Investigations on the occurrence of fungal root endophytes and an associated mycovirus in context with apple replant disease

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Abstract

Apple replant disease (ARD) negatively affects the production in nurseries and orchards worldwide. A biotic cause of the disease is most likely since soil disinfection can restore plant growth. Fungi have appeared to contribute to the complex of biotic factors, but up to now the actual cause of the disease remains unknown. Further, environmentally friendly and practically applicable mitigation strategies are missing. Fungal root endophytes were isolated in two central experiments of the ORDIAmur consortium. Dark septate endophytes (*Leptodontidium* spp.) were frequently isolated from apple roots. An abundant occurrence of Nectriaceae fungi (*Dactylonectria torresensis* and *Ilyonectria robusta*) was found in ARD roots. Reference sites displayed a different characteristic fungal community. In roots grown in irradiated soil, a reduction of the number of isolated fungi and a changed composition of the fungal community was found. To investigate the effect of fungal endophytes on apple plants a quick and soil-free bio test in Petri dishes was developed using perlite. Inoculated fungi isolated from ARD roots induced neutral (*Plectosphaerella, Pleotrichocladium,* and *Zalerion*) to negative (*Cadophora*, *Calonectria*, *Dactylonectria*, *Ilyonectria,* and *Leptosphaeria*) plant reactions. After re-isolation, most of the Nectriaceae isolates were confirmed as pathogens. Microscopic analyses of ARD-affected roots revealed necroses caused by an unknown fungus that forms cauliflower-like (CF) structures in diseased cortex cells. Two extraction methods, Harris Uni-Core punch and laser microdissection, were applied to further identify the fungus by PCR. Different Nectriaceae species were identified which form intracellular CF structures during the infection process. Both extraction methods can be used to identify also yet unculturable fungi from selected root areas of interest and help to avoid time-consuming isolations. Mycoviruses can influence their fungal hosts in several ways and may alter virulence (hyper- or hypovirulence) or toxin production. A hypovirulence-associated mycovirus has the potential to act as a sustainable control of fungal plant pathogens. Here, the sequence of a novel dsRNA virus originating from *Dactylonectria torresensis* is described, named Dactylonectria torresensis alternavirus 1 (DtAV1), which is a putative member of "*Alternaviridae*". In this work, Nectriaceae were demonstrated to be involved in ARD. Further investigations of microorganism and plant interactions are needed to clarify the cause of the disease, which will then help to develop targeted control strategies.

Keywords: Nectriaceae, bio test, mycovirus

Zusammenfassung

Die Apfel-Nachbaukrankheit (engl. apple replant disease; ARD) beeinträchtigt die Produktion in Baumschulen und Erwerbsobstanlagen weltweit. Da durch Bodendesinfektionen das Pflanzenwachstum wiederhergestellt werden kann, ist eine Beteiligung von biotischen Faktoren an der komplexen Krankheitsursache höchst wahrscheinlich. Insbesondere Pilze scheinen dazu einen Beitrag zu leisten, aber bis heute ist die tatsächliche Ursache der Krankheit unbekannt. Außerdem fehlen umweltfreundliche und praktisch anwendbare Kontrollstrategien. In zwei Zentralexperimenten wurden pilzliche Wurzel Endophyten isoliert. Dunkle, septierte Endophyten (*Leptodontidium* spp.) wurden häufig aus Apfelwurzeln isoliert. Außerdem konnte ein vermehrtes Vorkommen von Nectriaceae (*Dactylonectria torresensis* und *Ilyonectria robusta*) in Wurzeln aus ARD-Böden nachgewiesen werden. Verschiedene Referenzstandorte zeigten eine jeweils charakteristische Pilzgemeinschaft. Bei Wurzeln, die in bestrahltem Boden wuchsen, wurde eine Reduktion der Anzahl isolierter Pilze und eine veränderte Zusammensetzung der Pilzgemeinschaft festgestellt. Um den Einfluss von pilzlichen Endophyten auf Apfelpflanzen zu untersuchen, wurde ein bodenfreier Schnelltest in Petrischalen unter Verwendung von Perlit entwickelt. Inokulierte Pilze, welche aus ARD-Wurzeln isoliert wurden, führten zu neutralen (*Plectosphaerella*, *Pleotrichocladium* und *Zalerion*) bis negativen (*Cadophora*, *Calonectria*, *Dactylonectria*, *Ilyonectria* und *Leptosphaeria*) Pflanzenreaktionen. Nach Re-Isolierung konnten die meisten Nectriaceae-Isolate als Pathogene bestätigt werden. Mikroskopische Analysen von ARD-Wurzeln zeigten Nekrosen, die durch einen unbekannten Pilz verursacht wurden. Dieser bildet blumenkohlähnliche (engl. cauliflower-like, CF) Strukturen in erkrankten Rindenzellen. Zwei Extraktionsmethoden, Harris Uni-Core-Stanzung und Lasermikrodissektion wurden angewendet, um dann den Pilz mittels PCR weiter zu identifizieren. Dabei wurden verschiedene Nectriaceae-Spezies identifiziert, die während des Infektionsprozesses intrazelluläre CF-Strukturen bilden. Beide Extraktionsmethoden können dazu verwendet werden, auch noch nicht kultivierbare Pilze aus ausgewählten Wurzelbereichen zu identifizieren, und sie helfen zeitaufwändige Isolationen zu umgehen. Mykoviren können ihre pilzlichen Wirte auf verschiedene Weise beeinflussen und zum Beispiel die Virulenz (Hyper- oder Hypovirulenz) oder Toxinproduktion verändern. Ein Hypovirulenz-assoziiertes Mycovirus könnte als nachhaltige Bekämpfungsstrategie von pilzlichen Pflanzenpathogenen fungieren. Es wird die Sequenz eines neuen dsRNA-Virus beschrieben, welches aus *Dactylonectria torresensis* stammt und den Namen Dactylonectria torresensis Alternavirus 1 (DtAV1) trägt. Das Mycovirus ist der Familie "*Alternaviridae*" zuzuordnen. In dieser Arbeit wurde gezeigt, dass Nectriaceae an der Entwicklung von ARD beteiligt sind. Weitere Untersuchungen zur Interaktion von Mikroorganismen und Pflanzen sind nötig, um die Ursache der Krankheit aufzuklären. Letzteres hilft dann auch bei der Entwicklung von gezielten Bekämpfungsstrategien.

Schlagworte: Nectriaceae, Bio Test, Mykovirus

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Abbreviations

A	adenine
aa	amino acids
AaV-1	Alternaria alternata virus 1
ADD	alanine-aspartic acid-aspartic acid (motive)
AfMV	Aspergillus foetidus dsRNA mycovirus
AheAV1	Aspergillus heteromorphus alternavirus 1
AMF	arbuscular mycorrhizal fungi
AMV	Aspergillus mycovirus 341
appr.	approximately
ARD	apple replant disease
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	cytosine
cDNA	copy deoxyribonucleic acid
CE (1 or 2)	Central experiment
CF	cauliflower-like (structures)
CHV1	Cryphonectria hypovirus 1
CThTV	Curvularia thermal tolerance virus
DIC	differential interference contrast (microscopy)
DNase	deoxyribonuclease
dpi	days past inoculation
DSE	dark septate endophytes
DSMZ	German Collection of Microorganisms and Cell Cultures
dsRNA:	double-stranded Ribonucleic acid
DtAV1	Dactylonectria torresensis alternavirus 1
E	Ellerhoop
e.g.	exempli gratia; engl. example
engl.	English
ETI	effector triggered immunity
FgAV1	Fusarium graminearum alternavirus 1
FgV1	Fusarium graminearum virus 1
FiAV1	Fusarium incarnatum alternavirus 1
FpAV1	Fusarium poae alternavirus 1
G	guanine

G	gamma irradiation
GDD	glycine-aspartic acid-aspartic acid (motive)
H	Heidgraben
HIS	*histone 3* gene
HR	hypersensitive reaction
Hyp	hypothetical protein
ICTV	International Committee on Taxonomy of Viruses
i.e.	id est
ITS	*internal transcribed spacer*
LB	lysogeny broth (media for bacteria)
LMD	laser microdissection
MAMP	microbe associated molecular patterns
MEA	malt extract agar
MTI	MAMP- triggered immunity
n	number (e.g. of plants)
NA	no amplification
NCBI	National Center for Biotechnology Information
N ctrl	negative control
No.	number
nt	nucleotide
ORF	open reading frame
OTC	Oxytetracycline
PAMP	pathogen associated molecular patterns
PCD	programmed cell death
P ctrl	positive control
PR proteins	pathogenesis-related proteins
R	Ruthe
RACE	rapid amplification of cDNA ends
RdRp	RNA-dependent RNA polymerase
RFC	relative colonization frequency
RNase	ribonuclease
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
sf	symptom-free
sp.	species (singular)
spp.	species (plural)

SsHADV-1	Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1
ssRNA	single-stranded ribonucleic acid
syn.	synonym
T	thymine
TEF	*translation elongation factor 1-α* gene
TtV1	Thelephora terrestris virus 1
TUB	*partial β-tubulin* (*TUB*) gene
ut	untreated (soil)
UTR	untranslated region
vs.	versus
WGS 84	World Geodetic System 1984
γ	gamma irradiation

1.   General Introduction

1.1   Apple production

The cultivated apple, *Malus domestica* Borkh., is a worldwide important fruit crop. In 2017, the global production amounted 83.1 million t, 50 % of which were produced in China (FAOSTAT 2019). In Germany, apple is the most important fruit crop with a total consumption of 21 kg per person per year (Henrich 2019). The production in Germany was in 2012 800.000 to 1 million t of apple on an area of appr. 32.000 ha (Garming et al. 2015). Another important apple growing area for fresh market production in the EU is located in Italy. There apples are produced on an area of 60.000 ha with yields of appr. 2 million t per year.

The development of improved management strategies, increased planting densities and selected breeding programs for rootstocks and cultivars have intensified the apple production during the last decades (Robinson 2011). The average yields of an orchard can vary according to orchard localization and cropping system by around 30-40 t per ha (Garming et al. 2015). The planting of dwarfing rootstocks is a key factor in today's intensive apple cultivation. Especially the Malling series including rootstocks M9 and M26 is of worldwide importance (Volk et al. 2015). Already after 4 years, high density orchards can attain full productivity. By grafting scions on dwarfing rootstocks, the time to flower is reduced and plants invest more resources in fruit production instead of vegetative growth (Fazio et al. 2014). However, next to bacterial fire blight and collar rot these rootstocks are also prone to apple replant disease, which limits the productivity and therefore the possibility of cultivation in some areas (Robinson 2011). Additionally, dwarfing rootstocks have a limited economic lifespan of 12-16 years so that orchards need to be replanted more frequently (Volk et al. 2015).

Apple rootstock propagation is carried out in tree nurseries. The rootstocks are produced from rooted vegetative cuttings by layering or stooling (St. Laurent et al. 2010; Volk et al. 2015). Full production is reached after two years and can last for 15-25 years. Afterwards, rootstock liners are transplanted and grafting or budding of scions on the rootstock is performed (Volk et al. 2015). By the fact that production sites are often limited, also in tree nurseries replanting may occur. New, healthy field sites are not always available, since production often takes place in specialized growing areas and there is high competition for virgin leased land with other producers (e.g. plant production for bioenergy production) (Winkelmann et al. 2019). The replanting negatively affects tree quality like tree height, trunk diameter and average leaf area (Kviklys et al. 2008).

Apple replant disease is a crucial factor in apple plant production in tree nurseries as well as for the productivity of apple orchards.

1.2   Apple replant disease

1.2.1   *Symptoms and causes*

Apple replant disease (ARD) is a worldwide problem and develops when apple plants are replanted at the same site (Mai and Abawi 1981). Recently, ARD was defined as "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). Characteristics of ARD are the specificity for apple or closely related crops, decades of persistence in the soil, and immobility (Hoestra 1968; Klaus 1939; Savory 1966). Further, this phenomenon is reversible: Plantation in virgin soil or sterilization of the soil can restore plant growth (Mai and Abawi 1981; Mazzola 1998; Winkelmann et al. 2019; Yim et al. 2013).

Plants grown in ARD affected soils exhibit an uneven growth, delayed yields and poor fruit quality. In addition, shoot symptoms are stunting, shortened internodes and rosetted leaves. Also, the root system is severely affected and displays discolored roots, a reduction of functional root hairs and destructions of outer cell layers (Caruso et al. 1989; Hoestra 1968; Mai and Abawi 1981; Mazzola 1998; Mazzola and Manici 2012; Savory 1966). Already after two weeks of culture in replant affected soil, root tissues show blackening, necrosis and a reduction of cell vitality (Grunewaldt-Stöcker et al. 2019). All these symptoms result in a loss of productivity and can significantly affect the profitability of an orchard (Geldart 1994; van Schoor et al. 2009).

Molecular and physiological studies showed that phenolic compounds are accumulated in ARD roots and may act as antioxidants linking to oxidative stress (Henfrey et al. 2015). Additionally, genes of the secondary metabolism are upregulated in plants grown in ARD soil and phytoalexins (biphenyls and dibenzofurans) could be detected in high concentrations in the roots (Weiß et al. 2017a; Weiß et al. 2017b). The composition of root exudates has been proven to be genotype specific and can modulate the soil microbial community (Leisso et al. 2017; Winkelmann et al. 2019).

Soil disinfection experiments with broad spectrum biocides (e.g. Chloropicrin, Methyl Bromide or Dazomet), heat treatment and gamma irradiation can significantly improve plant growth, thereby linking to a biotic cause of the disease (Hoestra 1968; Jaffee et al. 1982; Mai and Abawi

1981; Mazzola 1998; Yim et al. 2013). Fungi are frequently mentioned as causal agents including *Cylindrocarpon*-like fungi and *Rhizoctonia* species. Further reported agents are bacterial genera like *Pseudomonas* and *Bacillus*, actinobacteria as well as oomycetous species of *Pythium* and *Phytophthora* (Manici et al. 2013; Manici et al. 2018; Mazzola 1998; Mazzola and Manici 2012; Otto et al. 1994; Tewoldemedhin et al. 2011a; Tewoldemedhin et al. 2011b; Utkhede et al. 1992). Nematodes, like plant-parasitic *Pratylenchus penetrans*, can enhance symptom development, but seem to have a minor role in the cause of the disease (Hoestra 1968; Mazzola 1998; Mazzola and Manici 2012). However, they might be involved by interacting directly or indirectly with other soil microbes (Kanfra et al. 2018; Winkelmann et al. 2019). A further difficulty is that many organisms have been described as the cause of ARD without scientific proof and results may differ from orchard to orchard and region to region (Mazzola and Manici 2012). ARD seems to be more of a disease complex rather than being based on independently acting, single pathogens. Environmental conditions such as climate and soil type are further components in this complex (Winkelmann et al. 2019), which could be an additional reason for the variation in observations for different sites (Mazzola and Manici 2012). Nevertheless, it is common to all ARD locations that the bacterial and fungal communities in the soil and rhizosphere undergo significant changes during apple cultivation (Caputo et al. 2015; Franke-Whittle et al. 2015; Rumberger et al. 2007). Toxic compounds possibly originating from root exudates might create long-lasting shifts in the soil microbial community rather than being biologically active for decades. Overall, the ARD soil - plant system seems to be in dysbiosis, and despite of many years of research, the exact etiology of ARD has not yet been discovered (Winkelmann et al. 2019).

1.2.2 *Soil and abiotic factors*

Frequent replanting, as it may be practiced in nurseries, leads to a faster induction of ARD in comparison to a permanent monoculture due to the repeated mixing of the soil (Winkelmann et al. 2019). Further, ARD has been described as a local phenomenon that could be detected in 0-30 cm depth of the soil (Hoestra 1968). In addition, the author showed that ARD cannot be washed off or be induced by soil leachates: ARD soil was filled in glass tubes and leached with water several times. The leachates were transferred to steamed ARD soil and were planted with seedlings after 6 weeks. The steamed soil with amended leachates did not show any growth reduction while the plants were still stunted after cultivation in the leached ARD soil (Hoestra 1968). Additionally, split root experiments have shown that ARD is not systemic and the mobility of causing agents seems to be restricted to roots directly in contact with the affected soil (Lucas et al. 2018).

Mazzola (1998) listed some abiotic factors that can contribute to tree growth problems, which are a low or high pH, phytotoxic compounds, an unbalanced soil nutrition, heavy metal contaminations, a poor soil structure and drainage, and cold or drought stress. But these abiotic factors are not likely to be the cause of ARD since this disease has a very specific nature and other non-rosaceous plant species are not impaired when cultivated in the affected soil. However, the soil properties mentioned above can further contribute to poor tree growth (Mazzola and Manici 2012).

1.2.3   Management strategies

Soil disinfection with broad spectrum biocides like Basamid (Dazomet) is highly efficient (Yim et al. 2013; Yim et al. 2017). The active substance is converted to gaseous methyl isothiocyanate, which is toxic to almost all soil organisms. Recently, a new registration for Basamid was given in Germany until May 2024 among others for the treatment of soil fungi, insects, nematodes and weeds in pome fruit (Certis 2019). However, since the use of Basamid still poses a potential risk to the environment, there is an urgent need for sustainable alternatives to managing replant disease (Winkelmann et al. 2019). The classical procedure of crop rotation is not applicable for ARD sites due to the long persistence in the soil. Further, the availability of healthy sites is very restricted in often specialized growing areas (e.g. Trentino in Italy or Bodensee region in Germany) (Garming et al. 2015; Winkelmann et al. 2019). Planting new trees in the former driving lanes may not be an option for all orchards due to construction systems, hail nets or irrigation systems (Kelderer et al. 2016; Leinfelder and Merwin 2006; Winkelmann et al. 2019). A physical method to overcome ARD is soil steaming. However, it is very time-consuming and requires large amounts of energy, which is why it is currently not suitable for practical use (Nitt et al. 2015; Winkelmann et al. 2019).

A promising approach is the biofumigation by using Brassicaceae plants, for example *Brassica napus, B. juncea or Sinapsis alba*. The plants are either incorporated into the soil or applied as seed meals (Mazzola et al. 2009; Mazzola et al. 2015; Yim et al. 2016). There, volatile glucosinolates are effective that are catalyzed among others to isothiocyanates by plant myrosinases (Yim et al. 2016). The application of biofumigation leads to shifts in the soil microbial community (Mazzola et al. 2015; Yim et al. 2017). Wang and Mazzola (2019) reported that a seed meal combination of *B. juncea* and *S. alba* was as efficient in disease control as chloropicrin treatment by reducing *Pratylenchus* infestations and suppression of *Pythium* infections. However, too high application rates of the seed meal combination led to phytotoxic reactions and tree mortality. In addition, the success of seed meal application

depends on further factors, which are plant genotype (Geneva lines perform better than the Malling series), time of application and content of glucosinolates of the seed meal (Wang and Mazzola 2019; Winkelmann et al. 2019). In addition, *Tagetes* plants produce thiophenes and terpenoids that have the potential to suppress nematodes and some soil-borne pathogens. When cultivated in ARD soil, *Tagetes* led to changes in the soil microbial community, but those were less pronounced as the effects of biofumigation treatments (Yim et al. 2017).

Arbuscular mycorrhizal fungi (AMF) may have the potential to promote plant health or act as biocontrol agents (Azcón-Aguilar and Barea 1996). For instance, AMF can increase the plant vigor due to additional uptake of water and nutrients. Čatská (1994) reported a growth stimulation of apple plants in ARD soil after inoculation with *Glomus fasciculatum*. This inoculation led also to changes in the rhizosphere composition. As well, Mehta and Bharat (2013) reported an increase of apple growth after inoculation with *G. fasciculatum* under replant conditions.

Inoculation with biocontrol microbes is an alternative strategy for mitigation of ARD (Winkelmann et al. 2019). Further, an increase of soil organic matter content and microbial biomass by the amendment of diverse composts can help to improve soil suppressiveness (Mazzola and Manici 2012). However, the outcome of these practices can vary widely.

The breeding of ARD resistant or tolerant rootstocks is another mitigation opportunity. So far, no resistant genotype has been described, but for example rootstocks G.11 and G.41 of the Geneva lines were reported as more tolerant than Malling rootstocks (e.g. M9 and M26) (Auvil et al. 2011; Wang and Mazzola 2019). The different rootstock genotypes create a distinct soil microbial community (St. Laurent et al. 2010), which might be induced by different root exudations of the rootstock genotypes (Leisso et al. 2017). The rootstock G.41 seems to be less susceptible to nematode infestation and oomycetes infections (Wang and Mazzola 2019). Also, *Malus* x *robusta* 5 (genetic source of G.41) is an interesting resource for rootstock breeding showing less susceptibility to ARD (Reim et al. 2019). In addition, this genotype is more tolerant to cold stress and to diseases like fire blight and powdery mildew (Wöhner et al. 2012).

Concluding, to perform a targeted control or mitigation of ARD, a better understanding of the disease induction and of the microbial interactions is needed (Berg et al. 2017). In the future, the focus for a sustainable mitigation of ARD should be on habitat quality together with the establishment of favorable soil microbial communities instead of potentially harmful soil disinfection treatments (Winkelmann et al. 2019).

1.3 Fungal Endophytes

Numerous reports have indicated that fungi appear to be involved in the yet unrevealed etiology of ARD. Therefore, fungal endophytes were in focus of investigations in this thesis.

1.3.1 Plant and fungal endophyte associations

Plants can be colonized by a variety of different fungi. Since the development of culture-independent sequencing methods, endophytes are now defined only by their habitat as microorganisms that colonize (at least in parts of their life cycle) the interior of plants (the so-called endosphere), independent of their function or interaction with the plant (Hardoim et al. 2015). In roots, they can be for example non-pathogenic, mutualistic associated like arbuscular mycorrhizal fungi (AMF), ectomycorrhiza, dark septate endophytes (DSE) or soil-borne pathogens (Mandyam and Jumpponen 2005). Thereby the relationship between the fungus and the plant can be commensal (without any known effect), mutualistic or pathogenic (Brader et al. 2017). The even variable nature of this interaction depends on the host genotype, the physiological and developmental state of the crop, biotic and abiotic environmental factors and other surrounding microorganisms (Brader et al. 2017; Redman et al. 2001; Schulz et al. 1999). Fungal endophytes have different lifestyles. The relationship to the plant can be obligate, e.g. mycorrhizal fungi which need the plant tissue to complete the fungal life cycle (Schüßler et al. 2001). Opportunistic endophytes only enter the plant roots episodically and exist most of the time outside the plant tissue (e.g. *Trichoderma* spp.) (Druzhinina et al. 2011). But, the majority of fungi are facultative endophytes that consume plant provided nutrients (Hardoim et al. 2015). Schulz and Boyle (2006) listed some characteristics of the interaction between plant roots and the fungal endophytes: Endophytic fungi mostly have a broad host spectrum, depending also on habitat and season. They actively colonize the root tissue through wounds or direct penetration of the cell wall. During the first stage of infection the nutrients are derived from storage material in spores, dead cortex cells, plant residues or host exudates. Later, components of the symplast and apoplast are used for nutrition. The growth of fungal endophytes can be inter- and/or intracellularly and only in some cases a colonization of upper plant parts is possible. Usually the vascular tissue remains free of colonization but if so, the reaction is in most cases pathogenic (Bacon and Hinton 1996; Schulz and Boyle 2005, 2006).

Hardoim et al. (2015) investigated a data set of 8,439 sequences (NCBI) of eukaryotic endophytic full-length *internal transcribed spacer* (*ITS*) regions. Most of the endophytes belonged to Glomeromycota (40 %), followed by Ascomycota (31 %), Basidiomycota (20 %), Zygomycota (0.1 %) and unidentified phyla (8 %). The Glomeromycota phylum contains

arbuscular mycorrhizal fungi (AMF) (Schüβler et al. 2001). For example, the genera *Glomus* and *Rhizophagus* are obligate symbionts forming associations with various host plants. The Ascomycota phylum contains a lot of endophytes, which vary in their function (commensal to pathogenic). In the phylum Basidiomycota, a large group of wood decaying fungi together with white and brown rot saprotrophs and beneficial ectomycorrhizal fungi is assigned to the class Agaricomycetes. Nevertheless, the function of an endophytic fungus often cannot be linked to its taxonomic position (Hardoim et al. 2015). Even strains of the same species may display interactions with the plant varying from mutualism to pathogenicity. For example, strains of *Fusarium oxysporum* have a narrow host specificity and are reported to be pathogenic to a lot of plant species (O'Donnell et al. 2009). But to non-hosts, most of the isolates do not exhibit pathogenicity or they may even act as biocontrol agents (Aimé et al. 2013).

Colonizing plants gives the microorganisms the advantages of a habitat protected from abiotic stress and a continuous supply of nutrients. The host plant can benefit from colonization through improved growth due to phytohormone production by e.g. a fungus and access to soil nutrients and minerals. Furthermore, induced disease resistance, biocontrol of plant pathogens and nematodes as well as improved tolerance to abiotic stress are plant performance improving mechanisms basing on endophytic microbial interactions (Hardoim et al. 2015; Schulz and Boyle 2006).

1.3.2   Disease Tetrahedron: Likelihood of a disease

The likelihood and development of a plant disease, including ARD, depends on various factors.

The interaction between fungi (pathogen), plant (apple) and environment has often been described as disease triangle (Agrios 2005). Recently, Brader et al. (2017) supposed the concept of a disease tetrahedron considering also biotic factors. The outcome and likelihood of a disease depends on the proportions and interplay of disease determining factors. On the plant (host) side, these factors are for example rootstock genotype (tolerant/susceptible), planting position in the orchard, growth stage and age. The pathogen is influenced by its fitness and adaptation to an environment. Moreover, survival, abundance and virulence are affecting the success of an infection. Also, environmental factors can favor or suppress disease development including temperature, precipitation, and soil parameters like organic matter, pH, nutrient content, toxic components (e.g., metals, salt, and pesticides). The fourth driving force consists of biotic factors including plant microbiota, alternate hosts, micro- and macro-fauna as well as vectors for pathogens (e.g. bacteria, fungi or viruses) (presence, adaption, fitness and association with microbiota) (Brader et al. 2017). Vayssier-Taussat et al. (2014) suggested the concept of a

pathobiome, since pathogens are acting in context of microbial communities. Additionally, the (rhizosphere) microbiome plays a crucial role for plant health and helps suppressing pathogens (Berendsen et al. 2012).

1.3.3 Plant response to fungal colonization

A broad range of endophytes, pathogens as well as non-pathogens, can produce enzymes and phytotoxic compounds that are required for host colonization and infection (Petrini et al. 1992; Schulz et al. 2002; Schulz and Boyle 2006; Sieber et al. 1991). Next to the cell wall - as physical barrier - the plant defense is based of two layers referred to as plant immune system (Jones and Dangl 2006). The first layer is based on the recognition of microbe- (or pathogen-) associated molecular patterns (MAMPs/PAMPs) which leads to a MAMP-triggered immunity (MTI) (Ausubel 2005; Jones and Dangl 2006). MAMPs are recognized by pattern recognition receptors localized on the plant surface, receptor-like kinases (localized on the plasma membrane) or by receptor-like proteins (Brader et al. 2017; Newman et al. 2013). The elicitors which trigger MTI serve for key functions among the microbes and are therefore conserved among pathogens and non-pathogens. This can be for example fungal chitin and β-glucans of oomycetes (Newman et al. 2013). Plants often respond with defense reactions to fungal colonization (Schulz et al. 1999; Schulz and Boyle 2005). For example, colonization by non-pathogenic *Fusarium oxysporum* (strain Fo47) resulted in an overexpression of defense genes in tomato roots (Aimé et al. 2013). Both beneficial and pathogenic fungi have developed mechanisms to evade plant defense and MTI. This is done by either modifying MAMP structures or actively by effector production leading to alterations in plant receptor function and structure (Lo Presti et al. 2015; Pel and Pieterse 2013).

The second plant defense layer after Jones and Dangl (2006) is the effector triggered immunity (ETI). It is derived from the recognition of microbial effectors by the plant. Plants respond to fungal effectors by producing pathogenesis-related (PR) proteins or indirectly act by using assessor proteins to perform ETI (Aoun 2017; Jones and Dangl 2006). For instance, manipulation of ETI is carried out by AMF colonization of *Medicago truncatula*: the mycorrhizal fungus *Rhizophagus irregularis* (previously *Glomus intraradices*) produces an effector (SP7) that interacts with the plant transcription factor (ERF19), which is regulating the expression of defense related genes resulting in suppression of defense gene expression (Brader et al. 2017; Kloppholz et al. 2011).

The activation of MTI or ETI leads to a signal transduction and expression of defense related genes. Plants respond to fungal colonization by production of secondary metabolites, oxylipins,

reactive oxygen species (ROS), defensins or by performance of hypersensitive reactions (HR) (cell death) (Brader et al. 2017). Or the fungi succeed in circumventing the plant's defense system in various ways and can colonize or infect the plant roots (Aoun 2017).

1.4 Mycoviruses

Mycoviruses may influence their fungal host in serval ways and might therefore be involved in soil microbial interactions as well as in interactions with the plant when infected by the host fungus. Also, fungal endobacteria might affect the interaction with either fungi, plant or other microorganisms, but in this work the focus was set on mycoviruses. So far, mycoviruses have not been studied in context with replant disease. Since pathogenic fungi seem to be involved in ARD disease development, the occurrence of mycoviruses was of interest. They may alter the virulence of a fungus causing hyper- or hypovirulence. If the latter is the case within isolates from ARD-infested apple roots, a sustainable mitigation strategy to control ARD-associated fungal pathogens might result from it.

1.4.1 Mycoviruses: Transmission, genome organization and effect on the host fungus

Several fungi can be infected by specific viruses, the so-called mycoviruses. Such infections were detected in all major phyla of fungi (Ghabrial et al. 2015). Like all viruses, mycoviruses depend on living host cells for replication. But in contrast to other viruses, mycoviruses lack movement proteins and an extracellular route for infection (Son et al. 2015). Further, this group of viruses can be transmitted intercellularly by cell division, horizontally by hyphal anastomosis and vertically by the distribution of spores (mostly conidia and sometimes meiotic spores). The natural host range is supposed to be restricted to related vegetative compatibility groups (Ghabrial et al. 2015; Son et al. 2015). Usually the incompatibility response leads to a programmed cell death (PCD) (Choi et al. 2012). However, it was reported that Cryphonectria hypovirus 1 (CHV1) was able to suppress this reaction by downregulation of host genes involved in PCD (Biella et al. 2002; Shang et al. 2008). Further, it is not known whether mycoviruses can be vector transmitted (Ghabrial et al. 2015). Petrzik et al. (2016) reported of a double-stranded (ds)RNA virus (Thelephora terrestris virus 1 (TtV1)) in the mycorrhizal fungus *Thelephora terrestris*. TtV1 was also detected by RT-PCR in soil oribatid mites (*Steganacarus carinatus*). However, no transmission trials were carried out.

Most of the described mycoviruses have a dsRNA genome that is packed in isometric particles. But genomes were also characterized with positive or negative single-stranded (ss)RNA as well

as DNA genomes (Ghabrial et al. 2015; Jiang et al. 2013; King et al. 2011; Li et al. 2020a; Liu et al. 2014; Yu et al. 2013). A mycovirus infection is not an uncommon incident, therefore it was suggested that 30-80 % of fungal species might be infected (Ghabrial and Suzuki 2009). Some viral families contain both plant- and mycoviruses, such as *Partitiviridae* and *Endornaviridae* (ICTV, King et al. 2011). Moreover, *Totiviridae* and *Chrysoviridae,* originally assigned to be mycoviruses, were also identified in plants (Roossinck 2012, 2014). To detect a mycovirus infection, often dsRNA extraction is used targeting the dsRNA directly or replicative intermediates. Multiple dsRNAs can represent segmented viral genomes or mixed infections (Pearson et al. 2009). For instance, one single isolate of *Rhizoctonia solani* was reported to be infected by at least 17 different mycoviral species that were detected by a deep sequencing approach (Bartholomäus et al. 2016).

In most cases a mycoviral infection does not cause any symptoms and remains latent. Symptoms can be abnormal pigmentation, irregular growth, and modifications in the sexual reproduction (Son et al. 2015). Most interestingly with regard to phytopathogenic fungi are deviations in the fungal virulence leading to hyper- or hypovirulence (Ahn and Lee 2001; Xie and Jiang 2014).

1.4.2   *Mycovirus mediated hypovirulence to control plant pathogens*

The use of mycoviruses that cause hypovirulence are an interesting approach to manage plant pathogenic fungi and thereby reduce crop losses (Xie and Jiang 2014). The most famous example is the control of the chestnut blight fungus *Cryphonectria parasitica* in orchards with the dsRNA *Hypovirus* Cryphonectria hypovirus 1 (CHV1) (Milgroom and Cortesi 2004; Shapira et al. 1991). Another example is a mycovirus-mediated hypovirulence in the plant pathogenic fungus *Fusarium graminearum*: Infections with Fusarium graminearum virus 1 (FgV1) led to decreased growth, altered pigmentation and reductions in mycotoxin production (Chu et al. 2002). Also, DNA viruses can mediate hypovirulence: the circular ssDNA virus Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) causes hypovirulence in *Sclerotinia sclerotiorum*, a worldwide distributed plant pathogen (Yu et al. 2010). Purified virus particles were applied extracellularly by spraying on plants (*Arabidopsis thaliana* and *Brassica napus*) and thus could infect the host fungus. Under field conditions, the virus particle application reduced disease severity and enhanced rapeseed yields (Yu et al. 2013). Xie and Jiang (2014) assumed that hypovirulent strains occupy the same niche as the virulent strain and grow well on the host plant. Thereby both strains come in contact and the virus can be transmitted to the plant pathogen. Moreover, the hypovirulent strains produce

the same PAMPs and/or effectors as the pathogen, thereby activating the hosts defense response specifically targeting the pathogen (Xie and Jiang 2014).

1.5   Project ORDIAmur

This work is part of the project ORDIAmur (**O**vercoming **R**eplant **D**isease by an **I**ntegrated **A**pproach). Aim of this project is the understanding of replant disease induction and to develop environmentally friendly, practicable and sustainable mitigation strategies. To achieve this, plant and soil science are combined with socio-economic studies also to enable a transfer of knowledge between science and practice (www.ordiamur.de). ORDIAmur is part of the BonaRes consortium that focus on the sustainable use of soils as limited resource (www.bonares.de).

1.6   Objectives and Hypotheses

Apple Replant disease is a complex phenomenon that has major impacts on the productivity of apple plants in nurseries as well as in orchards. The etiology of ARD remains unrevealed up to now. Moreover, there is still need of sustainable mitigation strategies.

The aim of this work was to characterize and quantify apple fungal root endophytes and their mycoviruses. Both endophytes and their associated mycoviruses may contribute to symptom development also by their absence. Moreover, mycoviruses may influence the virulence of the host fungus by causing hyper- or hypovirulence. Hypovirulence-associated strains might be used in future as sustainable mitigation strategy for ARD. Another objective of this work was to compare endophytes in apple roots growing in ARD- and control soils, which were obtained from two central experiments (CE1 and CE2). For a causal analysis of ARD, fungi needed to be isolated, identified and tested in a bioassay whether they infect or colonize apple roots.

Therefore, the hypotheses of this thesis are:

1. Fungi are involved in the disease development of ARD.

2. There are differences in the fungal community in apple plants growing in control soil compared to that in ARD soil.

3. Some fungal isolates are pathogens and have a negative influence on the growth of apple plants.

4. Mycoviruses are involved in the causal ARD complex.

2.   Fungal endophytes from apple replant diseased roots

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Abstract

Apple Replant Disease (ARD) affects the plant production in apple nurseries and orchards worldwide. Reductions in plant growth and yield result in economic losses. Shifts in the soil microbial community are characteristic for all ARD sites but the explicit cause remains yet undiscovered. ARD was induced at three reference field sites with traceable and comparable cropping histories. At the filed sites, samples were taken from plants in ARD plots and from plots were apple was planted for the first time (Apple New). The roots of sampled plants were surface disinfected prior to investigate the fungal endophyte community. Compared to Apple New plants, ARD plants displayed a clearly reduced shoot growth. An enrichment of Nectriaceae fungi, especially of *Dactylonectria torresensis* and *Ilyonectria robusta*, was found in ARD roots. *Leptodontidium* spp., belonging to the dark septate endophytes (DSE), was the most frequent isolated fungal endophyte from field plants. Furthermore, a biotest was conducted with soil from the field sites cultivating plants in untreated or gamma irradiated ARD and grass soil, respectively. Again, Nectriaceae species were most prevalent in ARD roots, while members of DSE belonging to Helotiales and Pleosporales seem to be reduced in their abundance. Each site displayed a characteristic fungal community. Plant growth was enhanced in gamma irradiated soils. These roots showed a reduction of the number of isolated fungi together with an altered fungal community. The role of Nectriaceae fungi as possible pathogens as well as of DSE in context with ARD should be addressed in further experiments.

2.1   Introduction

Apple Replant Disease (ARD) is a worldwide problem and 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). Plants exhibit growth diminutions together with reduced and delayed yields. This results in economic losses for plant producers (Geldart 1994). The root system - as interface of soil and plant - is reduced in size and displays discolored roots (Mai and Abawi 1981; Mazzola and Manici 2012; Winkelmann et al. 2019). Microscopic analyses of roots defined early diagnostic symptoms, which are necrosis and blackening along with cytoplasmic inclusion bodies and a loss in cell vitality. These changes in the root structure are often associated with fungal infections (Grunewaldt-Stöcker et al. 2019).

Soil disinfection by thermal, irradiation or chemicals treatments can restore the plant growth, which gives evidence of a biotic cause of the disease (Mai and Abawi 1981; Mazzola 1998; Yim et al. 2013). Several organisms were reported associated with the disease. These are nematodes, actinobacteria, chromista of the class Oomycetes (*Pythium* and *Phytophthora*), and fungi. Especially *Cylindrocarpon*-like fungi, *Fusarium* spp., and *Rhizoctonia* spp. are frequently reported to be involved in the etiology of ARD (Hoestra 1968; Manici et al. 2003; Manici et al. 2013; Manici et al. 2017; Mazzola 1998, 1999; Tewoldemedhin et al. 2011a; Utkhede et al. 1992). Various reports that claim to have found the causal agent are available in literature but lack profound experimental support. Moreover, the experimental designs are often difficult to compare, as the orchards usually have very individual cropping histories. This leads to many contradictory reports (Mazzola and Manici 2012). Hence, up to now the explicit cause of ARD is still undiscovered.

Mahnkopp et al. (2018) described the set up of three reference locations in the BonaRes project ORDIAmur: These locations have a defined, traceable and comparable cropping history with differences in their soil characteristics. ARD was induced in the field by frequent replanting cycles. Grass plots served as control. In the 5th replanting generation also one third of the grass plot was grown with apple for the first time (Apple New). For a biotest in the greenhouse the soil was taken from the field (ARD and grass soil) and sterilized by gamma irradiation or remained untreated.

Since the root system is in direct contact with the diseased soil, our focus was on fungal root endophytes. Due to cultivation independent analyses, endophytes are now defined by their

habitat only as "microorganisms inhabiting the interior of plants (endosphere) irrespective of the function in association with the plant" (Brader et al. 2017; Hardoim et al. 2015).

Our aim was to characterize and quantify the fungal endophyte community by a culture dependent approach. Therefore, fungal endophytes were isolated from surface disinfected roots either from the field experiment (ARD and Apple New) or from the biotest (ARD and grass soil, untreated or irradiated, respectively). Furthermore, we looked for differences in the fungal community of the three reference sites with different soil types.

2.2   Material and methods

2.2.1   Experiments and plant material

In the BonaRes project ORDIAmur three reference sites with traceable cultivation history were selected under the aspect that Rosaceae species were not cultivated before. For the field experiment CE1 (central experiment 1) ARD was induced by replanting the rootstock 'Bittenfelder Sämling' (hereafter given as Bittenfelder) in a cycle of two years. At the sites 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) four plots with apple were arranged and another four plots with grass cover were used as control, respectively. Detailed description of the experiment as well as soil characteristics are given by Mahnkopp et al. (2018). In April 2016 apple was replanted for the 5th time in Heidgraben and for the first time one third of the grass plots was grown with apple, too (referred to as Apple New). For the locations Ellerhoop and Ruthe the 5th replanting generation was in 2017. Samples were taken in Heidgraben in November 2016 and in Ellerhoop one year later in November 2017. Furthermore, fungal endophytes were isolated out of Bittenfelder roots before planting in the field (t0, April 2017, n= 3 plants). The plants used in CE1 were grown from seeds in showing beds and originated from a local nursery.

In a central greenhouse experiment in 2017, CE2, soil from ARD and grass plots of the three reference locations was brought to Hanover for a biotest (Mahnkopp et al. 2018). Either the soil remained untreated or it was sterilized by γ-irradiation ($\geq 10\,kGy$). ARD sensitive *in vitro* propagated *Malus domestica* 'M26' plants (hereafter referred to as M26) were grown for 8

weeks in the soil (Yim et al. 2013). Additionally, *in vitro* propagated Bittenfelder plants (clonal progeny of one plant) were grown in Ruthe soil.

2.2.2   *Surface disinfection and fungal endophyte isolation*

Adherent soil was removed by carefully washing the roots with tap water. The roots were dipped for 30 s in 70 % Ethanol followed by 7.5 min in 2 % NaOCl solution amended with Tween 20 and were washed five times in sterile distilled water. To control the success of the surface disinfection 100 µl of the last wash water was spread on 2 % malt extract agar plates (MEA) amended with Oxytetracycline (OTC, 50 µg mL^{-1}).

Fungal endophytes were isolated in CE1 7 month after planting in autumn 2016 from Heidgraben plants and in autumn 2017 from Ellerhoop plants (ARD and Apple New, n= 12 plants per treatment). Before starting the biotest CE2 2017, 4 plants were surface disinfected after acclimatization (t0). After 8 weeks of culture, 4 plants were sampled per treatment (untreated or γ-irradiated ARD and grass soil from three locations). Surface disinfected roots were cut and 4 1 cm-root segments were placed onto 3 MEA + OTC plates per plant. Plates were cultured for 2 to 7 days at 24 °C in the dark. Growing mycelium was separated and sub-cultured.

In CE2 2017, surface disinfected roots were additionally plated on 1.5 % water ager (penicillin 50 µg mL^{-1}, rifampicin 10 µg mL^{-1}, and pimaricin 25 µg mL^{-1}) to especially isolate members of Oomycetes. Sub-cultures were performed as described above on MEA.

2.2.3   *Endophyte identification*

Fungi from pure cultures were identified in a direct PCR using primers ITS 1 and 4 (White et al. 1990). PCR conditions for the identification of fungal cultures are described in Popp et al. (2019). Further, isolates of the Nectriaceae were investigated in detail performing a multi locus analysis (CE1 Ellerhoop 2017 and CE2 2017; Cabral et al. 2012a): Primer pairs CYLH3F and CYLH3R (Crous et al. 2004) for *histone H3* (*HIS*), T1 (O'Donnell and Cigelnik 1997), and Bt-2b (Glass and Donaldson 1995) targeting *partial β-tubulin* (*TUB*) gene as well as CylEF-1 (5'-ATG GGT AAG GAV GAV AAG AC-3'; J.Z. Groenewald, unpublished) together with CylEF-R2 (Crous et al. 2004) for *translation elongation factor 1-α* gene (*TEF*) were applied. PCR products were analyzed by Sanger sequencing using the sense primer of each amplification product (Microsynth Seqlab, Göttingen, Germany). Results were submitted to BLASTn analysis (Megablast, NCBI, Rockville Pike, USA) and are presented as first hit (sorted by max. score). The naming of Nectriaceae isolates is based on the *HIS* gene results.

2.3 Results

2.3.1 Identification of fungal endophytes in CE1

ARD was successfully induced in the field experiment at all three reference sites. The shoot fresh mass was significantly reduced comparing the 1st and 4th replant generation (Mahnkopp et al. 2018). There were clear differences regarding the shoot growth between the Apple New and ARD plants (5th replant generation, Fig. 2.1, Ellerhoop).

Fig. 2.1 Representative plants Ellerhoop CE1 2017. 'Bittenfelder' plants harvested 7 months after planting from plots Apple New (left side, apple planted for the first time in the same soil type) and ARD (right site, 5th replanting generation)

The total number of fungal endophytes isolated from surface disinfected Bittenfelder roots (n= 144 root sections of 12 plants per treatment) was n= 164 Apple New vs. n= 148 ARD, Heidgraben 2016 and n= 153 Apple New vs. n= 188 ARD, Ellerhoop 2017 (Fig. 2.2). Fungal endophytes were identified by *ITS*-PCR. A higher diversity of fungal genera (number of different fungal genera) was found in Apple New in comparison to ARD (29 vs. 19 for Heidgraben and 25 vs. 17 for Ellerhoop). For both reference sites, one third of the total fungal community was made up of *Leptodontidium* spp. belonging to the order Helotiales. In Heidgraben the second largest group were members of Hypocreales, especially Nectriaceae

fungi whose proportion of the total fungal community was increased in ARD (37 %) compared to Apple New (22 %). In addition, the percentage of isolates belonging to the order Pleosporales was reduced in ARD (12 %) compared to Apple New (20 %). Isolates identified as *Zalerion* sp. (Lulworthiales) had in both treatments a proportion of 8 %. About half of the fungal community of Apple New plants harvested from the reference site Ellerhoop were identified as Helotiales species followed by members of Pleosporales (like *Pleotrichocladium* and *Periconia*, 18 %) and Lulworthiales (*Zalerion* sp., 14 %). Nectriaceae species had a proportion of 13 % of the total fungal community. In comparison to that, the proportion of isolates identified as members of Helotiales (43 %), Lulworthiales (7 %) and Pleosporales (5 %) was reduced in ARD roots while the number of Hypocreales isolates (44 %) increased markedly, especially the abundance of *Ilyonectria* species. Only a few isolates assigned to Basidiomycota were isolated in CE1 (Fig. 2.2). These belonged mostly to the class Agaricomycetes. Next to other fungal species, Nectriaceae species were already identified in Bittenfelder nursery plants before planting in the field (Table 2.1; t0, CE1 2017 Ellerhoop).

Table 2.1 Fungal endophytes isolated from surface disinfected Bittenfelder roots before planting in CE1 Ellerhoop 2017 (t0). Plants originated from a nursery. Isolation of 37 fungi out of 36 root pieces from three plants. Identification by *ITS*-PCR and BLASTn search (first hit)

Plant	*ITS* Identification	Number
Plant 1	*Dactylonectria* sp.	2
	Ilyonectria sp.	1
	Nectriaceae sp.	1
	Psiloglonium sp.	2
	Mortierella sp.	1
	Paraphaeosphaeria sporulosa	1
	Pythium sylvaticum	1
Plant 2	*Bjerkandera* sp.	1
	Chaetomium sp.	1
	Fusarium sp.	1
	Ilyonectria sp.	1
	Plectosphaerella sp.	1
	Psiloglonium sp.	1
	Trichocladium sp.	1
	not identified	4
Plant 3	*Ilyonectria* sp.	5
	Nectriaceae sp.	2
	Cadophora sp.	4
	Leptodontidium sp.	3
	Mortierella sp.	1
	Psiloglonium sp.	1
	not identified	1

a   **Apple new n= 164**   **Heidgraben**   **ARD n= 148**

Legend (left):
■ *Leptodontidium*   ■ *Cadophora*   ■ *Mycochaetophora*
□ *Geomyces*   ■ *Ilyonectria*   ■ *Dactylonectria*
■ *Nectria*   ■ *Fusarium*   □ *Clonostachys*
■ *Trichoderma*   ■ *Zalerion*   ■ *Fusiconidium*
■ *Pleotrichocladium*   ■ *Prosthemium*   ■ *Paraphaeospaeria*
■ *Trematosphaeria*   ■ *Alternaria*   ■ *Dictyosporium*
■ Pleosporales   ■ *Exophiala*   ■ *Cladophialophora*
■ Chaetosphaeriales   □ *Clohesyomyces*   ■ *Gaeumannomyces*
□ *Monacrosporium*   ■ *Penicillium*   ■ *Psiloglonium*
■ *Ceartobasidium*   ■ *Phlebiella*   □ not identified

Legend (right):
■ *Leptodontidium*   □ *Cadophora*   ■ *Hymenoscyphus*
■ Helothiales   ■ *Ilyonectria*   ■ *Cylindrocladiella*
■ *Fusarium*   ■ *Trichoderma*   ■ *Zalerion*
■ *Prosthemium*   ■ *Pyrenocheata*
■ *Herpotrichia*   ■ *Paraphaeopspaeria*   ■ *Dendryphion*
■ *Fusiconidium*   ■ *Atractospora*   ■ *Plectosphaerella*
■ Agaricomycetes   □ not identified
■ *Pleotrichocladium*

b   **Apple new n= 153**   **Ellerhoop**   **ARD n= 188**

Legend (left):
■ *Leptodontidium*   ■ *Mycocheatophora*   ■ *Tetracladium*
■ *Hymenoscyphus*   ■ *Cadophora*   □ *Geomyces*
□ *Glarea*   ■ *Zalerion*   ■ *Ilyonectria*
■ *Dactylonectria*   ■ *Fusarium*   □ *Calonectria*
■ *Pleotrichocladium*   ■ *Periconia*   ■ *Prosthemium*
■ *Pyrenocheata*   ■ *Trematosphaeria*   ■ *Fusiconidium*
■ *Paraphaeosphaeria*   ■ *Paraphoma*   ■ Pleosporales
■ *Penicillium*   ■ *Phialophora*   ■ Agricales
■ Auriculariales   □ not identified

Legend (right):
■ *Leptodontidium*   □ *Cadophora*
■ *Pezicula*   ■ *Varicosporium*
□ Helotiales   ■ *Ilyonectria*
■ *Dactylonectria*   ■ *Cylindrocarpon*
■ Nectria not identified   ■ *Zalerion*
■ *Phoma*   ■ *Fusiconidium*
■ *Paraphaeosphaeria*   ■ *Paraphoma*
■ *Penicillium*   □ *Cladosporium*
■ *Pleotrichocladium*
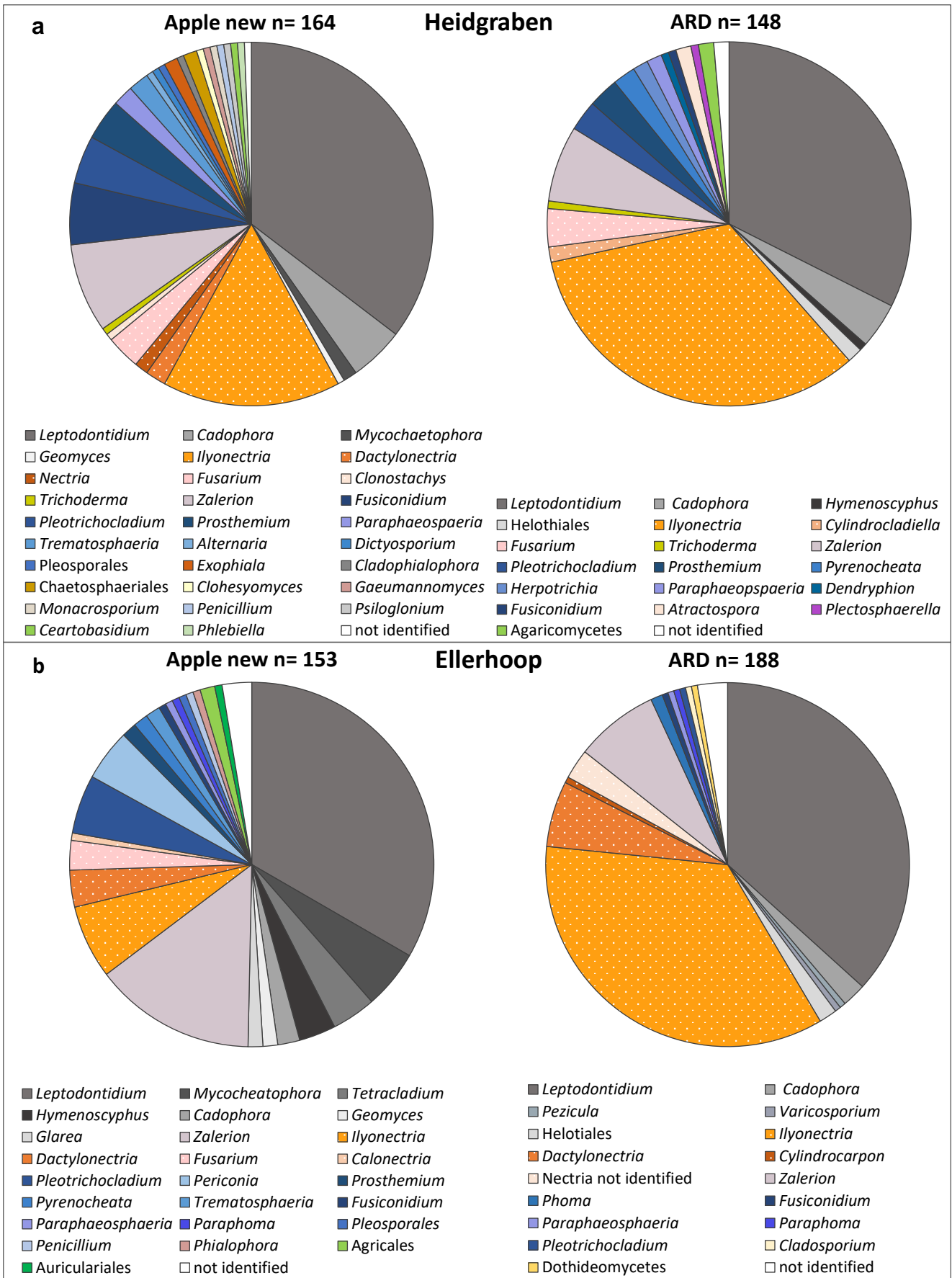■ Dothideomycetes   □ not identified

Fig. 2.2 (previous page) **Fungal endophyte isolation field experiment CE1.** Isolation from surface disinfected roots of 'Bittenfelder' plants 7 months after planting. Reference sites a) Heidgraben (planting in 2016) and b) Ellerhoop (planting in 2017). Soil variants are Apple new (left side, apple planted for the first time in the respective soil type) and ARD (right side, 5th replanting). n = number of isolated fungi of 144 root segments (from n = 3 plants out of 4 field plots, respectively). Identification by *ITS*-PCR and Sanger sequencing. Sectors denote members of Helotiales (grey); Nectriaceae (dotted/ orange); Lulworthiales (old rose); Pleosporales (blue) and Basidiomycota (green)

Since the proportion of Nectriaceae isolates was increased in the ARD fungal community, the isolates obtained from Ellerhoop roots in 2017 were identified to the species level by a multi locus analysis (Fig. 2.3). In Apple New, 20 isolates were assigned to Nectriaceae, which corresponds to 13 % of total isolated fungal isolates. Another 84 Nectriaceae isolates were gained from ARD roots (44 % of total isolated fungal endophytes). *Ilyonectria robusta* was only identified in ARD roots and represented the largest proportion of the Nectriaceae community in this treatment. Both soil treatments, Apple New and ARD, shared *Dactylonectria torresensis*, *Ilyonectria* sp. 1 AE-2001, *Ilyonectria crassa*, and *Ilyonectria europaea*. Only one isolate of *Calonectria* sp. and *Ilyonectria pseudodestructans* was gained from Apple New roots. Here, *Fusarium* was only detected in Apple New roots from Ellerhoop. Regardless, *Fusarium* spp. were isolated in equal proportions from both treatments of Heidgraben plants (2016) (Fig. 2.2).
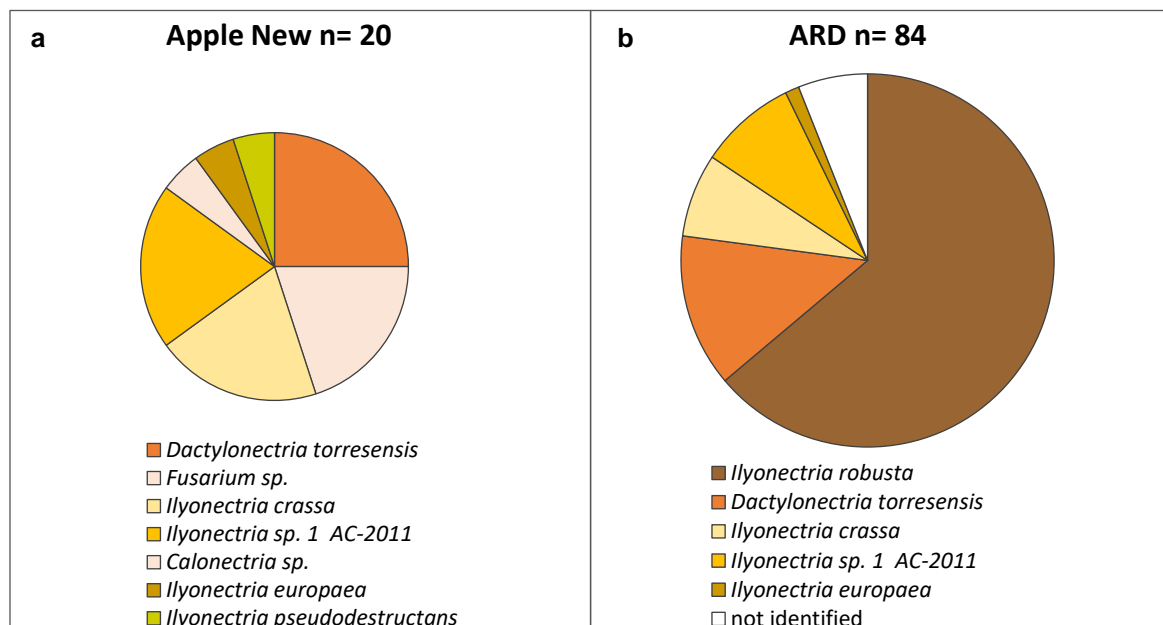

Fig. 2.3 Identification of Nectriaceae isolates CE1 Ellerhoop 2017. Isolation from surface disinfected roots of 'Bittenfelder' plants 7 months after planting. Soil variants are Apple New (a, apple planted for the first time in the same soil type) and ARD (b, 5th replanting). n = number of isolated Nectriaceae fungi of 144 root segments (from n = 3 plants out of 4 field plots, respectively). Identification by Sanger-sequencing of *histone 3* (*HIS*) gene

2.3.1   Identification of fungal endophytes in CE2

The greenhouse biotest CE2 confirmed the presence of ARD. Gamma irradiation led to an increase in shoot length after 8 weeks of cultivation compared to untreated ARD and grass soil variants (Mahnkopp et al. 2018).

Before starting the experiment, no fungi could be isolated from young, acclimatized M26 roots at t0. After eight weeks of cultivation, fungal endophytes were isolated from surface disinfected M26 roots and identified by means of *ITS*-PCR. Additionally, *in vitro* propagated Bittenfelder plants were grown in Ruthe soil in this experiment. The number of isolated fungi was almost the same for plants grown in untreated ARD and grass soil. In contrast, the number of fungal isolates obtained from roots grown in gamma treated soil was clearly reduced (Fig. 2.4).
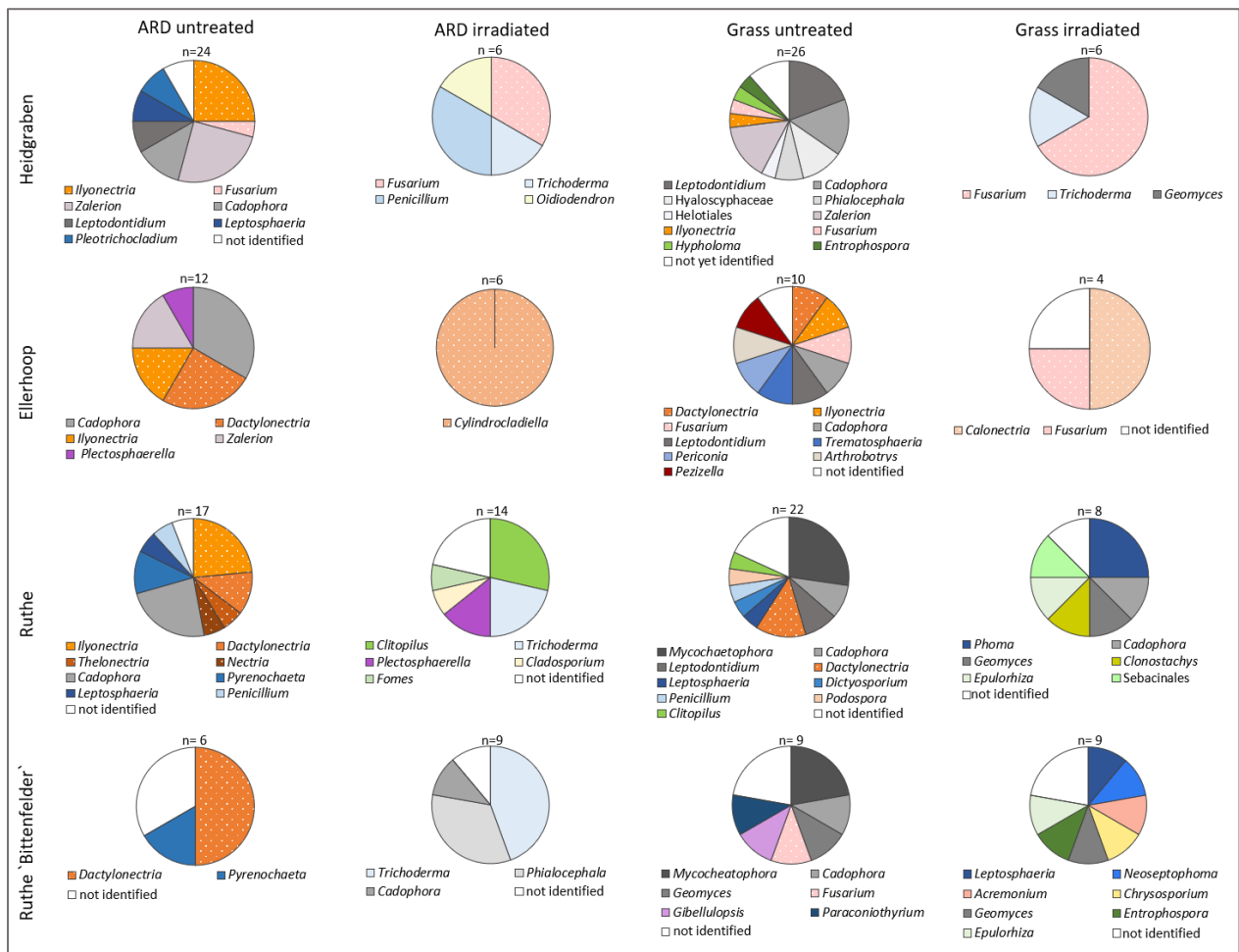

Fig. 2.4 Fungal endophyte isolation CE2 2017. Isolation from surface disinfected roots of M26 and 'Bittenfelder' plants after 8 weeks culture in untreated and γ-irradiated ARD soil and grass soil from the reference site locations Heidgraben, Ellerhoop and Ruthe. n = number of isolated fungi of 48 root segments (from n = 4 plants). Identification by *ITS*-PCR and Sanger sequencing. Sectors denote members of Helotiales (grey); Nectriaceae (dotted/ orange); Lulworthiales (old rose); Pleosporales (blue) and Basidiomycota (green)

The fungal community of roots grown in untreated ARD soils from the three reference sites consisted mainly of members of Hypocreales (especially Nectriaceae, *Ilyonectria* spp. and *Dactylonectria* spp.), Lulworthiales (*Zalerion* spp.) and Helotiales (e.g. *Cadophora* spp. and *Leptodontidium* spp.) (Fig. 2.4). Further, also some isolates of Pleosporales (*Leptosphaeria* spp., *Pleotrichocladium* spp. and *Pyrenochaeta* spp.) were found in roots cultured in untreated ARD soils. Only one *Fusarium* isolate was gained from roots grown in untreated Heidgraben ARD soil. The irradiation of ARD soil led to a completely different fungal community. Here, fast growing fungi like *Fusarium*, *Penicillium* and *Trichoderma* were found. In roots grown in irradiated ARD soil from Ellerhoop only *Cylindrocladiella* spp. was detectable. Roots of plants grown in irradiated Ruthe ARD soil revealed also Basidiomycota (*Clitopilus* spp. and *Fomes* spp.).

For roots grown in untreated grass soil of the sites Heidgraben and Ruthe the biggest proportion of the fungal community was formed by members of Helotiales (for example *Cadophora* spp., *Leptodontidium* spp., *Mycochaetophora* spp.). In exception to roots from Ellerhoop, only some Nectriaceae isolates were detectable in this treatment (Fig. 2.4). Also, the gamma irradiation of the grass soil led to a different community comparing to the untreated soil. Here, the community isolated from roots grown in Ruthe soil differed to those of Heidgraben and Ellerhoop. Additionally, in this experiment, *in vitro* propagated Bittenfelder plants were grown in Ruthe soil. Less fungal endophytes were isolated from Bittenfelder roots. Furthermore, both rootstocks display similarities in their fungal community.

Besides MEA also water agar was used in CE2 2017 to isolate especially oomycetes (see addendum Table 8.1). Less fungi were isolated with water agar compared to MEA, but similar fungal endophytes were identified in the different treatments. Here, only one *Pythium ultimum* isolate was gained from M26 roots grown in untreated ARD soil from Ruthe.

Summarizing, each location has a site-specific fungal community. But overall similarities were detectable, like the enrichment of Nectriaceae species in untreated ARD soils compared to untreated grass soils. The irradiation of the soil resulted in a reduction in the total number of fungal isolates and a different fungal community compared to the untreated soil (Fig. 2.4).
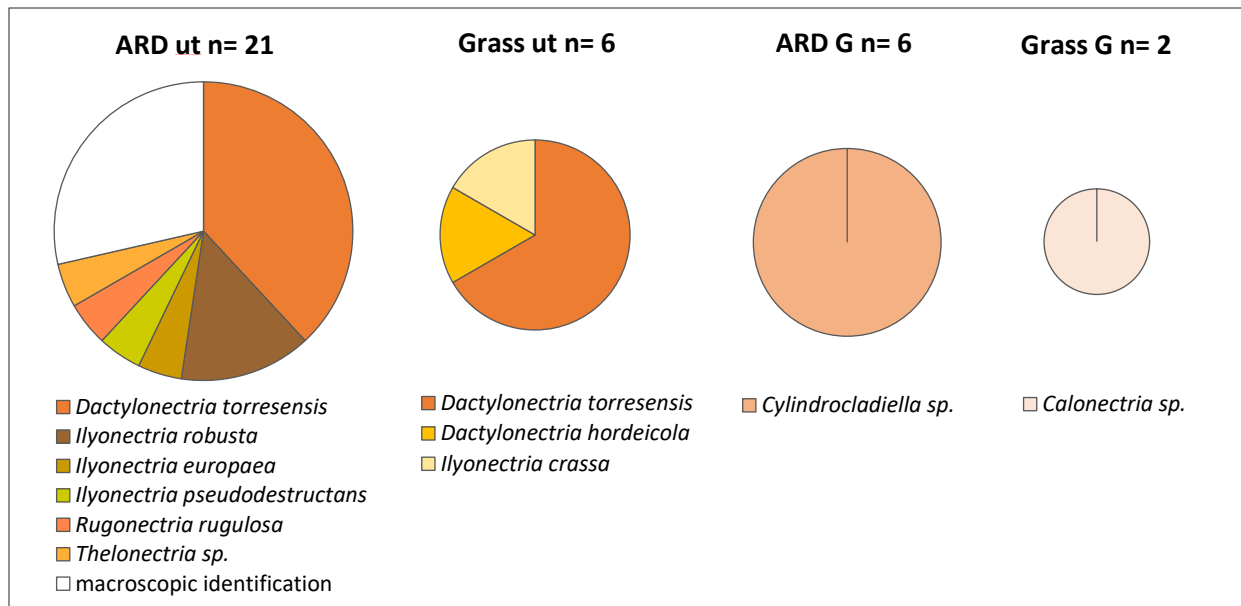
Fig. 2.5 Identification of Nectriaceae fungi in CE2. Isolation from surface disinfected roots of M26 and 'Bittenfelder' plants after 8 weeks culture in untreated (ut) and γ-irradiated (G) ARD soil and grass soil from all three reference site locations. n = number of isolated fungi of 48 root segments (from n = 4 plants). First identification by macroscopic features and *ITS* sequencing of Nectriaceae members (excluding *Fusarium*). In-depth species identification by Sanger sequencing of *histone 3* (*HIS*) genes

Nectriaceae isolates harvested in CE2 2017 were identified to the species level (Fig. 2.5). Comparing the treatments, most of Nectriaceae isolates were obtained from roots grown in untreated ARD soil. *D. torresensis* was most abundant in both untreated soils, ARD and grass. *I. robusta* could only be detected in root grown in untreated ARD soil like *I. europaea*, *I. pseudodestructans*, *Rugonectria rugulosa* and *Thelonectria* species. Next to *D. torresensis*, *D. hordeicola* and *I. crassa* isolates were obtained from roots grown in untreated grass soil. All six isolates harvested from roots out of irradiated ARD soil were identified as *Cylindrocladiella* species. From roots grown in irradiated grass soil two *Calonectria* isolates were found.

2.4   Discussion

ARD was successfully induced in the field experiment CE1. Clear differences in the shoot growth of ARD and Apple New plants were visible. Further, unexpected ARD effects resulting from small-scale soil heterogeneity could be excluded by using EMI soil sensing technology (Mahnkopp et al. 2018). The fungal endophyte community of apple consisted mainly of members of Helotiales, Hypocreales and Pleosporales. These groups of fungal endophytes are frequently reported to colonize plant roots (Knapp et al. 2012). For cost reasons, the Bittenfelder plants used in CE1 originated from a nursery and were raised from seeds in the field. Therefore,

already an initial fungal community could be detected in these plants (Table 2.1). However, it is supposed that plants raised in the same soil will accumulate a similar fungal community. Further, clear differences in the growth of plants in both treatments (Fig. 2.1) could be observed suggesting that the initial fungal community is not determining for the altered growth.

An enrichment of Nectriaceae fungi (former described as *Cylindrocarpon*-like fungi) in the fungal community of ARD plants could be found in both experiments (CE1 and CE2). Especially *I. robusta* and *D. torresensis* were most frequently identified in ARD roots. Nectriaceae fungi were often reported to be negatively correlated with plant growth and could be detected in ARD soils, rhizosphere soils and roots (Deakin et al. 2018; Franke-Whittle et al. 2015; Manici et al. 2013; Manici et al. 2018). In some experiments a pathogenicity of these fungi could be proved (Braun 1995; Dullahide et al. 1994; Mazzola 1998). In contrast to that, in other inoculation experiments Nectriaceae fungi only exhibited low infection rates and low to no pathogenicity although similar high colonization frequencies as found in our experiments were observed for roots grown in native soils (Manici et al. 2003; Manici et al. 2018; Tewoldemedhin et al. 2011a; Tewoldemedhin et al. 2011c). Apparently, there might be factors in the ARD soil that favor an infection of the roots by Nectriaceae fungi, which are missing under artificial inoculation conditions (Manici et al. 2018).

Compared to other members of Nectriaceae fungi, *Fusarium* spp. were not that frequently abundant. *Fusarium* species could be isolated from the field experiment CE1 and in the biotest CE2 from roots grown in untreated and irradiated soils. Results of other isolation experiments suggested that the *Fusarium* genus is irrelevant in context with ARD (Manici et al. 2003; Manici et al. 2013; Tewoldemedhin et al. 2011b).

The largest proportion of the fungal community of Bittenfelder plants in CE1 consisted of *Leptodontidium* spp., which is classified in the order Helotiales and seems to be a very common root endophyte (Lee et al. 2017; Nallanchakravarthula et al. 2014; Pecoraro et al. 2012; Upson et al. 2009b). In isolation experiments with strawberry and raspberry plants (both Rosaceae) in association with black root rot, *Cadophora/ Leptodontidium* spp. were the second most abundant group of fungal endophytes after Nectriaceae species (Weber and Entrop 2017). As reported there, especially *D. torresensis* was causing the black root rot. *Leptodontidium* belongs to the group of dark septate endophytes (DSE). Also, *Cadophora* spp. (Helotiales), *Alternaria* spp., *Herpotrichia* spp., *Periconia* spp., *Pyrenochaeta* spp. (Pleosporales) and *Zalerion* spp. (Lulworthiales) are referred to DSE. DSE frequently colonize plant roots in very different environments and ecosystems and are characterized by melanized, septate hyphae

(Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005; Newsham 2011). This group of fungal endophytes is polyphyletic and contains several taxa that belong to different fungal orders in Ascomycota (Jumpponen and Trappe 1998; Knapp et al. 2015). A clear demarcation of fungal species belonging to the DSE does not yet exist and depends on the definition by the authors (Knapp et al. 2012). This group of endophytes is worldwide distributed and often associated with environments under abiotic stress (Mandyam and Jumpponen 2005; Read and Haselwandter 1981). More than over 600 plant species are reported to be colonized by DSE, giving indications for no host specification (Jumpponen and Trappe 1998). The influence of the DSE colonization on the host plant is controversial (Mayerhofer et al. 2013): Inoculation experiments showed negative (Stoyke and Currah 1993; Tellenbach et al. 2011; Wilcox and Wang 1987) to positive plant responses (Newsham 1999; Upson et al. 2009a; Usuki and Narisawa 2007; Wu et al. 2010). Further, the ecological function of DSE is not yet understood (Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005). Therefore, different apple DSE isolates should be tested in the future in inoculation experiments for plant growth promoting or pathogenic effects, either alone or in combination with other fungal isolates or (ARD) soil organisms (e.g. collembola or nematodes).

The irradiation treatment of ARD soil in the biotest CE2 led to an increased plant growth (Mahnkopp et al. 2018). The reduction of the number of isolated fungi as well as a shift in the fungal endophyte community in irradiation treatments seemed to have a beneficial impact on plant growth. This further supports an involvement of fungal biotic factors in the disease complex. *Fusarium* spp. and *Trichoderma* spp., as typical saprobes and fast-growing fungi, were often reported as first colonizers in pasteurized or irradiated soils (Manici et al. 2013; Mazzola 1998).

The isolation experiments showed that some species or groups of fungal endophytes are common colonizers of apple plants. But there are also differences in the individual fungal endophyte community depending on the crops (ARD or grass soil) or soil type. Also, differences in the soil microbial communities between soils with grass cover and ARD affected soils were reported (Deakin et al. 2018; Radl et al. 2019). The fungal community of M26 and Bittenfelder plants, both cultivated in Ruthe soil in CE2, was quite similar. On the other hand, the soil origin had an effect on the individual root fungal community. Bonito et al. (2014) investigated the fungal root endophyte community in replicated plantings of *Populus*, *Quercus* and *Pinus* trees at three different field sites. There, the soil origin had a larger effect on the fungal community than the host species. The communities of fungal root endophytes seem to

be more influenced by the dispersal and biogeography than by the host availability (Bonito et al. 2014; Nallanchakravarthula et al. 2014).

Deviating to other reports, almost no *Rhizoctonia* or *Pythium* isolates were obtained in this isolation experiments. The few Basidiomycota isolates obtained belonged mostly to the Agaricomycetes. This class includes wood decaying fungi as well as white and brown rot saprophytes and beneficial ectomycorrhiza (Hardoim et al. 2015). Manici et al. (2013) conducted a biotest with rootstock M9 in ARD, fallow and sterilized (irradiated) soil. There, 87 % of total isolated root endophytes were *Fusarium* spp., *Cylindrocarpon*-like isolates, *Rhizoctonia* sp. and *Pythium* spp. Only in German orchards *Pythium* spp. prevailed as pathogen together with *Cylindrocarpon*-like fungi and therefore a site-specific effect of ARD pathogens could be demonstrated (Manici et al. 2013). Additionally, the authors reported that the occurrence of *Rhizoctonia* did not correlate with plant growth, suggesting a minimal role in the replant disease. Admittedly, the choice of the isolation medium as well as the duration of surface disinfection may have biased the number and species of isolated fungi. However, using water ager with a different combination of antibiotics in CE2 for isolation resulted in less isolated fungi but similar species. Further, only one *Pythium* isolate was obtained in CE2, suggesting that *Pythium* spp. are not the dominating pathogens at the ORDIAmur ARD field sites.

Fungal isolates obtained in the experiments CE1 and CE2 were identified by *ITS*-PCR. If any, most isolates could only be determined up to the genus level. One problem created by the increasing number of culture-independent investigations is the increase of uncharacterized microorganisms in the data bases together with ambiguous results caused by continuous changes in taxonomy by phylogenetic studies and old, remaining entries in the databases (Brader et al. 2017; Hofstetter et al. 2019). To give one example: Isolates assigned to *Zalerion* sp. were frequently isolated from apple roots. BLASTn search with the sequence in the NCBI data base gave also hits with the same max. score to the order Helotiales and Halenospora sp., which belongs to Helotiales. According to mycobank.ork one species, *Halenospora varia*, is recorded, but its current name is *Zalerion varia*. The latter belongs to a completely different order Lulworthiales. Regardless, taxonomy cannot necessarily be linked to function, since even stains of the same species might display different pathogenicity or other traits (Aimé et al. 2013; Hardoim et al. 2015). For this reason, inoculation experiments are indispensable to investigate the biology of fungal isolates.

2.5   Conclusion

Extensive isolation experiments from ORDIAmur ARD field sites with traceable and comparable cropping history revealed insights in the fungal root endophyte community. An enrichment of Nectriaceae fungi, especially of species *D. torresensis* and *I. robusta*, was found in ARD roots suggesting an involvement as pathogens in the replant disease complex. Further, members of DSE belonging to Helotiales and Pleosporales seem to be reduced in their abundance. Up to now, little is known of the ecology of this group of fungal endophytes. The role of Nectriaceae fungi as possible pathogens as well as of DSE in context with ARD should be addressed in further experiments.

3. A soil-free method for assessing pathogenicity of fungal isolates from apple roots

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4.   Molecular identification of Nectriaceae in infections of apple replant disease affected roots collected by Harris Uni-Core punching or laser microdissection

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5.   Sequence of an alternavirus from *Dactylonectria torresensis*

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Abstract

Dactylonectria torresensis isolate O6-1-A obtained from replant diseased apple roots was subjected to double-stranded RNA (dsRNA) extraction. Illumina sequencing was applied and *de novo* assembled sequences were confirmed by RT-PCR followed by 5'-RACE and 3'-end determination. Thereby a novel dsRNA virus was detected named Dactylonectria torresensis alternavirus 1 (DtAV1), which is a putative member of the newly proposed mycoviral family "*Alternaviridae*". The genome of DtAV1 is composed of three dsRNA segments, dsRNA1 (3578 bp), dsRNA2 (2668 bp) and dsRNA3 (2467 bp), each encoding one open reading frame. BLASTp analyses gave best hits to Fusarium poae alternavirus 1. Phylogenetic analyses further strengthened the affiliation of DtAV1 to "*Alternaviridae*". This is the first report of a mycovirus in *D. torresensis* that is a putative member of "*Alternaviridae*". The influence of the mycoviral infection on the host fungus and apple plants needs to be further investigated.

5.1   Introduction

Mycoviral infections are reported for all major taxonomic groups of fungi (Ghabrial et al. 2015). Mycoviruses with a double-stranded (ds) RNA genome are grouped according to the International Committee on Taxonomy of Viruses (ICTV) into seven families (*Chrysoviridae, Endornaviridae, Megabirnaviridae, Partitiviridae, Quadriviridae, Reoviridae and Totiviridae* and one genus *Botybirnavirus* (Kotta-Loizou and Coutts 2017). Kozlakidis et al. (2013) proposed the family "*Alternaviridae*" and the genus "*Alternavirus*" with Alternaria alternata virus 1 (AaV-1) (Aoki et al. 2009) as its type virus. So far, six more viruses are reported to be members of "*Alternaviridae*": Aspergillus mycovirus 341 (AMV) (Hammond et al. 2008), Aspergillus foetidus dsRNA mycovirus (AfMV) (Kozlakidis et al. 2013), Aspergillus heteromorphus alternavirus 1 (AheAV1) (Gilbert et al. 2019), Fusarium poae alternavirus 1 (FpAV1) (Osaki et al. 2016), Fusarium graminearum alternavirus 1 (FgAV1) (He et al. 2018) and Fusarium incarnatum alternavirus 1 (FiAV1) (Zhang et al. 2019).

Apple replant disease (ARD) is often associated with biotic factors as potential cause and especially fungi of the family Nectriaceae (also known as *Cylindrocarpon*-like fungi) became focus of investigations in the recent years (Manici et al. 2013; Manici et al. 2018; Popp et al. 2019; Tewoldemedhin et al. 2011c). Mycoviral infections can influence their host fungi in several ways. The virus may be able to modulate the virulence or toxin production of its fungal host and thus alter the interaction between fungus and plant (Hammond et al. 2008; Márquez et al. 2007). For example, Cryphonectria hypovirus 1 (CHV1) causes hypovirulence of the tree pathogen *Cryphonectria parasitica*, which causes chestnut blight (Shapira et al. 1991). On the other hand, a viral dsRNA up regulates the virulence of *Nectria radicicola* (current name *Ilyonectria destructans*, (Zinssm. Rossmann, L. Lombard & Crous, Studies in mycology 80: 217 (2015) [MB#810954]), the causal fungus of ginseng root rot (Ahn and Lee 2001). Further, a viral infection in *Fusarium graminearum* (strain DK21) reduced the mycotoxin production (trichothecene, 60-fold) besides other changes (Chu et al. 2002). In addition, mycoviruses with their potential as sustainable biocontrol agents of plant pathogenic fungi are getting more attention during the recent years (Xie and Jiang 2014; Yu et al. 2013).

Since sustainable mitigation strategies of the replant disease are still missing (Winkelmann et al. 2019), Nectriaceae isolates obtained from surface disinfected replant diseased roots were screened for mycoviral infections by dsRNA extraction. Here we describe a new alternavirus found in *Dactylonectria torresensis* by a deep sequencing approach of dsRNA and a phylogenetic analysis of its putative RNA-dependent RNA polymerase (RdRp). In addition, the

presence of tentatively named Dactylonectria torresensis alternavirus 1 (DtAV1) was confirmed by RT-PCR amplification performed on total nucleic acid extracts of *D. torresensis*.

5.2 Material and Methods

5.2.1 Fungal isolate

A total of 41 Nectriaceae isolates obtained in central experiment 2 (CE2) in 2017 (Mahnkopp et al. 2018) from apple roots grown in ARD soils were screened for mycoviral infections by dsRNA extraction. The fungal isolate of *D. torresensis* (O6-1-A, Fig. 5.1) was isolated from roots grown in ARD soil from the experimental site Ellerhoop (x-coordinate 53.71435; y-coordinate 9.770143 WGS 84, Schleswig-Holstein, northern Germany). Surface disinfected roots were plated on 1.5 % water agar amended with penicillin (50 µg mL^{-1}), rifampicin (10 µg mL^{-1}) and pimaricin (25 µg mL^{-1}). Sub-culturing was done on 2 % malt extract agar. The isolate was identified as *D. torresensis* by PCR amplification of *histone 3* gene (*HIS*) using primer pairs CYLH3F and CYLH3R (Crous et al. 2004) and *translation elongation factor 1-α* gene (*TEF*) (CylEF-1 (5′-ATG GGT AAG GAV GAV AAG AC-3′; J.Z. Groenewald, unpublished), and CylEF-R2 (Crous et al. 2004), followed by Sanger sequencing and NCBI BLASTn analysis (Popp et al. 2019).
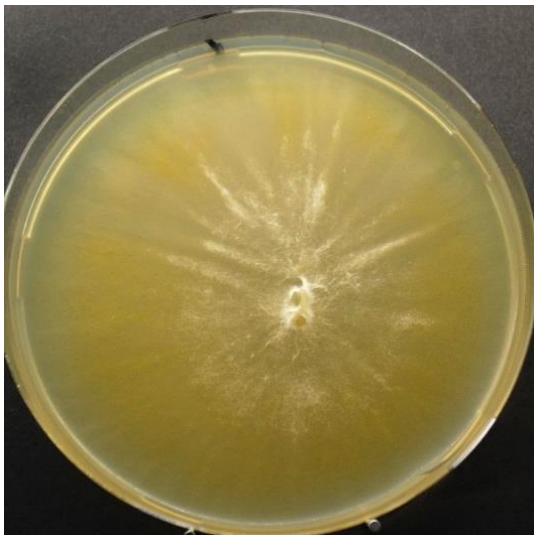

Fig. 5.1 Example of *Dactylonectria torresensis* isolate O6-1-A; 21 days after growth on malt extract agar

5.2.2   Extraction of dsRNA

Fungal mycelium was grown in 2 % malt extract broth for at least one week, grinded in liquid nitrogen and stored at -80 °C until use. For extraction a modified protocol of Morris and Dodds (1979) was applied as described in Lesker et al. (2013), besides using another cellulose (acid washed, powder for column chromatography, Merck, Darmstadt, Germany). For further purification of dsRNA, the eluate was digested with RNase T_1 and DNase I (Roche, Mannheim, Germany). Finally, the dsRNA pellet was suspended in 20 µl 5 mM Tris.

5.2.3   Illumina library preparation and de novo assembly

Illumina library preparation was performed as described in Knierim et al. (2019): ribosomal RNA was removed (RiboMinus Plant Kit, Invitrogen, Carlsbad, USA) followed by random cDNA synthesis with random octamer primers (RevertAid H Minus Reverse Transcriptase, Thermo Fisher Scientific, Waltham, USA), second strand synthesis (NEBNext, mRNA Second Strand Synthesis Module, NEB, Ipswich, USA), library preparation (Nextera XT Library Kit, Illumina, San Diego, USA), DNA quantification (Qubit dsDNA HS Assay Kit, Life Technologies, Carlsbad, USA) and quality analyses (High Sensitivity DNA Chips, Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, USA). The run was performed as paired-end reads on a MiSeq sequencer (Illumina 2x301, DSMZ, Braunschweig, Germany). Raw reads were trimmed (1 % error probability limit) and *de novo* assembled using Geneious software (Biomatters, Auckland, New Zealand). Consensus sequences were built out of the first 1000 contigs and translated into all possible frames. A local BLASTp was performed with these 6000 protein sequences against a local virus database to identify viral contigs.

5.2.4   RT-PCR and RACE

To confirm the results of the deep sequencing and to complete the sequences, primers were designed based on the *de novo* assembled virus sequences (all primers used are listed in Table 5.1). Total nucleic acids of the fungal isolate were extracted using silica particles (Menzel et al. 2002). For cDNA synthesis 3 µl total nucleic acid extract was incubated with 1 µl of anti-sense primer (primer No. 2, 4 or 6 for RNA1-3, respectively) at 99 °C for 3 min and rapidly cooled on ice. The cDNA mix consisted of 2 µl RT buffer, 0.5 µl dNTPs (10 mM, Roth, Karlsruhe, Germany), and 1 µl RevertAid reverse transcriptase (20 U µl^{-1}, Thermo Fisher, Waltham, USA) in a total volume of 10 µl and was incubated for 1 h at 42 °C. The PCR mix included 5 µl Phusion Flash High Fidelity PCR Master Mix (Thermo Fisher, Waltham, USA), 0.5 µl of each sense and anti-sense identification primer (primer No. 1-6) for the corresponding

RNA (10 µM) and 1 µl cDNA in a total volume of 10 µl. The cycle conditions were 98 °C for 15 s, followed by 34 cycles at 98 °C for 15 s, 62 °C for 5 s and 72 °C for 20 s, followed by a final elongation at 72 °C for 5 min. RT-PCR was performed in a C100 thermal cycler (BioRad, Hercules, USA).

Table 5.1 Primer designed to verify *de novo* assembled virus sequences by RT-PCR

Primer No.	Primer designation	Sequence
1	DacRNA1_ident_s	CGTATGAAGAACTGTTGGCTACCCG
2	DacRNA1_ident_as	CGACATCATCAGCACGATTGAGGG
3	DacRNA2_ident_s	CCGTGCCTTAACAAGCCTGGG
4	DacRNA2_ident_as	GCCTTGTCAGCAGATCCATGCC
5	DacRNA3_ident_s	CGCTTTCATGCCATCGGTGAG
6	DacRNA3_ident_as	CGGAAATCATTGACACCACGACC
7	Dac_1_5end	CGCTGCGCACCAACAAATTC
8	Dac_2_5end	GACGGCTACCGAGAGGAAGTTAGC
9	Dac_3_5end	GTACACCTCGTGCGTAGGATCG
10	Dac_1_5_nested	TCCTCCGCTTATTGATATGC
11	Dac_2_5_nested	TTGATATAGGACACCTTGCCAGTCTGAG
12	Dac_3_5_nested	TCGGCCTGATCAGGCATTTTGAAC
13	Dac_1_3_end	GCATGGGACAAGTTGATACCGC
14	Dac_2_3_end	CGTACTTGCAGCCGCCAACGC
15	Dac_3_3_end	GATGCGGCGTACCGTGCATCG
16	Poly-G15	CTCAAACAGTCACGGGGGGGGGGGGGGG
17	Poly-C14	ATCCTGCAGGCGCGCCCCCCCCCCCCCC
18	RACE-BOE1	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT(AGC)
19	RACE-BOE2	GACCACGCGTATCGATGTCGA

For rapid amplification of 5'-ends (RACE) cDNAs were synthesized as described above with primer No. 7-9 (Table 5.1). After purification with SureClean Plus (Bioline, London, UK), 3 µl of each cDNA was tailed in a 20 µl reaction volume with C and G (5 mM), respectively, using Terminal deoxynucleotidyl transferase (20 U) for 30 min at 37 °C. 3 µl of each tailed cDNA was used in PCR reactions using nested primers specific for RNA1-3 (primer No. 10-12) together with a poly-G or poly-C primer (primer No. 16 and 17). The 3'-ends were determined by using a modified oligo (dT) 16 primer (primer No. 18), a nested primer (primer No. 19) and a primer specific for RNA1-3 (primer No. 13-15).

5.2.5 *Phylogenetic analysis*

A complete alignment with full RdRp amino acid sequences of all known members of "*Alternaviridae*" was carried out by using Muscle software in MEGA 6 software (Tamura et al. 2013). The model with the smallest BIC value was applied (LG G) to build a maximum likelihood phylogenetic tree using the bootstrap method with 1000 replications. Hubei toti-like virus 7 was used as an outgroup according to Kotta-Loizou and Coutts (2017).

5.2.6 *Alignment*

Untranslated region (UTR) of the 5'-end sequences were aligned in ClustalX2.1. Amino acid motives of dsRNA1 of "*Alternaviridae*" described by Gilbert et al. (2019) were searched and marked by using the program SnapGene Viewer 4.3.7.

5.3   Results

A total of 41 Nectriaceae isolates were screened for mycoviral infections by dsRNA extraction. In seven isolates dsRNA fragments were visible after gel electrophoresis. Gel electrophoreses of the dsRNA extract obtained from *D. torresensis* isolate O6-1-A showed four fragments with approximately sizes of 3.5, 2.8, 2.5 and 1.5 kb (Fig. 5.2, lane 4).

After Illumina sequencing of dsRNAs, a total number of 204 000 reads was generated which were assembled into three contigs. All three contigs could be confirmed by RT-PCR out of total nucleic extracts. Following 5'-RACE and 3'-end determination RNA1 consists of 3578 bp, RNA2 of 2668 bp and RNA3 of 2467 bp excluding the poly-A tail (Fig. 5.3). No contig was generated for the fourth dsRNA fragment. So far, separate cloning and sequencing approaches with the fourth dsRNA delivered no sequence similarities after BLASTx with known viral sequences.

Each RNA contains one open reading frame (ORF). The ORF of RNA1 (nt position 63-3437) has a size of 1124 aa with an estimated molecular weight of 126.6 kDa. This corresponded with the frame sizes of segment 1 of AMV, AfMV and AheAV1 (Table 5.2). BLASTp analyses gave the best hit with the RdRp sequence of Fusarium poae alternavirus 1 (Ident 58.3 %). The ORF of dsRNA2 (nt position 67-2334) has a size of 755 aa with a predicted molecular weight of 81.8 kDa. The amino acid sequence of segment 2 fitted also best to Fusarium poae alternavirus 1 ORF2 (Ident 37.5 %). The ORF of dsRNA3 (nt position 76-2289) consists of 737 aa with a molecular weight of 79.7 kDa. BLASTp analysis revealed again the highest similarities to Fusarium poae alternavirus 1 ORF of RNA3 (Ident 42.1 %).
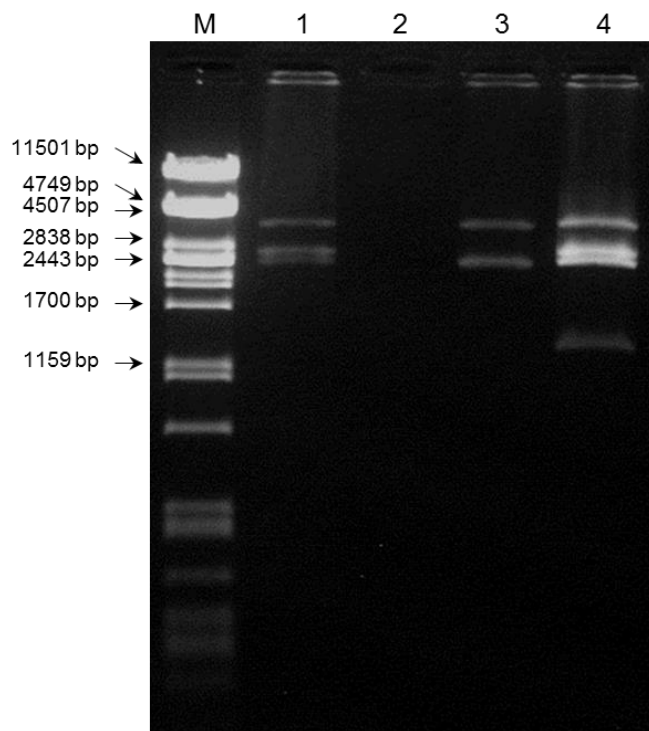
Fig. 5.2 Gel electrophoresis of dsRNA extracts. From the left: Size standard (M; *Pst*I digested Lambda DNA), lane 1 isolate 22-1-B (*Dactylonectria torresensis*), lane 2 isolate 20-1-B (*D. torresensis*), lane 3 isolate O16-2-D (*Ilyonectria robusta*) and **lane 4** isolate O6-1-A (*D. torresensis*)
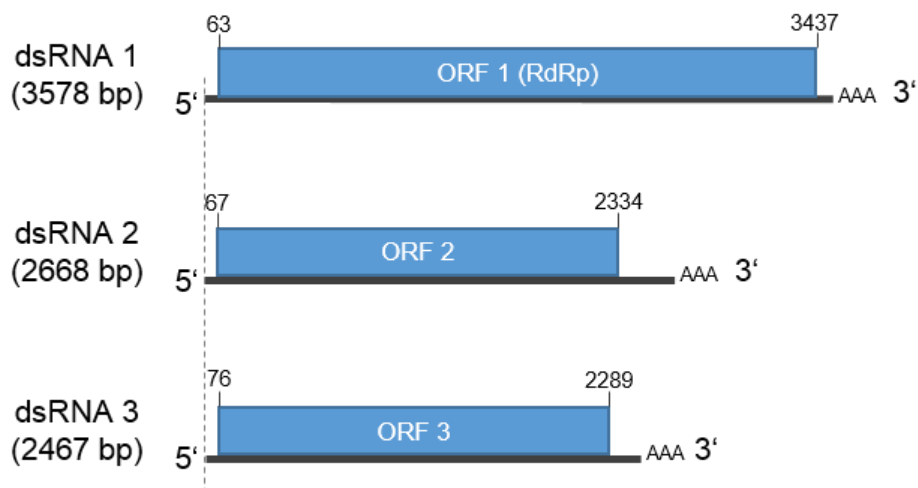

Fig. 5.3 Genome organization of Dactylonectria torresensis alternavirus 1: The genome consisted out of three dsRNA molecules each encoding one open reading frame (ORF) (blue box). Untranslated regions (UTR) are marked as single lines. ORF1 included motives of the RNA-dependent RNA polymerase (RdRp). ORF2 and 3 depicted hypothetical proteins. Each RNA had a poly(A) tail at the 3'-end.

Table 5.2 Overview of "*Alternaviridae*": Virus name and abbreviation as given in the GenBank (www.ncbi.nlm.nih.gov/) or original literature. Number of double-stranded RNA (dsRNA) segments; segment size in base pairs (bp); Open reading frame (ORF) size with nt= nucleotides, aa= amino acids and estimated molecular weight in kilo Dalton (kDa). Protein Function with RNA-dependent RNA polymerase (RdRp) and hypothetical protein (Hyp). Untranslated regions (UTR) length; 3'- UTR without poly A-tail

Virus name	Abbre-viation	Literature	Original host	dsRNA segments	Segment (bp)	Acession Nr.	ORF size (nt; aa; kDa)	Func-tion	UTR length (bp) 5'-UTR	3'-UTR
Alternaria alternata virus 1	AaV-1	Akoi et al. 2009	*Alternaria alternata* EGS 35-193	4	dsRNA1 (3613)	AB368492	3450; 1149; 129	RdRp	48	69
					dsRNA2 (2794)	AB438027	2535; 844; 91	Hyp	52	157
					dsRNA3 (2576)	AB438028	2280; 759; 82	Hyp	51	209
					dsRNA4 (1420)	AB438029	1182; 393; 41	Hyp	50	147
Aspergillus mycovirus 341	AMV	Hammond et al. 2008	*Aspergillus niger* 341	4	dsRNA1 (3,588)	EU289897	3,375; 1,124; 127	RdRp	51	145
Aspergillus foetidus dsRNA mycovirus	AfMV	Kozlakidis et al. 2013	*Aspergillus foetidus* IMI 41871	4	dsRNA1 (3,588)	HE588144	3,375; 1,124; 127	RdRp	51	145
					dsRNA2 (2,770)	HE588145	2,406; 801; 87	Hyp	48	280
					dsRNA3 (2,466)	HE588146	2,181; 726; 79	Hyp	50	187
					dsRNA4 (2,005)	HE647818	1,743; 580; 65	Hyp	50	168
Aspergillus heteromorphus alternavirus 1	AheAV1	Gilbert et al. 2019	*Aspergillus heteromorphus* isolate CBS 117.55	3	dsRNA1 3576	MK279437	3375; 1,124; 127	RdRp	42	142
					dsRNA2 2742	MK279438	2502; 833; 91	Hyp	48	184
					dsRNA3 2427	MK279439	2184; 727; 79	Hyp	49	184
Fusarium poae alternavirus 1	FpAV1	Osaki et al. 2016	*Fusarium poae* MAFF 240374	3	dsRNA1 3559	LC150613	3372; 1123; 126	RdRp	82	105
					dsRNA2 2496	LC150614	2271; 756; 84	Hyp	82	120
					dsRNA3 2482	LC150615	2232; 743; 81	Hyp	77	153
Fusarium graminearum alternavirus 1	FgAV1	He et al. 2018	*Fusarium graminearum* AH11	3	dsRNA1 3524	MG254901	3372; 1123; 126	RdRp	80	72
					dsRNA2 2470	MG254902	2271; 756; 84	Hyp	79	120
					dsRNA3 2485	MG697236	2232; 743; 81	Hyp	77	151
Fusarium incarnatum alternavirus 1	FiAV1	Zhang et al. 2019	*Fusarium incarnatum* LY003-07	3	dsRNA1 3548	MH899114	3372; 1123;126	RdRp	82	71
					dsRNA2 2514	MH899115	2271; 756; 84	Hyp	80	118
					dsRNA3 2498	MH899116	2220; 739; 81	Hyp	78	153

Phylogenetic analyses of dsRNA1 showed that the putative RdRp sequence of this mycovirus found in *D. torresensis* fitted very well in mycoviruses of the proposed family "*Alternaviridae*" (Fig. 5.4). It is more closely related to the virus found in *Fusarium* species compared to the viruses in other fungi (*Aspergillus* and *Alternaria*). Like AheAV1, FpAV1, FgAV1 and FiAV1 the genome of DtAV1 is tripartite.
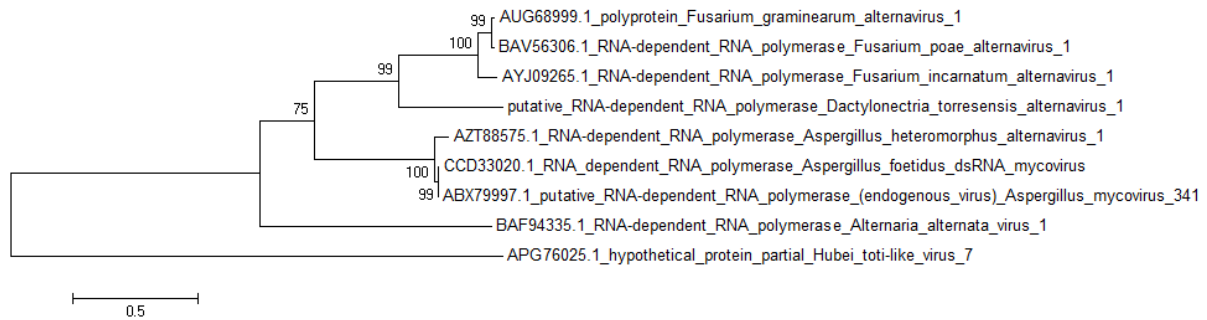

Fig. 5.4 Phylogenetic tree of the proposed family "*Alternaviridae*": Amino acid sequences of putative RdRp sequences of the viruses available in the NCBI GenBank were aligned with MUSCLE and a maximum likelihood phylogenetic tree was built using MEGA 6 software. Bootstrap values (%) obtained with 1000 replicates are indicated on branches and branch lengths correspond to genetic distance; scale bar at lower left corresponds to a genetic distance of 0.5. Accession Numbers: Alternaria alternata virus 1 (BAF94335.1), Aspergillus mycovirus 341 (ABX79997.1), Aspergillus foetidus dsRNA mycovirus (CCD33020.1), Aspergillus heteromorphus alternavirus 1 (AZT88575.1), Fusarium poae alternavirus 1 (BAV56306.1), Fusarium graminearum alternavirus 1 (AUG68999.1), Fusarium incarnatum alternavirus 1 (AYJ09265.1) and Hubei toti-like virus 7 (APG76025.1) as an outgroup (Kotta-Loizou & Coutts 2017)

Fig 5.5 Alignment of 5'-UTR of RNA1-3. Alignment in ClustalX2.1

Eight amino acid motives were found on RNA1 including the ADD motive for the RdRp of "*Alternaviridae*" (addendum Table 8.4). An alignment of the 5'-UTR sequences of RNA1-3 revealed conserved sequences which are GCT (T/C) TTA and TC (A/G) A (G/A) TAGGC (Fig. 5.5).

5.4   Discussion

Deep sequencing analysis of the dsRNA extract of *D. torresensis* isolate O6-1-A revealed that the fungus is infected by a mycovirus, which is a putative member of the proposed family "*Alternaviridae*". Although after gel electrophoresis of the dsRNA extract four fragments were visible on the gel the *de novo* assembly gave only hits for three dsRNA segments. According to its size of approximately 1.5 kb the smallest fragment of the dsRNAs would fit to RNA4 of AaV-1 and AfMV. In contrast, the fragment size is too small to associate it to a member of the *Chrysoviridae*. For this virus family four to five dsRNAs are reported. In *Fusarium* species the smallest fragment of the *Chrysoviridae* is always > 2 kb (Fusarium graminearum virus-ch9; dsRNA5 2423 bp (Darissa et al. 2011); Fusarium graminearum virus 2; dsRNA5 2414 bp (Yu et al. 2011); Fusarium oxysporum f. sp. dianthimycovirus 1; dsRNA4 2646 bp (Lemus-Minor et al. 2015). For "*Alternaviridae*" viruses with both four (AaV-1, AMV, AfMV) and three dsRNA segments (AheAV1, FpAV1, FgAV1, FiAV1) are described. Furthermore, it was reported that during mycovirus transfer the smallest dsRNA of AMV got lost and was not detectable (Hammond et al. 2008). Other mycoviral infections in this fungal isolate cannot be excluded, but no further RdRp sequences were found in the deep sequencing approach as well as no hits to sequences of RNA4 of other members of the "*Alternaviridae*".

Phylogenetic analyses revealed that DtAV1 fits well in the family of "*Alternaviridae*". It seems to be more related to the "*Alternavirus*" of *Fusarium* species, which are like *D. torresensis* members of the fungal family Nectriaceae. "*Alternaviridae*" are related to *Totiviridae* and *Chrysoviridae*, but there is a clear delineation between these viral families (Gilbert et al. 2019).

Gilbert et al. (2019) described eight conserved motives in the amino acid sequences of dsRNA1 for different members of "*Alternaviridae*". These motives could also be detected in the amino acid sequence of DtAV1 RNA1. Moreover, as with other members of "*Alternaviridae*", the RdRp domain ADD could be detected in motive VI instead of GDD, which is almost universally conserved in other virus genera (Gilbert et al. 2019). By aligning 5'-UTR sequences of RNA1-3, conserved domains were discovered. Viruses with a multipartite genome often have conserved sequences within the 5'-UTR (Gilbert et al. 2019). Conserved sequences in the 5'-UTR were also described for AaV-1 (Aoki et al. 2009), AfMV (Kozlakidis et al. 2013), AheAV1 (Gilbert et al. 2019) and FiAV1 (Zhang et al. 2019).

In summary, evidence that DtAV1 is a member of "*Alternaviridae*" is given by the fact that protein comparisons (BLASTp analyses) gave best hits to FpAV1. Phylogenetic analyses of the amino acid sequence of RNA1 further confirmed this. Members of "*Alternaviridae*" have an

ADD motive (RdRp) on RNA1 instead of GDD. Further amino acid motives similarities of RNA1 as described by Gilbert et al. (2019) were also found.

In *Alternaria alternata* the infection with AaV-1 led to an impaired growth phenotype that was correlated with high concentrations of dsRNA. The isolate exhibited reduced mycelia growth, aerial mycelia collapse, unregulated pigmentation and cytolyses. Abnormally enlarged vesicles resulted in the burst of mycelial cells (Aoki et al. 2009). In contrast to that, in *Aspergillus niger* the AMV infection had no marked effect on the host fungus (Hammond et al. 2008). For all other viruses of "*Alternaviridae*" virus-host interaction studies are missing. *D. torresensis* is a pathogen involved in the apple replant disease complex that infects the roots (Popp et al. 2019). Manici et al. (2018) investigated cultural filtrates of *D. torresensis* isolated from ARD diseased apple roots. The authors reported the production of toxins (tentoxin, HC toxin and zearalenone) and low molecular weight compounds (rabelomycin and nidulin) by the fungus together with phytotoxic activity of culture filtrates (reduced root elongation and tissue softening). Therefore, it is of great interest to study the effect of the mycoviral infection regarding a possible control strategy of the replant disease. Experiments are in progress to cure the fungal isolate and to investigate the effects of the virus infection on the host fungus *D. torresensis* and possible effects respecting the plant-fungus-virus interaction.

This is the first report of a virus belonging to "*Alternaviridae*" in *D. torresensis*. Therefore, we suppose the name Dactylonectria torresensis alternavirus 1 (DtAV1).

6.   General Discussion

6.1   Fungal Endophytes

6.1.1   Involvement of fungal endophytes in ARD

One aim of this work was to investigate whether fungal endophytes are involved in the development of ARD. Here, a culture-dependent approach was used to characterize and quantify the fungal endophyte community (Chapter 2). Fungal isolates are essential to investigate the relationship with apple plants for functions such as pathogenicity or plant growth promotion and for further interactions with other ARD organisms. On the other hand, culture-independent approaches would have allowed the detection of also yet unculturable microorganisms (Brader et al. 2017).

The isolations from experiments CE1 and CE2 confirmed the hypothesis that there are differences in the fungal root endophyte community comparing apple plants grown in control soil and ARD soil. An enrichment of Nectriaceae species, especially of *D. torresensis* and *I. robusta*, was found for roots grown in ARD soil compared to Apple New soil (CE1, Chapter 2). This could also be confirmed in experiment CE2: Only a few Nectriaceae isolates were obtained from roots grown in untreated grass soil. On the other hand, isolates assigned to Helotiales, like *Leptodontidium* spp., *Cadophora* spp., and *Mycochaetophora* spp., occurred frequently in this variant. Further, the latter isolates are characterized by dark, septate mycelium and can be considered as DSE fungi. In contrast, much more Nectriaceae isolates were gained from roots grown in untreated ARD soils, while on the other hand the number of DSE isolates were in most cases less frequently found in the untreated ARD soil variants. Same tendencies were observed for all reference sites, which have different soil types but share a defined cropping history (Mahnkopp et al. 2018). The gamma irradiation of the soil resulted in a clear reduction in the number of isolated fungi and a complete different fungal community compared to untreated soils, while the plant growth was significantly increased. This indicates that biotic agents are involved in the disease development of ARD. The perlite test (Chapter 3) has shown that some fungal isolates are able to directly infect apple roots leading to strong wilting of shoots and plant mortality. This further supports the hypothesis of an involvement of fungi in the development of ARD. Nectriaceae isolates of *Calonectria* sp., *D. torresensis, I. crassa, I. europaea* and *I. robusta* caused severe wilting and killed M26 plants in the test already after 14 dpi. These fungi were re-isolated and were thereby confirmed as pathogens for apple plants. In addition, the PCR analyses of Harris samples and LMD thin-sections (Chapter 4) have shown that a complex of Nectriaceae species produces intracellular CF-like structures during cell

infection with further tissue necroses. This validates the hypothesis that some fungal isolates are pathogens and have a negative influence on the growth of apple plants. But also, an isolate of *Cadophora luteo-olivacea* belonging to DSE fungi caused severe wilting, stunting and necrosis of M26 plants at 14 dpi in the perlite test. However, this isolate was not re-isolated. Other DSE isolates obtained from roots grown in untreated ARD soils in CE2, like *Leptodontidium* sp. or *Pleotrichocladium* sp. did not display such strong effects. The role of DSE and Nectriaceae fungi in context of ARD will be discussed in the following sub-chapters.

6.1.2   *The role of DSE fungi in the context of ARD*

In this work, DSE fungi were frequently isolated from apple roots. Especially *Leptodontidium* spp. seems to be a typical plant endophyte (Lee et al. 2017; Nallanchakravarthula et al. 2014; Pecoraro et al. 2012; Upson et al. 2009b). Manici et al. (2013) isolated also *Cadophora* spp. from apple, but these were not further discussed. In investigations of black root rot in other members of Rosaceae like strawberry and raspberry, *Cadophora* and *Leptodontidium* spp. were the second most abundant group of fungal endophytes after Nectriaceae species (especially *D. torresensis*) (Weber and Entrop 2017). In general, DSE are ubiquitous in occurrence and can often be traced in stressed environments (Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005; Read and Haselwandter 1981). DSE were reported in over 600 plant species and the effect of colonization is not clear and can vary from positive over neutral to negative (Jumpponen and Trappe 1998; Mayerhofer et al. 2013; Newsham 2011). In the perlite test (Chapter 3), the DSE fungi isolated from roots grown in untreated ARD soils in CE2 2017 showed varying effects after 42 dpi: M26 plants inoculated with *Pleotrichocladium* sp. (Pleosporales) and *Zalerion* sp. (Lulworthiales) appeared healthy, while *Leptodontidium* sp. (Helotiales) as well as *Pyrenochaeta* sp. (Pleosporales) led to shortened shoots and the latter also to mild wilting. On the other hand, inoculation with *Cadophora luteo-olivacea* (Helotiales) resulted in negative effects on the plant performance with shortened shoots and strong wilted and necrotic leaves. However, no re-infection trails were carried out with this group of fungi to confirm them as pathogens.

In general, DSE fungi are considered to be multi-functional (Mandyam and Jumpponen 2005): Like mycorrhizal fungi, DSE can facilitate the nutrient uptake from the rhizosphere, which can promote plant growth and increase plant fitness. This might further indirectly protect the plant by the reduction of carbon sources in the host rhizosphere for other pathogens. But in contrast to the arbuscules of AMF, DSE fungi lack a specialized interface for the transfer of nutrients between fungus and plant (Peterson et al. 2008). In addition, DSE can produce secondary

metabolites which are toxic to herbivores and which can promote plant growth (Mandyam and Jumpponen 2005; Newsham 1999, 2011). The melanin in the DSE fungal cell wall helps fungi to survive in harsh environments and to protect from stresses like oxidizing agents and ultraviolet light (Eisenman and Casadevall 2012). Mandyam and Jumpponen (2005) suggested that DSE fungi with their broad host spectrum may control plant community dynamics by the varying host responses to colonization.

Despite the very frequent isolations from surface disinfected root pieces, typical DSE structures such as microsclerotia and melanized hyphae were rarely described in the detailed microscopic and histological analyses of apple and rose roots conducted by Grunewaldt-Stöcker et al. (2019, unpublished data). In contrast to that, DSE isolates inoculated in the perlite test were able to build such structures (Chapter 3). The reason for the few observations of melanized hyphae might be that DSE fungi are also able to form non-melanized, hyaline hyphae in their plant hosts, which are hard to detected by microscopy (Haselwandter and Read 1982; Newsham 1999; Yu et al. 2001). These hyphae could not be stained with Trypan blue, a chitin targeting stain that is often used in the microscopy of roots, indicating that the colonizing fungus produces low amounts of chitin or develops a poor fungal cell wall (Barrow and Aaltonen 2001). For detection, the usage of a lipid specific stain, e.g. Sudan IV, together with DIC microscopy is necessary (Barrow and Aaltonen 2001). Yu et al. (2001) suggested that the hyaline hyphae produced by melanized DSE fungi often went unnoticed in microscopic studies and that this resulted in an underestimation of the true abundance of DSE. However, already after two weeks of cultivation in ARD affected soil, the roots of apple are extensively colonized by Nectriaceae fungi which may limit the establishment of DSE fungi in the roots (Grunewaldt-Stöcker et al., unpublished data). This might be another reason for the few DSE observations. Therefore, it would be interesting to expose a DSE pre-colonized apple plant to different Nectriaceae species. The DSE colonization might then reduce the available space for other endophytes (Mandyam and Jumpponen 2005). Co-inoculation experiments should be carried out to further investigate the interaction between DSE fungi, Nectriaceae and other ARD associated organisms like nematodes or collembola.

The diverse group of DSE fungi may contain pathogens as well as species with positive effects on apple plant similar to mycorrhizal fungi. But in contrast to the obligate symbiotic AMF, many DSE can be cultured axenically. This make these fungi very interesting for applications as biofertilizers (Mandyam and Jumpponen 2005), which may help to enhance the productivity in ARD affected apple orchards. So far, the role of DSE fungi in context of ARD remains elucidated and should be further investigated in the future.

6.1.3   The role of Nectriaceae in context of ARD

Nectriaceae, which include the former known group of *Cylindrocarpon*-like fungi, were frequently reported to be involved in the etiology of ARD (Braun 1995; Dullahide et al. 1994; Manici et al. 2013; Manici et al. 2018; Mazzola 1998). The fungal species *Cylindrocarpon destructans* (current name *Ilyonectria destructans,* former teleomorph *Nectria radicicola*) was often described to cause root rot in several plants, e.g. blackberry (Cedeño et al. 2004), grapevine (Rego et al. 2001), ginseng (Kernaghan et al. 2007), oak (Sánchez et al. 2002), and scots pine (Unestam et al. 1989). Samuels and Brayford (1990) detected morphological variations in a collection of *C*. *destructans*. Up to now, phylogenetic analyses revealed that *Cylindrocarpon destructans* is a species complex, and a lot of new species were established within the *Cylindrocarpon*-like fungi (Agustí-Brisach et al. 2016; Brayford et al. 2004; Cabral et al. 2012a; Cabral et al. 2012b; Chaverri et al. 2011; Crous et al. 2004; Lechat et al. 2010; Lombard et al. 2014, 2015; Salgado-Salazar et al. 2015; Salgado-Salazar et al. 2016; Zeng and Zhuang 2013, 2019). For the identification of ARD related Nectriaceae the *HIS* gene provided the best resolution and thus confirmed former results of Cabral et al. (2012a) and Lawrence et al. (2019).

In this work, different Nectriaceae were frequently isolated in experiments CE1 and CE2 from apple roots grown in ARD affected soils (Chapter 2). In another biotest, Manici et al. (2013) described results similar to those of CE2: *Cylindrocarpon*-like fungi were the major pathogens and negatively correlated with plant growth. Here, too, gamma irradiation led to an increase in plant growth as well as to an altered fungal community with lowest colonization of *Cylindrocarpon*-like fungi compared to untreated soil variants.

The perlite test has demonstrated that some Nectriaceae isolates can infect apple roots and have a pathogenic effect (Chapter 3). Our own previously conducted greenhouses experiments failed to confirm pathogenicity. Also, in other inoculation experiments difficulties in the reproduction of pathogenicity of Nectriaceae species were reported (Manici et al. 2003; Manici et al. 2018; Tewoldemedhin et al. 2011a). This indicates that native ARD soils contain factors that favor the disease and are absent under artificial conditions (Manici et al. 2018). In addition, Manici et al. (2018) reported that isolates of *D*. *torresensis* produced metabolites (tentoxin, HC toxin and zearalenone) that might be phytotoxic and may contribute to growth depressions as well as antibiotics (rabelomycin and nidulin) that may affect the bacteria and other fungi in the rhizosphere community. However, the involvement of those metabolites in pathogenicity of *D*. *torresensis* was not yet verified. Further, the development of Nectriaceae in the soil is yet

poorly understood. In experiments conducted by Radl et al. (2019) *Ilyonectria* species occurred in only very low abundance in the rhizosphere and there were no differences between apple plants grown in ARD soil and in grass soil from the site Ellerhoop. However, from apple roots, high frequencies of *Dactylonectria* and *Ilyonectria* were isolated (Manici et al. 2018). This confirms our results and underlines that as plant pathogens their essential habitat is inside the root.

A complex of Nectriaceae species was identified to be associated with the formation of CF structures leading to necrosis (Chapter 4). Different genera of Nectriaceae were identified in small tissue cubes of Harris Uni-Core Punch samples and also when samples of single laser-micro dissected CF structures were combined in one PCR cup. Co-infections of different Nectriaceae species seemed to appear on a very small scale. These differences also might be biased by taxonomic uncertainties in comparisons with GenBank information. So far it remains unclear whether gene transfer has occurred between the different Nectriaceae species described here. However, it is not entirely unlikely, because horizontal gene transfer has been reported in *Fusarium*: Two linage-specific chromosomes were transferred between otherwise genetically isolated strains and thereby converted a non-pathogenic stain into a pathogen (Ma et al. 2010). In general, horizontal gene transfer between fungi seem to play a role in the evolution of pathogens (Brader et al. 2017).

Histological analyses conducted by Grunewaldt-Stöcker et al. (unpublished data) demonstrated that Nectriaceae fungi can infect the roots directly, massively spread within the tissue and result in necroses. Further, the stele remains free of fungal colonization, which prevents a systemically distribution to other plant parts. This is in agreement with split root experiments that confirmed the local characteristics of ARD (Lucas et al. 2018). The split root experiment demonstrated that the disease cannot spread from a separated root part via the shoot to other parts of the plant, but is limited to the local root section, which is in direct contact with the ARD soil (Lucas et al. 2018).

In addition, in cortex tissue, intracellular fungal hyphae were also associated with the occurrence of black inclusion bodies. These infections together with the loss of cell vitality (Grunewaldt-Stöcker et al. 2019) have probably a considerable proportion on symptom development and thereby will lead to reductions in root and shoot growth and yield. However, so far it remains unclear what mechanisms promote disease caused by Nectriaceae. Factors, probably originating from the plant itself may shift the soil microbial community to a structure that favors infection and spread of Nectriaceae fungi.

6.1.4   ARD microbes and plant interactions

Fungal pathogens were often reported to be associated with ARD (Manici et al. 2013; Mazzola 1998; Mazzola and Manici 2012) and were also shown in this work to be involved in the disease development (Chapter 2-4). Transcriptome analyses revealed that genes related to biphenyl and dibenzofuran phytoalexin biosynthesis are upregulated in M26 plants grown in ARD affected soils (Weiß et al. 2017b). Also, inoculation experiments with *Pythium ultimum* led to an activation of phytoalexin biosynthesis genes (Shin et al. 2016). Phytoalexins are antimicrobial defense compounds in apple plants (Chizzali and Beerhues 2012). It was shown, that the defense pathway associated with biotic stress (bacteria and fungi) was activated but the outcome was not a sufficient disease defense. This might be due to an impaired exudation of these potentially phytotoxic compounds or a disturbed formation of reactive oxygen species (Weiß et al. 2017a). Furthermore, it should be examined whether other components of the ARD complex described here, especially the Nectriaceae species *D. torresensis* and *I. robusta*, would also lead to an upregulation of phytoalexin biosynthesis genes and in addition, which phytoalexins would be produced.

Reim et al. (2020) investigated candidate genes in the context of ARD: among others, biphenyl synthase genes (*BIS1-BIS4)* belonging to the phytoalexin biosynthesis pathway were upregulated in plants grown in ARD soil, and gene expression positively correlated with the total phytoalexin content of plants. Further, the authors reported that the less susceptible genotype MAL0595 accumulated less phytoalexins in comparison to susceptible M26 and B63 rootstocks (Reim et al. 2020). The hypothesis was made whether different ARD soils can lead to different phytoalexin patterns (Reim et al. 2020). Results from experiment CE2 showed that the soil from each location created an individual fungal root endophyte community (Chapter 2). Perhaps different fungal endophytes may result in varying phytoalexin production, which indeed might be an additional reason for contradictory reports for the different cropping regions.

The last decades of research indicated that ARD is a complex phenomenon dealing with microbial dysbiosis in the soil rather than the cause of a single group of fungal pathogens (Mazzola and Manici 2012; Winkelmann et al. 2019). Replanting experiments indicated that the changes in soil microbial communities might be induced by the plant itself: Five replanting cycles of 'Gala' seedlings in non-cultivated orchard soils induced similar changes in the bacterial and fungal communities together with growth reductions like in replant soil (Mazzola 1999). Interactions between plant and microbes can result in changes in the host physiology, leading to altered root exudation. This may also influence the composition and function of soil

and plant microbial communities as well as the interactions of microbes (Brader et al. 2017). The microbiome of the plant rhizosphere suppresses pathogens and plays a crucial role in plant health (Berendsen et al. 2012). Changes in the community composition may favor disease. It was shown, that different rootstock genotypes produce distinct root exudates, which lead to shifts in the soil microbial communities (Leisso et al. 2017; Winkelmann et al. 2019). Additionally, next to phytoalexins, also other phenolic compounds seem to play a role in ARD. The roots grown in ARD affected soils were reported to accumulate phenolic compounds potentially acting as antioxidants and thereby linking to oxidative stress (Henfrey et al. 2015). Also in root exudates, the phenolic compounds, e.g. phloridzin, were detectable (Hofmann et al. 2009; Leisso et al. 2018; Nicola et al. 2016). These phenolic substances can act against pathogens and help in the detoxification of reactive oxygen species (Emmett et al. 2014; Henfrey et al. 2015). In the replant disease of ginseng (*Panax ginseng*) the production of root exudates containing allelochemicals is related to root rot caused by Nectriaceae fungi: The phenolic acids, salicylic, cinnamic, and benzoic acid inhibited hyphal growth of *Cylindrocarpon destructans*, but stimulated the activity of hydrolytic and pathogenesis related enzymes like pectinase and cellulase (Sun et al. 2013). Further, it was reported that the virulent species *Ilyonectria mors-panacis,* which causes also root rot in ginseng, produces next to hydrolytic enzymes phenol oxidases to detoxify the accumulated polyphenols (Farh et al. 2018). Similar effects were also observed for *Fusarium oxysporum* f.sp. *niveum*, a fungus that causes Fusarium wilt in long-term watermelon monoculture: The watermelon root exudates containing allelochemicals seemed to promote the wilt. *In vitro* applications of cinnamic acid strongly inhibited hyphal growth and conidia production but activated hydrolytic enzymes (pectinase, proteinase, cellulase and to a lesser extend amylase) and highly stimulated the mycotoxin production (Wu et al. 2008). Although *Ilyonectria* species could only be detected in low abundance in the rhizosphere of apple plants grown in ARD soil (Radl et al. 2019) the phenolic root exudates may have reduced fungal growth but stimulated pathogenicity related enzyme activity or even toxin production. Further it was shown that in Sanqi ginseng (*Panax notoginseng*), the application of plant produced autotoxic ginsenosides altered the taxa composition in the fungal microbiome and stimulated soil-borne pathogens while potentially beneficial taxa were reduced (Li et al. 2020b). Whether the potentially phytotoxic phytoalexins produced by the apple plants or other phenolic root exudates have the same effect on the soil microbial community has not yet been demonstrated.

A deeper understanding of ARD associated microbe and plant interactions may help to modulate soil communities and facilitate the development of sustainable and ecologically friendly solutions in apple production.

6.2   Mycoviruses for mitigation of ARD

6.2.1   Mycoviruses of "Alternaviridae" and in Nectriaceae fungi

The hypothesis that mycoviruses are involved in the causal ARD complex could not be validated in this work. This requires further investigations. However, the description of the full-length sequence of DtAV1 is the first step for deeper analyses of the mycovirus biology and interaction studies with its host fungus *D. torresensis* and with apple plants. To investigate the effects of the DtAV1 infection, e.g. on sporulation, growth, virulence, and pigmentation, the fungus needs to be cured, for example by using cycloheximide (Aoki et al. 2009). The perlite test can help to examine the relationship between mycovirus, fungal endophyte and apple plant. In "*Alternaviridae*" only AaV-1 infection led to impaired growth and abnormal pigmentation in the host fungus *Alternaria alternata*. Cycloheximide treatment decreased the amount of dsRNAs and led to a normal growth and phenotype of the fungus (Aoki et al. 2009). Hammond et al. (2008) could not find any effects of AMV infection in *Aspergillus niger*. For all other members of "*Alternaviridae*" only the sequence was deposited in the GenBank. Therefore, it remains unclear whether members of "*Alternaviridae*" may cause hypovirulence and can be used in a mitigation strategy for ARD.

A crucial element for the use of a mycovirus to control fungal diseases is the establishment of the virus population at the site of action (Xie and Jiang 2014). One limiting factor in treating fungal diseases with hypovirulence associated mycoviruses is the fungal vegetative incompatibility that may inhibit virus transmission. Xie and Jiang (2014) listed some strategies to overcome this problem: This can be for example the amendment of chemicals, the use of mycoviruses with a strong infectivity and by discovering a universal mycovirus donor or by establishing vectors for transmission. According to Ghabrial et al. (2015) no vectors have been described for mycoviruses so far. One simple reason might be the additional effort for such studies. Recently, Petrzik et al. (2016) reported the detection of the dsRNA virus TtV1 in the mycorrhiza fungus *Thelephora terrestris* as well as in the soil oribatid mite *Steganacarus carinatus*. But unfortunately, no transmission trials were carried out. After the discovery of a hypovirulent mycovirus in a pathogenic fungus associated with ARD, its interaction with other

soil organisms would be very interesting to investigate. A lot of potential vectors for mycovirus transmission are available in ARD affected soil, for example collembola, mites and nematodes, which are currently investigated in the ORDIAmur project (Kanfra et al. 2018; Winkelmann et al. 2019, Michaelis et al. unpublished data). By feeding on soil fungi these vectors might help in virus transmission, overcoming the restriction to fungal related vegetative compatibility groups.

In general, investigations of mycoviral infections are still very limited. So far only one description of a mycovirus infection in *Cylindrocarpon*-like fungi reported a mycovirus in *Ilyonectria destructans* (former *Nectria radicicola*) with an upregulated virulence of the fungus (Ahn and Lee 2001) in ginseng (*Panax ginseng*). Ginseng is also susceptible to replant disease, and Nectriaceae fungi which cause ginseng root rot, are one of the major threats to ginseng production. Therein fungal strains contained up to four dsRNAs. Of those, the 6.0-kbp L1 dsRNA was responsible for virulence upregulation, also containing RdRp motives (Ahn and Lee 2001). This virus was assigned to *Partitiviridae* (Liu et al. 2012). In samples from replant diseased ginseng fields, the incidence of L1 dsRNA infected fungal strains was significantly increased compared to plant samples of first cropping fields (Ahn and Lee 2001). Whether in ARD fungi of the Nectriaceae might be associated with hypervirulence should be further examined. As Nectriaceae are root pathogens of various plants (Lawrence et al. 2019), it is not unlikely to detect a hypovirulent strain, which then might transfer a mycovirus to apple plant pathogens to decrease their virulence and also to overcome potentially existing hypervirulence in the fungal spectrum of ARD.

6.2.2   *Mycoviruses in fungal endophytes*

The interaction of mycoviruses with the plant and also other (soil) organisms is fairly unknown. At present, an increasing number of mycoviruses are being discovered by deep sequencing methods and often only sequences are stored in the GenBank without any further biological examination. The investigations are mainly concentrated on fungi with economic importance, but other fungal endophytes can also be an interesting subject of research (Roossinck 2014). Endophytic fungi of two plant species originating from a wild plant community were surveyed for viral infections. In the described system the viral diversity was the greatest, followed by a lower diversity of fungi and plants (Feldman et al. 2012). This indicated that the occurrence of mycoviruses is very common and that they may have potentially mutualistic effects for their host in the ecosystem. Especially in harsh environments the viruses may act in epigenetic aspects by providing additional genetic information (Bao and Roossinck 2013). A famous

example for a mutualism induced by a mycovirus is the three-way symbioses of tropical panic grass (*Dichanthelium lanuginosum*) with the fungal endophyte *Curvularia protuberata*. The infection with Curvularia thermal tolerance virus (CThTV) leads to enhanced thermal tolerance (Márquez et al. 2007). The symbiotic plants can bear a soil temperature of 65 °C but if grown separately, both the fungus as well as the plant cannot grow at temperatures higher than 38 °C. Only in the presence of CThTV, the fungus is able to confer heat tolerance to its host plant (Márquez et al. 2007). Therefore, it is of great interest to monitor also other fungal endophytes obtained from apple roots for the presence of mycoviruses, for example DSE fungi. It might be possible that mycovirus infections can be found, which could help the plant in adapting to the adverse conditions in ARD affected soils. Further, it would be interesting whether ARD pathogens and other apple endophytes share same mycoviruses and if the outcome of the infection will be the same for both fungal hosts.

6.3   Outlook

In this work, it was demonstrated that fungal endophytes are involved in ARD. Nectriaceae species were enriched in roots grown in ARD soil and were shown to have a negative effect on apple plants. In addition, fungal isolates assigned to DSE were also frequently isolated from apple roots but were reduced in the relative abundance in ARD. The effect of DSE fungi on apple plants needs to be elucidated. Co-inoculation experiments with Nectriaceae and DSE isolates should be carried out, for example in the perlite test system. Further, it should be investigated whether candidate genes like the biphenyl synthase genes are upregulated after inoculation with different Nectriaceae or DSE isolates, and if so what kind of phytoalexins are produced. Additionally, the effect of apple root exudates as well as phytoalexins on fungal growth and enzyme production would be of interest.

For mitigation of ARD different strategies are conceivable: For instance, some easy culturable DSE fungi might have plant growth promoting effects and may be used as biofertilizers. On the other hand, the effect of mycoviruses regarding hypovirulence or other potentially positive effects on fungal ARD endophytes or apple plants should be further considered.

7.   References

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8. Addendum

Table 8.1 Fungal endophyte isolations CE2 2017 using water agar. Surface disinfected roots of M26 and Bittenfelder were grown for 8 weeks in untreated or irradiated (Gamma) ARD or grass soil, respectively, from the reference sites Heidgraben, Ellerhoop and Ruthe. Relative Colonization Frequency (RFC) in % is the number of root pieces with fungal mycelium divided through the number of total incubated root pieces. Identification by *ITS*-PCR and Sanger sequencing and number (n) of isolates

Genotype	Location	Soil	Treatment	RCF (%)	Identification	n of isolates
M26	Heidgraben	ARD	untreated	25	*Nectria* sp.	3
					Cylindrocladiella sp.	1
					Bjerkandera sp.	1
					Leptodontidium sp.	1
					Zalerion sp	1
					not identified	4
		ARD	Gamma	4,2	*Calonectria* sp.	1
		Grass	untreated	18,8	*Fusarium* sp.	3
					Leptodontidium sp.	2
					Cadophora sp.	1
					Clohesyomyces sp.	1
		Grass	Gamma	0		
	Ellerhoop	ARD	untreated	12,5	*Dactylonectria* sp.	2
					Nectria sp.	2
		ARD	Gamma	7,1	*Trichoderma* sp.	1
					not identified	1
		Grass	untreated	6,3	*Dactylonectria* sp.	2
					Trichocladium sp.	1
		Grass	Gamma	3,6	not identified	1
	Ruthe	ARD	untreated	43,8	*Dactylonectria* sp.	5
					Doratomyces sp.	1
					Exophiala sp.	1
					Pyrenochaeta sp	1
					Plectospherella sp.	1
					Pythium ultimum	1
					not identified	4
		ARD	Gamma	8,3	*Trichoderma* sp.	1
					not identified	1
		Grass	untreated	15,6	*Cadophora* sp.	1
					Dactylonectria sp.	1
					not identified	4
		Grass	Gamma	4,2	*Phoma* sp.	1

Bittenfelder	Ruthe	ARD	untreated	18,75	*Dactylonectria* sp.	2
					Ilyonectria sp.	1
					Nectria sp.	1
					Cadophora sp.	1
					Doratomyces sp.	1
					not identified	2
		ARD	Gamma	0		
		Grass	untreated	0		
		Grass	Gamma	6,25	*Geomyces* sp.	1
					Leptosphaeria sp.	1

Table 8.2 Identification of endophytic root fungi harvested by punching with a Harris Uni-Core from pre-selected fine root samples with distinct infection sites or symptom-free tissue (sf). Fresh or fixed root samples of M26 plants grown in December 2018 (biotest 1) and May 2019 (biotest 3) for two to four weeks in untreated (ut) or gamma irradiated (γ) soils of apple replant diseased plots from sites Ellerhoop (E), Heidgraben (H) and Ruthe (R). NA= no amplification, Negative control (N ctrl) = only PCR reagents without root samples, Positive control (P ctrl) = fungal DNA of *D. torresensis* added to the PCR mix. Sanger sequencing of genes *histone 3* (*HIS*), and *translation elongation factor 1-α* (*TEF*) for identification, first hits of BLASTn analysis

Treatment	Plant no.	Sample ID	*HIS*	*TEF*
Biotest 1				
E-ARD-ut	1	314	NA	Hypocreales sp.[1]
E-ARD-ut	2	322	*Rugonectria rugulosa*	Hypocreales sp.
E-ARD-ut	3	331	*Rugonectria rugulosa*	NA
E-ARD-ut	3	332	*Rugonectria rugulosa*	Hypocreales sp.
H-ARD-ut	2	316	*Rugonectria rugulosa*	Hypocreales sp.
H-ARD-ut	2	318	*Rugonectria rugulosa*	NA
H-ARD-ut	3	327	*Dactylonectria torresensis*	NA
H-ARD-ut sf	1	334	*Dactylonectria torresensis*	NA
H-ARD-ut sf	1	335	*Ilyonectria europaea*	*Dactylonectria torresensis*
H-ARD-ut sf	2	306	*Rugonectria rugulosa*	NA
R-ARD-ut	1	311	*Rugonectria rugulosa*	NA
R-ARD-ut	1	312	*Dactylonectria torresensis*	NA
R-ARD-ut	2	319	*Rugonectria rugulosa*	*Ilyonectria venezuelensis*
R-ARD-ut	3	328	*Rugonectria rugulosa*	NA
N ctrl		N1	NA	NA
N ctrl		N2	NA	NA
P ctrl		P1	*Dactylonectria torresensis*	*Dactylonectria torresensis*
Biotest 3				
E-ARD-ut	1	345	*Rugonectria rugulosa*	NA
E-ARD-ut	1	347	*Ilyonectria robusta*	NA
E-ARD-ut	1	349	*Rugonectria rugulosa*	Hypocreales sp.[1]
E-ARD-ut sf	1	394	*Dactylonectria torresensis*	NA
E-ARD-ut	2	362	*Rugonectria rugulosa*	not tested
E-ARD-ut	2	364	*Dactylonectria torresensis*	not tested
E-ARD-ut	3	380	*Dactylonectria torresensis*	NA
E-ARD-ut	3	382	*Dactylonectria torresensis*	Hypocreales sp.
E-ARD-ut	3	385	*Rugonectria rugulosa*	Hypocreales sp.
H-ARD-ut	1	338	NA	Hypocreales sp.
H-ARD-ut	1	340	*Leptosphaeria* sp.	NA
H-ARD-ut	1	341	*Rugonectria rugulosa*	Hypocreales sp.
H-ARD-ut	1	342	*Leptosphaeria* sp.	NA
H-ARD-ut	2	358	*Dactylonectria torresensis*	Hypocreales sp.
H-ARD-ut	3	374	*Ilyonectria robusta*	NA
H-ARD-ut	3	377	*Dactylonectria torresensis*	NA
H-ARD-ut	3	378	*Dactylonectria torresensis*	NA
H-ARD-γ [s]	1	400	*Rugonectria rugulosa*	NA
H-ARD-γ [s]	2	403	*Ilyonectria robusta*	NA
H-ARD-γ [s]	3	405	*Dactylonectria torresensis*	NA
H-ARD-γ [s]	3	406	*Fusarium graminearum*	NA
H-ARD-γ sf	1	398	NA	NA
H-ARD-γ sf	1	399	NA	NA
H-ARD-γ sf	2	401	NA	NA
H-ARD-γ sf	2	402	NA	NA

H-ARD-γ sf	3	404	NA	NA
R-ARD-ut	1	350	*Rugonectria rugulosa*	NA
R-ARD-ut	1	351	*Conocephalum* sp.	not tested
R-ARD-ut sf	1	396	*Dactylonectria torresensis*	NA
R-ARD-ut	2	368	*Dactylonectria torresensis*	Hypocreales sp.
R-ARD-ut	2	369	*Ilyonectria robusta*	NA
R-ARD-ut	2	372	*Rugonectria rugulosa*	NA
R-ARD-ut	3	386	*Leptosphaeria* sp.	NA
R-ARD-ut	3	387	*Dactylonectria torresensis*	Hypocreales sp.
R-ARD-ut	3	391	1*. *Dactylonectria torresensis*, 2*. *Ilyonectria robusta*	NA
N ctrl		N3	NA	NA
N ctrl		N4	NA	NA

[1] Hypocreales sp. ICMP 13358, [s] light browning, *sequencing of two colonies in transformation

Table 8.3 Identification of endophytic root fungi collected by laser microdissection from cryo-sections of pre-selected areas with distinct fungal cauliflower-like structures (CF). Fixed tissue of M26 fine roots grown in 2018 (biotest 1) and 2019 (biotest 2) for two to four weeks in untreated (ut) apple replant diseased soil (ARD) from the sites Ellerhoop (E), Heidgraben (H) and Ruthe (R). Sanger sequencing of genes *histone 3* (*HIS*) or *translation elongation factor 1-α* (*TEF*) for identification, exemplarily after colony sequencing, first hits of BLASTn analysis

Treatment	Plant No.	Sample ID	Gene	Identification	Colony sequencing
Biotest 1					
E ARD ut	1	11	*HIS*	*Ilyonectria europaea*	
E ARD ut	2	13	*HIS*	*Cylindrocladiella* sp.	
E ARD ut	3	14	*HIS*	*Rugonectria rugulosa*	
E ARD ut	4	15	*HIS*	*Ilyonectria europaea*	
H ARD ut	1	6	*HIS*	*Dactylonectria torresensis*	
H ARD ut	2	6.1-2	*HIS*	*Dactylonectria torresensis*	
H ARD ut	3	18	*HIS*	*Cylindrocladiella* sp.	1. colony
				Ilyonectria europaea	2. colony
				Ilyonectria europaea	3. colony
H ARD ut	3	19	*HIS*	*Ilyonectria europaea*	1. colony
				Ilyonectria europaea	2. colony
				Cylindrocladiella sp.	3. colony
H ARD ut	4	30	*HIS*	*Ilyonectria europaea*	
R ARD ut	1	12	*HIS*	*Cylindrocladiella* sp.	
R ARD ut	2	17	*HIS*	*Ilyonectria europaea*	1. colony
				Dactylonectria torresensis	2. colony
				Dactylonectria torresensis	3. colony
Biotest 2					
E ARD ut	5	211	*HIS*	*Dactylonectria torresensis*	
H ARD ut	2	77	*HIS*	*Dactylonectria torresensis*	
H ARD ut	2	83	*HIS*	*Dactylonectria torresensis*	
H ARD ut	3	118	*HIS*	*Ilyonectria robusta*	
H ARD ut	3	119	*HIS*	*Ilyonectria robusta*	
H ARD ut	3	131	*TEF*	Hypocreales sp.[1]	
R ARD ut	1	103	*HIS*	*Dactylonectria torresensis*	
R ARD ut	3	139	*HIS*	*Rugonectria rugulosa*	
R ARD ut	3	136	*TEF*	Hypocreales sp.	

[1]Hypocreales sp. ICMP 13358 culture

MRSAFGVLNNAAKAANLFKHKAPHSPNLRWCLSDEDTYGVLSADVPALRSLASQWHQESFWISERARLGFPLLAFECYSEFVGAQRKLEERGLRVYMARNATELHDCLFRGVTLY
1   10   20   30   40   50   60   70   80   90   100   110

FGETLNGVRPFNAGRELCEELVNWLVDPWPLGCTHSDAYSKFVQHPLGITEDVWGELGAWAWGDFEELVRAAPKNHLPVDTSNWLRLCDSLAKDDRSSGLVALDALCQRQSRHKR
120   130   140   150   160   170   180   190   200   210   220   230

RHYFDVCTLYALCGETWDTQGQGDVFRRTARAVLYSGLRGGVGHERLYLTCFWLWTTPYWQDLCAFFLQWDLFSYGVDDYRAICKEITARVTASWMFPMSNVQHIASAYFLNAED
240   250   260   270   280   290   300   310   320   330   340

LTGFADNQESSGEVALEVLEFALNDFEYTAPGTLGRDFESYLSQFSSAVESMLEPLYREFRDRALPYEELLATRAAWGAGGVAGRRAREVLGTEKAPPGCTKAYAMVQAPAQDFA
350   360   370   380   390   400   410   420   430   440   450   460
        Motive I   Motive II

MDWGTTRNEIANKMDERGPPRCITATDMRDQISETFAFRHFSNRYPSIGLDIGESPTQAMARHAKLAGCTRGKRHYMVDGRLITAWDWMKWDHFYHCAEKVLVLQAMRRLTARWL
470   480   490   500   510   520   530   540   550   560   570
            Motive III   Motive IV

RGEVRGQLIAEIDDLISKHEEMVYRSQTYADAEYSKRVDELIAQHGDRASRLPGDAGATSVLIKRPNGQQSGRKTTLECNTIVGTARLLVRDAELLGRRAALGHRVALYTLNRAD
580   590   600   610   620   630   640   650   660   670   680   690
                                                            Motive V   Motive VI

DVAEVHATYAAGVAAVNKMIEQGHRANPKKQVAQWRSVVYLRNLYAGGSMRAFPPRAVYAAATGHPDKGAGSESSYMDKLRSVSKGLDMWMRRGGWMRMARCLYADAEKFFSSTR
700   710   720   730   740   750   760   770   780   790   800
Motive VI   Motive VII   Motive VIII

VWHDHGGVRRFSRVKIAVAVLRAAPENGGLGILPPGCYTYDYAVKCRTHPRYADIADAWEEQLAPYAKRTVGPGIHDLEGVGRKWLTDSTGLVPTDAEVKAYKDKWARGRMHQGG
810   820   830   840   850   860   870   880   890   900   910   920

DGQTRFLQRCAQVARSVKRWHRDVKDSVWGHRIHEHHIIRSTHALRDAIDVGAKRPDSSLPFDILRGIRGLPGYAMVEHLWHGYGQVMMGRRRLGPDALKSLCVRLASASQLGRD
930   940   950   960   970   980   990   1000   1010   1020   1030

FLALSRHWSAHAMADHLTGNLGPAGAWDKLIPPSWSGWFVGTVALCMASEIARNPKLTNTRYGLLKLRAQLSNVAAHFFVENFPELCLH
1040   1050   1060   1070   1080   1090   1100   1110   1120   1124

Table 8.4 Conserved amino acid motives of dsRNA1. Gilbert et al. (2019) described eight conserved motives in the open reading frame of RNA1 of *"Alternaviridae"* members. Here these motives were found in the amino acid sequence of RNA1 of DtAV1 (orange mark). The ADD motive VI (green mark) is a conserved RdRp motive of *"Alternaviridae"*

9. Acknowledgement

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11.   Publication list

Publications with peer review:

Popp C, Wamhoff D, Winkelmann T, Maiss E, Grunewaldt-Stöcker G (2020) Molecular identification of Nectriaceae in infections of apple replant disease affected roots collected by Harris Uni-Core punching or laser microdissection. J Plant Dis Prot 10.1007/s41348-020-00333-x

Popp C, Grunewaldt-Stöcker G, Maiss E (2019) A soil-free method for assessing pathogenicity of fungal isolates from apple roots. J Plant Dis Prot 126:329–341. 10.1007/s41348-019-00236-6

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Grunewaldt-Stöcker G, Popp C, Baumann A, Fricke S, Menssen M, Winkelmann T, Maiss E (submitted) Observations on early fungal infections with relevance for replant disease in fine roots of the rose rootstock *Rosa corymbifera* 'Laxa'. Sci. Rep.

Grunewaldt-Stöcker G, Popp C, Maiss E, Winkelmann T (in preparation): Novel fungal structures and other apple replant disease-associated microorganisms in infected tissue of apple fine roots.

Publications without peer review:

Presentations:

Popp C, Grunewaldt-Stöcker G, Maiss E (2018) Mycoviren in Apple Replant Disease (ARD) assoziierten pilzlichen Endophyten. 61. Deutsche Pflanzenschutztagung, Hohenheim

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Popp C, Weber RWS, Zahn V, Maiss E (2015) Optimierung eines PCR basierten Nachweisverfahrens für *Candidatus* Phytoplasma mali. 47. Tagung DPG Arbeitskreises „Viruskrankheiten der Pflanzen", Berlin

Poster:

Popp C, Grunewaldt-Stöcker G, Maiss E (2018) Contribution of Fungal Root Endophytes to ARD? BONARES Conference, Berlin

Popp C, Grunewaldt-Stöcker G, Maiss E (2018) Investigations of Fungal Root Endophytes and their Mycoviruses in context with Apple Replant Disease. 2nd Plant Microbiome Symposium, Amsterdam & 50. Tagung DPG Arbeitskreises „Viruskrankheiten der Pflanzen", Bad Herrenalb

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Master thesis:

„Optimierter PCR-Nachweis für '*Candidatus* Phytoplasma mali' und Untersuchung zur Verbreitung des Erregers an der Niederelbe". 2015, Institute of Horticultural Production Systems, Section Phytomedicine

Bachelor thesis:

„Einfluss von 'cool-morning' auf das Wachstum von *Cucumis sativus*". 2012, Institute of Horticultural Production Systems, Section Vegetable Systems Modelling