Bacterial endophytes in apple (*Malus domestica*) rootstocks grown in apple replant disease affected and non-affected soils

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Abstract

The apple replant disease (ARD) is a complex phenomenon, which affects apple orchards and nurseries worldwide. It occurs when apple is repeatedly planted at the same site, leading to growth depressions and reductions in fruit yield and quality. Despite decades of research, the etiology is poorly understood. However, since soil disinfection leads to improved plant growth, soil (micro)biota are known to be involved. Microbes can colonize roots from the rhizosphere, become so called endophytes, and can have a huge impact on plant growth, which may be negative or positive. But up to date, little is known about the role of endophytes in relation to ARD. In this study we hypothesized that the bacterial endophytic microbiome differs in roots of plants growing in replant affected soil from that in roots growing in non-affected soil.

Therefore, a greenhouse biotest was conducted in three years, in which M26 apple plants were planted either into untreated ARD soils or gamma irradiated ARD soils from three different sites. Results showed an increased plant growth in irradiated soil variants. To analyze the endophytic bacterial community structure in roots, surface disinfected roots from these plants were used for a culture independent 16S rRNA amplicon sequencing approach. Results showed that Proteobacteria were the dominant phylum in all variants. Interestingly, the genus *Streptomyces* was shown to be associated with ARD. Several amplicon sequence variants (ASVs) linked to this genus were highly abundant in roots grown in ARD soil from different sites and were negatively correlated to shoot length and shoot fresh mass. The same ASVs were also found in roots of plants growing in ARD soil in the field and were further found to be increasing in their abundance over time after planting in virgin soil. However, whether these members of the genus *Streptomyces* are causal part of the ARD complex or just opportunists remains unanswered.

Moreover, in a culture dependent approach, 150 different isolates were obtained, belonging to 29 different genera with 69 different bacterial species. With 25 different species, *Pseudomonas* was the dominant and most diverse genus. These isolates serve as future inocula to find not only possible ARD causal agents, but also plant growth promoting endophytes, which might help to overcome ARD.

Understanding the role of the endophytic bacterial community in the roots in the context of ARD will help to unravel the etiology of ARD and to develop possible countermeasures.

Key words: apple replant disease, biotest, endophytes, *Streptomyces*

Zusammenfassung

Die Apfelnachbaukrankheit (ARD) ist ein komplexes Phänomen, das Apfelplantagen und Baumschulen weltweit betrifft. Sie tritt auf, wenn Apfel wiederholt am gleichen Standort gepflanzt wird, was zu einer Reduzierung des Wachstums sowie des Ertrags und der Fruchtqualität führt. Trotz jahrzehntelanger Forschung ist die Ätiologie nur unzureichend verstanden. Da jedoch die Desinfektion des Bodens zu einem verbesserten Pflanzenwachstum führt, wird von einer Beteiligung des Boden(mikro)bioms ausgegangen. Organismen können Wurzeln von der Rhizosphäre aus besiedeln, werden dann zu so genannten Endophyten und können einen großen Einfluss auf das Pflanzenwachstum haben, sowohl negativ als auch positiv. Allerdings ist bis heute wenig über die Rolle der Endophyten in Bezug auf ARD bekannt. In dieser Arbeit wird die Hypothese geprüft, dass sich das bakterielle endophytische Mikrobiom in den Wurzeln von Pflanzen, die in von der Nachbaukrankheit betroffenen Böden wachsen, ivon dem in nicht betroffenen Böden unterscheidet.

Daher wurde in drei Jahren ein Gewächshaus-Biotest durchgeführt, in dem M26-Apfelpflanzen entweder in unbehandelten ARD-Boden oder in gammabestrahlten ARD-Boden von drei verschiedenen Standorten gepflanzt wurden. Die Ergebnisse zeigten ein besseres Pflanzenwachstum in den bestrahlten Bodenvarianten. Um die Struktur der endophytischen bakteriellen Gemeinschaft in den Wurzeln zu analysieren, wurden oberflächendesinfizierte Wurzeln dieser Pflanzen für einen kulturunabhängigen 16S rRNA-Amplikon-Sequenzierungsansatz verwendet. Es zeigte sich, dass Proteobakterien das dominierende Phylum in allen Varianten waren. Interessanterweise konnte gezeigt werden, dass die Gattung *Streptomyces* mit ARD assoziiert war. Mehrere „Amplicon Sequence Variants" (ASVs), die zu dieser Gattung gehören, waren in Wurzeln, die in ARD-Boden von verschiedenen Standorten wuchsen, sehr abundant und negativ mit der Sprosslänge und der Sprossfrischmasse korreliert. Die gleichen ASVs wurden auch in Wurzeln von Pflanzen gefunden, die im Feld in ARD-Boden wuchsen. Weiterhin wurde festgestellt, dass diese ASVs im Laufe der Zeit nach der Pflanzung in gesundem Boden in ihrer Abundanz zunahmen. Ob jedoch diese ASVs der Gattung *Streptomyces* ursächlicher Teil des ARD-Komplexes sind oder nur Opportunisten, bleibt unbeantwortet.

Zusätzlich wurden in einem kulturabhängigen Ansatz 150 verschiedene bakterielle Isolate gewonnen, die 29 verschiedenen Gattungen und 69 verschiedenen Arten zugeordnet werden konnten. Mit 25 verschiedenen Arten war *Pseudomonas* die dominante und diverseste Gattung. Diese Isolate dienen als zukünftige Inokula, um nicht nur mögliche ARD-Verursacher, sondern auch pflanzenwachstumsfördernde Endophyten zu finden, die bei der Überwindung von ARD helfen könnten.

Das Verständnis der Bedeutung der endophytischen Bakteriengemeinschaft in den Wurzeln im Zusammenhang mit ARD wird helfen, die Ätiologie von ARD zu entschlüsseln und mögliche Gegenmaßnahmen zu entwickeln.

Schlagwörter: Apfelnachbaukrankheit, Biotest, Endophyten, *Streptomyces*

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ARD	apple replant disease
ASV	amplicon sequence variant
BMBF	Bundesministerium für Bildung und Forschung
CE	central experiment
cv.	cultivar
DNA	desoxyribonucleic acid
Fig.	figure
G	gamma irradiated
IAA	Indole-3-acetic acid
ISR	induced systemic resistance
n	number
NaDCC	natriumdichlorisocyanurat
NCBI	National Center for Biotechnology Information
OTU	operative Taxonomic Unit
PGP	plant growth promoting
PGPB	plant growth promoting bacteria
qPCR	quantitative polymerase chain reaction
rRNA	ribosomal ribonucleic acid
sp.	species (singular)
spp.	species (plural)
Tab.	table
TMA	trimethylamine
UT	untreated
VOC	volatile organic compound
WP	work package

Contents

1. General introduction

1.1 The economic importance of apple

Apple (*Malus* x *domestica* Borkh. (Korban and Skirvin, 1984)) is the most important temperate tree crop worldwide (Brown, 2012). It is widely cultivated on all continents (except Antarctica) in temperate latitudes and even in high altitudes in the tropics (Luby, 2003). But due to late blooming and cold hardiness, apple has a broad northern range and is best adapted for the cool temperate zone (Kellerhals, 2009). In the year 2019, the worldwide harvested area was 4,717,384 ha (FAOSTAT, www.fao.org/faostat). Since more than 40 years, this total area has only changed marginally. However, in that time the amount of harvested apples has more than doubled, increasing by 127 % and reached 87,236,221 t in 2019. Reasons for this increase are intensified production methods, like dwarfing rootstocks, high planting densities, renewal pruning and the use of growth regulators, resulting in fewer and larger orchards (Luby., 2003; Robinson, 2011). However, the main reason is the increased production area in China, which increased 10-fold in that time period and accounted for around half of the global production in 2019, while the production areas in Europe and USA declined (FAOSTAT). With approx. 40 million t annually, China is also by far the largest apple consuming country.

After bananas and watermelons, apples are the third most produced fruit worldwide (FAOSTAT). The global apple market reached 78.8 billion dollars in 2019 (World - Apple - Market Analysis, Forecast, Size, Trends and Insights, https://fruitgrowersnews.com (Accessed: 27.01.2021)) which represents 16 % of the total fresh fruit revenue worldwide (Statista.com).

For Germany, the apple cultivated area reached 33,905 ha in 2020 (Destatis.de) and was almost constant compared to the last 17 years (FAOSTAT). This area yielded approximately one million t (1,023,316 t) apple fruits in 2020 (Destatis.de). The most important apple production sites in Germany are "Altes Land" and the region of "Lake Constance". In consequence, the highest number of apples was recorded in the federal state of Baden-Württemberg (403,235 t; 12106 ha), followed by Lower-Saxony (279,691 t; 8064 ha). With around 25.5 kg consumed per capita and year, apples are the most popular fruit in Germany (Statista.com). In 2018, 67 % of apples for consumption originated from national production. Regarding apple imports, Italy as the most important country accounted for 31% of all German apple imports of 2018 (Destatis.de).

Moreover, apple is also very important in German nurseries. Of the total fruit tree area of 974 ha (Destatis.de; Baumschulerhebung 2017), apple accounts for two thirds (A. Wrede, personal communication).

1.2 Symptoms and etiology of ARD

Apple replant disease (ARD) is a complex phenomenon affecting apple orchards and apple tree nurseries worldwide, leading to growth reductions and losses in fruit yield and quality (Mazzola and Manici, 2012; Manici et al., 2013; Winkelmann et al., 2019). Roots of affected plants show cell necrosis, blackening, impaired root hair development, and low cell vitality (Grunewaldt-Stöcker et al., 2019). Due to delayed precocity, reduced fruit yield and quality, orchards face severe economic losses during their lifetime (Mazzola, 1998; Van Schoor et al., 2009). Next to apple orchards, apple tree nurseries are highly affected as apple plants are replanted more frequently, leading to a rapid induction of ARD (Winkelmann et al., 2019). Recently, ARD was 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). This disease is species-specific and can persist for decades (Savory, 1966). It occurs, when apple or a closely related species is repeatedly planted at the same site. However, despite decades of research, the etiology of ARD is still not fully understood. It is known that soil disinfection leads to better growth indicating that biotic factors are the primary cause (Mai and Abawi, 1981; Yim et al., 2013; Spath et al., 2015; Mahnkopp et al., 2018). In addition to fungi belonging to the genera *Cylindrocarpon*, *Fusarium*, *Rhizoctonia* and *Cylindrocarpon*-like fungi (Nectriaceae) *Dactylonectria*, *Ilyonectria* and *Rugonectria*, a number of other taxa including various bacterial species such as members of Actinobacteria and the genera *Bacillus* and *Pseudomonas*, but also Oomycetes, such as *Phytophthora* and *Pythium* and even nematodes have been reported to contribute to ARD (Čatská et al., 1982; Utkhede and Li, 1988; Otto et al., 1993; Mazzola, 1998; Tewoldemedhin et al., 2011b; Manici et al., 2017; Kanfra et al., 2018; Popp et al., 2020; Popp, 2020). All these different potential causal organisms in these different studies suggest a disease complex rather than a single pathogen.

Based on molecular barcoding approaches, several studies confirmed not only changes in the abundance of specific pathogens in ARD-affected soils, but significant shifts in the microbiome

community structure of the bulk soil and the rhizosphere (Winkelmann et al., 2019). One of the earliest studies investigating the role of microorganisms in the rhizosphere of ARD-affected plants was conducted by Čatská et al. in 1982. They found an increased number of micromycetes and actinomycetes, whereas the overall number of bacteria, including fluorescent pseudomonads, decreased in the rhizosphere of ARD-affected plants compared to non-affected plants. A decrease of bacterial diversity when replanting apple was also confirmed by Sun et al. (2014). Further, several studies revealed a significant difference of the bacterial community structure in the rhizosphere between ARD-affected and non-affected apple plants (Rumberger et al., 2007; Yim et al., 2013; Sun et al., 2014; Jiang et al., 2017). This dysbiosis, the alteration of diversity and structure of the microbiome of ARD-affected plants compared to healthy plants, was also shown in split-root approaches, especially for the rhizosphere and rhizoplane (Lucas et al., 2018; Balbín-Suárez et al., 2020, 2021). Members of the genera *Streptomyce*s and *Variovorax* were especially enriched in the rhizosphere and rhizoplane in ARD-affected roots (Lucas et al., 2018; Balbín-Suárez et al., 2020, 2021). These significant shifts in the microbial diversity indicate an altered microbe "recruitment" of ARD-affected roots from ARD-affected bulk soil (Balbín-Suárez et al., 2020). Rumberger et al. (2007) showed that these (bacterial) rhizosphere communities were still significantly different from those of non-affected roots even three years after replanting. One reason for these shifted community structures are differences in root exudation patterns of ARD-affected plants. It was shown that under replant conditions the expression of genes responsible for phytoalexin biosynthesis are upregulated in roots (Weiß et al., 2017a; Reim et al., 2020; Rohr et al., 2020; submitted). This upregulation was shown to occur specifically and locally for roots grown in ARD soil (Rohr et al., 2020, submitted), and to be consistent with the phytoalexin content in the roots (Weiß et al., 2017b; Reim et al., 2020). Further, phloridzin was shown to be exuded by roots of apple seedlings at the onset of ARD symptoms (Hofmann et al., 2009) and was released by apple root debris (Nicola et al., 2017b).

Since it is known that plants can actively shape their microbiome, primarily through plant exudates (Hardoim et al., 2008; Bulgarelli et al., 2013; Philippot et al., 2013; Sasse et al., 2018), these apple-specific exudation patterns in the root rhizosphere of ARD-affected plants are responsible for microbe attraction, and consequently, for a different rhizo- and endosphere assembly.

1.3 Apple replant disease: Mitigation strategies and countermeasures

The most obvious strategy to circumvent ARD is simply to avoid replanting of apple in ARD-affected soil, e.g., using crop rotation (Mazzola and Gu, 2000). However, due to high orchard investments (e.g. hail nets, irrigation systems) and lack of areas (Winkelmann et al., 2019; Hanschen and Winkelmann, 2020), other strategies have to be developed.

Steaming of affected soils and exchange of soils are a possible solution, but highly laborious and costly. Planting trees in the inter-row (driving lane) is another way used in apple orchards to overcome ARD. It was shown to significantly increase plant growth (Rumberger et al., 2004; Kelderer et al., 2012). The influence of inter-row-planting on the rhizosphere microbial community structure was discussed controversy. While Kelderer et al. (2012) found no significant effect on rhizosphere bacteria, Rumberger et al. (2004) reported a significant difference of general rhizosphere bacteria and Actinobacteria between row replanted and inter-row-planted apple trees. Although this mitigation strategy is feasible, it can only be done once.

Another approach to overcome ARD is the use of the biocide Basamid ® containing the active ingredient Dazomet which, when getting in contact with water, is hydrolised to methylisothiocyanat. Plants grown in Basamid treated ARD soil showed better growth compared to untreated ARD soil under greenhouse and field conditions (Yim et al., 2013, 2016, 2017). However, since Basamid is toxic for organisms that get into contact with it, and its low degradation rate, its application is only permitted under strict conditions and the registration in Germany will end in May 2024.

Natural isothiocyanates are produced when incorporating Brassicaceae crops or seed meal into the soil. This so called biofumigation was shown to be a promising alternative countermeasure against ARD, recently reviewed by Hanschen and Winkelmann (2020). Several studies could demonstrate significant improvements in apple tree growth using biofumigation treatments (Mazzola and Mullinix, 2005; Mazzola et al., 2007; Mazzola and Brown, 2010; Mazzola et al., 2015; Yim et al., 2016; Yim et al., 2017; Wang and Mazzola, 2019a; Wang and Mazzola, 2019b). Biofumigation further results in a shift of the microbial community structure of the rhizosphere of apple plants (Mazzola et al., 2015; Yim et al., 2016; Wang and Mazzola, 2019a; Wang and Mazzola, 2019b). Mazzola et al. (2015) and Wang and Mazzola (2019a) could show that biofumigation treatments enrich bacterial and fungal taxa associated with pathogen suppression. However, despite promising results, the efficacy is highly site and

soil-dependent and varies depending on the starting material used (Brassicaceae plant species, fresh plant material or seeds, extent of tissue disruption, and amount incorporated into the soil (Hanschen and Winkelmann, 2020)). A similar effect can be observed when growing and incorporating *Tagetes* into the soil prior to apple planting (Yim et al., 2017). This method is commonly used in tree nurseries, but competes with the available area and growth period usually used for producing apple rootstocks and graftings.

Flooding is reported to reduce the ARD effect. The anaerobic conditions are thought to cause shifts in the soil microbiome. These anaerobic conditions were induced by Hewitharana et al. (2014) and Mazzola et al. (2020) by adding different carbon amendments, e.g. rice bran or grass, to the soil, which resulted in a reduced ARD effect. In growth chamber experiments, anaerobic soil disinfection was shown to retard the growth of the potential ARD causing organisms *Rhizoctonia solani* AG–5, *Pythium ultimum* and *Fusarium oxysporum* and reduce the densities of *Pratylenchus penetrans* at apple roots (Hewitharana et al., 2014). In sequence with biofumigation, anaerobic soil disinfection led to an altered fungal but not bacterial rhizosphere community in comparison to roots grown in untreated soils (Mazzola et al. 2020).

Similar effects were observed by Wang et al. (2019) by adding different amounts of biochar to replant diseased soil. Apple seedlings showed improved growth, which was attributed to increased diversity of the fungal community and suppression of the pathogen *Fusarium solani*. A similar approach to improve plant growth and alter the microbial community structure is to add compost to the soil prior planting. While Yao et al. (2006) and Rumberger et al. (2004) detected no increase in plant growth, Franke-Whittle et al. (2019) showed increased apple plant growth in 20 out of 26 evaluated compost variants. Compost amendments prior planting only had little effect on the bacterial community composition (Rumberger et al., 2004) or the effects were diminished in the second year after replanting (Yao et al., 2006) in comparison to control treatments. Changes in the bacterial rhizosphere community composition were rather attributed to different genotypes than to compost treatment in these studies (Rumberger et al., 2004; Yao et al., 2006). In contrast, Franke-Whittle et al. (2019) concluded that differences in plant growth were mainly attributed to changes in the microbial community composition introduced into the soil through the compost. However, the effect appeared to be compost- and soil-specific (Franke-Whittle et al., 2019).

A shift in the microbial community towards more diversity and a higher ratio of beneficial microorganisms was also aimed in various inoculation approaches. Via drench application, Utkhede and Smith (1982) inoculated the plant growth promoting bacterium *Bacillus subtilis* strain EBW-4 to the rootstock M26 grown in ARD-affected soil, which resulted in increased cross-sectional trunk area, total shoot growth, and fruit yield. Increased ARD-affected plant growth was also achieved by Čatská (1994), who inoculated the arbuscular mycorrhizal fungi *Glomus fasciculatum* and *Glomus macrocarpum* to apple seeds directly after sowing. Inoculation with *Glomus fasciculatum* resulted in increased shoot length, and shoot and root dry mass. Further, significant changes in the rhizosphere microbiome (regarding phytotoxic micromycetes and diazotrophic bacteria) in comparison to uninoculated controls were observed. Mehta and Bharat (2013) could confirm the growth promoting effect of *Glomus fasciculatum* on ARD-affected apple plants which, when inoculated, resulted in increased shoot length, and shoot and root dry mass in comparison to unioculated controls. Despite these examples of promising results, inoculants often do not have the expected rhizosphere competence, leading to poor establishing (Winkelmann et al., 2019).

In the long term, breeding of tolerant rootstocks might help to overcome ARD. Up to now, only a few genotypes are described to be less susceptible to ARD (Leinfelder and Merwin, 2006; St. Laurent et al., 2010; Robinson et al., 2012; Kviklys et al., 2016; Reim et al., 2019). New data suggest that this tolerance is soil specific. Further, several possible marker genes for ARD were already detected and can help in the breeding process (Reim et al., 2020; Rohr et al., 2020). However, conventional breeding of a tolerant rootstock will take decades, since the trait ARD tolerance has to be combined with other desired traits like dwarfing, pest resistance, drought tolerance, and the ability to compete with weeds for water and nutrients (Webster et al., 2000). In addition, tolerance test systems are difficult because of the mentioned soil specificity and the problem of proper controls.

Despite several promising approaches to mitigate ARD, none of the mentioned strategies showed consistent results while being both feasible and environmental friendly. Therefore, more research to unravel the etiology of ARD and to overcome ARD is needed.

1.4 The joint project ORDIAmur

This thesis is part of the project ORDIAmur (latin: Let's get started). ORDIAmur (Overcoming the Replant Disease by an Integrated Approach) is a project funded by the BMBF in the framework of BonaRes (soil as a sustainable resource) with the aim to unravel the etiology of ARD and to be able to develop and test environmentally friendly and practically feasible solutions for the challenging problem of ARD. This project started with a first phase in the end of 2015, is currently in the second phase until end of 2021 and will eventually be extended for further three years. In phase I, 16 project partners (plus project coordination) from all over Germany were involved. The ORDIAmur project is structured into five work packages (WPs, Fig. 1.): (WP1) Induction and etiology of ARD, (WP2) changes in rhizosphere and soil, (WP3) plant responses on replant diseased soils, (WP4) management and (WP5) socio-economic studies, which are all linked to each other. These include projects investigating root exudates, soil parameters, plant stress responses, breeding for ARD tolerance, bacterial and fungal endophytes, soil microbiome, nematodes, mesofauna organisms, phytoalexins, and socio-economic parameters. The heart of ORDIAmur are its central experiments. These large experiments are often conducted in cooperation of several different projects and enable sharing of samples among all partners. This gives the advantage of analyzing the same plants or samples by different partners.

This thesis is part of the project P9 which aims to characterize and quantify bacterial root endophytes in rootstocks grown in replant and non-replant soils. To do so, central experiments (biotests) in the greenhouse were conducted (Mahnkopp et al., 2018) from which samples were taken for histological analyses (Grunewaldt-Stöcker et al., 2019) and for culture independent analyses of the endophytic community structure (Mahnkopp-Dirks et al., 2020). Finally, bacterial root endophytes were investigated in roots grown in the field using culture independent and dependent approaches (Mahnkopp-Dirks et al., submitted).
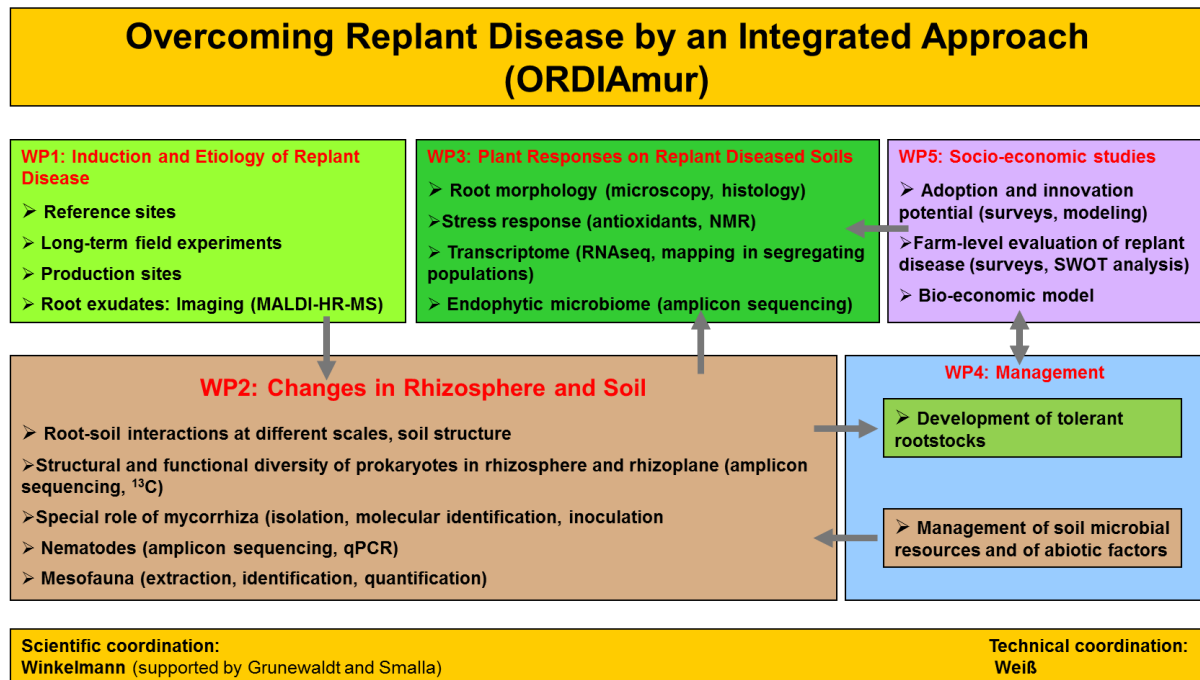
Figure 1: ORDIAmur work package structure

1.5   Bacterial endophytes

Today it is believed that every plant is colonized by at least one type of endophyte (Strobel and Daisy, 2003; Johnston-Monje and Raizada, 2011; Afzal et al., 2019). So far, endophytes have been found in every plant analyzed (Santoyo et al., 2016). Up to now, 16 phyla or more than 200 genera of bacteria have been reported as endophytes in numerous plant species (Kumar et al., 2020). In the last decade, endophytes gained much interest. However, many questions remain unanswered regarding natural history, biogeography, ecology, biodiversity and evolution (Harrison and Griffin, 2020). To date, only 1 – 2 % of all known (ca.) 300.000 plant species have been studied for their endophytic community (Strobel, 2018). A survey of 600 studies revealed that hosts from only 10.5 % of plant families were examined for bacterial endophytes (Harrison and Griffin, 2020).

1.5.1   Definition and ways of plant colonization

Since the term was first mentioned by Heinrich Friedrich Link in 1809 and Anton de Bary in 1866 the definition of endophytes has repeatedly been debated throughout the years. Classically they are defined as microorganisms, which can be isolated from surface disinfected

plant tissue and are not pathogenic (Hallmann et al., 1997; Santoyo et al., 2016). However, it was shown that some endophytes can live as latent pathogens inside the plant and become pathogenic under some circumstances (Kloepper et al., 2013; see chapter: "1.5.3 Effects of endophytes on plants"). Further, with the upcoming of new culture independent approaches employing next generation sequencing methods, isolations are not necessarily needed and pathogenicity assays not performed. This classical definition is therefore more suited for cultivated endophytes (Hardoim et al., 2015). Hardoim et al. (2015) claimed that the term "endophyte" should refer to their habitat, not function, and therefore proposed a more general definition that includes all microorganisms, which for all or part of their lifetime colonize internal plant tissue.

There are several ways how endophytes can colonize internal plant tissues. One way is to enter through the phyllosphere, e.g. through the stomata, but also via herbivorous insect vectors (Frank et al., 2017). But not only horizontal ways are known. Endophytes can also be transmitted vertically via seeds and pollen (Frank et al., 2017). However, soil is the main reservoir of microorganisms for the plant microbiome (Berg and Smalla 2009; Bonito et al. 2014; Lareen et al. 2016; Hartman and Tringe 2019). Most endophytes enter the plant through the rhizosphere, attracted by the presence of root exudates and rhizodeposits (Hardoim et al., 2008; Philippot et al., 2013). This chemotaxis-mediated response towards root exudates is an important factor for successful colonization of the rhizosphere and rhizoplane (Hardoim et al., 2008; Lugtenberg and Kamilova, 2009; Feng et al., 2018). According to Compant et al. (2010), endophytic bacteria represent "a class of specialized rhizobacteria that have acquired the ability to invade plant roots after establishing a rhizospheric population". The interior of roots can be colonized mostly through cracks formed during lateral root emergence and at root tips (Hardoim et al., 2008; Bulgarelli et al. 2013), but also through wounds caused by phytopathogens and soil herbivores (Hallmann et al., 1997). Endophytes entering the plant this way are often referred to as passive endophytes, which do not actively colonize the plant tissue, but do so as a result of stochastic events (Gaiero et al., 2013). But also more active ways of entering the plant interior, e.g. using cell wall degrading enzymes, are known (Hardoim et al., 2008). These facultative endophytes can multiply rapidly inside the plant and may colonize intercellular spaces locally (Hardoim et al., 2015) or reach the cortical zone with the endodermis which represents a barrier for a lot of bacteria (Compant et al., 2010). However, several bacteria were shown to be able to penetrate this barrier either passively,

when secondary root growth disrupts the endodermis, or actively using cell wall degrading enzymes (Compant et al., 2010). Systemic colonization can then take place using the xylem vascular system (James et al., 2002). However, distribution through plants can take several weeks, the reason for this slowly spread is unknown (Compant et al., 2005; Hardoim et al., 2015).

Next to intercellular colonization, endophytes are also known for intracellular colonization of plant cells (Kandel et al., 2017). However, this research area is still poorly understood (Kandel et al., 2017). Intracellular bacterial colonization of plant cells has been observed in roots of *Arabidopsis* (Van der Meij et al., 2018) and switch grass (White et al., 2014), shoot tips of banana (Thomas and Reddy, 2013), Scot pine buds (Pirttilä et al., 2000), and in micropropagated peach palms (De Almeida et al., 2009). Recently, Thomas and Franco (2021) revealed intracellular bacteria as a common phenomenon in different plant species by investigating healthy in vitro cell and callus cultures using fluorescent and confocal microscopy and 16S amplicon sequencing. However, the colonization pathway of intracellular endophytes is mostly unknown, but secreted cell wall degrading enzymes are thought to be involved (Kandel et al., 2017).

1.5.2 Effects of endophytes on plants

Endophytes can have various effects on plants ranging from neutral to positive and even negative effects. Quantitatively, most endophytes belong to the group of commensals (Hallman et al., 1997), which live on metabolites produced by the plant, but cause no effect regarding plant growth (Hardoim et al., 2015). It is expected to find so far unknown functions within this group (Hardoim et al., 2015).

But most importantly, endophytes can have positive effects on plant growth. Basically these effects can be classified into three main mechanisms: phytostimulation, biofertilization and biocontrol (Bloemberg and Lugtenberg, 2001). Phytostimulation is the production of phytohormones by endophytes which is probably the best studied plant growth promotion mechanism (Hardoim et al., 2015). Endophytes that promote plant growth by producing phytohormones like auxins and gibberellins are commonly found (Bastián et al., 1998; Long et al., 2008; Shi et al., 2009; Merzaeva and Shirokikh, 2010; Khan et al., 2012). But also, jasmonic and salicylic acid (Forchetti et al., 2007) and cytokinin producing endophytes are known.

However, although numerous bacteria known as endophytes were found to produce cytokinin in culture (Glick et al., 2012), limited evidence has been reported definitively linking bacterial cytokinin production to plant growth promotion (Ali et al., 2017). Next to the direct production of phytohormones, several endophytes were also shown to manipulate the endogenous plant phytohormone level by, for example, degradation of IAA (Spaepen et al., 2007), metabolizing abscisic acid (Belimov et al., 2014) or induction of salicylic acid and abscisic acid production (Wang et al., 2015). Further, various endophytes producing the enzyme ACC deaminase were described in literature (Ali et al., 2017). This enzyme cleaves the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), thus lowering the plant ethylene level. The phytohormone ethylene is involved in the germination of seeds, acts in response to various stresses and elevated levels can inhibit cell division, DNA synthesis and meristem growth in roots, shoots and axillary buds (Burg, 1973). When inoculating 25 different endophytes with ACC deaminase activity originally isolated from tomato seedlings, all of them showed canola seedling growth promotion compared to uninoculated seedlings (Rashid et al., 2012). Despite these effects, ethylene was found to be a key regulator in endophytic colonization (Iniguez et al., 2005). Ethylene insensitive *Medicago truncatula* mutants were shown to be hypercolonized by endophytic bacteria, while the addition of ethylene decreased the colonization (Iniguez et al., 2005). This suggest that the ACC deaminase supports the colonization of endophytes (Hardoim et al., 2015).

Another way endophytes promote plant growth is through biofertilization, which is the increased availability and accessibility of major nutrients to the plant (Bashan, 1998). A well-studied mechanism is nitrogen-fixation. This ability was found for several endophytic bacteria of the genera *Pseudomonas*, *Bacillus*, *Burkholderia*, *Sphingomonas*, *Microbacterium* etc. in different plant species (reviewed by Puri et al., 2018). Plant growth promoting endophytes are also known to increase the phosphorous availability through phosphorous solubilization (Gaiero et al., 2013). Forchetti et al. (2007) isolated and characterized endophytes of sunflower (*Helianthus annuus* L.) and found five strains that were, next to nitrogen-fixation, able to solubilize phosphates. By the use of plant growth promoting bacteria in plant nutrition experiments, Yazdani and Bahmanyar (2009) could reduce the need for phosphorous application for *Zea mays* by 50 % without significant grain yield loss. Moreover, endophytes are attributed to supply the plant with essential vitamins (Basile et al. 1985).

A further plant growth promoting mechanism by endophytes is the protection from phytopathogens, known as biocontrol (Gaiero et al., 2013). Biocontrol includes mechanisms similar as described for rhizosphere bacteria (Sturz et al., 2000; Ryan et al., 2008). These comprise competition for niches and nutrients, production of siderophores, antimicrobial substances and lytic enzymes, detoxification and degradation of virulence factors and induction of systemic resistance (Compant et al., 2005). It was shown in different studies that after endophyte inoculation, fungal, bacterial and viral diseases as well as nematode and insect damages were reduced compared to uninoculated plants (Kerry, 2000; Berg and Hallmann, 2006; Ardanov et al., 2011; Muvea et al., 2018). One important mechanism involved is the induced systemic resistance (ISR) which leads to a higher tolerance against pathogens (Kloepper and Ryu, 2006; Zamioudis and Pieterse, 2012). It is assumed that the plant growth promoting endophytes are at first recognized as potential pathogens, which trigger the plant immune response and at later stages are able to circumvent the plant defense response enabling successful colonization (Kloepper and Ryu, 2006; Zamioudis and Pieterse, 2012). This is well documented especially for members of the genus *Pseudomonas* and *Bacillus* (Zamioudis and Pieterse, 2012).

However, several endophytes are also known for their negative effects on plant health. Some of these facultative pathogens can shift their lifestyle depending on different factors such as environmental conditions, host and endophyte developmental stage or plant defense reactions (Schulz and Boyle 2005; Rosenblueth and Martínez, 2006). Rosenblueth and Martínez (2006) put forward the hypothesis of an equilibrium between plants and endophytes that under certain conditions gets unbalanced to the detriment of one of the partners. Schulz and Boyle (2005) termed this "endophytic continuum" and hypothesized that there are no neutral interactions between endophyte and plant host, but rather a balance of antagonism. Further, they hypothesized that endophytes, in contrast to known pathogens, have in general a higher phenotypic variability, leading to different stages like colonization, latency, pathogenicity and/or saprophytism. The disturbance of the endophyte plant equilibrium was demonstrated by Junker et al. (2012). Out of eight chosen endophytic isolates originally obtained from healthy *Arabidopsis thaliana* leaves, stems, and roots, five isolates showed pathogenic symptoms when colonizing the plant after reinoculation (under favorable conditions for the endophytes and stressful for the host plants). Kloepper et al. (2013) showed that the Fern Distortion Syndrome of the Leatherleaf fern (*Rumohra adiantiformis*) is caused

by latent infections of opportunistic endophytic fluorescent *Pseudomonas* spp., which contain virulence genes that are expressed when a minimum population is reached inside the plant. The importance of abiotic environmental conditions on this plant endophyte equilibrium was shown by Álvarez-Loayza et al. (2011) in the common palm tree *Iriartea deltoidea*. When seedlings grow under shady conditions, the endophytic fungi *Diplodia mutila* caused no symptoms. However, grown in direct sun light, the endophyte caused necroses in the palm tissue, probably by light-induced H_2O_2 production. This sensitive endophyte plant equilibrium is moreover thought to be an evolutionary driver (Schulz and Boyle 2005; Rosenblueth and Martínez, 2006). For example, Wheeler et al. (2019) reconstructed the evolutionary history of non-pathogenic endophytic populations of *Verticillium dahliae* in mint, mustard and grasses genetically and phenotypically and found that they evolved from pathogenic populations known in potato. Non-pathogenic endophytic populations differed genetically only marginally from the pathogenic populations. Another example is the fungal endophyte *Hymenoscyphus fraxineus*, which causes the European ash dieback. Interestingly, this fungal endophyte shows no pathogenicity to indigenous *Fraxinus* species in Asia (Cleary et al., 2016). However, after introduction of this endophyte in northern and central Europe, common ash trees (*Fraxinus excelsior*) show severe dieback symptoms (Cleary et al., 2016). Genetic diversity analyses of Asian and European *H. fraxineus* populations revealed a strong bottleneck in the European population (Cleary et al., 2016; McMullan et al., 2018), which was founded by just two divergent haploid individuals, resulting in reduced selection efficacy (McMullan et al., 2018).

In summary, endophytes can have various effects on plants. The results of Gorischek et al. (2013), who showed that the maternally inherited fungal endophyte *Epichloë elymi* can cause a shift of the sex ratio (more seeds, less pollen) in the grass *Elymus virginicus,* indicates that there are many more effects of endophytes in plants that wait to be uncovered.

1.5.3 Factors influencing the plant endophytic community

It is often described that the endophytic community is a subset of the rhizoplane community, which in turn is a subset of the rhizosphere community (Edwards et al., 2015; Sasse et al., 2018). One major factor influencing this community is the plant genotype. Different plant species and even cultivars grown in the same soil harbor different endophytic communities

(Granér et al., 2003; Aleklett et al., 2015; Afzal et al., 2019). Plants can actively shape their microbiome and selectively influence, which endophytes are favored before and after root colonization (Rosenblueth and Martínez, 2006; Hardoim et al., 2008; Gaiero et al., 2013). The root microbiome is shaped by the root physiology, root border cells and mucilage, as well as root exudates like sugars, amino acids, organic acids, fatty acids, and secondary metabolites (Bulgarelli et al., 2013; Sasse et al., 2018). These root exudates are diverse and dynamic and in turn are influenced by several factors. Mönchgesang et al. (2016) showed that plant exudation is defined by the genotype by comparing the exudation patterns of 19 *Arabidopsis thaliana* accessions. Further, plant exudation is influenced by the developmental stage of the plant suggesting that the plant can recruit microbes at different development stages for specific functions (Chaparro et al., 2014). Moreover, plant exudation is shaped depending on stressors. For example, plants show different exudation patterns in response to nutritional limitations (Carvalheis et al., 2013; Ziegler et al., 2016). Furthermore, different exudation patterns not only influence the microbiome but also the transcriptome of microbes as shown for the interaction between maize and the plant growth promoting bacteria (PGPB) *Bacillus amyloliquefaciens* FZB42 (Carvalheis et al., 2013).

Next to the plant genotype, the soil is described as the most important factor influencing the endophytic community (Berg and Smalla 2009; Bonito et al. 2014; Lareen et al. 2016; Hartman and Tringe 2019). Several soil environmental factors are of high importance for the structuring of soil bacterial communities (Fierer et al., 2017). These factors, which may be very heterogeneous, include salinity, soil texture and structure, nutrient availability, soil moisture, organic carbon quality and quantity, soil pH, soil O_2 and redox status (Fierer et al., 2017; Compant et al., 2019; Papik et al., 2020). The last three factors mentioned showed the highest relative importance in structuring the soil bacterial communities (Fierer et al., 2017). Because of this high soil dependent microbial diversity, plants of the same species grown in different soils contained different endophytic communities. Bulgarelli et al. (2012) and Lundberg et al. (2012) concluded after growing different *Arabidopsis thaliana* accessions in different soils under controlled environmental conditions that the effect of the soil on the plant root microbiome is more pronounced than that of the plant genotype indicating that the soil provides the initial inocula.

Besides the plant genotype and soil environmental factors, several other factors like climate, season, plant health, pathogen presence and human cultivation are also involved in shaping the plant endophytic microbiome (Bulgarelli et al., 2015; Compant et al., 2019).

1.5.4 Endophytes in apple

Several studies investigated the endophytic microbiome of apple, examining different organs, like leafs and stems (Afandhi et al., 2018: Liu et al, 2018, Liu et al., 2020), roots (Bulgari et al., 2012; Dos Passos et al., 2014), flowers (Shade et al., 2013) and fruits (Wassermann et al., 2019). 16S amplicon sequencing revealed that Proteobacteria are the dominant phylum in all organs (Bulgari et al., 2012; Liu et al, 2018; Wassermann et al., 2019). An exception are flowers, which are surprisingly dominated by the largely unknown phyla *TM7* and *Deinococcus-Thermus*. Interestingly, fruits contain approximately 100 million bacterial cells consisting out of Proteobacteria (80%), Bacteroidetes (9%), Actinobacteria (5%), and Firmicutes (3%), which were strongly reduced in diversity and evenness under conventional farm management compared to organic management. Via DGGE, Liu et al. (2020) could show that the factors tissue type (2_{nd} leaf, 3_{rd} leaf, green stem and woody stem), cultivar and site had the strongest influence on the endophytic microbiome, whereas the season (spring or autumn) had no significant effect. The influence of the rootstock on the scion endophytic microbiome was shown to be not significant (Liu et al, 2018). In addition, the more vigorously growing rootstock M.M. 111 harbored more plant growth promoting bacterial taxa compared to the dwarfing rootstock M.9 (Liu et al, 2018), indicating that, next to the plant genotype, the lack of plant growth promoting endophytes may contribute to the rootstock growth reduction effect.

In several studies, endophytic isolates were obtained from apple organs in culture dependent approaches (Bulgari et al. 2012; Dos Passos et al., 2014; Afandhi et al., 2018; Liu et al., 2020). Liu et al. (2020) isolated in total 783 bacterial (and 87 fungal) isolates from leaves and stems of which 19, belonging to the genera *Bacillus* and *Pseudomonas*, showed antagonism against the European Canker causing fungal pathogen *Neonectria ditissima*. Members of these two bacterial genera were also mainly identified by Bulgari et al. (2012) in *'Candidatus Phytoplasma mali'* infected and uninfected apple plants. By identifying 60 isolates each, from roots growing in conventional orchards and organic orchards, Dos Passos et al. (2014) found

more isolates capable of producing siderophores and indolic compounds, solubilizing phosphates, and having antagonistic activity against the bitter rot and leaf spot disease causing fungus *Colletotrichum gloeosporioides* in plants growing in organic orchards.

In relation to ARD, only a few studies have investigating the role of the endophytic community. *Rhizoctonia* spp. and *Cylindrocarpon* spp. were identified as endophytic root pathogens by Kelderer et al. (2012) in row (ARD-affected) and inter-row (control) planted apple trees. *Cylindrocarpon*-like fungi (*Ilyonectria* spp. and *Thelonectria* sp.) were also identified by Manici et al. (2013), next to *Pythium* spp. to be main causal agents of growth reduction in the rootstock M9 growing in ARD-affected soil. Numerous fungal root endophytes were isolated from ARD-affected apple roots and re-inoculated in a soil free biotest (Popp et al, 2019; Popp, 2020). Isolates of the genera *Dactylonectria*, *Ilyonectria, Cadophora*, *Calonectria*, and *Leptosphaeria* were described to have negative effects on plant growth. So far, only two studies considering the role of bacterial endophytes in relation to ARD were conducted (Tewoldemedhin et al., 2011a; Van Horn et al., 2021). In an approach to isolate and inoculate Actinomycetes, Tewoldemedhin et al. (2011a) identified 92 isolates from ARD-affected roots belonging to the genus *Streptomyces* and 4 to *Nocardiopsis*. Inoculation of selected isolates showed no effect on plant growth. Van Horn et al. (2021) characterized the endophytic community structure of rootstock genotypes reported to be tolerant (G210, G41, G890, and G935) and susceptible to ARD (M26 and M9) and found the strongest community differences between tolerant and susceptible ones. The most abundant endophytic bacteria were members of the genera *Arthrobacter*, *Halospirulina*, *Streptomyces*, and *Burkholderia*.

1.6 Objectives and hypotheses

As stated above, endophytes can have various effects (positive or negative) on plants. However, there is still a lack of knowledge of how endophytic communities, especially bacterial ones, are affected by ARD and how they can influence the plants grown in ARD-affected soils. Therefore, this thesis is focussed on bacterial endophytes. We hypothesize that

- the root endophytic microbiome of plants grown in ARD-affected soils differs from that of plants grown in non-affected soils.
- ARD causal agents are present in roots of different apple genotypes grown in different ARD soils from different sites.
- roots grown in ARD-unaffected control soil harbor plant growth promoting bacteria, which may help to overcome ARD.
- a possible causal agent will accumulate over time in roots after (re)planting.

Therefore, the aim of this work was to characterize and quantify the bacterial endophytes in apple roots growing in ARD-affected and non-affected soils. For that purpose, two main experiments were conducted to provide the basis for these analyses. In the first one, central experiment 1 (CE1), root samples were taken from apple plants from three different sites grown in (i) field plots where ARD was successfully induced by replanting apple every two years and (ii) plants grown in control plots where grass served as a cover. In the second experiment, central experiment 2 (CE2), conducted in three years each, control soil and ARD soil was taken from these sites and used either untreated or gamma irradiated. In vitro propagated plants of ARD susceptible rootstock genotypes were potted in these soil variants and grown for 8 weeks. Plant growth parameters were measured and root samples taken.

Based on these central experiments, for the first time, molecular barcoding approaches were used to characterize the bacterial endophytic community structure of apple roots grown in ARD-affected soils in comparison to non-affected soils. Furthermore, culture dependent methods were used in order to obtain a broad spectrum of bacterial apple root endophyte Isolates which will lay the basis for future inoculation experiments.

2.   Manuscripts

2.1   Induction and diagnosis of apple replant disease (ARD): a matter of heterogeneous soil properties?

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Eva Lehndorff	Contributed reagents/materials/analysis tools, contributed to the writing of the manuscript
Stefan Pätzold	Contributed reagents/materials/analysis tools, contributed to the writing of the manuscript
Andreas Wrede	Conceived and designed the experiments, performed the experiments, contributed to the writing of the manuscript
Traud Winkelmann	Conceived and designed the experiments, contributed reagents/materials/analysis tools, contributed to the writing of the manuscript

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Induction and diagnosis of apple replant disease (ARD): a matter of heterogeneous soil properties?

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ABSTRACT

Apple replant disease (ARD) occurs when apple or a closely related species are replanted at the same site leading to severe growth reduction and losses in fruit yield and quality. This complex disease phenomenon is a worldwide problem for tree nurseries and apple orchards. Its causes are not fully understood up to now and economically feasible and sustainable countermeasures do not exist. Research studies to unravel the etiology of ARD are in urgent need of sites differing in soil characteristics with a defined, comparable cropping history including appropriate control (healthy) plots. Thus, the first aim of this study was to induce ARD at three different sites with topsoils covering textures from sand, loamy sand to silt loam (Podzols to Luvisols). Grass plots served as controls. After eight years of repeatedly replanting the apple rootstock 'Bittenfelder', growth suppression was observed at the ARD plots on all three sites. Because until now no systematic correlation to soil parameters was approached, the second aim was to study the replant severity in the three different soils. Soil properties were investigated at different spatial scales. Significant differences occurred between sites in soil texture, pH, and C and N contents. Within plots, non-invasive soil sensing (EMI, gamma-spectrometry) helped to ensure homogeneous conditions and to exclude unexplained ARD effects due to small-scale soil heterogeneity. Soil from each site was submitted to a bio-test in which growth of in vitro propagated M26 plantlets in untreated and disinfected soil was compared to determine the ARD severity. Soil disinfection by gamma irradiation resulted in a significant increase in M26 shoot biomass, most pronounced in soil from ARD plots. Thus, on all soils, ARD was successfully induced with a negative correlation between ARD intensity and soil clay content, and a positive correlation with C/N ratio.

1. Introduction

Apple replant disease (ARD) is a worldwide problem in tree nurseries and apple production sites which leads to severe growth reduction and losses in fruit yield and quality (Mai and Abawi, 1981; Mazzola and Manici, 2012; Manici et al., 2013; Winkelmann et al., 2018). ARD occurs when apple is planted on a site where the same species or closely related species were grown before and was recently defined "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., 2018). The fact that disinfection of ARD soil leads to improved growth suggests that biotic factors are the primary causal factors of this disease (Mai and Abawi, 1981; Yim et al., 2013). Several groups of organisms like the fungi

Cylindrocarpon, *Fusarium* and *Rhizoctonia*, the oomycetes *Phytophthora* and *Pythium*, different bacteria like *Bacillus*, *Pseudomonas*, *Actinomycetes* and even nematodes like *Pratylenchus* are claimed to be involved (Utkhede et al., 1992; Mazzola, 1998; Tewoldemedhin et al., 2011; Manici et al., 2017). In addition, phytotoxins and cytotoxic compounds could also contribute to ARD (Manici et al., 2018). Nowadays, new sequencing techniques allow insights into microbial communities pointing to dysbiosis in ARD soils (Franke-Whittle et al., 2015; Yim et al., 2015, 2017, Peruzzi et al., 2017) and into the molecular responses in apple (Shin et al., 2016; Weiß et al., 2017; Weiß and Winkelmann, 2017). However, the exact etiology of this complex phenomenon is still unknown and sustainable counteractions are not at hand.

Apple replant disease appears in different soils to variable degrees

(von Bronsart, 1949; Franke-Whittle et al., 2018; http://www.leicesters.co.nz/specific-apple-replant-disease/). Some earlier studies intended to identify correlations between soil parameters and ARD. However, no consistent and generally valid relationship between causes and effects could be found, partly due to study designs which cover too many potential factors that often interact in complex ways (Li and Utkhede, 1991). Though, soil texture, i.e. grain size distribution, may influence the degree of ARD (von Bronsart, 1949), as it controls content and duration of soil moisture, oxygen supply in soil and, hence, the habitats of soil fauna and soil microbial community (Weil and Brady, 2016). Most likely, this impact leads to differential intensity of ARD at different sites. Beyond, soil texture is correlated with mineralogical composition (Heggemann et al., 2017) and pH value of soil. Consequently, soil texture can potentially serve as proxy for macro- and micro-nutrient availability, which again controls the microbial community (Willett et al., 1994; Jonkers et al., 1980; Utkhede, 2006). Accordingly, changes in pH were observed to correlate with changes in nutrient cycling in ARD soil (Fan et al., 2010; Fazio et al., 2012). Recently, Franke-Whittle et al. (2018) studied the effects of locally available compost as organic amendments to ARD soils in four countries and found them to reduce the ARD effects but in a soil and compost specific way.

In order to enable research studies to unravel the etiology of ARD in different soils, this study aimed at inducing ARD at three different sites in Northern Germany with different soil properties, where no Rosaceous species had been grown before. Second aim of this study was to examine relationships between ARD and soil properties at different scales: first, the different sites were compared. Second, soil heterogeneity at the field scale was examined, i.e., differences between experimental plots at a given site. Third, spatial patterns of soil sensing data within the experimental plots were detected to exclude unknown variation of soil properties that potentially cause unexplained effects. In the past years, non-invasive soil sensors basing on various physical principles have been introduced into soil science and precision agriculture applications (Kuang et al., 2012). These techniques allow to record soil information at high spatial resolution and low efforts and costs and were employed in this study. We hypothesize that soil properties at the selected test sites differed enough to show variable ARD severity. However, to achieve statistically significant results in view of the complex relationships between soil properties and ARD, soil heterogeneity at the plot-scale and at the m-scale was to be excluded. Thus, we further hypothesized that non-invasive sensor techniques are an appropriate tool to elucidate unknown soil heterogeneity at small scales and to ensure valuable results in this ARD study.

In brief, the objectives of this study were to (i) successfully induce ARD at three reference sites, (ii) evaluate the ARD severity on these three different soils after disinfection in a greenhouse experiment and (iii) analyze the relationship between field soil properties (texture, pH, SOC, and N_{total}) and ARD (expressed as apple growth reduction compared to disinfected soil).

2. Material and methods

2.1. Field design for induction of ARD

ARD was induced at the three reference sites located in Ellerhoop (x-coordinate 53.71435; y-coordinate 9.770143 WGS 84, Schleswig-Holstein, northern Germany), Heidgraben (x-coordinate 53.699199; y-coordinate 9.683171; WGS 84, Schleswig-Holstein, northern Germany) and Ruthe (x-coordinate 52.243668; y-coordinate 9.819700; WGS 84, near Hanover, Germany). At all sites, the cropping history recorded no Rosaceous species that had been grown before. Only for Heidgraben, the records were not complete for the time before 2005. In 2009, rootstock seedlings of the cultivar 'Bittenfelder Sämling' (hereafter referred to as Bittenfelder) were planted in four randomly arranged plots per site. Another four plots with grass cover served as "control" plots.

Besides these two variants (grass and ARD) being relevant for this study, on each site eight further variants with four replicates were replanted to represent the different subfamilies of the Rosaceae: *Rosa canina, R. corymbifera, R. multiflora, R. rubiginosa, R. majalis, Prunus avium, Pyrus communis* and *Spiraea* x *cinerea*. Sources of the plant material, its age and quality are listed in Supplementary Table A1. Plot sizes were 10 m x 10.05 m for Ellerhoop and Heidgraben, and 10 m × 4.55 m for Ruthe. Per ARD-Plot 880 (Ellerhoop, Heidgraben) or 380 seedlings (Ruthe) were planted with spacing of 25 cm within rows and 45 cm between rows.

Since 2009, apple seedlings were repeatedly replanted in a two year cycle which resulted in the 5th replanting generation on the field in 2017 (Fig. A1, Suppl.). Before planting, the soil tillage was carried out only by a power harrow to prevent mixing of soil between plots and the typical rotation of the top soil layers. After planting in March or April (depending on the weather conditions of the respective year), plants were allowed to grow for one vegetation period and then cut back to about 10 cm in length. The above-ground biomass of the second vegetation period was then recorded after leaf shedding in autumn of the second year by harvesting 50 central plants of each plot. In late autumn, all plants were uprooted, before the replanting of the next generation started the following spring. At sites Ellerhoop and Heidgraben, mineral fertilizers were applied in order to reach a mineral nitrogen amount of 70 kg N ha^{-1} a^{-1}, an extractable level of 48–87 mg P kg^{-1} soil and 124–166 mg K kg^{-1} soil, respectively (CAL method; Averdieck, 2006). At site Ruthe, due to a high nutrient status, fertilization was not necessary (for nutrition status and fertilization see Suppl. Tables A3 and A4). Weed control was performed according to common nursery practice with residual herbicides in early spring and contact herbicides during the rest of the year accompanied by manual weeding. Plant protection was necessary every year against downy mildew and aphids (for details see Suppl. Table A5).

The annual mean temperature for Ellerhoop and Heidgraben was 9.7 °C and 10 °C in 2016 and 2017, respectively, and for Ruthe it was 10.4 °C and 10.5 °C in 2016 and 2017, respectively. The annual precipitation added up to 833.3 mm (2016) and 1142.4 mm (2017) for Ellerhoop and Heidgraben, and to 517.1 mm (2016) and 682.5 mm (2017) for Ruthe.

2.2. Soil characterization

To elucidate any correlation between soil properties and plant development, soil properties were investigated at different spatial scales. First, the three experimental sites were selected in view of the variable parent materials and their largely differing topsoil properties. The sites Heid-graben, Ellerhoop, and Ruthe were located in different landscapes of Northern Germany and represented different soil conditions. In Heidgraben, an Entic Podzol (according to WRB 2015) had developed from aeolian sand. Soil in Ellerhoop was classified as an Endostagnic Luvisol from glacial till, whereas in Ruthe Haplic Luvisols developed from calcareous loess. Horizontation and major soil properties of one representative soil auger per site are given in Table 1.

Second, possible differences between the experimental plots at each site were to be unravelled. For this, eight plots (four ARD plots, four grass control plots) were sampled at each site to analyze soil properties such as pH, bulk density, texture (grain size distribution), and total C and N contents. Samples were taken from the Ap horizon (0–25 cm depth) using a Pürckhauer corer. At Ruthe, nine samples per plot were taken in order to achieve a representative mean value for each plot, whereas at Ellerhoop and Heidgraben, twelve samples per plot were taken because of the bigger plot size.

Soil bulk density (g cm^{-3}) was estimated in soil cores of 100 cm^3 after drying. Soil water content was determined gravimetrically. Soil pH was measured in 0.01 M CaCl$_2$ with a glass electrode. Total carbon (C) and nitrogen (N) contents were measured by elemental analysis (ISO 10694, 1995; ISO 13878, 1998) with an elemental analyzer

Table 1

Horizontation and major soil properties of the study sites Ellerhoop, Heidgraben, and Ruthe. Profile descriptions refer to selected representative soil augers from one plot per site (WRB = World Reference Base for soil resources; BD = bulk density; SOC = soil organic carbon; n.d. = not determined).

Study site	Depth cm	Horizons WRB	Texture WRB	BD g cm^{-3}	SOC g kg^{-1}	N$_{total}$ g kg^{-1}	pH (CaCl$_2$)	CaCO$_3$ g kg^{-1}
Ellerhoop	0-32	Ap1	Loamy sand	1.5	18.3	1.38	5.7	< 0.1
	32-60	Ap2	Loamy sand	n.d.	15.4	1.13	5.6	< 0.1
	60-75	II SgBt	Sandy loam	n.d.	4.52	0.35	5.7	< 0.1
	75-100	II Bw	Sandy loam	n.d.	5.62	0.41	5.7	< 0.1
Heidgraben	0-27	Ap	Sand (Medium sand)	1.3	25.4	1.45	5.3	< 0.1
	27-42	EB	Sand (Medium sand)	n.d.	20.9	1.22	4.6	< 0.1
	42-65	Bsh	Sand (Medium sand)	n.d.	10.1	0.63	4.9	< 0.1
	65-100	C	Sand (Medium sand)	n.d.	2.20	0.08	4.8	< 0.1
Ruthe	0-30	Ap	Silt loam	1,6	8.04	0.72	6.1	< 0.1
	30-40	E	Silt loam	n.d.	3.38	0.33	6.1	< 0.1
	40-70	Bt1	Silt loam	n.d.	2.77	0.35	6.5	< 0.1
	70-100	Bt2	Silt loam	n.d.	2.46	0.03	6.5	< 0.1

(MicroCube, Elementar Analysensysteme, Hanau, Germany). All top-soils were carbonate free; in consequence, total C corresponds to soil organic carbon (SOC). Soil texture was analyzed by wet sieving (sand fraction) and sedimentation (silt and clay fraction) after Köhn (ISO 11277, 2002).

However, soil properties can potentially vary at even smaller scales, resulting in high variation in data when sampling single plants. Therefore and third, we tested if soil properties were heterogeneous even at higher spatial resolution, i.e., at the sub-parcel scale within single plots. For this, we applied non-invasive soil sensing techniques (mobile gamma spectrometry; electromagnetic induction) to evaluate the degree of homogeneity in high spatial resolution at the meter scale (next section of the manuscript).

2.3. Sensor-based analyses giving information about soil heterogeneity at the m-scale

Two different non-invasive and mobile sensor techniques, electromagnetic induction (EMI) and gamma spectrometry, were used to detect eventual soil heterogeneity in high spatial resolution, i.e., within the single plots. Sensing campaigns were conducted at the following dates: 09.03.2016 (Heidgraben), 08.03.2016 (Ellerhoop), and 06.04.2016 (Ruthe), respectively. EMI delivers data on the apparent electrical conductivity (EC$_a$) and was conducted with the EM38 sensor (Geonics, Canada). The sensor was used in horizontal-dipole mode, capturing a measurement depth of 0–75 cm. Apparent electrical conductivity (EC$_a$) is influenced by a number of interacting soil properties, with clay content and actual soil moisture being dominant in most cases. The signal delivers integrated information over the measurement depth. Note that these two parameters are generally correlated, but cannot be separated when interpreting EC$_a$, neither in depth nor in their signal contribution. Combined with a GPS, the EM38 was mounted on a plastic sledge that was pulled over the plots with approximately 2.5 km h^{-1} and provided continuously measurements at 1 Hz, resulting in 25–35 data points per plot. Further methodological details and signal interpretation were given by Mertens et al. (2008).

Gamma spectrometry records the natural occurring gamma radiation from soil originating from the decay of radioactive isotopes. A spectrometer type RSX-1 (Radiation Solutions Inc., Canada) was mounted to a steel frame in the three-point linkage of a tractor. Driving velocity was adapted to the EMI measurements in order to achieve a similar data density (25–35 measurements per plot). The radioactive isotopes ^{238}U, ^{40}K, ^{232}Th as well as the total counts (TC; Total Counts) were recorded in counts per second [cps]. These parameters are correlated predominantly with soil mineralogy, and, in consequence, with soil texture in the uppermost 40 cm. To detect heterogeneity of gamma ray emissions, only the total counts were evaluated for this study. More

details about our approach concerning technical aspects, spectra acquisition and evaluation, and derivation of textural information are given by Heggemann et al. (2017).

2.4. Bio-test to verify ARD incidence and severity

In vitro propagated shoots of the ARD-susceptible rootstock genotype M26 were used for the bio-tests as described by Yim et al. (2013) which served as central experiment 2 (CE2) for the research consortium BonaRes ORDIAmur (www.ordiamur.de). Briefly, the shoots were propagated by axillary shoot formation every five weeks on Murashige and Skoog (MS) (1962) medium containing 3% sucrose, 4.4 µM BAP (6-benzylaminopurine), 0.5 µM IBA (indole-3-butyric acid) and 0.8% Plant Agar (Duchefa, The Netherlands) adjusted to pH 5.7. Rooting was achieved in 99% of the shoots on half-strength MS medium with 2% sucrose, 4.92 µM IBA and 0.75% Plant Agar (pH 5.7). In 2016, 1264 rooted plantlets were transferred to greenhouse conditions with an acclimatization success of 89% (in 2017, 1540 rooted plantlets with an acclimatization rate of 95%). After an acclimatization phase of 4 weeks, plantlets were freed from the adhering peat substrate and used for the bio-test.

From each reference site, soil was taken in a depth of 0–20 cm from the centres of (i) ARD plots and (ii) grass plots. Soils of the four plots were carefully mixed and sieved through 8 mm mesh. ARD and grass soil was treated as follows: untreated (UT) or gamma irradiated (G) with a minimal dose of 10 kGy (Yim et al., 2015). This resulted in an experimental design of four soil variants for each of the three reference sites, thus in a total of 12 variants.

The experiment CE2 was conducted twice: in 2016 and 2017. The different soil variants were supplemented with 2 g L^{-1} Osmocote Exact 3–4 M (16 + 9 + 12 + 2MgO) and filled into 1 l pots. Homogeneous acclimatized M26 plants were planted in the pots (one plant per pot) and placed randomized in three (2016) or four (2017) blocks in a greenhouse. Greenhouse conditions, irrigation and plant protection were carried out according to Yim et al. (2015). The mean daily temperature in 2016 was 20.2 °C ± 1.1, in 2017 21.1 °C ± 1.3. A 16 h photoperiod was achieved or supported by additional light (SON-T Philips Master Agro 400 W) with a set point of 25 klx.

Shoot length measurements from the surface of the soil to the base of the newly emerging leaves of the main shoot were done weekly. Fresh and dry mass (70 °C for 1 week) of the shoot and root were recorded after 8 weeks, when the experiment was finished.

2.5. Statistical analysis

Data for dry mass and shoot length for each reference site was analyzed using ANOVA (analysis of variance) and means were

compared by Tukey's HSD test (p < 0.05) assuming homogeneity of variance. Growth depression and soil parameters were correlated using the Pearson correlation. The program R-3.4.2 (R Development Core Team, 2017) was used for these analyses.

Differences between soil properties within study sites (ARD soil versus grass soil) and between the three study sites was tested by ANOVA with subsequent t-tests (SigmaPlot). Mean values and standard deviation were calculated to focus on differences between the three study sites. For sensor-based analyses, maps were calculated using IDW (ESRI, ArcGIS) to evaluate soil heterogeneity within sites.

3. Results

3.1. Soil characterization at different scales

Between the experimental sites, desired heterogeneity was clearly expressed. Soil properties as analyzed in the lab varied between the different sites, not only in the topsoil, but also in deeper horizons. Soil profile classification at one selected location per site led to the texture classes *sand* at Heidgraben (dominated by medium sand 200–630 μm, not shown), *loamy sand* to *sandy loam* (Ellerhoop), and *silt loam* (Ruthe), respectively (Table 1). More in detail, mean values of sand contents in the topsoil ranged from 92 g kg^{-1} (Ruthe, Loess) to 925 g kg^{-1} at Heidgraben (aeolian sand). Large differences also occurred for silt contents, ranging from 27 g kg^{-1} at Heidgraben and 770 g kg^{-1} at Ruthe. Clay contents were less variable (Table 2). Mean values of chemical soil properties (pH value, SOC, total N, and C/N ratio) were also significantly different between the three sites (Table 2).

Topsoil texture (sand) was, as expected, correlated with pH value (r^2 = 0.68), soil organic carbon (SOC; r^2 = 088) content, and C/N ratio (r^2 = 0.59) when compared between the three sites (Supplementary Fig. A2). It was remarkable that the C/N ratio was greater than expected from the SOC content at Heidgraben (Podzol from aeolian sand). In turn, the loess-derived Luvisol at Ruthe showed low SOC contents and a narrow C/N ratio.

Within the sites, i.e., at the plot scale, no important differences were stated (Table 2). However, at Ellerhoop and Heidgraben, small differences in pH, total C, and total N between grass control plots and ARD plots occurred. Due to the high sampling density, these results were statistically significant. Nevertheless, we consider these differences not relevant or explanatory for our experimental results, namely in view of the far bigger differences between the sites.

Within single plots, i.e., at the m-scale, non-invasively recorded sensor data served to evaluate spatial soil heterogeneity. Of course, the comparison of sensor data from different plots of a given site provides also information on variability within the sites. Note that no direct quantitative estimation of soil properties can be derived, because soil parameter variability and sampling density were too low to calibrate the sensor data. However, apparent electrical conductivity (EC$_a$ [mS m^{-1}]) and total gamma counts (TC [counts per second, cps]) allowed

for an evaluation of spatial heterogeneity of underlying soil properties (Fig. 1). At all sites, it was clearly stated that soil heterogeneity at the plot scale and the m-scale was generally small. The range of the legend units was narrow, hence, to enable a comparison at all scales, an overall classification of EC$_a$ and TC for all sites was selected.

Heidgraben revealed lowest EC$_a$ and TC values with small spatial variability. The EC$_a$ values mirrored the sandy texture down to 75 cm depth of the soil augering result (Table 1) and confirmed very similar profile composition throughout the whole site and within the single plots (Fig. 1a). At the sensing date in early March, soil revealed the typical seasonal high moisture contents that, obviously, did not largely vary over the field, because EC$_a$ variability was low. These findings were confirmed by the TC (Fig. 1b) that pointed to very low and homogeneously distributed gamma-emitting isotopes, i.e., a sandy, quartz-dominated topsoil.

At Ellerhoop, the results pointed to a more complex situation. EC$_a$ values are higher and less homogeneous between and within the plots. The profile description (Table 1) indicated a layered profile with a textural change towards higher clay contents in the subsoil. Subsoil horizons were formed from densely layered glacial till, while the topsoil still contained a certain amount of aeolian sand. Accordingly, stagnic patterns were visible in the SgBt and Bw horizons. The spatial distribution of EC$_a$ values indicated that in some plots the clay-rich layer likely appeared closer to the surface, leading to higher EC$_a$ values. This was notably true in the most Eastern part (see arrow in Fig. 1c), where, consequently, stagnic conditions are expected to be stronger (Hbirkou et al., 2011). Hence, EC$_a$ values also rise when soil of a given texture reveals higher moisture contents. The influence of clay and moisture on EC$_a$ cannot be distinguished, but as they are mostly correlated and act into the same direction, an interpretation with respect to overall conditions for plant growth is possible, anyway. These subsoil-induced conditions are not mirrored in the TC values that originated from the uppermost 40 cm and that revealed only small heterogeneity (Fig. 1d).

Finally, at Ruthe, EC$_a$ values yielded rather complex results, too. Generally, there seemed to be a trend towards higher EC$_a$ in the Western part of the test field. This was somehow surprising because loess as aeolian sediment tends to reveal a rather homogeneous spatial texture distribution. However, one single plot was conspicuous: the values around 9-10 mS m^{-1} in the control plot marked with an arrow (Fig. 1e) appeared somehow isolated between the neighboring plots. Note that EC$_a$ recording at Ruthe was conducted during the vegetation period (April). Hence the striking EC$_a$ values point to a vegetation influence. Variation in soil moisture due to variable water consumption of different plant stands generally lead to changing EC$_a$ values. As the observation holds true for most of the grass control plots, the result should not be regarded as texture-related phenomenon unless further investigation.

TC values for Ruthe (Fig. 1f) are highest among the three sites according to the highest clay contents in topsoil (Table 2). Small spatial variation occurs, but no clear spatial pattern is visible. This variability

Table 2
Properties of the topsoil (0–25 cm depth) at the study sites Ellerhoop, Heidgraben and Ruthe. Data are mean values from four ARD and four grass control plots per site. Each plot is represented by 9–12 samples (for details, refer to Section 2.2 Soil characterization).

Study site	plot	sand	silt (g kg^{-1})	clay	pH (CaCl$_2$)	Soil organic carbon (g kg^{-1})	N$_{total}$ (g kg^{-1})	C/N
Ellerhoop	ARD	725 ± 10[aA]	150 ± 6[aA]	99 ± 7[aA]	5.7 ± 0.11[aA]	17.3 ± 0.9[aA]	1.37 ± 0.07[aA]	12.6 ± 0.2[aA]
	control	721 ± 11[A]	150 ± 2[A]	103 ± 10[A]	5.8 ± 0.10[B]	19.4 ± 1.2[B]	1.58 ± 0.07[B]	12.4 ± 0.36[B]
Heidgraben	ARD	925 ± 3[bB]	27 ± 2[bB]	33 ± 9[bB]	5.3 ± 0.28[bC]	27.1 ± 3.6[bC]	1.48 ± 0.23[bC]	18.5 ± 0.78[bC]
	control	932 ± 8[B]	29 ± 4[B]	28 ± 3[B]	4,9 ± 0.25[D]	26.4 ± 3.2[C]	1.46 ± 0.18[C]	18.1 ± 0.21[C]
Ruthe	ARD	92 ± 13[cC]	770 ± 10[cC]	108 ± 1[cC]	6.0 ± 0.05[cE]	8.6 ± 0.2[cD]	0.75 ± 0.02[cD]	11.5 ± 0.56[cD]
	control	88 ± 10[C]	768 ± 17[C]	109 ± 3[C]	6.3 ± 0.27[F]	8.8 ± 0.8[D]	0.76 ± 0.09[D]	11.6 ± 0.51[D]

Lowercase letters indicate comparisons between ARD plots of the different sites. Capital letters indicate comparisons between ARD and control plots within distinct sites. Values marked by the same letters are not significantly different according to ANOVA and t-test, p < 0.05.
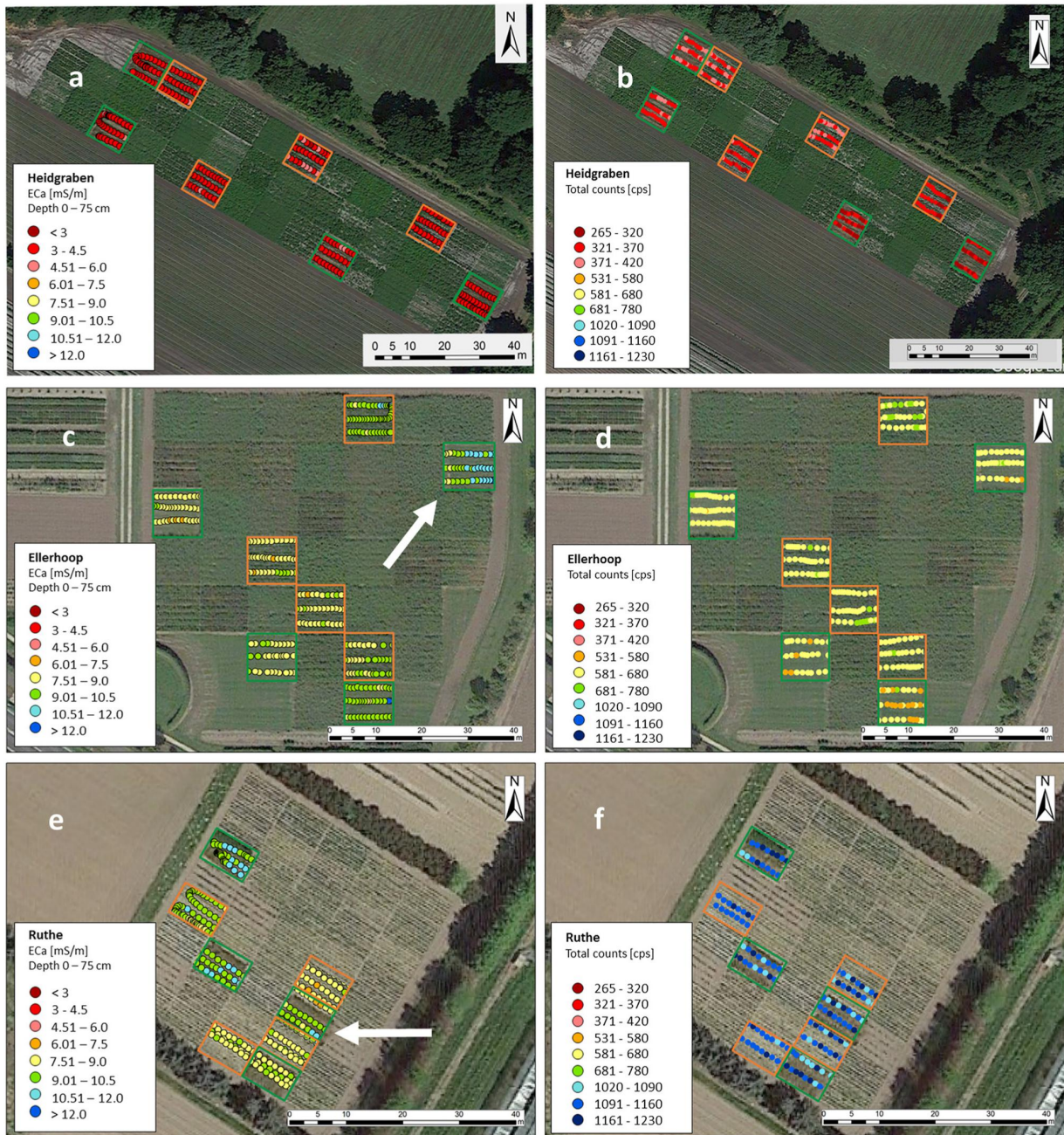
Fig. 1. Patterns of non-invasively recorded apparent electrical conductivity (EC$_a$ in mS m^{-1}; 0–75 cm depth; a, c, e) and total natural gamma radiation (TC in cps; 0–40 cm depth; b, d, f) of soil on the plots under study at the three experimental sites Heidgraben (a, b), Ellerhoop (c, d), and Ruthe (e, f). The legend units are uniform for all sites for better comparison. Orange frames indicate ARD plots, green frames grass control plots and arrows point to plots with high EC$_a$ values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cannot be interpreted, because gamma decay does not occur at a totally stable rate, but reveals a certain statistical temporal variability. A certain noise, notably at higher TC values, is not surprising.

3.2. Plant growth at the three reference sites over time

After each of the four replanting generations carried out up to now, the shoot fresh mass was recorded (Fig. 2). When growing apple for the first time on the sites, shoot fresh masses differed considerably between the sites with 280 g/shoot at Heidgraben and only 130 g/shoot at Ellerhoop. For the sites Heidgraben and Ruthe, already the first

replanting (= 2nd replanting generation) led to significantly reduced shoot growth by 66.9% and 71%, respectively. Over the following replanting generations, the shoot masses stayed more or less constant for these two sites (one exception being the increase in shoot mass in the 4th replanting generation at Heidgraben). For soil at site Ellerhoop due to very poor growth in the first replant generation only a slight decrease in shoot mass was observed (Fig. 2). However, if the 4th replant generation is compared to the 2nd, the shoot fresh mass was halved. Standard deviations indicating the variation among the four plots were small, except for Ruthe in the 1st and 3rd replant generation. This observation generally confirms the findings on small in-site soil
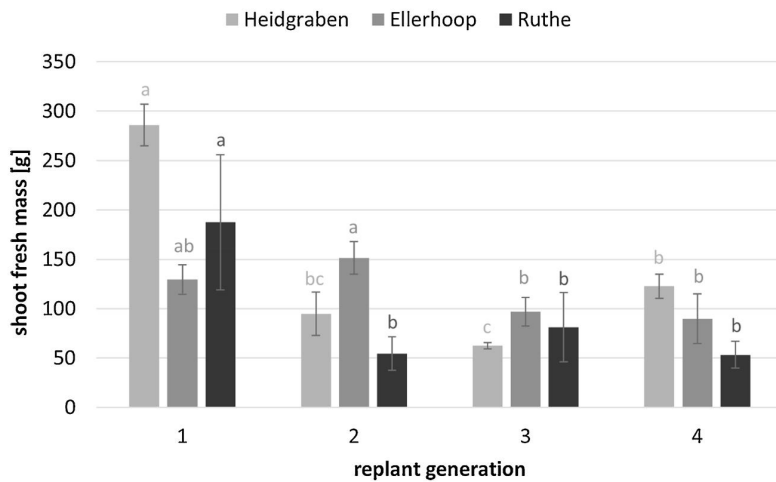
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Fig. 2. Mean shoot fresh mass of *Malus domestica* Bittenfelder plants at the three reference sites after different replanting generations. Per plot 50 shoots were cut above-ground at the end of the second vegetation period of each replanting period and weighed.

Same letters indicate no statistically significant difference within one site (Tukey test p ≤ 005; n = 4 and I = SD).

heterogeneity.

3.3. Bio-test to verify ARD incidence and severity

To evaluate the ARD severity at the reference sites, in vitro propagated M26 plantlets were planted in pots with ARD and grass soil, either untreated (UT) or gamma irradiated (G), in 2016 and 2017. Overall, the gamma irradiation led in most soils to a significant increase in shoot length and biomass. In both years, the lowest biomass was obtained in soil from untreated (UT) ARD plots, the highest in gamma irradiated (G) soil from grass plots, regardless of the site (Figs. 3 and 4). The effects of the gamma irradiation on the above-ground parts of the plants were higher than those on the root systems. The latter showed only in Heidgraben 2016 a significant increase of dry mass in the gamma irradiated ARD soil compared to untreated ARD soil (Fig. 4). In 2017, for sites Ruthe and Ellerhoop, the differences in shoot and root dry mass of plants grown in ARD UT compared to G were not significant (Fig. 4).

Gamma irradiation of ARD soil led in all variants (except for Ellerhoop in 2016) to a significant increase in shoot length compared to the untreated ARD soil after 8 weeks (Fig. 5 and Suppl. Fig. A3). The highest effect was observed in soil from Heidgraben (2017) with an increase of 332% in shoot length compared to untreated ARD soil. Moreover, in most soils the differences in shoot length in untreated and gamma irradiated ARD soil were already significant after 3–4 weeks, for soil from Ellerhoop in 2017 already after one week (Fig. 5).

Gamma irradiation also resulted in an increased shoot length of M26 plants growing in the control soils. After 8 weeks, the increase of shoot length of plants growing in G grass soil compared to UT grass soil varied for the different sites from 6.3% (Ruthe 2017) to 78.2% (Heidgraben 2016). But overall, this effect was much smaller compared to the increase due to gamma irradiation in the ARD soils (from 40.4% for Ruthe 2017 to 332% for Heidgraben 2017).

4. Discussion

4.1. Soil properties and ARD

Soil investigation at different spatial scales did not in all cases yield clear results. Most distinct effects were observed between the three sites that had been selected with respect to large differences in soil texture and related properties. In consequence, the chosen test sites form an appropriate investigation basis for this and further ARD studies. However, in-site heterogeneity (plot and m-scale) was mostly small and, consequently, no clear differentiations were achieved at that scale. This reduces the risk of unexplained variation in future studies. Further,

this statement could only be made that clearly after performing non-invasive sensing surveys in high spatial resolution.

Soil texture was supposed to affect ARD by direct and indirect effects. One important aspect is likely the influence on the habitats of microorganisms. For other soil biota like nematodes, strong texture effects have been confirmed (Hbirkou et al., 2011). However, a direct correlation between e.g. pore size and ARD was not yet found (von Bronsart, 1949). For this study, we selected three soils with clearly expressed, significant differences in texture to monitor ARD. This selection was insofar successful as ARD effects, i.e. growth depressions, differed between sites and were highest in the sandy soil at Heidgraben. Further, less difference to treated soils or grass controls occurred in the silty soils of Ruthe (Figs. 3 and 5).

For soil pH, controversial effects on ARD were reported in former studies. At low pH, ARD problems were found to be either less pronounced (Jonkers et al., 1980; Utkhede, 2006), or more developed (Willett et al., 1994). We here found a higher degree of ARD on sandy soils with low pH (5.0), however, there were no clear differences in soil pH between ARD and control (grass) soil, indicating that changes in pH were not induced by apple replanting. Whether this smaller pH in sandy soils was a factor that promoted ARD effects can only be hypothesized for future studies, such as in microcosms. However, pH is a rather unstable parameter and cannot alone explain ARD expression.

Soil organic matter is generally seen as positive for various soils functions, as it may provide nutrients and water to plants and soil microorganisms (Weil and Brady, 2016). Further, soil organic matter seems to act against strong ARD expression (Franke-Whittle et al., 2018). We here rather found a negative trend (Suppl. Fig. A2), i.e. soils with higher SOC were most affected by ARD. However, it seems likely that ARD was rather affected by soil texture (sand content, Suppl. Fig. A2), than by SOC, since differences between the sites were far stronger than within single sites, and no significant variation between ARD and control plots was observed. Further, soil texture influences SOC content, because sandy soils dry out more frequently, at least in the topsoil. During dry phases, microbial activity decreases, leading to organic matter enrichment in the long term. Vice versa, high available water capacity and long-lasting moist conditions generally lead to high microbial activity and subsequent organic matter decay.

A weak tendency may hide in the quality of organic matter. The C/N ratio as a measure for the processing of organic matter decreased from the most affected ARD site Heidgraben to the less affected ARD site Ruthe in the order 18.5 < 12.6 < 11.5 (Table 2). This may indicate that differences in organic matter quality may support development of ARD in sandy soil. Differences in C/N ratio and low pH values point to a less active microbial community and reduced C turnover (Weil and Brady, 2016) when compared to the other soils under study.

Fig. 3. M26 plants after 8 weeks culture in ARD or grass soil from the three reference sites Heidgraben, Ellerhoop and Ruthe which was either untreated (UT) or gamma irradiated (G) in 2017.

4.2. Sensor-based analyses giving information about soil heterogeneity

Although EMI data do not provide isolated information on distinct soil properties, surveys are widely carried out in terms of pattern recognition and can serve to explain growth differences of, e.g., yield or vegetation indices within arable fields (e.g., Sun et al., 2011; Rudolph et al., 2015). Thus, non-invasive recording of soil sensor data can potentially help to elucidate soil-ARD relationships at spatial resolution

and data density not yet known. That way, correlations between plant growth parameters and soil properties can potentially be elucidated that were not accessible with conventional methods. However, variability of soil properties within the examined plots was rather small. Although sensor application could not unravel deeper relationships between soil and ARD expression, these data help substantially to strengthen the outcome of the study. This is because it was assured that no unknown soil effects occurred, possibly blurring the results. Furthermore, the potential of such methods was demonstrated, and perspectives for future ARD studies arise when soil sensor application at high spatial resolution are taken into account as innovative methodology. Although the sensor data showed only a low heterogeneity in soil properties, the plant to plant variation in the field but also pot to pot variation in the bio-test require even smaller scale approaches. Again, soil sensor application may contribute to fulfill this requirement with an appropriate sensing design.

4.3. Induction of ARD at the three sites

By the observation of the growth of apple plants directly in the ARD plots of the three field sites (Fig. 2), we could show the severe growth reduction which is typical for ARD, thus indicating that ARD was successfully induced at the three sites, although to a different extent. At site Ellerhoop the growth decrease over time was lowest (Fig. 2). This rather small temporal variation might be explained with the stagnic conditions at Ellerhoop, because a certain oxygen deficiency might have reduced the development of ARD (see below). Surprisingly high was the early decline already in the 2nd replant generation. The data obtained in this field trial should, however, be considered with care, since plant growth in the field is strongly subjected to weather conditions, such as periods of drought or heat, strong frosts or water logging. Especially the conditions after planting may be decisive for plant growth. Weed control became increasingly difficult at the three sites, because the plots could not be ploughed. Moreover, the plants did not have identical sizes in the different years and seedlings are genetically inhomogenous material. Therefore, the data obtained in the bio-test which were obtained with clonally progagated and clean plant material and under controlled greenhouse conditions better reflect ARD incidence and severity.

It has to be mentioned that after the strong rainfalls in autumn and winter 2017/2018 flooding of plots was observed at the sites in Ellerhoop and Ruthe. Observations by growers are that anaerobic conditions due to water logging extenuate ARD, and also Hewavitharana and Mazzola (2016) suggest anaerobic soil disinfection as counteraction against ARD.

4.4. Bio-tests verify ARD incidence and allow an estimation of ARD severity

So far, the best indicator to prove ARD and to estimate its severity is the growth difference of plants grown in disinfected and untreated soil in a bio-test. Therefore, we conducted the bio-test described by Yim et al. (2013 and 2015) using in vitro propagated M26 plants, being free from pathogens and genetically identical. For most of the soils, already after 3 weeks shoot length was already significantly lower in UT than G variants (Fig. 5). In the case of Ellerhoop in 2017, these differences in shoot length were already significant after one week. When ranking the soils based on the data of 2017 for ARD severity, highest ARD incidence was recorded for the sandy soil from Heidgraben, followed by the loess soil from Ruthe and least ARD severity was observed in soil from Ellerhoop. Thus, the bio-test supported the observations made in the field (Fig. 2).

One advantage of gamma irradiation over other disinfection treatments is the low effect on chemical and physical soil properties (Trevors, 1996). The increased growth of apple plants in gamma irradiated soil (Figs. 3–5) supports the common opinion that biotic factors are the main cause of ARD. Moreover, beneficial and antagonistic
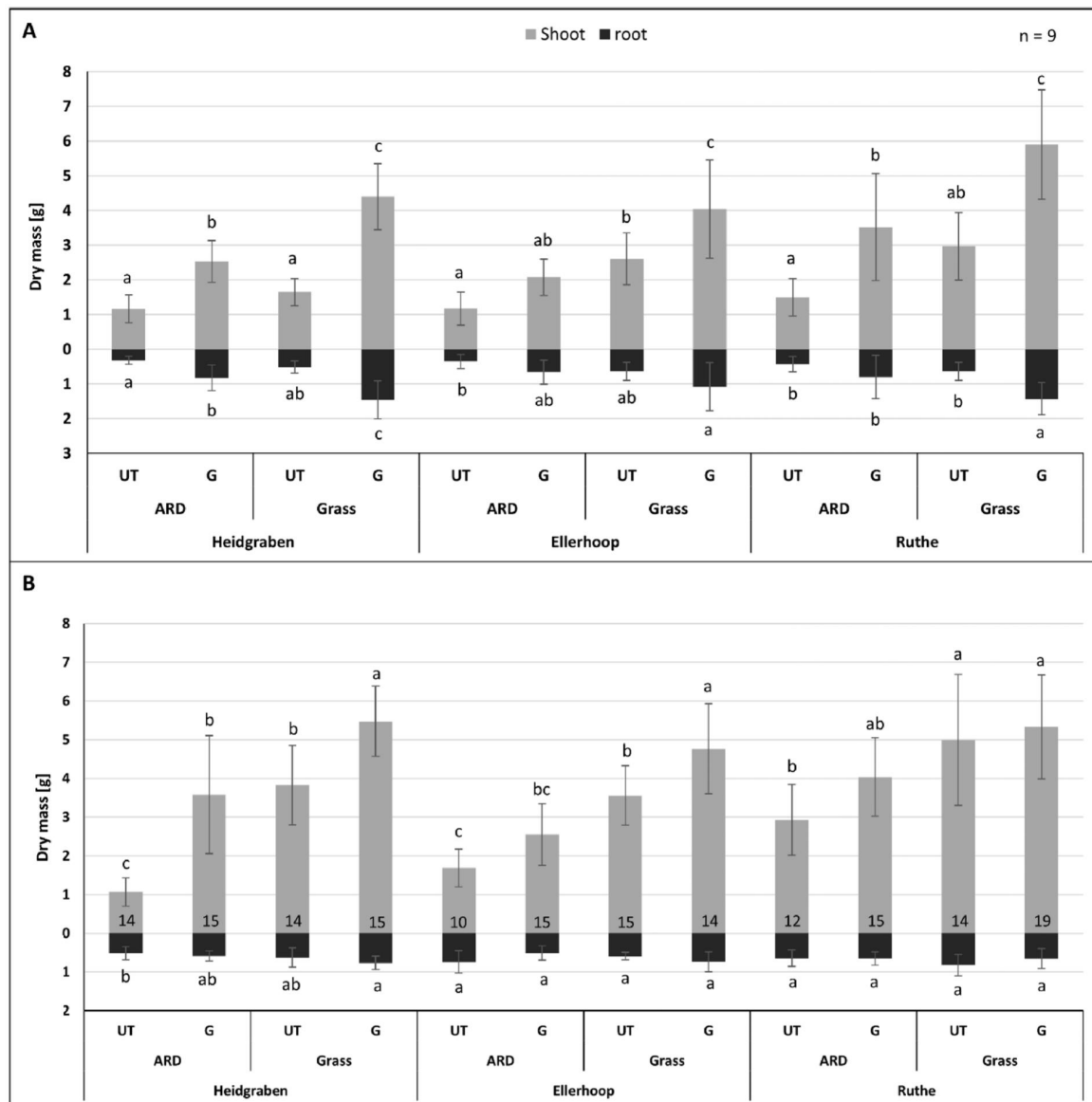
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Fig. 4. Shoot and root dry mass of M26 plants after 8 weeks of culture in ARD or grass soil from the three sites Heidgraben, Ellerhoop and Ruthe, which was either untreated (UT) or gamma irradiated (G) in 2016 (A) and 2017 (B). Same letters indicate no statistically significant difference within one site (Tukey test p ≤ 0.05; I = SD; n: A = 9, B = indicated in the columns).

microorganisms were found in lower abundance in ARD soils (Yim et al., 2015). For example, the plant growth promoting bacteria *Mucilaginibacter*, *Devosia*, and *Rhodanobacter* were significantly increased in the rhizosphere after an 8-week growth period of M26 plants in gamma irradiated ARD soil compared to untreated ARD soil (Yim et al., 2015).

Irradiation of grass soil also led to an increase of biomass of the M26 plants (Fig. 4). This could be due to three facts: (i) also in the grass soil detrimental microorganisms were present, (ii) gamma irradiated soils are quickly recolonized by plant growth promoting bacteria (Yim et al., 2015) or (iii) cell lesion of the microorganisms due to gamma irradiation leads to a release of nutrients which are easily accessible for the plant. McLaren (1969) described that irradiation induces a release of small amounts of ammonium, organic nitrogen, soluble carbon, phosphorus, manganese etc. from the soil microbes. Weiß and Winkelmann (2017) compared the nutrient content of M26 shoots grown in untreated and gamma irradiated soil and found no pronounced differences. To test for nutrient effects, we planted winter wheat in pots with

the same soils as used in the CE2 (data not shown). There were, however, no significant differences in wheat shoot dry masses indicating that there was no nutritional effect of the irradiation. Furthermore, no growth reduction of wheat in ARD soil confirmed the specificity of ARD. Thus, it is more likely that grass soils contained detrimental microorganisms as well and/or gamma irradiated soils are quickly recolonized by plant growth promoting bacteria. To proof this microbial analyses are necessary.

This raises the question of proper controls in ARD research. Disinfected soil represents a highly artificial situation which is rapidly changing due to recolonization. On the other hand, microbial communities (St. Laurent et al., 2008) and the soil mesofauna (Koehler and Born, 1989) are influenced by the grass cover.

4.5. ARD incidence is influenced by soil parameters

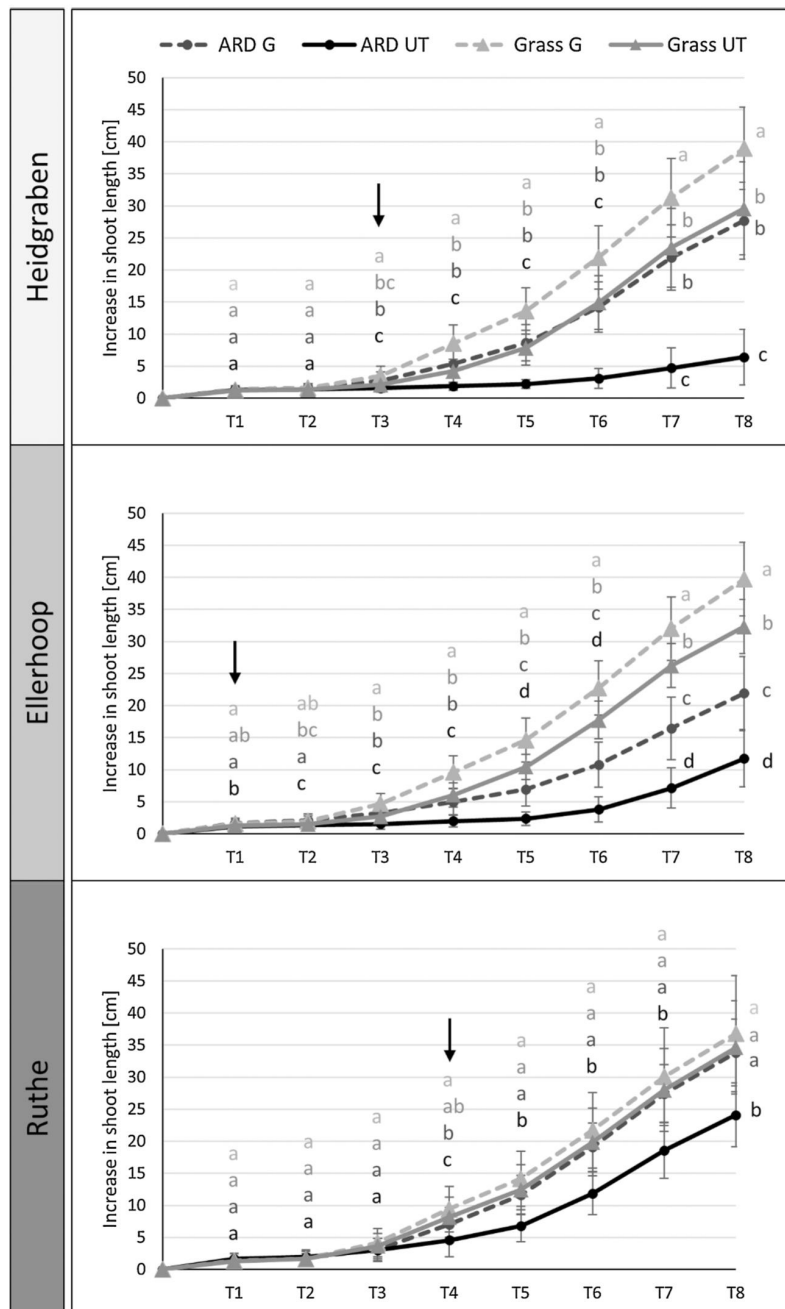Knowledge on how soil characteristics influence ARD incidence

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Fig. 5. Increase in shoot length of in vitro propagated M26 plants after transfer into either untreated (UT) or gamma irradiated (G) ARD and grass soil from the three reference sites Heidgraben, Ellerhoop and Ruthe. Shoot length measurements were performed weekly for the 8-weeks growth period in 2017. Arrows indicate first statistically significant difference between UT and G ARD soil. Same letters indicate no statistically significant difference within one time point and one site (Tukey test $p \leq 0.05$; I = SD, n = Supplementary Table A2).

would allow a better understanding of the etiology and enable predictions of risks of growth and yield reduction for given plots. Only one earlier study tried to correlate soil pH and nutrient content with ARD by analyzing apple seedling development in 568 soil samples from ARD sites (Li and Utkhede, 1991). However, measures for ARD severity should include the difference in plant growth in ARD soil and disinfected ARD soil. Since this comparison was lacking in the study of Li and Utkhede (1991), the observed effects on plant growth can not properly be linked to ARD. When trying to correlate soil abiotic factors to ARD in six soils from South Tyrol, Spath et al. (2015) compared untreated and pasteurized ARD soils. They concluded that the etiology

of ARD was not significantly influenced by soil pH, metals and most nutrients analyzed. However, this observation could have been caused by sampling soils which differed only marginally in these properties. Here, we investigated for the first time the relationship between different soil properties and growth depression being an expression of ARD severity at three ARD sites, which differ fundamentally in soil parameters but have the same cropping history and proper control plots. Regarding growth depressions, we could show a negative correlation (-0.714) with clay content and a positive correlation (0.703) with the C/N ratio. Of course, these correlations can only be takes as first indications as they are based only on two data points each for the three

sites. Further studies are needed to reveal a statistically relevant picture of the dependence of ARD etiology and severity on soil parameters.

5. Conclusion

We conclude that the given sites at Ellerhoop, Heidgraben and Ruthe represent a highly suitable soil set for future studies to analyse the relationship between soil properties and ARD, in particular due to their difference in soil texture. A future monitoring of soil properties within these sites may then reveal if soil organic matter quality and N status in soil develops differently in ARD versus control soils. Sensor application prior to establishing field experiments helps to exclude unexpected and/or unexplained effects because it delivers soil information at low efforts and costs. Further, with an appropriate study design, soil sensing can help to further unravel ARD-soil interaction, because soil information can be gained at very high spatial resolution.

Author contributions

Conceived and designed the experiments: TW AW.
Performed the experiments: FM AW MS.
Analyzed the data: FM MS.
Contributed reagents/materials/analysis tools: TW EL SP.
Contributed to the writing of the manuscript: FM TW AW MS SP EL.
The authors declare that there is no conflict of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2018.06.076.

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2.2 Diagnosis of apple replant disease (ARD): Microscopic evidence of early symptoms in fine roots of different apple rootstock genotypes

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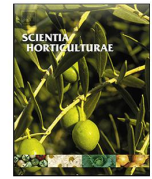
Diagnosis of apple replant disease (ARD): Microscopic evidence of early symptoms in fine roots of different apple rootstock genotypes

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ABSTRACT

Apple replant disease (ARD) is a problem of high economic relevance in tree nurseries and apple orchards worldwide. After replanting, plants show growth reduction, reduced yield, and damaged root systems. The disease's decade-long persistence in soil exacerbates the situation where alternative cultivation areas and ecologically safe soil decontamination measures are lacking. The etiology is still unclear and there are neither clear causal factors nor reliable parameters (except growth data from plant bioassays) for the early detection of the disease in plants or from samples from potentially affected soils. We report on greenhouse pot trials with the susceptible rootstock *Malus domestica* 'M26' grown in soils of ARD-affected as well as grass plots from three different field sites. Both gamma-irradiated ARD soils and grass soils served as control soils. The aim was to develop a diagnostic tool for the early detection of ARD. In fine roots of first to third order, we assessed the root structure, the root hair density, the cellular damages in the outer layers, and the cell vitality. After only two weeks in ARD soil, 'M26' reacted with cell necroses and blackening in a characteristic pattern, impaired root hair development, and low cell vitality. These symptoms were confirmed in *Malus domestica* 'Bittenfelder' and *Malus*-hybrid B63. Root systems grown in gamma-irradiated soils were almost symptom-free while those from grass soils showed different and only minor alterations. Thus, we suggest selected fine root symptoms to be used for an early diagnosis of ARD, e.g. in plant breeding selections and soil surveys.

1. Introduction

Apple replant disease (ARD) is a problem of high economic relevance in tree nurseries and apple cultivation areas worldwide. For decades, many studies have been published regarding the manifold aspects of the disease's occurrence, yield losses, geographic distribution, etiology-associated organisms and toxins, and attempts to overcome the problem through cultivation practices like inter-row cropping, soil decontamination, or soil additives like microbes and composts (Klaus, 1939; Savory, 1966; Hoestra, 1968; Utkhede and Li, 1989; Mazzola, 1998). Research in the field of replant disease has intensified with the exacerbation of the problem due to the lack of soils available for new plantations. Recent reviews give an overview of the current state of knowledge including control measures (Tewoldemedhin et al., 2011; Mazzola and Manici, 2012; Winkelmann et al., 2019). Yet, the etiology of ARD in its complexity is still unclear: Endophytic microorganisms, Actinobacteria, oomycetes, fungal pathogens, and nematodes as well as a dysbiosis of the microbiome are under discussion as

potential causes (Hoestra, 1968; Caruso et al., 1989; Braun, 1991; Otto et al., 1994; Manici et al., 2003, 2013; Yim et al., 2013; Caputo et al., 2015; Franke-Whittle et al., 2015; Manici et al., 2017). Root debris and root exudates may also be involved in the disease induction (Hofmann et al., 2009; Leisso et al., 2017). The recent development of new molecular tools allowed for new insights both into the response of apple in ARD soils on a molecular level, e.g. with the help of gene expression studies (Shin et al., 2016; Weiß et al., 2017a, b), and into microbial community compositions in ARD-affected soils (e.g. Tewoldemedhin et al., 2011; Caputo et al., 2015; Franke-Whittle et al., 2015; Yim et al., 2015, 2017). These topics are now in the foreground.

Many previous studies on the effects of ARD on plants have addressed growth reduction at field sites and focussed on the impact on aboveground biomass production. Damage to the root system is easily expressed as reduced shoot biomass. However, the fine root system - responsible for the essential absorption of water and nutrients - is the first part of the plant to be exposed to ARD-affected soil. Thus, the first, early impact of ARD on root growth and development needs a careful

investigation (e.g. Yao et al., 2006). Hoestra (1968) described ARD-symptoms of root samples from field and pot cultures as a complex of root tissue damage, discolorations and necrosis, root hair reduction in size and number, and root rot. After formation of new roots in a recovery phase, many of the new rootlets rotted away or grew only marginally or distinctly swelled. In contrast, fine roots of healthy plants grew undisturbed, were not discoloured, and were structurally intact. In association with fungal infections, Braun (1991) affirmed root symptoms of ARD as discolorations, black lesions and cortical decay. Emmett et al. (2014) found a relationship between root development and damage by ARD-associated pathogens based on the classification of root orders within the architecture of apple fine roots. Pathological changes in fine roots of first and second order were the results of a reaction to fungal infections and defence compounds allocated differentially across root branching orders. The root growth of different rootstock genotypes under ARD challenge has been of interest in plant breeding (St. Laurent et al., 2010). In analyses conducted by Emmett et al. (2014), rootstocks that were less susceptible to ARD than M26 showed higher rates of root growth and supported a lower abundance of ARD-pathogens. Likewise, Atucha et al. (2014) used the fine root classification to study the rootstock tolerance to ARD; root architecture, fine root lifespan, and root functions were analyzed in response to ARD in order to compare different rootstock genotypes. These publications gave us reason to investigate microscopically early reactions in the fine root region in more detail.

Surprisingly, the classical approach to study root tissue affected by ARD by means of microscopy (Caruso et al., 1989) has not been widely used up to now and root damages due to ARD were later described in broad terms rather than from a cellular point of view. As long as the complex cause of ARD is difficult to determine with general validity there is a need for rapid and reliable diagnosis of early disease symptoms. Within the German research program BonaRes ORDIAmur (www.ordiamur.de), three field sites were important sources for ARD soils with defined apple cropping history for the joint central experiments (CE2) of the research consortium (Mahnkopp et al., 2018). At the sites (hereinafter termed ARD-reference sites) 'Bittenfelder' apple plants provided first indications of injuries and tissue damages in fine roots (Mahnkopp et al., 2018). Due to differing sampling dates and weather and soil conditions, however, we could not obtain a sufficient amount of appropriate fine roots harvested per plant for extended microscopic assessments. Therefore, the already established greenhouse bioassay with sick soils from these fields and in vitro propagated apple plants (Yim et al., 2013, 2015) offered better conditions for studying the early ARD symptoms in fine roots. The present study aimed to identify reliable ARD symptoms for an early detection of the disease. Further, the results should lead to a diagnostic tool with practical relevance for testing potentially ARD affected soils and for supporting selection of ARD-tolerant rootstock genotypes.

2. Material and methods

2.1. Plant material

In all experiments, the indicator plant used for early detection of ARD was the susceptible apple rootstock 'M26' (hereinafter given as M26) with in vitro propagated plantlets potted four weeks after acclimatization (Yim et al., 2013, 2015). Additional rootstock genotypes tested were Malus domestica 'Bittenfelder' and the Malus hybrid genotype B63; both were propagated in vitro and acclimatized in the same way as described for M26. B63 is an offspring of the cross 4608 (M. purpurea 'Eleyi' x M. sieboldii) x M. pumila rootstock 'M9' derived from a breeding program aimed at resistance to apple proliferation disease (Jarausch, pers. comm.). Field-raised seedlings of 'Bittenfelder' with shoot lengths of 10 to 12 cm and received in June 2016 from a commercial nursery (Stahl Baumschulen GmbH, Tornesch, Germany) were used for a test in virgin soil.

2.2. Soil material

Three types of soils were collected in a depth of 0–20 cm at three different field sites (ARD-reference sites), sieved through 8 mm mesh and thus freed from thick roots and clumps of soil before used in greenhouse trials (Mahnkopp et al., 2018).

2.2.1. ARD-reference sites: locations, types and cropping history

The reference sites were Ellerhoop (E; x-coordinate 53.71435; y-coordinate 9.770143 WGS 84, Schleswig-Holstein, northern Germany), Heidgraben (H; x-coordinate 53.699199; y-coordinate 9.683171; WGS 84, Schleswig-Holstein, northern Germany), both of which were located at sites in the nursery area around Pinneberg, and Ruthe (R; x-coordinate 52.243668; y-coordinate 9.819700; WGS 84) near Hannover, Germany. The soil types were determined according to IUSS working Group WBR (2015) as an endostagnic luvisol (E), an entic podzol (H), and a haplic luvisol (R). Soil textures were classified as sand at Heidgraben, as loamy sand to sandy loam at Ellerhoop, and as silt loam at Ruthe, respectively (Mahnkopp et al., 2018). At these sites, the apple rootstock 'Bittenfelder' had been successively replanted 5 times since 2009 to induce ARD.

2.2.2. Soil treatments

The soil treatments were: (1) untreated ARD soil from apple plots where the apple rootstock 'Bittenfelder' had been successively replanted 5 times since 2009 (ARD ut), (2) untreated soil from the same site, taken from adjacent grass plots where no species of Rosaceae had been grown before (Grass ut), (3) soil of ARD and grass plots, respectively, γ-irradiated with a minimal dose of 10 kGy (Yim et al., 2015) (ARD γ, Grass γ). In addition, we used untreated sandy loam soil collected from a cereal field site in June 2016 (Aerzen, Lower Saxony; x-coordinate 52.087091, y-coordinate 9.200488; WGS 84) not previously exposed to a Rosaceae planting as virgin soil in an assay to determine the status of health in fine roots of 'Bittenfelder' seedlings compared to those grown in grass plot soils, and further to control microscopic methods.

2.3. Experimental set-up for greenhouse bioassays

2.3.1. Central greenhouse experiment CE2

The central greenhouse experiment CE2 for the ORDIAmur group was conducted twice, in 2016 and in 2017. The different soils were mixed with $2\,g\,L^{-1}$ Osmocote Exact Standard 3–4 M (16 + 9 + 12 + 2MgO, Everris International B.V., Geldermalsen, The Netherlands) and filled into 1 L-pots. One acclimatized M26 plantlet per pot was planted and the pots were randomized in three blocks with 10 replicates (CE2 2016) and four blocks with nine replicates (CE2 2017). Greenhouse conditions, irrigation schedule, and plant protection measures were chosen following Yim et al. (2015). The shoot length was measured weekly from the surface of the soil to the base of the newly emerging leaves of the main shoot. After 8 weeks, fresh and dry biomass (70 °C for 1 week) of the shoot and root system were weighed. Fresh biomass and shoot length data of the corresponding sites were log-transformed to achieve homogeneity of variances (tested by Levene's test) and submitted to an analysis of variance (ANOVA) followed by Tukeys` HSD test (p < 0.05). All statistical analyses were performed using R-3.4.2 (R Development Core Team, 2017).

During these experiments, two randomly selected plants per treatment were harvested after 2, 4, and 8 weeks for photo-documentation and microscopic root analyses. The number of plants studied was limited due to the limited time capacity for microscopy of fresh root material from a variety of treatments per harvest time (12 in CE2 2016 and 16 in CE2 2017).

2.3.2. Bioassay with Malus hybrid B63

In August 2017, the response of the rootstock Malus-hybrid B63 to

ARD soil was compared to that of M26 in a greenhouse bioassay with ARD soil from the field site Heidgraben (H). The experimental setup was similar to CE2 2017; only in order to save soil, two plants were cultivated per 1 L container. The experiment included untreated ARD soil (ARD ut) and γ-irradiated ARD soil (ARD γ). *In vitro* propagated plantlets were acclimatized for 30 days and potted at a mean shoot length of 0.88 ± 0.47 cm (M26, n = 115) and 2.54 ± 1.00 cm (B63, n = 115), respectively. Six randomly selected plants per treatment were harvested after 4 weeks for photo-documentation and microscopic examination.

2.3.3. Greenhouse-test in virgin soil with 'Bittenfelder' seedlings

Immediately after sampling, the virgin soil was filled into 1L-pots without further processing. Twenty field-raised seedlings of 'Bittenfelder' with a shoot length of 10–12 cm were cleaned with tap water, potted into the virgin soil, and cultured under greenhouse conditions of 24 ± 2 °C, 16-h photoperiod, and irrigated by hand when necessary. After 8 weeks, six randomly selected plants were photo-documented and their roots prepared for microscopy as described below.

2.4. Sample preparation and microscopy techniques

Plants were very gently rinsed in a beaker with tap water to avoid loss of lateral fine roots and epidermal tissue. Root systems were freed from adherent soil remnants with forceps (Dumont No 5) in a Petri dish filled with tap water. Fine roots of 0.1-0.7 mm in diameter from different parts of the root system of each plant (up to a maximum distance of 3 cm to the tip) were cut off and stained in FUN®1 cell stain (F-7030, Molecular Probes™, Thermo Fisher Scientific, Life Technologies, USA; 50 μM working solution in distilled water) for at least two hours in the dark. After subsequent clearing in sterilized distilled water, fine root samples were cut into 1 cm-segments and mounted on glass slides (Grunewaldt-Stöcker and von Alten, 2016). The discrimination of living from dead cells is based on the formation of moving cylindrical intravacuolar structures (CIVS) only in metabolically active cells. Staining with toluidine blue O (C.I. 52040, Merck; 0.03% in phosphate buffer pH 7.0) for 3–8 min followed by clearing in sterilized distilled water was applied to enhance the appearance of mycelial structures and to determine tissue components. Root segments were assessed with bright-field-DIC-microscopy 200-1000x, and epifluorescence microscopy (Zeiss AxioImager A2, extinction BP 485/20, beam splitter FT 510, emission LP 515; photo-documentation with Axiocam MRC).

2.5. Experimental design for microscopy

The microscopic assessments with M26 were based on thirty root segments randomly selected from each of two plants per treatment, soil treatment and harvest time after 2, 4, and 8 weeks in two independent experiments (CE2) in 2016 and 2017. Analyses of roots from 'Bittenfelder' in 2017 were restricted to soil from the field site Ruthe. In another experiment in 2017, the rootstock B63 was tested in addition to M26 in soil from site Heidgraben with two treatments (ARD ut and ARD-γ) and six plants per treatment assessed after 4 weeks. To determine the status of fine roots of 'Bittenfelder' plants grown in virgin soil, six plants were harvested for microscopic root analyses after 8 weeks of greenhouse cultivation. Ratings of each root segment regarded:

- its position in the root system (first to third order; Emmett et al., 2014),
- the intensity of root structural and surface damages in three classes (intact, slight damage, destructions),
- the occurrence of root hairs estimated in four classes (none; few: < 50; medium: > 50; many: > 100),
- cellular damages in the outer tissue layers in four classes (light

browning, necrosis, blackening, cytoplasmic inclusions),
- the vitality of rhizodermic, hypodermic, and, occasionally, first cortex cell layers, rated in five percentage classes representing 0, ≤ 25, 26–50, 51–75, > 75% of metabolically active cells per 1 cm-root segment,
- the occurrence of visible microorganisms (Actinobacteria, other bacteria, filamentous fungi, mycorrhizal fungi, oomycetes) and nematodes.

Plants from the different treatments were analyzed in a rotating scheme over the rating time of several days to maintain most equal test conditions. The rating data of CE2 with two plants were pooled and frequencies of symptoms were calculated for 60 randomized fine root segments, whereas in tests with B63 and with 'Bittenfelder' in virgin soil data of six plants per treatment were pooled and calculated for 180 randomized fine root segments. Since these are ratings and the data are based on a limited number of plants per treatment and harvest date, we have decided not to subject them to statistical significance tests, but to present absolute numbers of root segments in the various rating classes.

3. Results

3.1. Plant growth

In 2016 after 8 weeks of growth, M26 shoot fresh mass was significantly lower in untreated ARD soils vs. untreated grass treatments in soils of two reference sites (Ellerhoop and Ruthe), while in Heidgraben soil the difference was less pronounced (Table 1, ANOVA results are presented in the Supplement Tables S1 and S2). In the repetition trial 2017 (Table 2) this effect was significant for M26 and 'Bittenfelder' in soils of all tested sites. The irradiation treatment led to a significant increase in shoot fresh mass in all treatments (2016 and 2107), except in the grass soil of Ruthe soil (M26, 2017) when compared to plants in untreated soil (Tables 1 and 2). In all cases of both trials, the irradiation yielded a significantly higher shoot fresh mass in grass treatments than in ARD soils (except for Ruthe, M26 in 2017). The parameter root fresh mass, on the other hand, reflected the ARD effect less clearly, although M26 and 'Bittenfelder' root systems were visibly affected in untreated soils (Table 1; three-way ANOVA did not reveal an effect of the genotype: p = 0.284). Untreated ARD soils in 2016 led to less root fresh mass of M26 than did untreated grass soils of the three sites, but not with statistical significance. In addition, the irradiation treatment increased root fresh mass significantly only in M26 in two grass treatments (H and R; ut vs. γ). Comparing the root fresh mass of plants

Table 1
Plant biomass response of apple rootstock 'M26′ grown for 8 weeks in untreated soil (ut) and γ-irradiated soil (γ), sampled at three ARD-field sites from ARD plots and grass plots, respectively. Data of fresh masses of shoot and root, respectively, in experiment CE2 2016. Means with the same letter are not significantly different within the site (Tukey test p ≤ 0.05; n = 9).

CE2 2016			Fresh mass			
Site	Plot	Treatment	Shoot [g] mean ± SD		Root [g] mean ± SD	
Heidgraben	ARD	ut	4.64 ± 1.52	c	2.31 ± 0.80	b
		γ	8.25 ± 1.83	b	5.10 ± 2.48	b
	Grass	ut	6.55 ± 1.65	bc	3.50 ± 0.94	b
		γ	14.24 ± 2.78	a	9.42 ± 3.22	a
Ellerhoop	ARD	ut	4.00 ± 1.51	c	2.31 ± 1.16	c
		γ	6.59 ± 1.42	bc	3.22 ± 1.26	b
	Grass	ut	9.59 ± 2.86	b	3.88 ± 1.24	ab
		γ	13.65 ± 4.43	a	5.30 ± 1.89	a
Ruthe	ARD	ut	4.37 ± 1.59	c	2.23 ± 1.03	b
		γ	11.57 ± 4.32	b	4.40 ± 2.45	b
	Grass	ut	9.86 ± 3.02	b	3.26 ± 1.29	b
		γ	17.90 ± 4.25	a	7.64 ± 2.30	a

Table 2

Plant biomass response of apple rootstocks 'M26' and 'Bittenfelder' grown for 8 weeks in untreated soil (ut) and γ-irradiated soil (γ) sampled at three ARD field sites from ARD plots and grass plots, respectively. Data of fresh masses of shoot and root, respectively, in experiment CE2 2017. Means with the same letter are not significantly different within the site (Tukey test p ≤ 0.05).

Genotype	CE2 2017 Site	Plot	Treatment	N	Fresh mass Shoot [g] mean ± SD		Root [g] mean ± SD	
M26	**Heidgraben**	ARD	ut	14	2.85 ± 0.95	c	1.38 ± 0.31	c
			γ	15	10.77 ± 1.86	b	3.13 ± 1.30	b
		Grass	ut	14	12.38 ± 3.45	b	2.97 ± 0.91	b
			γ	15	18.05 ± 3.82	a	4.06 ± 0.72	a
	Ellerhoop	ARD	ut	10	4.78 ± 1.38	d	2.69 ± 0.85	ab
			γ	15	8.43 ± 2.34	c	2.98 ± 1.12	ab
		Grass	ut	15	11.25 ± 2.56	b	2.28 ± 0.60	b
			γ	14	16.76 ± 3.76	a	3.87 ± 1.72	a
	Ruthe	ARD	ut	12	8.49 ± 2.27	b	2.36 ± 0.96	a
			γ	15	12.80 ± 2.55	a	2.60 ± 0.77	a
		Grass	ut	14	13.98 ± 4.38	a	2.65 ± 1.26	a
			γ	19	16.36 ± 3.53	a	2.79 ± 1.48	a
'Bittenfelder'	**Ruthe**	ARD	ut	10	4.93 ± 1.48	c	2.22 ± 1.65	b
			γ	14	13.09 ± 2.52	b	3.90 ± 1.39	b
		Grass	ut	10	10.96 ± 1.56	b	2.96 ± 0.87	b
			γ	14	18.16 ± 4.43	a	6.45 ± 2.77	a

grown in irradiated soils, the treatment resulted in a significant greater level of growth improvement in grass soils from all sites in 2016 as to that observed in the ARD treatments. However, in 2017 this occurred only for M26 in Heidgraben soil and for 'Bittenfelder' in Ruthe soil (Table 2). In this repetition, the root fresh mass data showed even less differences between the treatments than in the test before. Overall, these growth data affirmed the induction of ARD in soils of the three reference sites in our trials, and shoot mass could better demonstrate growth depression by ARD than root mass.

3.2. Microscopic assessments of M26 and 'Bittenfelder'

Analyses of fine root segments of M26 in their unfixed original state were conducted after 2, 4, and 8 weeks in culture. Already after two weeks of contact with ARD soil, plants reacted with an impairment of several root features.

Regarding the outer appearance of the roots, growth reduction and thickening of newly developed first order roots led to a dense and poorly branched shape of the root systems of plants grown in untreated ARD soils. In contrast, in plants grown in soils of grass plots, and especially for those grown in irradiated soils, such disorders were not observed, irrespective of original soil site and plot (Fig. 1). Fine roots of 'Bittenfelder' although being more robust in structure than those of M26 showed similar reactions. Assessments of the fine root structure based on constrictions and swellings and of surface damages (i.e. injuries, fissures, and disrupted or missing tissue) showed pronounced changes of M26 in untreated ARD soils of all sites early on. These changes progressed over time, whereas root segments from grass soils (except from site Heidgraben) and irradiated soil treatments showed intact root surfaces in most of the segments (Fig. 2 A). 'Bittenfelder' roots only tested 2017 in soil from site Ruthe, reacted in the same way as M26 (Fig. 2 B).

The ratings of the root hair status (Figs. S1, S2) gave clear and identical results in both experimental repetitions (2016 and 2017): Root hairs were often missing in untreated ARD soils of three sites. This effect lasted for the entire duration of the test (Fig. 3) and was also observed, albeit less pronounced, in 'Bittenfelder' roots grown in untreated ARD soil from site Ruthe.

Further early symptoms were arrested growth of lateral fine roots and emerging root tips accompanied by a blackening of the epidermal
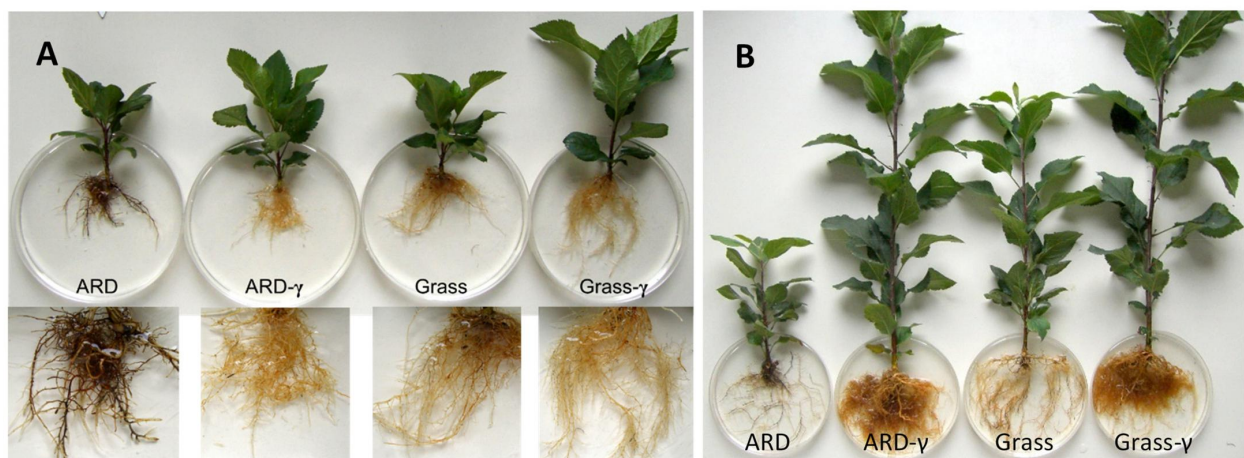

Fig. 1. *Malus domestica* M26 plants in CE2 (2016) in different soils: untreated ARD, ARD-γ, untreated Grass and Grass-γ, after 4 weeks culture in soil of reference site Heidgraben (A); after 8 weeks culture in soils of reference site Ruthe (B).
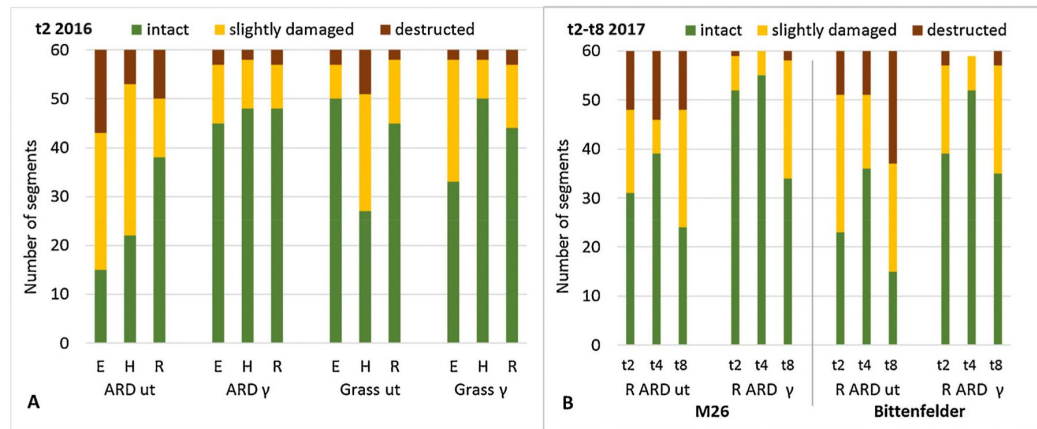
Fig. 2. Frequencies of fine root segments with structural and surface damages in three classes, n = 60 root segments. A: M26 after two weeks culture in soils of ARD plots and grass plots from Ellerhoop (E), Heidgraben (H), Ruthe (R), either untreated (ARD ut, Grass ut) or irradiated (ARD γ, Grass γ) in experiment CE2 2016. B: M26 and 'Bittenfelder' after two, four and eight weeks culture (= t2, t4, t8) in untreated ARD (ARD ut) and irradiated (ARD γ) soils from Ruthe (R) in experiment CE2 2017.

tissue (Fig. 4 A, B), constrictions in lateral fine roots, and disruption of outer cell layers at their base thus prematurely exposing the cortex tissue. Even primordia inside the root were discoloured and often root tips stopped elongation early on, resulting in several (brown to black) root tips clustered at one site. The most noticeable indicators of ARD were early cell damages in the rhizo- and hypodermis and even in deeper cell layers of the cortical parenchyma of elongated fine roots. Colour alterations in the epidermal tissue ranging from brown to black in individual cells as well as in areas of clustered necrotic cells, led to a patchy pattern of cell reactions (Fig. 4 C, D). Necrotic cell clusters increased with time and resulted in the typical symptom of blackening in fine roots, which was even visible to the naked eye (see Fig. 1 A). Black discoloration was even found in the intercellular space of cortex tissue (Figs. 4 E; and 5 A, B). The occurrence of cells with distinct black inclusions in the cytoplasm became most frequent after eight weeks in culture (Fig. 5 C). A slight browning of epidermal cells in younger root segments (in meristematic zones of root tips, elongation zones, and zones with and without root hairs), also accompanying the described damages, occurred in all treatments and sites with highest frequency (Figs. 5 D, 6). After the first symptoms had developed, the plants reacted to root damage with the formation of new pioneer and lateral roots. These new light-coloured rootlets stood out visually from impaired ones. However, the emerging lateral rootlets often stopped growing and eventually became necrotic. Moreover, lateral root primordia were often brownish. The microscopic observations are summarized in the frequencies of root segments in symptom rating classes

(Fig. 6).

Accordingly, cell damages like necroses, blackening and black cell inclusions occurring in roots grown in untreated ARD soils independently from the original soil sites are characteristic features of ARD, whereas light browning is not. These ARD symptoms appeared less frequently in untreated grass soil treatments, calling the grass soil into question as a control. In contrast, necroses, blackening, and black cytoplasmic inclusions were almost never present in treatments with irradiated soils. The comparison of M26 with 'Bittenfelder' in soils from site Ruthe in 2017 confirmed the previous findings (Figs. 4 F, G; and 6 C).

When investigating fine roots by means of brightfield and fluorescence techniques, microscopic analyses provided details of microorganisms occurring on the root surface and in the outer cell layers with prominent appearance of Actinobacteria and fungal infections (Fig. 7). FUN®1 cell staining indicated the cytoplasm of living bacteria with bright green fluorescence. There were clearly more segments with microorganism colonization in ARD-treatments of all locations than in the other treatments, and the irradiation of soils led to the aspired effect of eliminating microorganisms (Fig. 8). These observations were confirmed in 'Bittenfelder' in an equal manner (Fig. S3). Mycorrhizal colonization appeared constant after eight weeks in untreated soils. As in our previous trials, the occurrence of endoparasitic nematodes and of mycelia, oogonia and oospores of oomycetes was extremely low or nonexistent and therefore negligible.

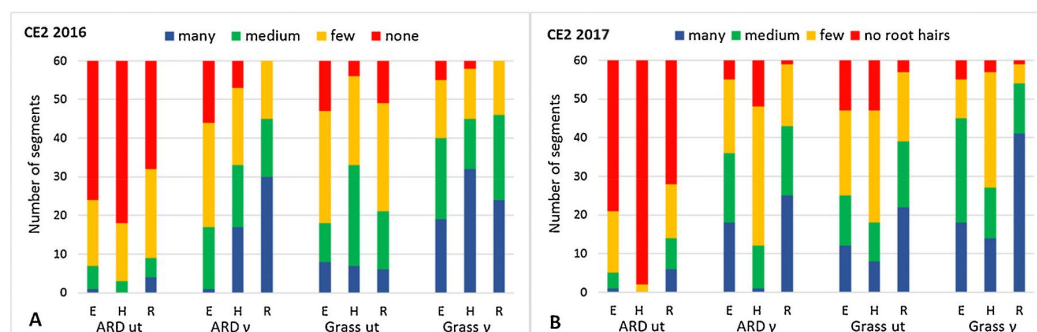By means of fluorescence microscopy after FUN®1 staining, the

Fig. 3. Frequencies of fine root segments with a root hair status in four classes, n = 60 root segments. A: M26 after eight weeks culture in soils of ARD plots and grass plots from Ellerhoop (E), Heidgraben (H), Ruthe (R), either untreated (ut) or irradiated (γ) in experiment CE2 2016, and B: data from experiment CE2 2017.
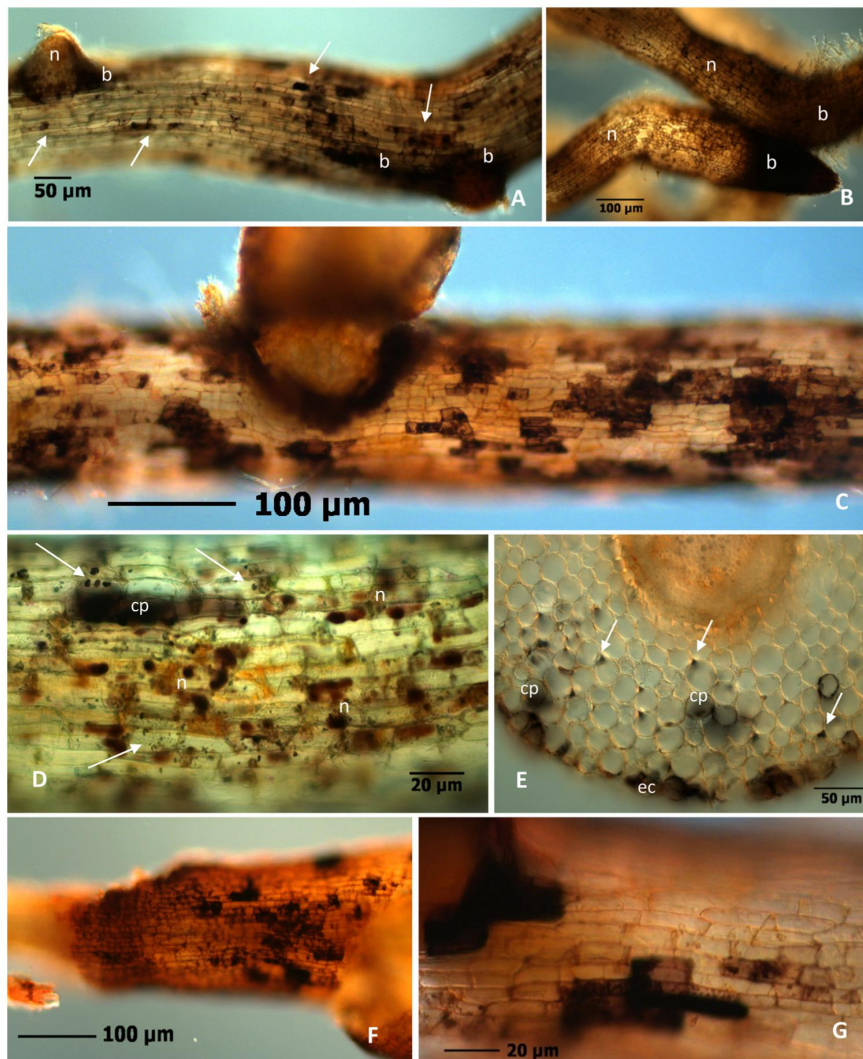
Fig. 4. Cell damages in fine roots of apple rootstock M26 (A–E) and 'Bittenfelder' (F–G), all brightfield-DIC-microscopy. A: Necrotic cells (arrows) and blackening (b) in the outer tissue layers of a branching fine root, and tip necrosis (n) of emerging lateral roots, after two weeks in untreated ARD soil (site Ellerhoop). B: Root tip blackening (b) and necrosis of rhizodermic cells (n) in lateral fine roots after eight weeks in untreated ARD soil (site Ruthe, 2016). C: Strong early necrotic reaction after two weeks in untreated ARD soil (site Ruthe, 2017). D: Differing cell damages like necrotic areas (n), brown-black cytoplasmic inclusions (arrows) in epidermal cells, and black cortex parenchyma cells (cp) of fine roots in untreated ARD soil (site Heidgraben). E: Cell damages in epidermal (ec) and cortex parenchyma cells (cp), and intercellular discoloration (arrows); unstained freehand cut cross section of a fresh root, treatment as in D. F: Clustered necrotic cells in a fine root with cortex damage after eight weeks in untreated ARD soil (site Ruthe, 2017), general view. G: close-up of F.

metabolically active cells of the outer cell layers were assessed to determine the cell vitality in fine roots (Fig. 9 A–C). FUN*1-staining enabled to discriminate living from dead cells by the formation of moving red cylindrical intravacuolar structures (CIVS). This ATP-dependent staining effect could be seen in root tissues in brightfield and in epifluorescence microscopy. Under brightfield microscopy, a vital cell was characterized by an uncompressed nucleus, cytoplasmic movement, and vacuoles with red coloured CIVS. Dead and disintegrated cells with a shriveled cytoplasm did not form CIVS. Metabolically active root cells were discernible by the bright green fluorescence of the nucleus in a light green cytoplasm and by the assembly of red fluorescent CIVS. Dead cell contents remained dark without staining. The monitoring of cell vitality of the first and second tissue layers is limited to the youngest root sections (first and second order) due to the succeeding cortex development. Extended tissue areas with vitality rated in class 5 (> 75-100% vital cells) occurred at the root tips and in elongation zones without root hairs. As a rule, in healthy root segments vital cell areas alternated with non-vital cells, and thus resulted in a spotted pattern of fluorescent cells. In both CE2 experiments with M26, the root segments obtained from untreated ARD soils had an evidently reduced vitality from the beginning (two weeks after being planted into ARD soil) until the end of the experiments after 8 weeks (Fig. 10, Fig. S4).

Under ARD conditions, fine root segments rated in vitality classes 4 and 5 occurred, but only in very low frequencies and they appeared primarily in newly formed, symptom-free lateral roots.

There were no pronounced differences due to the original location of the soil. The mean rating data from Ruthe soil treatments showed larger standard deviations only in 2017, but not so in 2016. Soil irradiation improved the vitality in ARD-γ treatments, whereas in Grass-γ treatments with a higher level of vitality in untreated soils there was no effect (Fig. 10; Fig. S4). In 2017 root segments of 'Bittenfelder' in the untreated ARD soil from the Ruthe site performed as poor as those of M26 with a mean cell vitality of ≤ 1.6 (Fig. S5). The method of cell vitality determination was validated with 'Bittenfelder' seedlings after 8 weeks culture in virgin cereal field soil. The randomly selected 60 fine root segments of each of six plants had intact fine root structures and overall well developed root hair zones. They hardly showed any signs of damage and had a mean cell vitality of 3.02 ± 0.2.

3.3. Microscopic assessments with Malus-hybrid B63

In an additional test in ARD soil from reference site Heidgraben, the rootstock genotype B63 reacted with similar ARD symptoms as previously demonstrated in M26 and 'Bittenfelder'. Early development of
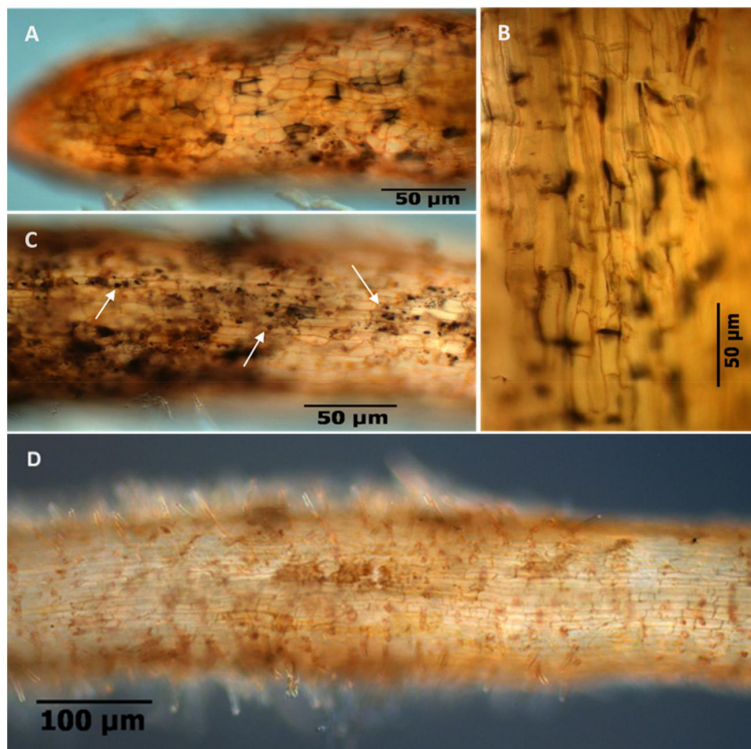
Fig. 5. Apple fine roots after four weeks culture in ARD-untreated soil (site Heidgraben): root tip with symptoms of individual black cells and black discoloration in the intercellular space (M26, A, B), root segment with black and brown cytoplasmic inclusions (arrows) in rhizodermal cells (B63, C), healthy root segment with light browning (M26, D), all examples with FUN*1 cell staining, brightfield-DIC-microscopy.

necroses, dark cell inclusions, and discoloration in the intercellular space with extension to the cortex tissue were characteristic in root segments from plants grown in untreated ARD soil. The comparison between B63 and M26 (Fig. 11) indicated that the root hair status was equally affected in both genotypes when grown in untreated ARD soil, but B63 formed much more root hairs in irradiated soil than M26. Cell damage symptoms were expressed in B63 and M26 in equal quality patterns, however, they occurred less frequently in B63; irradiated soil prevented the brown to black appearing cell damages, but not light browning. These findings led to the conclusion that B63 is also sensitive to ARD soil. The fresh mass data (means ± SD of ten plants) after 4 weeks in culture reflected these results: the shoot fresh mass in untreated ARD soil was 0.66 ± 0.17 g for M26 and 0.54 ± 0.13 g for B63 and in irradiated ARD soil 1.00 ± 0.28 g for M26 and 0.95 ± 0.17 g for B63. The root fresh mass in untreated ARD soil was 0.16 ± 0.08 g for M26 and 0.24 ± 0.08 g for B63 and in irradiated ARD soil 0.27 ± 0.07 g for M26 and 0.42 ± 0.20 g for B63.

To summarize, in several bioassays with untreated soils from three ARD-reference sites, characteristic major symptoms in the outer fine root tissue of three different rootstock genotypes could be detected only two weeks after planting and longer throughout the tests. Compared to corresponding fine roots grown in γ-irradiated soils and soils from grass plots, we classified the following as prominent early indicators of ARD: damage to the root structure, impairment of root hair formation, and cellular alterations in the outer tissue layers (blackening, necroses, black inclusions, discolorations in the apoplast).

4. Discussion

4.1. Greenhouse pot trials

In field studies as well as in greenhouse tests, plant growth reduction is used as the main indicator of the complex ARD phenomenon (Mazzola and Manici, 2012; Franke-Whittle et al., 2015). In order to

guarantee a more rapid diagnosis of ARD, clear and reliable alterations in the root tissue should be identified.

Starting microscopy with live fine roots of 'Bittenfelder' grown in ARD field plots at the reference sites and sampled in 2016 (Mahnkopp et al., 2018), symptoms of blackening and necroses were observed. But too much tissue damage, considerable loss of cortex tissue, and firmly adhering soil residues associated with a great deal of effort to obtain sufficient material, hindered a rapid and representative microscopic assessment of fine roots from field sites. Thus, to achieve our goal, it was preferable to examine apple plants with less destroyed roots in a short time interval under reproducible conditions, as given by a prior bioassay system (Yim et al., 2013). By analyzing the occurrence and extent of structural and cellular damages in apple fine roots, a data set of symptom frequencies was created allowing an evaluation of the relevance of individual symptoms for the presence of ARD. The striking visible alterations in sensitive M26 root systems appearing within the first two weeks after planting in ARD soils were identical to symptoms obtained in some roots of 'Bittenfelder' plants from ARD-reference sites. Thus, the plant material M26 and the bioassay system were suitable for our purposes.

The assessments of plant biomasses after eight weeks of culture at the end of the two central experiments confirmed this, as shoot fresh mass was an appropriate monitoring parameter for ARD. Nutrient status, pH and organic matter were not significantly different between grass and ARD plots at all three sites (Mahnkopp et al., 2018) and thus do not account for the differences in growth. But root fresh mass proved to be less suitable for that aim, because root fresh mass of ARD treatments and grass treatments both from untreated soils of three reference sites did not differ significantly in 2016 and 2017, with the exception of plants in Heidgraben in 2017. Overall, the growth data affirmed the induction of ARD in soils of the three reference sites in our trials, and shoot mass could better demonstrate growth depression by ARD than root mass.

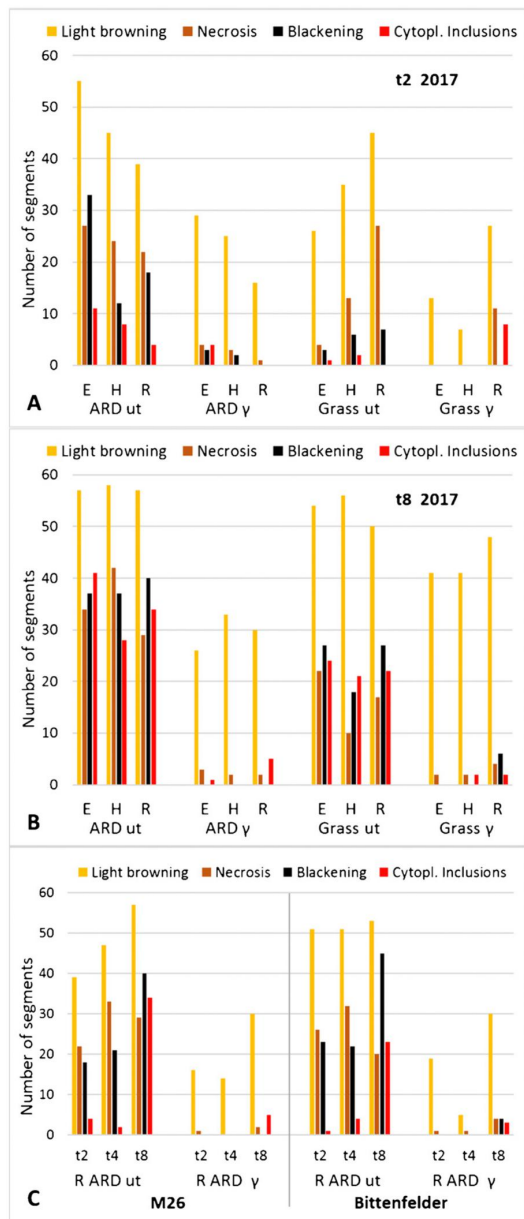Root systems grown in untreated soil of grass plots, however,

Fig. 6. Cellular damages in apple fine roots grown in soils of ARD plots (ARD) and grass plots (Grass) from Ellerhoop (E), Heidgraben (H), and Ruthe (R), either untreated (ut) or irradiated (γ) in experiments CE2 2017, n = 60 root segments. Frequencies of root segments in four symptom classes. A: M26, after culture for two weeks, B: M26, after culture for eight weeks, C: M26 and 'Bittenfelder' after two, four and eight weeks culture (t2, t4, t8).

showed considerably less visible damage than ARD treatments. The occurrence of blackening, necrosis and cell changes, albeit to a much lesser extent, made it clear that the grass plots in the immediate vicinity of the ARD-affected apple plots did not serve as an ideal control soil. They are a natural system in which various microorganisms including pathogens may be present, since the grass cover influences microbial communities (St. Laurent et al., 2008) as well as the soil mesofauna (Koehler and Born, 1989).

The increase in biomass by irradiation of soils from grass plots could be due to (1) detrimental organisms present in this soil, (2) nutrients

released by degraded killed microorganisms or (3) a quick recolonization with beneficial microorganisms. A test with wheat seedlings in untreated and gamma irradiated soil did not reveal any differences in shoot or root biomass (data not shown), thus the nutrient hypothesis can be ruled out.

The increased cell vitality and reduced cell damage symptoms obtained from roots in γ-irradiated soils indicated that biotic factors, eliminated by irradiation, play a major part as causal agents in the ARD disease complex – a generally accepted assumption that is affirmed with the mainly significantly raised shoot fresh mass data at the end of the experiments.

4.2. Microscopic evidence of symptoms

From the data obtained in the central experiments and described here, characteristic signs in the outer appearance of roots of affected plants arose which have so far mostly been described in general terms (Mazzola and Manici, 2012). Utkhede et al. (1992) used a rather coarse visual assessment of root symptoms in five root-rating classes ranging from healthy to fully rotten. Kelderer et al. (2012) applied a more differentiated scheme for visual scorings of root health in four growth percentage classes and four disease severity classes regarding discolorations, necroses, and severe root rot. Emmett et al. (2014) and Atucha et al. (2014), with their detailed analyses of root growth, fine root lifespan, root structure, and root pigmentation and damage due to ARD, provided essential information to base our microscopic evaluations on. Thus, our assessments focussed only on fine roots of first to third order. In this part of the root system, first and essential reactions for diagnostic aims were to be observed. They confirm symptoms which Caruso et al. (1989) showed in their early histological work on tissue alterations in ARD-affected roots from field grown apple trees with structurally intact healthy roots and partially sloughing away of the epidermal and cortical layers in diseased roots. We could affirm that root growth arrestment, thickening of first order roots, tissue destruction and loss, as well as a reduction in root hair numbers describe the outer appearance of fine roots from M26, 'Bittenfelder' and B63 in response to ARD. It seems that the plant is trying to avoid harmful factors in the soil by reacting with the formation of new pioneer and lateral roots to root damage in ARD affected soil. This reaction was only partially successful as roots became also discoloured and then necrotic, even though the plant survived.

Fine roots of 'Bittenfelder' grown in virgin soil were structurally intact, white to light brown in coloration, and had numerous root hairs. Through vital staining is was possible to discriminate areas of metabolically active epidermal tissue from dead cell areas. These studies helped evaluating and defining the symptom termed "light browning" which occurred in all tested genotypes. In line with the ratings of Atucha et al. (2014), we consider light browning as a sign of dead epidermal cells during the maturation of fine roots. This symptom appeared in all treatments irrespective of original soil site and treatment. Light browning characterized dead cells as they are occurring in any normal, healthy fine root of apple, along with a root lifespan of about four to five weeks (Atucha et al., 2014). In first order fine roots, rhizodermal cells form root hairs. Their short cell lifespan of only a few days was confirmed by cell vitality staining (data not shown) and characterized light brown cell coloration. Therefore, light browning is not classified as an early symptom of ARD.

The assessment of fine root features revealed that under ARD influence there was a distinct disturbance in the occurrence of root hairs in three different rootstocks and in replant soils from different locations. Due to our very gentle sample preparation, we assume that the loss of root hairs and epidermal tissue by washing was negligible, especially at the earliest harvest times t2 and t4. On the other hand, the observed excessive root hair formation in irradiated soils may indicate a plant reaction to acquire sufficient nutrients that otherwise are supplied by and through microbes. The ARD related decrease in the number of
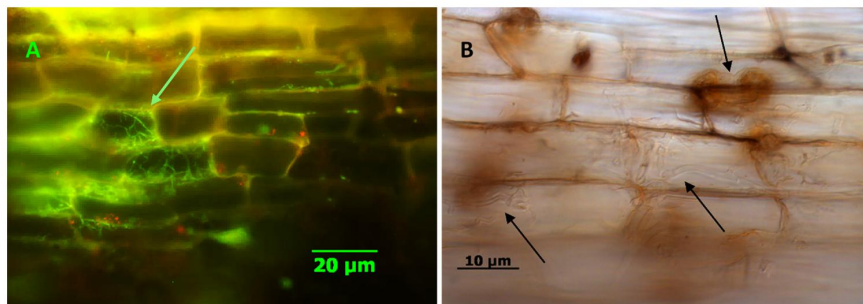
Fig. 7. Microorganisms in fine roots of M26 grown for four weeks in untreated ARD soil from field site Heidgraben (CE2 2017), FUN®1 cell staining. A: Green fluorescent Actinobacteria filaments (arrow) in dead root cells aside of living root cells, which are discernible by distinct fluorescent red intravacuolar structures and green nuclei (epifluorescence microscopy); B: dead cells with septate fungal hyphae (arrows), necrotic reactions and dark discolorations in the apoplast (brightfield-DIC-microscopy). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

root hairs was already mentioned by Savory (1966); Hoestra (1968), and Caruso et al. (1989) and needs further attention. Plants can counteract nutritional deficits such as phosphate or iron deficiency with an increased root hair formation and correspondingly increased absorptive surface area. This response is regulated, e.g., in Arabidopsis plants through systemic and local signalling pathways to which specific genes have been assigned to control root hair formation (Mueller, 2007). Apple plants suffering from ARD could react in the opposite way if such an adaptation by regulating genes is blocked. It would be interesting to examine which genes are altered in their expression resulting in the observed reduced root hair development. This kind of analyses should be complemented by numerical data on root hair density. Another hypothesis could be a locally effective impairment of trichoblasts with competence for root hair development, due to harmful biotic and/or abiotic factors in the ARD rhizosphere and/or ARD soil.

Damages to the outer cortex, tissue loss, and root rot are distinct injury symptoms that are easily detectable. They have already been attributed to ARD by Hoestra (1968) focussing on nematodes as causal agents but were not yet applied systematically for diagnostic purposes. We confirm these signs to be typical ARD symptoms for field and greenhouse grown fine roots. The strong blackening of individual or clustered rhizodermic, hypodermic, and underlying cortical cells, which was present after only two weeks of culture in ARD soils, represented another distinct symptom that was to be distinguished from single brown necrotic cells in damaged tissue areas with shed cell layers or bare lying cortex. The mixture of differently discoloured cells within small areas required a close examination but the cell shapes allowed for a clear classification in cortex and epidermal tissues. In brown discoloured roots of 'Golden Delicious' plants grown under replant conditions, Henfrey et al. (2015) associated the increased total phenol content with a reaction to biotic and abiotic stress factors. Previously, Emmett et al. (2014) differentiated phenolic compounds in their compositions and distribution within the tissue of replant diseased fine roots. The authors also suggested an induced production as defence or stress response to pathogen infection. One can assume that in our experiments, the appearance of blackening and necrosis in the epidermal tissue also points to such an accumulation of phenols as a plant reaction

to oxidative stress and/or pathogen infection.

Characteristic ARD symptoms also include prominent recognizable individual black cell inclusions, rounded and of differing sizes, in dead but not yet brown epidermal and cortical cells. This confirms the observation made by Caruso et al. (1989) of significant amounts of densely stained material in cortical cells of diseased apple roots. Further histological examinations of thin sections of fine root samples are in progress; they will provide information about the composition and chemical components of these inclusion bodies. The spotted distribution of black soluble substances in the cortical apoplast suggests a locally restricted cause. They may be released due to pathogen infections or toxins and will need further attention to clarify the disease etiology. Our own observations in diseased tissue suggest that members of Nectriaceae and *Fusarium* spp. are involved in the pathogen complex (Kelderer et al., 2012; Mazzola and Manici, 2012; Manici et al., 2017). However, as reported by Hoestra (1968) and Yim et al. (2013), the stele beyond the endodermis remained undamaged. Here, histological investigations will add information to the cellular symptomatology. Since the most striking finding of molecular responses of apple roots to ARD soil was a drastic accumulation of phytoalexins as early as three days after planting (Weiß et al., 2017b), their cellular localization and comparison to the symptoms described here will help in the understanding of their role in the response towards ARD.

Beyond the previously described characteristics, the investigation of the function of epidermal root cells was another attempt to record early plant reactions in replant disease. We chose the application of vital cell stain FUN®1 due to good experiences gained in previous experiments with root tissue of ericaceous plants (Grunewaldt-Stöcker and von Alten, 2016). Only in the presence of ATP, the stain is converted into fluorescent structures assembling in the vacuoles of intact cells (Millard et al., 1997). This staining method was not previously used for fine apple roots and the cell vitality of replanted diseased roots had not yet been examined microscopically. The assessment of cell vitality clearly demonstrated for the first time a reduction of metabolic activity in fine roots of ARD soils from all locations throughout the experiments, indicating the functional disturbance in ARD-affected root systems. Fine roots grown in irradiated soils had greater metabolic activity in the

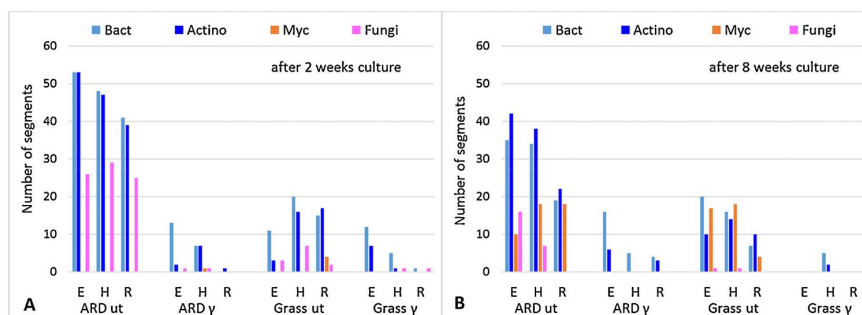**Fig. 8.** Frequencies of root segments with observed microorganisms (bacteria (Bact), Actinobacteria (Actino), mycorrhizal fungi (Myc), other fungi (Fungi)): M26 after two weeks (A) and eight weeks (B) culture in soils of ARD plots (ARD) and grass plots (Grass) from Ellerhoop (E), Heidgraben (H), and Ruthe (R), either untreated (ut) or irradiated (γ) in experiments CE2 2017, n = 60 root segments.
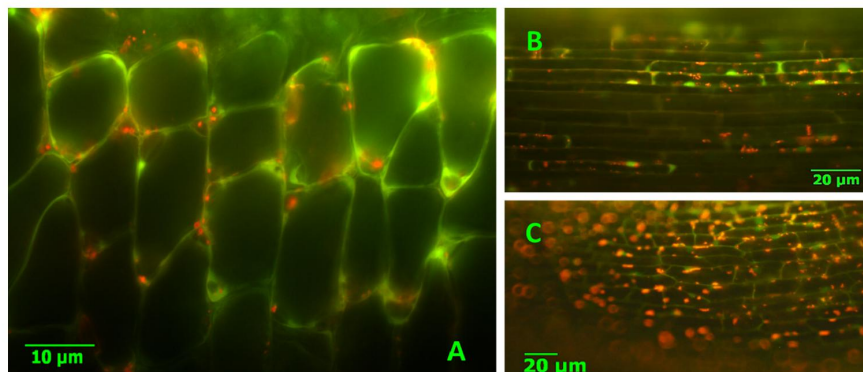
Fig. 9. Examples of cell vitality ratings in fluorescence analyses after FUN®1 cell staining: M26 fine root segments rated in cell vitality class 5 (A, C) and class 3 (B).

rhizodermal tissue: this may be due to the pronounced root hair formation and less tissue impairment. Fine roots with a light browning - occurring in all treatments - showed reduced cell vitality, thus confirming the results of Atucha et al. (2014) regarding the short lifespan of fine roots and root tissue in apple. Fine root sections with and without root hairs were not evenly distributed in the analyzed 1 cm-segments. This could be the reason why the mean value of the ratings for cell vitality per segment occasionally showed a high deviation between plants of the same treatment. Only by analyzing more plants and segments, this variability could be reduced. However, this would exceed the available time and working capacity for fresh root material within the bioassay and increase the costs due to expensive cell stains. Thus, the expensive and time-consuming quantification of cell vitality in fine roots will not become a routine test parameter. Nevertheless, the FUN®1 staining method is very well suited to examine living and dead cells in detail, e.g. in studies of infection processes and plant-endophyte-interactions.

In the selection of the early root symptoms, the reaction of three rootstock genotypes and three different ARD soils were taken into account. This means that our diagnostic results are based on more than the reaction range of M26. However, the sensitive M26 is ideally suited as a standard genotype for testing putatively affected soils and for comparison of symptoms in rootstock genotypes whose reactions to ARD are to be tested. This genotype was recently used by Rumberger et al. (2007); Yim et al. (2013, 2015), and Emmett et al. (2014) and its micropropagation is advantageous for the supply with homogenous test material. There was evidence that less susceptible rootstocks can support lower ARD pathogen abundance and an altered defence compound production in fine roots, and maintain higher rates of root growth in ARD soil (Emmett et al., 2014). The ongoing screening for such candidates in breeding programs for a selection of tolerance towards ARD (St. Laurent et al., 2010; Atucha et al., 2014; Fazio et al., 2015) could profit from the early diagnostic tool suggested in the present study by

saving time in a short-term bioassay in confirmed ARD soil. Microscopy of unfixed fine roots allows rapid detection of cell damages and the destruction, loss, and aberration of tissue as an immediate response to ARD challenge in such candidate genotypes. It can ideally complement the gene expression analyses of candidate genes proposed by Weiß et al. (2017a) and microbial community analyses by allowing a classification of roots according to the severity of damage.

5. Conclusions

The microscopy data collected made it possible to select relevant ARD symptoms. The first qualitative recording of the characteristic symptoms was followed by a frequency analysis of the root symptoms in order to enable a comparison between the test treatments. Our results showed that at least one-third of the root segments affected by ARD had impaired root hair development, structural damage, and cellular changes in structure and function in the outer tissue layers only two weeks after contact with diseased soil. It is to prove in further experiments with more genotypes and soil variants whether this can serve as a threshold value for the reliable determination of the disease. We demonstrate that these symptoms are valid for three rootstock genotypes and suggest these symptoms can be used for the early detection and diagnosis of ARD in routine biotest examinations as early as two weeks after planting when growth depressions are slowly appearing. Since root samples of 'Bittenfelder' taken from ARD-fields also showed the early symptoms, the early detection of ARD should be usable for field root material as well, provided that sufficient fine root material can be dug out for a reliable assessment.

Conflict of interests

The authors declare that they have no conflict of interest to report.

Fig. 10. Fine root cell vitality given as means and SD of ratings in five classes from 1.0 (0% vital cells) to 5.0 (> 75% vital cells): M26 after two weeks (A) and eight weeks (B) culture in soils of ARD plots (ARD) and grass plots (Grass) from Ellerhoop (E), Heidgraben (H), and Ruthe (R), either untreated (ut) or irradiated (γ) in experiment CE2 2017, n = 60 root segments.
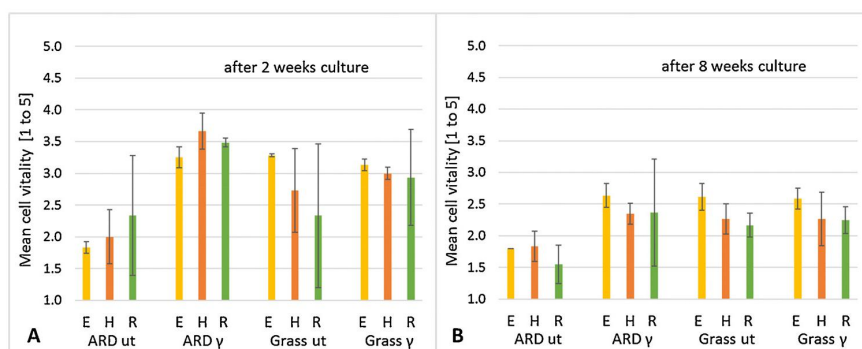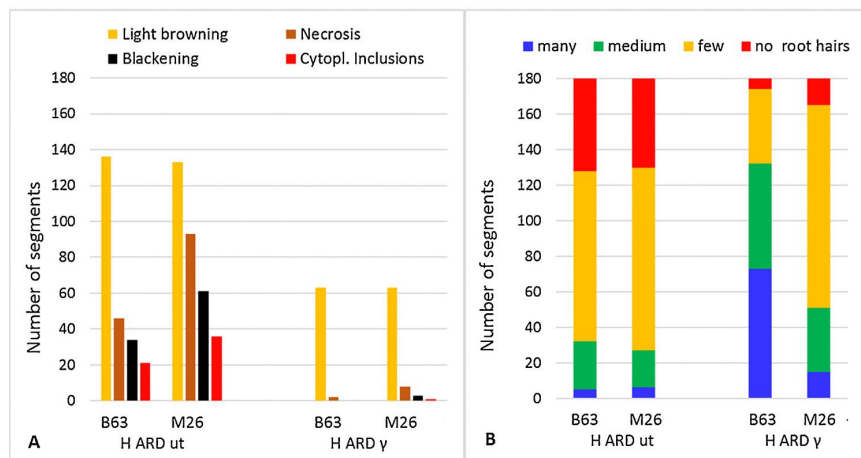
Fig. 11. Comparisons between rootstock genotypes B63 and M26 grown for four weeks in untreated (ARD ut) and γ-irradiated (ARD γ) soil from ARD plots at the reference site Heidgraben (H). A: Cell damage ratings in frequencies of four classes, B: Root hair status ratings in frequencies of four classes, n = 180 root segments.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2018.09.014.

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2.3   Molecular barcoding reveals the genus *Streptomyces* as associated root endophytes of apple (*Malus domestica*) plants grown in soils affected by apple replant disease

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Michael Schloter	Contributed reagents/materials/analysis tools, contributed to the writing of the manuscript
Traud Winkelmann	Conceived and designed the experiments, contributed reagents/materials/analysis tools, contributed to the writing of the manuscript

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RESEARCH

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Molecular Barcoding Reveals the Genus *Streptomyces* as Associated Root Endophytes of Apple (*Malus domestica*) Plants Grown in Soils Affected by Apple Replant Disease

Felix Mahnkopp-Dirks,[1] Viviane Radl,[2] Susanne Kublik,[2] Silvia Gschwendtner,[2] Michael Schloter,[2] and Traud Winkelmann[1,†]

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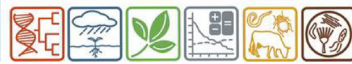[2] Helmholtz Zentrum München-German Research Center for Environmental Health, Neuherberg, Germany

ABSTRACT

Apple replant disease (ARD) occurs when apple is repeatedly planted at the same site, leading to growth reductions and losses in fruit yield and quality. Up to now, the etiology has been poorly understood; however, soil (micro)biota are known to be involved. Because endophytes often colonize plants via the rhizosphere, this study aimed at comparing the bacterial endophytic root microbiome in plants growing in ARD-affected and unaffected soils from three different sites based on greenhouse biotests using a molecular barcoding approach. The initial endophytic microbiome of the starting material (in vitro propagated plants of the apple rootstock M26) did not significantly affect the overall richness and diversity of the endophytic community in plants after 8 weeks of growth in the respective soils but some genera of the initial microbiome managed to establish in apple roots. *Proteobacteria* was the dominant phylum in all samples. No differences in diversity or number of amplicon sequence variants (ASVs) between plants grown in ARD soil and unaffected soil was observed. However, several ASVs of high abundance uniquely found in plants grown in ARD-affected soils were *Streptomyces* spp. In soil from all three sites, these *Streptomyces* spp. were negatively correlated with plant growth parameters. Future inoculation experiments using selected *Streptomyces* isolates have to prove whether bacteria from this genus are opportunists or part of the ARD complex. For the first time, the bacterial endophytic community of apple roots grown in ARD-affected soils was characterized, which will help us to understand the etiology of ARD and develop countermeasures.

Keywords: 16S rRNA amplicon sequencing, *Actinobacteria*, apple replant disease, endophytes, endophytic microbiome, greenhouse biotest, *Malus domestica*, microbiome, plants, soils, *Streptomyces*

Apple replant disease (ARD) is a complex phenomenon which affects apple tree nurseries and orchards worldwide, causing growth reductions and losses in fruit yield and quality (Manici et al. 2013; Mazzola und Manici 2012; Winkelmann et al. 2019). ARD occurs when apple or a closely related species is repeatedly planted at the same site and is 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). This disease is species specific and can persist for decades (Savory 1966). Because disinfection of the soil leads to better growth, it is generally accepted that biotic factors are the primary cause (Mahnkopp et al. 2018; Mai and Abawi 1981; Yim et al. 2013). Next to fungi belonging to the genera *Fusarium*, *Cylindrocarpon*, and *Rhizoctonia*, a number of other taxa, including oomycetes such as *Pythium* and *Phytophthora* spp., nematodes such as *Pratylenchus* spp., and various bacterial species such as members of the genera *Pseudomonas* and *Bacillus* as well as the phylum *Actinobacteria*, have been reported to contribute to ARD (Čatská et al. 1982; Manici et al. 2017; Mazzola 1998; Otto and Winkler 1993; Tewoldemedhin et al. 2011; Utkhede and Li 1988). However, despite decades of research, the etiology of ARD is still poorly known.

Based on molecular barcoding approaches in the last decade, many studies confirmed not only changes in the abundance of specific pathogens in ARD-affected soils but also significant shifts in the overall structure of the microbiome of the bulk soil and the rhizosphere (Winkelmann et al. 2019). These microbiome shifts

also affect major functional properties, including the potential to degrade aromatic compounds and functions for biocontrol (Radl et al. 2019).

However, despite their close interaction with host cells, surprisingly, there is still a lack of knowledge of how microbes colonizing the root interior (root endophytes) are affected by ARD. The interior of roots can be colonized mostly by rhizosphere microbiota (e.g., through cracks formed during lateral root emergence and at root tips) (Bulgarelli et al. 2013; Hardoim et al. 2008). Positive effects of endophytes on plants include direct or indirect provision of nutrients (Gaiero et al. 2013; White et al. 2019); production of plant hormones such as auxin, cytokinins, or gibberellins (Hardoim et al. 2015; Santoyo et al. 2016); increased tolerance against abiotic stress (Hardoim et al. 2015); and biocontrol due to competitive mechanisms or production of antimicrobial substances (Haas and Keel 2003). Yet there are also endophytes known for their negative effects on plant health. Some of these facultative pathogens can shift their lifestyle, depending on several factors such as host and endophyte development stage, plant defense reactions, or environmental conditions (Schulz and Boyle 2005). Rosenblueth and Martínez-Romero (2006) put forward the hypothesis of an equilibrium between endophytes and plants that, under certain conditions, gets unbalanced to the detriment of one of the partners.

Only a few studies have investigated the role of endophytes in ARD, focusing on potential fungal root pathogens. Manici et al. (2013) found the root endophytic Cylindrocarpon-like fungi (Ilyonectria and Thelonectria spp.) and Pythium spp. to be main causal agents of growth reduction in the rootstock M9 growing in ARD-affected soil. Cylindrocarpon spp. were also identified next to Rhizoctonia spp. as a pathogenic root endophyte by Kelderer et al. (2012) in row-planted (ARD-affected) and interrow-planted (control) apple trees. In addition, Fusarium solani and F. oxysporum were most abundant in roots but not considered pathogenic. Popp et al. (2019) isolated several fungal endophytes from ARD-affected apple roots and reinoculated them in a soil-free biotest. Cadophora, Calonectria, Dactylonectria, Ilyonectria, and Leptosphaeria spp. were reported to have negative effects on plant health. In contrast, studies on the effects of ARD on bacterial root endophytes are scarce. Thus far, only a targeted, cultivation-dependent approach has been published, where the focus was on the biocontrol properties of Actinobacteria isolates (mostly belonging to the genus Streptomyces) from the root interior of apple trees. However, no effect was observed when coinoculated to apple seedlings with Pythium irregulare and Cylindrocarpon macrodidymum (Tewoldemedhin et al. 2011).

Therefore, the aim of this study was to investigate the bacterial root endophytic community structure in plants growing in ARD-affected soils compared with ARD-unaffected soils based on greenhouse biotests using a molecular barcoding approach. In these biotests, the ARD-susceptible genotype M26 was planted into ARD-affected soil (untreated or γ-sterilized) and grass control soil (untreated or γ-sterilized). To go beyond a local response pattern, we used soils from three different sites from northern Germany in the frame of this study. For generating more robust data, we performed our study in two subsequent years to exclude specific effects of the used soils based on one particular season. We propose that a possible causal agent of ARD should be present in roots from all ARD-affected soils in all three sites.

MATERIALS AND METHODS

Experimental setting. Soil for these experiments was sampled from three different sites: Heidgraben (x-coordinate 53.699199;

y-coordinate 9.683171; WGS 84, Schleswig-Holstein, northern Germany), Ellerhoop (x-coordinate 53.71435; y-coordinate 9.770143; WGS 84, Schleswig-Holstein, northern Germany), and Ruthe (x-coordinate 52.243668; y-coordinate 9.819700; WGS 84, Lower Saxony, Germany). These sites differed in their climatic conditions and soil properties. The upper soil textures of the three sites were defined (based on World Reference Base for soil resources) as sand (Heidgraben), loamy sand (Ellerhoop), and silt loam (Ruthe) (Mahnkopp et al. 2018). Every site contained two different plot variants: (i) ARD plots, where ARD was successfully induced by repeatedly replanting Bittenfelder apple seedlings since 2009 in a 2-year cycle, and (ii) control plots, which were only covered with grass since then. ARD plots in Ruthe and Ellerhoop were replanted for the last time in spring 2015 and in Heidgraben in spring 2016.

Soils were sampled from all three sites at a depth of 0 to 20 cm at the end of 2015 and 2016. After sampling and sieving (8 mm), soils were either γ irradiated (G) at a minimal dose of 10 kGy or left untreated (UT), resulting in four variants per site: ARD untreated (ARD UT), ARD γ-irradiated (ARD G), grass untreated (grass UT), and grass γ-irradiated (grass G).

The ARD-susceptible apple rootstock M26, which was propagated and rooted in vitro (Weiß et al. 2017a), was acclimatized for 4 weeks and, afterwards, one plantlet each was planted in 1-liter pots containing the different soil variants. Soils were supplemented with Osmocote Exact 3-4M (16 + 9 + 12 + 2 MgO; https://icl-sf.com/de-de/products/ornamental_horticulture/8840-osmocote-exact-standard-3-4m/) at 2 g liter^{-1} to exclude nutrient effects. Shoot lengths were measured weekly. Plants were grown for 8 weeks in the greenhouse at a mean daily temperature of 21°C and a 16-h photoperiod achieved by additional light (SON-T Philips Master Agro 400 W) with a set point of 25 klx. Plant protection measures and irrigation were done according to Yim et al. (2015). At the first sign of insect pests, 0.3% NeemAzal was sprayed. During the night, a sulfur evaporator was used in order to prevent fungal diseases. The greenhouse experiment was conducted twice, in February 2016 and 2017 (Mahnkopp et al. 2018), with nine replicates per variant.

Acclimatized plants (before planting into the soil variants) were treated as described by Mahnkopp et al. (2018) and served as the source for "timepoint zero" (T0) samples in both years.

Sampling. After 8 weeks of cultivation in the greenhouse, 4 representative plants per variant were taken (48 per year, 96 in total) as biological replicates. Roots were washed carefully to get rid of the adhering soil. Shoot and root fresh mass were determined. For surface sterilization, roots were rinsed for 30 s in EtOH (70%), followed by stirring in 2% NaOCl for 7.5 min and, finally, washing five times in sterile deionized water. The final washing water was plated on 523 medium (Viss et al. 1991) and incubated at room temperature for 1 week. Plating resulted in <10 CFU/plate in all cases. Roots were stored in sterile 2-ml Eppendorf tubes at −80°C until DNA extraction.

DNA extraction and amplicon sequencing. Surface-sterilized roots (50 to 100 mg/sample) were homogenized under frozen conditions using steel beads (Ø 6 mm) in a mixer mill (MM400; Retsch, Haan, Germany) with a frequency of 23 Hz for 2 min using sterilized devices. DNA was extracted using the Invisorb Spin Plant Mini Kit (Stratec, Berlin, Germany) according to the provided protocol. DNA quality was checked using a spectrophotometer (Nanodrop 2000c; Peqlab, Erlangen, Germany).

The primer combination 335F (CADACTCCTACGGGAGGC)/ 769R (ATCCTGTTTGMTMCCCVCRC) (Dorn-In et al. 2015), including overhang adapter sequence, was used to amplify the V3-V4 region of the 16S ribosomal RNA (rRNA) gene. PCR assays contained 2× Phusion High-Fidelity Master Mix (1.5 mM MgCl$_2$,

200 μM each dNTP, and 0.2 U of Phusion DNA Polymerase [Thermo Fisher Scientific, Waltham, MA, U.S.A.]), 10 pmol of each primer, 5 ng of DNA template, and water to a final volume of 10 μl. The PCR cycling conditions consisted of an initial denaturation step of 98°C for 10 s; followed by 30 cycles involving 1 s of denaturation at 98°C, 5 s of annealing at 59°C, and 45 s of extension at 72°C; with a final extension of 1 min at 72°C. Triplicate PCR assays were pooled and purified using Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, U.S.A.). The purified products were quantitated using the Quant-IT PicoGreen dsDNA assay kit (Life Technologies Europe, Gent, Belgium). Sample indexing was carried out with Nextera XT Index Kit v2 Set A and B (Illumina, San Diego, CA, U.S.A.) in reaction mixtures containing 10 ng of purified PCR product, 2× Phusion High-Fidelity Master Mix (1.5 mM MgCl$_2$, 200 μM each dNTP, and 0.2 U of Phusion DNA Polymerase [Thermo Fisher Scientific]), 10 pmol of each indexing primer, and water to a final volume of 25 μl. The indexing PCR cycling conditions consisted of an initial denaturation step of 98°C for 30 s; followed by 8 cycles involving 10 s of denaturation at 98°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C; with a final extension of 5 min at 72°C.

Indexed samples were purified as described above. Equimolar concentrations of the purified indexed samples were prepared and diluted to a final concentration of 4 nM. The library was sequenced using the Illumina MiSeq platform with the MiSeq Reagent Kit v3 (600 cycle) (Illumina).

Bioinformatic and statistical analysis. FASTQ files were trimmed with a minimum read length of 50 and a minimum Phred score of 15 using AdapterRemoval (Schubert et al. 2016) without merging forward and reverse reads. Afterward, sequences were analyzed using the QIIME 2 software package release 2017.11 (Caporaso et al. 2010) with default parameters. The QIIME 2 plugin DADA2 (Callahan et al. 2016) was used for quality control with the following parameters: 10 bp were removed n-terminally, and reads were truncated at position 300 (forward) and 260 (reverse) for universal 16S rRNA genes. Expected error was adjusted to 2.

Taxonomic analysis of the resulting unique amplicon sequence variants (ASVs) was performed using primer-specific pretrained Naive Bayes classifiers of the SILVA_132_QIIME release 99% and the q2-feature-classifier plugin, setting the confidence threshold to 0.9. Because the PCR-negative control showed no ASVs, contamination during sample processing could be excluded. For further data analysis, unassigned reads and singletons (in sum, <0.03% of all reads) were excluded.

Raw sequence data were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession PRJNA647245.

16S amplicon sequencing of DNA extracted from the surface-sterilized M26 roots resulted after quality control in a total of 4,132,410 reads with a mean of 72,498 reads/sample in the biotest in 2016. After removal of chloroplast, archaea, and eukaryotic ASVs, sequence data were rarefied at a number of 28,817 reads (2016). In total, 5,898 ASVs were detected. Because rarefaction analysis (Supplementary Fig. S1) indicated that saturation was reached already with read numbers approximately 5,000 reads/sample, for 2017, the sequencing effort was reduced, resulting in in total 884,916 reads and a mean of 17,351 reads/sample (rarefied to 4,213 reads/sample after the removal of the chloroplasts). The total number of ASVs was 4,971, which were nearly all covered after rarefying at 4,813 reads/sample (Supplementary Fig. S2). In order to identify identical ASVs between the 2 years, alignments of the sequences of the 2 years on genus level were done using ClustalW Multiple Alignment (Thompson et al. 1994), with number of bootstraps set to 1,000 using BioEdit v7.2.5 (Hall 1999) followed

by calculating a sequence differences count matrix. ASVs from 2017 which were 100% identical to ASVs from 2016 were given the corresponding name of 2016 ASVs to improve comparability of figures and tables.

To calculate the relative abundance, the number of reads per ASV in the samples was divided by the sum of total reads per sample and multiplied by 100. The relative abundances of ASVs belonging to the same phylum or genus were combined to calculate the overall relative abundance of the corresponding phylum or genus. Species diversity (Shannon and Simpson) and richness (Chao1) indices were determined using the "Phyloseq" (McMurdie and Holmes 2013) and "Vegan" (Oksanen et al. 2019) packages of R v3.6.1 (R Development Core Team 2019) (http://www.R-project.org) and tested for normal distribution based on the Shapiro-Wilk test (Shapiro and Wilk 1965) and homogeneity of variance based on Levene's test (Levene 1960) using the program PAST3 v. 3.20 (Hammer et al. 2001). If the null hypotheses of normal distribution and equal variances were rejected, the Tukey test based on Herberich et al. (2010) was used at $P < 0.05$ to determine significant differences of the raw diversity and richness scores. In order to compare the relative abundance of different genera of the initial microbiome (T0 plants) between the years, all ASVs belonging to the same genus were merged. Because of unequal sample size and unequal variance, Welch's two-sample t test was used at $P < 0.05$ to determine significant differences. Nonmetric multidimensional scaling (NMDS) was performed with the program PAST3 v. 3.20 (Hammer et al. 2001) using the Bray-Curtis similarity index and analysis of similarity (ANOSIM) in order to visualize the community composition of the different samples. To indicate the influence of the different genera, vectors were added which show the correlation between the corresponding genus and the NMDS score. Spearman's correlation was used in order to correlate ASVs to shoot growth and fresh mass using the program PAST3 v. 3.20 (Hammer et al. 2001). Venn diagrams were designed using the Venn diagram tool of Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn).

To identify specific *Streptomyces* ASVs, their nucleotide sequences (414 bp) were blasted against the NCBI database (https://www.ncbi.nlm.nih.gov/) using BLASTn.

RESULTS

Root and shoot biomass. As expected, in both years, plants grown in untreated ARD-affected soils showed the lowest increase in shoot length and lowest shoot fresh mass in comparison with the other soil variants (Table 1). The γ irradiation of grass and ARD soil led to increased shoot length and shoot fresh mass compared with the respective untreated soils. Overall, plants grown in the biotest in 2017 showed a higher biomass in comparison with plants grown in 2016 but the response pattern to the different soils variants was comparable between both years. Detailed growth data for the full set of nine plants per variant can be found in Mahnkopp et al. (2018) whereas, in Table 1, only the data of the plants selected for the barcoding approach of this study are presented.

Endophytic bacterial community composition and diversity in plant roots grown in different soil variants. The mean number of observed ASVs per sample was 244 in 2016 (Table 2) and 201 in 2017 (Table 3). The highest number of observed ASVs were found in plants grown in ARD G soil from Ellerhoop ($n = 339 ± 93$) and the lowest number in the variant Heidgraben ARD G ($n = 148 ± 62$) (both in 2016). In both years, no significant differences in diversity or richness indices were recorded within or between the sites (Tukey's test, $P < 0.05$).

Proteobacteria was clearly the dominant phylum in all variants, with relative abundance ranging from 66.9% (Heidgraben ARD G) to 83.7% (Ruthe grass UT) (Supplementary Fig. S3) for the biotest performed in 2016. Phylum *Bacteroidetes* was of second most abundance, with a mean value of 10.3%, followed by *Actinobacteria* (5%) and *Firmicutes* (4.4%). Members of *Actinobacteria* appeared in higher relative abundance in roots grown in ARD UT compared with the other variants of the respective site. The strongest difference was observed in Heidgraben, where the abundance of ASVs linked to *Actinobacteria* in ARD UT variants (15.60%) was significantly higher than in ARD G (5.03%), grass UT (2.75%), and grass G variants (3.47%). The second biotest in 2017 showed similar shares for the different phyla (Supplementary Fig. S4). ASVs assigned to *Proteobacteria* ranging from 66.7 to

89.3% were dominant, followed by *Bacteroidetes*, with a mean value of 13.6% relative abundance; *Actinobacteria*, with 8.1%; and *Firmicutes*, with 2.2%. However, the higher abundance of ASVs linked to phylum *Actinobacteria* in the untreated ARD variants was not observed in this year.

Dynamics of endophytic bacteria during the biotest. T0 plants of 2016 had the highest diversity of all treatments over the years (Table 2). In 2017, T0 plants showed a significantly lower diversity compared with 2016 T0 plants (Supplementary Fig. S8). After cultivation for 8 weeks in the different soils, 9 of 12 variants of 2016 still had higher numbers in the observed ASVs compared with 2017. However, these differences were not significant (Supplementary Fig. S8).

TABLE 1
Shoot fresh mass and increase of shoot length of M26 apple plants grown for 8 weeks in the greenhouse biotest in 2016 and 2017[y]

Year[z]	Heidgraben				Ellerhoop				Ruthe			
	ARD		Grass		ARD		Grass		ARD		Grass	
	UT	G	UT	G	UT	G	UT	G	UT	G	UT	G
2016												
Length (cm)	5.9 ± 2.3 a	18.5 ± 1.5 c	13.5 ± 2 b	27.7 ± 6.3 c	7.5 ± 1 a	12.4 ± 3.8 ab	19.6 ± 1.6 b	25.9 ± 1.7 c	8.5 ± 1.4 a	25.2 ± 0.2 c	16.7 ± 1.9 b	32.0 ± 1.4 d
Mass (g)	2.8 ± 0.6 a	7.2 ± 0.6 b	5.9 ± 0.6 b	10.7 ± 1 c	3.2 ± 0.7 a	5.9 ± 1.3 ab	7.4 ± 1.1 b	14.4 ± 0.8 c	3.7 ± 0.4 a	11.4 ± 1 c	7.1 ± 0.1 b	17.6 ± 2.3 d
2017												
Length (cm)	5.7 ± 2.2 a	31.7 ± 1.3 b	29.8 ± 3.4 b	42.3 ± 3.9 c	13.4 ± 3.4 a	24.7 ± 0.8 b	31.6 ± 3.8 bc	43.0 ± 4.9 c	22.9 ± 1.9 a	34.3 ± 1.5 b	36.3 ± 4.9 bc	41.6 ± 1.7 c
Mass (g)	2.8 ± 0.6 a	11.7 ± 0.7 bc	9.4 ± 1.2 b	19.8 ± 3.9 c	4.8 ± 0.9 a	9.4 ± 0.3 b	9.7 ± 1.9 b	18.7 ± 3.6 b	8.3 ± 1.1 a	12.8 ± 1.4 b	11.8 ± 2.1 ab	15.9 ± 1 b

[y] Surface-sterilized roots of these plants were used for DNA extraction and amplicon sequencing. ARD = apple replant disease, UT = untreated, and G = γ irradiated. Shown is the mean and the standard deviation of *n* plants. Different letters indicate significant differences within the sites (Tukey's test, $P \leq 0.05$).
[z] Year and parameter: Length = increase in shoot length and Mass = shoot fresh mass.

TABLE 2
Richness and diversity of endophytic bacterial communities based on amplicon sequence variants (ASVs) in roots grown for 8 weeks in soils from different sites and treatments (ARD = apple replant disease, UT = untreated, and G = γ irradiated) of the biotest in 2016[z]

Site, soil	Treatment	*n*	Observed ASVs	Chao1	Shannon	Simpson
T0		4	290 ± 70	292 ± 70	5.12 ± 0.24	0.99 ± 0.00
Heidgraben						
ARD	UT	4	255 ± 106	257 ± 108	4.37 ± 0.50	0.97 ± 0.02
	G	4	148 ± 62	149 ± 61	3.73 ± 0.88	0.91 ± 0.08
Grass	UT	4	289 ± 119	294 ± 124	4.42 ± 0.50	0.97 ± 0.01
	G	4	329 ± 88	331 ± 89	4.88 ± 0.27	0.98 ± 0.01
Ellerhoop						
ARD	UT	4	303 ± 88	306 ± 89	4.86 ± 0.35	0.98 ± 0.00
	G	4	339 ± 93	344 ± 95	4.66 ± 0.52	0.97 ± 0.02
Grass	UT	4	202 ± 38	204 ± 39	4.15 ± 0.65	0.96 ± 0.03
	G	4	225 ± 110	228 ± 111	3.95 ± 0.65	0.94 ± 0.04
Ruthe						
ARD	UT	4	252 ± 118	264 ± 135	4.13 ± 0.72	0.94 ± 0.06
	G	4	211 ± 56	212 ± 56	4.33 ± 0.56	0.96 ± 0.02
Grass	UT	3	205 ± 18	205 ± 18	4.39 ± 0.13	0.97 ± 0.01
	G	4	167 ± 16	168 ± 17	4.00 ± 0.45	0.95 ± 0.02

[z] Additionally, acclimatized plants at timepoint zero (T0) before transferring into the soil variants are shown. There was no significant difference within and between the sites according to Tukey's test at $P \leq 0.05$. Shown are mean ± standard deviation of *n* replicates.

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On the phylum level, T0 plants grown in 2016 were dominated by *Proteobacteria* (79%, Supplementary Fig. S3), which did not change after 8 weeks of cultivation in the different soils. In 2017, this value increased to 89% for T0 plants but, here, this high relative abundance was found reduced by approximately 16% after the plants had been grown for 8 weeks in the different soil variants, regardless of the soil (Supplementary Fig. S4).

On higher phylogenetic levels, proteobacterial groups of the genera *Shewanella* and *Halomonas* belonged to the top three genera regarding relative abundance in T0 plants in both years (Supplementary Table S2). However, in total, nearly one-third (31.9%) of the genera showed significant differences in abundance between the years. In T0 plants from 2016, for example, *Ralstonia* was (with 6.6%) the most abundant genus but was not present in 2017 T0 plants. Even after growing for 8 weeks in different soils, this abundance pattern still remained for ASVs linked to *Ralstonia*. Similar contrasting abundance patterns were observed for genus *Pseudomonas* when both years were compared. Here, we could link 12.6% of all ASVs from T0 plants to this genus in 2017, which was more than four times higher than in 2016. However, in contrast to ASVs linked to *Ralstonia*, after 8 weeks of cultivation in the different soils, these initial differences in abundance of *Pseudomonas* were no longer detectable.

To analyze β-diversity, three-dimensional nonmetric multidimensional scalings were created for the untreated soils. In general, high variability within variants could be observed. In both years, T0 plants significantly separated from the other variants (ANOSIM with $P \leq 0.05$) (Fig. 1; Supplementary Fig. S5). Only for the biotest in 2016, significant differences between other variants were observed, especially for the treatments with soil from Ruthe (Fig. 1). ASVs assigned to genera *Pseudomonas*, *Rhizobium*, and especially *Streptomyces* were closely linked with ARD, whereas ASVs related to *Rhodanobacter*, *Dyella*, *Bradyrhizobium*, *Sphingomonas*, and *Rhizomicrobium* pointed to the untreated grass variants (Fig. 1). Most responsible for differentiation of T0 were ASVs which were linked to genera *Halomonas*, *Acinetobacter*, and *Shewanella*. In the

biotest in 2017, no clear clustering except for T0 was observed (Supplementary Fig. S5).

Identification of bacterial responders in the different treatments and correlation with plant growth. To further investigate ASVs responding to the different treatments of each site, Venn diagrams were designed. On the one hand, the number of ASVs shared by all four different variants per site, which we considered as the core microbiome of a given site, was surprisingly small. In 2016, only six ASVs (relative abundance >0.5%) in soil variants from Heidgraben, three in those from Ellerhoop, and four in those from Ruthe were present in all variants (Fig. 2). In 2017, these numbers were reduced to zero (Heidgraben), two (Ellerhoop), and one (Ruthe) (Fig. 3). On the other hand, the number of ASVs which were unique for each variant was high. In 2016, unique ASVs in untreated ARD variants ranged from 15 for Ruthe and 19 for Heidgraben to 28 for Ellerhoop (Fig. 2). In the untreated grass variants, the number of unique ASVs ranged from 23 for Ruthe to 26 for Ellerhoop and 29 for Heidgraben. Although the overall distribution was very similar in both years, for the soil from Ellerhoop, some variations were observed: the number of unique ASVs changed in untreated grass variants from 26 in 2016 to 9 in 2017. Results for soil variants from Heidgraben showed the lowest variation between the years, except for the unique ASVs for the grass variant sterilized by γ-irradiation, where 16 (2016) and 31 (2017) unique ASVs were observed.

In order to identify responders toward ARD, the unique ASVs of the untreated ARD variants were correlated with shoot growth and fresh mass of all variants of the three sites. In 2016, most noticeable were ASVs related to the genus *Streptomyces* (Fig. 2), which closely linked to plants grown in ARD-affected soils, confirming the overall observation that ASVs related to *Actinobacteria* were positively responding to the ARD-affected soils with increased levels in relative abundance. In Heidgraben ARD UT, 7 of 19 unique ASVs were linked to the genus *Streptomyces*, followed by

TABLE 3
Richness and diversity of endophytic bacterial communities based on amplicon sequence variants (ASVs) in roots grown for 8 weeks in soils from different sites and treatments (ARD = apple replant disease, UT = untreated, and G = γ irradiated) of the biotest in 2017[z]

Site, soil	Treatment	*n*	Observed ASVs	Chao1	Shannon	Simpson
T0		5	166 ± 42	170 ± 47	4.3 ± 0.30	0.97 ± 0.02
Heidgraben						
ARD	UT	4	149 ± 35	152 ± 36	4.07 ± 0.30	0.96 ± 0.01
	G	4	235 ± 84	239 ± 88	4.96 ± 0.28	0.99 ± 0.00
Grass	UT	4	152 ± 21	156 ± 24	3.94 ± 0.24	0.96 ± 0.02
	G	3	154 ± 49	154 ± 50	4.23 ± 0.49	0.97 ± 0.02
Ellerhoop						
ARD	UT	4	263 ± 50	275 ± 55	4.73 ± 0.35	0.98 ± 0.01
	G	2	206 ± 37	212 ± 33	4.84 ± 0.10	0.99 ± 0.00
Grass	UT	4	194 ± 60	200 ± 63	4.22 ± 0.42	0.94 ± 0.04
	G	3	165 ± 64	172 ± 64	4.26 ± 0.62	0.97 ± 0.02
Ruthe						
ARD	UT	3	193 ± 6	202 ± 10	4.3 ± 0.11	0.97 ± 0.01
	G	4	200 ± 25	201 ± 24	4.59 ± 0.24	0.98 ± 0.01
Grass	UT	4	253 ± 105	263 ± 117	4.59 ± 0.33	0.97 ± 0.01
	G	4	246 ± 148	261 ± 161	4.32 ± 0.74	0.96 ± 0.02

[z] Additionally, acclimatized plants at timepoint zero (T0) plants before transferring into the soil variants are shown. There was no significant difference within and between the sites according to Tukey's test at $P \leq 0.05$. Shown are mean ± standard deviation of *n* replicates.

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Ellerhoop ARD UT (4 of 28) and Ruthe ARD UT (3 of 15). Most of these ASVs were high in relative abundance. *Streptomyces* ASV66 in Heidgraben and *Streptomyces* ASV42 in Ruthe showed the highest relative abundance, with 4.48 and 4.10%, respectively. All *Streptomyces* ASVs were negatively correlated with the increase of shoot length and shoot fresh mass and some of them were even present in at least two sites. *Streptomyces* ASV21, which was present in all three sites as a unique ASV, showed the second highest negative correlation with both plant growth parameters (−0.54 and −0.58, respectively). This number was only surpassed by *Streptomyces* ASV70 and *Streptomyces* ASV76 (both present in Heidgraben and Ellerhoop), with a correlation of −0.59 to the increase of shoot length and −0.65 to shoot fresh mass.

A high number of other genera harboring unique ASVs were also negatively correlated with plant growth parameters. For example, *Novosphingobium* ASV92 and *Neorhizobium* ASV47 (highly abundant in Heidgraben and Ellerhoop) negatively correlated with

increase of shoot length (−0.59 and −0.53, respectively) and shoot fresh mass (−0.53 and −0.59, respectively) (Fig. 2).

In 2017, the overall number of negatively correlated ASVs was lower (Fig. 3). Nevertheless, several *Streptomyces* ASVs (also present in at least two sites) again were negatively correlated with plant growth. In Heidgraben and Ellerhoop, the relative abundance of *Streptomyces* ASV76 and *Streptomyces* ASV621 showed a correlation of −0.53 and −0.57 to increase of shoot length and −0.60 and 0.61 to shoot fresh mass, respectively.

We were further interested in unique ASVs of the untreated grass variants to identify possible plant-growth-promoting bacteria (PGPB), which could help to counteract ARD. In 2016, several ASVs of different genera were present in more than two sites (Supplementary Fig. S6). These included ASVs related to genera *Dyella*, *Massilia*, *Rhizobium*, *Rhodanobacter*, and unclassified species of family *Moraxellaceae*. In the biotest 2017, only two ASVs (assigned to genera *Rhizobium* and *Sphingobium*) were
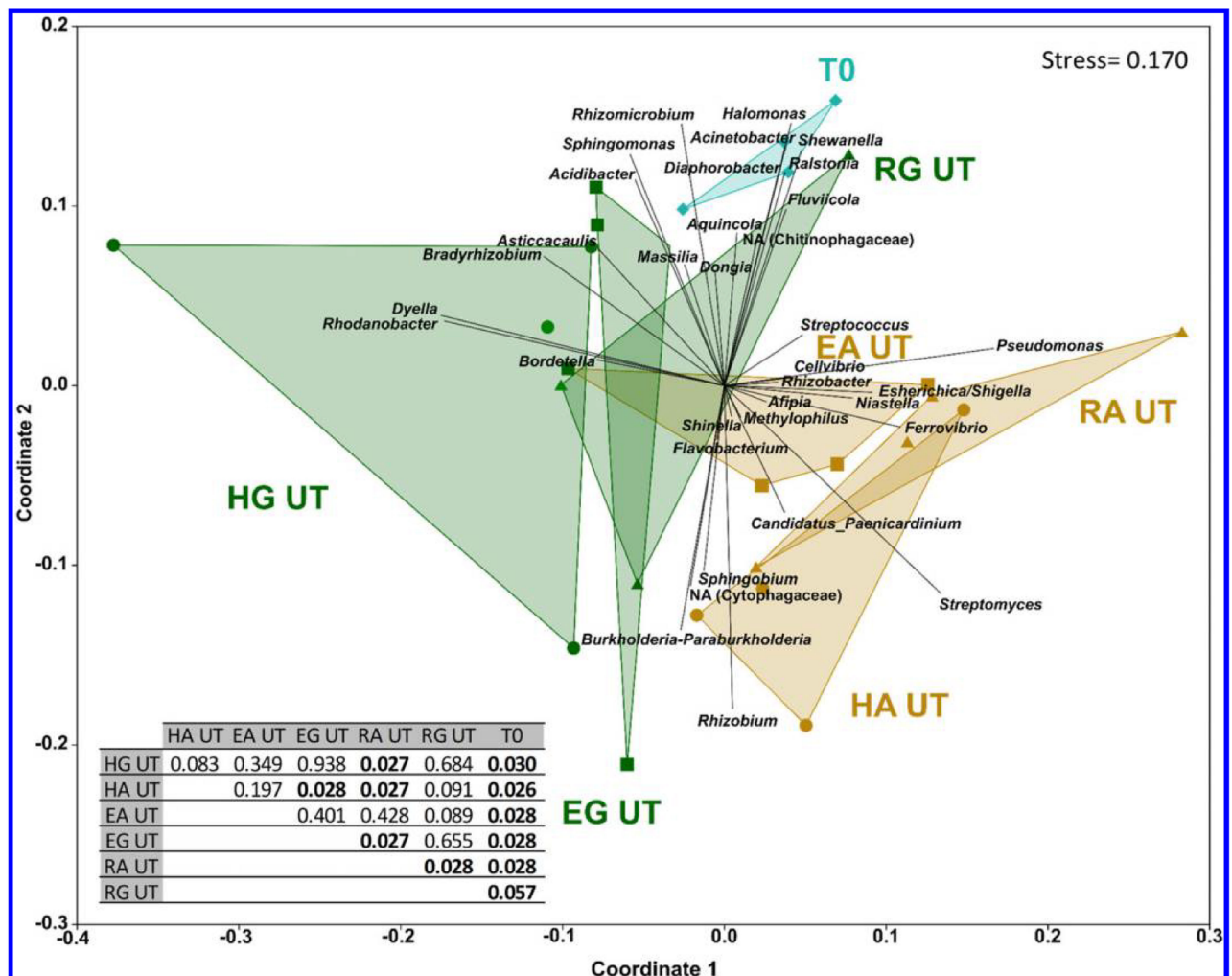
Fig. 1. Three-dimensional nonmetric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity of roots grown for 8 weeks in soils from different sites of the biotest in 2016. The γ-irradiated variants are not included. Vectors represent the correlation coefficient between the corresponding genus and the NMDS score. Relative lengths and the directions of the vectors indicate the influence of the respective genera (RA > 1%). The third axis is not shown. Results of the one-way analysis of similarities are shown in the lower left corner, and significant differences are highlighted in bold ($P \leq 0.05$). H = Heidgraben, E = Ellerhoop, R = Ruthe, A = apple replant disease, G = grass, and UT = untreated.

present at at least at two sites (Supplementary Fig. S7) However, none of them showed positive correlations with plant growth. The only positively correlated ASV was related to unclassified members of family *Rhizobiaceae* and found in 2017 with a relative abundance of 0.95% and a correlation of 0.32 and 0.30 to shoot growth and shoot fresh mass, respectively (Supplementary Fig. S7).

DISCUSSION

In the present study, we characterized the bacterial root endophytic community of apple plants grown in replant and nonreplant soil in order to understand the etiology of ARD and develop countermeasures.

Endophytic bacterial communities in apple roots were dominated by Proteobacteria. *Proteobacteria* was the dominant phylum in most studies where bulk soil or rhizosphere samples from ARD-affected sites had been analyzed (Franke-Whittle et al. 2015; Peruzzi et al. 2017; Sun et al. 2014; Tilston et al. 2018; Yim et al. 2015), with an average relative abundance of 35% (Nicola et al. 2018). The same was true for the root endophytes analyzed in our experiments in both years (Supplementary Figs. S3 and S4). However, in comparison with Nicola et al. (2018), the relative abundance of *Proteobacteria* in roots from plants grown in ARD UT was clearly higher (76% in 2016 and 71% in 2017 in an average of all three sites). This enrichment of *Proteobacteria* in the endosphere could be explained by selective recruitment or colonization or a higher competitiveness inside the plant. Members of this phylum are known for their various secretion systems (Preston et al. 2005), their fast growth, and their high metabolic activity and, therefore, they mostly predominate the endosphere (Lundberg et al. 2012; Reinhold-Hurek et al. 2015).
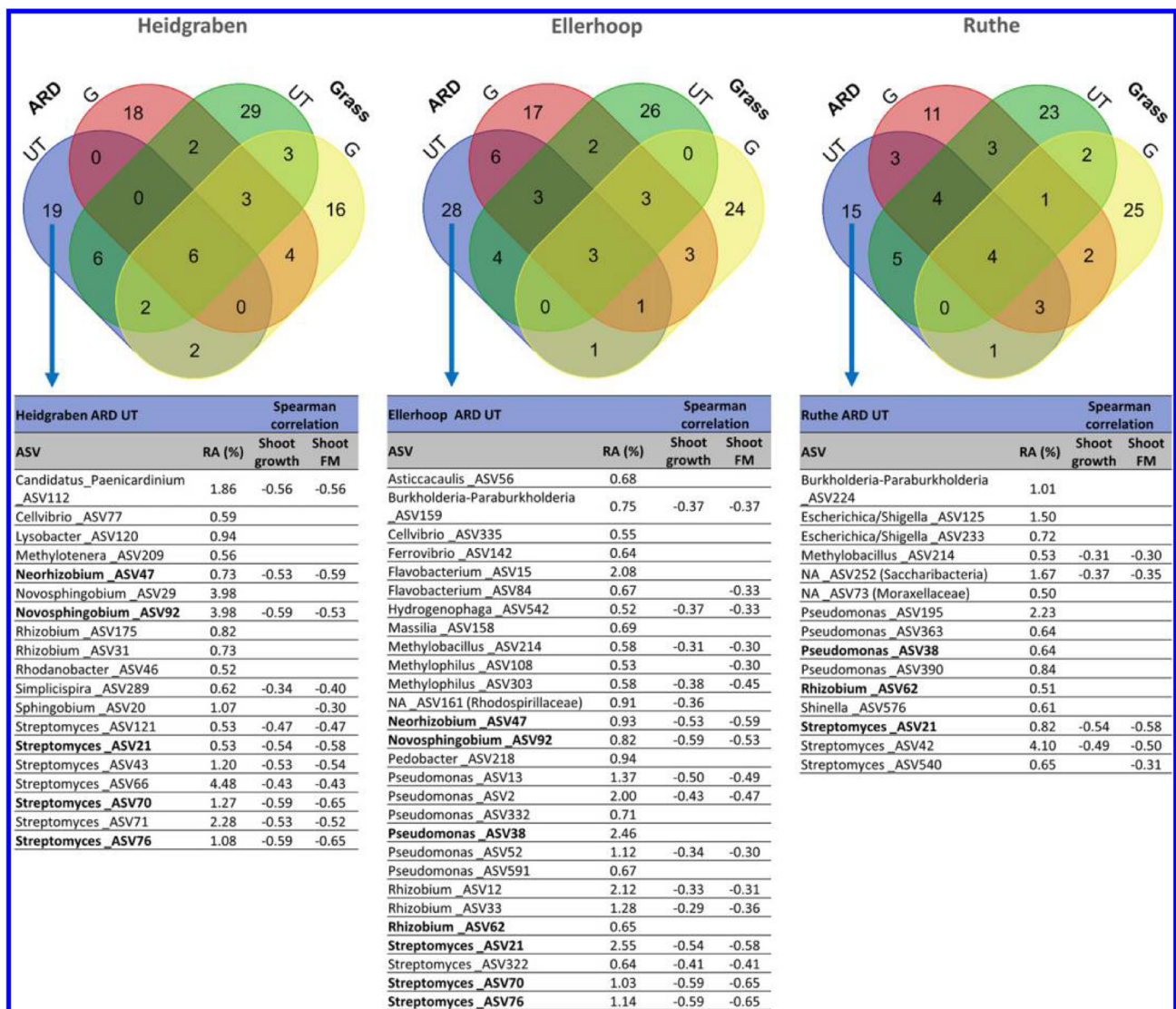
Fig. 2. Venn diagrams showing overlapping amplicon sequence variants (ASVs) (RA > 0.5%) of the different soil variants (ARD = apple replant disease, grass, UT = untreated, and G = γ irradiated) from Heidgraben, Ellerhoop, and Ruthe for the biotest in 2016. The three tables show the site-specific unique ASVs of ARD UT variants with their relative abundance and Spearman correlation with shoot growth (= increase in shoot length) and fresh mass (only significant correlations are shown; $P \leq 0.05$). ASVs highlighted in bold appear in at least two sites.

In many studies where rhizosphere and bulk soil samples of ARD-affected sites have been analyzed, *Actinobacteria* was among the most abundant phyla, with an average relative abundance of 16% (Nicola et al. 2018), which was slightly higher than in our study when we focused on root endophytes (10 and 11% in 2016 and 2017, respectively). In other studies, *Bacteroidetes* showed an average relative abundance of 14% (Nicola et al. 2018) and 13% (Tilston et al. 2018). In our study, values ranged between 8% (2016) and 15% (2017). As expected, members of *Acidobacteria*, which were also highly abundant in the rhizosphere and bulk soil in the abovementioned studies, were low in relative abundance in the root interior, due to the ecophysiological properties of these bacteria, including the use of complex organic compounds and their slow growth.

Differences between the outcome of the biotest of 2016 and 2017. Significant differences occurred in the results comparing biotests between 2016 and 2017. In 2016, *Actinobacteria* were significantly higher in relative abundance in root samples from ARD UT compared with the grass or γ-sterilized variants (Supplementary Fig. S3). Surprisingly, this was not the case in 2017 (Supplementary Fig. S4). Furthermore, there was a clustering of ARD variants apart from the grass variants in 2016 (Fig. 1) but not in 2017 (Supplementary Fig. S5). These differences in the outcome of the biotests could be due to various factors (e.g., higher shoot lengths of T0 plants of 2017 or variation in environmental factors). Another reason could be related to the soil. First of all, soil collection might have resulted in samples of different microbial composition due to patchy appearance of ARD in the field (Simon et al. 2020). Furthermore, at our reference sites, replanting takes place every second year, and was carried out in 2015 and 2017 at Ruthe and Ellerhoop and in 2014 and 2016 at Heidgraben. Soil for the first greenhouse experiment in 2016 was sampled at the end of 2015, where plants at Ruthe and Ellerhoop had been replanted for the fourth time in spring 2015, whereas plants in Heidgraben had just been uprooted. For the experiment in 2017, soil was collected at the end of 2016, when plants at Ellerhoop and Ruthe had been uprooted and at Heidgraben had been replanted for the fifth time in spring. It is known that the microbial community composition in the rhizosphere of different apple genotypes varies seasonally and among years (Rumberger et al. 2007). Also, replanting is known to have an influence of the rhizosphere community composition (Sun et al. 2014).

Heidgraben ARD UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Bordetella_ASV6403	1.03		-0.32
Burkholderia-Paraburkholderia_ASV6424	1.06		
Burkholderia-Paraburkholderia_ASV6476	1.55		
Burkholderia-Paraburkholderia_ASV6481	1.37		
Burkholderia-Paraburkholderia_ASV6501	0.90		
Burkholderia-Paraburkholderia_ASV6525	3.16		
Chitinophaga_ASV855	3.76		
Chitinophaga_ASV858	3.30		
Chitinophaga_ASV297	1.96	-0.34	-0.34
Dyella_ASV255	2.47		
Labrys_ASV4559	0.53		
NA_ASV4764 (Rhizobiaceae)	0.61	-0.47	-0.50
NA_ASV8160 (Enterobacteriaceae)	1.00	-0.48	-0.49
NA_ASV8179 (Enterobacteriaceae)	0.80	-0.45	-0.44
NA_ASV8190 (Enterobacteriaceae)	0.71		
NA_ASV9489 (Rhodanobacteraceae)	1.89		
Rhizobium_ASV4589	0.57		
Rhodanobacter_ASV9389	0.55		
Rhodanobacter_ASV9398	0.56		
Streptomyces_ASV76	0.61	-0.53	-0.60
Streptomyces_ASV621	0.58	-0.57	-0.61
Streptomyces_ASV121	0.61	-0.35	-0.34

Ellerhoop ARD UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Asticcacaulis_ASV3687	1.22		
Burkholderia-Paraburkholderia_ASV26	0.99		
Flavobacterium_ASV1140	0.69	-0.33	-0.39
Methylophilus_ASV7461	0.78		
Methylophilus_ASV7465	0.63		
NA_ASV8160 (Enterobacteriaceae)	1.07	-0.48	-0.49
Pseudoxanthomonas_ASV9569	0.56	-0.34	-0.38
Rhodanobacter_ASV9396	0.93		
Sphingobium_ASV5334	1.93		
Sphingobium_ASV199	2.09		
Streptomyces_ASV607	0.84	-0.36	-0.39
Streptomyces_ASV611	0.59		
Streptomyces_ASV121	0.60	-0.35	-0.34

| Ruthe ARD UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Arachidicoccus_ASV837	1.31	-0.35	
Arachidicoccus_ASV839	0.94		
Arachidicoccus_ASV840	0.58		
Asticcacaulis_ASV3668	0.55		
Bordetella_ASV6403	0.65		-0.32
Burkholderia-Paraburkholderia_ASV6501	0.66		
Cellvibrio_ASV7794	2.06		
Dyella_ASV255	0.97		
Methylobacillus_ASV7450	0.56		
NA_ASV5005 (Xanthobacteraceae)	0.59		
NA_ASV9489 (Rhodanobacteraceae)	0.76		
Rhodanobacter_ASV9396	1.38		
Rhodanobacter_ASV338	0.86	-0.33	-0.33
Rhodanobacter_ASV9402	1.07		
Rhodanobacter_ASV9404	0.66		
Rhodanobacter_ASV9410	0.78		
Streptomyces_ASV76	3.14	-0.53	-0.60
Streptomyces_ASV21	2.18	-0.55	-0.63
Streptomyces_ASV621	2.12	-0.57	-0.61
Wolbachia_ASV5191	0.80		

Fig. 3. Venn diagrams showing overlapping amplicon sequence variants (ASVs) (RA > 0.5%) of the different soil variants (ARD = apple replant disease, grass, UT = untreated, and G = γ irradiated) from Heidgraben, Ellerhoop, and Ruthe for the biotest in 2017. The three tables show the site-specific unique ASVs of ARD UT variants with their relative abundance and Spearman correlation with shoot growth (= increase in shoot length) and fresh mass (only significant correlations are shown; $P \leq 0.05$). ASVs highlighted in bold appear in at least two sites.

Another reason for these different outcomes of the biotest in the 2 years could be differences in the initial endophytic bacterial community composition of the starting material (T0) plants. In this study, a higher diversity and number of ASVs of the initial microbiome in the plant roots was found in 2016 compared with that in 2017. However, this difference between the 2 years was not significant after 8 weeks of growth in the soil. Therefore, a higher diversity and number of ASVs of the initial microbiome did not lead to higher number of ASVs and diversity in plant roots after 8 weeks of growth in the soil. Because soil is the main reservoir of microorganisms for the plant microbiome (Berg and Smalla 2009; Bonito et al. 2014; Hartman and Tringe 2019; Lareen et al. 2016), it is one of the major factors influencing the number of ASVs and diversity. However, some genera (e.g., *Ralstonia*) were present in the 2017 T0 plants and were still present in the plant roots after growth for 8 weeks in the soil (Supplementary Table S2). With this in mind, one strategy to help to overcome ARD could be to inoculate apple plants with PGPB before transferring them into the soil. This so-called microbiome engineering of plants was recently reviewed by Orozco-Mosqueda et al. (2018). Johnston-Monje and Raizada (2011) could show that green fluorescent protein-tagged *Enterobacter asburiaes* could systemically colonize the roots of maize and even the rhizosphere. This means that some genera of the initial endophytic microbiome may possess the ability to colonize not only the plant roots but also the rhizosphere. In order to have plants which are preinoculated with PGPB that can influence not only the endosphere but also the rhizosphere and, therefore, may be able to reduce the ARD effect, inoculation studies are needed.

Next to differences between the years, variations within the soil variants were observed. The observed ASVs and the different diversity indices within the variants showed high standard deviations (Tables 2 and 3). With the reanalysis of several studies of microbiomes of ARD-affected soils, Nicola et al. (2018) determined that the strongest factor for bacterial community variation were environmental variables. In our study, several factors responsible for variations were reduced to a minimum (soils mixed, clonally propagated plants, and the same greenhouse conditions). However, here, we were investigating the root endophytic community. Its selection is strongly controlled by the host plant and dependent on soil and several other factors such as stress and environmental conditions (Afzal et al. 2019). Although all plants and soils within a variant were treated in the same way, individual differences in the soil microbiome and, therefore, differences in root colonization cannot be excluded. To reduce these variations, future biotests should increase the number of analyzed plants.

Are streptomycetes part of the ARD complex? Our results show that nearly all *Streptomyces* ASVs were negatively correlated with increase in shoot length and shoot fresh mass (Figs. 2 and 3). Interestingly, the same ASVs were also identified in the roots of the rootstock cultivar Bittenfelder grown in ARD-affected soil in the three reference field sites Heidgraben, Ellerhoop, and Ruthe (results not shown). Therefore, regardless of the year, whether greenhouse biotest or field experiment, the site, or the apple rootstock genotype, *Streptomyces* ASVs were associated with apple roots grown in ARD soils. This raises the question whether *Streptomyces* spp. are a causative part of the ARD complex or just opportunistic or secondary colonizers.

Streptomyces is a well-studied genus and most famous for its production of antibiotics, with 80% of today's antibiotics being derived from *Streptomyces* spp. (de Lima Procópio et al. 2012). Next to this, traits such as production of antifungal substances and siderophores, solubilization of phosphate, synthesis of plant growth regulators, secretion of volatile compounds, biocontrol (competition for nutrients), and degradation of phytotoxins makes this genus

a potent PGPB, intensively reviewed by Olanrewaju and Babalola (2019), Sousa and Olivares (2016), Viaene et al. (2016), and Vurukonda et al. (2018). These reviews also highlight that genus *Streptomyces* is able to colonize a broad range of plant hosts. It is further believed that these plants can selectively recruit *Streptomyces* spp. (Viaene et al. 2016). However, the signals which attract them or the way of their entering and colonizing the roots are still unknown (Viaene et al. 2016; Vurukonda et al. 2018).

However, these various plant-growth-promoting effects of *Streptomyces* were not affirmed by our findings. Roots growing in soil from the grass variants showed better growth than those in ARD soils. Only two *Streptomyces* ASVs unique for at least two grass variants were found which had no correlation with increase in shoot length and shoot fresh mass (Supplementary Figs. S6 and S7). In contrast, in ARD variants, a clear negative correlation of the relative abundance of *Streptomyces* spp. and plant growth was shown (Figs. 2 and 3). On the one hand, this could indicate that genus *Streptomyces* is pathogenic and part of the replant disease. On the other hand, because *Streptomyces* has a saprophytic lifestyle, it could be an opportunist and degrade dead or damaged root material. Structurally damaged and partially necrotic root systems are typical symptoms for ARD-affected plants (Grunewaldt-Stöcker et al. 2019). *Streptomyces* spp. are able to break down organic remains of plants using several hydrolytic exoenzymes such as cellulases, lignocellulases, pectinases, xylanases, and cutinases (Chater 2016; Chater et al. 2010). *Streptomyces* was also shown to appear in higher abundance in the rhizosphere of *Arabidopsis thaliana* when plant exudated phenolic-related compounds such as salicylic acid were present (Badri et al. 2013; Lebeis et al. 2015) and can even grow on minimal media with only salicylic acid as a carbon source (Lebeis et al. 2015). Due to tyrosinase activity, some isolates were partially protected against plant-produced phenols, leading to increased colonization rates on *A. thaliana* roots (Chewning et al. 2019). Gene expression studies revealed that genes responsible for the production of phytoalexins (some of which belong to polyphenols) are upregulated in M26 roots growing in ARD-affected soils (Weiß et al. 2017a,b).

Overall, these reasons make it seem likely that genus *Streptomyces* finds favorable conditions and occurs in higher abundance in ARD-affected roots and, hence, is opportunistic. Yet pathogenicity cannot be excluded.

Of the 672 known *Streptomyces* spp. (Euzéby 1997; Parte 2018) (number as of 3 December 2020), only 10 have pathogenic features (Viaene et al. 2016). Most known are *Streptomyces scabies, S. acidiscabies*, and *S. turgidiscabies*, which cause common scab on roots and tuber crops. These species are able to directly penetrate plant cells and, in addition to necrotic scab lesions, lead to reduced growth, root stunting and browning, and a reduction of the complexity of the root system (Loria et al. 2003, 2006; Seipke et al. 2012) (i.e., symptoms that resemble the phenotype of ARD-affected roots). However, despite the large host range, none of these species were reported to infect woody plants, although the host range likely includes all higher plants, because dicot and monocot seedlings of several plant species have shown symptoms after inoculation with *S. scabies* (Leiner et al. 1996; Loria et al. 2006). One reason for this large host range is based on the assumption that genus *Streptomyces* is believed to have originated 400 million years ago, when green plants started to colonize the land (Chater 2016). Another reason for this flexibility is the fact that *Streptomyces* virulence genes are clustered on a pathogenic island which can be mobilized and, via conjugation, transferred to nonpathogenic relatives, which leads to the emergence of new plant-pathogenic streptomycetes (Lerat et al. 2009).

A closer look at the genus *Streptomyces* from our greenhouse experiment revealed that the *Streptomyces* ASVs which occurred in

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at least two sites (Figs. 2 and 3) shared a high similarity with the pathogen *S. turgidiscabies*. Blasting the sequences against the NCBI database (https://www.ncbi.nlm.nih.gov/) showed a similarity of 99.76% (ASV21 and ASV70), 99.51% (ASV76), and 99.52% (ASV621) (Supplementary Table S1). All of these ASVs showed a negative correlation with shoot fresh mass of approximately −0.60 or more, whereas ASV121, which showed the lowest negative correlation of −0.34 also shared the lowest identity with *S. turgidiscabies* (97.32%). However, for further comparisons to *Streptomyces* spp., the complete 16S rRNA sequence of the apple root endophytes identified in this study is necessary.

Nevertheless, the high similarity to pathogenic *Streptomyces* spp., the broad host range, and the ability for horizontal gene transfer of virulence genes may be arguments in favor of genus *Streptomyces* as a possible causative organism of ARD.

Role of genus *Streptomyces* in ARD. Several previous studies investigated *Streptomyces* in relation to ARD. However, they resulted in controversial conclusions. Genus *Streptomyces* is part of the order of *Actinomycetales*, members of which were first mentioned as a possible cause of ARD by Otto and Winkler (1977). The authors at that time could only identify the bacteria by their morphology at the level of the phylum, which was called "Actinomycetes" in those days. In their histological analysis, "Actinomycetes" species were found in damaged roots of apple seedlings with a frequency of 47.3% in replant affected soil but not (0.3%) in steamed soil (Otto and Winkler 1977). Also, in plants from our greenhouse experiments, *Actinobacteria* were histologically observed more frequently in roots in untreated ARD soils than in non-ARD soils (Grunewaldt-Stöcker et al. 2019). The so called "root-pathogenic Actinomycetes" (Otto et al. 1993) were observed in ARD-affected roots of apple seedlings. Thereafter, the degree of infestation increased with increasing shoot growth and decreased with stagnating growth. This led to the assumption that root exudates, which are influenced qualitatively and quantitatively by the growing buds, triggered the germination of persistent spores (Otto et al. 1993).

In contrast to a pathogenic role, *Streptomyces* spp. were considered to be plant growth promoters in other studies dealing with ARD. 16S rRNA pyrosequencing revealed that the genus *Streptomyces* was positively (0.64) correlated with shoot growth in plants grown in fumigated ARD soil (Nicola et al. 2017). A function in disease suppression was also associated with *Streptomyces* (Cohen and Mazzola 2006; Cohen et al. 2005; Mazzola et al. 2007), when the effect of seed meal amendments on the putative ARD-causing pathogens *Rhizoctonia solani* or *Pythium* spp. was investigated. Seed meal amendments resulted in increased populations of *Streptomyces*, which were able to suppress infections by *R. solani*. Disease suppression was attributed to a transformation of bacterial community structure and the production of nitric oxide (Cohen and Mazzola 2006; Cohen et al. 2005), which plays a role in the induction of plant systemic resistance. Most *Streptomyces* isolates recovered from the apple rhizosphere were able to produce nitric oxide (Cohen et al. 2005). By adding any of several *Streptomyces* strains, Cohen and Mazzola (2006) could restore disease suppressiveness in previously pasteurized soil. Next to disease suppression, promotion of root infection by *Streptomyces* spp. was also observed in apple (Zhao et al. 2009) and *Picea abies* (Lehr et al. 2007). Root infections were significantly elevated in the presence of *Streptomyces* spp. This may be a negative side effect, because genus *Streptomyces* is known to promote mycorrhizal formation by promoting fungal growth and by decreasing the plant defense response (Lehr et al. 2007; Tarkka et al. 2008; Vurukonda et al. 2018). *Streptomyces* sp. AcH 505 was shown to downregulate the peroxidase activity and pathogenesis-related peroxidase gene (*Spi2*)

expression (Lehr et al. 2007) of the host plant, thus promoting fungal root colonization.

Furthermore, two more traits of genus *Streptomyces* match the characteristics of ARD. First, like ARD, *Streptomyces* can persist for a very long time in soil. Due to no or minimal metabolic activity, spores can survive harsh conditions for years (Bobek et al. 2017). Second, *Streptomyces* is very sensitive to waterlogged conditions. *Streptomyces* is more abundant in drained soils (sandy loam) than in heavy soils (Gowdar et al. 2018) and, similarly, ARD is usually more severe in light soil compared with heavy soils (Mahnkopp et al. 2018; Winkelmann et al. 2019).

All of these findings indicate that *Streptomyces* spp. could be responsible for ARD or be part of it. However, to prove this, inoculation experiments are necessary, as was done by Tewoldemedhin et al. (2011). They isolated 92 *Streptomyces* strains from surface-sterilized roots from six ARD-affected sites in South Africa and inoculated 37 of them to 4-week-old apple seedlings to test pathogenicity. Moreover, 11 were coinoculated with the pathogens *Pythium irregulare* and *C. macrodidymum*. All tested streptomycetes had no effect on plant growth. At first, this seems to be a clear sign that these *Streptomyces* isolates were not pathogenic (directly or indirectly). However, these isolates had low identity (less than 98%) to known *Streptomyces* spp. and none showed close similarity to *S. turgidiscabies*, which had a high identity to our ASVs, with a negative correlation with plant growth. Also, in our experiments, not all *Streptomyces* spp. were negatively correlated with plant growth. In 2017, of 61 detected ASVs, only 6 showed negative correlations with shoot fresh mass (15 of 32 in 2016). Furthermore, inoculation trials were done in artificial soil (bark medium and sand [2:1]) (Tewoldemedhin et al. 2011), which means that potential "copathogens" were not present, unlike in ARD soil.

***Streptomyces* not present in T0 plants.** Only very few of the detected ASVs assigned to genus *Streptomyces* negatively linked to plant growth were present in T0 plants (data not shown). In 2017, only *Streptomyces* ASV611 was present in one of four replicates with a relative abundance of 0.38%. In 2016, ASV121 was detected in three of four replicates, one with a relative abundance of 1.39%. All other *Streptomyces* ASVs were not present in T0 plants. Because genus *Streptomyces* is widely distributed in soils (Ferrer et al. 2018; Olanrewaju and Babalola 2019; Seipke et al. 2012), plants in our experiments were most likely colonized after planting in the different soil variants. Based on molecular fingerprints of rhizosphere and bulk soil, Lucas et al. (2018) confirmed that *Streptomyces* is more abundant in ARD compared with grass control soil.

PGPB in plants grown in non-ARD soils. In order to find possible PGPB that may be used to overcome ARD, we also looked at the unique ASVs in the grass variants to find ASVs positively correlated with plant growth. However, in 2016, no ASV showed any significant positive correlation (Supplementary Fig. S6). In 2017, only one ASV (NA_ASV4691 [*Rhizobiaceae*]) showed a positive correlation with an increase in shoot length and shoot fresh mass, with 0.32 and 0.30, respectively.

Conclusion. Here, we showed, for the first time, the apple root endophytic community composition in plants grown on three replant-affected soils in comparison with non-replant-affected soils based on next-generation sequencing in 2 years. Although no PGPB to counteract ARD could be found, several ASVs with negative correlations with plant growth were associated with ARD. With *Streptomyces* spp. showing strong negative correlations and being present in all soils over the years, a potential key player for the cause of ARD may have been found. However, it remains to be clarified in future studies whether genus *Streptomyces* as root endophyte in ARD situations acts opportunistically or is pathogenic. *Streptomyces* spp. can grow saprophytically and just degrade plant material

and metabolize plant exudates; however, they can also play an essential role in the ARD complex by suppressing plant defense responses and, thereby, promote infection of fungal pathogens. Further inoculation studies with *Streptomyces* isolates in combination with fungal pathogens as coinoculants will help to answer this question.

ACKNOWLEDGMENTS

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2.4   Time course of the bacterial root endophytic microbiome of *Malus domestica* plants in field soils affected by apple replant disease

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Abstract

Apple replant disease (ARD) is a worldwide problem for nurseries and orchards leading to reduced plant growth and fruit quality. The etiology of this complex phenomenon is poorly understood, but shifts of the bulk soil and rhizosphere microbiome seem to play an important role. Since roots are colonized by microbes from the rhizosphere, studies of the endophytic microbiome in relation to ARD are meaningful. In this study, culture independent and culture dependent approaches were used in order to unravel the endophytic root microbiome of apple plants 3, 7 and 12 months after planting in ARD soil and grass control soil at two different field sites. The relative abundance of Actinobacteria increased over time in ARD and grass control plots. Furthermore, several ASVs linked to *Streptomyces*, which were shown in a previous greenhouse study to be negatively correlated to shoot length and fresh mass, were also detected in roots from both field sites. Especially in apples planted in grass control soil these ASVs increased in their relative abundance in roots over time. The isolation of 150 bacterial strains in the culture dependent approach confirmed the high diversity of members of the genus *Pseudomonas,* also detected by the molecular barcoding approach. only partial overlaps between the two approaches underline the importance of combining these methods in order to better understand this complex disease and develop possible counter measures. Overall, this study confirmed greenhouse data and suggests a key role of *Streptomyces* in the etiology of ARD.

Introduction

Apple replant disease (ARD) is a worldwide complex problem, which affects apple tree nurseries and orchards causing reductions in tree growth, fruit yield and quality (Mazzola und Manici, 2012; Manici et al., 2013; Winkelmann et al., 2019). It occurs when apple is repeatedly planted at the same site and is defined 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). The exact etiology of ARD is still not known but there is increasing evidence that, next to changes in the abundance of specific pathogens, shifts of the bulk soil and rhizosphere microbiome are an important driver of ARD (Winkelmann et al., 2019).

However, studies of the endophytic microbiome and its role in ARD are rare and most were focused on fungal pathogens in apple roots. Kelderer et al. (2012) identified *Cylindrocarpon* spp. and *Rhizoctonia* sp. as pathogenic root endophytes in row (ARD-affected) and inter-row (control) planted apple trees. *Fusarium oxysporum* and *Fusarium solani* were most abundant in roots in this study, but not considered as pathogens. Root endophytic *Cylindrocarpon*-like fungi (*Thelonectria* sp. and *Ilyonectria* spp.) were also found by Manici et al. (2013) next to *Pythium* spp. to be responsible for the growth reduction in the rootstock M9 growing in ARD-affected soil. Different species of Nectriaceae were as well found in ARD-affected cortex cells extracted by laser microdissection (Popp et al., 2020). Several fungal endophytes from ARD-affected apple roots were isolated and re-inoculated in a soil free biotest by Popp et. al. (2019). Negative effects on plant health were reported for *Cadophora*, *Calonectria*, *Dactylonectria*, *Ilyonectria*, and *Leptosphaeria*.

On the contrary, studies focusing on bacterial endophytes are rare. Up to now, only in one study endophytes were isolated (mainly *Streptomyces*) from apple roots and tested for their biocontrol activities in co-inoculations with the pathogens *Pythium irregulare* and *Cylindrocarpon macrodidymum*, but showed no effect (Tewoldemedhin et al., 2011). In a previous study (Mahnkopp-Dirks et al., 2020) we conducted a greenhouse biotest with apple plants of the ARD sensitive rootstock genotype M26 grown in ARD soil or grass soil and investigated the bacterial root endophytic community using a molecular barcoding approach. Results showed several Amplicon Sequencing Variants (ASVs) linked to *Streptomyces*, which were uniquely found in plants grown in ARD soil. Moreover, these ASVs were negatively correlated to shoot length and shoot fresh mass. These results were achieved under controlled

greenhouse conditions with in vitro propagated plantlets, which helped to reduce variability, but do not represent field conditions. To validate these results under field conditions, the same approach was used with the aim to investigate the bacterial root endophytic community structure in plants grown in ARD or grass control plots using a different seed propagated rootstock (cv. 'Bittenfelder Saemling') in different soils over a one year period. In order to investigate the bacterial endophytes over time and to analyze seasonal changes in the endophytic community structure, samples were taken over one year. We addressed the question, whether the same ASVs linked to *Streptomyces* that were detected in our previous greenhouse biotest study are found under field conditions, which would support their associated role in ARD. We further hypothesize that apple roots are colonized by different bacteria when growing in ARD soil compared to non ARD-affected control soil.

Moreover, we intended to obtain isolates to complement the culture independent data which couldserve as potential inoculants. Thus, we also used a culture dependent approach in order to isolate a broad spectrum of bacterial root endophytes, established pure cultures and identified them using Sanger sequencing of the 16S rRNA gene. This will enable us to study their effects on apple plants and finally to help and overcome the complex ARD phenomenon.

Material and Methods

Field sites and sampling

The field experiments were carried out at two different sites in northern Germany: Heidgraben (x-coordinate 53.699199; y-coordinate 9.683171; WGS 84, Schleswig-Holstein) and Ellerhoop (x-coordinate 53.71435; y-coordinate 9.770143 WGS 84, Schleswig-Holstein) which differed in their soil properties (Mahnkopp et al., 2018). Based on World Reference Base for soil resources, the textures of the top soil (0 - 20 cm) of the two sites were classified as sand (Heidgraben) and loamy sand (Ellerhoop) (Mahnkopp et al., 2018). Both sites contained two different plot variants in four replicates each: (i) ARD plots, where ARD was successfully induced by repeatedly planting ´Bittenfelder´ apple seedling rootstocks since 2009 in a two-year cycle and (ii) control plots which were only covered with grass since then. In spring 2016 in Heidgraben and spring 2017 in Ellerhoop, one third of these grass control plots were planted with Bittenfelder plants representing the first apple planting generation (hereafter referred to as grass plots). ARD plots in Ellerhoop were replanted for the last time in spring 2015 and in Heidgraben in spring 2016 representing the fifth replant generation at the time of sampling (Tab. 1).

Table 1: Time schedule of apple planting generations (gen.) and sampling time points (1,2,3) at the two sites Heidgraben and Ellerhoop (1 = sampling in July, 2 = sampling in November, 3 = sampling in April; planting: April, lifting: November)

Year	Plot	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Heid-graben	ARD	1. apple gen.	2. apple gen.		3. apple gen.		4. apple gen.		5. apple gen. 1 2 3		6. apple gen.
	Con-trol	Grass							1. apple gen.		
Eller-hoop	ARD	1. apple gen.		2. apple gen.		3. apple gen.		4. apple gen.		5. apple gen. 1 2 3	
	Con-trol	Grass								1. apple gen.	

In Heidgraben, plants of both plots were planted on 05. + 06.04.16 and in Ellerhoop on 10. + 11.04.17. At both sites, plants were sampled at three time points after planting: 3 months after planting (summer), 7 months after planting (late autumn) and one year after planting (spring). In Heidgraben sampling took place on 27.07.16 (1, summer), 16.11.16 (2, autumn) and 25.04.17 (3, spring) and in Ellerhoop on 25.07.17 (1, summer), 13.11.17 (2, autumn) and 23.04.18 (3, spring). Three plants were sampled per plot (in total 24 per site and season). Root fresh weight of plants taken after 12 months (spring) was measured after getting rid of adhering soil. In addition, samples of the planting material (plants taken before the transfer into the soil) were taken in Ellerhoop in spring 2017 served as "time point zero" (T0) plants. These plants were obtained as one year old seedlings from a specialized nursery (sawn in spring 2016, uprooted in autumn, and stored over winter in a cooling chamber).

Root surface disinfection

The following surface disinfection was performed as described in Mahnkopp-Dirks et al. (2020): To get rid of the adhering soil roots were washed carefully. Afterwards they were rinsed for 30 s in EtOH (70 %), followed by stirring in 2 % NaOCl for 7.5 min and finally washing 5 times in sterile deionized water. The final washing water was plated on 523 medium (Viss et al., 1991) and R2A Agar (Reasoner and Geldreich, 1985) and incubated for 1 week at room

temperature. Plating resulted in < 10 CFU per plate in all cases. Roots were stored in sterile 2 ml Eppendorf tubes at -80°C until DNA extraction for amplicon sequencing.

DNA extraction for amplicon sequencing

For Illumina sequencing, DNA was extracted as mentioned in Mahnkopp-Dirks et al. (2020) using the Invisorb Spin Plant Mini Kit (Stratec, Berlin, Germany) according to the manufacturer's instructions.

Amplicon sequencing

Amplicon sequencing was done using the primer combination 335F (CADACTCCTACGGGAGGC)/ 769R (ATCCTGTTTGMTMCCCVCRC) (Dorn-In et al. 2015) to amplify the V3 – V4 region of the 16S rRNA gene. Amplicon library preparation and bioinformatics analysis were described in detail in Mahnkopp-Dirks et al (2020). Briefly, PCR was performed using 2x Phusion High-Fidelity Master Mix (Thermo Fisher Scientific, Waltham, USA), 10 pmol of each primer and 5 ng DNA template in a final volume of 10 μL with PCR conditions: 98°C for 10 s, 30 cycles of 98°C for 1 s – 59°C for 5 s – 72°C for 45 s, 72°C for 1 min. After purification with Agencourt AMPure XP kit (Beckman Coulter, USA) indexing PCR (98°C for 30 s, 8 cycles of 98°C for 10 s - 55°C for 30 s – 72°C for 30 s, 72°C for 10 min) was performed using Nextera XT Index Kit v2 (Illumina, USA). Purified samples were equimolarly pooled to 4 nM and sequenced on Illumina Miseq platform. FASTQ files were trimmed using AdapterRemoval (Schubert et al., 2016) and analyzed using the QIIME 2 software package release 2017.11 (Caporaso et al., 2010) with default parameters. Quality control as performed via QIIME 2 plugin DADA2 (Callahan et al., 2016) with removing 10 bp n-terminally, length truncation at position 300 (forward) and 260 (reverse) and expected error of 2. Taxonomic assignment of the resulting amplicon sequence variants (ASVs) was performed using primer-specific pre-trained Naive Bayes classifiers of the SILVA_132_QIIME release 99% and the q2-feature-classifier plugin.

PCR negative control showed no ASVs, thus contamination during sample processing could be excluded. For further data analysis, unassigned reads, singletons, plastid sequences and sequences assigned to archaea and eukaryotes were removed (in sum 37 % of all reads), resulting in 4422 ASVs (over all samples), which were nearly all covered after rarefying at 4213 reads (Fig. S1). The relative abundance was calculated by dividing the number of reads per

ASV in the samples by the sum of total reads per sample and finally multiplied by 100. To calculate the overall relative abundance of the corresponding phylum/genus, ASVs belonging to the same phylum/genus were merged.

Isolation of bacteria from surface disinfected roots

In order to isolate bacterial endophytes four random 1 cm-pieces of surface sterilized fine roots (∅ < 2 mm) of each plant were placed per Petri dish containing 523 medium (Viss et al., 1991) and R2A Agar (Reasoner and Geldreich, 1985). For each plant, three Petri dishes per medium were prepared as replicates. After approximately 7 days at room temperature, different colonies were picked based on different morphology and streaked separately.

To avoid overgrowing of slow growing colonies, additionally 100 mg of surface disinfected roots were cut into small pieces and transferred into a 50 ml centrifuge tube containing 10 ml saline (0,85 % NaCl). Samples were shaken at 150 rpm and 4°C for 22 hours. 100 µl of the solution as well as dilutions up to $1:10^5$ were transferred onto three Petri dishes containing 523 medium and R2A medium respectively and evenly distributed. After 7 to 28 days, colonies were picked and streaked out. Selection of different colonies was based on different appearance and morphology with the aim of obtaining a broad spectrum of different bacterial root endophytes.

DNA extraction of bacterial isolates

Single colonies were transferred to liquid medium 523 (Viss et al., 1991) and incubated for 1 to 7 days at room temperature on a shaker at 150 rpm until growth was visible. 1 ml of this suspension was used for DNA extraction based on the protocol of Quambusch et al. (2014).

PCR amplification and sequencing of 16S rRNA gene

Partial sequences of the 16S rRNA gene of 140 isolates were obtained using the primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGYTACCTTGTTACGACTT) (Weisburg et al, 1991). Each PCR reaction (25 µl) contained 10 ng DNA, 1 x Williams Buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 20 mM $MgCl_2$; 0.01% gelatin), 200 µM dNTPs, 10 pmol of each primer and 1 U Biotaq DNA polymerase (Bioline, London, UK). The thermal cycler protocol started with an initial denaturation of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C

for 30 s, annealing of the primers at 52°C for 40 s and elongation at 72°C for 60 s and ended with a final elongation at 72°C for 5 min.

Fragments were separated via gel electrophoresis (1 x Tris-acetate-EDTA (TAE) buffer, Aaij and Borst, 1972; Hayward and Smith, 1972) and the 16S PCR products of about 1500 bp were excised from 1% agarose gels and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey & Nagel, Düren, Germany). The 16S rRNA gene fragments were sequenced with the Sanger method (Sanger et al., 1977) by Microsynth Seqlab (Göttingen, Germany) using the primers 27f and 1492r.

Out of the obtained sequences of the primers 27f and 1492r (reverse complement) a consensus sequence was created using the program BioEdit (version 7.2.5, Hall 1999). Sequences were blasted (Blastn) against the NCBI database (https://www.ncbi.nlm.nih.gov/). Identities and origins of the different isolates can be seen in Table S1. To detect identical sequences, alignments of the sequences belonging to the same species were done. Sequences were brought to the same length and compared in a sequence identity matrix (BioEdit, version 7.2.5, Hall 1999).

Data analysis

Phylogentic analyses based on 16S rRNA sequences of isolated strains

For phylogenetic analysis, an alignment of 16S nucleotide sequences of the isolates was done in BioEdit (version 7.2.5, Hall 1999). Out of 150 16S rRNA gene sequences those with at least 1300 bp were selected. Sequences with the same hit in the NCBI database were excluded, resulting in a total of 62 different sequences. *Flavobacterium oryzae* strain Jyi-05 (Accession no: NR_134036) was used as an outgroup to root the tree. All sequences were cut at 1320 bp before ClustalW multiple alignment (Thompson et al., 1994) was done with the number of bootstraps set to 1000. This alignment was used for phylogenetic tree construction with the program MEGA X (Kumar et al., 2018) using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993).

Amplicon data analyses

Determination of species diversity (Shannon, Simpson) and richness (Chao1) indices of the amplicon data was done using the "Phyloseq" (McMurdie and Holmes 2013) and "Vegan" (Oksanen et al. 2019) packages of R v3.6.1 (R Development Core Team (2008), http://www.R-

project.org). Normal distribution based on Shapiro-Wilk test (Shapiro and Wilk, 1965) and homogeneity of variance based on Levene´s test (Levene, 1960) were tested using the program PAST3 v. 3.20 (Hammer et al., 2001). If the null hypotheses of normal distribution and equal variances were rejected, the Tukey test based on Herberich et al. (2010) was used at $p < 0.05$ to determine significant differences of the diversity and richness scores. In order to compare the relative abundance of different phyla in different seasons and ARD variants to grass variants, a DESeq2 analysis using generalized linear models and pairwise comparisons ($p < 0.05$) were performed (DESeq2, Love et al. (2014)).

To link the culture independent approach and the culture dependent approach a local Blastn of the 16S rRNA sequences of the isolates against all ASVs obtained from amplicon sequencing was done using BioEdit (version 7.2.5, Hall 1999).

Results

Growth data

After 12 months of growing in ARD or grass plots, roots showed clear differences (Fig. 1). Roots of the plants in Ellerhoop growing in ARD soil had significant lower mass (21.64 ± 10.91 g) compared to roots from the grass soil (78.21 ± 33.49 g). In Heidgraben, roots from ARD soil had a lower mass (36.88 ± 15.65 g) than roots grown in grass soil (52.61 ± 25.34 g), but the differences were not significant (Welch Two Sample t-test p ≤ 0.05).

Figure 1: Apple roots in spring after grown for 12 months in grass soil (left) or ARD soil (right) in Ellerhoop (photo: Alicia Balbín-Suárez).

Culture independent approach

In order to compare the bacterial endobiome of roots growing in ARD-affected and non-affected soils, a metabarcoding approach using directly extracted DNA from the roots after surface disinfection and 16S rRNA gene amplification was performed. The highest number of ASVs was found in Heidgraben in autumn in the roots obtained from plants grown in ARD soil with 291 ± 96 and the lowest number of ASVs in Ellerhoop (spring) in roots from plants grown

in grass control soil (159 ± 28) (Tab. 2). In five out of six variants (site + season), roots from plants grown in ARD soil contained a higher richness (Observed ASVs, Chao1) compared to roots from plants grown in grass soil, but the difference was not significant due to high variation between samples. All ARD soils showed a higher diversity (Shannon, Simpson) than grass soils at the different sampling times. However, these differences in richness and diversity were not significant (Tukey's test at p ≤ 0.05). The only significant difference within a site was found in diversity (Shannon) in Ellerhoop ARD soil between the autumn and the spring sampling (Tab. 2).

Table 2: Richness and diversity of endophytic bacterial communities based on amplicon sequence variants (ASVs) in roots from Bittenfelder plants grown in ARD plots or grass plots from the sites Heidgraben and Ellerhoop. Additionally, T0 plants before planting at Ellerhoop are shown. Different letters indicate significant differences between the sampling times within one site (Tukey's test at p ≤ 0.05.) No letters indicate no significant differences. Significant differences between ARD and Grass are shown in bold (t.test at p ≤ 0.05; 0.001 '***' 0.01 '**' 0.05 '*'). Given are mean ± standard deviation of n replicates.

Site	Soil	n	Observed ASVs	Chao1	Shannon	Simpson
Ellerhoop	T0	3	244 ± 26	253 ± 30	4.6 ± 0.12	0.98 ± 0.00
Heidgraben (Summer 16)	ARD	3	260 ± 39	266 ± 41	5.0 ± 0.19	**0.99 ± 0.00**
	Grass	4	210 ± 78	222 ± 85	4.19 ± 0.47	**0.96 ± 0.02***
Heidgraben (Autumn 16)	ARD	4	291 ± 96	301 ± 104	4.82 ± 0.43	0.98 ± 0.01
	Grass	4	240 ± 95	248 ± 100	4.48 ± 0.59	0.97 ± 0.02
Heidgraben (Spring 17)	ARD	4	251 ± 41	264 ± 39	4.85 ± 0.31	0.99 ± 0.01
	Grass	4	236 ± 21	243 ± 19	4.76 ± 0.24	0.98 ± 0.01
Ellerhoop (Summer 17)	ARD	4	236 ± 72	245 ± 77	4.42 ± 0.61	0.97 ± 0.02
	Grass	3	166 ± 43	175 ± 43	3.89 ± 0.49	0.96 ± 0.02
Ellerhoop (Autumn 17)	ARD	2	160 ± 21	162 ± 22	4.05 ± 0.01[a]	0.96 ± 0.01
	Grass	3	171 ± 41	176 ± 37	4.04 ± 0.96	0.94 ± 0.06
Ellerhoop (Spring 18)	ARD	3	253 ± 77	263 ± 83	4.72 ± 0.17[b]	0.98 ± 0.00
	Grass	4	159 ± 28	161 ± 29	4.06 ± 0.73	0.92 ± 0.10

Proteobacteria were the dominant phylum with a mean relative abundance of 77.3 % over all samples (Fig. 2). Significant differences in relative abundance of this phylum between roots from plants grown in ARD and control soil were only detected in Ellerhoop (autumn). T0 plants had a lower relative abundance of Proteobacteria (61.4 %).

The second most abundant phylum was Bacteroidetes with a mean relative abundance over all samples of 12.8 %. Here no differences between plants grown in control soils and ARD-affected soils were detected. Roots of T0 plants had almost three times the abundance of Bacteroidetes (36.2 %). Actinobacteria had a mean relative abundance of 7.5 % over all samples. Interestingly, the abundance for this phylum was increasing over time in ARD and grass plots at both sites: In Heidgraben, the relative abundance in ARD plots started with 5.4 % 3 months after planting, was 12.9 % after 7 months and reached 14 % after one year. Roots of plants grown in grass soil showed a slightly lower abundance (3.5 %) after 3 months, reached 5.9 % after seven months and ended with an even higher abundance (20 %) than in roots from ARD plots. In Ellerhoop relative abundances of Actinobacteria were lower in ARD (1.8 % summer, 5.6 % autumn, 13.3 % spring) and grass plots (0.1 % summer, 5.3 % autumn, 10.2 % spring) but were also increasing over time. In pairwise comparisons of phyla abundance between ARD and grass plots, no significant differences were found except for Proteobacteria (autumn), Actinobacteria (summer) and Firmicutes (spring) in Ellerhoop.
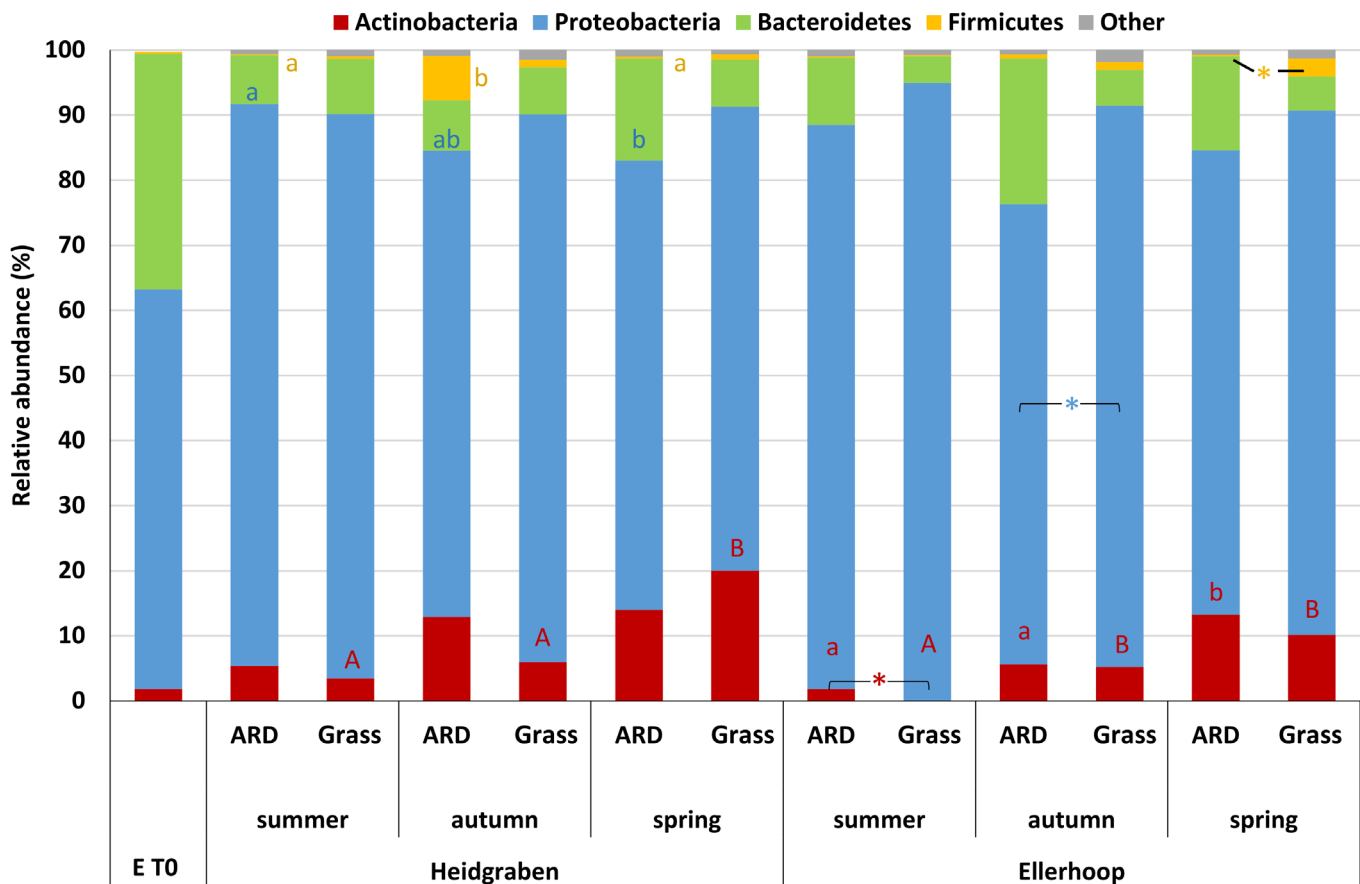
Figure 2: Relative abundance of dominant phyla in roots of Bittenfelder plants grown in ARD plots or grass plots in Heidgraben and Ellerhoop taken in summer and autumn after planting or the following spring. Different letters indicate statistically significant differences within one site in ARD plots (lower case) or grass plots (upper case) between the seasons (DESeq2 analysis using a generalized linear model and multiple comparisons with p ≤ 0.05). Significant differences between ARD and grass within one season are indicated by an asterisk (DESeq2 analysis using a generalized linear model and pairwise comparisons with p ≤ 0.05). Different colored letters belong to the respective phyla. N numbers are shown in Table 2.

In total, *Pseudomonas* was the most abundant genus (mean of relative abundance over all samples 20.1 %), followed by *Streptomyces* (5.9 %) and *Rhizobium* (4.5 %). A closer look at the genus level revealed, that in Heidgraben lots of different ASVs linked to *Pseudomonas* were present in roots grown in ARD and grass soil over all seasons (Fig. 3A). Based on relative abundance *Pseudomonas* was more dominant in roots grown in grass plots, especially at the early time points in summer and autumn. Most ASVs linked to *Rhizobium* were decreasing in relative abundance and number of ASVs over the time in grass as well as in ARD plots. The opposite pattern could be observed for ASVs linked to the genus *Streptomyces,* especially in

roots of plants grown in grass soil. In roots from both soils, the number of ASVs linked to *Streptomyces* with a relative abundance greater than 0.5 % was low (grass = 1, ARD = 2) as was their relative abundance 3 months after planting (summer). Until autumn the number of ASVs (RA > 0.5 %) increased to two (sum RA = 1.6 %) in roots grown in grass plots and to eight (sum RA = 10.4 %) in ARD plots. One year after planting (spring), this number further increased to six (grass) and nine (ARD). In terms of relative abundance, the genus *Streptomyces* was at this time the most abundant one in roots of both plots (grass = 11.7 %, ARD = 10.4 %).

In Ellerhoop, comparable patterns regarding *Streptomyces* ASVs were observed (Fig. 3 B). However, 3 months after planting (summer), no ASV linked to *Streptomyces* was present (over the threshold of 0.5 %) in roots of both plots. But after one year (spring) several ASVs linked to *Streptomyces* were present which were dominant in roots grown in ARD soil (sum of RA = 11.3 %) and less abundant in grass plots (sum of RA = 4.7 %). In roots grown in grass plots, all five ASVs linked to *Streptomyces* were increasing in their relative abundance over time.

ASVs linked to *Rhanella* were only found in autumn with a similar relative abundance of 19,9 % (grass) and 20 % (ARD).

Pseudomonas showed a different development in roots grown in grass soil compared to ARD soil over time. In grass soil, *Pseudomonas* was constantly the dominating genus at all sampling times (sum of RA in summer = 35.6 %, autumn = 34 %, spring = 31.1 %) whereas in ARD soil, the relative abundance decreased over time (sum of RA in summer = 20 %, autumn = 1.4 %, spring = 2.25 %).

27 ASVs linked to *Pseudomonas* were present in roots from T0 plants. Most of them disappeared over time in roots grown in ARD soil. After one year (spring), only 7 out of 27 ASVs were still present in roots grown in ARD soil. However, in roots grown in grass soil 16 ASVs were still detected, most of them being highly abundant. The most dominant genus in T0 plants, *Flavobacterium* (sum of RA = 23.9 %), strongly decreased over time in all soil variants (except in spring in ARD soil: 7 %). Four ASVs assigned to *Caulobacter*, which represented 13.8% of all ASVs detected in T0, were low abundant or not detected in autumn. However, in spring these ASVs were again all present with a mean relative abundance of 1.41 (± 0.35) %.
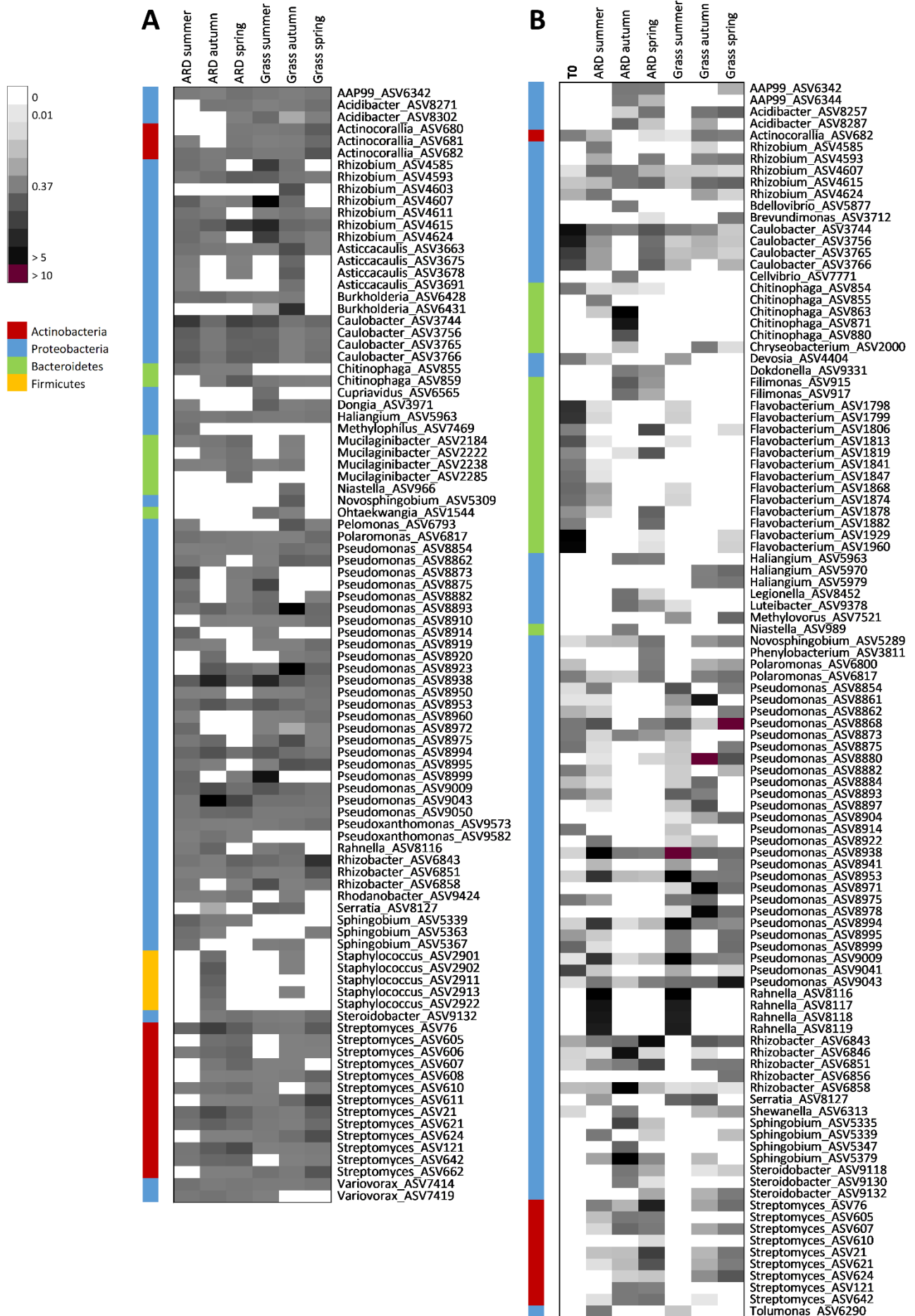
Figure 3: Heatmap showing the abundance of different ASVs (RA > 0.5 %) of roots of Bittenfelder plants from ARD and grass plots taken 3 months (summer), 7 months (autumn) and one year (spring) after planting in Heidgraben (A) in 2016/17 and Ellerhoop (B) in 2017/18. For each sampling time per site and soil, only ASVs with an abundance greater than 0.5 % were selected and their relative abundance compared with all other variants. The color code indicates the range from low relative abundance (light gray, 0.01 %), medium abundance (grey, 0.37 %) to high abundance (black, > 5 %, Purple, > 10 %). Different colors indicate the corresponding phylum of the ASVs.

Culture dependent approach

Next to the sequencing approach, a culture-dependent method was used in order to obtain a wide range of different endophytic bacterial isolates. In total, 150 isolates were obtained from both sites and sampling times (Fig. 4). 29 different genera with 69 different bacterial species were found. Most (25 out of 69) of the strains were classified as *Pseudomonas*. 31 species were only found in roots grown in ARD soil, 19 only in grass soil, and 19 in both soils. Species which were isolated most frequently, were *Rhanella aquatilis* (15), *Pseudomonas fluorescens* (12) and *Serratia plymuthica* (12).

Legend:
- = only ARD soil (yellow/orange)
- = only grass soil (green)
- = both soils (grey)

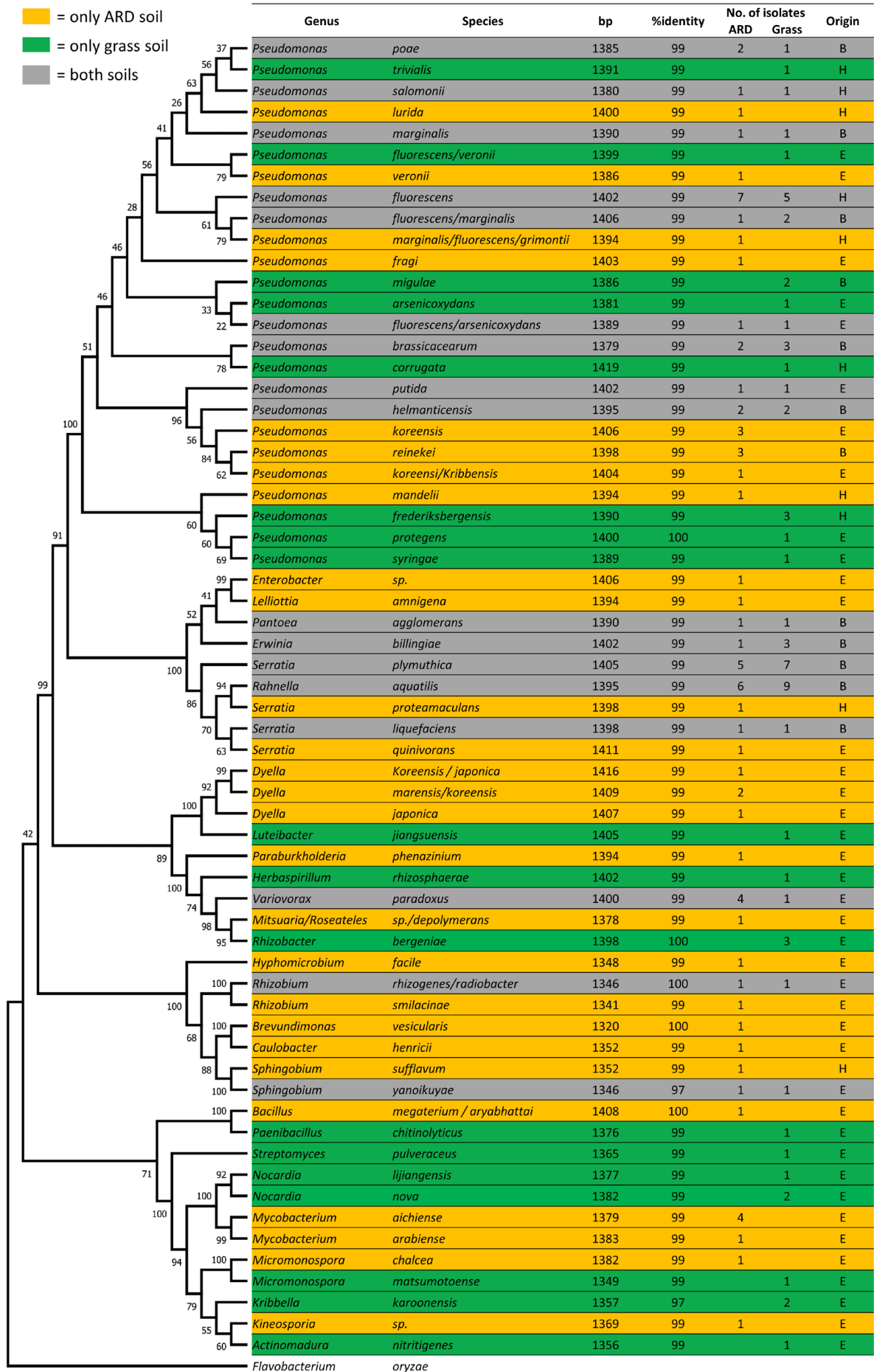| Genus	Species	bp	%identity	No. of isolates ARD	No. of isolates Grass	Origin
Pseudomonas	poae	1385	99	2	1	B
Pseudomonas	trivialis	1391	99		1	H
Pseudomonas	salomonii	1380	99	1	1	H
Pseudomonas	lurida	1400	99	1		H
Pseudomonas	marginalis	1390	99	1	1	B
Pseudomonas	fluorescens/veronii	1399	99		1	E
Pseudomonas	veronii	1386	99	1		E
Pseudomonas	fluorescens	1402	99	7	5	H
Pseudomonas	fluorescens/marginalis	1406	99	1	2	B
Pseudomonas	marginalis/fluorescens/grimontii	1394	99	1		H
Pseudomonas	fragi	1403	99	1		E
Pseudomonas	migulae	1386	99		2	B
Pseudomonas	arsenicoxydans	1381	99		1	E
Pseudomonas	fluorescens/arsenicoxydans	1389	99	1	1	E
Pseudomonas	brassicacearum	1379	99	2	3	B
Pseudomonas	corrugata	1419	99		1	H
Pseudomonas	putida	1402	99	1	1	E
Pseudomonas	helmanticensis	1395	99	2	2	B
Pseudomonas	koreensis	1406	99	3		E
Pseudomonas	reinekei	1398	99	3		B
Pseudomonas	koreensi/Kribbensis	1404	99	1		E
Pseudomonas	mandelii	1394	99	1		H
Pseudomonas	frederiksbergensis	1390	99		3	H
Pseudomonas	protegens	1400	100		1	E
Pseudomonas	syringae	1389	99		1	E
Enterobacter	sp.	1406	99	1		E
Lelliottia	amnigena	1394	99	1		E
Pantoea	agglomerans	1390	99	1	1	B
Erwinia	billingiae	1402	99	1	3	B
Serratia	plymuthica	1405	99	5	7	B
Rahnella	aquatilis	1395	99	6	9	B
Serratia	proteamaculans	1398	99	1		H
Serratia	liquefaciens	1398	99	1	1	B
Serratia	quinivorans	1411	99	1		E
Dyella	Koreensis / japonica	1416	99	1		E
Dyella	marensis/koreensis	1409	99	2		E
Dyella	japonica	1407	99	1		E
Luteibacter	jiangsuensis	1405	99		1	E
Paraburkholderia	phenazinium	1394	99	1		E
Herbaspirillum	rhizosphaerae	1402	99		1	E
Variovorax	paradoxus	1400	99	4	1	E
Mitsuaria/Roseateles	sp./depolymerans	1378	99	1		E
Rhizobacter	bergeniae	1398	100		3	E
Hyphomicrobium	facile	1348	99	1		E
Rhizobium	rhizogenes/radiobacter	1346	100	1	1	E
Rhizobium	smilacinae	1341	99	1		E
Brevundimonas	vesicularis	1320	100	1		E
Caulobacter	henricii	1352	99	1		E
Sphingobium	sufflavum	1352	99	1		H
Sphingobium	yanoikuyae	1346	97	1	1	E
Bacillus	megaterium / aryabhattai	1408	100	1		E
Paenibacillus	chitinolyticus	1376	99		1	E
Streptomyces	pulveraceus	1365	99		1	E
Nocardia	lijiangensis	1377	99		1	E
Nocardia	nova	1382	99		2	E
Mycobacterium	aichiense	1379	99	4		E
Mycobacterium	arabiense	1383	99	1		E
Micromonospora	chalcea	1382	99	1		E
Micromonospora	matsumotoense	1349	99		1	E
Kribbella	karoonensis	1357	97		2	E
Kineosporia	sp.	1369	99	1		E
Actinomadura	nitritigenes	1356	99		1	E
Flavobacterium	oryzae					

Figure 4: Phylogenetic tree based on 16S rRNA gene sequences of all endophytic isolates using the Maximum Likelihood method and Tamura-Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates (Felsenstein, 1985)) are shown next to the branches. The closest hit with species level and corresponding identity using the NCBI database is shown. Only isolates with more than 1300 bp were selected for alignment. *Flavobacterium oryzae* was used as an outgroup to root the tree. Isolates were obtained from roots grown only in ARD plots (yellow), grass plots (green) or both plots (grey) from the sites Heidgraben (H), Ellerhoop (E) or both (B) sites.

To link the isolates obtained from the culture dependent approach to the culture independent approach, their 16S rRNA gene sequences were blasted against the sequences obtained from amplicon sequencing using a local Blastn. Nearly all isolates showed a very high similarity to one or more of the ASVs (Tab. S2). However, only 20 Isolates out of 62 (>1300 bp) showed a 100 % identity to ASVs. The isolate *Kribella karoonensis* showed with 89,3 % (to NA_ASV568 (Nocardioidaceae)) the lowest identity to the amplicon data followed by *Actinomadura nitritigenes* with 93,2 % to NA_ASV677 (Nonomuraea). The isolates which were obtained frequently, e. g. *Pseudomonas fluorescens* (12x) or *Rhanella aquatilis* (15x) were only found in low abundance in the amplicon data.

Discussion

Community structure and relative abundance over time

In most studies, in which rhizosphere or bulk soils of ARD-affected sites were analyzed (Franke-Whittle et al. 2015; Perruzzi et al. 2017; Sun et al. 2014; Tilston et al. 2018; Yim et al. 2015), Proteobacteria were the dominant phylum with a mean relative abundance of 35 % (Nicola et al., 2018). In the greenhouse biotest (Mahnkopp-Dirks et al., 2020), plant roots grown in untreated ARD soil showed with 76 % in 2016 and 71 % in 2017 a cleary higher relative abundance of Proteobacteria as root endophytes. These results were confirmed in the present study in roots grown in the field at Heidgraben and Ellerhoop. At both sites, Proteobacteria showed in roots grown in ARD plots an average relative abundance of 76 % respectively (Fig. 2). However, in roots grown in grass soil the relative abundance was even higher with an average of 84 %. Due to their high metabolic activity and fast growth, members of this phylum are known to usually dominate the endosphere (Lundberg et al. 2012; Reinhold-Hurek et al. 2015).

During the season, the community structure changed over time. This was also observed by Rumberger et al. (2007) for the bacterial rhizosphere community of apple trees grown in ARD-affected sites using terminal restriction fragment length polymorphism (T-RFLP) analyses. The relative abundances of Actinobacteria were increasing over time in roots grown in ARD and grass plots at both sites (Fig. 2). In Heidgraben, 3 months after planting (spring), the relative abundance was even higher in roots grown in grass plots than in ARD plots. Microscopic analysis revealed Actinobacteria to be more often found in roots grown in ARD-affected soil than in unaffected soil (Grunewaldt-Stöcker et al., 2019). Further, typical affected blackish root tissue revealed a high frequency of Actinobacteria on the root surface and cortex in both sites (Grunewaldt-Stöcker et al., 2021). Actinobacteria were also found of higher abundance in the greenhouse biotest in roots grown in untreated ARD soil in comparison to controls in 2016 (Mahnkopp-Dirks et al. 2020). An increase of Actinomycetes in the rhizosphere was also observed by Čatská et al. (1982) with increasing age of apple trees grown in ARD-affected soils. They also observed a decline in "fluorescent Pseudomonads" in apple trees within 30 months after planting in ARD-affected soil but not in control soil. A reduction of *Pseudomonas* in the rhizosphere over years after replanting was also confirmed by Rumberger et al. (2007). This could also be observed for the endophytic root microbiome in the present study, especially in Ellerhoop in roots grown in ARD soil, where the total abundance of *Pseudomonas* ASVs was reduced to one tenth after summer but stayed nearly on the same level in roots grown in grass soil (Fig. 3). This also confirms the connection between the rhizosphere and endophytic community since the main way of entering the root interior is through natural cracks during lateral root emergence and root tips (Bulgarelli et al. 2013; Hardoim et al., 2008). Mazzola and Gu (2000) could show that a suppression of potential ARD causing pathogenic fungi was attributed to a transformation in composition of the fluorescent pseudomonad community in the apple rhizosphere in terms of an increase in proportion of *Pseudomonas putida* in the population and a decrease in recovery of *P. syringae* and *P. fluorescens*.

Streptomyces associated with apple plants in the field?

The majority of Actinobacteria reads were belonging to the genus *Streptomyces* which seems to play a role in ARD. In our greenhouse biotest (Mahnkopp-Dirks et al. 2020) we could show that in 2016 ten unique ASVs and in 2017 six unique ASVs linked to *Streptomyces* from ARD soil from three different sites (including Heidgraben and Ellerhoop) were negatively correlated

to shoot fresh mass and shoot length. Of these unique ASVs, 6 (Streptomyces_ASV76, 607, 611, 21, 121, 621) were now found in Heidgraben and 5 (Streptomyces_ASV76, 607, 21, 621, 121) in Ellerhoop in apple plants grown in the field in ARD soil. One of the most abundant unique *Streptomyces* ASVs in the greenhouse biotest, ASV76, which was present in 2016 in Heidgraben and Ellerhoop and 2017 in Heidgraben, was also the most abundant one in roots grown in ARD soil in the field sites Heidgraben and Ellerhoop. Overall, most ASVs linked to *Streptomyces* increased over time. Especially in roots grown in grass soil in Ellerhoop, where 3 months after planting in summer none of these *Streptomyces* ASV were present, *Streptomyces* represented in spring next year the second most abundant genus. With increasing root biomass over time, the total amount of root exudates is also increasing. It was shown that *Streptomyces* is highly abundant in the rhizosphere of *Arabidopsis thaliana* (Badri et al. 2013; Lebeis et al., 2015) and their root colonization rate is increased (Chewning et al. 2019) when plant exudates were present. Their accumulation could also lead to the assumption of pathogenicity of *Streptomyces*. After planting, their abundance is increasing over time. Even after removing the plant and planting non Rosaceae for several years, *Streptomyces* could remain in high amount in the soil due to their ability to form spores which can persist for years even under harsh conditions (Bobek et al., 2017). This would correlate whith ARD which is known to persist for decades after removing apple plants (Savory, 1966). After replanting apple, these highly abundant spores could germinate, triggered by plant material/exudates and therefore be a causative part of ARD. The question, whether *Streptomyces* is pathogenic and could be a key player in ARD is discussed in detail in Mahnkopp-Dirks et al. (2020). But, accumulating of *Streptomyces* over time in roots grown in grass soil and causing no ARD symptoms speaks against this hypothesis.   However, *Streptomyces* is known to be able to reduce the plant defense response (Lehr et al. 2007; Tarkka et al. 2008; Vurukonda et al. 2018) by reducing the peroxidase activity and pathogenesis-related peroxidase gene (Spi2) expression and to promote fungal root infections (Lehr et al. 2007). This could mean, that they enable easier colonization for potential fungal ARD pathogens, which are missing in grass soil.

Nevertheless, in this study we could show that the same *Streptomyces* ASVs from the biotest (Streptomyces_ASV76, 607, 611, 21, 621), which were negatively correlated to shoot length and shoot fresh mass, were also present in the field of Heidgraben and Ellerhoop during the season in both soils. Further, in comparison to the biotest, Bittenfelder seedlings were used

instead of the genotype M26. To summarize, these ASVs linked to *Streptomyces* were associated with ARD independent of the genotype (Bittenfelder or M26), field or greenhouse, at three different sites and independent of seasons or years.

Comparison of culture dependent and independent approach

Liu et al. (2017) summarized the proportion of different endophytic bacterial phyla in different plants based on 25 different references using culture independent and dependent methods. They found, that root endophytic bacterial communities are typically dominated by Proteobacteria (≈ 50% in relative abundance), Actinobacteria (≈ 10%), Firmicutes (≈ 10%) and Bacteroidetes (≈ 10%). By 16S amplicon sequencing of xylum tissue from different apple genotype shoots Liu et al. (2018) found the same four dominant different phyla, despite slightly different relative abundance (Proteobacteria (58.4%), Firmicutes (23.8%), Actinobacteria (7.7%), Bacteroidetes (2%)). In the present study, the culture independent 16S amplicon sequencing also revealed a root endophytic bacterial community dominated by Proteobacteria (80 %), Bacteroidetes (9,7 %), Actinobacteria (8,2 %) and Firmicutes (1,2 %, Fig. 2, mean of all plots and time points). The 150 Isolates obtained by the culture independent approach were comparably dominated by Proteobacteria (85,3 %), Actinobacteria (10 %) and Firmicutes (2 %). However, despite the so far similar phyla abundances between the culture independent and dependent approach, Bacteroidetes were not isolated.

In the culture independent approach, 4422 ASVs were found in total. These represent different sequences with at least 1 nucleotide difference, hence do not represent species level, which is often considered at a threshold of 97 % sequence identity. Since the sequences of the 150 isolates all have at least 1 nucleotide difference, they would represent 3.4 % of the total amount of ASVs found in the independent approach (sequencing errors cannot be excluded (Taq error rate ranges from 1.1×10^{-4} (Barnes, 1992) to 8.9×10^{-5} (Cariello et al., 1991) errors/bp)). The ASVs were linked to 473 different known genera. In the culture dependent approach, isolates belonging to 29 different genera were obtained, which represent 6.13 %. It is thought, that only 0.1 % - 10 % of the total diversity of an environment is culturable (Handelsman and Smalla, 2003). Other studies indicate that more than 99 % of all microorganisms are unculturable (Pham and Kim, 2012; Schloss and Handelsman, 2005; Vartoukian et al. 2010). Based on these numbers, the proportion of culturable bacteria in this study seems to be high. However, the total amount of 4422 ASVs did not fully represent the

total bacterial endophytic root community. Several biases in amplicon sequencing have an influence on the total bacterial endophytic root community (Reviewed by Pollock et al., 2018). For instance, the universal primer pair used in our study for amplicon sequencing was chosen because of minimal non-target DNA amplification like mitochondrial or chloroplast DNA (Dorn-In et al. 2015). However, despite being "universal", comparing the primer sequences to the 16S rRNA sequence collection of the Ribosomal Database Project (RDP, Cole et al., 2014) using "probe match" results in 1,122,475 hits out of 3,482,181 (32 %) sequences in the domain Bacteria (when using 0 mismatches; 1 mismatch = 1,596,717; 2 mismatches = 1,910,059). Next to the primer used, the DNA extraction protocol has a strong influence on the detected bacterial community composition (Carrigg et al., 2007; Pollock et al., 2018).

Even though the two different culture media used resulted in several different cultured isolates, the number of potentially culturable bacterial endophytes will definitely increase with the use of more different media. To also isolate obligate endophytes, the addition of plant extract to the medium might increase the number of different isolates (Eevers et al., 2015).

The most diverse genus in the culture independent approach was *Pseudomonas,* with 138 ASVs linked to it. Likewise, isolates obtained from the culture dependent approach belonging to the genus *Pseudomonas* were with 25 different species also the most diverse group. However, ASVs linked to the genus *Streptomyces* belonged to the most abundant ones, especially in roots grown in ARD soils, whereas in the culture dependent approach only one isolate could be obtained. One reason for this could be that the growth of *Streptomyces* was rather slow on the media used compared to other isolates which might have outcompeted them. Another reason is that the outgrowth of isolates took place at room temperature. The optimal growth temperature for *Streptomyces* species is described as 28 °C (Sheperd et al., 2010). Tewoldemedhin et al. (2011) were able to isolate 92 *Streptomyces* strains from surface disinfected roots from six ARD-affected sites in South Africa using Casein-Starch medium and water agar supplemented with cycloheximide at 27 °C for 4 weeks.

There were also some discrepancies in the abundance of some isolates compared to their corresponding ASVs (Tab. S2). Several isolates were isolated frequently from roots, like *Rhanella aquatilis* or *Pseudomonas fluorescens*, but their corresponding ASVs were not found in high abundance in the amplicon sequencing. Reasons mentioned above like primer selection or DNA extraction methods could select against these bacteria in the culture

independent approach. Since both of these isolates were found to be fast growing on the media used, it is more likely that the culture dependent approach selected for them. Both genera were also isolated in high abundance from apple roots and rhizosphere soil by Dos Passos et al. (2014). With *Kribella karoonensis*, there was also one isolate, whose genus were not found in the culture independent approach. The reason for this is probably that the primer 769R does not have any coverage in this genus based on 0 mismatches in the SILVA database. Several other isolates, like *Enterobacter*, *Lelliotti*, *Erwinia* or *Rhanella* were not found directly in the independent approach because the corresponding ASV sequences had several hits of different genera with the same score (resulting in NA), which means that the amplicon sequence might not be long enough to discriminate between these genera. Discrepancies between culture dependent and independent approaches were also observed in the phyllosphere of apple, where Actinomycetales were found only among isolates (Yashiro et al., 2011). In general, the culture dependent approach was rather used as a qualitative method rather than a quantitative one.

Conclusion

In this study we could confirm that the same six *Streptomyces* ASVs, which were found to be negatively correlated to shoot growth and fresh mass in a greenhouse biotest in a previous study, were also found in high abundance in roots grown in the field. Not only were they found in two different sites in two years, but in roots of 'Bittenfelder' seedlings also in a different rootstock. Interestingly, most of these ASVs were increasing over time especially in newly planted apple plants in grass soil leading to the assumption that the accumulation of these ASVs could be responsible for the induction of ARD. Next to the culture independent, approach, the isolation of 69 different bacterial strains showed on the one hand a comparable community structure with *Pseudomonas* being the most diverse genus. On the other hand, the discrepancies between these two approaches underline the importance of combining different methods.

Acknowledgement

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3. General discussion

3.1 Advantages and disadvantages of the biotest

To determine the presence and severity of ARD of the soil sampled from three sites, we conducted a biotest (Mahnkopp et al., 2018) originally developed by Yim et al. (2013, 2015). To do so, the ARD susceptible rootstock M26 was propagated in vitro and planted into pots with either untreated or gamma irradiated soil under greenhouse conditions. Based on the growth differences between these two variants, the ARD severity can be determined. So far, this method is the only reliable indicator for ARD besides the expression of candidate genes (Reim et al., 2020; Rohr et al., 2020; Rohr, 2020) and microscopic analyses of root material (Grunewaldt-Stöcker et al., 2019). In this project, we conducted a biotest in 2016, 2017 and 2018, respectively, with soil from the ORDIAmur reference sites Heidgraben, Ellerhoop and Ruthe. In all years, the growth differences showed nearly the same pattern (Fig. 2), which proves the reproducibility and reliability of this method. The differences in the absolute shoot length between the years were the result of growth differences of the starting material and slight variations in the climatic conditions. Biotests including gamma irradiation were also successfully conducted in different other studies including different genotypes and different soils (Yim et al., 2013, 2015; Weiß et al., 2017a; Reim et al., 2019; Rohr et al., 2020).

However, this method also has some drawbacks. Plating results done by ORDIAmur project partners (Balbín-Suárez, unpublished) showed that the gamma irradiation (min. 10 kGy) does not kill all microorganisms. Further, the water content of the soil prior gamma irradiation might affect the sterilization efficacy. Nevertheless, since increased growth can be observed in the gamma variants, the irradiation kills all or most of the ARD causing organisms. It is also known that fungi and Actinomycetes are more sensitive to gamma irradiation than bacteria (McNamara et al., 2003, 2007). Interestingly, irradiation of grass soil also led to increased plant growth. This could be due to three reasons: (i) grass soil also harbors detrimental microorganisms, (ii) gamma irradiated soils are quickly recolonized by plant growth promoting bacteria (Yim et al., 2015), or (iii) cell lesions of microorganisms due to gamma irradiation result in a release of nutrients that are easier accessible to the plant. McLaren (1969) showed that gamma irradiation causes a release of low amounts of ammonium, soluble carbon, organic nitrogen, manganese, phosphorous, etc. from soil microbes. Weiß and Winkelmann (2017) compared the nutrient contents of 7 and 56 days old shoots grown either in untreated

or gamma irradiated soil and found only marginal to no differences. To test a possible nutrient effect on plant growth, we included the winter wheat cultivar Tabasco in the biotest 2017 and found no differences in shoot length or fresh mass between plants grown in untreated ARD soil compared to gamma irradiated ARD soil after 6 weeks (Fig. 3). This not only disproved a possible nutrient effect on the plants, but also demonstrated the specificity of ARD, since no growth depressions or typical root symptoms were observed in wheat. Overall, compared to other disinfectants, like heating of the soil or chemical treatments, gamma irradiation has the least impact on soil properties (Trevors, 1996).

This also raises the question of what are proper control soils in comparison to ARD soil. On the one hand, gamma irradiation kills most of the (ARD causing) microorganisms (as described above) leading to rapid recolonization. Therefore, it represents an artificial situation. On the other hand, grass soil probably also contains detrimental microorganisms since gamma irradiation of grass soil leads to increased plant growth. Moreover, microbial communities as well as the soil mesofauna are influenced by the grass cover (Koehler and Born, 1989; St. Laurent et al., 2008)

Further, biotests are very labor intensive and time consuming. Due to high variation between single plants, high numbers are needed. After propagating the plants in vitro to the needed amount, the rooting and acclimatizing phases usually take four weeks each. After transferring the plants to the soil, the earliest time point to see differences is after 2 to 3 weeks, but usually evaluation takes place after 6 or 8 weeks.

Even with these drawbacks, it could be shown that the growth in the biotest is a very reproducible and reliable indicator for (the severity of) ARD. Moreover, it served as a basis for several other analyses, for example, of the endophytic bacterial and fungal community structure (Mahnkopp-Dirks et al., 2020; Popp, 2020), the identification of nematode-microbe complexes (Kanfra et al., submitted) or for the development of new ARD indicators (Grunewaldt-Stöcker et al., 2019; Grunewaldt-Stöcker et al., 2021; Rohr et al., 2020). A possible alternative for the biotest is described in "3.3.2 *Streptomyces* as indicator for ARD: An alternative for the biotest?". In future, a combination of these different and newly developed indicators might be the key to determine the presence and severity of ARD.
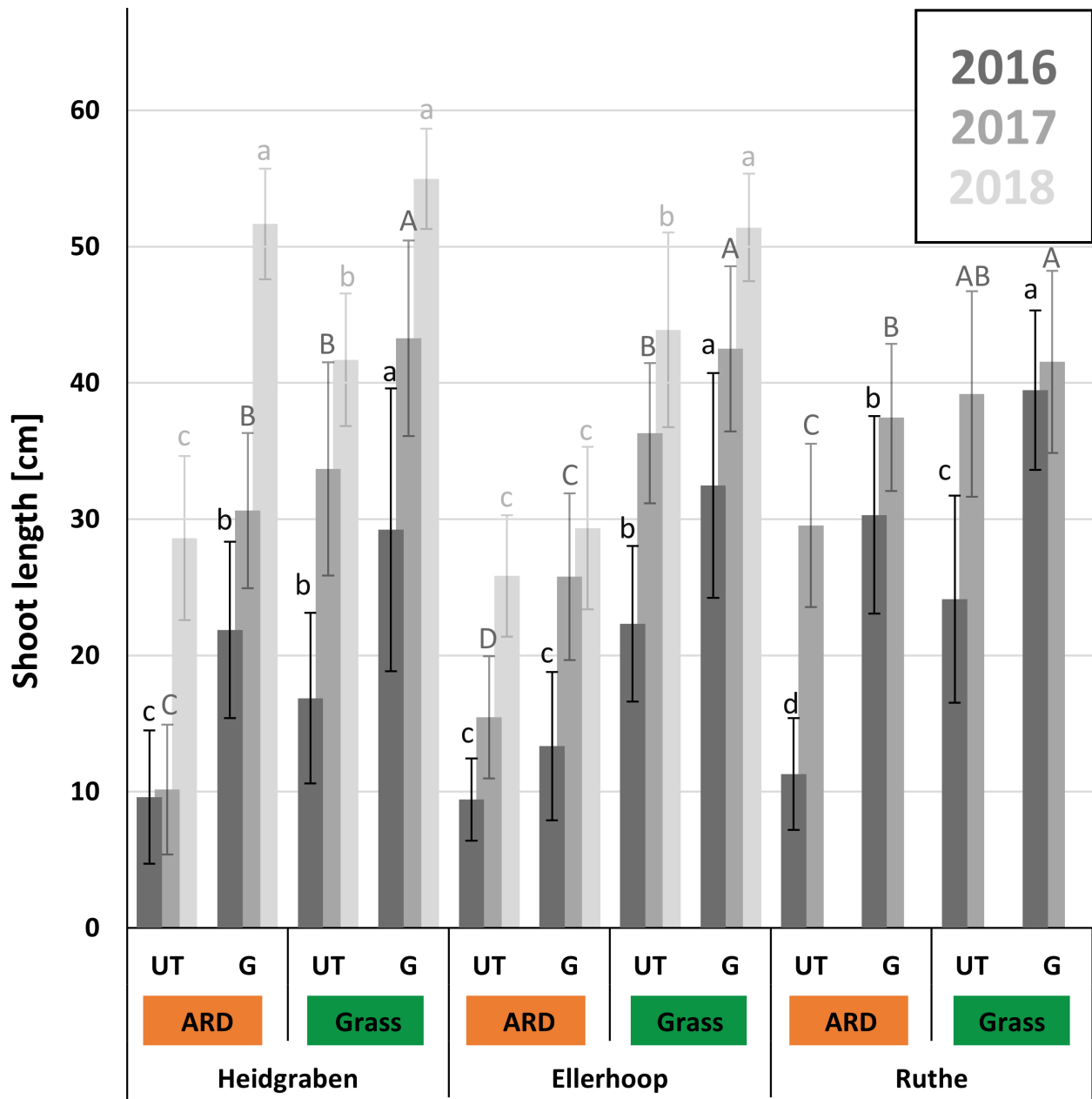
Figure 2: Shoot length of M26 plants after 8 weeks of culture in ARD or grass soil from the three sites Heidgraben, Ellerhoop and Ruthe, which was either untreated (UT) or gamma irradiated (G) in 2016, 2017 and 2018. Same letters (2016 = black lowercase; 2017 = gray uppercase; 2018 = light gray lowercase) indicate no statistically significant difference within one site and year (Tukey test p ≤ 0.05; I = SD, n = 9).
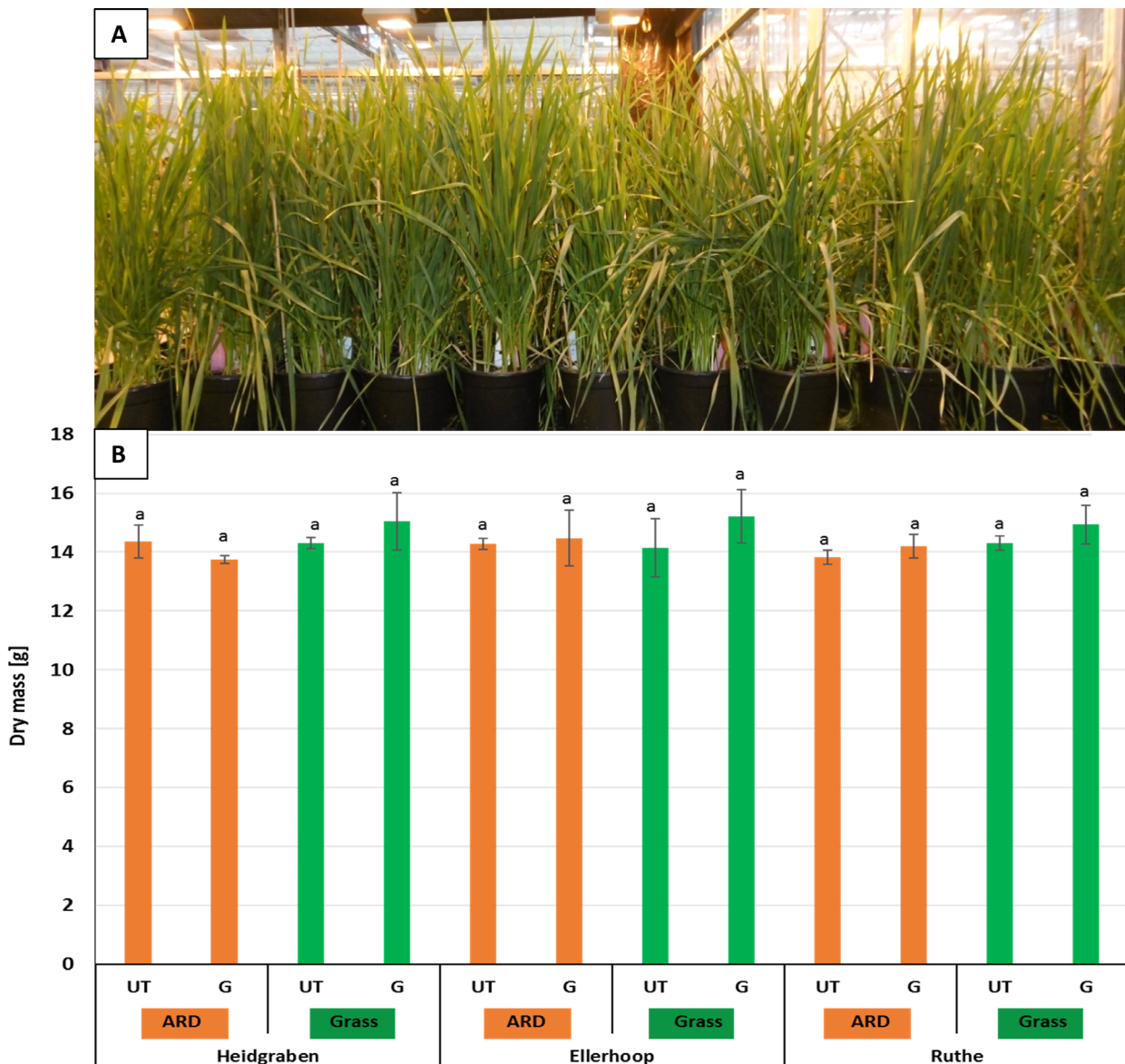
Figure 3: (B) Dry mass of winter wheat (*Triticum aestivum* cv. Tabasco) after 6 weeks of growth in ARD and grass control soil (untreated (UT) or gamma irradiated (G)) from the sites Heidgraben, Ellerhoop, and Ruthe. (A) Representative plants of the different soil variants. Different letters indicate statistically significant differences between all variants (Tukey HSD Test p ≤ 0.05, I=SD, n =3).

3.2 Plant growth promoting bacteria to overcome ARD

An additional aim of this thesis was to find possible growth promoting bacteria, which can be used to overcome ARD. However, plant growth promoting bacteria (PGPB) usually have a low rhizosphere competence (Compant et al., 2005). Moreover, their effects on the plant and their

rhizosphere competence can vary in different soils (Egamberdiyeva, 2007). Further, roots might grow beyond inoculation sites. Therefore, endophytes which can establish within the plant roots, can overcome these drawbacks. The ability to establish in the plant is one major advantage of endophytes in comparison to other rhizosphere bacteria. To overcome ARD, successfully inoculated and established endophytes could be part of an endobiome, which may protect the plant from ARD symptoms. This so called microbiome engineering has the potential to lead to biocontrol and plant growth promoting effects (Orozco-Mosqueda et al., 2018). The question whether endophytes in the starting material can be successfully established in the plant was answered in Mahnkopp-Dirks et al. (2020, submitted). In the biotest in 2016, several abundant ASVs, which were present in T0 plantlets were still present after 8 weeks of growth in ARD-affected soils from three different sites. However, in the biotest in 2017, the corresponding ASVs were not present in T0 plantlets and thus not present after 8 weeks of growth in the three different soils making it unlikely that they originated from the soils. For example, in T0 plantlets in 2016, the most abundant ASV (linked to *Ralstonia*) was still highly abundant in roots after 8 weeks of growth in all three ARD soils whereas in the biotest in 2017 no ASVs linked to the genera *Ralstonia* were present in T0 plants and therefore not present after 8 weeks of growth in the different soils. This phenomenon was also observed in plant roots at the field site in Ellerhoop (Mahnkopp-Dirks et al., submitted). ASVs linked to the genus *Caulobacter* were among the most abundant ones in T0 plant roots and were still present in roots after one year of growth. However, several other abundant genera in T0 plants, like *Flavobacterium*, decreased over time and were not present anymore after one year. This shows the importance of the competence for long-term establishment in roots of possible plant growth promoting endophytes if an inoculation of the start material is the envisaged strategy.

In the greenhouse biotest, the bacterial root endophytic microbiome was analyzed in grass variants in order to find possible PGPB. However, no ASVs positively correlated to shoot length or fresh mass were detected, except for one in Heidgraben in 2017 (NA_ASV4691 (Rhizobiaceae)), which could not be determined at genus level.

Several other ASVs were linked to genera, whose members are known to possess plant growth promoting abilities. For example, *Pseudomonas* was found in the culture independent approach to be the most diverse group representing the highest number of ASVs in both years

of the biotest (Mahnkopp-Dirks et al., 2020) and in plant roots grown in the field (Mahnkopp-Dirks et al., submitted), as well as in the culture independent approach with a multitude different species isolated. Interestingly, in three studies, the abundance of *Pseudomonas* was observed to decline in the rhizosphere of plants grown in ARD-affected soil, whereas no decline was observed in control soil (Čatská et al. 1982, Rumberger et al., 2007; Jiang et al., 2017). The same was also observed in roots grown in the field (Mahnkopp-Dirks et al., submitted). This may indicate that *Pseudomonas* could be involved in plant growth promoting activities (e.g. biocontrol). Members of *Pseudomonas* are known for their fast growth and good rhizosphere competence (Haas and Keel, 2003; Santoyo et al., 2012). Several strains were found to produce iron chelating siderophores (Santoyo et al., 2012; David et al., 2018) which can prevent potential phytopathogens from acquiring (enough) soluble iron, thus inhibiting their growth and proliferation (Kloepper et al., 1980; Loper and Henkels, 1999; David et al., 2018). Disease suppressive soils were associated with this trait of *Pseudomonas*, among others (Kloepper et al., 1980). Further, numerous members, which are also associated with plants, are able to produce antibiotics (Rosales et al., 1995; Raaijmakers et al., 1997; Haas and Keel, 2003; Paulsen et al., 2005).

Pseudomonas fluorescens was among the most frequently obtained isolates in the culture dependent approach (Mahnkopp-Dirks et al., submitted). This model organism, whose complete genome was sequenced in 2005 (Paulsen et al., 2005), was shown to induce systemic resistance in different plant species (Hol et al., 2013; David et al. 2018). For example, inoculation of pea roots with *Pseudomonas fluorescens* in an in vitro system resulted in an induced systemic resistance and inhibited the growth of *Fusarium* and *Pythium* (Benhamou et al., 1996), two organisms, which are often referred to contribute to ARD. Using consortial plant growth promoting fluorescent *Pseudomonas* strains, Sharma et al. (2017) were able to suppress the soil borne deleterious rhizobacterial and fungal population associated with ARD in rhizosphere soil from ARD-affected apple plants. Their fast growth and good rhizosphere colonization abilities are also the reason why strains of this well studied genus are part of several commercial available products (e.g. Cerall®, Proradix®, Rhizotech Plus ®).

Rhizobium is another genus well known for its growth promoting ability. Several unique ASVs were found in plants growing in grass soil in the biotests, which were present in more than one site (Mahnkopp-Dirks et al., 2020). This genus is most famous for its endosymbiotic nitrogen fixing mebers in legumes. But members of the genus *Rhizobium* are also capable of

other different direct and indirect plant growth promoting effects in different plant species, including the production of siderophores (Berraho et al., 1997), IAA and cytokinin (Dey et al., 2004; Noel et al., 1996), phosphate and heavy metal mobilization (Patil et al., 2017), secretion of antimicrobial substances (Joseph et al., 1983), induction of systemic resistance (Osdaghi et al., 2009) and increased abiotic stress tolerance (Patil et al., 2017). Catská and Hudská (1993) inoculated ARD-affected apple seedlings in greenhouse pot experiments and rootstocks in nurseries with *Rhizobium radiobacter* and could observe an enhanced growth. This effect was attributed to a shift in the rhizosphere community and reduction of phytotoxic micromycetes. However, no positive correlation to plant growth was observed in our biotests (Mahnkopp et al., 2020). Instead, two ASVs linked to *Rhizobium* even showed a negative correlation to shoot fresh mass. A negative correlation of *Rhizobium* to plant growth was also observed by Franke-Whittle et al. (2015), investigating rhizosphere soil samples of apple rootstocks grown in replant affected soils. However, in the culture dependent approach, only two different isolates were found, which could be due to suboptimal temperatures used instead of a higher optimal growth temperature of 25 – 30°C of most *Rhizobium* strains (Somasegaran and Hoben, 1994).

Another species often described in literature as plant growth promoting and frequently isolated in the culture dependent approach is *Serratia plymuthica*. Numerous studies and the complete genome sequences of three *Serratia plymuthica* strains (Cleto et al. 2014, Adam et al., 2016) revealed several growth promoting traits like production of siderophores (De Vleesschauwer and Höfte, 2003; Adam et al., 2016), IAA (Aisyah et al., 2019), induction of plant resistance (De Vleesschauwer and Höfte, 2003) and a broad-spectrum biocontrol activity against many phytopathogens. The latter includes the production of antibiotics, chitinases and proteases (Berg, 2000; De Vleesschauwer and Höfte, 2003; Adam et al., 2016). The biocontrol potential of different *Serratia plymuthica* strains was successfully used against commonly stated ARD causing organisms like *Pythium*, *Rhizoctonia*, *Phytophtora* and *Fusarium* (De Vleesschauwer and Höfte, 2003; Aisyah et al., 2019). However, first inoculation trials of the obtained isolate showed no positive effect on plant growth in ARD soil (Horn, 2020).

These are some examples of potential promising plant growth promoting bacteria identified in the present study. With *Bacillus*, *Burkholderia*, *Enterobacter* or *Sphingobium*, several other genera consisting of members that are commonly described as plant growth promoting were found in the culture independent and dependent approach, but are not discussed in detail

here. However, it has to be considered that not only different species within a single genus but also different strains within a species can show differences in their capabilities as plant growth promoting bacteria. Further, these growth enhancing abilities are often host specific, even on cultivar level (Hallmann et al., 1997; Akbari et al., 2020). Moreover, genera commonly known for their plant growth promoting abilities may also include pathogenic species.

The question arises, whether an inoculum consisting of a single plant growth promoting strain or even a mixture of some strains is capable of reducing ARD symptoms directly. A potential inoculum should also aim to shift the community structure and increase the microbial diversity of an ARD-affected plant, since results showed that the microbial diversity, richness, and evenness is reduced in ARD-affected rhizosphere soil compared to non-affected soil (Balbín-Suárez et al., 2021). Jiang et al. (2017) suggested a competition between beneficial and pathogenic microorganisms in the rhizosphere, whose ratio may vary under replant conditions. Therefore, a shift in the ratio towards beneficial microorganisms is desirable.

It has to be considered, that not only the plant growth promoting bacteria is important for a successful inoculum, but also the carrier material used. The carrier material (made of soil materials, organic materials, or inert materials) provides a suitable microenvironment for the bacteria and supports their survival and improves their performance (Malusá et al., 2012; Abd El-Fattah et al., 2013; Sohaib et al., 2020). Different carrier materials in combination with selected strains have to be tested in inoculation trials in order to find the most suitable material.

3.3 The importance of *Streptomyces*

In this work, *Streptomyces* was found to be one of the most important bacterial root endophytes associated with ARD. The following chapter describes some important general aspects of this genus, its lifestyle and way of plant colonization.

The genus *Streptomyces* belongs to the phylum Actinobacteria. Up to now 672 *Streptomyces* species are known (Euzéby 1997; Parte 2018, LPSN accessed 01.12.2020, validly published species names). This genus is well studied and is well known for its production of a wide range of bioactive secondary metabolites like antiviral, antifungal, antitumoral, anti-hypertensive, and immunosuppressive substances, but are most famous for their production of antibiotics (Ōmura et al., 2001; Patzer and Volkmar, 2010; Khan, 2011; De Lima Procópio et al., 2012). 80 % of today's antibiotics are derived from *Streptomyces* (De Lima Procópio et al., 2012).

Members of this genus are ubiquitous in soils (Seipke et al., 2012; Watkins et al., 2016; Ferrer et al., 2018; Olanrewaju and Babalola 2019;). This production of a multitude of antimicrobial compounds is one reason for their high competitiveness. Another reason is their saprophytical lifestyle, since they are able to degrade and metabolize various organic materials and substances using several hydrolytic exoenzymes like cellulases, lignocellulases, pectinases, chitinases, xylanases and cutinases (Chater et al., 2010; Chater, 2016). For example, the genome of the model organism *S. coelicolor* is with 7,825 predicted genes one of the largest of all sequenced bacteria (Bentley et al., 2002). It encodes a large number of secreted proteins (819), including 60 proteases, 13 chitinases/chitosanases, eight cellulases/endoglucanases, three amylases, and two pectate lyases (Bentley et al., 2002). Because of these degrading capabilities, *Streptomyces* has an ecological key role in turnover of organic material in soil ecosystems (Seipke et al., 2012; Chater, 2016). Another characteristic feature of *Streptomyces*, which is unusual for bacteria, is the filamentous growth. Members of these genus grow as branching aerial hyphal filaments to form fungi-like mycelium structures that differentiate into chains of single-celled spores (Seipke et al., 2012; Chater, 2016; Bobek et al., 2017). The ability to form spores, which can survive harsh conditions for several years, (Bobek et al., 2017) gives *Streptomyces* a competitive advantage over other microorganisms in soil ecosystems (Seipke et al., 2012; Vurukonda et al., 2018; Olanrewaju and Babalola, 2019).

Streptomyces are generally known as good rhizosphere colonizers and constitute a high amount of rhizosphere microbiota (Sousa and Olivares, 2016; Vurukonda et al., 2018). This genus was also found endophytic in several plant species. Vurukonda et al. (2018) reviewed the growth promoting abilities of *Streptomyces* as endophytes and found that they promoted the growth of 18 different plant species in several studies. In the plant, they mostly colonize the root system and the xylem (Vurukonda et al., 2018). However, little is known about the colonization process of *Streptomyces* of the rhizosphere and the plant endosphere (Viaene et al., 2016). Since members of this genus are able to metabolize plant exudates and were shown to appear in higher abundances in the rhizosphere of *Arabidopsis thaliana* when the roots exuded phenolic-related compounds like salicylic acid (Badri et al. 2013; Lebeis et al., 2015), it seems likely that they, similar to many other rhizobacteria, are attracted to plant exudates in the rhizosphere. Movement towards plant exudates in the rhizosphere is achieved by chemotaxis (Olanrewaju and Babalola 2019). As described in chapter "1.5.1 Definition and

ways of plant colonization" one of the most likely ways of bacteria entering the roots from the rhizosphere would be through cracks formed during lateral root emergence and at root tips (Hardoim et al., 2008; Bulgarelli et al. 2013). An alternative way could include a mild hydrolysis of cell walls and middle lamellae (Viaene et al., 2016). It is believed that the hyphae-like growth of *Streptomyces* sp. offers an easier entry and colonization of plant roots (Seipke et al., 2012, Viaene et al., 2016). GFP tagging of *Streptomyces* for studying the colonization was used for wheat seeds (Coombs and Franco, 2003) and lettuce (Bonaldi et al., 2015), but the transformation of *Streptomyces* is challenging (Viaene et al., 2016). Recently, high-resolution imaging with transmission electron microscopy demonstrated that *Streptomyces* strain coa1 is able to colonize *Arabidopsis* roots inter- and, surprisingly, also intracellularly (Van der Meij et al., 2018). However, further research is necessary to unravel the way of plant colonization.

3.3.1 *Streptomyces* as causal agent of the ARD complex?

Streptomyces was observed in increased abundance in roots grown in three ARD-affected soils in greenhouse biotests in two years and was shown to be negatively correlated to shoot length (increase) and shoot fresh mass (Mahnkopp-Dirks et al., 2020). Further, members of this genus were also observed in a different rootstock (Bittenfelder seedlings) grown in the field in two different ARD-affected sites, where their abundance was increasing over time (Mahnkopp-Dirks et al., submitted). Moreover, histological observations revealed a higher amount of Actinobacteria in ARD-affected roots than in non-affected roots (Grunewaldt-Stöcker et al. 2019, 2021). Since *Streptomyces* is capable of a saprophytic lifestyle and some members even of a pathogenic lifestyle, the question arises whether members of these genus are a causative part of ARD or just opportunists. This question was addressed thoroughly in Mahnkopp-Dirks et al. (2020).

As described above, *Streptomyces* is known for its saprophytical lifestyle and its ability to degrade plant material by a wide range of enzymes. Plants growing in ARD soil have severly damaged root systems, which show necrotic and blackish roots (Grunewaldt-Stöcker et al. 2019). This results in high amounts of freely available plant metabolites next to root exudates, which can be metabolized. Therefore, *Streptomyces* finds favorable growing conditions in this ARD situation.

On the other side, *Streptomyces* features several characteristics, which support a causative part of ARD, which are described in Mahnkopp-Dirks et al. (2020). In brief, pathogenic *Streptomyces* are able to **colonize a wide range of hosts** that likely includes all higher plants, since dicot and monocot seedlings of several plant species have shown symptoms after inoculation with *S. scabies* for instance (Leiner et al., 1996; Loria et al. 2006). The **symptoms** caused by the well-known pathogenic species *S. scabies, S. acidiscabies* and *S. turgidiscabies* are, next to necrotic scab lesions, typical ARD symptoms: Overall reduced growth, reduction of the complexity of the root system and root stunting and browning (Loria et al. 2003; Loria et al. 2006; Seipke et al. 2012). Further, virulence genes are clustered on a **pathogenic island**, which can be mobilized and transferred to nonpathogenic species via conjugation (Lerat et al., 2009). In in vitro assays, apple and Norway spruce (*Picea abies*) showed an increase in root infections of pathogenic fungi in the presence of some *Streptomyces* strains (Lehr et al., 2007; Zhao et al., 2009). This is attributed to the ability to **reduce the plant defence response** by downregulating the peroxidase activity and pathogenesis-related peroxidase gene (Spi2) expression of the host plant, thus promoting pathogenic fungal root colonization (Lehr et al., 2007). Another way of influencing the host defense response was demonstrated with the ability of *S. scabies* of producing coronafacoyl phytotoxins (Li et al., 2019). These have been shown to activate the jasmonic acid signaling pathway, hence inhibiting the antagonistic salicylic acid pathway, which is responsible for the defense reaction against biotrophic and hemibiotrophic pathogens (Glazebrook, 2005; Li et al., 2019). This inhibition of the salicylic pathway together with the downregulation of the peroxidase activity is presumably disturbing the hypersensitive reaction of plant cells and will subsequently results in easier fungal (pathogen) root colonization. Some coronafacoyl phytotoxins can also suppress the callose deposition (Geng et al., 2012; Li et al., 2019).

Several other traits of *Streptomyces* seem to match the characteristics of apple replant disease: *Streptomyces* appears in **higher abundance in sandy, well-drained soil** (Gowdar et al., 2018) which is similar to ARD, which is more severe in sandy soils than in heavy soils (Mahnkopp et al., 2018; Winkelmann et al., 2019). One reason for this is their **sensitivity to waterlogging conditions**, since Streptomycetes are aerobic bacteria. ARD is often considered to be less severe, when the soil is flooded, leading to anaerobic conditions. These anaerobic conditions were induced by Hewitharana et al. (2014) and Mazzola et al. (2020) in combination

with adding different carbon amendments to the soil, which resulted in a reduced ARD effect. Another aspect, which is similar to ARD, is its **persistence**. ARD is known to persist for decades (Savory, 1966). Due to their low metabolism, *Streptomyces* spores can as well survive for a long period (Bobek et al., 2017; Sawers et al., 2019). Although not experimentally proven, it is believed that *Streptomyces* spores, similar to other spore-forming bacteria, can survive for tens to thousands of years due to their non-growth and low respiration rate (Cano and Borucki, 1995; Sawers et al., 2019). Moreover, in several studies, biofumigation, the incorporation of **Brassicaceae plant materials** into soil leading to volatile glucosinolate breakdown products such as isothiocyanate formation, was shown to be a promising countermeasure against ARD (reviewed by Hanschen and Winkelmann, 2020). Recently, it was shown in a chamber bioassay that volatile emissions from macerated *Brassica* tissue were inhibiting the sporulation of *Streptomyces* isolates, but not hyphal growth, suggesting a significant influence on the *Streptomyces* abundance in the soil community (Gouws-Meyer et al., 2020).

To summarize, members of the genus *Streptomyces* share many traits which are characteristic for ARD and possess several pathogenic capabilities that, overall, make it likely that they play a role in the ARD etiology. However, *Streptomyces* sp. are in general considered as a plant growth promoting bacteria due to their biocontrol activities. Also in different ARD related studies, *Streptomyces* was positively correlated to shoot growth (Nicola et al., 2017a) and associated with disease suppression (Cohen et al., 2005; Cohen and Mazzola 2006; Mazzola et al., 2007). So far, in relation to ARD, members of the genus *Streptomyces* were in no studies considered to be a causative part of ARD. Only Actinomycetes (Otto and Winkler 1977; Otto et al., 1993) and Actinobacteria (Westcott et al., 1986; 1987; Grunewaldt-Stöcker et al., 2019, 2021), to which *Streptomyces* belongs, were histologically more frequently observed in roots grown in ARD-affected soil compared to non-affected soil suggesting a potential role in ARD. Tewoldemedhin et al. (2011a) followed the hypothesis of Actinobacteria, especially *Streptomyces*, being involved in ARD by inoculating 37 *Streptomyces* isolates directly to 4 weeks old apple seedling, but found no effect. The reason for this might be that *Streptomyces* is not directly causing ARD symptoms, but instead reducing the plant defense response, which subsequently leads to easier root colonization of pathogenic fungi. This plant defense reduction might be a negative side effect of *Streptomyces*, which is generally known as a

mycorrhiza helper bacterium able to promote mycorrhizal formation by promoting fungal growth and by decreasing plant defense response (Lehr et al. 2007; Tarkka et al. 2008; Vurukonda et al. 2018). Promotion of root infections was also observed by Zhao et al. (2009) in apple. Root infections of *Rhizoctonia solani* were significantly elevated in the presence of *Streptomyces vinaceaus*. Moreover, co-inoculation with *Streptomyces herbaricolor* resulted not only in significantly elevated root infections, but also in novel leaf symptoms, which were not observed in apple plants grown in soil with *Rhizoctonia solani* alone. Similar promoting effects were also observed by Lehr et al. (2007) when *Streptomyces* sp. AcH 505 was co-inoculated with the fungal pathogen *Heterobasidion abietinum* in *Picea abies*, which resulted in increased root infections. These two examples underline the importance of co-occurrence of *Streptomyces* together with potential fungal pathogens to potentially induce ARD symptoms. Recently, new insights of *Streptomyces* behavior were discovered, which support these hypotheses. Unlike many other bacteria, all *Streptomyces* cell types are characterized as non-motile (Jones et al., 2017). However, Jones et al. (2017) discovered that in the presence of fungi (*Saccharomyces cerevisiae*) *Streptomyces venezuelae* forms rapidly migrating so-called explorer cells, which are able to quickly transverse biotic and abiotic surfaces. Moreover, these cells are able to produce the volatile pheromone TMA (trimethylamine), which can induce exploratory growth in physically separated different *Streptomyces* species (Jones et al., 2017). These inter-kingdom interactions underline even further that the interplay between *Streptomyces* and fungi could be crucial for the induction of ARD. In the presence of certain fungi (even non-pathogenic), the distribution in soil along with root colonization of *Streptomyces* would be increased, leading to reduced plant defense, which promotes pathogenic fungal colonization. With several interacting organisms, this hypothetical example reflects the complexity of ARD, which is believed to be a disease complex (Winkelmann et al., 2019). *Streptomyces* in a key role as a "door opener" could also explain, why several studies report different pathogens be responsible for ARD. The fungi *Rhizoctonia* (Mazzola 1999; Mazzola and Manici, 2012; Manici et al., 2013), *Dactylonectria, Ilyonectria* (Manici et al., 2018; Popp et al., 2019, 2020), *Fusarium* (Tewoldemedhin et al., 2011b; Manici et al., 2017) and *Cylindrocarpon* (Mazzola 1999; Tewoldemedhin et al., 2011a; Franke-Whittle et al., 2015) as well as the oomycetes *Pythium* (Mazzola 1999; Tewoldemedhin et al., 2011a, 2011b; Tilston et al., 2018) and *Phytophthora* (Mazzola 1999; Tewoldemedhin et al., 2011a) are frequently reported. In the presence of plant defense reducing *Streptomyces*, these different pathogens

(which might be attracted by root exudates such as phloridzin) could be responsible for ARD symptoms depending of their presence in the soil.

The interplay of *Streptomyces* with different fungi could also explain the fact that the abundance of *Streptomyces* increases over time not only in roots grown in ARD-affected soil, but also in grass soil in the field where no ARD effects were observed (Mahnkopp-Dirks et al., submitted). The pathogens needed to co-occur with *Streptomyces* are not yet present in these non-ARD soils (in sufficient abundance), therefore causing no symptoms. The plant might have enough time to root and to establish before both *Streptomyces* and needed (fungal) pathogens reach a critical abundance, which cause symptoms. If replanted, the *Streptomyces*-pathogen-complex would already be present in high abundance and subsequently lead to replant symptoms.

3.3.2 *Streptomyces* as indicator for ARD: An alternative for the biotest?

Next to the expression of candidate genes (Reim et al., 2020; Rohr et al., 2020; Rohr, 2020) and histological analyses of root material (Grunewaldt-Stöcker et al., 2019), one of the most reliable and feasible way to determine the severity of ARD in soil is via biotest (see chapter "3.1 Advantages and disadvantages of the biotest"). However, all these methods are time consuming and laborious.

Since members of the genus *Streptomyces* were shown to be associated with ARD (Mahnkopp-Dirks et al., 2020) we designed qPCR primers to quantify the amount of *Streptomyces* in roots. Based on the sequence data of the first amplicon sequencing of samples from 2016 (Mahnkopp-Dirks et al., 2020) and the sequences of the Sanger sequencing of isolates (Mahnkopp-Dirks et al., submitted), a consensus sequence was generated from a total of 37 sequences. Specific qPCR primers were designed using Primer3 (http://primer3.ut.ee/). Their specificity was confirmed using the Probe Match of Ribosomal Database Project software 11.5 (Cole et al. 2014), SILVA TestPrime 1.0 (Klindworth et al., 2012) and NCBI. Finally, a total of 2 primer pairs could be designed (Tab. 1). Testing of these primer pairs as well as quantification of selected samples via qPCR should be approached in future studies.

Table 1: Primerpairs for the quantification of *Streptomyces*

Name	Nucleotide sequence (5´ - 3´)
Strepto_426_F	CTAGAGTGTGGTAGGGGAGATC
Strepto_610_R	ACACCTAGTTCCCACCGTTTAC
Strepto_508_F	GGATCTCTGGGCCATTACTGA
Strepto_610_R	ACACCTAGTTCCCACCGTTTAC

However, as shown in Mahnkopp et al. (2020) not all members (ASVs) of *Streptomyces* were negatively correlated to shoot length and fresh mass. In 2016, 15 out of 32 ASVs were negatively correlated and in 2017 only 6 out of 61 ASVs. Also in 2017, the abundance of total *Streptomyces* showed not the typical pattern of higher abundance in ARD-affected soil compared to non-affected soils. Nevertheless, all experiments showed several ASVs associated to ARD, independent of the cultivation system, site, season, or year. But to design qPCR primer, which are specific to these ASVs, is nearly impossible, since the obtained short sequences are almost identical to several other *Streptomyces* ASVs. To address this problem, non-16S-regions could be targeted which may be linked to pathogenicity or plant defense reduction (e.g. pathogenic islands).

Since soil is the main reservoir of microorganisms for the plant microbiome (Berg and Smalla 2009; Bonito et al. 2014; Lareen et al. 2016; Hartman and Tringe 2019) it is likely that *Streptomyces* shows a higher abundance not only in the roots, but also in the rhizosphere soil. Based on DGGE analysis in a split-root-approach, Lucas et al. (2018) confirmed this by finding mainly *Streptomyces* with an increased abundance in rhizosphere samples of the ARD variants. In the same split-root-approach, Balbín-Suárez et al. (2020) found several abundant *Streptomyces* OTUs in the rhizosphere and rhizoplane in ARD-affected soil. This means that roots and rhizosphere soil can serve as potential samples for a qPCR based quantification.

As hypothesized in "3.3.1 Streptomyces part of ARD?" (fungal) co-pathogens are presumably needed to induce ARD and cause symptoms. Therefore, it is advisable to identify the co-pathogens and also establish a qPCR based quantification method for them in order to quantify them together with *Streptomyces*.

4. Conclusions

In this thesis, the biotest with soil from different sites conducted in three consecutive years showed the successful induction of ARD and its severity, proofed the reproducibility of this method, and laid the basis for further analysis. For the first time, the community composition of bacterial root endophytes in apple plants growing in ARD-affected soils in comparison to non-affected soils was shown. Surprisingly, the core microbiome of apple roots was very small. Moreover, several ASVs linked to the genus *Streptomyces* were associated with ARD and were negatively correlated to shoot length and shoot fresh mass. The same ASVs were not only found in roots grown in soil from three different sites in two independent greenhouse experiments, but also in roots of a different genotype grown in two different field sites during the growth period of one year. This underlines the importance of members of the genus *Streptomyces* in the context of ARD, which might play a crucial key role in the etiology of ARD. Whether they are part of the disease complex or just opportunistic needs to be clarified. The quantification of this genus or better of selected strains could serve as a potential feasible indicator for ARD.

Although, no bacteria that were positively correlated to plant growth were found in this study, 150 isolates were obtained in the culture dependent approach, which can be further characterized and might serve as a source for future inoculants to help to overcome ARD.

5. Outlook

One of the major remaining questions is the role of *Streptomyces* in ARD. Are members of this genus part of the disease complex or are they simply opportunists? To answer this important question inoculation experiments with *Streptomyces* isolates are necessary. But up to now, only one isolate from surface disinfected root tissue is available. This work showed that only a fraction of ASVs of this genus was negatively correlated to shoot growth. Therefore, more different *Streptomyces* isolates are heavily needed. Next isolation approaches should have more favorable conditions for this genus. For example, the medium used should be optimized for best growth like SC-medium (Amoroso et al., 1998) or GYM-medium (DSMZ) and the optimal incubation temperature for most strains is reported to be 28°C (Kämpfer et al., 2014). Since *Streptomyces* is easily overgrown by faster growing bacteria, dilution approaches should be used. The obtained *Streptomyces* isolates could then be used in inoculation approaches. However, since it is hypothesized that deleterious *Streptomyces* strains decrease the plant defense response, ARD symptoms should occur only in soil, where other (fungal) pathogens are present. Therefore, prior *Streptomyces* inoculation, soils should be inoculated with different potential (fungal) pathogens.

Additionally, it would be interesting to test these isolates in interaction test with other endophytes (e.g. cross streak plate assays), since the genus *Streptomyces* is known for their vast production of secondary metabolites and antimicrobial substances. Further, analyses of volatile organic compounds (VOC) of these isolates would be of great interest, since many strains are known for their VOC production, which can influence their surrounding environment (Jones et al., 2017, 2019; Armin et al., 2021).

Because members of the genus *Streptomyces* seem to be at least associated with ARD, the development of a qPCR based quantification method was already started in order to develop an ARD indicator. The already designed primer pairs need further testing, especially for their efficiency and specificity. If genus specificity of the primer pairs is confirmed, several different ARD-affected and non-affected roots identified by microscopic indicators from different sites should be tested in order to confirm the *Streptomyces*-ARD association.

Further tasks to unravel the role of endophytes in ARD could also include their localization. The question, where certain endophytes are located within the roots or within the cells could be unraveled using histological techniques in combination with FISH (fluorescence in situ hybridization).

Another approach to further disentangle the role of endophytes in ARD could be to distinguish the endophytic community between healthy and diseased root parts of single plants using amplicon sequencing and/or plating techniques. First experiments were already done. However, the blackish diseased root parts got bleached during surface disinfection, making it impossible to distinguish between healthy and diseased root parts. Cutting roots prior surface disinfection resulted in killing of most endophytes because sodium hypochlorite entered most of the root tissue since the cutting segments of diseased root parts were very small and already damaged. Therefore, another approach using a gentler surface disinfectant like NaDCC seems promising. An alternative, but without surface disinfection, was conducted by Popp et al. (2020), who used Harris Uni-core punching in order to sample ARD-affected roots.

Moreover, comparing the endophytic community of good and poor growing apple plants in ARD plots could further unravel plant growth promoting endophytes. This approach was already started. 9 out of 12 isolates obtained from good growing ARD plants belonged to the genus *Pseudomonas*. However, only a small number of plants were tested, which should be increased in future. 16S amplicon sequencing could further reveal detailed endophytic community structure differences in good and poor growing ARD plants.

Finally, results indicate that ARD is soil specific. Even though roots grown in ARD-affected soils from three different sites were analyzed and showed members of *Streptomyces* as associated endophytes, more different ARD-affected soils from different sites are needed to be analyzed to confirm this *Streptomyces* association. The same is true for different genotypes. In this study, two different genotypes were analyzed. Currently in phase II of ORDIAmur, several other genotypes grown in ARD-affected and non-affected soils are analyzed for their endophytic bacterial root community. First results confirm a high abundance of *Streptomyces* in roots of these genotypes grown in ARD soils (N. Orth, unpublished). Recently, endophytic community structure differences were shown to be strongest between genotypes reported to be tolerant or susceptible to ARD (Van Horn et al., 2021). *Streptomyces* was stated as one of the most dominant endophytic group, but its abundance between the different genotypes was not reported further.

The apple replant disease and especially the role of the endophytic community still hold many secrets waiting to be unraveled.

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7. **Curriculum Vitae**

Name:	Felix Mahnkopp-Dirks
Date and place of birth	12.11.1988, Langenhagen, Germany

Academic education   Gottfried Wilhelm Leibniz Universität Hannover

10.2015 - today   **Ph.D.**
Section of Woody Plant and Propagation Physiology (Prof. Dr. Traud Winkelmann)

10.2013 - 09.2015   **M.Sc., Plant Biotechnology**

Master thesis, Section of Molecular Plant Breeding
(Prof. Dr. Thomas Debener):

Analysis of effector genes of *Diplocarpon rosae*

10.2010 - 10.2013   **B.Sc., Plant Biotechnology**

Bachelor thesis, Section of Woody Plant and Propagation Physiology (Prof. Dr. Traud Winkelmann):

In-vitro-propagation of *Dionaea muscipula* und *Drosera capensis*

Work experience

01.2019 - today   Scientific coordinator of the ORDIAmur project, Gottfried Wilhelm Leibniz Universität Hannover, Section of Woody Plant and Propagation Physiology

8. List of publications

Publications

Peer review:

Bartsch, M., Mahnkopp, F. And T. Winkelmann (2014): In vitro propagation of *Dionaea muscipula* ELLIS., Prop. of Ornam. Plants 14: 117-124

Pham, N.T., Meier-Dinkel, A., Höltken, A.M., Quambusch, M., Mahnkopp, F., & Winkelmann, T. (2017). Endophytic bacterial communities in in vitro shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans × intermedia*). Plant Cell, Tissue and Organ Culture (PCTOC), 130, 153-165.

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Grunewaldt-Stöcker, G., Mahnkopp, F., Popp, C., Maiss E. and Winkelmann, T. 2019. Diagnosis of apple replant disease (ARD): Microscopic evidence of early symptoms in fine roots of different apple rootstock genotypes. Scientia Horticulturae 243 (2019) 583–594. https://doi.org/10.1016/j.scienta.2018.09.014

Mahnkopp-Dirks, F., Radl, V, Kublik, S., Gschwendtner, S., Schloter, M. and Winkelmann, T. (2020): Molecular barcoding reveals the genus *Streptomyces* as associated root endophytes of apple (Malus domestica) plants grown in soils affected by apple replant disease. Phytobiomes Journal (in Press). https://doi.org/10.1094/PBIOMES-07-20-0053-R

Armin, R., Zühlke, S., Mahnkopp-Dirks, F., Winkelmann, T. and S. Kusari (2021): Evaluation of Apple Root-associated Endophytic *Streptomyces pulveraceus* Strain ES16 by an OSMAC-Assisted Metabolomics Approach, Front. Sust. Food Syst. (in Press).

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Mahnkopp, F., Winkelmann, T. und Bartsch, M. (2014): In-vitro-Vermehrung von *Dionaea muscipula* und *Drosera capensis*. hortigate.

Orth, N., Mahnkopp-Dirks, F., Hardeweg, B., Grunewaldt, J., Schimmel, J., Krüger, J. Und T. Winkelmann (2020): Der Apfelnachbaukrankheit auf der Spur – mit Biotest., Deutsche Baumschule 06/2020: 36-39

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Kanfra, X., Wrede, A., Mahnkopp-Dirks, F., Winkelmann, T. And H. Heuer (under revision): Networks of free-living nematodes and their associated microbiome involved in apple replant disease. Front. Plant Sci.

Armin, R., Zühlke, S., Grunewaldt-Stöcker, G., Mahnkopp-Dirks, F. and S. Kusari (submitted): Production of siderophores by an apple root-associated *Streptomyces ciscaucasicus* strain GS2 using chemical and biological OSMAC approaches. Chemoecology

Publications in preparation

Mahnkopp-Dirks, F., Radl, V., Kublik, S., Gschwendtner, S., Schloter, M. and Winkelmann, T. (2021): Time course of the bacterial root endophytic microbiome of Malus domestica plants in field soils affected by apple replant disease. (Manuscript)

Poster presentations

Felix Mahnkopp (2014): Entwicklung eines In-vitro-Vermehrungsverfahrens für Venusfliegenfalle (*Dionaea muscipula*). DGG 53. Gartenbauwissenschaftliche Tagung, "Nachhaltigkeit und Gartenbau". 05. - 08.03.2014, Dresden

Mahnkopp, F., Balbin-Suarez, A., Rauch, L., Winkelmann, T. and Smalla, K. (2016): Elucidating the etiology of Apple Replant Disease: a microbial ecology approach. XIV Meeting of the Working Group Biological control of fungal and bacterial plant pathogens, Biocontrol and Microbial Ecology, IOBC-WPRS, 12. - 15.09.2016, Berlin

Mahnkopp, F., Winkelmann, T. (2016): Bacterial endophytes in M26 apple rootstock growing in replant or virgin soils. 6. WeGa Doktorandentag, 06. – 07.10.2016, Veitshöchheim

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Wrede, A., Simon, M.T., Amelung, W., Lehndorff, E., Mahnkopp, F., Pätzold, S. Richartz, S. Und T. Winkelmann (2018): Long term field experiments (LTFE) to create and maintain experimental sites with replant disease. BonaRes Conference 2018. Soil as a Sustainable Resource. 26.- 28.02.18, Berlin

Pabst, S., Schöllhorn, A., Flachowsky, H., Baab, G., Klophaus, L. Mahnkopp, F., Winkelmann, T. Und M. Schmitz (2018): Specific stress responses of Malus under replant conditions as an indicator for the definition of tolerant apple rootstocks. Poster, BonaRes Conference 2018. Soil as a Sustainable Resource. 26.-28.02.18, Berlin

Mahnkopp, F., Winkelmann, T. (2018): Identifizierung von Bakterien in Apfelwurzeln, Die Nacht, die Wissen schafft. 10.11.2018, Hannover

Liu, B., Rohr, A.-D., Mahnkopp-Dirks, F., Weiß, S., Winkelmann, T., Beerhues, L (2019): Phytoalexin production and exudation by roots of Malus genotypes grown in ARD soils. BonaRes Statusseminar 2019, 19. - 21.02.2019, Leipzig

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Oral presentations

Felix Mahnkopp (2014). ADIVK Förderpreis: In-vitro-Vermehrung von *Dionaea muscipula* und *Drosera capensis.* 28. Mitgliederversammlung des ADIVK. 18. - 19.09.2014, Dresden

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Mahnkopp, F., Winkelmann, T. (2018): Bacterial endophytes in M26 apple rootstock growing in replant disease or healthy soils. BonaRes Conference 2018. Soil as a Sustainable Resource. 26.- 28.02.18, Berlin

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9. Danksagung

10. Supplementary material

The following information is available on the enclosed DVD:

1. Electronic version (PDF) of this thesis.

2. Abstract in English and German

3. Supplementary material of the manuscripts:

Chapter 2.1

Induction and diagnosis of apple replant disease (ARD): a matter of heterogeneous soil properties?

Chapter 2.2

Diagnosis of apple replant disease (ARD): Microscopic evidence of early symptoms in fine roots of different apple rootstock genotypes

Chapter 2.3

Molecular barcoding reveals the genus Streptomyces as associated root endophytes of apple (*Malus domestica*) plants grown in soils affected by apple replant disease

Chapter 2.4

Time course of the bacterial root endophytic microbiome of *Malus domestica* plants in field soils affected by apple replant disease