Eur. J. Hortic. Sci. *86*(1), 29–40 | ISSN 1611-4426 print, 1611-4434 online | https://doi.org/10.17660/eJHS.2021/86.1.4 | © ISHS 2021

Original article

Microscopic evidence of Nectriaceae and other microbes in infected fine root tissue of replant diseased apple plants

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Summary

Fine roots of apple rootstocks 'M26' and 'Bittenfelder Sämling' showing symptoms of apple replant disease (ARD) were histologically examined to clarify the relationships of microbial endophytes to tissue and cell damages. The plants were either grown on ARD-infested fields at three different locations in Northern Germany or cultivated in greenhouse biotests in untreated or γ-irradiated ARD soils from these three locations. Based on the previous microscopic detection of characteristic ARD symptoms in whole roots, thin sections of selected root segments were analyzed for specific tissue alterations like necrosis and blackening and for microbial colonization with a focus on fungi and Actinobacteria. Nectriaceae with cauliflower-like intracellular structures inducing cell necrosis in cortical tissue were frequently present in all tested root samples of ARD-affected plants and have recently been identified as representatives of *Ilyonectria, Dactylonectria* and *Rugonectria*. In this work, their cellular infection, intracellular development as well as plant reactions to this attack were clearly demonstrated by series of thin sections. In addition, mixed infections with filamentous, rod-shaped and coccoid bacteria and the colonization of mycorrhizal fungi revealed new insights into early stages of ARD development in root tissue. Other fungi and Oomycetes, which differ in their hyphal structure, in propagules or in their spread from Nectriaceae and arbuscular mycorrhizal fungi, were only rarely detected. The results from field samples confirmed those gained from biotest plants and emphasize the importance of Nectriaceae and Actinobacteria in further research on the etiology of ARD and in developing counter-measures.

Keywords
actinobacteria, ARD, etiology, histology, root symptoms, thin sectioning

Significance of this study

What is already known on this subject?

- The involvement of biotic causal factors in apple replant disease is the current state of knowledge, but microscopic examinations have rarely been carried out. Only in 2019 the microscopy of apple fine roots, already after two weeks of cultivation in ARD-affected soils, provided reliable symptom parameters for early detection of the disease.
- Recently, Nectriaceae fungi were validated with microscopic and molecular methods as pathogens contributing to ARD-specific tissue damage.

What are the new findings?

- Microorganisms were localized at the cellular level in diseased cortex tissue of apple fine roots. Nectriaceae with unique structures in necrotic host cells were frequently found in plants grown in soils of three ARD locations.
- This localization of defined endophytes is essential to assess their contributions to the development of ARD symptoms.

What is the expected impact on horticulture?

- Plant-pathogenic fungi of the Nectriaceae in interaction with other endophytes deserve high attention in further research on ARD etiology and the development of counter-measures.

Introduction

The problem of replant disease in Rosaceous plants is well known for a long time and does occur in nurseries and fruit orchards worldwide, but there is still the need to find sustainable mitigation strategies to minimize the dramatic economic losses (Yim et al., 2013; Winkelmann et al., 2019). Research on the elucidation of the etiology of replant disease, in particular with the focus on apple replant disease (ARD) was conducted (e.g., Mazzola, 1998; Tewoldemedhin et al., 2011a, b, c; Kelderer et al., 2012; Mazzola and Manici, 2012; Manici et al., 2013; Franke-Whittle et al., 2019), but the causes of the disease are not yet understood. In order to assess the impairment by ARD, a microscopic early diagnosis of diseased apple fine roots was suggested previously using greenhouse pot experiments with different rootstock genotypes and soils (Grunewaldt-Stöcker et al., 2019). Further, a laboratory infection test enabling a fast validation of isolated potential causative microorganisms was established (Perlite-assay; Popp et al., 2019). Otto et al. (1993, 1994) have addressed Actinobacteria (formerly Actinomycetes) as pathogens in ARD-affected apple roots. Besides nematodes and Oomycetes (e.g., *Pythium* spp.), several genera of fungi like *Rhizoctonia* spp. (Ceratobasidiaceae), *Fusarium* spp. (Nectriaceae), and especially *Cylindrocarpon*-like fungi (listed taxonomically within the Nectriaceae) were identified as part of the disease complex (Braun, 1991, 1995; Mazzola, 1998; Tewoldemedhin et al., 2011c; Kelderer et al., 2012; Manici et al., 2018). Most previous studies on ARD-associated pathogens focused on root damage and the resulting impaired

plant performance together with the cultivation and identification of the isolated fungi. Lately, molecular microbiome research studying bacterial and fungal biodiversity have been applied to better understand the ARD phenomenon (Rumberger et al., 2007; Yim et al., 2013, 2015; Sun et al., 2014; Franke-Whittle et al., 2015; Nicola et al., 2017, 2018; Radl et al., 2019). In addition to these in-depth molecular studies of complex microbial communities in soil, rhizosphere and plant roots using sophisticated new tools, it seemed important to investigate the localization and appearance of microorganisms within the root tissues of diseased apple plants. In earlier microscopic studies, Hoestra (1968) and Caruso et al. (1989) presented detailed descriptions of tissue damages. However, in recent decades a thorough histological evaluation of the infected tissue of replant diseased apple roots has not been continued. Following our studies on early root symptoms in *Malus* rootstocks 'M26' and 'Bittenfelder Sämling' (Mahnkopp et al., 2018; Grunewaldt-Stöcker et al., 2019), we considered the histological assessment of infected tissue as an important further step to illustrate and understand the symptoms and damages caused by different groups of pathogens and finally enlighten the etiology of ARD. In particular, it was the aim of our studies to gain more insights into infections by Nectriaceae recently identified with several genera and species in ARD-impaired apple roots grown in soils from different locations (Popp et al., 2020) and thus to extend previous reports on fungal infections. The microscopic visualization and differentiation of endophytes in root tissue should fill a gap in ARD research between general sequencing-based microbiome analyses and microbiological isolation tests on soil and plants. This localization of defined endophytes within diseased root tissue is essential to understand the infection pathway and to assess their contributions to the development of ARD-symptoms.

Materials and methods

Experimental set up of biotests and field trials and root sampling

Histological investigations were carried out to confirm and localize endophytic microorganisms involved in ARD-associated root tissue damage. We used apple fine roots from confirmed ARD-impaired 'M26' (herein named M26) and 'Bittenfelder Sämling' (herein named Bittenfelder) plants harvested in biotests and field trials with ARD-affected soils from three sites in Northern Germany, on which apple had been replanted at least four times and which differed in soil texture (Table 1, Mahnkopp et al., 2018).

In the field trials (CE1) of the joint project BonaRes-ORDIAmur (www.ordiamur.de) conducted in spring 2016 (Table 1), plants were harvested from ARD-affected plots of all three sites (Supplemental Information – Figure S1a, b). The induction of ARD had been proven by a significant reduction of shoot biomass in the fourth replant generation. The test conditions and the experimental design of the greenhouse biotests (CE2) conducted in 2016 and 2017 were described in detail by Mahnkopp et al. (2018). Briefly, *in vitro* propagated M26 plantlets were grown for 8 weeks in ARD-affected (ARD ut = untreated) soils from the three reference sites, named H (Heidgraben), E (Ellerhoop), and R (Ruthe). In parallel, M26 plants were cultivated in these soils after a disinfection treatment to eliminate the biotic causal agents of ARD by γ-irradiation (ARD-γ, minimal dose: 10 kGy) (Table 1; Supplemental Information – Figure S1c–f). The growth data revealed significantly reduced biomasses of plants in all ARD-ut variants compared to the respective ARD-γ variants (Mahnkopp et al., 2018). Root samples for microscopy were taken from two plants per variant after two, four and eight weeks of cultivation (t2, t4, t8) (Grunewaldt-Stöcker et al., 2019). This low plant number was due to the fact, that roots had to be microscopically examined freshly for symptoms, and the fixed material used for the thin sectioning was obtained from these samples. However, for quantitative analyses the data of the three time points were pooled resulting in a replicate number of six plants. In total, roots of plants grown in soils of three sites (each with ARD-ut and ARD-γ variants) of two independent experiments carried out in 2016 and 2017 were investigated in order to identify general ARD symptoms rather than soil-specific ones. Furthermore, to exclude reactions that are specific for M26 only, for soil R in CE2 2017, clonally propagated plantlets of one Bittenfelder genotype were included in the study.

The selection of fine root samples from ARD-impaired apple plants in both experiments CE2 2016 and 2017 and field trial CE1 based on the microscopic diagnosis of cellular damages such as blackening, necrosis, brown-black cellular inclusion bodies in the outer tissue layers and of microbial infection sites in whole mounted sensitive fresh fine roots (Grunewaldt-Stöcker et al., 2019). Additional segments were taken from plants from γ-irradiated ARD soils in the biotests (CE2) for comparison.

Root segment preparation for histology

Experiment CE2: Fifteen segments with ARD symptoms and ten symptom-free root segments from γ-irradiated soils were taken from two plants per soil and time point (Table 1)

Table 1. Overview of the origin of apple fine root samples for the histological studies (greenhouse pot biotests CE2, ARD field plots CE1, according to Mahnkopp et al. (2018) with soils of the three reference sites Ellerhoop (E), Heidgraben (H), and Ruthe (R), either untreated (ARD ut) or irradiated (ARD-γ). Harvesting in CE2 was done after two, four and eight weeks (t2, t4, t8).

Rootstock type	Greenhouse biotests CE2	Treatment	Field tests CE1	Harvest
M26	CE2 2016	E, H, R: ARD ut		t2, t4, t8
		E, H, R: ARD-γ		t2, t4, t8
M26	CE2 2017	E, H, R: ARD ut		t2, t4, t8
		E, H, R: ARD-γ		t2, t4, t8
Bittenfelder	CE2 2017	R: ARD ut		t2, t4, t8
		R: ARD-γ		t2, t4, t8
Bittenfelder			E, ARD	June 2016
			H, ARD	May 2016
			R, ARD	May 2016

and chosen for fixation, embedding and subsequent thin sectioning. Root segments of 1 cm length were fixed in alcohol-formaldehyde-glacial acetic acid (AFE; Gerlach, 1977), embedded in glycol methacrylate (GMA; Grunewaldt-Stöcker, 1985) and blocked with three to five specimen each. Thin sections of 5 µm (Reichert-Jung, Autocut 2040) were stained on the slides with toluidine blue before preservation (0.03% in phosphate buffer pH 7.0 (Gerlach, 1977). Three to five tissue sections per slide and four slides were evaluated microscopically per soil variant and time point (AxioImager.A2, Zeiss, Germany; 100× to 1,000× magnification, brightfield technique with Differential Interference Contrast (DIC)). The camera system AxioCam MRC (Zeiss, Germany) was used for the photo documentation.

Root segments of Bittenfelder plants from the field experiment CE1 at Ruthe, Heidgraben, and Ellerhoop sites (Table 1) were selected for histological analysis and treated like the samples from CE2. Less in number, they were used for a qualitative comparison with M26 root samples from the greenhouse tests regarding tissue symptoms and microbial colonization.

To study the intracellular development of Nectriaceae in detail, also Confocal Laser Scanning Microscopy (CLSM) was applied (Leica Microsystems, CLSM TCS SP2). Here, whole mount unfixed root samples were stained with acid fuchsine-lactophenol as formerly described (Grunewaldt-Stöcker et al., 2013).

Microscopic evaluation

One focus of the histological analyses was the detection and documentation of fungi and other microorganisms within the diseased plant tissue together with a presentation of microscopic details in illustrations. Nectriaceae with intracellular hyphae in connection to dense, cauliflower-like structures (CF structures) were of special interest due to their frequent occurrence in black root segments from ARD treatments (Supplemental Information – Figure S2). Fine hyphae without CF structures and without strong Nectriaceae mycelium were classified as "other fungi", whereas *Rhizoctonia* spp. and Oomycetes were differentiated from these due to their distinct mycelia and oospores. Arbuscular mycorrhizal fungi (AMF), known as useful root symbionts, were investigated with respect to their occurrence and developmental stages on the basis of runner hyphae, arbuscules and vesicles. Moreover, in addition to rod-shaped and coccoid bacteria, Actinobacteria with their characteristic, highly differentiated, branched, mycelium-like structures (frequently observed in uncut root samples by fluorescence microscopy (Supplemental Information – Figure S3) were included in the assessment of occurrence as an extra category by visual classification. The presence and localization of the different microbial structures were recorded as well as the occurrence of ARD tissue symptoms such as necrosis and dark intracellular inclusions.

All ratings were summarized in % frequency data on the basis of ratings per slide ($n = 12$ slides per treatment, $n = 4$ slides per harvest time*treatment with 3–5 thin sections each, in total 723 tissue sections in CE2).

The samples from plants in untreated ARD soils (in total 371 sections) cumulated over all harvest times were subjected to data analyses conducted with the statistic software R, v. 3.6.3 (R Core Team, 2020). For the binomial counting data, a deviance analysis for a generalized linear model under assumption of quasibinomial distribution was performed. For pairwise mean comparisons after Tukey the R software package "emmeans", v 1.5.3 was applied (Lenth, 2020). For Pearson correlation parameters the function "chart. Correlation" out of the package "PerformanceAnalytics", v. 2.0.4 (Peterson and Carl, 2020) was used.

Results

Histological analyses of roots from the greenhouse biotests CE2

The evaluation of the thin sections of ARD-impaired roots from soils of the three reference sites showed that early non-mycorrhizal fungal colonization in the outer tissue layers led to cell death in combination with a colour change. Additional segments of plants from γ-irradiated ARD soils taken for comparison were symptom-free. Fungi with coarse septate hyphae directly infected the roots and spread within the tissue forming intracellular CF structures in rhizodermal and cortical cells, which is characteristic of some Nectriaceae (Figure 1). The plants reacted by formation of callose deposits at the fungal penetration sites (proven by aniline blue staining, Supplemental Information – Figure S4), but these could not stop the cellular infections. However, the endodermis and the stele stayed free from infections. Using Confocal Laser Scanning Microscopy, the CF structures were shown to adhere to the plant cell wall at the fungal penetration site (Supplemental Information – Figure S5, related to Figure 1). In tissue areas with developing or already established CF structures, the ARD-symptomatic, drop-like dark coloured cellular inclusions of differing size could be detected in sections of plants grown in ARD soil from Ellerhoop and Heidgraben (Supplemental Information – Figure S6). The many individually occurring infection foci of Nectriaceae (identification according to Popp et al., 2020) with CF structures in samples harvested as early as two weeks after planting (t2) indicated an early and wide spread of these pathogens. At later time points, the formation of globose, thick-walled chlamydospores in such infected root tissue was observed.

Neither *Rhizoctonia* spp. nor non-septate Oomycetes were detected, which can be discriminated from Nectriaceae and AMF in their hyphal structure, in propagules or in their spread pattern. AMF appeared with typical hyphae and arbuscules in the cortex tissue of root samples from untreated soil not earlier than after four weeks of cultivation in both tested plant genotypes. In root segments of M26 from Heidgraben soil in 2016 and 2017, these mycorrhizal structures were found only after eight weeks (Table 2, Supplemental Information – Table S1). AMF colonized healthy tissue parts, but were also present in areas with other fungal infections, there with collapsed arbuscules (Figure 2a–c).

Actinobacteria were found in non-vital epidermal cell layers, in deeper necrotic areas (Figure 2d–f), but also in symptom-free tissue and they could even invade the root hairs. They colonized the same necrotic cell clusters as Nectriaceae, while other rod-shaped or coccoid bacteria mainly occupied the intercellular space and the root surface and less frequently destroyed cortex cells.

A combined infection of bacteria and fungi occurred in severely affected tissue areas. This mixture of intraradical localized microbes was observed after only two weeks of cultivation (Figure 3), and this fact made it difficult to reveal a sequence of infections by the different endophytes.

These multi-faceted observations were not isolated events. They are summarized in an overview as pooled rating data of both experiments (CE2 2016 and CE2 2017) on

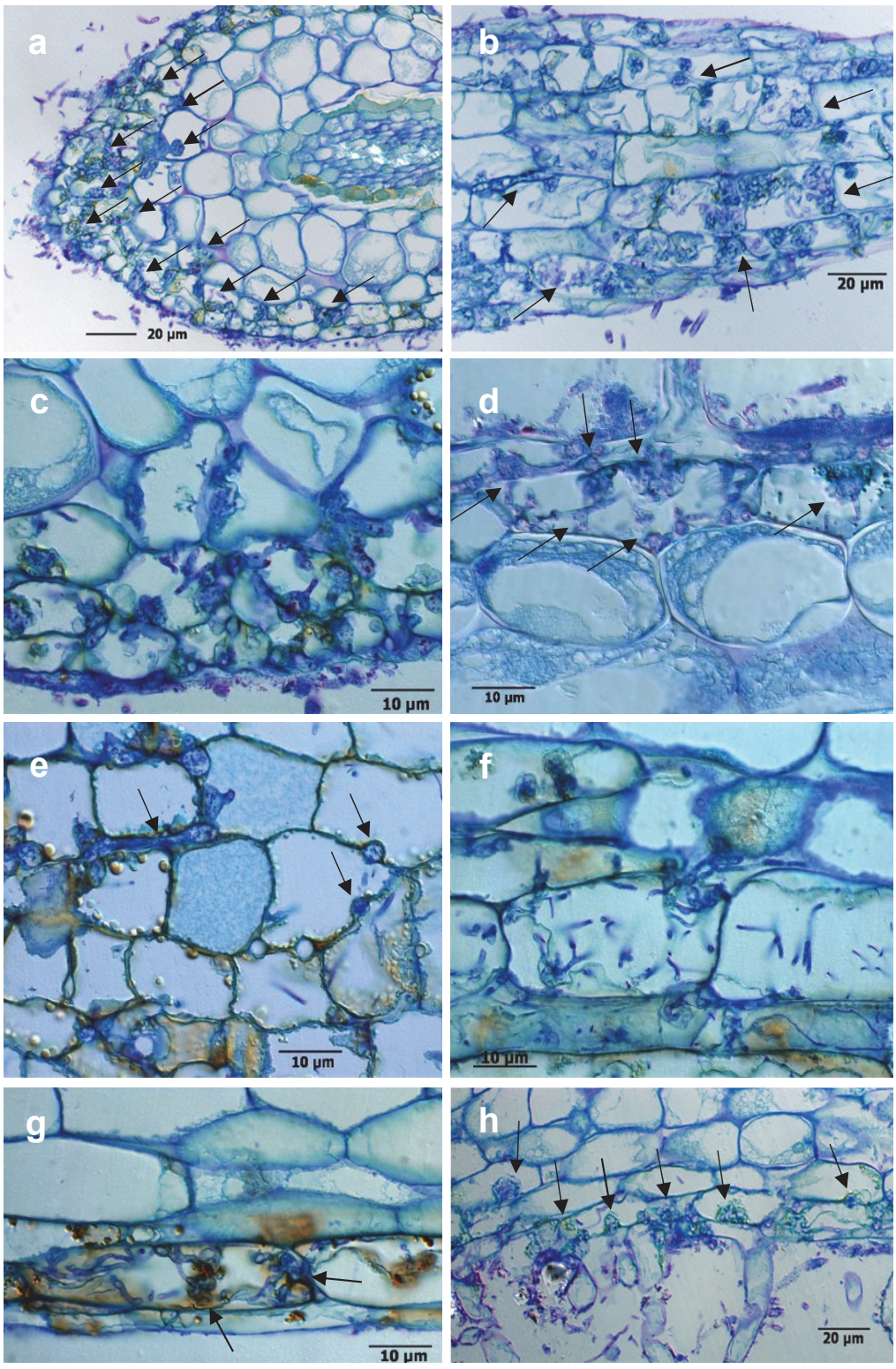


Figure 1. Fungal infections in cortex tissue of M26 fine roots stained with toluidine blue. a) Cross section of a lateral root with mycelium outside and infections in the rhizodermal and outer cortical layers. Intracellular, dense nodular cauliflower-like (CF) structures of Nectriaceae associated with plant cell necrosis (yellow staining of cytoplasma) after two weeks of cultivation in ARD soil from the Ruthe site, experiment CE2 2016. b) Longitudinal section of infected cortex tissue in M26 roots cultivated for eight weeks in ARD soil from the Heidgraben site (experiment CE2 2017), with many dense CF structures and mycelium stained blue. c) and d) Early fungal infections two weeks after cultivation in ARD soil from the Ruthe site with distinct formation of CF structures adherent to the plant cell wall at the penetration site (arrows). e) Thick intercellular Nectriaceae hyphae in necrotic tissue after two weeks (arrows), and f) Intracelluar mycelium and CF structures after four weeks in ARD soil from the Heidgraben site. g) Nectriaceae infection with CF structures (arrows) and necrotic cell reactions in rhizodermal and cortical tissue four weeks after cultivation in ARD soil from the Ellerhoop site, and h) In a root hair zone after two weeks in ARD soil from the Heidgraben site.

Table 2. Summary of histological findings on thin sections of M26 and Bittenfelder apple fine roots harvested two, four and eight weeks (t2, t4, t8) after starting the greenhouse biotests CE2 2016 and CE2 2017 in ARD soils (untreated ut, irradiated γ) from the sites Ellerhoop (E), Heidgraben (H), and Ruthe (R). Assessment data of single sections were pooled per treatment and harvest time regarding the following assessment parameters: Occurrence and localization of mycorrhizal fungi, Nectriaceae with cauliflower-like structures (CF), other fungi, Actinobacteria, other bacteria; occurrence and location of cell necroses, inclusion bodies (I), and yellowing (y). Filled cells represent the respective occurrence.

			Mycorrhizal fungi			Nectriaceae forming CF structures			Other fungi			Actinobacteria			Other bacteria		Necroses		
Soil and treatment	Time of harvest	No. of sections analysed	Intraradical mycelium	Arbuscules	Vesicles	Surface	Rhizodermis	Cortex	Surface	Rhizodermis	Cortex	Surface	Cortex	Stele	Surface	Cortex	Rhizodermis	Cortex	Endodermis
E ARD ut	t2	36																	
	t4	36																	
	t8	36															I		
E ARD γ	t2	32																	
	t4	20																	
	t8	40																	
H ARD ut	t2	32																	
	t4	36																	
	t8	36															I		y
H ARD γ	t2	36																	
	t4	36																	
	t8	36																	
R ARD ut	t2	36																	y
	t4	32																	
	t8	31																	y
R ARD γ	t2	32																	
	t4	32																	
	t8	28																	

the incidence of microbes and symptoms in Table 2 (and separated for both years in Supplemental Information – Table S1). Root sections from untreated ARD-affected soils of all experiments very often contained Nectriaceae identified by their CF structures, while other fungi with septate hyphae were only rarely detected (Figure 4). If the experiments of 2016 and 2017 are compared, the incidence of Actinobacteria, other bacteria and necroses did not differ significantly. However, there were differences ($p < 0.05$) between the soil origins regarding the occurrence of Nectriaceae (Figure 4). Furthermore, a pairwise comparison revealed for 2016 a significantly lower occurrence of Nectriaceae ($p < 0.05$) in roots cultivated in soil from Heidgraben than in 2017.

These Nectriaceae caused the majority of fungal infections and their appearance within the cortex tissue correlated significantly with the occurrence of cell necrosis (2017: $p < 0.001$, Pearson corr. $r = 1.00$; 2016 plus 2017: $p < 0.05$, Pearson corr. $r = 0.57$, Supplemental Information – Table S2). Bacteria, and especially Actinobacteria, were detected in many root samples (Figure 4, Supplemental Information – Table S1), and even early infections with Actinobacteria were often accompanied by necrotic plant reactions. However, no significant correlations of Actinobacteria or other bacteria with the occurrence of root tissue necrosis could be statistically proven in 2016 and in 2017 (Supplemental Information – Table S2).

AMF with intercellular hyphae, intracellular arbuscules and rarely vesicles were found in samples from all three ARD soil sites, but the frequencies of occurrence of AMF were not statistically different (Figure 4). Necrosis was shown in rhizodermal and cortical tissue of all samples from untreated ARD-affected soil, but only rarely in root tissue of plants from irradiated soils. The intracellular brown-black inclusion bodies (I; Table 2), which were found only in M26 in the experiment of 2017, were associated with browning or necrosis in the outer cell layers and with fungal infections (Supplemental Information – Figure S6). In all samples the endodermis and the stele remained free of damage (Figure 4; Supplemen-

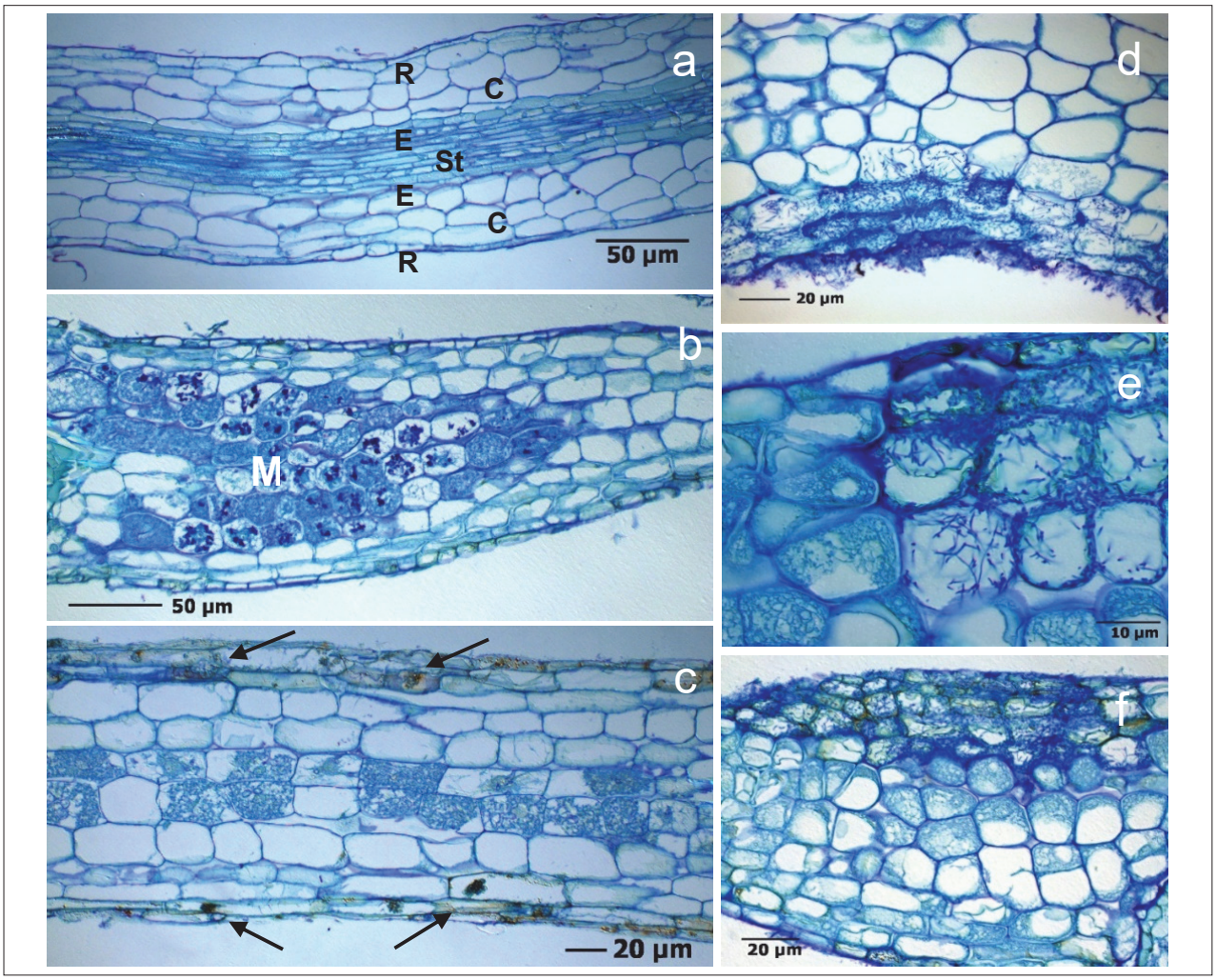


Figure 2. Colonization of M26 fine roots by mycorrhizal fungi and Actinobacteria in ARD soil from the Ellerhoop site. Longitudinal thin sections of fine root tissue harvested. a) Non-colonized, healthy tissue from γ-irradiated soil (t8, experiment CE2 2016) with rhizodermis (R), cortex (C), endodermis (E), and stele (St) with xylem parenchyma. b) From untreated ARD soil, healthy tissue with well-established mycorrhiza (M) in the cortex (t4, experiment CE2 2016). c) From untreated ARD soil with mycorrhizal arbuscules in the cortex tissue, and necrosis and infections (arrows) in the rhizodermal and outer cortex cell layers (t8, experiment CE2 2017). Actinobacteria (t8, in experiment CE2 2017) spread from the surface into the cortex tissue. d) Without signs of cell necrosis. e) and f) With progressive change in host cell colour to yellow green and development of necrosis.

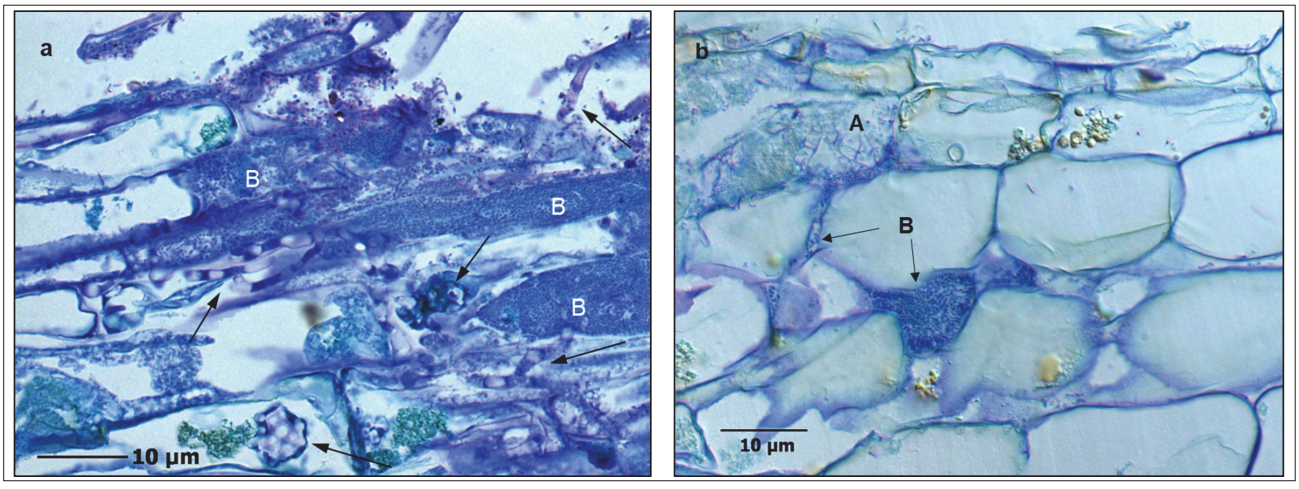


Figure 3. Examples of mixed infections in damaged fine root tissue of M26. a) After two weeks of cultivation in ARD soil from the Heidgraben site (experiment CE2 2017), Nectriaceae hyphae and nodular structures (black arrows), bacteria (B). b) Actinobacteria (A) and other bacteria (B) in the apoplast in impaired cortical tissue two weeks after cultivation in ARD soil from the Ruthe site (experiment CE2 2016).

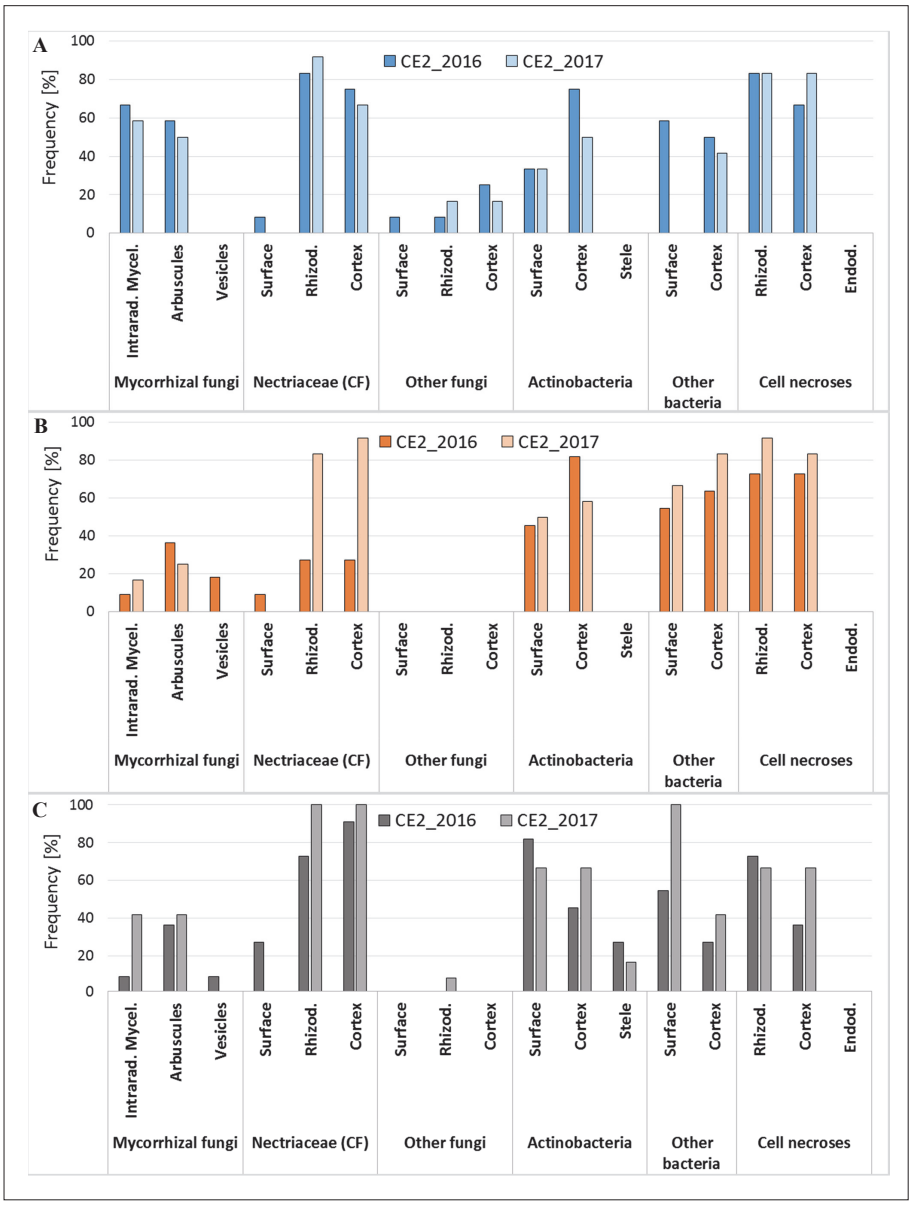


Figure 4. Comparison of microscopic ratings in experiments CE2 in 2016 and 2017, regarding the occurrence and localization of microorganisms and cell necroses in fine root tissue sections of replant diseased apple plants (M26) pooled from three harvest times at two, four and eight weeks of cultivation in ARD soils from three locations. Given are % frequencies, $n = 12$ slides per treatment (4 slides per harvest time, each slide with 3 to 4 tissue sections); data separated for soils from Ellerhoop (A), Heidgraben (B), and Ruthe (C).

tal Information – Figure S7), only rarely a light yellowing (y) was observed in this inner tissue (Table 2).

Tested root tissues of plants grown in irradiated soils from all three sites were largely free of microorganisms throughout the whole experimental period in both years (Figure 2a). Only in rare cases, coccoid and rod-shaped bacteria were found on the root surface or in rhizodermal cells (experiment 2017, Table 2, Supplemental Information – Table S1), and rarely necroses were located in cortex cells, partly in combination with bacteria, Actinobacteria or fungal mycelium. Both observations held true in root samples of M26 and of Bittenfelder obtained from irradiated soil of the Ruthe site. Comparing the findings in tissues of M26 and Bittenfelder roots grown in the untreated ARD soil from Ruthe in CE2 2017, there were no significant differences in the occurrence of Nectriaceae between the genotypes (Supplemental Information – Figure S8).

Histological analysis of roots from field-grown plants

Harvesting fine roots of Bittenfelder plants from the field plots of the three ARD reference sites proved to be difficult due to root losses or firmly adhering silicate and humus residues, which severely disturbed the process of sample thin sectioning. Histological examinations of sufficiently evaluable field samples showed similar patterns of cell damage and colonization with Nectriaceae as was found in the biotest samples. Starting from rhizodermal cells, fungal infections spread over necrotic browned cells and led to massive intracellular aggregations of septated short-celled hyphae. These infections were found frequently and to varying degrees and extended to the stele, but no further (Figure 5a). In such diseased root segments, the mycorrhizal state was disturbed in necrotic host tissue. Healthy sections with only mycorrhiza were rarely present; mostly, mixed infections were observed (Figure 5b–d). Rod-shaped and coccoid bacteria occupied the damaged tissue up to the stele, Actinobacteria were found both on the root surface and in the cortex, often in necrotic cells and accompanying collapsing mycorrhizal arbuscules (Figure 5d–f). In roots that appeared healthy, AMF colonization occurred at all stages of development: with intercellular long-distance runner hyphae, intracellular hyphae, arbuscules in expanding and collapsing stages, and vesicles in cortex tissue (Figure 5c). The histological studies of field grown root samples from replant diseased Bittenfelder plants qualitatively confirmed the occurrence of Nectriaceae infections and colonization by Actinobacteria, bacteria and AMF.

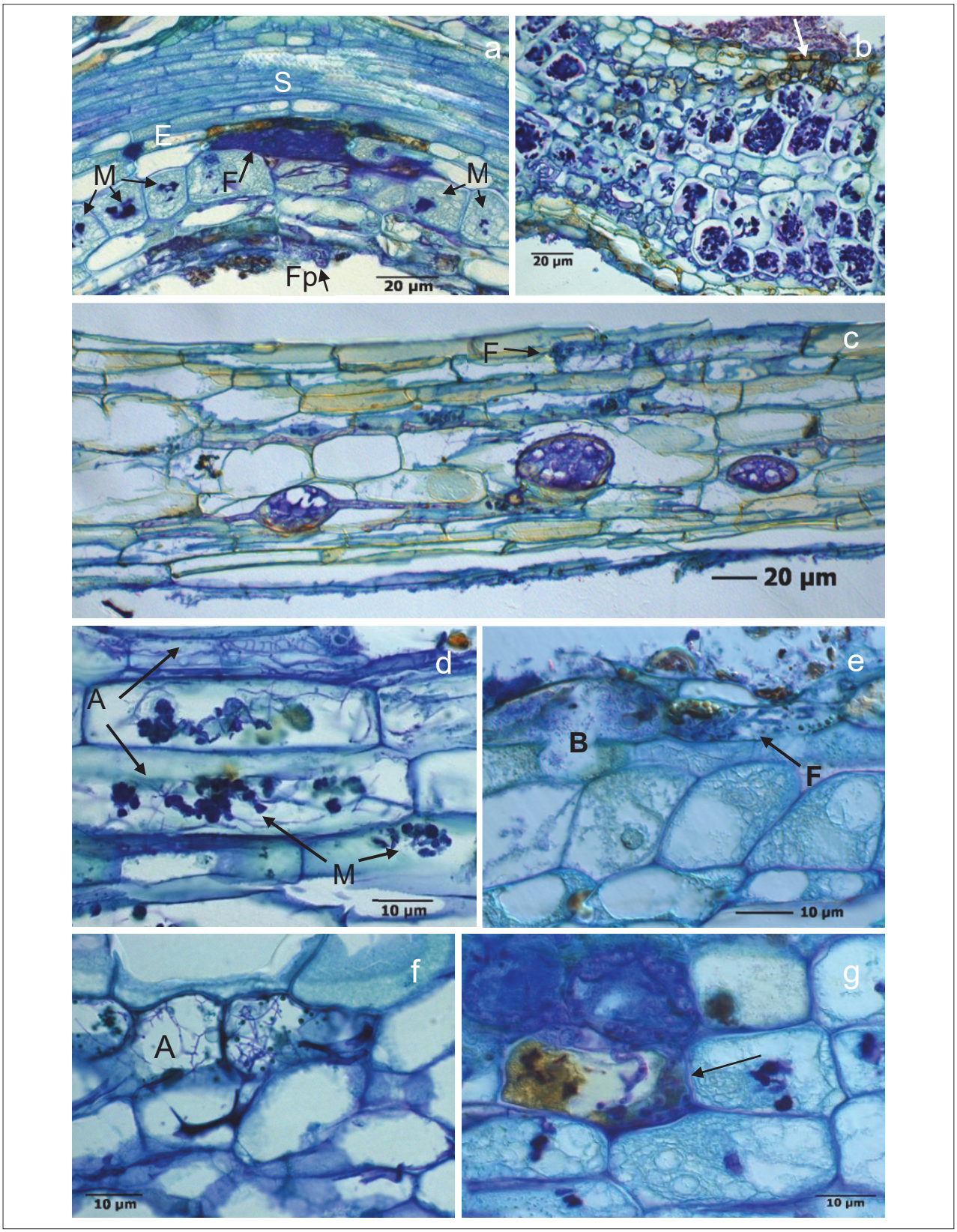


Figure 5. Microorganisms in ARD-symptomatic fine roots of Bittenfelder plants from field experiments, shown in thin sections of cortex tissue stained with toluidine blue. a) Penetration of a fungus (Fp) from the root surface, spreading through the cortex layers with mycorrhizal colonization (M) aside, up to the cushion-like hyphal assembly (F) in necrotic cells adjacent to the endodermis (E), whereas the stele (St) remains free of the infection. b) Mycorrhizal colonization with hyphae and arbuscules in cortex tissue, and a fungal infection in brown necrotic cells (arrow) starting from the rhizodermis. c) Oval shaped mycorrhizal vesicles in necrotic cortex tissue with fungal infection (F, arrow). d) Intracellular mycelium of Actinobacteria (A), even in cortex cells with collapsed arbuscules (M). e) Damaged cells filled with bacteria (B) and fungal hyphae (F) above intact cortex cells with cytoplasm, nucleus and vacuoles. f) Actinobacteria (A) in damaged cortex cells. g) Fungal hyphae around and in a brown necrotic cortex cell with mycorrhizal arbuscular residues (arrow).

Discussion

The evaluation of the thin sections clarified damages and infections at the cellular level of the apple root tissue and provided new detailed insights into microbial colonization and infections. Although root samples for microscopy based on only two plants per treatment and harvest time, the presented histological results support the close relation of microbial infections to the characteristic ARD root symptoms in an early diagnosis which have been described previously (Grunewaldt-Stöcker et al., 2019). The outstandingly early occurrence, high frequency and severity of Nectriaceae infections with CF structures in disturbed fine root segments and the correlation with cell necroses indicate the importance of these fungi for the development of ARD-characteristic root symptoms. Moreover, since the endodermis has proven to be an impassable barrier to infection of the stele, a systemic infection and further distribution into upper plant parts does not seem possible. This is in agreement with the local plant reactions to ARD-affected soils in a split-root experiment (Lucas et al., 2018).

The Nectriaceae that are responsible for the development of these CF structures, were recently identified using laser microdissection of cryo-fixed root thin sections and subsequent PCR analyses. Therein, clear evidence is presented that isolates of several genera and species, namely *Dactylonectria torresensis*, *Ilyonectria robusta*, *Rugonectria rugulosa*, *Cylindrocladiella* sp., and *Ilyonectria europaea*, formed these dense nodular structures (Popp et al., 2020). Thus, the histological results confirm the presence of Nectriaceae in two different rootstock genotypes grown in three different ARD-affected soils and under different growth conditions, although their identity was not further specified in the present study.

The function of the CF structures can currently only be speculated on. Staining revealed fungal plasma within the thick-walled CF structures near to the plant cell wall. Döbbeler (2005) described a haustorium for *Nectria hyperepiphylla* arising from the penetration point of the infection in an epiphyllous hepatic from New Caledonia, but with a much more delicate surface structure. The illustrated very thick wall of the CF structure, which is best visible in three-dimensional CLSM images (Supplemental Information – Figure S5), makes a haustorial mass transfer rather unlikely. Instead, a protective function for the inner fungal plasma must be assumed, maybe as a reaction towards a defensive host cell response. In addition to chlamydospores, these CF structures might serve long-term survival and thus support the durability of ARD. Therefore, further detailed studies are needed to clarify what contribution the plant and/or the fungus make to the development of the wall structure and for what type of function it is intended. It was never observed, that these structures changed to chlamydospores or to microsclerotia, but fungal hyphae could grow out from them.

In ARD research, other scientists had focused interests, e.g., on *Fusarium* spp. (Manici et al., 2003; Tewoldemedhin et al., 2011a; Caputo et al., 2015) or on *Rhizoctonia* spp. and *Pythium* spp. (Mazzola, 1997; Tewoldemedhin et al., 2011b), according to their observations or isolations made before. Contrary to the frequency of Nectriaceae fungi in our microscopic and histological samples, *Rhizoctonia* spp. and *Pythium* spp. which are visually identifiable due to distinct mycelia, sporangia and oospores, were not detected. It cannot be ruled out that other undefined fungi colonized the tested root segments, but they were very rarely observed in the tissue sections ("other fungi" in Figure 4). Our results indicate a differing prevalence of pathogens involved in ARD at different locations, thus confirming the statement of Manici et al. (2013). Moreover, Nectriaceae – formerly also described as *Cylindrocarpon*-like fungi – have been observed before within the complex of ARD fungal pathogens, and several former studies have confirmed their contribution to growth depression in apple plants (Braun, 1991, 1995; Mazzola, 1998; Tewoldemedhin et al., 2011c; Kelderer et al., 2012; Manici et al., 2018). This fungal group has also been noticed in molecular analyses of microbial communities within diseased apple roots and apple rhizospheres (Franke-Whittle et al., 2015; Wang and Mazzola, 2019), and in soil of orchard tree rows (Kelderer et al., 2012; Deakin et al., 2018), but was neither demonstrated to be responsible for the earliest cell infections associated with ARD root symptoms nor to form CF structures before Popp et al. (2020). The state-of-the-art molecular approaches of microbiome analysis provide insight into the specific differences between healthy and replant diseased root environments, but leave the concrete phytopathological determination of defined root infections unanswered. Therefore, a combined procedure with direct tissue sampling and subsequent molecular identification of the included fungi (as reported by Popp et al., 2020) delivers findings that are more precise at the plant and tissue level – at best in even earlier, time staggered samples. This could help to clarify the interactions of the frequently occurring Nectriaceae with other root microorganisms (including their metabolites) and with plant reactions including root exudates in the context of the complex replant disease. Important questions that still need to be answered, however, are what triggers infections with Nectriaceae in ARD situations and whether their chlamydospores occur more frequently in ARD-infested soils due to earlier apple cultures.

Open questions also apply to the detected Actinobacteria, which morphologically presumably belong to the group of soil-inhabiting Streptomycetes. In particular, these bacteria offer various approaches to evaluate their contribution to ARD in tests for their potentially positive, indifferent or harmful effects (Qin et al., 2011). In a biotest with apple plantlets in replant affected soils, Otto et al. (1994) noticed the very early colonization of fine roots by Actinobacteria resulting in epidermal and cortical cell damage. In our experiments, the early occurrence of Actinobacteria on the root surface and in the outer cell layers including root hairs could also be verified. However, the presence of unspecified Actinobacteria either in undamaged root sections or near or even in multiple infections with other bacteria and fungi does not necessarily confirm Otto's statement that Actinobacteria are the trigger of the specific apple replant disease. Moreover, this is supported by the fact that there was no significant correlation between the occurrence of Actinobacteria and cell necrosis. Since Actinobacteria, with their high biodiversity, can have beneficial, indifferent, or harmful effects to the host (Ventura et al., 2007; Singh and Dubey, 2018), studies are required to classify their pathogenicity more precisely, especially to identify the species involved in early root symptoms and to analyze features that differentiate non-pathogenic endophytes from pathogens. One could be tempted to speculate that they are attracted by high concentrations of phenolic substances (Hofmann et al., 2009; Henfrey et al., 2015) and phytoalexins (Weiß et al., 2017) produced early in ARD-affected roots. It could be possible that these secondary metabolites are triggered by Nectriaceae infections.

Damaged root systems consequently influence the attractiveness for further colonizers, and many endophytic and saprophytic bacterial species may have similar func-

tions in the decomposition of plant material, the triggering of plant resistance, or microbiome interactions by effector proteins (Brader et al., 2017). The observations of this study point to the complex network of plant pathogenic and plant non-pathogenic endophytes and its changes due to host plant reactions and environmental shifts. The interaction effects described apply in particular to the root microbiome associated with ARD. Even the observed presumably saprophytic bacteria on the surface, in injured tissue and in the apoplast may also play a role in this network.

Native AMF in healthy roots of biotest apple plants from untreated soils showed normal development in undamaged, well-functioning cortical cells as described earlier and in detail (Caruso et al., 1989). Here, they can contribute to plant health and performance. However, an assumed biocontrol or plant health-promoting potential of AMF (Čatská, 1994; Mehta and Bharat, 2013; Ceustermans et al., 2014) proved inefficient as demonstrated by the serious infections with Nectriaceae in the biotests with the three tested ARD soils from northern Germany. Collapsed arbuscules in impaired host cells in root sections with mixed infections confirmed this on the tissue level. As expected, fungal pathogens penetrated the young root cells much earlier and spread faster than AMF. From field samples it could be learned that the native AMF still colonized diseased plants, but their benefit for the plants was limited. It would therefore be interesting to test further AMF isolates in inoculation experiments for their efficacy as biocontrol agents against fungal root pathogens. In this case, according to our results, it seems important to give the AMF a time advantage over the challenge of pathogens.

Conclusions

The histological results clarified details of the incidence of early microbial infections in symptomatically altered, ARD-impaired young apple fine roots. The frequent occurrence of Nectriaceae in greenhouse biotests and their occurrence in fine roots of field grown plants gives reason to pay increased attention to this pathogen group in the etiology of the ARD. The previously unnoticed intracellular CF structures, which were significantly correlated with the induction of host cell necrosis, raise new questions about their function. In addition to chlamydospores, these CF structures might serve long-term survival and thus support the durability of ARD. Actinobacteria have also been associated with ARD symptoms in apple fine roots, however, their role in the development of the disease remains to be explained.

Acknowledgments

Our thanks go to Mrs. Ewa Schneider for providing *in vitro* propagated and acclimatized M26 plants and to Ms. Jenny Rebentisch for technical assistance. The German Federal Ministry of Research and Education funded this work in the project ORDIAmur (FKZ 031B0025A) within the framework of the BonaRes program.

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Received: Jun. 17, 2020
Accepted: Sep. 23, 2020

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Supplemental Information

For Supplemental Information, Tables S1–S2 and Figures S1–S8 see www.ishs.org/eJHS.