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Published in:
Applied Soil Ecology

DOI:
10.1016/j.apsoil.2021.104344

Publication date:
2022

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Topalovi, O., Santos, S. S., Heuer, H., Nesme, J., Kanfra, X., Hallmann, J., Sørensen, S. J., & Vestergård, M. (2022). Deciphering bacteria associated with a pre-parasitic stage of the root-knot nematode Meloidogyne hapla in nemato-suppressive and nemato-conducive soils. Applied Soil Ecology, 172, [104344]. https://doi.org/10.1016/j.apsoil.2021.104344

Download date: 14. jul., 2023
Deciphering bacteria associated with a pre-parasitic stage of the root-knot nematode *Meloidogyne hapla* in nemato-suppressive and nemato-conducive soils

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**ARTICLE INFO**

**Keywords:**
Root-knot nematode
Soil
Suppressive
Conducive
16S rRNA gene

**ABSTRACT**

Nematode-suppressive soils are characterized by the ability of soil microbial communities to reduce populations of plant-parasitic nematodes (PPN) either directly or by inducing systemic resistance in plants. Various microorganisms have been recognized as antagonists of PPN in suppressive soils using culture-dependent and culture-independent methods. However, the associations that PPN form with microorganisms in nematode-conducive soils have been poorly studied. Here we drenched tomato rhizospheres with microbial suspensions from nine different soils and followed their effects on plant growth and root invasion of the infective second-stage juveniles (J2) of the northern root-knot nematode species *Meloidogyne hapla*. Based on the number of invaded J2, four soils were determined as nematode-suppressive, while five soils were categorized as nematode-conducive. To reveal bacteria attached to the J2 cuticle in soils with varying suppressiveness, we incubated J2 in suspensions from three suppressive and three conducive soils, and analyzed J2-attached bacteria using amplicon sequencing of the V3-V4 region of 16S rRNA gene. Our results suggest that the soil origin had a major effect on the composition of J2-attached bacteria, while the highest bacterial abundance and richness were observed on J2 from two suppressive soils. In addition, the highest number of indicator amplicon sequence variants (ASVs) was associated with J2 in two suppressive soils, but they had a very distinct bacterial profile. Further studies are needed to resolve the complexity of nemato-microbial interactions in soil and to determine the exact function of nematode-attached microorganisms in suppressive and conducive soils and their role in nematode suppression and protection.

1. **Introduction**

Plant-parasitic nematodes (PPN) are very important pests of many cultural and natural plants (Wilschut and Geisen, 2020). A recent estimation of the yield losses caused by PPN worldwide is lacking, but it has been reported to reach billions of US dollars (Bernard et al., 2017). The root-knot nematodes (RKN) form a very important group of PPN due to their ubiquitous nature and wide host range, being able to reproduce sexually (amphimixis) and asexually (mitotic and meiotic parthenogenesis). Apart from the mature adult male, the infective second-stage juvenile (J2) of RKN is the only stage that resides in soil upon hatching (pre-parasitic stage). When J2 start feeding in the vascular cylinder of the host roots, they indulge in a sedentary lifestyle without the ability to move. During the sedentary phase, juveniles grow into a swollen appearance. Adult males, if present, regain a vermiform shape and mobility, whereas adult females remain swollen and sedentary and produce a large number of offspring. Although PPN are obligate biotrophs that cannot survive without the plant, they spend an extended amount of time in soil before migrating to the roots (the exophytic phase) (Vestergård, 2019). In this case, the subsequent development of the RKN population strongly relies on the capacity of the pre-parasitic J2 to overcome the adverse biological conditions in soil (predators, parasites, hyperparasites) prior to infecting the plant. Thus, the exophytic phase of RKN is important from the aspect of biological control, as
Various bacteria and fungi have been reported to antagonize J2 while residing in soil (Topalović et al., 2020b). The fungi parasitizing nematodes use different forms of traps (adhesive networks, hyphae and rings) or produce conidia and zoospores that attach to the nematode surface and germinate inside the nematode body (Moosavi and Zare, 2012). Some examples are the fungal genera *Arthrobostrys*, which forms adhesive networks, *Dactylellina*, which forms adhesive knobs and non-constricting rings, and *Drechslerella*, which forms constricting rings that trap nematodes (Moosavi and Zare, 2012). The most studied bacteria parasitizing nematodes belong to the genus *Pasteuria*. They attach to the nematode surface in soil using endospores and proliferate inside the nematode body when nematodes enter the plant roots (Chen and Dickson, 1998). However, other bacteria have also been reported to antagonize nematodes by producing toxins (*Pseudomonas* spp., *Rhizobium* spp., *Bacillus* spp., *Streptomyces* spp., and *Lysobacter* spp.), by inducing systemic resistance in plants (*Bacillus* spp., *Rhizobium* spp., and *Microbacterium* spp.), and by producing volatile organic compounds (*Arthrobacter* spp. and *Vartovarax* spp.) (Tian et al., 2007; Topalović et al., 2020a; Topalović et al., 2020b).

Soils comprising microbiomes that antagonize nematode performance in soil and on plants are referred to as nematode-suppressive (Raaijmakers and Mazzola, 2016). Stenberg et al. (2021) have proposed that soil suppressiveness, which establishes spontaneously and as a result of untargeted cultural practices, can be defined as natural biological control. Suppressively soils have been detected for various PPN species, including cyst-forming nematodes (*Westphal and Becker*, 1999; *Westphal and Becker*, 2001; *Olatinwo et al.*, 2006; *Westphal et al.*, 2011; *Hamid et al.*, 2017) and RKN (*Pyrowolakis et al.*, 2002; *Klein et al.*, 2012; *Adam et al.*, 2014b; Topalović et al., 2019b). The use of amplicon sequencing in recent studies has revealed that nematode suppressiveness is not always related to the presence and high abundance of only one antagonistic microbial species. Rather, a consortium of several microbial species can contribute to the decline of nematode populations in soil (Eberlein et al., 2016a; Castillo et al., 2017; Mwabhe et al., 2018; Eberlein et al., 2020; Silva et al., 2021). For instance, Castillo et al. (2017) reported that a negative correlation exists between the abundance of root-lesion nematode, *Pratylenchus penetrans*, and RKN, *Meloidogyne chitwoodi*, and bacterial genera such as *Bacillus*, *Arthrobacter*, and *Lysobacter* in potato agricultural soils. More in-depth studies on interactions between PPN and soil microorganisms have shown that soil-dwelling nematode stages attack a limited range of microorganisms to their surface (Adam et al., 2014b; Elhady et al., 2017; Topalović et al., 2019a). The microbial attachment to PPN depends on the nematode species and the soil origin (*Elhady et al.*, 2017), but plant exudation also plays a role in these interactions (Liu et al., 2017; Mohan et al., 2020; Elhady et al., 2021). Recently, Topalović et al. (2019b) have studied the suppressive ability of the microbiomes from different soils against the northern RKN species *Meloidogyne hapla*, which attacks almost all temperate vegetables and legume crops (CABI, 2021). Interestingly, the suppressive microbiomes impaired *M. hapla* infection and reproduction both via direct interactions and by inducing systemic resistance in plants. Additionally, some of the isolated bacterial strains attaching to *M. hapla* J2 in different soils antagonized *J2 in vitro* and in the greenhouse (Topalović et al., 2019a), while the J2-attached *Microbacterium* sp. strain triggered a different expression of pattern-triggered immunity (PTI)-responsive genes in plants upon nematode invasion of the roots (Topalović et al., 2020a). However, it remains elusive which microorganisms really antagonize RKN-suppressive from RKN-conducive soils. More in-depth understanding of the differences between microbial consortia attaching to RKN in suppressive and conducive soils could potentially aid the identification of agricultural soils that are at low or high risk, respectively, of devastating infection levels to susceptible crop species.

In the current study, we aimed to elucidate the effects of the microbiomes from nine different soils on the invasion of *M. hapla* J2 in tomato roots and on plant performance. In addition, we used amplicon sequencing to decipher the composition of bacteria attached to the J2 cuticle when incubated in microbial suspensions originating from the different soils. We hypothesized that the composition of J2-attached bacteria reflects their biological potential to suppress nematodes (in suppressive soils) or assist them during the protection from the antagonists and invasion into the roots (in conducive soils). Our study gives an insight into the nematode-microbial interactions in different soils and helps understand the role of nematode-attached bacteria in nematode suppression and survival, which would be of a benefit to the sustainable nematode control.

2. Materials and methods

2.1. Nematode extraction and plant growth for the greenhouse experiment

We extracted eggs of *M. hapla* from tomato roots and placed them on a modified Baermann tray for J2 hatching as described previously (Hooper et al., 2005). After hatching, we surface-sterilized J2s using 0.02% *HgCl*₂ for 3 min and a mixture of 10 mg l⁻¹ rifampicin, 25 mg l⁻¹ streptomycin-sulphate, and 1× CellCultureGuard (AppliChem, Darmstadt, Germany) for 4 h on a rotary shaker at 100 rpm. Nematodes were extensively washed on a sterile 5-μm sieve with sterile tap water to remove the traces of the chemical compounds after sterilization. We surface-sterilized seeds of tomato plants (*Solanum lycopersicum* L.) using 70% ethanol for 1 min and 3% *NaOCl* for 3 min. After sterilization, the seeds were extensively washed with sterile tap water and air-dried under sterile conditions. We planted one seed per 0.6 l pot containing autoclaved sand and clay pellets in a ratio of 10:1, and placed them in the glasshouse cabinet at 20°C with 16 h photoperiod. The plants were watered as needed with fertilizer solution, (WUXAL Super NPK fertilizer, 8-8-6 with micronutrients, AGLUKON, Düsseldorf, Germany). The sand/clay blend was autoclaved at 134°C for 10 min to kill the indigenous microbes and nematodes, followed by a 20-min dry cycle.

2.2. Preparation of soil microbial suspensions for the J2 invasion assay

We used the soils from a previous study (Topalović et al., 2019a) for preparing soil suspensions for J2 incubation (Table 1). We used Bödenatlas Deutschland (https://geoviewer.bgr.de) to determine the WRB types of the soils and World Geodetic System 1984 (https://epsg.io) to determine the coordinates. We sieved the soils through a 0.5-cm sieve and suspended 300 g of soil in 3 l of sterile tap water in 5 l glass bottles containing 1 mm metal beads. To release microorganisms from the soil particles, soil suspensions were shaken for 1 h at 120 rpm. After shaking, large soil particles were allowed to settle for 10 min and the supernatants were sieved through a series of 150-μm and 20-μm sieves. We centrifuged the flow-through at 4000 × g for 10 min (Sorvall Lynx 6000 Centrifuge, Fisher Scientific GmbH, Schwerte, Germany) and resuspended the pellet in 350 ml of sterile tap water. We drenched the rhizospheres of 3-week old tomato plants by evenly distributing 30 ml of soil suspensions around the roots (10 replicate pots for each soil suspension). One week later, 2 ml of sterile tap water containing 1000 surface-sterilized J2 of *M. hapla* were added to four holes around the stem (each hole receiving approx. 250 J2 in 500 μl of water). We arranged the pots in a randomized block design (10 replicate blocks × 9 soil treatments) in a glass house cabinet at 20°C with 16 h photoperiod. Pots were watered and fertilized as needed.

After seven days, we removed the plants from the pots, rinsed the root systems and measured the fresh weight of roots and shoots. We measured the dry weight of shoots after drying in the oven at 100°C overnight. We stained J2 inside the weighted roots with acid-fuchsin (Hooper et al., 2005) and counted the number of stained J2 per root under a stereomicroscope.
Table 1

| Location                  | Abbreviation | Soil texture | Humus content (%) | pH    | Coordinates          | WRB soil type          |
|---------------------------|--------------|--------------|-------------------|-------|-----------------------|------------------------|
| Geisenheim                | G            | Sandy clay   | 2.7               | 7.4   | 49°59′01″N, 7°57′25.5″E| Pararendzina           |
| Klein Wanzleben           | KWS          | Sandy        | 6.3               | 6.9   | 52°03′07.2″N, 11°23′13.2″E| Chernozem             |
| Dahnsdorf Schlag 2        | D2           | Less sandy loam | 1.1  | 5.9   | 52°06′16.1″N, 12°38′40.7″E| Luvisol                |
| Dahnsdorf BS1             | BS1          | Less sandy loam | 1.1  | 5.8   | 52°06′21.9″N, 12°38′13.7″E| Luvisol                |
| Quedlinburg JKI Schlag 5  | S5           | Less sandy loam | 2.5  | 6.9   | 51°46′9.5″N, 11°9′40.1″E | Chernozem-luvisol     |
| Quedlinburg JKI Schlag 9  | JKI          | Less sandy loam | 2.2  | 7.1   | 51°46′2.1″N, 11°9′3.6″E  | Chernozem-luvisol     |
| Sickte 10                 | S10          | Less sandy loam | 1.1  | 5.9   | 52°12′45.2″N, 10°38′20.7″E| Luvisol-luvisol       |
| Bundesallee               | B            | Less sandy loam | 1.4  | 6.2   | 52°17′57″N, 10°26′14″E  | Pseudogley             |
| Eldendorf                 | E            | Heavy sandy loam | 1.7  | 7.2   | 50°55′41.7″N, 6°33′56.8″E | Pseudogley             |

2.3. The J2 baiting in soil suspensions

Based on the data on root invasion by J2 in the glasshouse, we selected six soils (three suppressive and three conducive soils) for the J2 baiting experiment. We considered the soils as suppressive when the number of invaded J2 per root system was significantly different from the soil with the lowest J2 invasion (soils S10 (mean 5 J2/root), and G (mean 30 J2/root); see abbreviations in Table 1). We considered the soils as non-suppressive or conducive when the number of invaded J2 per root system was not significantly different from the soil with the highest J2 invasion (soils JKI (mean 92 J2/root), GWS (mean 65 J2/root), and E (mean 44 J2/root); see abbreviations in Table 1). The soils were sieved through a 1-mm sieve and 10 g of sieved soil was blended with 2 × 20 ml of sterile tap water for 1 min at a high speed (Stomacher®80, Seward, London, UK). We centrifuged the supernatant containing the released soil microorganisms for 5 min at 500 ×g to remove soil particles, after which we sieved the supernatant through a sterile 5-μm sieve to exclude indigenous nematodes. We used the flow-through as a microbial suspension for nematode incubation. Around 2000 surface-sterilized J2 were incubated in glass jars containing 30 ml of soil suspensions overnight. The incubations were performed using an incubator (Brünswick Scientific, Edison, New York, USA) at 30 rpm at room temperature. We prepared four biological replicates of each soil suspension for the baiting experiment. After incubation, we transferred nematodes to handmade 5-μm pore sieves, washed with 50 ml of sterile tap water to remove loosely attached microbes and transferred the nematodes to bead-beating tubes for DNA extraction.

2.4. DNA extraction and PCR amplification

We extracted total DNA from J2 of *M. hapla* and their attached microorganisms with the FastDNA Spin Kit for Soil (MP Bio, Heidelberg, Germany). To lyse the cells mechanically, the Fastprep FP120 bead beating system (MP Bio, California, USA) was used for 30 s at a high speed. For the high throughput sequencing of the J2-associated microorganisms, we amplified a fragment spanning the hypervariable regions V3-V4 of the 16S rRNA gene with an initial PCR step using primers Uni341F (5′-CCTAYGGGGRBGCASCAG-3′) and Uni806R (5′-GGACTACNNGGGATCTTAA-3′) originally published by Yu et al. (2005) and modified as described in Sundberg et al. (2013). The PCR conditions consisted of an initial denaturation step at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 20 s, and elongation at 72 °C for 1 min. The final extension step was performed at 72 °C for 10 min. In a second PCR reaction using the same cycling conditions but only 15 cycles, the primers additionally included Illumina specific sequencing adapters and a unique dual index combination for each sample. After each PCR reaction, amplicon products were purified using HighPure™ PCR Clean Up System (AC-60500, MagBio Genomics Inc., USA) paramagnetic beads using a 0.65:1 (beads: PCR reaction) volumetric ratio to remove DNA fragments below 100 bp in size and primers. Samples were normalized using the SequaPrep Normalization Plate (96 Kit) (Invitrogen, Maryland, MD, USA) and pooled using a 5 μl volume for each sample. The pooled samples library was concentrated using DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA, USA). The pooled library concentration was determined using the Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies). Before library denaturation and sequencing, the final pool concentration was adjusted to 4 nM before library denaturation and loading. Amplicon sequencing was performed on an Illumina MiSeq platform using Reagent Kit v2 (2 × 250 cycles) (Illumina Inc., CA, US). Raw sequencing data are deposited to the NCBI's Sequence Read Archive platform using Reagent Kit v2 [2].

2.5. Bioinformatic analysis

The DADA2 pipeline based on amplicon sequence variants (ASVs) was used for 16S rRNA gene amplicon sequence analysis (Callahan et al., 2016). Briefly, filtration and trimming (filterAndTrim - maxn = 0, maxee = 2, truncQ = 2) were performed to clean raw sequences, which then went through error learning (learnErrors), de-replication (derepFastq), and merging. By discriminating biological sequences from sequencing errors, ASVs were then inferred (dada). We merged the forward and reverse reads (mergePairs) for the construction of the sequence table (makeSequenceTable) and further removed chimera sequences (removeBimeraDenovo). Taxonomy classification of chimer-free ASVs was with the naive Bayesian RDP Classifier (Wang et al., 2007) with a bootstrap threshold of 80, by searching against the SILVA non-redundant database release 138 (Quast et al., 2013). ASV sequences identified as singletons were removed. The generated sample-wise ASV abundance table was used for further analysis.

2.6. Statistical analysis

For the plant growth parameters and J2 invasion, the data were log-transformed and analyzed in SAS 9.4 using GENMOD procedure with scale parameter Pearson and assuming a generalized linear model at alpha level p < 0.05. The multiple pairwise treatment effects were tested using Tukey-Kramer adjustments with the Least Square Means.

The statistical analyses of the sequencing data were conducted in the R Environment version 4.0.0 (R Core Team, 2020). We produced rarefaction curves of the observed 16S ASVs using the ‘rarecurve’ function of the package vegan on non-rarefied counts per sample. Barplots of the soil-ASVs relative abundance versus J2-associated ASVs were built with vegan package (Oksanen et al., 2020). Alpha diversity measures of J2-associated microbial assemblages were calculated per sample with vegan package, based on randomly subsampled read count data, to compensate for variation in read numbers across samples. We tested the effect of soil origin on alpha diversity measures with a one-way ANOVA followed by Tukey’s Honest Significant Difference (HSD) test for pairwise comparisons. To visualize the effect of soil suppressiveness on the J2-associated microbiome we performed canonical analysis of principal coordinates (CAP) on normalized ASV counts (variance stabilization - DESeq2 package: Love et al., 2014), with the “capscale” function of the vegan package. We assessed the statistical significance using the “permutes” function in the vegan package with 10⁴ permutations.
Additionally, soil origin and suppressiveness category was further tested by permutational analysis of variance (PERMANOVA) using vegan package, with 10^4 permutations.

To identify indicator ASVs of the J2-associated bacteria, indicator species analysis (indicspecies package; de Cáceres and Legendre, 2009) was used to calculate the point-biserial correlation coefficient (r) of an ASV’s positive association to one or a combination of soils. The analysis was conducted with 10^4 permutations and considered significant at p < 0.05. We used bipartite networks to visualize the significant (p < 0.05) indicator ASVs associations to one or more of the different soils in study. The networks were constructed using the Fruchterman-Reingold layout with 10^4 permutations as implemented in the R package igraph (v. 1.2.5; Csardi and Nepusz, 2006). To visualize the abundance distribution of the indicator ASVs across the different samples, we constructed a heatmap using the “heatmap.2” function in gplots package (Warnes et al., 2020).

3. Results

3.1. Effects of microbiomes from different soils on nematode invasion and plant growth

The soil origin of microbiomes affected nematode invasion 7 days past inoculation (DPI) (Tukey-Kramer, p < 0.05). The lowest number of invaded J2 (mean 5 J2/root) was recorded in tomato roots drenched with microbial suspension from soil S10 (Fig. 1). This was followed by soils BS1 (mean 23 J2/root), B (mean 24 J2/root), and G (mean 30 J2/root), respectively (Fig. 1). On the other hand, the microbiome from the JKI soil showed the least suppressive effect on nematode invasion, with an average of 92 J2 per root system. However, this effect did not differ significantly from those imposed by microbiomes from soils D2 (mean 76 J2/root), KWS (mean 65 J2/root), S5 (mean 45 J2/root), and E (mean 44 J2/root) (Fig. 1).

The soil microbiomes also affected growth of tomato plants differently (Fig. S1). At 7 DPI, the highest values for the dry shoot weight and fresh root weight were recorded for plants drenched with the KWS soil microbiome (mean 0.15 dry shoot weight; mean 0.58 g fresh root weight, respectively), which significantly differed from all the other treatments in this study. The lowest dry shoot weight was measured for the treatments BS1 and G (mean 0.044 g; mean 0.041 g), but this was significantly different from the treatments KWS, JKI and E (p < 0.05). In addition, fresh root weight was also low for the BS1 treatment (mean 0.1 g; p < 0.05), which was followed by the treatment B (mean 0.18 g; p < 0.05).

To see if the root weight affected nematode invasion into the roots, we also calculated the number of invaded J2 per g of fresh root weight (Fig. S2). The lowest nematode invasion per g of root was seen for the S10 treatment (mean 16 J2/g of root), while these mean values ranged from 120 J2 to 197 J2 per g of root for treatments E, K, B, G, and S5, respectively. Interestingly, while the fresh root weight was lowest for BS1 treatment, it showed the highest number of J2 per g of root (mean 248 J2) and did not differ significantly from the treatments D2 and JKI. However, as the J2 root invasion was not determined by the root mass, we decided to use the number of invaded J2 per root system as the measure for nematode suppression by microbiomes in this study.

3.2. Composition and comparison of M. hapla J2-associated bacterial communities in different soils

Sequencing of the 16S rRNA amplicon library resulted in 1602 ASVs in 30 samples (6 microbial suspensions × 4 replicates plus sequencing of the original 6 soil samples). Overall, 65,728 16S rRNA gene sequences (2191 ± 213; average ± standard error of the mean across all samples) were obtained with an average read length of 415 bp. Rarefaction curves evaluating the ASV richness per sample showed that bacterial variation was not covered completely in our sampling strategy for some samples (Fig. S3). When looking at the dataset of the J2-attached microbiome, samples recovered 54,940 16S rRNA gene sequences (2289 ± 261) that corresponded to 1213 ASVs.

The alpha diversity of bacterial ASVs, i.e. the number of observed ASVs and Shannon Index, attached to J2 of M. hapla varied for the different treatments (Fig. 2). The highest alpha diversity was found in J2 samples from two suppressive soils, BS1 and G, with significant differences observed for the Shannon index, when compared to the bacteria attached to the J2 inoculated with the conducive JKI soil microbiome (Fig. 2).

Comparative analysis of the J2-attached microbiomes with the bacterial soil origin revealed that only the suppressive soils G and BS1 clustered together with their respective J2-associated microbiomes (Fig. 3A). On the other hand, the non-suppressive soils (E, JKI and KWS) plus the S10 suppressive soil clustered together and separated from the J2 microbiomes (Fig. 3A). Across all samples, the most abundant taxa corresponded to six bacterial classes, i.e. Acidimicrobia, Actinobacteria, Alphaproteobacteria, Anaerolinaeae, Chloroflexi, and Gammaproteobacteria. In all microbiome suspensions, the J2 were predominantly colonized by Alphaproteobacteria, followed by Actinobacteria, except for the J2 incubated with the soil E microbiome, where a significant

![Fig. 1. Invasion of M. hapla J2 in tomato roots as affected by the microbiomes from nine different soils. Different letters above standard error bars represent statistical differences between the treatments at p < 0.05 (Tukey HSD test).](image-url)
decrease of Alphaproteobacteria was compensated by the enrichment of Actinobacteria (ANOVA, p < 0.05, Benjamini-Hochberg FDR). Acidimicrobia displayed a significantly higher relative abundance on J2 exposed to the conducive microbiomes from JKI and KWS soils, while Chloroflexi were significantly more enriched in samples associated with the suppressive G soil (ANOVA, p < 0.05, Benjamini-Hochberg FDR).

 Canonical analysis of principal coordinates (CAP) highlighted that the attachment of bacteria to the J2 cuticle was very specific for the individual soil origins based on the clear grouping of replicates for each soil, although there was some overlap between replicates from KWS, S10 and JKI treatments (Fig. 3B). Moreover, the J2-attached microbiomes of the BS1 and G treatments were more dissimilar and dispersed when compared with the other assemblages. PERMANOVA confirmed the significant effect of microbial suspensions on J2-attached microbiomes (Fig. 3B). However, there was no obvious pattern of sample grouping according to the degree of suppressiveness.

3.3. Indicator species analysis of J2-attached bacteria in different soils

Discriminant analysis was used to produce a network with M. hapla J2-attached ASVs that differed significantly across different soil microbiome treatments, by implementing indicator species analysis. A total of 40 ASVs that corresponded to 3.3% of the total ASVs, were identified as indicator taxa (Fig. 4A). The network shows that at a higher taxonomic resolution, the J2-attached microbiomes from S10 and G do not share indicator ASVs with any of the other treatments, while the other treatments do share a few indicator ASVs. In addition, BS1 and G samples have mostly Alphaproteobacteria and Actinobacteria as indicator species, while the indicator species in other treatments were mostly assigned to Proteobacteria and Gammaproteobacteria. Among these, 11 ASVs were specific to J2 in JKI treatment, 4 in KWS treatment, 15 in BS1 treatment, 1 in S10 treatment, and 12 in G treatment, while no indicator taxa were specific to J2 in E treatment.

To compare the relative abundances of different genera between the different treatments, the indicator taxa were further summarized at genus level in a heatmap of centered and scaled counts, showing the very specific attachment of bacteria to the J2 cuticle in each treatment (Fig. 4B). The most numerous indicator ASVs were associated with J2 from two nematode-suppressive soils, G and BS1, with one shared ASV from the genus Sphingomonas. The J2 from the G treatment were colonized by Alphaproteobacteria (genera Qi pang gua nia, Sphingomonas and Altererythrobacter, and families Devosia ceae and Sphingomonadaceae) and Actinobacteria (genus Nocardioi d e s and two Actinoplanes species, Actinoplanes digitatus and Actinoplanes italics). In the BS1 treatment, the J2-attached ASVs from the phylum Alphaproteobacteria were assigned to Sphingomonas and Methylobiog l ella ceae, while the members of Actinobacteria were more abundant and identified as Crossiella, Streptomyces ceae, Pseudarthrobacter, Micrococcaceae, Streptomyces, and Nocardia. In addition, two ASVs associated with M. hapla in the BS1 treatment were assigned to Chloroflexi and Clostridia. A low number of ASVs that were detected as attached to J2 after incubation in the S10 treatment was assigned to the genus Rhodococcus. In two non-suppressive soil microbiome treatments, KWS and JKI, two genera, Rhizobium and Devosia, attached to the cuticle of J2. In addition, the ASV corresponding to the order Burkholderiales was only found on the J2 from the JKI treatment.

4. Discussion

In this study we measured suppressive effects of microbiomes from nine different soils against the RKN species M. hapla by following J2 invasion in tomato roots. To make sure that only the microbial mienety of soils was tested against the nematode and to exclude the effects of varying physicochemical characteristics of different soils (Prot and van Gundy, 1981), we drenched the rhizospheres of tomato plants grown in sand and clay pellets with microbial suspensions extracted from different soils prior to nematode inoculation. We used this approach as it was shown that establishment of RKN was better in carrots grown in sand as compared to its dilutions with bed-soil (Kim et al., 2017). An enhanced soil aeration and low accumulation of plant growth inhibitory substances in sandy soils were considered important factors for successful nematode invasion and reproduction. Our results showed a
distinct capability of microbiomes originating from different soils to suppress nematode performance. The tomato roots drenched with microbiome from soil S10 had the lowest number of invaded *M. hapla* J2, which was followed by BS1, G and S soil microbiomes. In addition, our results indicate that microbiomes from soil JKI were least suppressive against J2 invasion in roots, followed by soils KWS, D2, E, and S5. J2 invasion of the roots did not depend on the size of the root system, as there was no indication that bigger roots hosted more nematodes than the smaller roots. Hence, we ascribe differences in J2 invasion of the whole root system between treatments to differences in the microbial suppression of the J2. In addition, a seven-day period used to assess J2 invasion was not long enough for observing significant nematode effects on the plant growth parameters in our study; thus, the differences in the plant growth between microbiome treatments should only be ascribed to different effects of the soil microbiomes. Indeed, the importance of soil bacteria in aiding the plant to uptake nutrients from soil and fight diseases, and in *de novo* synthesis of essential compounds for the plant growth has been repetitively reported (Hayat et al., 2010; Bakker et al., 2018).

It has previously been shown that the nematode surface can be an important source of nemato-antagonistic microorganisms in different soils (Adam et al., 2014b; Elhady et al., 2017; Orr et al., 2019; Topalović et al., 2019a). On the other hand, we know very little about the microorganisms that soil-dwelling stages of PPN carry on their surface for protection purposes (Topalović and Vestergård, 2021). So far, studies point to the surface coat, the carbohydrate-rich protein layer over the nematode epicuticle, as the main area for microbial attachment (Davies and Curtis, 2011). In the present study, we were able to decipher bacterial communities on the surface of *M. hapla* J2 that were incubated in microbial suspensions from six soils as analyzed by constrained analysis of principal coordinates (CAP) using Bray-Curtis distance. The principal coordinates 1 (CAP1) and 2 (CAP2) were plotted based on the soil origin. The statistical difference was determined using PERMANOVA assessed with $10^4$ permutations. Percentage of variation given on each axis refers to the explained fraction of total variation.

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**Fig. 3. Beta diversity of *M. hapla*-J2 associated bacteria.**

A) The relative abundance of bacterial ASVs associated with the different soils and J2-attached microbiomes, at class level. The labels below the barplots represent biological replicates of each treatment. The arrows next to the taxa show significant changes of their abundance for specific treatments (ANOVA, p < 0.05, Benjamini-Hochberg FDR). B) Bacterial communities attached to *M. hapla* J2 in microbial suspensions from six soils as analyzed by constrained analysis of principal coordinates (CAP) using Bray-Curtis distance. The principal coordinates 1 (CAP1) and 2 (CAP2) were plotted based on the soil origin. The statistical difference was determined using PERMANOVA assessed with $10^4$ permutations.
soil. We believe that the geographical position of the soil sites might have partly contributed to this effect, as soils JKI, KWS and S10 were geographically closest to each other. Notably, we observed a higher bacterial diversity and richness, even though not significant, on J2 recovered from two suppressive soils, BS1 and G, in comparison to other soils, while these alpha diversity parameters were lowest for the J2-attached bacteria from conducive JKI soil. The presence of more diverse bacterial assemblages on nematodes from suppressive soils could be correlated with the fact that the low occurrence rate of plant pathogens and parasites in soil is usually linked with a high microbial diversity (Workneh and van Bruggen, 1994; Garbeva et al., 2004; Zhou et al., 2019).

When considering a higher taxonomic level, a very low number of commonly shared ASVs between J2 from different treatments suggests that the surface-sterilization of J2 prior to their incubation in soil suspensions successfully removed most of the bacteria that were already present on the J2. In addition, we found the most numerous indicator ASVs on the surface of J2 from suppressive G and BS1 soils. Although the majority of these taxa belonged to Actinobacteria and Alphaproteobacteria, very few of the ASVs were common for these two treatments. Nevertheless, some of the G and BS1 originating J2-attached ASVs, including members of Sphingomonadaceae, Nocardioides, Devosia, Sphingomonas, Micrococcaceae, Actinoplanes, Streptomyces, Clostridium, and Nocardia, have been previously reported in association with different PPN species in soils with nematode-suppressive characteristics (Adam et al., 2014b; Eberlein et al., 2016b; Castillo et al., 2017; Elhady et al., 2017; Hu et al., 2017; Hussain et al., 2018). Members of Streptomyces and Clostridium produce compounds with nematicidal effects (Siddiqui and Mahmood, 1999), but there is no clear evidence as to if and how the rest of these bacteria can suppress nematodes.

The bacteria attached to J2 from conducive soils, KWS and JKI, including Devosia, Clostridium, Rhizobium, and Burkholderiales, have also been detected in studies on microbial attachment to Meloidogyne spp. (Adam et al., 2014b; Elhady et al., 2017). Clostridium, Rhizobium and Burkholderia have been reported as nematode antagonists (Reitz et al., 2000; Meyer et al., 2001; Adam et al., 2014a), but as microbiomes from KWS and JKI soils were conducive to J2 root invasion, we speculate whether bacteria attaching to the J2 could have protected the J2 rather than being only antagonists. Devosia, as such, has often been detected in cyst nematode-suppressive soils using amplicon sequencing (Eberlein et al., 2016b; Hu et al., 2017; Hussain et al., 2018), but it has never been tested as a nematode antagonist in vitro. On the other hand, both Devosia and Sphingomonadaceae have been associated with different life stages of M. incognita isolated from heavily infested tomato roots (Cao et al., 2015). As these bacteria have role in nitrogen fixation and cellulose degradation, it has been suggested that they may form a symbiotic...
association with nematodes and aid them during the plant root parasitism (Cao et al., 2015; Tian et al., 2015).

It thus appears that J2 exposed to microbiomes from soils with varying suppressiveness carry both putatively nemato-suppressive bacteria (e.g. Burkholderiales and Clostridiaceae) as well as bacteria that may facilitate J2 root invasion. This is an interesting phenomenon which should not come as a surprise, as nemato-parasites have a strong ability to attract nematodes (Jansson, 1987; Davies and Curtis, 2011), and here we show that they attached to J2 even in conducive soils. However, whether the attachment of bacteria that protect J2 against antagonists or facilitate their root invasion played a role for the high J2 root invasion in pots treated with conducive soil microbiomes is an intriguing question that warrants further investigation of the potential J2 protective role of microorganisms attached to the surface of infective stages of PPN. The application of nemato-antagonistic microbial strains has been proposed as a sustainable strategy for the management of PPN. However, the efficiency of a range of microbial control agents has been insufficient when applied in real agricultural soils. We propose that the attachment of indigenous nemato-protective microorganisms can partly explain the low efficacy of applied biocontrol strains (Topalović and Vestergård, 2021). It is important to note that Topalović et al. (2019a) have previously isolated bacteria attached to M. hapla J2 that were incubated in the suspensions extracted from the same soils used in the current study. The study reported a very high attachment rate of the strain Microbacterium sp. K6 in the re-attachment experiments with purified isolates, but no indicator ASV was assigned to this genus in our results. Thus, this confirms that nemato-bacterial interactions in different soils are very complex, and the estimation that only 1% of soil bacteria are culturable (Amanuel et al., 1995), adds up to this complexity.

Taken together, J2 of M. hapla, recovered from microbial suspensions with a varying potential to suppress the nematode, attached a specialized subset of bacteria, and their composition and abundance is mainly affected by the soil origin. In addition, bacterial colonizers on J2 from two suppressive soils were the most numerous and diverse, but further studies on nematode associations with microorganisms in conducive soils are needed in order to determine their function and possible role in nematode protection. This might give an explanation as to why we still lack microbial control agents with high and consistent efficiency against RKN.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104344.

Declaration of competing interest

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

Acknowledgements

This work was funded by the German Research Foundation grant DFG HE6957/1-1, the Independent Research Fund Denmark, grant no. 9041-00139B, and an Aarhus University Research Foundation Starting Grant. Graphical abstract created using Biorender.com.

References

Adam, M., Heuer, H., Hallmann, J., 2014a. Bacterial antagonists of fungal pathogens also control root-knot nematodes by induced systemic resistance of tomato plants. PLoS One 9, e90402. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0090402.

Adam, M., Westphal, A., Hallmann, J., Heuer, H., 2014b. Specific microbial attachment to root knot nematodes in suppressive soil. Appl. Environ. Microbiol. 80, 2679–2686. https://journals.asm.org/doi/10.1128/AEM.03905-13.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169. https://journals.asm.org/doi/10.1128/MBR.59.1.143-169.1995.

Bakker, P.A.H.M., Pieterse, C.M.J., de Jonge, R., Berendsen, R.L., 2018. The soil-borne legacy. Cell 172, 1178–1180. https://www.sciencedirect.com/science/article/pii/S009286741830165X?via%3Dihub.

Bard, G.C., Egnin, M., Bonti, C., 2017. Nematology: concepts, diagnosis and control. Inter. J. Complex Syst. 1695, 1–9.

Davies, K.G., Curtis, R.H.C., 2011. Cuticle surface coat of plant-parasitic nematodes. Annu. Rev. Phytopathol. 49, 135–156. https://www.annualreviews.org/doi/10.1146/annurev-phytopathology-111310-111406.

Eberlein, C., Heuer, H., Vidal, S., Westphal, A., 2016a. Microbial communities in g Nabodera pallida females raised in potato monocuscule soil, 106, 581–590. https://academic.asm.org/journals-apj/doi/10.1128/JP9.0117-15.

Eberlein, C., Heuer, H., Vidal, S., Westphal, A., 2016b. Microbial communities in g Nabodera pallida females raised in potato monocuscule soil, 106, 581–590. https://academic.asm.org/journals-apj/doi/10.1128/JP9.0117-15.

Eberlein, C., Heuer, H., Westphal, A., 2020. Biological suppression of populations of Heterodera schachtii adapted to different host genotypes of sugar beet. Front. Plant Sci. 11, 812. https://www.frontiersin.org/articles/10.3389/fpls.2020.00812/full.

Elbady, A., Ghanem, T., Topalović, D., Jacob, S., Sreesen, S.J., Sorbaj, F.U., Heuer, H., 2017. Microbiomes associated with infective stages of root-knot and lesion nematodes in soil. PLoS One 12, e0177145. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0177145.

Elbady, A., Topalović, O., Heuer, H., 2021. Plants specifically modulate the microbiome of root-lesion nematodes in the rhizosphere, affecting their fitness. Microorganisms 9, 679. https://www.mdpi.com/2076-2607/9/4/679.

Garbeva, P., van Veen, J.A., van Elsas, J.D., 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol. 42, 243–270. https://www.annualreviews.org/doi/10.1146/annurev.phytopathology.42.012604.135455.

Hamid, M.I., Hussain, M., Wu, Y., Zhang, X., Xiang, M., Liu, X., 2017. Successive soybean-mycorrhizal cropping assembles rhizosphere microbial communities for the soil suppression of soybean cyst nematode. FEMS Microbiol. Ecol. 93, fiw222. https://academic.oup.com/femsec/article/93/1/fiw222/2666422.

Hayat, R., Ali, S., Amara, U., Khalid, R., Ahsan, I., 2010. Biological bacteria and their role in plant growth promotion: a review. Ann. Microbiol. 60, 579–598. https://annalsmicrobiology.biomedcentral.com/articles/10.1007/s13213-010-0171-7.

Hooper, D.J., Hallmann, J., Subbotin, S.A., 2005. Methods for extraction, processing and detection of plant and soil nematodes. In: Luc, M., Sikora, R.A., Bridge, J. (Eds.), Nematology: concepts, diagnosis and control. InTech, Rijeka, Croatia. https://www.intechopen.com/chapters/55521.

Klein, E., Katan, J., Gamliel, A., 2012. Soil suppressiveness to Meloidogyne javanica as induced by organic amendments and solarization in greenhouse crops. Crop Prot. 39, 270. https://www.annualreviews.org/doi/10.1146/annurev-phytopathology-07-15-2018-08.

Kim, E., Seo, Y., Kim, Y.S., Park, Y., Kim, Y.H., 2017. Effects of soil textures on infectivity of root-knot nematodes on carrot. Plant Pathol. 66, 67–74. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5456757/.

Kleijn, E., Katzin, J., Gamliel, A., 2012. Soil suppressiveness to Meloidogyne javanica as induced by organic amendments and solarization in greenhouse crops. Crop Prot. 39, 26–32. https://www.sciencedirect.com/science/article/pii/S0261219412000506.

Liu, C., Timper, P., J.P., Mekete, T., Joseph, S., 2017. Influence of root-knot nematode on attachment of Paratextua penetrans to Meloidogyne arenaria. J. Nematol. 49, 304–310. https://www.exley.com/journal_of_nematology/doi/10.21307/jofnematology-2017-076.

Love, M.J., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.
