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Analysis of Microbial and Nematode Communities in the Apple Replant Disease Complex

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Abstract: Apple replant disease (ARD) is a complex issue caused by various fungal, oomycetes pathogens and parasites which can differ in their abundance between sites within a geographic region. Knowledge of the specific etiology of ARD at a particular site is required in order to develop commercially viable soil management strategies to combat specific/individual components of the disease. In this study we analyzed the soil from six ARD sites for the presence of fungal, bacterial and oomycetes communities and plant parasitic nematodes. Five fungal, and 17 bacterial classes were differentially represented in the microbiomes from different locations. Mortierellomycetes was the most abundant fungal taxa represented followed by Sordariomycetes. *Mortierella exigua* a fungal endophyte was the most abundant fungal amplicon sequence variant (ASV) in the core microbiome. Proteobacteria was the most prevalent phylum identified in orchard soils. Several potential phytopathogenic fungi involved in ARD and endophytes including *Fusarium oxysporum, F. solani, Nectria ramulariae, Ilyonectria robusta* and *Nectriaceae* were identfied in the orchard soil. *Pythium attrantheridium (Globisporangium attrantheridium), P. monospermum* and *P. ultimum* (*Globisporangium ultimum*) were the most abundant oomycete taxa identified in these soil samples. Six different groups of plant-parasitic nematodes were found across the six orchards soil. Root-lesion nematodes, *Pratylenchus* spp., which are commonly associated with ARD, were identified in all orchards soil at population densities ranging from 12 to 33/100 cm^3 soil. This research contributes valuable understanding of the ARD complex as the apple industry needs alternative approaches to combat the disease.

Keywords: apple replant disease (ARD); microbiome; fungal and oomycetes pathogens; nematodes; *Malus domestica*

1. Introduction

Apple replant disease (ARD) occurs as a result of the activity of pathogenic microorganisms and nematodes that have accumulated in orchard soils and subsequently infect newly established trees planted into these soils during orchard renovation/renewal. ARD is common in almost all apple and pome fruit growing regions of the world. Young apple trees affected by ARD have symptoms such as uneven growth, stunting, reduced yield, root necrosis and reduced root biomass compared to trees free of the disease (Mai and GS 1981). A number of studies have demonstrated that apple replant disease (ARD) is a complex of various fungal and oomycete pathogens and parasites which can differ in their abundance between sites within a geographic region (Winkelmann et al. 2019). In the past the use of broad spectrum, pre-plant agro-chemical soil fumigants have effectively controlled the disease

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in the Nova Scotia apple industry (Braun et al. 2010). With the recent loss of 1,3-dichloropropene and chloropicrin however, ARD has re-emerged as a major threat to the successful re-establishment of apple orchards. Knowledge of the specific etiology of ARD at a particular site is required in order to develop alternative, commercially viable soil management strategies to combat specific/individual components of the pathogen complex. These may include green and sustainable technologies with less chemical inputs such as biofumigation, anaerobic soil disinfestation (ASD), incorporation of organic amendments, semi-selective agrochemicals and immunization with useful endophytes.

Now widespread evidence suggests that the ARD complex has a number of components, some of which may be present or absent at any given old orchard site (Manici et al. 2003; Van Schoor et al. 2009). Braun (1991); Braun (1995) identified *Pythium irregulare* and *Cylindrocarpon lucidum* in combination, as causal pathogens of ARD in five old orchard soils of the Annapolis Valley apple growing region of Nova Scotia, Canada. In Europe, *Cylindrocarpon* spp. were reported as major fungal pathogens contributing to ARD in Germany, Austria and Italy, while *Pythium* spp. were found as major ARD causal pathogens in Germany (Manici et al. 2013). The composition of apple root colonizing fungi differed amongst orchards in South Tyrol, but not between row and inter-row locations within an orchard (Manici et al. 2003). *Fusarium solani* and *F. oxysporum* were the most represented species, that accounted for more than 50% of root colonizing fungi, although the pathogenicity of these fungi was not confirmed (Kelderer et al. 2012). The fungal pathogens *Cylindrocarpon* sp., and binucleate *Rhizoctonia* sp., were omnipresent in this study. Van Schoor et al. (2009) isolated *Cylindrocarpon, Pythium* and also *Fusarium* spp. from lesions in apple roots grown in six South African orchard soils, but again the pathogenicity of these isolates were not confirmed. These authors also assigned secondary roles to *Rhizoctonia* and plant parasitic nematodes *Pratylenchus* and *Xiphinema* spp. as causal agents of ARD.

More recently, the primary etiologic agents of the ARD c was described as a complex of fungal (*Cylindrocarpon, Rhizoctonia*) and oomycete (*Pythium, Phytophthora*) species which can vary in relative frequency or dominance from site to site (Kelderer et al. 2012; Tewoldemedhin et al. 2011c). Furthermore, disease severity due to this complex appears to be associated with the plant parasitic nematode *Pratylenchus penetrans* (Mazzola 1998). The involvement of bacterial pathogens in ARD is debatable and is not well investigated when compared with fungi and oomycetes. However, bacteria belonging to the *Actinomycetes*, *Bacillus* and *Pseudomonas* might also be a part of the disease-complex (Winkelmann et al. 2019). Composition of the *Pseudomonas* spp. community was shown to vary significantly between soils that were suppressive or conducive to ARD (Gu and Mazzola 2003). Also, some species of *Streptomyces* have been reported to reduce the incidence of *Rhizoctonia* root rot infections in apple (Zhao et al. 2009).

In summary, ARD has been reported by many as a complex of different types of microbial pathogens and plant parasitic nematodes which can differ in their relative abundance between sites within a geographic region (Kelderer et al. 2012; Mazzola 1998; Mazzola and Manici 2012; Tewoldemedhin et al. 2011c; Tewoldemedhin et al. 2011b). Accordingly, the aim of this research was to compare (i) the abundance and composition of soil fungal, bacterial and oomycete communities in six old orchard sites in the Annapolis Valley apple growing region of Nova Scotia, (ii) affirm the conventional work of Braun (1995) in identifying *Cylindrocarpon* and *Pythium* spp. as causal organisms of the ARD complex and identify other potential fungal and/or oomycete pathogens, (iii) determine the possible presence of a dominant player in the ARD complex and (iv) cast further light on the role of plant parasitic nematodes as contributors to the replant complex in ARD orchard soils.

2. Materials and Methods

2.1. Site Selection and Collection of Soils

Six orchard sites were chosen in the fall of 2020, representing the main apple growing regions within the Annapolis Valley of Nova Scotia: 1) ROE (Rockland East area, Sombric Ferro-Humic Podzol); 2) ROW (Rockland West area, Sombric Ferro-Humic Podzol); 3) CAN (Canard area - Sombric

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Ferro-Humic Podzol); 4) AYL (Aylesford area - Gleyed Sombric Brunisol); 5) KEN (Kentville area - Sombric Ferro-Humic Podzol); 6) BER (Berwick area - Sombric Ferro-Humic Podzol). Soil classifications were based on Agriculture Canada Expert Committee on Soil Survey (1987). At each site, soil was collected from the 0 – 30 cm depth by sub-sampling 10 locations under the tree canopy on the herbicide strip along each of 4 adjacent tree rows. The resulting sub-samples (~ 2 L each) were combined, thoroughly blended, and passed through an 8 mm screen on site to eliminate coarse organic material, stones and other debris from the sample. A ~1.5 kg composite soil sample was drawn from each bulk sample for the following analyses: i) a 300 mL sub-sample was stored at 4°C and shipped to the Agriculture and Agri-Food Canada Summerland Research and Development Centre for nematode analysis; ii) a 250 g sub-sample was stored at -80°C for DNA extraction; iii) the remaining sample was dried, ground and sieved through a 2 mm screen, and analyzed for soil physio-chemical properties using standard methods. Information on soil fertility, orchard characteristics, and land and physical soil characteristics are provided in Tables 1 and 2. All tools and equipment were sterilized with 75 % ethanol prior to sample collection at each site. The remaining bulk orchard samples were stored moist in totes lined with plastic at 5°C and used for ARD bio-assays in greenhouse.

Table 1. Soil fertility parameters of experimental orchards in the Annapolis Valley.

Orchard*	OM	pH	P205	K20	Ca	Mg	Al	CEC	Ca	Mg	K	H
	%	H_20	---------- kg ha^{-1} ----------					**	%	%	%	%
ROW	3.7	5.9	1097	275	2913	171	1571	10.2	71.7	7.0	2.8	18.7
BER	3.2	6.4	856	523	2863	455	1516	11.8	60.6	16.1	4.7	18.1
AYL	2.6	6.2	497	194	1715	382	1585	8.1	52.5	19.5	2.4	25.1
KEN	4.1	6.6	848	425	3885	512	1442	14.8	65.5	14.3	3.0	16.9
CAN	3.2	5.4	970	665	2239	520	1555	11.9	46.9	18.1	6.1	28.6
ROE	3.7	5.9	1097	275	2913	171	1571	10.2	71.1	7.0	2.8	18.7

*ROW (Rockland West), BER (Berwick), AYL (Aylesford), KEN (Kentville), CAN (Canard), ROE (Rockland East). ** meq 100 g^{-1}.

Table 2. Soil physical properties and characteristics of experimental apple orchards in the Annapolis Valley.

Orchard	Soil Texture	Drainage Class*	Soil EAM** (mm/30 cm)	Year Planted	Rootstock/ Variety***	Density Trees ha^{-1}	Row width (m)	ARD Severity (% R)****
ROW	Sandy	4	34.6	1989	RD/M26	683	6.1	235.0
BER	C. Loamy	3	23.2	1986	RC/MM111	236	6.5	267.4
AYL	C. Loamy	2	37.4	1964	Mac/M111	494	5.7	117.9
KEN	C. Loamy	4	18.5	1987	Mac/M26	694	4.8	166.8
CAN	C. Loamy	5	27.3	2008	GD/M9	2574	4.3	66.3
ROE	Sandy	4	12.6	1972	Mac/M111	249	7.3	168.5

* Drainage class: 5 = rapidly drained, 4 = well drained, 3 = moderately well drained, 2 = imperfectly drained; ** EAM = easily available moisture; ***RD: Red Delicious; RC: Royal Cort; Mac: MacIntosh; GD: Golden Delicious; M26, M111, M9: apple rootstocks of the Malling series; *** % R = percentage response, measured in a standard greenhouse bioassay.

2.2. Apple Seedling Bioassay in Greenhouse

Apple seedlings were used in bio-assays to confirm the presence of ARD and establish the relative degree of severity between the experimental orchard soils. Apple seeds (Malus domestica Borkh. Var. Golden Delicious) were germinated in seedling trays using a pasteurized, soilless growing medium (Promix®), and grown for 3 weeks until the plants were ~ 8 cm high with a good root-ball. Seedlings were subsequently selected for uniformity and transplanted to 6" nursery pots (one per pot) containing pasteurized (p) or non-pasteurized (np) orchard soil, and grown in the

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greenhouse for 9 weeks (April 8 – June 11, 2020). Pasteurization of the six orchard soils was accomplished by exposing the moist soil to a temperature of 70 °C for 2 hours across 2 cycles, each 24 h apart. Greenhouse settings for the plant growth period were 22 °C and 18 °C for day and night temperatures, respectively. Daylength during the spring growth period varied from 13 h on April 9, to 15.5 h on June 11. The trial was arranged in a randomized complete block design with 6 orchard soils, 2 levels of soil pasteurization (p, np) and 4 replications. All pots were watered frequently with de-chlorinated tap water and intermittently with complete, soluble nutrient solution at label rate. Aerial dry biomass accumulation after 8 weeks was used as the response variate to calculate seedling growth response to pasteurization of soil as follows (Gongshuai et al. 2018):

$$\% R = 100 * (x_p - x_{np}) / x_{np},$$

where x_p and x_{np} are the aerial dry biomass accumulations for the p and np soil treatments respectively. We ranked the severity of ARD in the experimental orchards as Severe (% R > 100%), Moderate (% R = 50 to 100%), and Low (% R < 50%).

2.3. DNA Extraction and Sequencing

DNA extraction from five replicates per orchard from 250 mg of well homogenized soils was carried out using DNeasy *PowerSoil* Kit (Qiagen) according to the manufacturer's protocol with slight modifications to increase the yield and obtain high-quality DNA (Wright et al. 2022). DNA quality and concentration were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA). At least 50 ng (10µL) of DNA from each sample were sent to the Dalhousie University CGEB-IMR (http://cgeb-imr.ca/) for sequencing and library preparation. The bacterial V6-V8, 16S rRNA gene was amplified using the primer set B969F (ACGCGHNRAACCTTACC) and BA1406R:(ACGGGCRGTGWGTRCAA) (Comeau et al. 2011) and the fungal internal transcribed spacer ITS2 region was amplified using the primer pair ITS86(F): (GTGAATCATCGAATCTTTGAA): ITS4(R) (TCCTCCGCTTATTGATATGC) (Op De Beeck et al. 2014). Samples were multiplexed using a dual-indexing approach and sequenced using an Illumina MiSeq with paired-end 300+300 bp reads. All PCR procedures and Illumina sequencing details were as previously described (Comeau et al. 2016). For oomycete sequencing a sub sample of DNA was sent to Molecular Technologies Laboratory at AAFC Ottawa Research and Development Centre. For oomycetes, the ITS region (oomITS) was sequenced using primer Oom_SSU-ITS: CGGAAGGATCATTACCACAC and Oom_lo5.8S47C: ATTACGTATCGCAGTTCGCA (Man in't Veld et al. 2002). Library preparation and sequencing was conducted as above. All sequences generated in this study are available in the NCBI sequence read archive under the accession numbers PRJNA968027, PRJNA968028 and PRJNA968029).

2.4. Sequencing Data Processing

Sequences were processed using QIIME2 and the Deblur plugin. We used the Microbiome Helper standard operating procedure to process and analyze the sequencing data (Comeau et al. 2017). Overlapping paired-end reads were stitched together using PEAR (Zhang et al. 2014). Paired sequences were then imported into a QIIME2 artifact (Bolyen et al. 2018) and filtered based on read quality and length using QIIME2's built in quality-filter q-score-joined script. Filtered reads were then processed with Deblur (Amir et al. 2017) using a trim length of 293 and 401 base pairs to obtain IT2 and 16S rRNA Amplicon Sequence Variants (ASVs). ASVs that contained fewer than 0.1% of the total sequences were filtered out in order to compensate for MiSeq run-to-run bleed-through (Comeau et al. 2016). 16S rRNA and ITS ASVs were classified taxonomically using a Naïve-Bayes RDP classifier and accessing the SILVA rRNA (Quast et al. 2012) and UNITE ITS database v7.2 (Nilsson et al. 2019), respectively. Reads annotated as mitochondria and chloroplast were filtered. For oomycetes reads processing, a standalone Nucleotide-Nucleotide BLAST+ (version 2.9.0+) search (mega blast) was performed to obtain the best high-scoring segment pair presenting at least 99% similarity and minimum 100-bp alignment length with the query sequence. Manually curated ITS sequences from Phytophthora-ID (http://phytophthora-id.org) (Grünwald et al. 2011; Robideau et al. 2011) were used as nucleotide reference database for BLAST. The annotation of all oomycetes ASVs was

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manually verified and updated using Nucleotide-Nucleotide BLAST against the NCBI nucleotide collection.

In brief, 166,769, 164,626 and 58,725 high-quality non-chimeric reads were obtained from 30 samples of 16S rRNA, fungal ITS2 and oomycetes ITS (oomITS) respectively. These sequences were clustered into 5,174 (16S rRNA), 1,026 (ITS2) and 282 (oomITS) ASVs. The datasets were normalized to the depth of 1,215, 1,628 and 1,192 reads resulting in the normalized datasets comprising 4,547 (16S rRNA), 1,006 (ITS2) and 258 (oomITS) ASVs, respectively.

2.5. Data Analysis

Alpha-diversity (Chao1 richness, Simpson evenness and Shannon diversity) and beta-diversity metrics were generated using QIIME2. Variations in sample groupings explained by weighted unifrac beta-diversity distances (Adonis tests, 999 permutations) were run in QIIME2 to calculate how sample groupings are related to microbial community structure. Non-metric Multi-dimensional Scaling (NMDS) plots were built based on Bray-Curtis distances using the Vegan package in (Oksanen et al. 2013) Visualization was done using the gglpot2 package in R (Gómez-Rubio 2017). Bacterial, Fungal and Oomycetal ASVs and taxa, differentially represented between orchards, were identified using analysis of composition of microbiomes (ANCOM) R package on non-rarefied ASV tables with 1% FDR (Gómez-Rubio 2017). ASVs, that were found in ≥80% of all samples, were assigned as core ASVs. Taxa represented in ≥80% of samples across each orchard were assigned as core families. Cytoscape 3.4.0 software was used to visualize the bacterial and fungal distribution and network of the core families (Shannon et al. 2003). The visualisation was represented as graphs with microbial groups and orchards as vertices/nodes and the edges as interaction representing the presence of the family in ≥80% of the samples in the orchard.

2.6. Nematode Counting

A wet sieving-sucrose centrifugation procedure was used to extract nematodes from triplicate 100 cm^3 subsamples from each composite sample (Carter and Gregorich 2007). Plant-parasitic nematodes in each extract were identified to genus and counted using an inverted microscope with a gridded counting dish.

3. Results

3.1. Orchard Site Characteristics

The physio-chemical properties of six ARD orchards analyzed in this study were different because of regional location and cultural practices at different orchards (Table 1 and Table 2). In the Annapolis Valley of Nova Scotia, apples are produced under rain-fed (non-irrigated) conditions in a maritime climate with long cold winters, short summers and generally wet autumns and springs. Droughty conditions can exist for short periods in the summer. In addition, soils are generally coarse, ranging from sandy to sandy loam in texture, with clay contents rarely exceeding 18 % clay (Holmstrom and Thompson 1989), and where they do, they are generally found in poorly drained depressions that collect run-off and seepage and are not suited to apple production. The result is that apples are produced on soils with a relatively narrow range of soil textures that are generally sandy to coarse loamy (Table 2). In these soils, water retention and internal drainage (Table 2), can often be better correlated with silt plus clay content rather than clay alone. This is in contrast to some orchard soils that occur in other apple growing regions of the world. For example, Mazzola et al. (1998) conducted ARD research on 5 Washington state orchard soils classified as “sandy loam” or “silt loam” which would likely have higher % (clay + silt) contents.

ARD severity was calculated as the ratio of aerial dry biomass produced in pasteurized and unpasteurized soil of the same orchard and the soils were classified into severe, moderate and low severity (Table 2). Five of the six soils were ranked as severe with only one orchard soil (CAN), as moderately severe. The latter site has also been in orchard for the fewest years (Table 2), while the

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site with the highest severity (BER) has the longest history of apple production, having had a previous orchard prior to its planting in 1986.

3.2. Fungal and Bacterial Microbiome Composition

ITS2 and 16S rRNA (V6-V8 region) amplicon sequencing was performed to evaluate fungal and bacterial communities in the soils, respectively. Taxonomic analysis revealed that Ascomycota had the highest relative abundance among fungal phyla in the orchard soils comprising 48% of all ITS2 reads identified in the study, followed by Mortierellomycota (37%) and Basidiomycota (10%). Mortierellomycetes, Sordariomycetes, Leotiomycetes, Tremellomycetes, Dothideomycetes, Eurotiomycetes, and Agaricomycetes were the most abundant fungal orders found in the ITS2 derived soil microbiome and were represented by 37%, 29%, 11%, 6%, 6%, 4%, and 3% of total ITS2 rRNA reads, respectively (Figure 1 right panel). Proteobacteria was the most prevalent phylum identified in orchard soils (38% of total 16S rRNA reads) with two classes Alphaproteobacteria (19%) and Gammaproteobacteria (15%) having the highest relative abundance among bacterial taxa (Figure 1 left panel). Bacteroidetes (Bacteroidia) was the third most relatively abundant class represented by 14% of total 16S rRNA reads. Actinobacteria and Acidobacteria were also highly represented at 16% of total 16S rRNA reads, each. Actinobacteria classes exhibiting the highest relative abundance included Thermoleophilia (8% of total 16S rRNA reads) and Actinobacteria (6%), while Subgroup6 (6%) and Acidobacteriia (5%) were present at the greatest relative abundance among Acidobacteria classes.

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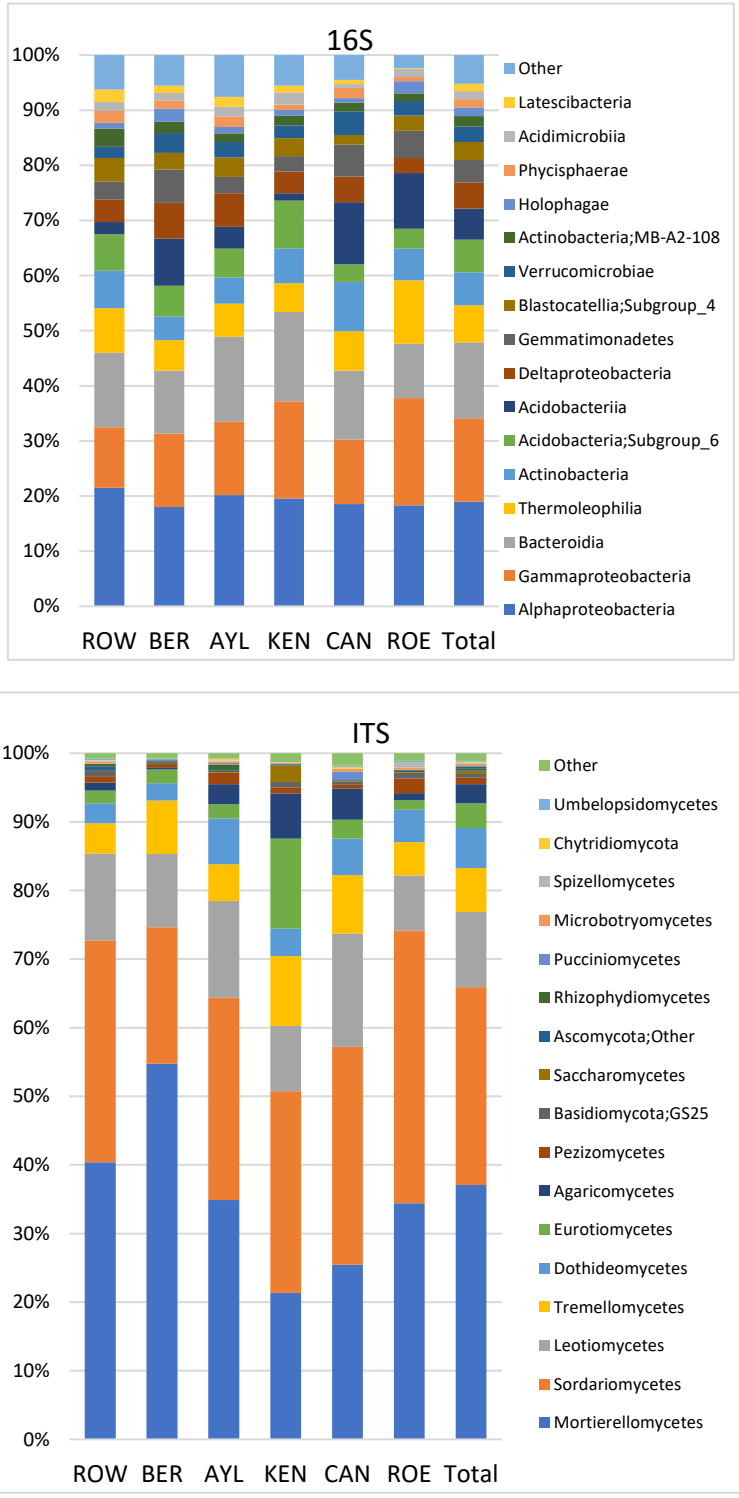

Figure 1. Bacterial and fungal community structure across Nova Scotia apple orchards. Bacterial (left panel) and fungal (right panel) annotated at class level and represented by at least 1% of total 16S RNA and 0.1% of total ITS2 reads are shown. Orchard locations in the Annapolis Valley of Nova Scotia in Canada: ROE (Rockland East area), ROW (Rockland West area), CAN (Canard area), AYL (Aylesford area), KEN (Kentville area) and BER (Berwick area).

3.3. Variation of Bacterial and Fungal Microbiome Structure across Apple Replant Orchard Soils

The analysis of strength and statistical significance of sample groupings (Adonis test based on weighted unifrac beta-diversity distances with 999 permutations) indicated a significant variation in community structure across different ARD orchard soils (p value < 0.001). Approximately 54% of

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bacterial and 55% of fungal community variation was explained by the site of sampling (orchard location). However, we did not detect a significant difference in fungal or bacterial alpha-diversity among sites (data not shown).

Three bacterial, and 3 fungal classes differed significantly in relative abundance between sic ARD orchards soil (Figure S1). Actinobacteria were over-represented in BER and CAN orchard, while unidentified Latescibacteria were least relatively abundant taxa in ROE soil, compared to other locations and Bacilli were over represented in CAN compared to all other five sites. Eurotiomycetes were over-represented in KEN, while Spizellomycetes were over-represented in ROE, compared to other locations. At genera level we identified 8 bacterial and 12 fungal taxa differentially represented between orchards. Fungal taxa Sordariomycetes *Lasiosphaeris*, 2 unidentified Eurotiomycetes *Chaetothyriales*, Saccharomycetes *Cyberlindnera*, and Dothideomycetes *Paraconiothyrium* were overrepresented in KEN soil (Figure 2), while Pezizomycetes *Peziza* and Dothideomycetes *Matsushimamyces* were more abundant in AYL. Gammaproteobacteria *Arenimonas* and *Chujaibacter*, Acidobacteriia Subgroup 2 and Firmicutes *Bacillus* were most abundant in CAN soil (Figure 3).

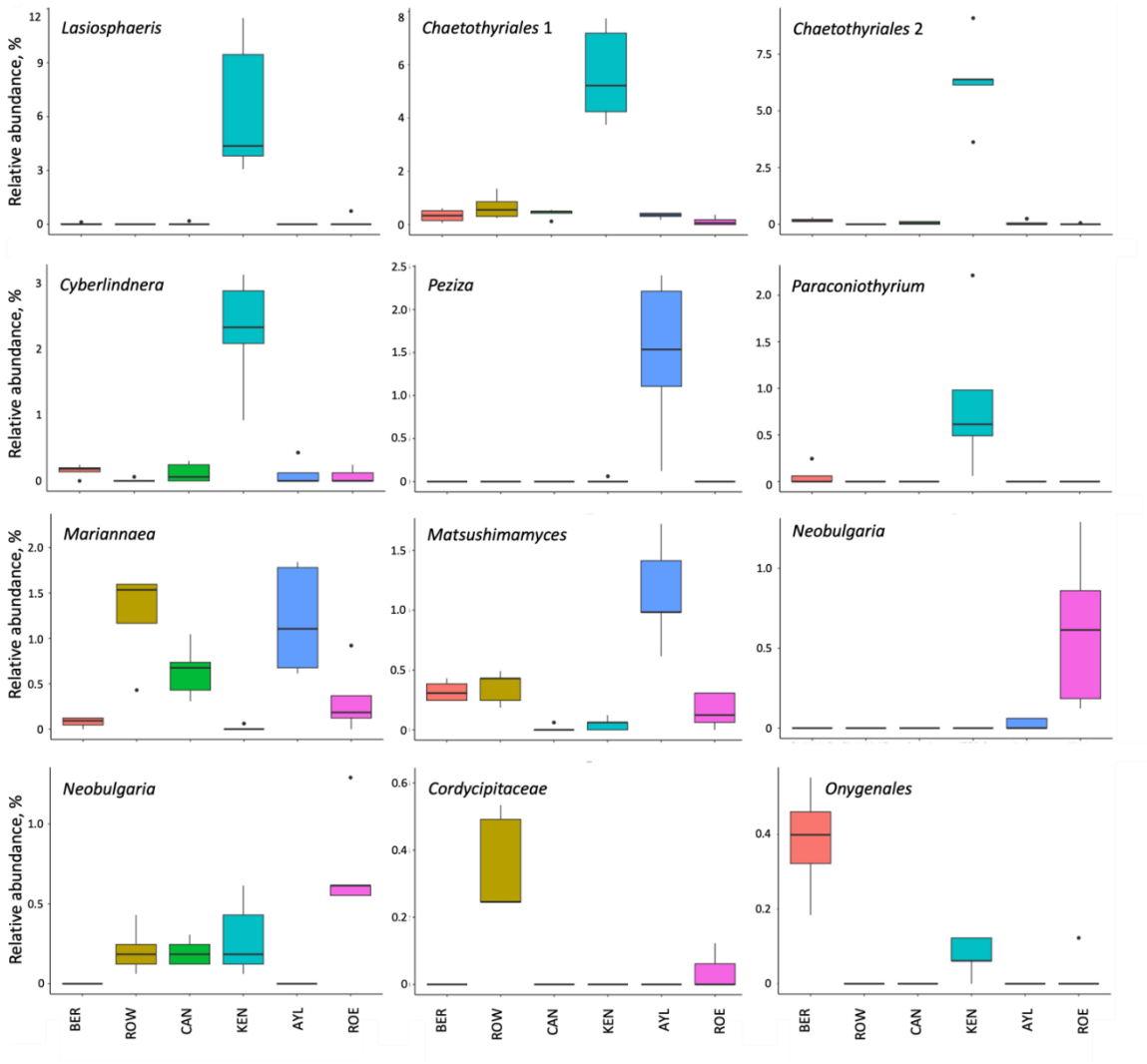

Figure 2. Differentially abundant fungal taxa across Nova Scotia orchards. Based on ANCOM test with 1% FDR. Orchard locations in the Annapolis Valley of Nova Scotia in Canada: ROE (Rockland East area), ROW (Rockland West area), CAN (Canard area), AYL (Aylesford area), KEN (Kentville area) and BER (Berwick area).

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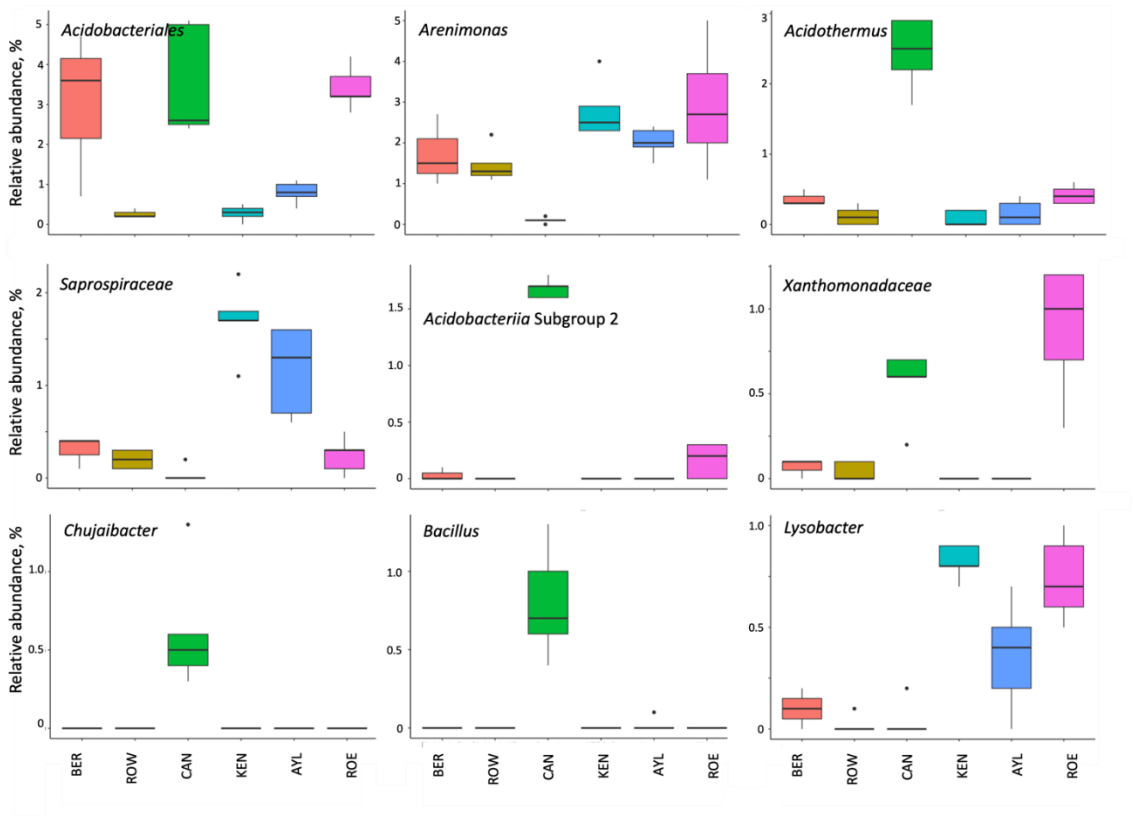

Figure 3. Differentially abundant bacterial taxa across Nova Scotia orchards. Based on ANCOM test with 1% FDR. Orchard locations in the Annapolis Valley of Nova Scotia in Canada: ROE (Rockland East area), ROW (Rockland West area), CAN (Canard area), AYL (Aylesford area), KEN (Kentville area) and BER (Berwick area).

3.4. Core Fungal and Bacterial ASVs

We extended our analysis to identify microbial ASVs that were stable across soil communities from different orchards and detected in at least 80% of all the samples. We identified 15 bacterial and 29 fungal ASVs, represented by 7% and 50% of total 16S rRNA and ITS2 reads, respectively (Table S1 and Table S2). Bacterial ASV's within this group were affiliated with Acidobacteria (4 ASVs), Bacteroidetes (4 ASVs) and Proteobacteria (5 ASVs). *Xanthobacteraceae* family and *Pseudolabrys* were the most abundant ASVs represented by 14% of total 16S rRNA reads. *Mortierella exigua* was the most abundant fungal ASV in the core microbiome, 9% of total ITS2 reads. A number of potential phytopathogenic ASVs were a part of core microbiome including *Fusarium oxysporum* (4%), *Fusarium solani* (1%), *Nectria ramulariae* (synonym *Cylindrocarpon ehrenbergii*) (0.5%) and *Ilyonectria robusta* (*Cylindrocarpon*-like asexual morphs) (1%), as well as *Nectriaceae* (0.4%) ASV annotated at family level. All together these ASVs were represented by 7% of total ITS2 reads.

Core microbial families. We also identified microbial families which were shared between orchard soils. Forty four bacterial and 15 fungal families were found in at least 80% of samples from each orchard (Table 3 and Table 4, Figure 4). These families were represented by 73% and 74% of total 16S rRNA and ITS2 reads, respectively. Core bacterial families included several taxa implicated in plant health and soil fertility such as Chitinophagaceae, Xanthobacteraceae, Nitrosomonadaceae, Sphingomonadaceae, Caulobacteraceae, Xanthomonadaceae, Pseudonocardiaceae, and Micromonosporaceae. The families Chitinophagaceae, Xanthobacteraceae, Nitrosomonadaceae, Xanthomonadaceae, and Sphingomonadaceae had high relative abundances in the total bacterial microbiome and were represented by 10%, 8%, 5%, 4%, and 3% of total 16S rRNA reads, respectively. Similarly core fungal families detected at high relative abundance included Mortierellaceae, Nectriaceae, Helotiaceae, Chaetomiaceae and Piskurozymaceae and were represented by 36%, 12%, 5%, 5%, and 4% of total ITS2 reads, respectively (Table 4).

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Table 3. Core bacterial families found in at least 80% of all samples in each orchard.

Family	Mean rel. freq.
Bacteroidetes; Bacteroidia; Chitinophagales; Chitinophagaceae	10%
Proteobacteria; Alphaproteobacteria; Rhizobiales; Xanthobacteraceae	8%
Acidobacteria; Subgroup_6; Other;	5%
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Nitrosomonadaceae	5%
Gemmatimonadetes; Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae	4%
Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	4%
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae	3%
Actinobacteria; Thermoleophilia; Gaiellales; uncultured	3%
Acidobacteria; Acidobacteriia; Acidobacteriales; uncultured	2%
Proteobacteria; Gammaproteobacteria; Xanthomonadales; Rhodanobacteraceae	2%
Actinobacteria; Thermoleophilia; Gaiellales; Gaiellaceae	2%
Acidobacteria; Blastocatellia (Subgroup 4); Pyrinomonadales; Pyrinomonadaceae	2%
Verrucomicrobia; Verrucomicrobiae; Chthoniobacterales; Chthoniobacteraceae	2%
Acidobacteria; Acidobacteriia; Solibacterales; Solibacteraceae (Subgroup 3)	1%
Planctomycetes;_Phycisphaerae;_Tepidisphaerales;_WD2101_soil_group	1%
Actinobacteria; MB-A2-108; Other; Other	1%
Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae	1%
Proteobacteria; Gammaproteobacteria; Gammaproteobacteria Incertae Sedis	1%
Actinobacteria; Actinobacteria; Propionibacteriales; Nocardioidaceae	1%
Actinobacteria; Thermoleophilia; Solirubrobacterales; 67-14	1%
Acidobacteria; Blastocatellia (Subgroup 4); Blastocatellales; Blastocatellaceae	1%
Bacteroidetes; Bacteroidia; Cytophagales; Microscillaceae	1%
Actinobacteria; Actinobacteria; Corynebacteriales; Mycobacteriaceae	1%
Actinobacteria; Actinobacteria; Pseudonocardiales; Pseudonocardiaceae	1%
Proteobacteria; Deltaproteobacteria; Myxococcales; Haliangiaceae	1%
Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae	1%
Proteobacteria; Deltaproteobacteria; Desulfarculales; Desulfarculaceae	1%
Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiales Incertae Sedis	1%
Verrucomicrobia; Verrucomicrobiae; Pedosphaerales; Pedosphaeraceae	1%
Actinobacteria; Actinobacteria; Micromonosporales; Micromonosporaceae	1%
Acidobacteria; Holophagae; Subgroup 7;Other	1%
Other; Other; Other; Other	1%
Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae	1%
Actinobacteria; Thermoleophilia; Gaiellales; Other	1%
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; TRA3-20	1%
Actinobacteria; Thermoleophilia; Solirubrobacterales; Solirubrobacteraceae	1%
Actinobacteria; Acidimicrobiia; IMCC26256; Other	1%
Proteobacteria; Alphaproteobacteria; uncultured; Other	<1%
Proteobacteria; Alphaproteobacteria; Rhizobiales; Beijerinckiaceae	<1%
Bacteroidetes;_Bacteroidia;_Chitinophagales;_uncultured	<1%
Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae	<1%
Actinobacteria;_Actinobacteria; Frankiales; Nakamurellaceae	<1%
Rokubacteria; NC10; Rokubacteriales; Other	<1%
Proteobacteria; Alphaproteobacteria; Elsterales; URHD0088	<1%

Table 4. Core fungal families found in at lease 80% of all samples in each study orchard.

Family	Mean rel. freq.
Mortierellomycota; Mortierellomycetes; Mortierellales; Mortierellaceae	36%
Ascomycota; Sordariomycetes; Hypocreales; Nectriaceae	12%
Ascomycota; Leotiomycetes; Helotiales; Helotiaceae	5%
Ascomycota; Sordariomycetes; Sordariales; Chaetomiaceae	5%
Ascomycota; Leotiomycetes; Helotiales; Helotiales fam Incertae sedis	4%
Basidiomycota; Tremellomycetes; Filobasidiales; Piskurozymaceae	4%

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Ascomycota; Sordariomycetes; Hypocreales; Hypocreaceae	2%
Ascomycota; Sordariomycetes; Glomerellales; Plectosphaerellaceae	2%
Basidiomycota; Tremellomycetes; Cystofilobasidiales; Mrakiaceae	1%
Ascomycota; Sordariomycetes; Hypocreales; Clavicipitaceae	1%
Basidiomycota; Tremellomycetes; Tremellales; Trimorphomycetaceae	1%
Ascomycota; Eurotiomycetes; Chaetothyriales; Herpotrichiellaceae	1%
Ascomycota; Sordariomycetes; Hypocreales; Bionectriaceae	1%
Unassigned; Other; Other; Other; Other	<1%
Ascomycota; Sordariomycetes; Hypocreales; Hypocreales fam Incertae sedis	<1%

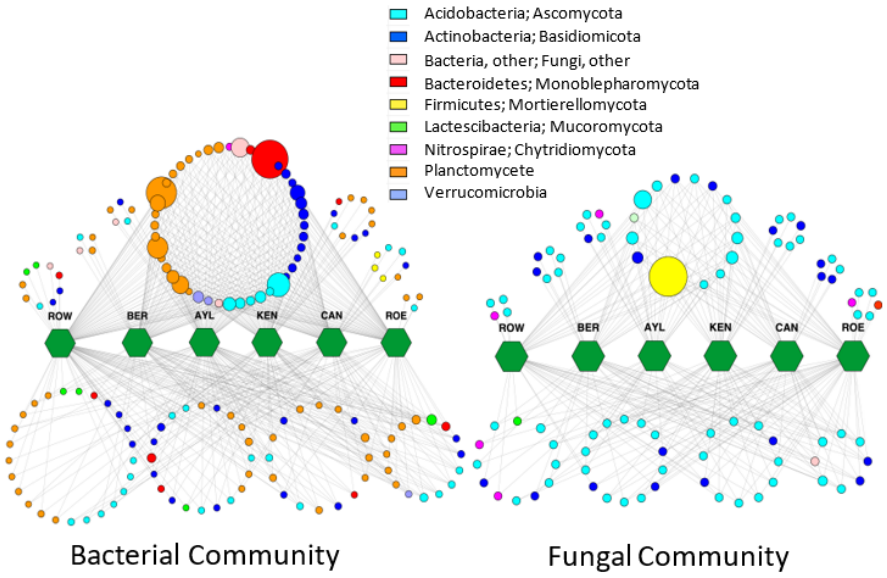

Figure 4. Distribution of bacterial and fungal taxa across orchards. Each node represents a sampling site (colored in green) or a family of the core microbiome (colored according to its phylum). The size of the nodes representing microbial families is proportional to each family's relative abundance across all samples. The lines (i.e., edges) connecting microbial and sampling site node represent the presence of the family in at least 80% of the samples in the orchard. The number of points in the microbial node clusters indicate the numbers of orchards where the families were detected. The node's clusters without numbers are unique families found only in one orchard. Orchard locations in the Annapolis Valley of Nova Scotia in Canada: ROE (Rockland East area), ROW (Rockland West area), CAN (Canard area), AYL (Aylesford area), KEN (Kentville area) and BER (Berwick area).

3.5. Composition of Oomycetal Microbiome

Pythium attrantheridium (Globisporangium attrantheridium), *P. monospermum* and *P. ultimum* (*Globisporangium ultimum*) were the most abundant oomycetal taxa identified in the analysis. They were represented by 50%, 12% and 12% of total oomITS reads, respectively (Figure 5). While the communities did not differ in Shannon diversity between the orchards (data not shown), location was a strong factor affecting oomycetal community structure. More than 60% of the community variation was explained by the place of sampling (Figure 5). This variation in community structure was reflected in the visual differences in the community profiles across the orchards (Figure 5). *P. attrantheridium (G. attrantheridium)*, *P. monospermum* and *P. ultimum* (*G. ultimum*) exhibited the most pronounced apparent variation across location. This variation was attributed to significant difference in relative abundances of three ASVs, which uniquely represented these species (Figure 6). ASV, annotated as *P. ultimum* (*G. ultimum*), was predominantly found in ROE soils and was represented by around 75% of total ROE oomITS reads. ASV annotated as *P. attrantheridium (G. attrantheridium)*,

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was overrepresented in CAN, KEN and AYL soil and *P. monospermum* ASV was predominantly found in ROW soil, comprising more than 20% of total ROW oomITS reads.

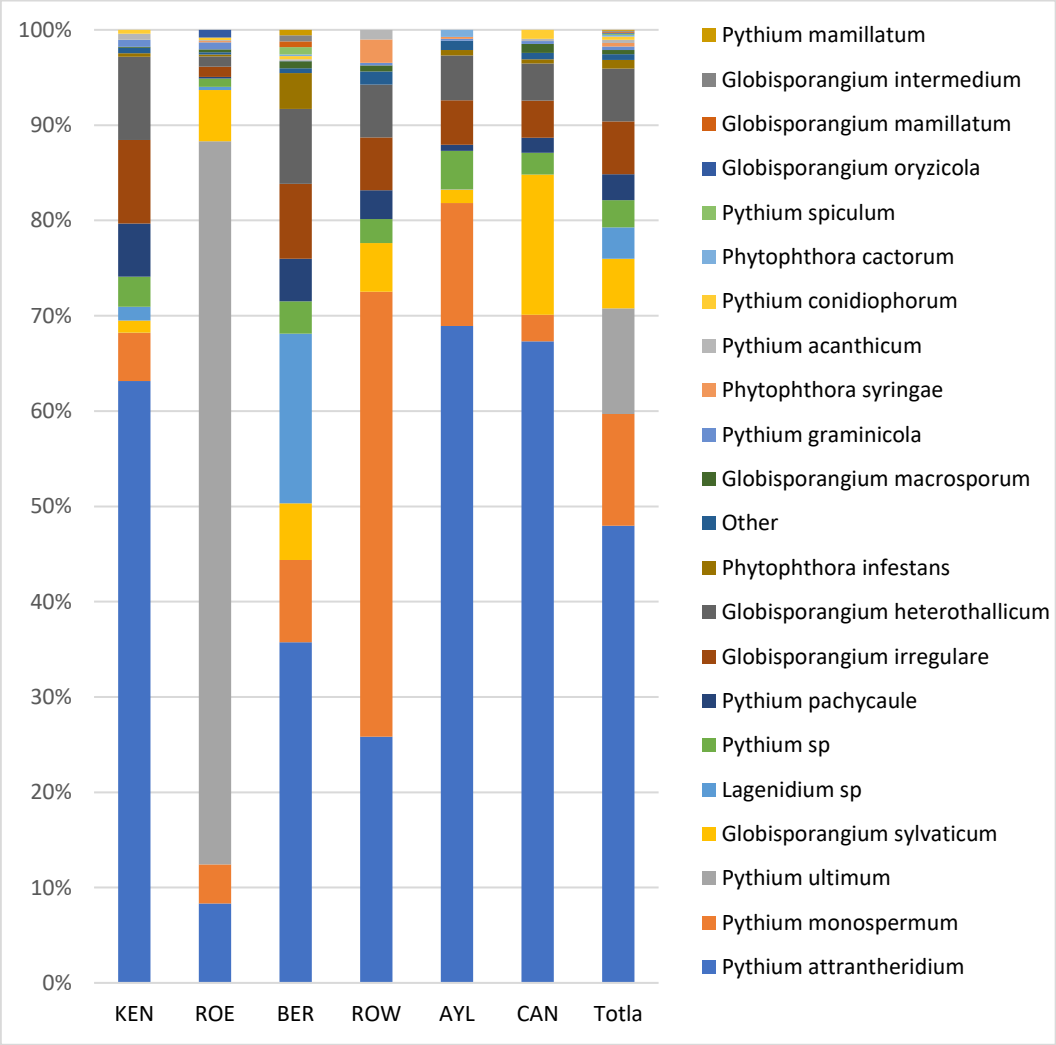

Figure 5. Oomycetes community structure across Nova Scotia apple orchards. Taxa represented by at least 1% of total ITS reads are shown.

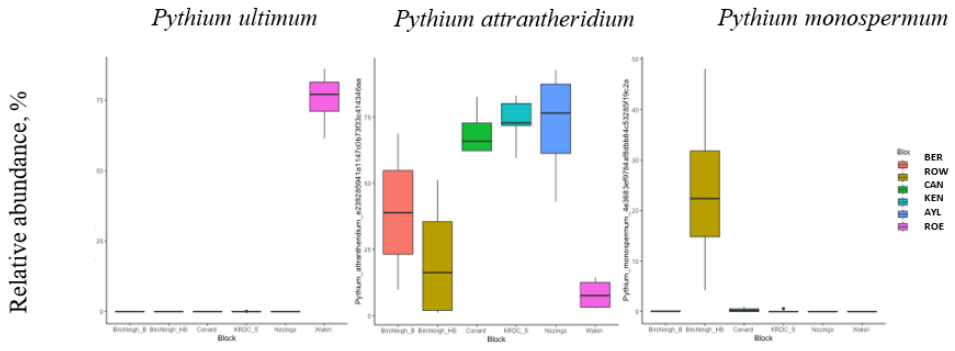

Figure 6. Oomycetes ASVs differentially represented across Nova Scotia apple orchards. Based on ANCOM test with 1% FDR. Orchard locations in the Annapolis Valley of Nova Scotia in Canada: ROE (Rockland East area), ROW (Rockland West area), CAN (Canard area), AYL (Aylesford area), KEN (Kentville area) and BER (Berwick area).

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3.6. Nematode Analysis

Six groups of plant-parasitic nematodes were extracted from the orchard soils: Root-lesion *Pratylenchus* spp.), dagger (*Xiphinema* spp.), ring (criconematidae), pin (paratylenchidae), spiral (hoplolaimidae) and root-knot (*Meloidogyne* spp.) (Table 5). Root-lesion nematodes were found at all six sites, at population densities ranging from 12 to 33 *Pratylenchus* / 100 cm^3 soil (Table 5). Dagger nematodes were found in five of the six sites and were at relatively high population densities, e.g. >100 *Xiphinema*/ 100 cm^3 soil, in two of the sites (Table 5). All dagger nematodes observed had morphological characteristics conforming to the *X. americanum*-complex of species.

Table 5. Population densities (nematodes/ 100 cm^3 soil) of genera of plant-parasitic nematodes extracted from experimental orchards in the Annapolis Valley.

	Nematodes / 100 cm^3 soil					
[x]**Orchard sites**	***Pratylenchus***	***Xiphinema***	***Mesocriconema***	***Paratylenchus***	*Hoplolaimidae*	*Meloidogyne*
ROW	14	149	21	0	2	0
BER	13	172	2	0	13	0
CAN	14	40	1	0	2	0
KEN	33	3	10	30	9	15
AYL	18	3	21	10	7	4
ROE	12	0	9	2	2	0

[x]Orchard sites were designated ROE (Rockland East Area), ROW (Rockland West Area), CAN (Canard Area), AYL (Aylesford Area), KEN (Kentville Area) and BER (Berwick Area).

4. Discussion

The etiology of the ARD complex is challenging to elucidate, as unlike many other soilborne diseases, numerous causal agents have been isolated from affected sites in different parts of the world. ARD has been reported by many as a complex of different types of microbial pathogens and plant parasitic nematodes which can differ in their relative abundances between sites within a geographic region (Kelderer et al. 2012; Mazzola 1998; Mazzola and Manici 2012; Tewoldemedhin et al. 2011c; Tewoldemedhin et al. 2011b). Some of the previous replant disease studies consider soil fertility and nutrient availability as major contributor to the disease (Liu et al. 2009). But other studies have negated this hypothesis as fertilization and an increase in soil nutrients were not able to eliminate ARD (Gongshuai et al. 2018; Manici et al. 2003). We measured several soil fertility parameters such as organic matter, available P, K and several other essential nutrients at the six ARD sites but did not find any correlation with the severity of ARD. However, I would like to mention that there were no "Low" disease severity sites identified in our study, only sites with "Moderate" or "Severe" disease potential were identified. These results are in agreement with study carried out in Bohai Gulf China by Gongshuai et al. (2018) as they also did not find any direct correlation between ARD severity and soil nutrient composition. Ad hoc field observations in the NS industry suggest that summer drought stress can aggravate the severity of the disease. Similar observations have been made in New York State (Rosenberger, 2023 personal communication). However, we did not find a correlation between the severity of ARD and the physical parameters of the six ARD soils that are linked with potential moisture stress such as soil texture, drainage class, and plant available moisture (Table 2). Our study provides evidence that ARD in Annapolis Valley is of biological origin as apple seedling growth in pasteurized soil was markedly improved when compared with non-pasteurized soil in a greenhouse bioassay, a finding that is consistent with previous studies reporting ARD as being of a biological nature (Gongshuai et al. 2018; Li et al. 2016; Tewoldemedhin et al. 2011b).

Soil microbial communities and soil biodiversity play a crucial role in soil ecosystem functions and plant health by decomposition of dead organic materials, nutrient recycling, nutrient availability to plants and suppression of soilborne disease causing pathogens and inducing plant disease resistance (Barrios 2007; Liu et al. 2014; Xu et al. 2012). We detected considerable variation in bacterial and fungal community composition and structure across ARD orchard sites. More than 50%

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variation was observed in bacterial and fungal community composition across ARD orchard study sites. Also, 3 bacterial, and 3 fungal classes were differentially represented at different ARD orchards sites. We did not observe any correlation between severity of ARD sites and fungal or bacterial alpha-diversity across Nova Scotia orchards.

The soils of these six orchards exhibited a remarkably stable core microbiome. The Ascomycota, Mortierellomycota and Basidiomycota were mainly identified phyla at the ARD sites that account for 95% of the total ITS2 reads. Ascomycota was revealed as the most dominant phylum present in ARD sites tested in this study. This finding is similar to previous studies that also reported Ascomycota as the most dominant phylum in ARD sites (Franke-Whittle et al. 2015; Gongshuai et al. 2018). Mortierellomycetes exhibited the greatest relative abundance in the ITS2 microbiome. Mortierellacea was the most dominant family representing 36% of the total ITS2 reads at the ARD sites in this study. The role of *Mortierella* sp., in ARD is not very well established. Some studies have reported these as causal agents of replant disease without isolating the organism or assessing whether or not they incite the disease (Mazzola and Manici 2012; Westcott III et al. 1987). Species of *Mortierella* have been reported as saprophytes in soil and are involved in decomposition of organic matter. Abundance of *Mortierella* spp., has been reported in healthy banana soil (Xue et al. 2015). Other studies indicated that *Mortierella* sp., have a mutualistic relationship with apple and have been associated negatively with the severity of ARD (Gongshuai et al. 2018) and positively related with plant growth (Franke-Whittle et al. 2015).

Fusarium oxysporum and *Fusarium solani* were the most relatively abundant core ASV in this study. *Fusarium* is a large genus that contains saprophytes, endophytes and plant and animal pathogens. In soil *Fusarium* are often associated with plant debris and most species are saprophytic and relatively abundant in the soil microbial community. *F. solani* and *F. oxysporum* were the most frequently isolated species followed by *Cylindrocarpon* spp. binucleate *Rhizoctonia* sp., and *Fusarium* spp. from apple replant orchards soil in Italy (Kelderer et al. 2012). No pathogenicity assays were performed for *Fusarium* spp. in their study as *Fusarium* were considered to be non-pathogenic on apple (Kelderer et al. 2012). Several species of the genus *Fusarium* also produce bioactive secondary metabolites that mediate positive interaction with host plants (Bacon and Yates 2006; Shalapy and Kang 2022). Both *F. solani* and *F. oxysporum* are considered as endophytes, as they are usually isolated from asymptomatic root tissue with high abundance not only from apple tree but several other crops (Macia-Vicente et al. 2008; Manici et al. 2003; Manici and Caputo 2010; Mazzola 1997; Mazzola 1998; Tewoldemedhin et al. 2011c). However more than 20 species of the genus *Fusarium* are pathogens of higher plants causing root rot, vascular wilt and storage rot (Shin et al. 2016). *Fusarium* is often isolated from diseased apple tree roots but most isolates representing several species did not prove to be pathogenic on apple (Dullahide et al. 1994; Mazzola 1998). Tewoldemedhin et al. (2011c) frequently isolated *Fusarium* from all orchards in the study, but most proved to be non-pathogenic towards apple seedlings and only two of the isolates (*F. avenaceum* and *F. solani*) were only weakly virulent on apple seedlings. Previous studies have shown that *F. solani* was either non-pathogenic, or had low virulence towards apple seedlings (Manici et al. 2003; Mazzola 1998). We also detected several other fungal genera in this study such as *Ilyonectria, Nectria* and *Nectriaceae* that fall into the group that were traditionally called *Cylindrocarpon*-like fungi (Chaverri et al. 2011). These genera are reportedly negatively involved in apple growth (Braun 1995; Franke-Whittle et al. 2015; Tewoldemedhin et al. 2011a). Our findings are in agreement with these previous studies and support the conventional work of Braun (1995) in identifying *Cylindrocarpon* as a causal agent of ARD.

Oomycete pathogens such as *Pythium and Phytophtora* species have been frequently isolated from ARD soil and apple roots with varying frequency or dominance from site to site (Kelderer et al. 2012; Mazzola and Manici 2012; Tewoldemedhin et al. 2011c). *Pythium* species were isolated as one of the ARD causal pathogens in the transnational European study of soils from Austria, Germany and Italy (Manici et al. 2003). *Pythium, Rhizoctonia and Phytophtora* were also reported as pathogens that play a direct role ARD in Washington (Mazzola 1999; Mazzola et al. 2002; Shin et al. 2016). In this study, *P. attrantheridium (G. attrantheridium), P. monospermum* and *P. ultimum* (*G. ultimum*) were the most relatively abundant oomycetal taxa. In a previous study, *Pythium irregulare* (*G. ultimum*) and

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Cylindrocarpon lucidum were identified as causal pathogens of ARD in five old orchards soils of the Annapolis Valley of Nova Scotia (Braun 1991; Braun 1995).

Several bacterial families containing potential plant growth promotion taxa were part of the core microbiome. Some members of the family Chitinophagaceae, which demonstrated high relative abundance in orchard soils have an ability to produce indole-3-acetic acid, solubilize phosphate, and possess ACC deaminase activity. Each of these attributes may function to promote plant growth (Madhaiyan et al. 2015). The Sphingomonadaceae include genera with plant growth-promoting activities. Some of these genera produce phytohormones salicylic acid, gibberellins, indole-3-acetic acid and abscisic acid (Yang et al. 2014) and induce host-plant systemic resistance (Chapelle et al. 2016; Hahm et al. 2012). The presence of Solibacteraceae in the plant rhizosphere was linked to plant resistance to *Fusarium* pathogens (Mendes et al. 2018) and Actinobacteria and Pseudonocardiaceae exhibit antimicrobial ability against some bacteria and fungi (Chaouch 2018).

Microorganisms that play an important role in the turnover of organic plant material and soil fertility were also a part of core microbiome. The family of *Xanthobacteraceae*, contains potential nitrogen fixers, and degraders of alkenes, halogenated aliphatic and aromatic compounds, terpenes, thiophenes, or polyaromatic compounds (Oren 2014). Members of family Nitrosomonadaceae contains species involved in nitrification, sulfur cycling and plant growth promotion (Prosser et al. 2014). Many strains belonging to family Micromonosporaceae can degrade chitin, cellulose, lignin, and pectin (Trujillo et al. 2014). In addition to plant beneficial microorganisms, potential phytopathogens were found in the core microbiome. The Xanthomonadaceae were highly abundant in these soils. Several species of the genus Lysobacter; that belong to this family have been shown to effectively control fungal and oomycete plant pathogens (Hayward et al. 2010; Kobayashi and Yuen 2007{Hayward, 2010 #155) and cyst nematodes (Yuen et al. 2018).

Of the six genera of plant-parasitic nematodes found in the orchard soils, only root-lesion and dagger nematodes are known to be pests of apple. Our analyses did not identify the species of root-lesion nematodes in each sample, but prior research has confirmed the widespread occurrence of *P. penetrans* in Nova Scotia orchards, including one of the orchards (KEN) sampled in this study (Forge et al. 2019). Root-lesion nematodes are known to cause economically significant damage to apple on their own (Ark and Thomas 1936; Bélair et al. 2019; King 2022), and population densities of 30 to 100 *P. penetrans* / 100 cm^3 soil have been proposed as approximate damage thresholds (e.g., http://www.omafra.gov.on.ca/IPM/english/apples/diseases-and-disorders/nematodes.html; https://pnwhandbooks.org/plantdisease/host-disease/apple-malus-spp-nematode-root-lesion) for apple replant. However, a recent field microplot study demonstrated apple growth reduction with an at-planting soil population density of 5.4 *P. penetrans*/ 100 cm^3 soil (King 2022), indicating that the *Pratylenchus* populations observed in these Nova Scotia orchard soils would likely have measurable effects on growth of apple. As migratory endoparasites of root cortical cells, *P. penetrans* cause cortical necrosis of fine feeder roots, making them vulnerable to infection by opportunistic fungal pathogens, suggesting a synergistic relationship with fungal pathogens and increasing the severity of the broader replant disease complex (Mazzola and Manici 2012).

The threshold for measurable dagger nematode damage to apple has been proposed to be 50 to 100 *Xiphinema*/ 100 cm^3 soil (e.g. Nematode | Intermountain Fruit | USU; Nematodes - Ontario AppleIPM (gov.on.ca), and we speculate that they would have affected tree growth in the three soils with population densities of 40, 149 and 172 *Xiphinema*/ 100 cm^3. *Xiphinema americanum* is also a vector of tomato ringspot virus which can be a problem in apple orchards.

Pin nematodes are known to parasitize apple but only cause damage at much greater population densities than those found in these orchard soils (e.g. >500 *Paratylenchus*/ 100 cm^3 soil). Ring nematodes, particularly the species *Mesocriconema xenoplax*, are known to be economically important parasites of *Prunus* fruit trees species (Ferris et al. 2004), but they are not often reported from apple orchards at high population densities, and there are no recorded controlled-inoculation studies of their host-parasite relationship with apple. No species of spiral nematodes have been demonstrated to be pests of apple and, similarly, no species of root-knot nematode known to exist in Canada that

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parasitize apple. We speculate that these nematodes are maintained in orchard soils via feeding on grasses and weeds in orchard alleys and are not of significance to apple.

5. Conclusions

Loss of access to pre-plant, broad-spectrum soil fumigants has resulted in the re-emergence of apple replant disease in Nova Scotia and in the rest of the world. The use of alternative technology and semi-selective agrochemicals to treat ARD orchard soils effectively in the future will require more detailed knowledge of disease etiology. We surveyed the soil microbial communities of six orchards with a long history of apple production in the Annapolis Valley, NS, to further elucidate the nature of disease complex. The previous work of Braun (1995) in identifying *Cylindrocarpon lucidum* as a causal pathogen in ARD, is consistent with our finding of Ascomycota as a dominant fungal phylum in the microbiome. Amongst the Oomycota we identified a high relative abundance of *P. attrantheridium (G. attrantheridium), P. monospermum, P. ultimum (G. ultimum)* and *P. irregulare* as reported by Braun (1995). We also did not find a significant presence of *Phytophthora* species that were reported by other studies as part of the ARD complex. Our study also confirmed a likely contribution of the plant parasitic nematodes *Pratylenchus penetrans* and *Xiphinema americanum* at soil counts consistent with the potential for economic damage. Future research should focus on identifying pathogens in the roots of young apple planting suffering from replant disease to resolve the root microbiome at the species level as a means of developing targeted approaches for combatting the disease.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1. Core 16S rRNA ASVs across 80% of all samples; Table S2. Core ITS2 ASVs across 80% of all samples.

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Data Availability Statement: The datasets generated in the current study are available in the [SRA NCBI] repository, and can be accessed from the following link (https://www.ncbi.nlm.nih.gov/ sra/PRJNA968027, PRJNA968028 and PRJNA968029).

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Conflicts of Interest: The authors declare no conflict of interests related to this study.

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