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

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Abstract

Apple replant disease (ARD) occurs when apple is repeatedly planted at the same site, leading to growth reductions and losses in fruit yield and quality. Up to now the etiology is poorly understood, but soil (micro)biota are known to be involved. Since endophytes often colonize plants via the rhizosphere this study aimed at comparing the bacterial endophytic root microbiome in plants growing in ARD affected and unaffected soils from three different sites based on greenhouse biotests using a molecular barcoding approach. The initial endophytic microbiome of the starting material (in vitro propagated plants of the apple rootstock M26) did not significantly affect the overall richness and diversity of the endophytic community in plants after 8 weeks of growth in the respective soils, but some genera of the initial microbiome managed to establish in apple roots. Proteobacteria were the dominant phylum in all samples.

No differences in diversity or number of amplicon sequence variants (ASVs) between plants grown in ARD soil and unaffected soil was observed. However, several ASVs of high abundance uniquely found in plants grown in ARD affected soils were *Streptomyces*. In soil from all three sites these *Streptomyces* were negatively correlated to plant growth parameters. Future inoculation experiments using selected *Streptomyces* isolates have to prove if bacteria from this genus are opportunists or part of the ARD complex. For the first time, the bacterial endophytic community of apple roots grown in ARD affected soils was characterized which will help to understand the etiology of ARD and develop countermeasures.

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

Introduction

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

Based on molecular barcoding approaches in the last decade, many studies confirmed not only changes in the abundance of specific pathogens in ARD affected soils, but significant shifts in the overall structure of the microbiome of the bulk soil and the rhizosphere (Winkelmann et al., 2019). These microbiome shifts are also affecting major functional properties including the potential to degrade aromatic compounds and for biocontrol (Radl et al., 2019).

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

Only a few studies have investigated the role of endophytes in ARD focusing on potential fungal root pathogens. Manici et al. (2013) found the root endophytic *Cylindrocarpon*-like fungi (*Ilyonectria* spp. and *Thelonectria* sp.) and *Pythium* spp. to be main causal agents of growth reduction in the rootstock M9 growing in ARD affected soil. *Cylindrocarpon* spp. was also identified next to *Rhizoctonia* sp. as a pathogenic root endophyte by Kelderer et al. (2012) in row (ARD affected) and inter-row (control) planted apple trees. In addition, *Fusarium solani* and *Fusarium oxysporum* were most abundant in roots but not considered pathogenic. Popp et al. (2019) isolated several fungal endophytes from ARD affected apple roots and re-inoculated them

in a soil free biotest. *Cadophora*, *Calonectria*, *Dactylonectria*, *Ilyonectria*, and *Leptosphaeria* were reported to have negative effects on plant health. In contrast, studies on the effects of ARD on bacterial root endophytes are scarce. So far, only a targeted cultivation dependent approach has been published, where the focus was given on the biocontrol properties of Actinobacteria isolates (mostly belonging to the genus *Streptomyces*) from the root interior of apple trees. However, no effect was observed when co-inoculated to apple seedlings with *Pythium irregulare* and *Cylindrocarpon macrodidymum* (Tewoldemedhin et al., 2011)

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

Material and Methods

Experimental setting

Soil for these experiments was sampled from three different sites: Heidgraben (x-coordinate 53.699199; y-coordinate 9.683171; WGS 84, Schleswig-Holstein, northern Germany), Ellerhoop (x-coordinate 53.71435; y-coordinate 9.770143 WGS 84, Schleswig-Holstein, northern Germany) and Ruthe (x-coordinate 52.243668; y-coordinate 9.819700; WGS 84, Lower Saxony, Germany). These sites differed in their climatic conditions and soil properties. The upper soil textures of the three sites were defined (based on World Reference Base for soil resources) as loamy sand (Heidgraben), sand (Ellerhoop) and silt loam (Ruthe) (Mahnkopp et al., 2018). Every site contained two different plot variants: (i) ARD plots, where ARD was successfully induced by repeatedly replanting ´Bittenfelder´ apple seedlings since 2009 in a two-year cycle and (ii) control plots which were only covered with grass since then. ARD plots in Ruthe and Ellerhoop were replanted for the last time in spring 2015 and in Heidgraben in spring 2016.

Soils were sampled from all three sites in a depth from 0 – 20 cm in the end of 2015 and 2016, respectively. After sampling and sieving (8 mm), soils were either gamma irradiated (G) at a minimal dose of 10 kGy or left untreated (UT) resulting in 4 variants per site: ARD untreated (ARD UT), ARD gamma (ARD G), grass untreated (Grass UT) and grass gamma (Grass G).

The ARD susceptible apple rootstock M26 which was propagated and rooted *in vitro* (for details see Weiß et al. 2017a) was acclimatized for 4 weeks and afterwards one plantlet each was planted in 1 L-pots containing the different soil variants. Soils were supplemented with 2 g L^{-1} Osmocote Exact 3–4M (16 + 9 + 12 + 2 MgO; https://icl-sf.com/de-de/products/ornamental_horticulture/8840-osmocote-exact-standard-3-4m/) to exclude

nutrient effects. Shoot lengths were measured weekly. Plants were grown for 8 weeks in the greenhouse at a mean daily temperature of 21°C and a 16 h photoperiod achieved by additional light (SON-T Philips Master Agro 400 W) with a set point of 25 klx. Plant protection measures and irrigation were done according to Yim et al. (2015). At the first sign of insect pests, 0.3 % NeemAzal was sprayed. During night, a sulfur evaporator was used in order to prevent fungal diseases. The greenhouse experiment was conducted twice, in February 2016 and 2017 (Mahnkopp et al. 2018) with 9 replicates per variant.

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

Sampling

After 8 weeks of cultivation in the greenhouse 4 representative plants per variant were taken (48 per year, 96 in total) as biological replicates. Roots were washed carefully to get rid of the adhering soil. Shoot and root fresh mass were determined. For surface sterilization, roots were rinsed for 30 s in EtOH (70 %), followed by stirring in 2 % NaOCl for 7.5 min and finally washing 5 times in sterile deionized water. The final washing water was plated on 523 medium (Viss et al., 1991) and incubated at room temperature for 1 week. Plating resulted in < 10 CFU per plate in all cases. Roots were stored in sterile 2 ml Eppendorf tubes at -80°C until DNA extraction.

DNA extraction and amplicon sequencing

50 to 100 mg surface sterilized roots per sample were homogenized under frozen conditions using steel beads (Ø 6 mm) in a mixer mill (MM400, Retsch, Haan, Germany) with a frequency of 23 Hz

for 2 minutes using sterilized devices. DNA was extracted using the Invisorb Spin Plant Mini Kit (Stratec, Berlin, Germany) according to the provided protocol. DNA quality was checked using a spectrophotometer (Nanodrop 2000c Peqlab, Erlangen, Germany).

The primer combination 335F (CADACTCCTACGGGAGGC)/ 769R (ATCCTGTTTGMTMCCCVCRC) (Dorn-In et al. 2015) including overhang adapter sequence were used to amplify the V3 - V4 region of the 16S rRNA gene. PCR reactions contained 2x Phusion High-Fidelity Master Mix (1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.2 U Phusion DNA Polymerase, Thermo Fisher Scientific, Waltham, USA), 10 pmol of each primer, 5 ng DNA template and water to a final volume of 10 µL. The PCR cycling conditions consisted of an initial denaturation step of 98 °C for 10 s, followed by 30 cycles involving 1 s of denaturation at 98 °C, 5 s of annealing at 59°C and 45 s of extension at 72°C, with a final extension of 1 min at 72°C. Triplicate PCR reactions were pooled and purified using Agencourt AMPure XP kit (Beckman Coulter, USA). The purified products were quantitated using the Quant-IT PicoGreen dsDNA assay kit (Life Technologies Europe, Gent, Belgium). Sample indexing was carried out with Nextera XT Index Kit v2 Set A and B (Illumina, USA) in reaction mixtures containing 10 ng purified PCR product, 2x Phusion High-Fidelity Master Mix (1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.2 U Phusion DNA Polymerase, Thermo Fisher Scientific, Waltham, USA), 10 pmol of each indexing primer and water to a final volume of 25 µL. The indexing PCR cycling conditions consisted of an initial denaturation step of 98 °C for 30 s, followed by 8 cycles involving 10 s of denaturation at 98 °C, 30 s of annealing at 55°C and 30 s of extension at 72°C, with a final extension of 5 min at 72°C.

Indexed samples were purified as described above. Equimolar concentrations of the purified indexed samples were prepared and diluted to a final concentration of 4 nM. The library was

sequenced using the Illumina Miseq platform with the MiSeq Reagent Kit v3 (600 cycle) (Illumina, USA).

Bioinformatic and statistical analysis

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

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

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

16S amplicon sequencing of DNA extracted from the surface sterilized M26 roots resulted after quality control in a total of 4,132,410 reads with a mean of 72,498 reads per sample in the biotest in 2016. After removal of chloroplast, archaea and eukaryotic ASVs sequence data was rarefied

200 at a number of 28817 reads (2016). A total of 5898 ASVs were detected. As rarefaction analysis
201 (Fig. S1) indicated that saturation was reached already with read numbers about 5,000 reads per
202 sample, for 2017 the sequencing effort was reduced, resulting in in total 884,916 reads and a
203 mean of 17351 reads per sample (rarefied to 4213 reads per sample after the removal of the
204 chloroplasts). The total number of ASVs was 4971 which were nearly all covered after rarefying
205 at 4813 reads per sample (Fig. S2). In order to identify identical ASVs between the two years,
206 alignments of the sequences of the two years on genus level were done using Clustal W Multiple
207 Alignment (Thompson et al., 1994) with number of bootstraps set to 1000 using BioEdit v7.2.5
208 (Hall et al., 1999) followed by calculating a sequence differences count matrix. ASVs from 2017
209 which were 100 % identical to ASVs from 2016 were given the corresponding name of 2016 ASVs
210 to improve comparability of figures and tables.
211 To calculate the relative abundance, the number of reads per ASV in the samples was divided by
212 the sum of total reads per sample and multiplied by 100. The relative abundances of ASVs
213 belonging to the same phylum/genus were combined to calculate the overall relative abundance
214 of the corresponding phylum/genus. Species diversity (Shannon, Simpson) and richness (Chao1)
215 indices were determined using the "Phyloseq" (McMurdie and Holmes 2013) and "Vegan"
216 (Oksanen et al. 2019) Packages of R v3.6.1 (R Development Core Team (2008), http://www.R-
217 project.org) and tested for normal distribution based on Shapiro-Wilk test (Shapiro and Wilk,
218 1965) and homogeneity of variance based on Levene´s test (Levene, 1960) using the program
219 PAST3 v. 3.20 (Hammer et al. 2001). If the null hypotheses of normal distribution and equal
220 variances were rejected, theTukey test based on Heberich et al. (2010) was used at $p < 0.05$ to
221 determine significant differences of the raw diversity and richness scores. In order to compare

the relative abundance of different genera of the initial microbiome (T0 plants) between the years, all ASVs belonging to the same genus were merged. Because of unequal sample size and unequal variance, Welch's Two Sample t-test was used at $p < 0.05$ to determine significant differences. Non-metric Multidimensional Scaling (NMMDS) was performed with the program PAST3 v. 3.20 (Hammer et al., 2001) using Bray Curtis similarity index and Analysis of Similarity (ANOSIM) in order to visualize the community composition of the different samples. To indicate the influence of the different genera, vectors were added which show the correlation between the corresponding genus and the NMMDS score. Spearman´s correlation was used in order to correlate ASVs to shoot growth and fresh mass using the program PAST3 v. 3.20 (Hammer et al., 2001). Venn-Diagrams were designed using the Venn-Diagram tool of Bioinformatics & Evolutionary Genomics (BEG, http://bioinformatics.psb.ugent.be/webtools/Venn).

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

Results

Root and shoot biomass

As expected, in both years, plants grown in untreated ARD affected soils showed the lowest increase in shoot length and lowest shoot fresh mass in comparison to the other soil variants (Tab. 1). Gamma irradiation of grass and ARD soil led to increased shoot length and shoot fresh mass compared to the respective untreated soils. Overall plants grown in the biotest in 2017 showed a higher biomass in comparison to plants grown in 2016, but the response pattern to the different soils variants was comparable between both years. Detailed growth data for the full set of 9 plants per variant can be found in Mahnkopp et al. (2018), whereas in Table 1 only the data of the plants selected for the barcoding approach of this study is presented.

Endophytic bacterial community composition and diversity in plant roots grown in different soil variants

The mean number of observed ASVs per sample was 244 in 2016 (Tab. 2) and 201 in 2017 (Tab. 3). The highest number of observed ASVs were found in plants grown in gamma irradiated (G) ARD soil from Ellerhoop with 339 ± 93 and the lowest one in the variant Heidgraben ARD G with 148 ± 62 (both in 2016). In both years no significant differences in diversity or richness indices were recorded within or between the sites (Tukey-test, $p < 0.05$).

Proteobacteria were clearly the dominant phylum in all variants with relative abundance ranging from 66.9 % (Heidgraben ARD G) to 83.7 % (Ruthe Grass UT, Fig. S3) for the biotest performed in 2016. Bacteroidetes were of second most abundance with a mean value of 10.3 % followed by

Actinobacteria (5 %) and Firmicutes (4.4 %). Actinobacteria appeared in higher relative abundance in roots grown in ARD UT compared to the other variants of the respective site. The strongest difference was observed in Heidgraben where the abundance of ASVs linked to Actinobacteria in ARD UT variants (15.60 %) was significantly higher than in ARD G (5.03 %), Grass UT (2.75 %) and Grass G variants (3.47 %). The second biotest in 2017 showed similar shares for the different phyla (Fig. S4). ASVs assigned to Proteobacteria ranging from 66.7 % to 89.3 % were dominant, followed by Bacteroidetes with a mean value of 13.6 % relative abundance, Actinobacteria with 8.1 % and Firmicutes with 2.2 %. However, the higher abundance of ASVs linked to Actinobacteria in the untreated ARD variants was not observed in this year.

Dynamics of endophytic bacteria during the biotest

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

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

On higher phylogenetic levels, proteobacterial groups of the genera *Shewanella* and *Halomonas* belonged to the top three genera regarding relative abundance in T0 plants in both years (Tab. S2). However, in total, nearly one third (31,9 %) of the genera showed significant differences in abundance between the years. In T0 plants from 2016 for example, *Ralstonia* was with 6.6 % the most abundant genus but not present in 2017 T0 plants. Even after growing for 8 weeks in different soils, this abundance pattern still remained for ASVs linked to *Ralstonia*. Similar contrasting abundance pattern were observed for *Pseudomonas* when both years were compared. Here we could link 12,6 % of all ASVs from T0 plants to this genus in 2017, which was more than four times higher than in 2016. However, in contrast to ASVs linked to *Ralstonia,* after 8 weeks of cultivation in the different soils these initial differences in abundance of *Pseudomonas* were no longer detectable.

To analyze ß-diversity, 3D Non-metric Multidimensional Scalings (NMDS) were created for the untreated soils. In general, high variability within variants could be observed. In both years T0 plants significantly separated from the other variants (ANOSIM with $p \leq 0.05$, Fig. 1, Fig. S5). Only for the biotest in 2016, significant differences between other variants were observed, especially for the treatments with soil from Ruthe (Fig. 1). ASV assigned to *Pseudomonas*, *Rhizobium* and especially *Streptomyces* were closely linked with ARD, whereas ASVs related to *Rhodanobacter*, *Dyella*, *Bradyrhizobium*, *Sphingomonas* and *Rhizomicrobium* pointed to the untreated grass variants (Fig. 1). Most responsible for differentiation of T0 were ASVs which were linked to *Halomonas*, *Acinetobacter* and *Shewanella*. In the biotest in 2017 no clear clustering except for T0 was observed (Fig. S5).

Identification of bacterial responders in the different treatments and correlation to plant growth

To further investigate ASVs responding to the different treatments of each site, Venn diagrams were designed. On the one hand the number of ASVs shared by all four different variants per site, which we considered as the core microbiome of a given site, was surprisingly small. In 2016, only 6 ASVs (relative abundance > 0.5 %) in soil variants from Heidgraben, 3 in those from Ellerhoop and 4 in those from Ruthe were present in all variants (Fig. 2). In 2017, these numbers were reduced to 0 (Heidgraben), 2 (Ellerhoop) and 1 (Ruthe) (Fig. 3). On the other hand, the number of ASVs which were unique for each variant was high. In 2016, unique ASVs in untreated ARD variants ranged from 15 for Ruthe and 19 for Heidgraben, to 28 for Ellerhoop (Fig. 2). In the untreated grass variants, the number of unique ASVs ranged from 23 for Ruthe to 26 for Ellerhoop and 29 for Heidgraben. Although the overall distribution was very similar in both years, for the soil from Ellerhoop, some variations were observed: the number of unique ASVs changed in untreated grass variants from 26 in 2016 to 9 in 2017. Results for soil variants from Heidgraben showed the lowest variation between the years except the unique ASVs for the grass variant sterilized by gamma-irradiation, where 16 (2016) and 31 (2017) unique ASVs were observed, respectively.

In order to identify responders towards ARD, the unique ASVs of the untreated ARD variants were correlated to shoot growth and fresh mass of all variants of the three sites. In 2016 most noticeable were ASVs related to the genus *Streptomyces* (Fig. 2), which closely linked to plants grown in ARD affected soils, confirming the overall observation that ASVs related to

Actinobacteria were positively responding to the ARD affected soils with increased levels in relative abundance. In Heidgraben ARD UT, 7 out of 19 unique ASVs were linked to the genus *Streptomyces* followed by Ellerhoop ARD UT (4 out of 28) and Ruthe ARD UT (3 out of 15). Most of these ASVs were high in relative abundance. *Streptomyces* ASV66 in Heidgraben and *Streptomyces*_ASV42 in Ruthe showed the highest relative abundance with 4.48 % and 4.10 %, respectively. All *Streptomyces* ASVs were negatively correlated to the increase of shoot length and shoot fresh mass and some of them were even present in at least two sites. *Streptomyces*_ASV21 which was present in all three sites as a unique ASV showed with -0.54 and -0.58 the second highest negative correlation to both plant growth parameters. This number was only surpassed by *Streptomyces* ASV70 and *Streptomyces* ASV76 (both present in Heidgraben and Ellerhoop) with a correlation of -0.59 to the increase of shoot length and -0.65 to shoot fresh mass.

A high number of other genera harboring unique ASVs were also negatively correlated to plant growth parameters. For example, *Novosphingobium* ASV92 and *Neorhizobium* ASV47 (highly abundant in Heidgraben and Ellerhoop) negatively correlated to increase of shoot length (-0.59 and -0.53) and shoot fresh mass (-0.53 and -0.59, Fig. 2).

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

We were further interested in unique ASVs of the untreated grass variants to identify possible plant growth promoting bacteria, which could help to counteract apple replant disease. In 2016, several ASVs of different genera were present in more than two sites (Fig. S6). These included ASVs related to *Dyella*, *Massilia*, *Rhizobium*, *Rhodanobacter* and unclassified Moraxellaceae. In the biotest 2017, only 2 ASVs (assigned to *Rhizobium* and *Sphingobium*) were present at least at two sites (Fig. S7) However, none of them showed positive correlations with plant growth. The only positively correlated ASV was related to unclassified Rhizobiaceae and found in 2017 with a relative abundance of 0.95 % and a correlation of 0.32 and 0.30 to shoot growth and shoot fresh mass (Fig. S7)

Discussion

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

Endophytic bacterial communities in apple roots were dominated by Proteobacteria

Proteobacteria were the dominant phylum in most studies, where bulk soil or rhizosphere samples from ARD affected sites had been analyzed (Franke-Whittle et al. 2015; Perruzzi et al. 2017; Sun et al. 2014; Tilston et al. 2018; Yim et al. 2015) with an average relative abundance of 35 % (Nicola et al., 2018). The same was true for the root endophytes analyzed in our experiments in both years (Fig. S3, S4). However, in comparison to Nicola et al. (2018) the relative abundance of Proteobacteria in roots from plants grown in ARD UT was clearly higher (76 % in 2016 and 71

374 % in 2017 in average of all three sites). This enrichment of Proteobacteria in the endosphere could
375 be explained by selective recruitment/colonization or a higher competitiveness inside the plant.
376 Members of this phylum are known for their various secretion systems (Preston et al., 2005), their
377 fast growth and their high metabolic activity and therefore mostly predominate the endosphere
378 (Lundberg et al. 2012; Reinhold-Hurek et al. 2015).
379 In many studies where rhizosphere and bulk soil samples of ARD affected sites have been analyzed
380 Actinobacteria were amongst the most abundant phyla with an average relative abundance of 16
381 % (Nicola et al., 2018), which was slightly higher than in our study when we focused on root
382 endophytes (10 and 11 % in 2016 and 2017 respectively). Bacteroidetes showed in other studies
383 an average relative abundance of 14 % (Nicola et al., 2018) and 13 % (Tilston et al., 2018). In our
384 study, values ranged between 8 % (2016) and 15 % (2017). As expected Acidobacteria, which were
385 also highly abundant in the rhizosphere and bulk soil in the above mentioned studies were low in
386 relative abundance in the root interior, due to the ecophysiological properties of these bacteria,
387 including the use of complex organic compounds and their slow growth.
388
389 Differences between the outcome of the biotest of 2016 and 2017
390 Significant differences occurred in the results comparing biotests between 2016 and 2017. In
391 2016, Actinobacteria were significantly higher in relative abundance in root samples from ARD UT
392 compared to the grass or gamma-sterilized variants (Fig. S3). Surprisingly, this was not the case in
393 2017 (Fig. S4). Furthermore, there was a clustering of ARD variants apart from the grass variants
394 in 2016 (Fig. 1) but not in 2017 (Fig. S5). These differences in the outcome of the biotests could
395 be due to various factors, e. g. higher shoot lengths of T0 plants of 2017 or variation in

environmental factors. Another reason could be related to the soil: First of all, soil collection might have resulted in samples of different microbial composition due to patchy appearance of ARD in the field (Simon et al. 2020). Furthermore, at our reference sites, replanting takes place every second year, and was carried out in 2015 and 2017 at Ruthe and Ellerhoop and in 2014 and 2016 at Heidgraben. Soil for the first greenhouse experiment in 2016 was sampled in the end of 2015, where plants at Ruthe and Ellerhoop had been replanted for the 4th time in spring 2015, while plants in Heidgraben had just been uprooted. For the experiment in 2017, soil was collected at the end of 2016, when plants at Ellerhoop and Ruthe had been uprooted and at Heidgraben had been replanted for the 5th time in spring. It is known that the microbial community composition in the rhizosphere of different apple genotypes varies seasonally and among the years (Rumberger et al. 2007). Also replanting is known to have an influence of the rhizosphere community composition (Sun et al., 2014).

Another reason for these different outcomes of the bio-test in the two years could be differences in the initial endophytic bacterial community composition of the starting material (T0) plants. In this study, a higher diversity and number of ASVs of the initial microbiome in the plant roots was found in 2016, as compared to that in 2017. However, this difference between the two years was not significant after 8 weeks of growth in the soil. Therefore, a higher diversity and number of ASVs of the initial microbiome did not lead to higher number of ASVs and diversity in plant roots after 8 weeks of growth in the soil. Since soil is the main reservoir of microorganisms for the plant microbiome (Berg and Smalla 2009; Bonito et al. 2014; Lareen et al. 2016; Hartman and Tringe 2019) it is one of the major factors influencing the number of ASVs and diversity. However, some

genera, e. g. *Ralstonia*, were present in the 2017 T0 plants and were still present in the plant roots after grown for 8 weeks in the soil (Tab. S2). With this in mind one strategy to help to overcome ARD could be to inoculate apple plants with plant growth promoting bacteria before transferring them into the soil. This so called microbiome engineering of plants was recently reviewed by Orozco-Mosqueda et al. (2018). Johnston-Monje and Raizada (2011) could show, that GFP tagged *Enterobacter asburiaes* could systemically colonize the roots of maize and even the rhizosphere. This means that some genera of the initial endophytic microbiome may possess the ability to not only colonize the plant roots but also the rhizosphere. In order to have plants which are preinoculated with PGPB that can influence not only the endosphere but also the rhizosphere and therefore may be able to reduce the ARD effect, inoculation studies are needed.

Next to differences between the years, variations within the soil variants were observed. The observed ASVs and the different diversity indices within the variants showed high standard deviations (Tab. 2 and 3). With the reanalysis of several studies of microbiomes of ARD affected soils Nicola et al. (2018) determined that the strongest factor for bacterial community variation were environmental variables. In our study several factors responsible for variations were reduced to a minimum (soils mixed, clonally propagated plants, same greenhouse conditions). However, here we were investigating the root endophytic community. Its selection is strongly controlled by the host plant and dependent of soil and several other factors like stress and environmental conditions (Afzal et al., 2019). Although all plants and soils within a variant were treated in the same way, individual differences in the soil microbiome and, therefore, differences

in root colonization cannot be excluded. To reduce these variations future biotests should increase the number of analyzed plants.

Are Streptomycetes part of the ARD complex?

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

Streptomyces is a well-studied genus and most famous for its production of antibiotics with 80 % of today's antibiotics being derived from *Streptomyces* (de Lima Procópio et al. 2012). Next to this, traits like production of antifungal substances and siderophores, solubilization of phosphate, synthesis of plant growth regulators, secretion of volatile compounds, biocontrol (competition for nutrients) and degradation of phytotoxins makes it a potent plant growth promoting bacterium intensively reviewed by Olanrewaju and Babalola (2019), Sousa and Olivares (2016), Viaene et al. (2016) and Vurukonda et al. (2018). These reviews also highlight that *Streptomyces* is able to colonize a broad range of plant hosts. It is further believed that these plants can selectively recruit *Streptomyces* (Viaene et al. 2016). However, the signals which attract them or

460 the way of their entering and colonizing the roots are still unknown (Viaene et al. 2016;
461 Vurukonda et al. 2018).
462 However, these various plant growth promoting effects of *Streptomyces* were not affirmed by our
463 findings. Roots growing in soil from the grass variants showed better growth than those in ARD
464 soils. Only two *Streptomyces* ASVs being unique for at least two grass variants were found which
465 had no correlation to increase in shoot length and shoot fresh mass (Fig. S6, S7). In contrast, in
466 ARD variants a clear negative correlation of the relative abundance of *Streptomyces* and plant
467 growth was shown (Fig. 2, 3). On the one hand this could indicate that *Streptomyces* is pathogenic
468 and part of the replant disease. On the other hand, since *Streptomyces* has a saprophytic lifestyle,
469 it could be an opportunist and degrade dead or damaged root material. Structurally damaged and
470 partially necrotic root systems are typical symptoms for ARD affected plants (Grunewaldt-Stöcker
471 et al. 2019). *Streptomyces* is able to break down organic remains of plants using several hydrolytic
472 exoenzymes like cellulases, lignocellulases, pectinases, xylanases and cutinases (Chater et al.,
473 2010; Chater 2016). *Streptomyces* was also shown to appear in higher abundance in the
474 rhizosphere of *Arabidopsis thaliana* when plant exudated phenolic-related compounds like
475 salicylic acid were present (Badri et al. 2013; Lebeis et al., 2015) and can even grow on minimal
476 media with only salicylic acid as a carbon source (Lebeis et al. 2015). Due to tyrosinase activity
477 some isolates were partially protected against plant produced phenols leading to increased
478 colonization rates of *A. thaliana* roots (Chewning et al. 2019). Gene expression studies revealed
479 that genes responsible for the production of phytoalexins (some of which belong to polyphenols)
480 are upregulated in M26 roots growing in ARD affected soils (Weiß et al. 2017a, 2017b).

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

Out of the 843 known *Streptomyces* species (Euzéby 1997; Parte 2018, LPSN accessed 18.04.2020) only 10 have pathogenic features (Viaene et al., 2016). Most known are *Streptomyces scabies, S. acidiscabies* and *S. turgidiscabies* which cause common scab on roots and tuber crops. These species are able to directly penetrate plant cells and in addition to necrotic scab lesions, lead to reduced growth, root stunting and browning and to a reduction of the complexity of the root system (Loria et al. 2003; Loria et al. 2006; Seipke et al. 2012), i.e. symptoms that resemble the phenotype of ARD affected roots. However, despite the large host range none of these species were reported to infect woody plants. Though, the host range likely includes all higher plants, since dicot and monocot seedlings of several plant species have shown symptoms after inoculation with *S. scabies* (Leiner et al., 1996; Loria et al. 2006). One reason for this large host range is based on the assumption that *Streptomyces* is believed to originate 400 million years ago when green plants started to colonize the land (Chater 2016). Another reason for this flexibility is the fact that *Streptomyces* virulence genes are clustered on a pathogenic island which can be mobilized and via conjugation transferred to nonpathogenic relatives which leads to the emergence of new plant pathogenic Streptomycetes (Lerat et al., 2009).

A closer look at the *Streptomyces* from our greenhouse experiment revealed that the *Streptomyces* ASVs which occur in at least two sites (Fig. 2, 3) shared a high similarity with the pathogen *S. turgidiscabies*. Blasting the sequences against the NCBI database

503 (https://www.ncbi.nlm.nih.gov/) showed a similarity of 99.76 % (ASV21 and ASV70), 99.51 %
504 (ASV76) and 99.52 % (ASV621) (Tab. S1). All these ASVs showed a negative correlation to shoot
505 fresh mass of around -0.60 or more, whereas ASV121 which showed with -0.34 the lowest
506 negative correlation also shared the lowest identity with *S. turgidiscabies* (97.32 %). However, for
507 further comparisons to *Streptomyces* species the complete 16S rRNA sequence of the apple root
508 endophytes identified in this study is necessary.
509 Nevertheless, the high similarity to pathogenic *Streptomyces*, the broad host range and ability for
510 horizontal gene transfer of virulence genes may be arguments in favor of *Streptomyces* as possible
511 causative organism of apple replant disease.
512
513 Role of *Streptomyces* in apple replant disease
514 Several previous studies investigated *Streptomyces* in relation to ARD. However, they resulted in
515 controversial conclusions: *Streptomyces* is part of the order of Actinomycetales, members of
516 which were first mentioned as a possible cause of ARD by Otto and Winkler (1977). The authors
517 at that time could only identify the bacteria by their morphology at the level of the phylum which
518 was called “Actinomycetes” in those days. In their histological analysis, “Actinomycetes” were
519 found in damaged roots of apple seedlings with a frequency of 47.3 % in replant affected soil, but
520 not (0.3 %) in steamed soil (Otto and Winkler 1977). Also in plants from our greenhouse
521 experiments, Actinobacteria were histologically observed more frequently in roots in untreated
522 ARD soils than in non-ARD soils (Grunewaldt-Stöcker et al. 2019). The so called “root pathogenic
523 Actinomycetes” (Otto et al. 1993) were observed in ARD affected roots of apple seedlings.
524 Thereafter, the degree of infestation increased with increasing shoot growth and decreased with

525 stagnating growth. This led to the assumption that root exudates which are influenced
526 qualitatively and quantitatively by the growing buds triggered the germination of persistent
527 spores (Otto et al. 1993).
528 In contrast to a pathogenic role, *Streptomyces* spp. were considered plant growth promoters in
529 other studies dealing with ARD. 16S rRNA-pyrosequencing revealed that the genus *Streptomyces*
530 was positively (0.64) correlated to shoot growth in plants grown in fumigated ARD soil (Nicola et
531 al. 2017). A function in disease suppression was also associated with *Streptomyces* (Cohen et al.,
532 2005; Cohen and Mazzola 2006; Mazzola et al., 2007), when the effect of seed meal amendments
533 on the putative ARD causing pathogens *Rhizoctonia solani* or *Pythium* spp. was investigated. Seed
534 meal amendments resulted in increased populations of *Streptomyces*, which were able to
535 suppress infections by *Rhizoctonia solani*. Disease suppression was attributed to a transformation
536 of bacterial community structure and the production of nitric oxide (Cohen et al. 2005; Cohen and
537 Mazzola 2006) which plays a role in the induction of plant systemic resistance. Most *Streptomyces*
538 isolates recovered from the apple rhizosphere were able to produce nitric oxide (Cohen et al.
539 2005). By adding any of several *Streptomyces* strains, Cohen and Mazzola (2006) could restore
540 disease suppressiveness in previously pasteurized soil. Next to disease suppression, promotion of
541 root infection by *Streptomyces* was also observed in apple (Zhao et al. 2009) and *Picea abies* (Lehr
542 et al. 2007;). Root infections were significantly elevated in the presence of *Streptomyces*. This may
543 be a negative side effect, since *Streptomyces* is known to promote mycorrhizal formation by
544 promoting fungal growth and by decreasing plant defense response (Lehr et al. 2007; Tarkka et
545 al. 2008; Vurukonda et al. 2018). *Streptomyces* sp. AcH 505 was shown to downregulate the

peroxidase activity and pathogenesis-related peroxidase gene (Spi2) expression (Lehr et al. 2007) of the host plant, thus promoting fungal root colonization.

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

All these findings indicate that *Streptomyces* could be responsible for ARD or be part of it. But to proof this, inoculation experiments are necessary as done by Tewoldemedhin et al. (2011). They isolated 96 *Streptomyces* strains from surface sterilized roots from six ARD affected sites in South Africa and inoculated 37 of them to 4 weeks old apple seedlings to test pathogenicity. Moreover, 11 were co-inoculated with the pathogens *Pythium irregulare* and *Cylindrocarpon macrodidymum*. All tested Streptomycetes had no effect on plant growth. At first this seems as a clear sign that these *Streptomyces* isolates were not pathogenic (directly or indirectly). However, these isolates had low identity (less than 98 %) to known *Streptomyces* species and none showed close similarity to *Streptomyces turgidiscabies*, which had a high identity to our ASVs with a negative correlation to plant growth. Also in our experiments not all *Streptomyces* were negatively correlated to plant growth. In 2017 of 61 detected ASVs only 6 showed negative correlations to shoot fresh mass (15 out of 32 in 2016). Furthermore, inoculation trials were done in artificial soil (bark medium and sand 2:1) (Tewoldemedhin et al., 2011) which means that potential “co-pathogens” were not present unlike in ARD soil.

568

569 *Streptomyces* not present in T0 plants

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

579

580 Plant growth promoting bacteria in plants grown in non-ARD soils

581 In order to find possible plant growth promoting bacteria (PGPB) that may be used to overcome
582 ARD, we also looked at the unique ASVs in the grass variants to find ASVs positively correlated to
583 plant growth. However, in 2016 no ASV showed any significant positive correlation (Fig. S6). In
584 2017, only one ASV (NA_ASV4691 (Rhizobiaceae)) showed with 0.32 and 0.30 a positive
585 correlation to the increase in shoot length and shoot fresh mass.

586

587 **Conclusion**

588 Here we could show for the first time the apple root endophytic community composition in plants
589 grown on three replant affected soils in comparison to non-replant affected soils based on next

590 generation sequencing in two years. Although no plant growth promoting bacteria to counteract
591 ARD could be found, several ASVs with negative correlations to plant growth were associated with
592 ARD. With *Streptomyces* showing strong negative correlations and being present in all soils over
593 the years, a potential key player for the cause of ARD may have been found. However, it remains
594 to be clarified in future studies, whether *Streptomyces* as root endophyte in ARD situations acts
595 opportunistic or is pathogenic. *Streptomyces* can grow saprophytically and just degrade plant
596 material and metabolize plant exudates, but can also play an essential role in the ARD complex
597 by suppressing plant defense responses and thereby promote infection of fungal pathogens.
598 Further inoculation studies with *Streptomyces* isolates in combination with fungal pathogens as
599 co-inoculants will help to answer this question.
600

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Tables

Table 1: Shoot fresh mass and increase of shoot length of M26 apple plants grown for 8 weeks in the greenhouse biotest in 2016 and 2017. Surface sterilized roots of these plants were used for DNA extraction and amplicon sequencing. Shown is the mean and the standard deviation of n plants (For n numbers see Table 2 and 3). Different letters indicate significant differences within the sites (Tukey test, $p \leq 0.05$). For the growth data of all plants see Mahnkopp et al. 2018.

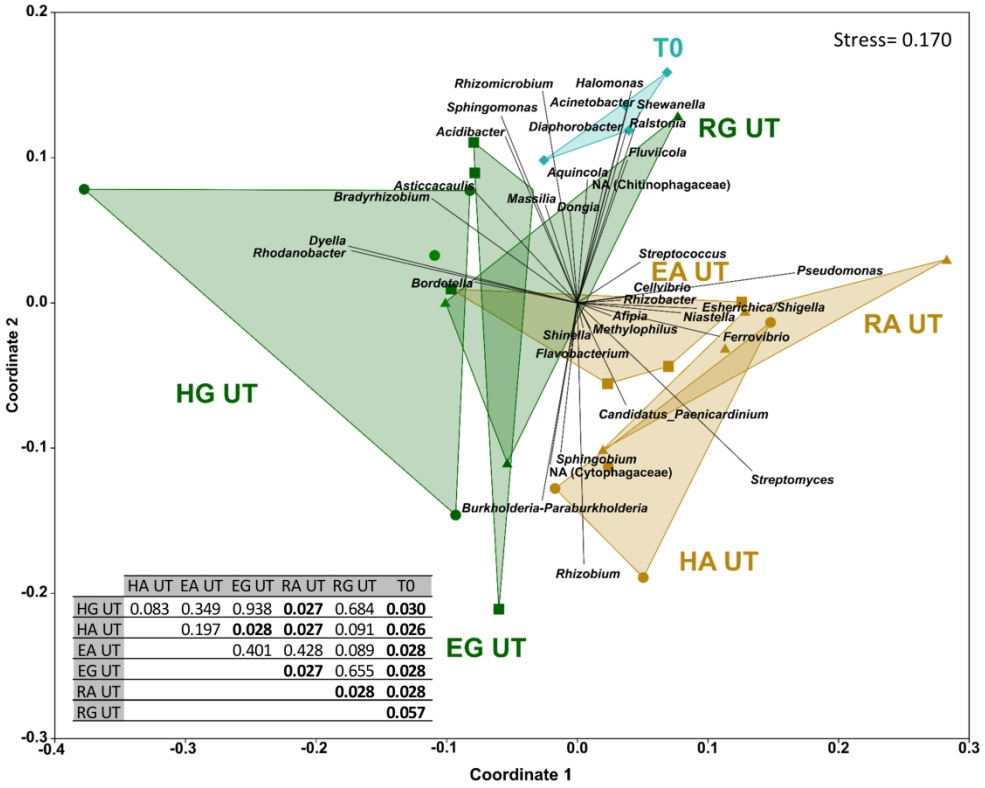
	Heidgraben				Ellerhoop				Ruthe			
	ARD		Grass		ARD		Grass		ARD		Grass	
	UT	G	UT	G	UT	G	UT	G	UT	G	UT	G
2016												
Increase in shoot length (cm)	5.9 ± 2.3 a	18.5 ± 1.5 c	13.5 ± 2 b	27.7 ± 6.3 c	7.5 ± 1 a	12.4 ± 3.8 ab	19.6 ± 1.6 b	25.9 ± 1.7 c	8.5 ± 1.4 a	25.2 ± 0.2 c	16.7 ± 1.9 b	32.0 ± 1.4 d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

Table 2: Richness and diversity of endophytic bacterial communities based on amplicon sequence variants (ASVs) in roots grown for 8 weeks in soils from different sites and treatments (UT = untreated. G = gamma irradiated) of the biotest in 2016. Additionally, T0 plants before transferring into the soil variants are shown. There was no significant difference within and between the sites according to Tukey's test at $p \leq 0.05$. Shown are mean ± standard deviation of n replicates.

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

Table 3: Richness and diversity of endophytic bacterial communities based on amplicon sequence variants (ASVs) in roots grown for 8 weeks in soils from different sites and treatments (UT = untreated. G = gamma irradiated) of the biotest in 2017. Additionally, T0 plants before transferring into the soil variants are shown. There was no significant difference within and between the sites according to Tukey's test at $p \leq 0.05$. Shown are mean ± standard deviation of n replicates.

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



	HA UT	EA UT	EG UT	RA UT	RG UT	T0
HG UT	0.083	0.349	0.938	**0.027**	0.684	**0.030**
HA UT		0.197	**0.028**	**0.027**	0.091	**0.026**
EA UT			0.401	0.428	0.089	**0.028**
EG UT				**0.027**	0.655	**0.028**
RA UT					**0.028**	**0.028**
RG UT						**0.057**

Figure 1: Three dimensional Non-metric Multidimensional Scaling (NMDS) using Bray-Curtis dissimilarity of roots grown 8 weeks in soils from different sites of the biotest in 2016. Gamma irradiated variants are not included. Vectors represent the correlation coefficient between the corresponding genus and the NMDS score. The relative lengths and the directions of the vectors indicate the influence of the respective genera (RA > 1 %). The third axis is not shown. Results of the one way analysis of similarities (ANOSIM) are shown in the lower left corner, significant differences are highlighted in bold ($p \leq 0.05$) . H = Heidgraben, E = Ellerhoop, R = Ruthe, A = ARD, G = Grass, UT = Untreated.

178x140mm (500 x 500 DPI)

Heidgraben ARD UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Candidatus_Paenicardinium _ASV112	1.86	-0.56	-0.56
Cellvibrio _ASV77	0.59		
Lysobacter _ASV120	0.94		
Methylotenera _ASV209	0.56		
Neorhizobium _ASV47	0.73	-0.53	-0.59
Novosphingobium _ASV29	3.98		
Novosphingobium _ASV92	3.98	-0.59	-0.53
Rhizobium _ASV175	0.82		
Rhizobium _ASV31	0.73		
Rhodanobacter _ASV46	0.52		
Simplicispira _ASV289	0.62	-0.34	-0.40
Sphingobium _ASV20	1.07		-0.30
Streptomyces _ASV121	0.53	-0.47	-0.47
Streptomyces _ASV21	0.53	-0.54	-0.58
Streptomyces _ASV43	1.20	-0.53	-0.54
Streptomyces _ASV66	4.48	-0.43	-0.43
Streptomyces _ASV70	1.27	-0.59	-0.65
Streptomyces _ASV71	2.28	-0.53	-0.52
Streptomyces _ASV76	1.08	-0.59	-0.65

Ellerhoop ARD UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Asticcacaulis _ASV56	0.68		
Burkholderia-Paraburkholderia _ASV159	0.75	-0.37	-0.37
Cellvibrio _ASV335	0.55		
Ferrovibrio _ASV142	0.64		
Flavobacterium _ASV15	2.08		
Flavobacterium _ASV84	0.67		-0.33
Hydrogenophaga _ASV542	0.52	-0.37	-0.33
Massilia _ASV158	0.69		
Methylobacillus _ASV214	0.58	-0.31	-0.30
Methylophilus _ASV108	0.53		-0.30
Methylophilus _ASV303	0.58	-0.38	-0.45
NA _ASV161 (Rhodospirillaceae)	0.91	-0.36	
Neorhizobium _ASV47	0.93	-0.53	-0.59
Novosphingobium _ASV92	0.82	-0.59	-0.53
Pedobacter _ASV218	0.94		
Pseudomonas _ASV13	1.37	-0.50	-0.49
Pseudomonas _ASV2	2.00	-0.43	-0.47
Pseudomonas _ASV332	0.71		
Pseudomonas _ASV38	2.46		
Pseudomonas _ASV52	1.12	-0.34	-0.30
Pseudomonas _ASV591	0.67		
Rhizobium _ASV12	2.12	-0.33	-0.31
Rhizobium _ASV33	1.28	-0.29	-0.36
Rhizobium _ASV62	0.65		
Streptomyces _ASV21	2.55	-0.54	-0.58
Streptomyces _ASV322	0.64	-0.41	-0.41
Streptomyces _ASV70	1.03	-0.59	-0.65
Streptomyces _ASV76	1.14	-0.59	-0.65

Ruthe ARD UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Burkholderia-Paraburkholderia _ASV224	1.01		
Escherichica/Shigella _ASV125	1.50		
Escherichica/Shigella _ASV233	0.72		
Methylobacillus _ASV214	0.53	-0.31	-0.30
NA _ASV252 (Saccharibacteria)	1.67	-0.37	-0.35
NA _ASV73 (Moraxellaceae)	0.50		
Pseudomonas _ASV195	2.23		
Pseudomonas _ASV363	0.64		
Pseudomonas _ASV38	0.64		
Pseudomonas _ASV390	0.84		
Rhizobium _ASV62	0.51		
Shinella _ASV576	0.61		
Streptomyces _ASV21	0.82	-0.54	-0.58
Streptomyces _ASV42	4.10	-0.49	-0.50
Streptomyces _ASV540	0.65		-0.31

Figure 2: Venn-Diagrams showing overlapping ASVs (RA > 0.5 %) of the different soil variants (ARD, Grass, UT = untreated, G = Gamma irradiated) from Heidgraben, Ellerhoop and Ruthe of the biotest in 2016. The three tables show the site-specific unique ASVs of ARD UT variants with their relative abundance and spearman correlation to shoot growth (= increase in shoot length) and fresh mass (only significant correlations are shown ($p \leq 0.05$). ASVs highlighted in bold appear in at least two sites.

255x220mm (500 x 500 DPI)

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

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

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

Figure 3: Venn-Diagrams showing overlapping ASVs (RA > 0.5 %) of the different soil variants (ARD, Grass, UT = untreated, G = Gamma irradiated) from Heidgraben, Ellerhoop and Ruthe of the biotest in 2017. The three tables show the site specific unique ASVs of ARD UT variants with their relative abundance and spearman correlation to shoot growth (= increase in shoot length) and fresh mass (only significant correlations are shown ($p \leq 0.05$). ASVs highlighted in bold appear in at least two sites.

254x199mm (300 x 300 DPI)

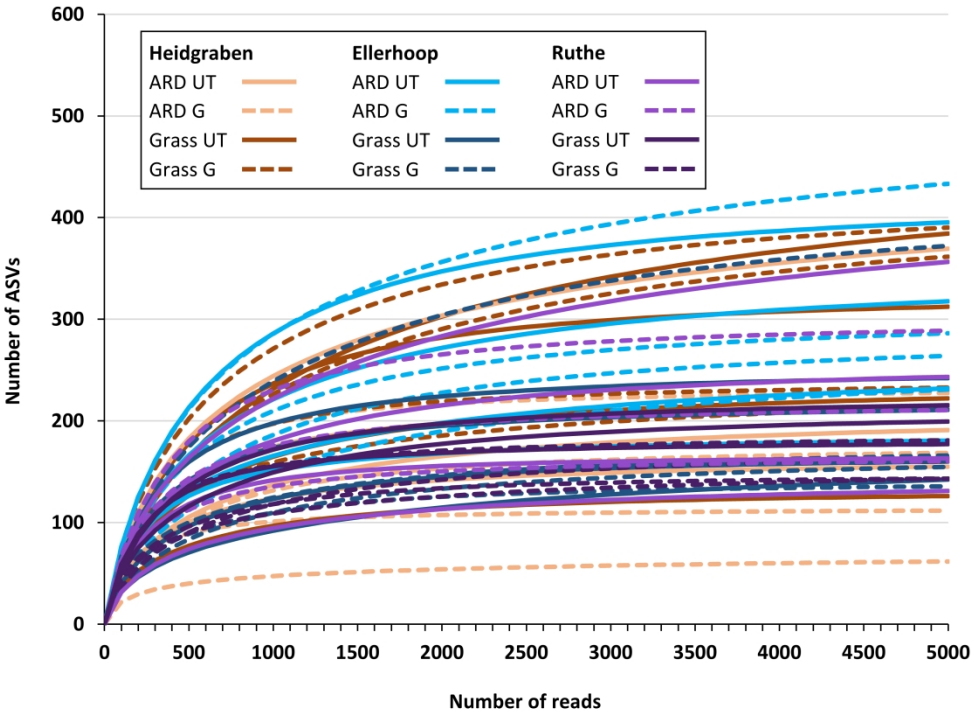


Figure S1: Rarefaction curves showing the number of observed ASVs in all samples of the biotest in 2016. Each line represents one sample of M26 roots taken after 8 weeks. All samples were rarefied at 28817 reads. UT = untreated, G = gamma irradiated.

178x129mm (500 x 500 DPI)

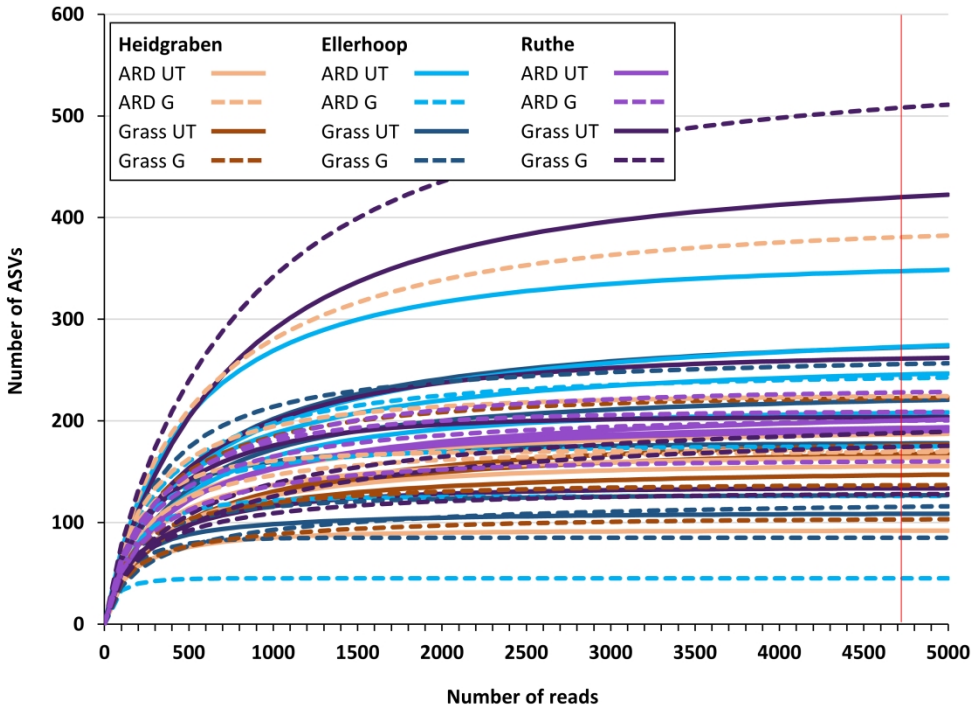


Figure S2: Rarefaction curves showing the number of observed ASVs in all samples of the biotest in 2017. Each line represents one sample of M26 roots taken after 8 weeks. All samples were rarefied at 4213 reads (red line). UT = untreated, G = gamma irradiated.

178x129mm (500 x 500 DPI)

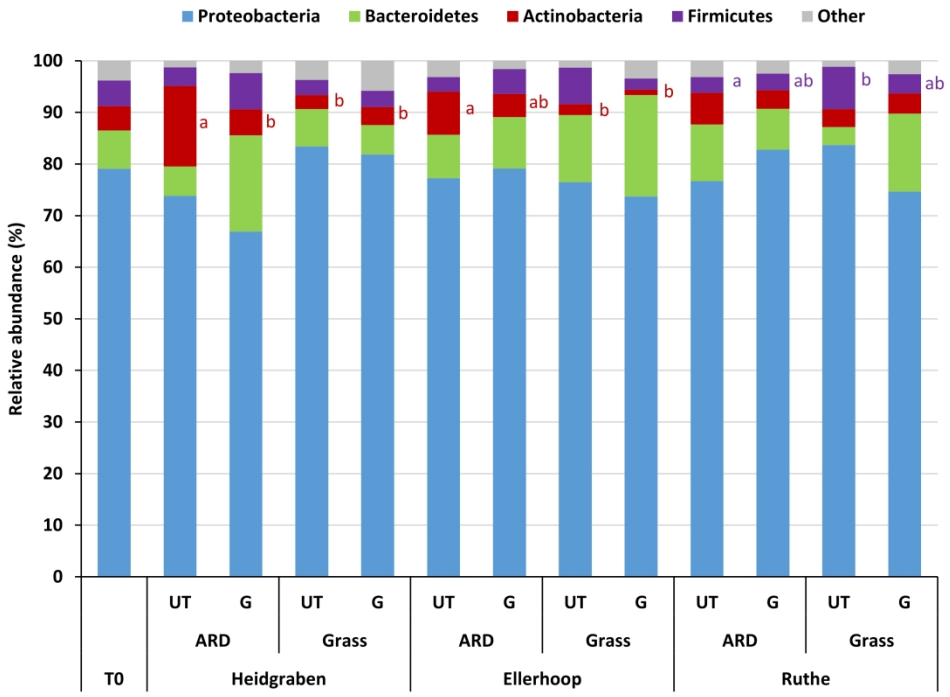


Figure S3: Relative abundance of dominant bacterial phyla in roots grown for 8 weeks in soils from different sites and treatments (UT = untreated. G = gamma irradiated) of the biotest in 2016. Different letters indicate significant differences within the sites (Tukey test, $p \leq 0.05$). Different colored letters belong to different phyla. No letters indicate no statistical difference. Replicate numbers (n) are shown in table 2.

178x129mm (500 x 500 DPI)

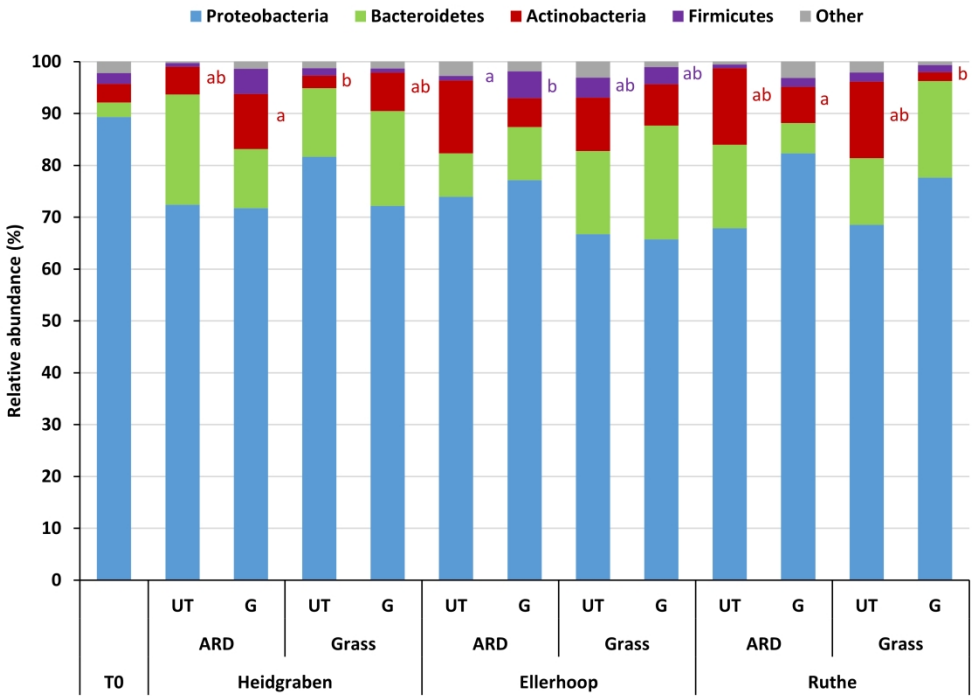
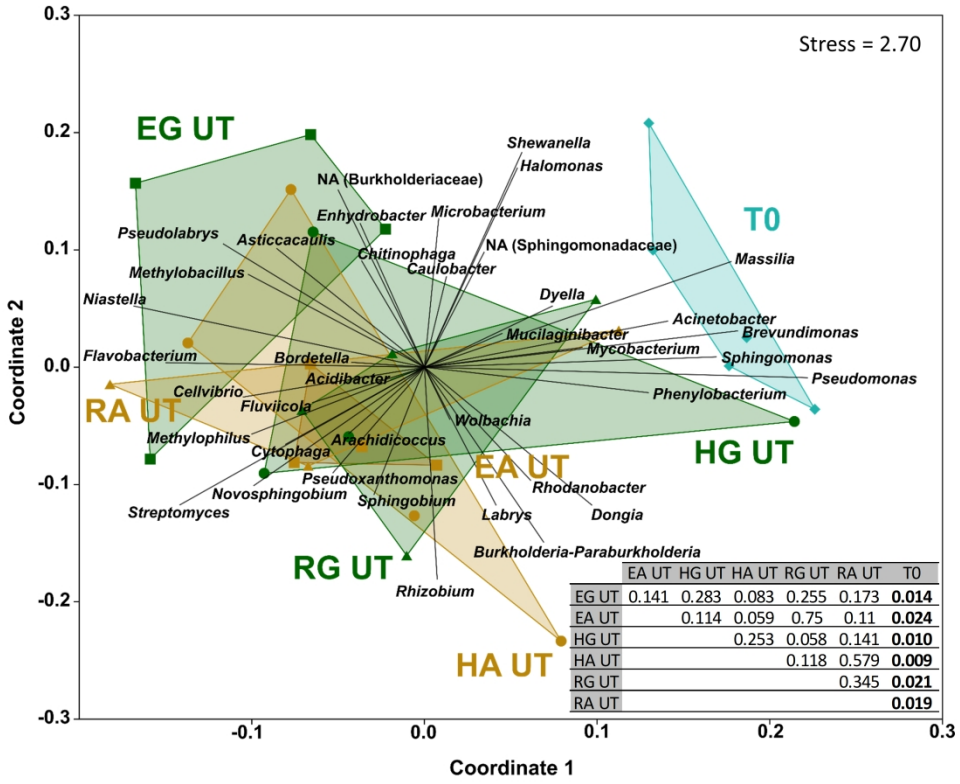


Figure S4: Relative abundance of dominant phyla in roots grown for 8 weeks in soils from different sites and treatments (UT = untreated. G = gamma irradiated) of the biotest in 2017. Different letters indicate statistically significant differences within the sites (Tukey test, $p \leq 0.05$). Different colored letters belong to different phyla. No letters indicate no statistical difference. N numbers are shown in table 3.

178x129mm (500 x 500 DPI)



	EA UT	HG UT	HA UT	RG UT	RA UT	T0
EG UT	0.141	0.283	0.083	0.255	0.173	**0.014**
EA UT		0.114	0.059	0.75	0.11	**0.024**
HG UT			0.253	0.058	0.141	**0.010**
HA UT				0.118	0.579	**0.009**
RG UT					0.345	**0.021**
RA UT						**0.019**

Figure S5: Three dimensional Non-metric Multi Dimensional Scaling (NMDS) using Bray-Curtis dissimilarity of roots grown 8 weeks in soils from different sites of the biotest in 2017. Gamma irradiated variants are not included. Vectors represent the correlation coefficient between the corresponding genus and the NMDS score. The relative lengths and the directions of the vectors indicate the influence of the respective genera (RA > 1 %). The third axis is not shown. Results of the one way analysis of similarities (ANOSIM) are shown in the lower right corner, significant differences are highlighted in bold ($p \leq 0.05$) . H = Heidgraben, E = Ellerhoop, R = Ruthe, A = ARD, G = Grass, UT = Untreated.

178x140mm (500 x 500 DPI)

Heidgraben Grass UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Arachidicoccus _ASV485	0.54		
Asticcacaulis _ASV56	2.57		
Asticcacaulis _ASV78	0.58		
Bordetella _ASV41	2.13		
Bradyrhizobium _ASV127	0.60		
Burkholderia-Paraburkholderia _ASV159	0.62	-0.37	-0.37
Burkholderia-Paraburkholderia _ASV202	1.74		
Burkholderia-Paraburkholderia _ASV348	0.58		
Dyella _ASV116	1.78		
Dyella _ASV141	0.64		
Dyella _ASV149	3.05		
Dyella _ASV232	0.82		
Dyella _ASV292	1.34		
Dyella _ASV343	1.11		
Dyella _ASV354	1.03		
Flavobacterium _ASV449	0.51	-0.30	-0.32
Massilia _ASV87	1.42		
Methylophilus _ASV108	1.66		-0.30
NA _ASV69 (Moraxellaceae)	0.75		
NA _ASV99 (Alcaligenaceae)	0.90		
Phenylobacterium _ASV196	0.55		
Rhizobium _ASV100	1.38		-0.30
Rhodanobacter _ASV162	1.34		
Rhodanobacter _ASV165	0.72		
Rhodanobacter _ASV50	1.22		
Shinella _ASV118	1.00	-0.47	-0.43
Sphingobium _ASV199	1.02		
Sphingobium _ASV272	0.62		
Sphingobium _ASV91	1.70		

Ellerhoop Grass UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Acidisoma _ASV222	1.25		
Asticcacaulis _ASV115	1.14		
Chitinophaga _ASV148	2.22		
Chitinophaga _ASV333	0.85		
Chitinophaga _ASV357	0.80		
Chitinophaga _ASV382	0.67		
Dyella _ASV141	0.90		
Flavobacterium _ASV174	1.06		
Halomonas _ASV30	0.63		
Massilia _ASV87	0.68		
NA _ASV124 (Cytophagaceae)	2.12		
NA _ASV317 (Cytophagaceae)	0.91		
NA _ASV470 (Paenibacillaceae)	0.50		
NA _ASV69 (Moraxellaceae)	6.16		
Pasteuria _ASV352	1.01		
Pasteuria _ASV448	0.66		
Pelomonas _ASV220	1.57		
Pelomonas _ASV248	1.32		
Pelomonas _ASV253	1.35		
Pelomonas _ASV96	3.75		
Pseudomonas _ASV223	2.51		
Rhizobium _ASV130	0.74		-0.29
Rhizobium _ASV134	0.71		
Rhizobium _ASV31	2.06	-0.36	-0.41
Rhizobium _ASV81	0.95		
Rhodanobacter _ASV46	1.41		

Ruthe Grass UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Asticcacaulis _ASV56	2.07		
Bradyrhizobium _ASV63	0.53		
Cellvibrio _ASV77	1.22		
Herbaspirillum _ASV429	0.65		
Mesorhizobium _ASV275	0.64		
NA _ASV566 (Moraxellaceae)	0.53		
Novosphingobium _ASV119	1.28	-0.37	-0.41
Novosphingobium _ASV129	1.39		
Novosphingobium _ASV279	0.57		
Novosphingobium _ASV54	0.72		
Pseudomonas _ASV22	0.53	-0.33	-0.31
Rhizobium _ASV100	1.31		-0.30
Rhizobium _ASV130	1.49		-0.29
Rhizobium _ASV134	1.46		
Rhizobium _ASV36	3.13		
Rhizobium _ASV81	1.83		
Rhodanobacter _ASV388	0.76		
Rhodanobacter _ASV46	5.34		
Shewanella _ASV67	0.69		
Staphylococcus _ASV144	1.20		
Staphylococcus _ASV286	0.65		
Streptomyces _ASV212	0.74		
Variovorax _ASV168	0.64		

Figure S6: Venn-Diagrams showing overlapping ASVs (RA > 0.5 %) of the different soil variants (ARD, Grass, UT = untreated, G = Gamma irradiated) from Heidgraben, Ellerhoop and Ruthe of the biotest in 2016. The three tables show the site specific unique ASVs of grass UT variants with their relative abundance and spearman correlation to shoot growth (= increase in shoot length) and fresh mass (only significant correlations are shown ($p \leq 0.05$). ASVs highlighted in bold appear in at least two sites.

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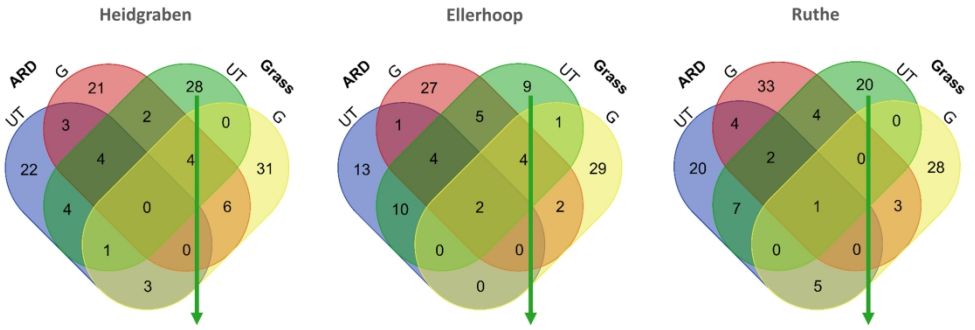

Heidgraben Grass UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
AAP99_ASV6339	1.60		
AAP99_ASV6345	3.57		
Acidibacter_ASV8343	0.69		
Bacillus_ASV2713	0.63		
Bordetella_ASV6401	0.58		
Duganella_ASV6584	3.99		
Duganella_ASV6587	2.62		
Duganella_ASV6618	3.27		
Duganella_ASV6619	2.48		
Duganella_ASV6620	1.55		
NA_ASV1173 (Chitinophagaceae)	1.26		
NA_ASV4691 (Rhizobiaceae)	0.95	0.32	0.30
NA_ASV4984 (Xanthobacteraceae)	0.64		
NA_ASV7202 (Burkholderiaceae)	0.52	-0.39	-0.42
NA_ASV7343 (Burkholderiaceae)	0.62		
NA_ASV7392 (Burkholderiaceae)	0.53		
Ohtaekwangia_ASV1522	0.52		
Pseudomonas_ASV8855	2.84		
Pseudomonas_ASV8863	2.65		
Pseudomonas_ASV8865	1.80		
Pseudomonas_ASV9034	1.18		
Rhizobacter_ASV6858	0.55		
Rhizobium_ASV4595	0.71		
Rhizobium_ASV81	2.28		
Rhodanobacter_ASV9390	0.52		
Sphingobium_ASV5335	1.60		
Sphingobium_ASV5379	1.92		
Streptomyces_ASV662	0.56		

Ellerhoop Grass UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Asticcacaulis_ASV3665	0.74		
Asticcacaulis_ASV3667	1.09		
NA_ASV7389 (Burkholderiaceae)	2.91		
NA_ASV7391 (Burkholderiaceae)	6.08		
Niastella_ASV980	1.05		
Niastella_ASV991	9.08		
Pseudolabrys_ASV4891	1.03		
Pseudolabrys_ASV4902	0.66		
Rhizobium_ASV4621	0.79		

Ruthe Grass UT		Spearman correlation	
ASV	RA (%)	Shoot growth	Shoot FM
Chitinophaga_ASV885	1.14		
Halomonas_ASV8617	0.57		
Methylophilus_ASV7463	0.53		
NA_ASV1414 (Amoebophilaceae)	1.04		
NA_ASV1416 (Amoebophilaceae)	0.76		
NA_ASV1417 (Amoebophilaceae)	0.81		
NA_ASV1418 (Amoebophilaceae)	1.50	-0.33	-0.36
NA_ASV8797 (Moraxellaceae)	1.03		
Novosphingobium_ASV180	0.55		
Pseudoxanthomonas_ASV9575	0.85		
Pseudoxanthomonas_ASV9583	0.85		
Pseudoxanthomonas_ASV9593	0.63		
Rhizobium_ASV4586	1.88		
Rhizobium_ASV4590	1.19		
Rhizobium_ASV4591	0.94		
Rhizobium_ASV81	1.97		
Sphingobium_ASV5347	0.72		
Sphingobium_ASV5379	1.42		
Wolbachia_ASV5193	0.69		
Wolbachia_ASV5993	0.68		

Figure S7: Venn-Diagrams showing overlapping ASVs (RA > 0.5 %) of the different soil variants (ARD, Grass, UT = untreated, G = Gamma irradiated) from Heidgraben, Ellerhoop and Ruthe of the biotest in 2017. The three tables show the site specific unique ASVs of grass UT variants with their relative abundance and spearman correlation to shoot growth (= increase in shoot length) and fresh mass (only significant correlations are shown ($p \leq 0.05$). ASVs highlighted in bold appear in at least two sites.

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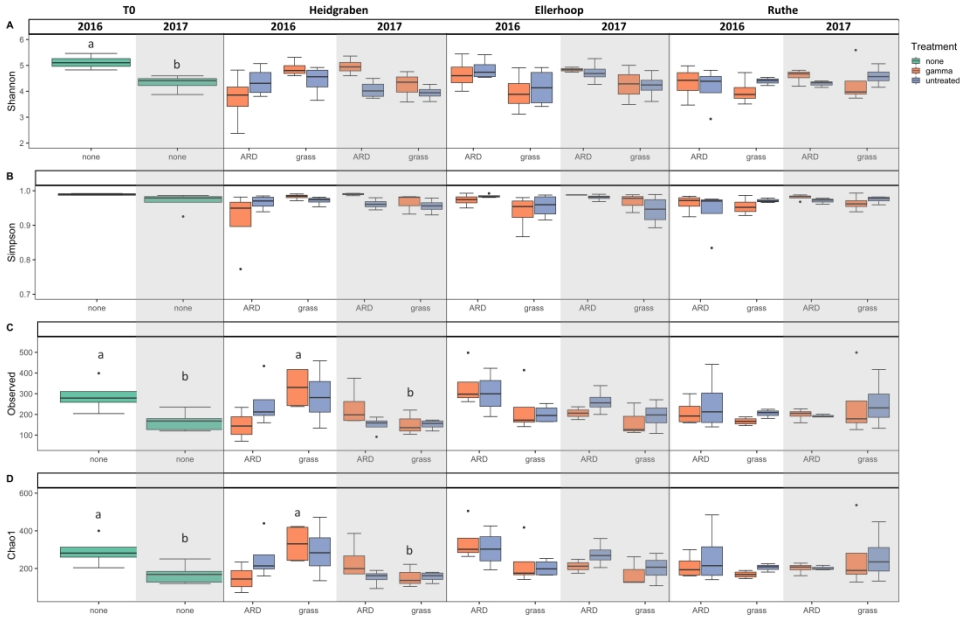

Fig. S8: Comparison of richness and diversity of bacterial communities based on amplicon sequence variants (ASVs) in roots grown for 8 weeks in soils from different sites and treatments (untreated, gamma irradiated) in the biotests in 2016 and 2017 . Shown are Shannon (A) and Simpson (B) indices, Observed ASVs (C) and Chao 1 (D). Additionally T0 plants before transferring into the soil variants are shown. Different letters indicate significant differences between the years of the corresponding treatment (Welch-Two-sample t-test, $p \leq 0.05$). No letters indicate no significant differences. Replicate numbers (n) are shown in Tab. 2 and 3.

315x199mm (500 x 500 DPI)