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Networks of free-living nematodes and co-extracted fungi, associated with symptoms of apple replant disease

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ABSTRACT

Apple replant disease affects tree nurseries and apple production globally. After repeated planting in the same soil, apple roots show accumulation of phytoalexins, stunting, and blackening. Recently, we showed that nematodes extracted from replanted soil and co-extracted microbes triggered these symptoms, while pathogens or plant-parasitic nematodes could not explain the early disease development. To identify nematode-microbe complexes that coincide with replant disease, apple rootstocks were grown in the greenhouse in soils from five replanted sites for eight weeks. Nematodes were extracted by floatation from pots with stunted or normal plant growth, washed on a 20-µm sieve, and used for DNA extraction. Nematode communities and co-extracted fungi and bacteria were analyzed by high-throughput sequencing of amplified ribosomal fragments. The experiment was repeated in the next year. Regardless of soil type or year, the nematode and fungal communities significantly differed between pots with differential plant growth. Bacteria were not significantly associated with growth depression. Plant-parasitic nematodes or pathogens were not abundant in numbers that could explain the observed root damage. Free-living nematodes *Prsimatolaimus, Acrobeles, Tylencholaimus, Acrobeloides,* and *Aphelenchus*, and associated fungi *Exophiala, Hohenbuehelia, Naganishia, Psathyrella,* and unidentified members of Orbiliales, Helotiales, and Rhytismataceae significantly correlated with reduced plant growth. Isolating and investigating such disease complexes will give a chance to understand external biotic stress of apple roots and design mitigation measures.

1. Introduction

A complex syndrome that causes growth depression in apple trees after replanting has been termed apple replant disease (ARD) (Ross and Crowe, 1973). Affected plants show significantly reduced shoot growth, necrosis and patchy blackening of root cells, impaired root hair development, and low cell vitality, which may lead to root death (Grunewaldt-Stöcker et al., 2019). Fruit yield and quality are significantly reduced by ARD (Mazzola, 1998). This is a worldwide problem that occurs in various apple-growing regions or different soil types yet the causes are still not clear (Mai and Abawi, 1981; Mai et al., 1994; Winkelmann et al., 2019). ARD has been observed to occur heterogeneously at a given orchard (Gebbers and Adamchuk, 2010) with soil physical or chemical properties playing a minor role in affecting the distribution of ARD. The disease also occurs patchily along the root of single plants (Lucas et al., 2018). Aside from that, changes in some defense genes in response to infected root tissues showed localized reactions restricted to the site of infection and were more frequent and pronounced than in leaves. These findings suggest that the disease is localized and not systemic (Reim et al., 2020). Countermeasures such as soil pasteurization or fumigation significantly improved the growth of apple plants which gave evidence that the disease is caused by biotic factors (Yim et al., 2013). Biotic agents including the oomycetes *Pythium* and *Phytophthora*, fungi of the *Cylindrocarpon- Ilyonectria* group, *Fusarium*, and *Rhizoctonia*, several bacterial genera, and the root-lesion nematode *Pratylenchus penetrans* have been implicated with ARD-related growth depression (Mazzola, 1998; Tewoldemedhin et al., 2011b; Manici et al., 2017; Otto, 2017). Accumulation of phenolic compounds or phytotoxins in disease-affected roots has been discussed to play a role in ARD (Weiß et al., 2017; Manici et al., 2018).

Nematodes have been assumed to contribute to ARD, as 50 °C treatment of affected soils (Yim et al., 2015), *Tagetes* as pre-plant (Yim

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et al., 2017), or application of nematicides (Jaffee et al., 1982; Johnson et al., 1982) ameliorated the disease. Different feeding groups of nematodes are abundant in soils, specialized to their food sources, and play an essential role in ecosystem functions and services (Yeates et al., 1993). They are involved in complex symbiotic, pathogenic, and predator-prey interactions with the soil microbiome. Some of the plant-parasitic forms invade roots, resulting in substantial crop losses, and therefore are well studied (Jones et al., 2013). On the contrary, little attention focuses on the associations of free-living nematodes with bacteria or fungi. Nematode-microbe associations have been shown to affect higher organisms as in the case of entomopathogenic nematodes, where the γ-Proteobacteria *Xenorhabdus* or *Photorhabdus* have symbiotic associations with *Steinernema* and *Heterorhabditis*, respectively, to kill insects (Dillman et al., 2012). Some human-associated bacteria and fungi are associated with free-living nematodes as well (Renker et al., 2003). Etiological relations between some nematodes and microbes as partners of soilborne disease complexes have been reported (Back et al., 2002; Morris et al., 2016).

A recent thorough reanalysis of studies on ARD suggested more complex disease causes than single pathogens citing a multivariate ecological ARD hypothesis (Nicola et al., 2018). The plant and the soil biome include numerous drivers that interact directly or indirectly with each other. These multitrophic interactions may involve nematodes, fungi, bacteria, archaea, and oomycetes that reside in the plant-associated soils but have hardly been investigated in the ARD complex. Recently, evidence showed that microorganisms associated with nematodes were the causal agents of ARD in the soil analyzed (Kanfra et al., 2018): In their study, nematodes and microbes were extracted from ARD soils or control soils not affected by ARD from plots of the same field and inoculated the extracted nematode and microbe fractions in combinations or singly to ARD-susceptible apple rootstocks in a sterile pot system. The washed nematode fractions from ARD soil but not from control soil induced ARD symptoms (reduced root weight, browning, and increased total phenolics in roots and antioxidant capacity in leaves, increased phytoalexin accumulation). The water-extractable microbial fraction from ARD soil and control soil enhanced the disease when added to the nematodes but had only minor effects without the nematodes. As we did not detect significant numbers of plant-parasitic nematodes in ARD soil and not more than in the control soil, we concluded that free-living nematodes in association with certain soil microbes caused the disease. In this study, we searched for such ARD-inducing nematodes and analyzed their associated microbes.

The objective of this study was to relate differences observed in plant growth of susceptible apple rootstocks grown in ARD soils to particular free-living nematode species and their associated fungi or bacteria to find candidate species complexes that cause ARD. The study was done with five orchard soils differing in soil type, cropping history, and agricultural management. The study hypothesized that (i) soils with plants showing high ARD symptoms harbor distinct free-living nematodes with associated fungi or bacteria compared to soils with plants showing less ARD symptoms; (ii) that co-occurrence of specific nematodes and the nematode-associated microbes play a role in the severity of ARD. For this purpose, nematodes were extracted from replanted soils (five and three ARD reference sites of the project BonaRes-ORDIAmur (www.ordiamur.de) in 2016 or 2017 respectively) in which susceptible plants had been cultivated for eight weeks in the greenhouse. We used high-throughput amplicon sequencing to characterize the nematode and nematode-associated microbial communities. The present study offers insights into the relationship between specific nematodes and the nematode-associated microbes linked to a reduction in plant growth.

2. Materials and methods

2.1. Field soil samples used for the bioassays

Soils were collected from ARD field sites; this included Heidgraben site one (H) and site two (HH) (53°41′57.5"N, 9°40′59.6″E), Ellerhoop (E) (53°0.71′43.5"N; 9°0.77′14.3″E), Ruthe (R) (52°24′36.7"N; 9°81′97.0″E) and Meckenheim (M) (50°37′8.5"N, 6°59′25.4″E). At Heidgraben, an Entic Podzol (according to WRB 2015) had developed from aeolian sand. The soil in Ellerhoop was classified as an Endostagnic Luvisol from glacial till, whereas in Ruthe Haplic Luvisol developed from calcareous loess. Meckenheim was classified as a Haplic Luvisol developed from loess. On the field plots, the apple rootstock 'Bittenfelder Sämling' was replanted starting in 2010 at the Heidgraben site, or in 2011 at the Ellerhoop and Ruthe fields, in a two-year cycle. There was four apple replanted plots interspersed with four additional plots covered with grass that served as "control" plots. The Meckenheim site has been in use for apple variety tests grafted on the rootstock M9 since 2006. Replanting took place in the years 2010 and 2017 (G. Baab and L. von Schoenebeck, personal communication).

2.2. Experimental set-up for greenhouse bioassays

From each site, soil samples were taken at a depth of 0–20 cm from the centers of ARD plots. Soils of the four plots were mixed and sieved through 8 mm mesh. Portions of the soils were gamma-irradiated with a minimal dose of 10 kGy or untreated. Treated or untreated ARD soils were filled into 1 l pots with 27 technical replicates of each of the two soil treatments for the five ARD field sites. The experiment was conducted separately in 2016 and replicated in 2017 and has been described thoroughly (Mahnkopp et al., 2018). Soils used for the first experiment in 2016 were denoted as "1" and soils used for the second experiment in 2017 were denoted as "2". In 2016, soils from Heidgraben site one and site two (H1 and HH1), Ellerhoop (E1), Ruthe (R1), and Meckenheim (M1) were used. In the 2017 experiment, soils from M and HH were exempted. The different soil variants were supplemented with 2 g l^{-1} Osmocote Exact 3–4 M (16 + 9 + 12 + 2 MgO) and filled into 1 l pots. Homogeneous, acclimatized in vitro propagated M26 apple rootstocks were planted in the pots (one plant per pot) and placed in randomized complete blocks in a greenhouse. Greenhouse conditions, irrigation, and plant protection were carried out as described by Mahnkopp et al. (2018). The mean daily temperature in 2016 was 20.2 ± 1.1 °C, in 2017 21.1 ± 1.3 °C. A 16 h photoperiod was achieved or supported by additional light (SON-T Philips Master Agro 400 W) with a set point of 25 klx. Shoot length measurements were taken from the base of the newly emerging leaves of the main shoot at 8 weeks after incubation. At eight weeks of plant growth, soil samples from pots were collected (Supplementary Table S1).

2.3. Soil sample collection and nematode extraction

In apple orchards, replanted apple plants show differential growth patterns linked to symptoms of ARD (Deakin et al., 2018). To explore the differences observed in plant growth of susceptible M26 apple rootstocks grown in subsamples of the field soils, nematodes (plant-parasitic and free-living) were extracted from 250 ml portions of the untreated ARD soils by centrifugal floatation using $MgSO_4$ at 1.18 specific density (Hooper et al., 2005). Nematodes were collected on a 20 μm sieve and thoroughly washed with sterile water.

2.4. Characterization of the nematode diversity

Total DNA from the washed nematode communities was extracted using the FastPrep FP120 bead beating system and FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, United States) as described by the manufacturer. The DNA was purified with GENECLEAN SPIN Kit (MP

Biomedicals) according to the manufacturer's instructions. To characterize the nematode diversity, we designed a forward primer targeting the beginning of the ribosomal RNA gene in an attempt to improve the resolution of the phylum Nematoda among eukaryotes. Using the ARB package, we searched for conserved sites and picked a 10mer oligo based on a core group of 18S rRNA sequences from the SILVA SSU 132 database (Quast et al., 2013). Using an in-house nematode sequence alignment, we designed a forward primer including the ARB probe at the 3′ end. We used our forward primer together with the D2B primer (Kaplan et al., 2000), which targets the rapidly evolving D2/D3 expansion segment of the 28S rRNA gene, in the attempt to amplify about 4 kb of the rRNA cistron. Together with primers NF1 and 18Sr2b (Porazinska et al., 2009), we developed a nested PCR amplification strategy. We evaluated the coverage and specificity of the primers used for this study (Supplementary Table S1). To minimize the formation of chimeric DNA molecules, we used the water-in-oil Micellula DNA Emulsion and purification kit (Roboklon, Berlin, Germany) with few modifications. Specifically, a 50 µl reaction emulsion PCR mixture comprised of ~73% Emulsion component 1, ~7% Emulsion component 2, and 20% Emulsion component 3, which was cool mixed for 1 min. The aqueous phase was a PCR reaction mix containing 10 µl of 5× Q5 reaction buffer (New England Biolabs), 7.5 µl 2 mM dNTP, 2.5 µl 2 mg ml^{-1} BSA, 10 µl Q5 High GC Enhancer (New England Biolabs), 0.5 µl 2 U ml^{-1} Q5 High-Fidelity DNA Polymerase (New England Biolabs), and 2.5 µl of each primer (10 µM). Template DNA and water were added to give a final volume of 50 µl for each sample. Water-in-oil emulsions were prepared by adding 50 µl of pre-cooled PCR reaction mix to 300 µl of the pre-cooled oil phase. The PCR was carried out using the following cycling conditions: the thermocycler block was heated for 1 min followed by an initial denaturation of 5 min at 95 °C, 20 cycles of (95 °C for 15 s; 54 °C for 30 s; 68 °C for 2 min) and a final extension of 68 °C at 4 min. Emulsions were broken by the addition of 1 mL 2-butanol and 400 µl of Orange-DX buffer (Roboklon) was added to the broken emulsion solution. This solution was centrifuged for phase separation. DNA purification was carried out according to the manufacturer's instructions. In the second PCR step, approximately 362 bp tagged PCR products were amplified from 18S rDNA using the forward primer NF1 5′-CGTATCGCCTCCCTCGCGCCATCAG-3′ and the reverse primer 18Sr2b 5′-CTATGCGCCTTGCCAGCCCGCTCAG-3′ (Porazinska et al., 2009), with Illumina 5′-overhang TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG or GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG for NF1 or 18Sr2b, respectively. PCR reactions of 50 µl contained 2.5 µl of 10× GoTaq buffer (Promega, Mannheim, Germany), 2 µl 25 mM $MgCl_2$, 5 µl of 2 mM dNTP each, 1 µl of each primer (10 µM), 2.5 µl of 2 mg ml^{-1} BSA, 4 µl of 50% acetamide, 0.4 µl of 5 U ml^{-1} GoTaq DNA polymerase (Promega). The following PCR cycler condition was used: initial denaturation of 5 min at 94 °C, 25 cycles of (94 °C for 45 s; 54 °C for 30 s; 72 °C for 1 min), and a final extension of 10 min at 72 °C. The resulting PCR product was purified using the High Pure PCR Purification kit (Roche Diagnostics GmbH) following the manufacturer's instructions. Barcoded amplicon sequencing of the 18S rRNA genes was done by 2 × 250 bp paired-end high-throughput sequencing on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, United States) at Novogene Europe (Cambridge, UK).

2.5. Characterization of the nematode-associated microbial diversity

The fungal community associated with the extracted nematodes was amplified using the primers gITS7 and ITS4 targeting the ITS2 region (Ihrmark et al., 2012). PCR was performed in a reaction mixture of 50 µl consisting of 10 µl of 10× GoTaq buffer (Promega, Mannheim, Germany), 5 µl of 25 mM $MgCl_2$, 5 µl of 2 mM dNTP, 2.5 µl of 2 mg ml^{-1} BSA, 1 µl of each primer (10 µM), 1 µl of 5 U $µl^{-1}$ GoTaq Flexi polymerase (Promega), and 1 µl of nematode community DNA. The following PCR cycler condition was used: initial denaturing of 5 min at 94 °C, followed by 30 cycles of (94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min) and final elongation at 72 °C for 5 min. To characterize the bacterial diversity associated with the extracted nematodes, the V3-V4 regions of 16S rRNA genes were amplified using the primers 341F (Sundberg et al., 2013) and 806R (Caporaso et al., 2011) in a 25 µl reaction volume containing 2.5 µl 10× of standard reaction buffer (NEB), 0.125 µl of 5 U $µl^{-1}$ NEB HotStart Taq polymerase, 2.5 µl of 2 mM dNTP, 1 µl of 2.5 mM $MgCl_2$, 2.5 µl of 2 mg ml^{-1} BSA, 1 µl of each primer (10 µM) and 1 µl of nematode community DNA. The following temperature steps were applied: 2 min at 94 °C, 30 cycles of 20 s at 94 °C, 20 s at 56 °C, 40 s at 72 °C, followed by a final elongation for 5 min at 72 °C. Amplicon sequencing of the ITS2 or 16S rRNA genes was done by 2 × 250 bp paired-end high-throughput sequencing on an Illumina HiSeq 2500 platform by Novogene (Cambridge, UK). The high-throughput amplicon sequencing data were submitted to NCBI SRA (accession number PRJNA669579).

2.6. Sequence analysis

The 18S rRNA, ITS, or 16S rRNA sequence demultiplexing was done using the MiSeq Controller Software and diversity spacers were trimmed using Biopieces (www.biopieces.org). The sequence reads for nematodes and nematode-associated bacteria were processed using USEARCH (v11.0.667). Raw reads were processed using the protocol established in the USEARCH pipeline followed by OTU (Operational Taxonomic Unit) clustering using UPARSE (Edgar, 2013). The nematode sequence read preparation and processing included paired-end merging with an overlapping minimum read length of 10 base pairs, filtering of low-quality sequences, removal of reads less than 200 bp, and dereplication to find unique sequences following default settings. OTU clustering and chimera removal was performed at a 97% identity threshold via the *cluster_otu* command implemented in the UPARSE algorithm. BlastN assigned taxonomic affiliations against the Silva SSU 132 database (Quast et al., 2013) with the Expect Value of 0.001, which was performed in a Galaxy workflow (Cock et al., 2013). Processing of the bacteria sequence reads included paired-end merging with an overlapping minimum read length of 10 base pairs and minimum merge length of 400 bp. Filtering of low-quality sequences and dereplication to find unique sequences was done following default settings. OTU clustering and chimera removal was performed at a 97% identity threshold via the *cluster_otu* command implemented in the UPARSE algorithm. OTU sequences were taxonomically classified by the software Mothur on the genus level and higher taxonomic ranks using RDP trainset 16 at 80% cutoff. For the fungi reads, overlapping regions within paired-end reads were aligned to generate “contigs” and primers removed from both ends of the sequences by PANDAseq using default settings (Masella et al., 2012). Taxonomic affiliations were assigned by BlastN against the UNITE database version 8.0 (Nilsson et al., 2019) (https://unite.ut.ee) with the Expect Value of 0.001, which was performed in a Galaxy workflow (Cock et al., 2013). Dereplication, singleton removal and clustering of sequences to operational taxonomic units (OTU, > 99% similarity) were performed using BLAST Parser (Antweiler et al., 2017) implemented in a Galaxy workflow (Cock et al., 2013).

2.7. Data analysis

The multivariate analyses on the nematode and the nematode-associated microbial OTU abundances were carried out with the R software version R3.6.3 (R Core Development Team) using the packages vegan (Oksanen et al., 2015), EdgeR (Robinson et al., 2010), labDSV (Roberts, 2016), indicspecies (de Cáceres et al., 2010), mvabund (Wang et al., 2012), SPIEC-EASI (Kurtz et al., 2015) and Emmeans (Searle et al., 1980). Plant growth data was normalized for each soil to account for soil-dependent differences in plant growth and was used for the analysis. Plant growth data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. The effect of years, soil, and plant growth on the relative abundances of the higher taxonomic level of

nematodes was calculated on the order level while the associated microbes were on the phylum level. This was evaluated using a multivariate analysis of variance (MANOVA) followed by a one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test. Diagnostic plots of residuals versus fitted values revealed the lack of significant heterogeneity of variance and Q-Q plots showed that assumptions of normality were justified. Further analysis was done on non-rarefied OTU data. To test the effect of the plant growth, the soil, and the years on the nematode or the nematode-associated microbial communities, we fitted a model using the 'manyglm' function in the mvabund package with a negative binomial probability distribution on the count OTU data. Significance of differences was carried out with the 'anova.manyglm' function using likelihood-ratio tests (LRT) (ANOVA, default pit-fall resampling, and 999 Bootstrap iterations). This function also provided a pairwise comparison adjusting for multiple comparisons (Tukey's HSD test via a free step-down resampling procedure). The community composition was visualized on log-transformed relative abundance data by non-metric multidimensional scaling (NMDS), 20 to 100 times randomly computed based on Bray-Curtis similarities using default settings (McCune et al., 2002). We analyzed the relationship between the plant growth and the community compositions, using canonical correspondence analysis (CCA). The analysis was carried out on the log-transformed relative abundances of OTU at 999 permutations. We applied the generalized linear model (GLM) and linear models (LM) on the CCA axes that explain most of the variation due to the plant growth. When the interaction of the plant growth and the soil type is significant, then we tested the influence of the soil type utilizing the 'emmeans' package. To test for significantly different abundant OTU, associated with plant growth, several approaches were utilized. These included: (i) a LRT under negative binomial distribution and generalized linear models, false discovery rate (FDR) - corrected $P < 0.05$ implemented in EdgeR, (ii) a LRT under negative binomial distribution, and generalized linear models with Tukey's stepwise adjusted P values implemented in "manyglm" function, (iii) an OTU indicator analyses with the multipatt function (option = 'r.g', 999 permutations from the package 'indicspecies' followed by FDR correction for multiple testing ($P \leq 0.05$), and (iv) a multiple linear regression with adjusted R squared ($P \leq 0.05$) model on the log-transformed relative abundances of the count data. Only OTU present in at least 50% of samples in each soil were considered. The correlation or contribution of the differentially significant OTU to the plant growth were tested and fitted on the CCA ordination by the 'Envfit' function at 999 permutations (Oksanen et al., 2015). We examined intra- and inter-kingdom nematode-microbial interactions following the SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) statistical package. Nematode, bacterial and fungal OTU were pooled, subjected to trimmed means of M transformation, and normalized as relative abundance counts per million using the "edgeR" package. We used OTU that were present in at least 50% of the samples and which had Spearman's rank correlations >0.7 for positive or > -0.7 negative correlations and p values <0.001. All the correlations were visualized using networks, and network properties were computed using the "igraph" package. All networks were subjected to Fruchterman-Reingold layout with 999 permutations.

3. Results

3.1. Induction of ARD symptoms in apple plants by soil biota

The severity of ARD at different sites was tested by growing M26 plantlets in pots containing gamma-irradiated or untreated ARD soils. After 8 weeks, the shoot length was significantly lower in the untreated ARD soils when compared to the respective gamma-irradiated soils (ANOVA, $P < 0.001$) (Fig. 1). The shoots were roughly 15 cm taller in sterilized ARD soil for all soils, while the growth varied between soils and years. In contrast to gamma-irradiated soil, roots in ARD soils were stunted and showed the typical brownish color of ARD. The patchy distribution of ARD symptoms observed in apple plant growth in the untreated ARD soils was explored and both the nematode communities and the nematode-associated microbial diversity were characterized.

3.2. Nematode community structure of ARD soils with plants showing differential ARD symptoms

The taxonomic composition of nematodes at the order level was soil-type dependent, changed in the two years and was affected by plant growth (Supplementary Table S2). The proportions of Rhabditida, Tylenchida, Diplogasterida, and Triplonchida were high (Fig. 2). Plant-parasitic nematodes represented a minor part of the nematode communities. A multivariate analysis of deviance using a likelihood ratio test (LRT) under negative binomial distribution revealed that regardless of

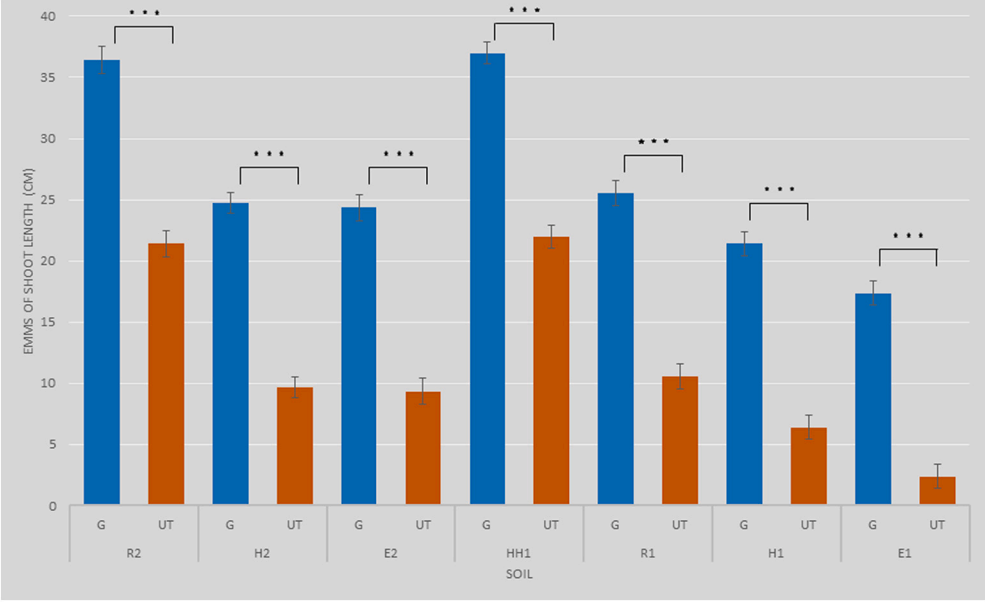

Fig. 1. Growth of apple plants in gamma-irradiated or untreated replanted soils from different apple growing areas. Soils R1, R2: Ruthe (2016, 2017); E1, E2: Ellhoop (2016, 2017); H1, H2: experimental field site Heidgraben (2016, 2017); HH1: nursery near Heidgraben (2016).

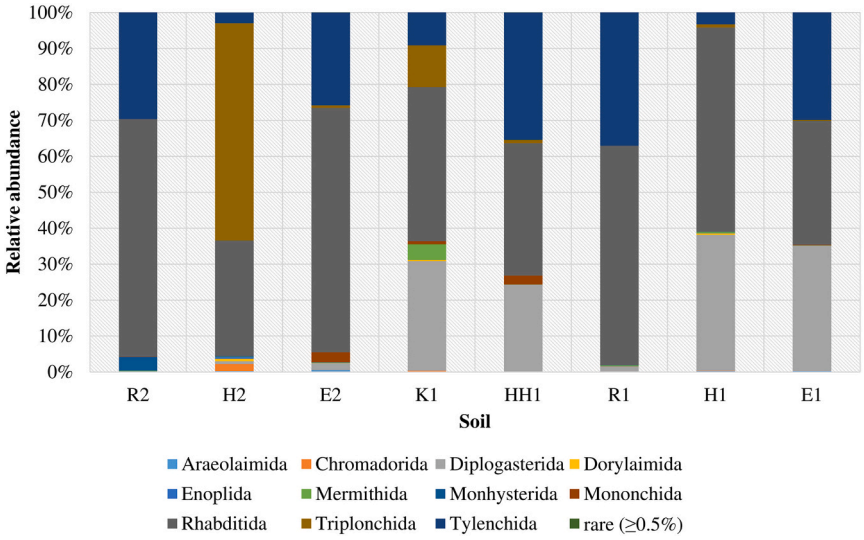

Fig. 2. Taxonomic profiles of nematode communities across all investigated soils. The average relative abundances are shown on the order level. Taxa with less than 0.5% relative abundance were grouped as rare.

the soil type or year, the nematode community significantly differed within the varying shoot lengths of plants in the untreated ARD pots (Table 1). This result was reflected by the non-metric multidimensional scaling (NMDS) ordinations that showed clusters of the nematode communities for the different soil types and to a lesser extent by the varying lengths of the shoot of plants measured for each soil (Fig. 3). However, the year did not contribute to the community differences observed. To examine the OTU vs. plant growth relationship, canonical correspondence analysis (CCA) and permutational tests were applied to the OTU table with shoot length as the environmental variable. Overall, the first CCA axis (CCA1) explained nearly 100% of the variance in the dataset. The CCA1 axis separated the nematode communities based on the differences in plant growth (Fig. 4) and to a lesser extent by the soil type (Supplementary Table S3).

3.3. Fungal and bacterial community structure of ARD soils with plants showing differential ARD

It was hypothesized that a shift in the microbial community

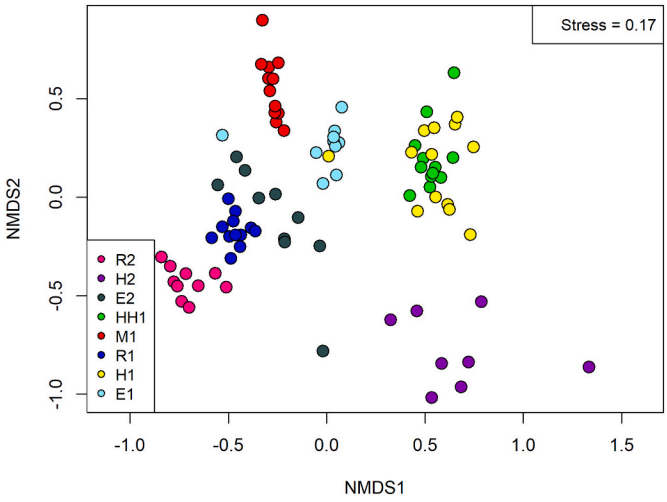

Fig. 3. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances of nematode communities in untreated ARD soils in pots with plants showing varying shoot growth. Nematodes were analyzed by high-throughput rDNA amplicon sequencing. Soils are color-coded while.

Table 1
Statistical association of plant growth and nematode community structure or nematode-associated microbial community structure analyzed by a multivariate analysis of the deviance of generalized linear models.

Soil biota	Factor	Res. Df[a]	Dev (LRT)[a]	*P*-value
Nematode community	Soil	79	13,158	0.001
	Year	78	0	0.846
	Plant growth	4	4906	0.001
	Soil*plant growth	4	0	0.150
Nematode-associated fungal community	Soil	80	3,400,888	0.001
	Year	87	1,325,283	1.000
	Plant growth	79	457,884	0.001
	Soil*plant growth	64	251.7	0.002
Nematode-associated bacterial community	Soil	76	23,793	0.001
	Year	83	32,953	0.001
	Plant growth	75	912	0.441
	Soil* plant growth	60	5613	0.112

[a] A Likelihood Ratio Test (LRT) after 999 bootstrap iterations assuming a negative binomial distribution was used, with a multivariate generalized linear model explaining the deviance (Dev) as a measure of the quality-of-fit as well as the residual degree of freedom (Res. Df).

associated with the extracted nematodes is linked to ARD symptoms. The taxonomic composition of fungal communities that were associated with the extracted nematodes varied by the soil type, followed by the year but not the plant growth. Overall, high proportions of Ascomycota, Basidiomycota, and Mortierellomycota dominated the fungal communities (Fig. 5) (Supplementary Table S2). Regardless of the year and soil, the varying plant growth measured in the untreated ARD pots was significantly associated with the fungal community structure (Table 1). To examine the species vs. plant growth relationship, CCA and permutational tests were applied to the fungal communities, with shoot length as the environmental variable. The fungal community co-extracted with the nematodes was significantly discriminated on CCA1 by the shoot length (Fig. 6).

The phylum composition of the bacterial communities that were co-extracted with the nematodes was significantly influenced by the year and by the soil type (Fig. 7). High proportions of Proteobacteria, Bacteroidetes, and Firmicutes dominated the communities. In contrast to the nematode-associated fungi, plant growth was not significantly

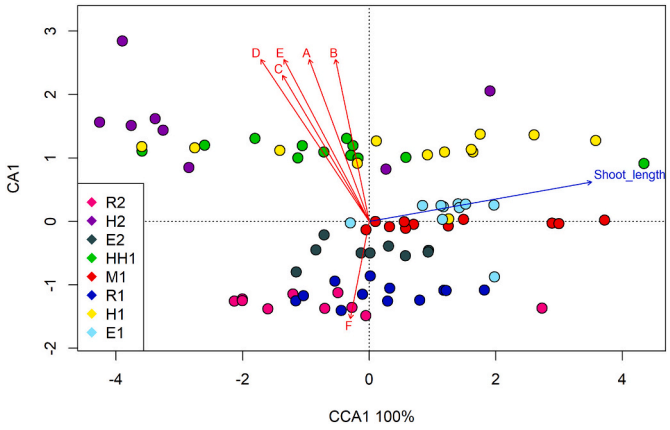

Fig. 4. Canonical correspondence analysis (CCA) showing the relationship between nematode community and "plant growth, corresponding to suppressed or normal growth, respectively. Differentially abundant OTU in soils negatively correlating with plant growth ($P < 0.05$) were fitted onto the ordination plots as vectors. Nematode OTU enriched with negative correlations: A: OTU18S_139 and C: OTU18S_174 (*Prismatolaimus*), B: OTU18S_388 (*Acrobeles*) and D: OTU18S_309 (*Acrobeloides*), F: OTU18S_682 (*Aphelenchus*), and E: OTU18S_123 (*Tylencholaimus*).

associated with the bacterial community structure. This result was reflected by the NMDS ordination that showed clusters of the bacterial communities for the different soil types (Supplementary Fig. S1).

3.4. Search for OTU of nematodes and nematode-associated fungi affecting plant growth

Using the CCA1 axis for univariate tests, the nematode communities were significantly discriminated within varying shoots of the plants for the soils analyzed (GLM, LRT, $P < 0.001$). Within the nematode communities, 11 OTU were sensitive to the varying plant growth in the untreated ARD pots (GLM, LRT, $P < 0.05$). The nematodes OTU18S_174, OTU18S_123, OTU18S_309, OTU18S_139, OTU18S_388, and OTU18S_682 significantly correlated with plant growth reduction (Supplementary Table S4). Among the nematode-associated fungal OTU, 17 were differentially enriched and associated with the varying plant growth (Supplementary Table S4). Among those, OTU_ITS63, OTU_ITS1347, OTU_ITS47, OTU_ITS2288, OTU_ITS1865, OTU_ITS1190, OTU_ITS179, OTU_ITS988, OTU_ITS283, OTU_ITS2577, and OTU_ITS2350 were significantly more abundant in the nematode fraction and correlated negatively with plant growth (GLM, LRT, $P < 0.05$).

3.5. Taxonomic affiliation of OTU linked to plant growth depression

The assignment of sequences of the nematode OTU linked to reduced plant growth was done by finding closely related sequences at the appropriate taxonomic level guided by available reference sequences (Supplementary Table S4). The best hit of the sequences of OTU_139 and OTU_174 was affiliated to sequences belonging to the genus *Prismatolaimus*. OTU_388 displayed a high sequence identity to species of the genus *Acrobeles*. OTU_309 was identified as *Acrobeloides*, OTU_682 as *Aphelenchus,* and OTU_123 as *Tylencholaimus*. The assignment of sequences of the nematode-associated fungi OTU_ITS47 and OTU_ITS63 displayed high sequence similarity to members of the order Orbiliales with OTU_ITS47 closely relating to *Hyalorbilia*. OTU_ITS988 was similar to members of Ventures, OTU_ITS1347 to members of Helotiales, OTU_ITS283 was similar to unidentified members of Rhytismataceae, and OTU_ITS1190 to an unidentified member of Sordariales. OTU_ITS179 was identical to *Exophiala,* OTU_ITS1865, and

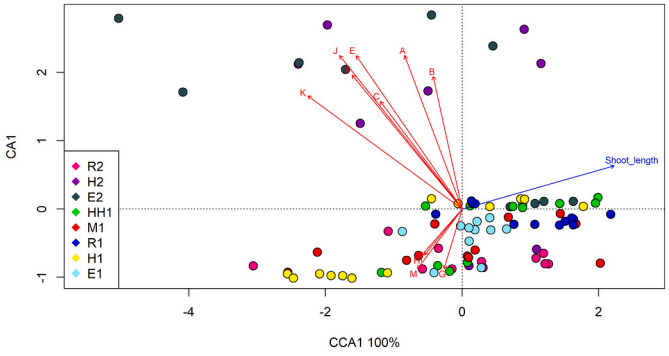

Fig. 6. Canonical correspondence analysis (CCA) showing the relationship between nematode-associated fungi and plant growth, corresponding to suppressed or normal growth, respectively. Differentially abundant OTU in soils of high or low plant response class ($P < 0.05$) were fitted onto the ordination plots as vectors. Fungi enriched and correlated negatively with plant growth are denoted as A: OTU_63 (Orbiliales), B: OTU_ITS1347 (Helotiales), C: OTU_ITS47 (*Hyalorbilia*), E: OTU_ITS2288 and F: OTU_ITS1865 (*Naganishia*), G: OTU_ITS1190 (Chaetomiaceae), H: OTU_ITS179 (*Exophiala*), I: OTU_ITS988 (Venturiales), J: OTU_ITS283 (Rhytismataceae), K: OTU_ITS2577 (*Hohenbuehelia*) and M: OTU_ITS2350 (*Psathyrella*).

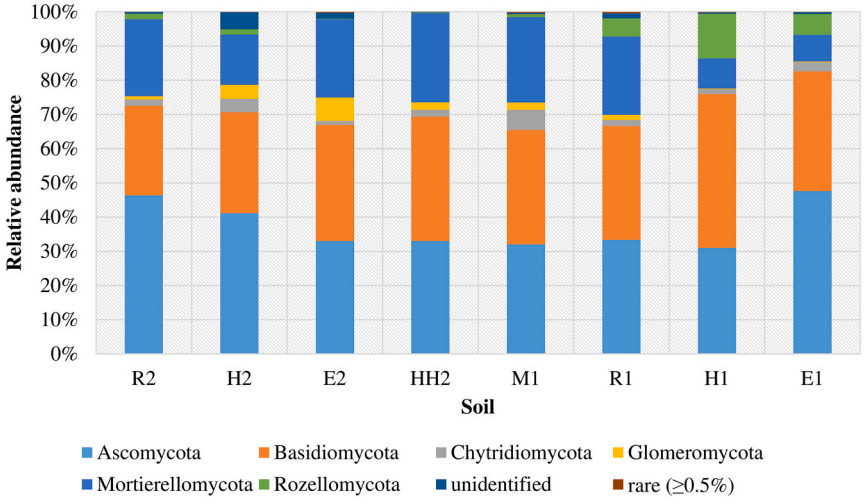

Fig. 5. Taxonomic profiles of nematode-associated fungal communities from all replanted soils analyzed. The average relative abundances are shown on the phylum level. Taxa with less than 0.5% relative abundance were grouped as rare.

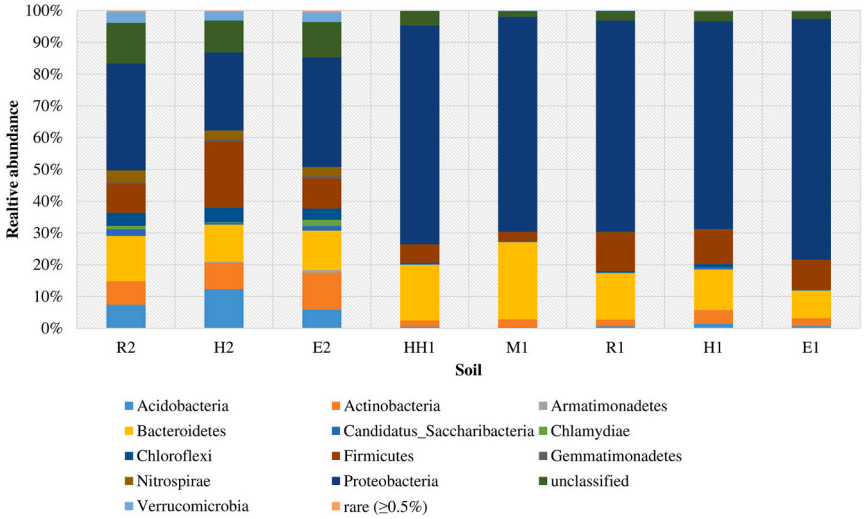

Fig. 7. Taxonomic profiles of nematode-associated bacterial communities from all replanted soils analyzed. The average relative abundances are shown on the phylum level. Taxa with less than 0.5% relative abundance were grouped as rare.

OTU_ITS2288 were similar to *Naganishia* and OTU_ITS2577 to *Hohenbuehelia* and OTU_ITS2350 to *Psathyrella*.

3.6. Co-occurrence network of nematodes and nematode-associated microbes

We analyzed co-occurrence networks to search for a putative disease complex of nematodes and their associated microbes that plays a role in ARD. The network revealed more intra kingdom associations and to a lesser extent, inter-kingdom associations (Fig. 8). This was evident among the OTU belonging to Proteobacteria with only negative associations (gray lines) as well as among the fungal and nematode OTU which had rather some fewer positive associations (red line). The nematode OTU established positive inter-kingdom associations with some bacteria OTU mostly belonging to the phyla Acidobacteria, Actinobacteria, or unclassified bacteria. In the network, three nematodes and seven bacteria keystones or hub OTU were identified (Supplementary Table S5). Among the nematode OTU that correlated negatively with plant growth, OTU18S_388 (*Acrobeles*) co-occurred positively with OTU16S_75 (unidentified Firmicutes) ($R^2 = 0.73$, $P \leq 0.001$) and OTU18S_139 (*Prismatolaimus*) positively with OTU16S_67 (*Flavobacterium*, KU204878.1 100%) ($R^2 = 0.75$, $P \leq 0.001$).

3.7. Discussion

ARD is characterized by retarded vegetative growth of apple trees

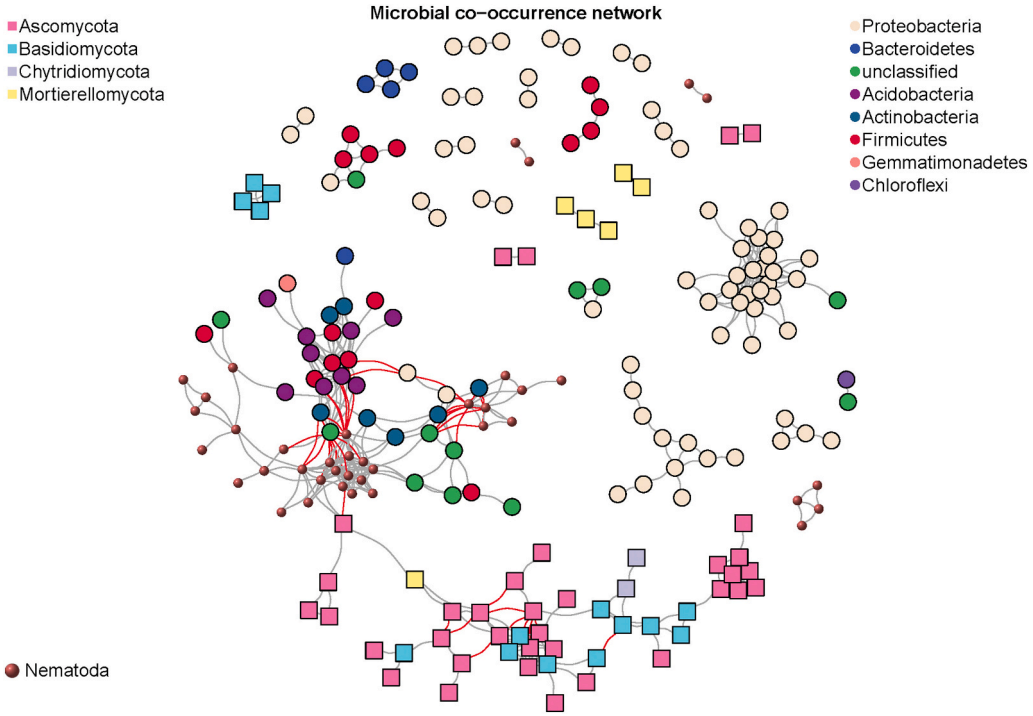

Fig. 8. Association network showing nematode and the nematode-associated microbial hubs in pots with plants showing differential ARD symptoms. OTU present in at least 50% of each soil was used to construct the network and are shown as nodes and co-occurrence as edges. Nematode fungal and bacterial nodes are represented as square, triangle, and circle symbols in the network, respectively.

and yield decline attributed to a yet unknown complex of soil biota (Winkelmann et al., 2019). Multitrophic interactions of biological agents were proposed to cause ARD but the understanding of the interactions and their implication to apple trees is lacking. Also, external stress factors such as phytotoxins or accumulation of phenolic compounds in disease-affected roots are being discussed to play a role in ARD (Weiß et al., 2017; Manici et al., 2018). Recently the involvement of free-living nematodes and their associated microbes in ARD was reported (Kanfra et al., 2018). In this study, further evidence was found that the abundance of distinct free-living nematodes and nematode-associated fungi were associated with ARD symptoms and that these species were common in the different ARD soil types, while nematode-associated bacteria seem to be less important in the disease complex but play a pivotal role in nematode microbial networks. Also, earlier studies found evidence that bacteria or plant-parasitic nematodes were not involved in ARD development, and chloramphenicol (targeting bacteria) or nematicides (targeting *P. penetrans*) failed to ameliorate ARD (Mazzola, 1998; Tewoldemedhin et al., 2011a). Fungicide application to ARD soil enhanced plant growth, and phytopathogenic fungi or oomycetes were frequently detected in diseased roots, including species of *Cylindrocarpon/Nectriaceae, Fusarium*, *Pythium, Phytophthora,* and *Rhizoctonia* (Mazzola, 1998; Mazzola and Manici, 2012; Popp et al., 2019; Popp, 2020). However, their presence and frequency largely varied among orchards (Tewoldemedhin et al., 2011b; Sun et al., 2017), and the contribution of some of the species such as *Pythium*, *Phytophthora,* and *Rhizoctonia* were not confirmed as causal agents to ARD (Wang et al., 2018). These previous studies assumed that the biotic cause of ARD must invade the root, thereby excluding chemical interference with biota in the rhizosphere. This study proposes that specific nematodes and nematode-associated fungi co-occur and interact synergistically in the induction of ARD symptoms. Care must however be taken in interpreting these findings as the functional evidence of these putative species has not yet been validated by Koch's postulates.

In this study, shoot length as a measure of plant growth has been used as a marker of ARD as previously cited as one of the best indicators of ARD severity (Mahnkopp et al., 2018). Plants grown in the untreated ARD soils had a lower shoot length compared to plants grown in the gamma-irradiated soils. This coincided with previous findings that biotic factors play an essential role in ARD (Yim et al., 2017; Winkelmann et al., 2019). Peculiar ARD symptoms were observed in the reduced root systems of plants grown in the untreated ARD soils. Histological analysis on the fine roots of plants grown in the untreated ARD soils showed noticeable indicators of the disease symptoms such as necrosis, blackening, and black cell inclusions (Grunewaldt-Stöcker et al., 2019).

Overall proportions of the nematodes belonging to the orders Rhabditida, Tylenchida, Diplogasterida, and Triplonchida were high in abundance across all the soils and constitute most of the species that contribute significantly to ecological functions and services. The orders Rhabditida, Diplogasterida, and Triplonchida constituting free-living nematodes were in high abundance compared to Tylenchida, which includes many plant-parasitic nematodes (Singh et al., 2015). These findings coincide with our previous studies where we showed that free-living nematodes were significantly more abundant in ARD soils than plant-parasitic nematodes (Kanfra et al., 2018). Besides, other studies observed high populations of free-living nematodes that varied among orchards (Isutsa and Merwin, 2014). The groups' role in the disease complex is subjective as the soil type mediates their distribution in affected orchards (Hoestra and Oostenbrink, 1962; Mai et al., 1994). In our study, plant-parasitic nematodes were low in abundance, and *P. penetrans* was not significantly associated with the varying plant growth. These findings are supported by studies by Manici et al. (2018), who suggested that plant-parasitic nematodes including *P. penetrans* do not cause the disease. In their studies using three affected orchard soils, they could not recover any plant-parasitic nematodes from the roots of the affected or symptomatic apple plants. Although Manici et al. (2013) previously reported the presence of root-lesion nematodes in affected roots, the low frequency of these nematodes did not indicate any significant contribution to the growth reduction in apples.

In our study, the microbial fractions associated with the extracted nematodes from ARD soils were analyzed. At the phylum level, Ascomycota, Basidiomycota, and Mortierellomycota dominated the fungal communities that were commonly associated with nematodes in all five ARD soils. The phyla Glomeromycota and Chytridiomycota were less dominant and varied among the soils. This is consistent with studies that analyzed fungal communities from apple root zones and found the above-mentioned phyla as most dominant in apple growing soils (Sun et al., 2017; Bintarti et al., 2020). The analysis revealed that the plant growth was less associated with the high order taxonomic community composition.

Previously, differences in nematode communities between ARD field plots and uncultivated grass plots were reported (Kanfra et al., 2018). The study related the decline in apple plant growth to a possible shift in the nematode community with an emphasis on the free-living nematode components. In this study, differences in species composition to differences in plant growth were shown regardless of the soil type, hence indicating that the nematode communities associated with plants showing differential ARD symptoms may be indeed unique and could be linked to the growth depression observed in these pots. It also further emphasizes that the heterogeneous distribution of ARD symptoms on the same apple replanted soil may not only be linked to soil properties (Simon et al., 2020) but probably to the patchy distribution of specific nematode-microbe assemblages.

We found that OTU closely related to the nematode genera *Prismatolaimus*, *Acrobeles*, *Acrobeloides*, *Tylencholaimus*, and *Aphelenchus* correlated with disease symptoms. These taxa are all bacteria feeding nematodes. These findings are in accord with previous studies that identified high abundances of bacterivorous nematodes including *Prismatolaimus*, *Acrobeles Acrobeloides, Tylencholaimus,* and *Aphelenchus* in apple or peach replanted soils (Pokharel et al., 2015).

The specificity of the microbiome associated with nematodes in soil has been shown for plant-parasitic nematodes (Elhady et al., 2017; Topalović et al., 2020). There have been few studies of host-associated microbes in free-living nematodes (Derycke et al., 2016; Dirksen et al., 2016; Schuelke et al., 2018). For the first time, the microbial fractions associated with the total nematode community have been characterized in this study. OTU closely related to fungi species of the genera *Psathyrella, Hohenbuehelia, Naganishia, Hyalorbilia* and *Exophiala* were preferably associated with the nematodes in soils and correlated negatively with plant growth which was evidenced by high disease symptoms of these plants. Notably, the genera *Exophiala* have been frequently associated with ARD soils from different orchards (Bintarti et al., 2020) and correlated negatively with plant growth in apple replant orchards (Franke-Whittle et al., 2015), which coincides with our findings. The genus *Psathyrella* was recently shown to play keystone taxa in microbial network analysis in untreated ARD soils (Zheng et al., 2018). *Hohenbuehelia* has a mycelium that adheres to and captures nematodes and has been shown to control deleterious nematodes in agricultural systems (Singh et al., 2013; Lubian et al., 2021). Members of the nematode-trapping fungi (Orbiliales) which includes the genus *Hyalorbilia* are well documented for their legacy trapping or parasitizing on important nematode pests such as the cyst and root rot nematodes (Witte et al., 2021). Members of the genus *Naganishia* can adapt and grow in freeze-thaw conditions (Schmidt et al., 2017).

In principle, many different biological agents may interact to cause ARD (Tewoldemedhin et al., 2011b). We constructed an association network among the nematode and the nematode-associated microbes to unravel the complex relationships among species and how this influences plant growth. In the network, both intra and inter-kingdom associations were observed. Clusters of fungal OTU displayed much more intra kingdom connections compared to the bacteria and nematode. Bacteria keystone genera, *Serratia* was reported to cooperate with nematodes to kill insects (Zhang et al., 2009; Abebe et al., 2010) as well

as antagonist activities against fungi (Berg, 2000) or nematodes (Schulenburg and Ewbank, 2004). *Pseudomonas*, *Bacillus,* and other Firmicutes are putative plant beneficial and typical rhizosphere inhabitants in fumigated apple replanted soils (Mendes et al., 2013; Nicola et al., 2017; Yim et al., 2017). The network suggested a few key associations between the nematodes and the nematode-associated bacteria, and indirectly with fungi. The findings in this study support the assumption that the ARD complex involves multiple biological players that are expected to interact either directly or indirectly with each other to affect apple plant health (Nicola et al., 2018; Winkelmann et al., 2019). Bintarti et al. (2020) recently investigated the relationship between apple root zone bacterial, archaeal, nematode, and fungal communities across 10 years old apple orchards. They could show interactions involving bacteria, fungi, and archaea but the sampled soil volume was not sufficient to analyze nematodes. They did not characterize the microbial community associated with nematodes, which is distinct from the abundant soil microbiome (Adam et al., 2014; Elhady et al., 2017). In our network, the nematodes belonging to the genera *Prismatolaimus* significantly connected the bacterial genus *Flavobacterium*. The nematode *Acrobeles* had a connection to an unidentified member of Firmicutes.

In conclusion, plants exhibiting differential ARD symptoms harbored distinct free-living nematodes and nematode–associated fungi that might synergistically interact to affect apple plant health. These nematodes together with their associated fungi should be analyzed in more reductionistic systems with in-vitro apple plantlets to test their potential to induce stress in the roots. Exploring the mechanism underlying the synergy between the nematodes and microbes will help to mitigate the disease.

Declaration of competing interest

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

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Appendix A. Supplementary data

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