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RESEARCH ARTICLE

Linking soil characteristics, rhizosphere microbiome composition, and plant defence reaction to apple replant disease severity

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Abstract

Introduction Apple replant disease (ARD) causes reduced growth and fruit yield and affects orchards and tree nurseries worldwide. A number of pathogens have been consistently identified as causal agents of ARD; however factors affecting disease-severity are not fully understood.

Aims We examined five soils from German tree nurseries and apple orchards featuring different soil characteristics and replant histories. We aimed to link the plant-soil interaction to replant disease severity.

Methods In a greenhouse experiment, young apple plants were grown for eight weeks on untreated and disinfected (control) soils. Growth parameters were recorded to evaluate the severity of ARD. The defence response of the plants was examined by expression analysis of ARD indicator genes (*BIS3*, *B4H* and *ERF1B*) and GC–MS-based detection of phytoalexins. The fungal and bacterial rhizosphere communities were investigated by ITS and 16S rRNA amplicon sequencing, respectively.

Results After eight weeks, ARD symptoms were observed on all soils. Growth depression was highest on soils that had faced intensive apple cultivation and lowest on a soil with only one year of apple cultivation prior to the experiment. These results correlated with increases in the *BIS3* expression level and the phytoalexin content in the roots. No bacteria and fungi commonly found in increased abundance in ARD soils were consistently detected in all soils.

Conclusions Replant history influenced disease severity more than soil characteristics. ARD symptoms correlated with *BIS3* expression and phytoalexin (PA) formation. PA exudation increased the relative abundance of bacterial genera with the potential ability to degrade phenolic compounds.

Keywords Apple replant disease · ARD · Rhizosphere microbiome · Phytoalexins · *Biphenyl synthase 3* · Molecular barcoding

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Introduction

Apple replant disease (ARD) occurs worldwide and has been described as a "harmfully disturbed physiological and morphological reaction of apple plants to soils that faced alterations in their (micro-)biome due to previous apple cultures" (Winkelmann et al. 2019). Another slightly different definition was introduced by Somera and Mazzola (2022) stating that ARD "occurs when plant-induced changes to the soil microbiome promote the infestation of multiple host-specific, soilborne pathogens". Symptoms commonly found are stunted growth, reduced fruit quality and yield as well as damages to the roots such as root tip necrosis, root blackening and reduced growth of root hairs (Grunewaldt-Stöcker et al. 2019; Mazzola and Manici 2012). The effectiveness of soil disinfection as a treatment to overcome ARD has consistently demonstrated biotic factors as the primary cause of the disease.

Numerous studies have found an altered abundance of several organisms in bulk and rhizosphere soils as well as apple roots in conjunction with ARD, including fungi, bacteria, nematodes and oomycetes (Balbín-Suárez et al. 2021; Franke-Whittle et al. 2015; Kanfra et al. 2018; Mahnkopp-Dirks et al. 2021; Manici et al. 2013, 2018; Mazzola 1998; Nicola et al. 2018; Otto and Winkler 1977; Popp et al. 2020; Utkhede and Li 1988). Somera and Mazzola (2022) stated that genera of known pathogens in the ARD complex generally occur consistently worldwide, while species composition and sometimes even occurrence may differ between studies conducted on ARD-affected soils from different regions. On the other hand, Manici et al. (2013) found *Pythium* spp. infections to be negatively correlated to plant growth in soils from three orchards in Germany but not in the soils from orchards in Italy or Austria examined in their study and Manici et al. (2003) found negative correlations between *Pythium* spp. and growth in Italy. Furthermore, different species of *Pythium* have been associated with ARD in different regions. Species commonly described in studies conducted in North America and Europe, such as *Pythium ultimum* and *Pythium intermedium* (Mazzola 1998; Sewell 1981; Tilston et al. 2018), were rarely identified as ARD agents in South Africa. For example, Moein et al. (2019) found only *Pythium ultimum* in their study, while Tewoldemedhin et al. (2011a) did not identify either species. Similar reports were published for fungi, e.g. the genera *Cylindrocarpon* and *Ilyonectria*. In Europe, *Ilyonectria robusta* and *Dactylonectria torrensis* were repeatedly identified in ARD-affected apple roots and subsequently shown to be pathogenic to apple plants (Manici et al. 2018; Popp et al. 2020). While *Ilyonectria robusta* has also been associated with ARD in North America (Wang and Mazzola 2019), *Dactylonectria torrensis* has, to the best of our knowledge, not been reported in studies on ARD outside of Europe. Tewoldemedhin et al. (2011b) identified *Cylindrocarpon macrodidymum* as a pathogen likely related to ARD in South Africa, while reports of this species in association with ARD are rare in other parts of the world. In summary, the occurrence and dominance of ARD-causing agents within the disease complex may vary at species level between different affected sites.

In their review, Somera and Mazzola (2022) also pointed out that a high abundance of certain microbial species, based on amplicon sequencing, in ARD-affected soils do not confirm their involvement in the ARD complex. Furthermore, Balbín-Suárez et al. (2021) suggested, alongside contributions from known causative agents, a dysbiotic state to be characteristic of ARD. This hypothesis is supported by the higher abundance of stress-related bacterial genes in ARD soils (Radl et al. 2019).

Even though biotic factors are agreed on to be the primary cause of ARD, soil and environmental factors strongly influence microbiota and may thus alter the abundance and relative dominance of causal microorganisms. For example, the pathogen complex may vary by region or even season depending on soil type, temperature and soil-water-content (Somera and Mazzola 2022), as those factors influence the microbiome in all terrestrial ecosystems. In a number of studies, the severity of ARD was observed to be lower in soils with higher clay content compared to sandier soils with less density and better aeration (Kviklys et al. 2016; Mahnkopp et al. 2018; Schimmel et al 2024). However, out of these studies, only Schimmel et al. (2024) were able to demonstrate the influence of clay in alleviating ARD-related growth reduction by treating soils with clay amendments. In contrast, opposing results have also been published, showing higher severity of ARD in soils with higher clay content (Sheng et al. 2019, 2020). Due to contradictory

statements based on a low number of soils in each study, more research in this field is needed.

As stated, it is evident that apple plants alter the soil microbiome during growth and leave the soil in a dysfunctional state for the repeated growth of apple. In addition to known causal agents, underlying mechanisms, which may contribute to the disease, remain to be elucidated. One proposed explanation is a dysbiosis caused by the exudation of secondary plant metabolites, which might either attract or eliminate specific organisms in the soil (Balbín-Suárez et al. 2021; Busnena et al. 2023). For example, a number of phytoalexins (PA) were produced in significantly higher amounts in apple plants grown in ARD soils compared to those in healthy soils (Busnena et al. 2021). Phytoalexins play an important role in the disease response of apple plants (Busnena et al. 2023). Genes involved in the production of phytoalexins, such as *BIS3* and *B4H*, are upregulated in roots shortly after facing ARD-affected soil (Weiß et al. 2017a, b). Rohr et al. (2020) showed significant upregulation of these genes as early as 72 h after contact of roots with ARD soil.

In summary, previous studies found that a shift in the soil microbiome, elicited by the repeated growth of apple plants, is an important factor for ARD. However, these shifts differ between sites and regions. Furthermore, the mechanisms that cause the shift as well as the plant reaction are still poorly understood. To better understand the effect of the soil, it is essential to collect data on the microbiome and the plant reaction from one and the same experiment, across a variety of ARD-affected soils with different histories and properties. This study examined five ARD-affected soils with differing replant histories, which were collected at German tree nurseries and apple orchards. We investigated a combined data set comprising soil properties and plant-available nutrients, plant growth parameters, gene expression of ARD indicator genes, phytoalexin production as well as a community analysis of fungi and bacteria in the rhizosphere. In a greenhouse experiment based on the biotest described by Yim et al. (2015), apple plants were grown on five different ARD-affected soils, either untreated or disinfected by gamma-irradiation. The difference in growth between the two variants of a given soil allowed an estimation of ARD severity in this respective soil.

Materials & methods

Soil sites

ARD-affected soils for this experiment were collected from five different regions with different replant histories in Germany: Site M (orchard, 50°37′8.5’N, 6°59′25.4’E, Meckenheim, North Rhine-Westphalia), Site C (tree nursery, 53°38′03.1’N, 9°42′22.1’E, Holm, Schleswig–Holstein), Site L (tree nursery, 51°49′57.1’N, 7°25′15.5’E, Dülmen, North Rhine-Westphalia), Site Ha-R (orchard, 53°28′55.1’N, 9°35′30.2’E, Jork, Lower Saxony) and Site ST (tree nursery, 53°40′42.6’N 9°43′53.3’E, Tornesch, Schleswig–Holstein). The soil type was classified based on the German classification system (Ad-hoc-Arbeitsgruppe Boden 2005). The classification and the cultivation history of the sites are displayed in Table 1.

Sampling took place in spring 2020. Soil samples were taken at different positions on each site from the upper 30 cm of the topsoil. The soils were homogenised using an 8 mm sieve. One half of each soil was left untreated (U) and the other half was disinfected using gamma-irradiation (G) (15.69—23.79 kGy; Synergy Health Radeberg GmbH, STERIS, Radeberg, Saxony, Germany), which served as a control. 100 g of each soil variant were sampled to determine grain size fractions, pH, organic matter and plant available nutrients. Until the start of the experiment, the soils were stored for one week at 4 °C.

Analyses of chemical soil properties

For soil analyses, samples were air-dried and sieved (< 2 mm). Grain size fractions (soil texture, Table 1) was determined by the pipette method for the clay and silt fractions and by wet sieving for the sand fraction (Gee and Bauder 2018). Soil pH (Suppl. Table 1) was measured in 0.01 M $CaCl_2$ solution (1:12.5 soil and solution). Plant available nutrients (Suppl. Table 1) in soil were analysed using the Mehlich III method (Mehlich 1984). Extraction solution (0.2 M CH_3COOH, 0.25 M NH_4NO_3, 0.015 M NH_4F, 0.013 M HNO_3, 0.001 M EDTA) was added to soil at a ratio of 1:3 and horizontally shaken for 5 min. After centrifugation at 2500 × *g* for 5 min, the supernatant was extracted and acidified (10% HNO_3) and elemental determination was performed by inductively

Table 1 Organic carbon (OC), total nitrogen (TN), particle size distribution, soil texture category, soil type (Ad-hoc-Arbeitsgruppe Boden 2005) and cultivation history of soil from the sites Meckenheim (M), Holm (C), Jork (Ha-R), Dülmen (L) and Tornesch (ST); untreated (UT) and gamma-irradiated (G)

Site	OC (%)	TN (%)	Clay (%)	Silt (%)	Sand (%)	Soil texture	Soil type	Cultivation history
M	1.27 (G) 1.40 (U)	0.14 (G) 0.14 (U)	21.3	71.8	7.0	Strong clayey silt	haplic Luvisol	Several generations of apple & pear for 5–20 years each since 1950
C	2.58 (G) 2.45 (U)	0.15 (G) 0.14 (U)	2.6	3.7	93.7	Sand	gleyic Podzol	2015–2018 three generations of *Malus*
Ha-R	1.56 (G) 1.69 (U)	0.13 (G) 0.14 (U)	4.1	50.3	45.6	Medium silty sand	stagnic Cambisol	Agricultural crops, including *Zea mays*, before 1995; 1995–2009 'Jonagold' on M9; 2010–2019 'Rubens' on M9
L	2.92 (G) 2.95 (U)	0.18 (G) 0.18 (U)	7.4	16.0	76.6	Slightly loamy sand	gleyic Podzol	1995–2009 *Malus* Pi 80; 2009–2018 a variety of Maloideae & drupe with periods of fallow or *Tagetes*
ST	1.35 (G) 1.59 (U)	0.10 (G) 0.11 (U)	5.9	42.0	52.1	Slightly loamy sand	stagnic Podzol	Until 2014 *Rosa*; 2015–2016 different grains & *Zea mays*; 2017 *Tagetes*; 2018–2019 M9

coupled plasma optical emission spectroscopy (ICP-OES, Varian 725-ES, Agilent Technologies, Santa Clara, CA, USA). The bulk organic C and N analyses (Table 1) were performed by dry combustion with a CNS-Elemental Analyzer (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany).

Greenhouse biotest

A greenhouse experiment, based on the biotest established by Yim et al. (2015), was carried out from March 17th to May 11th 2020 (19.3 °C ± 4.3). All soils were fertilised with 2 g L^{-1} Osmocote® Exact 3–4 M (16 + 9 + 12 + 2 MgO; ICL Specialty Fertilizers, Tel Aviv, Israel) at the start of the experiment. Previously in vitro propagated and rooted plantlets of the ARD-sensitive apple rootstock M26 were acclimatised for 4 weeks in a greenhouse prior to the start of the experiments as described in detail by Rohr et al. (2021). After removal of the peat substrate, which was used for acclimatisation, the plants were potted into 750 mL moist untreated (U) or gamma-irradiated (G) soil from the five different sites (M, C, Ha-R, L, ST). Each of the ten resulting variants, i.e. treatment-soil combinations (M-G, M-U, C-G, C-U, Ha-R-G, Ha-R-U, L-G, L-U, ST-G, ST-U), included 12 replicates, i.e. pots with one M26 plant each. The pots were placed randomised into four blocks in a greenhouse chamber.

During the growth period of 8 weeks, the plants were watered with tap water by hand as needed. Technical settings of the greenhouse in this period were as follows: heating at < 19 °C, ventilation at > 21 °C, shading at 25.0 klx (8 am—8 pm) and additional light (SON-T Philips Master Agro 400 W; 25.0 klx; 7 am − 11 pm). For pest control, a mix of 0.3% NeemAzal® (Trifolio-M GmbH; Lahnau, Germany) and 0.05% Gnatrol® (Valent BioScience LLC; Libertyville, IL, USA) solution was applied 1–2 times per week. Additionally, a sulphur evaporator was used at night to prevent powdery mildew infections.

Plant growth measurements and samplings

The shoot length (SL) growth (total SL increase) was calculated by subtracting the SL on day 0 from the SL after 8 weeks. At the end of the growth period, fresh mass (FM) and dry mass (DM) of shoots and roots were determined by oven drying of shoots and washed roots for at least 3 d at 70 °C. Six representative plants

of each soil and treatment, excluding the smallest and largest plant each, were chosen for molecular analyses. The following samples were taken from these six plants: For each untreated soil (U), rhizosphere samples were taken from six pots for 16S rRNA and ITS barcoding of the microbiome. The bulk soil was first removed by hand. Then, 1–2 mL of the remaining root adhering soil was collected in 2 mL reaction tubes and stored at −80 °C until DNA extraction. Furthermore, to determine concentrations of phytoalexins and phenolic compounds as well as the relative expression of ARD-indicator genes (Reim et al. 2020; Rohr et al. 2020; Weiß et al. 2017a, b), roots of the same six plants per U variant and an additional six plants from each G variant were sampled. The roots were deep frozen in liquid nitrogen and stored at −80 °C until they were processed further. In total, 30 samples were taken from U variants for each type of analysis and another 30 samples were taken from G variants for the analysis of phenolic compounds and gene expression.

RNA extraction and RT-qPCR for expression analysis of ARD indicator genes in roots

The root samples were homogenised on liquid nitrogen in a Retsch mill (MM400; Retsch, Haan, Germany) for 30 s at 27 Hz. About 60 mg were used for RNA extraction using the InviTrap® Spin Plant RNA Mini Kit (Invitek Molecular, Berlin, Germany) following the instructions of the manufacturer. Prior to cDNA-synthesis, genomic DNA was removed with the DNase I protocol provided by Thermo Fisher Scientific (Waltham, MA, USA). The cDNA was synthesised using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, USA) and oligo-dT primers.

Elongation factor 1-alpha (EF1a) and *elongation factor 1-beta 2-like (EF1b)* as well as *tubulin beta chain (TUBB)* were selected as reference genes based on the previous work of Weiß et al. (2017a). Following the results of Reim et al. (2020), *biphenyl synthase 3 (BIS3)* and *cytochrome P450 CYP736A12-like (biphenyl-4-hydroxylase, B4H)* were selected as the candidate genes reacting most specifically to ARD. Because both genes are involved in phytoalexin biosynthesis, *ethylene-responsive transcription factor 1B-like (ERF1B)* was also included as an indicator independent of the phytoalexin pathways (Reim et al. 2020; Rohr et al. 2020). The primer efficiency tests (technical replicates = 3) and qPCR (technical replicates = 2 (*BIS3*, *B4H*) or 3 (*ERF1B*, *EF1a*, *EF1b*, *TUBB*)) were conducted as described by Weiß et al. (2017a) using the CFX Connect™ qPCR cycler and CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA). Finally, the normalised gene expression was calculated according to Pfaffl (2001).

Phytoalexin (PA) extraction and quantification by gas chromatography-mass spectrometry (GC–MS)

Apple roots were lyophilised and homogenised to fine powder (29 Hz, 1 min; Mixer Mill MM400, Retsch, Haan, Germany). The pulverised roots were weighed and extracted with 1 mL of methanol supplemented with 25 µg of 4-hydroxybiphenyl (internal standard for relative quantification). The suspension was continuously vortexed using Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) at the maximum speed of 2700 rpm for 20 min. The resulting extract was centrifuged (13,439 g, 10 min) and the supernatant was dried under a stream of air in a reaction tube. The residue was re-dissolved in 1 mL dichloromethane:chloroform (1:1, v/v) and centrifuged at 13,439 g for 10 min. The supernatant was transferred to a new 1.5 mL reaction tube and evaporated to dryness under a stream of air. The residues were re-dissolved in 200 µL ethyl acetate and centrifuged at 13,439 g for 10 min. The clear supernatant was carefully transferred into a micro inlet sitting in an analytical 1.5 mL vial. The ethyl acetate was evaporated to complete dryness under a stream of air. The residue was re-dissolved in 50 µL N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA; ABCR, Karlsruhe, Germany) and silylated at 60 °C for 30 min. The silylated samples were analysed by an Agilent Technologies 6890 N (Agilent Technologies Deutschland GmbH, Böblingen, Germany) gas chromatograph coupled to an Agilent Technologies 5975B mass spectrometer (MS). The following MS settings were applied: ionisation voltage 70 eV, ion source temperature 230 °C and interface temperature 290 °C. The following temperature program was applied: 70 °C for 3 min, then increased at a rate of 10 °C min^{-1} to 310 °C and held at 310 °C for 5 min. For chromatographic separation, a Zebron ZB-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Phenomenex, Aschaffenburg, Germany) was used. Helium

was the carrier gas at a flow rate of 1 mL min^{-1}. The injection volume was 1 µL using a split ratio of 1:10. Relative quantification of the individual compounds using the added internal standard 4-hydroxybiphenyl (response factor 1) allowed the relative quantitative comparison of the levels of phytoalexins of all samples. A set of co-injected hydrocarbons (even-numbered C_{14}—C_{32}) was used to calculate the retention indices by linear extrapolation as described by Busnena et al. (2021).

DNA extraction and amplicon sequencing for microbial analyses of rhizosphere samples

DNA was extracted from 0.5 g of rhizosphere soil collected from the (U) samples using a protocol initially developed by Lueders et al. (2004) and subsequently modified by Stempfhuber et al. (2017). DNA was quantified using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). For the assessment of the bacterial community the '16S Metagenomics Sequencing Library Preparation' protocol (Illumina, San Diego, CA, United States) and quality guidelines by Schöler et al. (2017) were used. The V4 region of the 16S rRNA gene was amplified using primer pair 515F (Parada et al. 2016) and 806R (Apprill et al. 2015). Quality guidelines were followed as described in Schöler et al. (2017). PCR reaction mixtures contained 15 ng of DNA, 0.5 µL of 10 pmol of each primer (10 µM), 2.5 µL of 3% BSA, 12.5 µL of NEBNext® High-Fidelity 2×PCR Master Mix (New England Biolabs, Frankfurt am Main, Germany) and DEPC-treated water was added to bring the volume up to up to 25 µL. The amplification program for the 16S rRNA gene was initiated at 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, and terminated by a final extension at 72 °C for 5 min. PCR products were purified using MagSi NGSprep Plus beads (Steinbrenner, Wiesenbach, Germany) and quantified and quality checked using the Fragment Analyzer (Agilent Technologies, Santa Clara, CA, United States) and the NGS Fragment Kit (1–6,000 bp, Agilent Technologies, Santa Clara, CA, United States). Indexing PCR was performed in a reaction mixture consisting of 10 ng of the purified amplicon, 1.5 µL of each indexing primer (Nextera® XT Index Kit v2 Set B; Illumina, San Diego, CA, United States), 12.5 µL NEBNext® HighFidelity 2×PCR Master Mix and the volume was adjusted to 25 µL using DEPC-treated water. Afterwards, amplicons were purified and quantity as well as quality checked as previously described. The library was diluted to 4 nM and pooled equimolarly. For sequencing, the MiSeq®Reagent kit v3 (600 cycles) (Illumina, San Diego, CA, United States) was used for paired-end sequencing on the MiSeq® instrument (Illumina, San Diego, CA, United States).

For the assessment of fungal community, the ITS3 primer mix and ITS4 primer mix, which target the fungal internal transcribed spacer (ITS) were used (Tedersoo et al. 2015). PCR reaction mixtures contained 5 ng of DNA, 0.5 µL of 10 pmol of each primer, 2.5 µL of 3% BSA, 12.5 µL of NEBNext® High-Fidelity 2×PCR Master Mix (New England Biolabs, Frankfurt am Main, Germany) and DEPC-treated water up to 25 µL. The amplification program for the ITS gene was initiated at 95 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, and terminated at 72 °C for 10 min. Libraries were prepared in the same way as 16S rRNA amplicon libraries.

The demultiplexed sequences were processed using the Galaxy web server (www.usegalaxy.org; Afgan et al. 2018). FASTQ files were trimmed with minimum read length of 50 using Cutadapt v3.7 (Martin 2011) and quality control was done using FastQC v0.11.9 (Andrews 2010). Further read processing was performed using DADA2 pipeline (Callahan et al. 2016), as implemented in Galaxy Version 23.0.rc1, with the following trimming and filtering parameters: For both 16S and ITS, 20 bp were selected from the start of the sequence for forward and reverse reads. The expected error was 3 (forward) and 4 (reverse). Read length was truncated at 240 bp (forward, 16 s and ITS), 200 bp (reverse, 16S) and 190 bp (reverse, ITS), respectively. Taxonomy was assigned to the resulting unique amplicon sequence variants (ASVs) using the SILVA database (SILVA v138.1; Quast et al. 2013) for bacterial 16S rRNA gene sequences and the UNITE database (UNITE release s10.05.2021; Nilsson et al. 2019) for fungal ITS sequences. The minimum bootstrap confidence for assigning taxonomic level was 50. For bacteria, only 3.1% of all ASVs could not be assigned to a phylum, representing 0.5% of all reads. For fungi, 34% of ASVs could not be assigned at phylum level,

corresponding to the same percentage of overall unassigned reads. ASVs assigned to mitochondrial and chloroplast reads as well as Archaea were removed from the dataset. To exclude potential contamination, ASVs occurring in negative controls and singletons (ASVs represented by only one read) were also removed.

Statistical analyses

For growth parameters, normalised gene expression data and PA contents, an ANOVA as well as a Tukey test were performed in RStudio (RStudio ver. 2022.02.3 + 492; Boston, MA, USA), run on R (v4.3.1; R Core Team 2022), using the package 'agricolae' (v1.3–5; de Mendiburu 2021).

The analysis of the microbiome data was done in R (v4.3.1; R Core Team 2022). The data was normalised by scaling with ranked subsampling (Beule and Karlovsky 2020), using R package 'SRS' (v9.2.3, Heidrich et al. 2021). Alpha diversity indices (observed species richness, Pielou evenness and Shannon diversity index) were calculated using R-package 'microbiome' (v1.20.0; Lathi et al. 2019). Testing for significant differences of alpha diversity indices among different soils was done using Wilcoxon test implemented in the R package 'rstatix' (v0.7.2; Kassambara 2023) and corrected for multiple testing using 'Bonferroni'. Beta diversity was analysed via Bray–Curtis distance matrix. Principle coordinate analysis (PCoA) was used for ordination. Significant differences in community composition were tested applying PERMANOVA ($p < 0.05$), using function 'adonis2' implemented in R-package 'vegan' (v2.6–4; Oksanen et al. 2022), followed by a pairwise PERMANOVA using the R-package 'pairwise-Adonis' (v.0.4.1; Arbizu 2017) and the *p*-values adjusted using 'Bonferroni'. Relative abundance was estimated on the genus level, using transform ('compositional') function of R-package 'microbiome' (v1.20.0; Lathi et al. 2019). The heat map of the top 30 genera was plotted using the R-package 'MicroEco' (v1.6.0; (Liu et al. 2021a). Core microbiome analysis was performed with R-package 'ampvis2' (v2.7.33; Andersen et al. 2018) with an abundance cut-off of 0.1% and a frequency of min 80%. Testing for significance was done using Wilcoxon test implemented in the R package 'rstatix' (v0.7.2; Kassambara 2023) and corrected for multiple testing using 'Bonferroni'.

A Spearman correlation analysis was performed in R (v4.3.1; R Core Team 2022) using the 'corrplot' package (v0.92; Wei and Simko 2021). Bacterial and fungal genera were included using the same cut-off as described before. Only those PAs and genes were included which showed significant correlation ($p < 0.05$) with at least one growth parameter or microbial genus.

Results

After 8 weeks, severe growth reduction was observed in plants cultivated in ARD (U) soil compared to control (G) soil for all five soil types (M, C, Ha-R, L, ST). Previously reported ARD-related symptoms, such as stunted growth and root blackening, were apparent (Fig. 1B). Soils M and C caused the highest relative reduction in shoot fresh mass in ARD (U) compared to the control (G), i.e. 59% and 53%, respectively (Fig. 1A). The lowest reduction of shoot fresh mass (40%) was observed on soil ST. In contrast, root fresh mass was reduced most severely on Ha-R and L soils, i.e. 49% and 50%, respectively (Fig. 1C). The soils M and C showed comparatively lower reductions in root fresh mass (42% and 45%, respectively) than Ha-R and L, although these differences between U and G were not significant, likely due to relatively high standard deviations in M-U and C-U compared to Ha-R-U and L-U, in relation to their respective RFM.

Expression of ARD indicator genes

In addition to the evaluation of growth data, expression of three ARD indicator genes was analysed by quantitative RT-qPCR. The expression of the *BIS3* gene was significantly increased, i.e. 8- to 10-nfold, in roots grown in the ARD variants of soils M, C and L, compared to the respective disinfected (G) variants (Fig. 2A). The differences between ARD and G variants in Ha-R and ST soils were not significant. However, a clear increase in *BIS3* gene expression was seen in all U variants. The normalised expression level of *B4H* was lower than that of *BIS3* but showed a similar pattern (Fig. 2B). Only on ST soil, which resulted in the lowest fold-change of *B4H* expression, the difference between U and G variants was not significant. For both genes, the fold-change was highest in soil C, which also led to strong reduction in plant

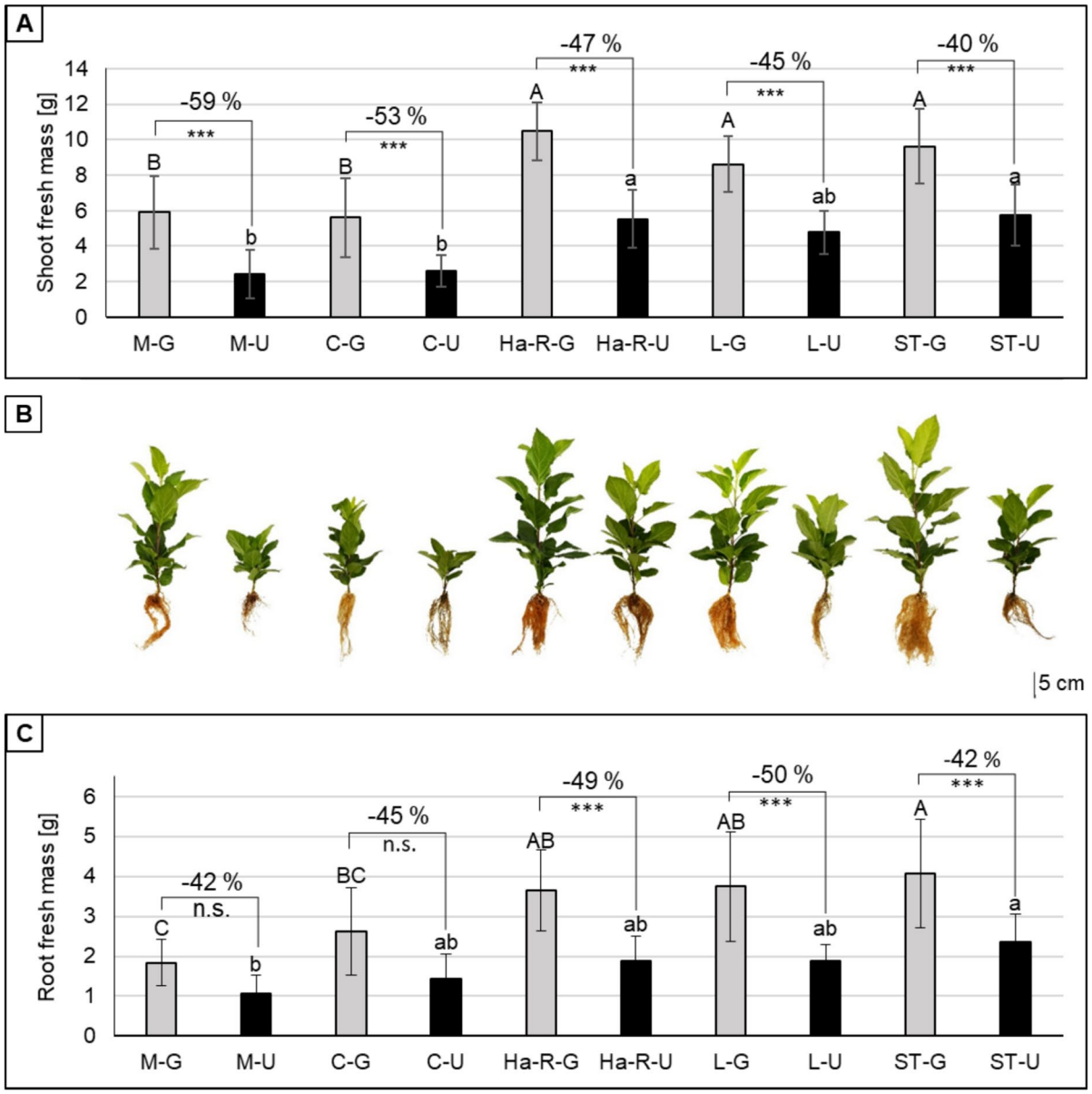


Fig. 1 Shoot fresh mass (**A**), representative photos (**B**) and root fresh mass (**C**) of M26 plants after 8 weeks on untreated (U) and gamma-irradiated (G) ARD soils from five different sites (M, C, Ha-R, L, ST; see Table 1). Percentage values indicate the respective, partial growth reduction (mass on G soil variants = 100%). Given are means ± SD ($n = 12$ plants). Stars indicate significant differences between G and U of the same soil in the Tukey test (*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$, n.s. = not significant). Capital letters indicate significant differences between G variants ($p < 0.05$); lowercase letters indicate significant differences between U variants ($p < 0.05$)

fresh mass, and it was lowest in ST soil with the weakest reduction in plant fresh mass. The relative expression level of the transcription factor *ERF1B* was generally lower than those of *BIS3* and *B4H* by the power of 10 or more (Fig. 2C). In most soils, *ERF1B* was not significantly higher expressed in the U variants, except for soil C.

When comparing the different soils with each other, there were no significant differences in the relative expression of the *BIS3* and *B4H* genes in the

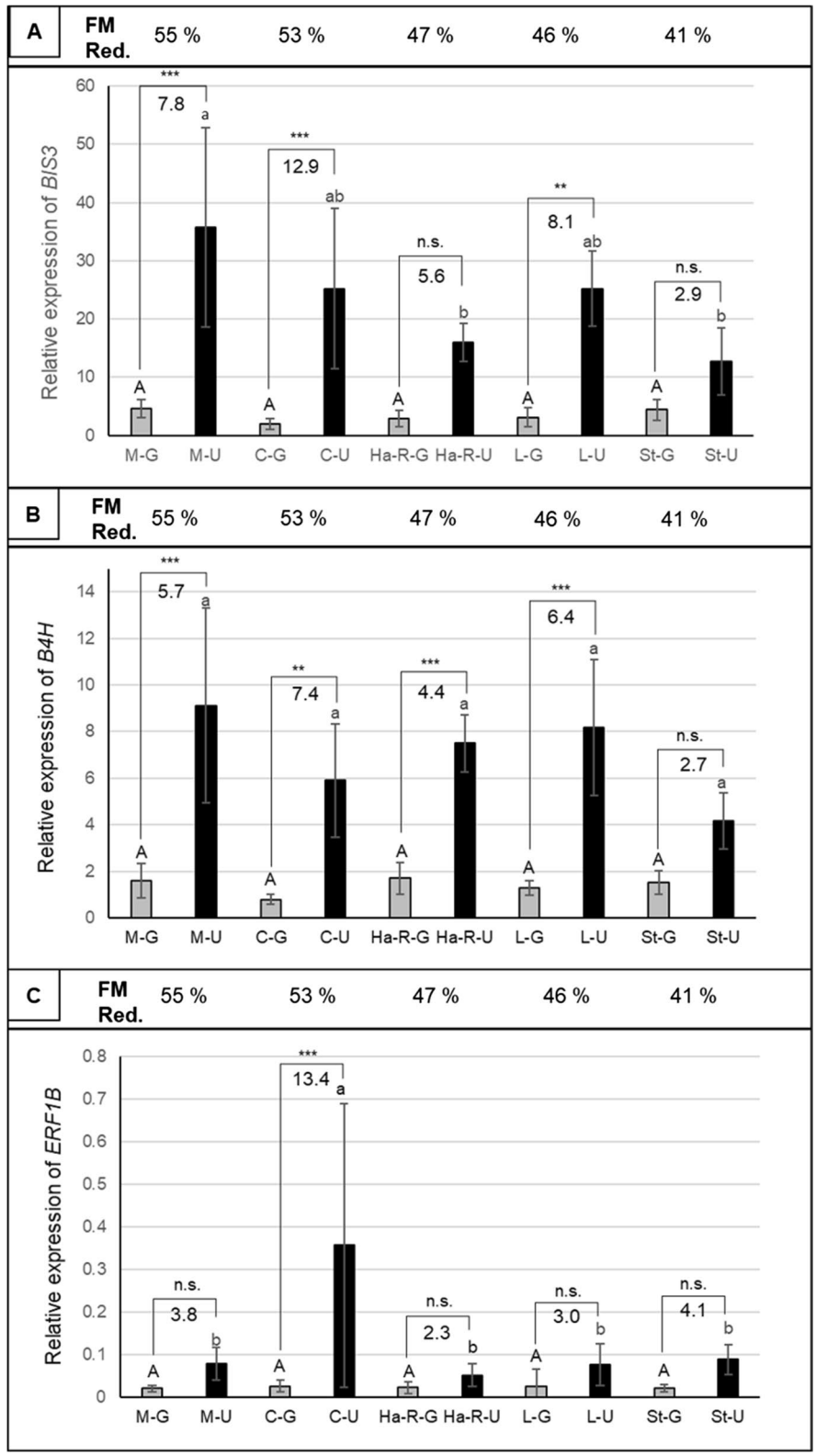


Fig. 2 Relative expression of the ARD indicator genes *biphenyl synthase 3* (*BIS3*, **A**), *cytochrome P450 CYP736A12-like* (*biphenyl-4-hydroxylase, B4H,* **B**) and *ethylene-responsive transcription factor 1B-like* (*ERF1B,* **C**) in M26 roots after 8 weeks on untreated (U) and gamma-irradiated (G) ARD soils from five different sites (M, C, Ha-R, L, ST). Reduction in plant fresh mass (FM Red.) between variants on the same soil is shown at the top of each figure and the fold-change between G and U of each soil is displayed below difference-indicating brackets. Given are means ± SD of $n = 6$ biological replicates. Stars indicate significant differences between G and U of the same soil in the Tukey test (*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$, n.s. = not significant). Capital letters indicate significant differences between G variants ($p < 0.05$); lowercase letters indicate significant differences between U variants ($p < 0.05$)

gamma variants of all soils. The same was true for the ARD variants (U). The expression of *ERF1B* was significantly increased in the U variant of soil C compared to the other soils.

Phytoalexin contents

In roots of the G variants, three phytoalexins, 2-hydroxy-4-methoxydibenzofuran (retention index (RI)=2131), hydroxyeriobofuran isomer 2 (RI=2331) and eriobofuran isomer 2 (RI=2346) were present at low levels. Their production in roots from untreated soils was highly increased and eight additional phytoalexins, i.e. 3-hydroxy-5-methoxybiphenyl (RI=1956), aucuparin (RI=2090), noraucuparin (RI=2121), eriobofuran (RI=2228), noreriobofuran (RI=2259), hydroxynoreriobofuran isomer 4 (RI=2365), methoxyeriobofuran isomer 4 (RI=2399) and hydroxyeriobofuran isomer 5 (RI=2474), were induced. As a result, the total PA contents in roots from all untreated soils were significantly higher than in roots from the respective gamma-irradiated soils (Fig. 3, Suppl. Table 2).

In the untreated soils, M26 roots from M soil contained the highest level of phytoalexins, whereas roots grown in ST soil contained the lowest level. The total phytoalexin contents in roots grown in L, Ha-R and C were similar and ranged between those of roots grown in ST and M soils (Fig. 3). The phytoalexins 3-hydroxy-5-methoxybiphenyl (RI=1956) and noraucuparin (RI=2121) were strongly induced in M, C and L soils, compared to the other two soils. In all untreated soils, the roots contained high levels of 2-hydroxy-4-methoxydibenzofuran (RI=2131) and hydroxyeriobofuran isomer 2 (RI=2331). In the gamma-irradiated soils, the PA contents did not significantly differ between the roots from the five soil origins.

Rhizosphere microbiomes

The microbial composition of the rhizosphere was analysed using 16S rRNA gene and ITS amplicon sequencing to detect bacteria and fungi, respectively.

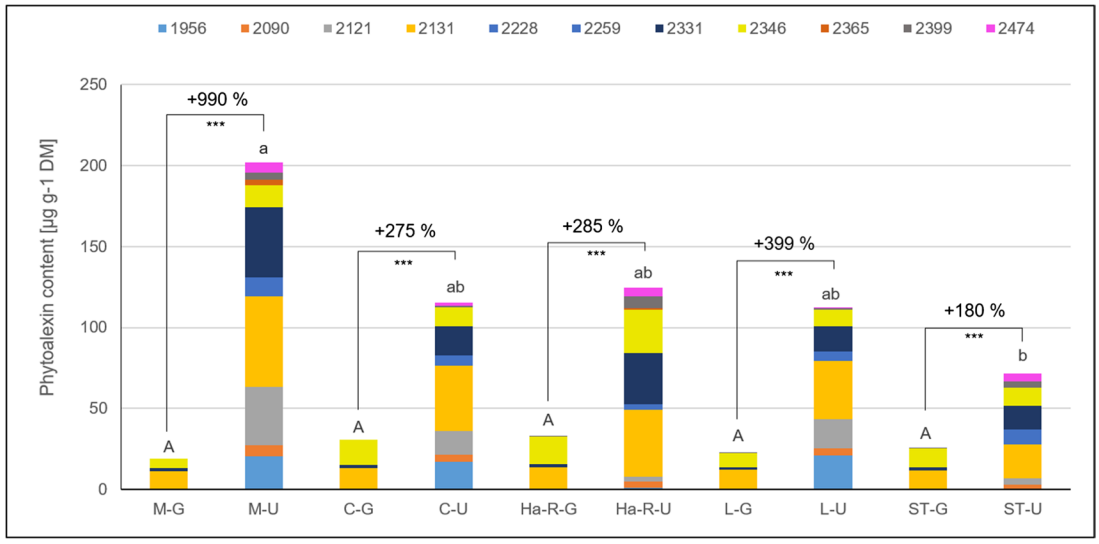


Fig. 3 Phytoalexin contents in roots of M26 plants grown for 8 weeks on untreated (U) and gamma-irradiated (G) ARD soils from five different sites (M, C, Ha-R, L, ST). The percentage between the bars shows the respective total phytoalexin increase (content in G variant=100%). Given are means of $n=6$ biological replicates. Stars indicate significant differences between G and U of the same soil in the Tukey test (***=$p<0.001$; **=$p<0.01$; *=$p<0.05$, n.s.=not significant). Capital letters indicate significant differences between G variants ($p<0.05$); lowercase letters indicate significant differences between U variants ($p<0.05$). Retention indices indicate the following compounds: 1956=3-hydroxy-5-methoxybiphenyl; 2090=aucuparin; 2121=noraucuparin; 2131=2-hydroxy-4-methoxydibenzofuran; 2228=eriobofuran; 2259=noreriobofuran; 2331=hydroxyeriobofuran isomer 2; 2346=eriobofuran isomer 2; 2365=hydroxynoreriobofuran isomer 4; 2399=methoxyeriobofuran isomer 4; 2474=hydroxyeriobofuran isomer 5

The Shannon index indicated that M-U had the highest bacterial alpha diversity, followed by C-U (Table 2a). In contrast, the highest fungal diversity was found for L-U, while M-U displayed the lowest species richness (Table 2b).

Beta-diversity analysis revealed that both bacterial and fungal communities in M-U were distinctive from those in the other soils (Fig. 4). Bacterial communities of Ha-R-U and ST-U clustered together. Additionally, fungal communities seemed to be more distinctive among soil sites compared to bacterial communities. The PERMANOVA analysis indicated an overall significant influence of the soil on both bacterial and fungal communities.

Considering the relative abundance (RA) of both bacterial and fungal genera, significant differences were recorded among the different soils (Fig. 5, Supplementary Tables 3, 4, and 5). *Fusarium*, *Gibberella*, *Gibellulopsis*, *Solicoccozyma*, *Humicola*, *Cladosporium*, *Mortierella*, *Trichoderma* and *Pseudogymnoascus* were identified within the top 30 abundant fungal genera in all soil sites (Fig. 5B, Supplementary Table 3). Genera of arbuscular mycorrhiza, including *Glomus*, *Diversispora*, *Entrophosphora*, *Rhizophagus* and *Paraglomus*, were detected in only low relative abundances in all soils.

In none of the five soils, a single bacterial genus reached a RA of 10% while fungal genera exceeded 10% RA in several cases, e.g. *Gibellulopsis* in L-U (20%) and *Solicoccozyma* in ST-U (15%). In contrast, low abundant bacterial taxa categorised as 'others' made up for 50% (ST-U) and 65% (M-U) of the total RA, whereas low abundant fungal taxa only accounted for 15% (ST-U) to 35% (L-U) of total RA. This difference is also reflected in the Pielou indices (Table 2a and b).

The core microbiome for the studied soils was calculated to show taxa that are present at all locations. To be regarded core, an ASV had to be present in 80% of the samples with a minimum RA of 0.1%. The analysis again corroborated the huge variation between the different soils. For bacteria, 22 genera built the core microbiome, which mainly belonged to Actinobacteria and Alphaproteobacteria (Fig. 6A). *Bacillus*, *Arthrobacter*, *Bradyrhizobium*, unknown KD4-96 uncultured bacterium, *Blastococcus* and *Terrabacter* were the most abundant taxa. With *Gaiella*, *Sphingomonas*, *Streptomyces* and *Nakamurella*, known candidates of apple rhizosphere inhabitants were also detected.

For fungi, similar effects were noted (Fig. 6B). Here, the core microbiome consisted of eleven genera belonging to Tremellomycetes, Leotiomycetes and Sordariomycetes, including *Solicoccozyma*, *Pseudogymnoascus*, *Humicola*, *Gibellulopsis*, *Tetracladium* and *Fusarium*. In contrast to the bacteria, however, each soil contained two dominant fungal genera, which made up almost 50% or more of the total RA of the core microbiome. These were *Tetracladium* (43.1%) and U. Ascomycota (23.1%) in M-U, *Solicoccozyma* (24.3%) and *Fusarium* (29.2%) in C-U, *Pseudogymnoascus* (36.6%) and *Humicola* (39.4%) in Ha-R-U, *Solicoccozyma* (13.9%) and *Gibellulopsis* (44.9%) in L-U and *Solicoccozyma* (32.7%) and *Pseudogymnoascus* (16.5%) in ST-U. This observation was also reflected in the lower Pielou indices in fungal communities compared to bacterial communities in this study (Table 2).

Table 2 Alpha diversity indices of bacterial (a) and fungal (b) communities in rhizospheres extracted after 8-week-growth of M26 plants in untreated (U) variants from 5 different sites (M, C, Ha-R, L, ST)

Soil	Shannon	Observed	Pielou
a) Bacterial communities			
M-U	6.98 ± 0.21^{a}	2007 ± 469^{a}	0.92 ± 0.00^{a}
C-U	6.86 ± 0.16^{a}	2270 ± 412^{a}	0.89 ± 0.01^{b}
Ha-R-U	6.42 ± 0.48^{a}	1351 ± 578^{a}	0.91 ± 0.02^{ab}
L-U	6.71 ± 0.50^{a}	1856 ± 800^{a}	0.91 ± 0.01^{b}
ST-U	6.53 ± 0.29^{a}	1402 ± 473^{a}	0.91 ± 0.01^{ab}
b) Fungal communities			
M-U	4.51 ± 0.39^{abc}	459 ± 85^{a}	0.74 ± 0.06^{abc}
C-U	4.37 ± 0.25^{ab}	569 ± 42^{a}	0.69 ± 0.04^{ab}
Ha-R-U	3.77 ± 0.15^{c}	490 ± 81^{a}	0.61 ± 0.03^{c}
L-U	4.64 ± 0.31^{a}	706 ± 153^{a}	0.71 ± 0.03^{a}
ST-U	3.94 ± 0.20^{bc}	494 ± 48^{a}	0.64 ± 0.03^{bc}

Given are means ± SD of 6 replicates. Significance was tested by Wilcoxon test and adjusted by 'Bonferroni' ($p < 0.05$). Different letters indicate significant differences of the same index between the variants

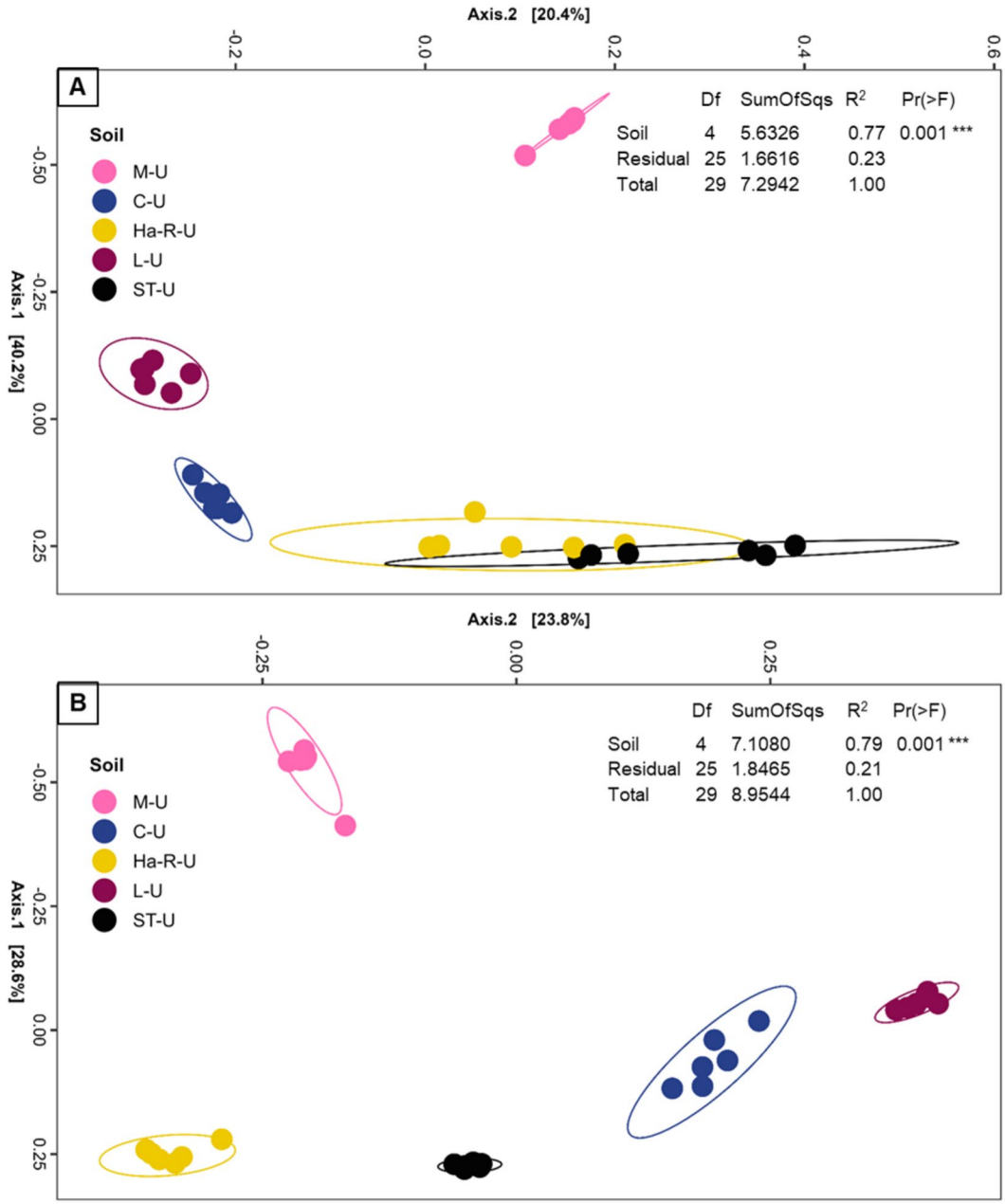


Fig. 4 PCoA clustering and PERMANOVA based on Bray–Curtis distance according to beta diversity of bacterial communities (**A**) and fungal communities (**B**) in rhizospheres extracted after 8-week-growth of M26 plants in untreated (U) ARD soils from 5 different sites (M, C, Ha-R, L, ST). Significant differences between two soils were tested by pairwise PERMANOVA and are displayed in Suppl. Table 6

Correlation analysis

In order to link the data of the different analyses, a correlation analysis was carried out. Of the three genes examined in this study, only the expression level of *BIS3* was significantly, negatively correlated with the growth parameters (Fig. 7). As previously shown, *B4H* showed a similar pattern of expression as *BIS3* in the different variants. However, *B4H* did not significantly correlate with growth. Most of the phytoalexins that were upregulated in the ARD variants correlated with both growth (negatively) and *BIS3* expression (positively).

In the rhizosphere, bacteria belonging to the genera *Bacillus*, *Bradyrhizobium* and *Nakamurella* were among those positively correlated with plant growth. Additionally, there were some unassigned genera that correlated positively with growth, namely U. Bacillales and U. Gaiellales as well as U. JG30-KF-AS9 and U. IMCC26256. In contrast, species belonging to *Sphingomonas*, *Gaiella*, *Nocardioides* and *Piscinibacter* were negatively correlated with growth.

None of the selected fungi in the rhizosphere correlated negatively with plant growth. This included the genera that were most abundant in the variants with the highest growth reduction (M-U and C-U). A few positive correlations with plant growth were observed for the genera *Pseudogymnoascus*, *Humicola*, *Pseudeurotium* and *Saitozyma* as well as unassigned genera of the family Heliotales.

In addition, correlations between microorganisms and phytoalexins were observed. Notably, bacteria of the order Bacillales, Gaiellales, IMCC26256 and JG30-KF-AS9 as well as the genus *Bacillus* correlated negatively with all phytoalexins included in this study, except for JG30-KF-AS9, which showed no significant correlation with 2-hydroxy-4-methoxydibenzofuran. In contrast, bacteria of the genus *Gaiella*, which are part of Gaiellales, were positively correlated with almost all phytoalexins. Furthermore, the genera *Sphingomonas* and *Nocardioides* were positively correlated with all phytoalexins and *Piscinibacter* was likewise positively correlated with most phytoalexins but aucuparin and 2-hydroxy-4-methoxydibenzofuran.

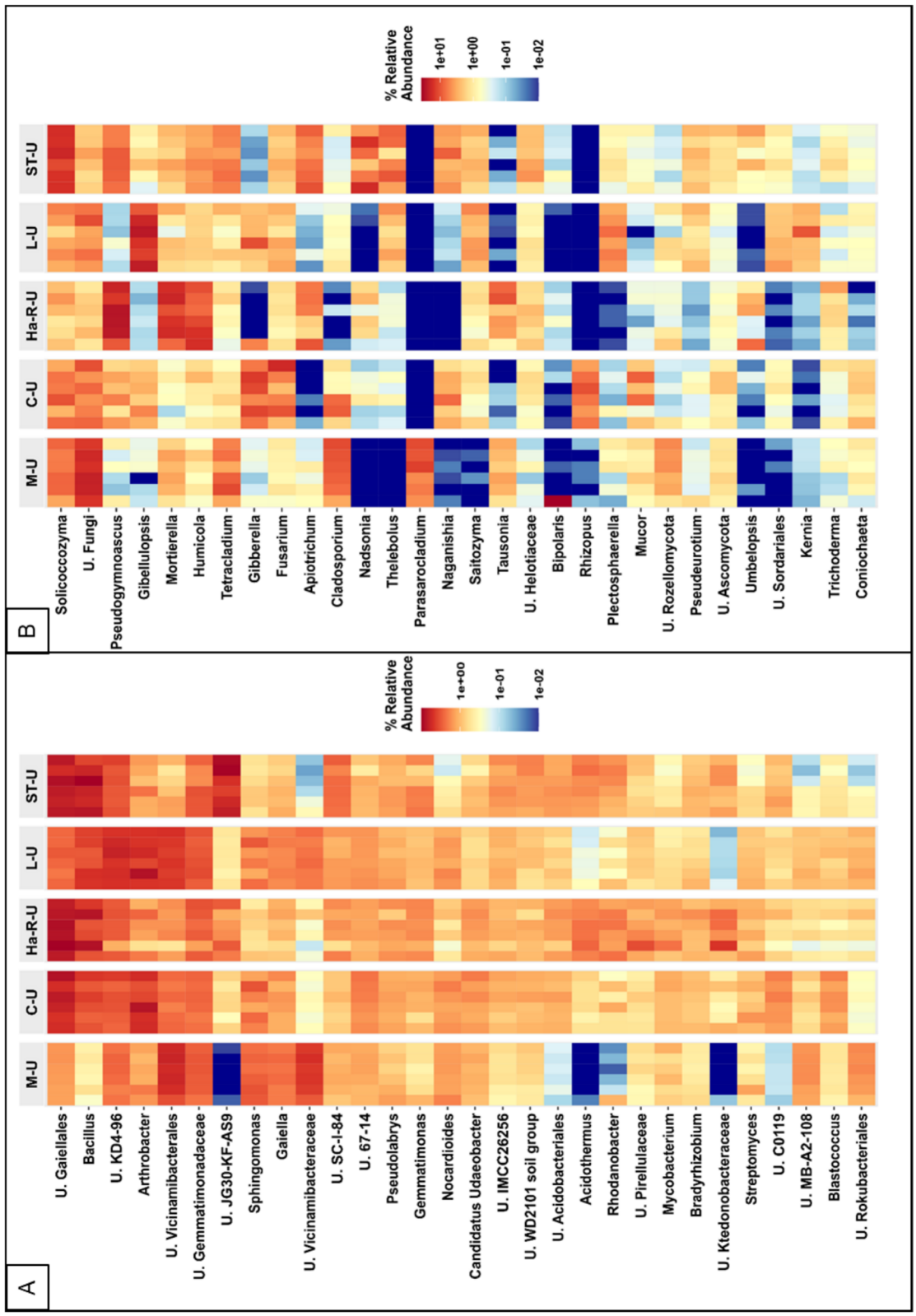
Fungi, which are part of the core microbiome, were less clearly correlated to phytoalexins compared to bacteria. Surprisingly, we mostly observed negative correlations, with only two exceptions: *Gibellulopsis* and *Tetracladium* correlated positively to 3-hydroxy-5-methoxybiphenyl and eriobofuran, respectively. Only *Pseudogymnoascus*, *Humicola* and U. Heliotales were negatively correlated with more than two phytoalexins, including 3-hydroxy-5-methoxybiphenyl, noraucuparin and eriobofuran for all three groups. 2-Hydroxy-4-methoxydibenzofuran was the only analysed phytoalexin that did not show a significant correlation with any of the fungi of the core microbiome.

Interestingly, some of the microorganisms were only correlated with the hydroxynoreriobofuran isomer 4; *Bradyrhizobium*, U. KD4-96, *Terrabacter*, *Oryzihumus* and *Saitozyma* correlated negatively, while *Tetracladium* correlated positively.

Discussion

In this study, significant growth reduction due to ARD was observed in the performed biotests for five soils with different apple replant histories. The shoot fresh mass reduction in untreated soils compared to gamma-irradiated soils ranged between 40 and 59%. This is in line with previous studies, which also observed shoot growth reductions for M26 under similar growing conditions in ARD-affected soils (Mahnkopp et al. 2018; Siefen et al. 2024; Yim et al. 2015). It also confirms the disease being present in the tree nurseries and apple orchards selected for this study. The root fresh mass was significantly reduced in U variants of three soils: L, Ha and ST. Although soils C and M led to lower, insignificant reductions in the root fresh mass, the highest shoot fresh mass reductions were observed for these two soils. This means that the severity of ARD in a soil does not necessarily affect shoot growth and root growth equally. In fact, the Pearson correlation (Fig. 7) showed only a medium positive correlation between shoot FM and root FM (+0.71).

Allometric growth in plants has been studied for a long time (Huxley and Teissier 1936). In previous studies, changes in the shoot:root ratio have been attributed to the compensation for constraints (e.g. root system damage), by aptly adjusting energy


A
M-U
C-U
Ha-R-U
L-U
ST-U
% Relative Abundance
1e+00
1e-01
1e-02
U. Gaiellales
Bacillus
U. KD4-96
Arthrobacter
U. Vicinamibacterales
U. Gemmatimonadaceae
U. JG30-KF-AS9
Sphingomonas
Gaiella
U. Vicinamibacteraceae
U. SC-I-84
U. 67-14
Pseudolabrys
Gemmatimonas
Nocardioides
Candidatus Udaeobacter
U. IMCC26256
U. WD2101 soil group
U. Acidobacteriales
Acidothermus
Rhodanobacter
U. Pirellulaceae
Mycobacterium
Bradyrhizobium
U. Ktedonobacteraceae
Streptomyces
U. C0119
U. MB-A2-108
Blastococcus
U. Rokubacteriales
B
M-U
C-U
Ha-R-U
L-U
ST-U
% Relative Abundance
1e+01
1e+00
1e-01
1e-02
Solicoccozyma
U. Fungi
Pseudogymnoascus
Gibellulopsis
Mortierella
Humicola
Tetracladium
Gibberella
Fusarium
Apiotrichum
Cladosporium
Nadsonia
Thelebolus
Parasarocladium
Naganishia
Saitozyma
Tausonia
U. Helotiaceae
Bipolaris
Rhizopus
Plectosphaerella
Mucor
U. Rozellomycota
Pseudeurotium
U. Ascomycota
Umbelopsis
U. Sordariales
Kernia
Trichoderma
Coniochaeta

◂**Fig. 5** Relative abundance (RA) of top 30 genera, sorted by mean RA across all soils, of the bacterial (**A**) and fungal communities (**B**) in rhizosphere soil extracted after 8-week-growth of M26 in untreated (U) ARD soils from five different sites (M, C, Ha-R, L, ST; 6 replicates each)

investments, (e.g. by upregulating plant defence mechanisms or root growth) (Mokany et al. 2006; Wilson 1988). A number of compounds with antimicrobial properties, including phloridzin (Börner 1960; Hofmann et al. 2009; Raa 1968) and phytoalexins (Reim et al. 2020; Weiß et al. 2017b), were reported to be produced in the roots of apple plants grown in ARD-affected soils. Yim et al. (2013, 2015) proposed that root browning and higher lignin contents in the outer layers of roots could be the consequence of the oxidation of phenolic substances in the roots.

Soil abiotic factors play an ancillary role

Different grain size fractions in soils might cause varying shoot:root ratios. For example, it is easier for plants to form roots in rather light, sandy soil than heavy, loamy soil (Poeplau and Kätterer 2017; Scandellari et al. 2010). Moreover, higher clay content has been repeatedly reported in conjunction with less ARD severity compared to sandy soils (Kviklys et al.

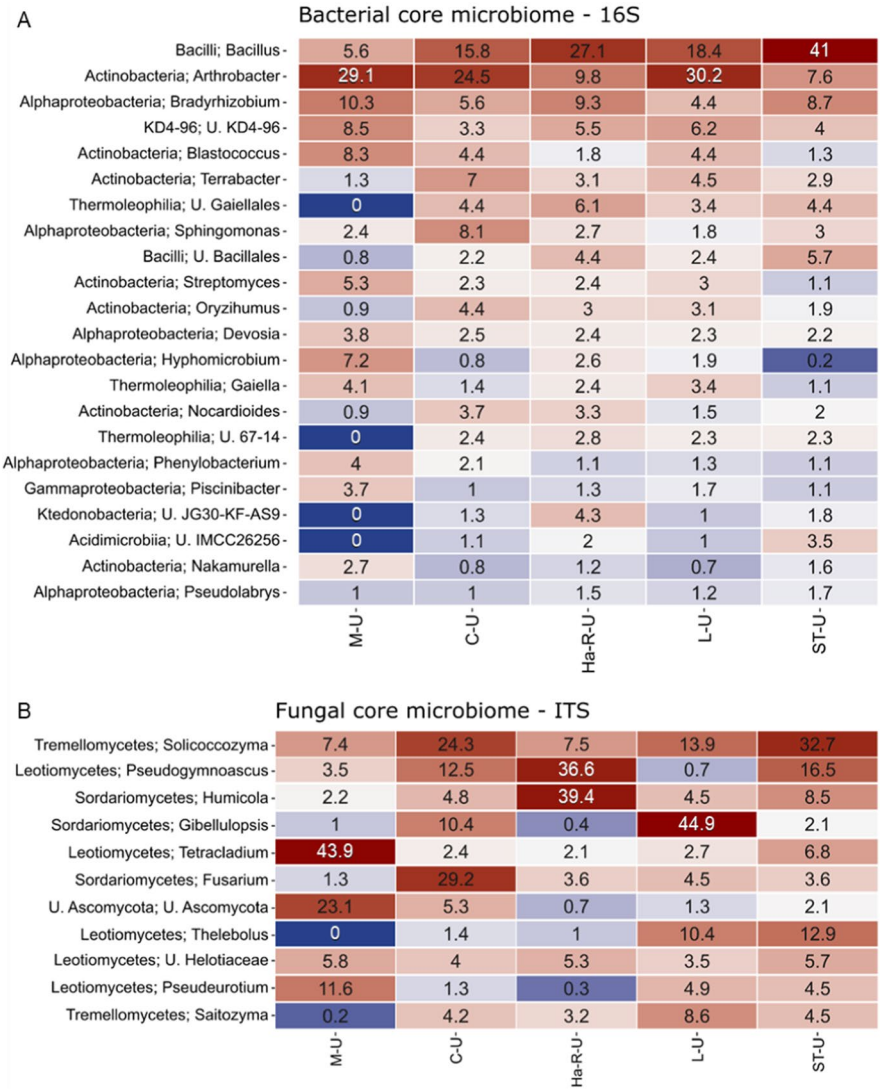


Fig. 6 Core microbiome taxa on genus level for bacterial 16S (**A**) and fungal ITS (**B**) partial sequences of microbial rhizosphere communities, extracted after 8-week-growth of M26 plants in untreated (U) ARD soils from 5 different sites (M, C, Ha-R, L, ST). The core microbiome was calculated using R-package 'ampvis2' (v2.7.33) with an abundance cut-off of 0.1% and a frequency of min 80%. Numbers displayed are relative abundances in percent of a taxon of the core microbiome in the respective soils

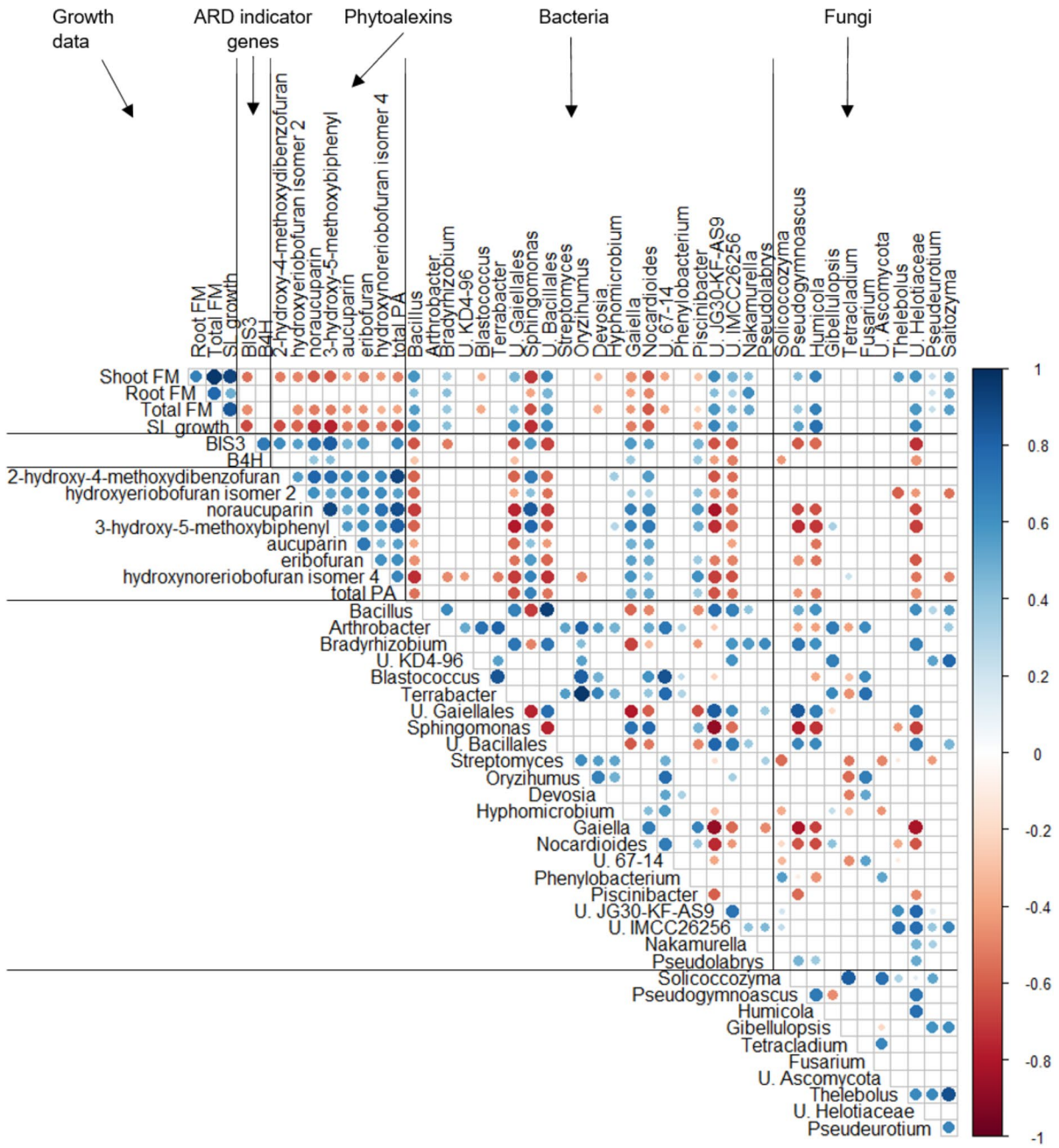


Fig. 7 Spearman correlations between growth data (shoot fresh mass = Shoot FM, Root fresh mass = Root FM, total fresh mass = total FM, shoot length growth = SL growth), relative expression of ARD indicator genes (*biphenyl synthase 3* = *BIS3*, *cytochrome P450 CYP736A12-like* = *biphenyl-4-hydroxylase* = B4H), phytoalexin contents (total phytoalexin contents = total PA) as well as relative abundances of bacterial and fungal genera in the rhizosphere (RA > 0.1%, > 80% frequency). Parameters are derived from the same 6 plants of each untreated (U) soil variant (M-U, C-U, Ha-R-U, L-U and ST-U), resulting in 30 data points per parameter. Significant positive correlations are shown as blue circles and significant negative correlations are shown as red circles ($p < 0.05$). Darker colours and larger circles indicate higher correlations

2016; Mahnkopp et al. 2018; Schimmel et al. 2024), although a conclusive statement on this topic cannot be made based on current literature. Soil texture can also strongly affect the soil–water-content, as loamy soils with fine pores are significantly more prone to waterlogging than sandy soils with comparatively large pores. The anaerobic conditions during waterlogging directly and indirectly affect root growth by changing the physiological conditions for the plant and the soil microbiome, respectively. In this study, while the relative abundances of bacteria and fungi comprising the core rhizosphere microbiome (present in 80% of the samples at > 0.1% RA) differed drastically between soils, a clear connection of certain strains to the severity of ARD could not be made.

The two soils with the highest total growth reduction, M and C, were on opposite sides of the spectrum in terms of grain size fractions. C was a very light soil with 93.7% sand and only 2.6% clay, while M consisted of only 7% sand but had a relatively high clay fraction of 21.3%. While these results seem to oppose previous reports on the effect of clay content at first glance, all these studies, including this one, are based on a comparison of a rather limited number of soils and thus should not be taken as conclusive. This study is also not the first one to report relatively high ARD severity in a soil with high clay content, as research by Sheng et al. (2019) showed similar results. Furthermore, the replant histories of the sites in this study align well with the severity of ARD. The soil ST had only faced one year of apple cultivation and also showed the lowest growth depression. Soils from sites M and C, which showed the highest growth depression, had been most extensively used for apple cultivation. Lastly, *Tagetes*, which was reported to somewhat alleviate ARD in some cases (Kanfra et al. 2021), was used in management of soils at the sites ST and L, possibly reducing the severity of ARD. This indicates that apple replant history and management mainly determined the development of ARD regardless of grain size fractions and other abiotic factors. However, the possibility of a joint effect by multiple abiotic factors, including grain size fractions, cannot be discarded. To approach a conclusive answer in this regard, an experiment with a large number of soils with heterogeneous soil properties is needed.

Higher expression of *BIS3* is reflected in higher phytoalexin contents

The phytoalexins detected in apple roots in this study can be divided into two biosynthetically related categories: biphenyls and dibenzofurans (Busnena et al. 2023). All biphenyls found, i.e. 3-hydroxy-5-methoxybiphenyl (RI = 1956), aucuparin (RI = 2090) and noraucuparin (RI = 2121), were only produced in roots grown in untreated soils. However, the amounts detected for these three phytoalexins were not equally distributed across all soils. Roots from the soils M-U, C-U and L-U contained considerably higher amounts of the biphenyls than roots from the other two untreated soils. These differences can be attributed to increased production of 3-hydroxy-5-methoxybiphenyl and noraucuparin in roots from the M-U, C-U and L-U soils, while the aucuparin contents were low and more or less the same in all untreated variants. Aucuparin is derived from noraucuparin by a single methylation reaction, catalysed by *O*-methyltransferase 2 (OMT2) (Khalil et al. 2015). This particular reaction step appears to be poorly efficient in the roots tested, resulting in a meager formation of aucuparin. For dibenzofurans, the formation of 2-hydroxy-4-methoxydibenzofuran and hydroxyeriobofuran isomer 2 was strongly stimulated in roots from all untreated soils.

Among the tested ARD indicator genes, both *BIS3* and *B4H* are part of the phytoalexin biosynthetic pathway, especially biphenyl formation (Busnena et al. 2023). Dibenzofurans are derived from biphenyls (Khalil et al. 2013), although the enzymes involved have not yet been published. Previous research showed consistent patterns of *BIS3* and *B4H* expression and phytoalexin contents in apple roots (Reim et al. 2020). In this study, the normalised expression levels of *BIS3* and *B4H* were significantly increased in roots from untreated M, C, and L soils. Among the tested ARD indicator genes, *BIS3* displayed the highest correlations to plant growth parameters and phytoalexin contents. Increased phytoalexin contents not only reflect increased concentrations inside the roots but also enhanced phytoalexin levels in the rhizosphere. Busnena et al. (2021) reported the exudation of phytoalexins by M26 roots into the surrounding soil. Interestingly, different phytoalexins were exuded to different

extents, indicating controlled mechanisms rather than simple tissue disintegration. Due to their antimicrobial activities (Busnena et al. 2024), the exuded phytoalexins appear to play an important role in shaping the soil microbiome, thereby influencing ARD severity.

The tested ARD indicator genes also included *ERF1B*. Expression of this gene was previously shown to be influenced by some abiotic stressors such as heat stress (Rohr et al. 2020). In the present study, its expression was not correlated to ARD severity, as indicated by the results for plant fresh mass reduction.

Low abundance of possible pathogenic members of the ARD complex

Members of the fungal phylum Ascomycota are often discussed as possible pathogens in the ARD complex. Among them, especially genera of the Nectriaceae, particularly *Fusarium, Cylindrocarpon*, *Ilyonectria*, *Nectria* and *Dactylonectria*, were often identified in studies on ARD-affected apple roots (Grunewaldt-Stöcker et al. 2021; Manici et al. 2017, 2018; Popp et al. 2020; Tewoldemedhin et al. 2011b). These taxa contain many known plant pathogens (Lawrence et al. 2019; Stępień 2022). Their ability to form long-lasting chlamydospores coincides with the persistence of ARD in soil (Hoestra 1968). Microscopic evidence of chlamydospores in ARD-affected root segments was previously published (Grunewaldt-Stöcker et al. 2020, 2021). In addition, molecular barcoding on diseased roots and their rhizosphere repeatedly revealed increased relative abundance of Nectriaceae in ARD-affected variants compared to control variants (Balbín-Suárez et al. 2021; Popp et al. 2020). However, some fungi that are usually described as pathogenic in conjunction with ARD, such as *Rhizoctonia* (Manici et al. 2003; van Schoor et al. 2009), *Fusarium* (Wang et al. 2018; Xiang et al. 2021) and *Cylindrocarpon/Ilyonectria* (Mazzola 1998; Mazzola and Manici 2012; Popp et al. 2020), were not found in significantly increased abundances in ARD in other studies (Radl et al. 2019; Sun et al. 2017).

In this study, no fungal genus was identified to consistently appear in high abundance in the rhizosphere across all five examined soils, even though apple plants in all soils showed typical above- and below-ground ARD symptoms. *Cylindrocarpon*, *Nectria* as well as *Dactylonectria* were not identified within the top 30 most abundant genera in the rhizosphere of untreated soils. While *Dactylonectria* appeared in all soils with mean relative abundances between 0.01% (ST-U) and 0.42% (M-U), *Cylindrocarpon* and *Nectria* were only present at low relative abundances in some soils (Suppl. Table 5). *Ilyonectria* was relatively low abundant in all soils, with the highest relative abundance in C (0.12%). *Fusarium* was detected in the rhizosphere of all soils but only occurred in relatively high abundance in C-U. *Rhizoctonia* was only detected in a single sample in Ha-R soil.

Bacterial genera, such as *Streptomyces*, *Sphingomonas*, *Rhodanobacter*, *Nocardioides*, *Bradyrhizobium* and *Bacillus*, which had previously been reported in ARD soil (Radl et al. 2019), were also detected among the top 30 genera in all soil sites (Fig. 5, Supplementary Table 3). Negative plant growth was correlated to *Sphingomonas*, *Gaiella*, *Nocardioides* and *Piscinibacter.* All four genera are commonly found in soil and can have diverse effects on plant health, depending on the species and environmental conditions through various mechanisms, including nutrient cycling, disease suppression, and interactions with plant roots (Wang et al. 2021). Only for *Nocardioides*, a clear link to ARD was described (Nicola et al. 2018). Research suggests that certain species of *Nocardioides* may play a role in the disease process, as exemplified by associations between increased abundance of Nocardioides and the presence of ARD symptoms in replanted apple orchards (Nicola et al. 2018). However, the specific mechanisms by which *Nocardioides* species contribute to ARD are not yet fully understood. Other bacterial genera that are often described as part of the ARD disease complex were not correlated with negative plant growth in our study.

Bacterial taxa, such as *Variovorax* (Mazzola 1999), *Rhizobium* (Spath et al. 2015) and *Streptomyces* (Lucas et al. 2018), and fungal ARD pathogen including genera, such as *Rhizoctonia* (Mazzola and Manici 2012), *Cylincdrocarpon/Ilyonectria* and *Dactylonectria* (Manici et al. 2018; Popp et al. 2020), which have been described to be enriched in ARD-affected soils were not found in high abundances in our study. This indicates that ARD is not always caused by the same conglomerate of pathogens but rather by soil-dependent groups that, despite compositional differences, exhibit similar pathogenic functionality.

Correlation of microorganisms and phytoalexin production

Phytoalexins are an integral part of the defence mechanisms in *Malus* spp. (Busnena et al. 2023). Increased production of phytoalexins in roots faced with replant diseased soil has been shown repeatedly (Busnena et al. 2021; Reim et al. 2020; Siefen et al. 2024; Weiß et al. 2017a, b). Furthermore, Busnena et al. (2021) reported compound-specific exudation rates for PAs, suggesting that these defence compounds may differentially inhibit soil microorganisms and hence be involved in shaping the microbial community. Since the production of PAs is mainly induced through plant–microbe interaction, it is interesting to link the production of certain compounds to the abundance of microbial responders in the soil. Because the production of PAs was also negatively correlated to plant growth, negative correlations between microorganisms and PA contents do not necessarily reflect a causal relationship between the occurrence of microorganisms and the absence of phytoalexin biosynthesis. Instead, either high abundance of plant growth promoting bacteria, leading to increased plant growth, or mere coincidence are the most likely explanations. The microorganisms, which occurred in high abundance while the PA production was lower, obviously did not induce the production of PAs. A pathogenic attack on the plant is thus unlikely. Furthermore, regulation of specific genes by plant beneficial bacteria was reported before. However, in case of PAs, downregulation of genes involved in the production of these compounds has, to the best of our knowledge, only been reported for pathogens—e.g. *Pseudomonas syringae* and *Pseudomonas phaseolicola* (Gnanamanickam and Patil 1977; Jakobek et al. 1993). On the other hand, non-pathogenic bacteria are generally reported to upregulate these genes instead. Some *Bacillus* spp. were shown to elicit induced systemic resistance (ISR) (Kloepper et al. 2004), i.e. production of PAs, via the mitogen-activated protein kinase based signalling (Xu et al. 2023) or by increasing the activity of phenylalanine ammonia lyase (Zhang et al. 2022).

In our study, negative correlations between most PAs and microorganisms of the core microbiome were observed for the bacterial taxa *Bacillus*, U. Bacillales, U. Gaiellales, U. JG30-KF-AS9 and U. IMCC26256 as well as the fungal taxa *Pseudogymnoascus*, *Humicola* and U. Helotiaceae. *Bacillus* spp. are widely known as plant beneficial bacteria (Kloepper et al. 2004). Aside from ISR, *Bacillus* spp. are also known for their ability to produce plant beneficial substances such as hormones (Ahmed and Hasnain 2010; Raddadi et al. 2008) and antibiotics (Cazorla et al. 2007; Leifert et al. 1995; Reyes-Ramírez et al. 2004). Little is known about JG30-KF-AS9 or IMCC26256 except for their common occurrence in farmland soil (Hu et al. 2018; Liu et al. 2021b; Wu et al. 2022; Xing et al. 2023). They are not known to be pathogens. Bacteria of the genus JG30-KF-AS9 seem to adapt well to acidic and heavy metal contaminated soils (Wu et al. 2022; Yu et al. 2022), which aligns well with their highest abundance in the most acidic soil in this study (Ha-R, Suppl. Table 1). *Pseudogymnoascus* was previously associated with nematodes in ARD soil (Kanfra et al. 2022). Furthermore, O'Donoghue et al. (2015) demonstrated the production of cuticle-degrading subtilisin peptidases by *Pseudogymnoascus destructans*, which are anti-fungal compounds utilised by some nematode-trapping fungi (Yang et al. 2007). *Humicola* are known for their extensive ability to metabolise complex carbon sources and produce a variety of bioactive compounds, including many antifungal and antimicrobial substances (Ibrahim et al. 2021). This might suggest some involvement in the development of disease suppression in the soil.

Among the most abundant bacteria, the genera *Sphingomonas* and *Nocardioides* were positively correlated with all tested phytoalexins in this study, followed by *Gaiella* and *Piscinibacter* which were positively correlated with all phytoalexins but 2-hydroxy-4-methoxydibenzofuran and, in case of *Piscinibacter*, also aucuparin. An enrichment of these genera is not surprising as both *Sphingomonas* (Jiang et al. 2022a, b; Zhou et al. 2016) and *Nocardioides* (Ma et al. 2023; Wang et al. 2018) were reported as genera that are highly capable of degrading polycyclic aromatic hydrocarbons, such as biphenyls and dibenzofurans. Although little is known about the functionality of *Gaiella* and *Piscinibacter*, both were previously debated as potential degraders of aromatics (Rutere et al. 2020; Zhang et al. 2023). Interestingly, the genus *Hyphomicrobium* correlated positively with only two phytoalexins: 2-hydroxy-4-methoxydibenzofuran and 3-hydroxy-5-methoxybiphenyl. The 2'-hydroxylation of 3-hydroxy-5-methoxybiphenyl and the subsequent intramolecular cyclisation to form

2-hydroxy-4-methoxydibenzofuran were proposed to be the branching point between the biphenyl and dibenzofuran biosynthetic pathways in apple (Busnena et al. 2023). The correlation of *Hyphomicrobium* and the branching point intermediates could hence suggest a specific plant response to one or more organisms in this genus. However, *Hyphomicrobium* spp. are commonly cited as degraders rather than pathogens (Meng et al. 2024; Moore 1981; Zhao et al. 2016). Thus, a role of *Hyphomicrobium* spp. in the degradation process of these specific compounds is more likely.

Of the most abundant fungi in the rhizosphere, only *Gibellulopsis* and *Tetracladium* showed a positive correlation with a single phytoalexin each, 3-hydroxy-5-methoxybiphenyl and hydroxynoreriobofuran isomer 4, respectively. *Gibellulopsis* was reported as both pathogen, e.g. in chrysanthemum (Kawaradani et al. 2013), and plant beneficial organism which can induce systemic resistance (Feng et al. 2023; Hao et al. 2022). The interaction with mitogen-activated protein kinase, and thus the production of PAs, is known (Feng et al. 2023) but not yet proven for apple. *Tetracladium* is mostly found as a decomposer in aquatic environments (Grossart et al. 2019) but is also present in and on terrestrial plants and soil (Selosse et al. 2008). The antifungal capacity of some of its species was previously proven (Sati and Arya 2010). Lazar et al. (2022) showed the recruitment of *Tetracladium* spp. by *Brassica napus* as root endophytes from the soil. While the exact role of *Tetracladium* in our study remains unclear, pathogenicity is unlikely. Based on previous research, it is more likely involved in decomposition of dying root material but further research is needed.

The plethora of different functions and traits associated with microbiomes shaped by ARD underpins the multicausal and multisymptomatic nature of the disease complex, in turn also calling for a complex management approach instead of targeting a single pathogen.

Conclusions

ARD symptoms such as black roots and severe growth depression were observed on all five soils examined in this study. Soil parameters, such as grain size fraction, play an ancillary role in the development of ARD, whereas our data stresses the importance of replant history over abiotic factors in the development of ARD. *BIS3* expression was clearly upregulated in ARD soils compared to the disinfected controls. Higher expression levels also coincided with more severe ARD symptoms, confirming the suitability of *BIS3* as ARD indicator. Consequently, the production of phytoalexins was also higher in roots of more severely affected plants. Interestingly, a number of PAs, namely 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, norerioboufuran, methoxyeriobofuran isomer 4 and hydroxyeriobofuran isomer 5, were only found in ARD-affected roots. This clearly indicates the specific induction of the involved enzymatic reactions by microorganisms enriched in ARD soils.

Although ARD severity was verified, it is very difficult to verify common microbial responders as causal agents of ARD. Furthermore, enriched bacteria and fungi found on the basis of amplicon sequencing were discussed based on previous publications, however, revealing their functionality and relevance needs more in-depth studies involving metagenomic finger printing, isolation of ARD-related strains and inoculation experiments. Bacterial responders to ARD, such as *Nocardioides* and *Sphingomonas*, are likely to be involved in the degradation of phenolic compounds, for example PAs, as highlighted by our correlation analysis. Previously suggested ARD-related pathogenic genera occurred only rarely in our experiment. Either the occurrence of these genera may be of secondary nature and is not necessary for initial disease formation or ARD is caused by soil-dependent groups of pathogens that, despite compositional differences, exhibit similar pathogenic functionality. Based on this and previous studies, we suggest an experiment involving a high number of ARD-affected soils to be more decisive for understanding this complex phenomenon. Furthermore, a more in-depth approach is needed to reveal the functionality of the microbiome in ARD soils.

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Author contributions TW designed the experiment. TW and NO conducted the experiment. NO drafted the initial manuscript. Sample analyses and data evaluation were split between authors as follows: growth parameters and correlation analysis by NO and TW, gene expression by NO, soil parameters by JK, GG and JB, phytoalexins contents by BL and LB, and rhizosphere microbiome analyses by SB, FM and MS. TW, GG, LB and MS also filled advisory roles. All authors helped interpreting the data and contributed to the manuscript by writing, reading and revising.

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Data availability Raw data of growth parameters and phytoalexin contents and the Cq-values of from the qPCR of the ARD-indicator and housekeeping genes is publicly available online at the BonaRes Repository: https://doi.org/https://doi.org/10.20387/bonares-jst2-ww4r

Microbiome data is available as raw reads in the Sequence Read Archive (SRA) of NCBI Database, under Bioproject number https://www.ncbi.nlm.nih.gov/sra/PRJNA1158719 with Biosample numbers SAMN43546314 to SAMN43546377.

Declarations

Competing interests The authors do not have any relevant financial or non-financial interests to disclose.

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