-CSN4 (Enzo, Shanghai, China) and a rabbit anti-CSN5 (ABclonal, Wuhan, China) polyclonal antibodies (1:1,000) to detect CSN4 and CSN5 proteins. After incubation with a goat anti-rabbit horseradish peroxidase (HRP)-linked antibody (1:,5000; Abcam, Shanghai, China), the complexes on the blot were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. A rabbit anti-actin polyclonal antibody (1:5,000; Abcam, Shanghai, China) was used as a loading control.

Determination of Lipid Peroxidation in Root Extracts

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) in the roots. Root samples of 0.5 g were harvested and grinded into homogenate in phosphate-buffered saline (PBS) buffer and then centrifuged at 12,000 rpm, 4°C for 10 min. Two milliliters of supernatant and 3 ml of 10% trichloroacetic acid containing 0.65% 2-thiobarbituric acid were mixed and heated at 95°C for 30 min and then centrifuged at 12,000 rpm for 10 min. The absorbance value of the supernatant was measured according to Zhou et al. (2012).

JA and JA-Ile Measurement

Root samples (100 mg) spiked with 100 ng ml^{-1} D_5-JA and D_5-JA-Ile (OlChemIm Ltd, Olomouc, Czech) as internal standards were used for the measurement of JA and JA-Ile. After shaking for 12 h in 1 ml of ethyl acetate in the dark at 4°C, the homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected, and the pellet was extracted again with 1 ml of ethyl acetate, being shaken for 2 h at 4°C. Both supernatants were evaporated to dryness under N_2 gas. The residue was resuspended in 0.5 ml of 70% methanol (v/v) and centrifuged at 12,000 rpm for 2 min at 4°C. The final supernatants were pipetted into glass vials and then analyzed by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) (Agilent Technologies, California, USA) according to a previously described procedure (Wang et al., 2016).

Y2H and BiFC Assays

Y2H assay was performed according to the method described previously (Hong et al., 2012). The full-length coding DNA sequences (CDS) of *CSNs* and *JAZs* were amplified by using the specific primers shown in **Supplemental Table S3**. The PCR products were digested and inserted into the pGBKT7 and pGADT7 vectors, respectively. Vectors were transformed into Y2H gold yeast strain. Yeast cells of 8×10^7 were loaded *per* spot on SD-Leu-Trp and SD-Leu-Trp-His-Ade plates to detect protein–protein interaction.

BiFC assay was performed as described by Zhou et al. (2018). The *CSN4* and *CSN5* CDS were inserted into p2YC to generate N-terminal in-frame fusions with the C-terminal side of yellow fluorescent protein (C-YFP), and *COI1* and *JAZ2* were ligated into p2YN to generate N-terminal in-frame fusions with the N-terminal side of YFP (N-YFP). The primers used for BiFC assays are listed in **Supplemental Table S4**. The resulting clones were verified by sequencing, and the resulting plasmids were transformed into *A. tumefaciens* strain GV3101. The transgenic *Nicotiana benthamiana*, which expresses the histone 2B fused with a red fluorescent protein (RFP) as a marker for nucleus location signals, was infiltrated into *A. tumefaciens* as previously described (Zhou et al., 2013). Infected tissues were analyzed 48 h after infiltration using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). The excitation and emission wavelengths were at 514 and 520–560 nm for YFP signal detection and at 561 and 580–620 nm for RFP signaling detection.

Phylogenetic Analysis

A phylogenetic tree was inferred from the full lengths of CSN protein sequences using the neighbor-joining method. Phylogenetic analysis was conducted in MEGA5 (Tamura et al., 2011). Bootstrap values from 1,000 replicates were used to assess the robustness of the tree. The protein sequences of *Arabidopsis* and tomato were obtained by downloading from The Arabidopsis Information Resource (https://www.arabidopsis.org/) and Sol Genomics Network (https://solgenomics.net/).

Statistical Analysis

At least three independent replicates were used for each experiment. Statistical analysis of the bioassays was performed using the SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA) statistical package. Experimental data were analyzed with Tukey's multiple range test at $P < 0.05$.

RESULTS

Expression Profiles and Protein Accumulation Analysis of CSNs Upon Defense Responses Against RKNs in Tomato Plants

To dissect the conserved relationships of CSN family members, CSN full-length amino acid sequences from *Arabidopsis* and tomato were plotted to construct a phylogenetic tree using the MEGA program (**Supplemental Figure S1**). The phylogenetic analysis showed that 10 CSN proteins which are divided into CSN1–CSN8 subunits have unique structures in each species but have close structural homology in *Arabidopsis* and tomato plants. Compared with two homologous CSN5 (AtCSN5A and AtCSN5B) and CSN6 (AtCSN6A and AtCSN6B) in *Arabidopsis*, there are two homologous CSN2 (SlCSN2A and SlCSN2B) and CSN5 (SlCSN5A and SlCSN5B) in tomato (**Supplemental Figure S1**).

To elucidate whether CSNs are involved in plant basal defense against RKNs, we examined the expression patterns of 10 *CSNs* in response to RKNs in wild-type (WT) tomato roots. As shown in **Figure 1**, the transcripts of these *CSN* genes were differentially induced after RKN infection, which were especially apparent for *CSN4* and *CSN5A* at 24 h post inoculation (hpi). Meanwhile, western blotting analysis indicated that the accumulation of CSN4 and CSN5 was significantly induced after RKN infection (**Figure 2**). The CSN4 protein levels increased by 160%, 132%, 76%, and 74% in RKN-infected roots compared to mock roots at 24, 48, and 72 hpi and 20 days post inoculation (dpi), respectively (**Supplemental Figure S2A**). Similarly, the CSN5 protein levels were increased by 82%, 71%,18%, and 15% in RKN-infected roots compared to mock roots at 24, 48, and 72 hpi and 20 dpi, respectively (**Supplemental Figure S2B**).

Involvement of CSN4 and CSN5 in Tomato Defense Against RKNs

To identify the roles of the CSNs in plant basal defense against RKNs, we silenced all eight CSN subunits including co-silencing of *CSN2A* and *CSN2B* or *CSN5A* and *CSN5B*, which share 88%

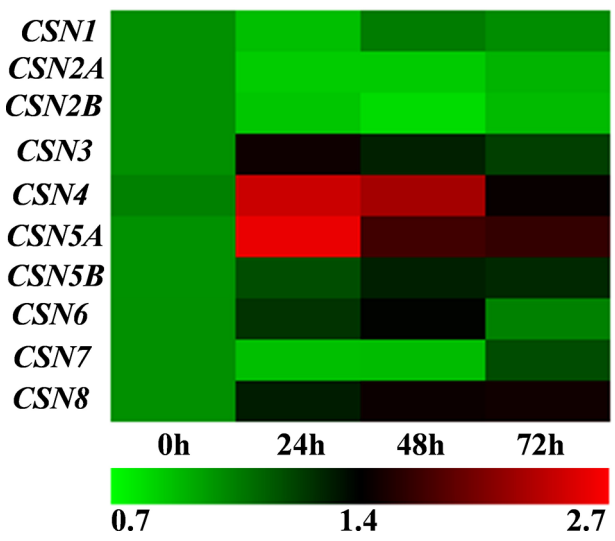

FIGURE 1 | Heat-map with the expression of COP9 signalosome (*CSN*) genes after root-knot nematode (RKN) infection in the roots of wild-type (WT) tomato plants. The labels 0 h, 24 h, 48 h, and 72 h indicate the time after RKN infection. Transcript levels were determined using quantitative real-time (qRT)-PCR, and cluster analysis was performed using MeV version 4.9. The color bar at the bottom shows the levels of expression. The data shown are the average of three biological replicates, and three independent experiments were performed with similar results.

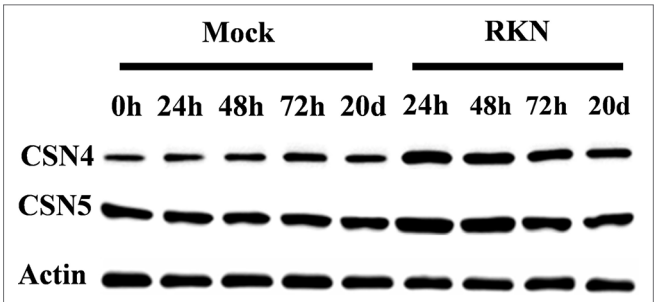

FIGURE 2 | The changes of CSN4 and CSN5 proteins in wild-type tomato plants at different time points after root-knot nematode (RKN) infection. Equal protein loading was confirmed using an anti-actin antibody. The labels 0 h, 24 h, 48 h, 72 h, and 20 d (h, hour; d, day) indicate the time after RKN infection. Three independent experiments were performed with similar results.

or 95% sequence identity at the amino acid level, respectively. qRT-PCR analysis indicated that the transcript levels of these CSN genes in the silencing lines were only 30% to 40% of those in empty vector (TRV) plants (**Supplemental Figure S3**). Nematode infectivity was determined by counting EM number *per* plant by using acid fuchsin staining in TRV and *CSN*-silenced plants at 4 weeks post inoculation (wpi) with RKNs (**Figures 3A** and **3B)**. There were approximately 111.1 EMs *per* plant in the TRV control roots (**Figure 3C**). The silencing of most *CSN* genes did not change plant resistance to RKNs. However, the silencing of *CSN4* or *CSN5* reduced basal defense in tomato plants against RKNs. The EMs were increased by 79.5% and 80.9% in the roots of TRV-*CSN4* and TRV-*CSN5* plants compared to TRV control roots, respectively (**Figure 3C**). Moreover, RKN infection also caused lipid peroxidation of cellular membrane in tomato roots (Veronico et al., 2017). The MDA content was significantly increased in infected roots of TRV control at 4 wpi relative to the untreated TRV control, and it was further aggravated in *CSN4*- or *CSN5*-silenced roots at 4 wpi with RKNs (**Figure 3D**). As shown with western blot analysis, RKN-induced accumulation of CSN4 and CSN5 proteins was compromised in TRV-*CSN4* or TRV-*CSN5* plants inoculated with RKNs, respectively (**Figure 4**). The results suggest that CSN4 and CSN5 are both required for basal defense against RKNs in tomato plants. The CSN4 protein levels increased by 155% and 160% in RKN-infected roots compared to mock roots in TRV and TRV-*CSN5* plants, respectively, but were compromised in TRV-*CSN4* plants (**Supplemental Figure S4A**). Similarly, the CSN5 protein levels were increased by 154% and 178% in RKN-infected roots compared to mock roots in TRV and TRV-*CSN4* plants, respectively, but were compromised in TRV-*CSN5* plants (**Supplemental Figure S4B**).

Requirement of CSN4 and CSN5 for JA Biosynthesis and Signaling Pathway in Response to RKN Infection

The CSN was identified to regulate JA biosynthesis in tomato response to insect herbivore and necrotrophic pathogens (Hind et al., 2011). To elucidate whether CSN4 and CSN5 are required for JA-dependent RKN resistance, we analyzed the contents of JA and JA-Ile in TRV control, *CSN4*- or *CSN5*-silenced roots at 24 hpi with RKNs. The contents of JA and JA-Ile were only 44.0% and 43.9% or 51.7% and 49.8% in *CSN4*- or *CSN5*-silenced roots, respectively, compared to TRV control roots under normal conditions (**Figure 5A**). After RKN infection, JA and JA-Ile contents significantly increased in TRV roots, but the elevation was compromised in *CSN4*- or *CSN5*-silenced plants (**Figure 5A**). Moreover, the transcript levels of two key enzymes of the JA biosynthetic pathway, lipoxygenase D (*LOXD*) and allene oxide cyclase (*AOC*), one JA signaling gene *COI1*, and one defense-response gene encoding the serine protease inhibitor (*PI-2*) in roots were significantly inhibited in *CSN4* or *CSN5* silencing compared to TRV roots under normal conditions (**Figure 5B**). Similarly, the RKN-induced expression of these genes was also compromised in *CSN4*- or *CSN5*-silenced roots (**Figure 5B**). These results indicate that CSN4 and CSN5 are involved in the regulation of JA biosynthesis and signaling pathway in response to RKN infection.

Interaction of CSN4 and CSN5 With JA Co-Receptor JAZ2

The CSN regulates the JA signaling pathway through interacting with the JA signaling component SCF^{COI1} in *Arabidopsis* (Feng et al., 2003). To gain insight into the relationship between CSN4/CSN5 and JA signaling components, we used a Y2H assay to identify whether there are interactions between CSN4/CSN5 and JA co-receptor JAZ proteins. The full length of *CSN4*, *CSN5A*, or *CSN5B* CDS were fused to the bait vector resulting in pGBKT7-CSN4, pGBKT7-CSN5A, or pGBKT7-CSN5B, respectively. The full length of each *JAZ* gene CDS was fused to the prey vector (pGADT7-JAZs). pGADT7-RecT and pGBKT7-53 were used as positive controls, and pGADT7-RecT and pGBKT7-Lam were used as negative controls (Li et al., 2018). By co-transforming the bait and prey vectors, we found that CSN4 and CSN5B both interacted with JAZ2 in yeast (**Figure 6A**).

To further determine the interaction between CSN4/CSN5 and JAZ2 *in vivo*, we performed BiFC assay in *A. tumefaciens*-infiltrated tobacco (*N. benthamiana*). The full length of *CSN4*, *CSN5A*, or *CSN5B* CDS was inserted to the C-YFP vector (CSN4-YFP^C, CSN5A-YFP^C, and CSN5B-YFP^C). The full length of *JAZ2* CDS were inserted to the N-YFP vector (JAZ2-YFP^N). When CSN4-YFP^C or CSN5B-YFP^C was co-expressed with JAZ2-YFP^N in tobacco leaves, YFP signals were detected in transformed tobacco cells (**Figure 6B**). Moreover, YFP signals were detected in positive control (SIZF3-YFP^N and CSN5B-YFP^C) as described by Li et al. (2018) but were not detected in negative control experiments (**Figure 6B**). These results suggest that CSN4 and CSN5B can interact with JA co-receptor JAZ2 to regulate JA signaling pathway.

To elucidate whether *JAZs* are involved in CSN-mediated RKN resistance, we examined the expression patterns of 12

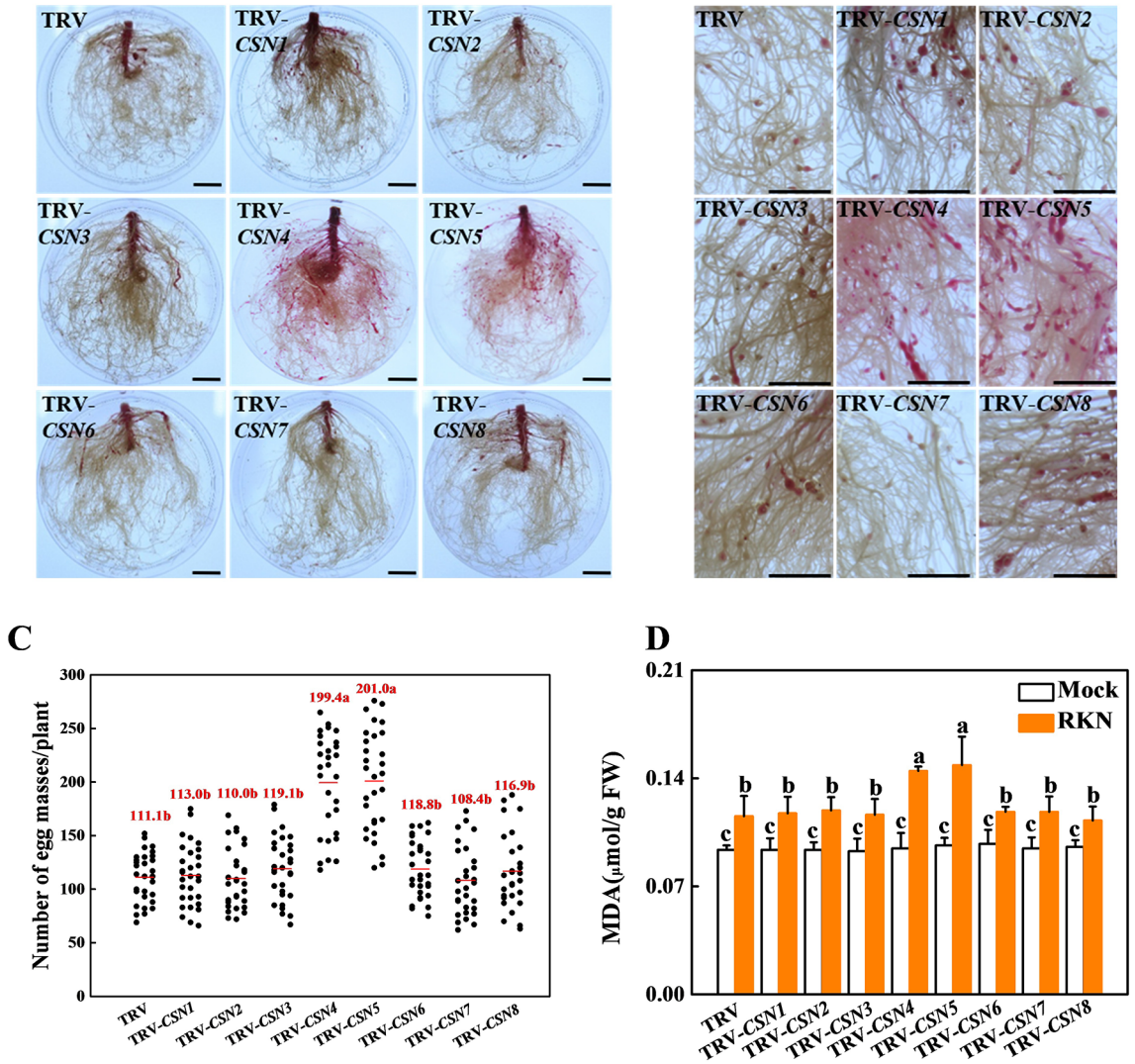

FIGURE 3 | Characterization of the COP9 signalosome (*CSN*)-silenced tomato lines against root-knot nematodes (RKNs). **(A** and **B)** Phenotype of RKN reproduction in different virus-induced *CSN* gene-silencing plants using acid fuchsin staining 4 weeks after RKN infection. Bars = 2 cm. **(C)** The number of egg masses on the roots at 4 weeks after infection with *Meloidogyne incognita*. The red number is the average number of egg masses *per* plant. Thirty plants *per* genotype were used in each experiment. Three independent experiments were performed with similar results. **(D)** Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) in the roots at 4 weeks after infection with *M. incognita*. The data shown are the average of three biological replicates, with the standard errors shown by vertical bars. Means denoted by the same letter did not significantly differ at $P < 0.05$, according to Tukey's test.

JAZ genes in response to RKNs in TRV control, TRV-*CSN4,* and TRV-*CSN5* roots at 24 hpi with RKNs. The expression of seven genes (*JAZ1*, *JAZ2*, *JAZ3*, *JAZ5*, *JAZ7*, *JAZ10*, and *JAZ11*) was induced, but one gene (*JAZ12*) was reduced, and four genes (*JAZ4*, *JAZ6*, *JAZ8*, and *JAZ9*) were not changed in TRV control roots by RKN infection (**Figure 7**). Importantly, among seven RKN-induced *JAZ* genes, only *JAZ2* expression was compromised in both *CSN4*- and *CSN5*-silenced plants after RKN infection (**Figure 7**). These results imply that CSN4 and CSN5 are required for the induction of *JAZ2* in the JA-dependent defense pathway against RKNs.

DISCUSSION

Although phytohormone JA has been suggested to play a critical role in basal defense against RKNs in tomato and other plants (Bhattarai et al., 2008; Nahar et al., 2011; Zhou et al., 2015), how JA signaling is regulated remains largely unknown. By silencing the expression of CSN subunits, we demonstrate that CSN4 and CSN5 play important roles in basal defense against RKN infection in tomato. Moreover, CSN4 and CSN5 regulate RKN-inducible JA biosynthesis and signaling response. Moreover, CSN4 and CSN5B can directly interact

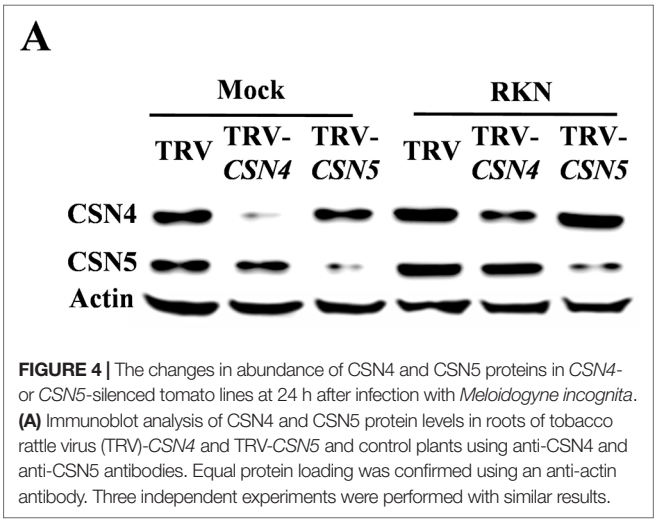

FIGURE 4 | The changes in abundance of CSN4 and CSN5 proteins in *CSN4*- or *CSN5*-silenced tomato lines at 24 h after infection with *Meloidogyne incognita*. **(A)** Immunoblot analysis of CSN4 and CSN5 protein levels in roots of tobacco rattle virus (TRV)-*CSN4* and TRV-*CSN5* and control plants using anti-CSN4 and anti-CSN5 antibodies. Equal protein loading was confirmed using an anti-actin antibody. Three independent experiments were performed with similar results.

with JA signaling component. Our work provides evidence that involvement of CSN in the RKN defense is associated with the JA signaling pathway.

CSN4 and CSN5 Regulate Plant Basal Defense Against RKNs

The CSN is best known as a regulator of the superfamily of CULLIN-RING E3 ubiquitin ligases (CRLs) which catalyze a key step in protein ubiquitination and degradation *via* the UPS (Hua and Vierstra, 2011; Jin et al., 2018). Therefore, it can be hypothesized that the CSN is involved in almost all processes of plant growth, development, and environmental response. In animals, COP9 signaling complex is known to be involved in immunity. However, the studies on the role of the CSN for plant defense are still scant. In tobacco, CSN4 subunit can interact with NbRar1 and NbSGT1, which are required for the *N* gene-triggered resistance response to *Tobacco mosaic virus* (TMV).

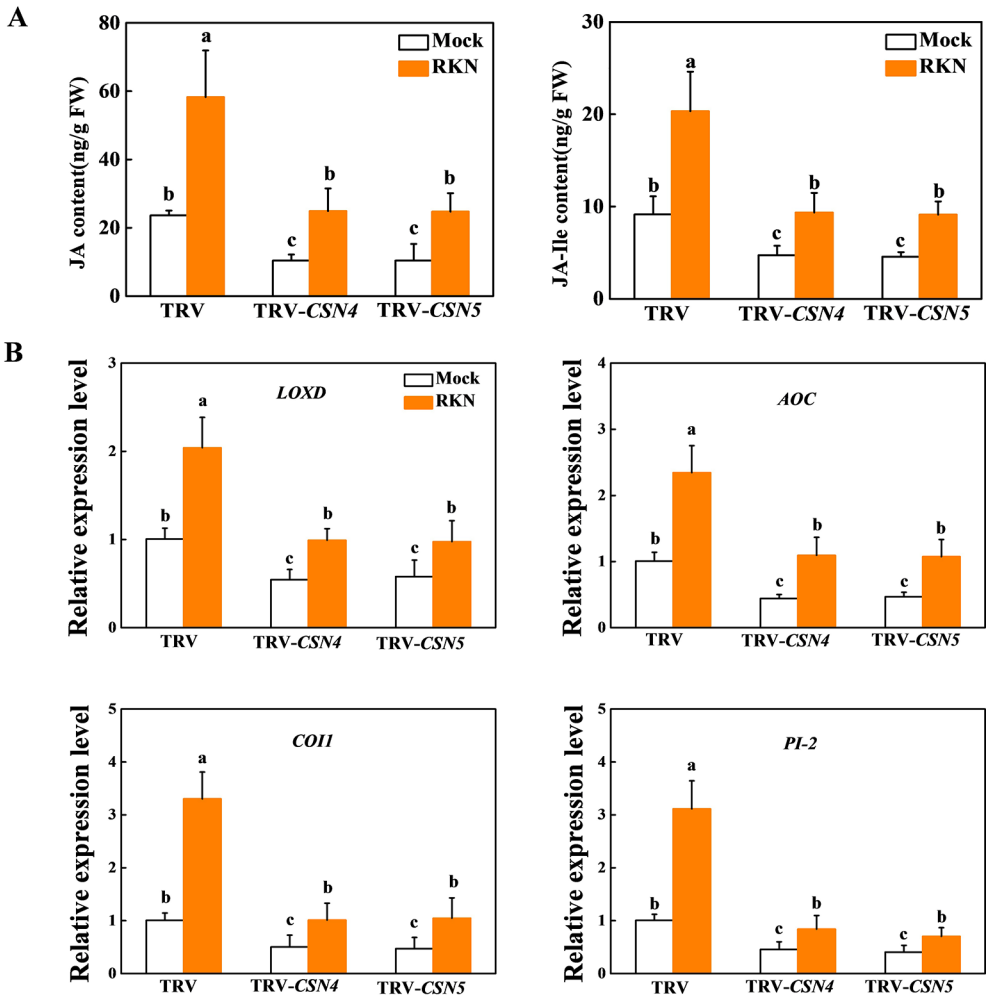

FIGURE 5 | The contents of endogenous jasmonic acid (JA) and JA-isoleucine (Ile) and the transcription levels of JA-related genes in *CSN4*- or *CSN5*-silenced tomato lines. **(A)** The contents of endogenous JA and JA-Ile were analyzed by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) in tobacco rattle virus (TRV) control, *CSN4*- or *CSN5*-silenced roots at 24 h after infection with *Meloidogyne incognita*. **(B)** The transcription levels of JA-related genes *LOXD*, *AOC*, *COI1*, and *PI-2* in tomato roots 24 h after infection with *M. incognita*. The data shown are the average of three biological replicates, with the standard errors shown by vertical bars. Means denoted by the same letter did not significantly differ at $P < 0.05$, according to Tukey's test.

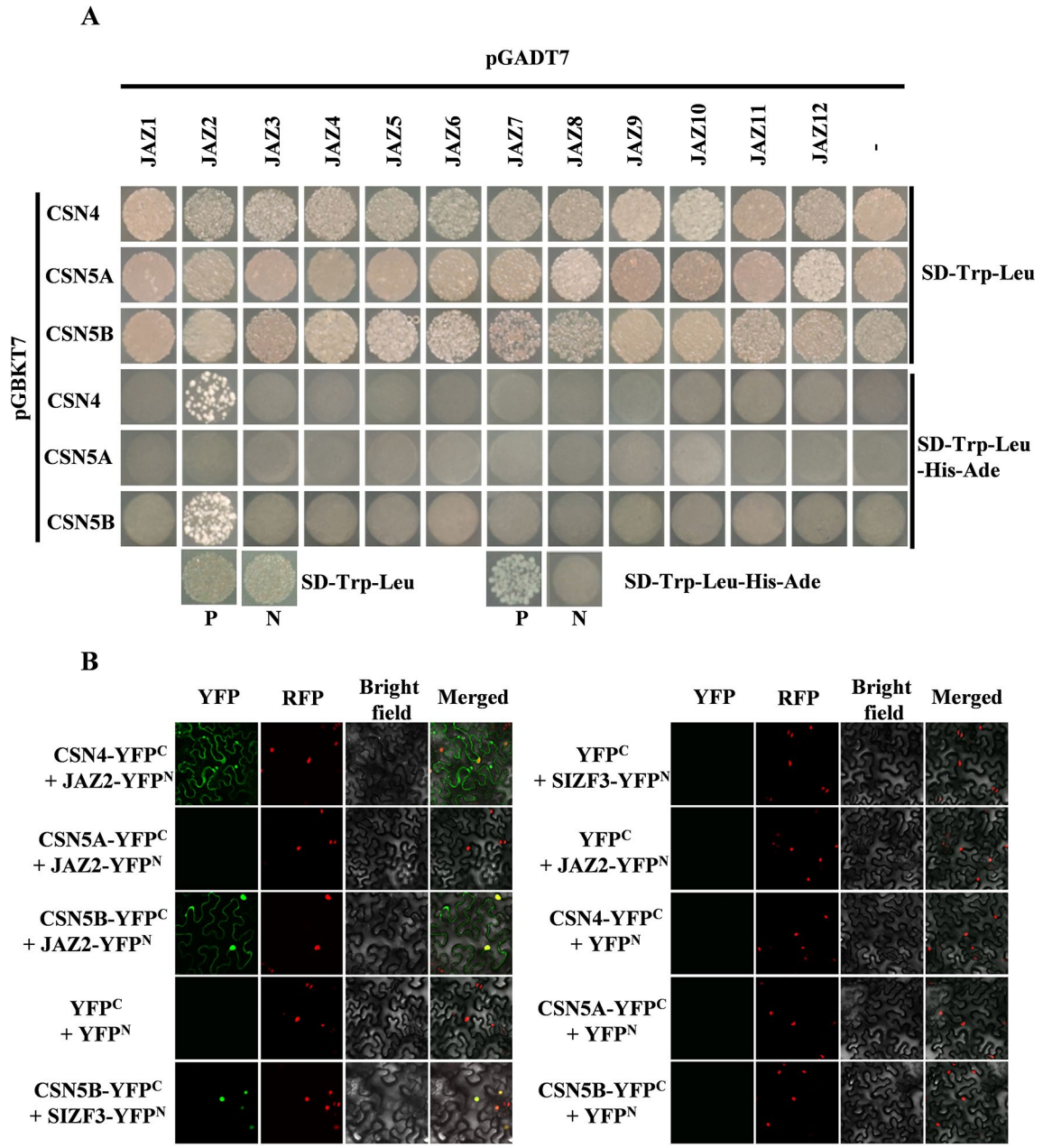

FIGURE 6 | Determination of the interaction between CSN4, CSN5A, CSN5B, and jasmonic acid (JA) co-receptor JAZ2 by yeast two-hybrid (Y2H) **(A)** and bimolecular fluorescence complementation (BiFC) assays **(B)**. Three independent experiments were performed with similar results. P, positive control (pGADT7-RecT + pGBKT7-53), N, negative control (pGADT7-RecT + pGBKT7-Lam). Bars = 20 μm.

Silencing of the *NbCSN3* or *NbCSN8* subunit compromises *N*-mediated resistance to TMV (Liu et al., 2002). Thus, CSN participates in *R* gene-mediated resistance signaling pathways. In contrast, silencing of the CSN does not increase defense gene expression and TMV infection in TMV-susceptible tomato plants which do not contain an *N*-gene or another *R*-gene to active ETI (Hind et al., 2011).

ETI is the host-specific defense response, whereas the much broader non-host or basal defense to pathogens is mediated by microbial-associated molecular patterns (MAMPs). However, the role of the CSN in plant basal defense is still unclear. In the present study, we found that the expression pattern of each CSN subunit was specifically regulated upon RKN infection in the roots of susceptible tomato. Only the expression of *CSN4* and *CSN5A* was induced at 24 to 48 hpi with RKNs. The proteins of CSN4 and CSN5 were also accumulated after RKN infection in tomato roots. Silencing of the *CSN4* or *CSN5* gene increased RKN infection in tomato roots (**Figures 1–3**).

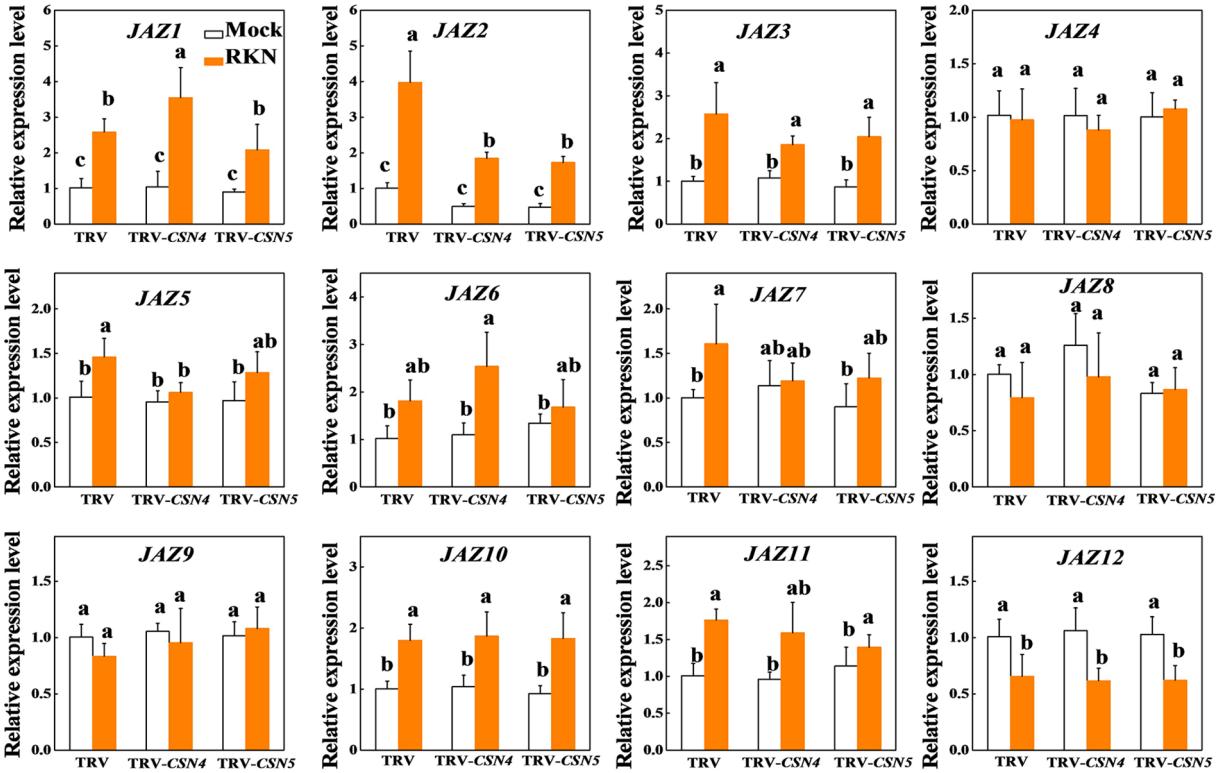

FIGURE 7 | The expression of *JAZ* genes in tomato roots 24 h after infection with *Meloidogyne incognita*. The data shown are the average of three biological replicates, with the standard errors shown by vertical bars. Means denoted by the same letter did not significantly differ at $P < 0.05$, according to Tukey's test.

Moreover, silencing of the *CSN4* or *CSN5* gene inhibited the accumulation of JA and JA-Ile and the expression of JA-related genes with or without RKN, suggesting CSN4 and CSN5 regulate JA-dependent basal defense pathway (**Figure 5**). These results indicate that the CSN subunits are not only involved in *R* gene-mediated ETI but can also regulate plant basal defense against RKNs.

CSN4 and CSN5 Are Required for JA Biosynthesis and Signaling Response

JA and its derivatives are a class of lipidic plant defense hormones. COI1 is identified as a JA receptor. In response to environmental signals, the active JA form JA-Ile accumulates and binds to COI1 and JAZ proteins which function as suppressors of JA-responsive transcription factors. JAZ proteins are subsequently ubiquitinated and degraded by the proteasome, and their degradation liberates JA downstream transcription factors to activate the JA signaling pathway (Thines et al., 2007; Zhang et al., 2017b). COI1 can assemble into the CRL SCF^{COI1}, which interacts with the CSN (Feng et al., 2003). *coi1* mutants or RNAi plants reduce JA-dependent defense responses to herbivorous insects and microbial pathogens in many plants (Li et al., 2004; Zhang et al., 2015; Zhang et al., 2017b). Therefore, the CSN can directly control COI1-dependent JA signaling pathway.

JA and its biosynthetic, signaling, and response genes are involved in plant basal defense against RKN (Islam et al., 2015; Zhou et al., 2015). In accordance with the involvement of CSN4 and CSN5 in basal defense against RKN in tomato, silencing of *CSN4* or *CSN5* resulted in the reduced concentration of JA and JA-Ile and the expression of JA biosynthetic, signaling, and response genes such as *LOXD*, *AOC*, *COI1*, and *PI2* after RKN infection. Thus, CSN4 and CSN5 regulate not only COI1-dependent JA signaling pathway but also JA biosynthesis in tomato against RKN infection. This is consistent with the report that MeJA induces the expression of JA biosynthetic, signaling, and response genes in WT tomato plant; however, the transcription of these genes is maintained at the basal level after MeJA treatment in *jai1-1* (*Jasmonate Insensitive 1*, a tomato COI1 homolog with 68% amino acid identity with COI1) mutant (Li et al., 2004). Similarly, *Arabidopsis coi1* mutant is significantly more susceptible to insects compared to WT plants (Wasternack and Hause, 2013), and its JA accumulation is also attenuated after wounding stress (Glauser et al., 2008). *Arabidopsis jaz* mutants exhibit stronger resistance to herbivores, and overexpression of the functional domain of *JAZ1* dramatically reduces plant resistance to insects (Chung et al., 2008; Campos et al., 2016; Major et al., 2017). Furthermore, JA accumulation is also strongly compromised in response to mechanical wounding by herbivorous *M. sexta* larvae in the *CSN*-VIGS tomato plants (Hind et al., 2011). Therefore, it seems that the CSN controls the degradation of SCF^{COI1}/JAZ to regulate JA signaling response. Moreover, the CSN may also regulate other CRLs and their

substrates to increase JA accumulation *via* positive feedback of JA biosynthetic genes in response to herbivorous insects and microbial pathogens.

CSN4 and CSN5 Affect the Stability of JA Co-Receptor COI1/JAZ2

The CSN physically interacts with SCF^{COI1} *in vivo*, and silencing of *CSN* expression results in decreased JA response (Feng et al., 2003; Hind et al., 2011). However, the relationship between the CSN and JAZ proteins is still unclear. Using Y2H and BiFC assays, we found that CSN4 and CSN5B physically interact with JAZ2 protein both *in vitro* and *in vivo* (**Figure 6**). Thus, CSN4 and CSN5 may directly regulate the degradation of JAZ2 *via* 26S proteasome. In tomato, CSN5A and CSN5B share 95% sequence identity at the amino acid level (Hind et al., 2011); however, the expression pattern in response to RKN infection and the protein function is different between these two CSN5 duplicates. Similarly, *Arabidopsis* CSN5A and CSN5B, which share over 85% identity at the amino acid level, have different expression levels in all plant tissues and organs and play unequal roles in plant development (Lorenzo et al., 2004). Furthermore, JAZ proteins association with COI1 leads to their degradation in a jasmonate-dependent manner. MeJA treatment induces expression of at least 10 *JAZ* genes, while MeJA does not affect JAZ1-GUS activity in *coi1* mutants (Thines et al., 2007), exhibiting COI1-regulated JAZ1 degradation. After RKN infection, we found that seven *JAZ* genes were induced in TRV control plants. In the present study, among seven RKN-induced *JAZ* genes, only *JAZ2* expression was compromised in both *CSN4*- and *CSN5*-silenced plants by RKN infection (**Figure 7**). These results suggest that the CSN may directly participate in the regulation of JAZ2 degradation in tomato plants. Whether the CSN plays a direct role in SCF^{COI1}-mediated degradation of JAZ proteins and what their function is in plant response to RKN infection are to be elucidated in further studies.

Many nematode effectors manipulate hormone signaling, protein modifications, redox signaling, and metabolism in host plants and accelerate nematode colonization and development (Holbein et al., 2016; Ali et al., 2017). Nematode effectors also interact with host proteins to interfere with plant growth. The potato cyst nematode (*Globodera pallida*) effector RHA1B, which encodes a ubiquitin ligase, triggers degradation of NB-LRR immune receptors to block ETI signaling and suppress PTI signaling *via* a yet unknown E3-independent mechanism in potato (Kud et al., 2019). Potato cyst nematode CLAVATA3/ENDOSPERM SURROUNDING REGION-like effector can interact with the potato CLAVATA2-like receptor, which controls the fate of stem cells in the shoot apical meristem, to promote nematode parasitism (Chen et al., 2015). Recently, Bournaud et al. (2018) reported that soybean CSN5 interacts with the *M. incognita* effector MiPM, which may trigger a host endocytosis pathway to penetrate the cell and may regulate the endosomal sorting of MiPM. Based on these results, we can speculate that the CSN may interact with more RKN effectors and disrupt the perception of nematode effector or effector-associated signaling pathways to interfere with RKN parasitism. The novel functions of the CSN between induction of plant defense and inhibition of RKN infection should be further unraveled.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

YS, JZ, and J-QY planned and designed the research. YS and KW performed experiments and analyzed data. YS and SS performed molecular cloning and analyzed data. YS and JZ wrote the article. All authors reviewed, revised, and approved the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01223/full#supplementary-material

SUPPLEMENTAL FIGURE S1 | Phylogenetic analysis of CSN proteins from tomato and Arabidopsis. Amino acid sequence alignment and tree construction were performed using the MEGA program.

SUPPLEMENTAL FIGURE S2 | Relative abundance of CSN4 and CSN5 proteins. **(A)** The relative abundance of CSN4/actin quantified according to **Figure 2**. **(B)** The relative abundance of CSN5/actin quantified according to **Figure 2**. The data shown are the average of three replicates using Quality One (Bio-Rad, Hercules, CA, USA), with the standard errors shown by vertical bars. Means denoted by the same letter did not significantly differ at $P < 0.05$, according to Tukey's test.

SUPPLEMENTAL FIGURE S3 | Relative messenger RNA (mRNA) abundance of COP9 signalosome (*CSN*) genes in virus-induced gene silencing plants. Total RNA was extracted 3 weeks after *Agrobacterium tumefaciens* infiltration for analysis of target gene silencing efficiency. The levels were expressed as percentages of the mean levels in control tobacco rattle virus (TRV) plants, which were defined as 100%.

SUPPLEMENTAL FIGURE S4 | Relative abundance of CSN4 and CSN5 proteins. **(A)** The relative abundance of CSN4/actin quantified according to **Figure 4**. **(B)** The relative abundance of CSN5/actin quantified according to **Figure 4**. The data shown are the average of three replicates using Quality One (Bio-Rad, Hercules, CA, USA), with the standard errors shown by vertical bars. Means denoted by the same letter did not significantly differ at $P < 0.05$, according to Tukey's test.

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Seasonal Variation and Crop Sequences Shape the Structure of Bacterial Communities in Cysts of Soybean Cyst Nematode

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Soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the number 1 pathogen of the important economic crop soybean. Bacteria represent potential biocontrol agents of the SCN, but few studies have characterized the dynamics of bacterial communities associated with cysts under different crop rotation sequences. The bacterial communities in SCN cysts in a long-term soybean–corn crop rotation experiment were investigated over 2 years. The crop sequences included long-term soybean monoculture (Ss), years 1–5 of soybean following 5 years corn (S1–S5), years 1 and 2 of corn following 5 years soybean (C1 and C2), and soybean–corn annual rotation (Sa and Ca). The bacterial 16S rRNA V4 region was amplified from DNA isolated from SCN cysts collected in spring at planting, midseason (2 months later), and fall at harvest and sequenced on the Illumina MiSeq platform. The SCN cyst microbiome was dominated by Proteobacteria followed by Actinobacteria, Bacteroidetes, and Verrucomicrobia. The bacterial community composition was influenced by both crop sequence and season. Although differences by crop sequence were not significant in the spring of each year, bacterial communities in cysts from annual rotation (Sa and Ca) or crop sequences of early years of monoculture following a 5-year rotation of the alternate crop (S1 and C1) became rapidly differentiated by crop over a single growing season. In the fall, genera of cyst bacteria associated with soybean crop sequences included *Rhizobacter*, *Leptothrix*, *Cytophaga*, *Chitinophaga*, *Niastella*, *Streptomyces*, and *Halangium*. The discovery of diverse bacterial taxa in SCN cysts and their dynamics across crop rotation sequences provides invaluable information for future development of biological control of the SCN.

Keywords: bacterial community, biological control, crop rotation, metabarcoding, soybean cyst nematode, *Heterodera glycines*

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is an important crop worldwide, and the Midwestern region of the United States, including states such as Illinois, Iowa, and Minnesota, produces over 80% of total soybean in the United States (Hartman et al., 2011). The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the primary yield-limiting factor in those regions (Wrather and Koenning, 2006). Planting resistant cultivars have historically been one of the primary approaches for managing the SCN (Chen et al., 2001). However, most of the resistant cultivars are developed from a single source of resistance (PI8898) and there is evidence and significant risk that SCN in the field is already breaking this resistance over years of continuous growth of resistant soybean (Koenning, 2004). Nematicides, which were also commonly used for control of plant-parasitic nematodes in the past, are not cost-effective for managing SCN (Chen et al., 2001), and many have been banned as they pose serious risks to both human health and the environment (Mereu and Chapman, 2010; UNEP, 2015). Crop rotation with a non-host crop such as corn (*Zea mays* L.) is an alternative approach. In Minnesota, a combination of annual rotation of resistant soybean with corn is the most common rotation. The annual corn rotation has some effectiveness in managing the SCN, but it may take up to 5 years of corn rotation to reduce the SCN population density to a level that does not cause significant damage to a susceptible soybean in northern climates (Chen et al., 2001). Biological control has been explored, and offers an attractive and environmentally sustainable alternative for SCN control as part of an integrated management plan (Chen and Dickson, 2012).

The effects of crop rotation on SCN populations and crop yield have been extensively studied in different soybean production regions (Ross, 1962; Crookston et al., 1991). The yield of both rotation crops was shown to increase in the first year after rotation (Howard et al., 1998). Crop rotation has also been found to influence soil microbial communities. Early research found that the total microbial community diversity in soil was greater in a wheat-soybean rotation than under monoculture (Lupwayi et al., 1998; Perez-Brandan et al., 2014). In a corn-soybean rotation system, it was reported that crop rotation affected the total microbial community based on phospholipid fatty acid analysis (PLFA; Vargas Gil et al., 2011). Furthermore, differences in the bacterial community composition were detected by denaturing gradient gel electrophoresis (DGGE), but this method was not sufficient to fully capture the diversity and richness of the community (Yin et al., 2003). More recently, metabarcoding studies have shown that the bacterial communities in soil may shift in response to tillage and crop rotation regimes (Yin et al., 2010).

Each growing season, under SCN susceptible soybean, the SCN produces abundant cysts, each containing hundreds of nematode eggs, which form a resistant overwintering structure that can protect the viability of nematode eggs in soil for up to a decade (Koenning, 2004; Chen, 2011). Thus, understanding the survival and dynamics of bacteria inhabiting the microenvironment of SCN cysts in agroecosystems is of ultimate importance for discovering new and effective biological control agents. However, there is very limited knowledge of how bacterial communities within SCN cysts are affected by crop rotation. Previous research has shown that the length of soybean monoculture impacted the bacterial communities inside SCN cysts, as distinct bacterial communities and taxonomic groups were found in cysts from short term (< 8 years) versus longer term (> 8 years) soybean monoculture (Zhu et al., 2013). A recent study demonstrated that the fungal communities in SCN cysts were also affected by crop rotation (Hu et al., 2018). With the emergence of metabarcoding approaching using high-throughput sequencing, there is growing demand and opportunity to explore the diversity and dynamics of microbial communities associated with the SCN cysts in agroecosystems in order to facilitate SCN management using biological control.

The bacteria found in SCN cysts have been shown to be highly diverse (Nour et al., 2003), and hundreds of bacterial species have been isolated from the cysts of the SCN. The dominant bacterial genera isolated from cysts from a field in southern Ontario, Canada, were *Lysobacter* spp. and *Variovorax* spp. (Nour et al., 2003). In addition, taxa belonging to Proteobacteria (Alpha, Beta, Delta, and Gamma-Proteobacteria) and Bacteroidetes were the dominant phyla detected by the DGGE method used in another study (Yin et al., 2003). Several sulfate-reducing bacteria and Actinobacteria in the genera *Actinomadura* and *Streptomyces* were also detected. Similarly, a greenhouse study of bacterial communities found in cysts grown in a long-term SCN suppressive soybean monoculture soil found that Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes were among the most abundant bacterial phyla present in SCN cysts (Hussain et al., 2018).

Bacteria isolated from cysts may have potential for control of SCN. Several species have been shown to inhibit egg hatch and to control the SCN in both *in vitro* and greenhouse assays (Bent et al., 2006). Rhizobacteria isolated from soybean roots, for example, reduced SCN egg hatch in the greenhouse, but gave inconsistent results in field trials (Tian and Riggs, 2000). *Bacillus* and *Pseudomonas* species have been shown to reduce SCN cyst numbers (Kloepper et al., 1992) and thus likely inhibit reproduction within soybean roots. The well-known biological control bacterium *Pasteuria nishizawae*, which is an obligate parasite of nematodes, was isolated from second-stage juvenile (J2) of SCN (Noel and Stanger, 1994). Although *Pasteuria* has been extensively studied in interactions with the root-knot nematode, there are only a few additional reports of its isolation from SCN in Minnesota (Chen and Dickson, 2012). Toxins or antibiotics produced by bacteria are in commercial use as nematode biopesticide products. Avermectins, produced by *Streptomyces avermitilis,* for example, can kill nematodes (Egerton et al., 1979) and form the active ingredient of the seed treatment Abermectin™ for SCN. Other known producers of antihelmenthic compounds include *Pseudomonas fluorescens*, *Bacillus chitinosporus*, and *Bacillus firmus* (Chen and Dickson, 2012). As the bacterial taxa within cysts have not been well characterized, it is likely that other bacterial taxa effective in reducing SCN populations remain to be discovered.

How crop sequences impact the microbial communities within the SCN cysts is also important because crop rotation is a common cultural practice for managing SCN. In order to investigate how crop rotation sequences influence the bacterial communities in cysts, we conducted research in a long-term field experiment that has been in soybean–corn rotation for over 30 years. Previous research at this site suggested that the soil ecology under corn and soybean crop rotation sequences were different based on changes in the nematode community (Grabau and Chen, 2016b). Moreover, among the nematodes that could affect soybean yield, the SCN was the most important at this site, and SCN density was shown to increase with increasing years of soybean monoculture over 5 years (Grabau and Chen, 2016a). In a previous companion study analyzing fungi associated with the same SCN cysts analyzed in this study, fungal communities were shown to vary by crop sequence as well as over the crop growing season (Hu et al., 2018). This study seeks to (1) characterize the key bacterial groups associated with the cysts of SCN in a soybean–corn rotation system, (2) to understand the influence of seasonal variation and crop rotation on these communities, and (3) to identify taxa enriched and associated with soybean crop sequences with potential for biological control.

MATERIALS AND METHODS

Field Experimental Design and Management

This study was performed in a field site (44°04′N, 93°33′W) at the University of Minnesota Southern Research and Outreach Center in Waseca, MN, United States. A corn–soybean crop rotation sequence was initiated in 1982. The experimental design of this field is detailed in Grabau and Chen (2016a). The factor of cropping sequence effects was examined in a complete randomized block design with four replicate plots. Each plot had six rows of crops.

This study focused on a subset of the corn-soybean crop rotation sequences in 2015 and 2016, sampled at three time points each season: spring (mid May), midseason (late July to early August), and fall (mid-October) (Hu et al., 2018). The 10 corn-soybean rotation treatments were: (1) continuous soybean monoculture since 1982 (Ss), (2) corn and soybean annual rotation (Ca, Sa), (3) first and second year corn (C1, C2) after 5 years monoculture of soybean, and (4) first through fifth year soybean (S1, S2, S3, S4, S5) after 5 years of corn monoculture, with the C indicating corn and the S soybean sequences. Pioneer P91Y90 (SCN-susceptible soybean) and DKC50-82 RIB (BT corn) were used for all soybean or corn plots. The plots were plowed with a field chisel after harvest at fall and before planting at spring each year. Nitrogen was only applied to the corn plots at a rate of 224.4 kg/ha. The herbicide glyphosate (Roundup) was applied for weed control. The insecticide Endigo (active ingredient: thiamethoxam and lambda-cyhalothrinm) was sprayed at 245 g/ha at midseason each year.

Cyst Sampling and Collection

Cyst sampling and collection were performed as detailed in Hu et al. (2018). Briefly, sucrose flotation and centrifugation method (Jenkins, 1964) was used to extract cysts from about 4 kg soil per plot. From each plot, six soil samples were collected with a shovel at a depth of 20 cm within 4 cm of plant growth in the center two rows and soaked in water prior to extracting cysts. A total of 50 individual intact mature (brown) SCN cysts were picked under an inverted microscope from each plot, surface-sterilized with 0.5% NaOCl for 3 min, and rinsed thoroughly with autoclaved water. The cysts were then stored at −80°C until subsequent DNA extraction. An insufficient number of cysts were collected from the S1 of midseason 2015 and this treatment was thus omitted from midseason 2015 analyses.

Cyst DNA Extraction and Metabarcoding

Surface-sterilized cysts were crushed in a small centrifuge tube using a pestle. DNA was isolated from the macerated cysts according to a modified CTAB protocol described in Hu et al. (2017). The DNA extracted from the 50 pooled cysts collected from each plot was used to construct a single metabarcode library, with four replicates per crop sequence at each collection time point. The PCR amplification, library preparation, and sequencing were conducted at the University of Minnesota Genomic Center, Saint Paul, MN, United States (Gohl et al., 2016). The universal bacterial primers targeting the 16S rRNA V4 region with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used. The Illumina index and flow cell adapters were amplified together with the V4 primers during the first step PCR, and dual-index barcode sequence was added at the second PCR step (Gohl et al., 2016). The samples from the same year were pooled and sequenced on one Illumina MiSeq lane with the 2 × 300 bp kit. For each paired-end lane of MiSeq, additional samples were included in order to assess the PCR and sequencing error in the downstream pipeline, including blank control samples, a mock community sample, which had equal amount of DNA isolated from pure bacterial cultures, and nine randomly chosen cyst samples for technical replicates.

Sequence Quality Control and Processing

The platform yielded 24,703,544 sequences that passed quality control filters. The make.contigs step in Mothur v.1.39.5 (Schloss et al., 2009) was used to pair the Illumina forward and reverse reads, and those sequences having less than 150 bp overlap were removed. Any remaining sequences which had more than 2 bp mismatch of V4 region primers were also removed. Sequences that had more than eight homopolymers, ambiguous bases, or were outside of the 200–400 bp length range were filtered out. Sequences that did not align to the Silva-based

bacterial V4 region were excluded. After the above filtering steps, the remaining high-quality sequences were then imported into QIIME V1.9.1 (Caporaso et al., 2010). The UCHIME (Edgar et al., 2011) pipeline was used to detect chimeras, and operational taxonomic units (OTUs; Blaxter et al., 2005) were picked utilizing a closed OTU picking approach with 97% similarity. Taxonomy was assigned to the OTU referred to the SILVA database (SILVA.123.1_SSURef_Nr99).

Analysis of OTU Diversity and Relative Abundance

The OTU tables generated by QIIME were imported into R version 3.4.2 (R Core Team, 2014) for downstream analyses. The OTUs that had less than 10 total counts across all the samples or could not be assigned to the domain Bacteria were removed prior to downstream analyses. Overall, an average of 65,219 high-quality sequences were generated per sample, with the highest 129,573 per sample and the lowest 2,245 per sample. One sample that had an extremely low OTU count (294) was excluded from the analysis. The data were not rarefied and raw read counts were used after filtering with these quality control steps. Two measures of alpha-diversity, observed OTUs and Shannon alpha diversity index, were calculated using the package "Phyloseq" (McMurdie and Holmes, 2013) and plotted in R using "ggplot2" (Wickham, 2009).

For the statistical analysis of the alpha diversity indexes, the data were normally distributed and not transformed. An ANOVA model was constructed, which included crop rotation sequences, sampling time points, and replicates as independent variables together with the interaction between year, crop sequences, and Season (Y = Year + CropSequences + Season + Year*CropSequences + Year*Season + CropSequences*Season + Year*CropSequences *Year). Because in the full model we observed interactions between year, crop sequences, and seasons, another set of analyses were conducted in which year was not included in the model as a repeated measure. Thus, data were analyzed separately within each year using the model Y = CropSeq + Season + Replicates + CropSeq*Season. This model was also used to test significant differences over sampling seasons within each year. Significant differences were reported at $P < 0.05$ throughout the manuscript, unless otherwise reported.

For beta diversity, the OTU counts were transformed to relative abundance of each sample and normalized by using a $\log_2$ transformation. A Bray–Curtis dissimilarity matrix was calculated using the R package "Vegan" (Oksanen et al., 2017), and the significance of Bray–Curtis dissimilarity matrix of cysts among all crop sequences, combined soybean vs. combined corn sequences, and among soybean sequences only was tested respectively by the Adonis function. The "Procrustes" function was used to detect changes in cyst bacterial communities over sampling time points and crop sequences.

In order to identify taxa specifically associated with cysts from crop sequences of a particular crop, several approaches were used. We analyzed the relative abundance of bacterial taxa at the Class and OTU levels using ANOVA. The relative abundance was transformed using $\log_2$ to improve the homogeneity. Additionally, a spearman rank correlation test was used to test for Classes and OTUs that were correlated with the increasing years of soybean monoculture (S1–S5). Finally, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm (Segata et al., 2011) was used to detect the OTUs that differed in abundance in cysts when compared between corn and soybean crop sequences. This method identifies both the consistency of association and enrichment of specific taxa with each crop (e.g., soybean and corn) within the framework of the hierarchical phylogenetic relationships between taxa. For this analysis, the three corn sequences were pooled as the corn treatment and the seven soybean sequences were pooled together as the soybean treatment. LEfSe uses a non-parametric factorial Kruskal–Wallis (KW) sum-rank test between these two groups, corn and soybean crop sequences, and taxa that differ significantly between the crops are further tested using the (unpaired) Wilcoxon rank-sum test across different crop sequences within corn and soybean internally. At the last step, LEfSe uses LDA to estimate the effect size of each taxa, and significance at each taxonomic level (e.g., phylum, class, order, genus, species). Taxa enriched and associated with corn and soybean, respectively, at phylum, class, and order level were displayed in cladograms generated using the LEfSe tools in Galaxy (Segata et al., 2011).

RESULTS

Taxonomic Composition of Bacteria in Cysts

Overall, when averaged across crop sequences and seasons, the predominant bacterial phyla in cysts were Proteobacteria, which comprised nearly half (48.4%) of all taxa found in cysts. These were followed by Actinobacteria (24.0%), Bacteroidetes (13.3%), Verrucomicrobia (4.0%), Planctomycetes (2.7%), Firmicutes (1.7%), Acidobacteria (1.6%), Chloroflexi (1.6%), Saccharibacteria (1.5%), and 24 other phyla which had less than 1% relative abundance (**Figure 1**). At the class level, Actinobacteria (23.6%) was the most abundant class, and comprised nearly all of the phylum Actinobacteria (**Figure 1**). Among the Proteobacteria, Alphaproteobacteria (19.8%), Betaproteobacteria (15.1%), Sphingobacteria (9.62%), Gammaproteobacteria (9.6%), and Deltaprobacteria (3.8%) were the dominant classes (**Figure 1**). Other classes comprising greater than 2% of total OTUs included Erisypelotrichia, Cytophagia, Thermoleophilia, Planctomycetacia, Acidobacteria, Spartobacteria, Verrucomicrobiae, Acidibacteria, Flavobacteria, Anaerolineae, Chloroflexia, Opitutae, Gemmatinonadetes, Phycisphaerae, and the vadiniHA49 group (**Figure 2**). Genera containing the largest percentage of OTUs in cysts corresponded to *Streptomyces*, *Rhizobacter*, *Rhizobium*,

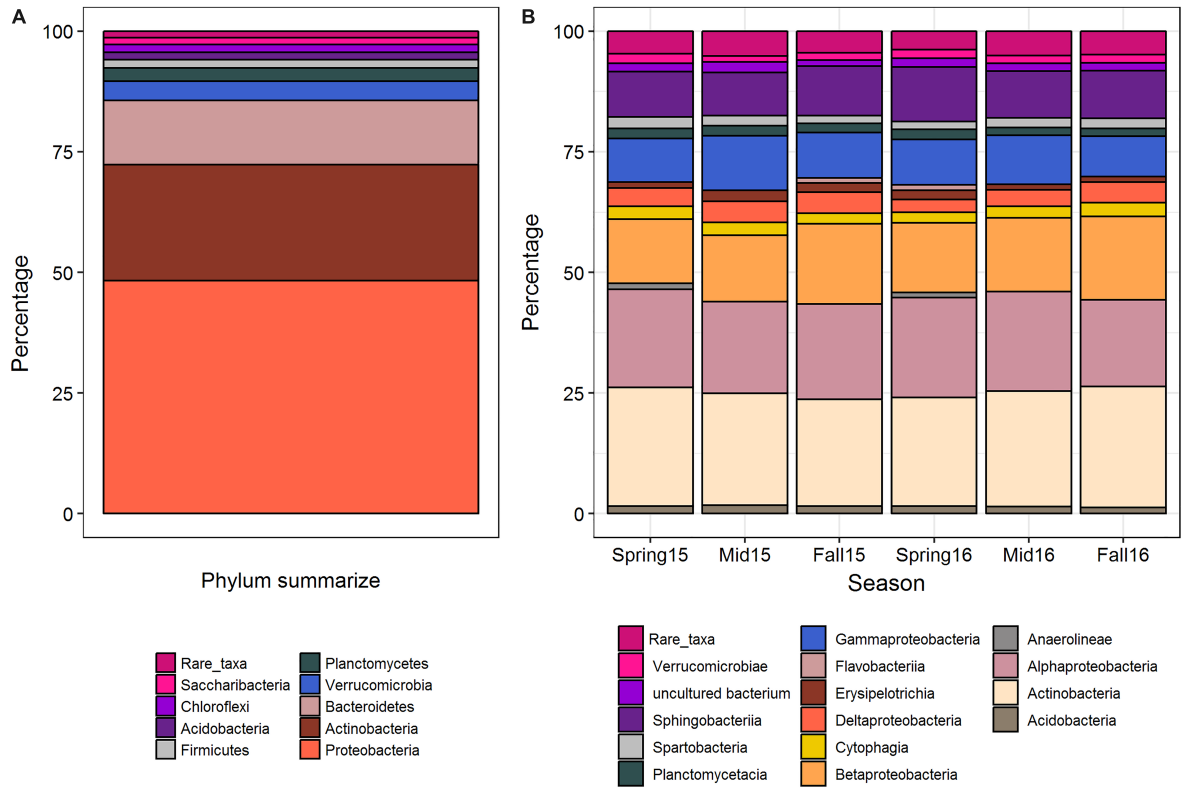

FIGURE 1 | Summary of the average percentage of OTUs at the phylum level across all cyst samples **(A)**, and average percentages at the class level across seasons **(B)**. Rare taxa include those phyla or class that contributed less than 0.1% to the whole community.

Aquincola, *Actinocoralia*, *Bradyrhizobium*, *Niastella*, *Massalia*, *Lechevelaria*, and *Halangium* (**Supplementary Figure S1**).

Variation in Abundance of Bacterial Taxa Across Season and Crop Sequences

Classes Actinobacteria, Acidimicrobiia, Alphaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria differed significantly ($p < 0.05$) in relative abundance across different seasons when pooled across crop sequences (**Figure 1** and **Supplementary Table S1**). A total of seventeen bacterial classes were found to differ significantly ($p < 0.05$) across crop sequences in at least four seasons (**Supplementary Table S2**). Among these, those that had the most significant (< 0.0001) differences in relative abundance across crop sequences in at least three seasons included Alphaproteobacteria, Betaproteobacteria, Planctomycetacia, Gemmatimonadetes, Acidimicrobiia, and Thermoleophilia (**Supplementary Table S2**). Among these, Alphaproteobacteria, Planctomycetacia, Gemmatimonadetes, and Thermoleophilia were generally more abundant in corn sequences, while Betaprotebacteria and Acidomicrobia were generally more abundant in soybean sequences (**Supplementary Table S2**). Other classes showing significant ($p < 0.05$) differences in relative abundance in at least three seasons included Holophagae, Thermomicrobia, Acidobacteria, KD4-96 group, Chloroflexia, Gammaproteobacteria, TK10 group, Clostridia, Phycisphaerae, Chlorobia, and Chlamydiae (**Supplementary Table S2**). However, no classes were found to significantly ($p > 0.05$) increase in relative abundance and positively correlate with increasing years of soybean monoculture in spearman rank correlation tests (data not shown).

Although 256 OTUs differed significantly ($p < 0.05$) in relative abundance across crop sequences (**Supplementary Table S3**), very few of these showed significant differences in more than a single season (**Supplementary Table S3**). Only 12 OTUs differed significantly across sequences in at least two seasons (**Supplementary Table S3**). One was identified to species, *Rickettsia felis* in Rickettsiales (Alphaproteobacteria), and several others could only be identified to genus, including *Kaistia* and *Bauldia* in Rhizobiales (Alphaproteobacteria), *Enterobacter* (Gammaproteobacteria), and *Longispora* (Actinobacteria), while the remaining belonged to the orders Acidobacteria Subgroup 17 (Acidobacteria), Chloroflexales (Chloroflexia), Rhizobiales (Alphaproteobacteria), Myxococcales (Deltaproteobacteria), the NKB5 group (Gammaproteobacteria), and Chthoniobacterales (Verrumicrobia) (**Supplementary Table S3**). A spearman rank correlation test identified six OTUs in fall of 2015 and five OTUs in spring of 2016 that were significantly correlated with increasing years of soybean monoculture (**Supplementary Table S4**). These included members of the Rhizobiales (Alphaproteobacteria)

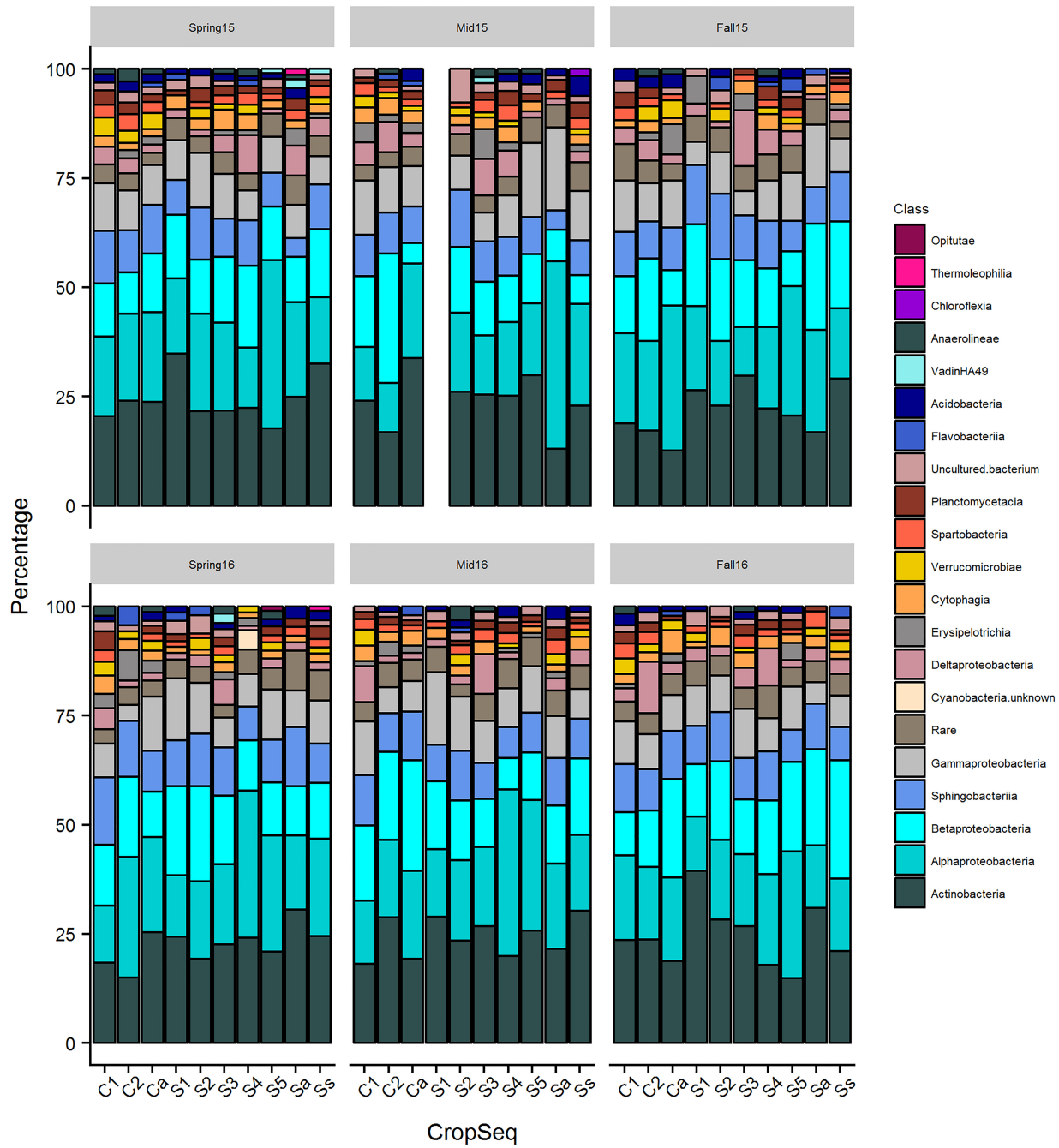

FIGURE 2 | The distribution of bacterial classes containing at least 2% of total OTUs across crop sequences for each season. Percentage of OTUs from each class out of the total from the cyst community is shown on *y*-axis and taxa with higher percentage of OTUs are shown toward bottom of each column.

in families Bradyrhizobiaceae, Rhizobiaceae, and Hyphomicrobiaceae, two OTUs in Sphingobacteriales (Sphingobacteria) in family Chitinophagaceae, one in Nitrosomadales (Betaproteobacteria), one in Planctomycetales (Planctomycetacia) in Planctomycetaceae, one in genus *Solirubrobacter* (Thermoleophilia), and one in the genus *Verrucomicrobium* (Verrucomicrobia) (**Supplementary Table S4**). Surprisingly, most of these were negatively correlated with years of soybean monoculture year except for *Verrucomicrobium* (**Supplementary Table S4**).

Alpha Diversity Affected by Seasons and Crop Sequences

Alpha diversity of bacterial communities was affected by both crop sequence and season in both years (**Table 1**). This effect

TABLE 1 | Bacterial community alpha diversity indexes across season.

Season	2015		2016	
	Observed	Shannon	Observed	Shannon
Spring	1071 a	4.42 a	1030 a	4.61 a
Mid	799 b	4.20 b	1039 a	4.26 b
Fall	1052 a	4.49 a	1034 a	4.61 a
ANOVA:				
CropSeq	<0.001***	<0.001***	0.86	0.060
Season	<0.001***	<0.001***	0.99	0.002**
CropSeq*season	0.14	0.4	0.52	0.006**

The data are means across all 40 samples, 10 crop sequences each with four replicates within each season. Significant differences are reported at (.) P < 0.1, (*) P < 0.05, (**) P < 0.01, and (***) P < 0.001. Different letters in a column indicate a significant difference between seasons according to the Least Significant Difference (LSD) test at P < 0.05.

of crop sequence and season was significant ($p < 0.001$) for observed species and Shannon index in 2015. Although the effect of crop sequence was marginally significant ($p = 0.06$) for the Shannon index in 2016, the effect of season was significant ($p < 0.01$) and an interaction was observed between crop sequences and season in 2016 (**Table 1**). However, observed OTUs did not show any significant effects for 2016 (**Table 1**). According to both observed OTUs and Shannon alpha diversity indexes, the bacterial community at midseason was less diverse than in spring or fall in 2015, whereas in 2016, only the Shannon index showed lower diversity at midseason compared to spring or fall (**Table 1**). Similarly, the soybean–corn crop sequences alone had a significant effect on the community diversity at spring and fall in both years, but not at midseason of either year (**Table 2**). In spring of 2015, C2 had the highest number of observed OTUs, while S1 had the lowest (**Table 2**). In fall of both years (**Table 2**), corn sequences (C1, C2, and Ca) tended to have a slightly higher diversity index (both observed OTUs and Shannon) than soybean crop sequences, although these differences were not significant between all corn and soybean crop sequences (**Table 2**). Overall, both season and crop sequences had some effect on the diversity of bacteria found in SCN cysts, with corn sequences generally having higher diversity compared to soybean sequences and diversity tending to be lower in midseason than in spring or fall of both years.

Beta Diversity Affected by Crop Sequences and Seasons

Adonis analyses using a complete model (Bray–Curtis ∼ Season + CropSequence + Year + Season*Year + Season*CropSequence + CropSequence*Year + Season*CropSequence*Year) showed that crop sequence was the most significant ($p < 0.0001$) factor affecting community composition (**Table 3**). While neither season nor year was significant by themselves, the interactions between season and crop sequences and crop sequences and year were also significant ($p < 0.0001$) (**Table 3**). Because of the interactions between crop sequence and season, the Adonis analyses were also performed separately for each season and the crop sequences had a significant ($p < 0.05$) effect on the bacterial communities in cysts at all sampling seasons (**Table 4**). However, when all communities from corn or soybean crop sequences were pooled, the effects of crop species (e.g., soybean vs. corn) were observed only at midseason and fall in both years, but not in spring of either year (**Table 4**).

The NMDS plot showed a consistent trend of the effect of crop sequences of each crop on the bacterial communities, with differentiation between cysts from crop sequences of corn and soybean increasing over the growing season, and showing the greatest divergence in the fall of each year (**Figure 3**). In spring, the centroids of communities from early years of soybean (S1 and Sa) clustered more closely with the second year of corn (C2) sequence, while the remaining soybean crop sequences clustered together at a different location on the NMDS plots (**Figure 3**). However, by fall, the cysts associated with S1 and Sa clustered more closely with other soybean crop sequences (S2, S3, S4, S5, and Ss), than with the corn crop sequences (C1, C2, and Ca), and bacterial communities were more clearly differentiated by crop species (**Figure 3**). The Adonis PERMANOVA analysis provided statistical support for this trend. Although differences between cysts across all crop sequences were significant ($p < 0.001$) at all sampling seasons in both years (**Table 4**), when cyst communities were compared between pooled crop sequences associated with each crop species (e.g., corn and soybean), no significant differences were observed in spring, but significant differences ($p < 0.001$) were observed in midseason and fall of both years (**Table 4**), indicating that differences between cyst communities from crop sequences associated with each crop became more pronounced over the course of the growing season (**Figure 3** and **Table 4**). In addition, soybean monoculture year (S1–S5, and Ss) significantly affected bacterial community structure at all sampling seasons, except in fall of 2016 (**Table 4**). In contrast, corn monoculture year (C1, C2, and Ca) only significantly affected bacterial community structure in spring of 2016 (**Table 4**). Beta-dispersion parameters, a measure of variation observed across samples of the same treatment, also varied significantly in midseason of 2015 and spring and fall of 2016 (**Supplementary Table S5**).

In pairwise comparisons of sampling seasons (e.g., spring vs. midseason and midseason vs. fall) within each year, the cyst bacterial communities were significantly different across all crop sequences according to the Procrustes test using the Bray–Curtis distance matrix (**Figure 4** and **Table 5**). However, the seasonal effects were not consistently observed across all crop sequences or the same crop sequence across both years (**Table 5**). The most significant ($p < 0.05$) differences observed between cyst communities in comparisons between spring and fall were between early years of crop rotation following monoculture (C1 in 2015, C2 in 2016) and annual rotation (Ca and Sa in 2015, and Ca in 2016) (**Table 5**). Overall, the earlier years of crop rotation (C1–C2 and S1–S), as well as annual rotation (Sa and Ca), showed more differences across season, while the long-term soybean monoculture crop sequence (Ss) appeared to have a more stable community composition, with no significant ($p < 0.05$) differences observed between seasons over the growing season in either year (**Table 5**).

TABLE 2 | Bacterial community alpha diversity indices across crop sequences within each season.

	2015 Spring		2015 Midseason		2015 Fall		2016 Spring		2016Midseason		2016Fall	
CropSeq	**Observed**	**Shannon**	**Observed**	**Shannon**	**Observed**	**Shannon**	**Observed**	**Shannon**	**Observed**	**Shannon**	**Observed**	**Shannon**
C1	908bc	4.32a	762ab	4.37a	1264a	5.03a	798a	4.13d	1027a	4.43a	1024a	4.87ab
C2	1440a	4.76a	1269a	4.52a	1313a	4.9ab	1102a	4.53bcd	1164a	4.83a	1157a	4.99a
Ca	1101abc	4.34a	837ab	4.44a	1276a	4.9ab	940a	4.24cd	1072a	4.53a	1202a	4.97a
S1	683c	4.46a	NA	NA	993ab	4.24cd	1147a	5.1a	1126a	4.34a	916a	4.17e
S2	919bc	4.17a	592b	3.95a	1067ab	4.27cd	1004a	4.18d	1017a	4.07a	1001a	4.41de
S3	1021bc	4.23a	750ab	3.95a	986ab	4.34cd	947a	4.17d	1062a	4.22a	976a	4.51cd
S4	927bc	4.23a	751ab	4.02a	794b	4.2cd	1013a	4.3cd	908a	3.89a	850a	4.54bcd
S5	1152ab	4.45a	777ab	4.13a	758b	4.06d	1045a	4.52cd	762a	4.36a	945a	4.48cde
Sa	1285ab	4.6a	745ab	4.23a	1051ab	4.54bc	1082a	4.98ab	1144a	4.28a	929a	4.4de
Ss	1136ab	4.43a	708ab	3.97a	1021ab	4.39cd	1179a	4.64bc	762a	3.63a	1163a	4.77abc
P-value	0.003**	0.18	0.06	0.48	0.01*	<0.001***	0.83	<0.001***	0.85	0.43	0.02*	<0.001***

Data were means of four replicates, significant values were reported at (*) P < 0.05, (**) P < 0.01, and (***) P < 0.001. Different letters in a column indicate significant difference across crop sequences within each season at P < 0.05 according to LSD test.

Bacterial Taxa Enriched and Associated With Cysts From Soybean Versus Corn Crop Sequences

The most significant differences of cyst bacteria between corn and soybean crop sequences were observed in fall of both years. In fall of 2015 and 2016, only about half of the observed OTUs in cysts (55.8 and 54.7% for 2015 and 2016, respectively) were shared between corn and soybean crop sequences, while 21.8 and 19.4% of cyst OTUs were unique to corn crop sequences and 22.4 and 25.9% of cyst OTUs were unique to soybean crop sequences in 2015 and 2016, respectively (**Supplementary Figure S2**).

Since few OTUs in cysts were found to be positively correlated or to significantly increase in relative abundance with increasing years of soybean monoculture, in order to detect bacterial taxa that were significantly different in abundance between cysts from each crop species (e.g., corn and soybean), we used a class-based biomarker detection method (LEfSe) (Segata et al., 2011). This approach detected bacterial classes and orders that showed a significant difference in relative abundance in cysts between soybean versus corn crop sequences (**Figures 5**, **6** and **Supplementary Table S6**). The fall sampling time points, where the greatest differences between cyst bacterial communities between corn and soybean were observed, were chosen for this analysis. When analyzed at the class level, the Deltaproteobacteria and Betaproteobacteria showed the strongest signal for significant enrichment in cysts associated with soybean crop sequences for both 2015 (**Figure 5A**; green) and 2016 (**Figure 6A**; green). A much larger number of bacterial classes were significantly associated with cysts from corn crop sequences, with the most significant enrichment in classes Alphaproteobacteria, Planctomycetacia, Acidobacteria, followed by Gammaproteobacteria (**Figures 5A**, **6A**; red and **Supplementary Table S6**). Here we focus primarily on taxa that were consistently enriched in cysts from soybean crop sequences and may have potential for biological control of the SCN. Orders contributing the most signal within the enriched classes for soybean crop sequences included the Burkholderiales within the Betaproteobacteria and Myxococcales within Deltaproteobacteria, which were enriched in both years (**Figures 5B**, **6B**; green and **Supplementary Table S6**). Streptomycetales within Actinobacteria were enriched only in 2016 (**Figure 6B**; green) and Methylophilales within Betaproteobacteria and a member of vadinHA64 (Opitutae class of Verrucomicrobia) were enriched only in 2015 (**Figure 5B**; green).

TABLE 3 | Adonis test of effect of year, season, and crop sequence on Bray–Curtis distance index.

Treatment	***F***	***P*-value**
Season	1.1997	0.13
CropSeq	2.6219	<0.0001***
Year	1.015	0.39
CropSeq*Season	1.2882	<0.0001***
Season*Year	1.1101	0.23
CropSeq*Year	1.4019	<0.0001***
SeasonRep*CropSeq*Year	1.2301	<0.0001***

Significant P values were reported at (*) P < 0.05 and (***) P < 0.001.

A much larger number of bacterial classes and orders were found to be enriched in cysts from corn crop sequences. Classes that were significantly ($p > 0.05$) enriched in both years included several classes in phylum Acidobacteria (Acidobacteria, Holophagae) and Proteobacteria (Alphaproteobacteria and Deltaproteobacteria), as well as Classes Thermoleophilia, Chlamydiae, Elusimicrobia, Gemmatinomnadetes, Planctomycetacia, Thermomicrobia, KD4-96 group, OPB35 soil group, and the SHA_109 group (**Figures 5**, **6** and **Supplementary Table S6**). Additional classes that were enriched only in 2015 included Acidomicrobia, Betaproteobacteria, Mollicutes, Negativicutes, Opitutae, and the S085 group, while those enriched only in 2016 included Acidimicrobiia, Chlorobia, Gammaproteobacteria, Phycisphaerae, Spartobacteria, the TK10 group, and a class within phylum Armatimonadetes (**Figures 5**, **6** and

TABLE 4 | *P*-value of Adonis test of Bray–Curtis dissimilarity matrix across crop sequence treatments within each season.

Treatment	Spring15	Mid15	Fall15	Spring16	Mid16	Fall16
All crop sequences[a]	<0.001***	<0.0001***	<0.001***	<0.001***	<0.001***	<0.001***
Soy vs. Corn	0.45	<0.001***	<0.001***	0.44	<0.001***	<0.001***
Soy only	<0.001***	0.04*	0.01*	<0.001***	0.04*	0.07.
Corn only	0.13	0.5	0.24	0.02*	0.29	0.44

[a]*All crop sequences included comparison across all 10 crop sequences; soy vs. corn compared between all pooled treatments from each different crop; soy/corn only compared among crop sequences with different number of years of monoculture of soybean or corn grown in each year. Significant differences are reported at FDR corrected (*) P < 0.05, (**) P < 0.01, and (***) P < 0.001.*

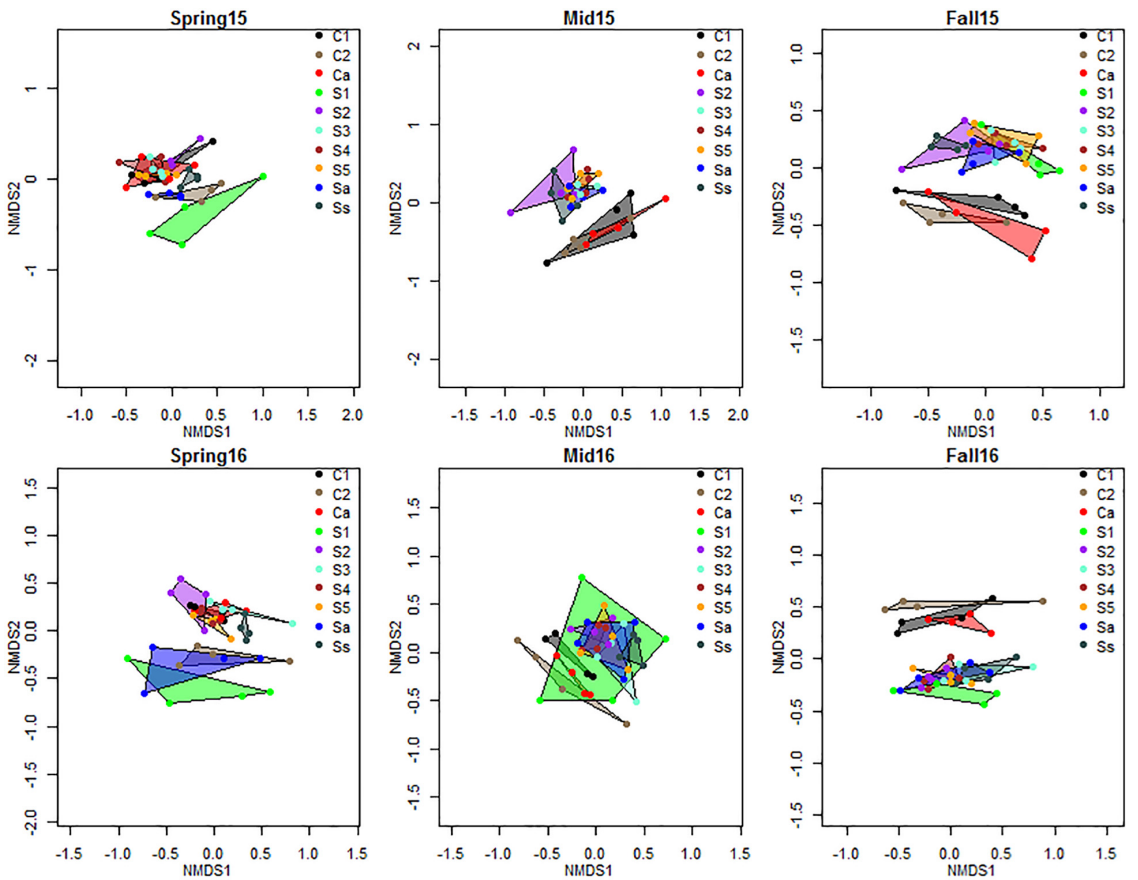

FIGURE 3 | NMDS plot of Bray–Curtis distance matrix of bacterial communities by crop sequences. Crop sequence treatments include first (C1) and second (C2) year of corn following 5-year soybean rotation, annual rotation of corn (Ca) following annual rotation of soybean (Sa), years 1–5 (S1–S5) of soybean following a 5-year corn rotation, and susceptible soybean (Ss) monoculture. Color coded shapes connect the four replicate datapoints representing communities from four replicate plots of each crop sequence.

Supplementary Table S6). Orders within these classes that were significantly enriched (corrected $p < 0.00001$) in both years included Subgroup6 (Acidobacteria), Acidimicrobiales (Acidimicrobiia), Solirubrobacterales (Thermoleophilia), Gemmatimonadales (Gemmatinomnadetes), Planctomycetales (Planctomycetacia), Rhizobiales and Rhodospirillales (Alphaproteobacteria), and Legionellales and Xanthomodales (Gammaproteobacteria).

The hierarchical structure of the LEfSe analyses also allowed us to analyze results at the genus and OTU level to detect specific OTUs within these higher level taxonomic groups enriched in cysts from soybean versus corn crop sequences. For soybean, these analyses identified taxa within several additional classes, including Cytophagia (Phylum FCB Group, Bacteroidetes), Alphaproteobacteria (Phylum Proteobacteria), and Spartobacteria (Phylum: PVC Group, Verrucombicrobia), that showed a significant association with cysts from soybean crop sequences (**Supplementary Table S6**). Within Burkholderiales (Betaproteobacteria), genera within the family Comamonadaceae (*Leptothrix*, *Rhizobacter*, and *Aquincola*) consistently showed the strongest signal for enrichment and association with soybean crop sequences across

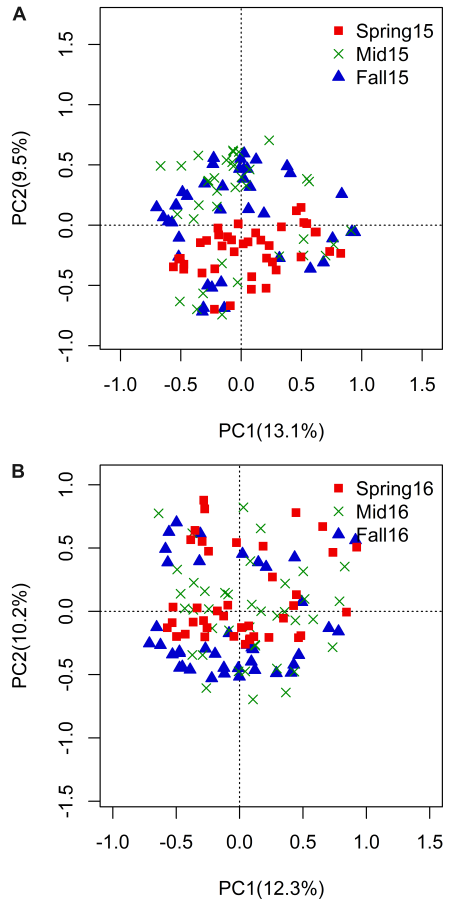

FIGURE 4 | PCoA plot of Bray–Curtis distance matrix by season within year 2015 **(A)** and 2016 **(B)**. Percentage of variation explained by season was shown on PC1 and PC2. Red squares = spring, green "x" = midseason, and blue triangles = fall.

both years (**Table 6** and **Supplementary Table S6**). In 2016, a symbiont of arbuscular mycorrhizal fungus (*Candidatus glomeribacter*) (Salvioli et al., 2016) within Burkholderiales was enriched in cysts from soybean crop sequences (**Supplementary Table S6**). Other genera that were enriched in both years included members of the Cytophagia (Bacteroidetes), including genera within the orders Cytophagales (*Cytophaga*) and Sphingobacteriales (*Chitinophaga* and *Niastella*). Within the Deltaproteobacteria, genera within order Myxococcales and family Haliangiaceae (*Helangium*) were enriched in both years (**Table 6** and **Supplementary Table S6**). Several Actinobacteria, including taxa within genera in the orders Streptomycetales (*Streptomyces*), Streptosporangiales (*Actinomadura*), and Micromonosporales (*Plantactinospora*), also showed enrichment in soybean crop sequences, but only in 2016 (**Supplementary Table S6**). The Alphaproteobacteria, which showed a less significant association with soybean crop sequences, included genera within the order Sphingomonadales (*Sphingomonas*). Within the Verrucomicrobia, an OTU mapping to *Candidatus xiphinematobacter*, known as a symbiont of the nematode genus *Xiphinema* (Vandekerckhove et al., 2000), was enriched in 2016, while an unknown species within order Opitutales was enriched in 2015 (**Supplementary Table S6**). Fewer taxa enriched in cysts in corn crop sequences could be identified to genera. Nonetheless, several genera that were enriched in both years included members of orders Rhizobiales (*Bradyrhizobium* and *Variibacter*) and Rhodospiralles (*Reyranella*) in Alphaproteobacteria, as well as orders Legionellales (*Aquicella*), Planctomycetales (*Planctomycetes*), and Solirubrobacterales (*Solirubrobacter*) (**Table 6** and **Supplementary Table S6**).

DISCUSSION

Because current management strategies for the SCN have significant limitations, an integrated management plan that includes use of biological control is essential for long-term and sustainable SCN management. However, only a limited number of bacterial species have been identified from cysts of the SCN based on traditional culture-based methods (Chen and Dickson, 2012), and even fewer have been tested in greenhouse or field trials or developed for their biological control potential, such as *P. nishizawae* (Clariva^{TM}) and *B. firmus* (PonchoVOTiVO^{TM}). Characterization of the microbial diversity of bacteria that could have activity against nematodes is useful for advancing management of this serious pathogen. This study utilized a high throughput metabarcode sequencing approach to uncover the diversity of bacterial taxa and the effects of crop host on bacterial communities directly associated with the cysts of SCN in field trials.

Taxonomic Composition of Bacterial Communities in Cysts

Overall, the dominant bacteria in SCN cysts belonged to Proteobacteria (Alpha-, Beta-, Delta-, and Gamma-Proteobacteria) and Actinobacteria, followed by Bacteroidetes and Verrucomicrobia, which is mostly consistent with previous studies based on DGGE and culture-based approaches (Nour et al., 2003) as well as culture-independent methods (Hussain et al., 2018). A recent study found that the dominant bacteria accumulating in cysts in an SCN-suppressive soil challenged with nematodes were Proteobacteria, followed by Actinobacteria and Bacteroidetes (Hussain et al., 2018). This same study found that, in addition to these three most abundant phyla, Verrucomicrobia, Planctomycetes, Chlamydia, and Firmicutes were enriched in cysts compared to the rhizosphere or the root endosphere communities (Hussain et al., 2018).

Changes in Bacterial Community Composition by Crop Sequence and Season

An understanding of the dynamics of microbes associated with SCN cysts in agroecosystems is also important for developing biocontrol agents that will be able to persist in commonly used crop rotation sequences. The bacterial community composition of cysts was significantly ($p < 0.001$)

TABLE 5 | Procrustes test of Bray–Curtis dissimilarity between seasons with FDR adjusted *P*-values < 0.1 (.), < 0.05 (*), < 0.01 (**), and < 0.001 (***).

	2015			2016		
Community	**Spring vs. mid**	**Mid vs. fall**	**Fall vs. spring**	**Spring vs. mid**	**Mid vs. fall**	**Fall vs. spring**
Overall	0.001**	0.001**	0.001**	0.001**	0.001**	0.001**
C1	0.05*	0.13	0.05*	0.14	0.17	0.14
C2	0.24	0.25	0.14	0.14	0.04*	0.05*
Ca	0.14	0.08	0.05*	0.24	0.08	0.05*
S1	NA	NA	1.0	0.33	0.42	0.10
S2	0.33	0.19	0.17	0.19	0.46	0.24
S3	0.05*	0.46	0.43	0.14	0.04*	0.19
S4	0.86	0.67	0.1.	0.33	0.67	0.48
S5	0.57	0.25	0.24	0.05*	0.08.	0.14
Sa	0.19	0.42	0.05*	0.24	0.08.	0.14
Ss	0.19	0.63	0.43	0.43	0.29	0.9

different when analyzed across all crops sequences according to the Adonis PERMANOVA analysis at nearly all sampling time points (**Table 4**), suggesting that crop sequences significantly affected bacterial communities in cysts. However, we also observed a strong interaction between crop sequences and season (**Table 3**), suggesting that the effect of crop sequence varied by season.

A previous companion study investigating the dynamics of fungal communities at the same field site identified several known biological control fungi whose relative abundance increased with increasing years of soybean monoculture, but did not show as strong a differentiation between corn and soybean crop sequences over a single growing season (Hu et al., 2018). In contrast, the current study revealed that the composition of bacterial communities within cysts from annual rotation (Ca, Sa) and early years of crop monoculture after 5 years of the alternate crop (C1, S1) changed more rapidly over the course of the growing season compared to fungal communities from long term monoculture (Ss). Although bacterial communities in cysts were not significantly different between corn and soybean crop sequences in spring of each year, at the end of a single growing season, they were clearly differentiated between corn and soybean crop sequences. This pattern was observed in the NMDS plot (**Figure 3**), and was supported statistically by the adonis PERMANOVA analysis (**Table 4**), where differences in communities in cysts from corn and soybean crop sequences were not significant in the spring, but became significant ($p < 0.001$) in the midseason and fall (**Table 4**).

Various factors could explain the more rapid changes observed in cyst bacterial communities compared to fungal communities over the course of the growing season. A faster rate of reproduction and turnover of bacteria compared to fungi in soil may be one factor. Another possible explanation is that the soybean plants may affect the bacterial communities in cysts, possibly through modifying the relative abundance of some bacterial taxa in the rhizosphere or inside roots of the crop plant that may serve as a source of inoculum and colonize newly formed cysts on the root. The hypothesis that root endophytes may infect SCN cysts forming on roots has been put forth previously for fungal parasites of nematodes (Carris et al., 1986, 1989). In support of this hypothesis, Hussain et al. (2018) reported that the bacterial community enriched in SCN cysts in a suppressive soil challenged with SCN showed more similarity with bacterial taxa enriched in the root endosphere compared to those in rhizosphere and bulk soil and also showed progressive enrichment of taxa from the rhizosphere to root and cyst communities. The use of susceptible soybean varieties in our study may also have increased the density of nematode cysts in soybean monoculture crop sequences. Some obligate parasites and pathogens of the SCN are known to show a density-dependent population dynamic in which the populations of the parasite and infection rates increase when populations of the nematode host are high (Persmark et al., 1996). Changes in other abiotic factors such as soil physio-chemical properties, rainfall, and temperature may also have contributed to changes in bacterial communities in cysts over the growing season.

Changes in Alpha Diversity by Crop Sequence and Season

Although patterns of alpha-diversity were not entirely consistent across the two years, potentially due to differences in weather and precipitation between years, or between all crop sequences, the cyst bacterial communities in corn crop sequences tended to be more diverse, especially by the Shannon index, than those from soybean sequences (**Table 2**). The higher overall diversity of bacterial communities from corn crop sequences may have contributed to the larger number of taxa that were enriched in cysts from the corn crop sequences in the LEfSe analysis (**Figures 5**, **6** and **Supplementary Table S6**).

The alpha diversity of the bacterial community in cysts also changed over the crop growing season, with midseason generally showing less diversity than either spring or fall, except for observed OTUs in 2016 (**Table 1**). The crop sequences also had a stronger influence on alpha diversity in spring and fall (**Table 2**). It is possible that at midseason, bacteria that had already colonized most of the available cysts prevented other bacteria from colonizing due to antagonism. Such "priority effects," where initial colonists inhibit new colonists is a phenomenon that has been demonstrated for fungi in SCN

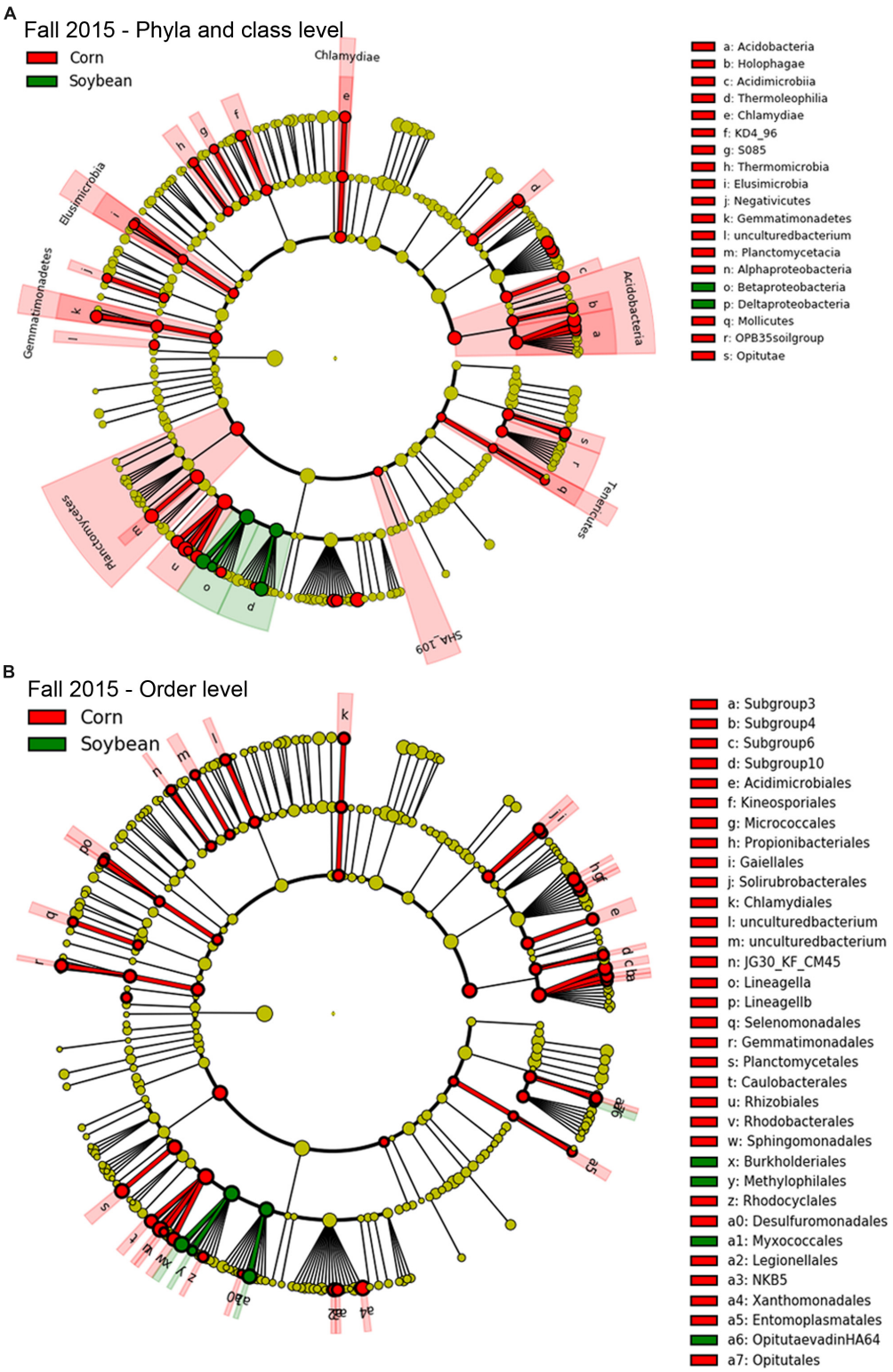

FIGURE 5 | Taxa enriched in cysts of corn and soybean crop sequences identified by LEfSe analysis in fall of 2015. Cladograph shows taxa at different phylogenetic ranks that are significantly discriminant in cysts from soybean (green) and corn (red) crop sequences in fall 2015 at level of both bacterial **(A)** phyla and classes and **(B)** orders. The colored wedges indicate phyla/class and orders that are significantly discriminant at that phylogenetic rank. Letters code the specific classes or orders shown in the legend to the right. Smaller colored circles in each ring indicate significantly discriminant taxa at each lower taxonomic hierarchical level below phyum [e.g., order, family, genus, and species (OTU)] within bacterial classes. A full list of taxa used to generate this cladogram is given in **Supplementary Table S6**.

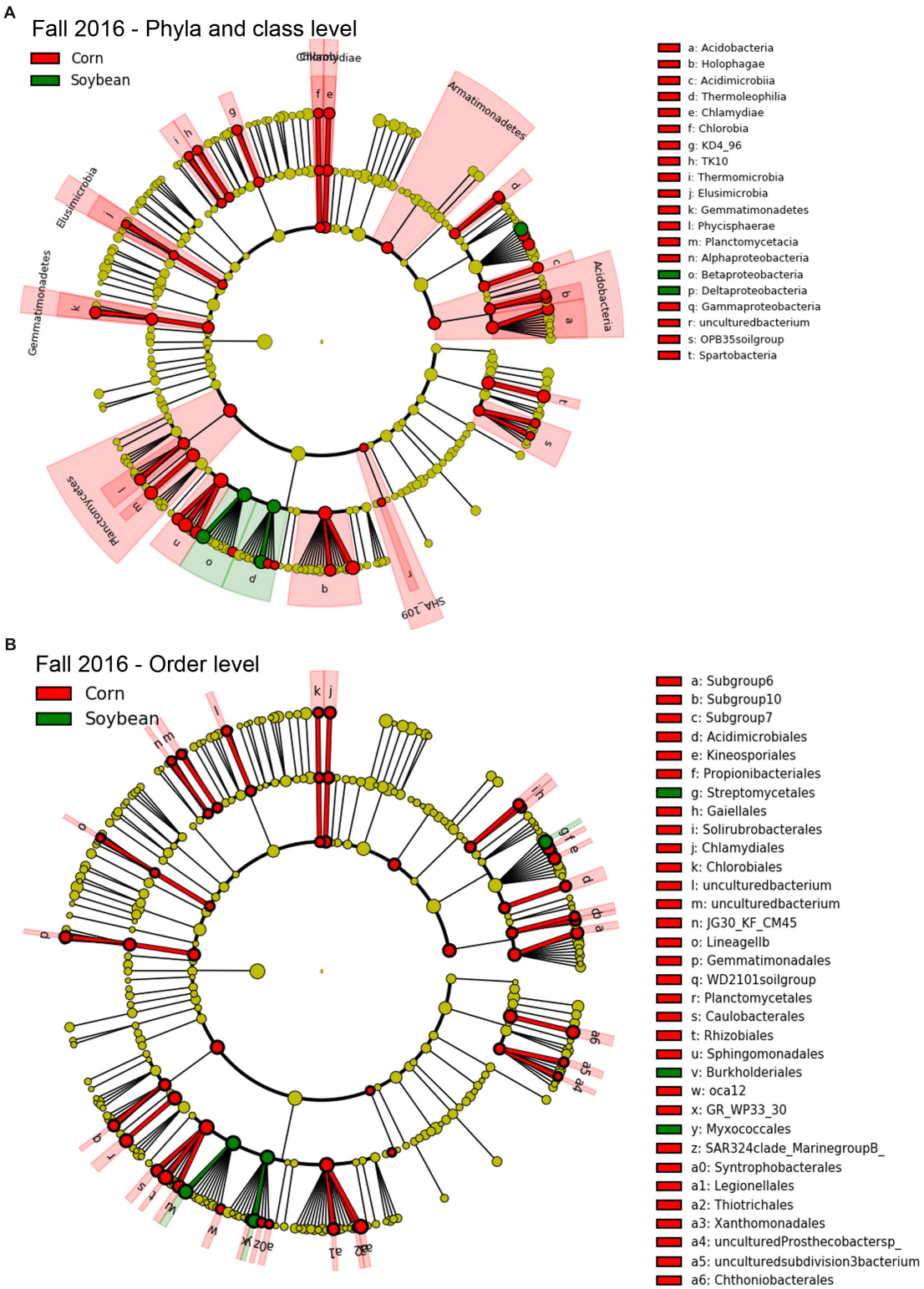

FIGURE 6 | Taxa enriched in cysts of corn and soybean crop sequences identified by LEfSe analysis in fall of 2016. Cladograph shows taxa at different phylogenetic ranks that are significantly discriminant in cysts from soybean (green) and corn (red) crop sequences in fall 2016 at level of both bacterial **(A)** phyla and classes and **(B)** orders. The colored wedges indicate phyla/class and orders that are significantly discriminant at that phylogenetic rank. Letters code the specific classes or orders shown in the legend to the right. Smaller colored circles in each ring indicate significantly discriminant taxa at each lower taxonomic hierarchical level below phyum [e.g., order, family, genus, and species (OTU)] within bacterial classes. A full list of taxa used to generate this cladogram is given in **Supplementary Table S6**.

TABLE 6 | Genera that were significantly ($P < 0.05$) enriched and associated with cysts from soybean and corn crop sequences in fall of both 2015 and 2016.

Soybean	Corn
Betaproteobacteria	**Alphaproteobacteria**
Bukholderiales	Rhizobiales
Comamonadaceae	Bradyrhizobiaceae
Rhizobacter	*Bradyrhizobium*
Leptothrix	Xanthobacteraceae
	Variibacter
	Rhodospirillales
	Rhodospirillales Incertae Sedis
	Reyranella
Cytophagia	**Gammaproteobacteria**
Cytophagales	Legionellales
Cytophagaceae	Coxiellaceae
Cytophaga	*Aquicella*
Deltaproteobacteria	**Planctomycetacia**
Myxococcales	Planctomycetales
Haliangiaceae	Planctomycetaceae
Haliangium	*Planctomyces*
Sphingobacteria	**Thermoleophilia**
Sphingobacteriales	Solirubrobacterales
Chitinophagaceae	Solirubrobacteraceae
Chitinophaga	*Solirubrobacter*
Niastella	

cysts (Chen and Chen, 2003). Between the mid and fall seasons, however, a second generation of new cysts would be produced and be available for colonization by diverse bacterial taxa. The previous study on the fungal communities in SCN cysts at this same site, for example, showed that diversity was the greatest in fall at harvest (Hu et al., 2018). It is also possible that higher temperature or other abiotic factors at midseason caused preferential proliferation of bacteria adapted to these conditions and reduced diversity between crops.

Bacterial Taxa Enriched in Cysts From Soybean and Corn Crop Sequences

Bacterial taxa enriched in cysts from the soybean crop sequences discovered in this study provide important information for directing future biocontrol screening efforts, as one of the key characteristics of a successful biological control agent is its abundance and ability to persist in crop rotations of the affected host (e.g., soybean). The biomarker approach used in this study was also able to pinpoint more precisely specific OTUs that contributed to differences of bacterial communities in cysts between corn and soybean crop treatments (**Table 6** and **Supplementary Table S6**), and may play an important role in SCN population management. This approach identified specific OTUs within these genera that were differentially abundant and associated with crop sequences of corn or soybean. Unfortunately, many of these OTUs could not be identified to species given current limitations in metabarcoding sequence regions and lack of representation of bacterial taxa in databases. These results suggest that identification of species or even strains that differ in abundance between crop sequences and treatments will be important to identify isolates with biocontrol potential and point to the need to improve taxonomic resolution of metabarcoding approaches for characterizing microbial communities.

Our results agree well with previous studies aimed at identifying bacterial taxa that are enriched in SCN suppressive or long-term soybean monoculture soils, but also identified some novel taxa enriched in cysts under crop sequences of each crop. Investigation of bacterial communities directly associated with control of nematodes has mostly focused on suppressive soil systems. A recent study found that several bacterial genera, including *Pseudomonas* (Gammaproteobacteria), *Burkholdaria* (Betaproteobaceria), *Chitinophaga* (Sphingobacteria), and *Streptomyces* (Actinobacteria), were enriched in the rhizosphere of soybean in long-term soybean monoculture fields that were also suppressive to SCN (Hamid et al., 2017). Another study found that when soil was amended with chitosan, a deacetylated form of chitin, taxa within *Streptomyces* increased in the rhizosphere of soybean grown in the presence of SCN and the nematode parasitic fungus *Hirsutella minnesotensis* (Mwaheb et al., 2017). Of the few studies that have investigated the effects of soybean crops sequences including long-term monoculture or SCN suppressive soils on bacterial taxa within cysts, an early study using a PCR-DGGE method and culturing approaches (Zhu et al., 2013) found that *Pseudomonas* and S*treptomyces*, as well as the nitrogen fixing rhizobacteria *Rhizobium* were enriched in cysts in a long-term soybean monoculture field. The recent greenhouse study of Hussain et al. (2018), looking at enrichment of bacterial taxa in the rhizosphere, root endosphere, and cysts in response to SCN suppressive soils and SCN challenge, found that the genera *Chitinophaga*, *Yersinia*, *Pseudoxanthomonas*, *Niastella*, *Pseudoxanthomonas*, and *Lentzea*, among others, were enriched in SCN cysts grown in SCN suppressive soil challenged with the SCN.

Plant growth promoting rhizobacteria, including several taxa within Pseudomonadales (Gammaproteobacteria) and Burkholdariales (Betaproteobacteria), are widely used in agriculture and horticulture to promote plant growth and protect plants from pathogens (Beneduzi et al., 2012; Gouda et al., 2018), including parasitic nematodes (Mhatre et al., 2019). Although we did not identify *Pseudomonas* as enriched in our study, several genera in the Burkholderiales (Betaproteobacteria) in the family Comamonadaceae (*Rhizobacter*, *Leptothrix*) were enriched in cysts from soybean crop sequences in both years (**Figures 5**, **6**, **Table 6** and **Supplementary Table S6**). Taxa within the genus *Rhizobacter* are intimately associated with the rhizosphere, and although to our knowledge have not been shown to play a role in nematode biocontrol, they have been shown to increase in relative abundance in a soil suppressive to a fungal wilt pathogen (Siegel-Hertz et al., 2018). *Leptothrix* was also identified in the previous study by Hussain et al. (2018) as being enriched

in cysts grown in an SCN suppressive soil. It is possible that some members of Comamonadaceae could also be nematode endosymbionts, as taxa within Comamonadaceae were shown to be dominant members of the endomicrobiota of another soil invertebrate, the springtail *Orchesella cincta* (L.) (Bahrndorff et al., 2018). Surprisingly, unlike Zhu et al. (2013), we did not find enrichment of *Rhizobium* or related genera of nitrogen-fixing rhizobacteria in cysts from soybean sequences (**Figures 5**, **6** and **Supplementary Table S6**). However, the LEfSe analysis identified several OTUs belonging to *Bradyrhizobium* as enriched in cysts from corn sequences in both years (**Figures 5**, **6** and **Supplementary Table S6**). The enrichment of *Bradyrhizobium* in cysts from corn sequences was surprising. We speculate that the SCN cysts could provide an alternative habitat for these bacteria to survive in the absence of their soybean host.

In agreement with previous studies, several genera of Actinomycetes (*Streptomyces* and *Actinomadura*) were also enriched in cysts under soybean crop sequences, but only in fall of 2016 (**Figure 6** and **Supplementary Table S6**). Taxa within *Streptomyces* have been shown in previous studies to be enriched in SCN cysts in long-term monoculture and SCN suppressive soils (Zhu et al., 2013; Hussain et al., 2018) and are well known for their production of secondary metabolite toxins or antibiotics. Some of these metabolites, including avermectins produced by the Actinomycete *Streptomyces avermitilis* (Cabrera et al., 2013) that are the active ingredient of the commercial nematicidal seed treatment Abamectin®, as well as others compounds may serve as nematode toxins or in antagonism of egg hatching (Liu et al., 2019). LEfSe analysis also identified OTUs within the genus *Haliangium* in the Myxococcales (Deltaproteobacteria) or "slime bacteria" to be enriched in cysts from soybean crop sequences in fall of both years (**Figures 5**, **6**, **Table 6**, and **Supplementary Table S6**). The Myxococcales are common soil bacteria and some species are known to produce secondary metabolites with bioactivity against eukaryotes (Weissman and Muller, 2009; Mulwa et al., 2018).

The ability to parasitize SCN females and eggs requires the enzymatic machinery necessary to penetrate the outer layer of the female, which is composed mostly of collagen, as well as the egg shell, which contains chitin (Burgwyn et al., 2003). However, once the female dies and the cyst matures, bacteria can enter the cysts freely through the fenestra, the opening used for the hatched SCN juveniles to exit the cyst. Few studies, however, have tested whether the bacteria colonizing cysts are able to directly penetrate eggs or the cuticle of the nematode juveniles inside eggs and parasitize live eggs. Presumably, enzymatic mechanisms or production of toxins are the main mechanism for these cyst-colonizing bacteria to penetrate and parasitize SCN eggs. Within the Sphingobacteria (Bacteroidetes), two genera within the family Chitinophagaceae, *Chitinophaga* and *Niastella*, were enriched in cysts from soybean crop sequences in both years in this study (**Figures 5**, **6**, **Table 6**, and **Supplementary Table S6**). Several OTUs mapping to *Chitinophaga* and *Niastella* were also found to be specifically enriched in cysts compared to rhizosphere soil and the root endosphere in SCN suppressive soil by Hussain et al. (2018). Similarly, the genus *Cytophaga* (Cytophagia) was enriched in cysts from soybean crop sequences in both years (**Table 6** and **Supplementary Table S6**). Some species within the genus *Chitinophaga* (*Chitinophaga japonensis*) have chitinolytic abilities (Sangkhobol and Skerman, 1981; Proenca et al., 2017), while the genus *Cytophaga* includes the well-studied cellulose degrading bacterium *Cytophaga hutchinsonii*. We speculate that the ability to degrade chitin and perhaps other carbon compounds found in either the outer cyst wall or egg shell may enable colonization of SCN cysts and eggs by taxa within these genera. Future studies should investigate taxa within these genera for their ability to degrade chitin and other carbon compounds found in SCN cysts or to directly parasitize SCN eggs. Interestingly, a *Chitinophaga* sp. found as an endosymbiont of *Fusarium keratoplasticum*, a seedborne fungus, was shown to significantly alter the carbon substrate usage of its fungal host (Shaffer et al., 2017). Species of *Fusarium* have been shown to be common inhabitants of SCN cysts (Carris et al., 1989; Haarith et al., 2019) and further investigation into potential bacterial–fungal symbioses that may promote colonization of SCN cysts and parasitism of nematode eggs by both partners is a promising area of future research.

CONCLUSION

This study characterized the taxonomic diversity of bacteria found within SCN cysts and showed that seasonal effects, crop sequences of corn and soybean, and the interaction of these factors shaped bacterial communities within cysts. The bacterial community structure within cysts showed a differentiation between corn and soybean over the course of a single growing season. The alpha diversity of bacterial communities in cysts was greatest in spring and fall and decreased slightly in midseason and diversity was slightly greater in corn sequences than in soybean sequences. Proteobacteria comprised a majority of the taxa found in cysts followed by Actinobacteria, Bacteroidetes, and Verrucomicrobia. A biomarker-based analysis (LEfSe) identified specific OTUs within these broader bacterial phyla that showed significantly greater abundance and a consistent association with SCN cysts under soybean cropping sequence, a desirable characteristic of an SCN biocontrol organism. Specific bacterial groups found to be enriched and associated with cysts from the soybean crop sequences included *Rhizobacter* and *Leptothrix* in the Betaproteobacteria, potential toxin producing taxa within Actinobacteria (*Streptomyces* and *Actinomadura*), *Helangium* in Deltaproteobacteria, and several genera in Bacteroidetes (*Chitinophaga* and *Cytophaga*). This study identified a diversity of bacteria found within SCN cysts that can be investigated as bacterial biological control agents of the SCN, for production of nematicidal metabolites, and for the discovery of novel chitin degrading enzymes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI accession number: PRJNA495048.

AUTHOR CONTRIBUTIONS

KB and SC co-conceived of and supervised the research. WH performed metabarcode sequencing of samples, designed metabarcoding analysis pipelines, analyzed the data, and co-wrote the manuscript. NS contributed data analysis pipelines and performed literature searches for classification of nematophagous fungi. DH assisted in collecting cyst samples and edited the manuscript. WH co-wrote the manuscript with KB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02671/full#supplementary-material

FIGURE S1 | Comparison of percent composition at the genus level between cyst communities found in all corn crop sequences and cyst communities found in soybean crop sequences at each season. Those genera containing greater than 2% of OTUs out of total OTUs are shown, with the genera containing the largest percentage ot OTUs at the bottom of each column.

FIGURE S2 | Venn diagram of OTUs shared in cysts from soybean and corn crop sequences and those unique to cysts from each crop species in **(A)** Fall 2015 and **(B)** Fall 2016.

TABLE S1 | Significant differences of bacterial classes across seasons.

TABLE S2 | Significant differences of bacterial classes across crop sequences at each season.

TABLE S3 | Significant differences of bacterial OTUs across crop sequences at each season.

TABLE S4 | Spearman rank correlation of bacteria OTUs with increasing years of soybean.

TABLE S5 | Differences in Beta dispersion parameters of bacterial communities across crop sequences.

TABLE S6 | LEfSe analysis results for fall 2015 and fall 2016.

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Shifts in the Active Rhizobiome Paralleling Low *Meloidogyne chitwoodi* Densities in Fields Under Prolonged Organic Soil Management

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Plants manipulate their rhizosphere community in a species and even a plant life stage-dependent manner. In essence plants select, promote and (de)activate directly the local bacterial and fungal community, and indirectly representatives of the next trophic level, protists and nematodes. By doing so, plants enlarge the pool of bioavailable nutrients and maximize local disease suppressiveness within the boundaries set by the nature of the local microbial community. MiSeq sequencing of specific variable regions of the 16S or 18S ribosomal DNA (rDNA) is widely used to map microbial shifts. As current RNA extraction procedures are time-consuming and expensive, the rRNA-based characterization of the active microbial community is taken along less frequently. Recently, we developed a relatively fast and affordable protocol for the simultaneous extraction of rDNA and rRNA from soil. Here, we investigated the long-term impact of three type of soil management, two conventional and an organic regime, on soil biota in fields naturally infested with the Columbian root-knot nematode *Meloidogyne chitwoodi* with pea (*Pisum sativum*) as the main crop. For all soil samples, large differences were observed between resident (rDNA) and active (rRNA) microbial communities. Among the four organismal group under investigation, the bacterial community was most affected by the main crop, and unweighted and weighted UniFrac analyses (explaining respectively 16.4% and 51.3% of the observed variation) pointed at a quantitative rather than a qualitative shift. LEfSe analyses were employed for each of the four organismal groups to taxonomically pinpoint the effects of soil management. Concentrating on the bacterial community in the pea rhizosphere, organic soil management resulted in a remarkable activation of members of the Burkholderiaceae, Enterobacteriaceae, and Pseudomonadaceae. Prolonged organic soil management was also accompanied by significantly higher densities of bacterivorous nematodes, whereas levels of *M. chitwoodi* had dropped drastically. Though present and active in the fields under investigation

Orbiliaceae, a family harboring numerous nematophagous fungi, was not associated with the *M. chitwoodi* decline. A closer look revealed that a local accumulation and activation of *Pseudomonas,* a genus that includes a number of nematode-suppressive species, paralleled the lower *M. chitwoodi* densities. This study underlines the relevance of taking along both resident and active fractions of multiple organismal groups while mapping the impact of *e.g.* crops and soil management regimes.

Keywords: organic soil management, active microbiome, rhizosphere, disease suppressiveness, *Meloidogyne chitwoodi*

INTRODUCTION

For decades, conventional soil management has resulted in consistent and high level of crop production by external inputs such as chemical fertilizers and pesticides. However, it is widely acknowledged that intensive monocropping has a number of downsides including soil degradation, leaching of nutrients, and biodiversity loss (Tsiafouli et al., 2014). Organic farming, an umbrella term for a wide range of management regimes having the abstinence of the use of mineral fertilizers and chemical pesticides in common, is a possible alternative that might alleviate the negative impact of crop production on soil ecosystems. In organic farming, most often organic manure is used to replenish the nutrient levels in the top soil and to maintain or increase the overall soil organic matter content. In addition, grain legumes are frequently part of the crop rotation because of their nitrogen binding capability. However, especially in Europe a wider application of grain legumes is currently hampered, by the relatively high level of variability in yield. This variation is thought to be due to the sensitivity of these crops to biotic and abiotic stressors (Cernay et al., 2015).

One of the key characteristics of sustainable soil management regimes should be the preservation of a relatively high level of soil biodiversity. In terms of biomass, bacteria and fungi are the most important biotic constituents of soils. Depending on soil type, cultivated soils typically harbor 0.2–0.7 mg of bacteria per g of dry soil, whereas the fungal community is represented by 0.01–0.2 mg per g (Kaczmarek, 1984). Protists and nematodes are major consumers of bacteria and fungi in soil ecosystems. Although the biomass of protists and nematodes is small compared to the primary decomposers (Bar-On et al., 2018), their impact on the turnover of bacteria and fungi is enormous. Protists alone are typically consuming >50% of the bacterial productivity (Foissner, 1999). Though it is a simplification of the biological reality, one could argue that the bacterial and fungal communities are shaped by (1) the quantity and nature of external C and energy inputs into the soil ecosystem, and (2) the activity of protist and nematode communities.

Being present in the soil ecosystem does not imply that a given organism is actively participating in the soil food web. On the contrary, many soil inhabitants are able to reduce their metabolic activity when unfavorable conditions occur, such as food scarcity or drought. This is especially relevant for bulk soils, where typically 80% of the cells, and 50% of the Operational Taxonomic Units (OTUs) are inactive (Lennon and Jones, 2011). Hence, it is essential to take both the resident and the active fractions into account when assessing the biological functioning of a soil ecosystem. A range of studies underlined the relevance of the distinction between resident and active soil biota (Baldrian et al., 2012; Nunes et al., 2018; Schostag et al., 2019). For taxonomic profiling, 16S or 18S ribosomal DNA (rDNA) is often used as molecular marker. Ribosomal RNA is frequently used to map the active microbial fractions (Ofek et al., 2014; De Vrieze et al., 2016). By the molecular characterization of both the resident and the active fractions of the bacterial, fungal, protist, and metazoan community, it is possible to assess the impact of soil management regimes on the soil food web (Harkes et al., 2019).

The rhizosphere of plants creates a center of high metabolic activity in soils. At the interface between the plant root and soil, the plant releases primary and secondary metabolites (Hinsinger et al., 2009; Reinhold-Hurek et al., 2015). With this blend of plant-derived components, the plant boosts a specific fraction of the soil biota. In return, stimulated microbiota increase the bioavailability of plant nutrients and/or they may contribute to the protection of the plants against pathogens (Lugtenberg and Kamilova, 2009; Berendsen et al., 2012; Turner et al., 2013). Especially in agricultural soil, the microbial community structure was shown to be distinct from the surrounding bulk soil (Sharma et al., 2005). Due to the application of fertilizers, root exudation is enhanced which on its turn affects the microbial community in the rhizosphere (Zhu et al., 2016).

Next to bacterivores and fungivores, the nematode community harbors a wide range of plant parasites. Most of them are relatively harmless root hair feeders and ectoparasites (Quist et al., 2015). Only a small subset may have a high impact in crop production. Root-knot nematodes (RKN), members of the genus *Meloidogyne*, are number one in terms of global crop damage by plant-parasitic nematodes (Jones et al., 2013). The highly polyphagous Columbian RKN *Meloidogyne chitwoodi* has a global distribution in temperate climate zones (Santo, 1989). In this study we investigated the long-term effects of three soil management regimes, conventional, integrated and organic, on the soil microbiome in fields naturally infested with *M. chitwoodi.* The legume *Pisum sativum* was used as main crop in these fields. Illumina MiSeq sequencing was used to characterize the active (rRNA) as well as the resident (rDNA) communities of bacteria, fungi, protozoans and metazoans both in bulk soil and in the rhizosphere. The main objectives of this study were (i) to characterize the resident and active microbial

community in the rhizosphere of pea with the underlying hypothesis that—besides being present—microbiota need to be active in order to be able to contribute to local changes in food web functioning, (ii) to map the effects of pea on the active and resident fractions of the four organismal group in the rhizosphere compared to the bulk soil under different soil management systems, and (iii) to identify microbial taxa which activities changed in parallel with distinct infestation levels of the root-knot nematode *M. chitwoodi*

MATERIALS AND METHODS

Study Sites

Samples were collected at the WUR experimental farm "Vredepeel", which is located in the south east of the Netherlands (51°32N and 5°51E). Experimental plots were situated on sandy soils (93,3% sand, 4.5% silt, 2.2% clay) with an organic matter (OM) content of 3%–5%. Three different soil management regimes were continuously applied from 2001 onwards: "ConMin," "ConSlu" and "Org." "'ConMin fields" solely received mineral fertilizer and processed organic fertilizer (liquid mineral concentrates), whereas "ConSlu fields" were supplemented with mineral fertilizer and slurry (pig/cow). In case of organic soil management ("Org fields"), farmyard manure and cow slurry were applied, and no pesticides were used. For further details of the set up and layout of the soil management experiments see the research reports (De Haan et al., 2018a; De Haan et al., 2018b).

Soil Sampling

Pea (*P. sativum*) is one of the main crops in the crop rotation system and was sown on the 10th of April 2018. Sampling was executed twice, during the vegetative stage (7th of May) and during the generative stage (31st of May). Each of the three no-tillage fields (ConMin, ConSlu, and Org) was divided in 6 subfields of 540 m^2 (**Supplementary Figure S1**). In each subfield, a bulk soil and a rhizosphere sample was taken, resulting in six pseudo-replicates. Rhizosphere composite samples were taken by harvesting all pea plants within a square of 20 x 20 cm. A spade of 20 cm was used to carefully lift the soil and take out the all pea plants, this was done in triplicate for each subfield. Excessive soil was removed by shaking the plants and whole plants were transported to the laboratory at the field site. At the field laboratory, the remaining soil that adhered to the roots was brushed off from 10 individual pea plants. Bulk soil was collected by combining three individual cores in the close vicinity of the rhizosphere sampling spot. This was done in between the pea rows with the use of an auger (ø1.5 cm, depth approximately: 15 cm). In total 36 samples (18 rhizosphere and 18 bulk) were taken at each time point, making a total of 72 samples.

Rhizosphere and bulk soil samples were frozen in liquid nitrogen and transported on dry ice to the laboratory, and stored at -80°C until further processing.

For nematode extraction a composite sample was collected in each field (ConMin, ConSlu, and ORG). In total two soil cores per subplot were randomly sampled (ø1.5 cm, depth approximately: 15 cm) resulting in 12 soil cores per field. Composite samples were mixed and stored at 4°C. Two days after collection, nematodes were extracted from 100 g soil, using an elutriator (Oostenbrink, 1960). This was done *in duplo* for each field resulting in six nematode suspensions.

DNA/RNA Extraction and cDNA Synthesis

Both DNA and RNA were simultaneously extracted from soil samples (2 g each), using a lab-made protocol based on phenol-chloroform-isoamylalcohol extraction (Harkes et al., 2019). Quality and quantity of the obtained RNA and DNA was measured with a Nanodrop and Qubit. The nucleic acid eluate was stored at -80°C until further processing. For synthesis of cDNA from extracted RNA, the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Thermo Fisher Scientific Inc., USA) was used according to the manufacturer's instructions. All individual DNA and cDNA samples were diluted to 1 ng/µl and 0.1 ng/µl, respectively, and used as template for PCR amplification.

To estimate the nematode density, a subsample of the nematode suspension (1/10 of each sample) was counted under a dissecting microscope. This was done in triplicate. Hereafter, nematode suspensions were concentrated and lysed according to Vervoort et al. (2012). This resulted in 100 µl purified DNA, which served as a template for quantitative PCR (qPCR).

PCR Amplification and Sequencing

The variable V4 region of bacterial 16S rRNA gene was utilized as target for the analyses of Illumina 16S rDNA sequencing and the V9, V7–V8, V5–V7 regions were utilized as targets for protozoa, fungi, and metazoan 18S rDNA sequencing, respectively. To prepare the samples for sequencing, a two-step PCR procedure was followed as described in (Harkes et al., 2019). In short, locus-specific primer combinations extended with an Illumina read area and the appropriate adapter were used to produce primary amplicons. This was done in triplicate for all samples and for each of the four organismal groups. PCR 2 was conducted on 40x diluted amplicons of PCR1 to attach the Illumina index and the Illumina sequencing adaptor. Products of PCR 1 and 2 were randomly checked on gel to ensure amplification was successful. Finally, PCR products of fungi, protozoa and Metazoa were pooled and sent for sequencing. Bacterial PCR products were sent separately in order to improve the sequencing resolution. Sequencing was done at Bioscience—Wageningen Research, Wageningen, Netherlands—using the Illumina MiSeq Desktop Sequencer (2*300nt paired-end sequencing) according to the standard protocols.

For analysis of the obtained nematode DNA from the 100g subsamples, 12 nematode taxa were selected for qPCR. 11 primer sets to asses a various set of plant parasitic nematodes—including *M. chitwoodi*—and one primer set to measure the total nematode density (see **Supplementary Table S1**).

Bioinformatics Framework

The composition of microbial communities of the soil samples was analysed based on the sequencing data obtained from the Illumina MiSeq platform. Reads were sorted into the experimental samples according to the unique combination of two index sequences. Thereafter, reads were sorted into the four organismal groups based on their locus-specific primer sequences.

Forward and reverse reads were paired for bacteria and fungi, while single-end (forward) sequences were analysed for protozoa and metazoan. The four taxonomical groups were quality trimmed by BBDUK and then merged *via* VSEARCH (Rognes et al., 2016; Bushnell, 2018). Resulting unique sequences were then clustered at 97% similarity by using the usearch_global method implemented in VSEARCH and a representative consensus sequence per *de novo* OTU was determined (Rognes et al., 2016). The clustering algorithm also performs chimera filtering to discard likely chimeric OTUs with UCHIME algorithm in *de novo* mode (Edgar et al., 2011) implemented in VSEARCH. Sequences that passed quality filtering were then mapped to a set of representative consensus sequences to generate an OTU abundance table. Representative OTU sequences were assigned to a taxonomic classification *via* BLAST against the Silva database (version 12.8) for bacteria, fungi, and metazoan and PR2 database (Guillou et al., 2013) for protozoa using SINA (Pruesse et al., 2012). Sequences belonging to chloroplasts, cyanobacteria, and mitochondria were discarded from the bacterial dataset; sequences not belonging to Fungi and Metazoa were removed for 18S Fungi and Metazoa datasets, respectively and Streptophyta, Metazoa, fungal, and unclassified Opisthokonta sequences were filtered for Protozoa dataset. Low-abundance OTUs (those with abundance of <0.005% in the total data set) were discarded prior to analysis (Bokulich et al., 2013). Samples were transformed using Hellinger transformation for all downstream analyses.

Processing and Analysis of Nematode Specific Sequences

For the nematode specific analysis, metazoan reads were blasted against a nematode database after quality trimming of the reads. Trimmomatic v.0.35 (Bolger et al., 2014) was used to trim poor quality bases (four base sliding window with a 13 ($p = 0.05$) average phred score cut-off), remove the locus specific primer and filter out short reads (<50 bases). The Blast database was based on the dataset of Holterman et al. (2017). Forward and reverse reads were blasted separately as the sequences did not overlap. Each read had one or multiple families assigned to it based on the best blast hit and any additional hits that differed by no more than one base pair from the best blast hit. Reads with less than 92% identity to the sequences in the database were considered not to be nematodes. Reads between 92% and 95% identity were counted as nematodes, but no family name was assigned to them. After this the results for the forward and reverse sequence of each mate pair was compared. Where possible, the results of both reads were combined to refine the family assignment. If the family assignments of both reads of a mate pair were in complete disagreement, the reads were discarded. In some cases, the amplified SSU fragment did not allow for the distinction between certain families, and the reads had to be pooled into a larger taxonomic unit, e.g. all members of the order Dorylaimida or members of the families Bastianiidae and Prismatolaimidae.

Statistical Analysis

Good's coverage was assessed (Good, 1953) in order to estimate what percent of the total species is represented in each sample. We explored β diversity patterns by performing principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity using QIIME software (Caporaso et al., 2012). Permutational multivariate analysis of variance (PERMANOVA) was used to compare the microbial community structure between soil managements taken from different sites and with different plant growth stages for active and resident community for four different taxa. This was performed with 1,000 permutations using the adonis function, based on Bray-Curtis distances using the "vegan" package (Oksanen et al., 2015) in R. In order to compare microbial community diversity in a phylogenetic context, UniFrac was performed with 1,000 permutations *via* the "phyloseq" package in R (Mcmurdie and Holmes, 2013). To assess variation in both relative abundance and presence/absence, we analysed both weighted and unweighted UniFrac distances (Lozupone et al., 2007). To investigate the indicator taxa involved in the differences between resident and active community, a linear discriminate analysis (LDA) effect size (LEfSe) was conducted in Microbiome Analyst (Dhariwal et al., 2017) to explore the differential microbial populations at the family level for the four different taxa (Segata et al., 2011). A significance level of $\alpha \leq 0.05$ was used for all biomarkers evaluated in this study.

To assess differences between family read abundances of nematodes, a Kruskal–Wallis test was conducted, followed by a Dunn's Test to test for significance between each of the three management types (IBM SPSS Statistics 25).

RESULTS

The long-term impact of organic soil management on four major soil organismal groups was monitored in experimental field where pea was grown as main crop. The bacterial and fungal communities were mapped as main primary decomposers, whereas protists and metazoa (mainly nematodes) were included as representatives of the next trophic level. For each of the four organismal groups resident and the active communities were characterized in (1) bulk and rhizosphere soil, (2) with three types of soil management, and (3) at two time points representing the vegetative and the generative growth stage of pea.

General Analyses of the Sequencing Data

Total DNA and RNA was extracted from 72 bulk soil and rhizosphere samples. MiSeq sequencing was performed on ribosomal DNA and cDNA fragments (16S for bacteria or 18S

for fungi, protists, and Metazoa). After filtering, a total of 22 million sequences with an average length of 250bp were retained, comprehending 208 samples for all taxa together. Comprehensive sampling of the microbial community was performed for all treatments, with average sequence coverage of 93%, 95%, 99%, and 99% for protozoa, bacteria, fungi, and metazoan, respectively determined by Good's coverage estimate.

Difference Between Resident and Active Communities In Bulk and Rhizosphere Soil at Organismal Group Level

To investigate whether contrasts could be observed between resident and active fractions of the individual organismal groups, and between bulk and pea rhizosphere soil, principal coordinate analysis (PCoA) ordinations on Bray-Curtis dissimilarity matrices were conducted (**Figure 1**). The effect of the variable Nucleic Acid (rRNA for active and rDNA for the resident community) is clearly visible. For all four organismal group there are distinct clusters for rRNA (red and ochre) and rDNA (blue and light blue) although this is somewhat less pronounced in case of Fungi. The effects of sample type (bulk *vs* rhizosphere) were easily observable as well. Especially for bacteria there is a clear separation of the two soil compartments (**Figure 1**).

To determine whether soil organismal groups were significantly affected by the four main variables included in this study three distinct methods to compare communities were used: Bray-Curtis dissimilarity, and weighted and unweighted UniFrac. Results of the PERMANOVAs are shown in **Table 1**. The R^2 values indicate how much of the observed variation is explained by each of the individual variables. Sample Type (bulk *versus* rhizosphere soil) was the dominant explanatory variable for the observed shifts in the bacterial communities. The large difference between the relevant R^2 values resulting from the Unifrac analyses (unweighted 16.4%, weighted 51.3%), points at a quantitative rather than a qualitative shift. For fungi, Sample Type was most important in the Bray

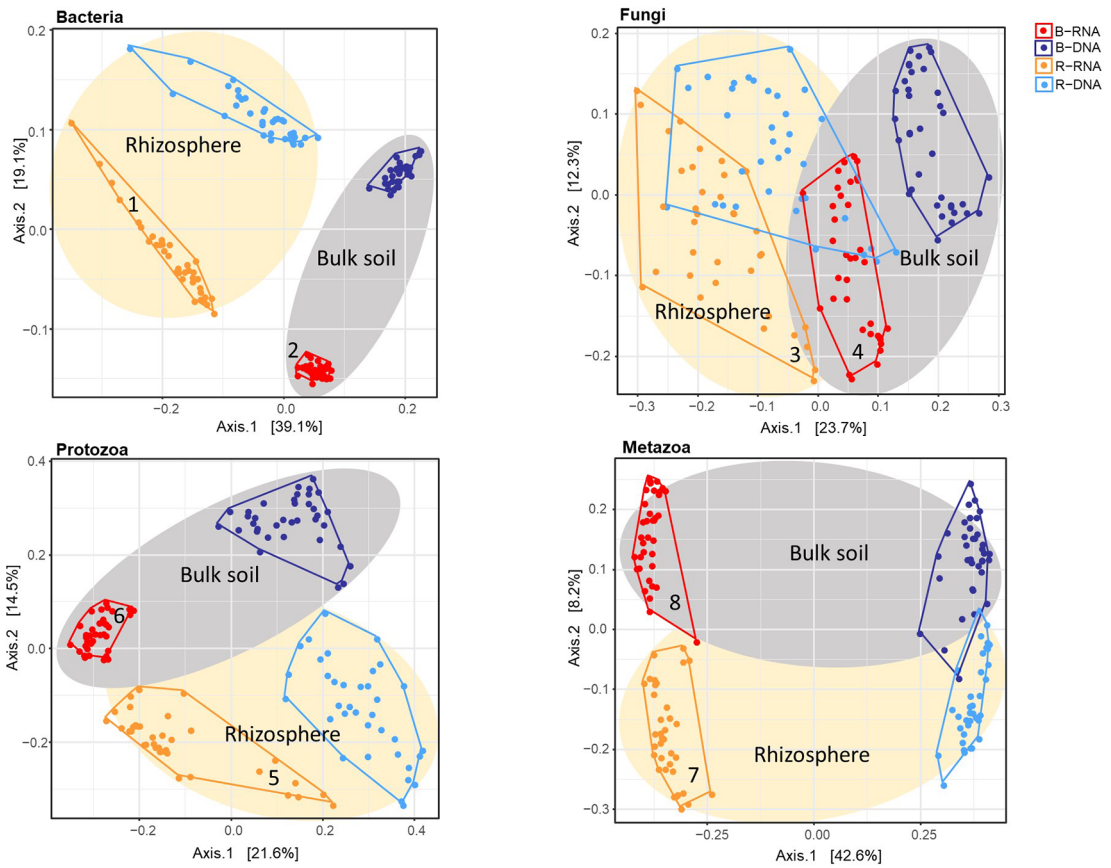

FIGURE 1 | Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix. Plots illustrate distances between communities [72 soil samples; for each sample both the resident (rDNA) and the active (rRNA) community were characterized] for each organismal group: **(A)** Bacteria; **(B)** Fungi; **(C)** Protozoa, and **(D)** Metazoa. Colours were used to distinguish between rRNA-bulk (red), rRNA-rhizosphere (ochre), rDNA-bulk (dark blue), and rDNA-rhizosphere (light blue). For all organismal groups: grey ellipses for bulk and ivory ellipses for rhizosphere. Numbers accompanying the active bacterial, fungal and protozoan communities (ochre and red) correspond to the numbers in the top left or top right corner of **Figure 2**.

TABLE 1 | The impact of four variables on four organismal groups in fields with three soil management regimes with pea as main crop.

	Bray-Curtis		UniFrac - Unweighted		UniFrac - Weighted	
Bacteria	R^2	P	R^2	P	R^2	P
Nucleic Acid	0.231	0.001	0.137	0.001	0.261	0.001
Treatment	0.076	0.001	0.086	0.001	0.042	0.001
Sample Type	**0.295**	0.001	**0.164**	0.001	**0.513**	0.001
Time Point	0.040	0.001	0.025	0.001	0.021	0.001
Residuals	0.357		0.589		0.163	
Fungi						
Nucleic Acid	0.108	0.001	**0.126**	0.001	**0.250**	0.001
Treatment	0.134	0.001	0.102	0.001	0.091	0.001
Sample Type	**0.172**	0.001	0.066	0.001	0.177	0.001
Time Point	0.045	0.001	0.028	0.001	0.039	0.001
Residuals	0.542		0.679		0.443	
Protozoa						
Nucleic Acid	**0.192**	0.001	**0.305**	0.001	**0.434**	0.001
Treatment	0.082	0.001	0.088	0.001	0.047	0.002
Sample Type	0.125	0.001	0.099	0.001	0.106	0.001
Time Point	0.091	0.001	0.039	0.001	0.083	0.001
Residuals	0.510		0.469		0.329	
Metazoa						
Nucleic Acid	**0.440**	0.001	**0.104**	0.001	0.072	0.001
Treatment	0.040	0.001	0.089	0.001	0.046	0.001
Sample Type	0.063	0.001	0.100	0.001	**0.216**	0.001
Time Point	0.022	0.001	0.023	0.001	0.059	0.001
Residuals	0.440	0.001	0.104	0.001	0.072	0.001

Summary of the PERMANOVA for Bray-Curtis dissimilarity distances as well as phylogenetic distances (UniFrac). This analysis tests differences in quantitative taxonomic composition of Bacteria, Fungi, Protozoa and Metazoa assemblages taking Nucleic Acid (cDNA/DNA), Sample type (Bulk/Rhizosphere), Treatment (ConSlu, ConMin, Org) and Time point (Vegetative/Generative) as main variables. For each of organismal group and each analysis method the variable with the highest R^2 is indicated in bold. Differences are considered significant if $P < 0.01$.

Curtis analysis only. Phylogeny-based distance methods identified Nucleic Acid—the difference between the resident and the active fungal community—as the most dominant variable explaining 12.6 and 25.0% of the observed variations. For the protozoa and the metazoa, Nuclei Acid was identified as the main explanatory factor as well (except for the weighted Unifrac in case of Metazoa).

Active Taxa Contributing to the Difference Between Bulk Soil and Rhizosphere

LEfSe was used to determine which active taxa have the highest contribution to the observed differences between bulk and rhizosphere. An LDA threshold of >2.5 was set, which resulted in 28 bacterial, 20 fungal, and 14 protozoan orders that gave rise to the differences between bulk and rhizosphere communities (**Figure 2**). It is noted that metazoans are not displayed. Soil samples of 2 g were analysed, and the sample size is too low to give a genuine impression about the composition of the micro and mesofauna. Metazoa were nevertheless taken along as co-extraction of their DNA is indicative for spatial association of microbial taxa and the detected metazoans.

The elevated activities of members of Rhizobiaceae and Labraceae (both Rhizobiales) were detected in the rhizosphere of pea. As *P. sativum* is nodulated by the N_2–fixing *Rhizobium leguminosarum*, an enrichment of the Rhizobiaceae was expected (**Figure 2**, panel 1). Moreover, increased activity was observed for number of bacterial families that harbour P-solubilizing members such as Rhizobiacea (including the genus *Rhizobium*), Enterobacteriaceae (including *Serratia*), Pseudomonadaceae (including *Pseudomonas*) and Burkholderiaceae (including *Burkholderia*) (Pii et al., 2015). In the bulk soil we observed a relatively high activity of the Nitrosophaeroceae (Archaea, Thaumarchaeota). Representatives of this family are known as ammonia oxidizers (Stieglmeier et al., 2014). In addition, a bacterial family that harbours members that initiate the oxidation of ammonia to nitrite, the Nitrosomonadaceae, showed enhanced activity. Regarding fungal families with upregulated activity in the rhizosphere at least two observation are noteworthy (**Figure 2**, panel 3). The Orbiliaceae harbour numerous nematophagous fungi, and this could affect the RKN *Meloidogyne chitwoodi* present in these experimental fields (Xie et al., 2010). Differential activity of two members of the Glomeromycota were observed in the two compartments. Whereas Glomeraceae (order Glomerales) showed enhanced activity in the rhizosphere, elevated levels of Gigasporaceae (order Diversisporales) activity were detected in the bulk soil (**Figure 2**, panels 3 and 4). This observation suggests that pea might interact with a member of the order Glomerales.

Members of the order Thecamoebida (**Figure 2**, panel 5) are known as large protists and voracious predators of bacteria and other protozoans (Melton et al., 2019), which explains their elevated activity in the rhizosphere. Another representative of the naked amoebae, the Echinamoebida, was highly active in the rhizosphere. Terrestrial Colpodea, small bacterivorous ciliates, are known as extreme r-strategists (Lüftenegger et al., 1985), and they can easily cope with fluctuating environmental dynamics, this might explain their enhanced activity in the rhizosphere (Foissner, 1993). The detection of active Pterocystida in bulk soil (**Figure 2**, panel 6) is remarkable as they belong to the heliozoan protists that are normally found in freshwater and marine environments, and only occasionally in soil (Cavalier-Smith and Chao, 2012). Also, the enhanced presence of active Chlamydomonadales, an order of green algae, is worth noting. We assume that these photosynthesizing protists were present at the very top layer of the bulk soil.

Difference Between Resident and Active Communities Under Three Distinct Soil Management Regimes at Organismal Group Level

To determine the level at which the soil management regimes ConMin, ConSlu, and Org had an effect on the four organismal groups, we analysed the rDNA sequence data separate from the rRNA data. As can be seen in **Table 2**, soil management ("Treatment") had a significant effect on both the composition of the communities, and their levels of activity for all four organismal groups (in all cases $P < 0.001$). These analyses also showed that the compartment effect, the contrast between rhizosphere or bulk soil, is consistently larger than the treatment effect.

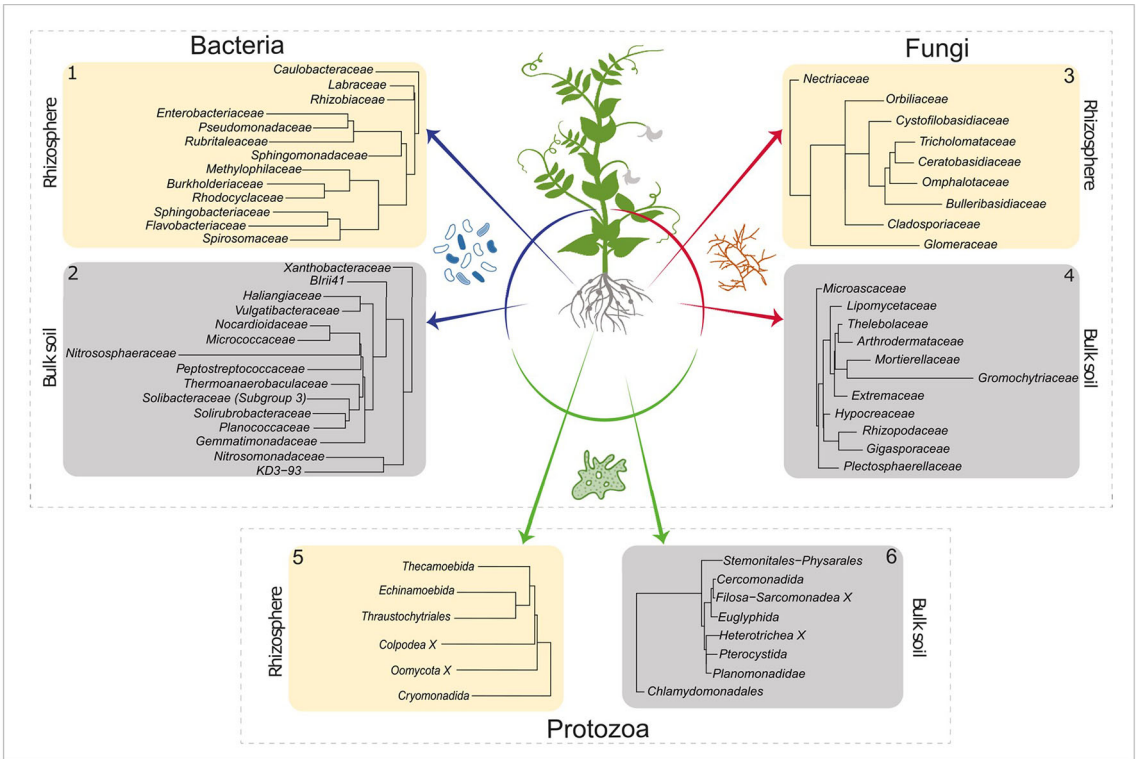

FIGURE 2 | LEfSe analysis of the active bacterial, fungal, and protists OTUs. Identifying taxa for which a major part of the population was active in rhizosphere (ivory square) or in bulk soil (gray square) (LDA score > 2.5). Numbers in the top left or top right corner correspond to the number next to the active communities (ochre and red) in **Figure 1**. LEfSe, linear discriminate analysis effect size; OTUs, Operational Taxonomic Units; LDA, linear discriminate analysis.

Principal coordinates analysis (PCoA) was used to visualize the effect of prolonged exposure to three distinct management regimes in bulk soil and rhizosphere on the active and resident communities of each organismal groups. As can be seen in **Figure 3**, soil management had a major impact on all four organismal groups. 'DNA bulk' shows the resident communities in absence of the main crop, and the communities in the fields under organic management were distinct from two conventional treatments, ConMin and ConSlu. In case of 'RNA bulk', the same trend was observed. Another interesting shift was observed within the two conventional treatments (ConMin in red and ConSlu in blue). Whereas the soil communities in bulk soil were fully separated in all three different management types, the two conventional treatments tend to overlap in their communities in the rhizosphere. It is noted that the highest percentage of the variation explained by the two axes was observed for "RNA rhizosphere" with an average of 44.4% for the four organismal groups.

Active Rhizosphere Taxa Contributing Most to the Observed Difference Between the Three Soil Management Regimes

Figure 4 shows the results of LEfSe analyses that revealed the taxa that contributed most to observed differences in the active pea rhizosphere communities under the three soil management systems. Regarding the bacterial community, families harbouring P-solubilizing members such as the Burkholderiaceae, the Enterobacteriaceae, and the Pseudomonadaceae showed high levels of activity in the organic fields. Members of the Nocardioidaceae showed elevated levels of activity under the ConMin regimes, the family was recently identified as being associated with the domestication of a pea relative, the common bean *Phaseolus vulgaris* (Perez-Jaramillo et al., 2017).

The Pezizaceae stood out as being active under the organic soil management regime. This fungal family harbours dozens of genera, and often grow on dung of animals. As the organic plots only received farmyard manure and cow slurry, the enrichment of this speciose fungal family can be seen in this perspective (Alexopoulos and Mims, 1979). Both Actinobacterial families, Intrasporangiaceae and Nocardioidaceae, showing a high level of activity in the conventional soil treatments ConSlu and ConMin (**Figure 4**). Both families are known as efficient secondary utilizers of cellulose-derived glucose under oxic conditions (Schellenberger et al., 2010). This observation points at enhanced cellulolytic activity in the pea rhizosphere under conventional soil management.

The families Nectriaceae and Chaetomiaceae were identified as fungal indicators for fields with the most conventional treatment (ConMin). The high activity of Nectriaceae might be related to the application of mineral fertilizer. A similar phenomenon was

TABLE 2 | The impact of three variables on the resident and the active fractions of four organismal groups in fields with three soil management regimes with pea as main crop.

	rDNA		rRNA	
Source	**R^2**	**P**	**R^2**	**P**
Bacteria				
Treatment	0.101	0.001	0.103	0.001
Sample Type	**0.378**	0.001	**0.428**	0.001
Time Point	0.048	0.001	0.063	0.001
Residuals	0.472		0.406	
Fungi				
Treatment	0.154	0.001	0.165	0.001
Sample Type	**0.218**	0.001	**0.208**	0.001
Time Point	0.047	0.001	0.063	0.001
Residuals	0.581		0.565	
Protozoa				
Treatment	0.139	0.001	0.132	0.001
Sample Type	**0.241**	0.001	**0.159**	0.001
Time Point	0.136	0.001	0.120	0.001
Residuals	0.484		0.589	
Metazoa				
Treatment	0.138	0.001	0.101	0.001
Sample Type	**0.168**	0.001	**0.200**	0.001
Time Point	0.064	0.001	0.067	0.001
Residuals	0.630		0.632	

This summary of the PERMANOVA for Bray-Curtis dissimilarity distances shows the impact of Treatment (ConSlu, ConMin, Org), Sample type (Bulk/Rhizosphere), and Time point (Vegetative/Generative) on four categories of soil inhabitants. For each of organismal group and each analysis method the variable with the highest R^2 is indicated in bold. Differences are considered significant if P <0.01. For Unifrac analyses see ***Supplementary Table S2****.*

observed in tropical rain forest plots treated with mineral fertilizer (Kerekes et al., 2013). The other upregulated member of the Sordariales, the family Chaetomiaceae, is known for its cellulolytic members (Wilhelm et al., 2017). This enhanced activity might have facilitated the elevated activity of members of the Intrasporangiaceae and Nocardioidaceae.

The Tubulinea orders Euamoebida and Nolandida were metabolically active in the organic fields. The first family was found in high relative abundances in grasslands and forest mineral soils, whereas the Nolandida is a relatively rare protist order in soils (Geisen et al., 2015). Xantophyceae, consisting of stramenopilan photoautotrophs, typify the ConMin fields. Other phototrophs showing enhanced activity in the ConMin fields were members of the green algae order Chlorellales, and the red algae order Cyanidiales.

Soil Management-Related Shifts in Nematode Communities

Microscopic nematode density counts, and qPCR nematode density data showed no significant differences in overall nematode abundances between the three soil management types (**Table 3**). By means of rRNA sequencing, taxonomic shifts in nematode communities were detected at family level (**Figure 5**). Five out of six bacterivorous families were found to be more abundant in Org fields. In case of the two fungivorous families Aphelenchidae and Aphelenchoididae, small but significant trends towards lower densities in fields under organic management were observed. The predator family Mylonchulidae was specifically upregulated in the ConSlu fields. With regard to the plant parasites, lower infestation levels for the families Telotylenchidae, Heteroderidae, and Meloidogynidae were observed in the organic fields.

MiSeq nematode community analysis does not allow for the detection of nematodes at species level, and therefore a species-specific qPCR assay was run to pinpoint the observed decrease of members of the genus *Meloidogyne*. qPCR analysis revealed that the *Meloidogyne chitwoodi* were significantly lower in the Org fields as compared to the two conventional treatments ($P< 0.05$). Soil samples were checked for the presence of other *Meloidogyne* species (**Supplementary Table S1**), and these were not present or at very low levels only. Therefore, we conclude that the observed difference in Meloidogynidae levels in **Figure 5** (based on rRNA data) can be attributed predominantly to the Columbian RKN *M. chitwoodi*.

Commonly used nematode extraction protocols exploit the mobility of nematodes to separate the roundworms from the soil matrix. Hence, non-active nematodes will not be extracted by these methods. Direct extraction of nematode DNA and RNA from soil does not include this selection step, and therefore we compared rRNA-based results (**Figure 5**) with results with rDNA (**Supplementary Figure S4**). Both figures show significant lower abundances of the monogeneric family Meloidogynidae in the organic fields. Hence, all three approaches, rDNA or rRNA-based sequencing and species-specific qPCR assays, demonstrate significantly lower RKN levels in Org fields.

DISCUSSION

Mapping of resident and active fractions of the primary decomposer community—bacteria and fungi—as well two major primary consumer groups—protists and nematodes—under three distinct soil management regimes revealed that pea exerts a large effect on the soil microbiome. Below we will discuss (1) how the current characterisation of the pea rhizobiome relates to other studies, (2) how our observations regarding the effect of soil management relate to previous findings, and (3) whether we can find plausible biological explanations for the observed sharp decline in RKN densities in fields under prolonged organic soil management.

How Does the Current Characterisation of the Pea Rhizobiome Relate to Previous Studies?

As exemplified by the impact of pea, lentil and chickpea, legumes have been shown to exert a large influence on the soil microbiome as compared to other crops such as cereals (Turner et al., 2013; Hamel et al., 2018). N rhizodeposition has been shown to comprise 13% of the total plant N for pea (Mayer et al., 2003), and presumably this has contributed to this large impact. The large overall effect of legumes could be corroborated by comparing the current study with a recent study on the barley rhizobiome that made use of the same experimental fields (Harkes et al., 2019). In case of barley, the compartment effect

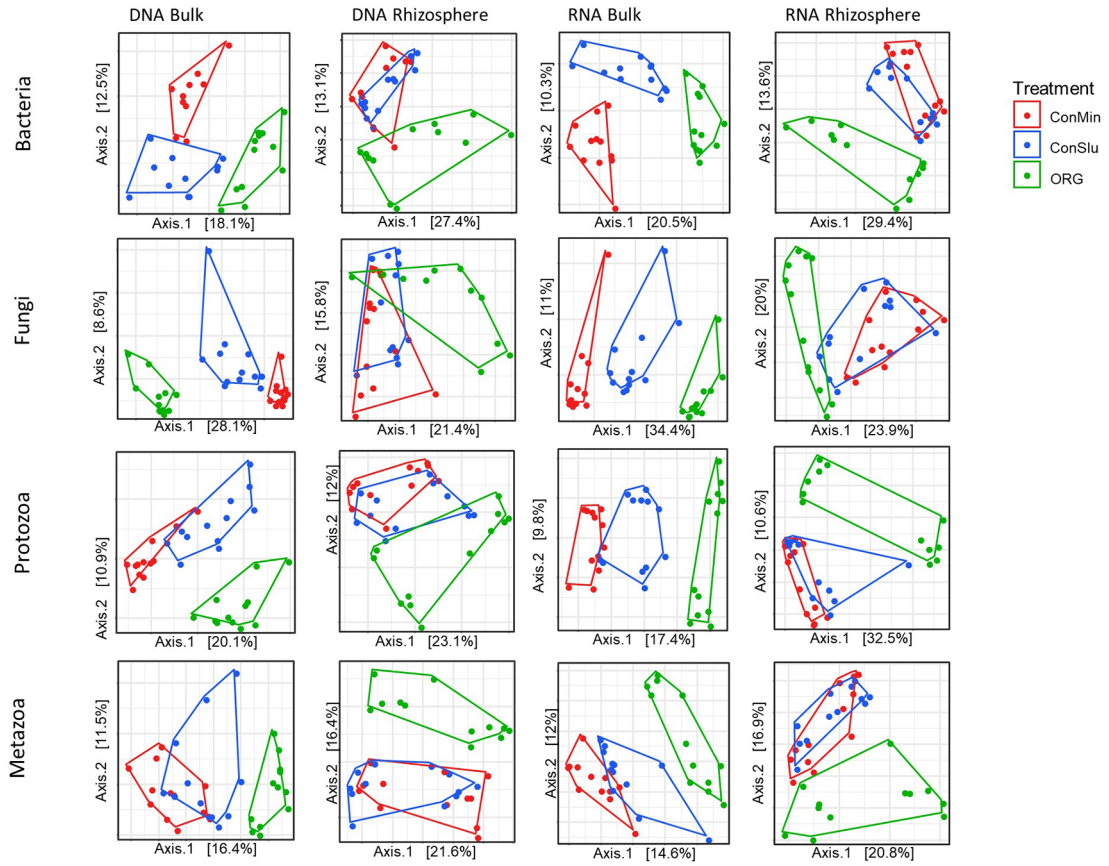

FIGURE 3 | Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix. Plots illustrate distances between communities for each of the organismal groups under three different soil management types [ConMin (red), ConSlu (blue), and Organic (green)]. Split plots per organismal group for DNA bulk, DNA rhizosphere, RNA bulk, and RNA rhizosphere.

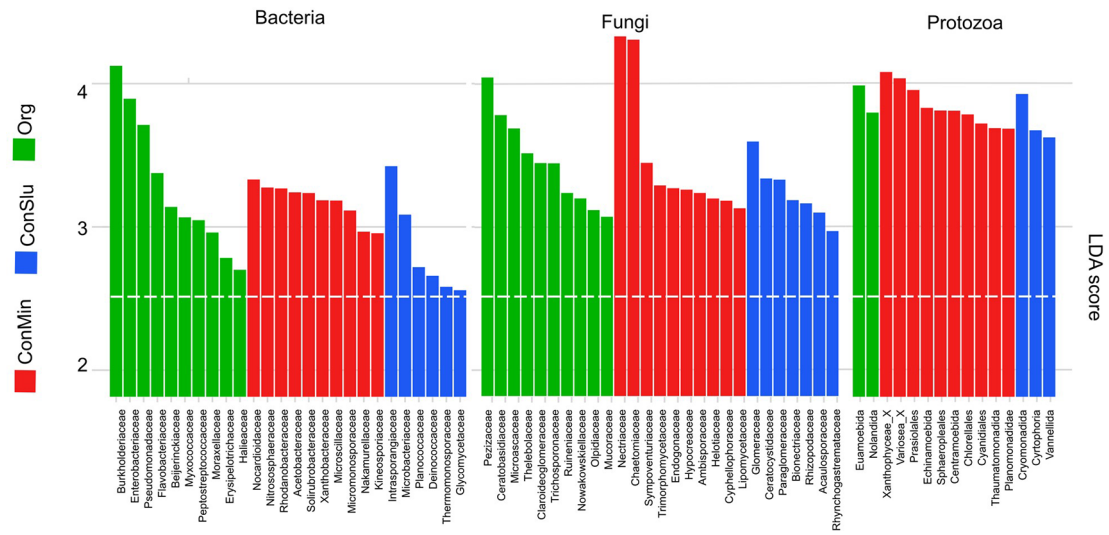

FIGURE 4 | Discriminant active bacterial, fungal, and protozoan taxa in the rhizosphere indicated by LEfSe analysis (LDA threshold of 2.5) resulting from distinct soil management types at location Vredepeel: ConMin (red), ConSlu (blue), and Org (green). LEfSe, linear discriminate analysis effect size; LDA, linear discriminate analysis.

TABLE 3 | Mean nematode densities and *M. chitwoodi* abundances (individuals per 100g soil) in the pea fields with three distinct soil management regimes.

	ConMin	ConSlu	Org
Nematode density (m)	3100	3210	2880
Nematode density (q)	1551	1622	1440
M. chitwoodi (q)	58[a]	65[a]	2[b]

Significant differences are indicated by lowercase a or b (Post-hoc Bonferroni P < 0.05). Samples were analyzed microscopically (m) or with quantitative PCR (q).

(bulk *vs* rhizosphere) explained a smaller percentage of the observed variation than impact of the soil management regime. For pea, on the contrary, all four organismal groups indicated the compartment effect to be larger than the soil treatment effect. Hence, under similar experimental conditions the compartment effect on the soil microbiome induced by pea (a legume) is stronger than the effect induced by barley (a cereal).

Recently, the effect of various frequencies pulse crop cultivation (including pea) on resident soil bacterial communities was mapped (Hamel et al., 2018). Increased frequency of pulse cultivation resulted in higher abundances of α-Proteobacteria in the rhizosphere, and a decrease in γ-Proteobacteria (although the latter was accompanied by an increased presence of *Pseudomonas*). Keeping in mind that the Rhizobiales (in our study Rhizobiaceae and Labraceae) belong to the subclass α-Proteobacteria, an overall increase of this subclass was to be anticipated. Moreover, Hamel et al. (2018) detected an increase *Pseudomonas* read in rotations involving pea. This might correspond to the increased activity of Pseudomonadaceae we observed in the pea rhizosphere (**Figure 2**, panel 1). We could not confirm the increased presence of Actinobacteria in the pea rhizosphere as observed in rotation systems with frequent inclusion of pulses (referred to as "3-pulse systems"). This phenomenon might only be observable after repeated cultivation of legumes.

We conclude that a number of parallels can be discerned between studies on the effect of pea on the rhizobiome. It is noted that differences in experimental approach (focus on resident or active soil biota) and set up (soil type, soil management practices) complicates the identification of generic effects of legumes on the soil living community.

How Does the Current Characterisation of the Effects of Soil Management on the Soil Microbiome Relate Other Studies?

In a long-term (>10 years) greenhouse experiment the effect of organic, integrated and conventional farming systems on the soil rhizobiome was investigated (Li et al., 2019). The authors identified a bacterial hub, a small number of highly interconnected taxa, consisting of *Bacillus* (Bacillaceae), *Sporosarcina* (Planococcaceae), *Hyphomicrobium* (Hyphomicrobiaceae), *Gaiella* (Gaiellaceae), as well as *Pirellula* and *Blastopirellula* (both Planctomycetaceae) that were significantly more abundant in soil from the organic management regime (Li et al., 2019). Another hub comprising of the genera *Rhizobium* (Rhizobiaceae), *Sphingobium* (Sphingomonadaceae), *Pseudoxanthomonas* (Xanthomonadaceae), and *Dyadobacter* (Cytophagaceae) was present in higher densities in the conventional and the integrated treatments. These findings show very little resemblance with the bacterial taxa that were shown to be activated under the organic or one of the two conventional soil managements systems in the present study (**Figure 4**). From this, we conclude that the plant effects can be stronger than the effect of soil management (variable "treatment" in **Tables 1** and **2**). Moreover, it is noted that the active bacterial community can be quite distinct from the resident bacterial communities mapped by Li et al. (2019) (**Figure 1**).

In another long-term field experiment (running for ≈ 15 years at time of sampling) fields were continuously exposed to either

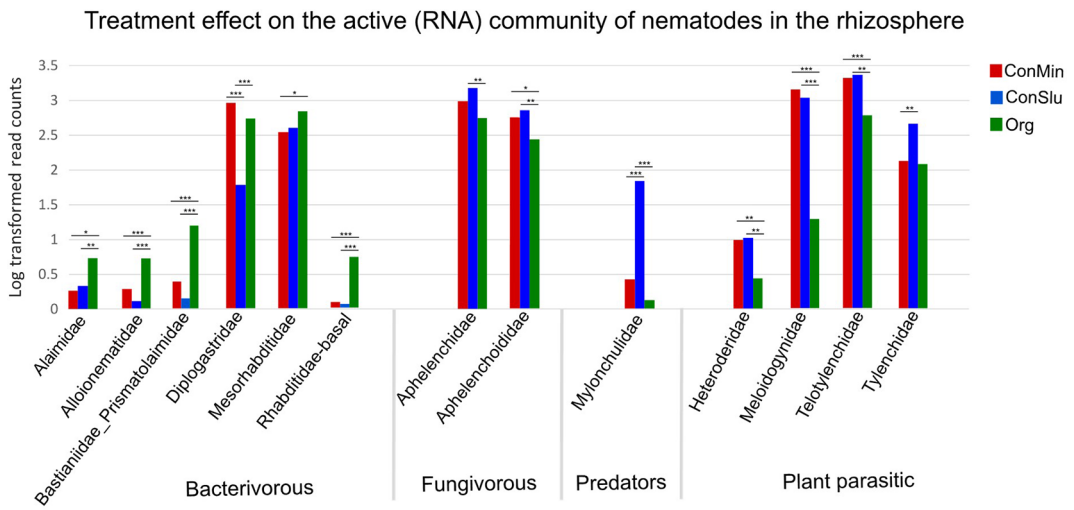

FIGURE 5 | Shifts in the active nematode communities in the rhizosphere for each of the three soil management regimes: ConMin (red), ConSlu (blue) and Org (green). Each bar represents the average of 36 data points (18 subplots were sampled at two time points). Kruskal-Wallis test, followed by Dunn's Test. (* = p < 0.05, ** = p <0.01, *** = p <0.001).

conventional or organic farming practices, and the impact of the practices on bulk soil were determined (Bakker et al., 2018). These authors showed a remarkable contrast between bacterial phyla with regard to the extent by which they were affected by the contrasting farming practices. More taxa showed higher abundances in organic as compared to conventional farming. Moreover, some bacterial phyla such as Chloroflexi, Firmicutes, and Gemmatimonadetes seemed to be unaffected by farming practice while others such as Proteobacteria, Acidobacteria, and Verrucomicrobia were. Our data only partly support this observation. The families Burkholderiaceae and Hyphomicrobiaceae (Proteobacteria) and the Peptostreptococcaceae (Firmicutes) were both more abundant and more active in bulk soil in organic fields (**Supplementary Figure S3**). Peptostreptococcaceae are one of the dominant family in the gut microbiome of earthworms (see *e.g.* Zeibich et al., 2018), and as such this result could point at an elevated presence of earthworms in fields under organic management. Our analysis of resident bacterial community in bulk soil under the organic regime, also showed an increase of members of the Acidobacterial family Blastocatellaceae (**Supplementary Figure S3**). In the most conventional soil management system (ConMin), the Verrucobacterial family Pedosphaeraceae was both abundant and highly activated. At high taxonomic level, this is in line with the observations presented by Bakker et al. (2018).

Hence, despite the fact that plant identity may have a stronger effect on the rhizobiome than soil management practices, the effect of these practices could be pinpointed at taxon level. Our data suggest that the working hypothesis saying that only a subset of the soil bacterial phyla is affected by conventional or organic soil management practices might be correct.

Can We Pinpoint Nematode-Suppressive Bacterial or Fungal Taxa That Might Underlie the *M. chitwoodi* Decline in Fields Under Organic Management?

In this study we investigated the soil microbiome of pea in fields naturally infested with *M. chitwoodi* under three different soil management regimes, conventional, integrated, and organic. *M. chitwoodi* is a highly polyphagous plant parasite infecting numerous mono- and dicotyledonous crops, including pea (*P. sativum*) (Oepp/Eppo, 1991), and it has a reputation as a major pest in potato. In all studied fields here, *M. chitwoodi* was already present for multiple years (Visser et al., 2014). As potato—a highly suitable host—was the main crop in the previous growing season, we expected the *M. chitwoodi* population to be physiologically fit at the onset of the pea growing season.

At the end of the growing season, *M. chitwoodi* densities in the two conventional soil management systems harbours ≈ 60 individuals per 100 g soil, whereas about two individuals per 100 g were detected in the organically managed system. We investigated whether a biological explanation could be found for this difference. Within the bacterial and fungal rhizosphere communities, families were detected that are known to harbor multiple nematode-trapping species. As shown in **Figure 2** (panel 3), the Orbiliaceae were shown to be active in the pea rhizosphere. This family comprises genera such as *Arthrobotrys*, *Dactylella*, and *Monacrosporium*. These genera are essentially saprophytic fungi but can become predatory under *e.g.* low nutrient conditions (Gray, 1987). As a response, the fungi will form traps (*e.g.* constricting rings, adhesive networks) which allow them to prey on nematodes (Xie et al., 2010). We verified whether Orbiliaceae activity was upregulated in the fields under organic management. This was not the case, and even a non-significant trend towards lower activity in organic fields was observed (**Supplementary Figure S2**). Elevated activity of another fungal family, the Olpidiaceae, typified fields under organic soil management. A member of this family, *Olpidium vermicola*, has been reported to parasitize eggs and females of endoparasitic nematodes such as *M. chitwoodi* (Esser and Schubert, 1983; Askary, 2015). However, other *Olpidium* species are virus-transmitting plant pathogens. Zoospores of *Olpidium virulentus* colonize roots of various plant species, and were demonstrated to accumulate in crop rotation with multiple pulses including pea (Niu et al., 2018).

The bacterial family Pseudomonadaceae was also identified as an indicator species for organic farming (**Figure 4**). Further analyses identified the genus *Pseudomonas* as main contributor to the indicator status of Pseudomonadaceae. The *Pseudomonas* species *P. aeruginosa, P. fluorescens, P. protegens, and P. chlororaphis* belong to ecologically most relevant nematode-suppressive bacteria in soil (Li et al., 2014). *Pseudomonas* species produce toxins which may inhibit hatching, survival and *M. chitwoodi*'s ability to penetrate plant roots (Thiyagarajan and Kuppusamy, 2014; Nandi et al., 2015; Kang et al., 2018).

The increased densities of bacterivorous nematode families might form an indirect explanation for the decrease of *M. chitwoodi* in organic soil management systems (**Figure 5**). As bacteria-grazing nematodes in the immediate vicinity of plant roots could locally improve nutrient availability *via* the excretion of easily uptakeable N and P. Plants could benefit from this in terms of improved growth and vitality, possibly making them less susceptible to plant-parasitic nematodes (Thoden et al., 2011).

Presumably multiple factors have contributed to significantly lower *M. chitwoodi* levels in the organic fields. This might have included nematode parasitic members of fungal genus *Olpidium* and/or the elevated activity of members of the Pseudomonadaceae. These results should be seen as potential leads for more detailed studies on the effect soil management regimes on the activity levels of nematode-suppressive bacteria and/or fungi.

Concluding Remarks

The development of a time-efficient and affordable protocol to extract total DNA and RNA from soil (Harkes et al., 2019) allowed us to monitor the effect of a legume, pea, on both resident and active communities of primary decomposers as well as primary consumers of bacterial and fungal assemblages. Pea was shown to exert a large effect on the rhizobiome, and this was not only true for the primary decomposers but also for the protist and metazoan community. For all four organismal groups, and irrespective of the algorithm used to assess community shifts, the variables "Nucleic Acid" and "Sample

Type"—representing respectively the differences between the resident and the active communities, and the effect of pea on the rhizobiome—had the highest impact on the soil microbiome. Notwithstanding this conclusion, soil management ("Treatment") had also a significant effect on both the primary decomposers and the two primary consumer groups. A number of taxonomic groups (mostly at family level) were identified as contributors to these contrasts. In some cases, these taxa could be linked to treatment or crop identity, but in other cases such families were highly speciose or barely characterized from a soil ecological point of view. In essence, this was also true regarding our efforts to find possible biological explanations for the remarkably low levels of the RKN *M. chitwoodi* under the prolonged organic management regime. Our data suggest that Pseudomonadaceae—here members of the genus *Pseudomonas*—could have played a role in the biological suppression of this notorious RKN species. It should be underlined that biological associations have been identified in this research, and it was by no means proven that one or more *Pseudomonas* species were actually responsible for the observed decline in RKN levels in fields under organic soil management.

In this study a broad approach was used to characterize shifts in the soil microbial community under various soil management regimes with a legume—pea—as main crop. In our analyses we mainly focused on the active fractions, and this allowed us to pinpoint target taxa associated with the various treatments for each of the four organismal groups. One of the main remaining hurdles for the fundamental understanding of shifts in soil microbial communities is our fragmented and often poor knowledge about the ecologies of soil inhabitants.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the: The raw sequences were submitted to the NCBI Sequence Read Archive (SRA) database under study accession numbers PRJNA561075 for bacteria, and PRJNA561072 for fungi, protozoa and metazoa.

AUTHOR CONTRIBUTIONS

PH, AS, JHa and JHe were responsible for the experimental design. PH, SE, AS, and MH were involved in sampling the rhizosphere. PH performed RNA/DNA isolation of the soil samples. PH performed the two step PCR reactions in order to prepare the sequence library. MH sorted the sequence data and analysed the nematode sequences. JS analysed the sequence data and performed the bioinformatics and statistical analysis. PH performed the statistical analyses on the nematode sequence data. PH and JHe wrote the manuscript; all others co-commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01697/full#supplementary-material

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Parallel Microbial Ecology of *Pasteuria* and Nematode Species in Scottish Soils

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Pasteuria spp. are endospore forming bacteria which act as natural antagonists to many of the most economically significant plant parasitic nematodes (PPNs). Highly species-specific nematode suppression may be observed in soils containing a sufficiently high density of *Pasteuria* spp. spores. This suppression is enacted by the bacteria *via* inhibition of root invasion and sterilization of the nematode host. Molecular methods for the detection of *Pasteuria* spp. from environmental DNA (eDNA) have been described; however, these methods are limited in both scale and in depth. We report the use of small subunit rRNA gene metabarcoding to profile *Pasteuria* spp. and nematode communities in parallel. We have investigated *Pasteuria* spp. population structure in Scottish soils using eDNA from two sources: soil extracted DNA from the second National Soil Inventory of Scotland (NSIS2); and nematode extracted DNA collected from farms in the East Scotland Farm Network (ESFN). We compared the *Pasteuria* spp. community culture to both nematode community structure and the physiochemical properties of soils. Our results indicate that *Pasteuria* spp. populations in Scottish soils are broadly dominated by two sequence variants. The first of these aligns with high identity to *Pasteuria hartismeri*, a species first described parasitizing *Meloidogyne ardenensis,* a nematode parasite of woody and perennial plants in northern Europe. The second aligns with a *Pasteuria*-like sequence which was first recovered from a farm near Edinburgh which was found to contain bacterial feeding nematodes and *Pratylenchus* spp. encumbered by *Pasteuria* spp. endospores. Further, soil carbon, moisture, bulk density, and pH showed a strong correlation with the *Pasteuria* spp. community composition. These results indicate that metabarcoding is appropriate for the sensitive, specific, and semi-quantitative profiling of *Pasteuria* species from eDNA.

Keywords: metabarcoding, nematode, *Pasteuria*, biocontrol, ecology

INTRODUCTION

Plant parasitic nematodes (PPNs) pose a major threat to global food security, with estimated combined crop losses due to PPNs equating to $80 billion USD each year (Nicol et al., 2011; Jones et al., 2013). However, broad spectrum chemical nematicides and soil fumigants which have been effective in PPN control are being steadily withdrawn from the market due to environmental concerns, such as ozone depletion by methyl bromide (Ristaino and Thomas, 1997), or their potential for negative effects on human health, such as the 1985 Aldicarb poisoning outbreak in the United States and Canada (Goldman et al., 1990). The withdrawal of these substances necessitates the development of sustainable alternatives to chemical PPN control. These alternatives may be: management practices, such as solarization (McGovern and Mcsorley, 1997; Freitas et al., 2000; Wang et al., 2006), or incorporation of organic amendments (Stirling et al., 2003; Jaffee, 2004; Walker, 2004; Bonanomi et al., 2007); breeding and growth of resistant cultivars, such as potato varieties which carry the *H1* resistance gene, preventing the development of *Globodera rostochiensis via* starvation of the infective juvenile stages within the root (Kort et al., 1977; Rice et al., 1985; Sobczak et al., 2005); and the incorporation or cultivation of PPN biocontrol agents (BCAs) in cropping soils, such as the nematophagous fungus *Pochonia chlamydosporia*, which parasitizes the eggs of some of the most impactful PPN species (Kerry et al., 1982; Yang et al., 2012; Manzanilla-López et al., 2013). Among BCAs specialist nematode parasites are the most effective as generalist nematode predators do not respond to large increases in PPN populations providing only a background level of biocontrol which may not be easily quantified (Stirling, 2014). Among the most specialized PPN BCAs are obligate hyperparasitic bacteria of the genus *Pasteuria*.

Pasteuria spp. are gram positive, endospore forming Firmicutes which suppress PPNs *via* two mechanisms. First, *Pasteuria* spp. endospores attach to the surface of the nematode hindering directional movement and, by extension, root access (Davies et al., 1991; Vagelas et al., 2012). Second, upon penetration of the nematode cuticle and colonization of the pseudocoelom, *Pasteuria* spp. are able to alter embryogenesis, sterilizing the host (Davies et al., 2011). *Pasteuria* spp. may be highly fastidious parasites, exhibiting host specificity which can be species or population specific (Davies et al., 2001; Davies et al., 2008; Duneau et al., 2011; Mohan et al., 2012). Cross generic attachment profiles have been described in *Pasteuria* spp. which are capable of attachment to both the pigeon pea cyst nematode (*Heterodera cajani*) and a potato cyst nematode (*G. pallida*) (Mohan et al., 2012), however these are both members of the Heteroderinae with similar life cycles. Specificity of *Pasteuria* spp. presents an advantage over broad spectrum chemical control and less targeted management practices such as soil solarization which may remove ecosystem services that are mediated by beneficial organisms, including BCAs (Wang et al., 2006). However, this host specificity also presents a challenge to the use of *Pasteuria* spp. as inundative or inoculative BCAs, as the interaction of a strain with a native PPN population cannot be easily predicted without prior testing. Inoculative and conservation biocontrol using *Pasteuria* spp. is hindered by a limited understanding of the impacts of soil properties and management practices on *Pasteuria* spp. populations.

oil characteristics, such as clay and organic matter content have been noted as a driver of *Pasteuria* biology (Dabiré and Mateille, 2004; Dabiré et al., 2007). Spores are non-motile and so require a degree of porosity in the soil in order to disperse and to come into contact with the nematode cuticle, allowing attachment and infection (Dabiré and Mateille, 2004). *Pasteuria* spp. endospores are robust, exhibiting resistance to extremes of temperature, and desiccation (Williams et al., 1989). However, they can be lost from the soil *via* leaching (Dabiré and Mateille, 2004; Cetintas and Dickson, 2005; Luc et al., 2010). Trudgill et al. (2000) reported attachment of *P. penetrans* was favored by decreasing coarse sand and increasing clay content in Senegal but decreasing clay and organic matter content in Burkina Faso, with no such observable environmental effects on populations from Ecuador. However, due to reduced porosity and the ability of spores to bind to colloids, the presence of clay has been shown to improve retention of spores in the upper soil profile (Dabiré et al., 2007). The vast majority of *Pasteuria* spp. ecology research to date has examined a single species, *Pasteuria penetrans*. This is with good reason as *P. penetrans* is a parasite of the most significantly damaging PPNs globally, the tropical apomictic root knot nematodes (RKN, *Meloidogyne* spp.) (Davies et al., 2011; Jones et al., 2013). Some variation between populations of this species is observable as noted above with regard to the impact of soil clay content on retention of endospores (Trudgill et al., 2000). Other factors are more consistent, for example rate of development of *P. penetrans* has been shown to increase linearly between 18° and 27°C in multiple studies (Giannakou et al., 1997; Serracin et al., 1997; Lopes et al., 2018). However, it is possible that focus on this species obscures a greater diversity of endospore properties and environmental interactions within the genus given the high diversity and global distribution of *Pasteuria* species (Chen and Dickson, 1998). For example, despite the apparent negative impact of leaching on *P. penetrans* endospore retention, Costa et al. (2006) were readily able to recover *Pasteuria* spp. directly from sandy soils in temperate dunes. *Pasteuria hartismeri,* a parasite of temperate RKN species (Bishop et al., 2007), may be expected to be better suited to the lower temperatures within its distribution. Understanding the diversity, or lack thereof, of the relationships between *Pasteuria* spp. and environmental characteristics may therefore be critical to its effective deployment as a BCA.

Morphological diversity within *Pasteuria* spp. endospores manifests in differences in the shapes of the sporangial walls resultant from the arrangement of parasporal fibers (Starr and Sayre, 1988). For example, *P. ramosa,* which parasitizes the water flea (*Daphnia magna*) forms a characteristic "tear-drop" shape, whereas *P. penetrans* possesses a distinctive "flying saucer" or "fried egg" shape resulting from an inferiorly collapsed sporangia

forming a concave cup like surface on the underside of the endospore (Starr and Sayre, 1988). The classical "flying saucer" shape of *P. penetrans* is common to a number of species which parasitize PPNs including *P. hartismeri* (Bishop et al., 2007) and *P. nishizawae*, which parasitizes the soybean cyst nematode (*H. glycines*) (Noel et al., 2005). *Pasteuria thornei,* which parasitizes semi-endoparasitic root lesion nematodes (*Pratylenchus* spp.) maintains a rigid rhomboidal structure (Starr and Sayre, 1988). Morphological characterization is, however, insufficient to identify most *Pasteuria* species and is therefore inadequate to predict interactions with the host. Molecular detection and characterization of *Pasteuria* spp. has been demonstrated using a variety of housekeeping genes, such as *GyrB* and *SigE*, (Schmidt et al., 2004; Nong et al., 2007; Mauchline et al., 2010; Mauchline et al., 2011) and the 16S rRNA gene (Duan et al., 2003; Atibalentja et al., 2004; Rao et al., 2012). Detection of single *Pasteuria* endospores was reported by Mauchline et al. (2010) using a commercial enzymatic digest followed by multiple strand amplification. PCR based detection has also been tested both in planta (Atibalentja et al., 2000; Schmidt et al., 2004; Rao et al., 2012) and in soils known to contain *Pasteuria* spp. (Duan et al., 2003). While these methods allow the molecular characterization of *Pasteuria* spp. populations and have provided the beginnings of a species 16S gene reference database, they are limited in both scale and depth. PCR and Sanger sequencing of individual samples with *Pasteuria* specific primers allows for few samples to be processed at any one time and, in complex populations, this method is unlikely to provide adequate sequencing depth to accurately characterize population diversity. We have developed a high throughput, high resolution method of determining *Pasteuria* spp. population structure which builds on existing molecular detection methods to increase their scale and depth. Here we demonstrate the utility of this method to assess the distribution and variation in *Pasteuria* spp. community structure in a range of Scottish soils and how such variation relates to the physical properties of the soils they inhabit. Further, we demonstrate that this approach can be combined with recent advances in high throughput nematode community profiling (Porazinska et al., 2009; Porazinska et al., 2010; Morise et al., 2012; Porazinska et al., 2012; Treonis et al., 2018; Waeyenberge et al., 2019) to assess the relationships that *Pasteuria* spp. may have on nematode community structure in agricultural soils. We describe significant species level variation in relationships with soil properties. The methodology described herein provides a framework which may be used or improved to study *Pasteuria* spp. ecology at scale and depth.

MATERIALS AND METHODS

Environmental DNA Samples

East of Scotland Farm Network

Nematode communities were extracted from soils in the 2014 re-sampling of the East of Scotland Farm Network (ESFN) (Hawes et al., 2010) (n = 560) using a modified Baermann funnel extraction method as described by Brown and Boag (1988) with 200 g of soil used for each extraction (Wiesel et al., 2015). Extracted nematodes were lyophilized before DNA extraction with a PureLink® Pro 96 well Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The concentration of extracted DNA from ESFN samples was not measured.

National Soil Inventory Scotland 2

The National Soil Inventory Survey 2 (NSIS2) comprises 406 soil samples collected at 195 sites between 2007 and 2010 (Lilly et al., 2011). Pits, one meter in depth, were dug at 20 km grid intervals across the Scottish Isles and mainland and soil samples were collected for each horizon. Satellite soil samples were taken at fixed distances from selected main sample pits. DNA was extracted from 0.25 g of each soil sample, quantified using Quant-iT PicoGreen dsDNA assay kit (ThermoFisher), and stored at −80°C. Extensive soil and environmental metadata was collected corresponding to each site (Lilly et al., 2011). One hundred thirty archived NSIS2 DNA samples were removed from storage at −80°C for inclusion in our analysis. These samples were selected based on the following criteria: only upper soil horizons were included; satellite samples, 4–16 m from the main sample pit, were selected where available allowing assessment of variation at a site; and 11 NSIS2 sample sites, indicated to contain *Pasteuria*-like sequences based on shallow non-specific 16S rRNA gene sequence analysis of the NSIS2 dataset (Freitag, unpublished data), were included in order to characterize populations at these sites. Each NSIS2 sample was diluted to a concentration of 10 ng μl^{-1} in high performance liquid chromatography (HPLC) grade water before amplification.

Pasteuria 16S rRNA Gene Copy Number Detection Limits

To determine an approximation of the sensitivity of our *Pasteuria* spp. PCRs, serial dilutions of *Pasteuria* spp. plasmids carrying cloned 16S rRNA gene PCR product were prepared. 16S rRNA gene sequences were amplified from *P. penetrans* genomic DNA and ESFN DNA. 16S rRNA gene product was amplified using primers 39F and 1166R (**Table 1**) as previously described (Mauchline et al., 2010) and ligated into pGEM-T easy plasmid (Promega) following the manufacturers protocol. Plasmids were used to transform electrocompetent DH5α cells from which individual clone colonies were cultured and plasmid extracted using the QIAprep spin kit (QIAGEN). The sequence of clone

TABLE 1 | Primers used in this study.

Primer	Sequence (5'-3')	Target gene	Reference
39F	GCGGCGTGCCTAATACA	16S rRNA	Atibalentja et al., 2000
1166R	CGCCGGCTGTCTCTCCAA	16S rRNA	Duan et al., 2003
PAS776F	CAGCATCTTTGTGCCGAAGG	16S rRNA	This Study
NF1	GGTGGTGCATGGCCGTTCTTAGTT	18S rRNA	Mullin et al., 2003
18Sr2b	TACAAAGGGCAGGGACGTAAT	18S rRNA	Mullin et al., 2003

plasmids was confirmed with Sanger sequencing using generic M13F and M13R primers. Plasmids were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The copy number of plasmid template μl^{-1} was calculated from the combined length of vector and insert and the concentration of each plasmid suspension (ng μl^{-1}) using the formula:

$$copy\ number = \frac{dsDNA\ mass\ (ng) \times (6.022 \times 10^{23})}{size\ of\ plasmid\ (bp) \times (650 \times 10^{9})}$$

To assess approximate gene copy number detection limits dilutions were prepared of *P. penetrans* and *P. hartismeri* 16S rRNA gene plasmid stocks to a concentration which was calculated to corresponded to ~10 million copies of each plasmid. This stock was then used to produce six replicate serial dilution series of each plasmid from an initial estimate of 10 million copies to a theoretical single copy of target sequence μl^{-1} of plasmid suspension. One µl of each plasmid dilution series was included as template in PCR reactions alongside NSIS2 and ESFN samples in *Pasteuria* PCR reactions as below and included in the final PCR product pool prepared for sequencing.

Amplification Strategy

Pasteuria spp. Primer Design

All primer sequences used in this study are presented in **Table 1**. Primers for the specific amplification of the *Pasteuria* 16S rRNA gene were designed based on published 16S rRNA gene data. All *Pasteuria* spp. 16S rRNA gene sequences (n = 272) were downloaded from the non-redundant nucleotide archive. These sequences were aligned using MAFFT (v7.407) (Katoh and Standley, 2013) and trimmed using a python script (this study) to those sequences containing 39F and 1166R primer sites allowing for a mismatch of up to 3 nt (n = 54) which excluded shorter sequence fragments. The remaining sequences were used as input in Primer-BLAST (Ye et al., 2012) as target sequences with primer 1166R as the input reverse primer and a maximum PCR product size of 500 bp. Primer-BLAST was then used to predict non-target amplification of candidate forward primer-1166R pairs vs the non-redundant nucleotide archive allowing up to six mismatches in total within the primer pair (Ye et al., 2012). The primer pair PAS776F (this study) and 1166R (Duan et al., 2003), generating a 333 bp fragment showed no *in silico* predicted non-target amplification. The reference set was again trimmed to the region between primers PAS776F and 1166R using python (this study) and aligned using MAFFT (v7.407) (Katoh and Standley, 2013) with the addition of 16S rRNA gene sequences for *P. ramosa*, which does not carry the primer binding sites, and *Thermoactinomyces daqus,* a related Firmicute (Ludwig et al., 2009). Using this alignment, it was determined that all previously described species were distinguishable by at least two bp in the target region. Identical sequences, and sequences unassigned to a particular species or host, were removed from the reference set leaving 16 representative sequences: *T. daqus*, (n = 1), *P. ramosa* (n = 1), *P. penetrans* (n = 3), *P. hartismeri* (n = 2), *P. nishizawae* (n = 2), *P. goettingianae* (n = 1), *P. usgae* (n = 1), *P. aldrichii* (n = 1), Plectid infective *Pasteuria* spp. isolates (n = 3), and *Pasteuria* HcP (n = 1). In addition, *Pasteuria*-like sequences aligning with 97% similarity to *P. hartismeri* were added which we have called here *Pasteuria* Luffness (n = 2) for the location of their initial recovery bringing the total reference set to 18 sequences. These sequences were used as the reference database for taxonomic assignment of *Pasteuria* spp. amplicons. The pairwise percentage identity of reference *Pasteuria* spp. sequences in the aligned region of the 16S rRNA gene ranged from a low of 84.7% (*P. ramosa* vs *P. usgae*) to a high of 99.3% (*P. penetrans* to *Pasteuria* HcP) with an average of 95% identity between all reference sequences. Maximum likelihood phylogeny was inferred from aligned reference sequences using IQ-TREE (v1.6.9) (Nguyen et al., 2014)

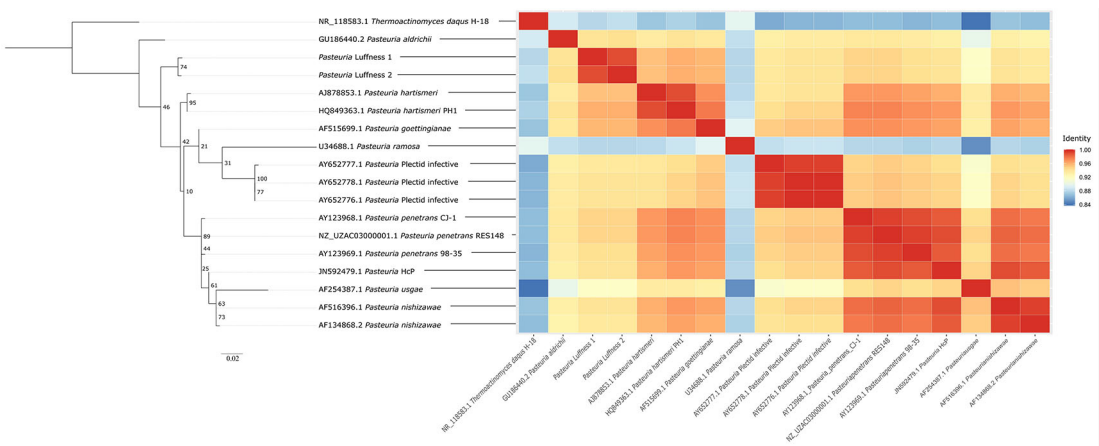

FIGURE 1 | Maximum likelihood phylogeny and pairwise sequence identity heatmap of *Pasteuria* spp. taxonomic reference sequences used in primer design and *Pasteuria* spp. ZOTU taxonomic assignment.

with bootstrapping (n = 100), and graphically represented in FigTree (v1.4.3) (Rambaut, 2012) (**Figure 1**). The pairwise similarity of reference sequences was plotted as a heatmap (**Figure 1**) using ggplot2 (Wickham, 2009).

Primer Barcode Design

A pairwise in-line primer barcoding strategy was devised to index PCR products. Barcode sequences (6 nt) were designed based on a Levenshtein distance of three using the EditTag python package (Faircloth and Glenn, 2012). These were trimmed to 32 tags (1024 pairwise combinations) based on minimizing penalty scores generated using the same software. Two adenine bases were appended to the 5′ end of each barcode to account for base loss at the beginning of a sequencing read. Pairwise combinations of barcoded primers were achieved by diluting the stocks from 100 µM to 0.6 µM and then adding 2.5 µl of each primer to a respective reaction, each individual primer in 32 unique reactions.

Amplification

PCR amplification of *Pasteuria* spp. 16S rRNA gene sequence was carried out using a semi-nested approach using 1 µl of ESFN, NSIS2, or plasmid dilution series DNA as template. Large fragment (1110 bp) 16S rRNA gene products were amplified using primers 39F (Atibalentja et al., 2000) and 1166R. This product was then diluted 1 in 10 and used as template for short, barcoded inner nest PCR that used primers PAS776F and 1166R. Outer nest PCR conditions were 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR conditions for *Pasteuria* inner nest reactions included an initial denaturation of 94°C for 5 minutes, followed by 15 cycles of 94°C for 15 seconds, 70°C for 20 seconds, and 72°C for 20 seconds, with a final extension at 72°C for 1 minute.

18S rRNA gene sequences were amplified from ESFN DNA samples using primers NF1 and 18Sr2b (Mullin et al., 2003; Porazinska et al., 2009). PCR conditions were 94°C for 5 minute, followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minute.

All reactions (20 µl) were carried out with Q5 High-Fidelity DNA Polymerase (NEB) with 1 µl of DNA as template. A high-fidelity polymerase with 3–5′ exonuclease activity was used in all PCR reactions to minimize artificial sequence variation generated by PCR errors.

Library Preparation From PCR Products

Each short PCR product (4 µl) was electrophoresed on a 2% agarose gel. Gels were visualized for bands of the expected size and each band was assigned a score from 1 to 4 based on its relative brightness: 1, not visible; 2, barely visible; 3, clearly visible; and 4, very bright. This scoring was used to approximately normalize PCR product concentrations, to avoid overrepresentation of concentrated reactions, as follows: 10 µl (scoring 1 or 2); 5 µl (scoring 3); and 1 µl (scoring 4) of products added to the final product pool. All products from PAS776 and 1166R PCRs of NSIS2, ESFN, and control DNA samples were pooled; all products from NF1 and 18Sr2b PCRs of ESFN DNA were pooled separately. Once pooled, products were concentrated by overnight incubation at −80°C with the addition of 1 volume of isopropanol, 0.2 volumes of 3M sodium acetate, and 20 ng of glycogen, followed by centrifugation at 10,600 g for 15 minutes at 4°C to pellet DNA. The supernatant was discarded, the pellet washed in 70% ice cold ethanol and allowed to air dry. Dried pellets were re-suspended in 105 µl of HPLC water. Pooled, concentrated PCR products were subjected to size selection using the MagJET NGS Cleanup and Size Selection Kit (Thermo Fisher Scientific). This size selection was carried out to exclude primers and, in the case of nested *Pasteuria* spp. 16s rRNA gene PCRs, to exclude outer nest PCR products. Binding buffer (400 µl) was used with both initial and final bead binding incubations which were extended to 15 minute, and intermediate binding was kept at 2 minutes to maximize product recovery. PCR product pools were combined and prepared for sequencing using the TruSeq PCR-Free Library Preparation Kit (Illumina), omitting shearing. The TruSeq PCR-Free Library Preparation Kit was selected to minimize sequence errors introduced by amplification. Sequencing was carried out at Edinburgh Genomics on Illumina's MiSeq platform with 300 base paired end reads.

Read Trimming and Overlap Merging

Initial sequence quality was assessed using FastQC v0.11.3 (Andrews, 2010). Trimmomatic v0.33 (Bolger et al., 2014) was used to trim reads with a minimum Phred quality score of 22 and a minimum length of 150 bp. Trimmomatic parameters were determined by incremental reduction in quality score cut-offs until >75% of all paired reads could be merged. PEAR v0.9.6 (Zhang et al., 2014) was used to merge quality trimmed paired end reads. Primer sequences were used to separate merged read pairs into amplification target groups, using primer regular expression matching with an acceptable ambiguity of 1 nt, and their orientation was then corrected using Python (this study). VSEARCH v2.1.1 (Rognes et al., 2016) was used to filter merged read pairs for a maximum number of expected base calling errors of less than 2 (Edgar and Flyvbjerg, 2015). Merged read pairs were binned in respective sample FASTQ files based on in-line primer barcode sequences, trimmed to exclude sequence up to and including primer sequences, to confine sequences to variable regions, then renamed to meet downstream requirements using Python (this study).

UNOISE3 from USEARCH v.10.0.240 (Edgar, 2013; Edgar, 2016) was used to generate zero radius operational taxonomic units (ZOTUs). ZOTU is a term specific to analysis with UNOISE referring to operational taxonomic units (OTUs) which are generated by an error correction algorithm as opposed to a sequence similarity clustering algorithm (Edgar, 2016). Raw merged read pairs were mapped back to ZOTUs using the otutab command in USEARCH.

Taxonomic Assignment

Taxonomy was assigned to ZOTUs using UCLUST (Edgar, 2010) *via* assign-taxonomy.py in QIIME v1.91 (Caporaso et al., 2010) to cluster ZOTU sequences iteratively between 100% and

90% identity to our curated *Pasteuria* spp. reference database for 16S rRNA gene products and to the SILVA-132 database (Quast et al., 2012) for the 18S rRNA gene products. All reference sequences were trimmed to the regions between the primer sequences allowing for a maximum mismatch of 1nt in each primer sequence. Iterative best hit assignment was used as each sequence being assigned taxonomy was a single representative of a putatively real biological sequence. The percentage match of each taxonomic assignment was appended to ZOTU identifiers as an integer between 0.9 and 1.0 (Quast et al., 2012). Iterative taxonomy tables were combined into a single table containing the best available match for each ZOTU sequence using Python (this study). Thereafter, ZOTU tables were combined with sample metadata and best taxonomic assignments using R scripts (this study).

ZOTU Table Quality Control

ZOTU tables with metadata were filtered for total ZOTU abundance (> 10) across the entire dataset to eliminate uninformative, low abundance ZOTUs. 18S rRNA gene products were also filtered for unwanted taxa to exclude ZOTUs outside of Nematoda. The number of reads generated for each sample was compared against the PCR band score to determine that observed amplification matched with the abundance of assembled products. Comparisons of these two independent estimates of PCR product mass were used to assess the likelihood of errors in the barcode sorting pipeline, pipetting of barcoded primers, sample storage, and cross contamination of barcode primer stocks. Samples which showed sustained mismatch between band scoring and merged paired read counts were removed.

Ordination and Statistical Analysis

Pasteuria spp. communities in the NSIS2 samples and nematode communities in the ESFN samples were ordinated using non-metric multidimensional scaling (NMDS) using Vegan (Dixon, 2003). ESFN samples which did not produce visible metazoan PCR product were excluded as uninformative. Soil metadata and ZOTU abundance were then fitted to these ordinations using Vegan's envfit. P values were adjusted for the false discovery rate using the Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995).

Pasteuria-Nematode ZOTU Interactions

Pasteuria and nematode ZOTU correlation was computed as the Spearman's rank correlation between each pairwise combination of ZOTUs. This was computed in R using a slightly modified version of a script described by Williams et al. (2014) designed to determine microbial co-occurrence patterns. P values were adjusted for the false discovery rate using the Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995).

Nematode Recovery

Recovery of nematodes from ESFN soils which returned a large 18S rRNA gene fragment PCR product was carried out to attempt to confirm the presence of *Pasteuria* endospores in soils which returned *Pasteuria*-like PCR products. This was done using a high density sucrose flotation method designed to optimize recovery of dense endospore filled nematodes (Hewlett et al., 1997). Two hundred grams of ESFN soils were suspended in 600 ml of tap water and the slurry was shaken vigorously for a period of 2 minutes then passed through a bank of sieves (250, 90, 25 μm). The retentate from the 25 μm sieve was re-suspended in approximately 30 ml of sterile distilled water and this was spun at 420 g for 5 minutes to pellet nematodes. The supernatant was discarded, and the pellet suspended in approximately 30 ml of sterile sucrose solution with an approximate density of 1.28 g ml^{-1}. This was spun again at 420 g for 1 minute and the supernatant passed through a 25 μm sieve. The retentate from this sieve was re-suspended in approximately 20 ml of SDW and 4 x 5 ml observed in a counting dish for the presence of endospores with an inverted microscope (Hund Wilovert®) at 50 and 200X magnification.

Immunofluorescence

Immunofluorescent labeling of *P. penetrans* spores on live nematodes recovered from soils was carried out using a previously described polyclonal antibody (Costa et al., 2006). A 1:1000 dilution of antibody was added to an equal volume of sterile distilled water containing the recovered nematode. This was left for 1 hour at room temperature or at 4°C overnight before being washed by successive transfer of individual nematodes to 5 ml of sterile distilled water. The resultant solution was then incubated with Goat anti-rabbit IgG bound to red fluorescent dye CyC at 4°C overnight. This was washed of unbound antibody as before and viewed under RFP fluorescent microscopy at 200 and 1,000x magnification with a Zeiss Axiosop microscope.

RESULTS

PCR

Of 560 ESFN nematode DNA samples 266 (47.5%) and 122 (21.8%) score of 2 or higher with metazoan and *Pasteuria* spp. primers, respectively. Considering ESFN samples which did not amplify as failed DNA extractions or PCR reactions, 45.9% of successful ESFN samples returned *Pasteuria* spp. PCR product. Of 144 NSIS2 soil DNA samples, 56 (38.9%) score 2 or higher using *Pasteuria* spp. PCR primers.

Size Selection

Extension of initial and final bead binding incubations, to the exclusion of primers and shorter products, and reduction of the intermediate binding, to the exclusion of larger PCR products, increased the efficiency of PCR product recovery from just 6% to 35% with an approximate input of 5 μg.

Sequence Pipeline

Out of a total of 8,945,443 raw paired end reads, 77.8% remained after trimming; of these 98.7% were merged. Merged read pairs produced 1,181,554 *Pasteuria* spp. 16S rRNA gene sequences; 2,100,892 metazoan 18S rRNA gene sequences; and 183,079

matching no primer sequence. Ninety-nine percent of the metazoan and 92% of the *Pasteuria* spp. merged read pairs remained after expected error and barcode sorting. Four percent of the *Pasteuria* spp. and 16% of the metazoan merged read pairs were unique sequences.

Nematode Primer Specificity

Nematode ZOTUs account for 72% of all ZOTUs and 85% of all merged read pairs within the ESFN 18S rRNA gene sample set. Annelid worms comprised 12% of the assembled read pairs and 4% of ZOTUs in this set. Fungi, Oomycetes, and Alveolata each comprised 1% of remaining assembled read pairs.

Pasteuria Detection Limits

Amplification and agarose gel electrophoresis demonstrated a typical detection limit of approximately 1000 target gene copies. Sequencing results largely reflected this with consistent detection of *P. penetrans* and *P. hartismeri* target sequences at 1,000 target copies and above

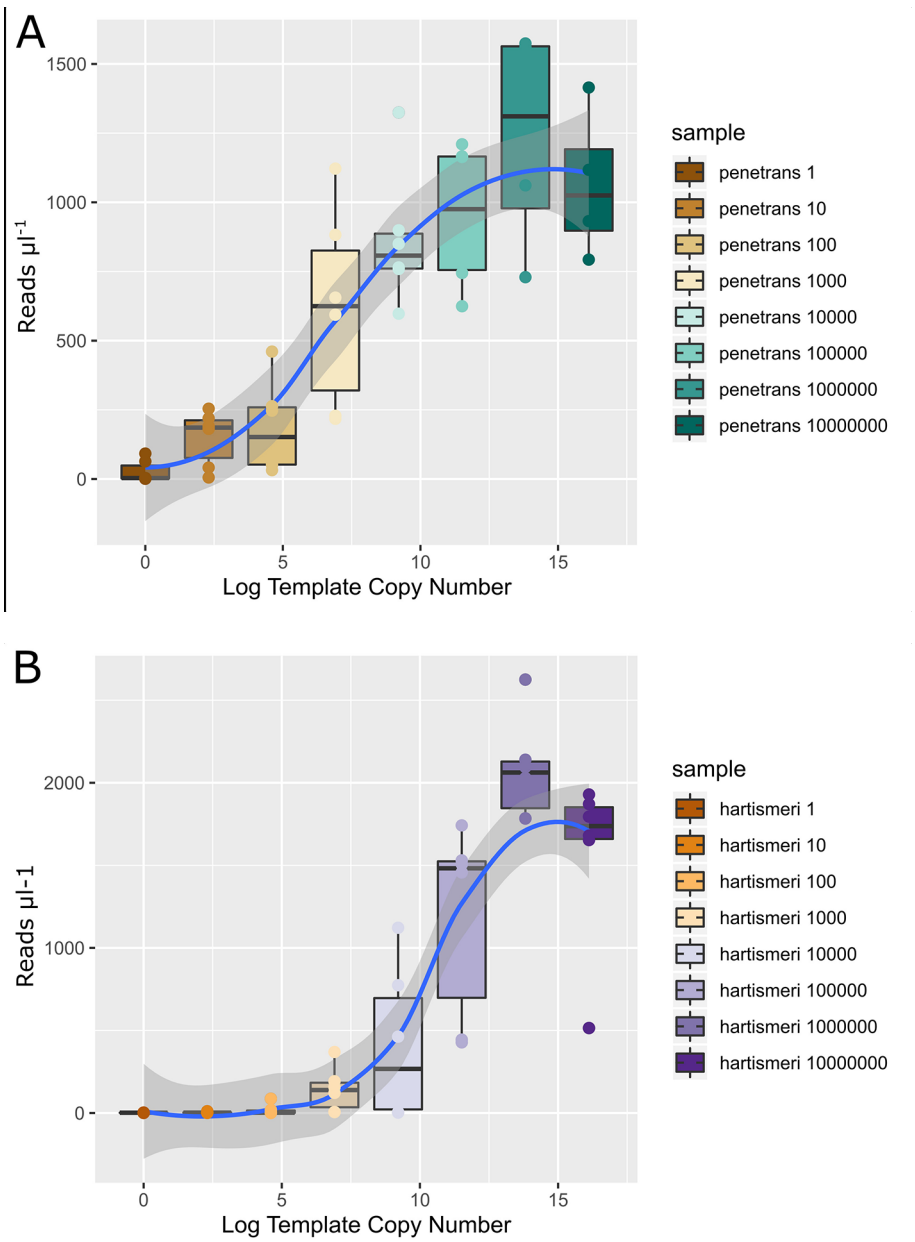

FIGURE 2 | Boxplots of *Pasteuria penetrans* **(A)** and *P. hartismeri* **(B)** 16S rRNA gene template copy number vs the merged paired read copy number ul^{-1} of product added to the final sequencing pool. Spearman's rank correlation is given as Rho and p. Loess curve of best fit is given as the blue trend line with dark grey shading representing uncertainty in this fit with a 95% confidence interval. Template copy number, representing the number of plasmids in each PCR reaction carrying the target gene, was log transformed as each input copy number was an order of magnitude greater or less than the next smallest or largest.

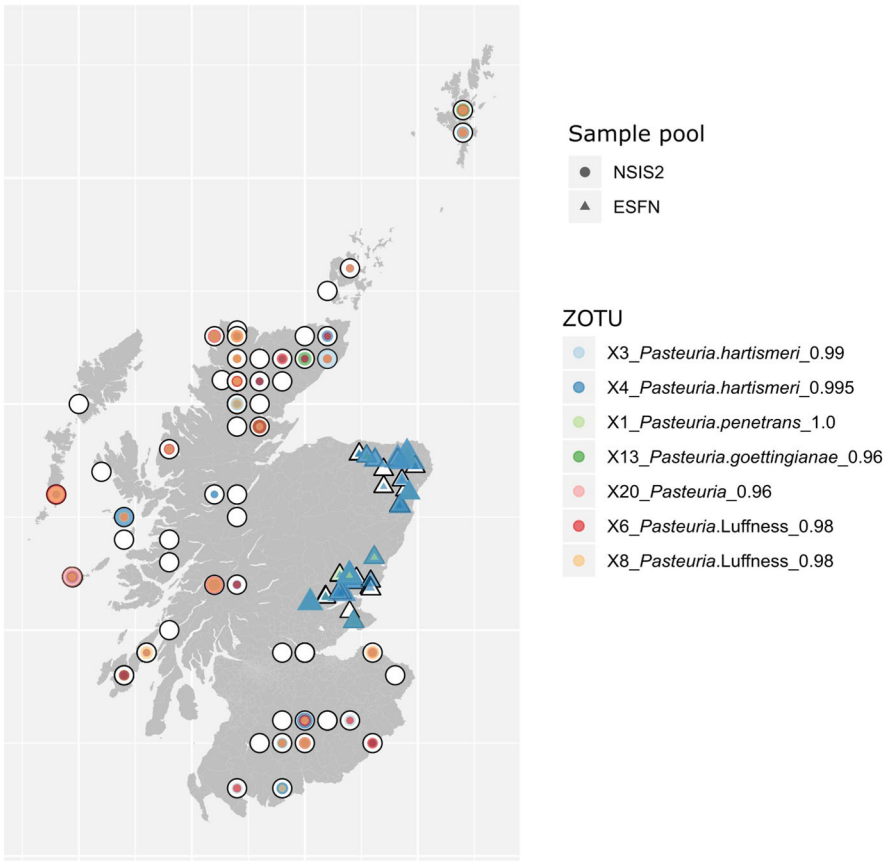

FIGURE 3 | Map of distribution of the most abundant *Pasteuria* spp. ZOTUs across both NSIS2 (circles) and ESFN (triangles) datasets. Plot points are colored by ZOTU and sized as a function of the total number of merged read pairs recovered for that ZOTU μl^{-1} of product added to the final pool from the corresponding sample.

(**Figure 2**). Reads obtained from sequencing μl^{-1} of PCR product do not increase beyond 1,000,000 target gene copies in the PCR reaction.

Diversity and Distribution

Soil samples from the NSIS2 which contained *Pasteuria* spp. were broadly distributed with no sequence or ZOTU variant bearing a significant relationship to easting or northing (**Figure 3**). The most common and widely distributed sequence variants recovered matched most closely to reference sequences for *Pasteuria hartismeri* and a sequence recovered at a farm near Edinburgh here referred to as "*Pasteuria* Luffness". ESFN samples were similarly dominated by these two sequences at most sites.

Pasteuria ZOTUs vs Soil Properties

Figure 4 shows the plotted NMDS ordination and associated metadata and species fits. Statistically significant relationships were observed between *Pasteuria* ZOTU sequence variants and near surface mineral horizon A, soil carbon, dry bulk density, pH, and field moisture (**Table 2**).

Major soil group peat, vegetation code ES1C (terminal phase blanket bog), clay content, and soil horizon O were significant factors pre-Benjamini-Hochberg correction, however, these drop from significance post-correction. Other soil horizons, major soil groups, and vegetation codes lacked statistically significant effects, both before and after Benjamini-Hochberg correction, on *Pasteuria* community composition.

NSIS2 *Pasteuria* spp. soil samples do not cluster into clear independent groups in the NMDS analysis. This suggests that the relationship between the above factors and the abundance of recovered ZOTUs is not binary but scalar. For example, ZOTU X3 assigned to *P. hartismeri* with 99% identity is more abundant in samples with less carbon, a higher pH, and a lower moisture content. These variables have strong pairwise Spearman's rank correlations (**Table 3**) and likely summarize the general properties of the soil horizon A in contrast to organic horizons L, H, and O. ZOTU X25, assigned with 96% identity to *Pasteuria goettingianae* has the inverse relationship with these variables, while X1, which is most similar to *P. penetrans* in the amplified region, shows no strong preference. However, each ZOTU is also

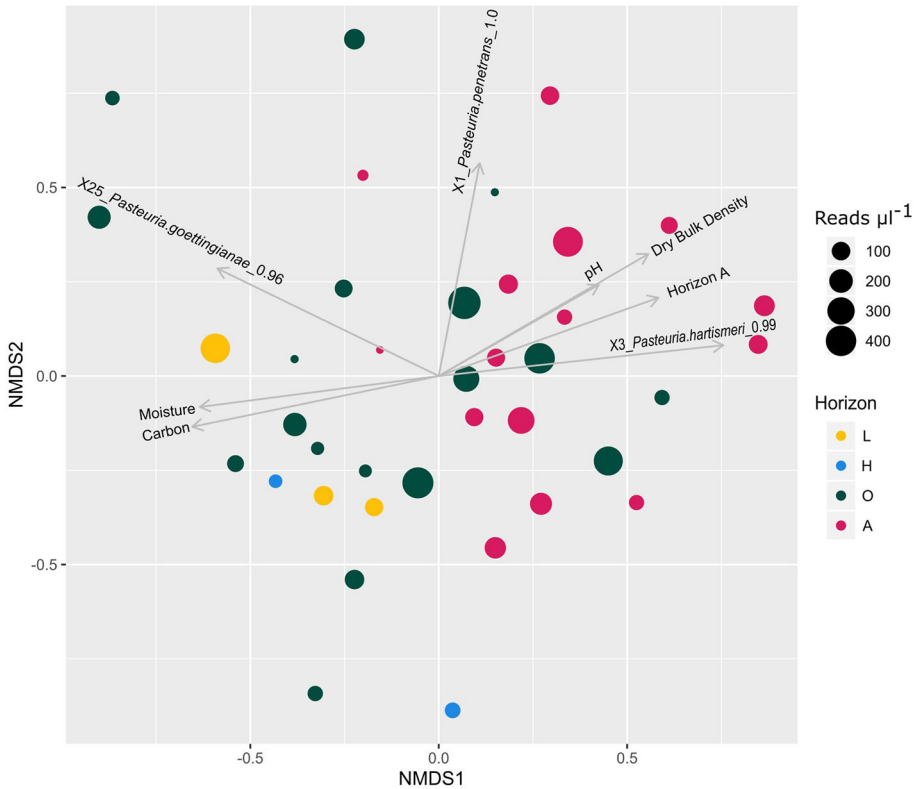

FIGURE 4 | NDMS ordination plot of NSIS2 *Pasteuria* spp. community composition. Points are colored by soil horizon where L = litter; H = humus; O = peaty material formed under wet, anaerobic conditions; and A = mineral horizon formed at or near the surface showing accumulation and incorporation of organic matter. Points are sized by the total number of merged read pairs μl^{-1} of PCR product added to the final pool from the corresponding sample. Stress = 0.245235.

present in organic and mineral soils/horizons at lower abundance.

Pasteuria-Nematode Interactions

ESFN Nematode Communities

Organic farming methods show a statistically significant relationship (P = 0.035) with nematode community structure pre-Benjamini-Hochberg correction although LEAF and conventional farms do not (**Figure 5**). No soil environmental factors tested in the ESFN dataset remained significant (P < 0.05) following Benjamini-Hochberg correction. However, the weight of sand (P = 0.009) and silt (P = 0.013) in each sample appeared to significantly influence the nematode community before correction.

Several ZOTUs bearing sequence similarity to PNN genera drive diversity in nematode community structure (**Figure 5**). *Pasteuria hartismeri*-like ZOTU X4 displays a negative fit to almost all statistically significant nematode ZOTU sequences. *Pasteuria* spp. ZOTU sequences X15 assigned to a *Pasteuria* spp. infective of bacterial feeding Plectidae, correlates with organic farms, silt weight, and *Longidorus elongatus* (Tzean and Estey, 1981).

Direct PCN J2 counts were available for 74 of the ESFN samples taken from a potato variety and nematicide incorporation trial conducted in two fields at a single farm.

TABLE 2 | Environmental variables with a statistically significant relationship to *Pasteuria* spp. community ordination before or after Benjamini-Hochberg correction.

	NMDS1	NMDS2	r	P	BH adjusted P
Horizon: A	0.583	0.208	0.38	0.001	0.01
Bulk Density	0.556	0.324	0.41	0.001	0.01
Carbon	–0.654	–0.135	0.45	0.001	0.01
Moisture	–0.634	–0.082	0.41	0.001	0.01
pH	0.425	0.242	0.24	0.006	0.05
Vegetation ES1C	–0.334	0.314	0.21	0.019	0.13
Clay	0.385	0.210	0.19	0.022	0.13
Major Soil Group: Peat	–0.438	0.005	0.19	0.033	0.17
Horizon: O	–0.388	0.041	0.15	0.053	0.24

TABLE 3 | Spearman's rank correlation of soil properties with statistically significant correlation to *Pasteuria* spp. community composition.

	Horizon A	Dry Bulk Density	Carbon	pH	Field Moisture
Horizon A	1	0.84	–0.85	0.84	–0.83
Dry Bulk Density	0.84	1	–0.78	0.78	–0.90
pH	0.84	0.78	–0.66	1	–0.75
Field Moisture	–0.83	–0.90	0.85	–0.75	1
Carbon	–0.85	–0.78	1	–0.66	0.85

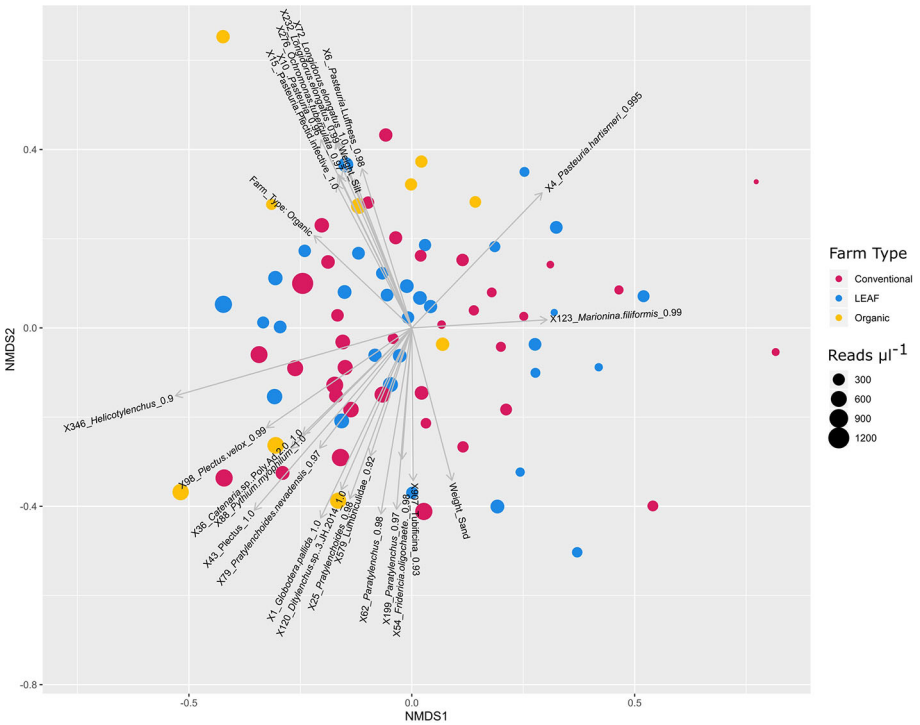

FIGURE 5 | NMDS ordination of ESFN nematode community ZOTUs. Each point represents a nematode community. Samples are colored by farm type and sized by the total abundance of nematode merged read pairs recovered μl^{-1} of PCR product added to the final pool. Arrows represent the statistically significant fits of Nematode ZOTUs, *Pasteuria* ZOTUs, non-nematode metazoan ZOTUs, and environmental factors where the length of the arrow reflects the effect size. For ease of visualization the number of significant nematode ZOTUs plotted has been reduced. Stress = 0.2482174.

Direct J2 counts and ZOTUs assigned to *Globodera* spp. showed statistically significant ($P = 3.25e^{-07}$) positive correlation. However, this correlation was weak (R = 0.67).

ZOTU Spearman's Rank Correlations

To test direct species to species interactions, rather than overall community interactions, the pairwise Spearman's rank correlation of all nematode and *Pasteuria* spp. ZOTUs was calculated. Several *Pasteuria* and nematode ZOTUs bear a statistically significant relationship (**Table 4**). However, the observed correlations are typically weak. *Pasteuria* spp. ZOTUs X1, X2, X3, and X4 display a significant negative correlation with metazoan ZOTUs assigned to *Paratylenchus* species. ZOTU X2, and X3 both assigned to *P. hartismeri* also displays a statistically significant negative correlation with *Heterodera* and *Meloidogyne* species.

Recovery of Endospore Encumbered Nematodes

Sucrose floatation of nematodes from ESFN soils returned *Pasteuria* endospore encumbered *Pratylenchus* spp. and free-living nematodes (**Figure 6**). Insufficient material was recovered to amplify endospore 16S rRNA gene sequences, however polyclonal anti-*Pasteuria penetrans* antibodies successfully recognized *Pratylenchus* spp. attached endospores (**Figure 6**).

DISCUSSION

Sensitivity

Sensitivity of our tests were high with detection limits of *Pasteuria* ranging from 10 to 1,000 copies of the target gene. Mauchline et al. (2011) hypothesized that the copy number of the 16S rRNA gene in *Pasteuria* spp. was likely to be low, one or two copies in contrast to those of related *Bacillus* spp., which typically have more than ten (Fogel et al., 1999). Genomic sequencing of *P. penetrans* RSE148 (Orr et al., manuscript in preparation) returned three SSU gene copies. Detection at this level would be theoretically sufficient to recover sequence from a single endospore filled juvenile nematode, which would typically contain ~500 mature endospores (Sturhan et al., 1994). These limits provide an indication of detection in an ideal sample where all bacteria are lysed, and which is free from inhibitors. However, spores of *Pasteuria* spp. are robust, being resistant or partially resistant to heat, desiccation, lysozyme, and SDS (Giannakou et al., 1997; Atibalentja et al., 2004; Mauchline et al., 2010). Environmental DNA samples are likely to contain inhibitors which would further increase the practical limits of detection relative to the inhibitor concentration within the starting material (Donn et al., 2008).

TABLE 4 | The top three most abundant *Pasteuria* spp. ZOTUs in the ESFN dataset and respective metazoan ZOTU Spearman's rank correlations which were statistically significant after Benjamini-Hochberg correction.

ZOTU 1	ZOTU 2	rho	P	BH corrected P
X4_*Pasteuria.hartismeri*_0.995	X701_*Filenchus.discrepans*_1.0	0.25	7.7E-05	2.4E-03
X4_*Pasteuria.hartismeri*_0.995	X851_*Ditylenchus.dipsaci*_0.98	0.23	2.1E-04	5.5E-03
X4_*Pasteuria.hartismeri*_0.995	X867_*Longidorus*_0.96	0.23	2.1E-04	5.6E-03
X4_*Pasteuria.hartismeri*_0.995	X845_*Pratylenchoides*_0.98	0.23	2.2E-04	5.8E-03
X4_*Pasteuria.hartismeri*_0.995	X58_*Diphterophora*_0.98	0.23	2.5E-04	6.5E-03
X4_*Pasteuria.hartismeri*_0.995	X70_*Diphterophora*_0.98	0.21	7.2E-04	1.5E-02
X4_*Pasteuria.hartismeri*_0.995	X72_*Longidorus.elongatus*_1.0	0.21	8.6E-04	1.8E-02
X4_*Pasteuria.hartismeri*_0.995	X876_Ecumenicus_0.98	0.20	1.8E-03	3.2E-02
X4_*Pasteuria.hartismeri*_0.995	X784_Cephalobidae_0.94	–0.20	1.2E-03	2.3E-02
X4_*Pasteuria.hartismeri*_0.995	X65_*Heterocephalobus.elongatus*_1.0	–0.21	6.6E-04	1.4E-02
X4_*Pasteuria.hartismeri*_0.995	X752_*Heterodera*_0.97	–0.23	3.3E-04	8.2E-03
X4_*Pasteuria.hartismeri*_0.995	X706_Tylenchidae_0.93	–0.23	2.5E-04	6.4E-03
X4_*Pasteuria.hartismeri*_0.995	X846_Tylenchoidea_0.93	–0.24	1.1E-04	3.1E-03
X4_*Pasteuria.hartismeri*_0.995	X657_Tylenchidae_0.91	–0.26	3.2E-05	1.1E-03
X4_*Pasteuria.hartismeri*_0.995	X800_*Eucephalobus*.cf.oxyuroides.JH.2004_0.97	–0.26	2.3E-05	8.5E-04
X4_*Pasteuria.hartismeri*_0.995	X55_*Thonus*.sp.JH.2004_1.0	–0.27	1.7E-05	6.6E-04
X4_*Pasteuria.hartismeri*_0.995	X641_Plectidae_0.92	–0.28	7.7E-06	3.2E-04
X4_*Pasteuria.hartismeri*_0.995	X5_*Meloidogyne*_1.0	–0.29	4.7E-06	2.1E-04
X4_*Pasteuria.hartismeri*_0.995	X68_*Eucephalobus*.cf.oxyuroides.JH.2004_0.995	–0.32	2.2E-07	1.3E-05
X4_*Pasteuria.hartismeri*_0.995	X56_*Aphelenchoides*.sp.JH.2004_0.97	–0.32	1.8E-07	1.1E-05
X4_*Pasteuria.hartismeri*_0.995	X75_Tylenchoidea_0.95	–0.35	9.9E-09	7.4E-07
X4_*Pasteuria.hartismeri*_0.995	X62_*Paratylenchus*_0.98	–0.37	1.8E-09	1.5E-07
X3_*Pasteuria.hartismeri*_0.99	X845_*Pratylenchoides*_0.98	0.31	4.0E-07	2.3E-05
X3_*Pasteuria.hartismeri*_0.99	X851_*Ditylenchus.dipsaci*_0.98	0.31	4.8E-07	2.6E-05
X3_*Pasteuria.hartismeri*_0.99	X701_*Filenchus.discrepans*_1.0	0.26	4.4E-05	1.5E-03
X3_*Pasteuria.hartismeri*_0.99	X867_*Longidorus*_0.96	0.24	9.7E-05	2.9E-03
X3_*Pasteuria.hartismeri*_0.99	X663_*Ditylenchus.dipsaci*_0.97	0.24	1.8E-04	4.9E-03
X3_*Pasteuria.hartismeri*_0.99	X70_*Diphterophora*_0.98	0.23	2.1E-04	5.6E-03
X3_*Pasteuria.hartismeri*_0.99	X798_*Ditylenchus.dipsaci*_0.995	0.22	4.0E-04	9.6E-03
X3_*Pasteuria.hartismeri*_0.99	X755_Tylenchidae_0.95	0.22	5.0E-04	1.2E-02
X3_*Pasteuria.hartismeri*_0.99	X549_*Neopsilenchus.magnidens*_0.97	0.21	1.0E-03	2.1E-02
X3_*Pasteuria.hartismeri*_0.99	X876_*Ecumenicus*_0.98	0.19	2.4E-03	4.0E-02
X3_*Pasteuria.hartismeri*_0.99	X784_Cephalobidae_0.94	–0.19	2.8E-03	4.5E-02
X3_*Pasteuria.hartismeri*_0.99	X706_Tylenchidae_0.93	–0.20	1.2E-03	2.3E-02
X3_*Pasteuria.hartismeri*_0.99	X5_*Meloidogyne*_1.0	–0.20	1.2E-03	2.3E-02
X3_*Pasteuria.hartismeri*_0.99	X56_*Aphelenchoides*.sp.JH.2004_0.97	–0.24	1.5E-04	4.1E-03
X3_*Pasteuria.hartismeri*_0.99	X55_*Thonus*.sp.JH.2004_1.0	–0.26	4.6E-05	1.5E-03
X3_*Pasteuria.hartismeri*_0.99	X641_Plectidae_0.92	–0.26	3.8E-05	1.3E-03
X3_*Pasteuria.hartismeri*_0.99	X657_Tylenchidae_0.91	–0.28	8.5E-06	3.5E-04
X3_*Pasteuria.hartismeri*_0.99	X68_*Eucephalobus*.cf.oxyuroides.JH.2004_0.995	–0.30	1.9E-06	9.3E-05
X3_*Pasteuria.hartismeri*_0.99	X75_Tylenchoidea_0.95	–0.30	1.5E-06	7.6E-05
X3_*Pasteuria.hartismeri*_0.99	X62_*Paratylenchus*_0.98	–0.32	3.2E-07	1.8E-05
X1_*Pasteuria.penetrans*_1.0	X887_Diphtherophorina_0.96	0.23	3.0E-04	7.5E-03
X1_*Pasteuria.penetrans*_1.0	X713_*Alaimus*.sp.PDL.2005_0.98	0.23	3.1E-04	7.7E-03
X1_*Pasteuria.penetrans*_1.0	X70_Diphterophora_0.98	0.21	6.5E-04	1.4E-02
X1_*Pasteuria.penetrans*_1.0	X615_*Nygolaimus*.cf.brachyuris.JH.2004_0.995	0.21	1.0E-03	2.1E-02
X1_*Pasteuria.penetrans*_1.0	X525_*Eumonhystera*.cf.filiformis.1.JH.2014_0.97	0.20	1.7E-03	3.1E-02
X1_*Pasteuria.penetrans*_1.0	X855_Aquatides_0.96	0.19	2.8E-03	4.5E-02
X1_*Pasteuria.penetrans*_1.0	X678_*Nygolaimus*.cf.brachyuris.JH.2004_0.99	0.19	3.0E-03	4.8E-02
X1_*Pasteuria.penetrans*_1.0	X56_*Aphelenchoides*.sp.JH.2004_0.97	–0.19	2.0E-03	3.5E-02
X1_*Pasteuria.penetrans*_1.0	X75_Tylenchoidea_0.95	–0.20	1.2E-03	2.3E-02
X1_*Pasteuria.penetrans*_1.0	X570_Plectus_0.94	–0.20	1.2E-03	2.3E-02
X1_*Pasteuria.penetrans*_1.0	X55_*Thonus*.sp.JH.2004_1.0	–0.22	5.8E-04	1.3E-02
X1_*Pasteuria.penetrans*_1.0	X657_Tylenchidae_0.91	–0.22	5.3E-04	1.2E-02
X1_*Pasteuria.penetrans*_1.0	X68_*Eucephalobus*.cf.oxyuroides.JH.2004_0.995	–0.23	2.9E-04	7.4E-03
X1_*Pasteuria.penetrans*_1.0	X641_Plectidae_0.92	–0.23	2.3E-04	5.9E-03
X1_*Pasteuria.penetrans*_1.0	X62_*Paratylenchus*_0.98	–0.24	1.8E-04	4.9E-03
X2_*Pasteuria*.Luffness_0.98	X92_*Xiphinema.pachtaicum*_1.0	0.26	2.4E-05	8.8E-04
X2_*Pasteuria*.Luffness_0.98	X867_*Longidorus*_0.96	0.24	1.3E-04	3.8E-03
X2_*Pasteuria*.Luffness_0.98	X734_*Longidorus*_0.97	0.21	7.2E-04	1.5E-02
X2_*Pasteuria*.Luffness_0.98	X701_*Filenchus.discrepans*_1.0	0.21	9.9E-04	2.0E-02
X2_*Pasteuria*.Luffness_0.98	X56_*Aphelenchoides*.sp.JH.2004_0.97	–0.19	2.6E-03	4.3E-02

(Continued)

TABLE 4 | Continued

ZOTU 1	ZOTU 2	rho	P	BH corrected P
X2_*Pasteuria*.Luffness_0.98	X657_Tylenchidae_0.91	–0.20	1.1E-03	2.2E-02
X2_*Pasteuria*.Luffness_0.98	X68_*Eucephalobus*.cf.oxyuroides.JH.2004_0.995	–0.21	6.8E-04	1.5E-02
X2_*Pasteuria*.Luffness_0.98	X62_*Paratylenchus*_0.98	–0.24	9.6E-05	2.9E-03
X2_*Pasteuria*.Luffness_0.98	X641_Plectidae_0.92	–0.25	7.4E-05	2.3E-03

Primer Specificity

UNOISE2 resulted in 148 16S rRNA gene ZOTUs across the entire dataset. Of these ZOTUs 75 (50.7%) were at least 90% similar to curated *Pasteuria* spp. reference sequences and only one sequence returned a best hit as low as 92% similarity. However, 100% of merged read pairs in the ESFN sample set and 93% of merged read pairs in the NSIS2 dataset were at least 90% identical to *Pasteuria* spp. reference sequences. The remaining 7% of NSIS2 assembled read pairs align predominantly with uncultured Acidobacteria. The lack of merged read pairs not matching to reference sequences suggests minimal off-target amplification from eDNA samples.

Nematode primers used in this study were not completely nematode specific, however ESFN DNA samples had been enriched for nematodes *via* Baermann funnel extraction. Nematode ZOTUs represented 581 (72%) of 803 18S rRNA gene ZOTUs and 85% of assembled read pairs illustrating that Baermann funnel extraction was an appropriate enrichment method. Sapkota and Nicolaisen (2015) reported a similar metabarcode study which enriched for nematode PCR products *via* initial semi-nested amplification using primers NemF and 18Sr2b from whole soil extracted DNA. These authors reported that 64.4% of OTUs recovered were taxonomically assigned to Nematoda (Sapkota and Nicolaisen, 2015). When contrasted with our results, PCR-based sample enrichment from whole soil extracted DNA appears to be less efficient (7.6% fewer nematode ZOTUs) than selection by Baermann funnel extraction. Similarly, *Pasteuria* spp. were amplified from a slightly higher proportion (45.9%) of ESFN samples compared to NSIS2 samples (35.9%), a difference of 7%. This slight increase in ESFN samples where *Pasteuria* spp. were detectable may be attributed to the greater volume of soil (200 g vs 0.25 g) which served as starting material for ESFN extractions. *Pasteuria* spp. may not be evenly distributed in soils and indeed the formation of microsites in soil may be important to effective parasitism (Stirling, 2014). Nematode enrichment from a sufficient volume of soil may also serve to enrich for *Pasteuria* species. Further, nested PCR reactions significantly increase the potential for well to well contamination, particularly within large sample sets. However, the proportional increase in *Pasteuria* spp. and nematode recovery in nematode enriched ESFN DNA extractions is slight; evaluating any statistical significance is not in the scope of this study. Further, it has been shown that an initial nested PCR reaction followed by a short number of barcoded primer cycles can reduce barcode bias effects and improve reproducibility in metabarcode studies (Berry et al., 2011).

Ordination and Soil Properties

The physical and chemical properties of soils had clear and statistically significant influence on *Pasteuria* spp. community structure, moreover, *Pasteuria* ZOTUs had separate and specific relationships with these properties. *Pasteuria hartismeri* appears to significantly associate with mineral A horizons in the NSIS2 soil DNA sample set. Soil organic carbon, pH, moisture, and bulk density are intrinsically linked (Vereecken et al., 1989; Kemmitt et al., 2006). Soil pH has previously been shown to correlate well with bacterial diversity and elevation (Shen et al., 2013). In Scotland, this relationship has a clear geographic implication in that altitude, rainfall, and pH vary significantly from North and West to South and East. No significant correlations were observed with either latitude or longitude. However, *Pasteuria* spp. communities in the ESFN appear broadly similar, being dominated by *P. hartismeri* and *Pasteuria* Luffness sequences. Soil pH has also been shown to directly affect attachment of *P. penetrans* to root knot nematodes (Afolabi et al., 1995). *Pasteuria* spp. found parasitizing *H. glycines* in China were found most commonly in high pH, low organic matter soils (Ma et al., 2005). In isolation the observed relationships between *Pasteuria* spp. and soil properties could be perceived as factors affecting the survival, attachment, and retention of endospores. However, it is likely that the single greatest influence on the abundance and diversity of *Pasteuria* spp. is the distribution of their hosts. Near surface mineral horizons are the location in the soil profile most likely to contain recorded root feeding nematode hosts for *P. hartismeri*, temperate *Meloidogyne* species (Bishop et al., 2007). Bulk density, pH, and organic matter content were also shown by Treonis et al. (2018) to have a statistically significant effect on nematode community structure in parallel metabarcode and morphological profiling within grain production systems in the mid-Atlantic USA. These researchers demonstrated that PNNs, likely hosts for *P. hartismeri*, were more abundant in conventional and zero tillage systems compared to organic farms (Treonis et al., 2018). Our findings in the ESFN nematode community structure dataset broadly support those of Treonis et al. (2018) except for pH which shows no statistically significant influence either before or after Benjamini-Hochberg error correction in the ESFN dataset. This is likely a reflection of the limited range of pH within this sample set (5.1–6.6).

Pasteuria-Nematode Community Relationships

Observed nematode-*Pasteuria* ZOTU correlations were not strong. This may be explained in part by successful nematode suppression. Where *Pasteuria* spp. are effectively parasitizing a nematode host, they may be difficult to recover as the number of juveniles in the community will be greatly reduced. They would

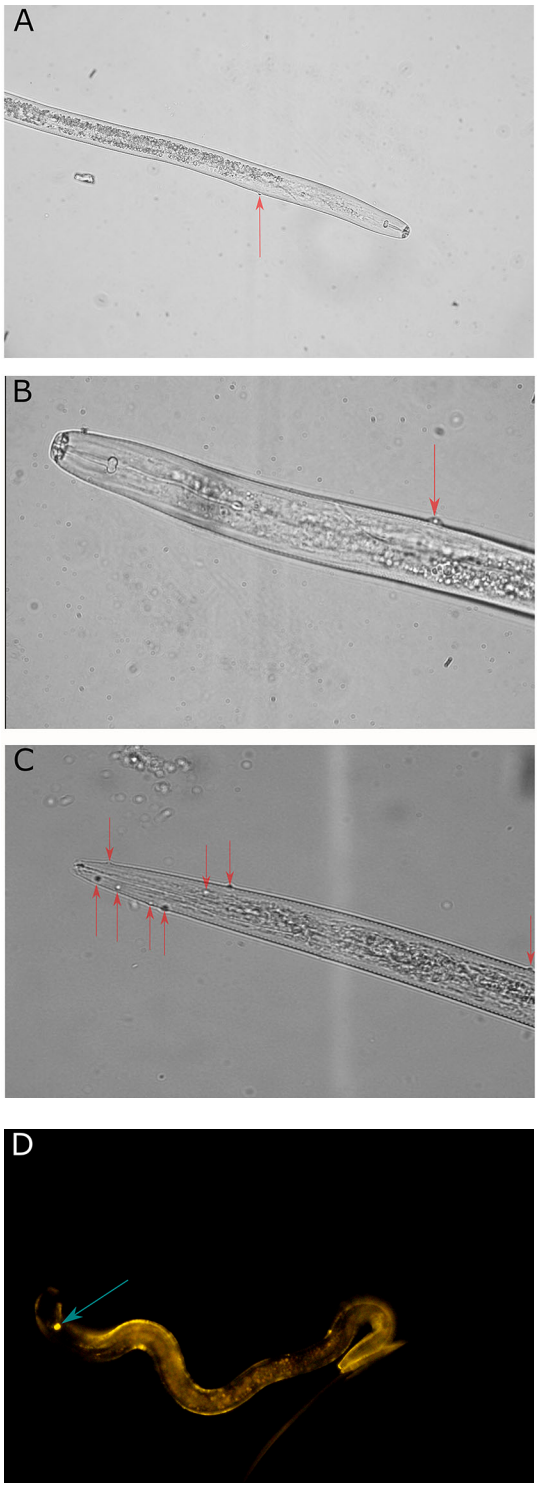

FIGURE 6 | (A) 400x magnification *Pratylenchus* spp. recovered from ESFN soil with *Pasteuria* spp. endospore attached (position indicated by red arrow). **(B)** 1000x magnification of *Pratylenchus* spp. recovered from ESFN soil with *Pasteuria* spp. endospore attached (position indicated by red arrow). **(C)** 1000x magnification of free living non-parasitic nematode recovered from ESFN soil with several *Pasteuria* spp. endospores attached (positions indicated by red arrows). **(D)** 1000x magnification of fluorescence image of *Pratylenchus* spp. recovered from ESFN soil with *Pasteuria* spp. endospore attached (position indicated by blue arrow), showing anti-*Pasteuria penetrans* antibody recognition.

also be less mobile due to the attachment of endospores to the cuticle, decreasing their recovery *via* methods which rely on the mobility of viable nematodes (Vagelas et al., 2012) and they may possess a much higher specific gravity, reducing their recovery *via* methods which rely on floatation (Oostendorp et al., 1991). Thus to successfully detect *Pasteuria* spp. from ESFN DNA samples both the bacteria and nematode had to be present; the nematode had to comprise enough of the total extracted metazoan community to detect; and the *Pasteuria* spp. had to be abundant enough to detect but not so abundant that the nematode could not be recovered. Despite this, the data provides some indication of *Pasteuria* spp. suppressive activity.

In ZOTU fits to the ESFN NMDS plots a *P. hartismeri*-like sequence which was prevalent in both datasets appeared to correlate negatively with almost all significant nematode species, including *Globodera* and *Pratylenchoides*. Suppression of *H. avenae* in UK soils by *Pasteuria*-like species has previously been reported (Davies et al., 1990). The dominance of *P. hartismeri* in Scottish soils is surprising. However, pairwise Spearman's rank correlations suggest a potential host range which is broader than it's currently described temperate RKN hosts (Bishop et al., 2007). Further, ZOTU X5, which is assigned with 100% identity to *Meloidogyne* spp. with exact matches to both *M. chitwoodi* and *M. fallax* with 99.7% identity to *M. minor* has a read abundance $\mu l^{-1} \geq 1$ in 9.2% of successfully amplified ESFN samples. Fleming et al. (2016) recently surveyed cereal and grassland soils in Northern Ireland, finding that *M. minor* was prevalent in 6% of soils tested. The abundance of this ZOTU is, however, typically low. This may reflect both primer bias and an insufficient depth of sequencing. Waeyenberge et al. (2019) recently demonstrated that *M. incognita* are up to 19.3 x underrepresented in metabarcoding data of defined communities depending on method of DNA extraction and primer selectionRecovery of endospore encumbered *Pratylenchus* spp. suggests that the *P. hartismeri*-like *Pasteuria* found in this study is capable of attachment to Pratylenchinae as well as temperate RKN species. *P. hartismeri* assigned ZOTU X4 has a significant negative Spearman's rank correlation ($P = 0.007$) with *Pratylenchus fallax* although this relationship is not significant post Benjamini-Hochberg correction. A number of other nematode ZOTUs also demonstrate a statistically significant negative correlation with this *P. hartismeri*-like sequence including ZOTUs assigned to *Heterodera* spp. and *Paratylenchus* species. *Pasteuria* spp. are broadly considered to be extremely fastidious in their attachment profile, however, cross-superfamily attachment of *P. penetrans* to *Meloidogyne* spp. and *Pratylenchus* spp. has been reported (Oostendorp et al., 1990; Sharma and Davies, 1996). De Gives et al. (1999) tested the attachment of five *Pasteuria* endospore isolates to a range of nematodes finding that, while the attachment profile of two isolates from *M. incognita* were fastidious within RKN, isolates from three *Heterodera* spp. were less stringent; attaching to populations of *Heterodera* spp., *Globodera* spp., *M. javanica*, *Pratylenchus* spp., *Aphelenchoides* spp., *Radopholus similis*, and *Rotylenchus reniformis*. Supporting this observation, Wishart et al. (2004) reported attachment of *P. nishizawae* endospores isolated from *H. glycines* to both *M. hapla* and *M. incognita*. Chen and Dickson (1998) listed 196 nematode species including free-living, predacious, plant-parasitic, and entomopathogenic nematodes which have been described encumbered with *Pasteuria* spp. endospores. However, the genetic diversity of *Pasteuria* spp. sequenced to date is relatively low. Mixed populations of *Pasteuria* spp. are often recorded (Davies et al., 1990), however, it is not often established that the endospores attached to each nematode species are distinct. Metabarcoding revealed surprisingly little genetic diversity of *Pasteuria* spp. in Scottish soils with two ZOTU sequences dominating in both agricultural and non-agricultural soils. However, we have captured only a fragment of the target gene, leaving the possibility that additional variability within these dominant ZOTU sequences has been overlooked. The range of nematodes to which attachment of well characterized *Pasteuria* spp. endospores has been tested is limited, normally restricted to root knot and cyst nematodes due to the focus on recovery of novel strains with biocontrol potential. While our understanding of the molecular mechanics of *Pasteuria* spp. and nematode surface coat interactions are incomplete, it may be premature to overstate the specificity of endospore attachment. Endospore attachment is one of the two mechanisms by which *Pasteuria* spp. may suppress a nematode population (Davies et al., 1991; Vagelas et al., 2012). Yet, in some cases, endospores attach and are then unable to germinate such as Noel et al. (2005) reported with *P. nishizawae* and *G. pallida*. This may account for indications of broad suppressive activity of our *P. hartismeri*-like sequence.

The near exact opposition of this *P. hartismeri*-like sequence and both *Catenaria*-like and *Pythium*-like sequences in NMDS ordination could indicate a competitive interaction for the same niche. *Catenaria* spp. are facultative parasites of a number of free living and PNNs (Stirling, 2014). *Pythium myophilum* (previously *Lagenidium myophilum*), the best taxonomic hit for ZOTU X88 is a parasite of shrimp (Nakamura et al., 1994). However, *Pythium* spp. have been recovered infecting of *Daphnia longispina* in several German lakes whose 18S rRNA gene sequence clustered with *P. myophilum* (Wolinska et al., 2009). *Daphnia* spp. are the only other recorded hosts outside of Nematoda for *Pasteuria* species (Duneau et al., 2011). Further, *Pythium monospermum*, a member of the same clade (Uzuhashi et al., 2010) is capable of parasitizing nematode eggs but not juveniles (Tzean and Estey, 1981; Wolinska et al., 2009; Duneau et al., 2011).

Limitations and Opportunities for Future Development

Although correlated, direct PCN J2 counts were not accurately reflected in the abundance of merged read pairs assigned to *Globodera* species. Over and under estimation of nematode abundance has been widely reported in nematode metabarcoding studies (Porazinska et al., 2009; Treonis et al., 2018; Waeyenberge et al., 2019). In the case of Heteroderinae, this may have been exacerbated by the presence of a single nucleotide mismatch in the NF1 primer sequence to the target DNA. Sample collection, enrichment, DNA extraction, storage, barcoding, amplification, library preparation, and sequencing each introduce unique biases which can be minimized but are difficult to eliminate (Waeyenberge et al., 2019). Pending further practical and/or computational developments, it is important to recognize that sequence variant analysis of nematode communities remains largely indicative; not

conclusive. Yet, several of the indicative relationships within the ESFN dataset corroborate previous literature (Treonis et al., 2018), or are corroborated in further investigation (**Figure 6**). This shows that, despite its limitations, metabarcoding can be a useful tool in uncovering biologically relevant trends in nematode communities.

The PCR barcoding strategy could be improved by double indexing of samples. This would account for possible primer dropout and allow for duplication of the assay in all samples. Alternatively, adapter indexes could be used (Glenn et al., 2016) which would preserve sequencing read length for PCR products. Waeyenberge et al. (2019) demonstrated that an adapted Holterman method (Holterman et al., 2006) offered improvements to complete lysis of nematode communities during DNA extraction. The use of multiple marker genes could provide a more robust method which is better able to discriminate taxa. Porazinska et al. (2009) found that sequencing of both 28S and 18S rDNA markers increased nematode taxon discrimination from 90% to 97% in complex artificial communities. However, an additional marker may reduce coverage or push up sequencing costs. Design of additional *Pasteuria* spp. markers is inhibited by a lack of robust sequence data from well characterized strains. Mauchline et al. (2010) identified several candidate markers such as *gyrB* and s*po0A* genes. A concerted effort to generate *Pasteuria* spp. reference sequences from diverse hosts and environments would be beneficial to understanding the true diversity of the genus and its evolutionary history. Further, conducting this assay in two stages, first assaying *Pasteuria* spp. populations from direct soil extracted DNA and then conducting deep sequencing of nematode communities, extracted using high density sucrose flotation, in a restricted number of interesting samples, may overcome some of the complications introduced by sampling strategy discussed above.

The influence of pH, bulk density, moisture, cation valence, and organic matter content on *P. penetrans* retention in the soil is well documented (Dabiré et al., 2001; Dabiré and Mateille, 2004; Dabiré et al., 2007; Mateille et al., 2009; Mateille et al., 2010). In addition, we have been able to show that these relationships may be species, or strain, specific and are likely driven by their effect on the host nematode. This method could also be used to conduct large scale assays of the effects of soil management practices in greater detail, such as investigation of tillage and organic amendment, on the cultivation of *Pasteuria* spp. specific suppression. Such an investigation may reveal a pathway to a more holistic integrated pest management strategy of which *Pasteuria* spp. are a part.

DATA AVAILABILITY STATEMENT

All code used to parse, analyze, and plot data presented in this article is available at: https://github.com/BioJNO/Pasteuria_Nematode_metabarcoding.

Paired end Illumina MiSeq data uploaded to the European Nucleotide Archive. Study accession: PRJEB34559.

Supporting data tables available on figshare: https://doi.org/10.6084/m9.figshare.9897374.

Figures available on figshare: https://doi.org/10.6084/m9.figshare.9849863.

AUTHOR CONTRIBUTIONS

JO, VB, PC, KD, RN, and TF contributed to the conception and design of the study. DR and TF were responsible for ESFN and NSIS2 DNA extractions respectively. JO carried out all other laboratory work and wrote the manuscript. JO and PC conducted all bioinformatic and statistical analyses. All authors contributed to manuscript revision, read, and approved the submission.

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Impacts of Root Metabolites on Soil Nematodes

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Plant parasitic nematodes cause significant crop damage globally. Currently, many nematicides have been banned or are being phased out in Europe and other parts of the world because of environmental and human health concerns. Therefore, we need to focus on sustainable and alternative methods of nematode control to protect crops. Plant roots contain and release a wide range of bioactive secondary metabolites, many of which are known defense compounds. Hence, profound understanding of the root mediated interactions between plants and plant parasitic nematodes may contribute to efficient control and management of pest nematodes. In this review, we have compiled literature that documents effects of root metabolites on plant parasitic nematodes. These chemical compounds act as either nematode attractants, repellents, hatching stimulants or inhibitors. We have summarized the few studies that describe how root metabolites regulate the expression of nematode genes. As non-herbivorous nematodes contribute to decomposition, nutrient mineralization, microbial community structuring and control of herbivorous insect larvae, we also review the impact of plant metabolites on these non-target organisms.

Keywords: plant parasitic nematode, attractant, repellent, hatching stimulants, non-target organisms, signaling, nematicide, gene expression

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INTRODUCTION

Plant parasitic nematodes cause serious damage and yield losses in a wide range of crops throughout the world estimated to cause >$80 billon losses annually (Nicol et al., 2011). Due to their adverse effects on human health and the environment, chemical nematicides are being banned worldwide, and there is therefore an urgent need for alternative and efficient control measures as well as improved agricultural practices to minimize crop losses.

Functionally, root feeding nematodes are categorized according to their feeding mode. All plant feeding nematodes feed on cell solubles drawn through a stylet (in Tylenchida) or odontostyle (in Dorylaimida) pierced through the cell wall. Epidermal cell and roothair feeders are probably relatively harmless, whereas ectoparasites and endoparasites are considered harmful. Endoparasites penetrate into and feed within the root tissue, whereas ectoparasites feed exclusively from the root surface. After root penetration, female sedentary endoparasites remain at a permanent feeding site within the root for their remaining life, whereas migratory endoparasites maintain mobility and move within and between roots (Yeates et al., 1993).

A variety of plant metabolites in roots and exuded from roots to the rhizosphere influence nematode behaviour, development, reproduction and even survival (Timper et al., 2006; Dandurand and Knudsen, 2016; Wang et al., 2018). Some metabolites thus facilitate plant parasitic nematode infection and damage, whereas others directly or indirectly reduce damage. There are still a lot of mechanisms to uncover, but for some plant-nematode interactions, the molecular mechanisms are well elucidated. Profound understanding of the chemical interactions between plant roots and plant parasitic nematodes can form the basis for novel pesticide-free strategies for reduced crop damage and losses to plant parasitic nematodes.

For instance, with the identification of nematicidal root metabolites plant-breeding programs can target the development of cultivars that produce high quantities of these specific metabolites. Agricultural practices can also be adjusted to optimize plant parasitic nematode management, e.g. through the choice of crop cultivars with nematode suppressive or repellent metabolic profiles, through intelligent crop rotations that include nematode suppressive cover crops or non-susceptible crops that produce sanitizing metabolites. Detailed knowledge on chemically induced nematode egg hatching or behavior may be the basis for targeted interference with nematode host identification and infection ability. Plant metabolites may even facilitate efficient rhizosphere colonization of nematode suppressive microorganisms (Elhady et al., 2018; Topalovic et al., 2019; Topalovic and Heuer, 2019). Hence, harnessing the full potential of microbial-assisted control of plant parasitic nematodes may depend on in-depth understanding of plant chemical influence on the tripartite interactions between plants, rhizosphere microbiomes, and nematodes.

Research on plant root metabolic impacts on plant parasitic nematodes is progressing, but the research is to a large extent still scattered and some results conflicting. In order to shed clarity on the field, we compiled current knowledge on aspects of the chemical interactions between live plants and plant parasitic nematodes in agroecosystems. We aim to review thoroughly the available literature to evaluate the evidence for specific root metabolites' impact on plant parasitic nematodes and to give an account of the different modes of action exerted by different root metabolites on plant parasitic nematodes. Further, we wish to identify gaps in current understanding and interpretation of plant chemical influence on plant parasitic nematodes. We focus on interactions between live plant roots and nematodes and refer to several excellent reviews that deal with biofumigation strategies based on cover crop soil incorporation (Fourie et al., 2016; Dutta et al., 2019). Further, we will evaluate to which extent metabolites that affect plant parasitic nematodes have unwanted effects on non-parasitic nematodes.

ROOT METABOLITES

About 5 to 20% of photosynthesis products are released to the rhizosphere through root exudates (Hütsch et al., 2002; Marschner, 2012). Roots deposit diverse metabolites into the rhizosphere, many of which are products of general metabolic plant processes. Deposition to the rhizosphere involves both active secretion and passive deposition of metabolites due to osmotic and concentration differences between cell and soil solutions. Several studies on model plants have identified metabolites released into the rhizosphere. For instance, 103 compounds were identified in root exudates collected from hydroponically cultivated *Arabidopsis thaliana* (Strehmel et al., 2014). In addition, root exudates of *A. thaliana* grown on MS media, were analyzed targeting primary metabolites, and 130 compounds identified (Chaparro et al., 2013). Furthermore, 289 putative secondary metabolites were quantified in *Arabidopsis* root exudates after elicitation with salicylic acid, jasmonic acid, chitosan, and two fungal cell wall elicitors (Walker et al., 2003). Chemical profiles of *Arabidopsis* thus show that a vast number of metabolites is released into the rhizosphere depending on growth condition.

In this review, we focus on the effects of specific root metabolites on nematodes ranging from plant parasitic to soil borne free-living nematodes. In **Tables 1–3** we present root exudates and specific root compounds that interact with plant parasitic nematodes and describe their effects on nematode taxa.

PLANT PARASITIC NEMATODES NAVIGATE *VIA* ROOT CHEMICAL CUES

Nematodes perceive their surrounding environment through chemosensory perception. Typically, plant parasitic nematodes locate their preferred host through root exudate signals (Bird, 2004). Several chemical gradients exist around physiologically active roots and it is likely that some chemicals constitute "long distance attractants", which help nematodes migrate towards root occupied soil volumes, whereas "short distance attractants" may aid nematode navigation to individual roots of a host (Perry, 2005). Infective J2 larvae of root knot nematodes *Meloidogyne incognita* and *M. graminicola* take the most direct route to their preferred host; however, they take the longest route towards poor hosts, which indicates that specific root metabolites act as attractants and repellants, respectively, and influence the movement patterns of the nematodes to find their suitable host (Reynolds et al., 2011).

Attractants

Under natural conditions, volatile compounds are long distance cues for infective root knot nematode J2 larvae location of suitable hosts. More locally in the root region, water soluble chemicals act as signaling cues (Curtis et al., 2009). For instance, *M. incognita* is able to perceive and utilize plant volatile organic compounds for host location (Kihika et al., 2017). Still, we know very little about the identity of compounds involved in nematode attraction to hosts, but recent studies have identified some host-elicited attractants (**Table 1**).

Five components [2-isopropyl-3-methoxypyrazine, 2-(methoxy)-3-(1-methylpropyl) pyrazine, tridecane, and α- and β-cedrene] were identified in the root-emitted volatiles of both

TABLE 1 | Root metabolites affecting nematode movement.

Plant species/ synthetic chemicals	Root metabolites	Assay concentrations	Test system	Nematodes affected	Effect	Reference
[1]Tomato *Solanum lycopersicum*	Methyl salicylate	40, 80, 160 ng/µl dissolved in hexane, hexane as negative control	Olfactory assay with sterilized sand	*Meloidogyne incognita*	Attractant	(Murungi et al., 2018)
[1]Spinach *Spinacia oleracea*	2-isopropyl-3-methoxypyrazine, tridecane	40, 80, 160 ng/µl dissolved in hexane, hexane as negative control	Olfactory assay with sterilized sand	*M. incognita*	Attractant	(Murungi et al., 2018)
[1]Pepper *Capsicum annuum*	Methyl salicylate, α-pinene, limonene and tridecane	20, 40, 80 ng/µl µl dissolved in hexane, hexane as negative control	Olfactory assay with sand	*M. incognita*	Attractant	(Kihika et al., 2017)
[3]Synthetic chemicals	Isoamyl alcohol, 1-butanol, and 2-butanone	Dissolved in sterile ethanol (0.05% v/v) final concentration NA, water as negative control	*In vitro* assay	*M. incognita*	Attractant	(Shivakumara et al., 2018)
[3]Synthetic chemicals	Salicylic acid	20, 50, 100, 200 µg/ml; controls consisted of the compound solvent (0.5% DMSO, 2.5 mM NaOH or distilled water), 1% acetic acid as a repellent and 0.5 M $CaCl_2$ as an attractant control.	*In vitro* assay	*M. incognita*	Attractant	(Wuyts et al., 2006)
[1]Tomato *S. lycopersicum*	Zeatin	4, 15.6, 62.3, 250, 1000 ng/µl dissolved in 2% DMSO; 2% DMSO as negative control and 100 ng/µlmethyl salicylate as positive control	Sand assay	*M. incognita*	Attractant	(Kirwa et al., 2018)
[3]Synthetic chemicals	Dopamine	20, 50, 100, 200 µg/ml; controls consisted of the compound solvent (0.5% DMSO, 2.5 mM NaOH or distilled water), 1% acetic acid as a repellent and 0.5 M $CaCl_2$ as an attractant control.	*In vitro* assay	*Radophulus similis*	Attractant	(Wuyts et al., 2006)
[1]Tomato *S. lycopersicum*	L-ascorbyl 2, 6-dipalmitate; 2, 6-Di-tert-butyl-p-cresol; dibutyl phthalate and dimethyl phthalate	0.5mM, 1.1mM, 2.2mM dissolved in 1% ethanol, 1% ethanol as control	*In vitro* assay	*M. incognita*	Dimethyl phthalate repellent, all four compounds nematicidal	(Yang et al., 2016)
[1]Castor *Ricinus communis*	Palmitic acid and linoleic acid	0.5,1, 2, 4 mM dissolved in methanol, methanol as control	*In vitro* assay	*M. incognita*	Repellent	(Dong et al., 2018)
[1]Tomato *S. lycopersicum*	Quercetin	4, 15.6, 62.3, 250, 1000 ng/µl dissolved in 2% DMSO; 2% DMSO as negative control, 100 ng/µl methyl salicylate as positive control	Sand assay	*M. incognita*	Low concentration act as attractant and higher concentration as repellent	(Kirwa et al., 2018)
[3]Synthetic chemicals	1-octanol	Dissolved in sterile ethanol (0.05% v/v), final concentration NA, water as negative control	*In vitro* assay	*M. incognita*	Repellent	(Shivakumara et al., 2018)
[2]Marigold *Tagetes patula*, [2]Pepper *Capsicum annuum*, and [2]Soybean *Glycine max*	Root exudate compounds	Extract doses in bioassays expressed as mg of tip section (mg eq) $volume^{-1}$, 1 to10 mg equivalent $dispenser^{-1}$; double distilled water as control	*In vitro* assay	*M. incognita* and *Heterodera glycine*	Repellent to root knot nematode and attractant to cyst nematode	(Wang et al., 2018)
[2]Pea *Pisum sativum*, [2]Snap bean *Phaseolus vulgaris*, and [2]Alfalfa *Medicago sativa* cv. Thor, cv. Moapa 69, cv. Lahonton	Root tip exudates	Border cells, root tip exudates, and border cells + root tip exudates with water as control	*In vitro* assay	*M. incognita*	Repellent to J2s; induced quiescence response, > 80% of the nematodes lost motility	(Zhao et al., 2000)
[1]Tomato *S. lycopersicum* and [1]Rice *Oryza sativa*	Small lipophilic molecules	Dissolved in 0.01% ethanol, final concentration NA, 0.01% ethanol and distilled water as control	*In vitro* assay	*M. incognita and Meloidogyne graminicola*	Repellent	(Dutta et al., 2012)
[3]Synthetic chemicals	p-coumaric acid, caffeic acid, ferulic acid, kaempferol, quercetin, myricetin	20, 50, 100, 200 µg/ml; controls consisted of the compound solvent (0.5% DMSO, 2.5 mM NaOH or distilled water), 1% acetic acid as a repellent and 0.5 M $CaCl_2$ as an attractant control		*M. incognita*	Repellent	(Wuyts et al., 2006)

(Continued)

TABLE 1 | Continued

Plant species/ synthetic chemicals	Root metabolites	Assay concentrations	Test system	Nematodes affected	Effect	Reference
[3]Synthetic chemicals	Protocatechuic acid, umbelliferone, caffeic acid, ferulic acid, luteolin, daidzein, genistein, kaempferol, quercetin, myricetin	20, 50, 100, 200 µg/ml; controls consisted of the compound solvent (0.5% DMSO, 2.5 mM NaOH or distilled water), 1% acetic acid as a repellent and 0.5 M $CaCl_2$ as an attractant control		*R. similis*	Repellent and nematicidal	(Wuyts et al., 2006)
[2]Potato *Solanum tuberosum*	Unknown volatile metabolites in root exudates	NA	Bioassay in sand	*Globodera pallida*	Attractants	(Farnier et al., 2012)
[3]Synthetic chemicals	Trans-cinnamic acid; p-coumaric acid	Trans-cinnamic acid 270 µM; p-coumaric acid 240 µM, water as control	*In vitro* assay	*M. incognita*	Repellent	(Fleming et al., 2017)
[3]Synthetic chemicals	Salicylic acid, ethephon, vanillic acid, gibberellic acid, indole-3-acetic acid, 6-dimethylallylamino purine, mannitol, arginine and lysine	Salicylic acid 100 µM; ethephon 1, 10, 50 µM; vanillic acid 240 µM; gibberellic acid 115 µM; indole-3-acetic acid 230 µM; 6-dimethylallylamino purine 200 µM; mannitol 5 mM; arginine 5 mM; and lysine, 5 mM; water as control	*In vitro* assay	*M. incognita*	Attractants	(Fleming et al., 2017)
[3]Synthetic chemicals	Salicylic acid, methyl jasmonate, ethephon, indole-3-acetic acid, mannitol	Salicylic acid 100 µM; methyl jasmonate 100 µM; ethephon 50 µM; indole-3-acetic acid 230 µM; mannitol 5 mM, water as control	*In vitro* assay	*G. pallida*	Attractants	(Fleming et al., 2017)
[1]Crown daisy*Glebionis coronaria*	Lauric acid	0.5, 1.0, 2.0, and 4.0mM dissolved in methanol, methanol as control	*In vitro* assay	*M. incognita*	Lauric acid (0.5, 1.0, 2.0 mM) acts as attractant and lethal trap and also act as repellent (4mM)	(Dong et al., 2014)
[2]Soybean *Glycine max* and [2]Arabidopsis *Arabidopsis thaliana*	Metabolites of ethylene pathway	NA	*In vitro* assay	*Heterodera glycines*	Ethylene (ET)-synthesis inhibitor and ET-insensitive mutations attractant to cyst nematode	(Hu et al., 2017)
[2]Thale cress *Arabidopsis thaliana*	Metabolites of ethylene pathway	NA	*In vitro* assay	*Heterodera schachtii*	Ethylene treated Arabidopsis more attractive to nematodes	(Kammerhofer et al., 2015)
[2]Thale cress *Arabidopsis thaliana* and [2]Tomato *S. lycopersicum*	Metabolites of ethylene pathway	NA	*In vitro* assay	*Meloidogyne hapla*	Ethylene (ET)-synthesis inhibitor and ET-insensitive mutations attractant while ET-overproducing mutants less attractive	(Fudali et al., 2012)
[2]Tomato *S. lycopersicum* and [2]barrel clover *Medicago truncatula*	Metabolites of ethylene pathway	NA	*In vitro* assay	*M. hapla, Meloidogyne javanica*, and *M. incognita*	Mutants defective in ethylene signaling of both hosts were found to be more attractive compared to wild type	(Čepulyte et al., 2018)

[1]study on effect of both plant and specific metabolites detected in root exudates of the plant, [2]study on effect of plant, [3]study on effect of synthetic compound on nematodes, NA indicate not applicable.

tomato and spinach, while three others (δ-3-carene, sabinene, and methyl salicylate) were specific to tomato roots volatiles. In bioassays, 2-isopropyl-3-methoxypyrazine and tridecane attracted *M. incognita* J2 larvae to spinach roots, but methyl salicylate was more attractive to the J2s than these two compounds, and repeated experiments confirmed that methyl salicylate renders tomato roots more attractive to *M. incognita* than spinach roots (Murungi et al., 2018). Similarly, among *Capsicum annum*-emitted root volatiles methyl salicylate exerted the strongest positive chemotaxis of infective *M. incognita* J2 larvae, followed by pinene, limonene, tridecane, and 2-methoxy-3-(1-methylpropyl)-pyrazine (Kihika et al., 2017). Hence, two studies (Kihika et al., 2017; Murungi et al., 2018) identify methyl salicylate as the most significant volatile attractant of *M. incognita* in the investigated Solanaceous plants. In a bioassay, salicylic acid attracted *M. incognita*, and dopamine attracted *Radopholus similis* (Wuyts et al., 2006).

We have limited information about compounds that attract cyst nematodes. Unknown volatile metabolites in potato root exudates attracted J2 larvae of the potato cyst nematode

TABLE 2 | Nematicidal and nematode inhibitory root metabolites.

Plant species/ synthetic chemicals	Root metabolites	Assay concentrations	Test system	Nematodes affected	Effect	Reference
[3]Synthetic chemicals	Purified VOC dimethyl disulfide (DMDS) and 3-pentanol	DMDS (100, 200, 300, 400, 500, 600, 700 ppm), 3-pentanol (200, 400, 600, 800, 1000, 1200 ppm), water as control	*In vitro* assay	*M. incognita*	Toxicity to J2s and reduced egg masses and gall formation	(Silva et al., 2018)
[3]Synthetic chemicals	DMDS and 3-pentanol	500 ppm and 1000 ppm, water as control	*In vitro* assay	*M. incognita*	Toxicity to eggs and J2s and reduced the number of galls and egg masses	(da Silva et al., 2019)
[3]Synthetic chemicals	DMDS	Actual DMDS concentration N/A	Field trials and pot experiment	*M. incognita, M. hapla, Pratylenchus penetrans, Heterodera* sp., *H. carotae, Globodera* sp.	Nematicidal	(Coosemans, 2005; López-Aranda et al., 2009; Curto et al., 2014; Leocata et al., 2014; Zanón et al., 2014; Myrta et al., 2018)
[1]Arugula *Eruca sativa*	Erucin	1 to 1000 ppm, water and mixture of methanol and aqueous Tween 20 at 0.3% (v/v) as control	*In vitro* assay	*M. incognita*	Nematicidal	(Aissani et al., 2015)
[1]Siam weed *Chromolaena odorata*	1,2-dehydropyrrolizidine alkaloids	7, 70, 350 ppm, water as control	*In vitro* assay	*M. incognita*	Nematicidal	(Thoden et al., 2007)
[1]Smooth Crotalaria *Crotalaria pallida*	Root exudates compounds - Proteinaceous papain inhibitor	Proteinaceous papain inhibitor 0.2, 0.5, 1 μg/μL, water and buffer as control	*In vitro* assay	*M. incognita*	Nematostatic and nematicidal	(Andrade et al., 2010)
[3]Synthetic chemicals	Butyric, caprylic, capric, lauric, myristic, palmitic, and oleic acids	100, 1000, 2000 μM dissolved in Tween-80/dimethyl sulfoxide/acetone/ water, Tween-80/dimethyl sulfoxide/ acetone/water as control	*In vitro* assay for mortality assessment, planted pot experiment for reproduction assessment	*M. incognita*	Mortality of J2s and reduced reproduction	(Zhang et al., 2012)
[3]Synthetic chemicals	Glucosinolates plus myrosinase, hydrolysis products gluconasturtiin, glucotropaeolin, glucoerucin, sinigrin, glucoraphasatin, gluconapin, glucoiberin, sinalbin, epi-progoitrin, glucoraphanin, glucoconringiin	0.0025 to 25 mM, phosphate buffer as control	*In vitro* assay	*M. incognita*	Nematicidal	(Lazzeri et al., 1993; Lazzeri et al., 2004)
[3]Synthetic chemicals	Glucosinolates plus myrosinase	Dehydroerucin, gloconapin, glucotropeolin, sinigrin; 0.005, 0.05, 0.5% (w/v), phosphate buffer as control	*In vitro* assay	*M. incognita*	Nematicidal	(Lazzeri et al., 1993; Lazzeri et al., 2004)
[1]Rattlepod *Crotalaria* sp. and [1]Ragwort *Senecio jacobaea*	1,2-dehydropyrrolizidine alkaloids	1, 0.1 and 0.01 mg/ml, water as control	*In vitro* assay	*M. incognita, H. schachtii, P. penetrans, Phasmarhabditis hermaphrodita, Rhabditis* sp.	Nematicidal and ovicidal	(Thoden et al., 2009a)
[1]Alfalfa *Medicago sativa*	Medicagenic acid	125, 250, 500, 1,000 μg/ml	*In vitro* assay	*M. incognita* and *Xiphinema index*	Nematicidal	(D'Addabbo et al., 2011)
[3]Synthetic chemicals	Sinigrin, glucosinalbin, gluconapin, epi-progoitrin, erucin, glucoiberin	0.05, 0.30, 1.0 and 2.0 mg/ml, water as control	*In vitro* assay	*H. carotae* and *X. index*	Nematicidal	(Avato et al., 2013)

(Continued)

TABLE 2 | Continued

Plant species/ synthetic chemicals	Root metabolites	Assay concentrations	Test system	Nematodes affected	Effect	Reference
[1]Rye *Secale cereale*	Benzoxazinoids (DIMBOA, DIBOA, MBOA and BOA	0, 0.9, 4.5, 9, 22.5, 45, 67.5, and 90 mg/ml, deionized water as control	*In vitro* assay	*M. incognita* and *Xiphinema americanum*	Nematicidal	(Zasada et al., 2005)
[1]Plume poppy *Macleaya cordata*	Sanguinarine, chelerytherine and allocryptopine	1, 5, 10, 25, 50, 100, 200 µg/ml, dissolved in 5% of acetone, 5% of acetone as control	*In vitro* assay	*M. incognita, Caenorhabditis elegans* and *Bursaphelenchus xylophilus*	Nematicidal	(Kui et al., 2015)
[3]Synthetic chemicals	Thymol, benzaldehyde	Thymol (0, 50, 100, and 150 ppm) to soil in combination with benzaldehyde (0, 50, and 100 ppm), dissolved in ethanol, ethanol as control	Planted pot experiment	*Meloidogyne areneria, Heterodera minor, Paratrichodorus minor,* Dorylaimoid nematode	Reduced root galling, cyst formation, suppressed population growth	(Soler-Serratosa et al., 1996)
[3]Synthetic chemicals	Geraniol	62.5, 125, 250, 500 and 1000 ppm dissolved in water with ethanol and Tween-20, distilled water and water with ethanol and Tween-20 as control	*In vitro* assay	*M. javanica*	Nematicidal	(Nasiou and Giannakou, 2018)
[3]Synthetic chemicals	Me pelargonate and ethylene glycol pelargonate	0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.5, 25, 50, 100 µl active ingredient l^{-1}, deionized water as control	*In vitro* assay	*M. javanica* and *H. glycines*	Reduced number of galls and cysts	(Davis et al., 1997)
[3]Synthetic chemicals	α-terthienyl and gallic and linoleic acids	0.00125, 0.0025, 0.005, 0.01 mg/ml, dissolved in DMSO, DMSO and water as control, carbufuran as positive control	*In vitro* assay	*Heterodera zeae*	Nematicidal	(Faizi et al., 2011)
[3]Synthetic chemicals	*Mixture of* glucosinolates and active myrosinase	0.00125, 0.0025, 0.005, 0.01 mg/ml glucosinolate stock, myrosinase 0.05, 0.3, 1.0 mg/ml, dissolved in DMSO, water and DMSO as controls	*In vitro* assay	*Globodera rostochiensis*	Nematicidal	(Buskov et al., 2002; Aires et al., 2009)
[2]Sticky nightshade *Solanum sisymbriifolium* and [2]Nightshade *Solanum nigrum*	Root exudates compounds	NA	*In vitro* assay	*Pratylenchus goodeyi*	Nematicidal	(Gouveia et al., 2014)

[1]study on effect of both plant and specific metabolites in root exudates of the plant, [2]study on effect of plant, [3]study on effect of synthetic compound on nematodes, NA indicate not applicable.

Globodera pallida (Farnier et al., 2012). In a bioassay, ethephon, methyl jasmonate, salicylic acid, indole acetic acid, and mannitol showed positive chemotaxis of *G. pallida* J2s (Fleming et al., 2017). In *in vitro* nematode infection assays, *Arabidopsis* mutants with a strigolactone signaling pathway deficiency reduced attraction and invasion by the cyst nematode *Heterodera schachtii* compared to the wildtype plant (Escudero Martinez et al., 2019).

Repellants

The identification of compounds that repel plant parasitic nematodes (**Table 1**) may be an important step towards better control measures. Second stage juveniles of three root knot nematodes (*Meloidogyne hapla, M. javanica*, and *M. incognita*), were highly attracted to root tips of both tomato plants and barrel clover (*Medicago truncatula*). However, ethylene signaling deficient mutants roots attracted more nematodes than the wild type (Čepulyte et al., 2018). Similarly, *Arabidopsis* in which ethylene synthesis was inhibited were more attractive to *M. hapla*, but ethylene-overproducing mutants roots were less attractive. Roots of an ethylene insensitive tomato mutant were also more attractive (Fudali et al., 2012). These examples suggest that either ethylene or products of ethylene-responsive pathways generally repel root-knot nematodes.

For cyst nematodes, the influence of ethylene is less clear-cut. Roots of soybean and *Arabidopsis* treated with ethylene synthesis inhibitor attracted more Soybean cyst nematodes (*Heterodera glycines*), and significantly more nematodes penetrated the roots of ethylene synthesis inhibited plants. On the other hand, ethylene insensitive mutants roots of *Arabidopsis* accessions were more attractive to *H. glycines* than the wild type (Hu et al., 2017). Ethylene-overproducing *A. thaliana* mutants roots were hypersusceptible to beet cyst nematode (*Heterodera schachtii*), and ethylene-insensitive mutants were less susceptible to *H. schachtii* (Wubben et al., 2001). Similarly, ethylene treated plant roots were more attractive to soybean cyst nematode and were infected much faster, resulting in a higher infection rate (Kammerhofer et al., 2015). Future studies should therefore aim to reveal, whether root-knot nematode repellence is governed directly by ethylene or by other compounds in ethylene responsive pathways. Further, more information on the impact of ethylene or ethylene pathways on cyst nematodes and other plant parasitic nematode taxa will disclose, if ethylene is a broad-spectrum repellant.

TABLE 3 | Root metabolites affecting nematode hatching.

Plant species/ synthetic chemicals	Root metabolites	Assay conditions and concentrations	Test system	Nematodes affected	Effect	Reference
[1]Welsh onion *Allium fistulosum*	4-hydroxybenzeneethanol	0.3, 0.6, 1.2, 2.4, 4.8, 9.6, and 19.2 mM dissolved in ethanol, sterile distilled water as control	*In vitro* assay	*M. incognita*	Hatching inhibitor	(Li et al., 2018)
[2]Tall fescue *Festuca arundinacea*	Root exudates compounds	Exudate concentrations 0.35, 0.70, 1.05, and 1.40% (w/v) diluted in sterile distilled water, sterile distilled water as control	*In vitro* assay	*M. incognita*	Hatching inhibitor	(Meyer et al., 2013)
[2]Sticky nightshade *Solanum sisymbriifolium* (cv. Sharp)	Root exudate compounds	Exudates of tested plants; root exudates of tomato and distilled water were used as controls, final concentrations NA	*In vitro* assay	Root knot nematodes	Hatching inhibitor of *M. arenaria, Meloidogyne chitwoodi, M. hapla* and *M. hispanica; J2s* not able to penetrate the plant and highly resistant	(Dias et al., 2012)
[2]Sticky nightshade *Solanum sisymbriifolium* (cv. Sis 4004)	Root exudate compounds	Exudates of tested plants; root exudates of tomato and distilled water were used as controls, final concentrations NA	*In vitro* assay	Root knot nematodes	Hatching inhibitor of *M. arenaria, M. hapla* and *M. hispanica; J2s* not able to penetrate the plant and highly resistant	(Dias et al., 2012)
[2]*Sticky nightshade Solanum sisymbriifolium* (cv. Pion)	Root exudate compounds	Exudates of tested plants; root exudates of tomato and distilled water were used as controls, final concentrations NA	*In vitro* assay	Root knot nematodes	Hatching inhibitor of *M. arenaria, M. hapla* and *M. javanica; J2s* not able to penetrate the plant and highly resistant	(Dias et al., 2012)
[1]Brown mustard *Brassica juncea*	2-propenyl isothiocyanate	0.002%	*In vitro* assay	*G. pallida*	Hatching inhibitor	(Brolsma et al., 2014)
[2]Potato *Solanum tuberosum*	Root exudate compounds	0.0001, 0.001, 0.01, 0.1, 1 mg/ml root leachate and water and buffer as control	*In vitro* assay	*G. rostochiensis* and *G. pallida*	Hatching stimulants and attractants	(Devine and Jones, 2003)
[3]Synthetic chemical	Picrolonic acid	0.4 to 4mM	*In vitro* assay	*Heterodera rostochiensis* (Syn. *Globodera rostochiensis*)	Hatching stimulant	(Clarke and Shepherd, 1966)
[1]Potato *Solanum tuberosum*	Alpha-solanine and alpha-chaconine, picrolonic acid, sodium metavanadate, sodium orthovanadate, and sodium thiocyanate	Dilution over a 10-concentration logarithmic series of 10 mg/ml stock solutions, water as control	*In vitro* assay	*G. rostochiensis* and *G. pallida*	Hatching stimulants	(Byrne et al., 2001)
[1]Tomato *S. lycopersicum* and [1]Potato *Solanum tuberosum*	Solanoeclepin A	NA	NA	*G. rostochiensis* and *G. pallida*	Hatching stimulant	(Schenk et al., 1999)
[2]Sticky nightshade *Solanum sisymbriifolium* and [2]Nightshade *Solanum nigrum*	Root exudate compounds	Cysts were soaked in tap water for 1 week and then exposed to 25 ml root diffusate	*In vitro* assay	*G. rostochiensis* and *G. pallida*	Hatching stimulants, reduced number of J2s per cyst and decreased population	(Scholte, 2000)
[2]Sticky nightshade *Solanum sisymbriifolium*	Root exudate compounds	Treatments included control containers with soil without plants, containers with soil with susceptible potato cv. Bintje and containers with soil with *S. sisymbriifolium*	Plant-soil greenhouse experiment	*G. pallida*	Hatching stimulants, reduced number of J2s per cyst and decreased population	(Timmermans et al., 2006)
[2]Sticky nightshade *Solanum sisymbriifolium* cvs	Root exudate compounds	Cysts were exposed to 5 mL root exudate of each plant cultivar, Potato (*S. tuberosum* cv. Désirée) root	*In vitro* assay	*G. rostochiensis* and *G. pallida*	Hatching stimulants; inhibit reproduction	(Dias et al., 2017)

(Continued)

TABLE 3 | Continued

Plant species/ synthetic chemicals	Root metabolites	Assay conditions and concentrations	Test system	Nematodes affected	Effect	Reference
Melody, Pion, Sis 4004 and Sis 6001		exudate as positive control, tap water as negative control.				
[2]Nightshade *Solanum nigrum*	Root exudate compounds	Cysts exposed to root exudate solutions collected 3 weeks after emergence (1:1, 1:10, 1:100, or 1:1000 exudate to distilled water).	*In vitro* assay	*Globodera tabacum*	Hatching stimulants	(LaMondia, 1995)
[1]Soybean *Glycine max* and [1]Kidney bean *Phaseolus vulgaris*	Glycinoeclepin A,	10^{-11}-10^{-12} g glycinoeclepin A mL^{-1}	*In vitro* assays	*H. glycines*	Hatching stimulants	(Fukuzawa et al., 1985; Masamune et al., 1987)
[2]Sunn hemp *Crotalaria juncea* and [2]Showy rattlepod *Crotalaria spectabilis*	Root exudate metabolites	One milliliter of root leachate added to 1 ml of egg suspension (600 eggs/ml), water as control.	Hatching assessed *in vitro*; penetration and development assessed in pot experiment, population response assessed in field trial	*H. glycines*	Hatching stimulants; stop nematode development in roots	(Kushida et al., 2003; Warnke et al., 2008)
[3]Synthetic chemical	Picrolonic acid	0.1 to 4mM	*In vitro* assay	*H. glycines, H. tabacum, H. cruciferae*	Stimulated hatching except cabbage cyst nematode	(Clarke and Shepherd, 1966)

[1]study on effect of both plant and specific metabolites in root exudates of the plant, [2]study on effect of plant, [3]study on effect of synthetic compound on nematodes, NA indicate not applicable.

Still, most specific compounds have only been demonstrated to repel a single nematode taxon in a single plant species (**Table 1**). It is therefore premature to draw general conclusions on which repellents most efficiently repel different species of plant parasitic nematodes.

Some plant metabolites that efficiently repelled plant parasitic nematodes in assays without plants could be objects of further investigation. For instance, *Capsicum annum* (pepper) root derived thymol, either alone or combined with other root volatiles of *C. annum* induced negative chemotaxis of both root-knot, cyst and stubby root nematodes (Kihika et al., 2017). Some flavonoids also repel plant parasitic nematodes, but for these compounds, the effect appears to be more species dependent. For instance, the flavonoids kaempferol, quercetin, and myricetin repelled *Radopholus similis* and *Meloidogyne incognita*, but not *Pratylenchus penetrans.* Other flavonoids, e.g. luteolin, daidzein, and genistein repelled *R. similis*, but had no effects on and *M. incognita* and *P. penetrans* (Wuyts et al., 2006).

NEMATICIDAL/INHIBITORY ROOT COMPOUNDS

Some plant taxa, e.g. *Tagetes* and Brassicaceae, are well-known for their production and release of nematode-defensive compounds (**Table 2**). Inclusion of plants with high contents of nematicidal or nematode inhibitory compounds in cropping systems as a sanitation strategy has thus received considerable research attention and is also applied in practice. Further, the application of purified nematicidal plant-derived compounds may be an efficient nematode management strategy (Zanón et al., 2014).

Allium species (e.g. leek, onion, and garlic) contain sulfur amino-acid precursors in their cytoplasm, which upon cellular degradation are broken down by the enzyme allinase to a new volatile organic compound, dimethyl disulfide (DMDS) (Haroutunian, 2015). Purified DMDS killed J2 juveniles and reduced egg masses and gall formation of *M. incognita* on tomato roots (Silva et al., 2018). DMDS is now available as a commercial biofumigant, which applied in tobacco field trials significantly reduced both *M. incognita* and *Heterodera* spp. infestation (Zanón et al., 2014). Similarly, DMDS was also effective against potato cyst nematodes and root knot nematodes on potato and tomato plants (Coosemans, 2005), *M. hapla* and *P. penetrans* on strawberry (López-Aranda et al., 2009), cyst nematode (*H. carotae*), and *M. incognita* on carrot (Curto et al., 2014), on tomato plants (Myrta et al., 2018), and on watermelon (Leocata et al., 2014).

Glucosinolates are one of the most frequently studied groups of defensive secondary metabolites in plants. Upon cellular disruption, e.g. wounding by nematodes, the thioglucoside linkage is hydrolyzed by endogenous enzymes (myrosinases), resulting in the formation of products (e.g. isothiocyanate, thiocyanate, nitrile, epithionitrile, oxazolidine-2-thione) that are active against herbivores and pathogens (Fahey et al., 2001; Lambrix et al., 2007; Santolamazza-Carbone et al., 2014). For instance, glucosinates purified from Brassicaceae (*Brassica napus, B. rapa, B. carinata, Lepidium sativum, Raphanus sativus,* and *Sinapis alba*) were not toxic to J2s of the cyst nematode *Heterodera schachtii* in their original form, but enzymatic hydrolysis products of glucosinolates (isothiocyanate, sinigrin, gluconapin, glucotropeolin, dehydroerucin) were lethal to

the nematode (Lazzeri et al., 1993). Similarly, 11 glucosinolates and their degradation products did not affect J2s of the root knot nematode *M. incognita*, but myrosinase hydrolysis products (gluconasturtiin, glucotropaeolin, glucoerucin, and sinigrin) were highly toxic (Lazzeri et al., 2004). Other studies also report *that* glucosinolates are only lethal to the cyst nematode *Globodera rostochiensis* in the presence of myrosinase (Buskov et al., 2002; Aires et al., 2009).

Pyrrolizidine alkaloids (PAs) are secondary metabolites in different species within the Asteraceae, Boraginaceae, Fabaceae, Convolvulaceae, Orchidaceae, and Apocynaceae (Rizk, 1991; Trigo, 2011), notably *Crotalaria* spp., *Ageratum* spp. and *Senecio* spp. (Asres et al., 2004; Flores et al., 2009; Thoden et al., 2009b; Stegelmeier, 2011). PA was found toxic to the plant parasitic nematodes *Meloidogyne incognita*, *Heterodera schachtii* and *Pratylenchus penetrans* (Thoden et al., 2009a). In a bioassay, a PA [Loline (N-formylloline)] in root exudates of Tall fescue, was reported nematicidal to J2s of *Pratylenchus scribneri* (Bacetty et al., 2009). Likewise, PA-containing *Ageratum houstonianum* and *Senecio bicolor* inhibited *M. hapla* reproduction totally, but *M. hapla* reproduced on other PA-containing species (Thoden et al., 2009b; Vestergård, 2019). Hence, adoption of PA-containing plants for the management of plant parasitic nematodes must rely on careful selection of suitable species.

α-terthienyl, usually abundant in marigold (*Tagetes* spp.) tissue, is one of the most extensively studied nematicidal compounds (Morallo-Rejesus and Decena, 1982; Nivsarkar et al., 2001; Hamaguchi et al., 2019). Although the negative effect of *Tagetes* on plant parasitic nematodes is not always achieved in the field (Hooks et al., 2010), many trials demonstrated the mitigating potential of *Tagetes*. For instance, *T. patula* reduced *P. penetrans* densities for three consecutive years and alleviated damage to strawberries from *P. penetrans*, and these effects lasted longer than the effect of chemical soil fumigation (Evenhuis et al., 2004). Likewise, intercropping tomato plants with *T. patula* reduced *Meloidogyne* reproduction and root galling (Tringovska et al., 2015). Under *in vivo* conditions, root diffusate of marigold (*Tagetes patula* cv. Single gold) did not affect hatching pattern, migration and penetration of *M. chitwoodi* and *P. penetrans* compared to tomato roots, but reduced *M. chitwoodi* and totally prevented *P. penetrans* reproduction (Nježić et al., 2014). Other bioactive *Tagetes* compounds may be involved in nematode suppression (Hooks et al., 2010), but there is no doubt that α-terthienyl is a potent nematicide. For instance, α-terthienyl exhibited 100% mortality of *Heterodera zeae* at concentrations of 0.125% after 24h (Faizi et al., 2011). The biological activity of α-terthienyl increases greatly upon near-UV exposure, resulting in the production of biocidal singlet oxygen (Marles et al., 1992). When the nematode penetrates the root, α-terthienyl is activated by root peroxidases in the absence of light (Gommers and Bakker, 1988), and it has therefore been assumed that α-terthienyl is only effective *in planta* and not active in the soil (Hooks et al., 2010). However, recently it has been reported that α-terthienyl actually was nematotoxic *ex planta* without photoactivation (Hamaguchi et al., 2019).

Root exudate benzoxazinoids, such as 2, 4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), mainly produced in rye and other cereals, have been found toxic against mixed stages of American dagger nematode *Xiphinema americanum* (Zasada et al., 2005). Rye cultivars with higher root concentrations of methoxy-substituted benzoxazinoids had the lowest numbers of *M. incognita* eggs. These cultivars were therefore suggested for soil incorporation as green manure to protect against root-knot nematodes (Zasada et al., 2007). In a greenhouse trial, soil infested with the root-knot nematode *Meloidogyne incognita* was treated with DIBOA (2,4-Dihydroxy-2H-1,4-benzoxazin-3(4H)-one) at concentrations ranging from 1.1 to 18 μg/g dry soil, and *M. incognita* egg production in cucumber roots decreased significantly at the highest concentration (Meyer et al., 2009).

PLANT DEFENSE ELICITED BY PLANT PARASITIC NEMATODES

Plant hormones are widely studied as defensive strategies against plant parasitic nematodes. The jasmonate (JA) plant hormones play a key role during early plant defense against the soybean cyst nematode *Heterodera schachtii* (Kammerhofer et al., 2015), the columbia root-knot nematode *Meloidogyne chitwoodi* (Vieira dos Santos et al., 2013), *M. incognita* (Fujimoto et al., 2011) and *M. hapla* (Gleason et al., 2016), the root lesion nematode *Pratylenchus neglectus* and the oat cyst nematode *Heterodera avenae* (Soriano et al., 2004). Jasmonate also cross talks with other plant hormones and defend the plants from nematode attacks. For instance, plants treated with Me-jasmonate and ethephon (an ethylene analogue) made plants more defensive against the rice root knot nematodes *Meloidogyne graminicola* compared to untreated plants (Nahar et al., 2011).

Low levels of salicylic acid (SA) may be sufficient for basal and Mi-1 resistance to root knot nematodes (Bhattarai et al., 2008). SA application induced resistance to the clover cyst nematode *Heterodera trifolii* in white clover (Kempster et al., 2001), and to *Meloidgyne incognita* (Molinari, 2016), *M. javanica* (Moslemi et al., 2016) and *M. chitwoodi* in tomato plants (Vieira dos Santos et al., 2013). Abscisic acid (ABA) plays a complex role in plant defense responses. While it promotes resistance in some plant–pathogen interactions, it enhances susceptibility in others (Lim and Lee, 2015). For instance, in one study, the reproduction of root knot nematode *M. incognita* on potato roots was much lower in ABA-sprayed plants compared to control plants (Karimi, 1995), whereas exogenous application of ABA on rice plants enhanced gall formation by *Meloidogyne graminicola* and did not impair nematode development (Kyndt et al., 2017). Similarly, exogenous ABA application reduced tomato plant resistance against *Meloidogyne javanica* (Moosavi, 2017). These varied responses to ABA application shows the complex role of ABA in plant defense against nematodes.

HATCHING STIMULATION/INHIBITION

Cyst nematodes (*Globodera* spp. and *Heterodera* spp.) generally have a very narrow host spectrum, and as active infective juveniles only have limited storage energy they will starve and die within a relatively short period without access to a suitable host. However, the encysted, dormant eggs stay viable for years to decades, and are triggered to hatch and re-activate by host-specific hatching stimulants. Application of hatching stimulants in the absence of host plants is therefore a promising strategy for efficient reduction of cyst nematode populations. A number of hatching stimulants have been identified (**Table 3**), e.g. picrolonic acid, which induce hatching of *Heterodera rostochiensis* (Syn. *Globodera rostochiensis*), *H. glycines* and *H. tabacum* (Clarke and Shepherd, 1966), glycinoeclepin, which induces *H. glycines* hatching (Masamune et al., 1987), and solanoeclepin, sodium thiocyanate, alpha-solanine, and alpha-chaconine, which induce hatching of *G. pallida* and *G. rostochiensis* (Schenk et al., 1999; Byrne et al., 2001).

MODES OF ACTION OF ROOT METABOLITES IN NEMATODES

For most plant-derived metabolites we know very little about their molecular mode of action in nematodes. Here we present examples of the few available studies on the effect of root metabolites on plant parasitic nematode gene expression.

Arabidopsis thaliana root exudates were found to affect gene expression in *M. incognita* J2 larvae, prior to physical contact and penetration of the root. Sixty three candidate genes were identified, which were differentially expressed within one hour of exudate exposure, providing the evidence that root exudates induce changes in *M. incognita* gene expression (Teillet et al., 2013). Later it was demonstrated that tomato root exudates differentially upregulated four candidate parasitism genes, namely calreticulin (*crt-1*), β-1,4 endoglucanase-1 (*eng-1*), cathepsin L cysteine protease (*cpl-1*), fatty acid retinol binding protein (*far-1*), and venom allergen-like protein (*vap-1*) in preparasitic *Meloidogyne hispanica* J2s (Duarte et al., 2015). However, the identity of the exudate compounds that elicit enhanced expression of these genes is still unknown. Their disclosure could potentially lead to the breeding of varieties with low levels of parasitism gene activators and thus new and improved strategies against root knot nematode infection.

Plant parasitic nematodes secrete plant cell wall degrading enzymes in order to penetrate the host (Mitsumasu et al., 2015). The first evidence that plant cell wall components and host root exudates regulate the expression of genes encoding such enzymes was published recently (Bell et al., 2019). Hence, *Pratylenchus coffeae* treated with cellulose or xylan or with root exudates of host plants up-regulated the gene expression of β-1,4-endoglucanase (*Pc-eng-1*) or β-1,4-endoxylanase (*Pc-xyl*) respectively. The study also confirmed that the expression of these two genes is important for root penetration (Bell et al., 2019).

Host exudate induction of cyst nematode egg hatching obviously involves exudate activation of genes in the dormant juvenile cyst nematode. For instance, in hydrated *G. rostochiensis* cysts, 8 h of potato root exudate exposure, resulted in up-regulation of a gene encoding for a transmembrane metalloprotease. This enzyme is known to activate/inactivate peptide hormones and may be involved in a cascade of events leading to hatching. After 48 h of exudate exposure, *G. rostochiensis* had 278 differentially expressed genes, several of which are known effector genes (Duceppe et al., 2017).

These studies on root knot and root lesion cyst nematodes demonstrate that root exudates influence gene expression of pre-parasitic phase/early phase of nematodes, but which exudate components are involved in gene regulation remains to be disclosed.

We know very little about the molecular responses elicited by nematode attractants, repellents, and toxins within the nematode body, but several studies demonstrate that root exudates regulate the expression of *flp* genes. *flp* genes encode FMRFamide-like peptides, a diverse group of neuropeptides, involved in nematode feeding, reproductive and locomotive behavior, and thus play a pivotal role in nematode chemotaxis. For instance, low concentrations (0.5–2.0 mM) of lauric acid from crown daisy (*Chrysanthemum coronarium*) root exudates attract *Meloidogyne incognita*, while higher lauric acid concentration (4.0mM) repels the nematode. This response is probably elicited by lauric acid's concentration-dependent regulation of *Mi-flp-18* gene expression (Dong et al., 2014). Moreover, two other active compounds, namely palmitic acid and linoleic acid derived from castor root exudates, was found to repel *M. incognita* and inhibited the expression of *Mi-flp-18* and *Mi-mpk-1* (mitogen-activated protein kinase) genes in a concentration-dependent manner (Dong et al., 2018). Silencing *G. pallida flp* genes (*Gp-flp-1, -6, -12, -14,* or *-18*) resulted in aberrant behavioral phenotypes, which further confirms that *flp* genes play key roles in motor function and suggests that *flp* gene silencing can be a novel plant parasite control strategy (Kimber et al., 2007). Furthermore, for *C. elegans* loss of *flp-1* and *daf-10* also disrupted different neurons in the neural circuits (Buntschuh et al., 2018).

Since marigold derived chemical α-terthienyl is expected to exert nematicidal action in the soil, a recent study investigated the molecular action of this chemical without photoactivation. This study revealed that α-terthienyl is nematicidal also in the dark, albeit the effect is higher when the compound is photoactivated. Further, it was established that α-terthienyl is an oxidative stress-inducing chemical that effectively penetrates the nematode hypodermis and suppresses *gst-4* (glutathione S-transferase) and *sod-1* (superoxide dismutase) gene expression. This results in restricted production of glutathione S-transferase and superoxide dismutase, which are necessary for nematode defense responses (Hamaguchi et al., 2019).

EFFECTS OF ROOT METABOLITES ON NON-TARGET NEMATODES

Due to their direct impact on crop yield and quality, agricultural researchers and practitioners pay more attention to and are more aware of plant parasitic nematodes than the many species of soil dwelling non-herbivorous nematodes. Nevertheless, non-

herbivorous nematodes perform functions that are essential to natural as well as agro-ecosystems.

With protozoa, microbial feeding nematodes are the principal microbial grazers in terrestrial ecosystems. They regulate the size, activity, and functioning of bacterial and fungal populations (Ingham et al., 1985; Rønn et al., 2012; Thakur and Geisen, 2019). The most important impact of nematode microbial grazing is the enhanced turnover and mineralization of plant nutrients, notably nitrogen, and thus stimulation of plant growth. Further, because bacterial taxa vary in terms of food quality and ingestibility for nematodes (Bjørnlund et al., 2012) nematode grazing changes the composition of bacterial communities (Xiao et al., 2014). Likewise, the quality as food for fungal feeding nematodes varies between fungal species (Chen and Ferris, 1999), and fungal feeding nematodes preferentially select certain fungal species (Ruess et al., 2000). Thus, nematode grazing on root-associated microorganisms probably modulates the plant-microbiome functional interactions.

Entomopathogenic nematodes, i.e. nematodes of the two genera *Heterorhabditis* and *Steinernema* are harmless to plants and under some circumstances even plant beneficial. *Heterorhabditis* spp. and *Steinernema* spp. are closely associated with species of insect lethal *Protorhabdus* and *Xenorhabdus* bacteria. The nematode enters the body cavity of the susceptible insect larvae, where the associated bacteria are released, multiply and eventually kill the insect. Bacteria growing on the insect cadaver then serve as food for the nematodes (Poinar and Grewal, 2012). Given the right conditions, entomopathogenic nematodes reduce the abundance of root detrimental insect larvae (Toepfer et al., 2009). Because non-herbivorous nematodes execute a variety of central functions in terrestrial systems, it is relevant to consider, if plant metabolites with adverse effects on plant parasitic nematodes similarly reduce the survival or performance of non-target nematodes.

Marigold (*Tagetes patula* cv. Single Gold) root exudates did not influence the migration rate of dauer juveniles of the entomopathogenic nematode *Steinernema feltiae* towards *Galleria mellonela* larvae. Even exposing dauer juveniles of *S. feltiae* for 24 hours to marigold root diffusate resulted in higher penetration rate of EPN compared to soil leachate (Nježić et al., 2010). In a bioassay, germinated seeds of marigold attracted *Steinernema carpocapsae*. Neither did aqueous root extracts of marigold adversely affect EPN infectivity, but synthetic α-terthienyl at concentrations of 20 and 40ppm significantly reduced the numbers of nematodes that infected insect hosts. This indicates that higher doses of this chemical may affect entomopathogenic nematodes (Kaya and Kanagy, 2010). *Caenorhabditis elegans*, a bacterial feeding nematode, was as sensitive as *Meloidogyne incognita* and 10 times more sensitive than *Pratylenchus penetrans* to α-terthienyl *in vitro* (Kyo et al., 1990; Hamaguchi et al., 2019).

In field trials, McSorley et al. (2009) and Wang et al. (2011b) assessed soil abundances of bacterial, fungal- and omnivorous/predatory nematodes as well as oribatid mites, predatory mites, and collembola after sunn hemp (*Crotalaria juncea*) and marigold cover crops and a fallow period. The comparison to a fallow treatment is not ideal for the evaluation of potential negative effects on microbial feeding nematodes and decomposer microarthropods, as the input of organic substrate for saprotrophic organisms is of course considerably lower in fallow than planted systems. It is therefore not surprising that sunn hemp mulching temporarily increased densities of bacterial and fungal feeding nematodes and microarthropods compared to fallow soil. The densities in marigold planted plots were as low as in the fallow plots at all sampling times, and could, although not unequivocally suggest that marigold prevented growth of microbial feeding nematodes. However, in other field experiments, densities of non-herbivorous nematodes were higher in marigold-planted than fallow plots and comparable to compost treated soil (Wang et al., 2011a; Korthals et al., 2014). Hence, the results from the limited number of field and *in vitro* studies on non-target effects of marigold root on non-herbivorous nematodes vary from negative effects over no effects to positive effects. Rigorously controlled experiments including the assessment of realistic and super-realistic concentrations of marigold metabolites on single nematode species and mixed communities are needed to reach more firm conclusions on their significance for the compositon and functioning of non-target nematode communities in practice.

The root exudates of green pea (*Pisum sativum*) induced reversible quiescence in all EPN species (*Heterorhabditis bacteriophora*, *H. megidis*, *Steinernema feltiae* and *S. carpocapsae)* tested. However, this response was concentration dependent, and diluted root exudates did not induce quiescence, but enhanced EPN activity and insect infectivity. The diluted root exudates still reduced the activity of the soybean cyst nematode *Heterodera glycines* and the root-knot nematode *Meloidogyne incognita* (Hiltpold et al., 2015). It is extremely difficult to determine which root exudate concentrations the nematodes are exposed to *in vivo*, but the authors presume that rhizosphere concentrations are below the level for quiescence induction (Hiltpold et al., 2015).

Soil incorporation of *Brassica carinata* reduced root-knot nematode *M. chitwoodi*, but also disrupted the ability of entomopathogenic nematodes *S. feltiae* and *S. riobrave* to control Colorado potato beetles (*Leptinotarsa decemlineata*). This study exposes the challenges of integrating biofumigation and biocontrol approaches in managing plant parasitic nematodes and other pests (Henderson et al., 2009). In a long-term field trial, incorporation of biofumigant Brassicaceae neither reduced plant parasitic nor total nematode abundances, but, as could be expected from the increased input of dead plant material to the soil, total densities of nematodes increased moderately (Korthals et al., 2014). Similarly, the biofumigant yellow mustard had none to slightly negative effects on plant parasitic nematodes and none to slightly positive effects on microbial feeding, omnivorous and predatory nematodes (Valdes et al., 2012).

Testing the effects of four different PAs, Thoden et al. (2009a) found that the mobility of bacterial feeding *Rhabditis* sp. was unaffected after 20 h exposure, but after a week's exposure, especially one PA (monocrotaline) reduced *Rhabditis* sp.

mobility. Further, monocrotaline repelled *Rhabditis* sp. Another PA (heliotrine) reduced *Rhabditis* sp. development and reproduction. The slug- and snail infecting nematode *Phasmarhabditis hermaphrodita* was completely unaffected by PAs (Thoden et al., 2009a; Thoden et al., 2009b). Further, it has been shown that the use of PA-producing *Crotalaria* species as soil amendment increases the abundance of free-living nematodes (Wang et al., 2007). In banana orchard, sunn hemp (*Crotalaria juncea* L.) thus consistently suppressed the population of the plant parasitic nematode *Radopholus similis*, while supporting the highest numbers of beneficial nematodes (bacterivorous, fungivorous, omnivorous, and predaceous species) (Henmi and Marahatta, 2018).

APPLICATION OF ROOT METABOLITES IN NEMATODE MANAGEMENT— CHALLENGES AND OPPORTUNITIES

A considerable number of bioactive root compounds with documented effect on the behavior, development or even survival (**Tables 1**–**3**) on plant parasitic nematodes have been identified. Potentially, this knowledge may be applied in practical control or management of plant parasitic nematodes. However, in most cases, the effects were only tested on a single nematode species, which is only natural given that the field is still at an early explorative stage. As compounds that affect a wider spectrum of plant parasitic nematode species and other pest organisms will of course be most interesting in practice, investigations of the effects on a broader spectrum of nematode taxa are highly pertinent. On the other hand, there is the risk that metabolites that are active against a broad spectrum of plant parasitic nematodes will also have unwanted negative effects on non-parasitic nematodes. Given the importance of microbivorous and entomopathogenic nematodes for nutrient turnover and the control of root herbivorous insects, respectively, priorities should be given to metabolites that are harmless to or even beneficial for non-target nematodes.

The ability to identify and find suitable hosts is essential for all plant parasitic nematodes. Thus, the nematodes navigate and differentiate between different plant species aided by attractant and repellent plant metabolites. A range of exudate metabolites have been identified as attractant of *Meloidogyne incognita* and a few as attractants of *Pratylenchus* and cyst nematode species. Potentially, intercropping susceptible crops with nematode-resistant or even nematode-suppressive highly attractant plants can alleviate damage of crop plants (Dong et al., 2014). We therefore foresee that continuous efforts aimed at identifying and screening for nematode chemical attractants exuded by plant species with additional desirable intercropping properties will contribute to improved management of plant parasitic nematodes.

Vice versa, the identification of quite a few compounds that repel several species of plant parasitic nematodes is of course interesting. Selection and breeding of plant cultivars that release high levels of repellents could prove a promising strategy for reduced nematode infection levels. With this perspective, it is interesting that repellent compounds have been identified in economically important host plants, where root knot nematodes are particularly problematic, e.g. tomato plants (Yang et al., 2016; Kirwa et al., 2018). In horticultural production, grafting is becoming a common alternative to lengthy breeding for pathogen resistance. Aboveground parts of cultivars with desirable traits, e.g. high yields and/or high fruit quality are grafted on rootstocks of close relatives that are resistant to specific soil-borne pests or pathogens, e.g. nematodes (Kawaide, 1985; Oka et al., 2004; Galatti et al., 2013; Thies et al., 2015). We propose that grafting on highly nematode repellent rootstocks could be a similar fast track to reduce yield losses caused by nematodes.

The list of nematicidal root compounds is long, and again, many were only tested in a single study. However, some compounds such as lauric acids, DMDS, pyrrolizidine alkaloids (PAs), α-terthienyl and products of myrosinase-catalyzed hydrolysis of glucosinolates have proved lethal against both root knot, cyst and lesion nematodes. Accordingly, plants with high levels of some of these compounds are integrated in strategies for nematode control. In practice, the efficacy of crops that produce nematicidal root metabolites can be quite unpredictive. For instance, the myrosinase-catalyzed hydrolysis of glucosinolates must be induced by cell wall disruption such as insect or nematode attack, or during the degradation of dead plant parts. Further, myrosinase activity is temperature-dependent (Ploeg and Stapleton, 2001; Lopez-Perez et al., 2005). Whether the elicited production and release to the rhizosphere soil is sufficient to significantly reduce populations of plant parasitic nematodes may thus be very context-dependent. The application of purified DMDS, the nematicide produced after enzymatic conversion of sulfur amino-acid precursors in *Allium* species (Zanón et al., 2014; Haroutunian, 2015) demonstrates that better and more reliable nematode control may be obtained by direct application of the bioactive phytochemical.

The long persistence time for encysted eggs poses a special challenge for the control of cyst nematodes. To our knowledge, the potential for applying hatching stimulants for cyst nematode control or eradication remains to be investigated. However, inclusion of hatching-stimulating non-host plants in crop rotation schemes or even termination of host crops before cyst nematode reproduction is a way to reduce the density of persistent cysts in infected soil (Scholte, 2000; Dandurand and Knudsen, 2016). Field trials, where *Solanum sisymbriifolium* (sticky nightshade) induce hatching of *Globodera*, which are unable to fulfill their life cycle on *S. sisymbriifolium*, demonstrate that hatching induction in the absence of susceptible hosts efficiently reduce the density of resting, viable cysts in the soil (Scholte and Vos, 2000).

For the majority of specific root compounds their effects on nematodes were demonstrated *in vitro* (**Tables 1**–**3**). *In vitro* experiments are obviously necessary to provide conclusive evidence for the effect of candidate compounds. Meanwhile, the extrapolation of results obtained *in vitro* to rhizosphere and *in planta* conditions is not straightforward. For many compounds, information about their concentrations in roots, and in particular

in rhizosphere soil, is sparse, and the concentration in the rhizosphere may easily be 1000 times lower than in the roots (Kudjordjie et al., 2019). We thus emphasize that effects detected at low concentrations, i.e. μM, ppm or lower, in assays covering a concentration gradient are more likely to reflect mechanisms that are relevant under realistic conditions.

It is evident that the physicochemical complexity of soil systems, be it in the greenhouse or in the field, can alter or altogether eliminate the effects exposed *in vitro*. For instance, NPK fertilization reduces PA concentrations in *Senecio* spp. roots (Hol, 2011) suggesting that plants regulate the production of defense compounds according to nutrient availability. Other abiotic factors such as soil moisture and structure modulate the diffusion and thus distribution of plant-derived volatile compounds in the soil matrix (Hiltpold and Turlings, 2008), and nematode responses to volatile cues therefore probably also depend on these factors. Hence, it will be interesting to see the outcome of more *in vivo* studies, e.g. assessing the impact of plant mutant lines that are impaired in or over-expressing specific metabolic pathways in different soil types and variable abiotic conditions.

Further, the production of many compounds that interfere with nematode performance are elicited by the presence of other above- or belowground herbivores. For instance, root exudates of potato plants exposed to the aphid *Myzus persicae* reduced egg hatching and interfered with cyst gene expression in *Globodera pallida* (Hoysted et al., 2018). Clearly, when plant chemical defense belowground depends on plant interactions with aboveground organisms it becomes difficult to forecast and rely on the efficacy of the belowground defense in practice. Moreover, symbionts also appear to regulate production of defense compounds; e.g. arbuscular mycorrhizal fungi reduced benzoxazinoid concentrations in wheat roots, while root infection by *Pratylenchus neglectus* was 47%–117% higher on mycorrhizal than non-mycorrhizal plants (Frew et al., 2018).

The biological complexity of soil systems is immense, and as such, root metabolites interact with multiple different soil organisms. Hence, most of the signaling and nematicidal compounds mentioned in this review also affect other pathogens. For instance, methyl salicylate and salycilic acids play multiple roles in plant defense against a long range of pathogens (Hammerbacher et al., 2019; Maruri-Lopez et al., 2019), and DIMBOA and other benzoxazinoids are also antagonistic towards insect pests and fungal pathogens (Fomsgaard et al., 2004). Plants that produce compounds that are antagonistic towards multiple pests and pathogens may be particularly valuable components in sanitation strategies.

The inevitable microbial turnover of exuded metabolites challenges the extrapolation of results from *in vitro* studies to soil conditions. For instance, while DIBOA proved an efficient nematicide in aqueous assays, the fast microbial turnover and possible absorption to soil particles reduced the nematicidal effect in soil (Meyer et al., 2009). Hence, microbial degradation may inactivate or reduce the activity of biocidal compounds in soil.

To complicate matters even more, the effects of some plant-derived chemicals vary between nematode species. Ethylene generally repels root knot nematodes, whereas at least some cyst nematodes are attracted by ethylene. Hence, in fields infested with both types of nematodes ethylene may on one hand reduce root-knot nematode infection, but on the other hand release cyst nematodes from competition from root-knot nematodes. Clearly, most research on root metabolite effects on nematodes has focused on the nematodes that are most damaging to economically important crops; i.e. root knot nematodes, particularly *M. incognita*, cyst nematodes and root lesion nematodes (Jones et al., 2013), and we know very little about the effects of root metabolites on ectoparasitic nematodes. Endo- and ectoparasitic nematode species may compete and dominate under different environmental conditions (Brinkman et al., 2004; Vestergård et al., 2004), and it is therefore worth considering if strategies targeting endoparasites can enhance populations of and crop damages exerted by ectoparasitic nematodes.

CONCLUDING REMARKS

With the current and past withdrawal of chemical nematicides, insights on root chemical impacts on plant parasitic nematodes are important contributions to alternative strategies for plant parasitic nematode management. This review exposes that a diverse array of root chemicals across a range of plant taxa are potentially involved in nematode host location, egg hatching, and survival. Some of this insight is already exploited in practice, e.g. the use of nematicidal plants or application of purified plant-derived nematicides, and the use of egg hatching stimulating plants for cyst nematode management. However, we believe that there are opportunities for improved exploitation of root metabolites for nematode management. Many plant metabolites that have clearly proved bioactive under highly controlled laboratory conditions are less reliable in the field. The discrepancy between *in vitro* and *in vivo* efficacy may reflect that compounds were tested at unrealistic concentrations in the lab, that soil physicochemical factors reduce their activity, that they are inactivated by fast microbial degradation etc. There is therefore a need for experimental investigations to bridge the gap between highly controlled laboratory experiments and realistic field conditions. Such experiments, e.g. plant-soil mesocosm experiments, should aim to clarify the fate and activity of key metabolites in the rhizosphere as a function of e.g. soil texture, structure, pH, temperature, and microbial activity to facilitate better prediction of the bioactive efficacy in different real-world contexts.

Within the last five years, the first studies to establish how root chemicals regulate genetic expression in plant parasitic nematodes have been published. We foresee that further disclosures of nematode molecular responses to specific root metabolites, and thus in-depth understanding of their modes of action will help us predict which compounds hold the largest potential for efficient control or management of plant parasitic nematodes.

In general, we propose to focus further developments on nematicidal plant species and compounds with biocidal activity against a broad spectrum of parasitic nematodes as well as other pathogenic and pest organisms to facilitate integrated

management of diverse plant pests. Hence, selection of and further breeding for cultivars that produce high levels of nematicidal or repellent metabolites can result in more nematode-resistant cultivars. In high value horticultural crops, e.g. tomato cultures grafting on rootstocks with high production of repellent or nematicidal metabolites could be a fast alternative to the lengthy breeding process.

As non-herbivorous soil nematodes contribute to decomposition processes, inorganic nutrient availability and even control of herbivorous insect larvae, it is relevant to consider if metabolites that are toxic to plant parasitic nematodes exert negative effects on these related non-target organisms. Only a limited number of studies assessed how plant metabolites that repress plant parasitic nematodes affect non-target nematodes, and with varied outcome. Therefore, we cannot draw any general conclusions on the sensitivity of microbivorous and entomopathogenic nematode species or communities to root metabolites.

AUTHOR CONTRIBUTIONS

MS and MV contributed equally to the conception and preparation of the manuscript.

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Effects of Pest Management Practices on Soil Nematode Abundance, Diversity, Metabolic Footprint and Community Composition Under Paddy Rice Fields

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The wide-scale adoption of transgenic crops has aroused public concern towards potential impacts to the ecological services of soil fauna, such as soil nematodes. However, few studies has examined whether the cultivation of transgenic rice would pose greater threats to soil nematode community and associated ecological functions than insecticides application. Moreover, what are determinants of soil nematode community in paddy fields remains unclear. During a 3-year field study, rhizosphere soil samples of transgenic-Bt rice, its counterpart non-Bt parental rice and not-Bt rice with insecticides application were taken at four times in the rice developmental cycle using a random block design with three replications for each treatment. We hypothesized that the effects of pest management practice on soil nematode abundance and metabolic footprint change with trophic group and sampling time. We also predicted there were significant differences in structure and composition of soil nematode community across the three treatments examined and sampling times. In agreement with our expectation, the effects of pest management practice on nematode abundance and metabolic footprints depend on trophic group and sampling time. However, pest management practice exerted no apparent effect on nematode diversity and community composition. Soil nutrient availability and C:N molar ratio are the primary regulating factor of soil nematode community in rice paddy fields. In conclusion, our findings implied that changes in abundance, diversity, metabolic footprints associated with the crop growth stage overweighed the application of *Bt* rice and insecticides. The cultivation of *Bt* rice Huahui-1 exerted no measurable adverse effect on soil nematode community in rhizosphere soil over 3 years of rice cropping.

Keywords: soil nematode community, management practice, *Bt* rice cultivation, insecticides application, paddy field

INTRODUCTION

As an important cereal crop worldwide, rice (*Oryza sativa* L.) provides staple food and nutrition for about 50% of the global population (Lu and Snow, 2005). However, the yield of rice in China suffers severe losses mainly from four major lepidopteran pests, namely, the rice striped stem borer *Chilo suppressalis* (Crambidae), the yellow stem borer *Scirpophaga incertulas* (Crambidae), the pink stem borer *Sesamia inferens* (Noctuidae) and the rice leaf roller *Cnaphalocrocis medinalis* (Crambidae) (Chen et al., 2011). To reduce the yield loss resulting from pest damage, China devoted great effort in developing insect-resistant rice using transgenic technology and has developed multiple *Bt* rice lines (Chen et al., 2011). Empirical evidence supported *Bt* rice can result in an evidently decreased application of pesticides, and thus benefit human health and the environment (Huang et al., 2005; Chen et al., 2011). However, the *Cry1Ab/Ac* protein, which was continuously produced within plant tissue of *Bt*-rice, could be released through root exudates during growth and persistent in the rhizosphere in paddy soils (Wang et al., 2018). Therefore, it might affect the activity, structure, diversity of soil fauna, and interactions among components of soil food webs, which in turn would influence soil fertility and plant productivity. Accordingly, there is a pressing need to understand the impacts of *Bt* rice on the sustainability of agricultural ecosystem.

Nematodes play a pivotal role in ecosystem functions because they hold a central role in soil food webs and actively participate in ecological processes, such as decomposition, nutrient cycling, and pest suppression (Neher, 2010). Moreover, they react rapidly to disturbances and enrichment (Bongers, 1999). Therefore, the abundance and community composition of soil nematode have been commonly used to indicate soil health condition and soil functions (Bongers and Ferris, 1999; Neher, 2001). Ample available evidence support that soil nematodes are related to soil physicochemical properties (Briar et al., 2011; Godefroid et al., 2013), microorganisms (Kaplan and Noe, 1993), management practice (Biederman et al., 2008; Neher, 2010; Naira and Ngouajiob, 2012; Liu et al., 2016), and stochastic factors. The frequency of pesticide application in fields of *Bt* rice is lower than that of its counterpart non-Bt rice (Li et al., 2014). However, the *Cry1Ab/Ac* of *Bt* rice can enter soil ecosystems through root exudations, pollen and plant residue inputs, and thus nematodes are likely to be exposed to bioactive proteins from transgenic *Bt* rice because of their trophic position in soil food webs (Ruf et al., 2013). Moreover, the *Cry1Ab/Ac* proteins of Bt rice might accumulate in rhizosphere soil (Liu et al., 2018a). However, no definite conclusions have been drawn about whether *Bt* rice can negatively affect soil nematode. Besides, there are compounding factors affecting the persistence and accumulation of Bt-toxins, and the activity, persistence, and accumulation of *Bt* protein would vary with Bt toxin origin, plant species, and environment condition (Icoz and Stotzky, 2008; Chen et al., 2017a). For example, the activity and persistence of the insecticidal protein varies depending on climatic conditions (Zwahlen et al., 2003) and soil properties (Tapp et al., 1994; Saxena et al., 1999). Thus, whether *Bt* rice cultivation would affect soil nematodes and associated ecosystem functioning remains elusive, particularly under paddy field condition.

The primary objectives of this study were: 1) to quantify the effects of contrasting pest management practices on soil quality with soil nematode community as integrative bio-indicator in rice soil under paddy field condition; and 2) to explore the main driving force of soil nematode community in paddy fields. We also hypothesized that the effect size of *Bt* rice cultivation on soil nematode abundance and metabolic footprints changes with trophic group and plant developmental stage (exactly sampling time), because the interaction strength between plant and soil nematodes might vary with trophic group and the microclimate as well as the quantity and quality of plant detritus entering soils might vary with plant phenology. We also hypothesized that the impact of pesticides application on abundance, diversity, and footprints of soil nematode community would be greater than that of *Bt* rice cultivation, since pesticides are of broad spectrum whereas the toxicity of *Bt* rice is specific to target insects.

MATERIALS AND METHODS

Site Description

A 3-year field experiment was carried out at the Jiangxi Academy of Agricultural Sciences, Jiangxi province (N28°21′91.4′′, E115° 55′49.7′′), where the research of transgenic plants is permitted. The mean annual temperature and the mean annual rainfall of this region were 17.6°C and 1,624.4 mm, respectively. The soil here is loamy sand, and the physicochemical properties of the field soils were as follows: total soil organic carbon (SOC), 21.79 g/kg; total nitrogen (TN), 1.17 g/kg; total phosphorous (TP), 0.84 g/kg; total potassium (TK), 21.01 g/kg; available nitrogen (AN), 84.82 mg/kg; available phosphorous (AP), 5.82 mg/kg; and available potassium (AK), 49.17 mg/kg.

Plant Materials

The transgenic *Bt* rice line (Huahui-1) and its corresponding non-transgenic counterparts (Minghui-63), which were provided by Huazhong Agricultural University, Wuhan, China were used in this experiment. The Huahui-1 is a certificate-granted insect-resistant rice line, it contains a fused *cry1Ab/1Ac* gene under the control of the rice actinI promoter showing a high level of expression of the δ-endotoxin, and thus is highly resistant to target insects under controlled conditions (Tu et al., 2000); while Minghui-63 is an elite Indica cytoplasmic male sterile restorer line developed in China in the early 1980s from the IR-30×Gui-630 hybrid combination.

Experimental Design

The experiment was conducted using a randomized block design with three treatments including *Bt* rice, non-Bt with normal pesticide application, and non-Bt rice without pesticide application. For each treatment, there were three replications. Seeds were sown in mid-May, and seedlings were transplanted in mid-June in 2012, 2013, and 2014. The distance between rice seedlings was approximately 30 cm, which is commonly used in fields by farmers in this region. Rice was cultivated using standardized agricultural management practices except for pesticide application during the growing season. Weeds were

controlled by hand-weeding every 3 weeks. The pests in *Bt* rice and one non-Bt fields were trapped by yellow sticky traps, whereas another non-Bt fields was controlled with insecticides spray.

Soil Sampling and Analysis

Soil samples were collected on June 11, August 11, September 11, and October 11 of 2012, 2013, and 2014 at the seedling, booting, heading, and maturing stage of rice. We also took soil samples on April 11 of 2013, 2014, and 2015 corresponding to prophase of sowing. Before each sampling time, the flood irrigation was stopped for 3 days. In each plot, eight soil cores (2.5 cm in diameter) were collected randomly between rice rows, mixed thoroughly and pooled as a composite sample. The samples were placed in plastic bags and stored in a portable cooler for transport to the laboratory. Each soil sample was divided into two subsamples of equal volume. One was passed through a 2-mm sieve to remove root fragments and other organic debris in soil and stored at 4°C before testing. This subsample was used to determine soil water content (SWC) and soil nematode community. The other subsample was air-dried and sieved before using for the analysis of SOC and other soil properties including TN, TP, AN, AP, and AK.

Nematodes were extracted from 100 g of field-moisture soil from each subsample using the minor modified cotton-wool filter method depending on the nematode mobility (Liang et al., 2009) with three technical replicates for each sample within 1 week after sampling. After a 48 h of extraction, nematodes were killed through heating, and thus fixed and preserved in 4% formaldehyde. Subsequently, 10% of the individuals (but not less than 200 individuals, if possible) were additionally identified to genus level based on nematode morphology of the stoma, stylet, basal bulb, and teeth characteristics following Bongers (1994) and Ahmad and Jairajpuri (2010) at a 400× or 1,000× magnification. When the individual number of nematodes in a sample was less than 200, all the specimens were identified. All identified specimens were assigned into bacterial feeders, fungal feeders, plant feeders, omnivores and predators (Yeates et al., 1993), and c-p classes (Bongers and Bongers, 1998).

SOC content was measured using the hot oxidation with potassium dichromate and sulfuric acid (Yeomans and Bremner, 1988). Soil TN was determined by the semimicro-Kjeldahl method after soil was digested by $HClO_4$ and HF. The contents of TP, TK, AK, and AP were assayed using inductively coupled plasma mass spectrometry (ICP-MS) analysis (IRIS Intrepid II XSP system; Thermo Electric Co., USA). The content of TP in soil was digested with H_2SO_4-HClO4 solution at 250°C and determined by the molybdenum-blue colorimetric method (Walker and Adams, 1958). The content of soil AP was extracted with 0.5 M $NaHCO_3$ solution and determined by the molybdenum-blue colorimetric method (Olsen and Sommers, 1982).

Statistical Analysis

The effect of pest management practice on abundance of soil nematodes was examined with generalized linear models, whereas that on diversity and metabolic footprints of soil nematode community was examined with general linear models. Subsequently, differences in composition of soil nematodes communities among treatments were investigated with non-metric multidimensional scaling (NMDS). Additionally, statistical differences in nematode community composition within plots of contrasting management practices and sampling times were assessed with "adonis" function based on 9,999 restricted permutations of the data. Finally, environmental variables which are related to the ordination of NMDS were selected with "envfit" function in vegan packages. The multivariate analyses including NMDS, adonis, and envfit, were performed with the "MASS" and the "vegan" package in R version 3.3.1 (R, 2016).

RESULTS

Abundance of Soil Nematodes

In 2012 and 2013, pest management practice exerts significant effects on abundances of all trophic groups and total nematode abundance (**Table 1**). In 2014, pest management practice exerts significant effects on the abundances of bacterivores (Wald χ^2 = 10.466, $P = 0.005$), omnivores (Wald $\chi^2 = 35.613$, $P < 0.001$), and total nematode abundance (Wald $\chi^2 = 15.847$, $P < 0.001$), whereas negligible effect on abundances of other trophic groups (**Table 1**). Specifically, the pesticide application greatly reduced nematode abundance, whereas *Bt* rice did not. Additionally, nematode abundances of the non-Bt rice fields with pesticides application were significantly lower in comparison with those of *Bt* rice fields. However, there were significant interactive effects of management *sampling time, suggesting the effect of pest management practice on nematode abundance depended on sampling time.

Diversity of Soil Nematode Community

Pest management practice was found be of no significant effect on the taxa richness, Margalef richness index, Shannon–Weaver diversity index, Simpson dominance index, and Pielou evenness index of soil nematode community (**Table 2**). The diversity indices vary greatly across sampling times, but they are independent of management practice.

Metabolic Footprints of Soil Nematodes

The effects of management practice on metabolic footprints of soil nematodes vary depending on year and trophic group examined. In 2012, no detectable difference in metabolic footprints examined among contrasting management practices of rice was observed. In 2013, herbivore footprint and predator footprint changed with management practice. In 2014, management practice exerted a significant effect on composite footprint, structure footprint, bacterivore footprint and omnivore footprint of soil nematode community. Additionally, metabolic footprints of soil nematodes varied with sampling time in most cases. However, the effects of management practice on metabolic footprints did not change with sampling time in most cases (**Table 3**).

Determinant of Soil Nematode Community in Paddy Fields

The nematode taxa richness in soils of rice paddy fields in the 2012, 2013 and 2014 growing season was 25, 21, and 22,

TABLE 1 | Summary of generalized linear models testing the effects of management practice, sampling time, and their interaction on abundance of soil nematodes in rice fields of contrasting management practices during 2012–2014 growing seasons.

Year	Variable	Parameter	Management	Sampling time	Interaction
	All	*df*	2	3	6
2012	Herbivores	Waldχ^2	52.651	1,280.983	31.362
		P	**<0.001**	**<0.001**	**<0.001**
	Bacterivores	Waldχ^2	5.487	579.862	98.021
		P	**< 0.0064**	**<0.001**	**<0.001**
	Fungivores	Waldχ^2	17.351	311.93	60.462
		P	**<0.001**	**<0.001**	**<0.001**
	Omnivores	Waldχ^2	6.657	16.856	33.12
		P	**0.036**	**0.001**	**<0.001**
	Predators	Waldχ^2	33.941	143.775	105.445
		P	**<0.001**	**<0.001**	**<0.001**
	Total	Waldχ^2	139.497	1,262.296	96.973
		P	**<0.001**	**<0.001**	**<0.001**
2013	Herbivores	Waldχ^2	95.563	254.174	25.206
		P	**<0.001**	**<0.001**	**<0.001**
	Bacterivores	Waldχ^2	93.906	509.785	106.725
		P	**<0.001**	**<0.001**	**<0.001**
	Fungivores	Waldχ^2	169.109	250.999	189.805
		P	**<0.001**	**<0.001**	**<0.001**
	Omnivores	Waldχ^2	115.414	483.906	19.799
		P	**<0.001**	**<0.001**	**0.003**
	Predators	Waldχ^2	339.198	1,176.536	51.586
		P	**<0.001**	**<0.001**	**<0.001**
	Total	Waldχ^2	824.096	1,215.524	109.763
		P	**<0.001**	**<0.001**	**<0.001**
2014	Herbivores	Waldχ^2	0.527	400.864	68.868
		P	0.768	**<0.001**	**<0.001**
	Bacterivores	Waldχ^2	10.466	274.138	21.468
		P	**0.005**	**<0.001**	**0.002**
	Fungivores	Waldχ^2	5.156	785.646	15.91
		P	0.076	**<0.001**	**<0.001**
	Omnivores	Waldχ^2	35.613	848.327	49.619
		P	**<0.001**	**<0.001**	**<0.001**
	Predators	Waldχ^2	2.541	297.212	86.6.
		P	0.281	**<0.001**	**<0.001**
	Total	Waldχ^2	15.847	1,668.931	155.559
		P	**<0.001**	**<0.001**	**<0.001**

The bolded results indicate difference in variables across treatments are statistically significant (p value < 0.05).

TABLE 2 | Summary of general linear models testing the effects of management practice, sampling time, and their interaction on the diversity of soil nematode community in rice fields during 2012–2014 growing seasons.

Year	Variable	Management practice			Sampling time			Interaction		
		df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
2012	S	2	1.353	0.277	3	8.885	**<0.001**	6	0.480	0.817
	SR	2	0.426	0.658	3	5.338	**0.006**	6	0.289	0.937
	H'	2	0.284	0.755	3	4.494	**0.012**	6	1.166	0.357
	λ	2	0.992	0.386	3	4.018	**0.019**	6	1.158	0.361
	J	2	0.885	0.426	3	2.566	0.078	6	1.074	0.405
2013	S	2	0.553	0.582	3	4.573	**0.011**	6	0.482	0.815
	SR	2	1.662	0.211	3	2.539	0.080	6	0.789	0.588
	H'	2	0.386	0.684	3	4.409	**0.013**	6	0.386	0.881
	λ	2	0.340	0.715	3	2.758	0.064	6	0.435	0.848
	J	2	0.925	0.410	3	3.348	**0.036**	6	0.665	0.679
2014	S	2	0.051	0.951	3	28.675	**<0.001**	6	0.624	0.709
	SR	2	0.107	0.899	3	13.517	**<0.001**	6	0.568	0.751
	H'	2	0.019	0.982	3	18.867	**<0.001**	6	0.418	0.860
	λ	2	0.096	0.909	3	14.538	**<0.001**	6	0.498	0.804
	J	2	0.032	0.969	3	13.684	**<0.001**	6	0.65	0.69

S, taxa richness; SR, Margalef richness index; H', Shannon–Weaver diversity index; λ, Simpson dominance index; J, Pielou evenness index.
The bolded results indicate difference in variables across treatments are statistically significant (p value < 0.05).

respectively (**Appendix A**). With exception of the 2013 growing season, sampling date ($P < 0.05$) rather than management practice ($P > 0.05$) affect community composition of soil nematodes (**Table 4**; **Figure 1**). In 2012, the community composition of soil nematodes in rice fields correlate with available nutrients (including available N, P, and K), TN and TP in soils (**Figure 2A**). In 2013, the community composition of soil nematodes in rice fields correlate with available N, SOC, TN, TP, and C:N molar ratio in soils and soil microbial biomass (**Figure 2B**). In 2014, the community composition of soil nematodes in rice fields correlate with available N, available K, SOC, TN, TP, and C:N molar ratio in soils and soil microbial biomass (**Figure 2C**).

DISCUSSION

The wide-scale adoption of *Bt* crops evidently reduces chemical insecticides application (Huang et al., 2005; Lu et al., 2012). However, *Bt* crops pose potential environmental risk to soil biota because they might change the quantity and quality of nutrient inputs and directly impact soil organisms with toxic activity of *Bt* protein. To date, whether the positive effects of the cultivation of *Bt* crop due to decreased application of chemical insecticides may outweigh its potential negative effects is unclear. Moreover, the responses of soil nematode to certain *Bt* transformation event was found to be context-specific, and thus a case-by-case study is necessary. China has developed multiple *Bt* rice lines to control target lepidopteran pests and boost agricultural productivity. For example, the Huahui-1 has been granted bio-safety certificates by the Chinese authorities since 2009. However, it has not been allowed to enter the Chinese agricultural system due to public concern towards its potential adverse environmental effects and food safety. Therefore, to enhance the communication about science related issues of *Bt* rice to public maybe a promising alternative of the commercial production of *Bt* rice in near future. The present study utilized the scare opportunity to figure out the potential environmental risks of the Huahui-1 on agricultural ecosystem.

No Apparent Impact of *Bt* Rice Cultivation on Soil Nematodes

In contrast to most of available studies addressing potential environment risks of *Bt* corn or cotton on soil nematode community in terrestrial ecosystems (Manachini and Lozzia, 2002; Griffiths et al., 2005; Höss et al., 2011; Karuri et al., 2013; Li and Liu, 2013; Neher et al., 2014; Yang et al., 2014; Höss et al., 2015; Liu et al., 2015; Čerevková et al., 2017; Liu et al., 2018b), we evaluated those of *Bt* rice in aquatic ecosystem. Our study revealed no significant adverse effect of *Bt* rice cultivation on soil nematode communities under paddy field. Our results are in agreement with other studies suggesting that no impact of *Bt* crops cultivation on either soil nematodes (Manachini and Lozzia, 2002; Griffiths et al., 2005; Höss et al., 2011; Karuri et al., 2013; Li and Liu, 2013; Neher et al., 2014;

TABLE 3 | Summary of general linear models testing the effects of management practice, sampling time and their interaction on the metabolic footprints of soil nematode community in rice fields during 2012–2014 growing seasons.

Year	Variable	Management practice			Sampling time			Interaction		
		df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
2012	CMF	2	0.598	0.558	3	2.014	0.139	6	0.176	0.981
	EMF	2	0.666	0.523	3	13.665	**<0.001**	6	0.315	0.923
	SMF	2	0.392	0.68	3	0.779	0.517	6	0.18	0.98
	HMF	2	1.858	0.178	3	28.639	**<0.001**	6	0.885	0.521
	FMF	2	0.363	0.699	3	4.642	**0.011**	6	0.87	0.531
	BMF	2	0.653	0.53	3	9.148	**<0.001**	6	0.221	0.966
	PMF	2	2.051	0.151	3	11.165	**<0.001**	6	1.350	0.274
	OMF	2	0.451	0.642	3	2.205	0.114	6	0.041	1.00
2013	CMF	2	2.479	0.105	3	5.797	**0.004**	6	0.224	0.965
	EMF	2	2.010	0.156	3	2.856	0.058	6	1.099	0.392
	SMF	2	2.381	0.114	3	6.301	**0.003**	6	0.236	0.96
	HMF	2	4.366	**0.024**	3	7.869	**0.001**	6	0.550	0.765
	FMF	2	3.004	0.069	3	2.940	**0.054**	6	1.098	0.392
	BMF	2	1.948	0.164	3	3.612	**0.028**	6	0.894	0.515
	PMF	2	3.864	**0.035**	3	7.91	**0.001**	6	0.684	0.665
	OMF	2	1.471	0.25	3	4.82	**0.009**	6	0.113	0.994
2014	CMF	2	13.256	**<0.001**	3	64.812	**<0.001**	6	9.164	**<0.001**
	EMF	2	2.86	0.077	3	85.813	**<0.001**	6	1.985	0.108
	SMF	2	13.192	**<0.001**	3	61.778	**<0.001**	6	9.174	**<0.001**
	HMF	2	0.604	0.555	3	44.736	**<0.001**	6	3.755	**0.009**
	FMF	2	0.563	0.577	3	113.882	**<0.001**	6	0.676	0.67
	BMF	2	4.026	**0.031**	3	43.949	**<0.001**	6	4.254	**0.005**
	PMF	2	0.465	0.634	3	10.072	**<0.001**	6	1.442	0.24
	OMF	2	21.684	**<0.001**	3	106.575	**<0.001**	6	13.422	**<0.001**

BMF, bacterivore footprint; CMF, composite footprint; EMF, enrichment footprint; FMF, fungivore footprint; HMF, herbivore footprint; OMF, omnivore footprint; PMF, predator footprint; SMF, structure footprint.

The bolded results indicate difference in variables across treatments are statistically significant (p value < 0.05).

TABLE 4 | Similarity of soil nematode community in Bt rice field without insecticide application, non-Bt rice fields with and without insecticide application.

Year	Source	*df*	*F*	*P*
2012	Management practice	2	1.02	0.399
	Sampling time	3	10.86	**0.001**
	Interaction	6	0.76	0.784
2013	Management practice	2	2.13	**0.042**
	Sampling time	3	4.93	**0.001**
	Interaction	6	0.48	0.988
2014	Management practice	2	1.53	0.168
	Sampling time	3	25.86	**0.001**
	Interaction	6	1.7	**0.053**

The bolded results indicate difference in variables across treatments are statistically significant (p value < 0.05).

Yang et al., 2014; Höss et al., 2015; Liu et al., 2015; Chen et al., 2017a; Chen et al., 2017b; Liu et al., 2018b) or other aquatic fauna (Wang et al., 2013; Li et al., 2014; Wang et al., 2014). One possible interpretation is that the concentration of *Cry* protein in field soils is lower than the threshold value which is adverse to the reproduction and growth rate of soil nematodes. There are several reasons for the low *Cry* protein concentration in the rice paddy soil. On one hand, the concentration of *Cry* proteins that enter soils is relatively lower or it is diluted in water. On the other hand, *Cry* proteins may be readily degraded (Wang et al., 2007; Liu et al., 2018b). In a study with the same rice cultivar (Huahui-1), the authors suggest that *Cry1Ab/Ac* proteins could not be detected in irrigation water of the rhizotrons (Liu et al., 2018a).

More Noticeable Change in Soil Nematodes Community With Time in Comparison With Application of Pesticides and *Bt* Rice

In contrast to most of available studies, which only focused on the difference in parameters of soil nematode community between *Bt* and non-Bt corn or cotton (Manachini and Lozzia, 2002; Griffiths et al., 2005; Höss et al., 2011; Karuri et al., 2013; Li and Liu, 2013; Neher et al., 2014; Yang et al., 2014; Höss et al., 2015; Liu et al., 2015; Čerevková et al., 2017; Chen et al., 2017a; Chen et al., 2017b), we set up another treatment that is the not-Bt crop field with chemical pesticides application when necessary. This is necessary because non-Bt crop refuge has been proposed as a promising strategy for delaying the resistance development towards *Bt* crop (Gould, 2000; Tabashnik and Carriere, 2017). However, previous studies reported that planthoppers move from *Bt* to adjacent non-Bt rice fields (Chen et al. 2003a; Wang et al., 2018) and that fungal diseases and non-target pests still required to be controlled with pesticides application when full yield is expected to be achieved (Wang et al., 2010). This indicates even if the cultivation of *Bt* rice cultivation has a great potential to reduce the use of broad-spectrum chemical insecticides (High et al., 2004), pesticides application in *Bt* rice is still required. However, pesticide sprays is neither effective nor environment-friendly (Frutos et al., 1999). For example, one study demonstrates that pesticide application results in biodiversity loss in rice-based ecosystems (Halwart, 2008).

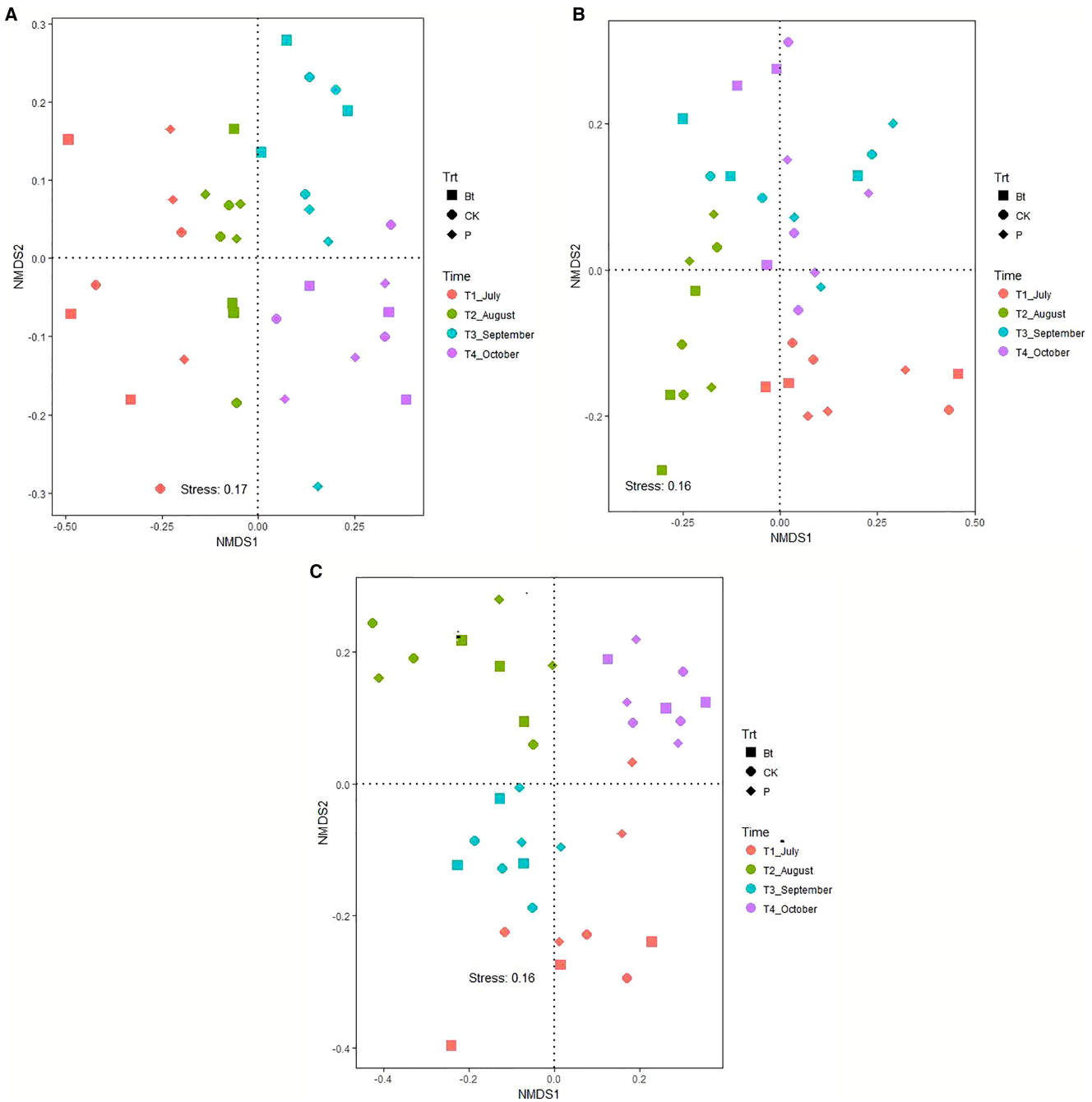

FIGURE 1 | Non-metric multidimensional scaling (NMDS) illustrating the divergence in community composition of soil nematodes in soils of rice fields under different mangement practices in 2012 **(A)**, 2013 **(B)**, and 2014 **(C)**. The plot of NMDS was produced using the Bray-Curtis distance. CK: non-Bt rice without pesticide application; Bt: Bt rice without pesticide application; P: non-Bt rice with pesticide application.

Another studies reported that pesticide (acetochlor) application at a high dose could reduce plant parasites (Waliyar et al., 1992), the *Helicotylenchus* (Todd et al., 1992), the *Pratylenchus* in soil (Shukla and Haseeb, 1996; Zhang et al., 2010). Chen et al. (2003a) indicated that pesticide (acetochlor) impacted the numbers of total nematode and trophic groups in a Chinese soybean field. Pen-Mouratov and Steinberger (2005) found pesticides application reduced the numbers of total nematodes, fungivores, and bacterivores in a desert system. Wada and Toyota (2008) reported pesticide effectively suppressed a *Pratylenchus penetrans*, but had little impact on free-living nematodes in a pot test. The discrepancy across studies may relate to pesticide type, application dose, and sensitivity of nematode species. Our findings highlight that the effects

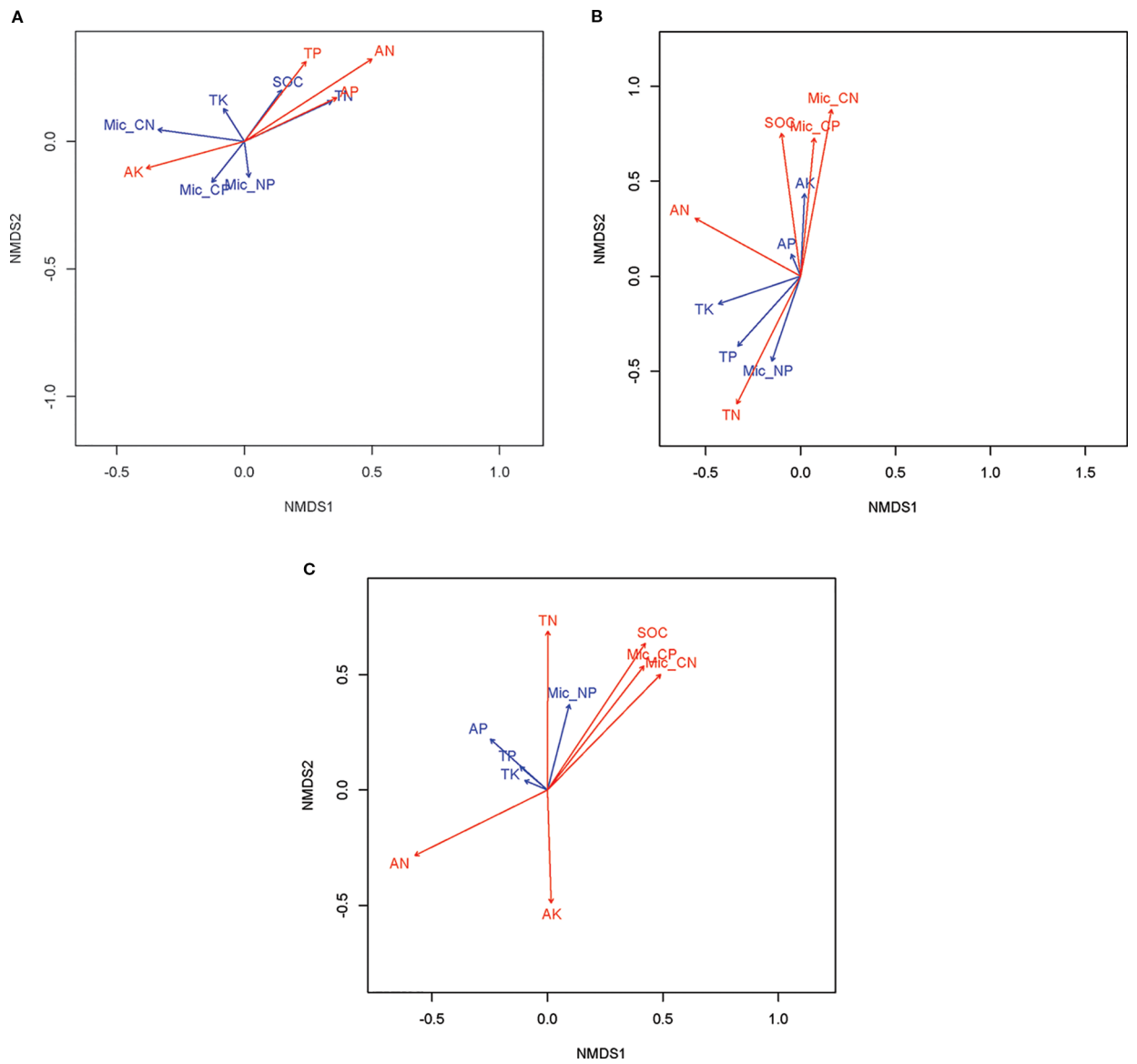

FIGURE 2 | Non-metric multidimensional scaling (NMDS) revealing the major shaping factors of soil nematode community composition in soils of rice fields under different mangement practices in 2012 **(A)**, 2013 **(B)**, and 2014 **(C)**. The plot of NMDS was produced using the Bray-Curtis distance e. The red vectors show the correlation between soil attributes and soil nematode community composition are significant at $P < 0.05$.

of year and sampling date were more pronounced than that of application of pesticide and Bt rice.

Factors Shaping Soil Nematode Community in Rice Fields Under Paddy Condition

Against our expectation and most studies that reporting agricultural management practice greatly impact soil nematode community (Sánchez-Moreno et al., 2009; Palomares-Rius et al., 2012), agricultural management did not change nematode community composition in the present study (**Table 4**). In agreement with the finding of similar issue focusing on plant parasites in Spain olive fields (Archidona-Yuste et al., 2020), soil was the following most influential factor driving nematode communities in rice paddy fields. Numerous studies suggest that nematode was closely related to soil physiochemical factors (Ferris et al., 1996; Wardle et al., 2004; Archidona-Yuste et al., 2020). For example, SOC and TN were found to be significantly associated with total nematodes abundance (Ma et al., 2018). In the present study, available N, SOC, and TN are important factors shaping soil nematode community in rice fields under paddy condition (**Figure 2**). Concerning the found close relationship between N and nematode, a reasonable explanation is that nematodes affect nitrogen availability both directly and indirectly (Ingham et al., 1985; Neher, 2010).

Regarding the observed close correlation between soil nematode community and soil C/N and microbial biomass, it is not surprisingly. After all, C/N is a promising soil quality indicator, and it has been reported as an important influencing factor of terrestrial nematode biodiversity (Mulder and Maas, 2017).

CONCLUSION

In summary, our results give the public the actual environmental risk of *Bt* rice cultivation to soil nematodes and their associated ecological functions. All the findings support that there is no deleterious effect of *Bt* rice cultivation on soil nematode community over 3 years. Additionally, the effects of year and sampling date on nematode variables examined in rhizosphere soil were more pronounced than that of application of insecticides and *Bt* rice. Soil nutrient availability and C:N molar ratio are the primary regulating factor of soil nematode community in rhizosphere soil of rice under paddy field condition.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

AUTHOR CONTRIBUTIONS

BY, FC and FG designed the experiment. QC, XL, YL and FC did the experiment. BY, WQ, and LH analyzed the data. All the authors wrote and improved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00088/full#supplementary-material

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Microbes Attaching to Endoparasitic Phytonematodes in Soil Trigger Plant Defense Upon Root Penetration by the Nematode

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Root-knot nematodes (*Meloidogyne* spp.) are among the most aggressive phytonematodes. While moving through soil to reach the roots of their host, specific microbes attach to the cuticle of the infective second-stage juveniles (J2). Reportedly, the attached microorganisms affect nematodes and reduce their performance on the host plants. We have previously shown that some non-parasitic bacterial strains isolated from the cuticle of *Meloidogyne hapla* in different soils affected J2 mortality, motility, hatching, and root invasion. Here we tested whether cuticle-attached microbes trigger plant defenses upon penetration of J2. In *in vitro* assays, *M. hapla* J2-attached microbes from a suppressive soil induced pathogen-associated molecular pattern-triggered immunity (PTI) in tomato roots. All tested PTI-responsive defense genes were upregulated after root invasion of J2 with attached microbes, compared to surface-sterilized J2, particularly the jasmonic acid-mediated PTI marker genes *TFT1* and *GRAS4.1*. The strain *Microbacterium* sp. K6, that was isolated from the cuticle, significantly reduced root invasion when attached to the J2. Attached K6 cells supported plant defense and counteracted suppression of plant basal defense in roots by invaded J2. The plant response to the J2-attached K6 cells was stronger in leaves than in roots, and it increased from 1 to 3 days post inoculation (dpi). At 1 dpi, the plant responded to J2-attached K6 cells by ameliorating the J2-triggered down-regulation of defense genes mostly in roots, while at 3 dpi this response was systemic and more pronounced in leaves. In a reactive oxygen species (ROS) assay, the compounds released from J2 with attached K6 cells triggered a stronger ROS burst in tomato roots than the compounds from nematodes without K6, or the metabolites released from strain K6 alone. Leaves showed a 100 times more sensitive response than roots, and the metabolites of K6 with or without J2 induced strong ROS bursts. In conclusion, our results suggest the importance of microorganisms that attach to *M. hapla* in suppressive soil, inducing early basal defenses in plants and suppressing nematode performance in roots.

Keywords: root-knot nematode, MAMP, pathogen-triggered immunity, induced systemic resistance, suppressive soil, antagonistic bacteria

INTRODUCTION

Plant-parasitic nematodes feed on many crops worldwide and, if not maintained below the damage threshold in soil, they can cause enormous yield losses with an estimation of 100 billion dollars a year (Coyne et al., 2018). The genus *Meloidogyne* [root-knot nematodes (RKN)] has gained substantial attention in science based on the damage it causes to plants (Jones et al., 2013). The RKN infective second-stage juveniles (J2) enter the host roots at the elongation zone and, upon moving through the apoplast, inject effector proteins through a hollow stylet into the host cells to manipulate their functions and suppress plant defense (Sijmons et al., 1991; Gheysen and Mitchum, 2011). In the vascular cylinder, the effectors induce a repeated mitosis of the surrounding cells without cytokinesis, resulting in the formation of 5 to 7 giant cells that become permanent feeding sites of the nematode (Gheysen and Mitchum, 2011; Siddique and Grundler, 2018). Up to the formation of permanent feeding sites, RKN J2 do not cause huge damage during the migration phase in roots (Williamson and Hussey, 1996). It was shown that, unlike cyst nematodes, RKN do not activate damage-associated molecular pattern-triggered immunity (Shah et al., 2017), but cause expression of certain defense genes (Williamson and Hussey, 1996; Teixeira et al., 2016). Thus, they employ insidious strategies to reduce their recognition by plant. However, the life cycle of RKN can be disrupted by beneficial soil microorganisms that induce plant defenses (Adam et al., 2014a). Root border cells and root exudates play a crucial role in shaping the microbial communities in the rhizosphere, eventually resulting in a positive plant-soil feedback (Bertin et al., 2003; Bais et al., 2006; Doornbos et al., 2012; De-la-Peña and Loyola-Vargas, 2014; Ma et al., 2017). Evidence is accumulating that certain beneficial microorganisms suppress plant-parasitic nematodes by inducing systemic resistance (ISR) in plants (Reitz et al., 2000; Munif et al., 2001; Siddiqui and Shaukat, 2004; Dababat and Sikora, 2007; Adam et al., 2014b; Selim et al., 2014; Martínez-Medina et al., 2017b; Elhady et al., 2018; Kang et al., 2018; Silva et al., 2018; Topalović and Heuer, 2019). In the initial phase of microbially induced plant defense the pattern recognition receptors localized on the plant cell membranes recognize molecular structures on the microbe/pathogen surface that are referred to as microbe/pathogen-associated molecular patterns (MAMP/PAMP) (Jones and Dangl, 2006). This leads to the activation of PAMP-triggered immunity (PTI) in plants, i.e. rapid release of reactive oxygen species (ROS) (Lamb and Dixon, 1997), callose deposition on the cell walls (Millet et al., 2010), mitogen-activated protein kinase (MAPK) signaling (Wu and Baldwin, 2010), and multiple transcriptional changes (Campos et al., 2014; Coninck et al., 2015). Phytohormones such as jasmonic acid (JA), ethylene, and salicylic acid (SA) orchestrate the plant defense responses often in an antagonistic manner (Pieterse et al., 2012), but their crosstalk seems to be essential to induce systemic resistance by beneficial microorganisms (Wees et al., 2008; Martínez-Medina et al., 2013; Martínez-Medina et al., 2017a). For instance, Martínez-Medina et al. (2017a) reported that the nematode-antagonistic fungus *Trichoderma harzianum* T-78 activates a higher expression of SA marker genes in the early stages of tomato infection by the RKN *Meloidogyne incognita*, but also inhibits the suppression of JA marker genes that are expressed in the absence of the fungus.

The idea for the current study came from the previous finding that a specific microbial portion attached to the cuticle of infective nematode stages in soils may induce a varying degree of suppressiveness against plant-parasitic nematodes (Adam et al., 2014b; Elhady et al., 2017). Recently, we have isolated bacterial strains attached to the J2 of the RKN *Meloidogyne hapla* in different soils, and showed their antagonistic effects against nematodes with respect to mortality, motility, and root invasion (Topalović et al., 2019). One of the soils that we used for the J2 incubation to isolate attached microbes was obtained from a glasshouse of Geisenheim University, in the southeastern part of Germany. Remarkably, the extracted microbiomes from this soil suppressed performance of two endoparasitic nematode species, *M. hapla* and *Pratylenchus neglectus*, on tomato plants (Topalović et al., 2020). In a split-root experiment with *M. hapla,* the microbiomes reduced nematode invasion by ISR in plants, while the progeny was reduced by ISR and a direct antagonism.

Based on the above, we aimed to test the hypothesis that nematode-attached microbes might induce defense reactions in plants when *M. hapla* penetrated the root. Either the associated microbes would release compounds from the cuticle of the nematode that would act as PAMP, or the J2-transmitted microbes are recognized by plant receptors to trigger PTI defense responses. To test these hypotheses, we compared the expression profiles of several PTI-responsive genes in tomato roots inoculated with J2 of *M. hapla* with and without attached microbes from the suppressive Geisenheim soil. In addition, we studied the ROS burst of tomato plants in response to J2 of *M. hapla* and their associated bacterial isolates. Finally, we studied the ability of J2-attached bacterial cells to induce changes in expression of PTI-like genes in tomato plants and to suppress J2 invasion into roots. Our results contribute to understanding the biological importance of the microbial attachment to the infective stages of endoparasitic nematodes and the ability of the nematode-attached microbiome to accelerate defense responses of the plant against further nematode attack.

MATERIALS AND METHODS

Surface-Sterilization of *M. hapla* J2 and Incubation in Soil Suspension

M. hapla J2 were extracted and surface-sterilized as previously described (Topalović et al., 2019). Briefly, eggs were extracted from egg masses on tomato roots using 1.5% sodium-hypochlorite and collected on 20 µm sieves. The eggs were transferred to a modified Baermann tray to allow hatching of J2 over a seven-day period. Freshly hatched J2 were collected every day and stored at 4°C until surface sterilization. J2 were surface sterilized using 0.02% $HgCl_2$ for 2 min, washed with sterile tap water, and incubated for 4 h in an antibiotic solution of

200 mg l^{-1} streptomycin sulfate, 25 mg l^{-1} rifampicin, and 10x Cell Culture Guard (PanReac AppliChem). After incubation, the J2 were extensively washed on a 5-µm sieve with sterile tap water. They were incubated for 2 days at room temperature to recover after surface sterilization.

To allow microbial attachment to nematodes, the *M. hapla* J2 were incubated in soil suspensions of a *M. hapla*-suppressive soil from Geisenheim (sandy clay soil with 2.7% humus, pH 7.4, 49°59'01" N, 7°57'25.5" E; Topalović et al., 2020). The soil was sieved through a 1 mm sieve and 30 g of sieved soil was blended with 120 ml of sterile tap water (10 g of soil with 2 × 20 ml) for 1 min at a high speed (Stomacher®80, LAB SYSTEM). The suspension decanted from Stomacher bags, was then centrifuged for 5 min at 500 *g* to remove soil particles. The supernatant containing released soil microbes was sieved through a sterile 5-µm sieve to remove native nematodes and retain larger particles. The flow through was centrifuged for 10 min at 5,000 *g* and the pellet was resuspended in 30 ml sterile tap water. In 3 ml of this suspension containing 10^7 CFU ml^{-1} bacteria and 10^4 CFU ml^{-1} fungi, 2,000 surface-sterilized J2 were incubated in 6-well plates overnight at 20°C on a shaker at 30 rpm. As a control, J2 were incubated in 3 ml of sterile tap water. Both treatments were replicated 10 times. After incubation, loosely attached microbes were removed by washing the J2 on sterile 5-µm sieves with 20 ml of sterile tap water. The soil suspension was plated on R2A media (Merck, Germany) supplemented with 100 mg l^{-1} cycloheximide and potato dextrose agar (Merck, Germany) supplemented with 50 mg l^{-1} streptomycin-sulfate, to determine the number of cultivable bacteria and fungi, respectively. The R2A plates were kept at 28°C for 2 days and the potato dextrose agar plates at room temperature for seven days before counting the colony forming units.

Incubation of *M. hapla* J2 With *Microbacterium* sp. K6

The J2 were surface sterilized as described above. The isolation and identification of *Microbacterium* sp. isolate K6 were done previously (Topalović et al., 2019). Bacteria were grown in liquid LB media at 28°C overnight, short-spinned for 30 s and resuspended in sterile tap water. Around 2,000 J2 were incubated in 1 ml of the bacterial suspension ($1.9 \cdot 10^7$ CFU) in 1.5 ml microtubes overnight. Loosely attached bacterial cells were removed by washing J2 on 5-µm sieves with 20 ml of sterile tap water.

Inoculation of Nematodes to Tomato Plants Growing in Sterile Media

Tomato seeds (*Solanum lycopersicum* cv. 'Moneymaker') were surface sterilized using 70% ethanol for 1 min and 2.5% NaOCl for 3 min. They were washed with sterile water and air-dried. The seeds were planted in MS media (Murashige Skoog, Duchefa Biochemie) supplemented with 2% Gelrite (Duchefa Biochemie) at pH 5.7. The plates with tomato seeds were kept in a climate chamber at 20°C with a 16 h photoperiod (Panasonic Versatile Environmental Test Chamber MLR-352H). To study if J2-attached microbes from soil suspension trigger expression of PTI-responsive genes in tomato roots, 350 J2 either with or without attached microbes were inoculated in a 10-µl drop at the root base and tip. Control plants were inoculated with 10 µl of sterile tap water. The plates (10 biological replicates) from each treatment were kept in a climate chamber for 3 days to let the J2 penetrate the roots. Similarly, to study whether attached bacterial cells of the strain K6 trigger expression of PTI-responsive genes in tomato leaves and roots, plants were inoculated with 350 J2 with or without attached bacterial cells as described above. In this experiment, each treatment was replicated twenty times and kept in a climate chamber for 1 or 3 days, respectively. Additional four replicates in each treatment were used to microscopically determine the number of invaded J2 in roots at 7 days post inoculation (dpi) after staining with acid fuchsin (Sigma Aldrich). For both experiments, small labeled bags were prepared from aluminium foil to quickly sample and freeze plants for RNA extraction. Using forceps, whole plants were carefully removed from the plates and adhering Gelrite was swiftly removed with a paper tissue. The plants were transferred to the bags, which were closed, immersed in liquid N_2, and then stored at −80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

Frozen roots and leaves were transferred to separate 2 ml microtubes and pulverized with a 5 mm metal bead in a TissueLyser II (Qiagen) twice for 20 s at the highest speed (30 Hz), keeping samples frozen in a pre-cooled block. Total RNA from plants was extracted using the FastRNA Pro Green Kit (MP Biomedicals). Briefly, 1 ml of RNApro solution was added to each tube and mixed well by inverting the tubes and pipetting to inactivate RNases and start chemical cell lysis. The suspension was transferred to a bead-beating tube and cells were mechanically lysed in a FastPrep-24 instrument (MP Biomedicals) for 35 s at speed level 6.0 m s^{-1}. After centrifugation at 4°C for 5 min at 14,000 *g*, 800 µl of the supernatant was extracted with 300 µl chloroform by vortexing for 10 s and incubation at room temperature for 5 min. After phase separation at 14,000 *g* for 5 min at 4°C, the aqueous phase was transferred to a new tube. RNA was precipitated by adding 500 µl cold absolute ethanol, inverting the tubes five times and incubation at −20°C for 30 min. The RNA was pelleted by centrifugation at 4°C for 15 min at 14,000 *g* and washed with 500 µl cold RNase-free 70% ethanol. The RNA was resuspended in 30 µl of RNase-free water (Invitrogen) and kept at −80°C until use. Remaining traces of DNA were removed from aliquots of 1 µg RNA by DNase I digestion followed by DNase inactivation and removal, using the DNA-*free* DNA Removal Kit (ThermoFisher Scientific). Purified RNA was reverse transcribed using Superscript IV according to the manufacturer's instructions (ThermoFisher Scientific). Briefly, 8 µl RNA (ca. 1 µg) were incubated with 1 µl 50 µM oligo dT(23), 1 µl dNTP (10 mM each) and 3 µl nuclease-free water at 65°C for 5 min and on ice for at least 1 min. Superscript IV (200 U in 1 µl), 4 µl 5× SSIV Buffer, 1 µl 100 mM DTT, and 1 µl RNase OUT were added and cDNA synthesized at 55°C for 10 min. Samples were heated to 80°C for 10 min and stored at −20°C.

Real-Time qPCR Analysis of Defense Gene Expression

Real-time amplifications were performed using a CFX Connect Real Time Detection System (Bio-Rad) in 20 µl reactions containing Standard *Taq* Reaction Buffer (New England BioLabs), 0.25 mM $MgCl_2$, 0.2 mM of each dNTP, 2× EvaGreen (Biotium), 1 µM of gene specific primers (**Table 1**), and 0.5 U Hot Start *Taq* DNA Polymerase (New England BioLabs). Thermocycles were as follows: initial denaturation at 94°C for 5 min, 40 cycles of a denaturation step at 94°C for 20 s, annealing step at 60°C for 30 s, an extension step at 68°C for 30 s, and 80°C for 30 s. The fluorescence was read at the 80°C step of each cycle. After each run, a melting curve was generated between 65° and 95°C. Ct values of defense genes were normalized to the ubiquitin gene expression, and their relative expressions in each sample were determined against gene expressions in control plants without nematodes, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Real-Time qPCR Analysis of *Meloidogyne* 18S rRNA in cDNA of Roots

Relative quantification of the 18S rRNA gene of *M. hapla* inside tomato roots infested with J2 with and without attached K6 strain were determined using 5 µl of cDNA samples. A 750 bp fragment of the *M. hapla* 18S rRNA of invaded J2 in roots was amplified in real-time qPCR using a CFX Connect Real Time Detection System (Bio-Rad) in 25 µl reactions containing Standard *Taq* Reaction Buffer (New England BioLabs), 0.25 mM $MgCl_2$, 0.2 mM of each dNTP, EvaGreen (Biotium), 1 µM of *Meloidogyne* spp. specific primers Melo1f (5′-AAGATATCTGGTTGATCCTGCCTGA-3′) and Melo723r (5′-GTTCAAAGTAAACTTGCAAYGMCTG-3′), 0.625 U Hot Start *Taq* DNA Polymerase (New England BioLabs). After initial denaturation at 94°C for 2 min, fragments were amplified in 40 cycles with a denaturation at 94°C for 20 s, annealing at 57°C for 20 s, extension at 72°C for 45 s, and fluorescence detection at 80°C for 30 s. Melting curves were generated between 65° and 95°C. Ct values were normalized to the ubiquitin gene expression, and 18S rRNA of J2 with attached bacterial strain K6 were determined relative to 18S rRNA of J2 without K6 using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

ROS Burst Assay

The *Microbacterium* sp. isolate K6 and *Acinetobacter* sp. isolate E1 were obtained in a previous study (Topalović et al., 2019). Around 10,000 surface sterilized J2 of *M. hapla* were incubated in 200 µl suspensions of the strains K6 or E1 (10^7 CFU ml^{-1}), or 200 µl of sterile tap water in 1.5 ml microtubes, at room temperature overnight without shaking. The bacterial suspensions were prepared as described previously (Topalović et al., 2019). The supernatants were sterile-filtered the following day and served as the surface-released compounds from the J2 (SC) with and without bacterial cell-free supernatants. The J2-bacteria pellets were suspended in 200 µl of sterile tap water to get suspensions of live J2 with and without the living bacteria.

To measure ROS burst we adapted previously published methods (Prince et al., 2014; Mendy et al., 2017). Briefly, the cotyledon leaves were excised from 12-days-old tomato plants and incubated in ddH_2O in the dark for 12 h. The leaf samples were then transferred to a 96-well plate that contained 80 µl of 0.1 µM luminol derivate 8-amino-5-chloro-2,3-dihydro-7-phenyl-pyrido[3,4-d] pyridazine sodium salt (L-012, Wako Chemicals). After another hour of incubation in the dark, 20 µl of 20 µg ml^{-1} horseradish peroxidase was added to each well followed by 50 µl elicitor, with 1 µM flagellin peptide (Flg22) as positive and sterile tap water as negative controls. The elicitor treatments were as follows: (a) Live J2, (b) SC, (c) Live J2 + K6 cells, (d)SC + K6 cell-free supernatant, (e) Live J2 + E1 cells, (f) SC + E1 cell-free supernatant. The assay was repeated three times with three technical replicates each. Relative light units of luminescence were measured in a 96-well microplate reader (Infinite® 200 PRO; TECAN) over 120 min. The data were analyzed using instrument software and Microsoft Office Excel.

Statistical Analysis

Fold changes in expression of each defense gene after root penetration of J2 with or without attached microbes, as related to expression without nematodes, were log-transformed to better fit linear models (smaller AIC). Generalized linear models were applied using the statistical software SAS 9.4. Class variables were MICROBES (1, 0) and GENE (*GRAS4.1*, *MPK1*, *PDF1.2*, *PR1a1*, *TFT1*, *WRKY28*) for the experiment with attached microbiome from Geisenheim soil, and additionally DPI (1, 3) and TISSUE (leaf, root) for the experiment with the attached bacterial strain K6. A *post hoc* Tukey-test was done to compare fold changes in expression of defense genes in plants invaded by J2, with or without attached microbes.

TABLE 1 | Primer pairs used in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
PR1a1	CTGGTGCTGTGAAGATGTGG	TGACCCTAGCACAACCAAGA	Harel et al., 2014
PDF1.2	GGCTAGCAAAATCACTTTCTGTG	CATGATCCTTATTTTTGCACCA	Tran et al., 2017
GRAS4.1	TTCGAATCCCCTGCTTCCAT	CCAGTTGGTGAATTGCTGCT	This study
MPK1	GCTGACAGATTGTTGCAGGT	TCCACCCCATAAAGATACATCA	Kandoth et al., 2007
WRKY28	ACAGATGCAGCTACCTCATCCTCA	GTGCTCAAAGCCTCATGGTTCTTG	Taylor et al., 2012
TFT1	GCCTCGTCCATCTGCTCCTG	GAATGCATCAGAAAAAGCATGCAG	Taylor et al., 2012
PTI5	ATTCGCGATTCGGCTAGACATGGT	AGTAGTGCCTTAGCACCTCGCATT	Taylor et al., 2012
Ubiquitin	GTGTGGGCTCACCTACGTTT	ACAATCCCAAGGGTTGTCAC	Bhattarai et al., 2008
Meloidogyne 18S rRNA	AAGATATCTGGTTGATCCTGCCTGA	GTTCAAAGTAAACTTGCAAYGMCTG	This study

RESULTS

J2-Attached Microbes From Geisenheim Soil Trigger Expression of PTI-Responsive Genes in Tomato Roots

To study if the microbiome attaching to the pre-infective J2 of *M. hapla* in Geisenheim soil induce PTI responses in tomato plants, the J2 were incubated in microbial suspensions overnight, washed, and nematodes with or without attached microbial cells were inoculated to 2-week old tomato plants growing in MS media. The differential expression of six PTI marker genes 3 dpi was determined compared to their expression in roots without invaded nematodes. All tested PTI marker genes were expressed in the roots infested with both, clean juveniles and juveniles carrying the attached soil microbes. However, the J2 with attached microbes triggered PTI responses higher than those of the surface-sterilized J2 alone (**Figure 1**). Overall, ANOVA revealed a significant effect of attached microbes on defense gene expression ($P = 0.001$). The response of the genes to J2 differed ($P = 0.0001$), while the interaction of GENE * MICROBES was not significant ($P = 0.55$). The average up-regulation of the plant defense genes by J2 with attached microbes was two-fold. However, data showed that J2 without attached microbes managed to down-regulate most of the defense genes (*GRAS4.1*, *MPK1*, *PR1a1*, *TFT1*), and that attached microbes counteracted this. The transcription factor *GRAS4.1* was significantly up-regulated 3 dpi when the roots were invaded by J2 with microbes attached to the cuticle, while in the absence of attached microbes this gene was repressed by the J2 ($P = 0.002$, Tukey test). Conversely, the transcription factor *TFT1* was down-regulated in both treatments, but the down-regulation was ameliorated by microbes attached to J2 ($P = 0.035$, Tukey test). Although microbial attachment to the J2 of *M. hapla* in Geisenheim soil led to the up-regulation of both the transcription factor *WRKY28* and the JA-regulated *PDF1.2* defensin gene, this was not statistically supported. Mitogen-activated protein kinase 1 gene (*MPK1*) and the marker gene of the SA pathway (*PR1a1*) were both down-regulated with minor differences between the two treatments. Independent soil sampling and incubation of J2 confirmed the effect of J2-attached microbes on defense gene expression. However, *TFT1* was significantly up-regulated by the attached microbes while down-regulated without microbes (**Supplementary Figure S1**).

Attachment of Bacterial Isolate K6 to *M. hapla* J2 Affected Root Invasion and Expression of PTI Marker Genes

The bacterial strain *Microbacterium* sp. K6 was isolated from the nematode cuticle after baiting of J2 in Geisenheim soil suspension. The effect of this strongly attaching strain on plant defense upon root penetration of *M. hapla* J2 was investigated. The expression profiles of six PTI-responsive genes were

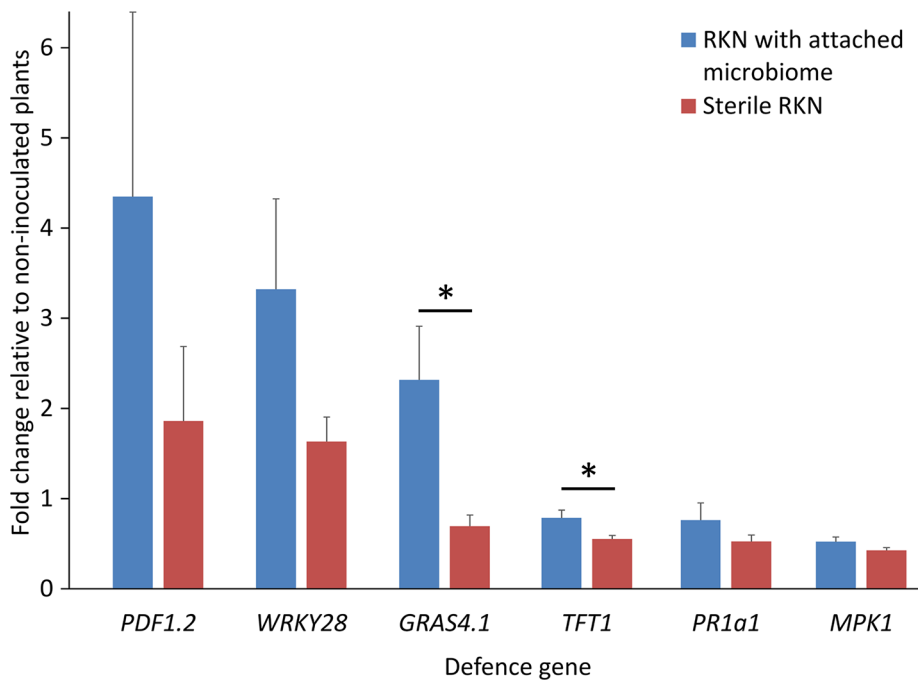

FIGURE 1 | Expression profiles of PTI-like genes in tomato roots in response to root penetrating juveniles of the root-knot nematode (RKN) *Meloidogyne hapla* with and without attached microbes from suppressive Geisenheim soil, 3 dpi. ANOVA by generalized linear models (see **Supplement Statistics 1**) revealed that the attached microbes significantly enhanced defense gene expression overall ($P = 0.001$), while the response of the various genes differed ($P = 0.0001$). Lines under an asterisk indicate significant differences between plants after invasion of nematodes with or without microbes attached to the cuticle ($P < 0.05$, Tukey test on log-transformed data). Error bars represent SE (n = 10 plants).

determined in roots and leaves of 2-week old tomato plants, 1 and 3 days after root inoculation of J2 with or without attached cells of strain K6 (**Figure 2**). Defense gene expression of invaded plants relative to uninoculated plants was normalized for ubiquitin transcripts. As the number of J2 invading the root and triggering plant defenses is reduced by strain K6, the defense gene expression was also adjusted to equal *M. hapla* 18S rRNA in roots. ANOVA showed that the attached bacteria significantly enhanced defense gene expression ($P < 0.0001$). At 1 dpi, four of the defense genes were down-regulated in roots by invading J2 (**Figure 2**). Attached K6 resulted in less down-regulation and in significant up-regulation of the mitogen-activated protein kinase 1 gene (*MPK1*) in roots. In leaves at 1 dpi, only a weak response of defense genes to J2 invasion in roots was detectable. J2-attached K6 resulted in a slightly higher expression of four of the defense genes, but a down-regulation of *PR1a1*. At 3 dpi, all six defense genes showed higher expression in roots and leaves in response to J2 with attached K6 compared to surface-sterilized J2. The attached bacteria caused a significant up-regulation of the PTI-genes in leaves and of the transcription factor *WRKY28* in roots, while surface-sterilized J2 kept PTI defense at a low level (**Figure 2**). *WRKY28* was down-regulated 1 dpi and up-regulated 3 dpi in both treatments, while the K6 cells significantly increased the up-regulation of this gene ($P < 0.0001$; Tukey test). In the ANOVA, the factors DPI and TISSUE had significant effects, and the effect of attached K6 was more pronounced 3 dpi than 1 dpi ($P = 0.002$ for the interaction effect K6 * DPI). Overall, the genes *WRKY28*, *GRAS4.1*, and *MPK1* in roots, and *MPK1* in leaves were significantly triggered by J2-attached bacteria ($P < 0.05$, Tukey correction).

To follow the effects of J2-attached K6 cells on nematode penetration, we determined the 18S rRNA of *M. hapla* in the same root samples that were used to study expressions of defense genes. The number of 18S rRNA increased from 1 to 3 dpi. The relative abundance of ribosomal RNA of J2 with attached K6 cells was 82% (± 22% SE) and 55% (± 15% SE), of the relative abundance of the J2 without attached bacterial cells at 1 and 3 dpi, respectively. The reduction became statistically significant at 3 dpi ($P = 0.018$, one sample t-test). With plants grown in parallel that were not sampled for RNA extraction, the number of J2 in roots was determined microscopically 7 dpi (**Figure 3**). The nematode penetration was significantly suppressed by the attached K6 cells. The number of invaded J2 in control roots was the double of those with attached K6 cells. Thus, constantly less J2 invaded the roots within the first seven days after inoculation, when bacterial cells were attached to the cuticle.

ROS Response of Tomato Tissue to *M. hapla* J2 With and Without Attached Bacteria

We tested whether PTI is triggered in tomato plants by living bacterial cells of *Microbacterium* sp. K6 and *Acinetobacter* sp. E1,

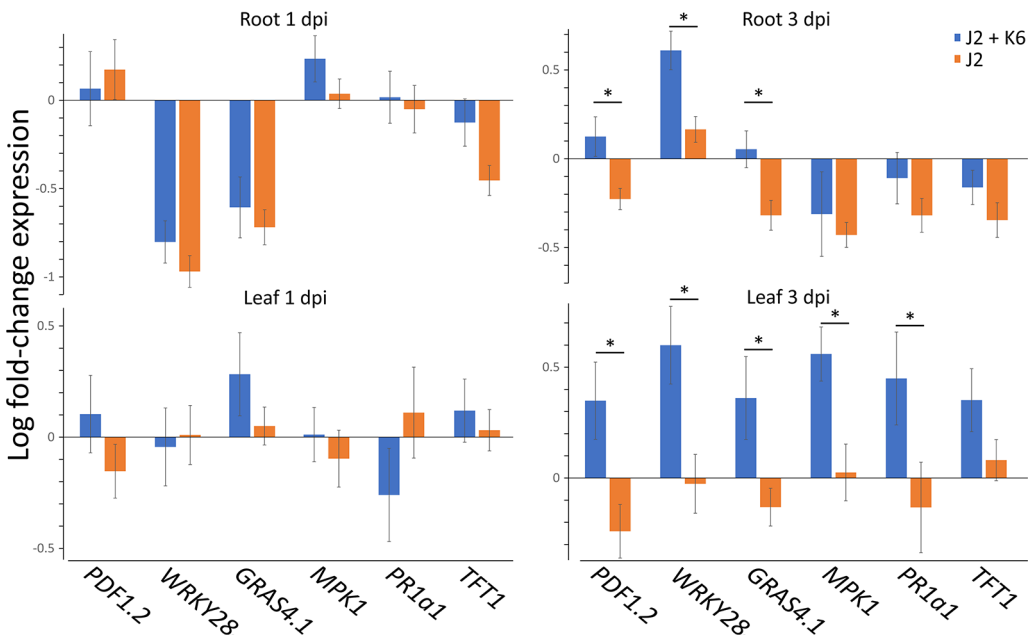

FIGURE 2 | Expression profiles of PTI genes in leaves and roots of tomato plants in response to *M. hapla* J2 with and without attached *Microbacterium* sp. K6. Defense gene expression of invaded plants relative to not inoculated plants, as determined in cDNA by Real-Time PCR, was normalized for ubiquitin transcripts and *M. hapla* 18S rRNA in roots, using the $2^{-\Delta\Delta Ct}$ method. Bars show means of log-transformed fold-changes in gene expression, one and three days after J2 inoculation to the roots (dpi). ANOVA by generalized linear models (see **Supplement Statistics 2**) revealed that the attached bacteria K6 (stages: with, without) significantly enhanced defense gene expression overall ($P < 0.0001$). The factors DPI (stages: 1 dpi, 3 dpi) and TISSUE (stages: root, leaf) had significant effects on gene expression. The effect of attached K6 was more pronounced 3 dpi than 1 dpi ($P = 0.002$ for the interaction effect K6 * DPI). Error bars represent SE (n = 10 plants).

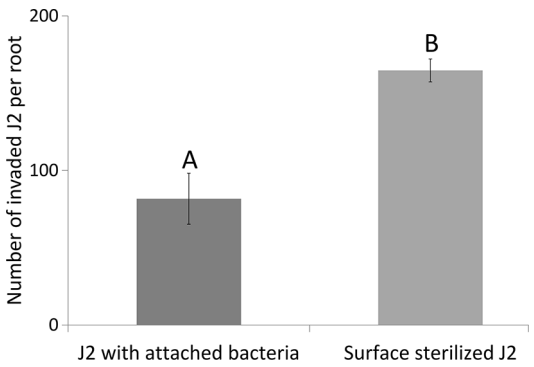

FIGURE 3 | Number of invaded J2 of *M. hapla* J2 in tomato roots 7 dpi, with or without attached *Microbacterium* sp. K6. Error bars represent SD. The letters above error bars indicate a significant difference between treatments ($P < 0.05$, Tukey test; n = 4 plants).

or by the compounds they release, or by the compounds that are released when J2 and bacteria are incubated together. The ROS patterns of leaves and roots of tomato plants were analyzed in response to the organisms and their surface-released compounds (SC), using a luminescence assay. To produce SC, nematodes and bacteria were incubated in water overnight alone or in combination, and the supernatant containing SC was separated from the organisms by centrifugation. Although all variants, living bacterial cells as well as their SC, nematodes and their SC, the live nematodes together with bacteria, and the SC from nematodes incubated with bacteria triggered a ROS peak in leaves within 25 min, differences were observed in their respective intensities (**Figure 4**). Both the SC from J2 and living J2 triggered a clear ROS burst, which was further amplified for variants including K6 or E1. Also in the absence of *M. hapla*, compounds of both bacterial strains were capable to elicit a strong ROS burst in the leaf tissue, while only the living cells of K6 induced a similar ROS pattern (**Figure 4**). Consequently, incubation with K6 or E1 led to

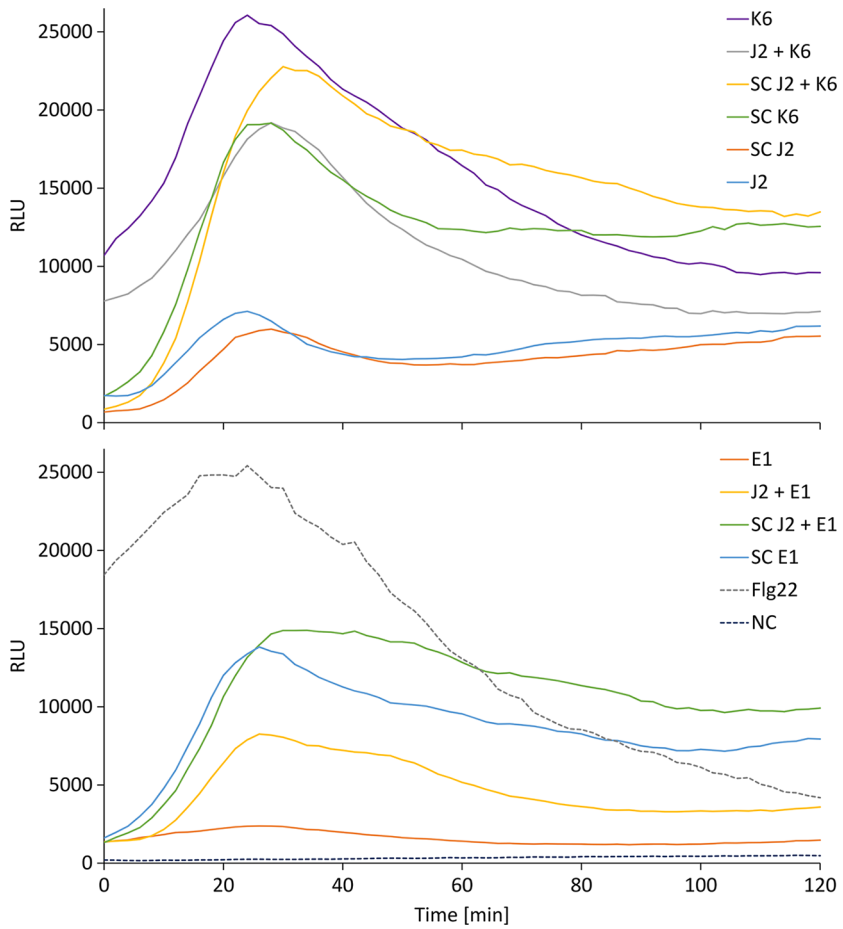

FIGURE 4 | Relative luminescence units (RLU) representing the ROS burst in tomato leaves over time for the different treatments. Treatments were K6: cells of *Microbacterium* sp. K6; J2 + K6: live juveniles of *M. hapla* (J2) + cells of K6; SC J2 + K6: released compounds from J2 incubated with K6; SC K6: released compounds from K6; SC J2: released surface compounds from J2; J2: live juveniles of *M. hapla* E1: cells of *Acinetobacter* sp. E1; J2 + E1: live juveniles + cells of *Acinetobacter* sp. E1; SC J2 + E1: released compounds from J2 incubated with E1; SC E1: released compounds from E1; Flg22: positive control; NC: water as negative control. Curves show means of 6 to 12 plants.

an intensified ROS response to the infective stage of the parasitic nematode and related SC (**Figure 4**). The compounds released from K6 and E1 were sufficient to induce a strong ROS burst in the leaf tissue (**Figure 5**). Also the SC from *M. hapla* J2 triggered a considerable ROS burst. The SC from J2 that were incubated with K6 or E1 showed a trend to induce a stronger ROS response than the SC from the respective bacteria alone, although not statistically significant (**Figure 5**). The sum of the signals from the SC of *M. hapla* J2 and the SC of bacteria were not more than the ROS signal from the combined treatments obtained in leaves (**Figure 5**). In roots, similar reactions were observed but at 100 times lower intensity (**Supplementary Figures S2**, **S3**). The SC of strain K6 triggered not significantly more ROS than SC of *M. hapla* J2, while the SC from J2 incubated with strain K6 induced a significantly stronger ROS burst in roots than SC of both organisms alone (**Figure 6**). In contrast, for strain E1 the effect of its SC did not differ from the effect of the SC from J2 + E1 that were incubated together.

DISCUSSION

DNA-based analysis of the microbiome associated with phytonematodes has gained attention over the recent years (Adam et al., 2014b; Cao et al., 2015; Elhady et al., 2017; Hussain et al., 2018). Characterization of microbial communities that are in a direct association with plant-parasitic nematodes in soil helps deepen the knowledge on the mechanisms that microbes deploy to antagonize these persistent plant parasites. However, previous studies mainly focused on the subtle differences in attached microbiota among different nematode stages or species and in different soils (Adam et al., 2014b; Elhady et al., 2017). Recently, we demonstrated that the soil microbiomes from a glasshouse at Geisenheim University reduced performance of *M. hapla* on tomato plants, while a split-root experiment revealed the contribution of microbially triggered ISR to this phenomenon (Topalović et al., 2020). In addition, we isolated bacterial strains from the cuticle of *M. hapla* and *Pratylenchus penetrans*, showing their antagonistic activity, but the ability of nematode-attached bacteria to induce systemic resistance in plants has not yet been investigated (Topalović et al., 2019). Our current study revealed that the early infection of tomato by *M. hapla* J2 alone resulted in the suppression of most of the analyzed PTI defense genes, but that the J2-attached microbes either ameliorated this effect, or triggered up-regulation of the genes. Plant-parasitic nematodes cause mechanical damage of the host cells, which respond by activating damage-associated molecular pattern triggered defense (Holbein et al., 2016; Shah et al., 2017). The opening wounds can also ease the entrance of pathogenic microorganisms that lead to secondary infections and disease complexes

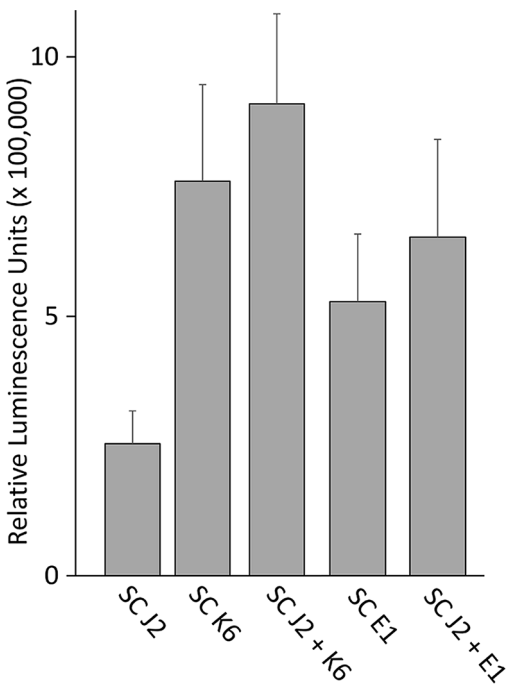

FIGURE 5 | ROS burst in tomato leaves triggered by surface-released compounds (SC) from juveniles of *M. hapla* (J2), bacteria isolated from the cuticle of J2 (*Microbacterium* sp. K6, *Acinetobacter* sp. E1), or J2 incubated with these bacteria. The sum of relative luminescence units over 2 h in a ROS assay with tomato leaves is shown. Differences among treatments were not significant ($P < 0.05$, Tukey test on log-transformed data). Error bars represent SE (n = 6 to 12 plants).

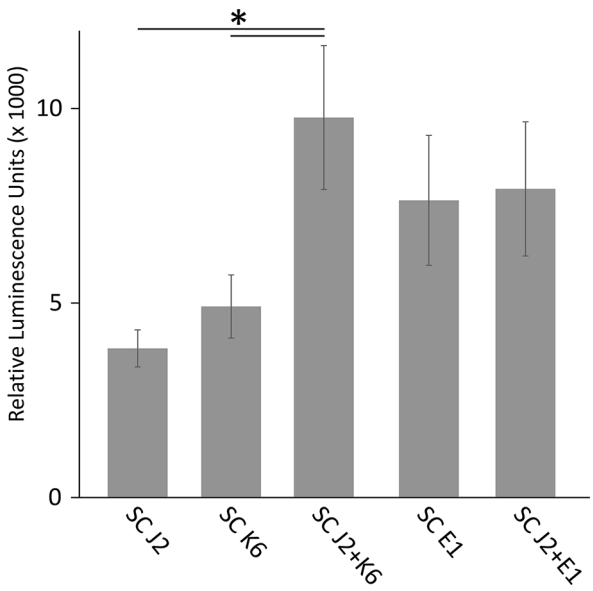

FIGURE 6 | ROS burst in tomato roots triggered by surface-released compounds (SC) from juveniles of *M. hapla* (J2), bacteria isolated from the cuticle of J2 (*Microbacterium* sp. K6, *Acinetobacter* sp. E1), or J2 incubated with these bacteria. The sum of relative luminescence units over 2 h in an ROS assay with tomato roots is shown. Lines under an asterisk indicate significant differences between treatments ($P < 0.05$, Tukey test on log-transformed data). Error bars represent SE (n = 6 to 12 plants).

(Karimi et al., 2000; Back et al., 2002). However, the RKN are well known to hide themselves from plant recognition, since the J2 move through the apoplast until establishing permanent feeding sites (Sijmons et al., 1994). Notably, it was shown that the cell wall degradation caused by cyst nematodes, while moving through the symplast, trigger activation of polygalacturonase inhibitor proteins (PGIP) in plants, which activate damage-associated molecular pattern triggered defense by production of oligogalacturonides (Benedetti et al., 2015; Shah et al., 2017). In contrast to cyst nematodes, Shah et al. (2017) could not detect activation of PGIP in response to the RKN infection, thus pointing out the ability of RKN to suppress early recognition by the host. In addition, several studies have shown down-regulation of PTI-like genes during the early events of RKN infection (Dubreuil et al., 2007; Barcala et al., 2010; Damiani et al., 2012). In our study, J2-attached microbes from the suppressive Geisenheim soil alleviated expression of several PTI-like genes, including *GRAS4.1*, *TFT1*, *WRKY28*, and *PDF1.2*. Interestingly, the transcription factor *TFT1*, a marker of JA-mediated PTI, was down-regulated in roots infected with J2. However, the down-regulation of this gene was suppressed by the J2-attached microbes. *TFT1* belongs to the tomato 14-3-3 family of acidic, phosphopeptide-binding proteins that are involved in metabolism, signal transduction, transcription, cell cycle, development, apoptosis, and stress signaling (Bridges and Moorhead, 2004; Smith et al., 2011). Taylor et al. (2012) showed that silencing of *TFT1* resulted in increased tomato susceptibility to the pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria*, and it was also required for the expression of some other JA- and SA-mediated defense genes, including *GRAS* and *WRKY*. The inhibited repression of *TFT1* by nematode-associated microbes in our study suggests that the plant recognized the transmitted microbes upon nematode invasion. The J2-attached microbes from Geisenheim soil also significantly elevated mRNA levels of the transcription factor *GRAS4.1*. The up-regulation of the *GRAS* gene was positively correlated with the expression of *TFT1* in establishing PTI against *X. campestris* pv. *vesicatoria* (Taylor et al., 2012). *GRAS* transcription factors are involved in plant development, gibberellic acid signaling, symbiotic processes, and stress signaling (Tian et al., 2004). This gene was also up-regulated in tomato and tobacco leaves infected by non-pathogenic *Pseudomonas fluorescens* as a reporter gene for assaying PTI (Nguyen et al., 2010).

The bacterial strain K6, which belongs to the Gram-positive genus *Microbacterium*, was previously isolated from the cuticle of *M. hapla* J2. It was firmly attached to J2, with more than 4,000 CFU/J2, and showed antagonistic effects on their viability, movement, and invasion into roots (Topalović et al., 2019). However, plant-mediated effects were more important for its antagonism against *M. hapla* as shown in a split-root experiment (Topalović et al., 2019). Similar to the attachment of microbes from the microbiome of the Geisenheim suppressive soil, the J2-attached strain K6 also ameliorated the J2-induced suppression of defense genes. The expression profiles of the genes differed 1 and 3 dpi in root and leaf tissues. At 1 dpi, K6 generally reduced down-regulation of defense genes in roots, while significantly up-regulating *MPK1*. This effect was stronger 3 dpi with several up-regulated genes, including *WRKY28*, *GRAS4.1*, and *PDF1.2*. However, at 1 dpi PTI activation seemed to be limited to belowground tissues as leaves hardly responded to the J2 infection in roots. At 3 dpi, K6 had a strong systemic effect in leaves and the defense response in roots increased. A similar pattern of belowground-aboveground response was observed by Wang et al. (2019). They reported root-shoot transmission of electrical and ROS signals in response of *Arabidopsis thaliana* to *Meloidogyne incognita* 1 dpi. This led to the activation of JA synthesis in leaves. Consecutively, JA systemically triggered the defense in the roots. In our study, the overall low plant responses to J2 at 1 dpi could also be attributed to the fact that *M. hapla* J2 are slow invaders in comparison to the more aggressive RKN *M. incognita*. Martínez-Medina et al. (2017a) reported that the fungus *T. harzianum* T-78 primed local and systemic tomato root tissues during the early stages of *M. incognita* infection by accelerating activation of SA-responsive genes and inhibiting suppression of JA-responsive genes. However, studies on the expression profiles of microbially induced PTI-like genes in roots, in response to nematode infection, are still scarce. It was shown that the expression of SA marker *PR* genes differed in roots and leaves in response to the RKN, but this seemed to depend on the nematode species and timing (Hamamouch et al., 2011; Dam et al., 2018). Interestingly, the transcription factor *WRKY28* was down-regulated in our study in response to both J2 alone and the J2-attached K6 cells at 1 dpi, while it was significantly up-regulated by the K6 cells 3 dpi. Although the RKN cause less damage during the early stages of infection, up-regulation of *WRKY28* by J2 alone confirms that the RKN *M. hapla*, nevertheless, also induce expression of defense genes (Williamson and Hussey, 1996). The up-regulation of *WRKY11* and *WRKY17* genes in *A. thaliana* roots infested with *M. incognita* suggested that these transcription factors behave as positive regulators of plant defense against RKN, as both *WRKY11* and *WRKY17* mutants showed significantly higher nematode reproduction in comparison to the wild type (Teixeira et al., 2016).

We showed a strong positive correlation between the activation of PTI by J2-attached K6 cells upon nematode penetration and a reduced number of invaded J2 over a 7-day period. Overall, our study suggests that microbes that attach to J2 in soil can antagonize nematodes by ISR in plants. The microbes themselves induced plant responses, so it seems unlikely that the microbial attachment alters the nematode surface, thereby indirectly triggering ISR in the plant. However, this needs further investigations. It has been suggested that the intensity and the duration of ROS burst in plants can determine the compatible or incompatible interaction with RKN (Melillo et al., 2011; Sato et al., 2019). A strong and a prolonged ROS burst caused an incompatible interaction between the plant and RKN, based on an increased plant defense against J2 penetration (Melillo et al., 2011). In our study, we investigated whether the two *M. hapla*-isolated bacterial strains, K6 and E1 induce or increase ROS burst in tomato plants exposed to *M. hapla* J2, and whether the released compounds of the studied organisms

induced a different reaction. In the ROS assay, the reaction of the plant was 100 times stronger in leaves than in roots. Moreover, the compounds released from the surface of J2 triggered a ROS burst. Likewise, Mendy et al. (2017) reported a strong induction of ROS burst in response to compounds released by two nematode species, *Heterodera schachtii* and *M. incognita*, which was accompanied by the expression of JA biosynthesis and signaling genes during the migratory phase in *A. thaliana*. In our study, K6 cells and cell-free compounds of both bacteria induced a much stronger ROS burst than the nematodes. Notably, the most prolonged ROS burst was recorded in the presence of compounds from J2 and strain K6 together, which might be evidence that active compounds are formed by an interaction of both organisms, for instance by degradation of the cuticle or J2-induced metabolites of strains K6 and E1.

CONCLUSIONS

Nematodes encounter a vast number of microorganisms in soil but specifically attach only a few of them (Adam et al., 2014b; Elhady et al., 2017). However, the antagonistic properties of nematode-attached microbes have been hardly investigated, with the exception of *Pasteuria* endospores. Recently, we have isolated bacterial strains that attached to the cuticle of J2 of *M. hapla* in different soils and demonstrated their effects against J2 viability and invasion into roots (Topalović et al., 2019). Our current study aimed to investigate whether the nematode-attached microbes can trigger ISR in plants and in this way contribute to soil suppressiveness against *M. hapla*. The results suggest that the microbes attaching to *M. hapla* J2 in suppressive Geisenheim soil triggered a differential expression of PTI-responsive genes in tomato roots, 3 dpi. In addition, it was also shown that the attachment of *Microbacterium* sp. K6 to J2 prior to their invasion into the roots induced expression of PTI-like genes in roots and leaves. The attachment of K6 cells to the J2 significantly reduced the J2 establishment, confirming a plant-mediated antagonistic effect of strain K6 against *M. hapla* (Topalović et al., 2019). Overall, our data suggest that nematode-attached microbes have a positive role in plant defense against the sedentary endoparasite *M. hapla*. Our study contributes to the current knowledge on the tripartite plant-nematode-microbe interactions in soil and on nematode suppression by microbially induced systemic resistance in plants.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

OT did the experiments, contributed to the ideas, and wrote the manuscript. SB and AS designed and performed the ROS assay together with OT. OT and HH statistically analyzed the data. HH initiated and supervised the research, and revised the manuscript. All authors read, revised, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00138/full#supplementary-material

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The Bacterial Microbiome of *Meloidogyne*-Based Disease Complex in Coffee and Tomato

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The *Meloidogyne*-based disease complexes (MDCs) are caused by the interaction of different root-knot nematode species and phytopathogenic fungi. These complexes are devastating several important crops worldwide including tomato and coffee. Despite their relevance, little is known about the role of the bacterial communities in the MDCs. In this study 16s rDNA gene sequencing was used to analyze the bacterial microbiome associated with healthy and infested roots, as well with females and eggs of *Meloidogyne enterolobii* and *M. paranaensis,* the causal agents of MDC in tomato and coffee, respectively. Each MDC pathosystems displayed a specific taxonomic diversity and relative abundances constituting a very complex system. The main bacterial drivers of the MDC infection process were identified for both crops at order level. While corky-root coffee samples presented an enrichment of Bacillales and Burkholderiales, the corcky-root tomato samples presented an enrichment on Saprospirales, Chthoniobacterales, Alteromonadales, and Xanthomonadales. At genus level, *Nocardia* was common to both systems, and it could be related to the development of tumor symptoms by altering both nematode and plant systems. Furthermore, we predicted the healthy metabolic profile of the roots microbiome and a shift that may result in an increment of activity of central metabolism and the presence of pathogenic genes in both crops.

Keywords: corky root, pathobiome, *Meloidogyne enterolobii*, *Meloidogyne paranaensis*, functional profile

INTRODUCTION

Plants harbor a myriad of microorganisms out and inside their tissues, constituting specific microbiomes. Plant-associated microorganisms, mainly endophytes, play an important role in plant health and productivity, since they provide their hosts with essential functions (Kiers et al., 2003; Kiers and van der Heijden, 2006; Bulgarelli et al., 2012; Vandenkoornhuyse et al., 2015). Pathogens attack, results in the disturbance of the plant microbiome balance, possibly promoting

diseases through cooperation or coinfection of different pathogens (Tollenaere et al., 2016; Vannier et al., 2019). The idea that disease could be caused by the combined action of more than one pathogen set the bases for the pathobiome concept, which describes the pathogen as a component of a complex microbial community (Ryan, 2013; Vayssier-Taussat et al., 2014; Lamichhane and Venturi, 2015; Jakuschkin et al., 2016; Brader et al., 2017).

The pathobiome theory considers the interaction between well adapted pathogens, known as keystone pathogens that modulate plant defenses and strongly affect plant microbiome composition; accessory pathogens, which find their niche in the pathobiome by providing nutritional or colonization support to keystone pathogens; and pathobionts, whose commensal or mutualistic relationship switches to pathogenic, in response to changes in the plant microbiome (Brader et al., 2017).

Considering this definition, the pathobiome concept contradicts the Koch and Hill's fundamental postulate "one microbe-one disease" (Ryan, 2013; Vayssier-Taussat et al., 2014), stating that disease is driven by a highly complex network in which microorganisms interact with each other influenced by diverse external factors (Lamichhane and Venturi, 2015; Brader et al., 2017).

Sedentary endoparasitic nematodes such as *Meloidogyne* spp. are very harmful plant parasites due to their high pathogenicity and their ability to overcome resistance in many hosts (Jones et al., 2013; Elling, 2013). Interactions of root-knot nematodes (RKN) with other phytopathogenic microorganisms can cause syndromes known as *Meloidogyne*-based disease complex (MDC), severely affecting world agricultural production (Wolfgang et al., 2019). MDC symptoms are characterized by the initial infection of RKN, causing the typical root swellings and knots. As the infection progresses, the concomitant presence of fungi and bacteria is observed along with nematodes in root tissues, which present more severe symptoms such as hyperplasia with deep and cracked cortical root tissues, having a cork-like appearance, leading to necrosis and atrophy of the root system (Koike et al., 2006; Taher et al., 2017; López-Lima et al., in press; Wolfgang et al., 2019). These symptoms, in the advanced stages of infection, are also commonly known as corky-root disease in coffee (Bertrand et al., 2000; Lopez-Lima et al., 2015; Villain et al., 2018). Consequently, water and nutrient flows are altered, causing significant damage to the plant including chlorosis, defoliation, necrosis of tip branches, and decreased production (Hajji-Hedfi et al., 2018).

The bacterial community is an important component of MDC, perhaps favoring disease development. Some groups of bacteria like *Herbaspirillum*, *Rickettsia*, *Chitinophaga,* and *Pedobacter* have been reported as symbionts of nematodes (Tian et al., 2011; Baquiran et al., 2013; Cheng et al., 2013). In contrast, other bacterial species such as *Pseudomonas* and *Commamonas* have been reported reducing nematode populations by inhibiting hatching or by nematicidal action (Siddiqui et al., 2006; Wolfgang et al., 2019). Root bacterial microbiome structure has been studied in some relevant crops affected by RKN and other pathogens with the aim of understanding their function in the agroecosystems and developing better management strategies (Busby et al., 2017; Finkel et al., 2017; Toju et al., 2018; Toju and Tanaka, 2019).

Particularly, severe effects of MDC have been reported in two crops of world importance: tomato (Sasanelli et al., 2008; Ganaie and Khan, 2011; Rizvi et al., 2015; Mwangi et al., 2019) and coffee (Alvarado, 1935; Carneiro et al., 1996; Carneiro et al., 2004; Villain et al., 2013; Lopez-Lima et al., 2015; Monteiro et al., 2016; Villain et al., 2018). In tomato MDC is caused by *M. incognita* and/or *M. enterolobii* interacting with *F. oxysporum*, *F. solani*, *Pyrenochaeta lycopersici,* and *Rhizoctonia solani* (Sasanelli et al., 2008; Ganaie and Khan, 2011; Rizvi et al., 2015; van Bruggen et al., 2016; Mwangi et al., 2019). In Mexico, *M. enterolobii* causes significant losses in due to its high virulence, which leads to plant death before completing the crop cycle. This is the case of cultivars carrying the resistant gene *Mi-1*, which confers resistance to *M. javanica*, *M. arenaria* and *M. incognita* (Kiewnick et al., 2008; Rosa et al., 2014; Martínez-Gallardo et al., 2015; Martínez-Gallardo et al., 2019).

In coffee, MDC is caused by the RKN *Meloidogyne paranaensis* or *M. arabicida* interacting with phytopathogenic fungi such as *Fusarium oxysporum* and *F. solani* (Bertrand et al., 2000; López-Lima et al., in press). In Mexico, Brazil and Guatemala, *M. paranaensis* is the RKN responsible for MDC, affecting both Arabica and Robusta crops (Carneiro et al., 1996; Carneiro et al., 2004; Villain et al., 2013; Lopez-Lima et al., 2015; Villain et al., 2018).

Notwithstanding, little is known about the bacteria associated with these MDCs and their role in the development of the disease in tomato and coffee crops. *M. enterolobii* and *M. paranaesis* are emerging species causing severe damage in tomato and coffee crops, respectively (Elling, 2013). These two pathosystems differ greatly, since tomato is a short-cycle crop mainly grown under greenhouse conditions and subjected to intensive management, while coffee is a woody perennial crop mainly grown within agroforestry systems.

In order to understand the pathogenicity process of a plant disease, the pathobiome components must be characterized. We are particularly interested in investigating the bacterial microbiome associated with the diseases caused by a root-knot nematode-fungi complex: *Meloidogyne*-based disease complex in tomato and coffee. The study aims were to: i) investigate the bacterial community of tomato and coffee, infested by MDC, and ii) determine whether bacterial community composition and function differ between healthy and infected tissues, as well as in different life stages involved in RKN and rhizospheric soil. This work will allow us to establish a fundamental understanding of the dynamics and ecological role of microbes in the pathobiome of *Meloidogyne*-based disease complex.

MATERIALS AND METHODS

Study System and Sample Collection

Tomato (*Solanum lycopersicum)* samples were collected from a five months-old crop, cultivar Red Grape, located in Culiacan, Sinaloa, Mexico (24°55'59" N, 107°26'34" W at 67m.a.s.l). Coffee (*Coffea arabica*) samples were collected from a four years-old

plantation, cultivar Costa Rica 95 in Jilotepec, Veracruz, Mexico (19°35'42" N, 93°53'01" W at 996 m.a.s.l). Plants were classified according to the progress of MDC symptoms and the root galling index (RGI, Coyne and Ross, 2014) from 1: no visible galling damage to 5: severe galling damage. 1) No symptoms: roots with no galling damage (RGI level 1, healthy root tissues), 2) Initial symptoms: roots with slight galling damage (RGI level 2), separated knots but without rot or necrosis (root nodule tissue), and 3) advanced symptoms: roots with heavy galling damage (RGI level 4–5), hyperplasia, continuous nodules, with deep and cracked cortical tissues and presence of rot and necrosis (corky-root tissues). Five plants were collected per plant species, from them healthy root tissue, root nodule tissue, corky-root tissue (in this study, corky-root refers to severely damaged root tissues in the last stage of the disease), female nematodes, egg masses, and rhizospheric soil were obtained (**Figure S1**, **Table S1**). Female nematodes and egg masses were obtained by dissecting corky roots. Nematodes were identified by species-specific SCAR markers (Randig et al., 2002; Tigano et al., 2010). *Meloidogyne enterolobii* and *M. paranaensis* were identified from tomato and coffee samples, respectively.

Females and eggs were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.4) by slow pipetting. PBS females and eggs washing solution, containing external bacteria, was recovered (**Figure S1**, **Table S1**). All samples were stored at −20°C until processing.

DNA Extraction, 16S rRNA Gene Amplification, and Sequencing

Female nematodes or eggs were ground using a sterile pestle in 1.5 mL tube after adding 1 mL of cold Extraction Buffer I (10 mM Tris, 60 mM NaCl, 5% Sucrose, 10 mM EDTA, pH = 7.8, sterilized and stored at 4°C). Then, 200 µL of Extraction Buffer II (300 mM Tris, 1.25% SDS, 5% Sucrose, 10 mM EDTA pH = 8.0) was added and mixed. Samples were then incubated for 30 min at 65°C. Afterward, 60 µL 3 M Potassium Acetate (pH = 4.8 to 5.2) were added, and the samples were incubated at −20 °C for 20 min, thawed at room temperature for 5 min and centrifuged at maximum speed. The recovered pellet was washed with 70% ethanol. Samples were air dried and resuspended in 35 µL of sterile molecular grade water. Root and soil DNA extraction was conducted using the Quick-DNA ™Fecal/Soil Microbe Miniprep kit (Zymo Research, Cat. No. D6010) following the manufacturer protocol.

To determine bacterial diversity, 16S ribosomal primers were designed from variable regions three and four and used for sequencing in an Illumina platform (Illumina, 2013) in the FISABIO foundation (Valencia, Spain). PCR reactions were conducted using total genomic DNA and Kappa polymerase (Kappa HiFi Hotstart Ready Mix, cat. No. KK2602, Kappa Biosystems). PCR products were cleaned up using magnetic beads (BeckmanCoulter Agencourt Ampure XP, cat. No. A63881) and resuspended in 10 mM Tris pH 8.5 or Resuspension buffer (RSB, Illumina Inc.).

Libraries were constructed using Nextera XT adapters (Illumina Inc.) using the same polymerase and cleaned again using magnetic beads.

Libraries were quantified by fluorescence using a Qubit DNA HS kit, library quality was analyzed using a Tapestation 2200 or Bioanalyzer 2100 and pooled to an equimolar ratio to be sequenced in a 2X300 bp MiSeq Run. The raw data was deposited in NCBI´s sra archive under bioproject accession number PRJNA591631.

Bioinformatic and Statistical Analysis

All the reads with a quality below 20 were filtrated by Prinseq-lite program (Schmieder and Edwards, 2011). R1 and R2 fastq files were joined by fastq-join from the ea-tools suit. Sequences were then checked for chimeras with Mothur (Schloss et al., 2009) using Greengenes 13-8 database (http://greengenes.lbl.gov/). The reads were clustered at 97% in OTUs (Operational Taxonomic Units) using uclust algorithm as implemented in the script pick_open_reference_otus.py from QIIME 1.9.1 (Caporaso et al., 2010; Lan et al., 2012). The taxonomy assignment was done by RDP method (Lan et al., 2012) against Greengenes 13-8 database. Each sample was further manually filtered to remove OTUs missing in at least four out of the five samples in the same treatment, with a frequency lower than 0.01% and with homology to the chloroplast, archaea, and mitochondrial sequences (Supporting Information **Table S2**). Alpha diversity estimations were calculated using the next scripts: multiple_rarefactions.py, alpha_diversity.py, collate_alpha.py and make_rarefaction_plots.py from QIIME 1.9.1. Rarefaction curves were generated by subsampling the OTU table with step increments of 10 and the sampling depth of 1,500 reads. The alpha diversity indexes Chao1, Shannon, and Simpson and the number of OTUs by Treatment, were plotted by "*vegan*" and "*stats*" for R v.3.3.1 (Oksanen et al., 2016; R Core Team, 2016). We applied the Pairwise Wilcox test tested to determine significant differences between the diversity indexes of the treatments.

The relative taxonomic abundance of the Samples and the Treatment was represented by collapsed histogram plotted by "*RColorBrewer*" and "*ggplot2*" libraries for R v.3.3.1 (Wickham, 2009; Neuwirth, 2014). The data table contained the relative frequency of the most frequent OTUs and the collapse frequency of the others by its closest common taxa. To visualize the relative abundance of the most frequent OTUs a heat map per treatment was constructed by "*ape*", "*gplots*", "*vegan*" and "*RColorBrewer*" libraries for R v.3.3.1 (Paradis et al., 2004; Warnes et al., 2016). The OTUs with a frequencies higher than 0.1% were represented and the soil samples were not considered.

To represent dissimilarities across the samples, we performed a nonmetric multidimensional scaling (NMDS) using the Bray-Curtis distances matrix. In the NMDS plot, the distance between objects represents the relative dissimilarity between them. The measure of the goodness of fit of the final NMDS plot was represented by the *stress* value, which indicates the match between inter-object distance and dissimilarity. The closer the *stress* value is to zero, the better the ordination (Manly, 1986; Quinn and Keough, 2002). Finally, the effect of crop and sample type was evaluated with a permutational multivariate ANOVA (PERMANOVA) using the Bray-Curtis dissimilarities (Anderson,

2001; Anderson and Walsh, 2013), the significance threshold for PERMANOVA was set at $p < 0.001$. All the analyses were performed using "*vegan*" and "*mass*" for R v.3.3.1 (Oksanen et al., 2016; Venables and Ripley, 2002).

We predict the metagenome functional profiling of the different samples base on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway by PICRUSt. To assess the accuracy of the PICRUSt predictions, the Nearest Sequenced Taxon Index was calculated for each sample and treatment (Langille et al., 2013; **Tables S3** and **S4**). The effect of crop and sample type on KEGG orthology (KOs) was evaluated with a PERMANOVA using a Bray-Curtis dissimilarities matrix, which was previously calculated considering the relative abundance of functional categories in all samples. The significance threshold for PERMANOVA was set at $p \leq 0.001$. When PERMANOVA was significant, differences between samples were determined with multiple pairwise comparisons using a Wilcoxon test with FDR correction set at $p \leq 0.05$. All the analyses were performed using "*vegan*" and "*mass*" for R v.3.3.1.

We generated two cluster dendrograms based on the Bray-Curtis distance matrix estimating of the OTUs taxonomic abundance filtered at 0.01%, the KOs abundance predicted by PICRUSt by "*vegan*" for R v.3.3.1.

We made a graphical representation of the main drivers of the healthy and Corky/knot conditions using relative abundance data at order level.

Based on the functional dendrogram results we defined three main clusters, and we built Venn diagrams of the specific or shared KOs between corky clusters and the healthy cluster, and among coffee and tomato corky clusters and the soil cluster.

RESULTS

Diversity and Species Richness of Bacterial Community

A total of 2,131,578 reads were sequenced by Illumina PE platform. The quality check removed 20% of the reads obtaining an average of 53,846 quality read filters per sample (max = 2,002,726, min = 1,426) (**Table S1**). The sequencing depth quality was confirmed by estimating the Chao, Shannon, and Simpson alpha diversity indexes and rarefaction curves of the number of observed OTUs, where all the curves reflected a saturated sampling (**Figure S2**).

The OTUs richness and diversity were calculated using Simpson and Shannon indexes. In general, in both crops the number of observed OTUs differed significantly among sample types (ANOVA, Tomato: $F_{(7,\ 40)} = 8.72$, $p = 5.77e\text{-}06$; Coffee: $F_{(7,\ 40)} = 25.97$, $p = 1.73e\text{-}11$), with higher values in soil samples than in root samples, and higher values in root samples than in nematode samples (Supporting Information **Figure S3**). Moreover, the coffee samples exhibited significantly higher values than tomato samples, with a maximum value of 310.45 ± 19.45 (observed OTUs) for coffee soil samples and a minimum value of 81.8 ± 51.9 (observed OTUs) for female wash solution from tomato samples ($F_{(7,\ 40)} = 4.43$, $p = 0.0394$). Regarding the diversity indexes, significant differences were only detected across coffee samples (Shannon: $F_{(7,\ 40)} = 12.58$, $p = 1.25e\text{-}07$, Simpson: $F_{(7,\ 40)} = 2.83$, $p = 0.206$), registering the maximum diversity values in soil samples (7.147 ± 0.277) and the minimum in female wash solution samples (4.252 ± 0.609) (Supporting Information **Figure S3**).

The high-quality reads were clustered over 881 OTUs, most of them belonging to the phylum Proteobacteria (49%), Bacteroidetes (14%), and Actinobacteria (10%). The relative abundance of major genera and families by treatment is shown in **Figure 1** (Supporting Information **Figure S4**). The genera *Pseudomonas* and *Flavobacterium* had the highest relative abundance with a total of 8.07% and 5.67%, respectively. The genera shared for all samples were *Pseudomonas* and *Bacillus*, while at OTUs level 560886 and 961783 (*Pseudomonas*) were the only ones common to all samples. The core microbiome of the tomato samples consisted of the genera *Cellvibrio* and *Rhodoplanes*, while *Microbacterium*, *Achromobacter*, *Pedobacter,* and *Flavobacterium* were the genera present in core microbiome of coffee. The OTUs 558264 (*Achromobacter*) and NCR.OTU928383 (*Pedobacter*) were shared by all the coffee samples (**Figure 2**). No common core microbiome was found among nematode samples. Root samples however, shared the genera *Novosphingobium*, *Sphingobacterium,* and *Janthinobacterium.* In the case of *Janthinobacterium*, we found 10 OTUs with a relative frequency lower than 0.1%. Specifically, the corky root had the genera *Nocardia* in common, although in low frequencies, 0.001% in corky root tomato and 0.009% in corky root coffee. Soil and Corky tomato samples shared 22 OTUs belonged to the genera *Pseudomonas, Sphingobacterium, Flavobacterium, Rhodoplanes, Cellvibrio, Shinella, Luteolibacter, Brevundimonas, Janthinobacterium, Chitinophaga, Bacillus,* and *Agrobacterium,* while Soil and Corky coffee samples shared 10 OTUs belonged to the genera *Pedobacter, Janthinobacterium,* and *Candidatus Solibacter*.

Permutational ANOVA (PERMANOVA) and NMDS analysis (NMDS) of the bacterial community revealed that both factors, crop species and sample type, clearly determine the bacterial community assemblage (**Figure 3**, **Table 1**). The microbiome structure of all coffee samples differed significantly from the all the tomato samples (PERMANOVA, $F_{(1,\ 80)} = 213.987$, $R^2 = 0.27180$, $p = 0.001$; **Figure 3**). Moreover, the soil samples associated to each crop, differed from the other samples (Tomato: $F_{(1,\ 40)} = 27.584$, $R^2 = 0.85783$, $p = 0.001$; Coffee: $F_{(1,\ 40)} = 25.753$, $R^2 = 0.84925$, $p = 0.001$; **Figure 3**). A split analysis of all tomato or coffee samples showed significant differences between root and nematode samples (**Figure S5**) and among the different disease developmental stages (knots vs. corky tissues) and the nematode stages (eggs vs. females) (**Figure S5**).

The microbiome structure was modified by nematode infection, leading to the depletion of Pseudomonadales (Gammaproteobacteria) and Sphingobacteriales (Sphingobacteria) and the enrichment of Actinomycetales (Actinobacteria), in both crops. The microbiome structure variation of both crops differed in the taxonomic orders. The corky-root coffee samples exhibited an enrichment of Bacillales (Bacilli) and Burkholderiales (Betaproteobacteria) in comparison with healthy samples, while in corky-root tomato samples

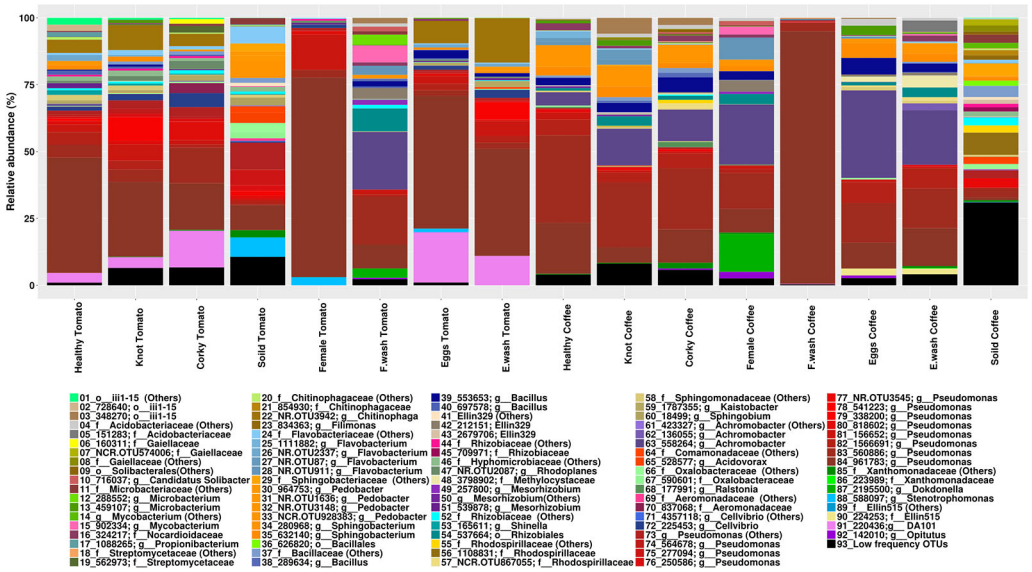

FIGURE 1 | Relative taxonomic abundance by treatment represented by the most frequent OTUs, the least frequent taxa are collapsed in others OTUs.

Saprospirales (Saprospirae), Chthoniobacterales (Spartobacteria), Alteromonadales (Gammaproteobacteria), and Xanthomonadales (Gammaproteobacteria) were enriched in comparison with healthy samples and Rhodobacterales were slightly depleted in corky-root samples. The pairwise comparisons of relative abundance between Healthy, Knot, and Corky coffee samples within the order Rhodobacterales were significant with a p-value of 0.03 (**Figure S6**).

Predictive Functional Analysis of the Bacterial Microbiome

The association of the microbial community with a metabolic profile was assessed by PICRUST analysis. We predicted the gene content of the bacterial microbiome and construct a dendrogram based on taxonomic and metabolic dissimilarities (**Figure 4**, **Tables 1** and **2**). The results showed that, while at a taxonomic level the samples grouped primarily based on the crop species (tomato vs. coffee plant) and within each of both groups, based on the origin of the sample (nematode stage vs. root source); at a functional level (KO), in both cases (tomato and coffee plants), the PBS wash solution eggs and the corky root samples grouped together, with the particularity that within the coffee cluster, knot samples were also present in this cluster and that in the tomato samples, egg samples belonged to this cluster. The healthy root samples of both crops clustered together along with knot tomato samples. Regarding coffee and tomato soil samples, at a taxonomic level they both clearly separated and clustered with their relative root and nematode crop samples, whereas at a functional level (KO), they clustered together and with the coffee infested roots and nematode stages samples.

The Healthy Root Cluster (healthy tomato roots, healthy coffee roots, and tomato root-knot) had 6,071 KOs, the Coffee Cork Cluster (corky roots, root-knots, wash solution egg) had 6,211 KOs and the Tomato Cork Cluster (corky roots, eggs, wash solution egg) had 5,264 KOs shared 3,147 KOs and had 135, 220, and 9 specific KOs, respectively. The metabolic biomarkers that defined the healthy root stage were defined by the presence of 135 KOs, which were classified principally in the following functional categories: Membrane transport (42 KOs), Carbohydrate metabolism (13 KOs), Cellular processes (eight KOs), Glycan biosynthesis and metabolism (eight KOs), Metabolism of cofactors and vitamins (seven KOs), and Poorly characterized functions (26 KOs) (**Figure 5**).

Moreover, the samples of the Healthy Cluster had 285 KOs with higher relative frequency and 135 KOs with a lower significant relative abundance compared to the Corky Coffee Cluster, while the Healthy Cluster samples had 113 KOs with significantly higher relative abundance and 50 KOs with a significantly lower relative abundance compared to the samples of the Corky Tomato Cluster (**Table S5**, **Figure 6**). The 285 mentioned KOs with higher relative frequency in the healthy cluster vs. in the corky coffee cluster classified principally in the following functional categories: Membrane transport (94 KOs), Amino acid metabolism (36 KOs), Transcription (36 KOs), Glycan biosynthesis and metabolism (26 KOs), Carbohydrate metabolism (25 KOs), Cellular processes and signaling (11 KOs), and Unclassified (61 KOs). The other 135 with lower relative frequency classified in almost the same functional categories: Membrane transport (105 KOs), Carbohydrate metabolism (61 KOs), Amino acid metabolism (50 KOs), Transcription (45 KOs), Cellular processes and signaling (13 KOs), and Unclassified (83 KOs). Only the metabolic functions Replication and repair and Glycan biosynthesis and metabolism showed higher numbers in the Healthy Cluster samples (15 and 26 KOs, respectively) than in the Corky Coffee Cluster samples (9 and 14 KOs,

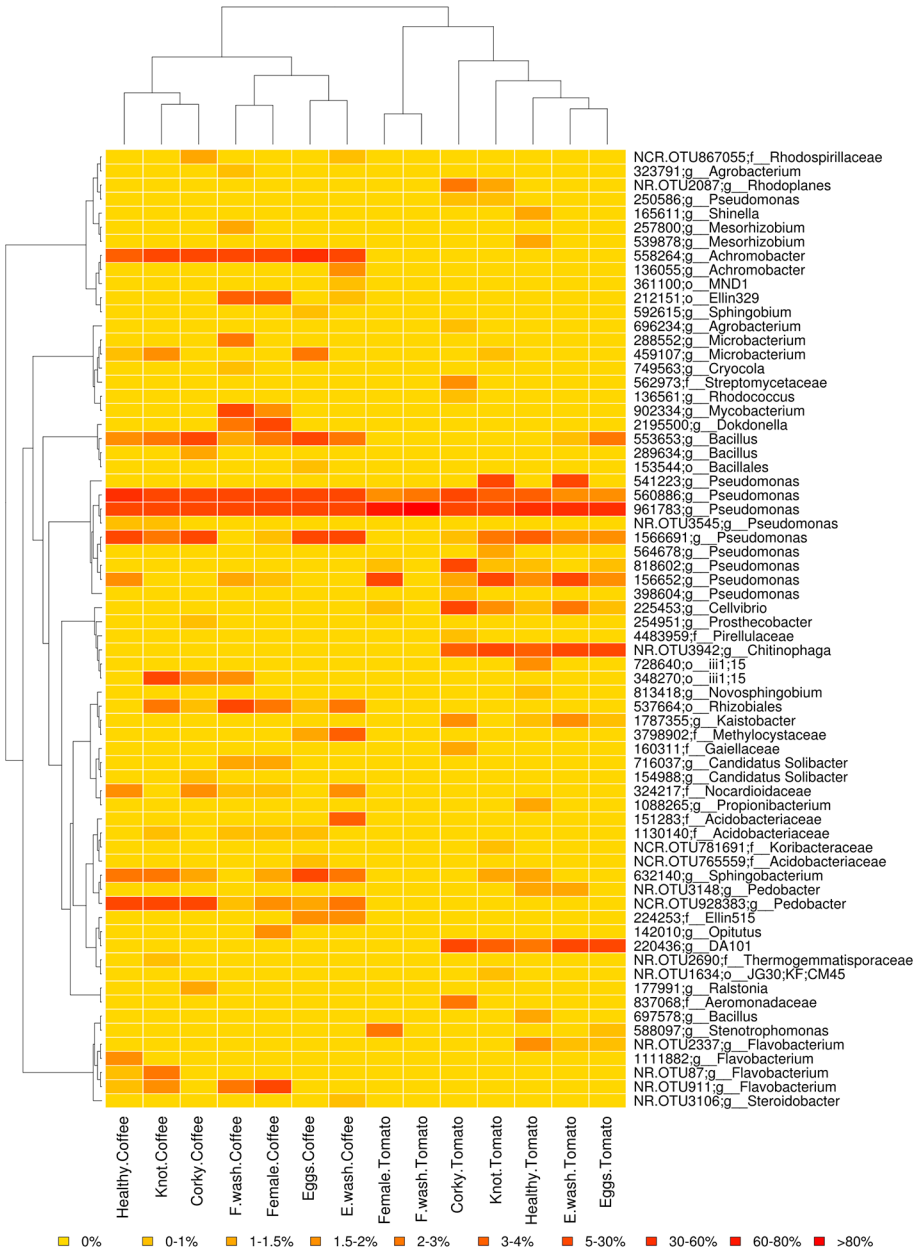

FIGURE 2 | Relative abundance heat map of the OTUs with a frequency higher than 0.1% by treatment. The soil samples are not taken into account. The dendrogram on the left shows the phylogenetic relationship between the OTUs, the dendrogram on the top illustrate the relationship between the samples based on Ray distance. Yellow, low relative abundance; orange, high relative abundance.

respectively). Regarding the 113 KOs with higher frequency in Healthy Cluster compared to Corky Tomato Cluster, they classified mainly in the functional categories: Membrane transport (20 KOs), Carbohydrate metabolism (20 KOs), Amino acid metabolism (19 KOs), Xenobiotics biodegradation and metabolism (19 KOs), Cellular processes and signaling (four KOs), and Unclassified (19 KOs), while the other 50 KOs with lower frequency classified on Metabolism of cofactors and vitamins (six KOs), Amino acid metabolism (five KOs) Cellular processes and signaling (six KOs), and Unclassified (six KOs). While the metabolic profile of the Corky Coffee Cluster samples was defined by the increase in the KOs number of the different metabolic categories compared to the Healthy Cluster samples, the Corky Tomato Cluster samples were defined by a decrease of the same functional categories.

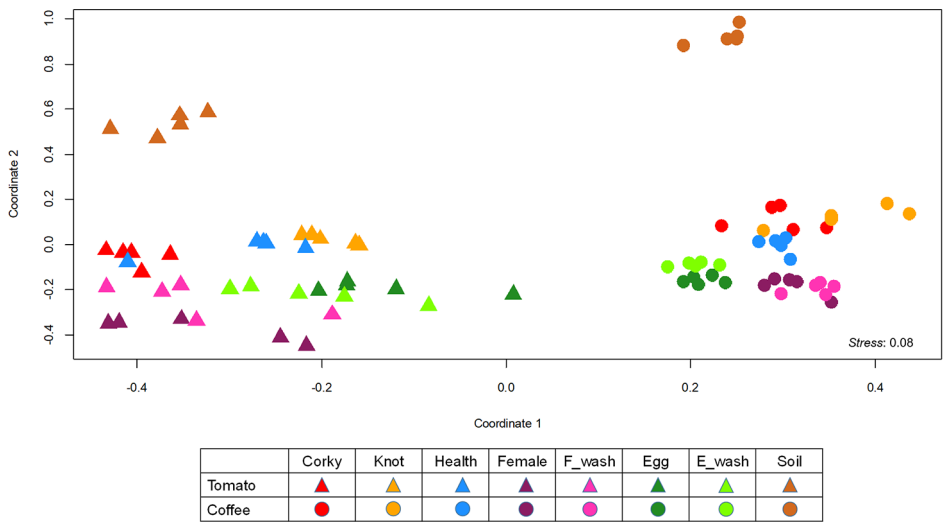

FIGURE 3 | Non-metric multidimensional scaling (NMDS) analysis of all the samples. NMDS plot for Bray–Curtis dissimilarities of the bacterial communities associated with tomato and coffee.

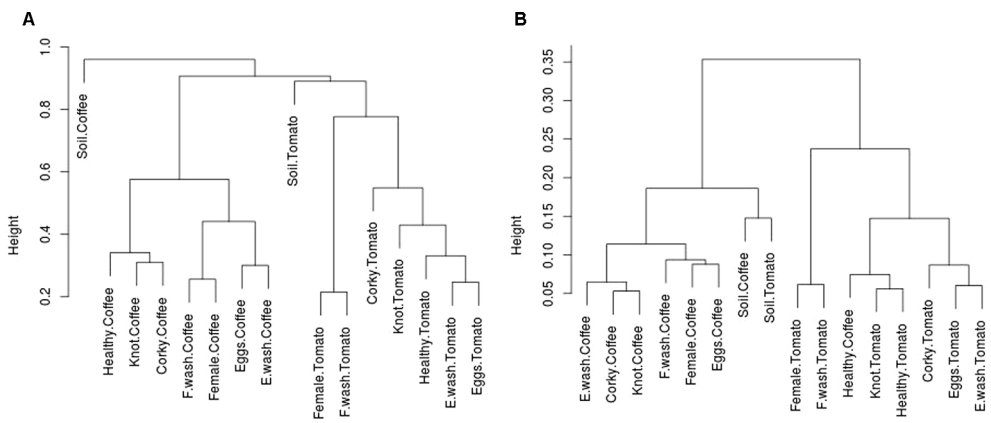

FIGURE 4 | Cluster dendrogram of dissimilarity matrix of **(A)** OTUs relative abundance and **(B)** KO's abundance predicted by PICRUSt.

TABLE 1 | Permutational multivariate ANOVA of the bacterial communities associated with different sample types of coffee and tomato.

Factor[a]	*F*	R^2	*P*
Global			
$Crop_{1, 64}$	206,690	0.27095	0.001
$Treatment_{7, 64}$	41,087	0.37703	0.001
$Crop:treatment_{7, 64}$	29,218	0.26812	0.001
Tomato			
$Treatment_{7, 32}$	26,627	0.85347	0.001
Coffee			
$Treatment_{7, 32}$	26,277	0.85181	0.001

[a]*Subscript numbers indicate the degrees of freedom and residuals of each F test.*

Corky stage has been characterized by the presence of 10 specific KOs, which were classified principally in the following functional categories: Cellular processes and signaling (KOs 4), Membrane transport (3), Unclassified (3). Compared to Corky Coffee stages, only 220 specific KOs were found and classified in: Membrane transport (34 KOs), Carbohydrate metabolism (23 KOs), Amino acid metabolism (15 KOs), Cellular processes and signaling (14 KOs), Enzyme families (13 KOs), Xenobiotics biodegradation and metabolism (seven KOs), and Unclassified (57 KOs). In Corky Tomato stages, only nine specific KOs were found, involved principally in Membrane transport (three KOs), Cellular processes (signaling, 2) and Unclassified (four KOs).

TABLE 2 | Permutational multivariate ANOVA of the metabolic functionality Kyoto Encyclopaedia of Genes and Genomes categories of the bacterial communities associated with different sample types of coffee and tomato crops.

Factor[a]	*F*	R^2	*P*
Global			
$Crop_{1, 64}$	2.7409	0.17921	0.001
$Treatment_{7, 64}$	5.4902	0.25139	0.001
$Crop:treatment_{7, 64}$	3.291	0.15069	0.001
Tomato			
$Treatment_{7, 32}$	4.9528	0.52002	0.001
Coffee			
$Treatment_{7, 32}$	2.9209	0.38985	0.001

[a]*Subscript numbers indicate the degrees of freedom and residuals of each F test.*

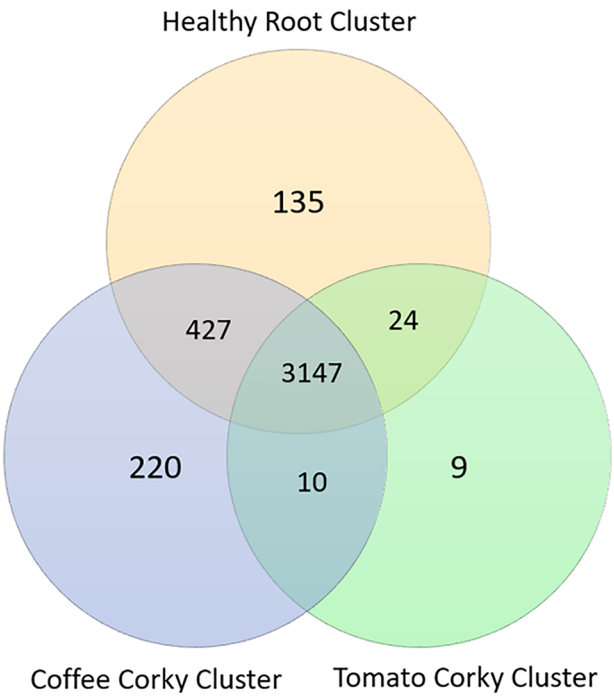

FIGURE 5 | Venn diagram of the KO numbers specific to ot shared by the coffee and tomato corky root clusters and the healthy root cluster.

A specific analysis of the metabolic profile shared by the Soil Clusters and both Cork Cluster, showed that Cork coffee Cluster and Soil Cluster shared 290 KOs, with 14.14% (67 KOs) involved in Environmental Information Processing and 60.34% (286 KOs) involved in Metabolism. The Cluster Cork Tomato and Soil Cluster shared 128KO's which were involved in Environmental Information Processing 35,96% (64 KOs) and 35,96% (64 KOs) involved on Metabolism (**Figure S7**).

DISCUSSION

In this study, we used 16S rDNA gene Illumina sequencing to analyze the microbial communities associated with *M. enterolobii* and *M. paranaensis* and different disease stages of the MDC in tomato and coffee field plants. The microbiome isolated from soil, healthy roots, infested roots, and different nematode developmental stages of each crop showed a very complex and distinct structure.

The two crop systems (tomato vs. coffee) differed in their bacterial community, alpha diversity and OTUs richness, all these variables were higher in coffee than in tomato samples. Similarly, the crop type clearly affected the composition of MDC bacterial microbiome, as revealed by NMDS and PERMANOVA. Coffee samples came from perennial plantations, grown under agroforestry systems, holding several tree shade species and with minor use of fertilizers and pesticides (Gordon et al., 2007; González-Zamora et al., 2016). Moreover, tillage is not employed, leading to the establishment of many soil microbiota species (Legrand et al., 2018; Dube et al., 2019). By contrast, tomato, an annual crop with short cycle, is known by its intensive cropping systems, with high demand of agrochemicals (both fertilizers and pesticides), applied before and during the growing cycle (Pérez-Olvera et al., 2011; Pratt and Ortega, 2019). Previous studies have shown that plant management or status has a clear effect on the associated microbiome (Coleman-Derr et al., 2016; Hartman et al., 2018; Compant et al., 2019) and that intensively grown crops have a lower diversity of associated plant and soil microbiota than agroecosystems subjected to less aggressive agricultural practices (Rathore et al., 2019; Saleem et al., 2019).

We consider that the significant differences of microbiota diversity found between the two studied crop systems could be related with the management practices, leading to the establishment of a more diverse bacterial community in the coffee agroecosystem.

Moreover, we found significantly higher alpha diversity and OTUs richness in root samples than in nematode samples. However, for both crops, there was no significant differences between disease stages or nematode life cycle stages. These results differ with those observed by Wolfgang et al. (2019), who describe decreasing alpha diversity during the root disease process. Conversely, we observed a non-significant increasing diversity in disease stages in both crops.

We identified Pseudomonadales, Sphingobacteriales, and Actinomycetales as the main common drivers of the MDC implicated in the infection process for both crops. Additionally, we observed crop-specific drivers of the MDC infection process: Bacillales and Burkholderiales in coffee samples and Saprospirales, Chthoniobacterales, Alteromonadales, and Xanthomonadales in tomato samples. Few similarities with the results published by Tian et al. (2015) and Wolfgang et al. (2019) were observed. Our results and the two cited studies only agree in the richness reduction of Pseudomonadales in the corky-root samples in comparison with the healthy ones. The order Pseudomonadales contains species listed as opportunistic parasites (Castillo et al., 2017); however, other species in the same order have shown nematicide effects by the production of compounds such as cyanide (Siddiqui et al., 2006). The discrepancies regarding

FIGURE 6 | Venn diagram of the KO numbers **(A)** with higher significant relative frequency, and **(B)** with lower significant relative frequency, on the healthy root cluster relative to coffee corky root and tomato corky root clusters.

the main drivers observed in these studies may be due to cultivar differences (Rybakova et al., 2017). These differences could be explained by the diverse sets of available microorganisms with varied abilities to attach to the nematode cuticle. We also consider that the rhizobiome composition could be affected by the physicochemical conditions of the soil (Bonito et al., 2019; Saad et al., 2019). Wolfgang et al. (2019) proposed that changes in physiological conditions of plant cells is the cause by a microbial structure shift, and this is influenced by the ability of some bacteria to adhere to the nematode cuticle (Elhady et al., 2017). In addition, it has been shown that the rhizosphere microbiome may influence the severity of RKN infection by promoting pathogens in infested soils and suppressing pathogens in uninfected soils (Zhou et al., 2019).

At the genera level, only *Nocardia* was shared by the corky-root samples. Species of this genus are found worldwide in soils rich in organic matter, some of which are plant pathogenic (Xing et al., 2011; Bai et al., 2016; Conville et al., 2017). *Nocardia* sp. is the causal agent of the disease known as tobacco false broomrape, which causes cracked tumors in the main and secondary roots, resembling the hyperplasia produced in the advanced stages of the MDC in tomato and coffee (Morán et al., 2013; Morán-Gómez et al., 2018). Moreover, *Agrobacterium* was externally recovered from *M. enterolobii* female and tomato corky-roots. Species of this genus have virulence genes that affect the response of the plant cells to growth regulators (auxins and cytokinins) and induce uncontrolled cell division, which results in tissue proliferation and gall formation (Bourras et al., 2015; Lacroix and Citovsky, 2019). Thus, the genera *Nocardia* and *Agrobacterium*, associated with RKN microbiome reported in this and other studies (Cao et al., 2015), may contribute to the formation of corky root symptoms in the MDC.

Additionally, *M. enterolobii* produces the MeTCTP (translationally controlled tumor protein) effector that promotes parasitism by suppressing programmed cell death in host plants (Zhuo et al., 2017). The combined effect of the tumor caused by the nematode and the bacteria could explain the remarkably wide range of hosts and severity of symptoms observed in plants infested with this nematode (Elling, 2013; Jones et al., 2013); however, this requires further investigation.

The grouping pattern based on the microbiome metabolic prediction differs from the grouping pattern based on the taxonomic information. At taxonomic level we observed two big clusters based on the crop of origin (Tomato vs. Coffee) and the type of sample (Roots vs. Nematodes), leaving the soil samples alone as an external group. By contrast, we observed three metabolic clusters: the Healthy Cluster (Healthy coffee and tomato roots plus Tomato Root-Knot samples); the Coffee Corky cluster (Coffee Corky-Roots and Root-Knots plus Coffee PBS washout nematode Eggs samples); and the Tomato Corky cluster (Tomato Corky-Roots plus Tomato nematode Eggs and PBS washout Eggs samples).

Metabolic functions of the microbiota associated with the soil samples of both crops were comparable, as well as the metabolic profile of healthy roots of both crops. The metabolic functional categories enriched in Corky samples versus soil samples, considering the KOs analysis, were associated to pathogenicity, and include cell signaling and regulation and membrane transport, and other categories like carbohydrate metabolism, amino acid metabolism, cofactor and vitamins metabolism, family enzymes, and transcription factors related with the increase in the metabolic activity in the nematode feeding site. Our metagenomics analyses conducted in the two phathosystems (Tomato- *M. enterolobii*, and Coffee- *M. paranaensis*) suggest that root infection by the nematode is, on the contrary, not linked to a specific microbiome community, but is associated

with a metabolic profile shift characterized by an increase in pathogenicity and central metabolism functional categories, which is consistent with Tian et al. (2015) observations.

Additionally, one of the defense mechanisms of the coffee plant toward the attack of *Meloidogyne* spp. is the accumulation of phenolic compounds at the feeding site to limit their reproduction (Albuquerque et al., 2010; Silva et al., 2010). Our results, indicate that corky-roots of coffee plants had more metabolic profiles that encode enzymes involved in the pathway of benzoate degradation (aminobenzoate and benzoate) than healthy coffee roots. Benzoate is an intermediate in the pathway of anaerobic metabolism of aromatic compounds, i.e. phenolic compounds (Carmona et al., 2009). It is possible that some of the bacteria associated with coffee corky-root prevent the accumulation of phenolic compounds at the feeding site of *M. paranaensis* and contribute to the infection process, as occurs in other pathosystems (Cheng et al., 2013).

Our results indicate that, during nematode pathogenesis and nematode feeding site induction in plants, the MDC infection process could be mediated by particular metabolic microbial categories at a functional level, even though the two MDCs studied here involved different RKN species and unspecific associated bacterial taxa. Nonetheless, further studies are required to gain a deeper understanding of the bacterial functional roles in the MDC infection process.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI using accession number PRJNA591631.

AUTHOR CONTRIBUTIONS

GC, DL-L, ArL designed the experiments. DL-L and LV collected and prepared the samples. DL-L, ArL extracted the DNA. AM and AmL performed the sequencing. ArL and AA performed the bioinformatic analysis. DD performed the statistical analyses. GC, ArL, DD, DL-L, LV, and AA-S wrote the manuscript. GC, ArL, DD, DL-L, LV, AA-S, AM, AmL read, revised, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00136/full#supplementary-material

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REVIEW
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Plants and Associated Soil Microbiota Cooperatively Suppress Plant-Parasitic Nematodes

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Disease suppressive soils with specific suppression of soil-borne pathogens and parasites have been long studied and are most often of microbiological origin. As for the plant-parasitic nematodes (PPN), which represent a huge threat to agricultural crops and which successfully defy many conventional control methods, soil progression from conducive to suppressive state is accompanied by the enrichment of specific antagonistic microbial consortia. However, a few microbial groups have come to the fore in diminishing PPN in disease suppressive soils using culture-dependent methods. Studies with cultured strains resulted in understanding the mechanisms by which nematodes are antagonized by microorganisms. Recent culture-independent studies on the microbiome associated with soil, plant roots, and PPN contributed to a better understanding of the functional potential of disease suppressive microbial cohort. Plant root exudation is an important pathway determining host-microbe communication and plays a key role in selection and enrichment of a specific set of microbial antagonists in the rhizosphere as first line of defense against crop pathogens or parasites. Root exudates comprising primary metabolites such as amino acids, sugars, organic acids, and secondary metabolites can also cause modifications in the nematode surface and subsequently affect microbial attachment. A positive interaction between hosts and their beneficial root microbiota is correlated with a low nematode performance on the host. In this review, we first summarized the historical records of nematode-suppressive soils and then focused on more recent studies in this aspect, emphasizing the advances in studying nematode-microbe interactions over time. We highlighted nematode biocontrol mechanisms, especially parasitism, induced systemic resistance, and volatile organic compounds using microbial consortia, or bacterial strains of the genera *Pasteuria*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Streptomyces*, *Arthrobacter*, and *Variovorax*, or fungal isolates of *Pochonia*, *Dactylella*, *Nematophthora*, *Purpureocillium*, *Trichoderma*, *Hirsutella*, *Arthrobotrys*, and *Mortierella*. We discussed the importance of root exudates in plant communication with PPN and soil microorganisms, emphasizing their role in microbial attachment to the nematode surface and subsequent events of

nematode parasitism. Comprehensive understanding of the plant-beneficial microbial consortia and the mechanisms underlying disease suppression may help to develop synthetic microbial communities for biocontrol of PPN, thereby reducing nematicides and fertilizers inputs.

Keywords: disease-suppressive soils, plant-parasitic nematodes, root exudates, rhizosphere microbiome, root endophytes, nematode antagonists, induced systemic resistance

INTRODUCTION

Disease suppressive soils are the conspicuous prototype of a microbe-mediated plant defense against pathogen infection. In general, suppressive soils are those in which soil-borne pathogens and parasites do not establish or persist, establish but cause limited or no disease at all, or establish and cause disease onset initially before it abates (Baker and Cook, 1974; Weller et al., 2002). By contrast, agricultural soils in which pathogens and parasites infect plants and cause diseases are referred to as non-suppressive or conducive soils (Weller et al., 2002; Garbeva et al., 2004). By definition, general soil suppressiveness, a typical epitome of cumulative soil microbiome competitive activities, is supposed to act against a wide range of soil-borne diseases (Cook and Baker, 1983; Borneman and Becker, 2007). These soils operate on the principle of "seed, feed, and weed" such as it could be initiated by the addition of organic matter in the soil or on the presence of seed and root exudates (seed), which results in the uptake of nutrients by diverse microorganisms (feed), and consequently limiting the outbreak of pathogens and parasites (weed). Therefore, general soil suppressiveness is reduced by soil steaming, but cannot be transferred by small amounts of suppressive soil to conducive soil (Cook and Baker, 1983; Weller et al., 2002). On the other hand, specific soil suppressiveness is typically induced in field soils during crop monoculture after a disease outbreak. It relies on the antagonistic activities of specifically enriched microbial consortia that disrupt the life cycle of plant pathogens or parasites (Raaijmakers and Mazzola, 2016). Specific soil suppressiveness can be eliminated by soil sterilization and biocide treatments and is transferable to a conducive soil with small amounts of a suppressive soil (0.1–10%) (Yin et al., 2003a; Borneman and Becker, 2007). The criterion of transferability implies that the transferred antagonistic microbes can sufficiently multiply in the conducive soil to reach suppressive densities. This depends on interactions with soil biota, roots, or for specialized antagonists on the density of the pathogen. The distinction between general and specific soil suppressiveness may thus be rather related to the diversity of antagonists than the mechanisms of suppressiveness.

Soils with specific suppressiveness have been reported for plant-parasitic nematodes (PPN) from distinct geographical locations worldwide. Importantly, the life cycle of PPN is distinct from those of pathogenic fungi and bacteria, and diverse microbes were characterized to prey and parasitize different stages of nematodes (Li et al., 2015). Consequently, most of the studies were mainly focused on identifying the microbes that can directly kill PPN, while the plant-mediated microbial suppression of nematodes was often overlooked. In soil systems, nematophagous fungi and bacteria have diverse strategies to attack PPN, e.g., nematode-trapping fungi form adhesive hyphal traps, endoparasitic fungi and bacteria use spores, egg- and female-parasitizing fungi use hyphal tips, and several fungi and bacteria produce toxins to prevent plant roots from nematode invasion (**Figure 1**). Recently, using the next-generation technologies several studies have come to the fore signifying the role of soil and root microbiota that disturb the performance of PPN. Thus, a detailed understanding of the microbiome associated with the soil, plant roots, and distinct life stages of PPN may enable us to engineer (synthetic) core microbial consortia that can act as a sustainable alternative to control nematode diseases and to enhance crop productivity. Herein, we will review the studies highlighting the contribution of microbes in inhibiting nematodes in disease suppressive soils using culture-dependent and culture-independent methods. In addition, we will highlight the importance of root-associated microbiota in plant-nematode interactions. Finally, we will tackle some important points on the role of root exudates in nematode infection and their effects on the nematode surface coat (SC), which influence nematode-microbe interactions in soil. This review will give insights into the plant-nematode-microbe interactions in suppressive soils.

HISTORY OF NEMATODE-SUPPRESSIVE SOILS

The chronological records of nematode-suppressive soils reliably show the progress that has been made in understanding nematode-microbe interactions. The decline of PPN populations in field soils was first reported in 1962 by Collingwood for the cyst nematode *Heterodera avenae* in the United Kingdom under intensive cropping of cereal hosts (Collingwood, 1962; Kerry, 1982). Since then, suppressive soils have been reported for several nematode species including *H. avenae* in the United Kingdom (Gair et al., 1969; Williams, 1969; Kerry, 1975), *Heterodera schachtii* in Netherlands, United Kingdom, and United States (Heijbroek, 1983; Crump and Kerry, 1987; Westphal and Becker, 1999), *Globodera rostochiensis* in Germany (Roessner, 1987), *Globodera pallida* in the United Kingdom and Germany (Crump and Flynn, 1995; Eberlein et al., 2016), *Heterodera glycines* in the United States and China (Chen et al., 1996b; Sun and Liu, 2000; Hamid et al., 2017; Hussain et al., 2018), the false root-knot nematode *Nacobbus aberrans* in Mexico (Zuckerman et al., 1989), root-knot nematodes *Meloidogyne* spp. in Mexico, United States, Spain, and Germany (Bird and Brisbane, 1988; Zuckerman et al., 1989; Chen et al., 1994b; Pyrowolakis et al., 2002;

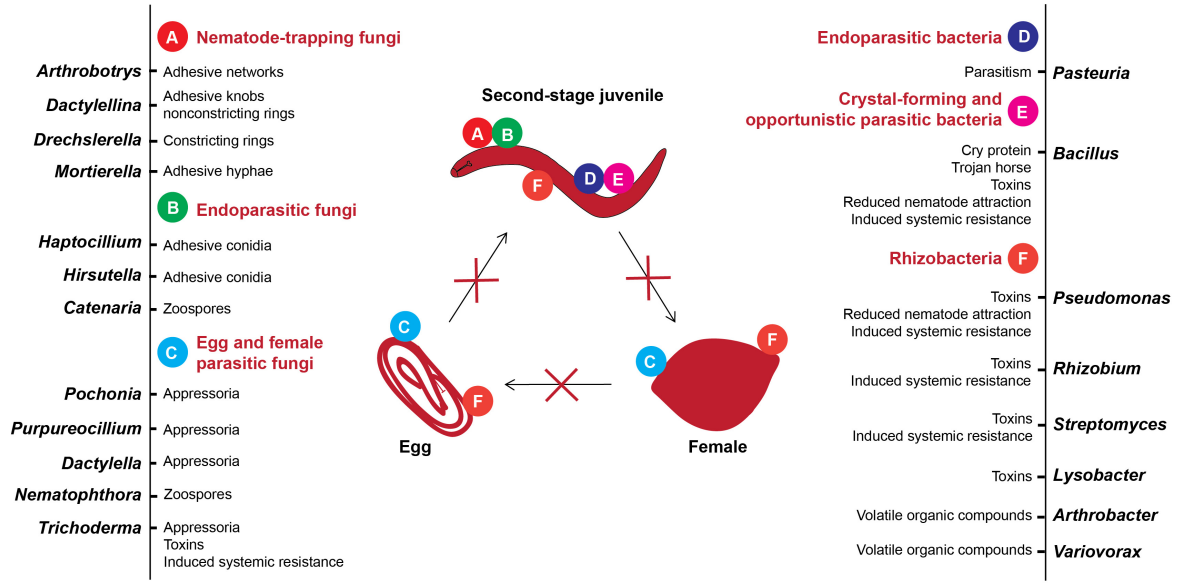

FIGURE 1 | Microbial antagonists of plant-parasitic nematodes and mechanisms of their antagonism.

Bent et al., 2008; Adam et al., 2014b; Giné et al., 2016), and the ring nematode *Mesocriconema xenoplax* in the United States (Kluepfel et al., 1993, 2002).

General and specific suppression of soil-borne pathogens and parasites is most often of microbiological origin. In 1960s, the formalin application was the only factor known to reduce soil suppressiveness against *H. avenae* in agricultural fields (Williams, 1969). It was suggested that the application of formalin inhibited the parasitic fungi *Nematophthora gynophila* and *Pochonia chlamydosporia* and thereby resulted in an increased population density of *H. avenae* (Kerry et al., 1980; Kerry, 1988). Later, several studies demonstrated the microbial involvement in soil suppressiveness by soil autoclaving or heating (Bird and Brisbane, 1988; Zuckerman et al., 1989; Kluepfel et al., 1993; Chen et al., 1996b; Weibelzahl-Fulton et al., 1996; Westphal and Becker, 1999; Sun and Liu, 2000; Bent et al., 2008; Adam et al., 2014b; Eberlein et al., 2016; Giné et al., 2016; Hamid et al., 2017; Bhuiyan et al., 2018), by the application of biocides (Kerry et al., 1980; Crump and Kerry, 1987; Westphal and Becker, 1999; Sun and Liu, 2000; Westphal and Becker, 2001; Pyrowolakis et al., 2002; Yin et al., 2003a,b; Bent et al., 2008; Song et al., 2017), and through soil transplantation (Mankau, 1975; Stirling and Kerry, 1983; Kluepfel et al., 1993; Westphal and Becker, 2000; Sun and Liu, 2000; Yin et al., 2003a,b; Chen, 2007; Bent et al., 2008). Notably, the soil suppressiveness was also observed to be transferred by egg-suspensions of the root-knot nematode *Meloidogyne incognita* (Orion et al., 2001) and cysts of the sugar beet cyst nematode *H. schachtii* (Westphal and Becker, 2001). Some soils with specific suppressiveness have been characterized and underlying microbial mechanisms were proposed, including parasitism and antibiosis. The role of parasitism in nematode-suppressive soils has extensively been studied for root-knot nematodes (Mankau, 1975), cereal cyst nematodes (Stirling and Kerry, 1983), sugar beet cyst nematodes (Olatinwo et al., 2006b; Becker et al., 2013), and soybean cyst nematodes (Chen, 2007). Antibiosis is an important mechanism of biocontrol, in which the antagonist produces metabolites, such as lytic enzymes, toxins, antibiotics, or volatile compounds, which potentially disrupt the pathogen invasion. Unlike the essential increased initial concentration of parasites for a successful nematode control, it has been noted that nematode suppression by some rhizobacteria, employing different modes of action including antibiosis, can be accomplished at lower microbial densities (Kluepfel et al., 1993). This was the case with ring nematode (*M. xenoplax*)-suppressive soils, where *Pseudomonas* sp. producing salicylic acid were found to dramatically alter the development of eggs and to be involved in the inhibition of egg hatch (Kluepfel et al., 2002).

More and more studies on soil microorganisms associated with nematode decline have unraveled an array of new microbial species with an antagonistic potential against PPN. In the following lines we will review bacteria and fungi that were either isolated from the diseased or dead nematodes, or that were identified in nematode-suppressive soils by next-generation sequencing. The culture-dependent and culture-independent approaches in determining nematode antagonists will be compared and discussed.

CULTURE-DEPENDENT STUDIES ON MICROBIOTA IN NEMATODE-SUPPRESSIVE SOILS

As specific soil suppressiveness is determined by the activity of certain antagonistic microbial species that are main culprits

for the decrease in nematode population density, the cultivation of such organisms on growth media is an essential step for their large-scale multiplication and prolonged utilization in nematode control. As discussed in the next section, many studies using culture-independent approaches unraveled a high diversity of the microbiome associated with PPN in suppressive soils. However, it has been proposed that only 1% of microorganisms in nature are cultivable (Amann et al., 1995). This significantly decreases the range of nematode antagonists that can be mass-produced. Moreover, cultivation of nematode obligate parasites represents an additional challenge since it often requires the presence of the nematode host. Antagonistic effects of diverse microorganisms were detected in *in vitro* studies on the biology of free-living nematodes (Huang et al., 2005; Rae et al., 2008), or they appeared as by-products of studies on soil suppressiveness against other pathogens and parasites of plants (Adam et al., 2014a). Nevertheless, there are still numerous microbial species that were isolated from nematodes in suppressive soils (Bird and Brisbane, 1988; Chen et al., 1996a; Weibelzahl-Fulton et al., 1996; Borneman et al., 2004; Borneman and Becker, 2007; Chen, 2007; Adam et al., 2014b; Eberlein et al., 2016; Giné et al., 2013, 2016). In this review we focused on the microorganisms associated with the most important group of PPN, sedentary endoparasites (root-knot and cyst nematodes). We assigned these microbes to two different groups: (1) microbial species associated with suppression of migratory stages of sedentary endoparasitic nematodes and (2) microbial species associated with suppression of sedentary stages of sedentary endoparasitic nematodes.

Microbial Species Associated With Suppression of Migratory Stages of Sedentary Endoparasitic Nematodes

The most studied microorganism associated with migratory stages of PPN in suppressive soil is the bacterium *Pasteuria* (Mankau, 1975). The obligate nature of *Pasteuria* spp. makes them a promising candidate for biocontrol of PPN. Several species of *Pasteuria* have been reported to parasitize nematodes. *Pasteuria penetrans* parasitizes *Meloidogyne* spp. (root-knot nematodes), *Pasteuria nishizawae* parasitizes *Globodera* spp. and *Heterodera* spp. (cyst nematodes), *Pasteuria thornei* parasitizes *Pratylenchus* spp. (root-lesion nematodes), and *Pasteuria usgae* parasitizes *Belonolaimus* spp., a sting nematodes (Preston et al., 2003; Noel et al., 2005). The attachment of *Pasteuria* spp. endospores to the nematode cuticle is a first step during parasitism. In case of *P. penetrans* parasitizing *Meloidogyne*, the spores first attach to the infective second-stage juveniles (J2) in soil, and then, as J2 enter the roots, bacteria produce microcolonies inside the nematode's pseudocelom. Eventually, the development of eggs within females is disrupted (Li et al., 2015). However, in other PPN, endospores can germinate through the cuticle and complete the bacterial life-cycle with new endospores forming in the nematode juvenile (e.g., *Pasteuria* on *H. avenae*) (Davies et al., 1990). *Pasteuria* spp. were repeatedly isolated from soils that exhibited suppressiveness against different nematode species (Stirling and Wachtel, 1980; Stirling and White, 1982; Bird and Brisbane, 1988; Chen et al., 1994b; Weibelzahl-Fulton et al., 1996; Stirling et al., 2017; Bhuiyan et al., 2018). However, the presence of *Pasteuria* endospores in soil does not always guarantee nematode parasitism and small numbers of attached spores may not lead to the infection of PPN. For instance, Stirling et al. (2017) have detected *Pasteuria* spp. in 56% of the sugarcane fields, but only 5% of the observed nematodes had attached spores. In one field, *Pasteuria* sp. was suggested to be responsible for the suppression of *Meloidogyne javanica*, but only in case when the inoculated J2 had to cross a distance of more than 4 cm to reach the roots. The low parasitism rate in some cases appears as a result of a very high nematode-bacterium specificity (Chen and Dickson, 1998). Concentration of bacterial endospores in soil and persistence of antagonism were tested by Bhuiyan et al. (2018). It was shown that reproduction of *M. javanica* was reduced in a concentration-dependent manner, and the level of parasitism was very high with a 96% decrease in reproduction at 6 months post inoculation, to a 81% decrease in reproduction at 20 months post inoculation. Amongst other microbial antagonists isolated from suppressive soils that showed high antagonistic effects against PPN, *Streptomyces costaricanus* was effective against a wide range of nematode species (Dicklow et al., 1993; Esnard et al., 1995). The fungus *Hirsutella* spp. was demonstrated to produce conidia that adhere to the cuticle of J2 of cyst nematodes, and thereafter penetrate, digest and kill the nematode before root invasion (Stirling and Kerry, 1983; Hussain et al., 2016; Wang et al., 2016a,b). Topalović et al. (2019) have isolated several bacterial genera from the cuticle of the root-knot nematode *Meloidogyne hapla* in soils with a varying degree of suppressiveness. These were assigned to *Microbacterium*, *Sphingopyxis*, *Brevundimonas*, *Acinetobacter*, and *Micrococcus*. Although these are bacteria not obligate parasites of PPN, they showed antagonistic effects against J2 by increasing mortality, reducing motility, or reducing J2 invasion into the roots. One of the strains reduced hatching of J2 from the eggs.

While a direct antagonism of microbes to PPN was mostly regarded as the main mechanism of soil suppressiveness, more recently the plant is understood as a holobiont in association with its microbiome (Hassani et al., 2018). Using split-root systems of tomato plants, Adam et al. (2014a) showed that for *Bacillus* isolates, although selected as potential biocontrol strains based on their ability to produce nematicidal and fungicidal compounds, the main mechanism suppressing root-knot nematodes was not direct but plant-mediated. Microorganisms do not only target mobile stages of PPN in soil, but can also colonize the roots to parasitize the sedentary stages of endoparasitic nematodes, which is brought up in the following lines.

Microbial Species Associated With Suppression of Sedentary Stages of Endoparasitic Nematodes

Amongst the first isolated microorganisms that exhibited parasitism against sedentary stages of PPN were the fungi *N. gynophila* and *P. chlamydosporia* (former *Verticillium chlamydosporium*) that lead to the decline of the cyst nematode *H. avenae* (Kerry, 1982; Stirling and Kerry, 1983).

P. chlamydosporia is a saprophytic fungus in soil whose association to nematode eggs and cysts in soils is common (Giné et al., 2016; Song et al., 2017; Hu et al., 2018, 2020). Suppression of *H. schachtii* in Californian soil has been extensively studied, and transfers of amounts as small as 0.1% of this soil to a conducive soil have resulted in a significant decline of nematode reproduction (Westphal and Becker, 2000). Soil suppressiveness was correlated with a high presence of the fungi *Dactylella oviparasitica* and *Fusarium oxysporum* in nematode cysts and eggs (Westphal and Becker, 2001). However, involvement of *F. oxysporum* in nematode suppression is still speculative since in several subsequent studies it failed to cause a nematode decline (Olatinwo et al., 2006a,b; Gao et al., 2008; Becker et al., 2013). In addition, Becker et al. (2013) have pointed out that, although *D. oviparasitica* was highly effective in egg parasitism, viable eggs still remained resistant to this fungus. Since it is also able to parasitize J2, they proposed J2 as a target when applying *D. oviparasitica* as a biocontrol agent against cyst nematodes. Egg parasitism by certain strains of *Pseudomonas* and *Bacillus* species isolated from suppressive soils is also important to mention in case of ectoparasitic PPN (Westcott and Kluepfel, 1993; Kluepfel et al., 2002; Colagiero et al., 2017). Kluepfel et al. (2002) have detected several candidate genes in an isolated *Pseudomonas* strain to be responsible for egg toxicity.

Consortia of other fungi were also isolated from eggs and females of root-knot or cyst nematodes in several studies and for some of them antagonistic effects were confirmed in greenhouse assays (Marban-Mendoza et al., 1992; Chen et al., 1994a, 1996a; Crump and Flynn, 1995). Using microbial strains isolated from diseased nematode stages, different mechanisms to antagonize the nematodes were shown, such as parasitism, production of toxins and traps, or plant-mediated mechanisms. The mass production of antagonists is preceded by their isolation, thus a culture-dependent approach to study nematode-suppressive microbes is of enormous importance. However, microbial identification using DNA-based methods gives a more accurate representation of microbial consortia associated with suppression of PPN, and the following sections cover studies in this aspect.

CULTURE-INDEPENDENT STUDIES ON MICROBIOTA ASSOCIATED WITH PPN IN NEMATODE-SUPPRESSIVE SOILS

Although several potential microbial antagonists are known to regulate nematode population densities under laboratory and controlled greenhouse conditions, most of them failed to antagonize PPN in the field environment at a distinct geographical location. This inconsistency of microbes to fully express their antagonistic characteristics have been attributed to the lack of their survival within complex microbial communities in soil, or to their inability to better colonize plant roots under different environmental conditions (Alabouvette et al., 2009). Besides, soil suppressiveness is established because of the activity of microbial consortia rather than of just a single species (Costa et al., 2012; Raaijmakers and Mazzola, 2016). Therefore, several cultivation-independent approaches, including community profiling by oligonucleotide fingerprinting of rRNA genes (OFRG), denaturing gradient gel electrophoresis (DGGE), and high-throughput sequencing of PCR amplified taxonomic marker genes have been employed to decipher microbial cohort contributing to disease suppressiveness within the complex microbial communities, and to infer microbial antagonistic activities operating in nematode-suppressive soils.

Microbes Identified in a Direct Association to PPN

Several studies have focused on the structure and diversity of microbial communities associated with different life stages of PPN. Since nematode mobile stages reside in soil until they find the suitable roots for feeding, their association with soil microorganisms has been studied the most. A microbial community analysis of root-knot nematode infective J2 revealed a dominance of the bacterial genera *Sphingomonas*, *Micrococcus*, *Bacillus*, *Methylobacterium*, *Rhizobium*, and *Bosea*, and of the fungal genera *Davidiella* and *Rhizophydium* in soils that were suppressive against *M. hapla* (Adam et al., 2014b). Also, J2 of *M. incognita* were enriched by fungi of the genera *Malassezia*, *Plectosphaerella*, *Gibellulopsis*, and *Lectera* in a soil suppressive against this nematode species (Elhady et al., 2017). Notably, Elhady et al. (2017) also found an association of the bacterium *Bacillus thuringiensis* and the fungus *Plectosphaerella cucumerina* with *M. incognita* J2 in soil. *B. thuringiensis* produces proteinaceous protoxin crystals (called crystal protein or Cry protein) that cause lysis of the intestine and the nematode's death (Griffitts et al., 2005; Vachon et al., 2012). *P. cucumerina* was also isolated from the egg masses of *M. incognita* (Yu and Coosemans, 1998) and eggs of *G. pallida* (Kooliyottil et al., 2017), and has been found to infect eggs of *G. rostochiensis* (Atkins et al., 2003). The microbial community analysis using bacterial 16S rRNA gene and ITS amplicon sequencing from *G. pallida* females showed a dominance of diverse microbiota such as *Burkholderia*, *Bosea*, *Rhizobium*, *Devosia*, *Ralstonia*, and *Streptomyces*, and the fungal genera *Davidiella*, *Hirsutella*, *Malassezia*, *Microdochium*, *Monographella*, and *Penicillium* in potato monoculture soil (Eberlein et al., 2016). Some of the bacterial genera have been previously associated with infective stages of root-knot and lesion nematodes in suppressive soils (Adam et al., 2014b; Elhady et al., 2017).

The adult females of cyst nematodes first appear on the roots and finally develop into mature brown cysts which end up in the surrounding soil. The eggs inside the cysts are colonized by a number of microbes (Nour et al., 2003), and it has been reported that the cysts can transfer suppressive microbes into a nematode-conducive soil (Westphal and Becker, 2001). The earliest effort to characterize bacterial and fungal microbiota inhabiting the cysts in suppressive soil using OFRG identified major taxonomic groups of bacteria (α-*Proteobacteria*, β-*Proteobacteria*, γ-*Proteobacteria*, *Cytophaga-Flexibacter-Bacteroides*, and *Actinobacteria*) and fungi (*D. oviparasitica*, *F. oxysporum*, and *Lycoperdon* spp.), and

were then coupled with quantitative PCR to highlight the association of *Rhizobium*- and *D. oviparasitica*-like rDNA groups with the suppressiveness against *H. schachtii* (Yin et al., 2003a,b). In addition, using high-throughput amplicon sequencing to examine fungal and bacterial communities from cysts in soil suppressive to *H. glycines*, Hu et al. (2017) demonstrated that suppressiveness is associated with the change in relative abundance of diverse bacterial and fungal taxa, especially *P. chlamydosporia*, *Exophiala* spp., and *Clonostachys rosea*. Similarly, the investigation of fungi from egg masses of *M. incognita* revealed a total of 11 phylotypes, including *P. chlamydosporia*, that were proposed to be involved in regulating root-knot nematode populations (Bent et al., 2008). Recently, Hussain et al. (2018) highlighted the interaction between *H. glycines* cysts and soybean root microbiota using high-throughput sequencing of the V4 region of the bacterial 16S rRNA gene. Specifically, they showed that the microbial consortia enriched upon nematode infection in the rhizosphere and root endosphere also colonized nematode cysts. This points out that cysts are able to serve as an inoculum source for nematode suppressiveness. Moreover, the *H. glycines* cyst bacterial community was established by the consecutive selection of bacterial taxa from the root endosphere. Bacterial microbiota including, *Chitinophaga*, *Yersinia*, *Lentzea*, *Niastella*, and *Pseudoxanthomonas* were dominating the cyst bacterial community in suppressive soil (Hussain et al., 2018). *Chitinophaga* is a chitin-decomposer with chitinase activity (Sangkhobol and Skerman, 1981; McKee et al., 2019) and the chitin is an essential element of the nematode eggshell (Gortari and Hours, 2008). Chitinases could have an effect on egg viability and hatching (Chen and Peng, 2019). Further, the chitinase-generated chitooligosaccharides could induce the expression of parasitism-related genes in egg-parasitizing fungi (Escudero et al., 2016) inhabiting the cysts (Hu et al., 2018, 2020). Thus, microbiota directly associated with PPN stages in suppressive soils showcasing the ability of nematode suppression elucidate the potential role of diverse microbial consortia in this enticing microbiological milieu.

Microbes Detected in Soils With a Reduced Nematode Performance

The recent use of cultivation-independent technologies has provided deep insights into the microbial community composition of rhizosphere soils and their contribution in the suppression of PPN. Diverse bacterial and fungal taxa were reported to be enriched in soils with a low presence of PPN or with a poor PPN performance on the host plants grown in these soils. For instance, Giné et al. (2016) investigated the structure of bulk soil microbiota using bacterial and fungal community fingerprinting by DGGE. They found that the bacteria assigned to *Lysobacter*, *Flavobacterium*, *Chryseobacterium*, *Flexibacter*, *Steroidobacter*, and *Methylobacterium*, and the fungi assigned to *Cladosporium*, *Fusarium*, *Mortierella*, *Preussia*, and *Stachybotrys* were frequently detected in soils with a low record of root-knot nematodes. Consequently, the fungus *P. chlamydosporia* was isolated and verified to parasitize *Meloidogyne* eggs (Giné et al., 2016). In another study, *P. chlamydosporia*, together with the fungus *Purpureocillium lilacinum* and the bacterium *Pseudomonas* sp., were also marked as major taxa inhabiting the rhizosphere of soybean plants that were grown in soils from north-eastern China, and that were suppressive against *H. glycines* (Hamid et al., 2017). These two egg-parasitic fungi showed a strong geographical preference. The bacterium *Pseudomonas* sp. had a higher relative abundance in the suppressive than in the conducive soils across all geographical locations (Hamid et al., 2017). It was hypothesized that soils with the ability to suppress specific diseases have a memory of previously encountered pathogens (Raaijmakers and Mazzola, 2016). Those microbial consortia that responded to pathogen attack in a previous plant generation are probably the key drivers of soil memory against specific pathogens (Berendsen et al., 2018). Thus, plants can adopt a "cry for help" strategy during pathogen invasion, leading to the selective enrichment of a specific set of microbes in the soil (Bakker et al., 2018). Furthermore, the high-throughput sequences of the fungal ITS locus showed that both *P. chlamydosporia* and *P. lilacinum* were increased in the rhizosphere upon combined soil application of the nematode endoparasitic fungus *Hirsutella minnesotensis* and chitosan to suppress *H. glycines* (Mwaheb et al., 2017). Similarly, soils with low population densities of *Pratylenchus neglectus* and *Meloidogyne chitwoodi* were enriched by the rhizobacteria *Arthrobacter*, *Bacillus*, and *Lysobacter* as revealed by high-throughput sequence analysis of the V4 region of the bacterial 16S rRNA gene (Castillo et al., 2017). With regards to the bacteria, Hussain et al. (2018) using high-throughput sequencing of the V4 region of the bacterial 16S rRNA gene identified less than 30 genera, including *Pasteuria, Pseudomonas*, and *Rhizobium*, that were enriched in the rhizosphere and/or root endosphere of soybean grown in suppressive soil, and associated with the suppression of *H. glycines* under a long-term crop monoculture.

Importantly, we summarized the nematode biocontrol mechanisms of the most commonly detected antagonistic microbial consortia from suppressive soils such as the fungi *Pochonia*, *Dactylella*, *Nematophthora*, *Purpureocillium*, *Trichoderma*, *Hirsutella*, *Haptocillium*, *Catenaria*, *Arthrobotrys*, *Dactylellina*, *Drechslerella*, and *Mortierella*, and the bacteria *Pasteuria*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Streptomyces*, *Arthrobacter*, *Lysobacter*, and *Variovorax* (**Figure 1**). With respect to the fungi, a wide range of the antagonistic species has been described with the ability to trap, parasitize, or intoxicate nematodes, or suppress endoparasitic PPN by inducing systemic resistance in plants (Yang et al., 2007; Szabó et al., 2012; Askary, 2015; Martínez-Medina et al., 2017; Zhang et al., 2019). Likewise, bacterial antagonists often have more than one mode of action. Although the parasitism of nematodes by *Pasteuria* has been the most extensively studied, the efficiency of nematode antagonism by bacterial toxins, production of volatile organic compounds and nematode repellence from the roots by bacterially induced systemic resistance in plants has also been studied (Siddiqui and Mahmood, 1999; Wei et al., 2003; Sikora et al., 2007; Tian et al., 2007; Li et al., 2015). The results of recent studies

on characterization of microbiota associated with soil, plant roots, and nematodes in suppressive soils are summarized in **Table 1**.

To get further insights into the active microbiota and to identify other antagonistic microbial traits involved in nematode-suppressive soils, shotgun metagenomic (Chapelle et al., 2016), metatranscriptomic (Chapelle et al., 2016), and metabolomic (Hayden et al., 2019) approaches might be applied or combined. By combining metagenomics and metatranscriptomics, Chapelle et al. (2016) found that upon fungal invasion the stress-related genes were up-regulated in bacterial families inhabiting the rhizosphere of sugar beet grown in a suppressive soil. The fungal pathogen *Rhizoctonia solani* secreted phenylacetic and oxalic acid during plant root colonization, leading to oxidative stress in plants and rhizosphere microbes. This oxidative stress response caused a shift in bacterial composition and activated antagonistic traits that confined pathogen infection. Furthermore, Hayden et al. (2019) using metabolomic and metatranscriptomic analyses revealed that sugar molecules were more abundant in the *R. solani*-suppressive than in *R. solani*-conducive soils. They found that the most abundant compound associated with suppressive soils was the antimicrobial secondary metabolite macrocarpal L. These studies demonstrated that the combination of various approaches could provide us with a detailed understanding of the microbes and mechanisms involved in disease-suppressive soils. To acknowledge the enormous plant contribution in nematode antagonism by beneficial microbes in soil, in the next two sections we will focus on nematode-microbe interactions in the rhizosphere and on the involvement of root exudations in this regard.

IMPORTANCE OF THE RHIZOSPHERE MICROBIOTA IN PLANT-NEMATODE INTERACTIONS

The most intense interactions between the mobile stages of PPN and soil microorganisms occur in the rhizosphere. Nematodes sense environmental signals using different sensilla, but the most studied are paired anterior sensory organs, called amphids. They are positioned in the nematode head region and consist of a glandular sheath cell, a socket cell, and the secretions that are produced by a sheath cell and that surround many dendritic processes (Perry, 1996). Amphids are responsible for navigating PPN to the host roots, providing their positive interaction with the chemical cues released from the roots. Reynolds et al. (2011) proposed that the host range of a certain nematode species determines whether they respond to the general or more specific plant cues. Gene expression in *Pratylenchus coffeae* was influenced, in a host-specific manner, by cell wall components that were either secreted by the plant or released by degradation of root tissue (Bell et al., 2019). Cellulose or xylan from host plants upregulated the level of β-1,4-endoglucanase or β-1,4-endoxylanase genes, respectively. Plants can interfere with PPN signaling (Manohar et al., 2020), and it is likely that also microbial activities modulate and mediate plant-nematode communication. Blocking plant cues or nematode chemoreceptors results in nematode repellence from the roots (Zuckerman, 1983). However, plant root components alone do not entirely affect nematode attractiveness to the roots and a subsequent invasion. In order to reach the roots, nematodes need to travel through a one to several millimeters long soil space in the close vicinity of the root that is called rhizosphere. This is a very active zone along the growing roots with a constant water and nutrient uptake, root exudations and microbial activities (Berg and Smalla, 2009). It was estimated that soil acidity is 10-fold higher in the rhizosphere than in bulk soil, suggesting very pronounced root effects on chemical and biological characteristics of the surrounding soil (Hübel and Beck, 1993). Both soil type and plant genotype contribute to the composition of the microbial communities in the rhizosphere (Micallef et al., 2009). The composition of exudates varies among plant species (Badri and Vivanco, 2009; Eisenhauer et al., 2016) and genotypes (Mönchgesang et al., 2016). The modulation of the microbiome by root exudates induces a feedback of the microbiome. This plant-soil feedback can support growth and/or health of the plant and of the next plants growing in the same soil (van der Putten et al., 2016; Brinkman et al., 2017). Plant-soil feedback effects varied among plant species, but the number of PPN that fed on the roots of a particular plant species correlated with a negative plant-soil feedback (Wilschut et al., 2019). Plants rely on their root microbiome when they are under attack by pathogens and parasites, leading to a selective enrichment of plant-protective microbes and microbial activities in the rhizosphere (Bakker et al., 2018). The importance of the rhizosphere microbiome in plant-nematode interactions has been extensively reviewed (Kerry, 2000; Griffiths et al., 2007; Sikora et al., 2007; Nyaku et al., 2017; Topalović and Heuer, 2019). Importantly, some recent studies have demonstrated that the transfer of the rhizosphere microbiome from one crop to another significantly alleviated nematode infection and enhanced the plant resistance to PPN (Elhady et al., 2018; Zhou et al., 2019). This effect depended on the plant species. The plant's own microbiome protected it better from root invasion of PPN than the bulk soil microbiome or a foreign microbiome, with the notable exception of the highly suppressive maize microbiome (Elhady et al., 2018). Furthermore, Hussain et al. (2018) revealed that the same subset of microbial OTU was commonly enriched in the rhizosphere and root endosphere upon nematode challenge in suppressive soil. This points out a strong communication between plants and their associated microbiota when under a threat by PPN. In this case, the rhizosphere microbiota can act as a first line of defense against invading PPN, while the root endosphere microbiota can act as a second line of defense against successfully invaded nematodes. Depending on the microorganisms associated with the roots, PPN can be antagonized by parasitism, intoxication, production of volatile organic compounds, or by microbially induced systemic resistance in plants (**Figure 2**). Most often, the united efforts of more than one mode of action are employed by soil microbiota in nematode suppression. Topalović et al. (2020b) have shown in a split-root experiment that microorganisms from a suppressive soil induced systemic resistance in tomato plants

TABLE 1 | DNA-based characterization of the bacterial and fungal microbiota inhabiting the soil, plant roots, and nematode stages (mobile and sedentary) in disease suppressive soils.

Nematode (Reference)	Microhabitat	Technique	Microbial genera/species
Meloidogyne hapla (Adam et al., 2014b)	J2/Bulk soil	ITS – DGGE	*Malassezia, Aspergillus, Cryptococcus, Chaetomium, Eurotium, Ganoderma, Cladosporium, Davidiella, Mortierella, Cylindrocarpon, Rhizophydium*
		16S – DGGE	*Bradyrhizobium, Sphingomonas, Staphylococcus, Micrococcus, Bacillus, Propionibacterium, Methylobacterium, Streptococcus, Solirubrobacter, Janthinobacterium, Rhizobium, Pedomicrobium, Ochrobactrum, Nitrospira, Devosia, Kaistia, Magnetospirillum, Bosea, Rhodobacter, Pseudomonas*
		16S – amplicon	*Micrococcus, Rothia, Geobacillus, Streptococcus, Anaerococcus, Peptoniphilus, Clostridium, Mycoplasma, Ochrobactrum, Hirschia, Haematobacter, Paracoccus, Malikia, Janthinobacterium, Neisseria, Vogesella, Shigella, Acinetobacter, Acinetobacter, Enhydrobacter, Pseudomonas*
Meloidogyne incognita Pratylenchus penetrans (Elhady et al., 2017)	Infective stage/bulk soil	16S – DGGE	*Burkholderia, Fusicatenibacter, Burkholderia, Oscillatoria, Curvibacter, Acinetobacter*
		16S – amplicon	*Paraburkholderia, Ralstonia, Streptococcus, Staphylococccus, Bacillus thuringiensis, Streptococcus, Acinetobacter, Gemmatimonas, Anaerococcus, Pelomonas, Burkholderia, Neorhizobium*
		ITS – amplicon	*Plectosphaerella, Penicillium, Lectera, Tetracladium, Chaetomium, Petriella, Malassezia, Taphrina, Alternaria, Stemphylium, Cladosporium, Aspergillus, Gibellulopsis*
Globodera pallida (Eberlein et al., 2016)	Females	16S – amplicon	*Actinophytocola, Aquabacterium, Bosea, Bradyrhizobium, Brevundimonas, Burkholderia, Dermacoccus, Devosia, Moraxella, Pantoea, Pelomonas, Ralstonia, Rhizobium, Rhodobacter, Sphingopyxis, Streptomyces, Zoogloea, Flavobacteria*
		ITS – amplicon	*Davidiella, Hirsutella, Malassezia, Microdochium, Monographella, Penicillium, Colletotrichum*
Heterodera schachtii (Yin et al., 2003a,b)	Cyst	Bacteria – OFRG	Actinobacteria, Cytophaga-Flexibacter-Bacteroides, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, *Rhizobium*
		Fungal – OFRG	*Dactylella oviparasitica, Fusarium oxysporum, Lycoperdon*
Heterodera glycines (Song et al., 2017)	Cyst	ITS – DGGE	*Geomyces, Aureobasidium, Fusarium, Penicillium, Aspergillus, Cladosporium, Setosphaeria, Alternaria, Mortierella, Cryptococcus, Trichosporon, Galactomyces*
Heterodera glycines (Hu et al., 2017)	Cyst	ITS – amplicon	*Trichoderma, Leptosphaeria, Clonostachys, Purpureocillium, Penicillium, Pochonia, Fusarium, Exophiala, Mortierella, Microstroma, Typhula, Phoma, Oudemansiella, Saksenaea, Melanospora, Xylaria, Orbilia, Entoloma*
		16S – amplicon	*Streptomyces, Enterobacter, Acidovorax, Pseudomonas, Variovorax, Rhizobium, Serratia, Massilia, Dactylosporangium, Lentzea, Amycolatopsis, Mesorhizobium, Actinoplanes, Asteroleplasma, Nocardia, Bradyrhizobium, Actinocorallia, Micromonospora, Streptosporangium, Kribbella, Phyllobacterium, Devosia, Nonomuraea, Actinomadura, Aminobacter, Sphingomonas, Shinella, Chitinophaga, Niastella, Steroidobacter, Kineosporia, Luteolibacter, Lysobacter, Rhodanobacter, Echinococcus*
Heterodera glycines (Hussain et al., 2018)	Cyst	16S – amplicon	Proteobacteria (*Devosia, Ferrovibrio, Sphingopyxis, Phaselicystis, Sphingomonas, Aquabacterium, Steroidobacter, Lysobacter, Albidiferax, Ideonella, Pseudoduganella, Tahibacter, Bosea, Yersinia, Pseudoxanthomonas, Pseudoduganella, Pseudomonas, Steroidobacter*), Actinobacteria (*Actinophytocola, Actinocorallia, Actinomadura, Nocardia, Planosporangium, Actinoplanes, Promicromonospora, Streptosporangium, Kribbella, Streptomyces, Saccharothrix, Amycolatopsis, Lentzea*), Bacteroidetes (*Taibaiella, Ohtaekwangia, Dyadobacter, Chitinophaga, Candidatus paenicardinium, Niastella), Chlamydiae (Candidatus rhabdochlamydia*), Verrucomicrobia (Verrucomicrobium, Haloferula), Planctomycetes (*Planctomyces*)
Meloidogyne incognita (Bent et al., 2008)	Egg masses	Fungal – OFRG	Pochonia, Fusarium, Plectosphaerella, Microdochium, Saccharomyces, Tetracladium, Geomyces, Monacrosporium, Ceratobasidium, Auricularia
Meloidogyne spp. (Giné et al., 2016)	Bulk soil	16S – DGGE	*Lysobacter, Flavobacterium, Chryseobacterium, Flexibacter, Steroidobacter, Methylobacterium, Candidatus Solibacter*
		ITS – DGGE	*Pseudaleuria, Fusarium, Preussia, Ctenomyces, Mortierella, Cladosporium, Stachybotrys, Pseudallescheria, Psathyrella, Heydenia*
Heterodera glycines (Hamid et al., 2017)	Rhizosphere	ITS – amplicon	*Mortierella, Purpureocillium, Fusarium, Pochonia, Clonostachys, Scleroderma, Penicillium, Aspergillus, Corynespora, Guehomyces, Humicola, Eupenicillium, Cryptococcus, Monographella, Tetracladium, Geomyces, Stachybotrys, Ilyonectria, Myrothecium, Monodictys, Arthrobotrys, Dactylellina, Drechslerella, Haptocillium, Hirsutella, Trichoderma, Acremonium, Penicillium, Nematoctonus, Catenaria*

(Continued)

TABLE 1 | Continued

Nematode (Reference)	Microhabitat	Technique	Microbial genera/species
		16S – amplicon	*Pseudomonas, Massilia, Arthrobacter, Rhizobium, Sphingomonas, Burkholderia, Chitinophaga, Streptomyces, Mesorhizobium, Novosphingobium, Variovorax, Enterobacter, Bradyrhizobium, Bacillus, Niastella, Mucilaginibacter*
Meloidogyne chitwoodi Pratylenchus neglectus (Castillo et al., 2017)	Rhizosphere	16S – amplicon	*Pasteuria, Brevibacillus, Bacillus, Pseudomonas, Agrobacterium, Arthrobacter, Burkholderia, Brevundimonas, Clostridium, Corynebacterium, Flavobacterium, Hydrogenophaga, Lysobacter, Methylobacterium, Mycoplana, Phyllobacterium, Rhizobium, Sphingobacterium, Stenotrophomonas, Streptomyces, Variovorax*
Heterodera glycines (Hussain et al., 2018)	Rhizosphere	16S – amplicon	Proteobacteria (*Pseudomonas, Ensifer, Shinella, Rhizobium, Aquabacterium*), Firmicutes (*Pasteuria*), Bacteroidetes (*Candidatus paenicardinium*), Verrucomicrobia (*Haloferula*)
Heterodera glycines (Hussain et al., 2018)	Root endosphere	16S – amplicon	Proteobacteria (*Phyllobacterium, Aquamicrobium, Rhizobium, Rhodomicrobium, Luteimonas, Cupriavidus, Bdellovibrio, Devosia, Pseudomonas, Aquabacterium, Hydrogenophaga, Shinella, Ensifer, Pseudoxanthomonas, Bosea*), Actinobacteria (*Agromyces, Micromonosporaceae, Lentzea, Streptomyces, Glycomyces, Microbacteriaceae*), Firmicutes (*Pasteuria, Fictibacillus*), Bacteroidetes (*Candidatus paenicardinium*), Planctomycetes (*Planctomyces, Rhodopirellula*)

against *M. hapla*, but a combination of a direct antagonism and induced resistance provided a better protection to the plants. They also showed in a sterile system that the microbes attaching to the cuticle of *M. hapla* in the suppressive soil induced systemic resistance in the plant upon nematode invasion (Topalović et al., 2020a). In addition, in recent years scientists have intensively explored the role of plant and microbial volatile organic compounds on nematode parasitism (Huang et al., 2010; Yang et al., 2012; Barros et al., 2014; Xu et al., 2015; Estupiñan-López et al., 2018; Silva et al., 2018; Pedroso et al., 2019). Thus, the potential of root microbiota to assist plants in fighting PPN is enormous. In the next section, the role of root exudates on nematode-microbe interactions is more comprehensively discussed.

THE ROLE OF THE NEMATODE SURFACE COAT AND ROOT EXUDATES IN PLANT-NEMATODE-MICROBE INTERACTIONS

When it comes to the interactions with soil microorganisms, the most intriguing feature of the nematode's body is the SC. This is a carbohydrate-rich protein layer secreted over the epicuticle by the hypodermis (Curtis et al., 2011), or by the excretory and nervous system (Lin and McClure, 1996). The SC is probably involved in recognizing the lectin-like protein molecules on the microbial surface by its glycohydrate epitopes (Bird, 2004; Davies and Curtis, 2011). Bird (2004) produced a very concise and detailed review on the recognition and consequences of nematode-microbe interactions. Depending on the microorganism, the association between the two can result in parasitism, e.g., *Anguina* sp. and a parasitic *Rathayibacter* sp. (Bird, 1985; Riley and Reardon, 1995), or *Meloidogyne* sp. and a parasitic *Pasteuria* sp. (Davies, 2009), commensalism, e.g., *Longidorus* sp., *Xiphinema* sp., *Trichodorus* sp., and *Paratrichodorus* sp. as virus vectors (Taylor and Brown, 1997), mutualism, e.g., *Steinernema* sp. and their endosymbiotic bacteria *Xenorhabdus* sp. (Lacey and Georgis, 2012), or a phoretic association, e.g., *Steinernema* and the bacterium *Paenibacillus* sp. (El-Borai et al., 2005). Some studies have shown that root exudates and some phytohormones, like indole-acetic acid, can alter the SC of PPN (Akhkha et al., 2002; Curtis, 2008) and affect the subsequent attachment of microorganisms to the nematode surface (Singh et al., 2014; Liu et al., 2017). Similarly, López de Mendoza et al. (2000) recorded an increased uptake of the fluorescent lipid analog 5-N-(octadecanoyl) aminofluorescein (AF18) by the SC of J2 of *M. incognita* and the animal parasitic nematode *Haemonchus contortus* after exposure to tomato root exudates for 30 min. Root exudates from different plant species have a variable effect on attachment of endospores of *P. penetrans* to J2 of root-knot nematodes (Singh et al., 2014; Liu et al., 2017). This suggests that a correlation exists between the host type of root exudates and nematode-microbe interactions in soil. It was found that the attachment of *P. penetrans* endospores to J2 of *M. incognita* was increased when J2 were exposed to the root exudates in the presence of soil microorganisms (Singh et al., 2014). As nematode secretions and the SC components are first perceived by the plant when encountering nematodes (Curtis, 2008; Mendy et al., 2017), masking these nematode receptors upon microbial attachment to the nematode in soil might reduce nematode recognition by the plant. On the other hand, some endoparasitic nematodes, like *Meloidogyne* spp., hide from the strong plant defense responses by moving through the apoplast until reaching permanent feeding sites (Sijmons et al., 1991; Williamson and Hussey, 1996; Shah et al., 2017). It was recently shown that microorganisms attaching to the J2 of *M. hapla* in suppressive soil before J2 penetration into the roots increase their recognition by the plant by up-regulating several PTI-responsive defense genes (Topalović et al., 2020a). Studying the microbially induced chemical and metabolic changes in nematode perception by the plant would better elucidate the exact mechanisms in this tripartite interaction. In

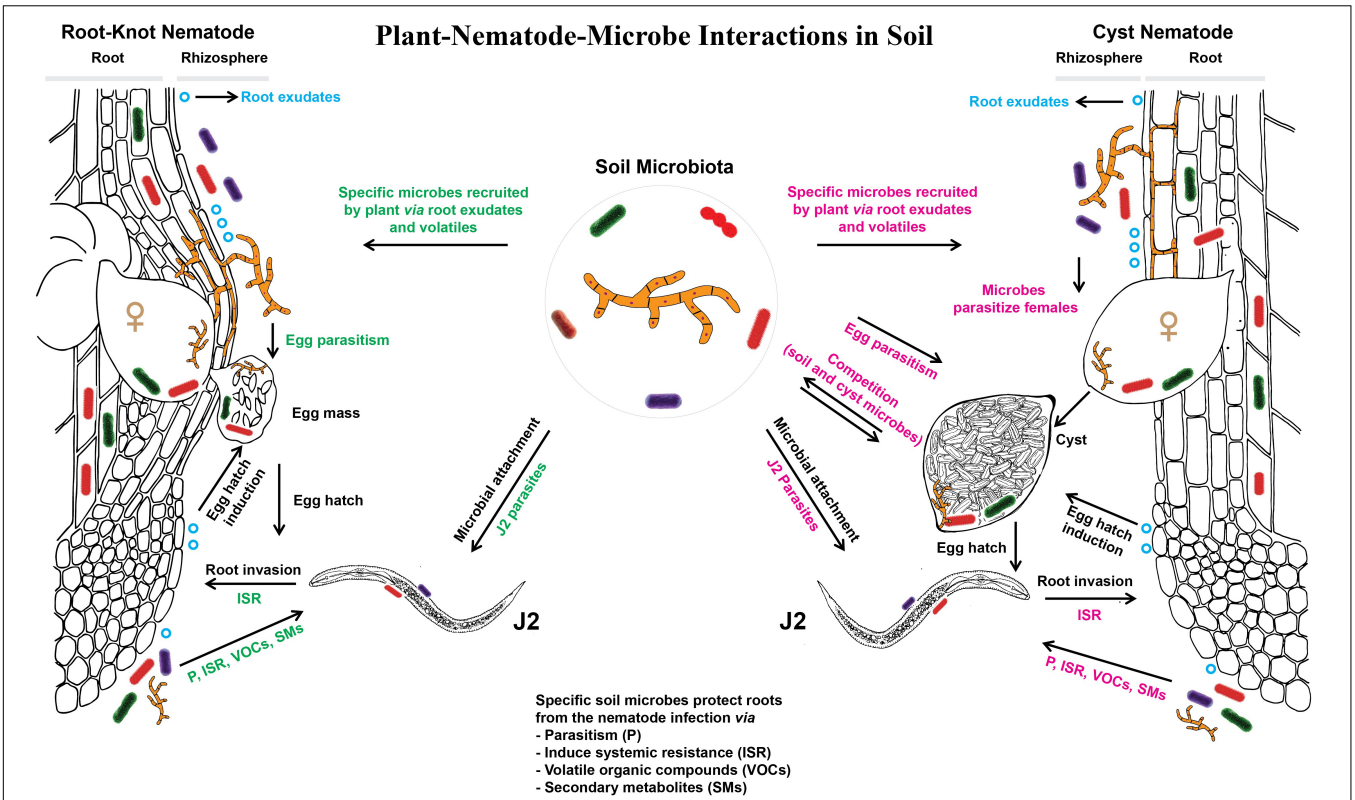

FIGURE 2 | Below-ground communication between plant roots, associated microbiota, and plant-parasitic root-knot or cyst nematodes.

addition, the nematode SC is subjected to a constant turnover and renewal (Spiegel and McClure, 1995; Spiegel et al., 1995; Gravato-Nobre et al., 1999; Curtis et al., 2011). A study with animal parasitic nematodes showed that the SC changed minutes after nematodes are exposed to the conditions equivalent to those inside the host, excluding the influence of molting in such a short time (Proudfoot et al., 1993). The discarded SC deposits inside the plant or animal host can trick plant immune effectors and keep them away from mobile nematodes that are in search for a more stable feeding site (Blaxter et al., 1992). Thus, although the root exudates are important in nematode attraction to the roots, they can also directly affect nematode interactions with soil microorganisms by inducing changes in the surface of PPN. The nature of these interactions and the type of soil microorganisms and root exudates determine whether PPN will evade the plant immune responses or fail to infect the plant.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plant-parasitic nematodes cause considerable losses to vegetables and agronomic crops worldwide. Notably, most research has been done on a few species of the endoparasitic sedentary genera *Meloidogyne*, *Heterodera*, and *Globodera*. Investigations on other PPN will give the opportunity to generalize conclusions on the interactions of PPN within the phytobiome and to acknowledge species or genus specific differences in life strategies (Topalović et al., 2020b). Microorganisms contributing to the natural suppression of PPN in soils have been studied using culture-dependent and culture-independent methods. Specific suppression of soils is mainly attributed to the antagonistic activities of selective microbial consortia against eggs, juveniles, or females of PPN. The *in vitro* effects of isolated microbial strains often fail to reproduce upon reintroduction of strains in conducive field soils, due to their inability to colonize roots and fully express putative modes of action. While isolation of specific microbial antagonists is essential for their mass production and application in integrated pest management of PPN, the DNA/RNA-based characterization of the microbiomes associated with plant and nematode is important for a more comprehensive understanding of the interactions between nematodes and their natural enemies in soil, rhizosphere and plant. In parallel, the success of the nematode infection depends on how the signaling molecules from the nematode surface are perceived by the plant roots. Semiochemicals exuded from the plant with root exudates are not only important for the communication between plants and nematodes, but they also directly affect nematode-microbe interactions by modulating components of the nematode surface. Depending on the nematode host range and the microbial composition in soil, endoparasitic nematodes can evade plant defense responses and successfully establish

inside the roots, or they can be antagonized inside (root endosphere) or outside (rhizosphere) the plant. Thus, plants infinitely rely on their root microbiome during nematode invasion, leading to the enrichment of a specific subset of plant-protective microbes in the rhizosphere and root endosphere (Hussain et al., 2018). Moreover, a thorough understanding of interkingdom microbe-microbe interactions in soil ecosystems may help to enrich the abundance and activities of indigenous keystone microbial taxa by agricultural management practices such as crop rotation (Hu et al., 2018) or tillage (Hu et al., 2017), or through soil amendments like chitin (Cretoiu et al., 2013) or chitosan (Mwaheb et al., 2017). Unraveling the microbiome structure and functions in nematode-suppressive soils and understanding their relationship with the plant will provide us with more knowledge on the mechanisms responsible for nematode suppression, and may help to develop synthetic microbial communities or manage the soil biome for biocontrol of PPN.

AUTHOR CONTRIBUTIONS

OT and MH conceived, developed the theme, and wrote the manuscript. MH prepared figures. HH contributed to ideas and provided critical revisions of the manuscript.

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Response of Tomato Rhizosphere Bacteria to Root-Knot Nematodes, Fenamiphos and Sampling Time Shows Differential Effects on Low Level Taxa

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A factorial taxonomic metabarcoding study was carried out to determine the effect of root-knot nematodes (*Meloidogyne incognita*, RKN) and the nematocide fenamiphos on the rhizosphere microbiome of tomato. Plants inoculated (or not) with RKN second-stage juveniles (J2), and treated (or not) with the nematocide, were tested in a 6 months greenhouse assay using a RKN-free soil proceeding from an organic crop. Rhizosphere soil was sampled at J2 inoculation, 3 months later (before the second nematocidal treatment), and again after 3 months. At each sampling, the RNAs were extracted and the 16S rRNA V4 regions sequenced with a Next Generation Sequencing (NGS) protocol. Changes in bacteria metagenomic profiles showed an effect of the treatments applied, with different representations of taxa in samples receiving nematodes and fenamiphos, at the two sampling times. In general, a tendence was observed toward an increase number of OTUs at 6 months, in all treatments. β-Proteobacteria were the most abundant class, for all treatments and times. When compared to soil before transplanting, the presence of tomato roots increased frequency of Actinobacteria and Thermoleophilia, reducing abundance of Solibacteres. At lowest taxonomic levels the samples clustered in groups congruent with the treatments applied, with OTUs differentially represented in relation to RKN and/or fenamiphos applications. *Bacillus*, *Corynebacterium, Streptococcus,* and *Staphylococcus* were more represented at 6 months in samples inoculated with RKN. The nematodes with the nematocide application increased the emergence of rare OTUs or reduced/enhanced the abundance of other taxa, from different lineages.

Keywords: 16S rRNA, fenamiphos, *Meloidogyne*, metagenome, nematode, soil

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INTRODUCTION

Bacteria underpin several specialized functions in soil food webs, including nutrient recycling, detoxification and regulation of pests, which may lead to macroscopic effects including pest or pathogens suppression (Esnard et al., 1995; Westphal and Becker, 2001; Westphal and Xing, 2011). Several species, moreover, have a biotechnological potential, including antibiosis

(Schloss and Handelsman, 2003). The diversity and densities of the operational taxonomic units (OTUs) present in the rhizosphere depend, among other factors, also on the bacteria specialization and trophism. Moreover, factors such as the aboveground vegetation and soil physiochemical properties affect the composition and stability of belowground bacterial communities, as well as reflecting farming practices (Leff and Fierer, 2013). Diversity of soil bacteria is also influenced by local microhabitat conditions, which may vary over very small scales. Other significant factors, however, have direct anthropic origins, including a number of agricultural practices such as the addition of fertilizers and organic matter, the application of chemicals for pest and weed management and the accumulation of related by-products (Foster, 1988; Van Elsas et al., 2008; Souza et al., 2013). The telluric microbial diversity is in the order of thousand OTUs $\cdot$ g^{-1} of soil, of which only a small fraction (0.1–1%) is suitable for cultivation and isolation. This leaves the effects of several taxa mostly undetermined (Kellenberger, 2001; Rosello-Mora and Amann, 2001; Roesch et al., 2007).

This situation requires effective and sensitive techniques to identify the services provided by soil bacteria or to evaluate the impact of factors such as common agricultural practices and presence of pests. The study of diseased roots may yield informations on the shifts in bacterial community composition related to parasitism (Tian et al., 2015). Similarly, also the chemicals applied for disease or pest management may affect the rhizosphere bacterial diversity or induce changes in the physiology of roots, with indirect effects on specialized rhizosphere taxa such as growth promoters or biocontrol agents.

Nematodes are a key component of soil food webs, including pest species that have a direct impact on plants productivity and yields. For decades nematode pests have been controlled through the application of a wide range of chemicals, including fumigants, carbamates or organophosporous compounds. Due to many inconvenients related to the use of chemical nematocides, including soil, groundwater contamination and toxicity to higher animals including man, several chemicals have been banned or limited for use. Among organophosphorous products, fenamiphos has been used as a nematocide and insecticide. It has been widely applied against nematodes at concentrations varying around 10 kg of active ingredient (a.i.) $\cdot$ ha^{-1}. Actually, in the EU it may be only applied in permanent greenhouses through drip irrigation, halting treatments at least 60 days before starting harvesting. Fenamiphos, that is effective systemically or by contact against most nematode pests through acetylcholinesterase inhibition (Ravichandra, 2018), may be degraded by a number of soil bacteria (Megaraj et al., 2003; Cáceres et al., 2008; Cabrera et al., 2010).

The relationships of nematodes with bacteria likely cover all type of interactions, ranging from symbiosis to antagonism. Although the studies on these interactions are, given the large rhizosphere bacterial diversity, still incomplete, a number of taxa have been reported in microbiome studies of nematodes (Ladygina et al., 2009; Tian et al., 2015; Elhady et al., 2017; Hussain et al., 2018; Hu et al., 2019). The diversity of clades associated to soil nematodes has been mostly explored using the 16S ribosomal rRNA gene sequence. Variations in the microbiome composition related to parasitism were shown by comparing nematode-parasitized and healthy tomato roots (Tian et al., 2015). Metagenomics of the pinewood nematode (PWN) *Bursaphlenchus xylophilus* showed that mutualistic symbiotic bacteria provided a detoxification function, allowing PWN to survive the host pine defense reaction (Cheng et al., 2013). Bacteria directly linked to nematodes include species found in association to free-living or plant parasitic species, such as *Pseudomonas* spp., Verrucomicrobia, or endosymbionts such as *Cardinium*, *Wolbachia,* or *Pasteuria* spp. (Noel and Atibalentja, 2006; Haegemann et al., 2009; Zou et al., 2015; Hussain et al., 2018). Members of Bacillales in association to nematodes include *Bacillus nematocida*, described from free-living *Panagrellus redivivus* (Huang et al., 2005), or species active against *Caenorhabditis elegans* and *Pristionchus pacificus* (Rae et al., 2010).

This study was motivated by the need to enlarge actual knowledge on the interactions among rhizosphere bacterial communities, nematode pests and nematocides applied for their management. Starting hypothesis was that either the nematode presence and the application of a nematocide affect the composition, reproduction and activity of rhizosphere bacterial communities. One possibility considered was a change in the abundance of unknown taxa, directly acting as nematode endosymbionts or antagonists, as well as related to plant parasitism, or indirectly linked to nematode metabolism. A second effect hypothesized was an effect on bacterial groups in relation to a nematocide treatment or an increase of taxa involved in its decomposition. Finally, it is not known whether and at which extent (i.e., acting on single species or on entire clades) these effects could be observed, as well as how long they persist in time.

In particular, we aimed at identifying the effect of the root-knot nematode (RKN) *Meloidogyne incognita* and/or fenamiphos on the diversity of bacteria in tomato rhizosphere. We examined the changes induced on the bacterial community composition in the rhizosphere of plants growing in a previously untreated and pest-free soil, proceeding from an organic farm. Being culture-independent, taxonomic metabarcoding techniques allow a broad identification range and a sufficient sampling depth, representing the standard for analysis of microbial communities in many environments (Handelsman, 2004; Patrick and Handelsman, 2005; Susannah and Edward, 2005; Caporaso et al., 2010). A taxonomic metabarcoding NGS-based approach was hence applied to study the changes in bacterial diversity in treated plants, in a long lasting (more than 6 months) greenhouse experiment, by measuring rhizosphere OTUs abundance, at two different sampling times.

MATERIALS AND METHODS

Soil (53.9% sand, 14.4% silt, 31.7% clay, pH 7.8) was collected in July 2012 at 20–30 cm depth from a single field sampling site, ca. 1 m wide, from a parcel not cultivated for 2 years in an organic horticultural farm located at Mesagne (Brindisi, Italy), and only superficially labored to remove weeds. Soil depth was

chosen to get soil as uniform as possible, avoiding parts exposed to sunlight or dry, presence of debris, and irregularities in the soil surface profile. The samples were checked for presence/absence of RKNs by the soil sieving and decanting technique, suspending a 2 l soil sub-sample in tap water and filtering with a set of 500 and 75 μm sieves. The filtered suspension was then examined in a Hawksley counting chamber with a Leitz Orthoplan light microscope at 50×, in three replicates. After no RKN nematode stage was detected (only free living nematodes were found), the remaining soil was mixed, distributed in 20 cm diam. pots, kept in a greenhouse and all planted with seedlings of *Solanum lycopersicum* L. cv Tondino previously germinated in turf and washed free of the substrate residues.

The assay consisted of four treatments sampled at two intervals of 3 months each (**Supplementary Figure S1A**). The treatments were: (i) soil untreated and free of RKN (control); (ii) the same soil inoculated with RKN juveniles (J2); (iii) the same soil inoculated with the J2 and treated with the nematocide fenamiphos and (iv) the same soil treated only with fenamiphos. The nematocide commercial product used (Nemacur® 240 CS, with 23.1% a.i.) is a micro-encapsulated formulation applied for vegetable crops such as tomato at 42 $l \cdot ha^{-1}$. At each treatment, 0.13 ml of product were applied per pot, calculated for the pot surface (10 cm radius) using the 4.2 $ml \cdot m^{-2}$ dose indicated by the producer, corresponding to 0.03 ml of a.i. per pot.

The J2 used (*M. incognita* population L4, descending from a single egg mass and proceeding from Leverano, Lecce) were multiplied on tomato plants cv Tondino in artificially infested soil, originally sterile. To obtain the J2, fragmented root galls were placed in flasks with sterile water (SW) and aerated with a peristaltic pump to allow eggs hatching. The freshly hatched J2 were washed and superficially sterilized in 0.5% NaOH hypochloride for 5 min, followed by washing in an antibiotic solution (0.1% streptomycin, 0.1% ampicillin, 0.1% chloramphenicol) for 10 min, and then in SW for further 10 min. Before seedlings inoculation, the soil of two control pots was stirred before collecting the first (time 0 = T_0) 2 g samples, that were then frozen in liquid nitrogen and stored at −80°C, for subsequent RNA extraction. The nematode inoculations were then carried out by adding 5000 J2 per pot through gentle pipetting. The J2 number was adjusted in 25 ml of sterile water suspension containing 200 J2· ml^{-1}, that were pipetted around the plant base in three equally spaced points, at a depth of about 8–10 cm. Each treatment was planned in five replicated pots, using three of them for the taxonomic metabarcoding analyses. The test was carried out in a greenhouse under controlled conditions (26 ± 2°C, RH 40–60%), with two further sampling times, T_1 = 90 days post inoculation (dpi) and T_2 = 180 dpi, after the initial baseline T_0 (**Supplementary Figure S1A**).

One month after inoculation, the plants were checked for galls to confirm RKN infestation. At this point, the seedlings of treatments including the nematocide application were treated twice (at end of month 1 and 2) with fenamiphos, as described. One month later, the second series of rhizosphere soil samples (T_1) was collected from roots. The roots were excavated and exposed in each pot using a sterile steel spatula, collecting 2–3 g of soil particles and aggregates at a distance within 5 mm from the exposed root and/or gall surface, avoiding any emerging tip (for sampling timing see experimental scheme in **Supplementary Figure S1A**). Data on gall index, plant length, root weight, density of eggs · g^{-1} of roots were collected. The assay continued by transplanting in each pot new tomato seedlings at the third leaf stage, collecting the third and final rhizosphere soil samples after three further months (T_2).

Total RNA was extracted from three replicated rhizosphere soil samples (each from a single pot), per treatment and time, with the PowerSoil Kit (MoBio Laboratories, CA), following the manufacturer's instructions. The RNA concentration was determined with a Nanodrop™ spectrometer at 260 nm. The extracted material was subjected to reverse transcription according to the Illumina™ sequencing protocol, using SuperScript III (Invitrogen, United States), following the manufacture's protocol. The material obtained was then purified using the QIAquick PCR Purification Kit (Qiagen®, United Kingdom).

The nucleic acids integrity was checked by electrophoresis on 1.5% agarose gel. The subsequent taxonomic metabarcoding analyses relied on the bacterial 16S ribosomal rRNA (rRNA) gene sequence data, produced from the 16S hypervariable regions that may be amplified using universal primers with affinity for flanking conserved motifs (Van de Peer et al., 1996; Baker et al., 2003; Clarridge, 2004). In the 16S rRNA nine "hypervariable regions" (V1–V9) are present that show enough diversity to discriminate at the species or genera levels (Kataoka et al., 1997; Bertilsson et al., 2002; Becker et al., 2004).

MiSeq System™ Illumina platforms, provided by commercial services (IGA-Technology, Udine and Genomix4life, Salerno, Italy), were used for sequencing the samples, in separate runs. Both ends of the V4 16S rRNA hypervariable region were used (Yang et al., 2002). They are considered capable to yield sufficient informations for taxonomic classification (Liu et al., 2007, 2008; Caporaso et al., 2011). In all bacteria the V4 region consists of 254 nt differing only for a few base pairs. According to the Illumina protocol this region was amplified with 515F (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806R (5′ GGACTACVSGGGTATCTAAT 3′) primers, with anchored adapters unique for each sample, for subsequent identification.

For the bioinformatic assembly of the single read contigs, PandaSeq[1] was used (Masella et al., 2012), with the following parameters/conditions: filtering sequences with unidentified nucleotides; minimal length of overlapping region = 25 nt; contig lengths (min-max) = 245–265 nt (Claesson et al., 2010). For each sample, the single fasta format file of assembled sequences obtained, by merging data, was then used as first input for data processing with QIIME, running in a Linux emulator (Virtual box), within a Windows 7 environment.

QIIME was applied to analyze the OTUs assigned through the implementation of UCLUST, applying a 97% identity threshold to discriminate at the species level (Caporaso et al., 2010). An OTU table was then constructed using *pick_de_novo_otus.py* and a combined fasta file with labels, generated by a metadata mapping file with *add_qiime_labels.py*. A biom-formatted OTU-table was

[1]https://github.com/neufeld/pandaseq

then obtained, and used for analyses. Selected representative sequences of each OTU were classified using a QIIME-based wrapper of the Ribosomal Database Project (RDP) classifier, using a 0.80 confidence threshold for taxonomic assignments and the RDP core set (Wang et al., 2007; Cole et al., 2009; Edgar, 2010). β-diversity estimates were calculated with QIIME using weighted Unifrac distances between samples (Lozupone and Knight, 2005), at a depth of 6000 sequences per sample. Jackknifed principal coordinates (PCoA) were then computed. PAST (Hammer et al., 2001) was used to calculate a set of OTU α-diversity indices.

The OTU biom format file with sequence abundance per sample and treatments was analyzed with the graphical interface provided by STAMP (Statistical Analysis of Metagenomic Profiles ver. 2.1.3)[2] (Parks and Beiko, 2010; Parks et al., 2014). To compare sample pairs or samples organized into two or more groups identified by treatment and/or other traits provided with the mapping file (such as sampling time, presence/absence of nematodes or nematocide), the entire samples were used as parent level with different profile levels, applying a two tailed Student's *t*-test, with other comparative statistics. To keep unclassified OTUs and their higher levels in the analyses, the latter were identified in the hierarchy (and eventually represented in plots) by using the corresponding OTUs ID as tags for the higher taxonomic levels. Heatmap plots of only active features (ANOVA, with 0.95 *post hoc* Tuckey–Kramer test, filtering threshold: $P \leq 0.05$) were produced with the average neighbor UPGMA algorithm and a 0.65 dendrogram clustering threshold. Two groups comparisons were performed applying a two sided, equal variance *t*-test ($P \leq 0.05$, effect size as ratio of proportions = 0.8). Metagenome ring-charts by treatments and sampling time were produced with Krona (Ondov et al., 2011). Further analyses were performed using R (R Core Team, 2013), with Bioconductor libraries *Biobase*, *BiocParallel*, *BiocVersion* (Ihaka and Gentleman, 1996), *ggplot2* (Wickham, 2016) and package *mctoolsr*, ver. 0.1.1.2[3].

RESULTS

Bacterial Microbiome Composition and Diversity

A total of $13.5 \cdot 10^6$ single reads were obtained from the 25 samples analyzed, yielding $6.6 \cdot 10^6$ qualitatively valid sequences with an average length of 93 bp and 56% GC content (for raw data inventory see NCBI Bioproject PRJNA371772)[4]. PandaSeq yielded 483687 contigs that were analyzed with QIIME, yielding a total 294597 sequences, that were used to get the taxonomic assignments in each sample.

The 4127 OTUs obtained (including 79 redundants) were represented in the various samples with different frequencies. Only 93 (2.3%) of them were classified at the species level. The OTUs belonged to 179 families, 125 orders, 79 classes and 28 bacterial phyla, with Archea only represented by phylum Crenarchaeota (**Supplementary Figure S2**). A trend toward an increased number of total and unique genera was observed at T_2 in all treatments, with fenamiphos plus RKN showing the highest score (**Supplementary Figures S1B, S3**). Similarly, also the total number of OTUs progressively increased during the 6 months of the experiment, as did the number of unique OTUs, that increased from T_1 to T_2, in all treatments (**Figures 1A–C**).

β-Proteobacteria were the most abundant class, for all treatments and times. The most represented lineages were α-, β-, γ-, and δ-Proteobacteria, which accounted for 66% of sequences at T_0, followed by Actinobacteria, Acidobacteria, Gemmatimonadetes and others. When compared to soil before transplanting, the presence of tomato roots increased frequency of Actinobacteria and Thermoleophilia, reducing abundance of Solibacteres (**Table 1** and **Supplementary Figure S2**). After rarefaction at 800 sequences, which kept all samples, the first ten taxa with highest relative abundance (mean% of total) showed different frequencies per treatment and time, with a number of dominant genera. Most represented in the control samples were genera *Thiobacillus* (at sampling time T_0), and the archean *Ca.* "Nitrososphaera" (at T_1 and T_2). *Cellvibrio* showed a dramatic increase in time in treatments with fenamiphos, whereas *Bacillus* was dominant at T_2 in the samples inoculated with RKN only, followed by *Oscillospira* and *Corynebacterium* (**Figure 2A**). When considering presence/absence of RKN, *Bacillus* again was dominant in presence of RKN, whereas *Ca.* "Nitrososphaera" and *Cellvibrio* were most abundant in samples without RKN inoculation, followed by *Corynebacterium* and *Thiobacillus* (**Figure 2B**).

Sequence data pooled by treatment showed *Ca.* "Nitrososphaera" and *Thiobacillus* as most prevalent in control, *Cellvibrio* and *Corynebacterium* in fenamiphos, *Rubrobacter* in fenamiphos with RKN, and *Bacillus* with *Corynebacterium* and *Oscillospira* in samples with only RKN (**Figure 2C**). Comparing samples by presence/absence of the nematocide showed *Cellvibrio* and *Steroidobacter* as more represented in presence of Fenamiphos, whereas *Bacillus*, *Ca.* "Nitrososphaera" and *Thiobacillus* were more represented in the other samples (**Figure 2D**).

Mean α-diversity indices, calculated by PAST for treatments and times, showed higher values at T_2 for the treatments with fenamiphos plus RKN and RKN only, either in terms of number of taxa or Chao-1, the latter indicating higher prevalence of rare OTUs. This was also reflected by a higher evenness variability, as indicated by Shannon H and dominance, with all taxa more equally present in the other treatments (**Figure 3**). The extent of the β-diversity among all samples showed distinct clusters, formed by individual samples rather than treatments groupings, mostly visible on the PCoA plan formed by the first two axes (accounting for 56% of variance), with a higher aggregation for the RKN-inoculated samples on the other two factorial plans (**Supplementary Figures S1C–E**).

Effects of Treatments on Plants

In the conditions of the assay, the fenamiphos applications did not significantly affect the tomato plants performance, apart

[2]http://kiwi.cs.dal.ca/Software/STAMP

[3]https://github.com/leffj/mctoolsr/

[4]https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP099116

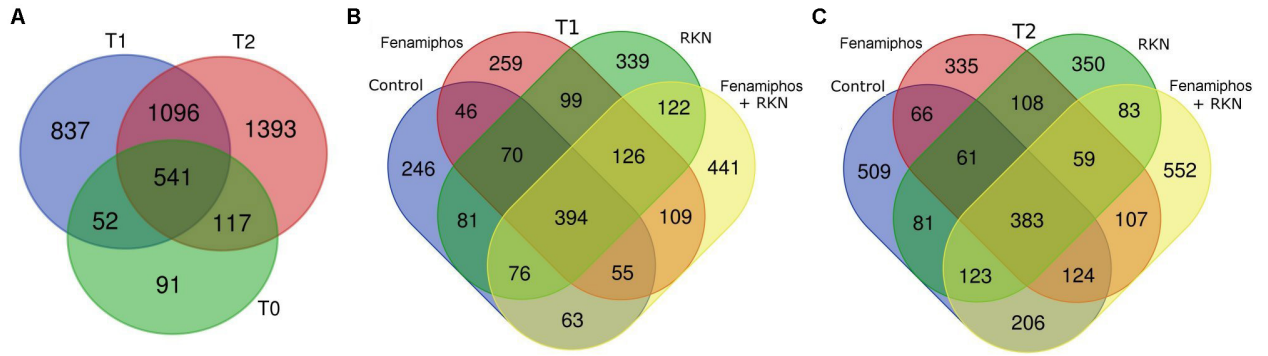

FIGURE 1 | Venn diagrams showing the number of OTUs in all samples before planting (T_0) and at 3 (T_1) and 6 months (T_2) **(A)**. Number of unique and shared OTUs by treatment, 3 (T_1, **B**) and 6 months (T_2, **C**) after pre-transplant sampling.

TABLE 1 | Relative abundance (% of total) of sequences for most common bacterial phyla and classes, by treatment and time.

Phylum	Class	Treatments*									Mean
		Control			RKN		Fenamiphos		Fenamiphos and RKN		
		T_0	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	
Proteobacteria											
	α-Proteobacteria	6	4	6	5	3	5	5	11	6	5.67
	β-Proteobacteria	51	31	13	11	19	16	11	10	37	22.11
	γ-Proteobacteria	3	5	6	7	7	9	48	6	9	11.11
	δ-Proteobacteria	6	2	5	2	3	6	3	7	6	4.44
Actinobacteria											
	Actinobacteria	4	16	12	12	12	13	10	11	8	10.89
	Acidimicrobiia	2	2	4	4	5	3	4	3	8	3.89
	Thermoleophilia	2	18	13	10	5	6	2	5	4	7.22
	Rubrobacteria	5	0.5	1	0.7	0.4	0.8	0.3	2	2	1.41
Gemmatimonadetes											
	Gemm-2	0.1	0.4	0.8	0.4	1	0.2	0.2	1	0.9	0.56
	Gemm-3	0.3	0.4	0.6	0.6	0.9	0.3	0.3	0.6	0.4	0.49
	Gemm-5	0.2	1	2	2	3	0.9	1	0.6	1	1.30
	Gemmatimonadetes	1	2	7	3	5	3	2	0.9	5	3.21
Armatimonadetes											
	Fimbriimonadia	0.1	0.04	0.09	0.1	0.06	0.04	3	0.7	0.1	0.47
	c_0319-6E2	0.05	0.06	0.3	0.7	–	0.6	–	1	0.01	0.39
Acidobacteria											
	Acidobacteriia	0.07	0.2	0.2	1	0.1	6	0.03	2	0.1	1.08
	Solibacteres	15	3	10	13	6	7	2	10	6	8.00
	[Chloracidobacteria]	0.2	1	5	8	0.08	7	0.2	10	0.09	3.51
Chloroflexi											
	Chloroflexi	0.03	0.3	0.9	0.8	0.02	0.6	0.007	0.9	0.01	0.40
	Ellin6529	0.03	0.1	0.3	0.5	0.03	0.5	0.03	0.8	0.005	0.26
	Thermomicrobia	0.08	0.6	1	2	0.1	2	0.07	3	0.09	0.99
Nitrospirae											
	Nitrospira	1	1	2	7	3	5	0.7	4	3	2.97
Firmicutes											
	Clostridia	–	0.04	0.06	0.1	5	0.01	3	0.04	0.02	1.03
	Bacilli	1	0.7	1	1	16	1	1	1	1	2.63

**Each treatment mean of three replicates, except Control at T_0 and Fenamiphos with RKN at T_2, with two replicates each.*

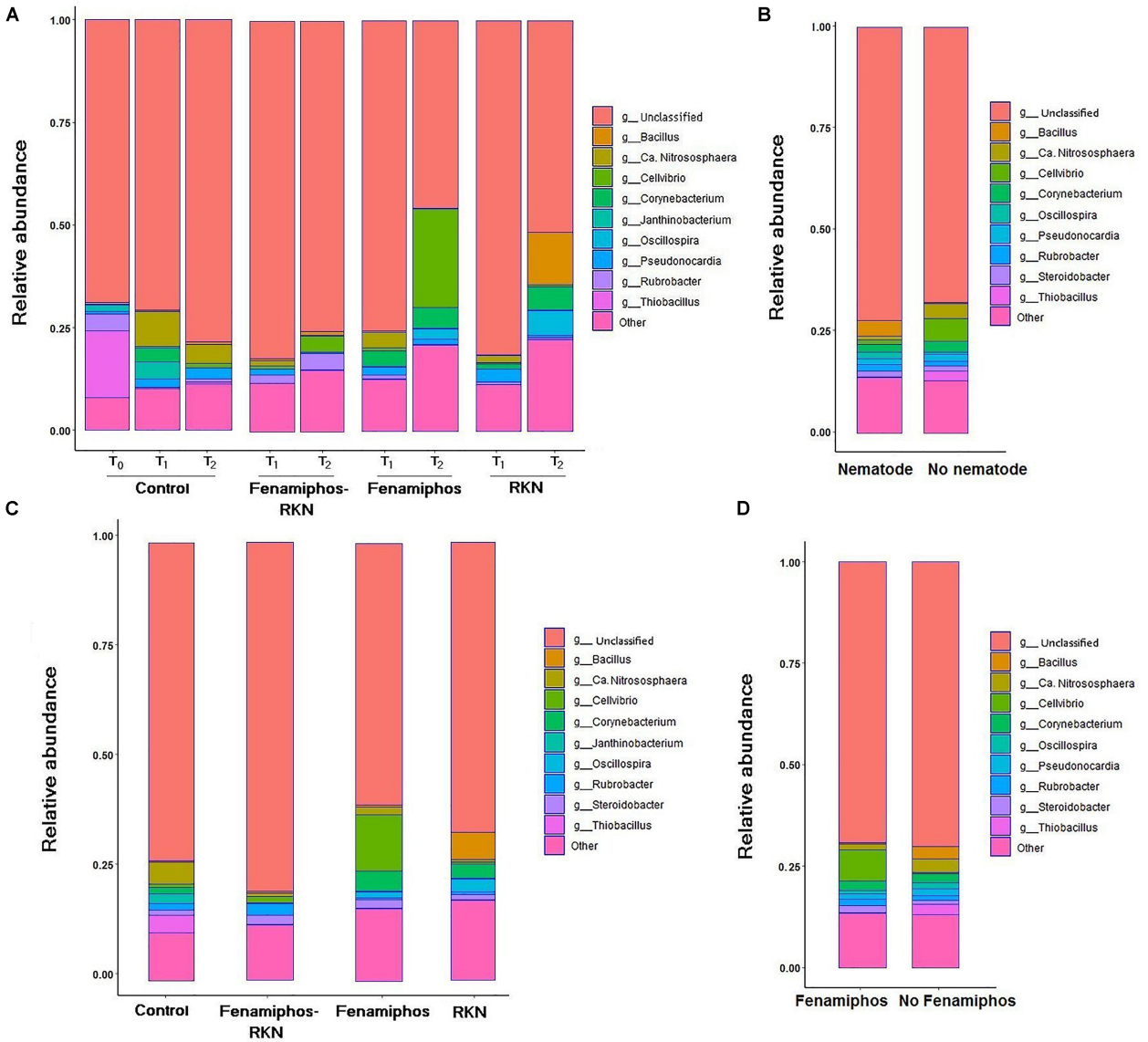

FIGURE 2 | Relative abundance of the ten most represented genera, by treatment and sampling time **(A)**, when considering pooled samples with or without *Meloidogyne incognita* (RKN) inoculum **(B)**, pooled by treatment **(C)**, and with or without fenamiphos application **(D)**.

from an increase observed for the root weight at 3 months ($P < 0.05$). In the treated and RKN-inoculated plants, the number of RKN eggs and root gall index (RGI) decreased at T_2, with an opposite trend in the plants inoculated only with nematodes. Application of the nematocide increased plants height at T_1 (fenamiphos only), and at T_2 in the treated samples, with nematode inoculation (**Supplementary Figure S4**).

Bacterial Microbiome Evolution in Time

The abundance of taxa showed a number of changes in relation to time and treatments, when compared with the proportions in the pre-treatment control (T_0) (**Supplementary Table S1**). Archea (Crenarchaeota), that were not detected at T_0, were found at 3 months in control (8% of total sequences), in one sample treated with fenamiphos and, with lower proportions (0 – 1%), in other samples. They persisted at 6 months (T_2) only in the untreated control, although with a mean reduction to 4% of total sequences. When Archea were present, *Candidatus* "Nitrososphaera" always accounted for more than 80% of their sequences (for Krona diagrams with mean proportions of taxa by treatments and times see **Supplementary Figure S2**).

No significant change (statistical test: ANOVA; applied filtering criteria: $P \leq 0.05$, effect size = 0.8, min. 1% of sequences at least in one sampling time) was found for the relative abundance of Bacteria in the control samples along the three sampling times, both at the phylum and class levels. At the order level, only six taxa forming congruent clusters were found along time accounting, however, for <0.3% of total sequences

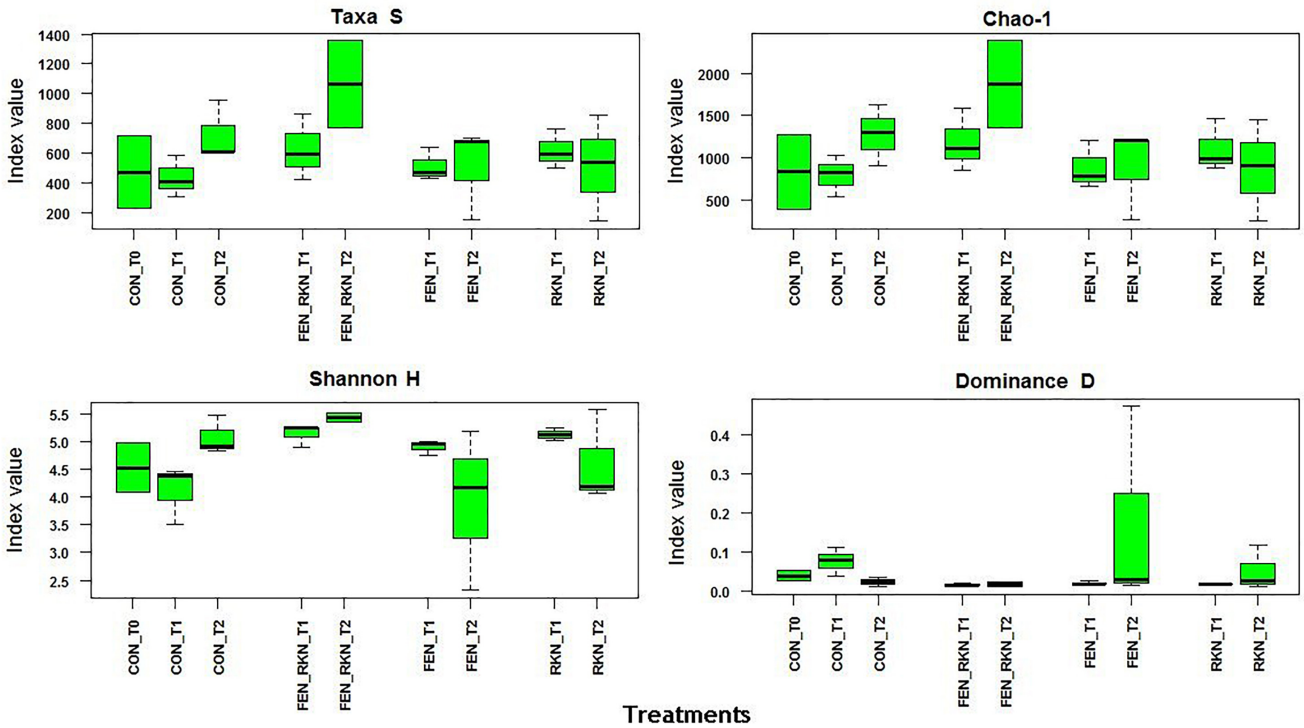

FIGURE 3 | Alpha diversity indices selected for treatment and sampling times. Treatments codes: CON, untreated control; RKN, inoculated with *Meloidogyne incognita*; FEN, treated with fenamiphos; FEN-RKN, inoculated with *M. incognita* and fenamiphos. Boxes show the quartile range (75th to 25th). The median is shown as a line within the box. Whiskers indicate the most extreme data point within 1.5 · (75th – 25th percentile) of the median.

(**Figure 4A**). Significant differences were observed in time for β-Proteobacteria and Bradyrhizobiaceae (α-Proteobacteria) which, differing from the control, declined with fenamiphos, showing a more resilient response and an increase at T_2, when RKN were also present (**Figure 5**).

During the 6 months of the assay, RKN treatment showed an alternated trend for Pseudonocardiaceae (Actinobacteria) and, in combination with fenamiphos, increased Gemmatimonadetes and Rhodocyclaceae (β-Proteobacteria) (**Figure 4B**). Differing from control, the nematocide also had a detrimental effect on Solibacteres (**Figure 4B**), with OTU ID1111565 characterized by a more resilient response, when RKN were also inoculated (**Figure 6**). An opposite trend in time was shown by *Nitrospira* and OTU ID4472017 which, differing from control where their changes in time were not significant, sharply increased in presence of fenamiphos with RKN accounting for 1.8% of total sequences at T_2 (**Figure 6**). When considering all samples together, number of unique OTUs was found mostly in this treatment, although with a low relative abundance (<1% of total sequences) (**Supplementary Table S2**). At the genus level the samples showed clusters distinct from control and, in the case of RKN inoculation, congruent with treatments and sampling times (**Figures 7A,B**).

Effect of Treatments

STAMP analysis showed changes in the relative abundance of taxa, reflecting the treatments and the time of sampling. Heatmaps showed samples that often clustered in groups congruent with treatments. In general, more taxa were identified at T_2 as differentially represented among treatments, mostly at the genus level. Comparison with control of the fenamiphos-treated samples (two sided *t*-test equal variance, $P \leq 0.05$, sequence filter maximum = 3–10; effect size filter as ratio of proportions = 0.8) showed differential abundance only at the genus level, at both sampling times, but for different taxa (**Figure 8**). Compared to control, the RKN-inoculated samples showed at T_2 a higher relative abundance for *Corynebacterium*, *Streptococcus* and *Staphylococcus*, together with additional OTUs (**Figure 9**). A higher abundance of taxa was also observed at T_2 in the samples treated with fenamiphos and inoculated with nematodes, when compared to control, involving mostly Proteobacteria, with a higher representation for Burkholderiales, with family Comamonadaceae (**Figure 10** and **Supplementary Table S3**).

When fenamiphos-treated samples differed for RKN inoculation, most differences were observed at T_2 for classes Nitrospira, Solibacteres, Gemmatimonadetes and Gemm-2, with a higher number of genera differentially represented at T_2, and different from those observed at T_1 (**Figure 11**). Similarly, a higher number of differentially represented taxa were observed at T_2 when comparing the samples with RKN, differing for addition of fenamiphos, with a higher representation for Burkholderiales and other orders at T_2, including Rhodospirillales and the order

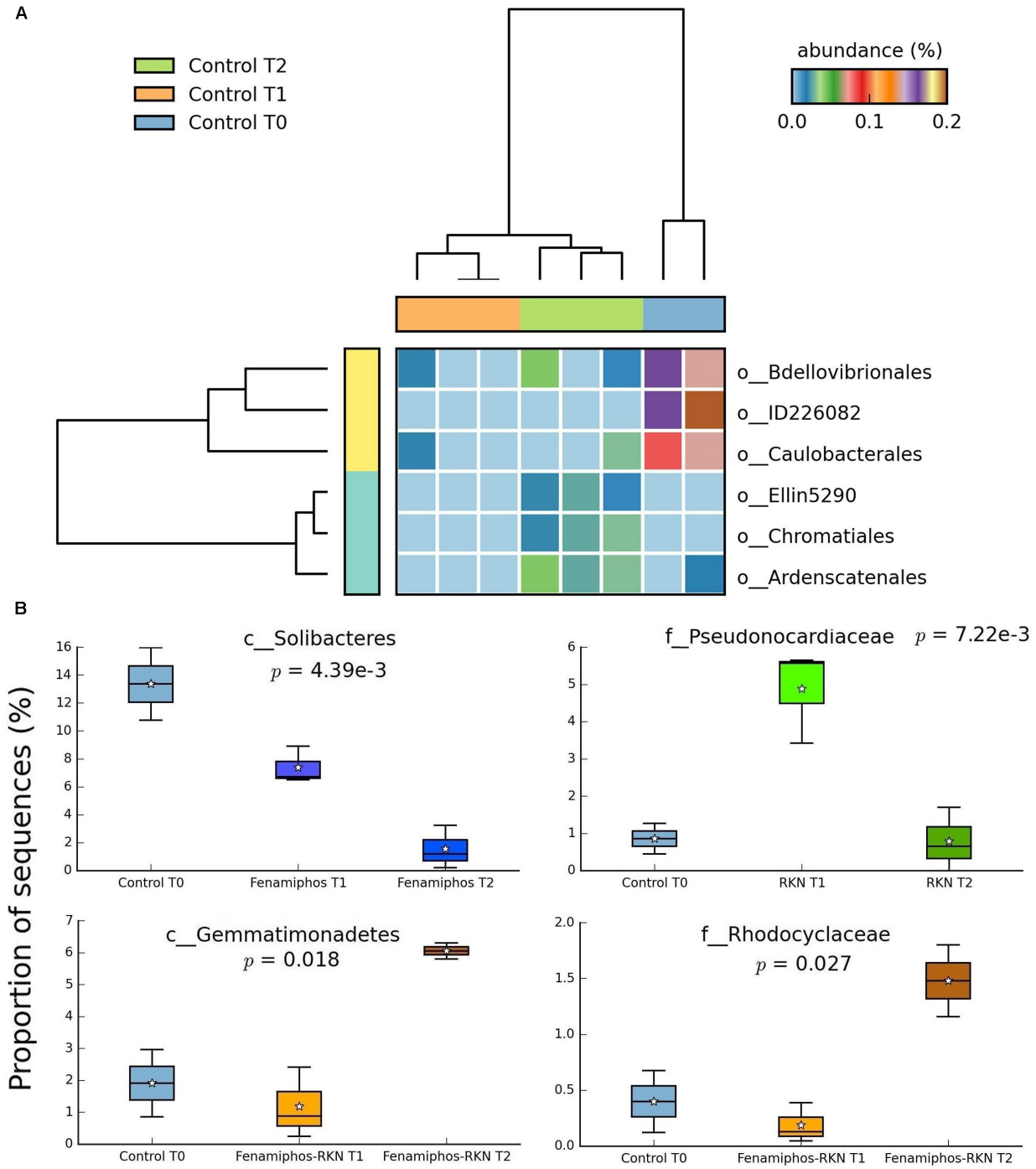

FIGURE 4 | Significant changes ($P < 0.05$; effect size, ratio of proportions = 0.8) observed at the three sampling times for control soil samples, at the order level **(A)**, and changes in proportion of sequences at class and family levels **(B)**. For whisker plot details see **Figure 3** legend.

of OTU ID778795 (Gemmatimonadetes) (**Figure 12** and **Supplementary Figure S5**). Congruent clustering was also observed when comparing fenamiphos-treated samples with those inoculated with RKN, although for four genera only, whereas no difference was found at T_2, apart of a higher relative abundance for *Streptococcus*, in the samples with nematodes (**Supplementary Figure S6**).

When comparing all samples pooled by treatment ($P < 0.05$; sequence filter maximum = 10; effect size filter as ratio of proportions = 0.8), a higher differential

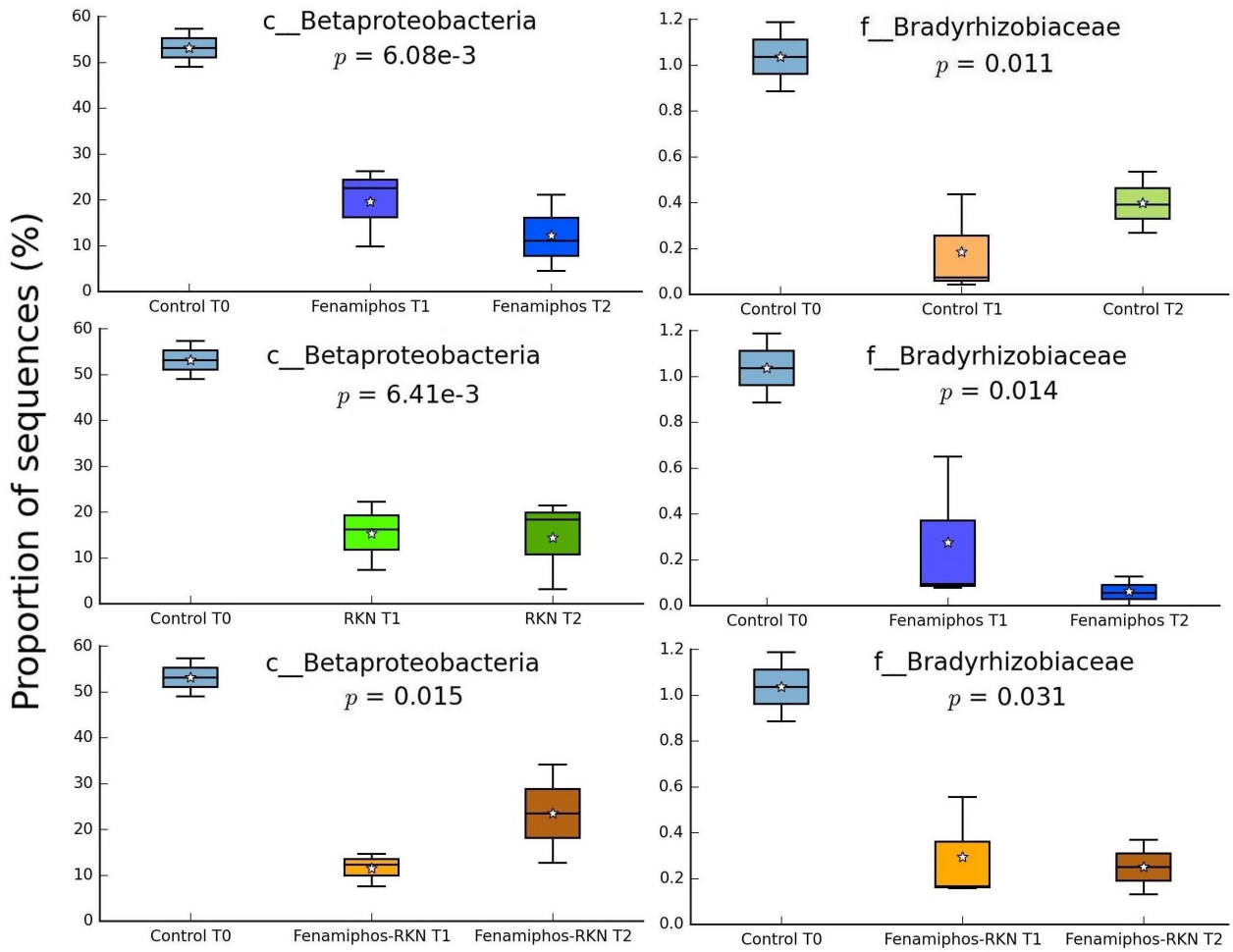

FIGURE 5 | Changes of proportion of sequences (% of total) for β-Proteobacteria and Bradyrhizobiaceae, by treatment and sampling times. Boxes show the quartile range (75th to 25th). For whisker plot details see **Figure 3** legend.

abundance was observed for *Streptococcus*, *Staphylococcus*, OTU ID134102 and others, in RKN samples *vs.* control. A detrimental effect of fenamiphos, in comparison to control, was found on *Lysobacter*, *Blastococcus* and OTU ID261264, with an increase for *Luteimonas* (**Supplementary Figure S7**). A higher number of taxa was differentially represented when comparing all controls to all samples with fenamiphos and RKN, including *Solirubrobacter*, *Afifella*, *Devosia*, and several other unclassified OTUs (**Supplementary Figure S8**).

Finally, four of the five genera reported as fenamiphos-degrading bacteria (Cabrera et al., 2010) were found, with low abundance, among the examined samples. At 6 months, *Cupriavidus* was found in four samples, one for each treatment. *Microbacterium* was present only in one sample with fenamiphos and nematode inoculum. *Sinorhizobium* occurred only in one sample treated with fenamiphos, whereas *Ralstonia* was found in a RKN-inoculated and a control sample.

DISCUSSION

Metabarcoding data showed changes in the bacterial abundance profiles related to the treatments applied to rhizosphere soil samples that had a common origin (a single site in an organic farm field). This result, not assumed *a priori*, was supported by quantitative data and appeared with different dataset profiles and mostly at lowest taxonomic depths. Morever, data indicated that the changes in soil bacterial abundance was mainly due to rare and unclassified taxa, in particular for the samples treated with fenamiphos and inoculated with RKN, as also shown by the Chao-1 index. Hence, the microbiome appeared sensitive to factors related to (or deriving by) the nematocide and/or the nematode additions, mostly operating at lowest and unclassified taxonomic levels, in particular after 6 months.

Rare taxa sensitivity to these drivers suggest that d chemical treatments and pest management may affect their abundance and, indirectly, the services these bacteria provide.

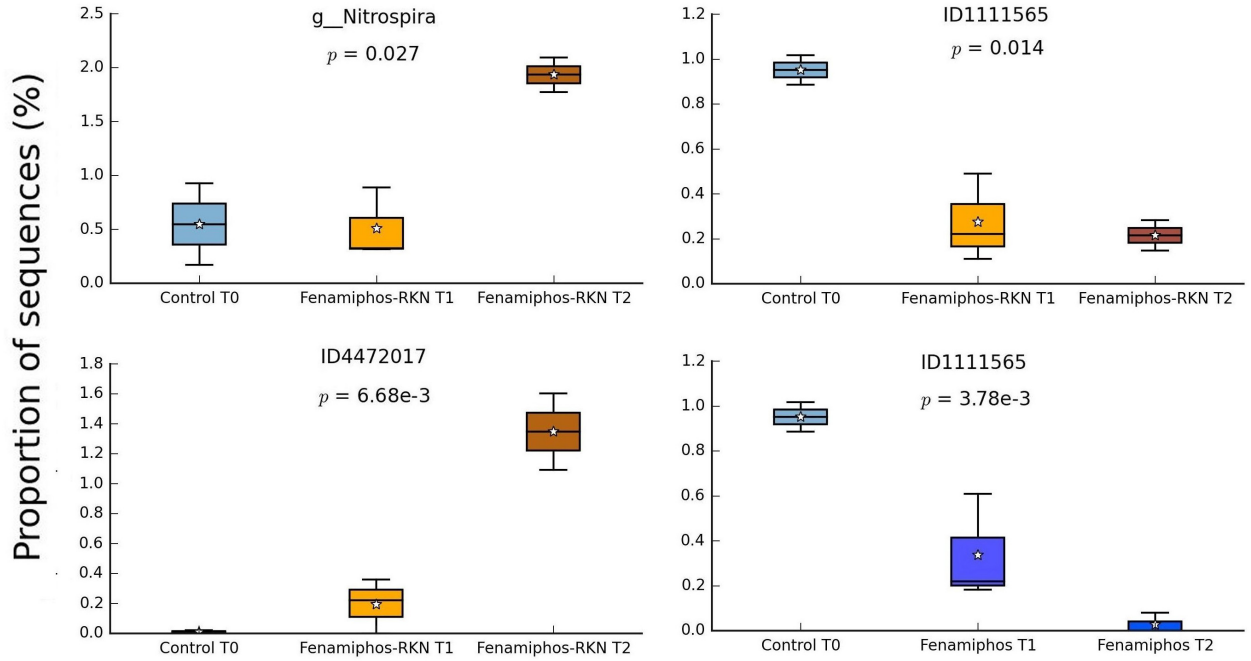

FIGURE 6 | Changes in time of proportion of sequences (% of total; $P \leq 0.05$; effect size, ratio of proportions = 0.8) for fenamiphos with RKN treatment, and comparison of OTU ID1111565 with trend observed in presence of fenamiphos only. Boxes show the quartile range (75th to 25th). For whisker plot details see **Figure 3** legend.

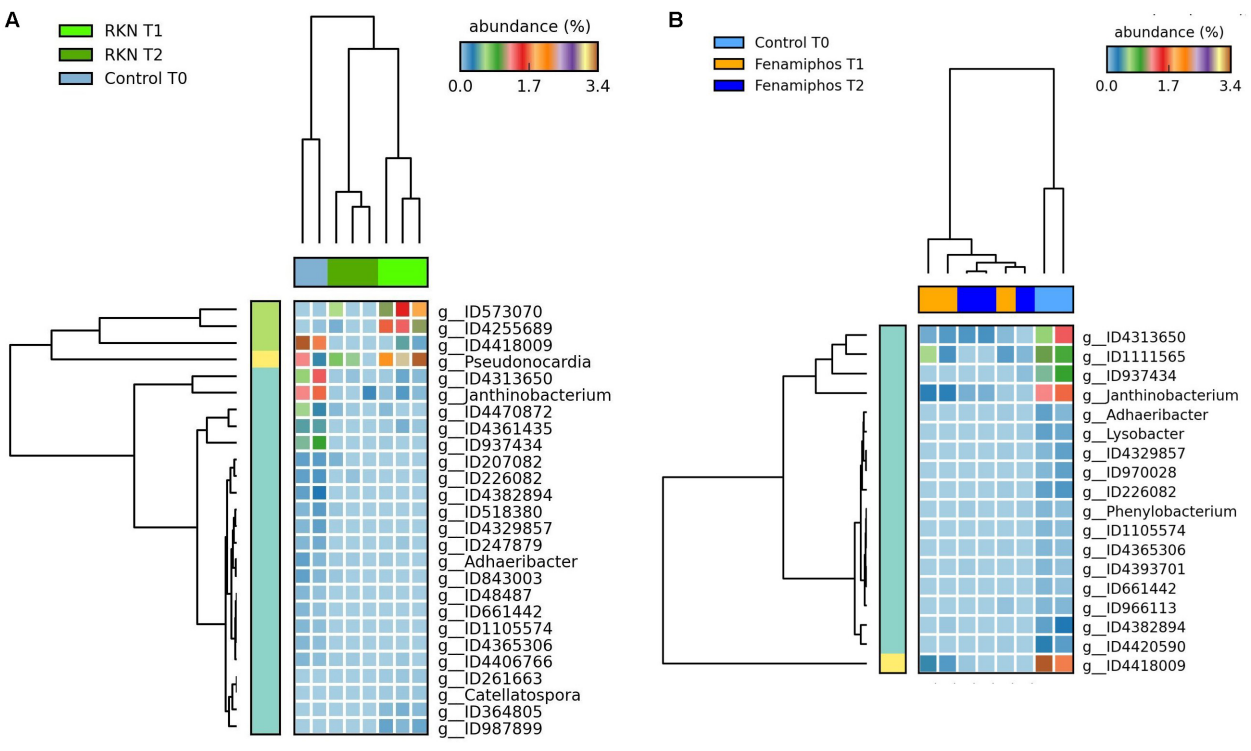

FIGURE 7 | Heatmaps showing significant changes (% of total; $P \leq 0.05$; effect size, ratio of proportions = 0.8) in abundance for genera significantly different at T_1 and T_2 from initial control (T_0), for RKN-inoculated **(A)** and fenamiphos-treated samples **(B)**.

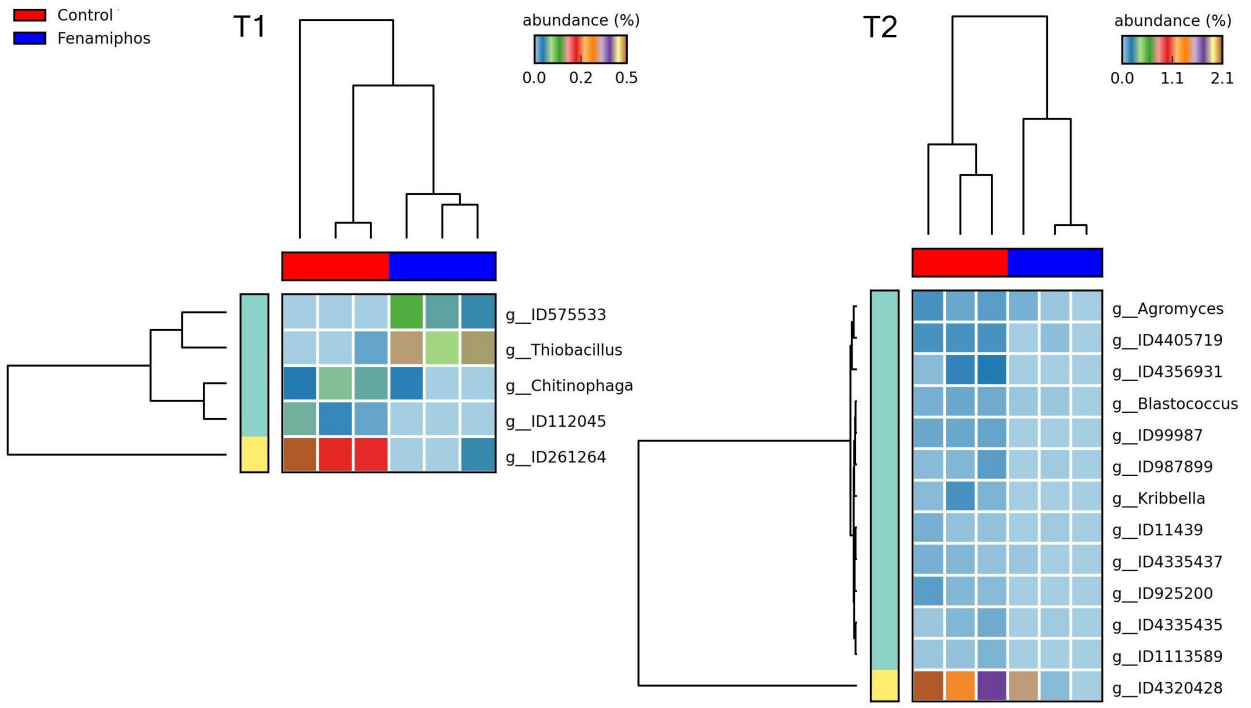

FIGURE 8 | Heatmaps showing changes in abundance for genera significantly different, when comparing fenamiphos treatment with control, at T_1 and T_2.

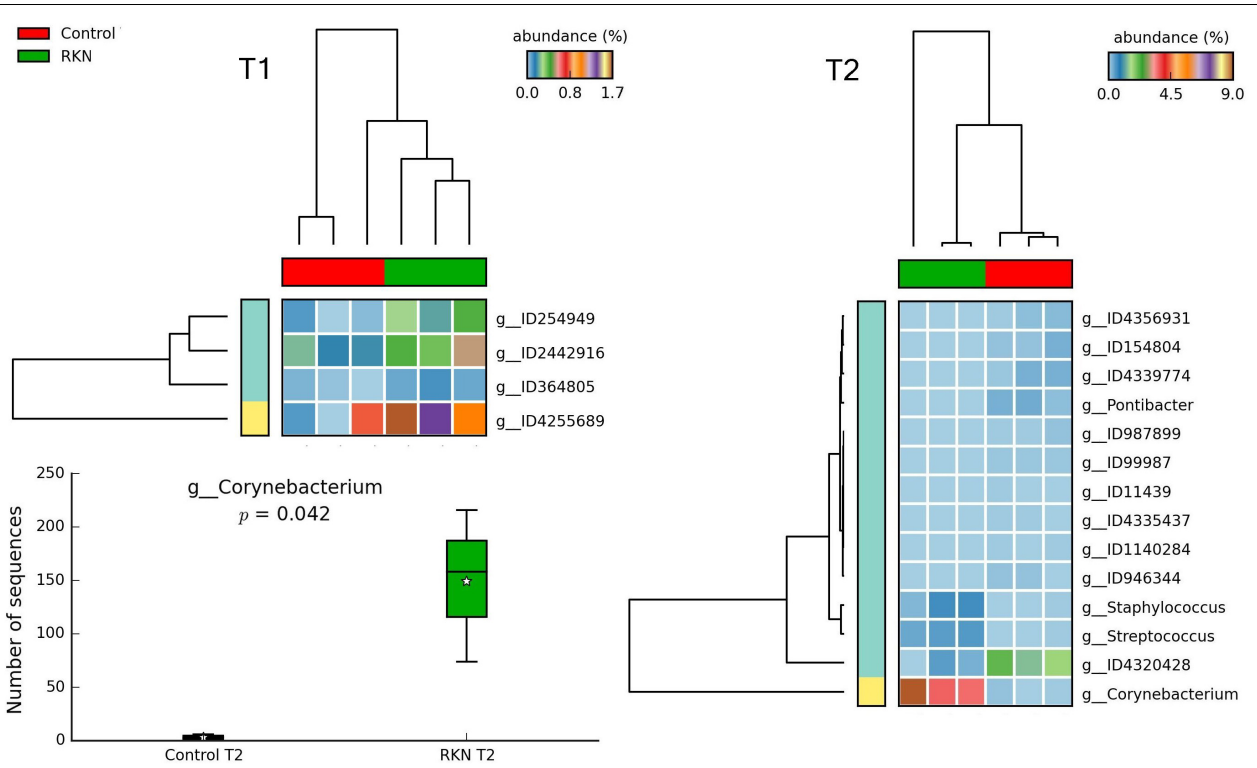

FIGURE 9 | Heatmaps showing clustering of samples by treatment, with significant changes for abundance of genera (% of total; $P \leq 0.05$; effect size, ratio of proportions = 0.8), when comparing RKN inoculation with control, at T_1 and T_2. Box plot shows differential abundance in the number of *Corynebacterium* sequences, at T_2. For whisker plot details see **Figure 3** legend.

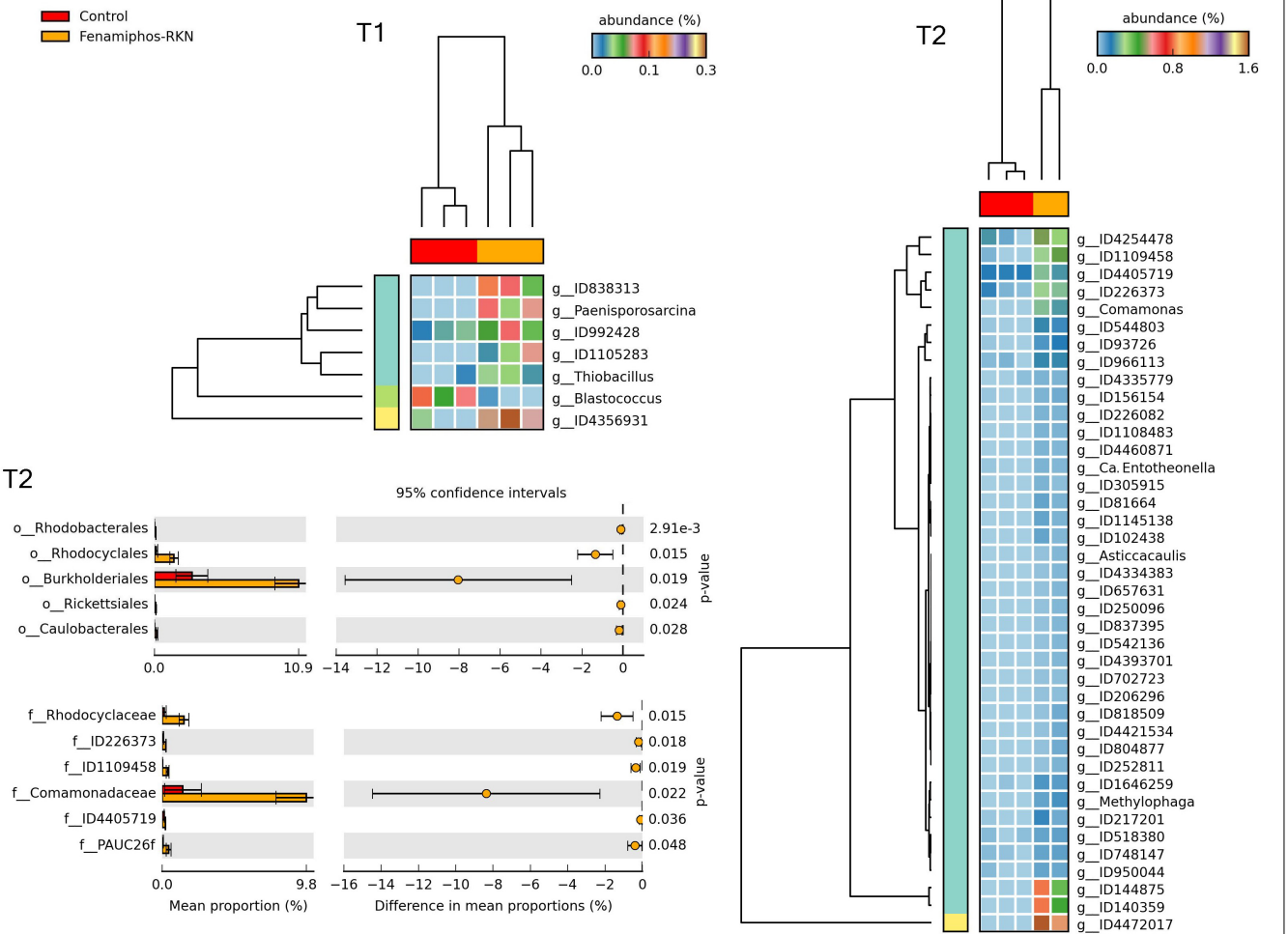

FIGURE 10 | Heatmaps showing samples clustering by treatment with significant changes for abundance of genera (% of total; $P \leq 0.05$; effect size, ratio of proportions = 0.8), when comparing samples of control vs. fenamiphos with RKN inoculation, and most significant changes in mean proportion (%) of sequences at the order and family levels, at T_2 (equal variance *t*-test, two sided; bars show SD).

RKN and fenamiphos affected OTUs that were different at the two times, not intercepted nor available for sequencing in other treatments and/or at T_1, because present at low initial numbers. The selective increase of specific taxa in the presence of nematodes (i.e., Nitrospirales, *Bacillus*, *Corynebacterium*, *Streptococcus*, *Staphylococcus*) may suggest an effect of novel substrates and/or of previously scarce molecules (i.e., chitin, polysaccharides) that increased due to the presence of J2, eggs and egg mass matrix. Similarly, also secondary metabolic by-products may have been involved in these changes. Some of these taxa, i.e., *Streptomyces* (Actinobacteria) were also found to be enriched in cysts of the soybean cyst nematode *Heterodera glycines* or in soil suppressive to *M. incognita* (Elhady et al., 2017; Hu et al., 2019). Other lineages that were enriched in cysts, i.e., Cytophagales (Bacteriodetes) were found at a doubled abundance in RKN and also in control samples at T_2 (0.5 and 0.4% of total sequences, respectively), decreasing or remaining unchanged in the other treatments (**Supplementary Figure S2**). These data suggest a specificity in the lineages of nematode-associated bacteria, in part reflecting the soil type and/or the nematode species involved.

As the soil taxonomic metabarcoding profiles also changed by the sampling times, this effect was possibly integrated by the long-term activation and germination of durable, resting propagules, in particular for *Bacillus* spp. (Nicholson, 2002). This mechanism, however, occurred independently among treatments, but was mostly found in samples with addition of RKN and nematocide.

A second mechanism underpinning the shifts observed in the microbiome profiles appears linked to the biology of the organisms involved. The presence of *M. incognita* eggs and J2 likely influenced the abundance and dispersal of specific nematode-associated bacteria, through mechanisms such as passive transport onto the cuticle (Standing et al., 2006). Although the nematodes were surface sterilized before inoculation, the J2 possibly mobilized rare bacterial species associated to chitin or to damaged root tissues, as well as other soil organisms such as nematophagous fungi or protozoa

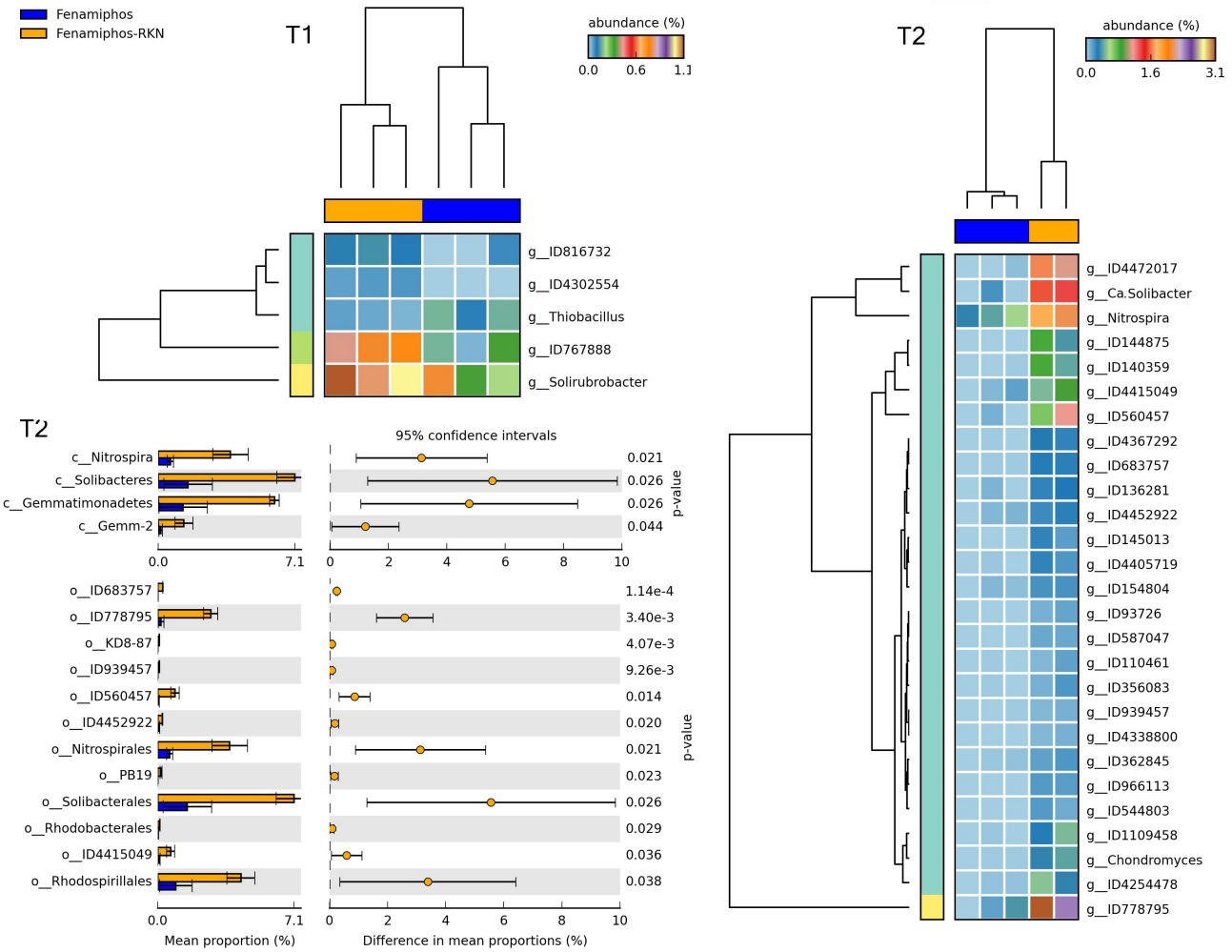

FIGURE 11 | Heatmaps showing samples clustering by treatment with significant changes for abundance of genera (% of total; $P \leq 0.05$; effect size, ratio of proportions = 0.8), when comparing samples of fenamiphos vs. fenamiphos with RKN inoculation, and significant changes in mean proportion (%) of sequences, at the class and order levels, at T_2 (equal variance *t*-test, two sided; bars show SD).

and, indirectly, their associate microflora. The introduction of additional cuticle-associated durable endospores cannot be, furthermore, excluded. Similarly, we cannot exclude an effect due to the changes in root architecture, in particular as related to the formation of galls, an enhanced number of tips and the release of exudates by the stressed roots (Saleem et al., 2018; Hartman and Tringe, 2019).

RKN inoculation likely allowed also the introduction or mobilization of bacteria or endosymbionts associated to nematode oral region, gut and/or intestine (Noel and Atibalentja, 2006; Haegemann et al., 2009; Ladygina et al., 2009; Berg et al., 2016) or associated as endophytes in roots. All nematodes co-exist with specific bacterial groups in their environments (Poinar and Hansen, 1986), and their interactions also affect the surrounding soil structure and/or microbial communities (De Mesel et al., 2004). An enrichment, shown by taxonomic metabarcoding data in root endophytic bacteria was reported in RKN-parasitized tomato roots, with increased bacterial activities in galls related to polysaccharides degradation, carbohydrate/protein metabolism and nitrogen fixation (Tian et al., 2015). Finally, many bacteria are known to produce a variety of compounds such as antibiotics, nutrients, exoenzymes and signal molecules (Pierson and Pierson, 2007), affecting the density and composition of their surrounding microflora.

A partial effect of the nematocide application was observed at T1 with a significant increase in the root weight, and at T2 with a lower (although not significant) egg number (**Supplementary Figure S4**). The nematocide effect was, however, not evident when considering other variables, at both sampling times. This appeared related to fenamiphos half life (21 days), to the time of first nematocide application (1 month after initial RKN inoculation) and/or to its run off by irrigation water. The RKN persisted during the assay and no long-term extinction occurred at 6 months. This was likely the effect of a density dependent reaction, as the nematode populations rebound after a

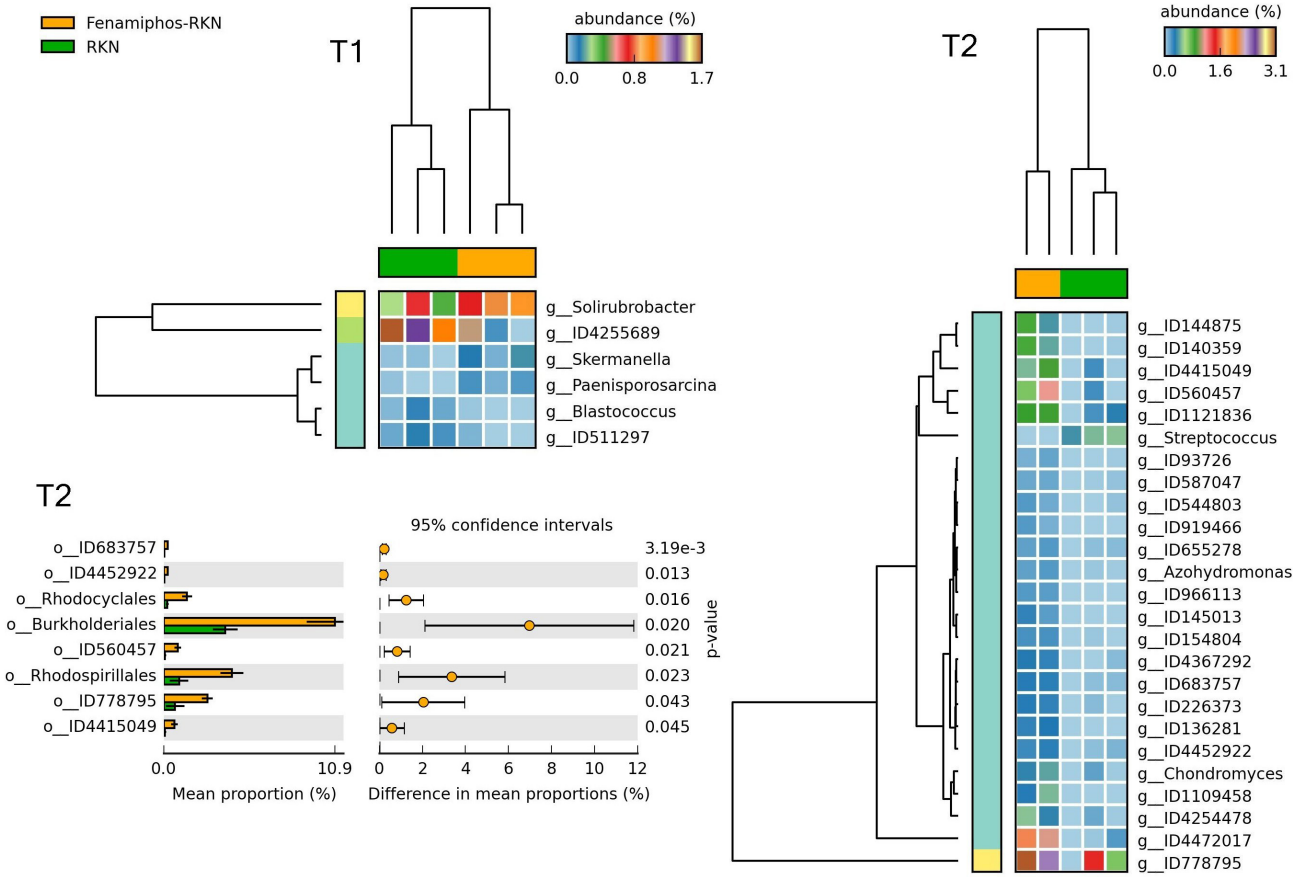

FIGURE 12 | Heatmaps showing samples clustering by treatment with significant changes for abundance of genera (% of total; $P \leq 0.05$; effect size, ratio of proportions = 0.8), when comparing samples of RKN inoculated samples vs. fenamiphos with RKN inoculation, and significant changes in mean proportion (%) of sequences, at the order level, at T_2 (equal variance *t*-test, two sided; bars show SD).

reduction caused by an external factor, i.e., a chemical treatment (Seinhorst, 1967). The nematocide effect was instead more visible in relation to the bacteria taxonomic metabarcoding profiles in the treated samples, also suggesting a possible effect of some degradation compounds.

In this study the organic farm soil was used to eliminate other factors, i.e., any effect related to previous applications of pesticides or residues, as occurs in conventional farming. A similar rationale was applied for RKN, so that soil was chosen also because of the absence of RKN, to avoid interference by already indigenous RKN populations. Most differences observed in bacteria diversity then reflected the RKN and nematicide treatments that were applied in the assay.

This study showed that adding further components to a soil food web likely alters one or more links established among functional groups, resulting in a complex of effects visible in time at the taxonomic metabarcoding scale. Similar considerations hold for the nematocide applied, considering its activity in relation to the presence of J2 cadavers, and its degradation and metabolism as well. Considering the cadavers, the J2 hatching, persistence in soil (usually 24–48 h when close to roots) and death are not synchronous, and a different experimental approach (i.e., time samplings at weekly or few days regular intervals) had be applied to test the contribution of cadavers to the observed changes in bacteria diversity. As concerns the nematocide degradation, although changes in OTUs abundance in presence of fenamiphos do not directly demonstrate a degrading capacity for taxa with higher abundance that were differentially represented in this treatment only, they may, however, reflect indirect effects due to the nematocide activity and/or changes in RKN numbers. Services of soil bacteria include also the metabolism of pesticides such as fenamiphos, including its degradation after long-term applications (Johnson, 1998; Cabrera et al., 2010).

Fenamiphos degradation by soil bacteria was shown in a wide range of soils with radio-labeled molecules, with varying recovery rates related to the soil types and temperature (Simon et al., 1992). Specialized soil bacteria degrading fenamiphos and its metabolites were identified by Cabrera et al. (2010). Other assays showed that a *Microbacterium esteraromaticum* isolate also degraded related toxic oxidation products such as fenamiphos sulfoxide and sulfone (Cáceres et al., 2008). Other fenamiphos-degrading species include a *Brevibacterium* sp., capable to rapidly hydrolyze a high fraction of the molecules

adsorbed to an organo-clay complex (Singh et al., 2003; Singh and Walker, 2006). The insurgence of bacterial groups capable of degrading pesticides and related by-products was also observed for other active compounds, such as fenitrothion, whose introduction in soil increased *Burkholderia* spp. and other methylotrophs degrading its methanol derivative (Itoh et al., 2014). The degrading capacity of pesticides by soil bacteria represents a fundamental service, useful not only in case of pollution but also in sibling contamination issues such as the continuous agricultural practices. In the soil used in the assay there was a number of fenamiphos-degrading taxa that were found, however, at low abundance, suggesting either they did not increase because of the nematocide or that they had a transient effect, not revealed during the sampling intervals.

CONCLUSION

The changes observed in the bacterial microbiome reflected abundance, reproduction and survival of species such as *Bacillus* spp., initially present at very low densities and increasing at 6 months in presence of RKN. Changes in relative abundance were observed at any taxonomic level and mostly involved unclassified OTUs, with a clustering of samples-by-treatments visible at lowest taxonomic levels. Sample clusterings indicated that the re-organization of the bacterial communities following treatments mainly concerned a range of specialized taxa, often belonging to distant lineages, shifting in time. Results also indicated a clear link of rhizosphere bacteria with phytoparasitic nematodes. Encompassing unclassified species, the data enlarge our knowledge on the functional role of rare or unculturable OTUs, as well as on their potential for nematode management.

DATA AVAILABILITY STATEMENT

All taxonomic metabarcoding datasets generated for this study are available at NCBI Bioproject PRJNA371772, https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP099116.

AUTHOR CONTRIBUTIONS

AC, MC, and LS planned and designed the research. MC and LR performed the experiments. MC, AC, DC, and LR analyzed the data. AC and MC wrote the manuscript. All authors reviewed, revised, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00390/full#supplementary-material

FIGURE S1 | A schematic drawing **(a)** of the experimental design applied, showing the timing (in months) of chemical treatments (dots) and samplings for RNAseq and nematode analyses (arrows). OTUs genera counted in RNAseq data **(b)**, by treatment and sampling times. Plots of OTUs β-diversity by treatments **(c–e)**, as shown by Principal Coordinate Analysis (all samples, see colors in legend for treatments. Treatments codes: CON, untreated control; RKN, inoculated with *Meloidogyne incognita*; FEN, treated with fenamiphos; FEN-RKN, inoculated with *M. incognita* and fenamiphos).

FIGURE S2 | Interactive ring-charts (html format) produced with Krona, showing the mean taxonomic repartitions and relative abundance of taxa resulting from the RNAseq analyses, by treatment and sampling times. For treatments codes see legend of **Supplementary Figure S1**. Files constructed using the mean of three replications, except CON at time T_0 (prior to transplants), and FEN-RKN at T_2 (6 months), with two replicates each. Unclassified taxa were retained in the analyses.

FIGURE S3 | Venn diagrams showing the number of genera per treatment, at 3 (T_1) and 6 months (T_2). For control, the number of genera before planting (T_0) is also shown.

FIGURE S4 | Effect of treatments on *Meloidogyne incognita* egg density on roots **(A)**, root gall index (RGI, **B**), tomato root weight **(C)** and plant height **(D)**, 3 (T_1) and 6 months (T_2) after inoculation. Means ± SE from five replicates. Asterisks on bars show significant differences from corresponding controls at the same sampling time, as indicated by Student's *t*-test, for $p \leq 0.05$ (*) and $p \leq 0.01$ (**). For treatments application see **Supplementary Figure S1a**.

FIGURE S5 | Proportion of sequences (% of total) for selected OTUs at the three sampling times, by the treatments applied. Boxes show the quartile range (75th to 25th). The median is shown as a line within the box, a star indicates the mean. Whiskers indicate the most extreme data points within 1.5 · (75th – 25th percentile) of the median.

FIGURE S6 | Differential representation and congruent clustering for significant changes found when comparing pooled samples for RKN and fenamiphos treated samples, at 3 months (T_1). Differential abundance of *Streptococcus* 6 months (T_2) after *Meloidogyne incognita* inoculation. Horizontal line across bars show the mean values.

FIGURE S7 | Differential representation and congruent clustering for significant OTUs found in heatmaps and relative sequence proportions when comparing pooled samples for control vs. *Meloidogyne incognita* **(A)** and fenamiphos **(B)** (equal variance *t*-test, two sided; bars show SD).

FIGURE S8 | Differential representation and clustering for significant OTUs shown as heatmap and relative sequence proportions when comparing pooled samples for control vs. fenamiphos with *Meloidogyne incognita* (equal variance *t*-test, two sided; bars show SD).

TABLE S1 | Significant changes observed by sampling time for relative abundance of Bacteria (% of sequences), per treatment.

TABLE S2 | Significant changes observed by sampling time for relative abundance of Bacteria (% of sequences) for all pooled samples and treatments.

TABLE S3 | Significant changes observed for relative abundance of Bacteria (% of sequences) comparing control samples vs. samples treated with fenamiphos and inoculated with RKN.

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Mediation of Potato–Potato Cyst Nematode, *G. rostochiensis* Interaction by Specific Root Exudate Compounds

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Potato (*Solanum tuberosum*) is a widely consumed staple food crop worldwide whose production is threatened by potato cyst nematodes (PCN). To infect a host, PCN eggs first need to be stimulated to hatch by chemical components in the host root exudates, yet it remains unknown how most root exudate components influence PCN behavior. Here, we evaluated the influence of eight compounds identified by LC-QqQ-MS in the root exudate of potato on the hatching response of the PCN, *Globodera rostochiensis* at varying doses. The eight compounds included the amino acids tyrosine, tryptophan and phenylalanine; phytohormones zeatin and methyl dihydrojasmonate; steroidal glycoalkaloids α-solanine and α-chaconine and the steroidal alkaloid solanidine. We additionally tested two other Solanaceae steroidal alkaloids, solasodine and tomatidine, previously identified in the root exudates of tomato, an alternative host for PCN. In dose-response assays with the individual compounds, the known PCN hatching factors α-chaconine and α-solanine stimulated the highest number of eggs to hatch, ∼47 and ∼42%, respectively, whereas the steroidal alkaloids (aglycones), solanidine and solasodine and potato root exudate (PRE) were intermediate, 28% each and 21%, respectively, with tomatidine eliciting the lowest hatching response 13%. However, ∼60% of the hatched juveniles failed to emerge from the cyst, which was compound- and concentration-dependent. The amino acids, phytohormones and the negative control (1% DMSO in water), however, were generally non-stimulatory. The use of steroidal glycoalkaloids and their aglycones in the suicidal hatching of PCN offers promise as an environmentally sustainable approach to manage this pest.

Keywords: ***Globodera*** **spp., hatching factors, potato root exudates, semiochemicals, steroidal glycoalkaloids**

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INTRODUCTION

Potato cyst nematodes (PCN) have been of great economic importance to potato production since their introduction to Europe in the mid 1880's. They originate from the Andean region in South America (Grenier et al., 2010) but have subsequently spread to other potato-growing regions of the world (Hockland et al., 2012). The PCN species *Globodera rostochiensis* and

Globodera pallida are among the most regulated quarantine nematode pests globally, with a potential to cause potato (*Solanum tuberosum*) yield losses of up to 70% (Turner and Subbotin, 2013). In the United Kingdom, PCN alone causes up to 9% potato production losses, corresponding to a market value of around €43 million annually (Sasaki-Crawley, 2013). In the rest of Europe, the market value of losses is approximately seven times higher (€300 million annually) (Moxnes and Hausken, 2007).

Recently, both species of PCN have been detected in Kenya (Mwangi et al., 2015; Mburu et al., 2018), where potato is the second most important food crop (CIP, 2019). Potato, a crop which is grown mainly by smallholder farmers, provides more calories per cultivated area than any other crop and a source of income generation to millions of people (VIB, 2019). However, in recent years, potato production has experienced a 61% decline, even though the potato cultivation area has increased (Food and Agriculture Organization [FAO], 2017). Several factors including the lack of quality seed, poor agronomic practices and PCN and diseases are major contributors to this decline (Kaguongo et al., 2014). Recently, the potential impact of *G. rostochiensis* has been further highlighted, which has now been confirmed to be widespread across Kenya (Mwangi et al., 2015; Mburu et al., 2020) and present within regions in Rwanda (Niragire et al., 2019) and Uganda (Cortada et al., 2020). As such, PCN poses a major threat to potato production on a regional level (Mburu et al., 2018).

Infection by PCN diverts the host plant nutrients to the nematode and causes physical damage to plant tissue as the nematodes migrate through the roots, leading to potential secondary infections (Jones et al., 2013). This effect translates to reduced water and nutrient uptake, resulting in decreased crop yield. To ensure effective penetration of root tissue, the nematodes secrete effectors, comprised of proteins, which can alter the host cell structure. Additionally, they suppress host plant defenses (Haegeman et al., 2012; Yang et al., 2019), increasing the susceptibility of the plant to attack by other pests and pathogens.

Current PCN management strategies include the use of botanicals, synthetic chemical nematicides, biological control agents, genetic resistance, crop rotation, trap crops, fallow rotation, organic soil amendments and/or intercropping. These management strategies have been implemented with varying degrees of success, while PCN remain a persistent and economic threat to the potato industry. Most synthetic chemical nematicides that were previously effective have been withdrawn from use due to their adverse environmental and toxic effects (Khalil, 2013). Despite resistant cultivars having been identified as the most effective method for cyst nematode management, the species-specific resistance response of some of the resistance genes (*R*-genes) to certain nematode species allows other PPN species, when present, to reproduce. Cyst nematodes tend to have a limited host range and require specific chemical cues from host plants to stimulate hatch and for host location. A characteristic feature of cyst nematodes is their ability to endure periods of adverse conditions or the absence of hosts, as a cyst. This diapausing structure limits the effectiveness of crop rotation or fallowing, or even chemical management as the nematodes are inactive and are protected by the cyst structure, making them particularly pervasive pests.

Given the importance of potatoes in East Africa, there is an urgent need to establish PCN management options that are more effective and environmentally sustainable for small holder farmers. Innovative solutions, such as exploiting semiochemical based tools to disrupt the nematode life cycle could prove effective. One promising avenue appears to be the induction of 'suicidal hatch' using naturally occurring phytochemicals (Devine and Jones, 2000, 2001; Devine et al., 2001). The hatching of the infective juveniles (J2s) from encysted eggs is triggered by chemical signals originating from the host plant roots. The hatch is initiated by alteration of the eggshell membrane, which is a calcium-mediated process that involves either hatching factors from the host root exudates binding or displacement of internal calcium ions. Although, some studies have identified compounds in host root exudates, such as the triterpenoid solanoeclepin A that stimulate PCN hatch (Mulder et al., 1996), their practical application for PCN management is yet to be realized. The structural complexity of solanoeclepin A makes it expensive and challenging to synthesize in sufficient amounts for use in the field (Tanino et al., 2011).

Other PCN hatching factors identified from potato root exudates include the steroidal glycoalkaloids (SGA) α-solanine and α-chaconine (Devine et al., 1996). Generally, potato and other Solanaceae plants are known to produce steroidal glycoalkaloids, their aglycones (steroidal alkaloids) and other classes of compounds (Shakya and Navarre, 2008; Distl and Wink, 2009). However, most of these compounds are yet to be screened for PCN hatching response. In the current study, we profile the chemistry of the root exudate of a PCN-susceptible and widely grown potato cultivar, 'Shangi,' in Kenya (Mburu et al., 2020), and screen specific identified compounds for induction of PCN suicide hatch.

MATERIALS AND METHODS

Plants

Seed potatoes of cv. 'Shangi' were planted in 2 L plastic pots (13 cm base diameter, 17 cm top diameter and 15 cm deep) perforated at the base and filled with sterilized sand, autoclaved for 40 min at 121°C (Astell Scientific autoclave, United Kingdom). The plants were grown in a screenhouse at the International Centre of Insect Physiology and Ecology (*icipe*) Duduville Campus, Nairobi, Kenya (1.2219° S, 36.8967° E) at 23 ± 3°C and relative humidity 60–70%. The plants were watered twice a week using a nutrient solution prepared according to Lambert et al. (1992) for 5 weeks before use in the study.

Potato Root Exudates

Potato root exudates of 5 weeks old plants, were collected using the dipping method (Kirwa et al., 2018). Briefly, the plants were gently removed from the soil, the roots washed in tap water to remove sand particles and then rinsed in distilled water. Batches of five plants were combined to constitute one replicate and the roots immersed in 500 ml of distilled water in a 2 L glass beaker to collect the potato root exudates. This was done in three replicates. The beaker was wrapped with aluminum foil

to prevent photodegradation and the setup left for 24 h. The collected exudates were filtered and freeze-dried (SP Scientific, VirTis Advantage) to dryness and weighed. The potato root exudate was kept at −80°C until used for chemical analysis and hatching bioassays.

Nematodes

Potato cyst nematodes *G. rostochiensis* samples were obtained from soil from a freshly harvested farm in Nyandarua County, Kenya (00.78537° S, 036.60429° E). The soil was air-dried and cysts extracted by floatation using a Fenwick can (EPPO, 2013). The cysts were collected on a milk filter paper, dried, hand-picked under a stereomicroscope (LEICA M125, Singapore) and sorted based on color. Freshly formed cysts were used for the hatching experiments, which, under Kenyan conditions appear to be hatching without the need for diapause and which is currently being investigated further.

Chemicals

LC-MS grade methanol (LC-MS LiChrosolv®, Merck (≥99.97%), formic acid (98–100%), water (LC-MS Chromasolv), *trans*-zeatin (≥97%), solasodine (≥95%), tomatidine hydrochloride (≥85%), α-solanine (≥95%), solanidine (≥95%), phenylalanine (≥98%), tyrosine (≥98%), tryptophan (≥95%), methyl dihydrojasmonate (≥96%), and DMSO (≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, United States) and α-chaconine (≥95%) (PhytoLab, Germany).

Chemical Analysis of the Potato Root Exudates

To determine the chemical composition of potato root exudates, 10 mg of the freeze-dried root exudates was dissolved in 1 ml of 30% methanol in ddH_2O. The sample was then vortexed for 10 s, sonicated for 30 min, and centrifuged at 14,000 rpm for 10 min. The supernatant was diluted to 1 mg/ml and transferred into a sample vial and 0.1 μl analyzed using an Ultra Performance Liquid Chromatography coupled to a triple quadrupole tandem mass spectrometry (UPLC-QqQ-MS/MS). Chromatographic separation was performed on a ACQUITY UPLC I-class system (Waters Corp., Milford, 151 MA) fitted with an ACQUITY UPLC BEH C18 column (2.1 mm × 150 mm, 1.7 μm particle size; Waters Corp., Wexford, Ireland), that was heated to 45°C. The autosampler tray was cooled to 5°C. The mobile phase comprised of water acidified with 0.01% formic acid (solvent A) and methanol (solvent B) and followed a gradient system. The gradient system used was 0–2 min, 5% B, 2–4 min, 40% B, 4–7 min, 40% B, 7–8.5 min 60% B, 8.5–10 min 60% B, 10–15 min, 80% B, 15–19 80% B, 19–20.5 min, 100% B, 20.5–23 min, 100% B, 23–24 min 95% B, 24–26 min, 95% B. The flow rate was held constant at 0.2 ml/min.

The UPLC was interfaced with an electrospray ionization (ESI) Waters Xevo TQ-S operated in full scan MS in both positive and negative ionization modes. Data were acquired over the m/z range 100–2,000 with a capillary voltage of 0.5 kV, sampling cone voltage of 30 V, source temperature 150°C and desolvation temperature of 120°C. The nitrogen desolvation flow rate was 600 L/h.

Data was acquired using MassLynx version 4.1 SCN 712 (Waters). Potential assignments of compounds were determined after the generation of the mass spectrum for each peak, establishing the molecular ion peaks using adducts, common fragments, literature and where available, confirmed with authentic samples through co-injections. All the samples were analyzed in triplicate, with each replicate collected from different batches of plants.

Hatching Assays

We tested 10 synthetic standards: the steroidal alkaloids solanidine, solasodine, and tomatidine hydrochloride; steroidal glycoalkaloids α-solanine and α-chaconine; amino acids, tryptophan, tyrosine and phenylalanine; and phytohormones, zeatin and methyl dihydrojasmonate. PCN *in vitro* hatching bioassays were conducted according to the protocol used by Twomey et al. (1995). The standards were diluted to five concentrations: 0.2, 0.4, 0.6, 0.8, and 1 μg/ml from a stock of 1 mg/ml in 10% DMSO. A 200 μl aliquot of each test solution was added to each well of a Linbro® 96 multi-well sterile plate containing five cysts per well, pre-soaked in water for 5 days (Twomey et al., 1995). Each concentration was tested in six replicates. Potato root exudate and 1% DMSO in water were used as the positive and negative control, respectively.

The 96-well plate was covered and placed in the dark at 20 ± 3°C. The emerging J2 were counted weekly and the test solutions replenished following each count. After 5 weeks, the remaining eggs within each cyst were assessed for viability using Nile blue A stain [(5-aminobenzo[a]phenoxazin-9-ylidene)-diethylazanium;sulfate - Sigma-Aldrich, India], which stains dead/non-viable eggs but not live ones (Ogiga and Estey, 1974).

Hatching activity for each experimental treatment was expressed as cumulative percentage (%) of hatched J2 recovered from the wells and the encysted hatched J2 recovered from the cysts. The total number of viable eggs was the sum of hatched J2 plus the viable eggs recovered from the cyst.

Data Analysis

A hatching index (HI) for all tested compounds was calculated according to the formula:

$$HI = (TH - CH)/(TH + CH) \times 100$$

where, TH is the number of J2 hatching stimulated by a test compound and CH is the number of J2 stimulated by the positive control, potato root exudate. A positive value in the hatching index indicates that a higher proportion of the J2 hatched on stimulation by the compounds, compared to the control, whereas a negative value indicates the opposite effect. Plots of hatching index were made separately for the J2 that emerged from the cyst and those that remained encysted. The data on the hatched J2 were analyzed using a generalized linear model with a binomial distribution. Using the control potato root exudate as a reference category, the odds ratios (ORs), a measure of the likelihood that PCN responded to the other treatments instead

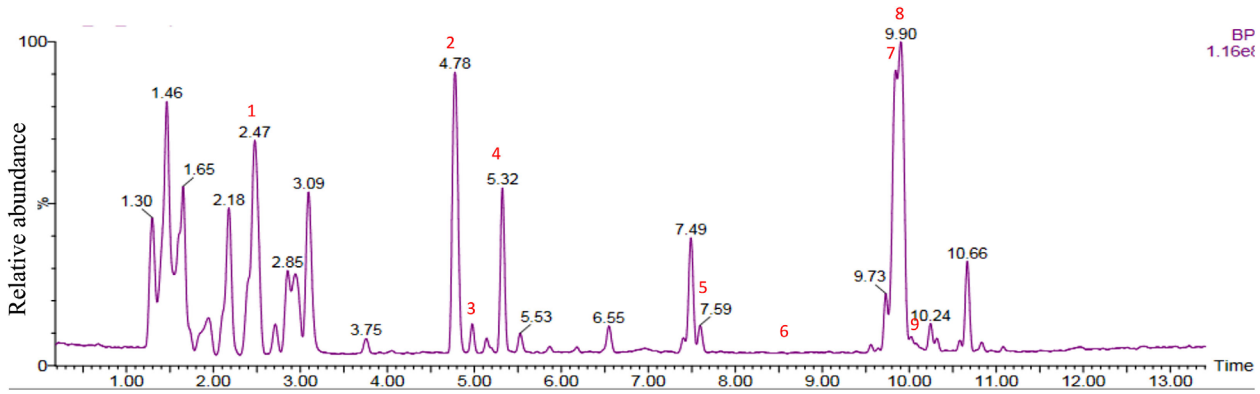


FIGURE 1 | LC-QqQ-MS chromatogram of potato root exudates from 5-week-old plants with identified compounds.

of the control were estimated including Confidence Interval (CI) and corresponding *p*-values. With OR for the control set at 1, values above this indicates better hatching response and values below underperformance of the treatments, relative to the positive control. Differences between the compounds were evaluated using a test of proportions. Significant differences between the emerged/encysted J2s hatching indices and the positive control was determined using one-sample *t*-test and one-sample wilcoxon signed rank test depending on the normality of the data. All the analyses were conducted on R version 3.5.2 using a 5% significance level.

RESULTS

Chemical Analysis of Potato Root Exudates

Chemical analysis of the potato root exudates, by LC-QqQ-MS, identified 9 compounds belonging to four classes; amino acids, phytohormones, triterpenoid and steroidal alkaloids (**Figure 1** and **Table 1**). Additionally, the chemical profile of the root exudate showed several unidentified components (**Figure 1**).

The amino acids identified included tyrosine (1), phenylalanine (2) and tryptophan (3). Phytohormones, zeatin (4) and methyl dihydrojasmonate (5) and a tetranortriterpenoid, solanoeclepin A (6) was also identified. The steroidal glycoalkaloids (SGA) α-chaconine (7) and α-solanine (8) with their aglycone, solanidine (9) were also detected in the potato root exudate. All the compounds were identified in both positive and negative ionization modes with key fragments illustrated in **Table 1**.

Hatching Response of PCN to the Synthetic Compounds

Potato cyst nematode *G. rostochiensis* hatching response over the range of concentrations tested was highest in SGAs α-chaconine (7) and α-solanine (8), ∼47 and ∼42%, respectively, intermediate for the steroidal alkaloids (aglycones), solanidine (9), solasodine [**Figure 2** (10)] and tomatidine [**Figure 2** (11)] and PRE, ∼28, ∼28, ∼13, and 21% respectively, and lowest for the phytohormones zeatin (4) and methyl dihydrojasmonate (5), amino acids tyrosine (1), phenylalanine (2) and tryptophan (3) and the negative control (1% DMSO in water), <10%. For the SGAs, PCN was more sensitive to lower concentrations of α-chaconine (7) at 0.2 μg/ml ($\chi^2 = 10.39$, df = 1, $p < 0.05$) and at 0.4 μg/ml ($\chi^2 = 90.12$, df = 1, $p < 0.001$) than α-solanine (8) (**Figures 3**, **4** and **Table 2**).

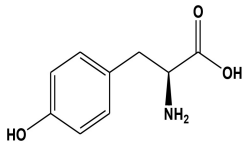
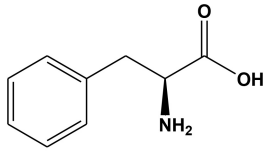
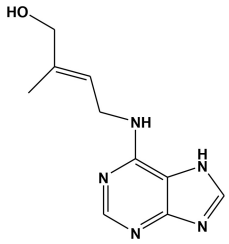
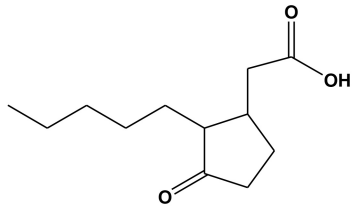
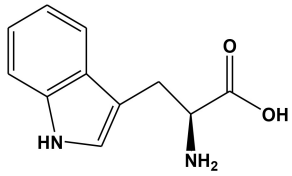
Potato cyst nematodes hatching index for the aglycones varied with the concentration of the compound. Solasodine (10), elicited a higher emerged J2 response across the different concentrations than solanidine (9): at 0.2 μg/ml; ($\chi^2 = 100.25$, df = 1, $p < 0.001$), at 0.4 μg/ml; ($\chi^2 = 32.70$, df = 1, $p < 0.001$), with tomatidine (11) at 0.2 μg/ml; ($\chi^2 = 37.92$, df = 1, $p < 0.001$); and 0.6 μg/ml; ($\chi^2 = 4.96$, df = 1, $p < 0.05$) eliciting the lowest response (**Figure 3**). However, PCN response to the aglycones was dominated by encysted than emerged J2, with comparable responses to solasodine and solanidine (**Figure 4** and **Table 2**).

Of the amino acids, phenylalanine (2) stimulated a higher J2 hatch compared to tyrosine (1) and tryptophan (3) but only for the emerged J2 (**Figure 3** and **Table 2**), whereas there was no detectable stimulation of encysted J2 hatch for all the amino acids. A similar pattern was observed for the phytohormones and the negative control (1% DMSO in water) (**Figures 3**, **4** and **Table 2**).

DISCUSSION

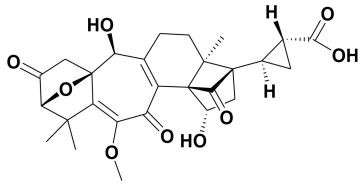
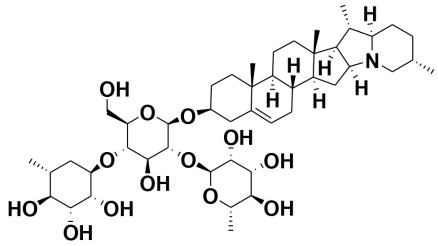
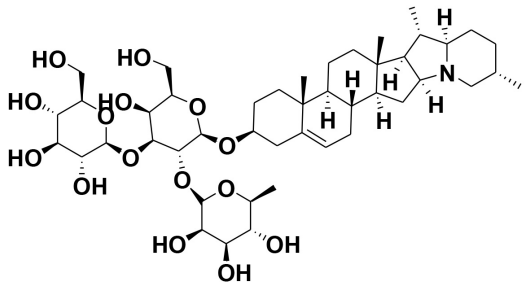
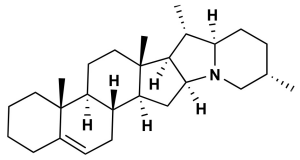
Root exudate is an important factor that influences interactions with organisms in the rhizosphere (Koo et al., 2005; Canarini et al., 2019). It consists of organic compounds that include both primary (e.g., sugars, amino acids, and organic acids) and secondary metabolites (e.g., alkaloids, flavonoids, terpenes, and phenolics) that are passively released into the rhizosphere through the roots of living plants across a concentration gradient (Grayston et al., 1997; Vranová et al., 2013). Root exudates are utilized by soil dwelling microbes for various biological processes and they can also function as semiochemicals (chemical attractants, repellants, hatching factors, and hatching inhibitors), growth inhibitors and growth promoters, among

TABLE 1 | LC-QqQ-MS fragments of identified compounds.

Peak No.	t_R (min)	Compound	Structures	Class of compound	(M + H)	(M − H)	Positive mode Fragmentation	Reference
1	2.41	Tyrosine[b]		Amino acid	182.2	180.1	119.0, 136.0, 147.0, 165.1	El Aribi et al., 2004; Zhang et al., 2019
2	4.76	Phenylalanine[b]		Amino acid	166.1	164.0	103.0, 120.0, 149.1, 131.0	Piraud et al., 2003
3	5.18	Zeatin[b]		Phytohormone	220.3	218.2	202.2, 137.0	Kirwa et al., 2018
4	5.31	Tryptophan[b]		Amino acid	205.2	203.1	188.2, 146.1, 159.2	El Aribi et al., 2004; Zhang et al., 2019
5	7.57	Methyl-dihydrojasmonate[b]		Phytohormone	227.3	225.1	209.2, 167.1	Zeng et al., 2018

(Continued)

TABLE 1 | Continued

Peak No.	t_R (min)	Compound	Structures	Class of compound	(M + H)	(M − H)	Positive mode Fragmentation	Reference
6	8.67	Solanoeclepin A[b]		Tetranortriterpenoid	499.6	497.1	–	Sasaki-Crawley, 2013
7	9.92	α-Chaconine[a]		Steroidal glycoalkaloid	852.6	850.9	706.7, 560.7, 398.6	Stobiecki et al., 2003; Cataldi et al., 2005
8	9.90	α-Solanine[a]		Steroidal glycoalkaloid	868.8	866.9	722.9, 706.7, 560.7, 398.6	Stobiecki et al., 2003; Cataldi et al., 2005
9	10.01	Solanidine[a]		Steroidal alkaloid	398.6		–	Shakya and Navarre, 2008

[a]*Identified by co-injection with authentic samples.*
[b]*Identified by comparison to literature.*

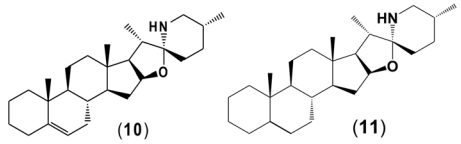


FIGURE 2 | Structures of steroidal alkaloids solasodine (10) and tomatidine (11).

other functions (Walker et al., 2003). Our chemical analysis of potato root exudate identified different classes of compounds, including amino acids, phytohormones, steroidal glycoalkaloids and steroidal alkaloids reflecting the findings of previous studies (Koo et al., 2005; Li et al., 2013; Kirwa et al., 2018), and several unidentified components, whose identities and biological roles would require further research.

In the present study, α-solanine (7) and α-chaconine (8) elicited the highest PCN hatching response, consistent with the previous findings (Devine et al., 1996; Byrne et al., 1998; Devine and Jones, 2000). Structurally, α-solanine and α-chaconine are chemically similar. They bear the same aglycone, solanidine, which is attached to three different sugar moieties in the two different SGAs. For instance, the triose of α-solanine consists of two six-carbon sugars, D-glucose and D-galactose and one five-carbon sugar L-rhamnose. On the other hand, the triose of α-chaconine consists of one six-carbon sugar D-glucose and two five-carbon sugar L-rhamnose (Ghisalberti, 2006). The fact that the two SGAs elicited a similar hatching response pattern, which varied with concentration suggest that the sugar type linked to the aglycone may influence J2 stimulation response. Although the two SGAs are highly oxygenated and polar, the sugar types and their configuration may determine their binding to receptors via hydrogen bonding to stimulate J2 hatch (Torto et al., 2018a). It appears that α-chaconine may have the correct configuration to bind to the receptors to stimulate J2 hatch at lower concentrations, whereas higher concentrations of α-solanine may be needed to saturate and bind to the hatching receptors. Since a sialoglycoprotein binding site for hatching in the eggshell membrane of *G. rostochiensis* has been identified (Atkinson and Taylor, 1983), it would be interesting to identify SGA binding sites to determine the optimal concentrations needed for PCN to discriminate between different SGAs. However, it is interesting that the majority of J2 that hatched in response to the SGAs remained encysted. Several reasons may account for this observation. Firstly, this suggests that additional chemical cues are needed to stimulate optimal emergence (e.g., hatching and post-hatch movement) and to complete the host location process of J2s. Secondly, the SGAs could be binding to the emerged J2 receptors, thereby activating a feedback signal that blocks additional emergence of the encysted J2s. Thirdly, the SGAs could also stimulate a sudden burst in the hatching of J2s, leading to overcrowding and movement through the vulval fenestra of the cyst. Future work should investigate all these possible scenarios. Previously, SGAs have been associated with growth inhibition of various bacterial (Seipke and Loria, 2008) and fungal species (Munafo and Gianfagna, 2011). The fact that we found SGAs in the potato root exudate of plants grown in sterilized autoclaved sand, suggests that the plant may be priming itself against microbial attack. As such, it appears that the PCN has, over time, evolved to 'eavesdrop' into the plant defense priming system to hatch in response to varying concentrations of SGAs, as demonstrated in the present study.

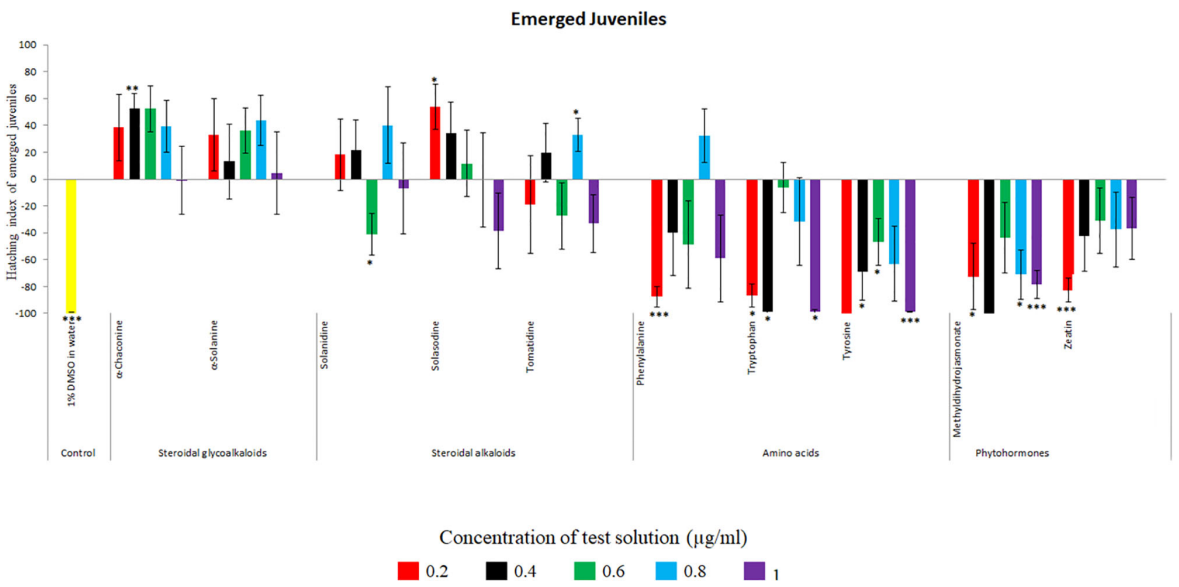


FIGURE 3 | Hatching index of emerged J2s of PCN, *G. rostochiensis* in response to ten compounds compared to the positive control, potato root exudates at five concentrations (µg/ml). Error bars indicate standard error of the mean (SEM). Treatments with an asterisk above the bars indicate a significant difference from the positive control. The level of significance is indicated by * for $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

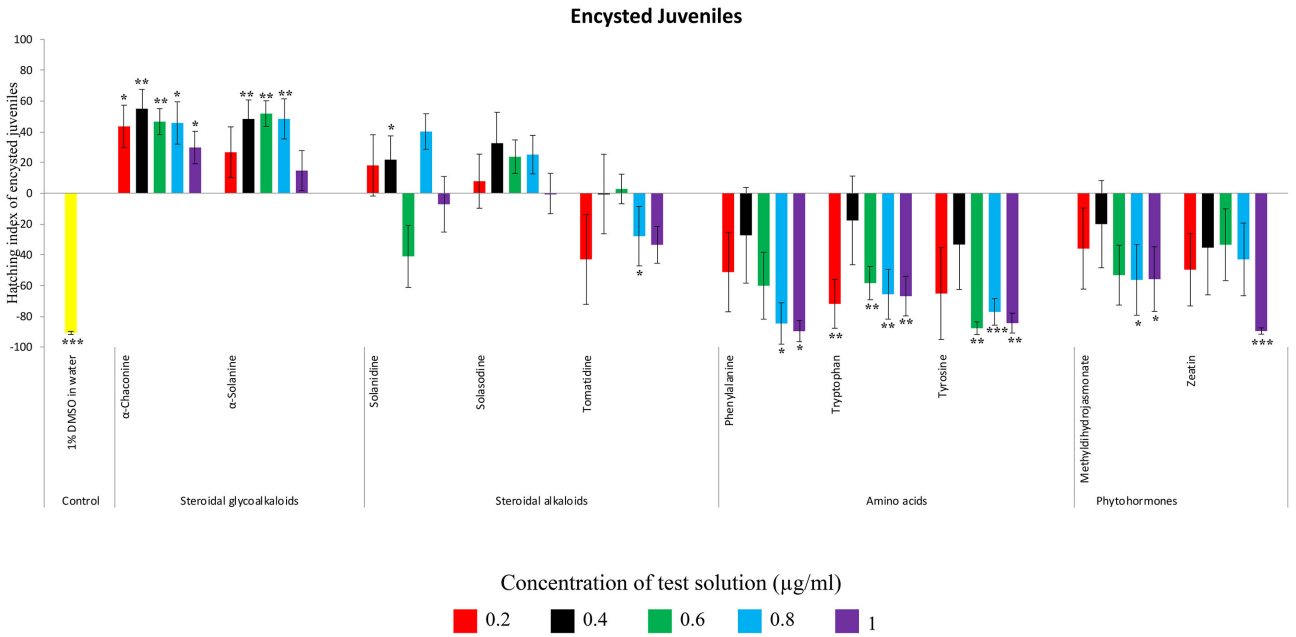


FIGURE 4 | Hatching index of encysted hatched J2s of PCN, *G. rostochiensis* in response to ten compounds compared to the positive control, potato root exudates at five concentrations (µg/ml). Error bars indicate standard error of the mean (SEM). Treatments with an asterisk above the bars indicate a significant difference from the positive control. The level of significance is indicated by * for $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLE 2 | Comparisons of PCN hatching response to 10 compounds relative to potato root exudate (positive control).

Treatment		Concentration (µg/ml)				
		0.2 µg/ml	**0.4 µg/ml**	**0.6 µg/ml**	**0.8 µg/ml**	**1 µg/ml**
α-Solanine	*p*-value	0.108	0.046	0.000	0.001	0.390
	OR (95% CI)	2.14 (0.86–5.61)	2.78 (1.05–8.00)	5.25 (2.62–11.19)	5.15 (2.02–14.29)	1.38 (0.67–2.88)
α-Chaconine	*p*-value	0.002	0.000	0.000	0.013	0.461
	OR (95% CI)	4.19 (1.69–10.90)	5.75 (2.25–16.30)	5.28 (2.59–11.20)	3.44 (1.34–9.57)	1.32 (0.64–2.74)
Solanidine	*p*-value	0.466	0.035	0.497	0.092	0.389
	OR (95% CI)	1.42 (0.55–3.79)	2.93 (1.11–8.44)	1.32 (0.60–2.95)	2.34 (0.89–6.58)	0.74 (0.34–1.60)
Solasodine	*p*-value	0.485	0.028	0.084	0.159	0.444
	OR (95% CI)	1.41 (0.54–3.76)	3.07 (1.17–8.81)	1.95 (0.93–4.25)	2.06 (0.77–5.83)	0.51 (0.22–1.13)
Tomatidine	*p*-value	0.032	0.954	0.808	0.705	0.102
	OR (95% CI)	0.20 (0.03–0.76)	1.03 (0.33–3.26)	0.91 (0.38–2.11)	0.79 (0.25–2.51)	0.36 (0.15–0.84)
Phenylalanine	*p*-value	0.000	0.767	0.010	0.008	0.021
	OR (95% CI)	0.12 (0.03–0.34)	0.79 (0.14–3.86)	0.29 (0.10–0.70)	0.27 (0.09–0.66)	0.09 (0.02–0.27)
Tryptophan	*p*-value	0.000	0.192	0.100	0.003	0.000
	OR (95% CI)	0.06 (0.00–0.23)	0.23 (0.01–1.64)	0.51 (0.22–1.11)	0.23 (0.08–0.57)	0.06 (0.01–0.18)
Tyrosine	*p*-value	0.001	0.195	0.004	0.010	0.000
	OR (95% CI)	0.06 (0.00–0.23)	0.23 (0.01–1.64)	0.13 (0.02–0.43)	0.24 (0.07–0.65)	0.03 (0.00–0.12)
Methyl dihydrojasmonate	*p*-value	0.035	0.006	0.074	0.039	0.000
	OR (95% CI)	0.31 (0.10–0.88)	0.25 (0.09–0.64)	0.42 (0.15–1.05)	0.15 (0.01–0.71)	0.09 (0.02–0.25)
Zeatin	*p*-value	0.007	0.010	0.209	0.619	0.000
	OR (95% CI)	0.15 (0.03–0.52)	0.28 (0.12–0.72)	0.57 (0.23–1.34)	0.76 (0.24–2.27)	0.15 (0.02–0.26)

Values are the p-values, odds ratios (OR) and confidence intervals (CI) from a comparison of mean percentage hatch of 6 replicates from the test solutions and potato root exudate at different concentrations.

Although PCN hatched in response to the steroidal alkaloids (aglycones), it was lower than for the SGAs. This is likely because they are less oxygenated and therefore less polar than the SGAs. As with the SGAs, most of the hatched J2 remained in the cyst, confirming that additional cues are required to stimulate optimal emergence. However, of the aglycones, tomatidine (11) elicited the lowest hatching response. Structurally, tomatidine is less polar than solasodine and solanidine because the latter two bear an olefinic bond in ring B of the molecule. Consequently, future studies should elucidate structure-activity relationships, such as the influence of oxygenation and presence of olefinic bonds in triterpenoids identified in the potato root exudate and the exudates of other host plants in PCN hatching response. A recent study identified solasodine and tomatidine in the root exudate of tomato, an alternative host for PCN, which induced significant stylet thrusting in the plant parasitic nematode, *Meloidogyne incognita,* even though they did not elicit attraction (Kirwa et al., 2018). In another study, pre-exposure of *M. incognita* to the quinolone alkaloids waltherione A and waltherione E, isolated from the aerial part of *Triumfetta grandidens*, significantly inhibited egg hatch (Jang et al., 2015). These findings demonstrate the parsimonious role of specific host root exudate compounds on the behavior of different plant parasitic nematodes- specific SGAs and their aglycones as hatching factors in *G. rostochiensis*, but also serve as egg hatching inhibitors and J2 stylet thrusting stimulants in *M. incognita*. Hence, it would be interesting to further examine these compounds at the same concentrations, either individually or in a blend, to elucidate their full role in the behavior of different species of plant parasitic nematodes.

Of the four classes of compounds detected in potato root exudates, the amino acids and phytohormones had the least effect on egg hatch. Although these compounds are also polar in nature, they are associated with the root exudates of several plants, including tomato (Kirwa et al., 2018); maize (Carvalhais et al., 2011), sorghum and cowpea (Odunfa, 1979), cotton (Sulochana, 1962), rice (Bacilio-Jiménez et al., 2003), and peanut (Li et al., 2013) among others. As such, they may be perceived by the PCN as non-specific chemo-stimulants. However, it is possible that they may contribute as important background signals to the SGAs and their aglycones in the root exudate to stimulate PCN hatching, which should be investigated. Further testing of the naturally occurring concentrations of the SGAs, aglycones, amino acids, phytohormones and unidentified compounds may shed more light on the role of the potato root exudate and its metabolites in PCN hatching.

CONCLUSION

The results of the present study have significant implications for PCN management in East Africa. Since potato production in East Africa is dominated by small holder farmers, who find it difficult to adopt the available PCN management strategies, use of Solanaceae plants such as the indigenous edible African nightshade species *Solanum villosum* and *Solanum scabrum* can be promising trap crops for PCN and in the context of subsistence agriculture in SSA. In field trials, these two species were found to reduce PCN densities by up to 80% after three seasons (Chitambo et al., 2019). We recommend further studies on these trap crops to elucidate the underlying mechanisms as to how they work. A previous study has shown that the African nightshade species *Solanum sarrachoides* contains high levels of SGAs (Jared et al., 2015), which could potentially be used as a trap crop for PCN. Additionally, such plants can also be used as organic amendments, processed plant products or incorporated in a crop rotation system to reduce PCN levels in farmer crop fields (Torto et al., 2018b). Finally, synthetic standards of the SGAs and steroidal alkaloids could be used to stimulate suicidal hatch in PCN infested fields before farmers plant their potato crops.

In summary, the current study has shown that understanding chemo-ecological interactions of PCN with host plants provides opportunities for identifying semiochemicals that can be exploited for their management.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

BT and LC designed the experiments. JO performed the experiments. JO and LC wrote the first draft of the manuscript. DC, BT, AH, MN, LC, and JO edited the manuscript and wrote the final manuscript. BT, LC, and DC contributed with the reagents and laboratory space where the experiments were carried out.

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Biological Suppression of Populations of *Heterodera schachtii* Adapted to Different Host Genotypes of Sugar Beet

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Productivity of sugar beet and brassica vegetable crops is constrained by the nematode *Heterodera schachtii* worldwide. In sugar beet cropping areas of Central Europe and North America, *H. schachtii* is managed by crop rotation, and cultivation of resistant brassica cover crops. The recently released nematode-tolerant sugar beet cultivars suffer less damage than susceptible cultivars at high initial population densities of *H. schachtii*. Many tolerant cultivars allow for less nematode reproduction than susceptible cultivars. Monoculture of susceptible hosts can facilitate the evolution of suppressive soil. Objectives of this study were to determine if susceptible hosts are required for this process, and if monoculture with sugar beet genotypes of different host status (susceptible, resistant, tolerant) impact this capacity. Additionally, we tested if amending soil with the cyst nematode pathogens *Pasteuria nishizawae* or *Hyalorbilia* sp. strain DoUCR50 favored the establishment of soil suppressiveness. In 4-year microplot studies with *H. schachtii* Schach0 or Schach1, one susceptible, one Schach0-resistant, and one tolerant sugar beet genotype were monocultured. In 2010, plots were amended with *P. nishizawae* or DoUCR50, the last being introduced into non-treated soil for Schach0, and into previously biocide-treated soil for Schach1. In 2011, respective Schach0 plots received a second amendment with DoUCR50. Nematode population densities and growth and yield parameters were determined annually. Effects of *P. nishizawae* and DoUCR50 on populations of *H. schachtii* were limited and not consistent. Starting in the second year of the monoculture, eggs of both *H. schachtii* pathotypes became diseased. Up to 90% of the total eggs were encumbered by the third cropping cycle, under the susceptible, resistant, and tolerant cultivar. In all years, the tolerant genotype produced the highest and most stable white sugar yields while yields of the other cultivars slowly improved during the monoculture. Results of this study suggested the presence of egg-infecting factors in this sugar beet monoculture that dramatically increased the proportions of diseased eggs. The tolerant cultivar allowed establishment of soil suppressiveness without the initial yield decline observed when susceptible sugar beet genotypes are grown in monoculture.

Keywords: augmentation, biocontrol, *Heterodera schachtii*, sugar beet cyst nematode, soil suppressiveness

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INTRODUCTION

The soil environment is the place of tri-trophic interactions of the plant, its parasites/pathogens, and potential beneficial organisms/hyperparasites that interfere with activities of the second trophic level. In many cases, these interactions can result in suppression of the detrimental organisms allowing flourishing growth of host plants despite the presence of the parasites. In extreme cases of degraded soils, the balance of parasites and their antagonists is offset, and severe activities of detrimental organisms with resulting disease expression occur (Sikora, 1992; Stirling, 2014). Some suppression of parasitic activity by biological balancing is expected in most soils (Stirling, 1991; Mazzola, 2004; Janvier et al., 2007). The discovery of soils where this antagonistic potential or soil suppressiveness is noticeably high has fueled interest in biological control mechanisms in the soil environment. Several studies have reported such soils, and methods of their detection and description have long been proposed (Weller et al., 2002; Westphal, 2005; Borneman and Becker, 2007).

The currently accepted hypothesis is that monoculture of a susceptible host is mandatory for generating such pathogen-suppressive soils. This train of thought was based on results from early studies on take-all decline soils that established under wheat monoculture (Cook, 1981), and also from investigations of cereal cyst nematode suppressive soils in the United Kingdom (Gair et al., 1969). The recommendation of using a susceptible host seemed supported by the lack of increase of population densities of the antagonists under a resistant cereal crop (Kerry and Andersson, 1983). Monoculture of a susceptible host has repeatedly been viable to generate suppressive soil, presumably when beneficial organisms for induction of this phenomenon were present at the beginning of such strategy (Gair et al., 1969; Baker and Cook, 1974; Westphal and Becker, 1999; Chen, 2007). It appeared that at least one of the target pathogens needed to be present at onset of such trials to result in suppressive soil (Westphal and Xing, 2011).

The practical value of generating suppressive soil by monoculture of susceptible hosts was quickly negated because of the expected yield losses in such strategy that rendered this approach non-viable for commercial producers (Kerry, 1990). The role of resistant cultivars as impacting this balance within this tri-trophic network in suppressive soil has been studied (Westphal and Becker, 2001). In that study, resistant cultivars of *B. vulgaris* or *Raphanus sativus* were able to preserve suppressiveness against the sugar beet cyst nematode while a double crop of the non-host *Triticum aestivum* reduced soil suppressiveness against *Heterodera schachtii* (Westphal and Becker, 2001). It was speculated that some activity of the nematode under the host crop was necessary for maintenance of this beneficial soil function. The hypothesis that tolerant cultivars may aid in the monoculture approach of host plants was formed many years ago but not tested so far.

In sugar beet production, recently cultivars tolerant to *H. schachtii* damage have been released, and quickly have become the preferred sugar beet genotypes in high-production areas. These cultivars can withstand higher population densities of *H. schachtii* than standard susceptible (and sensitive) cultivars. Under high population densities but also under very low population densities, tolerant sugar beet cultivars are able to have high yields, and almost identical to non-tolerant high yielding cultivars under non-infested conditions (Heinrichs, 2013; Kaemmerer et al., 2014). In contrast, resistant cultivars have a lower yield potential than tolerant or susceptible cultivars, especially in the absence or under low population densities of the respective nematode, where they can yield 15% less compared to susceptible cultivars (Schlinker, 2010, 2012; Bürcky, 2013). Tolerant cultivars do permit some reproduction higher than resistant cultivars, but less than susceptible ones (Westphal, 2013; Kaemmerer et al., 2014). Such genotypes potentially offer opportunity to overcome the yield decline in the "establishing phase" of soil suppressiveness.

It was our hypothesis that these tolerant genotypes would not suffer as severe yield losses in the initiating time of the suppressiveness as susceptible lines while permitting significant increases of suppressive principals in the soil. This was challenging the current concept of mandatory susceptible crop monoculture. As representative model organisms for an obligate parasite, *Pasteuria nishizawae* was included in these trials. *Hyalorbilia* aff. *multiguttulata* DoUCR50 (DoUCR50, NCBI GenBank accession number JQ638668), closely related to *Hyalorbilia oviparasitica* (formerly: *Dactylella oviparasitica*) represented highly effective but non-obligate fungal antagonists (Olatinwo et al., 2006b; Baral et al., 2018). The specific objectives of this study with *H. schachtii* pathotypes Schach0 (wild type with no virulence on any resistance sources) and Schach1 (virulent on sugar beet genotypes including those that carry the $Hs1^{pro-1}$ gene for resistance to *H. schachtii*; Müller, 1998) were to determine: (A) if monoculture of sugar beet genotypes of different host suitability to *H. schachtii* (susceptible, resistant, tolerant) had similar effects on the development of soil suppressiveness and (B) if the obligate bacterial hyperparasite *P. nishizawae* or the facultative fungus DoUCR50 reduce nematode population densities and protect yield of susceptible, resistant, and tolerant cultivars.

MATERIALS AND METHODS

A multi-year study was conducted in outside microplots of 1 m^2 surface area containing sandy soil (88.2% sand, 7.4% silt, 4.4% clay, 2.4% O.M., pH 6.9) that had been originally infested with *H. schachtii* pathotype Schach0 (16 microplots) or Schach1 (16 microplots) at Münster, Germany. Plots had been used for crop rotation and nematode management research on *H. schachtii* for multiple years before initiating the current project. For each pathotype, a different experiment was conducted. Each experiment was arranged as a split-plot design with the entire microplots serving as mainplots, further divided into three subplots with a total of four replicates. In April 2010, main plots infested with Schach0 received the following treatments: (i) untreated control (ii) *P. nishizawae* amendment, (iii) DoUCR50 amendment, and (iv) an experimental nematicidal seed treatment (only applied in 2010). Mainplots infested with Schach1 received the following treatments: (i) untreated control,

(ii) *P. nishizawae* amendment, (iii) Dazomet + DoUCR50 amendment, and (iv) Dazomet—DoUCR50 (no amendment). On 7 April 2010, dazomet at 500 kg/ha (tetrahydro-3,5-dimethyl-1,3,5-thiadiazine-2-thione; Basamid, BASF, Germany) was applied to the soil surface and incorporated following general label instructions before covering the plots with 0.04 mm thick black PE tarp (Polydress). This was done to perturb soil microbial communities. These differences in treatment among the two experiments were done because the Schach1 population was expected to be more difficult to suppress on two of the three sugar beet cultivars being used. On 30 April 2010, microbial treatments were applied. Within each mainplot, the three parallel subplots (33 × 100 cm) for one three-plant row each were established in split-plot design. For microbial amendments, a soil core of 19-cm diameter and 10-cm depth was removed from each of the three planting sites per subplot, and the total 8.5 L were transferred into a 20-L bucket. The soil was mixed and amended with either 150 mL DoUCR50 suspension (the equivalent of three 3-week-old 9-cm potato dextrose agar culture plates incubated in the dark at room temperature; approximately 3.5×10^7 CFUs per subplot), or 3.04 g *P. nishizawae* spores (4.25×10^9). DoUCR50 suspension was produced according to the method by Olatinwo et al. (2006b). *P. nishizawae* inoculum was commercially provided (Pasteuria Bioscience, Inc., Alachua, FL, United States). The respective soil mixes were then evenly distributed and replaced into the three planting sites per subplot. Two days after the amendments, 0.3 m × 1.0 m subplots were randomly assigned to *B. vulgaris* L. 'Beretta' (susceptible to *H. schachtii*), 'Sanetta' (resistant to Schach0), or 'Pauletta' (tolerant to *H. schachtii*). At each of the three planting sites per subplot, six seeds of the respective cultivar were hand seeded. For control plots without microbial amendments, the same soil mixing procedures were followed. In 2011, in Schach0-infested plots, plots amended with DoUCR50 received a second amendment (3.5×10^7 CFUs per subplot), whereas plots that were planted to treated sugar beet seeds in 2010 were amended with DoUCR50 (3.5×10^7 CFUs per subplot). Both experiments, Schach0 and Schach1, were cropped in monoculture of sugar beet with the same sugar beet genotypes in each subplot until harvest 2013. During the vegetation period, the patterns of temperature (14.1–14.9°C) and precipitation (290–480 L/m^2) varied between years (**Supplementary Figure S1**). Plots were carefully monitored, and at beginning of water stress, supplement irrigation was administered as needed to sustain unimpeded plant growth.

In both experiments, approximately three weeks after sowing at the cotyledon stage with initial true leaves (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie, BBCH 10; Meier et al., 1993), one seedling per planting site was removed, so that from each replication, three seedlings per cultivar in each treatment were removed for staining. One seedling was maintained per planting site in the plots, the rest was discarded. After staining the roots with acid fuchsin (Byrd et al., 1983), root penetration by second-stage juveniles (J2) was determined. The root lengths of the seedlings were measured with a ruler and penetration was reported as J2 per centimeter of root. Every year prior to sowing and during the growing season, plots were fertilized with varying combinations of NPK (12% N, 5.2% P, 6.6% K), ammonium nitrate (27% N) and thomaskali (3.5% P, 12.5% K) fertilizers to deliver N rates ranging from 40 to 120 kg ha^{-1}, P rates of 120 kg ha^{-1}, and K rates of 170 kg ha^{-1}. Plants were maintained following standard regional cultivation recommendations including watering as needed, and fungicide and insecticide applications. To control insect pests, 0.05% Fenpropathrin (Rody, Sumitomo Chemical Co. Ltd., Japan) was sprayed, and Imidacloprid (Confidor 70 WG, Bayer CropScience, Germany) was applied as soil drench. These insecticides are not known to reduce fungal activities. To control fungal pathogens, 1 L/ha Difenoconazol/Fenpropidin (Spyrale, Syngenta Crop Protection AG, Switzerland) was sprayed, care being taken to avoid run-off into the soil. Plant growth was monitored and the perpendicular diameter of the plant canopy was measured 6–9 weeks after sowing. At harvest, fresh weights of washed sugar beet taproots were determined. Sugar content was determined by standard procedures (Buchholz et al., 1995) and reported as white sugar yield (WSY) per plant.

To determine initial population densities of *H. schachtii* prior to sowing, soil samples of 12 2-cm diameter cores were collected per subplot from the upper 30 cm with a soil corer. No such samples were processed for the experiments in 2013. To determine final nematode population densities at harvest, four 2-cm diameter soil cores were taken from the root zone of each of the three plant sites of one subplot. Twelve soil cores per subplot were separated into 0–30- and 30–60-cm depth samples. Subsamples of 400 g of fresh soil were used for extracting cysts by density centrifugation with $MgSO_4$ (Müller, 1980). Cysts were counted under a binocular before being crushed in a custom-made tissue grinder to release the eggs and juveniles. These nematode stages were suspended in water and counted in duplicate under an inverted transmitted light microscope (63× magnification), using 2 × 1 mL aliquot portions. During counting, eggs and juveniles were classified into healthy (normally developed, intact J2 inside) or diseased (abnormal development or obviously colonized by microbes).

Statistical Analysis

Analysis of variance was carried out using a four-factor split-plot model including experimental year in SAS (version 9.4, SAS Institute, Cary, NC, United States). Analyses were performed for each experiment separately. In the spring, nematode numbers were only from 0 to 30 cm depth (only 2010–2012 data), at harvest, numbers of the depth 0–30 and 30–60 cm were included in the model as a strip factor within the split-plot model. Count data was $\log_{10}$-transformed [$\log_{10}(x + 1)$]. For each year, pooling of error terms was done where possible to simplify models in Proc GLM. Regression analysis was conducted with PROC REG and slope comparison was done in PROC GLM (Rasch and Verdooren, 2004). To compare nematode populations, growth, and yield parameters between years, data were analyzed as repeated measurements using the GLIMMIX procedure of SAS (version 9.4). Specific comparisons were tested and their *P*-values adjusted with the Edwards and Berry's simulation method. Statistical significance was set at $P \leq 0.05$. Results obtained from Proc GLIMMIX are presented as back-transformed lsmeans ± lsmse.

RESULTS

The main factors year and cultivar significantly impacted all parameters measured (**Tables 1**, **2**). Egg population densities and yields changed over the years and the factor year interacted with several other factors as described in more detail below. In both experiments, nematode population densities were stratified by depth, which was also impacted by year (**Tables 1**, **2**). In Schach1, population of healthy eggs at the 0–30 cm soil depth significantly decreased over time (from 8365 eggs/100 g of soil to 266 eggs/100 g of soil), with a decline to less than half in the second year of the experiment. In the last two years, number of healthy eggs were close to the detection level (62–266 eggs/100 g of soil; data not shown). At the 30–60 cm soil depth, population of healthy eggs decreased following the same pattern of eggs at the 0–30 cm depth, but with initial numbers much lower than at the 0–30 cm soil layer (3074 eggs/100 g of soil; data not shown). In both nematode pathotypes, populations of total eggs at both soil depths decreased in the third year of the experiment to then reach final numbers by the end of the experiment that were still lower than the initial numbers in 2010, with the exception of eggs at the 30–60 cm depth in Schach0 with final numbers similar to those in 2010 (data not shown). Because the depth factor only interacted with the year factor, foremost the average of nematode numbers in 0–30 and 30–60 cm depth were used for illustrating data in more detail below.

Population Densities of *Heterodera schachtii* Under the Different Cultivars

In both experiments, differences in the final population densities at harvest were observed among susceptible (Beretta), resistant (Sanetta), and tolerant (Pauletta) cultivars according to their known host status (**Tables 1**, **2**).

In Schach0, final population densities of total eggs decreased over time under Beretta, but remained similar under Sanetta and Pauletta (**Figure 1**). Overall, numbers of total eggs were higher under Beretta than Sanetta and Pauletta, the last two showing similar numbers, except in 2012, where numbers were higher under Pauletta than Sanetta (**Figure 1**). The proportion of healthy eggs decreased per year under the three cultivars after the first year (**Table 1** and **Figure 1**). In Beretta, this reduction in healthy eggs was more pronounced from the first to the second year (**Figure 1**). In 2012 and 2013, healthy eggs under all cultivars were close to detection levels (**Figure 1**). Separate graphs for number of healthy, diseased, and total eggs are provided as **Supplementary Figure S2**.

In Schach1, final populations of total eggs decreased over time similarly under Beretta, Sanetta, and Pauletta (**Figure 2**). In the first two years, numbers of total eggs were higher under Beretta than under Sanetta and Pauletta, the latter with lower numbers than Sanetta (**Figure 2**). In the last two years, numbers of total eggs among Beretta and Sanetta were similar and higher than under Pauletta (**Figure 2**). Under all cultivars, the proportion of healthy to diseased eggs declined over time (**Table 2** and **Figure 2**). The reduction in healthy eggs was most pronounced under the susceptible cultivar Beretta (**Figure 2**). In 2012 and 2013, numbers of healthy eggs were close to the detection limit (**Figure 2**). Separate graphs for number of healthy, diseased, and total eggs are provided as **Supplementary Figure S3**.

Population Densities of *Heterodera schachtii* Under the Different Soil Treatments

When averaging across cultivars and examining treatment effects for Schach1, final populations of total eggs decreased over time under the untreated control, dazomet + DoUCR50, and dazomet – DoUCR50, but remained similar under the treatment with *P. nishizawae* (**Figure 3**). Whereas in the first year, numbers of total eggs were higher after treatment with dazomet – DoUCR50, compared to dazomet + DoUCR50, from the second until the last year, numbers were similar among these two treatments (**Figure 3**). When considering the interaction between cultivar and treatment over time, numbers of total eggs in the first year were higher after treatment with dazomet – DoUCR50 than after inoculation with DoUCR50 but only under Sanetta (data not shown). Under Beretta, total eggs were numerically higher after treatment with dazomet – DoUCR50 than after dazomet + DoUCR50 (data not shown). Numbers of total eggs after amendment with *P. nishizawae* were similar to the untreated control in all years (**Figure 3**). Proportion of healthy eggs diminished over time, being close to the detection level during the last two years (**Figure 3**). Proportions of diseased eggs increased over time (**Figure 3**). Separate graphs for number of healthy, diseased, and total eggs are provided as **Supplementary Figure S4**.

White Sugar Yield of Sugar Beet in Monoculture of Three Different Cultivars

The cultivars had different yield potential in the nematode-infested soil. Average yields increased over years, and did so differently for the different cultivars. Whereas WSY was stable throughout the years for Pauletta, different dynamics with a yearly increase of WSY for Beretta and Sanetta were determined. Treatment effects on yield were somewhat limited.

In Schach0, WSY was stable and higher in Pauletta than in the other two cultivars and did not change over time (**Figure 4**). WSY of Beretta declined slightly from 2010 to 2011 to then increase steadily until the last year of the experiment (**Figure 4**). In 2010, the lowest WSY was found in Sanetta. To the second year, Sanetta WSY increased and remained constant at that level (**Figure 4**). In 2011, Sanetta WSY was higher than in Beretta. In 2012 and 2013, WSY was similar in Beretta and Sanetta (**Figure 4**). Only for Beretta, a significant negative linear regression for WSY and healthy nematode eggs at planting was ascertained [Beretta $f(x) = -25.4395\ x + 158.6060$, $R^2 = 0.64$; $P < 0.01$; **Figure 5**). There was only a non-significant trend line with a negative slop for Sanetta and Pauletta's non-significant trendline (**Figure 5**). There were no significant differences in WSY among the soil treatments (**Table 1**).

In Schach1, the WSY in Pauletta remained constantly high over time above both other cultivars (**Figure 6**). WSY of Beretta and Sanetta were on a similar level in 2011 and 2012 but

TABLE 1 | Summary of ANOVA *P*-values of four-factor effects on early plant growth, early root invasion by nematodes, initial and final egg population densities and health, and sugar yield in an experiment with sugar beet and *Heterodera schachtii* Schach0 at Münster, Germany from 2010 to 2013[a].

			Egg population density						
	Early season		Planting			Harvest			White sugar yield
Factor	Diameter	J2/root	Healthy	Diseased	Total	Healthy	Diseased	Total	
Treatment (TRT)	0.0986	0.0855	0.2211	0.2071	0.1705	0.1745	0.2471	0.1564	0.0337
Cultivar (CV)	<0.0001	<0.0001	0.0010	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
TRT × CV	0.4200	0.1390	0.2473	0.1865	0.1740	0.0425	0.0200	0.0411	0.0010
Depth (D)	–	–	–	–	–	0.0031	0.0017	0.0015	–
Year (Y)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
TRT × Y	0.0990	0.0212	0.4019	0.0002	0.3459	0.1031	0.3284	0.3526	0.9597
CV × Y	0.1443	0.5123	0.2601	0.3274	0.9112	0.4503	0.0110	0.0056	<0.0001
TRT × CV × Y	0.8763	0.4627	0.8806	0.6526	0.8466	0.6873	0.8038	0.6409	0.6440
D × Y	–	–	–	–	–	0.1309	<0.0001	<0.0001	–

[a]*Interactive factors that were non-significant for all parameters in both experiments were not shown.*

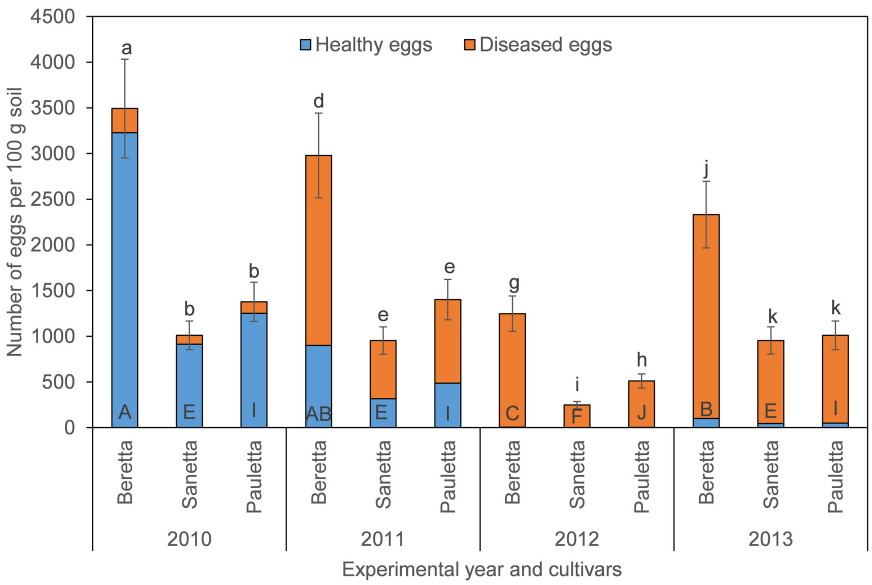

FIGURE 1 | Final population densities of eggs of *Heterodera schachtii* Schach0 in Münster from 2010 to 2013 under sugar beet cropping (Beretta, Sanetta, Pauletta), and averaged across treatment and soil depth. Data are presented as back-transformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–c: 2010; d–f: 2011; g–i: 2012; j–l: 2013. Bars within each cultivar over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: Beretta; E–H: Sanetta; I–L: Pauletta.

increased in Beretta over time while staying on a similar level in Sanetta throughout the monitoring time (**Figure 6**). Only for Beretta, a negative linear regression trend for WSY and healthy nematode eggs at planting was observed [Beretta $f(x) = -10.1271\ x + 102.5928$, $R^2 = 0.20$; $P = 0.0808$; **Figure 7**). There was only a non-significant trend line with a negative slop for Sanetta, and a non-significant level to slightly positive trendline for Pauletta (**Figure 7**). In this experiment, in the first year after Dazomet treatment before inoculation with DoUCR50, higher WSY compared to the non-treated control was found in Beretta, but numbers were similar to those under the Dazomet treatment only (data not shown). Yields did increase in the non-treated control in Beretta after 2010, and then were on a similar level with the other treatments for the remainder of the experiment. After amendment with *P. nishizawae*, WSY in the susceptible cultivar Beretta increased by the last year (data not shown).

Sugar Beet Root Penetration and Impact on Early Canopy Diameter

Overall, the three cultivars followed a linear regression with a positive slope for the relationship of root penetration in relation to at planting-egg population densities in the soil. In the description of the relationship between juvenile root penetration and canopy diameter, a negative relationship was found.

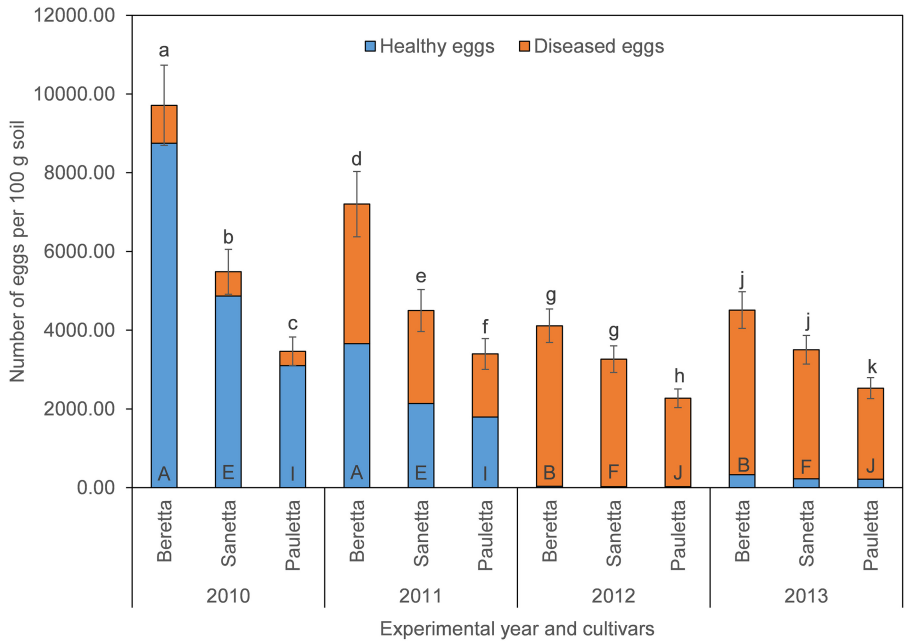

FIGURE 2 | Final population densities of eggs of *Heterodera schachtii* Schach1 in Münster, from 2010 to 2013 under sugar beet cropping (Beretta, Sanetta, Pauletta), and averaged across treatment and soil depth. Data are presented as backtransformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–c: 2010; d–f: 2011; g–i: 2012; j–l: 2013. Bars within each cultivar over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: Beretta; E–H: Sanetta; I–L: Pauletta.

TABLE 2 | Summary of ANOVA *P*-values of four-factor effects on early plant growth, early root invasion by nematodes, initial and final egg population densities and health, and sugar yield in an experiment with sugar beet and *Heterodera schachtii* Schach1 at Münster, Germany, from 2010 to 2013[a].

			Egg population density						
	Early season		Planting			Harvest			White sugar yield
Factor	Diameter	J2/root	Healthy	Diseased	Total	Healthy	Diseased	Total	
Treatment (TRT)	0.5088	0.7216	0.0029	0.0001	<0.0001	0.0041	0.0029	0.0006	0.6305
Cultivar (CV)	<0.0001	<0.0001	0.3166	<0.0001	<0.0001	0.0008	<0.0001	<0.0001	<0.0001
TRT × CV	0.5098	0.2886	0.5375	0.2658	0.0182	0.2966	0.0157	0.0032	0.5657
Depth (D)	–	–	–	–	–	0.0011	<0.0001	<0.0001	–
Year (Y)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0019
TRT × Y	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0554	0.0902	<0.0001	0.2179
CV × Y	0.6028	0.4845	0.1448	0.0869	0.0086	0.3932	0.4490	0.0207	0.0003
TRT × CV × Y	0.0976	0.0426	0.6974	0.6029	0.0835	0.2279	0.4540	0.0001	0.0037
D × Y	–	–	–	–	–	<0.0001	0.0044	<0.0001	–

[a]*Interactive factors that were non-significant for all parameters in both experiments were not shown.*

In Schach0, averaged across treatments and years (2010–2012), the increase of root penetration with increasing numbers of eggs of *H. schachtii* in soil at planting was significant at $P = 0.05$ in Beretta but was not statistically different from the slopes in Sanetta and Pauletta that were only significant at $P = 0.10$ [Beretta $f(x) = 0.8723\ x - 2.1558$; $R^2 = 0.38$; $P = 0.0189$; Sanetta $f(x) = 0.4806\ x - 0.9552$; $R^2 = 0.25$; $P = 0.0563$; Pauletta $f(x) = 0.4057\ x - 0.7972$; $R^2 = 0.22$; $P = 0.0689$]. This interaction followed the linear regression $f(x) = 0.7590\ x - 1.8267$; $R^2 = 0.50$; $P < 0.01$ (**Figure 8**). Juvenile root penetration had only limited effects on the early canopy diameter (log-transformed) with just slight non-significant trends of decreasing diameters with increasing nematode population densities of the roots (data not shown). In all years, root penetration was numerically increased in Beretta than Sanetta and Pauletta (**Supplementary Table S1**), although this increase was not significantly different when considering the interaction between year and cultivar (**Table 1**). Overall, canopy diameter was numerically larger in Pauletta than Beretta and Sanetta (**Supplementary Table S1**) but not significant (**Table 1**).

In Schach1, averaged across treatments and years 2010–2012, root penetration followed the same linear regression for all three

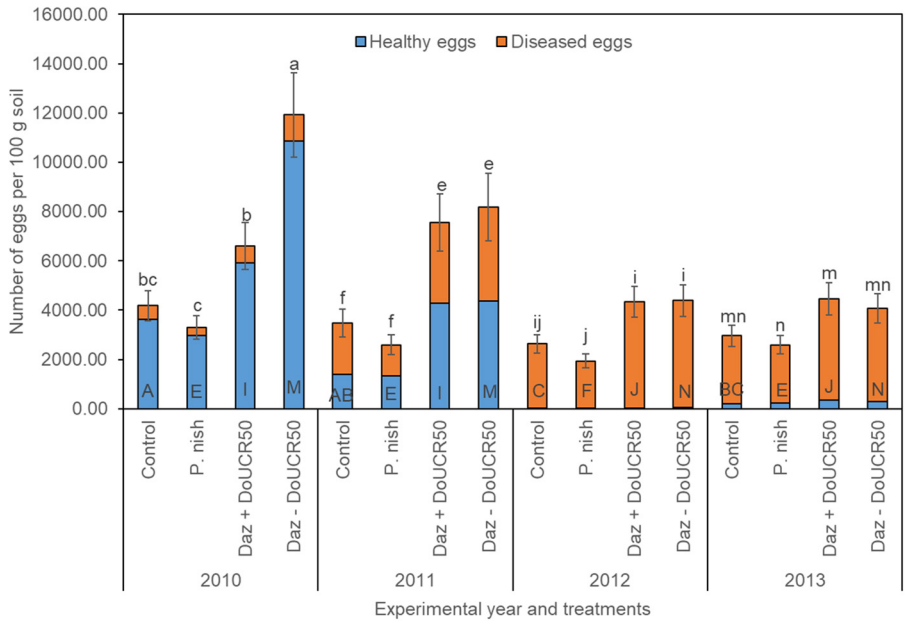

FIGURE 3 | Final population densities of eggs of *Heterodera schachtii* Schach1 in Münster, from 2010 to 2013 under four different treatments averaged across cultivar and soil depth. Data are presented as backtransformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–d: 2010; e–h: 2011; i–l: 2012; m–p: 2013. Bars within each treatment over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: untreated control (Control); E–H: *P. nishizawae* (*P. nish*); I–L: Dazomet + *Hyalorbilia* sp. strain DoUCR50 (Daz + DoUCR50); M–P: Dazomet - *Hyalorbilia* sp. strain DoUCR50 (Daz – DoUCR50).

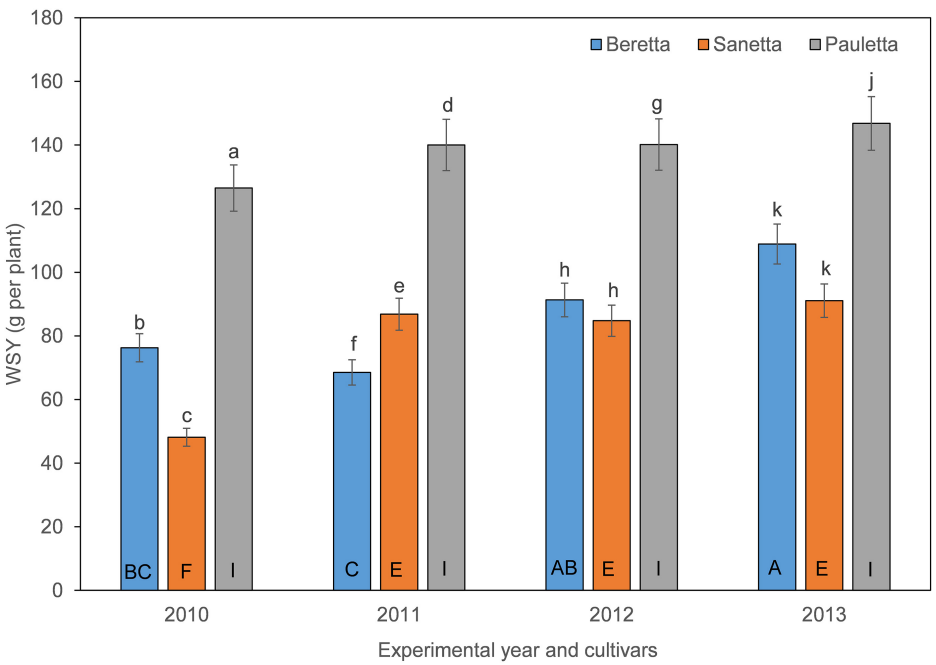

FIGURE 4 | White sugar yield (WSY) of three different cultivars of sugar beet (Beretta, Sanetta, Pauletta) grown in microplots infested with *Heterodera schachtii* Schach0 in Münster from 2010 to 2013. Data are averaged across treatment and are presented as back-transformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–c: 2010; d–f: 2011; g–i: 2012; j–l: 2013. Bars within each cultivar over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: Beretta; E–H: Sanetta; I–L: Pauletta.

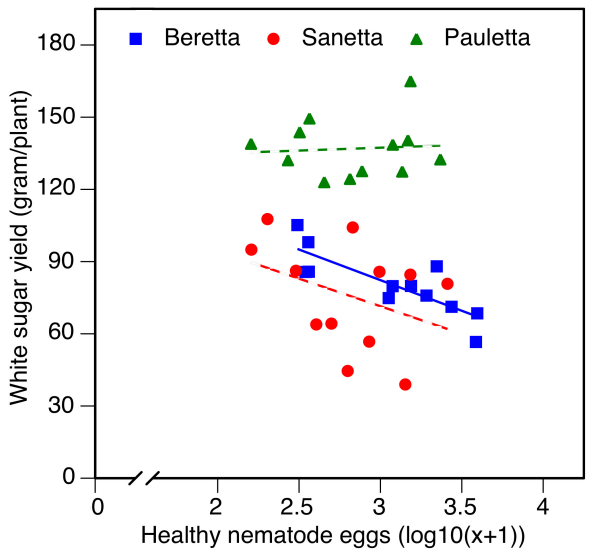

FIGURE 5 | White sugar yield of sugar beet in relation to healthy nematode eggs at planting in *Heterodera schachtii* Schach 0-infested soil in 2010–2012. Data are averaged across treatment. Solid line: Beretta $f(x) = -25.4395\ x + 158.6060$, $R^2 = 0.64$; $P < 0.01$; hatched line: trendlines for Sanetta and Pauletta.

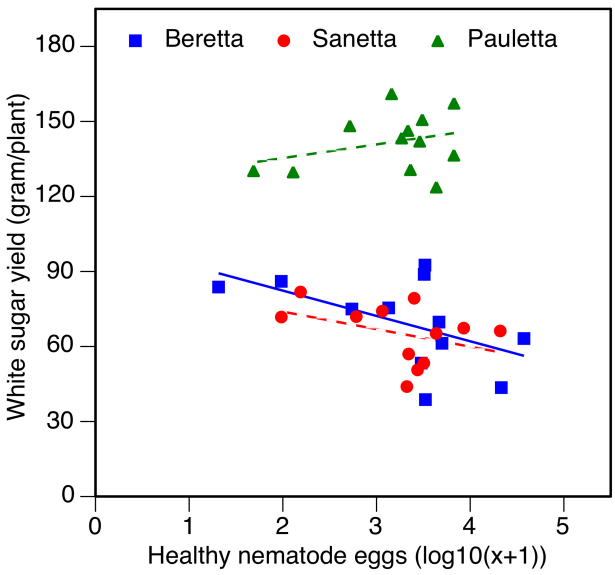

FIGURE 7 | White sugar yield of sugar beet in relation to healthy nematode eggs at planting in *Heterodera schachtii* Schach1-infested soil at Münster in 2010–2012. Data are averaged across treatment. Beretta $f(x) = -10.1271\ x + 102.5928$, $R^2 = 0.20$; $P = 0.0808$.

cultivars $f(x) = 0.8152\ x - 2.3290$; $R^2 = 0.45$; $P < 0.01$ (**Figure 9**). Juvenile root penetration had a strong negative impact on canopy diameter (log-transformed). The mutual linear regression was described by $f(x) = -0.3630\ x + 1.8503$; $R^2 = 0.61$; $P < 0.01$ (data not shown). In all years, root penetration was more numerous in Beretta than Pauletta (**Supplementary Table S1**), although

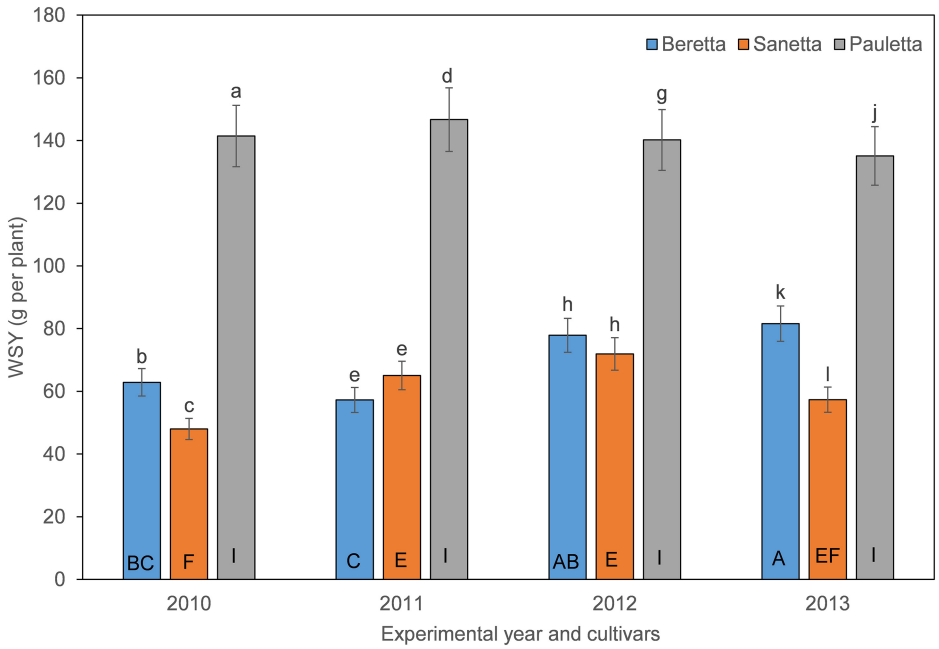

FIGURE 6 | White sugar yield (WSY) of three different cultivars of sugar beet (Beretta, Sanetta, Pauletta) in microplots infested with *Heterodera schachtii* Schach1 in Münster from 2010 to 2013. Data are averaged across treatment and are presented as backtransformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–c: 2010; d–f: 2011; g–i: 2012; j–l: 2013. Bars within each cultivar over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: Beretta; E–H: Sanetta; I–L: Pauletta.

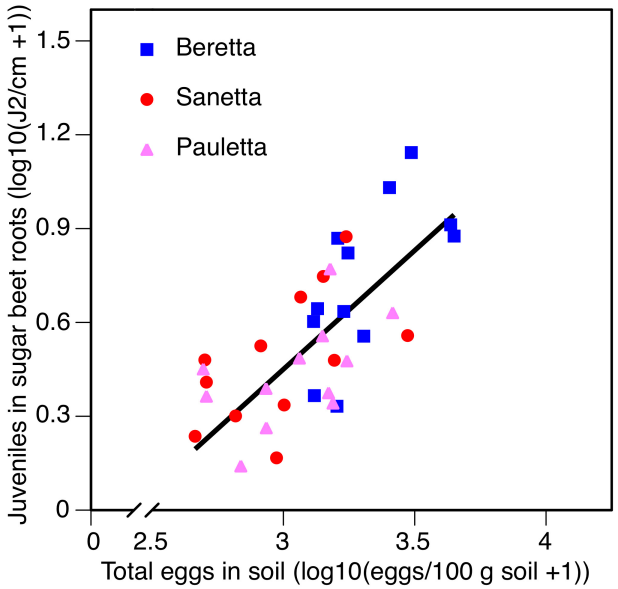

FIGURE 8 | Number of juveniles (J2) in sugar beet roots in three sugar beet cultivars after four soil treatments in relation to total numbers of eggs of *Heterodera schachtii* at planting in Schach0-infested soil at Münster, Germany in 2010–2012.

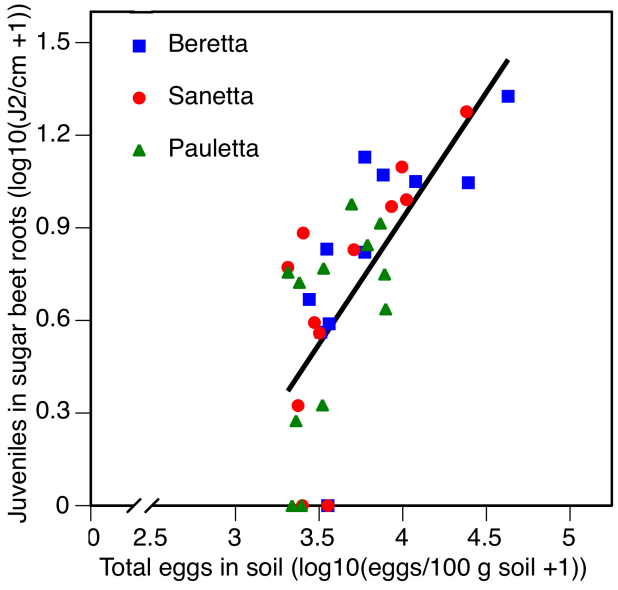

FIGURE 9 | Number of juveniles (J2) in sugar beet roots in three sugar beet cultivars after four soil treatments in relation to total numbers of eggs of *Heterodera scchachtii* at planting in Schach1-infested soil at Münster, Germany in 2010–2012.

these differences were not significantly different at the year and cultivar interaction (**Table 2**). In every year, canopy diameter was numerically larger in Pauletta than Beretta and Sanetta (**Supplementary Table S1**), but not significantly (**Table 2**).

DISCUSSION

In this study, the monoculture of a *H. schachtii*-susceptible, resistant, and tolerant sugar beet cultivar led to severely declining health status of eggs of the nematode independent of the cultivar genotype. As expected, the tolerant cultivar produced the highest yields in plots of both pathotypes. The tolerant cultivar Pauletta maintained high yields throughout the monoculture while egg population health deteriorated. These data support the hypothesis that a tolerant cultivar is useful to protect from yield losses when initiating a host plant monoculture for developing soil suppressiveness. The hypothesis that some nematode activity was important for maintenance of suppressiveness (Westphal and Becker, 2001) and the need for the pathogen to be present (Westphal and Xing, 2011) seemed supported by these findings. The nematode health decline in all plots allowed for some increasing yields in the susceptible and resistant cultivars further illustrating the development of a suppressive soil.

Decline of *H. schachtii* population densities under a sugar beet monoculture was observed by Thielemann and Steudel (1973) in a field at Elsdorf, Germany, and by Heijbroek (1983) in the Netherlands. In the United Kingdom, Crump and Kerry (1987) found that there was little population increase of *H. schachtii* in the untreated soil during a three year-field trial. In our study, after four years of monitoring, the decline of Schach0 egg final populations under the susceptible cultivar (20%) was similar to the decline observed by Thielemann and Steudel (10%) and by Heijbroek (30%). In Schach1 however, eggs final populations under the susceptible cultivar decreased by 70% after four years. On the other hand, diseased eggs presenting fungal hyphae or physiologically disordered content that represented around 10% in 2010, increased by 2013 to >90% in Schach0 and Schach1. This pronounced increase in diseased eggs in both pathotypes suggests the presence of controlling agents in this sugar beet monoculture soil. High numbers of diseased eggs were also observed by Bursnall and Tribe (1974) in *H. schachtii*, by Morgan-Jones et al. (1981) in *H. glycines*, and by Dackman (1990), Eberlein (2017) and Eberlein et al. (2016) in potato cyst nematodes.

Westphal and Becker (2001) found that soil suppressiveness against *H. schachtii* was reduced after a double crop of the non-host *T. aestivum* but preserved after cropping of resistant cultivars of sugar beet or oil radish. Cotton, watermelon, and melon resistant cultivars to their respective Fusarium wilt, some of them grown in monoculture, have been shown to induce soil suppressiveness against the causal agent of this disease (Katan et al., 1983; Hopkins et al., 1987; Sneh et al., 1987). Hopkins et al. (1987) also found that the suppressiveness that developed after monoculture of a resistant watermelon cultivar was effective on all cultivars. It seems that resistant cultivars not only have the ability *per se* to reduce the population densities of a pathogen, but also indirectly by enhancing microbial antagonists that are able to survive under reduced populations of the pathogen, thus inducing the development of soil suppressiveness.

To enhance the potential for establishment success, plots of Schach1 were pretreated with dazomet to remove biological buffering before amending with DoUCR50. Such treatment had previously been useful to disturb microbial populations without

eliminating cyst nematodes from field plots (Xing and Westphal, 2009). The lack of soil receptivity had been expected when introducing non-native organisms to soil, especially in such a different agro-ecological environment considering its' presumed niche in California. Parasitism, competition for nutrients, and microbiostasis can negatively impact the establishment of nematode-parasitic fungi (Cook and Baker, 1983; De Boer et al., 2003; Monfort et al., 2006). In 2010, the Dazomet treatment resulted in a reduction of final populations of total eggs under the resistant cultivar, compared to the corresponding control (dazomet-treated plots without the fungus). Amendment with DoUCR50 reduced *H. schachtii* population densities in the first growing year of application compared to their respective Dazomet control in Schach1. This find confirmed the positive suppressive effects against *H. schachtii* of DoUCR50 in fumigated soil under greenhouse and microplot conditions (Olatinwo et al., 2006a,b). In California, *H. oviparasitica* was considered the major agent in suppressing root-knot nematodes in a peach orchard, and its' close relative DoUCR50 was shown to be one of the most abundant fungi in *H. schachtii* cysts from the suppressive 9E soil (Stirling et al., 1979; Westphal and Becker, 2001; Yin et al., 2003). In the microplots discussed here, this difference was not maintained in the subsequent cropping cycles, suggesting that the suppressiveness due to DoUCR50 did not become continually established in this context. There was no indication for long-term establishment of this fungus within the nematode populations because no additional differences in egg health or population densities compared to other treatments were detected. In its geographical origin, a single application of the fungus led to a stable *H. schachtii* suppressiveness over the entire experimental period (Olatinwo et al., 2006a). That success of DoUCR50 in parasitizing nematodes was associated with its ability to occupy the rhizoplane of host plants (Olatinwo et al., 2006a). Recolonization of the soil by microorganisms after the biocidal treatment may have interfered with the persistence of DoUCR50 although some suppressive capacity of the fungus was found when it was co-inoculated with the nematode (Olatinwo et al., 2006b). Even in its original ecosystem in California, low levels of DoUCR50 in *H. schachtii* cysts from field suppressive soils were found by Yang et al. (2012), suggesting that other microorganisms competed with DoUCR50 in the cysts and eventually replaced this fungus. Also, this fungus was exposed to overwintering conditions of freezing soil temperatures in Germany that it presumably never experiences in its original California niche. The incapacity for microbial establishment after amendment with inocula is not new. For example, populations of rhizobacteria introduced on seed or into the soil to persist in time after a successful establishment, and their eventual decrease, illustrates the great impact of biological buffering (Kluepfel, 1993; Weller and Thomashow, 1994).

The obligate hyperparasite *P. nishizawae* did not establish in these experiments, and had limited measurable initial effects following application. A slight yield improvement over time was only observed for Schach1 in the susceptible cultivar. This bacterium had first been isolated from *Heterodera glycines*, a close relative of *H. schachtii*, but the sensitivity of the nematode and the capacity of the bacterium to complete its lifecycle was not comprehensively studied (Noel et al., 2005). Thus, its efficacy may have been encumbered by its lack of infectivity on *H. schachtii*. The release of the bacterium into a quite different environment than its original niche in the Midwest of the United States may also have influenced its lack of establishment capacity. *P. nishizawae* is endoparasitic, and thus it does not grow outside of the nematode but its spores are subjected to myriads of soil organisms. Chen and Dickson (1998) have suggested the possibility that microfauna feed on spores of *Pasteuria penetrans* in field soil, especially at high spore density. Talavera et al. (2002) suggested that amoebae and rotifers could have fed on spores of the close relative *P. penetrans*. Although watering can have a positive distribution effect on spores of *P. penetrans* (Talavera et al., 2002), their downward dispersal with percolating water resulting from rainwater or irrigation can lead to a depletion of spores in the top 15–20 cm of soil if they are not continuously amplified in that soil layer (Cetintas and Dickson, 2005). Leaching of endospores is also greater in sandy than in clay soils. Under a drip system, 76% of endospores leached 10 cm after 24 h in sand. With increasing clay content fewer endospores leached, since spores got trapped within clay aggregates (Dabire and Mateille, 2004). We did not trace the re-distribution of spores of *P. nishizawae* but 4.4% of clay content at Münster was below the percentages considered to be optimal for biological control with *P. penetrans* (10–30%).

The generally expected parasite–host plant interaction remained in place that initial host plant root penetration was related to number of nematode eggs in the soil. The effects of these numbers on early plant growth were less clearly related than reported for the same sugar beet genotypes when healthy nematode populations were used (Westphal, 2013). In Westphal's studies, early juvenile penetration of sugar beet roots predicted the early canopy expansion. Canopy diameter was also predictive of final yield for the susceptible Beretta. Here, this lack of association of nematode population densities and early plant measure, and then final yield further illustrate the reduction of nematode infectivity and damage potential throughout the monitored growing seasons.

CONCLUSION

A suppressive effect of the monoculture was evident by the rapid and dramatic increase in diseased eggs that constituted over 90% of the total eggs by the third cropping cycle in both pathotypes. Irrespective of the cultivar that was cropped, this pronounced increase in diseased eggs suggests the compatibility of controlling factors with different sugar beet genotypes. Amendments with DoUCR50 had only transient effects on *H. schachtii* population densities or yield while the naturally developing suppressiveness appeared stable and more effective in supporting yields. The obligate bacterium *P. nishizawae* failed to be active under the conditions described here. The use of resistant and especially tolerant cultivars supported the development of suppressiveness, and the use of tolerant cultivars could overcome the yield

penalty of generating soil suppressiveness by monoculture of susceptible hosts.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CE obtained partial funding, designed the experiments, conducted the research, wrote the draft, edited the manuscript, and conducted analysis. HH edited the manuscript. AW designed the project, conducted the research, mentored the first author, conducted part of the analysis, edited the manuscript, and obtained the research effects.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00812/full#supplementary-material

FIGURE S1 | Monthly and vegetation period (April to October) averages and sums **(a)** air temperature, and **(b)** precipitation in Münster, Germany during 2010–2013. Data obtained from WetterKontor GmbH, online: wetterkontor.de; accessed: May 28, 2020.

FIGURE S2 | Final population densities of **(a)** healthy eggs, **(b)** diseased eggs, and **(c)** total eggs of *Heterodera schachtii* Schach0 in Münster from 2010 to 2013 under sugar beet cropping (Beretta, Sanetta, Pauletta), and averaged across treatment and soil depth. Data are presented as back-transformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–c: 2010; d–f: 2011; g–i: 2012; j–l: 2013. Bars within each cultivar over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: Beretta; E–H: Sanetta; I–L: Pauletta.

FIGURE S3 | Final population densities of **(a)** healthy eggs, **(b)** diseased eggs, and **(c)** total eggs of *Heterodera schachtii* Schach1 in Münster, from 2010 to 2013 under sugar beet cropping (Beretta, Sanetta, Pauletta), and averaged across treatment and soil depth. Data are presented as backtransformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–c: 2010; d–f: 2011; g–i: 2012; j–l: 2013. Bars within each cultivar over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: Beretta; E–H: Sanetta; I–L: Pauletta.

FIGURE S4 | Final population densities of **(a)** healthy eggs, **(b)** diseased eggs, and **(c)** total eggs of *Heterodera schachtii* Schach1 in Münster, from 2010 to 2013 under four different treatments averaged across cultivar and soil depth. Data are presented as backtransformed lsmeans ± lsmse. Bars within each year indexed with the same letter were not significantly different at $P = 0.05$; a–d: 2010; e–h: 2011; i–l: 2012; m–p: 2013. Bars within each treatment over year indexed with the same letter were not significantly different at $P = 0.05$; A–D: untreated control (Control); E–H: *P. nishizawae* (*P. nish*); I–L: Dazomet + *Hyalorbilia* sp. strain DoUCR50 (Daz + DoUCR50); M–P: Dazomet – *Hyalorbilia* sp. strain DoUCR50 (Daz – DoUCR50).

TABLE S1 | Lsmeans ± lsmse of early plant growth and early root invasion by nematodes in an experiment with sugar beet and *Heterodera schachtii* Schach0 and Schach1 at Münster, Germany, from 2010 to 2013.

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Infection of *Medicago truncatula* by the Root-Knot Nematode *Meloidogyne javanica* Does Not Require Early Nodulation Genes

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Because of the developmental similarities between root nodules induced by symbiotic rhizobia and root galls formed by parasitic nematodes, we investigated the involvement of nodulation genes in the infection of *Medicago truncatula* by the root knot nematode (RKN), *Meloidogyne javanica*. We found that gall formation, including giant cell formation, pericycle and cortical cell division, as well as egg laying, occurred successfully in the non-nodulating mutants *nfp1* (*nod factor perception1*), *nin1* (*nodule inception1*) and *nsp2* (*nodulation signaling pathway2*) and the cytokinin perception mutant *cre1* (*cytokinin receptor1*). Gall and egg formation were significantly reduced in the ethylene insensitive, hypernodulating mutant *skl* (*sickle*), and to a lesser extent, in the low nodulation, abscisic acid insensitive mutant *latd/nip* (*lateral root-organ defective/numerous infections and polyphenolics*). Despite its supernodulation phenotype, the *sunn4* (*super numeric nodules4*) mutant, which has lost the ability to autoregulate nodule numbers, did not form excessive numbers of galls. Co-inoculation of roots with nematodes and rhizobia significantly reduced nodule numbers compared to rhizobia-only inoculated roots, but only in the hypernodulation mutant *skl*. Thus, this effect is likely to be influenced by ethylene signaling, but is not likely explained by resource competition between galls and nodules. Co-inoculation with rhizobia also reduced gall numbers compared to nematode-only infected roots, but only in the wild type. Therefore, the protective effect of rhizobia on nematode infection does not clearly depend on nodule number or on Nod factor signaling. Our study demonstrates that early nodulation genes that are essential for successful nodule development are not necessary for nematode-induced gall formation, that gall formation is not under autoregulation of nodulation control, and that ethylene signaling plays a positive role in successful RKN parasitism in *M. truncatula*.

Keywords: Autoregulation, abscisic acid, cytokinin, ethylene, nodulation signalling, rhizobia, root gall, root knot nematode

INTRODUCTION

Plant parasitic nematodes are destructive parasites of most crop plants, estimated to cause annual crop losses of more than $1 billion world-wide (Nicol et al., 2011). This is due to the lack of effective and non-toxic control methods, but also because plant parasitic nematodes often cause unspecific above-ground symptoms that are recognized too late after infection (Moens et al., 2009; Nicol et al., 2011). Sedentary endoparasitic nematodes are thought to be the most destructive parasites as they require a living host and initiate the formation of a feeding site inside the host root, in which the female develops and consumes host resources until it lays numerous eggs that later hatch to infect new plants (Jones et al., 2013).

Root knot nematodes (RKNs) are one kind of sedentary endoparasite that cause world-wide crop losses and target hundreds of crop species. The agriculturally most relevant species include *Meloidogyne incognita*, *M. hapla*, *M. javanica, M. arenaria,* and *M. graminicola* (Moens et al., 2009; Atkinson et al., 2012). The reason for their relative lack of host specificity is unknown, but is at least partly due to the multitude of nematode effectors the nematodes inject into the host plant to produce a feeding site (Williamson and Gleason, 2003; Bird, 2004). RKNs hatch from eggs laid in a gelatinous egg mass on the root surface by mature female nematodes. The juveniles rapidly hatch and develop into the infective second stage juveniles (J2). J2s are attracted to host roots, although it is not clear which root exudates are the essential chemoattractants. Some root exudates and volatiles, including phenolic acids and flavonoids, have been shown to alter nematode movement, and some mediate repulsion of nematodes from the root (Chin et al., 2018; Sikder and Vestergård, 2020). The plant hormone ethylene is also implicated in regulating the attractiveness of the root to parasitic nematodes. Several studies found that plant mutants defective in ethylene signaling are less attractive to root knot nematodes, while ethylene overproduction reduces their attraction (*e.g.* Fudali et al., 2013; Čepulyte et al., 2018).

J2s enter the host root just behind the tip and travel intercellularly to the root tip, where they reverse direction and travel into the vascular cylinder of the root (Jones, 1981). Within 24 h, the nematode typically starts to initiate a feeding site by injecting a multitude of effectors into vascular parenchyma cells (Mejias et al., 2019). Often, several cells are targeted by one female (Bird, 1959). Some of the effectors play a role in controlling host defense responses (*e.g.* Sato et al., 2019), while others control the development of the feeding site (Davis et al., 2008; Viera and Gleason, 2019). The target cells undergo endoreduplication, which leads to the formation of multinucleate 'giant cells' (Goverse et al., 2000; Favery et al., 2002). The expansion of the giant cells inside the vascular cylinder is accompanied by multiple divisions of pericycle and cortical cells in the surrounding cell layers, which leads to the formation of a root gall, in which the female develops (Jones, 1981).

The formation of root galls shows some developmental similarities to the formation of root nodules in legumes (Mathesius, 2003; Bird, 2004). Nodulation is initiated by Nod factors produced by symbiotic bacteria called rhizobia. Nodule development in legumes starts with cell cycle activation and cell division in the pericycle and cortex of the host root (Xiao et al., 2014) and leads to the formation of differentiated nodules, in which rhizobia fix atmospheric nitrogen (Oldroyd, 2013). However, giant cell formation is unique to gall formation. Nodule formation has been studied in detail in several legumes, and many of the genes required for the infection of rhizobia and the development of nodules have been characterized (Roy et al., 2020). The symbiosis starts with the exudation of specific root flavonoids by the host, which activates the synthesis of nodulation (Nod) genes in compatible rhizobia (Peters et al., 1986). Nod gene activation results in the synthesis of Nod factors, which are perceived by specific receptors on the root surface, encoded by LysM receptor kinases (Radutoiu et al., 2003). In the model legume *Medicago truncatula*, these are encoded by *NFP1* and *2* (*NOD FACTOR PERCEPTION1/2*) (Amor et al., 2003; Arrighi et al., 2006). This activates a signaling cascade that involves calcium spiking and the activation of transcription factors inside the infected root hair that are necessary for successful nodulation. Essential genes for nodulation include the transcription factors *NIN* (*NODULE INCEPTION;* Vernié et al., 2015) as well as *NSP1* and *2* (*NODULATION SIGNALING PATHWAY1/2;* Kaló et al., 2005), which activate downstream genes, including the gene encoding the cytokinin receptor *CRE1* (Gonzalez-Rizzo et al., 2006; Plet et al., 2011). Mutation of *NFP1/2*, *NIN1* or *NFP1/2* completely abolishes nodulation, while mutation of *CRE1* significantly reduces nodule numbers, but does not completely prevent the formation of nodules (Murray et al., 2007; Plet et al., 2011). Infection and nodule number are further under negative control of ethylene signaling (Larrainzar et al., 2015; Guinel, 2015). The ethylene insensitive mutant *skl* (*sickle*; Penmetsa and Cook, 1997), which encodes the ethylene signaling protein EIN2 (Penmetsa et al., 2008), is characterized by both hyperinfection by rhizobia as well as hypernodulation. Interestingly, the *skl* mutant is also hyperinfected by other organisms including the pathogenic fungus *Rhizoctonia solani*, the oomycete pathogen *Phytophthora medicaginis* as well as symbiotic mycorrhizal fungi (Penmetsa et al., 2008). The likely explanation for those phenotypes is that ethylene plays an important role in plant defense (Broekgaarden et al., 2015).

Another plant hormone that interferes with nodule development is abscisic acid (ABA), which inhibits Nod factor signaling, including calcium spiking responses that occur following Nod factor perception, as well as the induction of several early nodulation genes (Ding et al., 2008). Nodule numbers, as well as lateral root numbers, are significantly reduced in the ABA-insensitive *latd/nip* (*lateral root organ defective/numerous infections and polyphenolics*) mutant of *M. truncatula* (Bright et al., 2005; Liang et al., 2007). However, it was also found that LATD/NIP encodes a nitrate transporter and thus the exact function of LATD/NIP in nodulation remains unclear (Yendrek et al., 2010). Nevertheless, the mutant shows generally defective root organ numbers and root apical meristem

defects and is thus an interesting mutant to study during the formation of nematode galls, which also involve the initiation of cell divisions, similar to other root organs.

Both nodules and galls develop into nutrient sinks that consume host resources to some extent (*e.g.* Carneiro et al., 1999; Voisin et al., 2003). Legumes have evolved an autoregulation of nodulation mechanism that limits nodule numbers to levels adjusted to the nitrogen need of the plant. This mechanism involves the synthesis of small peptides of the CLE (CLAVATA3/ESR-RELATED) family following Nod factor perception and *NIN* activation (Ferguson et al., 2019). The CLE peptide CLE12 is further arabinosylated by RDN (ROOT DETERMINED NODULATION; Kassaw et al., 2017) and travels to the shoot, where it is perceived by a receptor kinase named SUNN (SUPER NUMERIC NODULES) in *M. truncatula* (Schnabel et al., 2005). This leads to the generation of one or more inhibitory signals travelling to the root to inhibit further nodule formation. These signals include changes in auxin transport in *M. truncatula* (van Noorden et al., 2006), altered cytokinin transport in *Lotus japonicus* (Sasaki et al., 2014) and shoot-to-root transport of a microRNA (miR2111) in *L. japonicus* (Tsikou et al., 2018).

So far, it is not clear whether genes involved in nodule development are also required for gall formation and *vice versa*. Some of the genes involved in gall formation are also activated during nodule formation, including genes playing a role in meristem activation (*PHAN* and *KNOX*), the early nodulin *ENOD40* and the cell cycle regulator *CCS52* (Koltai et al., 2001). The dividing cells of nodules and galls also show similarities in auxin responses and in the activation of flavonoids, which may play a role in the control of auxin transport or defense during the interactions (Hutangura et al., 1999; Mathesius, 2003). However, while flavonoids are essential for nodule development through their action on Nod gene activation and auxin transport (Wasson et al., 2006), flavonoid-deficient transgenics roots of *M. truncatula* still form galls, although somewhat smaller (Wasson et al., 2009). A transcriptome comparison of genes expressed in nematode-induced galls and the nodule zone II, which is the zone actively infected by rhizobia, showed a significant overlap in the expression of many genes in *M. truncatula* infected by its symbiont *Sinorhizobium meliloti* or the RKN *M. incognita* (Damiani et al., 2012). In the model legume *L. japonicus*, it was observed that nematodes cause similar root hair deformations, accompanied by cytoskeletal changes, as rhizobia, and it was hypothesized that nematodes make Nod factor-like molecular termed Nem-factors, although their existence has so far not been confirmed (Weerasinghe et al., 2005). In addition, the *L. japonicus* Nod factor perception mutants *nfr1* and *nfr5* showed reduced gall numbers after infection with *M. incognita*, suggesting a possible overlap in the signal transduction required for both nodules and root galls (Weerasinghe et al., 2005). In addition, the *L. japonicus* supernodulation mutant *har1* was found to form twice the number of galls, although not in an expanded root zone as typically found for nodulation (Lohar and Bird, 2003). However, so far little progress has been made in the elucidation of common gene necessary for both interactions.

Here, we examined a number of nodulation mutants of the model legume, *M. truncatula*, which are either defective in nodule initiation or nodule number control. We show that none of the genes analyzed that are essential for nodulation are necessary for gall or giant cell formation, although some mutations did affect the total number of galls and eggs produced by the female infecting nematodes. We also investigated the interaction of rhizobia with RKN on the same plants, as in the field, legumes are often co-infected by both organisms. We found that in wild type plants, rhizobia inhibited gall formation, but that this relationship was altered in some of the nodulation mutants.

METHODS

Plant Preparation and Growth Conditions

Wild type (WT) *Medicago truncatula* Gaertn. cv. Jemalong A17 seeds were purchased from the South Australian Research and Development Institute (SARDI), Adelaide, Australia. Nodulation mutants, *nfp1-1, nsp2-2,* and *nin1* were received from Giles Oldroyd (Cambridge University), *cre1* from Florian Frugier (Université Paris-Saclay), *latd/nip* from Jeanne Harris (University of Vermont), *skl* from Douglas Cook (University of California Davis), *and sunn4* from Julia Frugoli (Clemson University).

Seeds were lightly scarified using fine sand paper and surface-sterilized in 6% (w/v) sodium hypochlorite for 10 min. Seeds were then rinsed five times with sterile MilliQ water, spread on a 10% (w/v) water agar in a 15 cm diameter round plate (Corning, USA) and stratified in the dark at 4°C for two days. Germination was initiated by inverting and incubating the plates at 25°C for 16 h. Seedlings with ~0.5–1 cm radicle length were transferred onto round 15 cm diameter plates containing Fåhraeus medium (Fåhraeus, 1957) enriched with 1.5 mM potassium nitrate (hereafter called 'enriched Fåhraeus medium'), which supported both nodulation and RKN infection. Seedlings of the *skl* mutant were transferred onto 245 mm × 245 mm square petri dishes (Corning, USA) containing enriched Fåhraeus medium because of their longer root length. The bottom half of the plates was covered with black paper and plates placed in a semi-vertical position in a plastic tray. Plants were maintained in a controlled temperature room at 25°C with 16 h light and 8 h dark cycle with 150 μE light intensity.

Meloidogyne javanica Preparation

The *Meloidogyne javanica* population was isolated from field samples collected in Kiola, New South Wales, Australia. These were identified morphologically through perineal pattern observation and genetically based on PCR with primers specific to *M. javanica* (Zijlstra et al., 2000). Axenic cultures of *M. javanica* were amplified and maintained on *M. truncatula* A17 in enriched Fåhraeus medium. Egg masses were collected from infected *M. truncatula* A17 roots using a pair of forceps and surface-sterilized with 0.06% (v/v) sodium hypochlorite for 4 min with intermittent shaking. The eggs were centrifuged at 1,467 g for 5 min and were rinsed thoroughly with 1 ml sterile

MilliQ water three times. This was followed by sterilization with a cocktail of 60 mg/µl penicillin, 250 mg/µl streptomycin, 20 mg/µl kanamycin, and 10 mg/µl amphotericin B in 1 ml solution for 4 h with 15 rpm vertical rotation. Thereafter, the eggs were centrifuged and the rinsing step was repeated. The solution containing the eggs was pipetted as 300 µl droplets on a sterile 8 cm diameter round petri dish (Corning, USA), and eggs were hatched by incubating at 25°C for one week. Hatched J2s were collected from the droplets and were counted in three aliquots of 5 µl. Ten to 15 J2s were inoculated per root (unless otherwise specified) by directly pipetting the J2s onto the root tip.

Rhizobia Preparation

Sinorhizobium meliloti strain 1021 was grown overnight on Bergersen's Modified Medium (BMM) (Rolfe et al., 1980) in the dark at 28°C. A colony of bacteria was transferred to a 15-ml sterile Falcon tube containing 9 ml of liquid BMM and incubated at 28°C overnight on an orbital shaker. The optical density of the resulting suspension was adjusted to 0.1 (at 600 nm) with sterile water before pipetting onto plant roots.

Plant–Nematode Interaction Studies

J2s were inoculated on the root tips of one-week-old seedlings. At 35 days post inoculation (d.p.i.), the number of galls was counted using a stereomicroscope (Nikon SMZ745, Nikon, Japan). Five galls from each genotype were embedded in 3% (w/v) agarose and cross-sectioned at 110 µM thickness on a Vibratome 1000 plus (Vibratome Company, USA). Sections were viewed under a Leica DMLB microscope (Leica, Germany) under bright field illumination with a mounted CCD camera (RT Slider, USA). The remaining galls were used to harvest egg masses for nematode egg counts. The gelatinous matrix of egg masses was dissolved in 0.6% (w/v) sodium hypochlorite and processed as mentioned above. Eggs were counted one week after harvesting, and counts were based on three aliquots of 10 µl from the original suspension of 1 ml using a microscope (Leica Laborlux 11, Leica, Germany). Unhatched eggs and hatched J2s were counted for each aliquot to calculate total offspring and hatching rate (% of hatched J2s/total number of eggs).

Plant–Nematode–Rhizobia Interaction Studies

A multi-factor experiment was designed to assess the phenotype of the selected *M. truncatula* nodulation mutants to *S. meliloti* and *M. javanica* infection. The two organisms were inoculated alone or in combination to establish whether these interact *via* the plant, potentially changing the phenotype.

Wildtype A17 and nodulation mutants growing on enriched Fåhraeus medium in petri dishes were each inoculated with 5 µl suspension of *S. meliloti* at OD = 0.1 (R treatment), 5 µl nematode suspension containing five *M. javanica* J2 (N treatment) or with both the rhizobia and the nematodes, inoculated immediately after one another (NR treatment). Inoculation was done by pipetting the suspensions directly onto the root tip. Each treatment was replicated 25 times.

After three weeks, numbers of galls and nodules of each replicate were recorded. Five plants of each treatment were further processed for visualization of nematodes in root tissue to ascertain whether genotypes differed in being able to be infected. For this, nematodes in root tissue were stained red through an adaptation of the Acid Fuchsin staining protocol (Byrd et al., 1983), adjusted for very fine roots. Briefly, whole roots were collected from the plates, transferred to 1.5 ml microtubes, and completely covered with a boiling solution of Acid Fuchsin stain. The stain solution was prepared by adding 10.5 ml of the stain stock solution (1.75 g Acid Fuchsin, 125 ml acetic acid, 375 ml distilled water) to 315 ml distilled water. The microtubes were left to cool overnight and roots were then rinsed in tap water to remove the stain and kept in glycerol:lactic acid (1:1). Nematodes inside roots were observed using a stereomicroscope, and their developmental stage was ascertained according to Triantaphyllou and Hirschmann (1960). Numbers of fully-developed females, or J2 through to J4 nematodes were recorded for each observed root.

Statistical Analysis

Data were tested for normal distribution and homogeneity of variances prior to ANOVA. One-factor designs were analyzed by one-way ANOVA and pairwise comparisons were done by the *post-hoc* Tukey test. Assays with combined rhizobia and nematode inoculations were analyzed by two-way ANOVA, and significant differences caused by main effects or their interactions compared by the Bonferroni *post-hoc* test. All analyses were performed in GraphPad Prism 5.0 (GraphPad Software, San Diego, California).

RESULTS

RKN Infection in *M. truncatula* Nodulation Mutants

First, we tested the ability of a number of nodulation mutants to establish feeding sites (galls) following infection by *M. javanica*. All nodulation mutants, including the non-nodulating mutants *nfp1-1*, *nin1,* and *nsp2-2*, the low nodulation mutants *cre1* and *latd/nip*, the hypernodulating mutant *skl,* and the supernodulating mutant *sunn4* successfully formed galls (**Figures 1** and **2A**). Gall morphology was similar across the different mutants, and all genotypes supported the formation of giant cells and of pericycle and cortical cell divisions surrounding the giant cells (**Figure 1**). Despite their ability to successfully form galls, the genotypes differed quantitatively in their ability to host the RKNs through the formation of galls and the production of eggs (**Figure 2**). None of the non-nodulating mutants were compromised in their ability to form galls (**Figure 2A**). The lowest number of galls was formed in the hypernodulating *skl* mutant (**Figure 2A**), and this was even lower after normalizing gall numbers by root weight (**Figure 2B**).

The supernodulating mutant *sunn4* did not form significantly increased numbers of galls, suggesting that gall formation is not under autoregulation control in *M. truncatula*. To confirm that this result was not due to a limitation in the number of infecting J2, we conducted an additional experiment with WT and *sunn4*

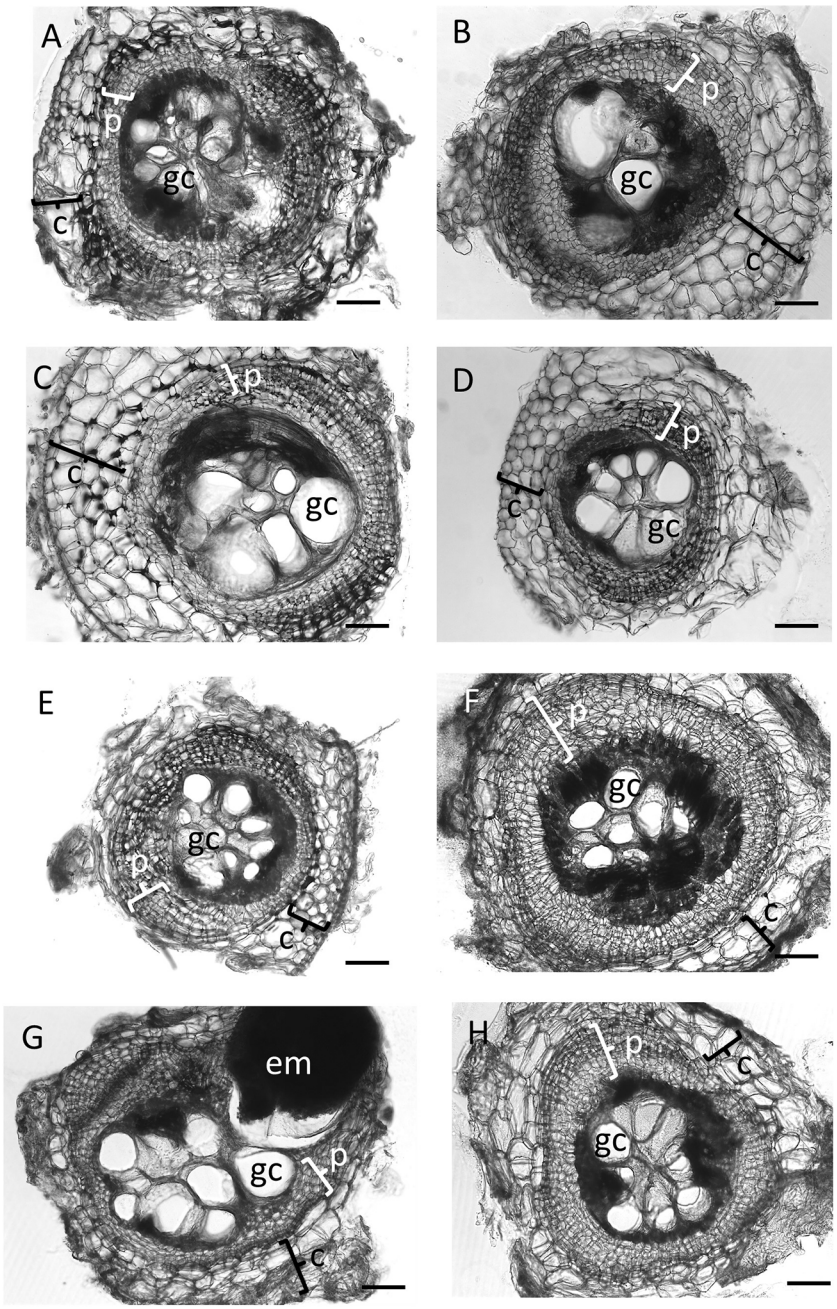

FIGURE 1 | Cross sections of galls of wild-type *Medicago truncatula* A17 and nodulation mutants at 35 days post inoculation with *Meloidogyne javanica.* All photos were taken at the same magnification and the scale bar represents 200 µm. In total, five galls were sectioned from every genotype, and similar results were found for all five galls; thus these examples are representative of the replicates. **(A)** Gall section of *M. truncatula* A17, **(B)** *nfp1*, **(C)** *nin1*, **(D)** *nsp2-2,* **(E)** *cre1*, **(F)** *latd/nip*, **(G)** *skl* **(H)** *sunn4* mutants. Typical features of a gall are labeled as follows: c, cortex, occasionally showing divisions; em, egg mass; gc, giant cell (only one giant cell is labeled per section); p, pericycle, showing multiple layers following division.

mutant plants, in which we increased the number of J2s to up to 50 nematodes per root. While both WT and *sunn4* mutants showed increased numbers of infecting nematodes and galls with increased numbers of inoculated J2s, the *sunn4* mutant still did not form more galls than the WT, in fact, gall numbers were lower than WT across inoculum levels (**Figure S1**). Gall numbers reached an average maximum of around five galls per root even with inoculation of 50 J2s, indicating that not all inoculated nematodes infect or form galls. Overall, gall numbers in the *sunn4* mutant roots varied somewhat between experiments relative to WT roots, but were never significantly higher than in WT roots in any of the experiments.

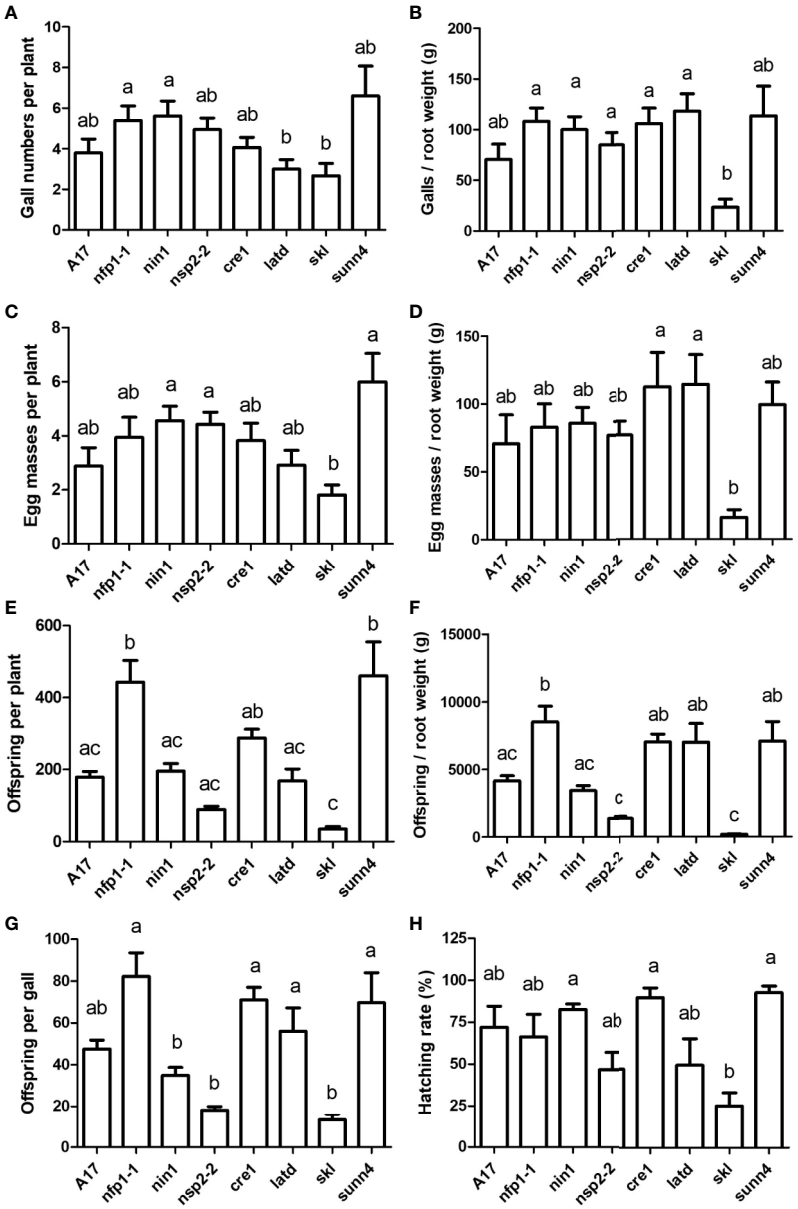

FIGURE 2 | Nematode infection phenotypes of *Medicago truncatula* wild type and nodulation mutants. J2s of *Meloidogyne javanica* grown initially on WT plants were hatched and inoculated onto roots of different *M. truncatula* genotypes. All phenotypes were recorded at 35 days post inoculation. **(A)** Numbers of galls per plant. **(B)** Number of galls normalized against root weight. **(C)** Number of egg masses per plant. **(D)** Number of egg masses normalized against root weight. **(E)** Total offspring per plant (sum of unhatched eggs and hatched J2s after one week of incubating the harvested egg masses). **(F)** Total offspring normalized against root weight. **(G)** Total offspring per gall. **(H)** Hatching rate calculated as the numbed of hatched J2s/total number of eggs and J2s after one week of incubating the harvested egg masses. N = 17–25 plants for each genotype, with n = 5 for *sunn4*. Graphs show means and standard errors. Means with different letters differ significantly from each other with $p < 0.05$ (one-way ANOVA with Tukey's post-test).

Galls of all genotypes also supported the formation of egg masses. Egg mass numbers per plant followed a similar pattern to gall number per plant, as most galls formed one egg mass. Again, the *skl* mutant showed significantly reduced eggs masses per root and egg masses per root weight compared to other genotypes (**Figures 2C, D**). Because egg masses contain variable number of eggs, we quantified the number of offspring (total of eggs and hatched J2s at one week after harvesting egg masses) and found significant differences between genotypes, with the *skl* mutant again showing the least number of offspring, while the *nfp1-1* and the *sunn4* mutants produced the highest numbers of offspring per plant and the *nfp1-1*, cre1, *latd/nip* and *sunn4* mutants supporting the highest number of offspring per root weight (**Figures 2E, F**). Similar figures were recorded when

considering eggs produced per gall (**Figure 2G**). The percentage of eggs that resulted in hatched J2s were again the lowest for the *skl* mutant (**Figure 2H**).

To test whether the hatched J2s were infective, we inoculated the hatched J2s originating from each genotype onto a new generation of WT roots. There were no significant differences between egg origin from different genotypes in the number of galls per plant formed in the subsequent generation (**Figure 3A**). The number of egg masses per plant was higher when J2s originated from the *latd/nip* mutant, but this was only significant in comparison with the *skl* and *sunn4* mutants (**Figure 3B**). Overall results confirmed that eggs produced by nematodes on all genotypes were viable.

Co-Inoculation of Roots With *M. javanica* and *S. meliloti*

As legumes are typically infected by nodule-forming rhizobia in the field, we tested the interaction of nematodes and rhizobia on the same root system (**Figure S2**). Concurrently, controls with inoculation of roots with rhizobia only or nematodes only were set up. We harvested these plants at three weeks post inoculation, which was late enough to ensure galls and nodules were clearly visible, but early enough to avoid large nutritional effects due to nitrogen fixation, which would be low at this early stage. Co-inoculation of wild type plants with RKN and rhizobia together significantly reduced the number of galls compared to RKN-only infected roots (**Figure 4A**). A much smaller, and not statistically significant drop was recorded in the *nfp1*, *nsp2-2*, *cre1*, *latd/nip*, *skl* and *sunn4* mutants (**Figure 4A**). We also examined the total number of RKN that had infected the root because not all nematodes that infect also succeed in causing gall formation

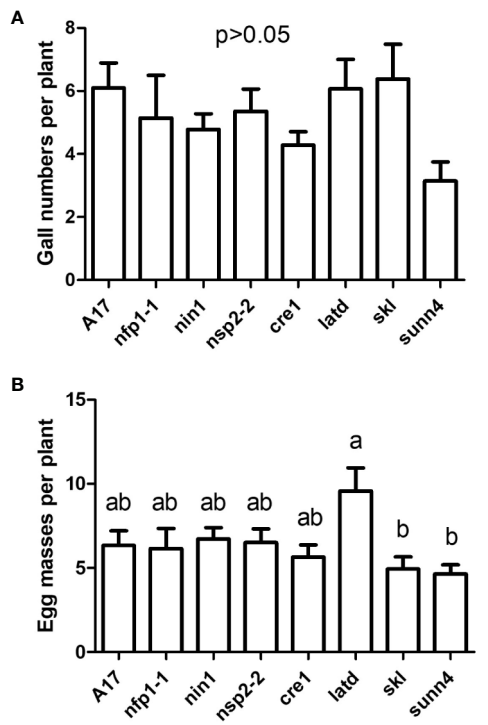

FIGURE 3 | Nematode infection phenotypes of *Medicago truncatula* wild type roots infected with J2s hatched from eggs harvested from different nodulation mutants. **(A)** Number of galls per plant. No significant differences were found between egg origin from different genotypes. **(B)** Number of egg masses per plant. N = 14–21 plants for each genotype. Graphs show means and standard errors. Means with different letters differ significantly from each other with $p < 0.05$ (one-way ANOVA with Tukey's post-test).

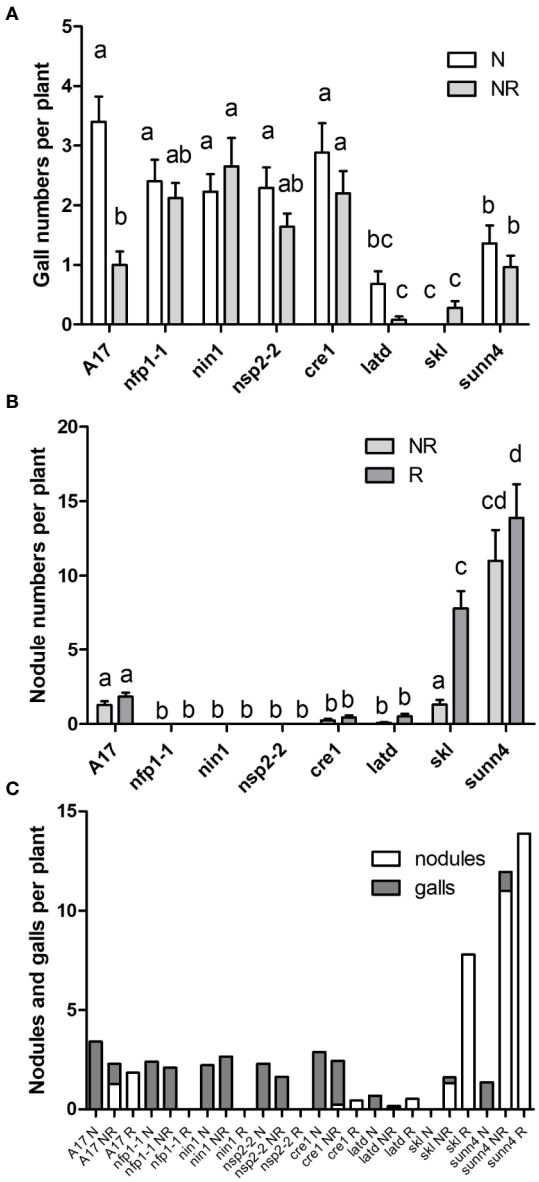

FIGURE 4 | Gall and nodule formation in *M. truncatula* WT and nodulation mutants. Plants were either infected by nematodes only (N), by rhizobia only (R), or by rhizobia and nematodes at the same time (NR). Phenotypes were recorded 3 weeks after inoculation. **(A)** Number of galls per plant. **(B)** Number of nodules per plant. **(C)** Total number of galls and nodules. N = 25 plants for each genotype. Graphs show means and standard errors. Means with different letters differ significantly from each other with $p < 0.05$ (two-way ANOVA with Bonferroni post-test).

(**Figure S3**). We did not find any evidence that RKNs were present in roots with low gall formation, *e.g.* in the *skl* mutant (**Figure 5**), suggesting that reduced gall formation in the *skl* mutant is due to reduced numbers of nematodes entering the root, or remaining in the root after initial infection without being able to initiate a gall.

Co-inoculation of wild type plants with RKN and rhizobia together did not significantly reduce the numbers of nodules in WT plants compared to rhizobia-only inoculated roots (**Figure 4B**). Nodule numbers were very low and did not significantly change in the *cre1* or *latd/nip* mutants in co-inoculated plants. In contrast, in the hypernodulating *skl* mutant, co-inoculation with nematodes reduced the numbers of nodules significantly compared to rhizobia-only inoculated roots, with a lesser, but not statistically significant, drop in nodule numbers in the *sunn4* mutant (**Figure 4B**). The total number of galls and nodules in the different genotypes is shown in **Figure 4C**. This figure shows that there was no clear relationship between the ability of genotypes to host either nodules or galls.

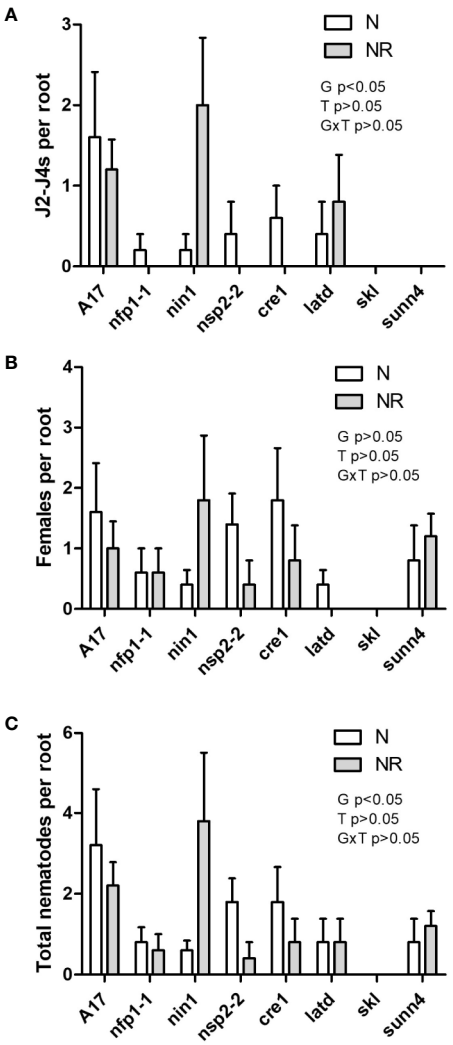

FIGURE 5 | Quantification of nematodes localized inside *M. truncatula* roots. Roots were stained with Acid Fuchsin and both juvenile and adult stage *M. javanica* were counted in each root (examples of these stages are shown in **Figure S3**). **(A)** Number of J2 to J4 stage nematodes. **(B)** Number of adult stage nematodes. **(C)** Total number of juveniles and adults per root. N = 5. Graphs show means and standard errors. Significant effects of genotype (G), treatment (T), or their interaction (G × T) are shown in each panel but as interactions were not significant, no pairwise comparisons could be made (two-way ANOVA with Bonferroni post-test).

DISCUSSION

Our aim was to characterize the interaction of RKN with a number of *M. truncatula* mutants defective in different stages of nodule formation. Firstly, we found that all nodulation mutants were able to form galls with well-formed giant cells, and that females in galls formed on all the nodulation mutants produced viable eggs. This shows that none of these nodulation genes are essential for successful feeding site formation by RKN. Our results did not support previous findings in *L. japonicus* showing that the non-nodulation mutants *nfr1* and *nfr5*, defective in Nod factor perception, showed reduced numbers of galls and mature females in *L. japonicus* roots infected with *M. incognita* (Weerasinghe et al., 2005). It is possible that the different species of plants and nematode species or the growth conditions used could explain this difference, but both studies agreed with the observation that Nod factor perception and early signal transduction genes were not absolutely required for gall formation.

As some of the early nodulation genes are required for rhizobial infection, and the infection mechanisms of rhizobia (*via* infection threads through root hairs; Murray, 2011) and RKN (intercellularly between cells; Jones, 1981) are quite different, the finding that early nodulation genes are not essential for nematode infection might not be surprising. On the other hand, early nodulation genes also control immune responses, and quantitative differences in infection of nodulation mutants with pathogens have been observed previously. For example, the *nfp1* mutant is more susceptible to infection by the oomycete *Phytophthora palmivora* (Rey et al., 2015), the oomycete *Aphanomyces euteiches* and the pathogenic fungus *Colletotrichum trifolii* (Rey et al., 2013). In contrast, the *latd* mutant was less susceptible to *P. palmivora*, while the *nin1* and *nsp2* mutants were similarly infected as the WT (Rey et al., 2015). The NFP receptor perceives *N*-acetylglucosamine derivatives like Nod factors. The presence of related signals (Nem factors) by RKN has been hypothesized because of the presence of *NodC* genes in RKN genomes and because RKNs have been observed to cause root hair curling, similar to the effect of rhizobial Nod factors (Weerasinghe et al., 2005); however Nem factors have so far not been identified.

While Nod factor perception is necessary for rhizobial infection and nodule development, legumes can activate the nodule development program in the absence of rhizobia and without Nod factor perception through spontaneous nodule formation, for example in alfalfa (Joshi et al., 1991). Ectopic overexpression of *NIN* (Vernié et al., 2015) or activation of the cytokinin signaling pathway (Tirichine et al., 2007; Heckmann

et al., 2011; Gauthier-Coles et al., 2019) also induces nodule initiation by activating cortical cell divisions, suggesting that the nodule development program does not require Nod factor perception but does require cytokinin signaling. If gall formation requires similar signaling, the formation of pericycle and cortical cell divisions as part of the gall development program would therefore have been expected to require Nod factor signaling through NIN and CRE1, as well as NSP2 (which acts downstream of CRE1), but not NFP1 (Madsen et al., 2010). It is possible that nematodes directly target cytokinin signaling in the root to establish feeding sites, as RKN can synthesize cytokinins (de Meutter et al., 2003), although this might not be sufficient for maintaining sustained cell divisions.

While early nodulation genes were not essential, the nodulation mutants did differ quantitatively in their ability to host galls and eggs produced by infecting females. The one mutant that showed repeatable and significant differences compared to other genotypes was the *skl* mutant, which formed significantly fewer galls, hosted fewer nematodes inside roots, with females producing fewer eggs with a lower hatching rate. Our observations in *M. truncatula* were consistent across two different experiments and were found in the absence and presence of rhizobia. This result was surprising because the *skl* mutant is hyperinfected by both rhizobia as well as symbiotic mycorrhizal fungi and root pathogens, including *Phytophthora* and *Rhizoctonia* (Penmetsa et al., 2008), and this is thought to be a result of reduced defense responses in ethylene-insensitive plants (Broekgaarden et al., 2015; Berrabah et al., 2018). When RKNs infect host roots, host defense responses are initially observed, although there is also evidence that the infective females inject effectors into target cells that prevent defense responses at the stage of giant cell initiation (Goto et al., 2013; Ji et al., 2013; Goverse and Smant, 2014). In tomato roots, a comparative transcriptomics study showed that ethylene-related responses were more strongly induced in resistant tomato genotypes in response to *M. incognita* than in a susceptible genotype, suggesting a role for ethylene in RKN defense (Shukla et al., 2018).

Ethylene is also required for induction of some of the systemic plant defense responses activated by jasmonic acid, and application of the ethylene releasing compound ethephon to leaves of rice was shown to reduce infections of rice roots with *M. graminicola* (Nahar et al., 2011). To what extent lack of ethylene signaling regulates the local defense responses to nematodes is less well studied, but our results agree with observations that *M. javanica* induced ethylene production in tomato roots and more strongly so in susceptible cultivars, and that application of ethylene inhibitors reduced infection, suggesting that ethylene is required for successful RKN infection (Glazer et al., 1983; Glazer et al., 1985). Ethylene has also been implicated at an earlier stage of the interaction by affecting chemotaxis of nematodes towards the root. Ethylene-insensitive mutants of Arabidopsis and tomato were previously shown to attract more nematodes (Fudali et al., 2013). However, we suggest that this is unlikely to be a factor influencing our results because nematodes were inoculated directly at the root tip and thus did not have to move towards the root. In addition, if the *SKL* mutation acted similarly to other ethylene signaling defects in other species, the *skl* mutant should have attracted more, rather than fewer RKNs. In future work, it would be interesting to compare RKN infections in a number of ethylene-related mutants that vary in the parts of the ethylene pathway that is defective.

While lack of ethylene signaling was expected to enhance nematode infection, we expected that lack of cytokinin signaling would reduce gall formation. Cytokinin is critical for the control of cell division and the cytokinin response gene *ARR5* was induced during the early stages of giant cell formation and in surrounding dividing cells of *L. japonicus* and tomato roots infected by RKN (Lohar et al., 2004). In Arabidopsis, cytokinin synthesis genes and cytokinin receptors were also activated during gall formation (Dowd et al., 2017) and the Arabidopsis cytokinin perception (double) mutants *ahk2/3, 2/4* and *3/4* were characterized by reduced numbers of galls (Dowd et al., 2017). The *M. truncatula CRE1* gene is a homolog of *AtAHK4* (Gonzalez-Rizzo et al., 2006). It is likely that the normal gall formation in the *cre1* mutant was observed because other Medicago cytokinin receptors were able to compensate for the lack of *CRE1*. Partial redundancy of cytokinin receptors has also been observed during nodulation (Held et al., 2014).

The results observed in the *latd/nip* mutant varied slightly between experiments. While the first experiment did not show significant reductions of gall numbers in the *latd/nip* mutant (**Figure 2**), reduced galls and infecting RKNs were observed in the second experiment (**Figures 4** and **5**). It is possible that this was due to different harvest times and number of nematodes used for infection. The *latd/nip* mutation leads to ABA insensitivity, although this is likely not the only reason for its meristem defect because LATD/NIP encodes a nitrate transporter. In rice and tomato, addition of ABA increased root susceptibility to RKN, but the detailed role of ABA in plant-RKN interactions will need to be investigated in more detail in the future.

Despite its supernodulation phenotype, the autoregulation mutant *sunn4* did not show increased numbers of galls, while increased nodule numbers in the *sunn4* mutant were observed under the same conditions, as expected (Schnabel et al., 2005). This suggests that signals that trigger autoregulation of nodulation are not activated by *M. javanica* and agrees with the lack of requirement for Nod factor signaling genes during RKN infection. In *M. truncatula*, Nod factor signaling through NIN, NSP2 and CRE1 is required for activation of autoregulation (Mortier et al., 2010; Mortier et al., 2012), but the *nin1, nsp2-2* and *cre1* mutants were not defective in gall formation in our study, indicating that this signaling pathway is not activated by RKN. Our finding was in contrast to the observation in *L. japonicus* that the supernodulating mutant *har1*, defective in the ortholog of SUNN, formed higher number of galls (Lohar and Bird, 2003). It is possible that the different plant species behave somewhat differently, especially because the *nfp* mutants of *L. japonicus* also showed reduced numbers of galls (Lohar and Bird, 2003).

A further question of our study was to explore the interaction of rhizobia and nematodes in the same root system. A previous study found a correlation between the ability of different ecotypes

of *M. truncatula* to form galls and nodules (Wood et al., 2018). This suggests common genetic regulation of both interactions, although the molecular basis for this is not understood. However, both organisms also affect each other indirectly through their effects on the host or each other. Rhizobia as well as a number of other soil bacteria often contribute to preventing plant infection by parasitic nematodes, and this can involve changes in plant (systemic) defenses, root exudates that affect both organisms, while some bacteria and fungi can also directly kill or trap nematodes (Topalović et al., 2020). Thus, we were interested to see what effect rhizobia had on nematode gall formation and if this was controlled by any of the selected nodulation genes.

We found that the number of galls formed were influenced by concurrent inoculation with rhizobia, however, this was only significant in WT plants. The observed drop in gall numbers in rhizobia-coinoculated roots could be due to changes in defense, exudation or other responses in the root induced by rhizobia (Zamioudis and Pieterse, 2012; Topalović et al., 2020), but it could also be due to resource competition of the established organs, as both nodules and galls act as nutrient sinks in the root (Carneiro et al., 1999; Voisin et al., 2003). While resource competition between nodules and galls could account for the drop in galls in WT roots, this drop was not seen in other genotypes that nodulated even better, e.g. the *skl* and *sunn4* mutants. The second explanation is the possible alteration of defense-related responses in rhizobia-inoculated roots. However, this is difficult to explain in view of the inconsistent responses in the different mutants, *i.e.* both hyper- and non-nodulating mutants lacked a drop in gall numbers in the presence of rhizobia. Our experiments were additionally complicated by the fact that plants grown on agar plates typically do not nodulate as well as soil-grown plants, and this involves the induction of ethylene in the plate (Smith and Long, 1998), which is likely to affect gall numbers as well. Therefore, the interpretations of our results need to be viewed with some caution and will require further studies in soil-grown plants. Moreover, the nodulation mutants, in particular *cre1*, *latd/nip*, *skl* and *sunn4* have other root architecture phenotypes, which could influence the results indirectly (Penmetsa and Cook, 1997; Bright et al., 2005; Schnabel et al., 2005; Desbrosses and Stougaard, 2011; Plet et al., 2011). For example, differences in speed of root growth, number of lateral roots (providing entry points for nematodes) and differential C allocation to roots, galls and nodules could all indirectly influence the RKN infection phenotypes analyzed here. In addition, the interaction between gall and nodule numbers could be influenced by the differences in the size of the root system of different mutants. For example, the root system of supernodulation mutants is typically much smaller than in WT plants, and this changes with nodulation status (*e.g.* Wopereis et al., 2000; Schnabel et al., 2005; Goh et al., 2019). Not all of these phenotypes were captured in our experiments. Thus, a systematic study of changes to the whole root architecture in these mutants in the presence and absence of different parasites and symbionts would be fruitful, especially in soil and field-grown plants.

Co-inoculation of RKNs and rhizobia also resulted in changes to nodule numbers, although the only significant reductions in nodule numbers were seen in the hyper-nodulating *skl* mutant. This suggests that, even though RKN induced very low numbers of galls in the *skl* mutant, the RKN must have induced responses in the plants that interfered with nodulation. The *skl* mutant is thought to generally show reduced ethylene-mediated defense responses (Penmetsa and Cook, 1997; Penmetsa et al., 2008), but it is possible that this is compensated for by activation of defense responses that are not mediated by ethylene, and this would be interesting to investigate in the future. Resource competition between galls and nodules is also an unlikely explanation in this experiment because we did not observe a significant reduction in nodule numbers in the *sunn4* mutant, which would have had a strong carbon sink due to its high nodule numbers.

A drop in nodule numbers in nematode-infected root system has also been observed in other legumes (e.g. Khan et al., 2018). However, results are likely to be strongly influenced by the age and nutritional status of the plant, the time of harvest and the age of the galls and nodules. Additional complex interactions could be due to the contribution of fixed nitrogen from nodules, which we tried to minimize here by phenotyping the plants as early as possible. Future experiments could try to unravel these complex interactions in plants grown under a number of different conditions.

In summary, we found that none of the examined early signaling genes required for nodulation were essential for successful RKN parasitism. The autoregulation of nodulation gene *sunn4* was similarly not involved in controlling the numbers of galls in *M. truncatula*. We found evidence for a positive role of ethylene signaling in successful gall formation and egg production, in contrast to its negative role in nodulation, mycorrhization and other pathogen interactions. We found a protective effect of rhizobia on RKN parasitism although the mechanism of this protection remains unclear. A negative effect of RKN on nodule numbers was also observed, but only in the *skl* mutant, suggesting a role for ethylene signaling in the interaction of rhizobia and RKN in the root. The molecular mechanism for the action of ethylene signaling in RKN infection and feeding site formation will be an important goal in the future.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

SRC and SC carried out the experiments. All authors contributed to the conception of the study and analysis of data. UM wrote the manuscript with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01050/full#supplementary-material

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Bacillus firmus Strain I-1582, a Nematode Antagonist by Itself and Through the Plant

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Bacillus firmus I-1582 is approved in Europe for the management of *Meloidogyne* on vegetable crops. However, little information about its modes of action and temperature requirements is available, despite the effect of these parameters in its efficacy. The cardinal temperatures for bacterial growth and biofilm formation were determined. The bacteria was transformed with GFP to study its effect on nematode eggs and root colonization of tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) by laser-scanning confocal microscopy. Induction of plant resistance was determined in split-root experiments and the dynamic regulation of genes related to jasmonic acid (JA) and salicylic acid (SA) by RT-qPCR at three different times after nematode inoculation. The bacteria was able to grow and form biofilms between 15 and 45°C; it degraded egg-shells and colonized eggs; it colonized tomato roots more extensively than cucumber roots; it induced systemic resistance in tomato, but not in cucumber; SA and JA related genes were primed at different times after nematode inoculation in tomato, but only the SA-related gene was up-regulated at 7 days after nematode inoculation in cucumber. In conclusion, *B. firmus* I-1582 is active at a wide range of temperatures; its optimal growth temperature is 35°C; it is able to degrade *Meloidogyne* eggs, and to colonize plant roots, inducing systemic resistance in a plant dependent species manner.

Keywords: *Cucumis sativus*, induced resistance, nematode antagonist, *Meloidogyne incognita*, root-knot nematodes, *Solanum lycopersicum*

INTRODUCTION

Plant parasitic nematode (PPN) management is a challenge for reducing crop yield losses all across the world. These parasites are responsible for annual yield losses reaching ca. 10% of life-sustaining crops and 14% of economically important crops (Agrios, 2005), varying greatly between areas due to specific nematode-plant-environment interactions. Root-knot nematodes (RKN) belonging to the genus *Meloidogyne* are the most damaging PPN worldwide due to their

wide range of plant hosts, worldwide distribution and high reproductive capacity (Jones et al., 2013). The potential damage of some *Meloidogyne*-crop combinations has been summarized by Greco and Di Vito (2009). Currently, RKN management strategies tend to reduce dependence from chemical nematicides by encouraging alternative control methods that promotes both the incompatible plant-RKN relationship by the use of plants bearing resistance genes (*R*-genes) and/or by microbe-inducing plant resistance, and the antagonistic potential of soils against the nematode.

During the course of a compatible infection, second-stage juveniles (J2) of *Meloidogyne* enter the root near the elongation zone and migrate intercellularly to circumvent the endodermal barriers and establish a permanent feeding site into the vascular cylinder, which induces the formation of giant cells and root galls. The J2 secrete effectors that affect cell wall architecture, plant development, plant defenses and metabolism to complete their life cycle successfully (Shukla et al., 2018). Subsequently, J2 become sedentary and molt three times before achieve the mature adult female stage. The most frequently encountered and damaging tropical species, *M. arenaria, M. incognita,* and *M. javanica*, reproduce parthenogenetically. The female lays a large number of eggs in a gelatinous matrix, known as the egg mass, located on the surface or inside galled roots. In an incompatible plant-RKN relationship due to plant *R*-genes, expression of plant genes related to phytohormone-mediated resistance responses, signal transduction pathways and secondary metabolite-mediated resistance responses are activated leading to suppression of nematode development and reproduction (Shukla et al., 2018). Plant hormones play a regulatory role in the induction of plant resistance by microorganisms (Pieterse et al., 2014). Primed plants can improve resistance to stressing agents during their life cycle and the effects can be maintained over generations (Mauch-Mani et al., 2017).

The antagonistic potential of a soil is the capacity to reduce the spread of deleterious agents to plants through biotic factors (Sikora, 1992). Several nematode antagonists can occur naturally in agricultural soils providing some degree of suppressiveness (Giné et al., 2016; Elhady et al., 2017). High levels of soil suppressiveness can be achieved under favorable interactions between plant-RKN-antagonists, cultural practices, and abiotic factors (Giné et al., 2016). In soils with no or low levels of soil suppressiveness, nematode antagonists can be introduced by the application of biological-based nematicides. Currently, three biological-based nematicides are approved in Europe: *Bacillus firmus* I-1582 (Bf I-1582), *Purpureocillium lilacinum* (= *Paecilomyces lilacinus*) strain 251 and *Pasteuria nishizawae* Pn1. Among them, only Bf I-1582 and *P. lilacinum* strain 251 are approved for use against RKN in vegetable crops (European Commission, 2019).

Vegetables are important components of the human diet and are cultivated worldwide in both open field and under protected structures. Rotation sequences including fruiting solanaceous and cucurbit crops are very common because they represent the main source of income for many growers. Unfortunately, all of them are affected by RKN (Hallman and Meressa, 2018). Some attempts to manage RKN using *P. lilacinum* strain 251 have shown a high percentage of fungal egg parasitism in both *in vitro* and pot experiments (Khan et al., 2004, 2006; Kiewnick and Sikora, 2006a,b; Kiewnick et al., 2011). However, limited efficacy have been reported in field experiments under Mediterranean conditions (Anastasiadis et al., 2008; Kaşkavalci et al., 2009; Giné and Sorribas, 2017) probably due to sustained sub-optimal soil temperatures experienced during the cropping seasons and/or the inhibition of fungal enzymes by components of the formulation (Giné and Sorribas, 2017). In the case of Bf I-1582, some reports have shown its effectivity against several PPN including RKN under different experimental conditions, from *in vitro* to field experiments on different crops (Giannakou et al., 2004, 2007; Mendoza et al., 2008; Terefe et al., 2009; Castillo et al., 2013). This bacterial strain reduced egg hatching and the viability of nematodes in *in vitro* experiments, and suppressed nematode reproduction and disease severity in pot and field experiments. Moreover, Bf I-1582 triggered systemic resistance in soybean (*Glycine max*) infected with *Heterodera glycines,* and in cotton (*Gossypium hirsutum*) parasitized by RKN in split-root experiments, but not in corn (*Zea mays*) infected with *M. incognita* (Schrimsher, 2013; Gattoni et al., 2018). The interactions of *Bacillus* species and plants with special reference to induced systemic resistance (ISR) have been reviewed (Choudhary and Johri, 2009). It seems that specific transduction pathways promoted during ISR by *Bacillus* spp. vary depending on the bacterial strain, the host plant and the plant pathogen (Kloepper et al., 2004). Extensive work has been done with several *Bacillus* spp., but information about *B. firmus* in particular remained limited until 2013 (Wilson and Jackson, 2013). Since then, novel information concerning nematicidal virulence factors (Geng et al., 2016; Zheng et al., 2016; Marin-Bruzos and Grayston, 2019) and induction of plant resistance in cotton (Gattoni et al., 2018, 2019) has been reported.

Optimal use of microbial antagonists against RKN on vegetable crops cultivated under irrigation has to consider the cardinal temperatures of microbial growth, the effect on the most abundant and vulnerable nematode development stages, and the plant-microbe interaction, especially the putative induction of plant resistance against nematodes. In this manuscript, all these aspects are presented: (i) cardinal temperatures for Bf I-1582 growth and biofilm formation; (ii) effect of Bf I-1582 transformed with green fluorescent protein gene (Bf I-1582 -GFP) on RKN eggs; (iii) colonization of tomato and cucumber roots by Bf I-1582 -GFP; and (iv) putative induction of systemic resistance and the dynamic regulation of genes related to the jasmonic acid (JA) and salicylic acid (SA) pathways in tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*).

MATERIALS AND METHODS

Sources of Planting Material and Nematode and Bacteria Inoculum

Tomato cv. Durinta and cucumber cv. Dasher II, both susceptible to RKN (Giné et al., 2016) were used in this study. For all the experiments seeds were surface-sterilized in a 50% bleach solution (40 g l^{-1} NaOCl) for 2 min, washed three times in

sterilized distilled water for 10 s each. The seeds were then sown in a tray containing sterile vermiculite and maintained in a growth chamber at 25°C ± 2°C with a 16 h:8 h (light:dark) photoperiod.

The *Meloidogyne incognita* isolate Agropolis used in this study came from a nematode population obtained from tomato cv. Durinta roots cultivated in a plastic-greenhouse in 2010 in which tomato and cucumber were previously cultivated. Twenty tomato plants cv. Durinta grown in 200 cm^3 pots filled with sterile sand were inoculated with a single egg mass each and maintained in a growth chamber at 25 ± 2°C and 16 h:8 h photoperiod for 45 days. The adult females developing from a single egg mass were identified as *M. incognita* by the morphology of perineal pattern and by SCAR markers (Zijlstra et al., 2000). The offspring were maintained on tomato cv. Durinta for experimental purposes. J2 were used as inoculum. Eggs were extracted from tomato roots by blender maceration in a 5% commercial bleach (40 g l^{-1} NaOCl) solution for 10 min (Hussey and Barker, 1973). The egg suspension was filtered through a 74 μm aperture sieve to remove root debris, and eggs were collected on a 25 μm sieve and placed on Baermann trays (Whitehead and Hemming, 1965) at 25°C ± 2°C. J2 were collected daily using a 25 μm sieve for 7 days and stored at 9°C until use.

The Bf I-1582 strain isolated from the VOTiVO® formulation (Bayer CropScience) was used in the cardinal temperatures and biofilm formation experiments, as well as for the bacterial transformation with the green fluorescent protein (GFP) gene to study plant root and nematode egg interactions. The commercial formulate VOTiVO® was used in the induction of plant resistance experiments and gene expression analysis.

Bf I-1582 GFP Transformation

Bf I-1582 was transformed with the GFP gene with a pAD43-25 plasmid (Dunn and Handelsman, 1999) kindly provided by the *Bacillus* Genetic Stock Center. Bacterial protoplasts were prepared according to Aono et al. (1992) with slight modifications. Three ml of Bf I-1582 cells grown overnight in Penassay medium (1.5 g l^{-1} beef extract, 1.5 g l^{-1} yeast extract, 5 g l^{-1} peptone, 1 g l^{-1} glucose, 3.5 g l^{-1} NaCl, 3.6 g l^{-1} dipotassium phosphate and 1.32 g l^{-1} monopotassium phosphate) were harvested by centrifugation at 3000 g for 10 min at 4°C. The pellet was suspended in 0.1 ml of SMMP medium consisting of SMM (0.5M sucrose, 0.02 M maleic acid and 0.02 M $MgCl_2$) in double-strength Penassay media pH 6.4, supplemented with 40 mg of lysozyme, and incubated at 37°C with gentle shaking for 75 min. The *B. firmus* protoplasts were recovered by centrifugation at 1000 g for 30 min at 10°C, washed twice with SMMP medium and finally suspended in 0.1 ml of SMMP media.

Transformation of Bf I-1582 was performed according to Chang and Cohen (1979). Briefly, 150 ng of purified plasmid pAD43-25 were mixed with 5 μl of 2X strength SMM buffer and 50 μl of *B. firmus* protoplasts obtained as explained above. Then, 150 μl of 40% polyethylene glycol 8000 (Scharlau) were added, and 0.5 ml of SMMP media were added after 2 min of incubation. Protoplasts were recovered by centrifugation at 2500 rpm for 10 min. Protoplasts were suspended in 0.1 ml of SMMP media and kept at 30°C with gentle shaking for 4 h. Finally, cells were plated in solid Luria-Bertani (LB) supplemented with 20 μg ml^{-1} chloramphenicol and incubated at 37°C. After 2 days, GFP expression of pAD43-25 of the transformed Bf I-1582 colonies was assessed in a Hertel and Reuss CN-hF microscope with appropriate filters.

To corroborate that the species transformed with the pAD43-25 plasmid was *B. firmus,* one stable colony transformant was selected. This colony was inoculated in LB and growth O/N before performing a genomic DNA extraction and a PCR with BLS342F (5′ CAGCAGTAGGGAATCTTC 3′) and 1392R (5′ ACGGGCGGTGTGTACA 3′) primers following the conditions described in Blackwood et al. (2005). The 1050 bp PCR product obtained was sequenced and aligned against nr using nBLAST with default settings.

Live-Cell Imaging of Tomato and Cucumber Roots and *M. incognita* Eggs Colonized by Bf I-1582-GFP

Tomato and cucumber seeds were surface-sterilized, as described previously, and were placed on a moist chamber at 25°C until the main root was ca. 2 cm long (5–7 days). Seedlings were transferred to an Erlenmeyer flask filled with 80 ml of 0.5X Murashige and Skoog (MS) semisolid agar medium (0.07% agar) and inoculated with 20 ml of the Bf I-1582-GFP overnight culture grown at 35°C in liquid LB supplemented with 20 μl ml^{-1} chloramphenicol (Sigma). Seedlings were incubated at 25°C for 5 and 10 days, respectively (Hao and Chen, 2017). Afterward, roots were washed with distilled water to eliminate non-adhered bacteria and 10 fragments of the root system (ca. 1 cm long) were examined with laser-scanning confocal microscopy. To determine the spatial pattern of root colonization by Bf I-1582-GFP, roots were imaged using a Leica TCS 5 STED CW microscope (Leica Microsystem) equipped with hybrid detectors and with a 40x 1.25NA HCX Pl Apo CS Leica objective. A 488 nm laser was used for fluorescence excitation. GFP fluorescence was detected at 505–530 nm and autofluorescence of root cell walls at 580–620 nm, as described in Macià-Vicente et al. (2009). Stacks of 8–13 μm, step size of 0.2–0.3 μm, were acquired. Z projection-images and Z stack movies are shown at **Figure 2** and **Supplementary Video S1**, respectively. All image analysis was performed using Fiji (Schindelin et al., 2012).

Nematode eggs were surface-sterilized as in McClure et al. (1973). A 200 μl suspension containing ca. 100 eggs were dispensed in a 1.5 ml microcentrifuge tube containing 50 μl of bacterial culture grown on LB supplemented with 20 μg ml^{-1} chloramphenicol (Sigma) at 35°C for 2 days. After 3, 5 and 10 days of incubation at 35°C ± 2°C eggs were examined with laser-scanning confocal microscopy using a Leica TCS 5 STED CW microscope (Leica Microsystem) equipped with hybrid detectors and with a 63x 1.4NA HCX Pl Apo CS Leica objective. A 488 nm laser was used for fluorescence excitation and transmission-light detection. GFP fluorescence was detected at 505–530 nm and egg autofluorescence was detected at 580–620 nm (Escudero and Lopez-Llorca, 2012). Stacks of 8–13 μm,

step size of 0.2–0.3 μm, were acquired. Z projection-images are shown in **Figure 3**. A three-dimensional (3D) reconstructed Z-stack (**Supplementary Video S2**) of an *M. incognita* egg after 10 days from bacterial inoculation was created using Huygens software (Huygens SVI, Netherlands).

Cardinal Temperatures of Bf I-1582 and Biofilm Formation

Cardinal temperatures of Bf I-1582 were determined. Sets of three Petri dishes containing nutrient agar (3 g l^{-1} beef extract, 5 g l^{-1} peptone, 5 g l^{-1} NaCl, 15 g l^{-1} agar, pH 7) were inoculated with ca. 200 bacteria colony-forming units (CFU) and incubated in the dark at 4, 9, 20, 25, 30, 35, 40, 45, and 50°C for 96 h before counting.

Growth kinetics in liquid media were determined inoculating 10^6 CFU in Erlenmeyer flasks containing 200 ml of LB. Sets of three Erlenmeyer flasks were incubated at 10, 15, 20, 25, 30, 35, 40, 45, and 50°C. Cultures were maintained with shaking and one aliquot of 3 ml was taken at 0, 2, 4, 6, 8, 10, 12, 24, 30, 36, and 48 h to measure the optical density at 590 nm ($OD_{590\ nm}$) in an UV-Vis Evolution 300 spectrophotometer (Thermo Fisher Scientific, United States).

Sets of three Petri dishes (40 mm diameter) with 10 ml of Schaeffer's sporulation medium plus glucose and glycerol (SGG) (5 g l^{-1} beef extract, 10 g l^{-1} peptone, 2 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4$ $7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2{\cdot}4\ H_2O$, 1 μM $FeSO_4$, 1 g l^{-1} glucose, and 1% glycerol) were inoculated with 10^6 CFU and incubated at 10, 15, 20, 25, 30, 35, 40, 45, and 50°C in the dark for 2 days to determine the effect of the temperature on the Bf I-1582 biofilm formation (Hsueh et al., 2015).

Induction of Plant Resistance by Bf I-1582 Against *M. incognita*

Tomato and cucumber were grown in a split-root system as described in previous studies (Ghahremani et al., 2019). In this system, the main root is divided into two halves transplanted in two adjacent pots: the inducer, inoculated with Bf I-1582 and the responder inoculated with 200 J2 of *M. incognita*. Briefly, the main root of 5-day-old seedlings was excised and plantlets were individually transplanted into seedling trays containing sterile vermiculite and maintained in a growth chamber at 25 ± 2°C and 16 h:8 h photoperiod for 3 weeks for tomato and 2 weeks for cucumber. Afterward, plantlets were transferred to the split-root system by splitting roots into two halves planted into two adjacent 200 cm^3 pots filled with sterilized sand. The experiment consisted of three treatments: Bf I-1582/*M. incognita* (Bf-Mi) and *M. incognita* (None-Mi) to assess the capability of Bf I-1582 to induce plant resistance against *M. incognita*, and a control non-inoculated with neither bacteria or nematode to assess the effect of the split-root system on each root half development. Each treatment consisted of ten individual plants. Two experiments were conducted with tomato, and one with cucumber.

The inducer pot was inoculated with a liquid suspension of 10^9 CFU of BF I-1582 one week after transplantation. One week after bacterial inoculation, the responder pot was inoculated with a liquid suspension to achieve a soil density of 1 J2 of *M. incognita* per cm^3. The treatment None received the same volume of water. The plants were maintained in a growth chamber under the same conditions described previously in a completely randomized design for 43 days. Plants were irrigated as needed and fertilized with Hoagland solution twice per week. Soil temperatures were recorded daily at 30 min intervals with a PT100 probe (Campbell Scientific Ltd.) placed in the pots at a depth of 4 cm. At the end of the experiment, plants were uprooted and fresh root weight of both inducer and responder halves from the non-inoculated control treatment were measured. Roots from responder pots inoculated with *M. incognita* were immersed in a 0.01% erioglaucine solution for 45 min to stain the egg masses (Omwega et al., 1988) before counting them. Afterward, the nematode eggs were extracted from the roots according to the Hussey and Barker (1973) procedure in a 10% commercial bleach solution (40 g l^{-1} NaOCl) for 10 min and counted.

Endophytic root colonization by Bf I-1585 was estimated at the end of the first and second experiment with cucumber and tomato, respectively. Bacterial DNA was quantified by qPCR from three individual biological samples from the Bf-Mi treatment. Each composite biological sample consisted of the inducer half of the roots from three plants. For the composite inducer sample, half of the roots (of three plants) were surface sterilized using 50% commercial bleach solution (40 g l^{-1} NaOCl) for 2 min and washed three times with sterile distilled water for 10 s each, and then blotted onto sterile paper. The DNA was extracted from each biological replicate following the López-Llorca et al. (2010) procedure. The qPCR reactions were performed using the Brilliant Multiplex QPCR Master Mix (Agilent Technologies) in a final volume of 25 μl containing 50 ng of total DNA and 0.5 μM of each primer (5′–3′ direction): Votivo-2F (forward) CTCCAATTCCTAATATCCTGCAAAG, Votivo-2R (reverse) GGAAAGTCACGGGACAGTTAT (Mendis et al., 2018). Negative controls containing sterile water instead of DNA were included. Reactions were performed in duplicate in a Stratagene Mx3005P thermocycler (Agilent Technologies) using the following thermal cycling conditions: initial denaturation step at 95°C for 15 min, then 39 cycles at 95°C for 30 s, and 58°C for 1 min. DNA of Bf I-1582 was used to define a calibration curve ranging from 5 pg to 50 ng. PCR specificity was verified by means of melting curve analysis and agarose gel electrophoresis. The Bf DNA referred to the total DNA biomass (50 ng) is expressed as a proportion.

Dynamic Expression of JA and SA Related Genes by Bf I-1582 and *M. incognita* in Tomato and Cucumber

Two-week-old cucumber seedlings and 3-week-old tomato seedlings were transferred to 200 cm^3 pots filled with sterilized sand, and maintained in a growth chamber as previously described. The assessed treatments were: non-inoculated plants (Control), plants inoculated with Bf I-1582 (Bf), plants inoculated with *M. incognita* (Mi) and plants co-inoculated with both organisms (Mi+Bf). Bf I-1582 treatments were

inoculated with 10^9 CFU 1 week after transplantation, and those *M. incognita* treatments were inoculated with 200 J2 2 weeks after transplanting. The expression of the genes related to JA and SA pathways was evaluated at 0 days after nematode inoculation (DANI), at 7 DANI, when the nematode infected the roots, and at 40 DANI. Root infection was determined by staining the nematode into the root with acid fuchsin (Byrd et al., 1983). At 40 DANI, the nematode eggs were extracted by the Hussey and Barker (1973) from roots of three individual tomato or cucumber plants from all inoculated treatments. Total tomato and cucumber root colonization by Bf I-1585 was estimated from three different plants of each Bf treatment by qPCR at 0 and 40 DANI. Roots were washed three times in sterilized distilled water for 10 s each and then blotted onto sterile paper. The DNA extraction and qPCR were conducted as described under section "Induction of Plant Resistance by Bf I-1582 Against *M. incognita*."

For the expression study, three biological replicates were assessed at each sampling time. Each biological replicate consisted of a composite sample of roots from three plants that were pooled. Roots excised from the aboveground plant parts were washed three times with sterile distilled water, blotted on sterile filter paper, and immediately frozen with liquid nitrogen. Samples were stored at −80°C until used. RNA isolation and retrotranscription were conducted according to Ghahremani et al. (2019). Dynamic regulation in the JA pathway was determined by the expression of the lipoxygenase D (*LOX-D*) gene for tomato (Fujimoto et al., 2011) and lipoxygenase 1 (*LOX1*) gene for cucumber (Shoresh et al., 2004). In the SA pathway we evaluated the expression of the pathogenesis-related 1 (*PR1*) gene for tomato (Gayoso et al., 2007) and the phenylalanine ammonia-lyase (*PAL*) gene for cucumber (Shoresh et al., 2004). The ubiquitin (*UBI*) gene was used as a reference gene for both plant species (Yang et al., 2012; Song et al., 2015). Relative gene expression was estimated with the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001). The sequences of the primers used in the RT-qPCR are shown in **Supplementary Table S1**. The qPCR reactions were performed in a final volume of 20 μl with 1 μl of cDNA, 0.3 mM primers and 1X Fast SYBR Green Master Mix (Applied Biosystems) in a 7900HT Fast Real Time PCR System thermocycler (Applied Biosystems). Reactions were performed with two technical replicates per each biological replicate using the following conditions: 20 s at 95°C followed by 40 cycles of 30 s at 95°C and 1 min at 60°C for tomato (Gayoso et al., 2007) and 20 s at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C for cucumber (Shoresh et al., 2004). PCR specificity was verified by means of melting curve analysis and agarose gel electrophoresis.

Statistical Analysis

Statistical analyses were performed using the JMP software v8 (SAS institute Inc., Cary, NC, United States). Both data normality and homogeneity of variances were assessed. When confirmed, a paired comparison using the Student's *t*-test was undertaken. Otherwise, paired comparisons were conducted using the non-parametric Wilcoxon test ($P < 0.05$).

RESULTS

Cardinal Temperatures of Bf I-1582 and Biofilm Formation

Bf I-1582 grew in temperatures ranging from 15 to 45°C, with 35°C being the optimal growth temperature in both solid (**Figure 1A**) and liquid media (**Figure 1B**). Similarly, biofilm formation was observed between 15 and 45°C, being thicker and uniform at 35°C (**Figure 1C**).

Tomato and Cucumber Roots Colonization and *M. incognita* Eggs Degradation by Bf I-1582-GFP

Both tomato and cucumber roots were colonized by *B. firmus* (**Figure 2**). In tomato, Bf I-1582GFP was observed on root hairs and epidermal cells at 5 days after inoculation (DAI) (**Figure 2a**). At 10 DAI bacterial colonies were observed in root hairs and some lone bacteria were found inside the root (**Figure 2c**). In cucumber, few bacteria were observed on epidermal cells at 5 DAI (**Figure 2b**). There were no bacteria found inside the root at 10 DAI (**Figure 2d**) with the few cells observed being attached to the surface of the root section, as shown in **Supplementary Video S1**.

M. incognita egg-shell degradation and egg colonization by Bf I-1582-GFP were studied with confocal-scanning laser microscopy (**Figure 3**). At 3 DAI, bacteria were surrounding and degrading the nematode egg and embryo (**Figure 3a**); at 5 DAI, bacterial colonies were adhered to the egg-shell and some bacteria were found inside the egg (**Figure 3b**); at 10 DAI bacterial biofilms adhered to the egg-shell and bacteria inside the egg were observed (**Figure 3c**). Egg-shell erosion and egg colonization by Bf I-1582-GFP can be observed in the **Supplementary Video S2**.

Bf I-1582 Induces Plant Resistance to *M. incognita* in Tomato but Not in Cucumber

Fresh root weight of the two halves of the non-inoculated split-root system did not differ significantly ($P < 0.05$; data not shown) in either tomato or cucumber, proving that the treatment did not hamper root development.

Lower number ($P < 0.05$) of egg masses and eggs per plant were recorded in the responder part of the tomato root of the Bf-Mi treatment compared to the None-Mi, irrespective of the experiment (**Figures 4A,B,D,E**). However, no effect was detected in cucumber (**Figures 4C,F**).

The standard curve for qPCR used for estimating the bacterial DNA density was: Ct = −3.1413 * log10 DNA concentration + 24.522 ($R^2 = 0.9591$). *B. firmus* colonized roots of both plant species endophytically, but ca. 61% higher ($P < 0.05$) density of bacterial DNA was recorded in tomato than in cucumber roots (**Figure 5**).

Dynamic Regulation of JA and SA Genes by Bf I-1582 Is Plant-Dependent

The regulation of genes related to JA and SA pathways varied between tomato and cucumber (**Figure 6**). In tomato, at 0 DANI,

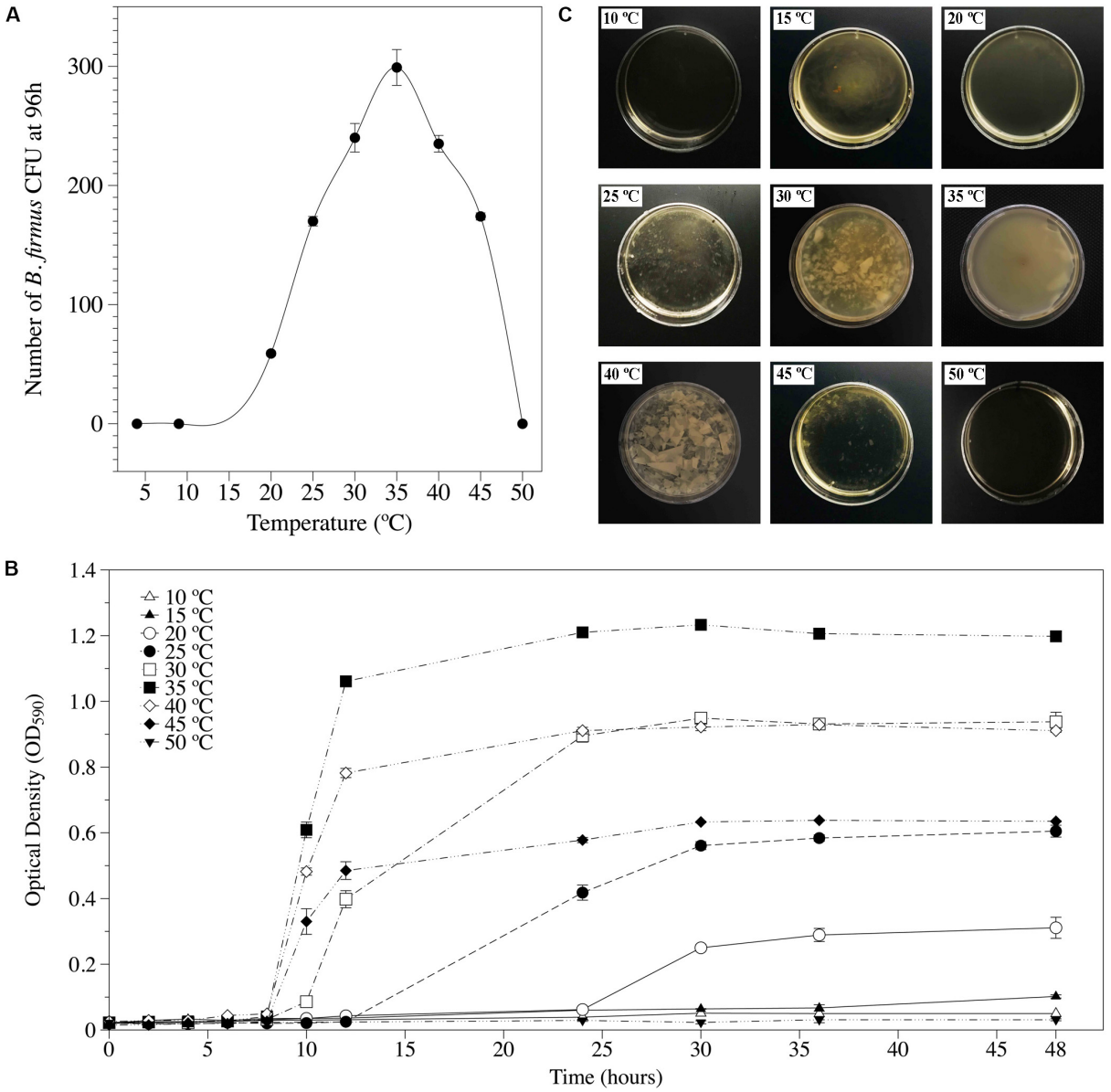

FIGURE 1 | Cardinal temperatures for *Bacillus firmus* I-1582 growth and biofilm formation. **(A)** Number of bacterial CFU after 96 h of incubation in solid nutrient agar at different temperatures. Each value is mean ± standard error of three replicates. **(B)** Bacterial growth kinetics incubated for 0, 2, 4, 6, 8, 10, 12, 24, 30, 36, and 48 h in Luria-Bertani (LB) liquid media at different temperatures. Each value is mean ± standard error of three replicates. **(C)** Biofilm formation in Schaeffer's sporulation medium plus glucose and glycerol (SGG) media 48 h after being inoculated with 10^6 bacterial CFU and incubated at different temperatures.

which corresponds to 7 days after Bf I-1582 inoculation and just after nematode inoculation, both JA (*LoxD*) and SA (*Pr1*) pathways were up-regulated in plants inoculated with the bacteria in comparison to non-inoculated plants (**Figure 6A**). At 7 DANI, when nematode infection was established, only the plants co-inoculated with nematode and bacteria showed an up-regulation of the JA related gene (*Lox D*) (**Figure 6B**). At 40 DANI, when J2 hatching began and new root infections occurred, tomato plants co-inoculated with the nematode and Bf I-1582 had repressed the JA related gene (*LoxD*). Meanwhile the gene related to the SA pathway (*Pr1*) was up-regulated in plants co-inoculated and also inoculated with Bf I-1582 alone, but was suppressed in plants inoculated only with the nematode (**Figure 6C**).

In cucumber plants, no differences between treatments were found at 0 DANI (**Figure 6D**). At 7 DANI *Pal I* was up-regulated both in the *M. incognita* inoculated plants and those co-inoculated with the bacteria and the nematode (**Figure 6E**). At 40 DANI, both JA and SA pathways were suppressed in plants inoculated with *M. incognita* but only JA in the co-inoculated plants (**Figure 6F**).

Nematode reproduction in tomato co-inoculated with bacteria was reduced ($P < 0.05$) in a 53%, but did not differ between

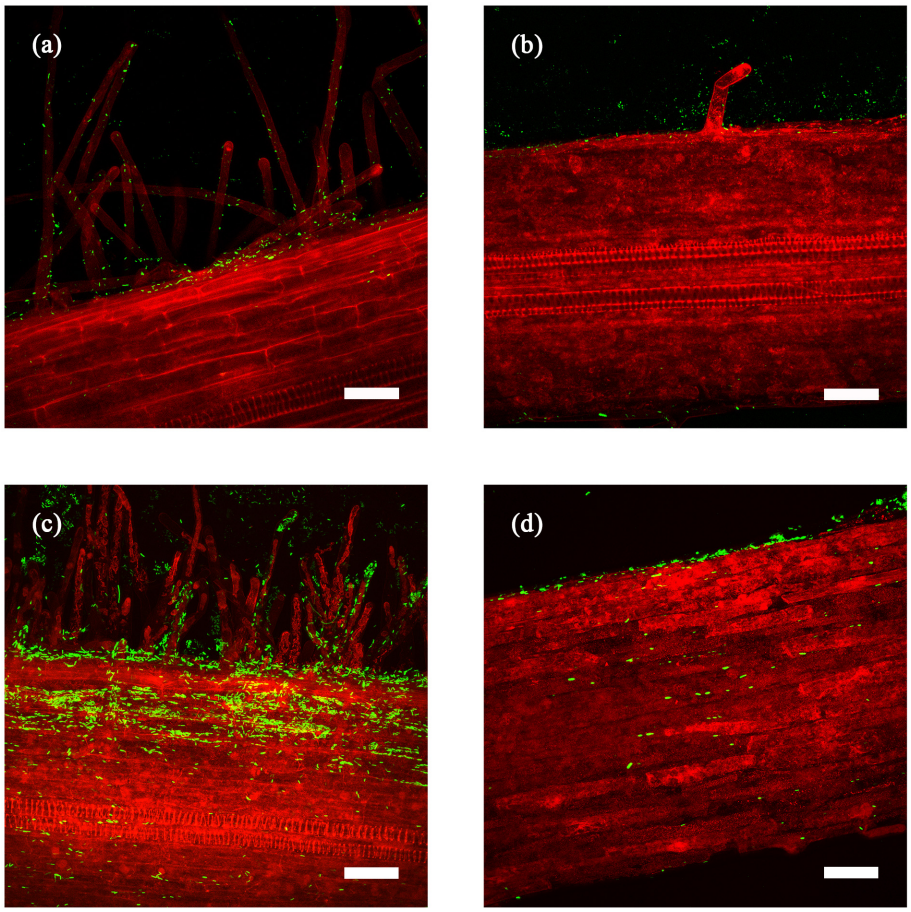

FIGURE 2 | Z projection of laser-scanning confocal microscopy images of *Bacillus firmus* I-1582 transformed with the green fluorescent protein gene (Bf I-1582GFP) colonizing tomato **(a,c)** and cucumber **(b,d)** roots after 5 **(a,b),** and 10 **(c,d)** days after bacterial inoculation and incubation at 25°C. Scale bar: 50 μm. Autofluorescence of roots is shown in red.

treatments in cucumber. Bacterial colonization of tomato roots was ca. 65% higher in tomato than in cucumber at 0 and 40 DANI (**Figure 7**).

DISCUSSION

The results of this study contribute to better understanding of *B. firmus*-tomato or cucumber interactions, two economically important fruiting vegetables cultivated worldwide; and *B. firmus-Meloidogyne* eggs interaction, the most abundant nematode developmental stage in soil and in plant roots. In addition, determining the cardinal temperatures of bacterial growth and biofilm formation provide valuable information to optimize the use of *B. firmus*–based formulations to maximize nematode control efficacy.

B. firmus was able to colonize both tomato and cucumber roots, but it grew more efficiently on tomato. The GFP-transformed bacteria colonized the rhizoplane of both plant species, and the observation of attenuated fluorescence indicates that some bacteria could colonize tomato roots endophytically. This finding was corroborated by qPCR, showing that the proportion of bacteria inside or on the surface of tomato roots was ca. 61–65% higher than that found inside or on cucumber roots. It is known that *B. firmus* is able to colonize roots of other economically important crops such as corn, soybean and cotton (Mendis et al., 2018; Gattoni et al., 2019). Root colonization by bacteria could prevent nematode infection and production of viable inoculum by affecting the nematode cuticle and the egg-shell. Some reports pointed out the capability of *B. firmus* to inhibit J2 hatching, motility and viability (Mendoza et al., 2008; Terefe et al., 2009; Xiong et al., 2015; Gattoni et al., 2018). In the current study, the degradation of nematode eggs by the GFP-transformed bacteria was observed by laser-scanning confocal microscopy. Serine proteases have been reported in several fungal and bacterial nematode antagonists as a key factor affecting directly the plant-parasitic nematode physical barriers (Bonants et al., 1995; Segers et al., 1996; Lian et al., 2007; Iqbal et al., 2018). Recently, Geng et al. (2016) identified the serine protease Sep 1 from *B. firmus* DS-1, which is capable of degrading multiple cuticle and intestinal-associated proteins. Thus, bacterial colonies growing on or inside the roots could affect the nearest infective

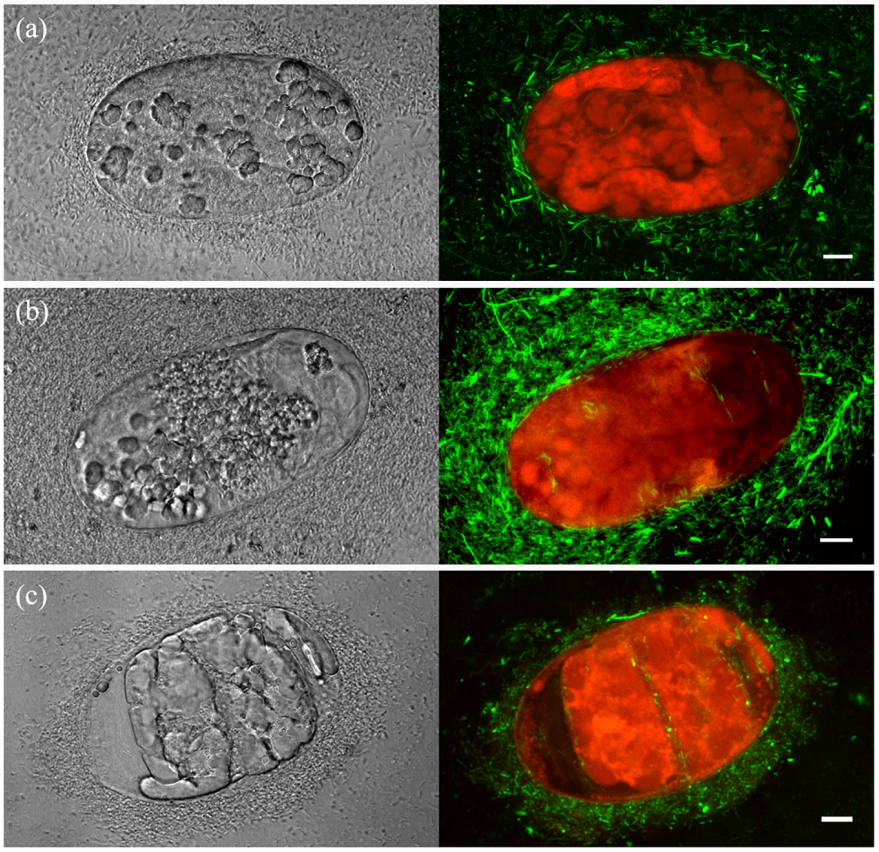

FIGURE 3 | Laser-scanning confocal microscopy images of *Bacillus firmus* I-1582 transformed with the green fluorescent protein gene (Bf I-1582GFP) damaging *Meloidogyne incognita* eggs after 3 **(a)**, 5 **(b),** and 10 **(c)** days of incubation at 35°C. Z projection images of transmitted (left) and fluorescence (right) channels. Scale bar: 10 μm. Autofluorescence of nematode eggs is shown in red.

or sedentary nematodes and eggs by some of the nematicidal virulence factors exhibited by *B. firmus* (Geng et al., 2016; Zheng et al., 2016; Marin-Bruzos and Grayston, 2019). Further investigation of this type of interaction is required.

In the present study, Bf I-1582 induced systemic resistance against *M. incognita* in tomato but not in cucumber in split-root experiments. Previous experiments showed that this bacterial isolate was able to induce systemic resistance in cotton cv. Phytogen 333 WRF (Gattoni et al., 2018) but not in an unspecified corn cultivar (Schrimsher, 2013). Thus, these results support the hypothesis that this phenomenon is plant species dependent at least, because all these studies were conducted using only one cultivar of each plant species. Further studies should be conducted to determine the ability of Bf I-1582 to induce resistance in a range of cultivars from economically important crops to determine its putative use to manage RKN and other PPN through the plant. In our study, the relative expression of genes related to SA and JA pathways was assessed in tomato and cucumber at 7 days after bacterial inoculation and just after nematode inoculation (0 DANI), after root infection by the nematode (7 DANI), and when the offspring reinfected plant roots (40 DANI). We observed some differences between plant species. Tomato plants were primed by SA and JA at 0 DANI, and the nematode infection was reduced, measured as the number of egg masses recorded at the end of the split-root experiment. However, no differences in the expression of SA and JA related genes was observed in cucumber plants inoculated with bacteria as well as in the number of egg masses produced in cucumber roots. Martinez-Medina et al. (2017) and Ghahremani et al. (2019) considered that SA primed plants affect nematode infection, corroborating the results of the tomato experiments conducted in this study. At 7 DANI, *Lox D*, gene related with JA biosynthesis was up-regulated in tomato and could affect nematode development and reproduction, as proposed by Martinez-Medina et al. (2017) and Ghahremani et al. (2019). Nonetheless, *PAL*, a key regulatory enzyme in the synthesis of SA that can be activated by the JA/ethylene pathway (Martinez et al., 2001; Shoresh et al., 2004) was up-regulated in cucumber in treatments inoculated with *M. incognita*. Our results are in concordance with those reported by Shukla et al. (2018) who observed *PAL* up-regulation during disease development in tomato plants inoculated with *M. incognita*. In cucumber, no effect on nematode infection and/or nematode reproduction was observed at the end of the split-root or co-inoculation experiments. Time elapsed between bacterial and nematode inoculations could explain this delay in plant response, since

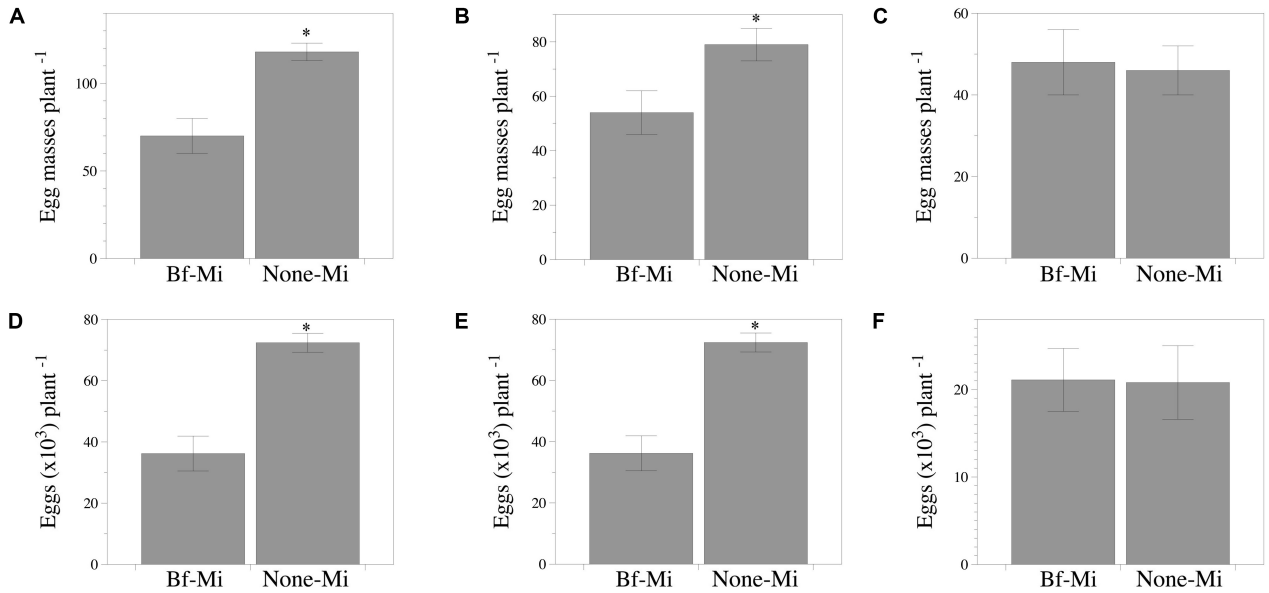

FIGURE 4 | Capability of *Bacillus firmus* I-1582 to induce systemic resistance in tomato cv. Durinta and cucumber cv. Dasher II against *Meloidogyne incognita* in two split root experiments. The inducer part of the root was inoculated with 10^9 CFU of *B. firmus* I-1582 and the responder part of the root was inoculated with 200 J2 of *M. incognita*. In the responder part of the root the number of egg masses per plant (infectivity) and the number of eggs per plant (reproduction) were assessed 43 days after nematode inoculation. Number of egg masses per plant **(A–C)** and total nematode eggs per plant **(D–F)**. Tomato experiment 1 **(A,D)**; tomato experiment 2 **(B,E)**; cucumber experiment **(C,F)**. Data are means ± standard error of 10 replicates. The asterisk indicates within each graph and for each experiment, that treatments are significantly different ($P < 0.05$) according to the non-parametric Wilcoxon test.

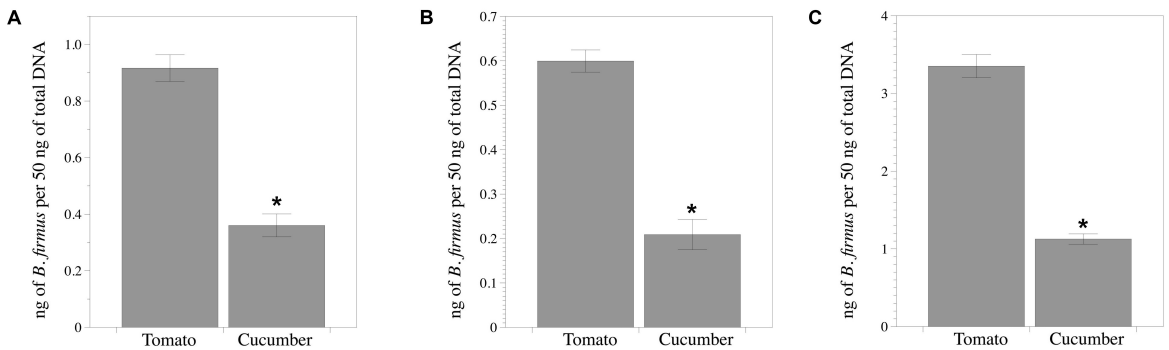

FIGURE 5 | *Bacillus firmus* I-1582 colonizes tomato and cucumber roots. **(A)** Endophytic bacterial DNA biomass in relation to the total DNA biomass of the sample from the inducer half of the root 50 days after being inoculated with 10^9 CFU and 43 days after nematode inoculation with 200 *Meloidogyne incognita* juveniles in the responder half of the root of the split-root experiment. The inducer part of the root was surface sterilized with 50% commercial sodium hypochlorite for 2 min and washed three times with sterile distilled water for 10 s each prior to the DNA extraction. Each value is mean ± standard error of three biological replicates composed of three plant roots each. **(B)** Bacterial DNA biomass in relation to the total DNA biomass of the sample 7 days after bacterial inoculation at a rate of 10^9 CFU per plant and just after nematode inoculation with 200 J2 (0 DANI) and **(C)** at 40 DANI. Each value is mean ± standard error of three biological replicates composed of one plant root each. Asterisk indicates significant difference between plant species according to the non-parametric Wilcoxon test ($P < 0.05$).

bacterial colonization was less efficient in cucumber than in tomato roots, as assessed by laser-scanning confocal images and qPCR measuring of bacterial DNA. At 40 DANI, the SA related genes were down-regulated in both tomato and cucumber plants inoculated only with the nematode agreeing with previous results reported by Shukla et al. (2018). However, it was up-regulated in tomato plants co-inoculated with the bacteria and the nematode, according to the incompatible nematode-plant interaction (Shukla et al., 2018) while no effect was observed in cucumber. Regarding the JA related gene, it was down-regulated in both plant species co-inoculated with the bacterium and the nematode. The dynamic regulation of genes related to SA and JA induced by *B. firmus* I-1582 and *B. amyloliquefaciens* QST713 in cotton against *M. incognita* has been studied recently (Gattoni et al., 2019). At 1 week after inoculation, the gene related to SA was up-regulated by both bacterial strains, and the authors proposed that a long-term SA-dependent systemic response was responsible for nematode suppression. According

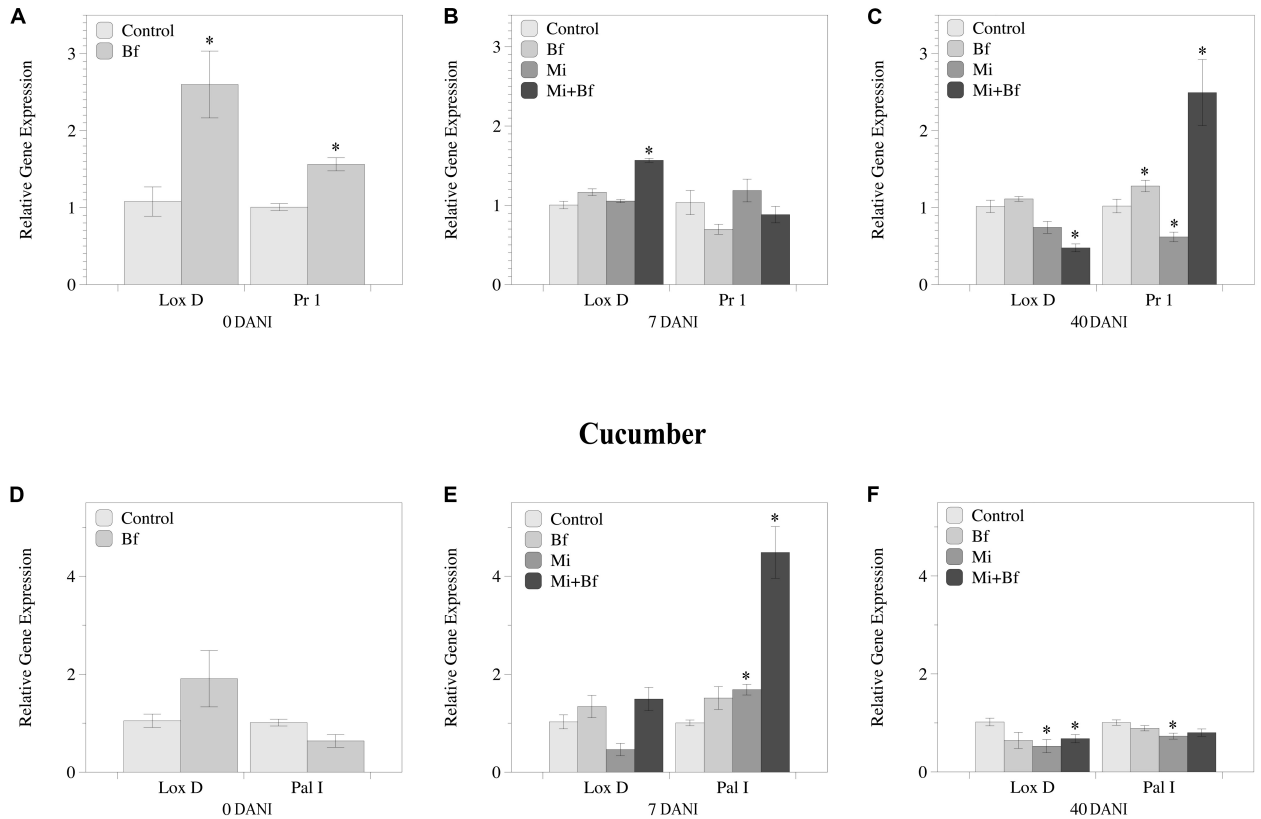

FIGURE 6 | Dynamics of the relative gene expression of genes related to the jasmonic acid (*Lox D*) and the salicylic acid (*PR1/Pal I*) pathways in tomato **(A–C)** and cucumber **(D–F)** plants that were non-inoculated (Control), inoculated with *Bacillus firmus* I-1582 (Bf), inoculated with *Meloidogyne incognita* (Mi) and co-inoculated with both nematode and bacteria (Mi+Bf). Bf I-1582 treatments were inoculated with 10^9 CFU 1 week after transplanting, while *M. incognita* treatments were inoculated with 200 juveniles 2 weeks after transplanting. The relative expression of the genes was evaluated at 0 days after nematode inoculation (DANI), at 7 and at 40 DANI. The ubiquitin (*UBI*) gene was used as a reference gene for both plant species. Each value is mean ± standard error of three biological samples with two technical replicates each. Each biological replicate consisted of the roots from three plants pooled together. Asterisk indicates significant differences with respect to the Control using the non-parametric Wilcoxon test ($P < 0.05$).

to our results, *B. firmus* I-1582 induces a response in plants but its effects on nematodes vary depending on the host plant species. In plant species for which the nematode is suppressed, a shift from SA to JA regulation genes affects nematode infection and reproduction, as it has been reported for *Trichoderma harzianum*-tomato (Martinez-Medina et al., 2017) and *Pochonia chlamydosporia*-tomato interactions (Ghahremani et al., 2019). Conversely, regulation of plant defense genes can also occur but at a time at which no effect on nematodes is observed, as we found for cucumber. The result of the *B. firmus*-cucumber interaction could be explained by the influence of root exudates, such as *p*-coumaric acid, on the growth of the bacteria in the rhizosphere, in turn affecting the concentration of bacterial compounds, such as surfactin, reported as plant resistance elicitors (Cawoy et al., 2014; Zhou et al., 2018).

The results of our study have shown that *B. firmus* I-1582 is a nematode antagonist that can act by itself and through the plant by induction of plant defenses, a mechanism that is plant species dependent. These findings are particularly interesting for the development of strategies aimed to maximize the efficacy of *B. firmus* I-1582-based formulations against RKN. Indeed, the application of formulates to the substrate of tomato plants seven days before transplantation will allow root colonization and early induction of resistance to nematode infection and reproduction. Moreover, the bacterium could also act by itself reducing nematode egg viability and consequently the potential of inoculum. In this regard, even if the bacterium would not have an effect on cucumber, it might have a direct effect when applied to the soil. In Greece, broad application of the *B. firmus* formulate significantly suppressed the number of females in roots and disease severity and/or J2 in soil after cropping cucumber in greenhouses from May to September at environmental temperatures ranging from 20 to 45°C (Giannakou et al., 2004). Giannakou et al. (2007) assessed the efficacy of the bacterial formulate alone and in combination with soil solarization against *M. incognita*. The number of

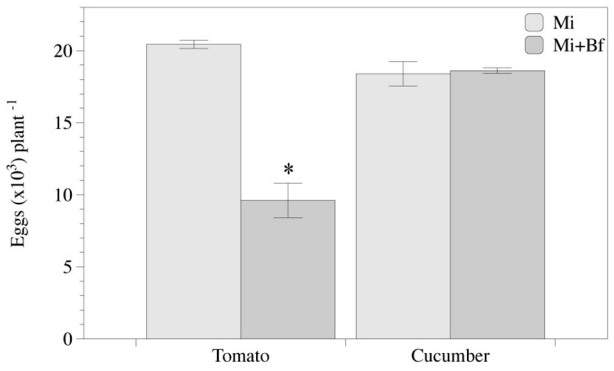

FIGURE 7 | *Meloidogyne incognita* reproduction is affected in *Bacillus firmus* I-1582 primed tomato plants but not in cucumber. Number of eggs in tomato and cucumber plants 40 days after inoculation with *M. incognita* (Mi) or co-inoculated with 10^9 CFU of Bf I-1582 one week after transplanting and with 200 juveniles of *M. incognita* 2 weeks after transplanting (Mi+Bf). Each value is mean ± standard error of three replicates. Asterisk indicates significant differences between treatments per each plant species according to the non-parametric Wilcoxon test in tomato or Student's *t*-test in cucumber ($P < 0.05$).

nematodes in roots and the galling index of the following cucumber crop were reduced in *B. firmus* I-1582 treated plots compared to the control, but these parameters were higher than in plots treated with the chemical nematicide Basamid® (active ingredient: dazomet). Nonetheless, no differences were found between plots treated with the chemical nematicide and the application of the bacterial strain just after soil solarization for 30 days with soil temperatures ranging from 32 to 40.5°C at 15 cm depth. In a pot experiment, the viability of J2 and eggs was suppressed after daily exposure at 35 and 40°C for 4 h followed by 20 h at 27–30°C for 2 and 4 weeks, respectively. In our study, the optimal temperature for bacterial growth and biofilm formation was recorded at 35°C. Thus, the application of the bacteria in summer when soil temperatures are around the optimal for bacterial growth and biofilm formation, will be the best time for nematode management in soil, reducing nematode densities and consequently the disease severity and crop yield losses.

CONCLUSION

In conclusion, *B. firmus* I-1582 is a nematode antagonist able to degrade and colonize *Meloidogyne* eggs by itself, and also able to induce plant systemic resistance. However, this second effect varies depending on the host plant. This bacterial isolate is active across a wide range of temperatures, with an optimum of 35°C. This temperature is suboptimal for *Meloidogyne,* which could further enhance its antagonistic activity. Additional studies are required for maximizing its antagonistic potential and to design successful RKN management strategies, such as the bacterial capability to induce resistance in other important vegetable crops frequently included in rotation sequences; the putative effect of the induced resistance against virulent nematode populations to selected *R*-genes, as observed with the additive effects of resistance induced by *Trichoderma asperellum* isolate T34 in tomato with the Mi1.2 gene against a virulent *M. incognita* population (Pocurull et al., 2020) and the optimal timing for *B. firmus* I-1582-based formulations application under field conditions, among others.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

FS and NE conceived, designed, supervised the experiments, the data collection, and analyses. ZG performed the experiments, analyzed the data, and wrote the draft of the manuscript. NE and DB-A transformed the bacterial strain. NE, MC, JA, and PL-A performed the nematode-bacteria and plant-bacteria laser-scanning confocal microscopy study. NE, ES, and ZG performed the molecular analysis. TG provided the reagents, materials, and advice. NE, ES, MC, PL-A, TG, and FS wrote the final version of the manuscript. All authors edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00796/full#supplementary-material

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SYSTEMATIC REVIEW
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A Systematic Review of the Effects of Arbuscular Mycorrhizal Fungi on Root-Lesion Nematodes, *Pratylenchus* spp.

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Root-lesion nematodes (*Pratylenchus* spp.) and arbuscular mycorrhizal fungi (AMF) occupy the same ecological niche in the phytobiome of many agriculturally important crops. Arbuscular mycorrhizal fungi can enhance the resistance or tolerance of a plant to *Pratylenchus* and previous studies have been undertaken to investigate the relationship between these organisms. A restructuring of the AMF phylum Glomeromycota has reallocated the species into genera according to molecular analysis. A systematic review of the literature was synthesized to assess the interaction between *Pratylenchus* spp. and AMF using the revised classification. Plants inoculated with AMF generally exhibited greater tolerance as demonstrated by increased biomass under *Pratylenchus* pressure. Species of AMF from the order Diversisporales tended to increase *Pratylenchus* population densities compared to those from the order Glomerales. Species from the genera *Funneliformis* and *Glomus* had a reductive effect on *Pratylenchus* population densities. The interaction between AMF and *Pratylenchus* spp. showed variation in responses as a result of cultivar, crop species, and AMF species. Putative mechanisms involved in these interactions are discussed.

Keywords: arbuscular mycorrhizal fungi, *Pratylenchus*, root-lesion nematodes, phytobiome interactions, Glomeromycota, systematic review

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INTRODUCTION

Pratylenchus spp. or root-lesion nematodes, are migratory endoparasites (Singh et al., 2013). They feed and move through the root cortex, penetrating parenchyma cells with their stylet, excreting cell degrading enzymes, ingesting the cellular contents, and destroying cortical tissue. This results in necrotic lesions, loss of root function and consequently, reductions in plant vigor, and yield of economic products (Jones et al., 2013).

Root-lesion nematodes are polyphagous and have the broadest host range of all plant-parasitic nematodes. They are responsible for substantial yield losses of many important crop species including cereals, legumes, sugarcane, coffee, banana, potato, vegetables and fruit trees (Castillo and Vovlas, 2007). There are over 68 recognized species of *Pratylenchus* associated with the phytobiome and they are distributed in diverse habitats worldwide (Castillo and Vovlas, 2007). Historically, *Pratylenchus* spp. were distinguished on the basis of their morphometric characteristics. With the advent of molecular techniques, differences in the sequences of ribosomal DNA can distinguish

between species despite high levels of intraspecific variation in some *Pratylenchus* spp. High levels of intraspecific variability occur within some *Pratylenchus* spp. such as *P. coffeae* and *P. penetrans* while other species exhibit less intraspecific internal transcribed spacer (ITS) variation, for example, *P. goodeyi* and *P. vulnus* (de Luca et al., 2011; Jones et al., 2013).

Arbuscular mycorrhizal fungi (AMF), from the phylum Glomeromycota are a ubiquitous group of soil microorganisms associated with the phytobiome. Arbuscular mycorrhizal fungi form a complex symbiosis with land plants which originated in the Ordovician period 400 million years ago (Parniske, 2008). They have remained morphologically unchanged since then, forming an intrinsic part of ecosystem functionality (Powell and Rillig, 2018). These obligate biotrophs form beneficial mutualistic associations with the roots of an estimated 80% of land plants including many agriculturally important crop species with the notable exception of most species in the families *Brassicaceae* and *Chenopodiaceae* (Lambers and Teste, 2013). Their characteristic arbuscules (microscopic tree-like structures) within the root cortical cells of compatible plants enable the photosynthetically derived organic compounds supplied by the plant to be exchanged for inorganic nutrients and water supplied by the fungus from the soil. The fungus also aids in the stabilization of soil aggregates through hyphal binding and exudation of glomalin (Smith and Read, 2008; Leifheit et al., 2014). It is estimated that up to 20% of the photosynthetic carbon of plants is allocated to maintaining the fungal association (Smith and Read, 2007). This carbon cost to the plant is outweighed by the many benefits conferred by the fungi, foremost of which are improved acquisition by the fungal hyphae of immobile nutrients from the soil such as phosphorus (P) and zinc (Zn) (Parniske, 2008).

Arbuscular mycorrhizal fungi have been promoted as a natural tool to maintain and promote sustainable agriculture due to their role as natural biofertilizers; increasing the levels of nitrogen (N), P and Zn in the crop (Thompson, 1993; Parniske, 2008; Smith et al., 2011; Baum et al., 2015; Berruti et al., 2016). They also play a role in drought tolerance (Zhao et al., 2015) and as bio-protectants against fungal, bacterial, and nematode pathogens (Whipps, 2004; Pozo and Azcón-Aguilar, 2007; Veresoglou and Rillig, 2012; Yang et al., 2014).

Early classifications defined species within the order Glomerales of the phylum Glomeromycota on the basis of spore morphology (Morton and Benny, 1990). Schüßler and Walker (2010) restructured the phylum Glomeromycota according to molecular phylogenies based on the small subunit (SSU) rRNA gene, the large subunit (LSU) rRNA gene, β-tubulin sequence data and the ITS region. Consequently, the current classification of the order Glomerales consists of two families — the *Glomeraceae* and the *Claroidoglomeraceae*. A number of *Glomus* species have been transferred to the genera *Funneliformis* and *Rhizophagus*. **Table 1** shows the phylum Glomeromycota and the subdivisions into the orders Glomerales, Diversisporales, Archaeosporales, and Paraglomerales (Redecker et al., 2013).

Plant-parasitic nematodes are classified according to their feeding strategies. These include (i) ecto-parasitic nematodes which feed externally on root cells and remain in the rhizosphere such as *Tylenchorhynchus* spp., (ii) migratory endo-parasitic nematodes which enter the plant root, feed, and move through the root tissues destroying cells as they migrate such as *Pratylenchus* spp., and, (iii) sedentary endo-parasitic nematodes which convert vascular cells into specialized feeding cells where they remain, such as the root-knot nematodes (*Meloidogyne* spp.) and the cyst nematodes (*Heterodera* and *Globodera* spp.) (Decraemer and Hunt, 2013).

TABLE 1 | Classification of the phylum Glomeromycota according to Redecker et al. (2013).

Order	Family	Genus*
Diversisporales	Diversisporaceae	*Tricispora*
		Otospora
		Diversispora
		Corymbiglomus
		Redeckera
	Acaulosporaceae	***Acaulospora***
	Sacculosporaceae	*Sacculospora*
	Pacisporaceae	*Pacispora*
	Gigasporaceae	***Scutellospora***
		Gigaspora
		Intraornatospora
		Paradentiscutata
		Dentiscutata
		Centraspora
		Racocetra
Glomerales	Claroideoglomeraceae	***Claroideoglomus***
	Glomeraceae	***Glomus***
		Funneliformis
		Septoglomus
		Rhizophagus
		Sclerocystis
Archaeosporales	Ambisporaceae	*Ambispora*
	Geosiphonaceae	*Geosiphon*
	Archaeosporaceae	*Archaeospora*
Paraglomerales	Paraglomeraceae	*Paraglomus*

**Genera in bold were considered in this review.*

The coexistence of AMF and nematodes in the phytobiome has prompted a number of investigations into their interactive effects on plants (reviews: Pinochet et al., 1996; meta-analyses: Borowicz, 2001; Hol and Cook, 2005; Veresoglou and Rillig, 2012; Yang et al., 2014). Published meta-analyses describe the generally suppressive effect that AMF have on nematodes (Veresoglou and Rillig, 2012; Yang et al., 2014). These analyses included nematodes belonging to different genera and they grouped plant-parasitic nematodes into their feeding modes (sedentary or migratory). AMF reduced the numbers of the sedentary endo-parasitic nematodes (*Meloidogyne, Heterodera*, and *Globodera* spp.) and the ectoparasitic nematodes (*Tylenchorhynchus* spp.). However, some analyses showed an increase in migratory

endo-parasitic nematode numbers on inoculation with AMF (Borowicz, 2001; Hol and Cook, 2005). Grouping the nematodes into their broad feeding modes has the effect of obscuring the data on interactions of AMF with *Pratylenchus* spp. and those with other migratory endo-parasites including *Radopholus* spp. and *Hirschmanniella* spp.

Due to the ubiquitous distribution and the great economic importance of *Pratylenchus* spp. to agricultural crops worldwide, this systematic review examines the relationship exclusively between *Pratylenchus* spp. and AMF taking into account the current classification of AMF genera. All life stages of *Pratylenchus* spp., adults, juveniles, and eggs occupy the same root cortex tissue as the AMF structures of hyphae, arbuscules, and vesicles (Pinochet et al., 1996) and co-occur with AMF extraradical hyphae and spores in the rhizosphere soil.

The aims of this review are to determine (a) the responses in *Pratylenchus* population densities to AMF, (b) the effects of AMF on the growth of plants infested with *Pratylenchus* and, (c) the effects of degree of AMF colonization on *Pratylenchus* population density. The outcomes of the systematic review are discussed in relation to putative mechanisms involved in the interaction between *Pratylenchus* spp. and AMF. These mechanisms may include: (a) enhanced plant tolerance to *Pratylenchus* as a result of increased nutrient uptake and altered root morphology, (b) direct competition between *Pratylenchus* and AMF for resources and space, (c) effects on *Pratylenchus* through plant defense mechanisms such as induced systemic resistance in the plant from AMF colonization, and (d) altered rhizosphere interactions (Pozo and Azcón-Aguilar, 2007; Schouteden et al., 2015).

METHODS

Selection of Studies

A systematic review of the literature was performed according to PRISMA systematic review guidelines (Moher et al., 2009). Studies investigating interactions between *Pratylenchus* spp. and AMF were obtained from the databases,—Web of Science (www.webofknowledge.com), SCOPUS (https://www.scopus.com) and Google Scholar (https://scholar.google.com/).

The search parameters included the following terms, "*Pratylenchus*," "arbuscular mycorrhizal fungi" AND "root-lesion nematode." The papers were further screened to select original research with quantitatively measured data of the following response variables: (a) effects of AMF on *Pratylenchus* population densities, (b) effects of *Pratylenchus* spp. on degree of AMF colonization in the roots (mycorrhization), and (c) effects of both organisms on plant biomass. Other pre-requisites for eligibility for inclusion in the review were (a) studies with one or more AMF species, but not mixed treatments with other beneficial organisms, (b) studies with *Pratylenchus* species alone not mixed with other plant-parasitic nematodes, and (c) studies with a non-inoculated control. Reviews, meta-analyses and book chapters were excluded from the analyses, but the original research papers cited within were cross referenced and assessed for suitability for inclusion.

Analyses of Response Variables

The "nematode response" was calculated using the following formula:

$$nematode\ response = \frac{(Pratylenchus - Pratylenchus\ plus\ AMF)}{Pratylenchus} * 100 \quad (1)$$

where "*Pratylenchus*" is the final population density of *Pratylenchus* in nematode only treatments and "*Pratylenchus plus AMF*" is the population density of *Pratylenchus* in co-inoculated AMF and nematode treatments.

The "biomass response" was calculated using the following formula:

$$biomass\ response = \frac{(Pratylenchus\ biomass - Pratylenchus\ plus\ AMF\ biomass)}{Pratylenchus\ biomass} * 100 \quad (2)$$

Where "*Pratylenchus biomass*" is the plant biomass in nematode only inoculated treatments and "*Pratylenchus plus AMF biomass*" is the plant biomass in co-inoculated AMF and nematode treatments. Biomass data were expressed as shoot, root and total biomass where available.

The "AMF response" was calculated using the following formula:

$$AMF\ response = AMF\ \%\ colonisation - AMF\ \%\ colonisation\ plus\ Pratylenchus \quad (3)$$

where "*AMF % colonization*" is the percentage of mycorrhization of plants with AMF alone and "*AMF % colonization plus Pratylenchus*" is the percentage of mycorrhization of plants co-inoculated with AMF and nematodes.

The effect of inoculation with AMF on the *Pratylenchus* population density was categorized as decrease, no effect, or increase based on statistical significance ($P<0.05$) of studies in the original publications. A chi-squared test for independence was performed to assess the relationship between order of AMF (Glomerales and Diversisporales) and effect on *Pratylenchus* population densities. Chi-squared values were calculated from two-way contingency tables (Steel and Torrie, 1960) of AMF order by *Pratylenchus* density effect for the 56 studies using the following function:

$$\chi^2 = \Sigma\{(\text{observed number} - \text{expected number})^2 / \text{expected number}\} \quad (4)$$

The percentage AMF colonization of the roots of the plants in these three categories of AMF effects on *Pratylenchus* population densities for the studies with relevant data was subjected to one-way analysis of variance (ANOVA) using GenStat (VSN International, 2014).

The data were examined under other independent groupings such as (a) restructured AMF genera according to the current classification by Schüßuler and Walker (Schüßler and Walker, 2010) and (b) host plant functional group (grasses, trees, herbs, shrubs).

RESULTS

The initial search conducted on all available literature in the three databases provided 519 potential papers for inclusion. Further screening by removing duplicates and ineligible papers resulted in 22 full text articles selected for the systematic review (**Table 2**). Experiments within papers were treated as separate studies when; (a) two or more AMF species were studied independently, (b) more than one plant cultivar was included, and (c) more than one time of inoculation was used. If there were various times of assessment for plant biomass over multiple years, the most recent data set was used. In total, 60 studies were analyzed (**Supplementary Table 1**).

Table 3 shows the response of *Pratylenchus* sp., arbuscular mycorrhizal fungi (AMF) and plants to co-inoculation of AMF and *Pratylenchus* sp. compared to *Pratylenchus* sp. alone in glasshouse and microplot experiments. The data is statistically significant as stated in the original papers. The majority of the crops assessed were agriculturally or horticulturally important with the exception of dune grass (*Ammophilia arenaria*). In general, the experiments were undertaken in glasshouses with some transplanting of pre-inoculated AMF colonized plants to field microplots. There were 14 individual species of AMF used

TABLE 2 | PRISMA Flow Diagram for eligible articles to include in the qualitative review.

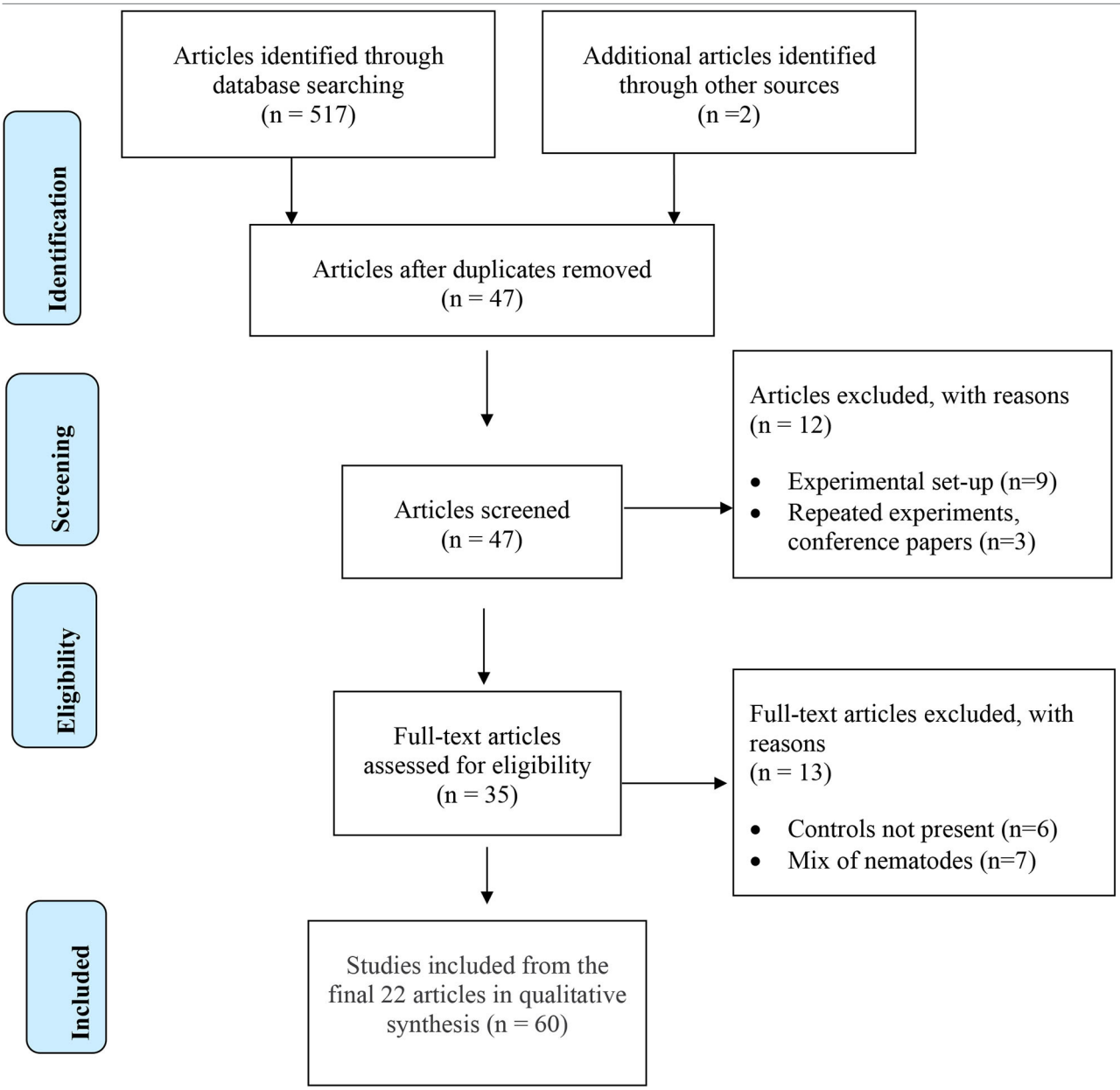

TABLE 3 | Response of *Pratylenchus* spp., arbuscular mycorrhizal fungi (AMF) and plants to co-inoculation of AMF and *Pratylenchus* spp. compared to *Pratylenchus sp.* alone in glasshouse and microplot experiments.

Plant species (common name)		*Pratylenchus* species	Response to AMF-*Pratylenchus* interaction (%)					
	AMF species		Nematode	AMF	Biomass	Shoot wt	Root wt	Reference
GRASS								
Triticum aestivum (wheat)	Mix: *Claroideoglomus etunicatum, F. coronatum, Rhizophagus irregularis, F. mosseae*	*P. neglectus*	47 to 117[1]	ns	↓30 to ↓40[1]	ND	↓31 to ↓44[1]	Frew et al., 2018
Zea mays (maize)	*R. clarus*	*P. brachyurus*	990	ND	ND	ns	ND	Brito et al., 2018
	Dentiscutata heterogama		353	ND	ND	ns	ND	
	Gigaspora rosea		447	ND	ND	ns	ND	
	C. etunicatum		441	ND	ND	ns	ND	
	G. margarita		353	ND	ND	ns	ND	
	S. calospora		900	ND	ND	ns	ND	
Ammophila arenaria (dune grass)	*Glomus* sp.	*P. dunensis*	↓38[2]	ns	↓44[2]	ND	ND	Rodríguez-Echeverría et al., 2009
	Glomus sp.	*P. penetrans*	↓67[2]	ns	ns	ND	ND	
	Mix: *Glomus* spp., *S. castanea*	*P. penetrans*	↓47 to ↓86[3]	ns	ns	ND	ns	de La Peña et al., 2006
Tree								
Cydonia oblonga (quince)	*R. intraradices*	*P. vulnus*	ns	↓26	ND	65	51	Calvet et al., 1995
Malus domestica (apple)	*C. claroideum*	*P. penetrans*	ns	ND	ND	ND	ns	Ceustermans et al., 2018
	Acaulospora longula		ns	ND	ND	ND	ns	
	C. claroideum, A. longula		ns	ND	ND	ND	165	
	R. intraradices		ns	ND	ND	ND	ns	
	AMF species mix (13)		↓97	ND	ND	ND	ns	
	C. etunicatum		ns	ns	ns	8	ns	Forge et al., 2001*
	R. aggregatus		ns	ns	ns	ns	ns	
	R. clarus		ns	ns	ns	ns	ns	
	F. mosseae		ns	ns	19 to 45[1]	9 to 54[1]	1 to 32[1]	
	R. intraradices		ns	ns	19 to 43[1]	12 to 49[1]	5 to 37[1]	
	G. versiforme		ns	ns	ns	47	ns	
Malus silvestris (crab apple)	*F. mosseae*	*P. vulnus*	↓51	ns	ND	201	142	Pinochet et al., 1993
Pyrus communis (pear)	*R. intraradices*		↓57	ns	ND	403	209	Lopez et al., 1997
	F. mosseae		↓63	ns	ND	341	202	
Prunus mahaleb (cherry)	*R. intraradices*		ns	ns	ND	89	78	Pinochet et al., 1995a
Prunus persica (peach)	*F. mosseae*		↓42	ns	ND	ns	ns	Pinochet et al., 1995b
Prunus cerasifera X *P. munsoniana* (Prunus rootstock)	*R. intraradices*		ns	↓14	ND	ns	28	Pinochet et al., 1998
	F. mosseae		ns	↓14	ND	ns	ns	
Prunus cerasifera (cherry plum)	*F. mosseae*		ns	↓34[1]	ND	ns	86[1]	Camprubi et al., 1993
Herb								
Musa sp. (banana)	*F. mosseae*	*P. coffeae*	↓76[1]	ns	ND	175 to 433[1]	192 to 310[1]	Elsen et al., 2003a
	F. mosseae		↓79 to ↓80[1]	↓17 to ↓24[1]	ND	ns	ns	Elsen et al., 2003b
	F. mosseae	*P. goodeyi*	ns	ND	ND	16	ns	Jaizme-Vega and Pinochet , 1997
	R. aggregatus		ns	ND	ND	14	ns	
	R. intraradices		ns	ND	ND	8	ns	
Phaseolus vulgaris (common bean)	*R. fasciculatus*	*P. penetrans*	ns	ND	ND	ND	ND	Elliott et al., 1984

(Continued)

TABLE 3 | Continued

Plant species (common name)	AMF species	*Pratylenchus* species	Response to AMF-*Pratylenchus* interaction (%)					Reference
			Nematode	AMF	Biomass	Shoot wt	Root wt	
Daucus carota (carrot)	*F. mosseae*	*P. penetrans*	↓48	ns	207	ND	ND	Talavera et al., 2001
Lycopersicon esculentum (tomato)	*F. mosseae*		↓87	ns	ND	ns	ns	Vos et al., 2012
Ananas comosus (pineapple)	*Glomus* sp.	*P. brachyurus*	↓24 to ↓74[4]	↓9 to ↓32[4]	ND	105 to 359[4]	50 to 269[4]	Guillemin et al., 1994
Shrub								
Gossypium hirsutum (cotton)	*Gigaspora margarita*	*P. brachyurus*	↓66	ND	ND	556	544	Hussey and Roncadori, 1978
Coffea arabica (coffee)	*A. mellea*		1049[3]	↓32[3]	946[3]	ND	ND	Vaast et al., 1997
	R. clarus		432[3]	↓26[3]	504[3]	ND	ND	

*[1]Cultivar dependent; [2]AMF, country of origin dependant; [3]Time of inoculation dependent; [4]Cultivar and time of inoculation dependent; ns, non-significant result; ND, not determined. Nematode response, difference between Pratylenchus alone and co-inoculated with AMF; AMF response, difference between percentage mycorrhization of AMF alone and co-inoculated with Pratylenchus; Biomass response; difference between Pratylenchus alone and co-inoculated with AMF; ↓ indicates negative effect of AMF x Pratylenchus interaction; *Glasshouse data only.*

in 43 studies, one undetermined species in ten studies, and a mix of AMF species in seven studies. These species came from both the order Glomerales which included the genera *Rhizophagus, Glomus, Funneliformis, Claroideoglomus,* and the order Diversisporales, which included the genera *Acaulospora, Dentiscutata, Gigaspora*, and *Scutellospora.*

The studies involved seven *Pratylenchus* spp. namely *P. penetrans, P. vulnus, P. neglectus, P. coffeae, P. goodeyi, P. brachyurus* and *P. dunensis*. These species reviewed are many of the species of *Pratylenchus* causing the most economic damage worldwide (Jones and Fosu-Nyarko, 2014).

Responses in *Pratylenchus* Population Densities to AMF

The effects of AMF inoculation on *Pratylenchus* population densities varied from a decrease in population densities ($n = 22$), no effect on *Pratylenchus* population densities ($n = 28$), to an increase in *Pratylenchus* population densities ($n = 10$).

The taxonomic order of AMF species used had an effect on *Pratylenchus* densities, whereby inoculation with species from the order Glomerales tended to decrease *Pratylenchus* population densities compared with species from the order Diversisporales which tended to increase *Pratylenchus* population densities (**Table 4**). Although there were fewer studies with comparisons for Diversisporales than for Glomerales, the differences in response between these groupings were highly significant (**Table 4**). Within the Glomerales, inoculation with the genera *Glomus* and *Funnelifomis* had a neutral to reductive effect on *Pratylenchus* population densities.

Increases in *Pratylenchus* population densities due to AMF inoculation in studies subdivided in relation to the host plant functional group were predominantly found in the grasses (increases in 8 out of 15 studies). No increase in *Pratylenchus* population densities were found in trees (0 increases in 24 studies), or herbs (0 increases in 16 studies).

Effects of AMF on the Growth of Plants Infested With *Pratylenchus*

Plant shoot biomass increased when AMF were co-inoculated with *Pratylenchus* compared with infection with *Pratylenchus* alone. From the 34 studies with data providing comparisons on shoot biomass, 24 showed an increase in shoot biomass while 10 had no effect. No studies showed a reduction in shoot biomass. Most studies calculated shoot biomass ($n = 35$) and root biomass independently ($n = 41$), with fewer reporting results on total biomass ($n = 28$). From these 28 studies, eight showed an increase in total plant biomass, and three studies a decrease in total plant biomass with 17 having no significant effect.

The change in root biomass between plants inoculated with *Pratylenchus* and the plants co-inoculated with AMF and *Pratylenchus* is shown in **Table 3**. The majority of the studies showed an increase in root biomass when inoculated with AMF ($n = 22$) in the presence of *Pratylenchus* with the exception of two studies by Frew et al. (2018).

Effects of Degree of AMF Colonization on *Pratylenchus* Population Density

There were 58 studies with data on the degree of AMF colonization of the roots. In most studies there was a decrease ($n = 21$) or no effect ($n = 27$) on *Pratylenchus* population densities, which were associated with relatively high percentage AMF colonization of the roots (43.9 and 42.2% respectively), compared to an increase in *Pratylenchus* population densities ($n = 10$), which were associated with a significantly lower percentage AMF colonization (20.1%) (**Table 5**).

DISCUSSION

This review is the first to examine the effects of specific genera and order of AMF acting on *Pratylenchus* population densities and demonstrates that the taxonomic order of AMF

TABLE 4 | Number of studies investigating AMF-*Pratylenchus* interaction included in the systematic review and the effect of AMF order on *Pratylenchus* populations.

Order	Genus	Effect on *Pratylenchus* populations			Total studies
		Increase	No effect	Decrease	
Glomerales	*Rhizophagus*	2	12	1	15
	Glomus	0	2	8	10
	Funneliformis	0	8	8	16
	Claroideoglomus	1	2	0	3
	AMF mix (*Claroideoglomus*, *Rhizophagus*, *Funneliformis*)	2	0	1	3
	Total	5	24	18	47
Diversisporales	*Acaulospora*	1	2	0	3
	Dentiscutata	0	1	0	1
	Gigaspora	2	0	1	3
	Scutellospora	2	0	0	2
	Total	5	2	2	9
		$\chi 2 = 10.43$ with 2 d.f. $P < 0.01$			

TABLE 5 | Effects of AMF inoculation on change in *Pratylenchus* population densities in relation to degree of AMF colonization in the roots.

Change in *Pratylenchus* population density	Number of comparisons	AMF % colonization in presence of *Pratylenchus*		
		$\log_e$	SE[a]	BTM (%)[b]
Decrease	21	3.7818	0.1419	43.9
No effect	27	3.7421	1.1252	42.2
Increase	10	2.9994	0.2057	20.1

Fprobability, 0.006 from ANOVA of the transformed data.
[a]*SE, standard error.*
[b]*BTM, back-transformed mean.*

has a significant influence on *Pratylenchus* population densities. Previous reviews and meta-analyses showed a varied response of AMF on migratory endo-parasites ranging from a suppressive (Veresoglou and Rillig, 2012; Yang et al., 2014) to a stimulatory effect (Borowicz, 2001; Hol and Cook, 2005).

Variation in functionalities between AMF families has been reported (Smith et al., 2004). Members of the Glomeraceae are typically fast colonizers, concentrating their hyphae within the plant roots and can increase P uptake and promote plant growth under pathogen attack and drought stress (Klironomos, 2000; Hart and Reader, 2002; Maherali and Klironomos, 2007; Yang et al., 2015; Seymour et al., 2019). Members of the Diversisporales are typically slower to colonize roots, concentrating hyphae externally to the plant root in the soil and are effective at enhancing plant phosphorus uptake (Klironomos, 2000; Hart and Reader, 2002; Maherali and Klironomos, 2007). However, from the studies in this review, there was lack of data on the percentage of AMF colonization of the controls in the order Diversisporales ($n = 2$) therefore it remains unclear if Diversisporales are slower to colonize from these studies.

From our review, species from the genera *Glomus* or *Funneliformis*, in the order Glomerales decreased or had no significant effect on the *Pratylenchus* population densities compared with *Rhizophagus* and *Claroideoglomus*. The difference in effects that AMF genera have on *Pratylenchus* population densities could be due to differences in the secondary metabolites produced under the symbiotic relationship. For example, in tomato, although the metabolic pathways altered by the AMF symbiosis were similar, different metabolites were produced, depending on inoculation with *F. mosseae* or *R. irregularis* (Pozo et al., 2002). An increase in the accumulation of bioactive forms of jasmonic acid was found in roots colonized by *F. mosseae* (Rivero et al., 2015). Jasmonic acid and its derivative methyl jasmonate play a role in plant defense against herbivores and they can reduce susceptibility of plants to infestation by *Pratylenchus* (Soriano et al., 2004). Root metabolites may influence populations of plant parasitic nematodes by acting as attractants, repellents or affecting hatch rates of nematodes (Sidker and Vestergård, 2019). Mycorrhizal colonization can increase phenolics such as ferulic acid and gallic acid in the host plants (López-Ráez et al., 2010; Li et al., 2015). Ferulic acid inhibits mobility and is toxic to the burrowing nematode *R. similis* but is ineffective against *Pratylenchus penetrans* (Wuyts et al., 2006). Gallic acid acts as a nematicide to the root-knot nematode *M. incognita* (Seo et al., 2013). High constitutive total phenol contents were found in synthetic hexaploid wheat genotypes resistant to *P. thornei* combined with high levels of induced phenol oxidases (Rahaman et al., 2020). These studies indicate that the biochemical responses of host plants to both inoculation with AMF and infestation by plant-parasitic nematodes are highly complex.

Even within populations of a single species of AMF, there is a high genetic variability which may affect the host/fungal relationship (Koch et al., 2006, 2017). Variations in the effects that a single species of AMF have on *Pratylenchus* population

densities were observed in the studies by Elsen et al. (2003b) and Jaizme-Vega and Pinochet (1997). Both studies used the same cultivar of banana and the same species of AMF, but obtained different results depending on the *Pratylenchus* sp. tested. Elsen et al. (2003b) stated that it was difficult to explain the contrary results, however, the AMF strain and the environmental conditions differed between experiments. As a different isolate of *F. mosseae* was used as inoculum, it is important to emphasize the traceability of isolates that are used in experiments. A similar observation was made in dune grass whereby *Pratylenchus* sp. were only reduced in the interaction with a community of AMF isolated from Wales and not from an AMF community isolated from Belgium (Rodríguez-Echeverría et al., 2009). This highlights the need to study interactions between specific crops, cultivars and AMF species or communities.

Plant functional group influenced *Pratylenchus* population densities in grasses but not in herbs and trees. Interestingly, response to AMF can be attributed to plant functional groups in which non-nitrogen fixing forbs and woody plants, and C4 grasses benefit more in plant growth by the fungal association, compared to nitrogen fixing plants and C3 grasses (Hoeksema et al., 2010). However, Yang et al. (2016) concluded that nitrogen fixing plants had a greater mycorrhizal growth response only when the host plant was a forb and not woody. A practical application to improve tolerance, or plant growth, when *Pratylenchus* is present may therefore be to pre-inoculate tree species with AMF prior to transplanting into orchards, taking into account the interaction between cultivars, their mycorrhizal dependency and AMF species used as inoculum sources (Pinochet et al., 1996). The potential of AMF inoculum conferring benefits to crop production in high economic value vegetable crops has been reviewed by Baum et al. (2015). These include advantages such as increases in yield, increases in commercial quality of the crop, protection against nematodes and other pathogens, tolerance to drought and other abiotic stressors and nutrient uptake. As the interaction between host, AMF inoculum and environment can be very specific, future research is needed to optimize the inoculation protocols to target specific crop production limitations.

The outcomes of the present systematic review, in relation to putative mechanisms involved in the interaction between *Pratylenchus* spp. and AMF, are discussed below.

Enhanced Plant Tolerance

Plant shoot biomass increased when AMF were co-inoculated with *Pratylenchus* compared with infection with *Pratylenchus* alone. A number of studies investigated tolerance to *Pratylenchus* spp. as a reflection of increasing vegetative plant nutrition. AMF can increase the uptake of P and other nutrients such as Zn from the soil (Parniske, 2008; Seymour et al., 2019). This increase in nutrition can lead to a greater plant biomass response conferring a compensatory effect against the damage done by nematodes. Previous studies have shown that AMF confers tolerance to *Pratylenchus* spp. by compensating for root damage caused by *Pratylenchus* spp. through increasing the uptake of P and other micronutrients, such as Fe, Mn, Zn, and Cu (Calvet et al., 1995; Pinochet et al., 1998). However, improvement in the nutritional status of the plant is not believed to be wholly responsible for the biocontrol effect of AMF (Bødker et al., 1998; Jung et al., 2012).

Tolerance conferred by AMF to a crop under *Pratylenchus* pressure has been described in the majority of the reviewed papers ($n = 41$) with the exception of the following; peach, *Musa* sp., maize, tomato, dune grass and wheat (Pinochet et al., 1995b; Elsen et al., 2003a; Rodríguez-Echeverría et al., 2009; Vos et al., 2012; Brito et al., 2018; Frew et al., 2018). This may be a reflection of the mycorrhizal dependency of the cultivars assessed as some tomato and wheat cultivars have a low mycorrhizal dependency (Smith et al., 2009) while cultivars of maize, *Musa* sp. and peach generally have higher mycorrhizal dependency (Pinochet et al., 1995b; Kaeppler et al., 2000; Elsen et al., 2003a). A study by Martín-Robles et al. (2018) found that domesticated crops benefit more from the symbiosis with AMF under P limiting conditions. It is worthwhile to note that most of the studies analyzed in this review were undertaken in low P experimental conditions where AMF function most efficiently (**Supplementary Table 1**).

The studies assembled in **Table 3** demonstrate the predominantly beneficial effects AMF have on crop species, alleviating the damage to the root and shoot biomass caused by *Pratylenchus*. There were only three studies where AMF decreased total biomass and root weight when co-inoculated with *Pratylenchus*. These studies were on wheat and dune grass, both C3 crops (Rodríguez-Echeverría et al., 2009; Frew et al., 2018). Variations in root morphology between C3 and C4 grasses determine their dependency on the mycorrhizal symbiosis (Hetrick et al., 1991), which may help explain the reduction in biomass. Wheat has a low to intermediate dependency on mycorrhiza depending on genotype (Lehnert et al., 2017) and modern plant breeding may contribute to a reduction in dependency on the mycorrhizal symbiosis by screening and selecting new varieties in high phosphate or highly fertile soils (Hetrick et al., 1993). However, a modern wheat cultivar Batavia was found to have high dependency on AMF colonization under drought conditions on a field site infested with *P. thornei* (Owen et al., 2010). Dune grass forms an association with AMF promoting plant growth (Tadych and Blaszkowski, 1999). de La Peña et al. (2006) suggested that evidence of biomass reduction in dune grass was related to a species-specific interaction between a geographically unique community of AMF from Wales and the species of *Pratylenchus* (*P. dunensis*) studied. Biomass reduction was not significant in another study of the interaction between AMF and *P. penetrans* on dune grass (de La Peña et al., 2006).

Previous reviews have also demonstrated this positive effect that AMF have on increasing plant growth under attack by migratory nematodes (Hol and Cook, 2005; Yang et al., 2014). This is contrary to the study by Borowicz (2001) that concluded AMF increased the negative effects of nematodes on plant biomass, indicating a reduced nematode tolerance.

The majority of studies showed an increase in root biomass in the presence of *Pratylenchus* when inoculated with AMF. *Pratylenchus* infestation negatively impacts root biomass, resulting in a reduction in the quantity and length of root branches (Fosu-Nyarko and Jones, 2016). Colonization by AMF can also result in alterations to root morphology, causing either an increase or decrease in root branching (Hooker et al., 1992;

Sikes, 2010). A study on morphological changes within the root system in *Musa* sp. under *Pratylenchus* pressure showed that AMF increased root branching counteracting the negative consequences of *Pratylenchus* infection (Elsen et al., 2003a). Berta et al. (1995) also demonstrated in cherry plum (*Prunus cerasifera)* that AMF increased the branching of all root orders. However, there were variable effects on root diameter depending on which genera of AMF were used.

Baylis (1975) hypothesized that plants with extensive fine root systems with long dense root hairs were less reliant on the mycorrhizal symbiosis in comparison to coarsely rooted plants. However, recent evidence suggests that coarse roots are not necessarily a good predictor of crop dependency on the AMF symbiosis (Maherali, 2014). A meta-analysis by Yang et al. (2016) found that although plants with fibrous roots responded less to mycorrhizal colonization than tap rooted plant species, this was only evident for C3 and not C4 grass species. Notwithstanding this, plants that have a highly branched root system may still benefit from the AMF association via other ecosystem functions such as pathogen protection (Newsham et al., 1995).

Competition for Space Between *Pratylenchus* and AMF

Degree of AMF colonization had an effect on the population densities of *Pratylenchus*. Inoculation with AMF that resulted in low levels of AMF colonization was associated with increases in *Pratylenchus* population densities compared with other cases with high levels of AMF colonization that were associated with decreases or no effects on *Pratylenchus* population densities. The nematode population density could also affect the rate of colonization by AMF indicating a competition between species. Both AMF and *Pratylenchus* occupy the same ecological niche within the root cortical cells as described in various crop species, for example, quince, cherry, peach, pear, banana, plum, and coffee (Calvet et al., 1995; Pinochet et al., 1995a,b, 1998; Lopez et al., 1997; Vaast et al., 1997; Elsen et al., 2003b). *Pratylenchus* sp. and AMF were considered to have competed for space within the cortical cells in quince, coffee, banana and dune grass (Calvet et al., 1995; Vaast et al., 1997; Elsen et al., 2003b; de La Peña et al., 2006).

Arbuscules are the metabolically active sites of exchange between the plant and the fungus and a mature mycorrhizal colonization of the plant, as evidenced by the production of arbuscules, has been thought to be the prerequisite for a biocontrol effect (Khaosaad et al., 2007). It has been hypothesized that a greater colonization of AMF in plant roots would lead to a greater biocontrol effect on nematodes.

Pratylenchus can affect the quantity and morphology of AMF within the root cortical cells. For example, in quince, AMF increased the production of arbuscules reflecting a metabolically active state under *Pratylenchus* infestation, compared to an increase in the production of vesicles in the absence of infestation (Calvet et al., 1995). In banana, nematodes reduced the frequency of colonization but not the intensity (Elsen et al., 2003b). In pineapple, although nematodes reduced the frequency of arbuscules when applied at a later time point during transplanting, they did not affect the efficiency of the symbiosis (Guillemin et al., 1994).

The time of inoculation was not a factor in how the nematode population densities responded to AMF inoculation. AMF was applied to the plants prior to nematode inoculation in the majority of studies ($n = 42$), which gave the symbiosis a chance to establish before being challenged with *Pratylenchus*. However, this established symbiosis was not reflected in a decrease in nematode population density, but may have aided the plant in tolerance to nematode infestation through increased vegetative growth as previously discussed.

Plant Defense and Induced Systemic Resistance

Mycorrhiza-induced resistance that can operate systemically can be effective against plant-parasitic nematodes and may contribute toward the biocontrol effect of AMF (Jung et al., 2012). Induced systemic resistance has no association with pathogenesis related proteins or salicylic acid but is regulated by jasmonic acids and ethylene (Pieterse et al., 1998).

There is little available research on induced systemic resistance by AMF against *Pratylenchus* as compared to other plant pathogens. However, using split root experiments, the systemic biocontrol effects of the AMF species *F. mosseae* and *R. irregularis* on *Pratylenchus* were demonstrated in banana and tomato. *Rhizophagus irregularis* induced a systemic suppression of *P. coffeae* and *R. similis* in banana, though the pathways involved in this suppression were not determined (Elsen et al., 2008). In tomato, inoculation with *F. mosseae* reduced the number of females of *P. penetrans* through a localized mechanism and the number of juveniles through a systemic mechanism (Vos et al., 2012). Contrary to this, only a localized suppression of *Pratylenchus* population densities was observed in dune grass (de La Peña et al., 2006).

Investigations into the metabolomics of AMF showed that AMF colonization increased the production of AMF plant signaling compounds and anti-herbivory defenses (Hill et al., 2018). There is still very little research available on the interactions between *Pratylenchus* and AMF on effects on the metabolome. Frew et al. (2018) reported that AMF reduced plant defense metabolites, specifically benzoxazinoids, which accounted for an increase in *P. neglectus* population densities in wheat. Studies involving root organ cultures of carrot showed significant suppressive effects of AMF on *P. coffeae* female population densities believed to be a result of biochemical changes in the mycorrhized root (Elsen et al., 2003c). Exudates from AMF can reduce the motility and penetration of sedentary nematodes (Vos et al., 2012) but little research has been done on their effects on migratory endo-parasites. An *in-vitro* chemotaxic assay on the migratory endo-parasite *R. similis* demonstrated that the exudation of a water-soluble compound, produced by mycorrhizal roots, reduced attraction at a pre-infection stage (Vos et al., 2012), but there is little information on how exudates affect *Pratylenchus* spp. Further research is needed to assess the mechanisms of AMF in influencing *Pratylenchus* population densities.

Alterations in the Rhizosphere

Alterations in chemical compounds in the rhizosphere as a result of interactions between plant-parasitic nematodes and AMF have been reviewed (Schouteden et al., 2015). These involve changes in exudation of sugars, organic acids, amino acids, phenolic compounds, flavonoids and strigolactones in AMF colonized plants as compared to non-AMF plants. AMF exudations into the rhizosphere promote beneficial microorganisms such as plant-growth promoting rhizobacteria (PGPR) (Jung et al., 2012; Javaid, 2017) and resultant changes can be induced systemically, influencing the bacterial community structure (Marschner and Baumann, 2003). This enhanced microbial activity around plant roots has been termed the mycorrhizosphere effect (Linderman, 1988). Plant growth promoting rhizobacteria have been implicated in nitrogen fixation, phosphate solubilization, modulating phytohormone levels and the production of antibiotics and lytic enzymes (Glick, 2012). Cameron et al. (2013) proposed that AMF and PGPR act together to increase plant defenses against biotic stressors in mycorrhiza-induced resistance. Studies on multipartite interactions between *Pratylenchus*, AMF, PGPR and crop hosts are lacking in the literature.

Species of PGPR in the genera *Pseudomonas*, *Bacillus*, *Streptomyces* and *Lysobacter* have been implicated in reducing *Pratylenchus* population densities (Walker et al., 1966; Stirling, 2014; Castillo et al., 2017), and some research has been conducted on the interaction between AMF and these PGPR. In strawberry, *Pseudomonas chlororaphis* suppressed populations of *P. penetrans* (Hackenberg et al., 2000) while extracts from the AMF species *R. irregularis* stimulated the growth of *Pseudomonas chlororaphis in vitro* (Filion et al., 1999). *Streptomyces* spp. can reduce *Pratylenchus* population densities (Meyer and Linderman, 1986; Samac and Kinkel, 2001) and they can also stimulate spore germination in *F. mosseae* and *Gigaspora margarita* (Tylka et al., 1991). This indicates a link between the three types of phytobiome organisms, though further research is needed to assess AMF and PGPR combined effects on *Pratylenchus* population densities.

LIMITATIONS OF THE REVIEW AND FUTURE RESEARCH

The crops assessed in this review were agriculturally or horticulturally important with the exception of dune grass (*Ammophilia arenaria*). Most studies looked at a single species of AMF alone and not in combination with species from different orders and genera of AMF, or other beneficial microbes such as PGPR. The taxonomic orders of AMF used in the studies reviewed were limited to the Glomerales and Diversisporales. Other orders such as the Archaeosporales and the Paraglomerales are also present in soils, though they are under-represented in experimental work. A study by Gosling et al. (2014), found a wide distribution of the Paraglomerales in agricultural soils in the UK. AMF species such as *F. mosseae* and *R. irregularis* have a tendency to be over represented in this type of experimental work due to their ease of multiplication in trap cultures. The studies in this review were undertaken in low P soils, predominantly in glasshouses, with some transplantations to microplots. Arbuscular mycorrhizal fungi function most efficiently under low to moderately high P conditions, and therefore the benefit of AMF in improving plant nutrition and plant biomass under *Pratylenchus* pressure could be overstated for agricultural systems receiving continued high rates of P fertilizers. Better matching of P fertilizer inputs to crop removal is required in some agricultural systems to avoid excessive levels of available P in soils for better harnessing of AMF functions, stewardship of global P supplies and environmental quality (Gianinazzi et al., 2010).

The number of studies in this highly specific review of the interaction between *Pratylenchus* spp. and AMF was limited to only 60 studies suitable for inclusion. Further research needs to be undertaken in the area, using a broad range of crop cultivars and AMF species from diverse orders to further increase our understanding of the relationship between these organisms in the rhizosphere.

Further research needs to be done in assessing the mechanisms involved in the effect of AMF on *Pratylenchus* population densities through investigations into induced systemic resistance and changes in the metabolome. As research is lacking on the effects of AMF, *Pratylenchus* and beneficial bacteria in the rhizosphere, more studies need to be undertaken on multipartite interactions between these organisms in crop hosts.

CONCLUSION

The interactions between *Pratylenchus* and AMF reveal some unique effects as influenced by crop species, crop cultivar, AMF order and AMF genus. Our review showed increased *Pratylenchus* densities in plants inoculated with species from the order Diversisporales. Inoculation with the AMF genera *Glomus* and *Funneliformis* from the order Glomerales, reduced or had no effect on *Pratylenchus* densities in host roots. AMF aids the tolerance of plants to *Pratylenchus* through increased vegetative growth. The biocontrol effect of AMF is likely to be a combination of increasing host tolerance, competition between organisms, and systemic resistance, though further research is needed to identify the mechanisms involved. Further studies will need to take into account the specific interactions between crop, cultivar and AMF species in both glasshouse and field trials.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JT and EG conceptualized the paper. EG performed the database search, collated the data, and drafted the manuscript. JT and EG conducted the statistical analyses. EG, KO, and RZ integrated information on tables. All authors contributed to revising

the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00923/full#supplementary-material

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Host-Induced Silencing of FMRFamide-Like Peptide Genes, *flp-1* and *flp-12*, in Rice Impairs Reproductive Fitness of the Root-Knot Nematode *Meloidogyne graminicola*

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Hada A, Kumari C, Phani V, Singh D, Chinnusamy V and Rao U (2020) Host-Induced Silencing of FMRFamide-Like Peptide Genes, flp-1 and flp-12, in Rice Impairs Reproductive Fitness of the Root-Knot Nematode Meloidogyne graminicola. Front. Plant Sci. 11:894. doi: 10.3389/fpls.2020.00894

Rice (*Oryza sativa* L.) is one of the major staple food crops of the world. The productivity of rice is considerably affected by the root-knot nematode, *Meloidogyne graminicola*. Modern nematode management strategies targeting the physiological processes have established the potency of use of neuromotor genes for their management. Here, we explored the utility of two FMRFamide like peptide coding genes, *Mg-flp-1* and *Mg-flp-12* of *M. graminicola* for its management through host-induced gene silencing (HIGS) using *Agrobacterium*-mediated transformation of rice. The presence and integration of hairpin RNA (hpRNA) constructs in transgenic lines were confirmed by PCR, qRT-PCR, and Southern and Northern hybridization. Transgenic plants were evaluated against *M. graminicola*, where phenotypic effect of HIGS was pronounced with reduction in galling by 20–48% in the transgenic plants. This also led to significant decrease in total number of endoparasites by 31–50% for *Mg-flp-1* and 34–51% for *Mg-flp-12* transgenics. Likewise, number of egg masses per plant and eggs per egg mass also declined significantly in the transgenics, ultimately affecting the multiplication factor, when compared to the wild type plants. This study establishes the effectiveness of the two *M. graminicola flp* genes for its management and also for gene pyramiding.

Keywords: root-knot nematodes, FMRFamide-like peptide, RNAi, *Oryza sativa*, transgenics

Abbreviations: FLPs, FMRFamide-like peptides; HIGS, Host-induced gene silencing; dsRNA, Double-stranded RNA; hpRNA, Hairpin RNA; PPNs, Plant-parasitic nematodes; RKN, Root-knot nematode; MF, Multiplication factor.

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple cereal "global grain" constituting everyday meal of more than three billion people around the world. Although rice is popularly grown across different parts of the world, the tropical parts of Asia and South-East Asian countries produce approximately 90% of the global rice output (Mantelin et al., 2017). In the present era of modern farming, plant-parasitic nematodes (PPNs) pose a major threat to the agricultural food production and rice is also attacked by a wide array of nematodes (Prasad et al., 1992; Jones et al., 2013). Among the major PPN species attacking rice, the root-knot nematode (RKN) *Meloidogyne graminicola* Golden and Birchfield, 1965 alone has been reported to inflict up to 50% yield loss under different conditions (Lorenzana et al., 1998; Bridge et al., 2005). This economically important RKN species is widespread in almost all the rice growing areas in the world, and has been well documented to cause extensive yield and quality losses (Dutta et al., 2012; Kyndt et al., 2012). The infective pre-parasitic second-stage juveniles (J2s) of *M. graminicola* enter the roots and develop permanent feeding cells (giant cells) resulting in typical hook shaped galls (De Waele and Elsen, 2007).

Various strategies are used for management of PPNs, and the traditional practices are mostly relied on the use of cultural/physical methods and chemotherapeutics. However, due to the adverse effects of most of the nematicidal chemicals on environment, non-target organisms and human health, they were either discontinued or restricted for use in agricultural fields (Bridge et al., 2005; De Waele et al., 2013). Hence, there has been a continuous demand for development of environmentally benign target-specific nematode management approach, and genetic engineering based techniques have gained promising popularity in this regard (Roderick et al., 2018; Dutta et al., 2019). Genetic improvement of rice against PPNs through breeding programs faces a major challenge due to scant availability of suitable resistant sources (Soriano et al., 1999; Cabasan et al., 2012). Considerable natural resistance against *M. graminicola* has been reported in *Oryza glaberrima* and *O. longistaminata*, but limited resistant source has been reported in *O. sativa* (Soriano et al., 1999; Cabasan et al., 2012; Kumari et al., 2016; Kumari et al., 2017; Hatzade et al., 2020). The recent advancements in PPN genomics and transcriptomics have enabled us to identify the propitious molecular targets in nematodes that can be exploited for their management and also for drug designing (Danchin et al., 2013; Taylor et al., 2013; Shivakumara et al., 2019). The availability of draft genome can also offer a supportive platform to identify and validate the candidate genes responsible for rice–*M. graminicola* interaction (Somvanshi et al., 2018).

Nematode neuropeptides play a key role in controlling and modulating the physiological processes like host recognition, navigation, infection, secretion, reproduction etc., and have been proved to be potential drug targets (Maule et al., 2002; Kimber et al., 2007; Warnock et al., 2017). The FMRFamide-like peptides (FLPs) constitute a large and diverse group of neuropeptides in nematodes, governing the basic behavioral functions by coupling to the G-protein coupled receptors (GPCRs) (McVeigh et al., 2006; Atkinson et al., 2013). Similar results have also been reported due to silencing of *flp* genes that resulted in locomotory defects, reduced penetration, aberrant behavior and reduced reproduction in *Meloidogyne incognita* and *Globodera pallida* (Dalzell et al., 2010; Dong et al., 2013; Papolu et al., 2013; Banakar et al., 2015). However, scant knowledge is available to date with respect to performance of the *flp* gene repertoire against *M. graminicola* in rice through HIGS. Of late, HIGS has emerged as an effective and successful strategy to silence the genes in plant-parasitic nematodes for functional validation (Fairbairn et al., 2007; Danchin et al., 2013). In the present study, we have selected two *M. graminicola* FLP coding genes, *Mg-flp-1* and *Mg-flp-12*, for evaluation based on their reported effect on juvenile penetration and infectivity in rice. Previously, Rao et al. (2013) found that the translated sequence of *Mg-flp-12* putatively contains a N-terminal secretion signal peptide indicating its involvement in extra cellular signal transduction functioning like G protein coupled receptor (GO: 0004930); and similar finding was noted for *Mg-flp-1*, which contains the highly conserved LFRGR motif. These observations putatively indicated the potential of the genes as molecular target(s) against *M. graminicola*. Subsequently, Kumari et al. (2017) characterized nine *flp* genes from *M. graminicola* including *flp-1* and *flp-12*, and *in vitro* silencing of the said genes resulted in significantly reduced penetration of J2s and their infection potential in rice. Additionally, Masler (2008) showed that disruption of *flp-1* and *flp-12* results in various neuromuscular dysfunctions in other nematodes, and *flp-12* has also been proved to be a potential target against pine wood nematode, *Bursaphelenchus xylophilus* (Huang et al., 2010). Hence, to explore the potential of *flp-1* and *flp-12* as molecular targets, transgenic rice plants were developed expressing the dsRNA constructs of the two genes, and their effectiveness were evaluated against *M. graminicola* using soil-less Pluronic media and soil system. Thus, the present study strengthens our knowledge on the effect of *M. graminicola flp-1* and *flp-12* in nematode reproduction and plant parasitism potential, when applied through HIGS in rice.

MATERIALS AND METHODS

Nematode Culturing

Pure culture of an Indian isolate of *M. graminicola* Golden and Birchfield, 1965 was maintained on rice (*O. sativa* cv. PB 1121) in a glasshouse at ICAR–Indian Agricultural Research Institute, New Delhi, India. Galls were handpicked from washed infected roots, and infective second-stage juveniles (J2s) were hatched using modified Baermann's assembly (Whitehead and Hemming, 1965). The freshly hatched J2s were used for all the experiments.

Preparation of Hairpin RNA (hpRNA) Constructs of Target Genes and Generation of Transgenic Plants

Previously, *flp-1* and *flp-12* were reported from *M. graminicola* by Rao et al. (2013) and subsequently Kumari et al. (2017) performed their molecular characterization and showed *in vitro*

silencing effect of the genes on *M. graminicola* against rice. The partial sequence of *Mg-flp-1* (214 bp) and *Mg-flp-12* (299 bp) were then subjected to BlastN search against GenBank nonredundant (nr) databank and *M. graminicola* genome database (Altschul et al., 1990). Further, off-target sites in the targeted dsRNA were investigated at http://dsCheck.RNAi.jp/ for their presence, if any (Naito et al., 2005).

RNAi Gateway vector ph7GWIWG2(II) was obtained from VIB-UGent Center for Plant Systems Biology, Ghent University, Ghent, Belgium. Partial sequence of *Mg-flp-1* (214 bp), *Mg-flp-12* (299 bp) and an unrelated gene *gfp* (375 bp; green fluorescent protein used as non-native negative control) were PCR amplified from the corresponding recombinant pGEM-T clones (Accessions: KC250005, KC250006 and HF675000), sub-cloned into pDONR 221 entry vector, followed by subsequent cloning into ph7GWIWG2(II) destination vector using the LR recombination in sense and antisense orientation, using GATEWAY recombination cloning kit (Invitrogen, Carlsbad, CA, USA). The recombinant vectors were transformed into *E. coli* (DH5α), mobilized into *A. tumefaciens* (LBA4404), and confirmed the orientation of target genes by PCR using gene specific primers, CaMV 35S promoter and attB2, CaMV 35S terminator and attB2, *hptII* marker specific primers. Primer details are given in **Table S1**.

For *Agrobacterium* primary culture preparation, the positive clones were inoculated into 5 ml of liquid Yeast Extract Mannitol (YEM) medium (Sambrook et al., 1989) supplemented with 100 mg L^{-1} spectinomycin (Sigma Aldrich, St. Louis, Missouri, USA) and 30 mg L^{-1} rifampicin (Sigma Aldrich, St. Louis, Missouri, USA), and incubated at 28°C for 48 h at 200 rpm in an incubator shaker (New Brunswick Innova 44; Eppendorf, Hamburg, Germany). Primary culture was re-inoculated into 100 ml of antibiotic supplemented YEM medium, incubated overnight at aforesaid conditions, until the A_{600} (*Agrobacterium* suspension cells) reaches at 0.8. The culture was centrifuged for 20 min at 5,000×*g* to pellet the cells which were re-suspended in Murashige–Skoog (MS) medium (MS salt 4.41 g L^{-1}, sucrose 1.5%; pH 5.4) containing 150 μM of acetosyringone and used for co-cultivation.

O. sativa cv. Taipei 309 was used for *Agrobacterium*- mediated transformation. The seeds were procured from Division of Genetics, ICAR-Indian Agricultural and Research Institute, New Delhi, India. Healthy seeds were de-husked and surface sterilized with 70% ethanol for 90 s, followed by washing with 50% (v/v) sodium hypochlorite solution for 20 min. Seeds were then washed with autoclaved double distilled water thrice until traces of sodium hypochlorite vanished completely. About 50 surface sterilized rice seeds were inoculated aseptically on callus induction medium (MCI) and incubated at 26 ± 2°C in dark for development of embryogenic calli. The calli were then co-cultivated by immersing them in *Agrobacterium* suspension for 20 min, excess moisture removed using a sterilized filter paper (Whatman Grade 4, Whatman International, UK), transferred on to the co-cultivation medium (MCCM, pH 5.8) containing 150 μM of acetosyringone and incubated in dark for 48 h.

After co-cultivation, the infected calli were washed with sterilized double distilled water for 25 min, containing 300 mg L^{-1} cefotaxime (HIMEDIA, Mumbai, India) and 200 mg L^{-1} ticarcillin (Sigma Aldrich, St. Louis, Missouri, USA). Calli were then transferred onto the selection medium-I (MSM I) and incubated for 15 days in dark. After primary selection, the healthy calli were transferred to fresh selection medium twice at 14 days interval for further proliferation. The newly proliferated macro-calli were grown on regeneration medium-I (MSRM-I, pH 5.8) for 7 days at 26 ± 2°C in dark and subsequently sub-cultured on fresh MSRM-I before exposing to light (light:dark photoperiod 16:8 h). Thereafter, the healthy regenerated shoots were transferred to rooting medium (RM) for root development, and later rooted plants were grown in ½ strength Yoshida's nutrient solution (Yoshida et al., 1976) for 7 days. Plants with well-established roots were hardened in Soilrite-mix and moved to the green house for further development and seed setting. T_0 seeds were harvested and used for further studies by raising T_1 generation plants.

Molecular Characterization of Transgenic Rice Plants

Genomic DNA (gDNA) was isolated from young leaf tissues of primary transformants and wild type (WT) plants using Nucleospin Plant II DNA extraction kit (Macherey-Nagel, Düren, Germany). Presence of transgenes was primarily confirmed by PCR using target genes specific primers. T_1 generation plants were raised in autoclaved soil and DNA was isolated from the fresh leaves as stated above and PCR confirmation of the transgenes was done using different sets of primers (**Table S1**).

For determination of T-DNA integration and transgene copy number, gDNA was isolated from young leaf tissues of T_1 of both sets of transgenics expressing *Mg-flp-1*; *Mg-flp-12* and WT plants; and Southern hybridization was performed. About 12 μg of gDNA was digested with *Sac*I (20 U μl^{-1}) (New England Biolabs, Massachusetts, USA) at 37°C for 16 h. Digested DNA was then resolved on 0.8% high resolution Meta Phor agarose gel and transferred to nitrocellulose membrane (Bio-Rad, Hercules, California, USA). Probe synthesis (*Mg-flp-1*: 214 bp and *Mg-flp-12*: 299 bp), digoxigenin (DIG) labeling, hybridization, detection and blot development was carried out as described previously (Papolu et al., 2013).

Following Southern blot analysis, Northern hybridization was performed to check the presence of siRNA in the transgenics. For this, total RNA (small and large RNA in single fraction) was extracted from young leaves of T_1 plants using NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany). Separation of RNA, membrane transfer, probe preparation (*Mg-flp-1*: 214 bp and *Mg-flp-12*: 299 bp), DIG labeling, hybridization and detection was carried out as described earlier (Papolu et al., 2013).

Quantification of Expression of Target Genes in Plants and Nematodes

Transgenic plants, after being confirmed by PCR and Southern blotting, from different lines of the two genes were subjected to quantitative real-time PCR (qRT-PCR) to analyze the transcript abundance of *Mg-flp-1* and *Mg-flp-12*. Total RNA was extracted from young leaves of T_1 plants using Nucleospin plant II RNA

kit (Macherey-Nagel, Düren, Germany), and assessed for quality and quantity using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Approximately 500 ng of purified RNA was reverse transcribed (Superscript VILO, Invitrogen, Carlsbad, CA, USA), and qRT-PCR was carried out using gene specific primers in realplex^2 thermal cycler (Eppendorf, Hamburg, Germany). Gene expression was normalized using *O. sativa 18S rRNA* (Accession: AF069218). Three biological and three technical replicates were kept for each sample, and data were analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

For analysis of expression abundance of *Mg-flp-1* and *Mg-flp-12* in the nematodes feeding on T_1 transgenic lines, mature females were extracted from the transgenic and WT plants. RNA extraction, cDNA preparation and qRT-PCR analysis were done as described earlier (Shivakumara et al., 2019). All analyses were based on three biological and three technical replicates and nematode *18S rRNA* (Accession: HE667742) was used for normalization.

Bioefficacy Analysis of T_1 Transgenics Against *M. graminicola*

The T_1 plants expressing dsRNA constructs of *Mg-flp-1*, *Mg-flp-12*, and the *gfp* and WT controls were initially evaluated against *M. graminicola* using Pluronic gel medium, PF-127 (Wang et al., 2009; Kumari et al., 2016). Four day-old rice seedlings (T_1, WT and reference control plants) were used for evaluation. Approximately, 30 *M. graminicola* J2s were inoculated at the root tip of each seedling, and assays were performed as described earlier (Kumari et al., 2016). There were five replicates for each event and the parameters like total number of galls, endoparasites, number of egg masses and eggs per egg mass per plant were used for disease scoring. Nematode multiplication factor was derived as described earlier (Kumari et al., 2017).

The transgenic lines, after being evaluated on PF-127, were further screened in soil system under greenhouse conditions. Five replicates were kept for each event keeping the WT and *gfp*-control plants as reference. Twenty day-old seedlings raised singly in pots filled with autoclaved soil were inoculated at the rate of 2 J2s per g of soil and plants were grown in greenhouse as described earlier (Hatzade et al., 2020). Plants were uprooted carefully at 45 days post inoculation (dpi), roots washed free of soil and scored for total number of galls, endoparasites, number of egg masses and eggs per egg mass. Nematode multiplication factor was derived as described earlier (Kumari et al., 2017). Photographs were taken using a ZEISS SteREO Discovery V20 microscope.

Statistical Analyses

The bioassay data were subjected to one way analysis of variance (ANOVA) using completely randomized design (CRD), and statistical significance was determined at P = 0.05 and P = 0.01. Values of mean of total replications from each treatment were taken for statistical analyses for individual assays.

RESULTS

Target Identification of *Mg-flp-1* and *Mg-flp-12* for Generating HIGS Constructs

The amplified sequences of *Mg-flp-1* (214 bp) and *Mg-flp-12* (299 bp) neither showed similarity with any sequence at nr database nor showed homology with any *M. graminicola* sequence. The target dsRNA sequences were queried in dsCheck database to identify the potential off-target sites, and no exact match could be found for the processed siRNAs (19 nucleotides) in the existing database (**Figure S1**). Therefore, silencing of the aforesaid genes may not induce any off-target effects on other organisms.

RNAi Gateway vector ph7GWIWG2(II) was used to clone *Mg-flp-1*, *Mg-flp-12* and *gfp* genes flanked by attB1 and attB2 sites in sense and antisense orientation (**Figure S2**). PCR analysis confirmed the orientation of the target genes (**Figure S3**).

Validation of RNAi Vectors of *Mg-flp-1* and *Mg-flp-12* in *O. sativa* cv. Taipei 309 Using *A. tumefaciens*

The embryogenic calli of rice cv. Taipei 309 were used for *Agrobacterium*-mediated genetic transformation with hairpin constructs of *Mg-flp-1*, *Mg-flp-12* and *gfp*, separately. *Agrobacterium*-infected calli were proliferated, histo-differentiated and transferred to MSM-I (**Table S2**). Following incubation, some calli remained healthy-creamish, whereas others turned brownish. The healthy calli were transferred to fresh MSM-II for further proliferation and later-transferred to MSRM-I to induce shoot development (**Figure S4**). The regenerated plants were hardened in soilrite-mix. A regeneration frequency of 47% was observed in the transformed calli (**Table S3**).

Molecular Characterization of Transgenic Rice Plants Harboring RNAi Constructs

Ten primary transgenic events (T_0) of each of *Mg-flp-1* and *Mg-flp-12* were genotyped by PCR using gene specific, CaMV 35S promoter and attB2, CaMV 35S terminator and attB2, and *hptII* primers. No amplification was observed in the WT plants while transgenic plants yielded expected amplicons (**Figures S5A, B**). Independent transformed events were confirmed by presence of transgenes conferring hygromycin resistance with an overall transformation efficiency of 6.4% (**Table S3**).

The T_1 progeny plants were generated by selfing the selected T_0 plants and re-validated by PCR using the aforesaid pair of primers, which showed amplification of expected fragments in all the tested lines (**Figures S6A, B**).

In order to confirm the integration and copy number of transgenes in various transgenic lines, PCR positive T_1 plants were analyzed by Southern blot hybridization. It was found that the lines A2-3, A3-5, A5-3, A6-1, A9-1, and A10-3 for *Mg-flp-1*, and lines B2-1, B5-7, B6-4, B7-1, B9-2, and B11-3 for *Mg-flp-12* showed positive integration. WT plants were used as control (**Figures 1A, B**).

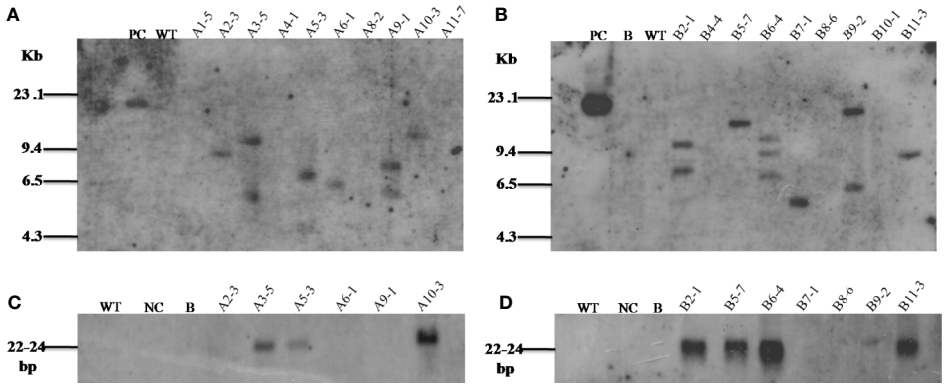

FIGURE 1 | Southern and Northern blot analyses for transgenic lines harboring dsRNA constructs of *Mg-flp-1* and *Mg-flp-12*. **(A)** Southern blot analysis of *Mg-flp-1* expressing lines; A1-5, A2-3, A3-5, A4-1, A5-3, A6-1, A8-2, A9-1, A10-3, A11-7, and **(B)** *Mg-flp*-12 expressing lines; B2-1, B4-4, B5-7, B6-4, B7-1, B8-6, B9-2, B10-1, B11-3. **(C)** Northern blot analysis for *Mg-flp-1*-specific sRNA in selected transgenic lines; A2-3, A3-5, A5-3, A6-1, A9-1, A10-3, and **(D)** *Mg-flp-12*-specific sRNA in selected transgenic lines; B2-1, B5-7, B6-4, B7-1, B8-6, B9-2, B11-3. (PC, positive control; NC, negative control; B, blank; WT, wild type plant).

As a key component of HIGS, expression of siRNAs of *Mg-flp-1* and *Mg-flp-12* was established by Northern blot analysis in the transgenic lines. The siRNAs were detected in the representative samples of *Mg-flp-1* (A3-5, A5-3, A10-3) and *Mg-flp-12* (B2-1, B5-7, B6-4, B11-3) transgenic lines (**Figures 1C, D**), confirming the possibility for HIGS.

Transgenic plants expressing *Mg-flp-1* and *Mg-flp-12* dsRNA constructs were further confirmed at mRNA level by qRT-PCR. The results indicated variable level of expression of the transgenes in all the selected lines. The line A5-3 of *Mg-flp-1* and B5-7 of *Mg-flp-12* had the highest expression, whereas line A10-3 of *Mg-flp-1* and B8-6 of *Mg-flp-12* showed least expression (**Figure S7**). However, no expression was detected in the WT plants.

In order to investigate the effect of HIGS in suppressing target genes in nematode, qRT-PCR was performed with adult females of *M. graminicola* extracted from the transgenic plants of both genes. *Mg-flp-1* extracted females exhibited down regulation in the range of 0.63 ± 0.2–1.71 ± 0.2 fold while that of *Mg-flp-12* showed 0.04 ± 0.1–1.80 ± 0.2 fold reduction ($P \leq 0.05$) (**Figure 2**).

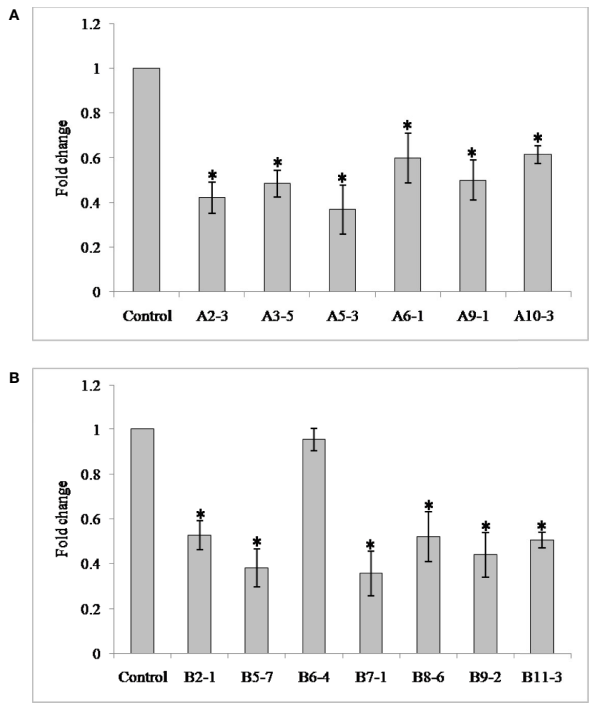

FIGURE 2 | Transcript levels of *Mg-flp-1* and *Mg-flp-12* in *M. graminicola* females extracted from dsRNA expressing transgenic lines. **(A)** *Mg-flp-1* lines; A2-3, A3-5, A5-3, A6-1, A9-1, A10-3, **(B)** *Mg-flp-12* lines; B2-1, B5-7, B6-4, B7-1, B8-6, B9-2, B11-3. Expression was quantified as fold change values calculated by $2^{-\Delta\Delta CT}$ method, and *18S rRNA* gene was used as reference. Each bar represents the mean ± SE of $n = 3$, and asterisks indicate significant difference at $P < 0.05$.

Bioefficacy Analysis of T_1 Transgenics of Rice Against *M. graminicola*

Performance of the transgenic plants was initially assessed against *M. graminicola* on Pluronic F-127 medium. Number of galls, endoparasites and egg masses developed per plant, and eggs per egg masses were counted at 18 dpi. The results showed reduction in average galling by 13–44% for *Mg-flp-1* (**Figure 3A**) and 30–48% for *Mg-flp-12* in different transgenic lines (**Figure 4A**). This observation was corroborated with reduced number of endoparasites which was 28–46% for *Mg-flp-1* and 36–57% for *Mg-flp-12* transgenic lines. Likewise, a significant reduction was seen in number of egg masses (25–55%) and eggs per egg mass (19–35%) in *Mg-flp-1* transgenics. Similarly, *Mg-flp-12* silencing decreased number of egg masses by 34–60% and eggs per egg mass by 29–47%. Finally, derived multiplication factor was reduced in *Mg-flp-1* and *Mg-flp-12* silenced plants by 40–68% and 53–78%, respectively when compared to the WT and negative control plants (**Figure 5A**).

The independent T_1 lines harboring RNAi constructs were further assessed for performance in soil by inoculating 20-day

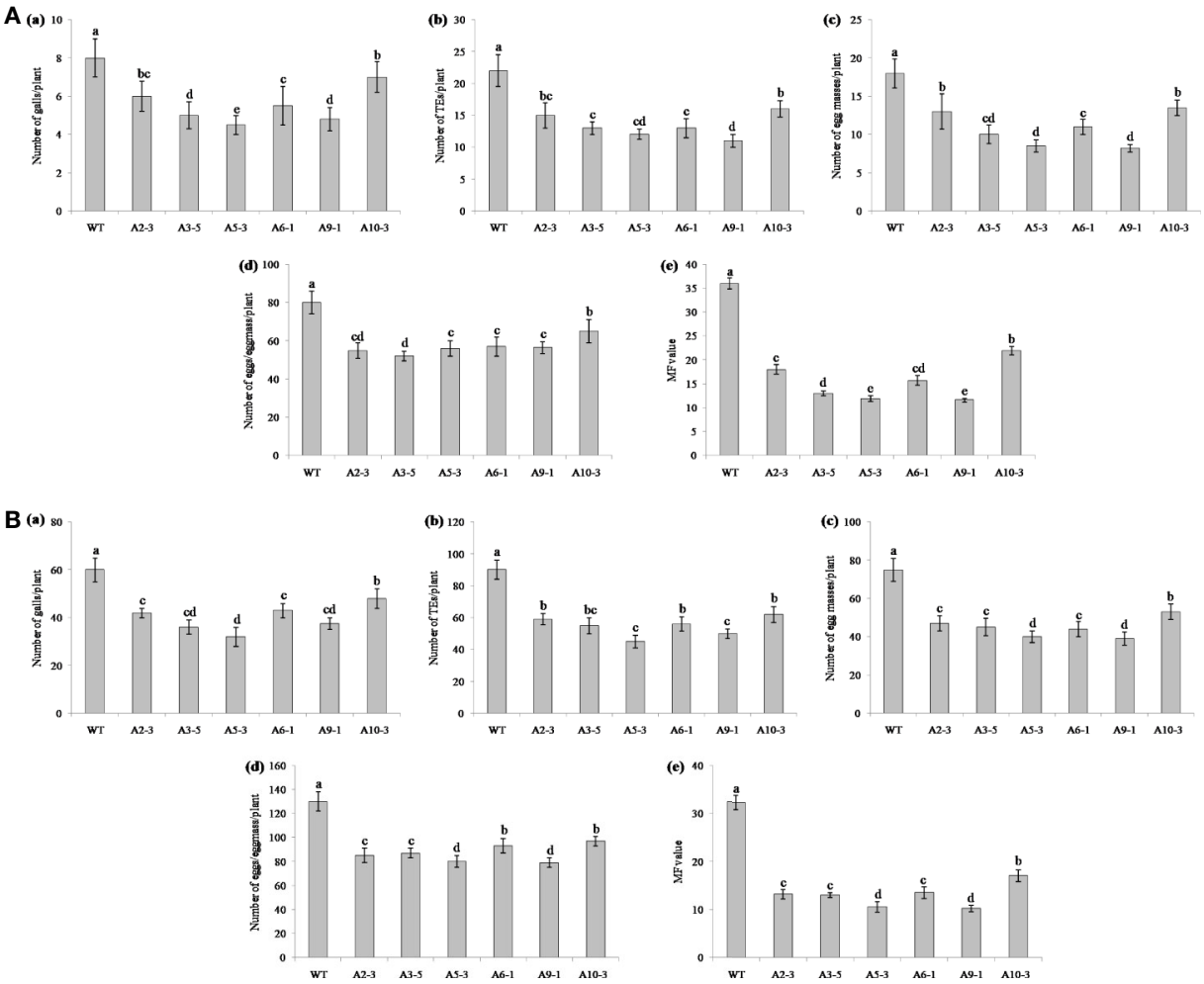

FIGURE 3 | Effect of host induced silencing of *Mg-flp-1* on development and reproduction of *M. graminicola*. Performance of transgenic rice plants was assessed on **(A)** Pluronic F-127, and **(B)** soil medium. Relative number of galls (a), total number of endoparasites (b), egg masses (c), eggs per egg mass (d), and the respective multiplication factor (e) were determined in different transgenic lines (A2-3, A3-5, A5-3, A6-1, A9-1, A10-3) and wild type plants at 18 and 45 dpi. Each bar represents the mean ± SE of $n = 5$, and bars with different letters (within each parameter) denote significant difference at $P < 0.05$.

old plants with freshly hatched J2s. The plants were harvested after completion of two successive life cycles of nematode. The results demonstrated that transgenic plants of different lines showed significant reduction ($P \leq 0.05$) in gall number compared to WT plants and control plants. Line A10-3 of *Mg-flp-1* (**Figure 3B**) and B8-6 of *Mg-flp-12* (**Figure 4B**) showed highest number of galls but also showed significant reduction in total endoparasites (31–37%), egg masses (30–40%) and eggs per egg mass (25–33%). The derived multiplication factor was found to be reduced by 47–60% (**Figure 5B**).

DISCUSSION

In the present study, we have evaluated HIGS of two FMRFamide-like peptide (FLP) genes of *M. graminicola*, *Mg-flp-1* and *Mg-flp-12* in rice. Nematode neuropeptides, especially the FLPs are associated with numerous physiological functions including host recognition, feeding, sensory perception, navigation, reproduction and parasitism (Kimber et al., 2007; Morris et al., 2017; Warnock et al., 2017). Hence, these neuropeptides could be potential targets for designing safe and specific nematode management schedule (Peymen et al., 2014; Warnock et al., 2017). Several FLPs have been identified from the PPNs, and FMRFamide-like immune reactivity has also been observed in the nervous systems of some PPNs like *G. pallida* and *G. rostochiensis* (Kimber et al., 2001). To date, 19 *flp* genes have been identified in the most notorious nematode species *M. incognita*, and HIGS of *flp-14* and *flp-18* in tobacco provided significant reduction in reproductive potential and parasitism of the nematode (Papolu et al., 2013). Rice production is largely affected by *M. graminicola* (Jones et al., 2013), and earlier observations demonstrated that *in vitro* silencing of nine *flp* genes (*flp-1*, *flp-3*, *flp-6*, *flp-7*, *flp-11*, *flp-12*, *flp-14*, *flp-16* and *flp-*

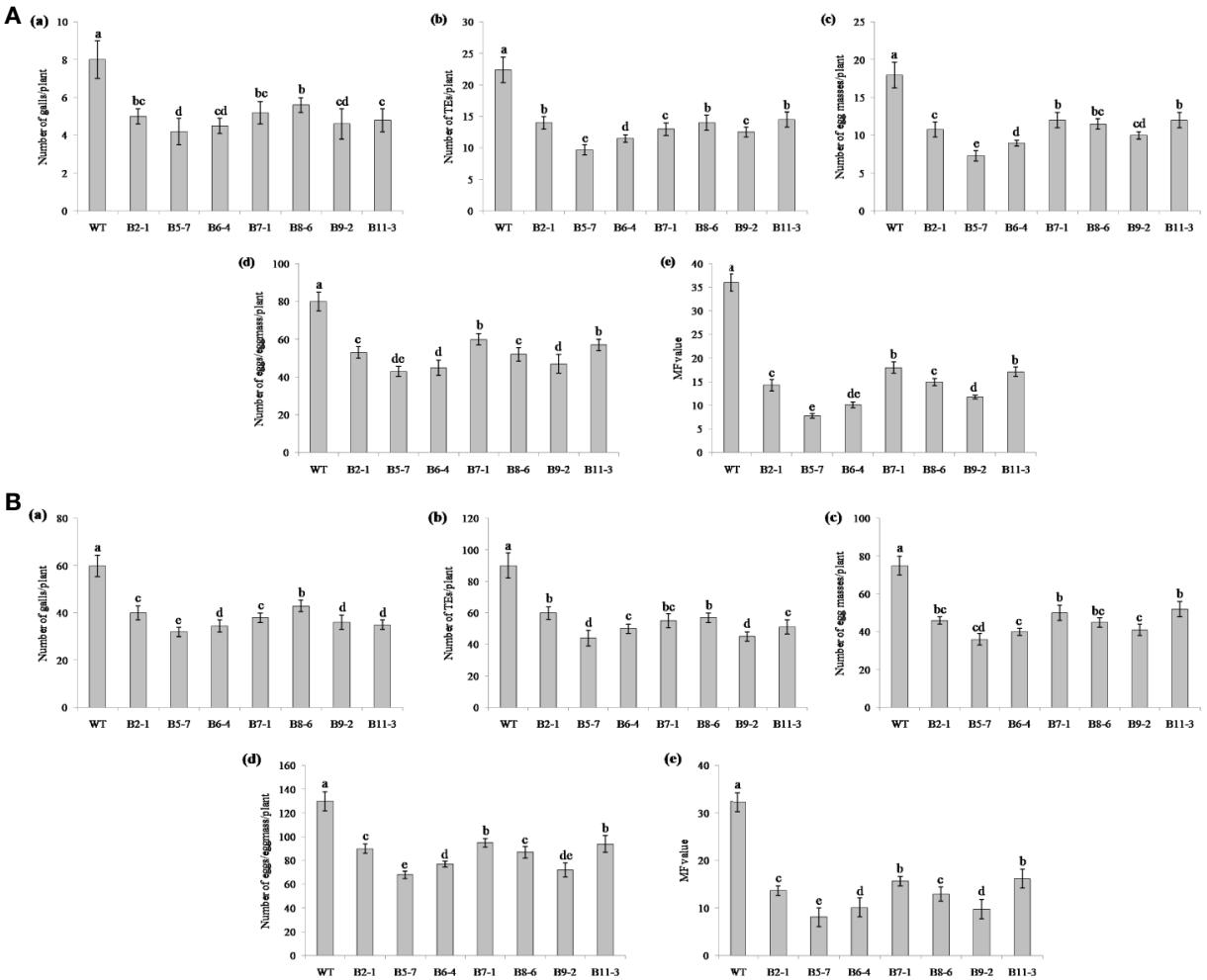

FIGURE 4 | Effect of host induced silencing of *Mg-flp-12* on development and reproduction of *M. graminicola*. Performance of the transgenic rice plants was assessed on **(A)** Pluronic F-127, and **(B)** soil medium. Relative number of galls (a), total number of endoparasites (b), egg masses (c), eggs per egg mass (d), and the respective multiplication factor (e) were determined in different transgenic lines (B2-1, B5-7, B6-4, B7-1, B8-6, B9-2, B11-3) and WT plants at 18 and 45 dpi. Each bar represents the mean ± SE of $n = 5$, and bars with different letters (within each parameter) denote significant difference at $P < 0.05$.

18) in *M. graminicola* J2s significantly retarded their penetration and reproduction potential while infecting rice (Kumari et al., 2017). Further, results involving *G. pallida*, *G. rostochiensis*, *Heterodera glycies*, *H. schachtii*, *M. incognita*, *M. javanica*, *Pratylenchus* spp., etc. suggest that HIGS of parasitism genes provide a better platform for nematode management and also strengthens the findings of *in vitro* RNAi (Urwin et al., 2002; Chen and Roberts, 2003; Vanholme et al., 2004; Dubreuil et al., 2007; Adam et al., 2008; Bakhetia et al., 2008; Tan et al., 2013; Chaudhary et al., 2019; Shivakumara et al., 2019). In this regard, the present study convincingly supports the earlier findings of Kumari et al. (2017), and also validates the efficiency of *M. graminicola flp-1* and *flp-12*, as potential targets for nematode management through HIGS in Asian rice *O. sativa*.

Molecular analyses by PCR and Southern hybridization revealed the insertion, integration and inheritance of the T-DNA harboring dsRNA constructs of *Mg-flp-1* and *Mg-flp-12* independently in most of the transformed lines. However, the expression of hpRNA coding transgenes quantified by qRT-PCR was found to be variable among different lines. This suggests that the transgenes were randomly integrated at diverse transcriptionally active sites in the rice genome. Additionally, the nematode gene sequences showed no similarity or homology with any short stretches of similar sequences in rice genome, which reduces the risk of any non-target effect.

The results of nematode bioassays revealed significant reduction in development and reproduction in most of the transgenics lines, containing dsRNA construct of *Mg-flp-1* and *Mg-flp-12*, when compared to the WT plants. Simultaneously, galling and multiplication factor also declined significantly in the transgenic plants. No apparent morphological variation was observed in transgenic lines compared to the WT plants, indicating the focused RNAi effect. In addition, the detection of target gene specific siRNAs in transgenic lines provided authentic evidence for the HIGS of *Mg-flp-1* and *Mg-flp-12* genes, which resulted in

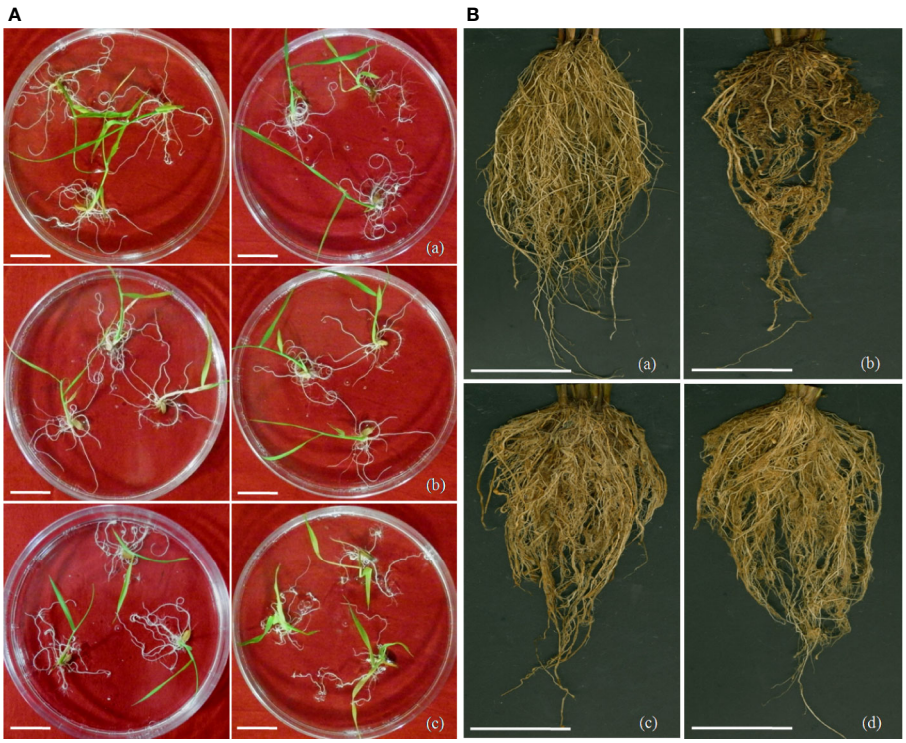

FIGURE 5 | Screening of transgenic rice lines for nematode infection. **(A)** Screening of *M. graminicola* infected plants in PF-127 medium at 18 dpi; representative nematode infected plants of (a) *Mg-flp-1* transgenic lines, (b) *Mg-flp-12* transgenic lines, (c) wild type plants. Typical hook-like galls on roots with greater number of them in WT compared to transgenic lines; scale bar = 0.5 cm, **(B)** Comparison of *M. graminicola* infection in transgenic plant roots and WT plants at 45 dpi; representative roots of (a) healthy WT plants, (b) nematode infected WT plants, (c) nematode infected *Mg-flp-1* transgenics, and (d) nematode infected *Mg-flp-12* transgenics. Intensity of galling was comparatively higher in WT plants than the transgenics; scale bar = 5 cm.

reduced virulence and reproduction of the nematode species. A previous study has showed that *in vitro* silencing of both these genes reduced penetration and infection of the *M. graminicola* juveniles (Kumari et al., 2017). This finding can be corroborated with some other studies (Kimber et al., 2007; Papolu et al., 2013), where silencing of *flp* genes also showed similar results. dsRNA mediated silencing of *flp-1*, *flp-6*, *flp-12*, *flp-14* and *flp-18* genes in *G. pallida*, led to aberrant behavioral phenotypes (Kimber et al., 2007). Host induced silencing of *flp-14* and *flp-18* in tobacco affected development of *M. incognita* (Papolu et al., 2013). In *B. xylophilus*, *flp-4* and *flp-18* might coordinate with NPR-4 receptor, and *flp-3*, *flp-18*, *flp-7* and *flp-11* activate NPR-10 and FRPR-3 receptors, thus forming an integral part of complex neuronal network controlling nematode movement through motor and sensory neurons (Kimber et al., 2007). The reduced development, reproduction and parasitism as a result of silencing *Mg-flp-1* and *Mg-flp-12* might also involve a complex neuromuscular system in *M. graminicola* that affects the rice-nematode interaction. Studies with *Ascaris suum* revealed that silencing of *flp-14* and 15 other *flps* inhibit nematode ovijection by shortening oviduct length, relaxing the circular muscles and cessation of contractile activity simultaneously (Moffett et al., 2003). Similar physiological anomalies might take place upon silencing of *Mg-flp-1* and *Mg-flp-12*, which possibly result in reduction in nematode's egg laying capacity. Similar findings were also recorded in the model nematode *Caenorhabditis elegans*, where loss of function mutants of *flp-1* showed altered locomotion and egg laying (Chang et al., 2015).

In order to assess the HIGS of *Mg-flp-1* and *Mg-flp-12*, the adult females were extracted from the T_1 transgenic lines and WT plants and qRT-PCR was performed to assess the transcript abundance. A significant reduction in gene expression (up to 1.8 folds) was observed in the nematode females extracted from the transformed lines. These findings unequivocally support that HIGS of both *flp* genes in rice could effectively control the RKN species *M. graminicola* independently, and are adequately substantiated by the inclusion of negative controls during *in planta* RNAi studies.

Although there are several reports of efficacy of *flp* genes disrupting the neuromotor functions in nematodes, this is an established report to demonstrate the effectiveness of *Mg-flp-1* and *Mg-flp-12* genes in the management of *M. graminicola* using HIGS in rice. The significant reduction of nematode multiplication factor in the transgenic plants has proved the potential of RNAi silencing of both genes. Based on the environmental effects, agronomic conditions and biology of the nematodes, absolute resistance is very hard to achieve against these pests (Fuller et al., 2008; Rosso et al., 2009). In this direction, *Mg-flp-1* and *Mg-flp-12* genes would be highly effective to withstand the initial population

toll in the field, and will also help bringing down the population build up that will help in reducing the initial nematode pressure in soil for successive crop(s). Thus the early crop growth won't get affected. Further, sustainable management can also be achieved by pyramiding few promising *flp* genes together to efficiently interfere different physiological processes required for successful nematode parasitism.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

UR conceived, designed, and supervised the experiments. AH and CK performed the experiments. DS help in plant transformation. AH analyzed the data, and wrote the draft of the manuscript. UR, VP, and VC reviewed and wrote the final draft of the manuscript. All authors contributed to the article and approved the submitted version.,

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00894/full#supplementary-material

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Organic Fertilization Assembles Fungal Communities of Wheat Rhizosphere Soil and Suppresses the Population Growth of *Heterodera avenae* in the Field

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Heterodera avenae (cereal cyst nematode, CCN) infects wheat and other cereal crops and causes severe losses in their yield. Research has shown that CCN infestations can be mitigated by organic fertilization in wheat fields, but the mechanisms underlying this phenomenon are still largely unknown. In this study, the relationships among CCN, soil properties, and soil fungal communities with organic fertilizer (OF) or chemical fertilizer (CF) and without fertilizer (CK), were investigated for two years in a wheat field in Henan province, China. Our results showed that the concentrations of soil total N, total P, available P, available K, and organic matter were all promoted by the OF treatment at the jointing stage of wheat, coinciding with the peak in egg hatching and penetration of wheat root by CCNs. Soil total N correlated positively ($R^2 = 0.759$, $p < 0.05$) with wheat yields but negatively ($R^2 = 0.663$, $p < 0.01$) with Pf/Pi (index of cyst nematode reproduction), implying the regulated soil property contributes to suppressing CCN in the OF treatment. Furthermore, fungal community α-diversity (Shannon and Simpson) and β-diversity (PCoA) of rhizosphere soil was improved under the organic fertilizer treatment. The fungal genera negatively associated with the Pf/Pi of CCN were highly enriched, which included *Mortierella* and *Chaetomium*, two taxa already reported as being nematophagous fungi in many other studies. These two genera were heavily surrounded by much more related fungal genera in the constructs co-occurrence network. These results suggested that the OF treatment shifted soil fungal community functioning towards the suppression of CCN. Taken together, the suppressed cyst nematode reproduction with the assembly of fungal communities in the rhizosphere led to greater wheat yields under organic fertilization. These findings provide an in-depth understanding of the benefits provided by organic fertilization for developing sustainable agriculture.

Keywords: *Heterodera avenae*, organic fertilizer, microbiome assembly, rhizosphere, soil fungi communities, suppression, population growth, wheat field

INTRODUCTION

Heterodera avenae (cereal cyst nematode, CCN) is among the most important soil-borne pathogens infesting cereal crops worldwide. The wheat yield losses caused by *H. avenae* can range from 10% up to 100% in some infected fields (Bonfil et al., 2004; Smiley et al., 2017; Yang et al., 2019). In modern intensive agriculture, usually a synthetic nematicide is used to manage this nematode and safeguard wheat yields (Zhang et al., 2016), and while its control efficacy is significant, such nematicides are expensive and they also pollute the local environment (Peng et al., 2009), which over the long-term is unsustainable (Bridge, 1996). Furthermore, intensive agriculture systems based on the continuous use of mineral fertilizers have led to reductions in soil biodiversity and aggravated the infestation of CCN (Hu et al., 2014; Matute et al., 2018). Thus, taking an environmentally friendly view of fertilization in crop management, which could reduce both soil pollution and the damages from CCN, is urgently needed for developing truly sustainable agroecosystems.

Research has revealed that the diversity and density of soil nematodes can change depending on the fertilization type used in fields (Liang et al., 2009; Diacono and Montemurro, 2010; Al-Hazimi and Dacbah, 2014; Hu et al., 2014). Nitrogen fertilization significantly enhances the contents of soil ammonium and nitrate nitrogen, which have the potential to negatively impact soil nematodes (Zhao et al., 2014; Song et al., 2015). Similarly, negative effects upon soil nematodes were observed when a high level of phosphate fertilizer was applied in forest soils (Zhao et al., 2014). Specifically, adding urea or calcium superphosphate alone can significantly inhibit the abundance of CCN (Yang et al., 2008; Al-Hazimi and Dacbah, 2014). Ammonium sulfate fertilizer was found to significantly limit the abundance of soil nematode communities (Ikoyi et al., 2020), whereas potassium sulfate promoted the occurrence of CCN-associated disease in wheat plants (Yang et al., 2008). Different fertilization types may change the main food sources of soil nematodes, thereby changing the community structure of soil nematodes (Bjørnlund et al., 2006; Zhao et al., 2014). In particular, organic inputs to soil tend to change the proportion of plant parasitic nematodes most significantly (Liu et al., 2016), and some studies have demonstrated that not only can organic fertilizers fertilize the soil and increase wheat yields but also effectively reduce the damage to the crops caused by CCN (Diacono and Montemurro, 2010; Hu et al., 2014). However, such research on how different fertilization treatments affect soil nematodes are usually limited to investigating the changed chemical properties of treated soil. The key factors, abiotic and biotic, driving CCN infestations under different modes of fertilization are still largely unknown.

A large abundance of nematode antagonistic fungal consortia will accumulate on plant parasitic nematode suppressive soil (Raaijmakers and Mazzola, 2016; Hamid et al., 2017; Botelho et al., 2019; Topalovic et al., 2020). For instance, the fungi *Cladosporium* and *Syncephalastrum* were significantly enriched in suppressive coffee farm soils infested with the root-knot nematode *Meloidogyne exigua* (Botelho et al., 2019). Both *Purpureocillium* and *Pochonia* were more abundant in soybean cyst nematode suppressive soils under long-term monoculture conditions (Hamid et al., 2017). Other taxa of nematode antagonistic fungi, such as *Dactylella*, *Nematophthora*, *Trichoderma*, *Hirsutella*, *Haptocillium*, *Catenaria*, *Arthrobotrys*, *Dactylellina*, *Drechslerella*, *Chaetomium*, and *Mortierella* were also detected in suppressive soils (Kerry and Crump, 1980; Ashrafi et al., 2017; Topalovic et al., 2020). Apart from those specific fungi, there is evidence that communities of fungal consortia can inhibit the growth and activity of soil-borne pathogens in disease suppressive soils (Weller et al., 2002; Dudenhöffer et al., 2016). Additionally, substrate-mediated shifts in soil microbial communities were found associated with the transition of *Verticillium* wilt-conducive soils to -suppressive soils, suggesting that soil-borne pathogen suppressive soils can arise *via* manual amendment (Mueller and Sachs, 2015; Inderbitzin et al., 2018). Recently, nitrate fertilization improved the resistance of cucumber to *Fusarium* wilt disease alongside the assembly of fungal communities in the plant's rhizosphere (Gu et al., 2020), pointing to a underappreciated factor by which fertilization engenders disease suppressive soils. Although belowground associations between plant parasitic nematodes and soil fungal communities have been widely studied, the mechanisms underpinning fertilization-driven shifts in fungal community functioning towards suppression of the CCN pathogen are less known.

Here, we investigated the different effects of organic versus inorganic fertilizers on (i) the population growth of cereal cyst nematode (CCN) in wheat plants, and (ii) the soil fungal communities in a wheat field. Based on our results, we then attempt to clarify the relationships between CCN and soil fungal communities under the application of organic fertilizers. Finally, we comment on feasible technologies and approaches to alleviate CCN damage to staple crops to increase their productivity in sustainable agricultural systems.

MATERIALS AND METHODS

Experiment Design and Soil Sampling

The experimental site was located at the Kai-Feng Experimental Station of China Agricultural University in Henan Province, North China Plain (34°76′ N and 114°27′ E), where there is a high incidence of *Heterodera avenae* disease (Yuan et al., 2014). The mean annual temperature is 16.5°C, ranging from a minimum of 2°C in January to a maximum of 29.5°C in July, and mean annual precipitation is approximately 621 mm, of which 60% occurs from July to September.

The fertilization experiment was designed to test three treatments: *organic fertilizer* (OF: applied at 15 t ha^{-1}, according to the report by Hu et al. (2014), soil plant-parasites nematodes could be suppressed under this dosage; mostly chicken manure, with a mean nutrient content of N = 18.63 g kg^{-1}, total P = 15.67 g kg^{-1}, total K = 18.51 g kg^{-1}, and organic matter = 363.56 g kg^{-1}), *chemical fertilizer* (CF: 234 kg ha^{-1} of N, 72 kg ha^{-1} of P_2O_5, 99 kg ha^{-1} of K_2O; local traditional

fertilization), and a *control group* (CK: no fertilization). Each treatment had three replications. Each replication consisted of a plot 8 m × 8 m in size (L × W), with all 9 plots laid out in a completely randomized arrangement. All plots were planted with wheat (*Triticum aestivum* cv. 'Zhoumai 22') in winter (from October to June) and with maize (*Zea mays* L.) in summer (from June to October) on an annual rotation, which is the typical cropping system used in this region. For the OF treatment, organic fertilizer was applied on the day before wheat sowing. As to the CF treatment, which is the conventional mode of fertilization used by farmers in this region, 2/3 of the total fertilizer was applied prior to wheat sowing and the other 1/3 applied at the end of February. Except for the different fertilizers, all field management practices were the same, and no fertilizer was applied during the planting of maize. The fertilization experiment was conducted from October in 2017 to June in 2019.

For soil fungal community analysis, rhizosphere soil samples were collected at seven time-points: before sowing, and the seedling, wintering, regreening, jointing, heading, and harvest stages of wheat. To obtain the rhizosphere soils, excess soil was first removed by manually shaking the roots of wheat, leaving an approximately 2-mm layer of soils still attached to the roots. Then, using a sterilized brush, the root-attached soil material was collected as a rhizosphere soil sample (Edwards et al., 2015). This material from nine random sampling points [each sampling point contains an area 15 cm × 15 cm × 20 cm in size (L × W × D)] in each plot was then pooled into one sample (approximate 60 g soils); hence there were three replicated samples per fertilizer treatment. In addition, from each sample, 20 g of rhizosphere soils were immediately frozen in liquid nitrogen for the fungal community analysis, and the remainder (approximate 40 g soils) used for the analysis of their chemical properties. The wheat grain weight in an area 1 m × 2 m in size (L × W) per plot was measured as crop yield and recorded.

CCN Cyst Investigation and Chemical Properties' Analysis

To investigate number of cysts of CCN, bulk soil samples before wheat crop's sowing and after its harvest were collected. There were three soil samples were collected for per plot, each soil sample contained three randomly sampling sites, each site was sampled in an area 15 cm × 15 cm × 20 cm (L × W × D). There were 27 soil samples (3 samples × 9 plots = 27) were collected. 500 g soil from each soil sample was used to extract the cysts by following the methodology of Krusberg et al. (1994). Next, the number of recovered cysts per sample was counted under a stereoscope (Olympus, Japan). An index Pf/Pi of cereal cyst nematodes was calculated by cyst numbers at the harvesting of wheat comparing to that before sowing.

Soil's pH and nutrient concentrations (organic matter, available P, total P, available K, total K, and total N) were measured according to the *Soil Physicochemical Analysis Handbook* (Bao, 2000), using air-dried rhizosphere soil samples. Briefly, a 10 g soil sample was placed in a 200 ml flask with 50 ml of distilled water and shaken for 3 min and then filtered. A 20 ml aliquot from the filtrate was used to determine the pH value with a pH meter (METTLER TOLEDO, Switzerland). The organic matter was digested from 0.2 g soils with 0.4 mol L^{-1} $K_2Cr_2O_7$-H_2SO_4 applying additional heat (170°C) in oil for 5 min and determined by titrimetric analysis. The available P was extracted from 2.5 g soils with 0.5 mol L^{-1} $NaHCO_3$ for 30 min and total P was extracted from 1.0 g soils with $HClO_4$-H_2SO_4 digested for 1 h, then the digestive solutions were analyzed using a spectrophotometer (LASPEC, China). The available K was extracted from 2.5 g soils with 1.0 mol L^{-1} NH_4OAc for 30 min and total K was extracted from 0.2 g soils with 2.0 g solid granular NaOH applying additional heat (720°C) for 15 min, then the digestive solutions were analyzed using a flame photometer (Alpha Chemika, England). Total N was determined with 1.0 g soils by a semi-micro Kjeldahl digestion followed by ammonium distillation and titrimetric determinations.

Fungal Diversity and Community Analysis

To extract the total DNA from each rhizosphere soil sample (0.5 g), the FastDNA® Spin Kit for Soil (MP Biomedicals, USA) was used by following the manufacturer's instructions. The purity and concentration of all extracted DNA were both quantified by a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). The ITS1-ITS2 hypervariable region of fungi was amplified using the specific primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') (Adams et al., 2013). In brief, the PCR system consisted of 4 μl of 5×FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu polymerase (TIANGEN, China), 0.2 μl of BSA, and 10 ng of template DNA, which was amplified in a total volume of 20 μl. The thermal-cycling conditions used: denaturation at 95°C for 3 min, then 35 cycles at 95°C for 30 s, annealing at 55°C for 30 s, with an extension at 72°C for 45 s, followed by 10 min at 72°C. The PCR products were purified using a PCR Clean-UpTM kit (MO BioLabs, USA) and then sent to the Majorbio Company (Shanghai, China) for sequencing using the Illumina MiSeq PE300.

Bioinformatics Analysis

Barcodes and primers were removed after quality control, any low-quality sequences were filtered out using Trimmomatic (v0.33, http://www.usadellab.org/cms/?page=trimmomatic) and the remainder merged using the Flash tool (v1.2.11, https://ccb.jhu.edu/software/FLASH/). Those merged sequences having > 97% similarity were assigned to the same operational taxonomic unit (OTU), using Uparse software (v7.0.1090, http://www.drive5.com/uparse/) (Edgar et al., 2011). The OTUs' abundance levels were normalized based on the sample having the least number of sequences, which was 31,639. To ensure robust comparisons among the samples, all subsequent analyses were made using this normalized data set. Taxonomic characterization of the representative sequences of fungal OTUs was performed using the Unite database (v8.0, https://unite.ut.ee/), by applying the BLAST method with a 0.7 similarity threshold (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The richness estimators (Sobs and Chao1), diversity indices (Shannon and Simpson) and coverage indices (Coverage) were determined

using Mothur software (v1.30.1 http://www.mothur.org/). The FUNGuild approach was used for making fungal community predictions (Nguyen et al., 2016).

Statistical Analysis

To test for differences in fungal α-diversity (Sobs, Chao1, Shannon, Simpson, or Coverage) among the three treatments, the Student's t test was used. In the β-diversity analyses, principal coordinate analyses (PCoA) utilizing the bray-curtis distances were used to evaluate the differences among treatments. Meanwhile, redundancy analysis (RDA) was relied upon to examine the relationships among a subset of selected soil environmental properties and the soil fungal communities. PCoA, analysis of similarities (ANOSIM), and RDA were all carried out using the "vegan" package in R (v.3.5.1; https://cran.r-project.org). Linear discriminant analysis (LDA) coupled with effect size determination (LEfSe) was conducted to identify significantly different fungal taxa among the OF, CF, and CK treatments. The LDA score threshold was set to 2.5, and this analysis done online to obtain the LEfSe (http://huttenhower.sph.harvard.edu/galaxy/).

To construct the co-occurrence network of fungal genera in each treatment, only the abundant genera were used (the 50 most abundant). Because the data did not conform to the assumptions of the general linear model, significant correlations between any two genera were calculated using Networkx software (http://networkx.github.io/), with Spearman's $|r| > 0.7$ and a p-value < 0.05 following the previously described method (Ye et al., 2020). Networks visualization was calculated with the interactive platform Gephi (https://gephi.org/). Considering the same nodes existed in the 50 most abundant genera of different treatments, the genera enriched in the OF treatment (vs CF and CK) were grouped together as module 1; the nodes of module 2 and 3 respectively indicated positive and negative correlations to module 1 nodes; the module 4 nodes had no significant correlation with module 1 nodes. Significant differences in each of the soil properties, yield, and Pf/Pi among the three fertilization treatments was determined by ANOVA (at $p < 0.05$).

RESULTS

Pf/Pi of CCN Cysts Is Negatively Correlated With Wheat Yield and Soil Total N

Given the hysteresis of fertilization for shifting soil fertility, soil chemistry properties were investigated in the 2nd year trial. After continuously applying the organic fertilizer for two years, the soil total N, total P, available P, available K, and organic matter were 1.19 g kg^{-1}, 1.44 g kg^{-1}, 115.8 mg kg^{-1}, 275.9 mg kg^{-1}, and 22.96 g kg^{-1} at wheat jointing stage, respectively (**Table 1**). These soil properties were significantly enhanced over those in treatments with (CF) or without (CK) chemical fertilizer. Therefore, wheat yield was significantly increased by the OF treatment. Interestingly, the index of Pf/Pi of cereal cyst nematodes (its cyst numbers at the harvesting of wheat comparing to that before sowing) was markedly lower in the OF treatment (**Table 1**).

To understand the relationships among Pf/Pi, soil chemical properties, and wheat yield, a heatmap of bivariate Pearson correlations was generated (**Figure S1**). The Pf/Pi had significant curvilinear negative correlation with soil total N (TN) ($R^2 = 0.6631$, $p < 0.01$) and yield of wheat ($R^2 = 0.7680$, $p < 0.01$) (**Figures 1A, B**). The yield of wheat had a significant curvilinear positive correlation with soil TN ($R^2 = 0.759$, $p < 0.05$) (**Figure 1C**).

General Analyses of the Hi-Seq Sequencing Data

After filtering out any chimeric sequences and mismatches, the number of ITS region-reads obtained from the 126 samples totaled 7,836,371. Based on a 97% similarity threshold, a total of 2631 OTUs were obtained for fungi across all the samples. Rarefaction curves of most samples tended to be flatten (**Figure S2**), suggesting that a reasonable sequencing depth was achieved (although extra rare fungal taxa were likely present in the samples). This robust sampling extent was further supported by the high coverage estimates obtained (**Tables 2** and **S1**).

Influence of Organic Fertilizer on α-Diversity and β-Diversity of Fungal Communities

The α-diversity of fungal communities in each year trial were compared among OF, CF, and CK treatments at different wheat growth stages (**Tables 2** and **S1**). Significant differences were observed from the seedling through to jointing stages of wheat. At the seedling stage, community richness indices (including Sobs index and Chao1 index) were lower in the OF than CK treatment in both years. After continuous organic fertilizer application, higher fungal community diversity (including Shannon index and Simpson index) was mainly observed under this treatment at the jointing stage of wheat in the 2nd year trial (**Table 2**). Additionally, PCoA was done at the OTU level to infer the similarity of soil fungal communities at wheat's

TABLE 1 | Soil properties at jointing stage of wheat, wheat yield and Pf/Pi of cereal cyst nematode treated by chemical fertilizer (CF), organic fertilizer (OF) or without fertilizer (CK) treatments in the 2nd year.

Treatment	pH	Organic Matter (g/kg)	Available P (mg/kg)	Total P (g/kg)	Available K (mg/kg)	Total K (g/kg)	Total N (g/kg)	Yield (kg/ha)	Pf/Pi
CK	7.48 ± 0.01^{a}	18.34 ± 0.55^{b}	34.92 ± 1.46^{b}	1.168 ± 0.021^{b}	181.8 ± 9.2^{b}	6.624 ± 0.182^{a}	0.857 ± 0.071^{b}	5503 ± 421^{c}	3.046 ± 0.779^{a}
CF	7.34 ± 0.06^{b}	18.62 ± 1.11^{b}	44.61 ± 2.09^{b}	1.183 ± 0.043^{b}	153.9 ± 13.1^{b}	6.606 ± 0.362^{a}	0.949 ± 0.019^{b}	7066 ± 172^{b}	1.664 ± 0.293^{b}
OF	7.37 ± 0.01^{ab}	22.96 ± 1.41^{a}	115.8 ± 7.0^{a}	1.440 ± 0.045^{a}	275.9 ± 37.4^{a}	7.026 ± 0.250^{a}	1.189 ± 0.061^{a}	7857 ± 110^{a}	1.160 ± 0.237^{c}

Values are mean ± SE. Different letters indicate significant differences from one another (P < 0.05) as identified by one-way ANOVA.

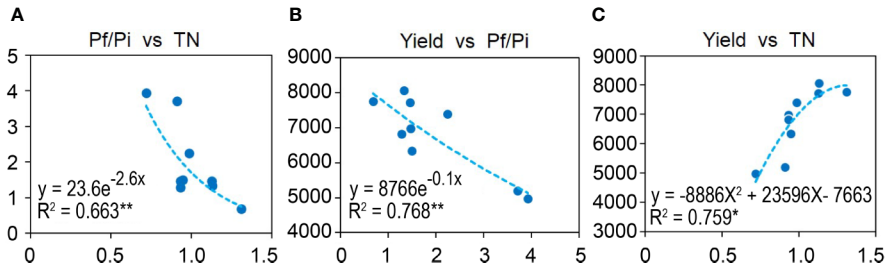

FIGURE 1 | Correlations between **(A)** Pf/Pi and TN, **(B)** Yield and Pf/Pi, or **(C)** Yield and TN. Curvilinear of soil and wheat yield in the 2nd year. * represents the $p < 0.05$, ** represents the $p < 0.01$.

TABLE 2 | Richness and diversity estimation of the ITS sequencing libraries in CK, CF, and OF treatments in the 2nd year trial.

α Diversity		Wheat growth stages						
		Before sowing	Seedling	Wintering	Regreening	Jointing	Heading	Harvest
Sobs	CK	425 ± 12	474 ± 14	541 ± 31	476 ± 12	405 ± 54	537 ± 40	484 ± 24
	CF	451 ± 48	392 ± 88	452 ± 18*	499 ± 47	457 ± 3	473 ± 22	421 ± 38
	OF	423 ± 53	385 ± 36*	414 ± 90	428 ± 23*	469 ± 40	446 ± 57	479 ± 31
Chao1	CK	548 ± 2	644 ± 12	653 ± 9	650 ± 16	547 ± 43	664 ± 68	618 ± 40
	CF	560 ± 66	542 ± 130	578 ± 48	642 ± 58	596 ± 32	652 ± 65	537 ± 39
	OF	538 ± 60	512 ± 54*	575 ± 132	552 ± 56	596 ± 85	589 ± 65	614 ± 41
Shannon	CK	3.66 ± 0.04	3.70 ± 0.16	4.08 ± 0.34	3.72 ± 0.13	2.34 ± 0.51	3.81 ± 0.36	3.94 ± 0.29
	CF	3.51 ± 0.26	2.76 ± 1.20	3.58 ± 0.23	3.91 ± 0.17	3.46 ± 0.24*	3.55 ± 0.26	3.66 ± 0.20
	OF	3.53 ± 0.37	2.84 ± 0.30*	3.29 ± 0.42	3.66 ± 0.05	3.59 ± 0.36*	3.54 ± 0.34	3.89 ± 0.31
Simpson	CK	0.057 ± 0.005	0.051 ± 0.009	0.044 ± 0.016	0.059 ± 0.012	0.351 ± 0.115	0.063 ± 0.028	0.057 ± 0.023
	CF	0.095 ± 0.027	0.248 ± 0.228	0.080 ± 0.019	0.047 ± 0.011	0.087 ± 0.050*	0.082 ± 0.032	0.057 ± 0.010
	OF	0.074 ± 0.059	0.155 ± 0.077	0.092 ± 0.031	0.055 ± 0.005	0.077 ± 0.045*	0.075 ± 0.033	0.057 ± 0.025
Coverage	CK	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001
	CF	0.997 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.997 ± 0.001
	OF	0.997 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001	0.996 ± 0.001

*CK, without fertilizer; CF, chemical fertilizer; OF, organic fertilizer. Values are mean ± SE. Asterisks indicate statistically significant differences compare to CK in same column by t-test: *p < 0.05.*

jointing stage among different treatments (**Figure 2**). The OF samples were obviously separated from that of CF or CK in both years. The ANOSIM analysis revealed a clear separation of treatment ($R = 0.2333$, $p < 0.05$) and time ($R = 0.9609$, $p < 0.001$).

Factors Influencing Shifts in Soil Fungal Communities and CCN Under Organic Fertilizer

RDA was used to determine the correlation of soil properties (**Table 1**) with fungal community structures at the wheat jointing stage (**Figure 3**). These soil variables evidently influenced the fungal community, with the first two axes explaining 82.76% and 8.51% of variance in the species' compositional data, respectively. This revealed that total N, total P, available P, and available K were significantly and positively correlated with fungal community structure in the OF treatment. Particularly, both available P and total P were the two most important contributors ($r^2 = 0.905$ and 0.827, respectively) to variation in the fungal communities (**Table S2**). Interestingly, soil fungal communities treated with OF showed significant negative correlations with Pf/Pi (**Figure 3**) which was consistent with the down-regulation of Pf/Pi of cereal cyst nematode found in the OF treatment. Additionally, of the 12 most abundant OTUs, six were positively related to the OF treatment. This number was more than that of positively related OTUs in CF or CK treatment.

Negative Correlations Between the Enriched Genera of Fungi and Pf/Pi of CCN in the OF Treatment

To investigate the distinct taxonomic profiles in OF treatment at the wheat jointing stage in the 2nd year trial, the abundance enriched fungal taxa in each treatment against the others were identified by LDA measurements (**Figures 4** and **S3B**). The numbers of enriched taxa were much higher in the OF treatment than those of either the CF or CK treatments. The significantly enriched taxa numbers in the OF, CF, and CK treatments respectively were 19, 3, and 0. Under the OF, *Volutella* (the genus), *Hapsidospora* (genus), *Chaetomiaceae* (the family and its genus *Chaetomium*), *Schizothecium* (genus), *Microascales* (the order, and its family *Microascaceae* and genus *Lophotrichus*), *Thelebolales* (the order, and its family *Pseudeurotiaceae* and genus *Geomyces*), *Laboulbeniomycetes* (from class to genus), and *Mortierellomycetes* (class to genus) were all enriched. In stark contrast, only *Exserohilum* (genus)

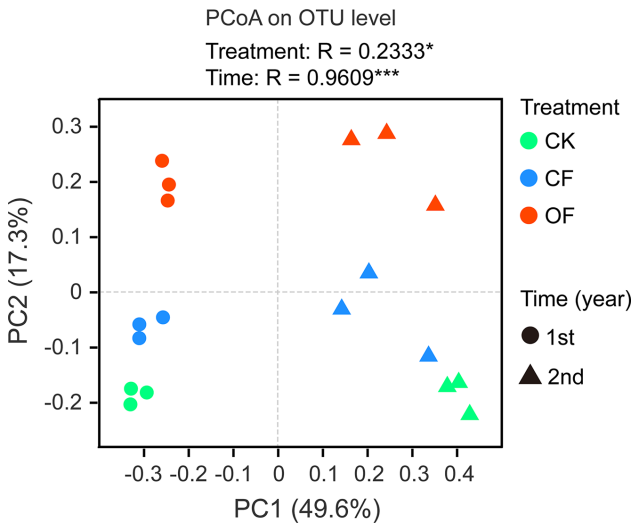

FIGURE 2 | Principal co-ordinate analysis (PCoA) utilizing the bray-curtis distances on operational taxonomic unit (out) level at wheat jointing stage in two years trial. CK, without fertilizer; CF, chemical fertilizer; OF, organic fertilizer. Asterisks indicate statistically significant differences by ANOSIM analysis: $*p < 0.05$, $***p < 0.001$.

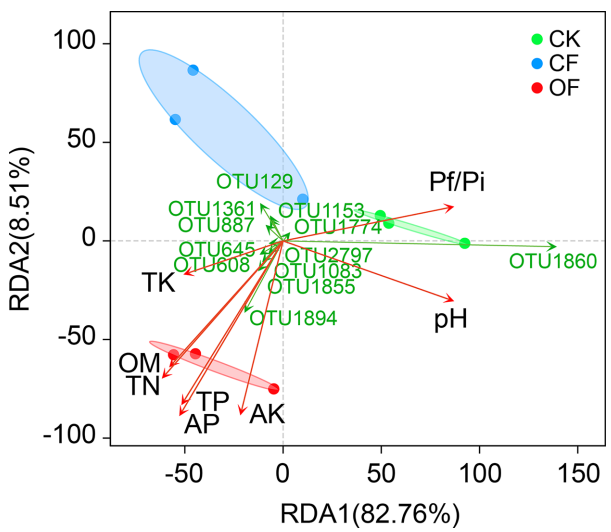

FIGURE 3 | Redundancy analysis (RDA) on operational taxonomic unit (OTU) level, soil properties, and Pf/Pi of cereal cyst nematode at wheat jointing stage in 2nd year trial. CK, without fertilizer; CF, chemical fertilizer; OF, organic fertilizer. Soil properties: TN, total N; TP, total P; TK, total K; AP, available P; AK, available K; OM, organic matter. The OTUs represent the 12 most abundant species.

and *Arthopyreniaceae* (family to genus) were enriched by the CF treatment.

To determine the relationships between Pf/Pi of cereal cyst nematode and the relative abundance of enriched genera observed in the OF treatment, linear correlation analyses were conducted (**Figure 5**). The relative abundances of the eight enriched genera, presented in **Table S3**, were negatively correlated with the Pf/Pi of CCN, except those of *Hapsidospora* and *Pyxidiophorales_XX*, which occurred only in the OF treatment at a relative abundance of 0.08% and 0.34%, respectively. Notably, the abundances of genera *Chaetomium* (4.9%) and *Mortierella* (19.6%), showed a significantly negative correlation with the Pf/Pi of CCN. Interestingly, the *Lophotrichus*, *Mortierella*, and *Geomyces* genera were influenced by organic fertilization greatly, as reflected by their identical enrichment in both trial years (**Figure S3**).

To parse the fungal community datasets by ecological guild, FUNGuild was used, an efficient and widely used method. In comparison with the CK and CF treatments, trophic mode (pathotroph, saprotroph, symbiotroph) and guild (especially that of pathogen) of the eight enriched fungal genera in the OF treatment were analyzed (**Table 3**). Except for the unmatched genus *Pyxidiophorales_XX*, the other unique genus *Hapsidospora* in OF treatment was designated a saprotroph. Additionally, genera *Geomyces*, *Lophotrichus*, and *Schizothecium* also belonged to the saprotroph guild. Mortierellomycota was classified into both saprotroph and symbiotroph. Interestingly, *Chaetomium* and *Volutella* were each a pathotroph. The FUNGuild database predicted *Chaetomium* as an animal pathogen; hence it could inhibit soil animals such as nematodes.

More Taxa Correlated With the Enriched Genera in the OF Treatment

The 50 most abundant soil fungal genera, constituted an intense community network that differed considerably among the OF, CF, and CK treatments (**Figure 6**). Among the eight fungal genera enriched by organic fertilization, those of *Geomyces*, *Lophotrichus*, *Chaetomium*, and *Mortierella* belonged to the groups of 50 most-abundant genera. To analyze associations between the enriched genera and others, the former were designated as module 1 nodes (red color), for which the nodes of module 2 and 3 respective indicate the positive and negative correlations, while the module 4 nodes had no significant ($p < 0.05$) correlation with module 1. This network analysis revealed that more genera were significantly related to module 1 nodes in the OF treatment than in the CF and CK treatments (44, 39, and 25 genera, respectively). Under the OF treatment, the number of positive and negative genera were 39 and 5, respectively. Meanwhile, the percentage of positive relation among different nodes in OF was 81%, much higher than CF's 52% or CK's 65%. The higher relative abundance—as conveyed by the size of each node—of module 1 nodes and its 39 significant positively correlated fungal genera constituted a more intense community network in the soils treated with the organic fertilizer.

DISCUSSION

Organic Fertilization Reduces Pf/Pi of CCN to Enhance Wheat Yield

Applying organic fertilizers (OF) is able to improve soil fertility and wheat yield, but it can also effectively reduce the damage to

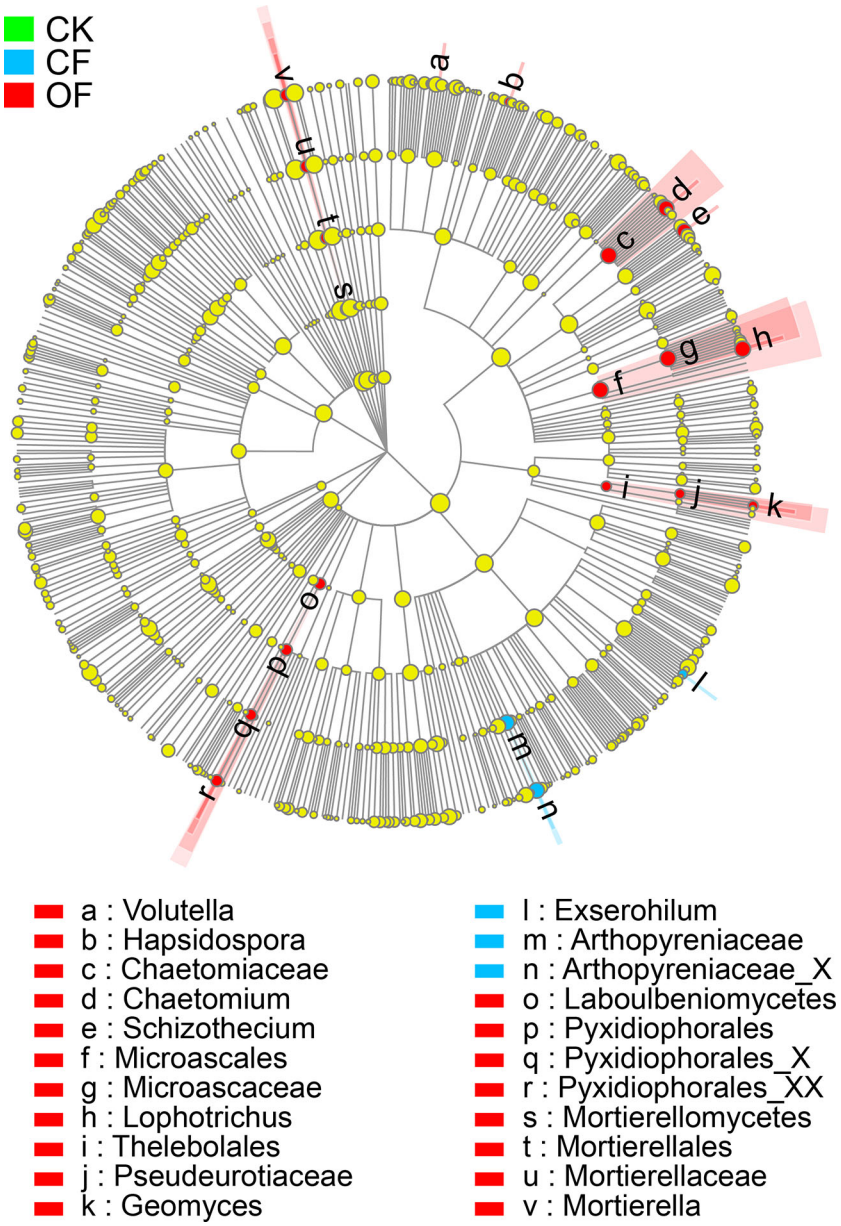

FIGURE 4 | Cladogram showing the phylogenetic distribution of fungal lineages in soils. The taxa of fungi with significantly enriched abundances treated with organic fertilizer (OF), chemical fertilizer (CF), or without fertilizer (CK) are represented by red, blue, or green dots, respectively. The taxa with the absolute LDA scores >2.5 and $p < 0.05$ are shown.

crops from cereal cyst nematode [CCN] (Diacono and Montemurro, 2010; Hu et al., 2014). A similar phenomenon was found in our present study. Compared with CF, the OF treatment significantly enhanced soil organic matter and improve other soil properties, while decreasing the Pf/Pi of CCN (**Table 1**). Some studies have pointed out that a few different elemental fertilizers can exhibit various effects on CCN populations. For instance, nitrogen and phosphate fertilizers, especially urea or calcium superphosphate, are effective at suppressing the abundance of CCN or plant parasitic nematodes (Al-Hazimi and Dacbah, 2014; Zhao et al., 2014; Song et al., 2015). Potassium sulfate promotes whereas ammonium sulfate alleviates the damage to plants caused by CCN (Yang et al., 2008; Ikoyi et al., 2020), thus implicating potassium a plausible key factor promoting CCN's abundance in fields (Yang et al., 2008). In our study, the integrated shifts of soil properties in the OF treatment might have reduced the Pf/Pi of CCN, with soil nitrogen having the strongest effect (significant

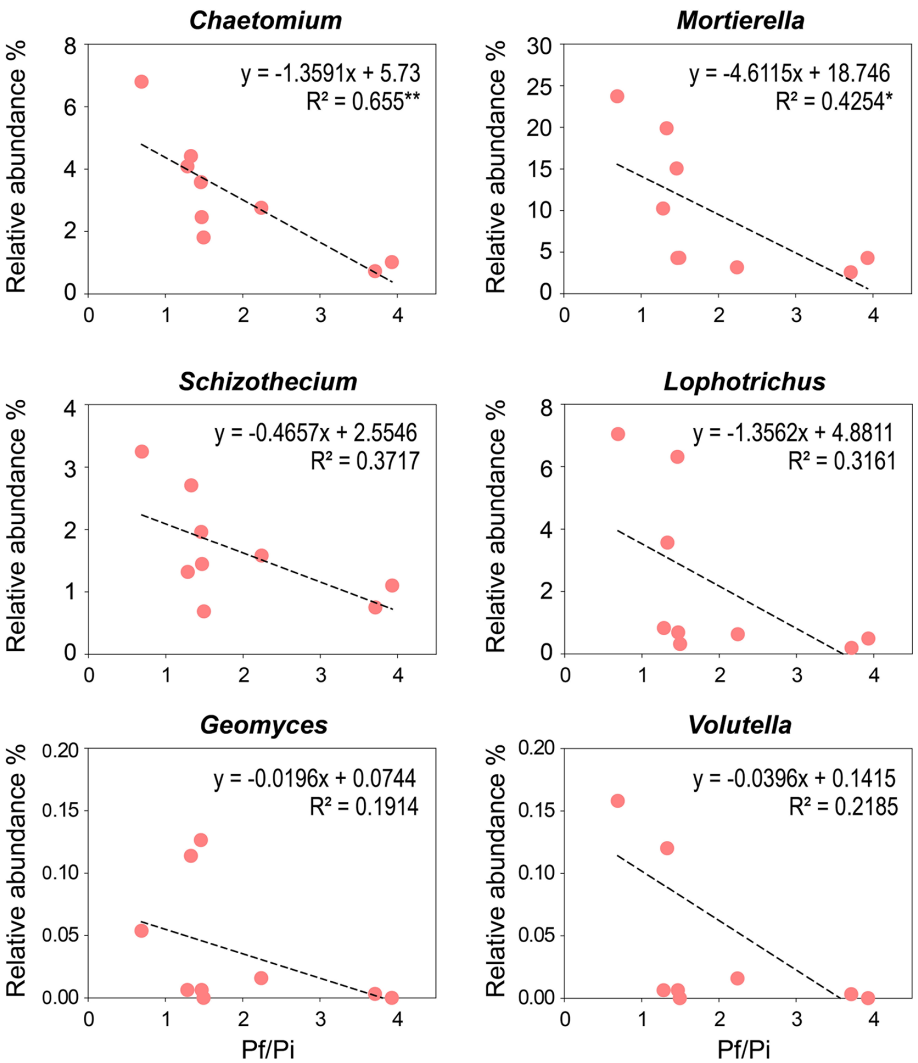

FIGURE 5 | Negative correlations between Pf/Pi of cereal cyst nematode and the enriching genus (*Chaetomium, Mortierella, Schizothecium, Lophotrichus, Geomyces*, and *Volutella*) observed in organic fertilizer treatment at wheat jointing stage in 2nd year trial. * represents the $p < 0.05$, ** represents the $p < 0.01$.

negative correlation with Pf/Pi) on suppressing CCN reproduction. Ultimately, the suppressed abundance of plant parasitic nematodes will likely ensue with the addition of organic materials over the long-term (i.e., more than 10 years) in such fertilization experiments (Li et al., 2010). In addition, in wheat fields treated with a gradient of nitrogen applications, plant parasitic nematodes attained their highest numbers at 100 kg N ha^{-1} application, but decreased at 300 kg N ha^{-1} application (Song et al., 2015). Hence, the quantity of application of fertilizers may also affect the community structure of soil nematodes, as well as the abundance of those that are plant parasitic nematodes.

The type of organic materials used in fields may also influence differential suppression of plant parasitic nematodes in soils (Li et al., 2010; Roth et al., 2015). For example, sugarcane bagasse and sugarcane refinery sludge were used as organic inputs in a banana field, where their application suppressed the relative abundance of plant parasitic nematodes more so than the application of plant residues (Tabarantab et al., 2011), and the latter's effects on plant parasites also differed from those of animal manure. Recently, Li et al. (2018) found that the relative abundance of plant parasites was highest in a polar leaf addition, followed by maize straw, yet it was lowest in cow manure treatments. Therefore, it seems that a more suitable soil environment for plant parasitic nematodes is created by plant residues application. Nevertheless, different effects from various kinds of animal manures upon CCN, such as chicken manure versus cow manure, have yet to be characterized.

Organic Fertilization Promotes the Enrichment of Fungal Communities Negatively Related to CCN

Fertilization of soil, through organic or chemical fertilizers, may affect rhizosphere fungal communities by changing their

TABLE 3 | Functional profile of the enriched genus in organic fertilizer treatment predicted by FUNGuild.

Phylum	Genus	Trophic Mode	Guild	References
Ascomycota	*Chaetomium*	Pathotroph/ Saprotroph/ Symbiotroph	Animal Pathogen/ Dung/Plant/Wood Saprotroph/ Endophyte/Epiphyte	Bills et al. (2013) Tedersoo et al. (2014) Massimo et al. (2015) Busby et al. (2016) David et al. (2016)
Ascomycota	*Volutella*	Pathotroph	Plant Pathogen	Tedersoo et al. (2014)
Ascomycota	*Hapsidospora*	Saprotroph	Undefined Saprotroph	Sterkenburg et al. (2015)
Ascomycota	*Geomyces*	Saprotroph	Soil Saprotroph	Minnis and Lindner (2013) Tedersoo et al. (2014)
Ascomycota	*Lophotrichus*	Saprotroph	Undefined Saprotroph	Tedersoo et al. (2014)
Ascomycota	*Schizothecium*	Saprotroph	Dung Saprotroph	Bills et al. (2013) Tedersoo et al. (2014)
Mortierellomycota	*Mortierella*	Symbiotroph/ Saprotroph	Endophyte/Litter/Soil Saprotroph	Cannon and Kirk (2007) Purahong et al. (2016)
Ascomycota	*Pyxidiophorales_XX*	–	–	–

'–' indicates no matched function in FUNGuild data base.

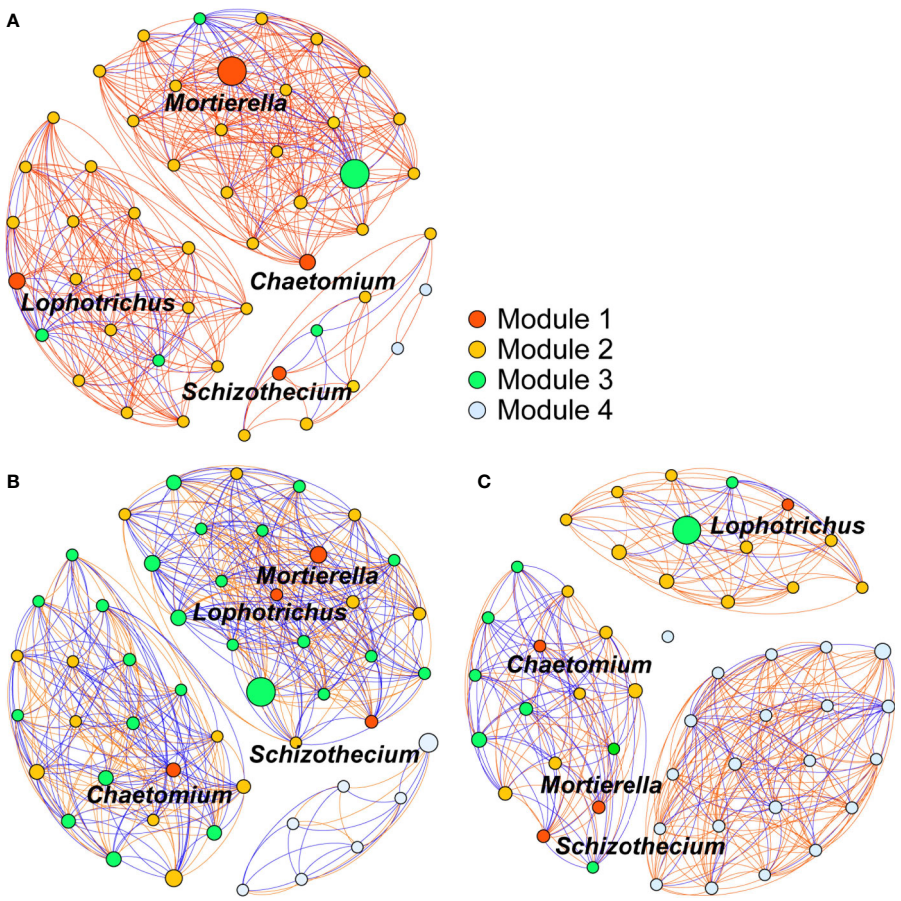

FIGURE 6 | Co-occurrence network of the 50 most abundant soil fungi (genus level) treated with organic fertilizer **(A)**, chemical fertilizer **(B),** or without fertilizer **(C)**. Module 1 nodes indicate the enriched genus observed in organic fertilizer treatment. Nodes of module 2 and 3 indicate the positive and negative correlations to module 1 nodes, respectively. Module 4 nodes indicate no significant ($p < 0.05$) correlation with module 1. Each edge stands for (Spearman's $|r| > 0.7$) correlations. Red edges represent positive correlations, blue edges represent negative correlations. The size of each node is relative abundance of the genus.

food or energy sources in terms of the quality and quantity of root exudates (Kerry, 2000; Nuland et al., 2016; Zhang et al., 2017). In this study, the induced changes to soil fungal communities mainly occurred from the seedling through jointing stage of wheat (**Tables 2** and **S1**). Higher fungal community diversity was not observed until the jointing stage in the 2nd year trial, a result that may be explained by the hysteresis of continuous organic fertilization. Similarly, fungal community structures under the OF treatment were distinct and unlike the other treatments in the 2nd year trial (**Figure 2**), indicating a significant influence on the rhizosphere ecosystem from continuous organic fertilization over two successive years. Interestingly, it precisely the wheat jointing stage that has the most suitable soil temperature for cereal cyst nematodes' egg hatching and juvenile penetration into wheat roots, thus presenting the highest risk of parasitism to wheat roots (Yuan et al., 2014; Song et al., 2015). Therefore, the jointing stage is not only a critical period for wheat yield but also a critical period for the interaction between CCN parasitism and soil fungal communities.

Organic fertilization offers a valuable way to restore soil biodiversity that is essential for developing sustainable agriculture, which has been severely undermined by intensive farming based on the widespread use of synthetic pesticides and chemical fertilizers (Glen-Karolczyk et al., 2018). Organic inputs, as a critical source of organic matter, can enhance the abundance of plant beneficial fungi that have been inhibited from the excessive use of chemical fertilizer during intensive agriculture (Ai et al., 2015), In our study, applying organic fertilizer significantly enhanced the α-diversity (Shannon and Simpson) and resulted in distinct community composition (PCoA) of rhizosphere fungal communities (**Table 2** and **Figure 2**), and promoted positive correlations between fungal community structures and key soil chemical properties (total N, total P, available P, available K) at wheat jointing stage (**Figure 3**). Furthermore, much more enriched fungus taxa were observed in the wheat rhizosphere (**Figure 4**), suggesting that organic inputs contributed to a suitable growth substrate or environment for fungi (Bernard et al., 2014). This finding is consistent with the view that soil fungal communities could be altered through an enhancement of soil chemical properties by fertilization practices (Ai et al., 2015; Yao et al., 2018).

Soil fungal community composition, diversity, and functioning have proven links to the occurrence of plant soil-borne disease (Shen et al., 2019). A large abundance of microorganisms will accumulate on pathogen-suppressive soil, and they are negatively correlated with pathogens' abundance (Raaijmakers and Mazzola, 2016; Hamid et al., 2017). Similarly, corresponding to the down regulation the Pf/Pi of CCN, the soil treated with organic fertilizer harbored fungal community structures which showed significantly negative correlations with the Pf/Pi of the nematode (**Figure 3**). Among the eight fungi genera enriched by organic fertilization, *Chaetomium* and *Mortierella* had negative correlations with the Pf/Pi of CCN (**Figure 5**). Further, a more intense community network of rhizosphere fungi formed under the OF treatment (**Figure 6**), suggesting these fungi may have functions for suppressing CCN. Generally, natural disease suppression does not rely on a single taxon but rather the high abundance of several microbial taxa, or special functional groups of microorganisms, corresponding to disease suppression (Sanguin et al., 2009; Mendes et al., 2011; Chapelle et al., 2016). Yet rare populations may also exert a disproportionately large effect on a community's functional stability if they provide a common good, such as secreted extracellular enzymes or essential growth factors that other members are incapable of synthesizing (Konopka et al., 2015). Accordingly, the unique existence of genera *Hapsidospora* and *Pyxidiophorales_XX* in the OF treatment should not be overlooked, despite their very low relative abundance of 0.08% and 0.34%, respectively.

The Assembled Soil Fungi Are Candidate Biocontrol Agents for CCN

Nematophagous fungi have been extensively investigated as biological control agents of nematodes since the 20th century (Barron, 1977). Two of the enriched soil fungi genera in the OF treatment, *Mortierella* and *Chaetomium*, are known to have the ability to suppress many pathogens (Edgington et al., 2014; Melo et al., 2014; DiLegge et al., 2019). Overall, their functional mechanisms are related to antibiotics' synthesis and internal parasitism. For example, *M. alpina* can synthesize alkaloid antibiotics and possess marked biocontrol activity against some human pathogens (Melo et al., 2014). These compounds have been isolated from the endophytes *Neotyphodium* spp. of grasses and then used to enhance the protection of plants against worms and phytopathogens (Schardl et al., 2004; Zhang et al., 2012). Furthermore, both *M. alpina* and *M. signyensis* are apt at killing insect pests, such as the wax moth (*Galleria mellonella* L.) and housefly (*Musca domestica* L.), by inoculation or injection (Edgington et al., 2014). As to another ecological function of *Mortierella*, the species *M. globalpina* was confirmed as pathogenic against root-knot nematodes by trapping them, then penetrating and digesting the nematodes' cellular contents (DiLegge et al., 2019).

Besides *Mortierella*, species of the genus *Chaetomium* have been also investigated as potential biological control agents against soil-borne diseases (Tagawa et al., 2010). The *Chaetomium* members are important candidate nematode-controlling fungi, distinguished by their biosynthesis of chaetoglobosins, which are effective preventive agents against plant parasitic nematodes (Nitao et al., 2002; Hu et al., 2013). *Chaetomium* is a widespread genus, with 95 known species from around the world (Kirk et al., 2008), which can produce a diverse array of secondary metabolites: not only chaetoglobosins, but also xanthones, anthraquinones, terpenoids, depsidones, and steroids having anticancer, antioxidant, antimicrobial, and cytotoxic properties, among others (Zhang et al., 2012). *C. globosum* is reportedly an effective biocontrol agent for several plant pathogenic

microorganisms (Qin et al., 2009; Shanthiyaa et al., 2013), and its metabolites have been tested and confirmed for nematicidal activities against *Meloidogyne incognita* and *H. glycines* (Nitao et al., 2002; Hu et al., 2013). Chaetoglobosin A is the major component of the chaetoglobosins synthesized by *C. globosum* (Qin et al., 2009; Kawahara et al., 2013). A temporary inhibition of mobility was observed when chaetoglobosin A was tested against *Caenorhabditis elegans* and *H. filipjevi* (Ashrafi et al., 2017), and it has toxic, lethal effects against *M. incognita* (Hu et al., 2013).

Along with the *Mortierella* and *Chaetomium*, the *Geomyces* and *Lophotrichus* are noteworthy; together, these were the four most abundant genera enriched by OF (**Table S3**). In our network analysis, the hubs indicated the most important nodes, which may be interpreted as being the key taxa inside a connected community (Gu et al., 2020). We found these four genera negatively related to the Pf/Pi of cereal cyst nematode, and they were surrounded by much more closely associated genera, suggesting that organic fertilization assembles the fungal communities that suppress CCN in the rhizosphere of wheat plants.

CONCLUSION

Our study provided an integrative view of the relationship between cereal cyst nematode (CCN) and soil fungal communities as shaped by organic fertilizer applications in wheat field of the Kaifeng district, in China's Henan province. The organic fertilizer treatment enhanced soil total N, as well as soil organic matter, total P, available P, and available K at the jointing stage, when both egg hatching and J2 penetrating into root of wheat plant by CCN is greatest. We found that soil total N was positively correlated with wheat yield and soil fungal community structures, but negatively correlated with the Pf/Pi of cereal cyst nematode. Our results implied that integrated shifts of soil properties are a key factor contributing to the reduced index of Pf/Pi of CCN under OF; in particular, the negative effect of soil nitrogen was the most powerful at suppressing CCN. Further, organic fertilization improved α-diversity and β-diversity of rhizosphere fungal communities, and enriched several fungal genera, namely *Mortierella*, *Chaetomium*, *Lophotrichus*, *Schizothecium*, *Volutella*, and *Geomyces*, whose abundances were negatively related to the Pf/Pi of CCN. Furthermore, an intense fungal community network, characterized by much more closely related fungi surrounding the nematophagous fungal genus *Mortierella* and *Chaetomium*, suggested that CCN-suppressing fungal community structures were stimulated by organic fertilization. Taken together, organic fertilization enhances soil fertility and assembled fungal communities capable of suppressing cereal cyst nematode reproduction in the rhizosphere, resulting in wheat yield's increase. Our results help to understand deeply the relationships among fertilization, soil-borne disease, and soil fungal communities.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject number PRJNA639921.

AUTHOR CONTRIBUTIONS

WQ, QL, and HJ designed the experiments. WQ and HS performed most of the experiments. KJ and LY participated in part of the soil analysis. WQ wrote the manuscript. HS, QL, and HJ revised the manuscript. All authors discussed the results and commented on the manuscript. HJ provided funding for this work as the corresponding author.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01225/full#supplementary-material

TABLE S1 | Richness and diversity estimation of the ITS sequencing libraries in CK, CF, and OF treatments in the 1st year trial. CK: without fertilizer; CF: chemical fertilizer; OF: organic fertilizer. Values are mean ± SE. Asterisks indicate statistically significant differences compare to CK in same column by t-test: * $p < 0.05$.

TABLE S2 | Explanation of environmental factors affecting fungal community structure at wheat jointing stage in 2nd year trial. OM, organic matter; AP, available P; TP, total P; AK, available K; TK, total K; TN, total N. Pf/Pi, index of cereal cyst nematode reproduction.

TABLE S3 | Relative abundance of the enriching genus observed in organic fertilizer treatment at wheat jointing stage in 2nd year trial. CK: without fertilizer; CF: chemical fertilizer; OF: organic fertilizer. 'n' indicates no abundance.

FIGURE S1 | Pearson relationship among soil properties, wheat yield and Pf/Pi of cereal cyst nematode. * represents the $p < 0.05$. TN: total N; TK: total K; TP: total P; AK: available K; AP: available P; OM: organic matter.

FIGURE S2 | The rarefaction analysis of all samples within the Shannon index (A) and Sobs index (B).

FIGURE S3 | A linear discriminant analysis effect size (LEfSe) method identifies the significant different abundant taxa of fungi in the soil of organic fertilizer (OF), chemical fertilizer (CF), or without fertilizer (CK) treatments at wheat jointing stage. The taxa with the absolute LDA scores > 2.5 and $p < 0.05$ are shown. Red letters indicate the same taxa enriched in two-year trials.

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Hatching of *Globodera pallida* Induced by Root Exudates Is Not Influenced by Soil Microbiota Composition

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Plant-parasitic nematodes are among the most harmful pests of cultivated crops causing important economic losses. The ban of chemical nematicides requires the development of alternative agroecological approaches to protect crops against nematodes. For cyst nematodes, egg hatching is stimulated by host plant root exudates. Inducing "suicide hatching" of nematode second-stage juveniles (J2), using root exudates in the absence of the host plant, may constitute an effective and innovative biocontrol method to control cyst nematodes. However, before considering the development of this approach, understanding the effect of soil biotic component on cyst nematode hatching by root exudates is a major issue. The effectiveness of this approach could be modulated by other soil organisms consuming root exudates for growth as soil microbiota, and this must be evaluated. To do that, four different native agricultural soils were selected based on their physicochemical properties and their microbiota composition were characterized by rDNA metabarcoding. To disentangle the effect of microbiota from that of soil on hatching, four recolonized artificial soils were obtained by inoculating a common sterile soil matrix with the microbiota proceeding from each agricultural soil. Each soil was then inoculated with cysts of the potato cyst nematode, *Globodera pallida*, and low or high doses of potato root exudates (PREs) were applied. After 40 days, viable J2 remaining in cysts were counted to determine the efficiency of root exudates to stimulate hatching in different soils. Results showed that (i) when physicochemical and microbiota compositions varied among native soils, the hatching rates remained very high albeit small differences were measured and no dose effect was detected and (ii) when only microbiota composition varied among recolonized soils, the hatching rates were also high at the highest dose of PREs, but a strong dose effect was highlighted. This study shows that abiotic and biotic factors may not compromise the development of methods based on suicide hatching of cyst nematodes, using root exudates, molecules inducing J2 hatch, or trap crops.

Keywords: microbial composition, plant-parasitic nematodes, suicide hatching, potato, metabarcoding

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INTRODUCTION

Crop pest management is one of the major challenges to ensure agricultural production and to cope with the expected increase of the world population. During the past decades, chemical control has been a standard and an efficient practice to control pests and prevent economic losses (Popp et al., 2013). However, the intensive use of these agrochemical products has led to the detection of chemical in the environment, loss of biodiversity in agrosystems, as well as a rise of human and animal health risks in some agricultural production areas (e.g., Carvalho, 2017). These environmental and human health concerns are at the heart of civil society debates and public health policies, leading to the withdrawal of several chemicals in many countries. It has become necessary to search for new alternative and agroecological solutions for plant protection. This is the case, in particular, for the control of plant parasitic nematodes for which many nematicides have been recently removed from the market in Europe. As a result, several crops and vegetable industry sectors are left with few solutions.

Plant-parasitic nematodes represent a worldwide concern in food security, as they often cause nonspecific symptoms resulting in lower crop yields with significant economic losses (Nicol et al., 2011; Jones et al., 2013). Root-knot (*Meloidogyne* spp.) and cyst nematodes (*Globodera* and *Heterodera* spp.) are the two most economically important and damaging of cultivated crops (Molinari, 2011). Cyst nematodes are obligatory root endoparasites, with the ability to produce a thick survival structure, the cyst, formed by the dead female body. Cysts can contain up to 200–500 juveniles that adapt to the absence of the host by remaining in dormant eggs until their host returns. Dormancy can last for several years, which thus make these pests hard to eradicate. Basically, the second-stage juveniles (J2) hatch from the cyst depending on climate conditions and perception of chemical mediators [i.e., hatching factors (HFs)] released from the host roots. The level of dependence on root exudates varies considerably between species of cyst nematodes. For instance, *Globodera pallida*, one of the most harmful pathogens of potato (*Solanum tuberosum*) and quarantine organism worldwide, is very specific to the Solanaceae family. Indeed, this species hatches only after the perception of HFs, some of which have been identified in potato root exudates (PREs), such as the solanoeclepin A (Masler and Perry, 2018; Sikder and Vestergård, 2020).

Rhizodeposits, substances released by plant roots into their surrounding environment, include water-soluble exudates (amino acids, organic acids, sugars, phenolics, and other secondary metabolites) and water-insoluble products (sloughed cells and mucilage; Badri and Vivanco, 2009). This exudation is influenced by many plant characteristics, such as plant species and plant age, and also by many other biotic and abiotic factors (Jones et al., 2004). Through the root exudates, plants mediate a number of beneficial but also deleterious interactions with soil organisms (Dakora and Phillips, 2002; Bais et al., 2006; Baetz and Martinoia, 2014). Indeed, a range of diverse soil parasites, such as bacteria, fungi, oomycetes, nematodes, protists, or insects, have synchronized their life cycle to one of their hosts to enhance their chances of infection and survival (Perry and Clarke, 1981; Chippendale, 1982; Brown and Tellier, 2011; Zwanenburg et al., 2016). Host detection mainly occurs through a broad range of signaling molecules released by roots. Some authors suggest to use this chemical signal, as biocontrol methods, to lure the parasites and thus to decrease their population in soil, as for example, for controlling parasitic weeds (Zwanenburg et al., 2016), pathogens (Rashid et al., 2013; Balendres et al., 2016), or nematodes (Devine and Jones, 2000a; Lettice and Jones, 2015). The principle of this strategy, namely suicide hatching, is to apply hatching stimulants into the soil to induce hatching of the parasite in absence of the host plant and thus its starvation and death. For instance, in potato cyst nematodes, it was evaluated that hatched J2 cannot survive more than 2 weeks in the absence of a host plant (Robinson et al., 1987). In general, biocontrol methods are very promising in laboratory studies, but they are less reliable in the field where ecological interactions, in particular with microbial communities, are much more complex than those occurring in controlled conditions (Nicot et al., 2011; Le Mire et al., 2016; Sikder and Vestergård, 2020). Some bacteria and fungi in the soil feed on carbon compounds contained in root exudates and thus could alter the perception of chemical mediators by parasites, preventing the hatching. In this way, before developing biocontrol methods based on the suicide hatching principle, using root exudates, molecules inducing J2 hatch, or trap crops, many studies are first needed in the laboratory and in the field to measure the effects of physicochemical properties and soil microorganisms on the transmission and perception of hatching chemical mediators. Devine and Jones (2001) showed that the suicide hatching of the potato cyst nematode *G. pallida* induced by the incorporation of tomato root exudates in the soil was better in sandy soils than in clay and peaty soils under field conditions without considering the effect of soil microbiota.

In this study, we evaluated the effect of soil microbiota (and physicochemical properties) on the hatching of cyst nematodes. To do so, native agricultural soils differing by their physicochemical and microbial characteristics were selected to evaluate the global soil effect. To disentangle the microbiota effect from that of soil physicochemical properties, a sterile soil matrix was used and inoculated with the microbiota proceeding from different native soils (NSs). In parallel, metabarcoding analyses were carried out to describe the bacterial and fungal compositions. Each soil was artificially inoculated with cysts of *G. pallida*, and two different doses of PREs were added by exogenous applications. At the end of the experiment, the viable J2 remaining in the cyst were counted in order to determine the efficiency of root exudates to stimulate cyst hatching according to the different soil properties.

MATERIALS AND METHODS

Soil Materials and Inoculation

Native Soils

To measure the effect of soil (biological and physico-chemical effects), 10 soil types were sampled in fields from the main potato production areas in France. In each field, soil samples were collected at 10–30 cm below the surface at several sampling

points distributed along a diagonal and were pooled to obtain 25 kg of soil. The soils were homogenized and subsequently stored in containers at ambient temperature in the dark. Physical and chemical properties of each soil were determined at the Capinov laboratory of agri-food, agricultural, and environmental analysis (29,800 Landerneau, France; **Table 1**). A principal component analysis (PCA) was performed on the properties of the 10 sampled soils (dudi.pca function, "ade4" package; Dray and Dufour, 2007; fviz_pca_var function, "factoextra" package; Kassambara and Mundt, 2017) to explore variations in the dataset according to factors known as some of the main drivers of microbial community diversity in soils. These were namely organic matter (OM), pH, and soil texture (silt, clay, and sand; Girvan et al., 2003; Lauber et al., 2008; Baker et al., 2009; **Supplementary Figure S1**). Geographic location was also used as a discriminant factor to avoid selecting two soil samples from the same area. Out of the 10 soils, four were retained to achieve the suicide hatching experiments. They were A (Normandy, France), D (Hauts-de-France, France), F (Brittany, France), and J (Auvergne-Rhône-Alpes, France).

Recolonized Soils

To measure the effect of soil microbiota, the microbial communities of the four NSs A, D, F, and J were inoculated in a common sterile soil matrix. The soil used as matrix, named La Gruche, was collected at the INRAE experimental site (Pacé, 48°08′24.468" N, 01°48′0.99" W). The topsoil (0–5 cm) was removed and the layer between −5 and −30 cm was harvested, homogenized, sieved at 4 mm and mixed up with silica sand (1/3 sand and 2/3 soil). The soil was then sterilized with gamma radiation at 35 kGy (IONISOS, Pouzauges, France) to eradicate indigenous microorganisms and kept for 2 months in 5 kg plastic containers for stabilization at 18°C in the dark. The physicochemical properties, listed in **Table 1**, were not affected by the gamma radiation.

After 2 months, this soil was inoculated and incubated according to the method described by Lachaise et al. (2017). Briefly, the inoculation process consisted of inoculating 5 kg of the sterile soil matrix (1 container) with 90 g of NS (A, D, F, or J), re-suspending in 500 ml of deionized and twice autoclaved water. Four inoculations for each NS, corresponding to four containers, were performed per NS. Containers were incubated at 18°C in the dark to allow both bacterial and fungal growth. All containers were shaken for 5 min (Turbula®, Willy A. Bachofen) and opened for 3 min under sterile conditions (laminar flow cabinet) several days a week to facilitate microbial respiration and recolonization. Four additional containers (one of each inoculated microbiota) were added to check the biotic capacities of the recolonized soils (RSs) based on colony forming units (CFUs) count method. At the end of the recolonization process (14 days), the four containers for each NS were pooled to obtain 20 kg of RSs and pH of the four RSs were measured using the method of Sumner (1994). In brief, each soil was first dried in a stove for 48 h at 105°C. Then, 8 g of dry soil was suspended in 8 ml of CaCl2 (1:1 dilution) and agitated at 250 rpm for 30 min, and measurements were performed with a PHM220 pH meter (MeterLab®, Germany).

Characterization of Bacterial and Fungal Communities

Microbial DNA Sample Preparation

Soil DNA extractions were performed from three replicate samples per NS and per RS collected at the end of the recolonization process according to the Genosol protocol (INRA UMR Agroecology, Dijon, France) as described by Plassart et al. (2012) and modified by Ourry et al. (2018). Each sample was lyophilized under a 170-h program (−40°C/40h, −20°C/60, and then −10°C/70 h) and stored at −20°C. DNA was extracted from 1 g of freeze-dried soil in 5 ml of lysis buffer containing 100 mM of Tris-HCl (pH 8), 100 mM of EDTA (pH 8), 100 mM of NaCl, 2% SDS, and sterile ultrapure water in a 15 ml Lysing Matrix E tube (MP Biomedicals, Santa Ana, CA, USA, containing 1.4 mm ceramic spheres, 0.1 mm silica spheres, and eight 4 mm glass beads). Then, tubes were shaken manually and then for 30 s at 4 $m \bullet s^{-1}$ with a FastPrep® 24 (MP Biomedicals, Santa Ana, CA, USA) grinder for three times. Following vortex agitation, tubes were heated at 70°C during 30 min in a water bath (vortex agitations at 15 and 30 min) and then centrifuged at 3,500 rpm for 10 min at 20°C to prevent SDS solidification. For the next steps of DNA extraction, samples were duplicated in order to obtain a higher DNA concentration. De-proteinization was

TABLE 1 | Physicochemical properties of the native agricultural soils. The bold lines correspond to the selected NS (A, D, F, and J) and the sterilize matrix (La Gruche), which was used for recolonization process.

Soils	pH	CO (g/kg)	OM (g/kg)	P_2O_5 (g/kg)	K (g/kg)	Ca (g/kg)	Mg (g/kg)	Clay (g/kg)	Silt (g/kg)	Sand (g/kg)	Texture
A	**7.4**	**16**	**27.6**	**0.19**	**0.2**	**4.97**	**0.1**	**271**	**544**	**144**	**Silty Clay**
B	8.1	14.6	25.3	0.27	0.39	11.66	0.13	297	481	187	Silty sandy clay
C	6.9	10.4	17.9	0.31	0.2	2.17	0.07	136	696	141	Silt
D	**8.2**	**10**	**17.3**	**0.16**	**0.22**	**0.44**	**0.11**	**92**	**263**	**620**	**Silty Clay**
E	6.2	24.8	42.9	0.47	0.28	1.46	0.14	157	527	258	Clayey sandy silt
F	**6.8**	**20.4**	**35.3**	**0.42**	**0.44**	**3.39**	**0.15**	**157**	**656**	**141**	**Silt**
G	7.2	20.9	36.2	0.26	0.19	2.15	0.12	124	733	94	Silt
H	6.2	6	10.4	0.42	0.12	0.73	0.08	78	110	797	Sand
I	7	10.3	17.8	0.12	0.17	2.26	0.07	160	738	77	Silt
J	**6.1**	**19.7**	**34**	**0.17**	**0.06**	**1.3**	**0.06**	**122**	**167**	**668**	**Clayey Sand**
La Gruche	**5.75**	**7.34**	**12.67**	**0.04**	**0.20**	**3.71**	**0.63**	**106**	**470**	**424**	**Sandy silt**

performed by adding 1/10 volume of 3 M potassium acetate (pH 5.5) to 1 ml of supernatant (two tubes per sample), and then the tubes were homogenized by turnaround and incubated 10 min on ice before being centrifuged for 10 min at 4°C and 14,000 *g*. For the precipitation step, supernatant (around 900 µl) was collected in a 2 ml tube and 900 µl (1:1) of ice-cold 100% isopropanol were added. Tubes were shaken by turnaround and kept at −20°C for 30 min. The supernatant was eliminated after centrifugation at 13,000 rpm at 4°C for 30 min, and the DNA pellets were washed with an addition of 400 µl of ice-cold 70% ethanol. Tubes were then centrifuged at 13,000 rpm at 4°C for 5 min and the supernatants were removed. The remaining traces of ethanol were eliminated in a stove at 60°C during 15–20 min. Then, the DNA pellets were suspended with 100 µl of sterile ultrapure water. The duplicated samples were pooled and stored at −20°C prior to purification. Soil samples were purified twice. The first purification required PolyvinylPolyPirrolidone (Sigma Aldrich) Microbiospin (Bio-Rad, Hercules, CA, USA) columns, which were prepared according to the protocol described by Ourry et al. (2018). For this purpose, 100 µl of DNA solution were deposited on top of a column (previously transferred to a clean tube) and centrifuged at 1,000 *g* at 10°C during 4 min after incubation on ice for 5 min. The second purification was performed using the Geneclean® Turbo kit (MP Biomedicals). For this, five volumes of Geneclean Turbo GNomic Salt Solution (GTGNSS) was added to the obtained DNA and homogenized by pipetting. The mixture was deposited on the top of a purification column from the kit, centrifuged at 10,000 *g* at 10°C during 10 s, and tubes were emptied. Then, 500 µl of Geneclean Turbo Wash (GTW) were added into the column and tubes were centrifuged (10,000 *g* at 10°C for 10 s) and emptied. This washing step was realized twice. Empty columns were centrifuged at 10,000 *g* at 10°C for 4 min. Columns were put in a new tube, 30 µl of Geneclean Turbo Elution (GTE) solution were deposited into them, and then samples were incubated on ice for 5 min before being centrifuged at 10,000 *g* at 10°C during 1 min. The GTE, incubation on ice and centrifugation steps were repeated twice to finally obtain approximately 60 µl of clean DNA. Samples were then stored at −20°C until further analysis.

DNA quantification was performed using a Quantus™ Fluorometer (Promega, Madison, WI, USA) and the Quantifluor kit (dsDNA: E26710). PCR amplifications and sequencing were performed at the GenoScreen platform (Lille, France) using the Illumina MiSeq "paired-end" 2*250 bp sequencing technology. PCR were conducted according to the sequencing platform protocol using primers pairs: Forward_479 (5′-CAGCMGCYG CNGTAANAC-3′) and Reverse_888 (5′-CCGYCAATTCMTT TRAGT-3′), and also Forward_FF390 (5′-CGATAACGAAC GAGACCT-3′) and Reverse_FR1 (5′-ANCCATTCAATCGG TANT-3′) to amplify 16S and 18S rDNA genes, respectively.

Sequence Processing

Fastq files (read 1 and read 2) were processed with DADA2 package version 1.10.1 (Callahan et al., 2016) on the R software (R Development Core Team, 2019). The default parameters proposed by the DADA2 workflow were retained except from truncLen argument, which was set at 200. Moreover, only sequences which lengths ranging from 369 to 374 and from 312 to 324, for bacteria and fungi, respectively, were kept. Taxonomy affiliations for the amplicon sequence variants (ASVs) obtained with DADA2 were performed using a naive Bayesian classifier (Wang et al., 2007) on the Silva ver. One hundred thirty-two databases (Callahan, 2018) for bacteria and the Silva 18S ver. One hundred twenty-eight databases (Morien and Parfrey, 2018) for fungi. Unclassified phyla were removed from the dataset. Then, rarefaction curves were realized using the "ggrare" function of "ranacapa" package (Kandlikar et al., 2018) to verify that the sequence coverage was sufficient to accurately describe the bacterial and fungal composition of each sample. Data were normalized based on sequencing depth using a rarefaction procedure (rarefy_even_depth function, "phyloseq" package) at 12,614 and 14,873 per sample for bacteria and fungi, respectively. At the end of this workflow, output files obtained were an ASV table and a taxonomy table.

Microbial Analyses

Alpha Diversity

Bacterial and fungal richness and diversities, characterized as number of ASVs found in each sample and the Shannon index (on normalized data), were determined with the diversity function of R "vegan" package (Oksanen et al., 2019). For the analysis, richness and diversities were compared between NS and RS using a Wald chi-square test on a Linear Mixed-Effects Model, (lmer function, "lme4" package; Bates et al., 2015) taking into account the soil (A, D, F, and J) and the type of soil (NS and RS), as fixed factors, and the replicate, as random factor. Pairwise comparisons of estimated marginal means (EMMs) were performed (emmeans function, "emmeans" package; Lenth, 2019), applying the false discovery rate (FDR) correction for *p*-values.

Beta Diversity

The analyses of beta diversity were performed on normalized, filtered (using a 1‰ threshold) and log2-transformed ASVs tables. A Bray-Curtis dissimilarity matrix was calculated from ASV data using the vegdist function from the R "vegan" package. To compare the bacterial and fungal community compositions, a distance-based redundancy discriminant analysis (dbRDA) was performed on the matrix data (dbrda function, "vegan" package). Then, a type II permutation *F*-test for constrained multivariate analysis was performed on the dbRDA to evaluate the contribution of each factor (i.e., soil, type of soil, and interaction between them) to microbial community composition ("RVAideMemoire" package; Herve, 2019).

Taxonomic Rank Analyses

To identify phyla and genus differences among soils, normalized counts were analyzed using Likelihood Ratio Test (LR Test) on Generalized Linear Model (distribution: quasipoisson, link function: log), and pairwise comparisons of EMMs were then computed. To visualize community taxa and map similarities/differences among NS and RS, heat trees were realized with the "heat_tree_matrix" function from the R "metacoder" package (Foster et al., 2017). Heat trees were realized on normalized and filtered (genera higher than 50/10,000) data, which were transformed into presence/absence data.

Suicide Hatching Assay

Biological Material

Nematodes

The *Globodera pallida* population "Chavornay", multiplied in 2018 on potato cv. Désirée, was used to perform the suicide hatching experiments. Cysts were extracted from soil by a Kort elutriator and stored at 5°C in the dark before furthers experiments. Cysts were sieved between 400 and 500 µm diameters and an estimation of initial viable J2 contained per cyst (Pi) was done. To do so, and because this is a destructive method, 50 cysts were randomly selected from this pool of cysts: each cyst was crushed in 1 ml of tap water and the number of viable J2 was counted under a stereomicroscope. From this count, Pi was estimated at 290 viable J2 per cyst. Then, for the experiment, 10 cysts were put in a tulle bag that was inserted in the different treatments. The tulle mesh allowed the movement of J2 from the cyst to the soil.

Root Exudates

Potato root exudates were produced using 400 pre-germinated tubers of potato cv. Désirée. Tubers were suspended on grids onto 10 cans (40 tubers per can) containing 10 L of tap water each. The distance between tubers and water was approximately 0.5 cm; this close proximity of tubers to water meant that roots produced by the tubers were immediately immersed in water. Three weeks after incubation in the dark at 20°C, root exudates were collected (i.e., all the solution in the can), pooled, filtered at 0.20 µm to eliminate potential bacterial contamination and stored at −20°C. Carbon concentration of PRE, determined at the Centre Mondial de l'Innovation (CMI) Groupe-Roullier (Saint Malo, France), was 66.4 mg of C per g of dry matter. Then, as the HFs were generally C-based molecules, two doses of root exudates were tested based on the C concentration: 15 ml containing 0.13 mg of C (dose 1) and 2.5 ml (six times less) containing 0.021 mg of C (dose 2) completed with sterile permuted water at 15 ml. These two doses were selected from previous experiments performed in pots showing that applications of PRE at 30 and 15 mg of C per g of dry matter achieved a high level of hatching (unpublished data, Dr. B. Ngala and N. Mariette). So, to explore a dose effect in our experiment, we divided six times the lowest dose previously tested.

Hatching Assay

Suicide hatching experiments were conducted in a climatic chamber at 18/14°C day/night with 16 h photoperiod. Soils (550 g for NS and 650 g for RS) were packed in pots of 9*9*9.5 cm with a bag of 10 cysts inserted at a depth corresponding to one-third of the pot (from the bottom). Pots were disposed in randomized way into three blocks to buffer the possible inequalities of light and temperature within the chamber. Temperature and humidity of soils were measured during all the experiment, respectively, by thermic probes (External Soil Temp Sensor 3,667, Spectrum Technologies Inc.) and soil moisture sensors (WaterScout SM 100 Sensor 6,460, Spectrum Technologies Inc.) plugged into measurement station (WatchDog micro station 1,000 series, Spectrum Technologies Inc.). Temperature in climatic chamber was also measured with three thermochron buttons (iButton ds1921G, maxim integrated).

Three treatments per soil were tested: dose 1 of PRE (0.13 mg of C), dose 2 of PRE (0.021 mg of C), and negative control (sterile permuted water). Each treatment was replicated nine times (three blocks and three repetitions per block). The experiment took place over 40 days and every 4 days, pots were weighed and watered to reach 80% of their field capacity, with (i) dose 1 of PRE, dose 2 of PRE, or water as per treatment at days 0, 4, 8, 12, and 16 (five applications) and (ii) water at days 20, 24, 28, 32, and 36 (five applications).

At the end of the experiment (day 40), the bags of cyst were retrieved from each pot. Bags were opened, and cysts were crushed to count under a stereomicroscope the number of remaining (unhatched) viable J2 per cyst (Pf) as described above for Pi assessments. The hatching rate for each treatment was then computed as the ratio of difference between the mean Pi and Pf values to the mean Pi value [(Pi-Pf)/Pi] and expressed as percentages.

Statistical Analyses

The effects on hatching of the factors soil (A, D, F, and J), treatment (dose 1, dose 2, and control), and interaction between soil and treatment were tested using a Linear Mixed-Effects Model, (function lmer, "lme4" package; Bates et al., 2015) with parametric analysis of variance (ANOVA) provided by R package "car" (Fox et al., 2012). Block effect was introduced as random effect in the model. Normality and homogeneity of variances were checked by performing Quantile-Quantile (QQ) plots of the residuals and the fitted models using function plotresid ("RVAideMemoire" package; Herve, 2019). Pairwise comparisons of EMMs were performed ("emmeans" package; Lenth, 2019), applying the false discovery rate (FDR) correction for values of p.

RESULTS

Diversity and Structure of Microbial Communities

The suicide hatching experiments were conducted on four NSs and four RSs obtained by inoculating the same soil matrix with the microbiota from the NSs. Two factors have been considered: (i) the type of soil, namely NS and RS, and (ii) the soil, namely A, D, F, and J corresponding to the global properties (biotic and abiotic) for NSs and to the microbiota for RSs.

After normalization of metabarcoding data by rarefaction, 12,614 and 14,873 reads per sample were obtained for bacteria and fungi, respectively. Alpha diversity of native and recolonized soils was assessed by evaluating the number of ASV and calculating the Shannon index (**Supplementary Figure S2**). In bacterial communities, the number of ASVs was influenced by the type (NS and RS) of soil (χ^2 = 335.56, df = 1,

$p < 0.0001$), the soil ($\chi^2 = 23.48$, df = 3, $p < 0.0001$), and the interaction between type of soil and soil ($\chi^2 = 21.96$, df = 3, $p < 0.0001$). Indeed, there were significantly more ASVs in NS (mean of 950 ± 37.21) than in RS (mean of 493 ± 19.10; **Supplementary Figure S2A**). Moreover, among NS, soil F contained the highest number of ASVs (1,122 ± 13.33) and soil A the lowest (836 ± 42.40). The number of ASVs among RSs was not significantly different. The Shannon index of bacterial communities was also influenced by the type of soil ($\chi^2 = 1960.43$, df = 1, $p < 0.0001$), the soil ($\chi^2 = 82.44$, df = 3, $p < 0.0001$) and the interaction between type of soil and soil ($\chi^2 = 33.72$, df = 3, $p < 0.0001$) with the NS (6.38 ± 0.05) containing significantly more diverse bacterial communities than RS (5.11 ± 0.05; **Supplementary Figure S2B**). Among NS, F was the most diverse, followed by D, J, and A, the least diverse one. In fungal communities, the number of ASVs was influenced by the type of soil ($\chi^2 = 15.41$, df = 1, $p < 0.0001$), the soil ($\chi^2 = 27.55$, df = 3, $p < 0.0001$), and the interaction between type of soil and soil ($\chi^2 = 20.21$, df = 3, $p < 0.0001$). Among the eight soils, the J NS contained significantly more ASVs (193 ± 7.42) than the others (**Supplementary Figure S2C**). Furthermore, among NS, J contained significantly more ASVs (193 ± 7.42) than D and F (132 ± 4.18 and 140 ± 1.76, respectively). For RS, no significant difference was observed. The Shannon index of fungal communities was impacted by the type of soil ($\chi^2 = 31.19$, df = 1, $p < 0.0001$) and the interaction between type of soil and soil ($\chi^2 = 23.04$, df = 3, $p < 0.0001$). Among NS, no significant difference was observed, whereas among RS, J was significantly less diverse (1.86 ± 0.22) than F (3.39 ± 0.04; **Supplementary Figure S2D**).

Beta diversity, i.e., reflecting bacterial community composition between soils, was driven by the type of soil ($F = 49.18$, df = 1, $p = 0.001$), the soil ($F = 15.10$, df = 3, $p = 0.001$) and the interaction between type of soil and soil ($F = 9.00$, df = 3, $p = 0.001$). Our model explained 88.36% of the total constrained variance, with 40.76% supported by axis 1 and 23.76% by axis 2, which could be explained by the type of soil (NSs vs. RSs) and the soil, respectively (**Figure 1A**). The bacterial community composition was different among NSs with F close to J and A to D, whereas for RSs, all bacterial communities have converged toward a more similar composition. The fungal community composition was driven by the type of soil ($F = 13.72$, df = 1, $p = 0.001$), the soil ($F = 13.83$, df = 3, $p = 0.001$), and the interaction between type of soil and soil ($F = 2.25$, df = 3, $p = 0.004$). Our model explained 79.48% of the total constrained variance, with 31.92% supported by axis 1 and 25.60% by axis 2, explained by the type of soil (NS vs. RS) and the soil, respectively (**Figure 1B**). Composition of fungal communities among NS varied, and differences were also observed among RS (unlike for bacterial communities). In both cases, for bacterial and fungal communities, the three replicates were grouped, indicating homogeneity in sample profiles.

Taxonomic Composition of Microbial Communities

According to the taxonomic composition, bacterial communities were composed of 7,552 ASVs considering all NSs and RSs. The three main phyla were Proteobacteria (2,814 ASVs, 57.6% of reads), Bacteroidetes (1,214 ASVs, 13.3% of reads), and Actinobacteria (795 ASVs, 9.6% of reads). α-Proteobacteria, Bacteroidia, and Termoleophilia were the most abundant classes in NS (**Figure 2A**) and γ-Proteobacteria, Bacteroidia, and Actinobacteria in RS (**Figure 2B**). A total of 428 bacterial genera were detected. For NS, the most abundant genera were *Reyranella* (α-Proteobacteria), *Bacillus* (Firmicute), *Terrimonas* (Bacteroidetes), *Gaiella* (Actinobacteria), and *Acidibacter* (γ-Proteobacteria; **Figure 3A**). All soils were significantly different in terms of abundance for these genera (**Supplementary Table S1A**). Moreover, only J soil contained genus *Rhodomicrobium* (α-Proteobacteria; **Supplementary Figure S3A**). Recolonized soils had a different bacterial composition than NSs. Indeed, the most abundant genera were *Massilia* (β-Proteobacteria), *Pseudomonas* (γ-Proteobacteria), *Pedobacter* (Bacteroidetes), *Lysobacter* (γ-Proteobacteria), and *Pseudarthrobacter* (Actinobacteria; **Figure 3B**). Only relative abundances of *Massilia*, *Pseudomonas*, and *Lysobacter* were significantly different among the four RSs

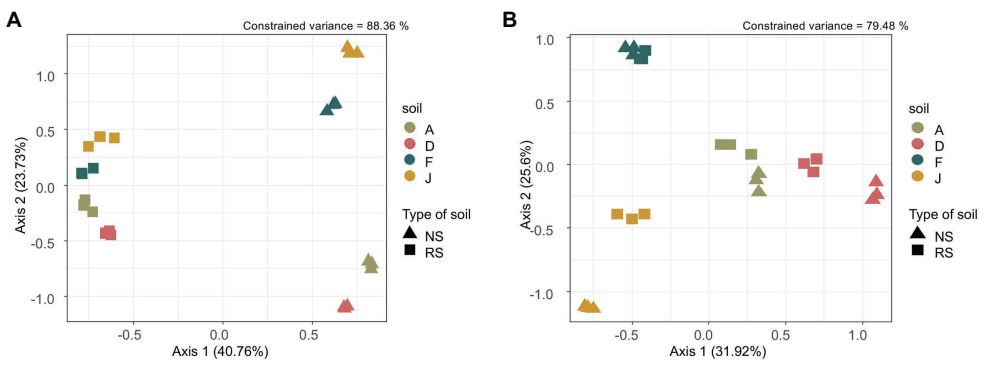

FIGURE 1 | (A) Bacterial and **(B)** fungal community composition represented by an ordination plot and analyzed using a distance-based redundancy discriminant analysis (dbRDA) performed on the Bray Curtis distance index.

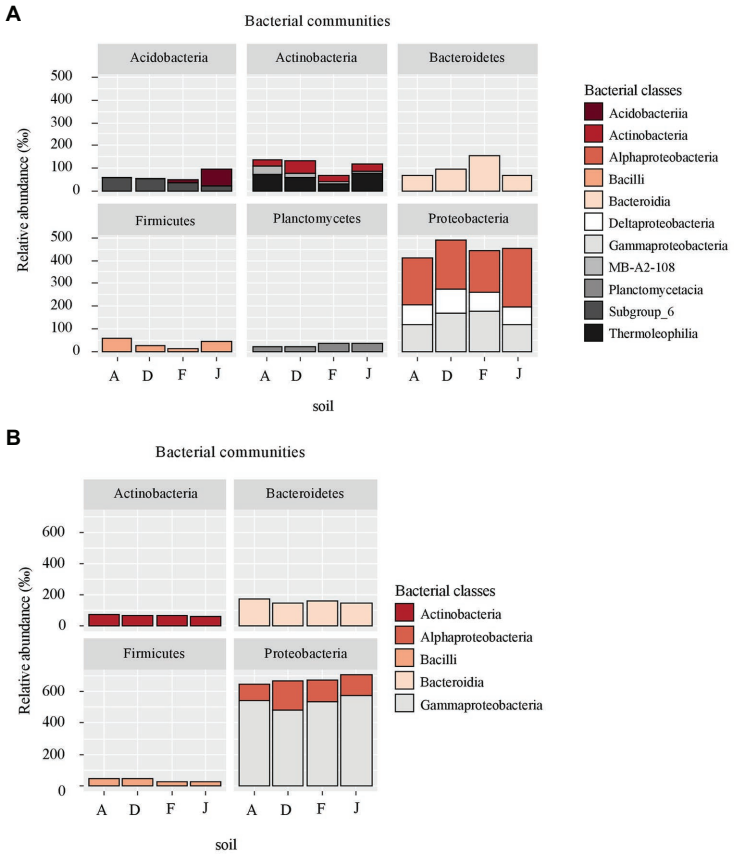

FIGURE 2 | Principal bacterial classes (higher than 60‰ relative abundance) and associated phyla for **(A)** native soil (NS) and **(B)** recolonized soil (RS).

(**Supplementary Table S1B**). Also, only RS J contained genus *Collimonas* (γ-Proteobacteria; **Supplementary Figure S3B**).

Fungal communities were composed of 960 ASVs, considering all native and recolonized soils. The three main phyla were Ascomycota (391 ASVs, 54.5% of reads), Basidiomycota (180 ASVs, 8.8% of reads), and Mucoromycotina (80 ASVs, 33.1% of reads). Sordariomycetes, Tremellomycetes, and Mucoromycotina were the most abundant classes (**Figure 4**) for both NS and RS. Considering all soils, a total of 208 fungal genera were detected. In NSs, the most common genera were *Mortierella* (Mucoromycotina), *Chaetomium* (Ascomycota), *Bionectria* (Ascomycota), *Cryptococcus* (Basidiomycota), and *Fusarium* (Ascomycota; **Figure 5A**). All these genera were significantly different in terms of relative abundance among NSs (**Supplementary Table S2A**). Moreover, some genera were observed only in one soil (**Supplementary Figure S4A**), namely *Ambispora* (Glomeromycota) for soil A; *Stachybotrys* (Ascomycota), *Torula* (Ascomycota), and *Cochliobolus* (Ascomycota) for soil D; *Ustilago* (Basidiomycota) and *Scopulariopsis* (Ascomycota) for soil F; and *Metarhizium* (Ascomycota), *Atractospora* (Ascomycota), *Rhodocybella* (Basidiomycota), and *Dictyostelium* (Basidiomycota) *Mariannaea* (Ascomycota) for soil J. The fungal genus compositions of RS were nearly similar to NS. Indeed, *Mortierella* (Mucoromycotina), *Chaetomium* (Ascomycota), *Pseudogymnoascus* (Ascomycota), *Cryptococcus* (Basidiomycota), and *Fusarium* (Ascomycota) were the most common genera in both soil types (**Figure 5B**). All these genera were significantly different in terms of abundance among RSs except for *Pseudogymnoascus* (**Supplementary Table S2B**). Moreover, private genera were *Stachybotrys* (Ascomycota), *Torula* (Ascomycota) for RS D and *Otidea* (Ascomycota), *Ustilago* (Basidiomycota), and *Dendryphion* (Ascomycota) for RS F (**Supplementary Figure S4B**).

Alpha and beta diversity, with taxonomic composition, indicated a different microbiota composition among the four NS and RS.

Effect of Soils on Suicide Hatching of Cyst Nematodes

After 40 days inside each NS or RS and application of PREs at two doses (0.13 and 0.021 mg of C), all bags of 10 cysts were retrieved from the soils and hatching rates were determined. Results clearly showed that application of PREs significantly stimulated *G. pallida* hatching, whatever the type of soil (**Figure 6**).

For NSs (**Figure 6A**), in the control (i.e., no application of root exudates), a mean of 25.95% ± 3.08 of J2 hatched spontaneously (hatch in water). For any given soil, application

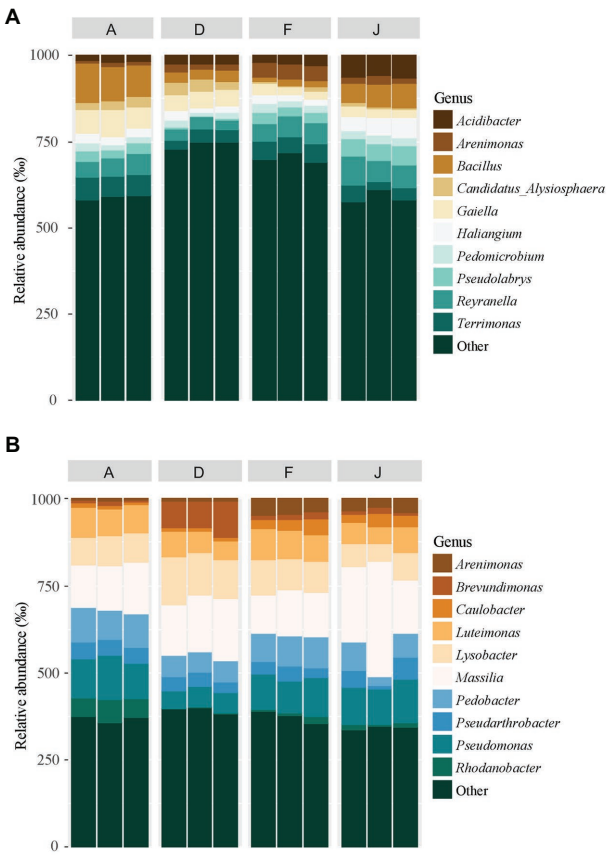

FIGURE 3 | Principal bacterial genera for **(A)** NS and **(B)** RS. Unclassified genera were removed from the plot.

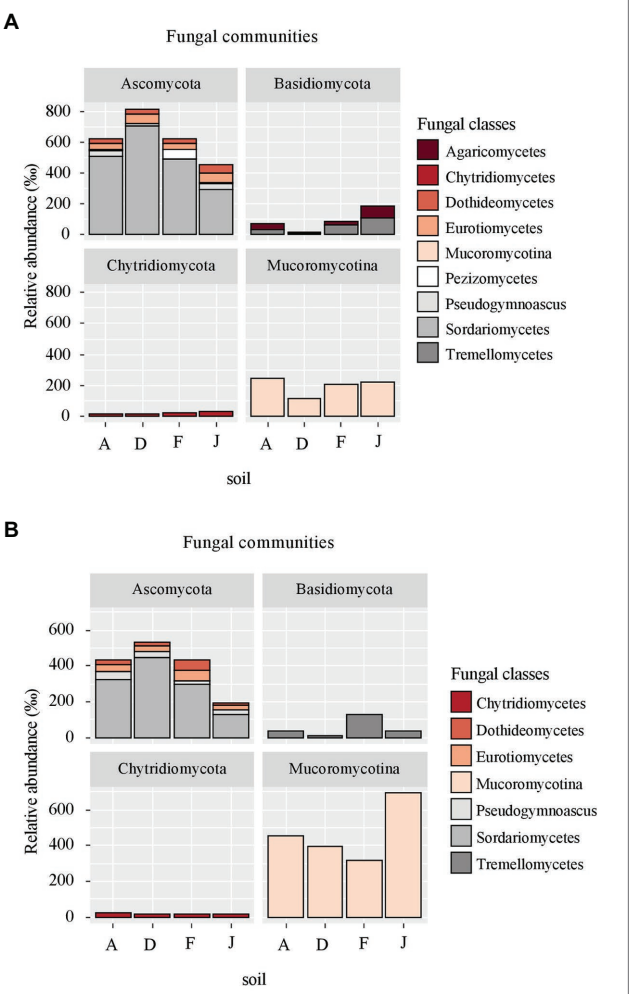

FIGURE 4 | Principal fungal classes (higher than 60‰ relative abundance) and associated phyla for **(A)** NS and **(B)** RS.

of PREs, dose 1 (0.13 mg of C per application) or dose 2 (0.021 mg of C per application) induced statistically the same level of hatching rate among NSs with slight but significant differences. Results indicated that, despite microbiota and physicochemical properties, which were different among NSs, the suicide hatching of the cysts was only slightly affected by the global properties of soils with no impact of the concentration of root exudates tested.

For soils that were inoculated (RS; **Figure 6B**), the hatching rates were not significantly different among controls (18.68% ± 3.14), dose 1 (91.37% ± 3.09), or dose 2 (61.63% ± 3.05). Nevertheless, dose 1 or dose 2 of PREs induced statistically higher hatching of nematodes than in control, and higher hatching rates were observed with dose 1 than dose 2. Thus, as RSs were different in bacterial and fungal community composition, the microbiota does not affect the hatching of cysts, in the experimental conditions tested.

DISCUSSION

The main goal of this study was to evaluate the impact of NSs (physicochemical properties and microbiota) and soil microbiota on the hatching of *G. pallida* induced by PREs. Our results showed that the global properties of soils and the microbiota did not affect, in our conditions, the efficiency of root exudates to induce hatching of *G. pallida*, in the absence of host plants.

The first step of this study was to select four contrasting native agricultural soils in terms of soil texture, physicochemical properties, and microbiota. Physicochemical properties of soils were used as discriminant factors to select soils, as previous studies have revealed that soil texture, organic C content, and pH are the main drivers of microbial communities (Fierer and Jackson, 2006; Lauber et al., 2008; Naveed et al., 2016). For instance, regarding soil characteristics to explain the distribution of soil bacteria richness in France, pH is positively correlated to richness, but clay content, C:N ratio, and total K negatively correlated, according to the French Soil Quality Monitoring Network (Terrat et al., 2017). Moreover, other parameters such as climate conditions or land use can influence microbial distribution (Dequiedt et al., 2011; Maestre et al., 2015). Thus, the geographical location was also considered. Consistently with

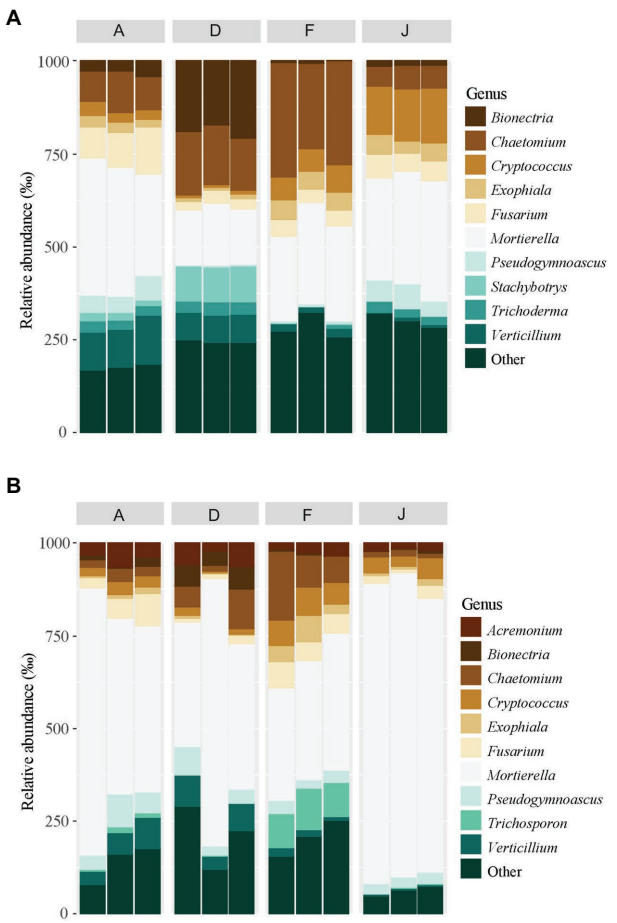

FIGURE 5 | Principal fungal genera for **(A)** NS and **(B)** RS. Unclassified genera were removed from the plot.

this hypothesis, the rDNA metabarcoding analysis confirmed differences in microbiota composition among the NSs in terms of diversity, abundance, and bacterial and fungal communities.

The second step was to obtain soils with the same physicochemical properties and different microbiota. For this, a common sterile soil matrix was inoculated with microbiota from the four selected NSs. All the four RSs shared, hence, the same physicochemical properties. The process of recolonization slightly affected the physicochemical properties of the matrix as exemplified for pH, which initially was 5.75 for the sterilized matrix and ranged between 5.83 and 6.07 after recolonization. However, both bacterial and fungal communities were significantly, but contrastingly, affected by habitat changes. The major modification occurred for bacteria communities with a decrease of specific richness and species diversity in recolonized compared to NSs. Moreover, the relative abundances of the same bacterial genera, such as *Massilia* (β-Proteobacteria), *Pseudomonas*, and *Lysobacter* (γ-Proteobacteria) increased during the recolonization process and as a consequence, a convergence of bacterial composition from all RSs was observed. These genera are excellent competitor microorganisms and can colonize rapidly an uncrowded environment (Bowen and Rovira, 1976; Ofek et al., 2012). Conversely, the fungal communities were less impacted in terms of alpha diversity and conserved a similar composition as in NSs but some slight differences, i.e., an increase of *Mortierella* (Mucoromicota) relative abundances, were observed. Regarding the whole microorganism communities (bacteria and fungi), four different microbiota were obtained during the recolonization process.

Our main goal was to anticipate potential fluctuations of hatching rate according to both physicochemical properties and microbiota or microbiota only. The hatching process of cyst nematodes is a complex chain reaction, which depends, according to the considered nematodes species on several

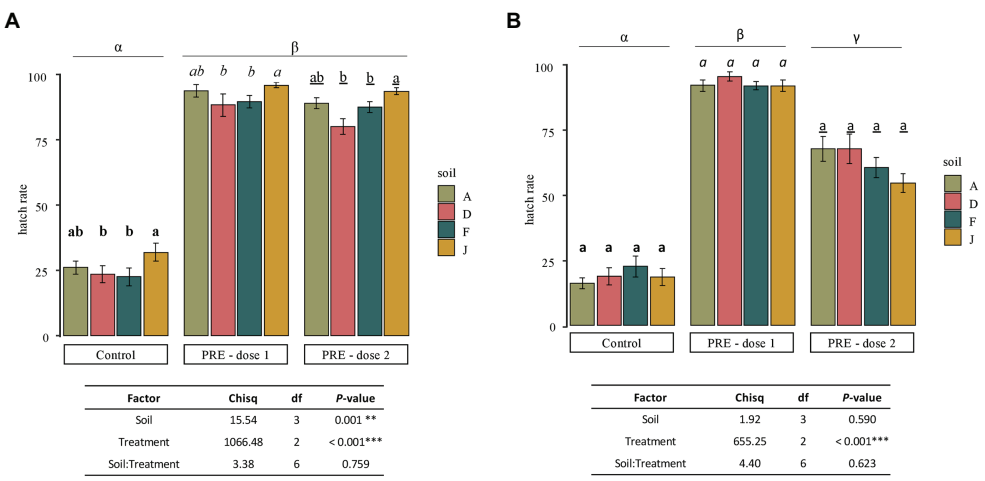

FIGURE 6 | Mean of hatching rate of *Globodera pallida* juveniles (mean values ± SEM) at the end of the experiment (after 40 days) for control (water application), dose 1 (PRE application at 0.13 mg of C), and dose 2 (PRE application at 0.021 mg of C) treatments, in **(A)** NS and **(B)** RS. Letters represent significant differences between soil for a given treatment and the Greek letters among treatments.

different stimuli and environmental conditions, such as temperature, pH, soil moisture, and texture. These abiotic parameters are not the only factors, which influence the hatching but also the host plant cultivar, plant age, or the period of exudates production (Evans, 1983; Perry and Gaur, 1996). In our assay, some of these factors were controlled to favor the hatching. Indeed, temperature and moisture were maintained in a range close to the optimum described in the literature (see Masler and Perry, 2018; for a review): 14–18°C for temperatures and 80% of field capacity for moisture, and PRE was produced during 3 weeks after potato germination.

In NS, results showed a very high level of suicide hatching of *G. pallida*, compared to the control (water applications) when PRE was applied regardless of tested dose and soil. Five applications of dose 1 (0.13 mg C) or 2 (0.021 mg C), every 4 days, induced the same level of hatching. Hatching factors are present in very small amounts in root exudates. For example, Fukuzawa et al. (1985) found 1.25 mg of glycinoeclepin A as its bis(p-bromophenacyl) ester (HF for *Heterodera glycines*, the soybean cyst nematode) in 1058 kg (dry weight) of bean roots. Also, Devine and Jones (2000b) found a HF in PRE at less than 2.9×10^{-5}% of recovered OM, active *in vitro* at less than 2.1×10^{-8} M. Some authors reported a dose-response relationship between PRE and the stimulation of hatching (Devine et al., 1996; Fukuzawa, 2007). Due to the high specificity of *G. pallida* to PRE, it seems to be interesting to test even smaller doses to highlight an effect and determine the sensitive threshold. However, slight but significant differences were observed among soils. Indeed, for dose 1, dose 2, or control, soil J had a minor but significantly higher hatching than soils D and F. Some authors highlighted the role of microorganisms on nematode hatching in soils and suggested a tritrophic interaction between host, microorganisms, and cyst nematodes (Ryan et al., 2003; Ryan and Jones, 2003). Ryan and Jones (2003) demonstrated that the spontaneous hatching of potato cyst nematodes (in the absence of plant or PRE) was higher in sand compared to *in vitro*, suggesting the occurrence of other HF produced by microorganisms. Lettice and Jones (2015) showed that isolated rhizobacteria, including *Bacillus sp.*, induced higher levels of *G. pallida* hatching in the absence of host plant than control. As soils A and J contained higher levels of *Bacillus*, we cannot exclude the hypothesis that soil microorganisms play a role in hatching and may be implicated, for an undetermined proportion, in the spontaneous hatch of cyst nematodes.

In the recolonized matrix, our results showed a high hatching compared to the control with no effect of microbiota origin but a non-negligible impact of tested doses. Indeed, dose 2, characterized by smaller quantity of C than dose 1, induced a lower hatching of *G. pallida*. Root exudates HF include C-based molecules, such as solanoeclepin A (C27H30O9), glycinoeclepin A (C25H34O7), α-solanine (C45H73NO15), or α-chaconine (C45H73NO14), which constitute an important source of food for microorganisms living in soil (Bertin et al., 2003; Bais et al., 2006; Mendes et al., 2013). Moreover, microorganisms can be characterized according to their life-history strategy: copiotroph (r-strategist) or oligotroph (K-strategist). According to Fierer et al. (2007), copiotroph microorganisms have high growth rates in response to availability of abundant C resources and are able to colonize rapidly an unexploited environment. In contrast, oligotrophs have slow growth rate and are good competitors in low nutrient environment due to their high affinity for resources. Our experimental strategy, i.e., colonization of a germ-free environment with higher nutrient resources than in NSs, created a favorable habitat to select copiotroph microorganisms. Many members of the β- and γ-Proteobacteria, Bacteroidetes, and Actinobacteria are classified as copiotrophs (Fierer et al., 2007, 2012) and they were the main phyla in RSs. The increase of copriotroph bacteria in RS compared to NS, with more equilibrates ecological attributes (copiotroph-oligotroph continuum), could explain the dose effect observed. The C from PRE present in soils was probably largely metabolized by the selected bacterial r-strategists, leaving few hatching signals for the nematodes. Another hypothesis is the physicochemical properties or the structure of the soil matrix that we used, a sandy silt soil, could influence the availability of HFs in the soils. For this, it would be interesting to test another structure of soil matrix.

To conclude, our study provides key elements for the development of suicide hatching, as a promising eco-friendly control strategy, against *G. pallida* in NSs. Even if our RSs are artificial soils, they indicate that applications of a small C amounts can decrease the HF efficiency in presence of many copiotroph microorganisms. The selection of copiotroph microorganisms could occur in relation to soil management (tilling and organic amendment) or during different stresses which reduced microbial biomass or diversity (Ho et al., 2017; Chen et al., 2018). For furthers studies, it would be interesting to evaluate the impact of root exudates on microbial communities in relation to nematode hatching rate.

DATA AVAILABILITY STATEMENT

The raw metabarcoding data sets are available on the European Nucleotide Archive database system under the project accession number PRJEB36768. Soil sample accession numbers range from ERS4307830 to ERS4307877 and sequencing runs from ERR3908564 to ERR3908611. An Excel file containing the raw data of the suicide hatching, in native and recolonized soils, is available at data.inrae.fr (doi: 10.15454/THNCAC).

AUTHOR CONTRIBUTIONS

CG, LM, SF, A-YG-E, CP, JL and LL performed the experiments according to a protocol elaborated jointly by CG, SF, JM, J-CY, EN-O, CM, and LL. CG, LM, SF, JM, and LL analyzed the data. CG, SF, JM, CM and LL wrote the text and prepared the figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.536932/full#supplementary-material

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