Effects of biofumigation on plant growth and microbial communities in replant disease soils

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Abstract

When a chemical soil disinfectant or a proper soil management is not applied, nurseries producing apple and rose rootstock plants, apple orchards as well as rose production enterprises often experience replanting problems after several cultivations on the same site. The etiology of apple and rose replant problems is most likely caused by soil-borne pathogens, defined as 'replant disease' (RD). Replanting symptoms are typically visualized as a reduction in shoot and root growth, a smaller leaf area, a significant decrease in plant biomass, yield and fruit quality and a shorter life span. In the present study, three sites, at which rootstocks of rose (sites K and M) and apple (site A) plants had been replanted, were subjected to different soil treatments under field conditions. The treatments included Basamid® granules, biofumigation (a soil-borne pest and pathogen suppression approach due to liberated products, mainly from Brassicaceae plants) with *Brassica juncea* and *Raphanus sativus* for one and two years as well as *Tagetes patula*.

The study aimed at (1) identifying and quantifying glucosinolates in different plant organs of *B. juncea* and *R. sativus*, (2) determining glucosinolate degradation products and their concentrations in the biofumigated and methyl-isothiocyanate in the Basamid® treated soils, (3) investigating bacterial and fungal community structures and responders that were affected by the different soil treatments and (4) evaluating the effectiveness of the different soil treatments based on the performance of the indicator plant growth.

Employing ultra-high-performance liquid chromatography with diode array detection, glucosinolates in all plant organs were identified and quantified. The highest concentration was found in inflorescences followed by leaves of both biofumigant plant species with no differences between sites. *B. juncea* and *R. sativus* differed in their glucosinolate profiles, e.g. in all organs 2-propenyl (allyl) glucosinolates were dominant in *B. juncea* whereas 4-(methylthio)-3-butenyl glucosinolates were dominant in *R. sativus*.

In soils treated with *B. juncea*, *R. sativus* and Basamid, 2-propenyl, 4-(methylthio)-3-butenyl and methyl isothiocyanates, respectively were detected by gas chromatography-mass spectrometry. The isothiocyanate concentrations measured in both biofumigated soils were much lower than those in the Basamid treated soils, and they were site-dependent.

Treatment- and site-dependent effects on the bacterial and fungal community compositions were evident as revealed by both denaturating gradient gel electrophoresis fingerprints (studied for all sites) and Miseq® Illumina® sequencing (studied for sites K and A) of 16S rRNA gene and ITS fragments. All soil treatments showed stronger shifts in fungal than in bacterial community composition, especially at site K. For RD soils cropped with *Tagetes* changes in both bacterial and fungal communities were least pronounced compared to biofumigation and Basamid treatments.

The bacterial phyla *Actinobacteria* and *Bacteroidetes* were significantly enhanced in relative abundance after biofumigation with *R. sativus* at sites K and A. Common responders were recorded for the bacterial

genera *Arthrobacter* and *Curtobacterium* in *R. sativus* and Basamid treated soils, respectively (sites K and A). For fungi, the genera *Podospora*, *Monographella* and *Mucor* significantly proliferated in soils treated with *B. juncea* and *R. sativus* (sites K and A).

Based on the performance of the apple rootstocks M26 and M106 that were evaluated as indicator plants under greenhouse and field conditions, respectively, the effects of the different soil treatments were deemed site- and treatment-dependent. The effects of biofumigation, Basamid and *Tagetes* treatments were evident at site K. Differences in plant growth were neither observed between one- and two-year biofumigation nor between biofumigant plant species (*B. juncea* and *R. sativus*; sites K, A and M). Furthermore, biofumigation effects on plant growth were comparable to Basamid and *Tagetes* treatments at all sites.

The effects of biofumigation possibly resulted from e.g. the combination of improving soil structure, suppressing soil-borne pests and pathogens, changing the soil microbial community compositions and additional nutrients from the incorporated biomass.

Key words: Apple, Basamid® granules, biotest, *B. juncea*, biofumigation, Denaturating gradient gel electrophoresis, 454-pyrosequencing, Miseq® Illumina® sequencing, *R. sativus*, replant disease, Rosaceae, rose, *Tagetes*

Zusammenfassung

Ohne Einsatz chemischer Bodendesinfektionsmittel oder bei fehlendem Bodenmanagement, kommt es nach wiederholter Kultivierung auf gleichen Anbauflächen zu Nachbauproblemen in Apfel- und Rosenunterlagen-Baumschulen, Apfelplantagen, sowie Rosenproduktionsunternehmen. Die Ätiologie der Apfel- und Rosennachbauprobleme wird höchstwahrscheinlich durch bodengebürtige Krankheitserreger verursacht und ist definiert als „Nachbaukrankheit" (engl. replant disease, RD). Nachbaukrankheitssymptome treten oft als Verringerung des Trieb- und Wurzelwachstums, und in Form einer kleineren Blattfläche, einer signifikanten Abnahme der Pflanzenbiomasse, Ernte und Fruchtqualität sowie einer kürzeren Lebensdauer auf. In der vorliegenden Studie wurden drei Standorte, an denen Wurzelunterlagen von Rosen (Standorte K und M) und Apfelpflanzen (Standort A) kultiviert wurden, unter Feldbedingungen mit verschiedenen Bodenbehandlungen untersucht. Die Behandlungen umfassten Basamid®-Granulat, Biofumigation (eine Methode zur Unterdrückung bodenbürtiger Schädlinge und Krankheiten durch die Freisetzung von Wirkstoffen ausPflanzen, hauptsächlich aus der Familie der Brassicaceae) mit *Brassica juncea* und *Raphanus sativus* für ein und zwei Jahre sowie *Tagetes patula* als Zwischenfrucht.

Die Studie hatte die folgenden Ziele: (1) Identifizierung und Quantifizierung von Glucosinolaten in verschiedenen Pflanzengeweben von *B. juncea* und *R. sativus*, (2) Bestimmung von Glucosinolat-Abbauprodukten und deren Konzentrationen in den mit Biofumigation behandelten und Basamid® (Wirkstoff: Methylisothiocyanat) behandelten Böden, (3) Untersuchung der bakteriellen und pilzlichen Gemeinschaften in den Böden und von Respondern, die von den verschiedenen Bodenbehandlungen betroffen waren, und (4) Bewertung der Wirksamkeit der verschiedenen Bodenbehandlungen auf Grundlage des Wachstums der Indikatorpflanzen.

Ultra-Hochleistungs-Flüssigkeitschromatographie mit Diodenarray-Detektion wurde zur Identifizierung und Quantifizierung von Glucosinolaten in allen Pflanzenorganen verwendet. Die höchste Konzentration wurde in Blütenständen gefunden, gefolgt von Blättern beider Biofumigationspflanzenarten ohne Unterschied zwischen den Standorten. *B. juncea* und *R. sativus* unterschieden sich in ihrer Glucosinolat-Zusammensetzung, z.B. waren in allen Organen 2-Propenyl-(allyl)glucosinolate dominierend in *B. juncea*, während 4-(Methylthio)-3-butenylglucosinolate bei *R. sativus* dominierten. In Böden, die mit *B. juncea*, *R. sativus* und Basamid behandelt wurden, wurde jeweils 2-Propenyl-, 4-(Methylthio)-3-butenyl- bzw. Methylisothiocyanat durch Gaschromatographie-Massenspektrometrie nachgewiesen. Die Standortabhängigen Isothiocyanat-Konzentrationen, die in beiden Biofumigationsvarianten gemessen wurden, waren deutlich niedriger als die in den mit Basamid behandelten Böden.

Behandlungs- und Standortabhängige Effekte auf die Zusammensetzung der Bakterien und Pilzgemeinschaften zeigten sich nach der denaturierenden Gradienten-Gelelektrophorese (untersucht für alle Standorte) als auch die Miseq® Illumina®-Sequenzierung (untersucht für die Standorte K und A) des 16S-rRNA-Gens und der ITS-Fragmente. Alle Bodenbehandlungen zeigten eine stärkere Verschiebung in der Zusammensetzung der pilzlichen Gemeinschaften im Vergleich zu Bakterien, insbesondere am Standort K. Für RD-Böden, die mit *Tagetes* bepflanzt wurden, waren Veränderungen für sowohl Bakterien- als auch Pilzgemeinschaften im Vergleich zu Biofumigations- und Basamid-Behandlungen am wenigsten ausgeprägt.

Die bakteriellen Phyla *Actinobacteria* und *Bacteroidetes* waren nach Biofumigation mit *R. sativus* an den Standorten K und A signifikant erhöht. Als „Responder“ konnten die Bakteriengattungen *Arthrobacter* und *Curtobacterium* in jeweils *R. sativus* bzw. Basamid-behandelten Böden nachgewiesen werden (Standorte K und A). Für Pilze waren die Gattungen *Podospora*, *Monographella* und *Mucor* in *B. juncea* und *R. sativus* behandelten Böden (Standorte K und A) signifikant abundant.

Basierend auf der Wachstumsleistung der Apfelunterlagen M26 und M106, die als Indikatorpflanzen unter Gewächshaus- und Feldbedingungen ausgewertet wurden, waren die Effekte der verschiedenen Bodenbehandlungen standort- und behandlungsabhängig. Die Auswirkungen von Biofumigation, Basamid- und *Tagetes*-Behandlungen zeigten sich am Standort K. Unterschiede im Pflanzenwachstum wurden weder zwischen ein- und zweijähriger Biofumigation, noch zwischen Biofumigation Pflanzenarten (*B. juncea* und *R. sativus*; Standorte K, A und M) beobachtet. Darüber hinaus waren Biofumigationseffekte auf das Pflanzenwachstum vergleichbar mit denen der Basamid- und *Tagetes*-Behandlungen an allen Standorten.

Die Auswirkungen der Biofumigation resultierten möglicherweise aus der Kombination aus z.B. Verbesserung der Bodenstruktur, Unterdrückung von Schädlingen und Krankheiten, Veränderung der mikrobiellen Bodengemeinschaftszusammensetzungen und zusätzlichen Nährstoffen aus der eingearbeiteten Biomasse.

Schlagwörter: Apfel, Basamid® Granulat, Biotest, *B. juncea*, Biofumigation, Denaturierende Gradientengelelektrophorese, 454-Pyrosequenzierung, Miseq® Illumina® Sequenzierung, *R. sativus*, Nachbaukrankheitsboden, Rosaceae, Rose, *Tagetes*

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Abbreviations

ARD	apple replant disease
ACC	1-aminocyclopropane-1-carboxylate
ANOVA	analysis of variance
ANOSIM	analysis of similarity
CFU	colony forming unit
Con	control
DGGE	denaturing gradient gel electrophoresis
DM	dry mass
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
ET	ethylene
Gamma	treatment with gamma irradiation
GC-MS	gas chromatography - mass spectrometry
GS	glucosinolate
H50	temperature treatment at 50°C
IAA	indole-3-acetic acid
ITC	isothiocyanate
ITS	internal transcribed spacer
JA	jasmonic acid
log	logarithm
LSD	least significant different test
NCBI	national center for biotechnology information
NO	nitric oxide
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PCoA	principal coordinate analyis
PGP	plant growth promoting
qPCR	quantitative real-time polymerase chain reaction
rRNA	ribosomal RNA
RD	replant disease
RF	response factor
RDM	root dry mass

ROS	reactive oxygen species
SA	salicylic acid
SD	standard deviation
SDM	shoot dry mass
SFM	shoot fresh mass
SIM	selected ion monitoring
SL	shoot length or plant height
TC-DNA	total community DNA
TIC	total ion count/ full scan mode
UHPLC-DAD	ultra high-performance liquid chromatography with diode-array detection
UPGMA	unweighted pairwise grouping method using arithmetic means

1 General introduction

1.1 Plant microbe interactions in soil

Soil microbes comprise soil bacteria, archaea, fungi and oomycetes (Lugtenberg 2015). A subset of soil microbes lives closely associated with plants as 6 - 20 % of the carbon fixed by plants is estimated to be exuded from plant roots (Lugtenberg 2015). In the rhizosphere, the number of microbes was estimated to occur at approximately 10- to 100-fold higher density than in bulk soil (Lugtenberg 2015), and about10^{11} microbial cells per gram root material was reported (reviewed by Berendsen et al. 2012). They may exhibit neutral, positive and negative effects on plant growth (Forge et al. 2001; Wu et al. 2015; van der Wolf and De Boer 2015; de Wit 2015; Thomashow and Bakker 2015). Soil types, cultural practices and plant species as well as genotypes all shape the composition and activity of soil microbes as reported in several earlier studies (St. Laurent et al. 2010; Schreiter et al. 2014; Neumann et al. 2014; Bakker et al. 2015; Uroz et al. 2016).

Plant disease-causing microbes often carry several virulence factors including production of plant cell-wall degrading enzymes, phytotoxins that cause damage to plant cells or change metabolism and physiology of plants as well as effector molecules that can be injected into plants cells to suppress the host response (van der Wolf and De Boer 2015; de Wit 2015). Upon pathogen infections, plant defense at the cellular level involves the accumulation of reactive oxygen species (ROS), nitric oxide (NO), hormone modulation (SA, salicylic acid; JA, jasmonic acid and ET, ethylene) and biosynthesis of antimicrobial secondary metabolites, callose deposition and cell wall modifications (reviewed by Zhu et al. 2014).

Plant growth promotion by microbes can be caused by several mechanisms such as N cycling, phosphor solubilization, production of plant hormones and siderophores, stress alleviation, rhizoremediation, enhanced plant defense response and antagonist effects against soil-borne pests and pathogens (Berendsen et al. 2012; Lugtenberg 2015).

1.2 Apple and rose production

All apple trees (genus *Malus*, a member of the Rosaceae family) are known to grow on propagated rootstocks because they influence the size of the trees, maturity, yield and fruit quality, labor for pruning and picking, tolerance for soil and climate conditions and disease resistance (Lauri et al. 2006; Tworkoski and Miller 2007; Kviklys et al. 2012; Fazio et al. 2015). Compared to the standard size grown from seedling (about 9 m height), apple rootstocks are grouped into four main categories extreme dwarf, dwarf, semi-dwarf and vigorous or standard size (Atkinson et al. 1999). Rootstock names consist of an abbreviation of type’s name (breeding station) followed by the selection number, e.g. Bud or B (Budagovsky); CG or G (Cornell/Geneva); M (Malling); MM (Malling Merton); MARK (Michigan Apple Rootstock Clones) and

EMLA (East Malling/ Ashton Long) (http://treefruit.wsu.edu/varieties-breeding/rootstocks/, accessed on 13.02.17). Commercially available apple rootstocks are propagated via hardwood cutting (Hartmann et al. 1965; Dvin et al. 2011), stooling or mound-layering (Akbari et al. 2015) and in rare cases micro- or *in vitro* propagation (reviewed by Dobránszki and de Silva 2010). Rootstocks for vigorously growing trees are propagated via seeds, i.e. *Malus* 'Bittenfelder' (Winkelmann pers. communication 2017).
Also for most roses (genus *Rosa*, also a member of the Rosaceae family), budding or grafting onto rootstocks is preferred because of a better nutrient uptake, growth and yield of flowers as well as tolerance to both biotic and abiotic stresses (Spethmann and Otto 2003; Niu and Rodriguez 2008; 2009; Nazari et al. 2009; Balaj and Zogaj 2011).
Regarding the production, the United States are the second largest apple producer after China with about 11 – 15 million apple trees are planted every year (FAO 2016). In Germany, about 31,334 ha were reserved for apple production resulting in1,032,913 t in 2016 (Statistisches Bundesamt 2016). The area for rootstock production of fruit trees was approximately 176 ha in 2012 (Statistisches Bundesamt 2016). In contrast, rootstock production for roses took place on about 197 ha in 2012 in Germany (Statistisches Bundesamt 2016).

1.3 Replant problems and replant disease in apple and rose

After several cultivations on the same site, nurseries producing apple and rose rootstock plants, apple orchards and rose production centers often experience replant problems when a chemical soil disinfectant or a proper soil management is not applied (Klaus 1939; Hoestra 1994; Spethmann and Otto 2003; Hofmann et al. 2009). Based on literature reviews, studies on rose replant problems have been less documented compared to apple replant problems which were reported worldwide (Kandula et al. 2010; St. Laurent et al. 2010; Mazzola and Manici 2012; Sun et al. 2014, Franke-Whittle et al. 2015; Nicola et al. 2017).
The etiology of replant problems is so far unclear and it is most likely caused by both biotic and abiotic factors (Hoestra 1994; Politycka and Adamska 2003; Mazzola and Manici 2012). Utkhede (2006) defined 'replant disease or RD' to be caused by biotic factors, and it is considered to be one of the components of replant problems. In Europe, 'RD' is sometimes called 'soil sickness' (Winkelmann pers. communication). However, according to Spethmann and Otto (2003) in Germany nursery men considered replant problems as a broad term that included macro- and micronutrient deficiencies, nematode damages, structural changes in the soil, toxin accumulation and an imbalance in microorganism populations.
Overall, replanting symptoms are visualized as a reduction in shoot and root growth, a smaller leaf area, a significant decrease in biomass, fruit quality and yield, and a shorter life span (Jaffee et al. 1982; Brown and Koutoulis 2008; Hofmann et al. 2009; Yim et al. 2013; Henfrey et al. 2015; Nicola et al. 2017; Weiß et al. 2017).

Phytotoxicity resulting from root exudations and decomposition of apple residues were recently suggested as abiotic factors contributing to RD (Yin et al. 2016; Nicola et al. 2016). Yin et al. (2016) reported the three main phenolic compounds benzoic acid, vanillic aldehyde and phlorizin to be detected in high concentrations in soils with apple replant problems. Other phenolic compounds such as the phytoalexins 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzofuran, 2'-hydroxyaucuparin and noreriobofuran were also recently discovered in apple roots grown in replant soil at significantly higher concentrations compared to roots cultivated in sterilized replant soil (Stefan Weiß, unpublished data, Leibniz Universität Hannover).

Phlorizin is a typical phenolic exudate by apple roots which has been detected also in earlier studies (Börner 1959; Hofmann et al. 2009; Emmett et al. 2014). This compound as well as total phenolic compounds were found significantly higher in apple roots suffering from replant problems compared to those grown in sterilized soils (Emmett et al. 2014; Henfrey et al. 2015). Nicola et al. (2016) demonstrated the toxicity of phlorizin on apple seedlings when they were grown on a soil mixed with ground apple roots. Thus, a role of this phenolic compound in apple replant problems cannot be excluded. However, further observations by Nicola et al. (2016) revealed that after leaving the soil mixtures for three months under natural conditions before planting, the biomass of apple plants was comparable to the mass in untreated soil, indicating phlorizin degradation. Hofmann et al. (2009) assumed two functions of phlorizin exuded from roots of apple seedlings, either as a defense against soil microbes or as a beneficial host-signal compound for plant pathogens.

Klaus (1939) reported that even after 30 years replant problems were still observed, although the sites were abandoned, especially for Rosaceae species. Therefore, the etiology of apple replanting problems most likely is caused by soil-borne plant pathogens and it is called 'replant disease or RD' as already postulated in several other studies because the disease symptoms were reduced when apple plants grew in sterilized soil that was achieved by either heat, gamma irradiation or chemical treatments (Mai and Abawi 1978; Jaffee et al. 1982; Hoestra 1994; Parchomchuk et al. 1994; Utkhede 2006; Brown and Koutoulis 2008; Hofmann et al. 2009; Yim et al. 2013; Henfrey et al. 2015; Weiß et al. 2017; Nicola et al. 2017). Changes in total bacterial and fungal community composition were previously reported in RD soils treated with 50 and 100°C (Yim et al. 2013) and soil fumigant Basamid® granules (Nicola et al. 2017) which all of the mentioned treatments provided the best growth and healthy apple root. Apple roots grown in RD soil showed darker brownish color and were necrotic (Yim et al. 2013, Figure 1.1). The highly stained cortical layer and more lignified vascular tissue of apple roots in untreated soil could possibly be a response of plant roots to pathogens (Zhu et al. 2014).

Cultivation dependent approaches indicated several soil microbes as possible RD causing agents including actinomycetes (Otto et al.1994), *Pythium* spp. (Hoestra 1994; Emmett et al. 2014), *Cylindrocarpon* spp.,

Phytophthora spp. and *Rhizoctonia solani* (Mazzola 1998; Tewoldemedhin et al. 2011; Kelderer et al. 2012) as well as nematodes, e.g. *Pratylenchus penetrans* (Mai et al. 1994). Total community DNAs based studies tried to identify these pathogens, but rather showed microbial community shifts in RD soils after soil treatments that restored apple growth (Yim et al. 2013; Sun et al. 2014; Franke-Whittle et al. 2015; Nicola et al. 2017). However, several bacterial genera such as *Gp5*, *Gp6*, *Gp9*, *Geobacter* (Nicola et al. 2017), *Gemmatimonas*, *Devosia*, *Sphingomonas* (Franke-Whittle et al. 2015), *Phenylobacterium* and *Lysobacter* (Sun et al. 2014; Franke-Whittle et al. 2015) and fungal genera *Cryptococcus*, *Mortierella* and *Tricharina* (Nicola et al. 2017) were identified to be linked with apple RD incidence.

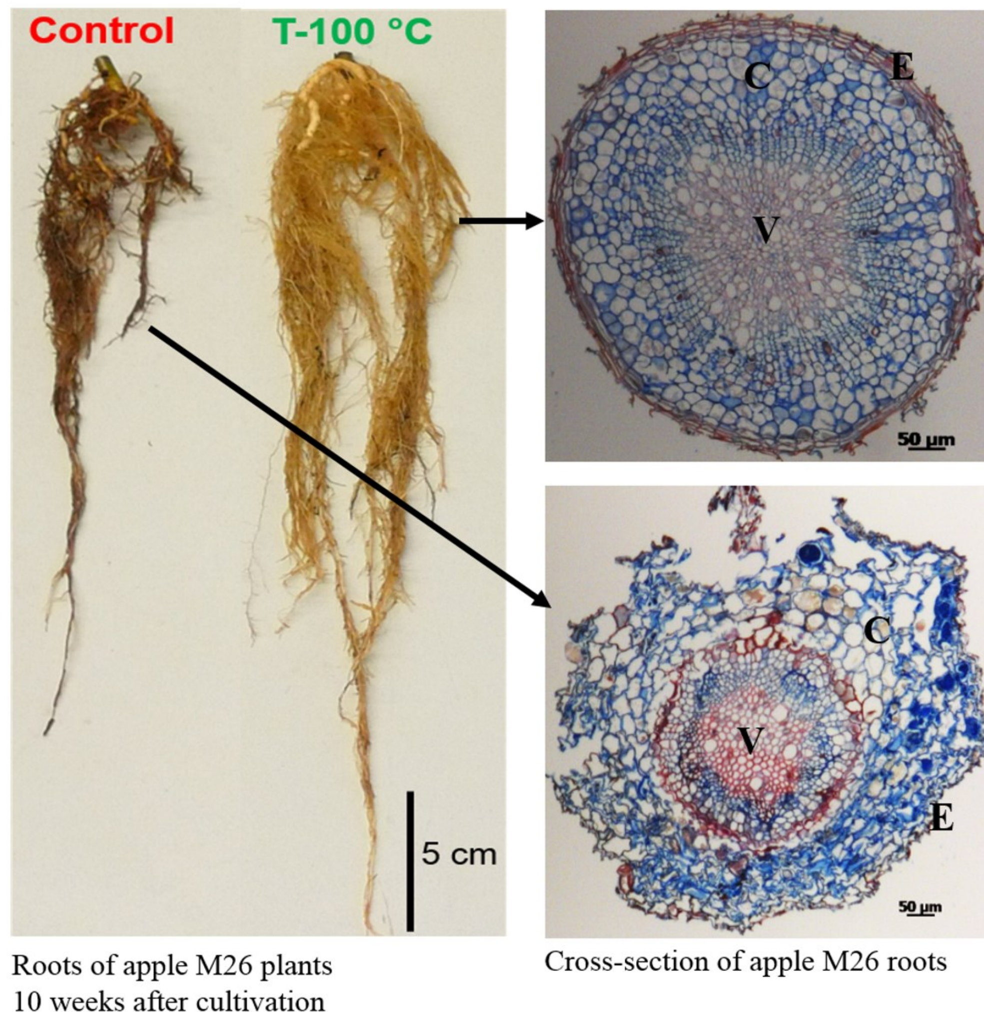

Figure 1.1: Morphological and histological observation on apple roots grown in untreated and thermal treated (100°C) replant disease soil (Yim et al. 2013). V, vascular tissue; C, cortical layer and E, epidermis of the apple root.

1.4 Biotest to evaluate replant disease soil

The RD soils can be identified via a biotest that compares shoot and root growth of plants in untreated and heat, gamma irradiation or chemical soil fumigant treated soils (Mai and Abawi 1978; Hoestra 1994; Utkhede 2006; Yim et al. 2013; Weiß et al. 2017). An increase in plant biomass in treated- compared to untreated-RD soils by 100%, 50-100% or less than 50% was considered as severe, moderate or no RD, respectively (Gilles 1974).

Yim et al. (2013) developed a fast and reproducible biotest using homogeneous acclimatized *in vitro* propagated apple rootstock M26 plants. The period of plant growth in the greenhouse could be reduced to 10 weeks in a small soil volume of 2 L. The reaction of M26 plants to RD incidence can be visualized five weeks after cultivation. Figure 1.2 shows an experimental set up in the greenhouse to evaluate RD soils.

Figure 1.2: Experimental set up (biotest) performed in the greenhouse to evaluate replant disease (RD) soil. A, acclimatized *in vitro* propagated apple rootstock M26 plants, 23 days old; B, acclimatized M26 plantlets were sorted for homogeneity before planting (the substrate was removed from plant roots before planting); C, M26 plants in differently treated RD soils eight weeks after planting in 3 L pots and D, differences in root and shoot growth of M26 plants in untreated and treated RD soils eight weeks after planting. Untreated RD soil (Control) and treatments at 50°C (T-50 °C) and with gamma irradiation (Gamma) of RD soils. The bar indicates 5 cm.

1.5 Counteractions to apple replant disease

Several approaches were previously reported to reduce RD symptoms on apple plants. In case the problems occurred due to soil nutrient deficiencies, fertilization with monoammonium phosphate (MAP) showed promising results on apple plant growth (Neilsen et al. 1991; 1994; Wilson et al. 2004). Incorporating composts into RD soils either into planting holes or wide spread (Autio et al. 1991; Moran and Schupp 2005) was another option to suppress apple RD incidence. However, inconsistent results were reported, for instance, by Moran and Schupp (2005) who stated that improved growth of apple plants was not evident in apple RD soils supplemented with MAP. Likewise, supplementing apple RD soil with compost did not result in effects on apple rootstock plant growth (Wilson et al. 2004).

Other approaches include replanting of new apple plants in inter-rows (Kelderer et al. 2012; Yin et al. 2016), inoculating apple roots with arbuscular mycorrhizal fungus species *Glomus mosseae* (Forge et al. 2001), treating RD soils with heat (Yim et al. 2013; Henfrey et al. 2015), soil fumigants (Mai and Abawi 1978; Brown and Koutoulis 2008; Yim et al. 2013; Nicola et al. 2017), biofumigation of RD soils using Brassicaceae seed meals (Mazzola et al. 2015) and using tolerant rootstocks (Isutsa and Merwin 2000; Mazzola et al. 2009; Rumberger et al. 2004; St. Laurent et al. 2010) could reduce apple RD incidence.

Conventional chemical soil disinfectants such as chloropicrin, 1.2 dichloropropane, 1.3 dichloropropene, methyl bromide and Basamid® granules with broad spectrum antimicrobial, fungicidal, herbicidal, insecticidal and nematicidal effects were shown to be most effective against apple RD (Mai and Abawi 1978; Brown and Koutoulis 2008; Yim et al. 2013; Nicola et al. 2017). Basamid® granules are an alternative product developed after a phasing out of methyl bromide (Ruzo 2006). The active ingredient is 99% dazomet (tetrahydro-2H-3,5-dimethyl-1,3,5-thiadiazine-2-thione) that releases the methyl-isothiocyanate after incorporation into soil (Ruzo 2006; Eo and Park 2014). However, those chemical substances are environmentally toxic and their application is no longer allowed in many countries (Ruzo 2006; Porter et al. 2010).

1.6 Biofumigation to control soil-borne plant pathogens

Biofumigation was defined as a soil disease suppression method using products from Brassicaceae such as seed meal, growing plants on site followed by incorporation of the total plant biomass into diseased soils (as intercropping or rotation, Figure 1.3) or as a liquid formulation via a foliar spray or a drip irrigation (Brown et al. 1991; Kirkegaard and Sarwar 1998; Mazzola et al. 2007; Mattner et al. 2008; De Nicola et al. 2013; Rongai et al. 2009). The effects result from the plant secondary metabolites glucosinolates (GS) that are hydrolyzed mainly by plant myrosinase enzymes (reviewed by Halkier and Gershenzon 2006). In plant cells, the GSs are stored in vacuoles and myrosinases are adjacent to GSs (Kissen et al. 2009). Once, they

get in contact in the presence of water (hydrolysis) several compounds are liberated such as isothiocyanates (ITCs), nitriles, thiocyanates, epithionitriles and oxazolidine-2-thiones (Brown et al. 1991; Kirkegaard and Sarwar 1998, Figure 1.4). Halkier and Gershenzon (2006) reported that nitrile formation was favored by a lower soil pH, the presence of Fe^{2+} ions and the epithiospecifier protein.

Figure 1.3: Biofumigation using onsite growing plants from *B. juncea* and *R. sativus*. A, biofumigant plants at mid-flowering stage suitable for biofumigation; B, aboveground biofumigant plants were cut and crushed by Humus WM Flail mulchers (Humus®, Bermatingen, Germany); C, damaged biofumigant plant tissues prior to incorporation into soil and D, incorporation of biofumigant plant tissues into soil by a common rotary cultivator followed by soil layering using the rolls of the sowing machine. B, C and D were taken by Dr. Andreas Wrede and Heike Nitt.

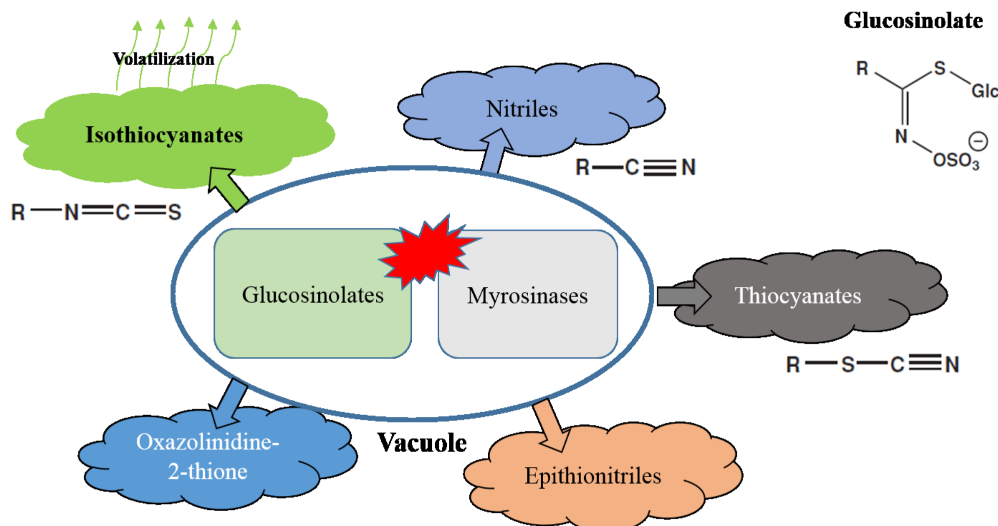

Figure 1.4: Schematic overview of glucosinolate degradation products after hydrolysis (Grubb and Abel 2006).

The GS and ITC profiles depend on *Brassica* species and cultivars (Table 1.1), and were classified into three groups, aromatic, aliphatic and indolic GSs (Kirkegaard and Sarwar 1998; Antonious et al. 2009; Ishida et al. 2014). Variation of ITC concentrations measured in amended soils depend on several factors such as incorporated biofumigant plant species/cultivar, levels of plant tissue disruption, soil moisture, temperature, pH and Fe ions, soil enzymes, soil organic matter and soil microbiome (Gimsing et al. 2006; Neubauer et al. 2014; Hanschen et al. 2015).

Among liberated products, volatile ITCs received more attention and they were shown to be responsible for the suppression of weeds (Sarwar et al. 1998; Mattner et al. 2008; Malik et al. 2008) and soil-borne plant pests and pathogens in different crops (Borek et al. 1998; Peterson et al. 1998; Matthiessen and Shackleton 2005; Bones and Rossiter 2006; Mazzola et al. 2007; Mattner et al. 2008; Agerbirk and Olsen 2012; Aires et al. 2009; Neubauer et al. 2014).

Different *Brassica* spp. have different effects on soil-borne plant pathogens due to their differences in released ITCs of respective species as presented in Table 1.1 (Mazzola et al. 2007; 2009; 2015; Handiseni et al. 2016). Aromatic (benzyl and 2-phenylethyl) ITCs showed an increased effect against tested pathogens when they were dissolved in agar compared to aliphatic (methyl, 2-propenyl, 3-butenyl and 4-pentenyl) ITCs (Sarwar et al. 1998). Another study also showed that benzyl and 2-phenylethyl ITCs were more toxic against the potato cyst nematode *Globodera rostochiensis* cv. Woll (Buskov et al. 2002) and *Verticillium dahliae* (Neubauer et al. 2014) compared to several other aliphatic ITCs.

Within the aliphatic ITCs, a shorter side chain or a lower molecular weight resulted in higher toxicity levels against tested pathogens. Almost two times the concentration of 4-methylsulfinyl-3-butenyl ITC was needed to obtain the same effect like methyl or 2-propenyl ITCs on *Verticillium dahliae* (Neubauer et al.

2014). Not only ITCs, but also other compounds, i.e. nitriles released from macerated roots of the *B. rapa/ B. napus* biofumigation crops were hypothesized to be involved in the suppression of weeds and strawberry pathogens (Mattner et al. 2008).

Besides, Bassicaceae plants, i.e. *B. juncea*, were also used for phytoremediation to extract heavy metals from polluted soils, e.g. Cd, Pb, Zn, Cu and Ni (Purakayastha et al. 2008; Bhuiyan et al. 2011) and to degrade the herbicide atrazine (Khan and Gaikwad 2013) as well as toxic polychlorinated biphenyls (Pino et al. 2016). Regarding phytoremediation, extraction of heavy metal Pb was also possible with *R. sativus* plants (Kapourchal et al. 2009).

Table 1.1: Biofumigant crops and their respective main glucosinolates and liberated isothiocyanates

Species	GS	ITC	Reference
B. juncea **(brown mustard)**	2-propenyl or allyl	2-propenyl or allyl	Neubauer et al. (2014), Mazzola et al. (2015)
B. napus **(oilseed rape)**	3-butenyl	3-butenyl	Mazzola et al. (2001), Neubauer et al. (2015)
B. rapa **(turnip)**	3-butenyl, 4-pentenyl	3-butenyl, 4-pentenyl	Doughty et al. (1996), Padilla et al. (2007)
R. sativus **(radish)**	4-methylthio-3-butenyl	4-methylthio-3-butenyl	Neubauer et al. (2014)
Sinapis alba **(white mustard)**	4-hydroxybenzyl	4-hydroxybenzyl	Neubauer et al. (2014; 2015)

GS, glucosinolate; ITC, isothiocyanate; B., *Brassica* and R., *Raphanus*

1.7 Project introduction

The study is part of a joint research project entitled 'Effects of biofumigation on plant growth and microbial communities in replant disease soils', in which the Leibniz Universität Hannover (LUH), the Chamber of Agriculture Schleswig-Holstein, the Julius-Kühn-Institut (JKI) Braunschweig, the Leibniz Institute of Vegetable and Ornamental Crops (IGZ) Großbeeren and partners from tree nurseries have been working together. Within the initiation of the Federal Program for Ecological Farming and other Forms of Sustainable Agriculture (BÖLN), the study aims to contribute to the development of an approach to overcome replanting problems in Rosaceae, according to the guideline 7.7.2011, 2.1.2 for environmental friendly plant cultivation and risk reduction in plant protection, particularly through non-chemical and biological plant protection means.

This study compared effects of different soil treatments including biofumigation with *B. juncea* and *R. sativus* and the nematode repellent plant *Tagetes* to the conventional soil fumigant Basamid® granules at

three sites with apple or rose RD soils. The project lasted from September 2012 – August 2015 and was supported by three nurseries K (53° 41′ 58.51″ N, 9° 41′ 34.12″ E), A (53° 42′ 18.81″ N, 9° 48′ 16.74″ E) and M (53° 44′ 25.21″ N, 9° 46′ 55.18″ E) located in the region of Pinneberg, Northern Germany. The sites had different soil physical and chemical properties (see manuscripts) and documented problems due to replanting of rose (sites K and M) and apple (site A) rootstocks. An experimental plot of 1000 m² per site was split into sub-plots for seven treatments. The design regarding treatments, three replicates each, was completely randomized (Figure 1.5).

Treatments 2, 3, 4 and 7 were carried out in two years, namely in 2012 and 2013. The plots 1, 5 and 6 were treated only in 2013 (grass was growing in 2012). A more detailed description of treatments 1, 2, 3 and 4 was described by Yim et al. (2016), chapter 2.2. Briefly, for treatment 1 grass was grown in 2012 and 2013 to maintain the RD status and in August 2013 the commercial soil fumigant Basamid® granules was incorporated at a dose of 400 kg ha^{-1} as recommended by the manufacturer (ProfiFlor GmbH, Stommeln, Germany). Treatment 2 served as the control plots and was divided into three sub-plots for comparison of different rootstocks from apple and rose (intensified RD plots). Treatments 5 and 6 (a one-year biofumigation) were similar to treatments 3 and 4 (a two-year biofumigation), except for being carried out once. The cultivars *B. juncea* 'Terra Plus' 12 kg h^{-1} (3 or 5) and *R. sativus* 'Defender' 30 kg h^{-1} (4 or 6) were sown for biofumigation (P. H. Petersen Saatzucht Lundsgaard GmbH, Germany). Biofumigation for treatments 3 and 4 was carried out four times, in spring and summer of 2012 and 2013 as described in the manuscript. For treatment 7, the nematode repellent *Tagetes patula* 'Nemamix' (Hooks et al. 2010), 10 kg ha^{-1} was sown. The treatment was scheduled in April/May 2012 and 2013, and plants were grown until November (2012/2013) before they were ploughed into the soils using a common rotary cultivator. Two weeks after total plant biomass incorporation, *Avena nuda* 12 kg ha^{-1} was sown as a winter cover crop on plots 3, 4, 5, 6 and 7 in 2012 and 2013 (P. H. Petersen Saatzucht Lundsgaard GmbH, Germany).

The analyses of the project aimed to identify alternative approaches besides conventional soil fumigant, i.e. Basamid® granules, in counteracting RD in apple and rose, and to link the plant growth status with bacterial and fungal taxa that were affected by treatments.

	Bed 1	Bed 2	Bed 3	Bed 4	Bed 5	Bed 6	Bed 7
30 m	1a	2a 'Laxa' 2a 'Bitt.' 2a M111	5a	6a	3a	4a	7a
30 m	3b	4b	6b	7b	5b	1b	2b M111 2b 'Laxa' 2b 'Bitt.'
30 m	7c	6c	3c	2c 'Bitt.' 2c M111 2c 'Laxa'	4c	5c	1c
	1.5 m						
	10.5 m						

Figure 1.5: Field experimental design in an area of 1000 m² with seven treatments (1, 2, 3, 4, 5, 6 and 7) and three replicates per treatment (a, b and c). Treatment 1, Basamid® granules in 2013; 2, split-plots cultivated with rootstocks *Rosa corymbifera* 'Laxa' and *Malus* 'Bittenfelder' and M111 in 2013, served as untreated intensified replant disease soils; 3 and 4, a two-year biofumigation with *B. juncea* and *R. sativus*, respectively in 2012 and 2013; 5 and 6, a one-year biofumigation with *B. juncea* and *R. sativus*, respectively in 2013 and 7, treatment with *Tagetes* in 2012 and 2013. Each replicate bed is 45 m², except the sub-plots in treatment 2 (15 m²).

1.8 Thesis objectives

This study aimed to investigate effects of biofumigation using *B. juncea* and *R. sativus* as well as the nematode repellent plant *Tagetes* in comparison to the conventional soil fumigant Basamid® granules at three sites with apple or rose RD soils.

The specific objectives of the thesis were:

- to identify and quantify GSs in different plant organs of *B. juncea* and *R. sativus*,
- to identify and quantify GS degradation products after biofumigation and to quantify methyl ITC in the Basamid® treated soils,
- to investigate bacterial and fungal community structures affected by different soil treatments using denaturating gradient gel electrophoresis fingerprints (DGGE) and next generation amplicon-sequencing approaches and
- to evaluate the effectiveness of different soil treatments revealed by the growth of indicator plants.

The studies were performed with four cooperating partners. The Chamber of Agriculture Schleswig Holstein (Gartenbauzentrum Ellerhoop) carried out the field experiments. The plant secondary metabolite GS in the biofumigant plants and their breakdown products in amended soils were analyzed in the Department of Plant Quality, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Großbeeren, Germany. The effects of different soil treatments on indicator plant growth were examined in the greenhouse at the Leibniz Universität Hannover. The molecular analyses regarding the effects of different soil treatments on bacterial and fungal community structures, richness, diversity and responders were performed in cooperation with the Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics at the Julius Kühn-Institut (JKI), Braunschweig.
The thesis is comprised of three manuscripts (chapter 2), an overall discussion (chapter 3) that focuses on aspects not covered by the manuscript discussion sections including appendix, outcomes of the study and future prospects (chapter 4) and ends with conclusions.

2 Publications and manuscripts

2.1 Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis – contribution to improved aboveground apple plant growth?

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Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis – contribution to improved aboveground apple plant growth?

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Replant disease (RD) severely affects apple production in propagation tree nurseries and in fruit orchards worldwide. This study aimed to investigate the effects of soil disinfection treatments on plant growth and health in a biotest in two different RD soil types under greenhouse conditions and to link the plant growth status with the bacterial community composition at the time of plant sampling. In the biotest performed we observed that the aboveground growth of apple rootstock M26 plants after 8 weeks was improved in the two RD soils either treated at 50°C or with gamma irradiation compared to the untreated RD soils. Total community DNA was extracted from soil loosely adhering to the roots and quantitative real-time PCR revealed no pronounced differences in 16S rRNA gene copy numbers. 16S rRNA gene-based bacterial community analysis by denaturing gradient gel electrophoresis (DGGE) and 454-pyrosequencing revealed significant differences in the bacterial community composition even after 8 weeks of plant growth. In both soils, the treatments affected different phyla but only the relative abundance of *Acidobacteria* was reduced by both treatments. The genera *Streptomyces*, *Bacillus*, *Paenibacillus,* and *Sphingomonas* had a higher relative abundance in both heat treated soils, whereas the relative abundance of *Mucilaginibacter*, *Devosia,* and *Rhodanobacter* was increased in the gamma-irradiated soils and only the genus *Phenylobacterium* was increased in both treatments. The increased abundance of genera with potentially beneficial bacteria, i.e., potential degraders of phenolic compounds might have contributed to the improved plant growth in both treatments.

Keywords: biotest, apple replant disease, DGGE, qPCR, pyrosequencing, bacterial community composition, bacterial diversity

INTRODUCTION

Intensive production of perennial and annual crops in the same area might lead to replant problems which become evident by low yields and growth reduction (Hoestra, 1994). The replant problems are potentially caused by both biotic and abiotic factors (Hoestra, 1994; Politycka and Adamska, 2003). The decline in plant growth evoked by biotic factors is called "replant disease" (RD; Utkhede, 2006). The RD was already reported by Klaus (1939), and the disease incidence is typically resident and persistent. It is most likely that biotic factors including soilborne pathogens play a major role since significantly improved growth after heat or chemical soil treatment was reported compared to the growth in untreated soil (Mai and Abawi, 1978; Hoestra, 1994; Parchomchuk et al., 1994; Utkhede, 2006; Yim et al., 2013). The important role of soilborne organisms in apple RD was recently discussed in a review by Mazzola and Manici (2012). As reported in various studies, possible causes of apple RD differed widely between regions and included actinomycetes (Westcott et al., 1986; Otto et al., 1994), *Pythium* sp. (Hoestra, 1994; Emmett et al., 2014), *Cylindrocarpon* sp., *Phytophthora* sp. and *Rhizoctonia solani* (Mazzola, 1998; Tewoldemedhin et al., 2011; Kelderer et al., 2012) as well as nematodes, e.g., *Pratylenchus penetrans* (Mai et al., 1994).

Bacteria and fungi associated with apple RD were traditionally identified after isolation from the respective soils. However, the soil microbial diversity is highly complex and can be only partially evaluated by traditional cultivation techniques as a large proportion does not form colonies on solid media after plating. It was estimated that less than 14% of the bacterial cells per gram of soil can be cultured (Janssen et al., 2002; Janssen, 2006). The analysis of soil total community (TC-) DNA or RNA helped to overcome this limitation. Gomes et al. (2005) reported no pronounced differences between DNA and cDNA based fingerprints when working with soils. In many studies, fingerprinting and amplicon sequencing methods have been applied to study soil bacterial communities based on 16S rRNA gene fragments amplified from soil TC-DNA (reviewed by Berg and Smalla, 2009).

In the study by Yim et al. (2013), a biotest was developed to determine the degree of apple RD in a given soil. It involved soils untreated and treated at 50°C and at 100°C. It is assumed that the treatment at 50°C primarily affects nematodes, bacteria and fungi sensitive to this temperature, while the treatment at 100°C strongly reduces the total soil microbiota (Pullman et al., 1981; Cabos et al., 2012). A comparison of the growth of *in vitro* propagated apple rootstock M26 in these three soil variants under greenhouse conditions for 10 weeks clearly indicated the level of the RD. The DGGE analysis of 16S rRNA gene fragments amplified from soil TC-DNA revealed a distinct bacterial community composition of the soils depending on the treatments at the end of the biotest (Yim et al., 2013); the DGGE fingerprints did not provide more detailed information on the taxonomy of bacterial responders.

In the present study, a modified biotest was employed by using a gamma irradiation treatment instead of a heat treatment at 100°C since gamma irradiation has less influence on soil physical and chemical properties (Trevors, 1996). This study aimed to investigate the effects of soil disinfection treatments on plant growth and health in a biotest in two different RD soil types and to link the plant growth status with the bacterial community composition and diversity at the time of plant sampling. We hypothesized that differences in apple rootstock growth and symptoms observed resulted from changes in the microbial community composition and diversity in RD soils caused by their heat or gamma irradiation treatment. At the end of the biotest, the bacterial community composition of soil loosely adhering to the roots was analyzed by DGGE and pyrosequencing of 16S rRNA gene fragments amplified from TC-DNA. Statistical analysis of the 454 pyrosequencing data allowed us to identify responders to the treatments. In addition soil samples taken before the biotest were included in the DGGE analyses.

MATERIALS AND METHODS

Soil Characteristics

Two RD soils were obtained from two private nurseries, Kle (53°41′ 58.51″ N, 9° 41′ 34.12″ E) and Alv (53° 42′ 18.81″ N, 9° 48′ 16.74″ E) in the Pinneberg area in Germany. Both soil types had different cropping and management histories. The Kle site soil had been mainly cultivated with rose rootstock plants from 1980 until 2011, and crop rotation with *Tagetes* started in 2002. In the Alv soil, apple rootstock plants had been planted for several years until 2009. Then, *Prunus domestica* and *Cydonia oblonga* were grown in 2010 and 2011, respectively. In May 2012, the apple rootstock 'M4' was planted in both soils. Supplementary Table S1 shows the characteristics of both soil types.

Biotest

In October 2012, approximately 100 L of the RD soils were taken from each nursery, Kle and Alv, at a depth of 0–25 cm from three field replicates. For the treatments, the soils were mixed manually, and one third of the total soil volume per soil type remained either untreated (Con) or was treated at 50°C (H50) or with gamma irradiation (Gamma). The 1-h treatment at 50°C was performed in a dry air oven, using 2 L autoclavable bags. Timing for 1 h was started when the core soil temperature in the bag had reached 50°C which was checked by an inserted thermometer and it took approximately 1 h and half for the two soils used in this study. The soil disinfection with gamma irradiation was applied at a minimal dose of 10 kGy (McNamara et al., 2003) in 15 L autoclavable bags (with no influence of the soil volume and duration). Acclimatized *in vitro* apple rootstock M26 plants, 20 days old, were planted as a susceptible genotype (Kviklys et al., 2008; St. Laurent et al., 2010; Yim et al., 2013), to evaluate the effects of the different RD soil treatments. The experiment was carried out with 10 replicates per treatment in 3 L pots with supplementation of 2 g L^{-1} Osmocote-Exact 3-4M [16 + 9 + 12(+2)], a slow release fertilizer[1]. In total, 60 plants were cultivated in all soil variants (Kle or Alv).

The biotest was set up in a greenhouse during winter time (November 2012) at 20 ± 2°C and a 16 h photoperiod supplied

[1]http://www.scottsprofessional.com

by additional light (Philips Master Agro 400W). The irrigation was applied on a daily basis. Spraying against pests and diseases on aboveground plant parts such as aphids, thrips or spider mites was carried out weekly according to horticultural practices. For data collection the aboveground shoot length (SL) was measured weekly, and after 8 weeks the plants were harvested to determine shoot fresh mass (SFM) and dry mass (SDM) as well as root dry mass (RDM).

For statistical analysis, the homogeneity of variance of SL, SFM, SDM, and RDM was checked prior to the analysis by a Dunnett's test to check the differences between the control and the treatments. The Tukey test was applied to reveal differences between the three treatments of every measured parameter with $p < 0.05$ using R3.1.0[2] software.

Analyses of Soil Bacterial Populations

Soil Sampling and Processing

The soil samples used for the bacterial community analyses were collected at the end of the biotest, after the apple M26 plants had been growing for 8 weeks in the greenhouse. Among the 10 replicates per treatment, the soils were taken from eight replicates of the biotest (no. 1–8). Soil attached to roots of the plants was collected by vigorous shaking. Then, soil from two plants was pooled and used as one biological replicate (about 27.2 ± 7.3 g wet soil). In total, 24 soil samples were analyzed (four replicates x three treatments per soil type) after sieving with a mesh size of 2.0 mm. For the DGGE analyses, another eight soil samples taken before the biotest were included (four replicates per soil type).

Soil TC-DNA Extraction and Purification

The TC-DNA isolation was accomplished by direct extraction from 500 mg soil from each replicate by bead beating of the FastPrep® Instrument from mpbio (MP Biomedicals, Santa Ana, CA, USA). The extracted nucleic acids were then purified with GENECLEAN SPIN Kit from qbiogene (Qbiogene, Inc., Carlsbad, CA, USA) followed by centrifugal precipitation in 50 μl GENECLEAN® SPIN elution solution according to the protocol described by the manufacturer (MP Biomedicals, Heidelberg, Germany).

Amplification of Bacterial 16S rRNA Gene Fragments for Real-Time PCR Analysis

The bacterial 16S rRNA gene copy numbers were quantified using a 5′ Nuclease assay in the real-time quantitative PCR (qPCR). The qPCR reaction mixture (50 μl) consisted of 1x PCR TrueStart™ buffer (Fermentas GmbH, Darmstadt, Germany), 0.2 mM dNTPs, 3 mM $MgCl_2$, 5 μg BSA (Bovine Serum Albumin), 1.2 μM BACT1369F as forward primer (5′-CGGTGAATACGTTCYCGG-3′), 1 μM PROK1492R as reverse primer (5′-GGWTACCTTGTTACGACTT-3′), 0.5 μM TM1389F as probe (5′-CTTGTACACACCGCCCGTC-3′), 1.25 U TrueStart™ Taq (Fermentas GmbH, Darmstadt, Germany) and 1 μl TC-DNA (ca. 3 ng). The thermal cycling programs were as previously described by Suzuki et al. (2000).

[2] http://www.r-project.org

Amplification of Bacterial 16S rRNA Gene Fragments for DGGE Analysis

Amplification of bacterial 16S rRNA gene fragments (GC-PCR) for DGGE fingerprints analysis was carried out as described by Yim et al. (2013), except that 0.5x PCR GoTaq® buffer, 3.75 mM $MgCl_2$ and 1.25 U GoTaq® (Promega GmbH, Mannheim, Germany) were used for the PCR reaction (25 μl). The PCR amplification was conducted at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 2 min and finally 72°C for 10 min.

Amplification of Bacterial 16S rRNA Gene Fragments for 454-Pyrosequencing Analysis

For pyrosequencing of 16S rRNA gene fragments, all TC-DNA samples which had an absorbance ratio A260/A280 between 1.9 and 2.4 (Nanodrop2000c, Spectrophotometer, PEQLAB Biotechnologie GmbH, Erlangen, Germany) were sent for sequencing to the Biotechnology Innovation Center (Roche Life Sciences, BIOCANT, Cantanhede, Portugal). The amplification and sequencing of hypervariable V3-V4 regions of the 16S bacterial ribosomal genes were carried out through the 454 Genome Sequencer FLX platforms according to Roche-Life Sciences using primers 338F (5′-ACTCCTACGGGAGGCAG-3′) and 802R (5′-TACNVRRGTHTCTAATYC-3′) which were fused to the 454 A and B adapters, respectively (Huse et al., 2008; Vaz-Moreira et al., 2011). The PCR reaction mixture (50 μl) contained 5 U of Fast Start Polymerase (Roche Diagnostics, Penzberg, Germany), 3 mM $MgCl_2$, 6% DMSO, 0.2 μM of each primer, 200 mM dNTPs and 2 μl of TC-DNA (ca. 3 ng μl^{-1}). The PCR conditions were 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 44°C for 45 s, 72°C for 1 min and finally at 72°C for 2 min elongation (Ding et al., 2012).

Data Analysis

The digital images of silver-stained DGGE gels were analyzed by GelCompar II 6.5 (Applied Math, Sint-Martens-Latern, Belgium). The analysis was based on Pearson correlation coefficients of pairwise similarity measure of two lanes in one gel from the absolute intensity signal in each electrophoresis lane. The UPGMA (unweighted pairwise grouping method using arithmetic means) was applied to obtain a similarity and hierarchical cluster of the lanes. For statistical tests, we used the Pearson similarity matrices from the UPGMA and performed a Permutation test. The test statistics calculated the differences (d-value) between the average of all correlation coefficients within the group (within treatment) and the average over all correlation coefficients of different groups (different treatments). Thus, the d-value indicated the differences in the bacterial community composition between the soil treatments or soil variants (Kropf et al., 2004).

To check the effect of soil types and of treatments on the bacterial 16S rRNA gene copy numbers by qPCR, ANOVA and Tukey test were applied using R3.1.0[2] software with $p < 0.05$, respectively.

The analysis of pyrosequencing data was done using Mothur 1.30. software (Schloss et al., 2009). Briefly, the barcode and primer sequences were removed and only those sequences

with a length of more than 200 bp were included in the analysis. The trimmed sequences (>200 bp) were aligned to the SILVA 16S rRNA gene database (Pruesse et al., 2007) and the sequence errors were removed by *chimera.uchime*. Classification of sequences into an operational taxonomic unit (OTU) based on 97% sequence similarity for an OTU level report (containing domain, phylum, class, order, family, and genus) according to their taxonomy as well as number of sequences for each of the samples were done as described in Ding et al. (2012). Data were transformed by $\log(n/N * 100 + 1)$ (n, the number of sequences for each OTU and N, the total number of sequences from the sample) for the following analyses. The effect of different soil treatments on bacterial relative abundances was checked by the statistical software R3.1.0[3] using the transformed data and applying Tukey's honest significant test. Rarefaction analysis was performed to compare the diversity of detected sequences between treatments of both soils based on OTUs defined at 97% similarity. Invsimpson's diversity index of each sample replicate was used to reveal significant differences of the bacterial diversity between the treatments, applying Tukey test, $p < 0.05$ using R3.1.0.

The principal coordinate analysis (PCoA) using the Bray–Curtis distance metric was carried out with the OTU composition from the dominant phyla (>1% of total sequences in the sample) and only with those OTUs which were identified at the genus level, with Past 3 (3.02). By one-way and two-way ANOSIM tests the differences in the relative abundance of bacterial OTUs between the soil treatments and soil types were tested for significance.

Pyrosequencing data were deposited at the NCBI Sequence Read Archive under the accession number PRJNA276496.

RESULTS

Biotest to Reveal Replant Disease Soils

For both RD soils apple M26 plants showed a significantly improved growth in treated (H50, Gamma) compared to untreated (Con) soils (**Figure 1**, **Table 1**). The first significant deviations of SLs between Con and H50/Gamma were noted in Kle soil already 2 weeks after planting (**Figure 1A**). The SL significantly increased by about 81% in H50 soil and by up to 131% in Gamma soil compared to Con soil. In Alv soil, significant deviations of SLs were first recorded 5 weeks after planting (Con vs. H50/Gamma) (**Figure 1B**). In contrast to Kle soil, there was no significant difference for SLs between AlvH50 and AlvGamma (**Figure 1B**). Growth enhancement after gamma irradiation was observed in both soils in comparable extents, since the SLs in AlvGamma was 148% of that in Con soil (**Figure 1**). However, overall growth of M26 plantlets was much higher in Kle than in Alv soil (**Figure 1**).

The shoot fresh and dry mass correlated with the SL. The H50 and the Gamma treatment increased the shoot dry mass 1.5- and 1.8-fold, respectively, in Kle soil compared to the control (Con). However, in Alv soil the observed increase of the shoot dry mass was higher in H50 (1.7-fold) than in Gamma-treated soil (1.5-fold). The biomass of roots was not significantly influenced by the treatments in both soils. However, the plants in Con soils showed smaller root systems that were darker brownish in color, and some parts of the roots were necrotic and rotten compared to the roots from H50 and Gamma treatments in both soils (Supplementary Figure S1). In both soils the root-to-shoot ratio was significantly higher in Con soil compared to H50 and Gamma soil (**Table 1**).

[3]http://www.r-project.org

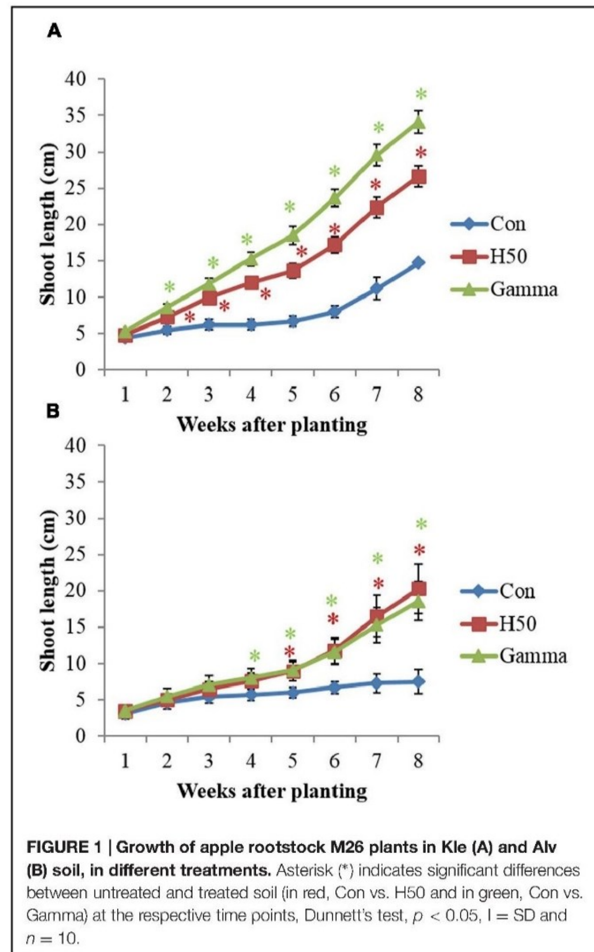

FIGURE 1 | Growth of apple rootstock M26 plants in Kle (A) and Alv (B) soil, in different treatments. Asterisk (*) indicates significant differences between untreated and treated soil (in red, Con vs. H50 and in green, Con vs. Gamma) at the respective time points, Dunnett's test, $p < 0.05$, I = SD and $n = 10$.

Gene Copy Numbers of 16S rRNA Genes Amplified from Soil TC-DNA

The qPCR analysis in soil TC-DNA collected 8 weeks after the biotest showed that approximately 10^9 16S rRNA gene copy numbers per gram soil were detected with no significant differences in both Con soils (**Figure 2**) (KleCon vs. AlvCon, ANOVA, $p < 0.05$). Only in Kle soil, significantly reduced 16S rRNA gene copy numbers in the Gamma-treated soil were recorded, while the H50 treatment did not influence the numbers in both soils (**Figure 2**).

TABLE 1 | Biomass of apple rootstock M26 plants grown for 8 weeks in replant disease (RD) soils after different treatments.

Soil	Treatment	SFM (g/plant)	SDM (g/plant)	% (fold) of increases of SDM	RDM (g/plant)	RDM to SDM ratio
Kle	Con	4.3 ± 1.2^{a}	3.1 ± 0.4^{a}		2.7 ± 0.6^{a}	0.9^{a}
	H50	9.2 ± 0.8^{b}	4.5 ± 0.3^{b}	45 (1.5)	3.0 ± 0.5^{a}	0.7^{b}
	Gamma	12.7 ± 1.5^{c}	5.7 ± 0.4^{c}	84 (1.8)	3.1 ± 0.5^{a}	0.5^{b}
Alv	Con	2.5 ± 1.1^{a}	1.5 ± 0.4^{a}		3.3 ± 0.1^{ab}	2.2^{a}
	H50	6.2 ± 2.4^{b}	2.6 ± 0.8^{b}	73 (1.7)	3.4 ± 0.2^{a}	1.3^{b}
	Gamma	5.7 ± 2.0^{b}	2.3 ± 0.6^{b}	53 (1.5)	3.2 ± 0.3^{b}	1.4^{b}

Mean ± SD within same parameter and soil followed by different letters indicates significant differences, Tukey test, $p < 0.05$ and $n = 10$. Shoot fresh mass (SFM), shoot dry mass (SDM), and root dry mass (RDM).

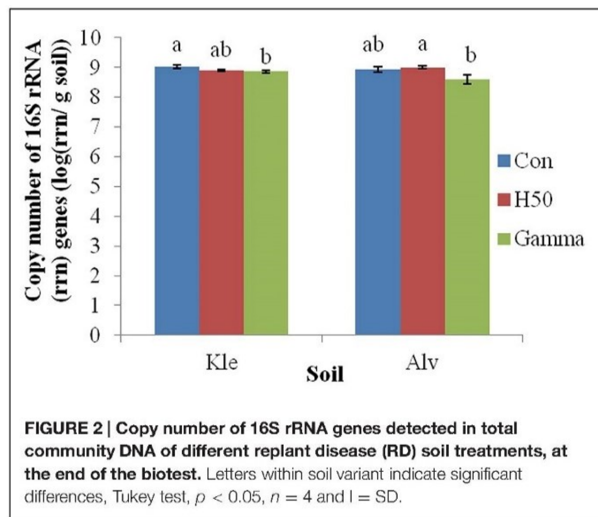

FIGURE 2 | Copy number of 16S rRNA genes detected in total community DNA of different replant disease (RD) soil treatments, at the end of the biotest. Letters within soil variant indicate significant differences, Tukey test, $p < 0.05$, $n = 4$ and I = SD.

DGGE Analysis of 16S rRNA Genes Amplified from Soil TC-DNA

The bacterial DGGE fingerprints of 16S rRNA gene fragments amplified from soil TC-DNA before the biotest revealed significant differences between both soils (KleT0 and AlvT0) which were indicated by dissimilarities in the Permutation test (*d*-value) of 10.6%, $P = 0.03$ (**Table 2**). This demonstrated distinct bacterial community compositions in the two soil types. In both soils, the bacterial community compositions have changed by 5.7 and 5.3% in Kle and Alv soils, respectively, when comparing between sampling time (T0) and at the end of the biotest (Con) after 8 weeks (**Table 2**).

Denaturing gradient gel electrophoresis analysis of 16S rRNA gene fragments amplified from TC-DNA of soil collected at the end of the biotest revealed that the treatments significantly changed the bacterial communities in both soils (**Table 2**). The *d*-values indicated that the H50 treatment resulted in less pronounced shifts in the bacterial community compositions compared to the Gamma treatment, since smaller *d*-values were observed between Con and H50 than between Con and Gamma (**Table 2**).

Pyrosequencing Analysis of 16S rRNA Genes Amplified from Soil TC-DNA

The pyrosequencing analysis of the V3–V4 region of 16S rRNA gene amplified from soil TC-DNA of samples taken at the end of the biotest resulted in a total of 187,602 sequences with more than 200 bp per sequence from the 24 samples after filtering out low quality or chimeric sequences. All sequences were affiliated to the domain Bacteria. The number of classified sequences ranged between 4,228 and 10,005 sequences per sample, and thus relative abundances were used in the analysis. The sequences were binned based on 97% sequence identity resulting in 10,227 OTUs.

The Permutation test using the pyrosequencing data confirmed the results of the DGGE analyses that after 8 weeks of M26 plant growth the Gamma treatment led to a significantly higher difference of the bacterial community composition compared to Con soil than the H50 treatment (**Table 2**).

Rarefaction curves allowed a comparison of detected bacterial community diversity with the top curve representing the highest diversity. The bacterial community composition of all treatments of Kle soil was more diverse than that in Alv soil (**Figure 3**). Invsimpson indices of Kle soil (34.9–44.0) were significantly higher than those of Alv soil (11.4–16.3) at $p < 0.001$ (Supplementary Table S4). Within each soil type, the bacterial

TABLE 2 | Treatment-dependent differences of bacterial communities in RD soils before planting and after 8 weeks of the biotest with apple M26 plants (DGGE and pyrosequencing).

Comparison	DGGE		Pyrosequencing	
	***d*-value**	***p*-value**	***d*-value**	***p*-value**
KleT0 vs. AlvT0	10.06	0.03	n.a.	n.a.
KleCon vs. KleT0	5.7	0.03	n.a.	n.a.
AlvCon vs. AlvT0	5.3	0.03	n.a.	n.a.
KleCon vs. KleH50	19.2	0.03	22.1	0.01
KleiCon vs. KleGamma	57.6	0.03	29.2	0.02
AlvCon vs. AlvH50	7.6	0.03	7.2	0.04
AlvCon vs. AlvGamma	11.2	0.03	21	0.01

Percent dissimilarity (d-value), average within-group pairwise Pearson's correlation – average between-group pairwise Pearson's correlation, $p < 0.05$ and $n = 4$ (Kropf et al., 2004). Abbreviations: T0, samples before planting apple rootstock M26 and Con, H50 and Gamma, samples collected 8 weeks after the biotest (after planting M26); n.a., not analyzed.

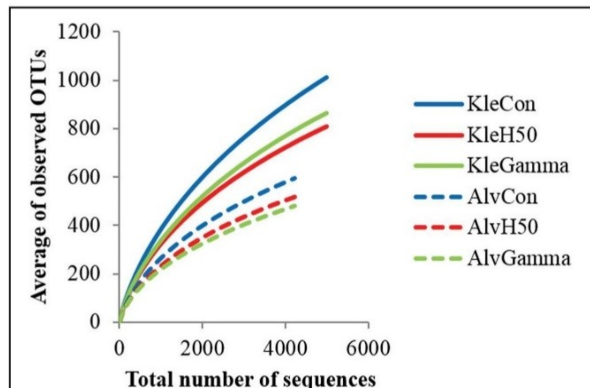

FIGURE 3 | Rarefaction curves indicating the observed number of operational taxonomic units (OTUs) of bacterial communities. Diversity of detected sequences, in RD soils, Kle and Alv, 8 weeks after planting apple rootstock M26 plants.

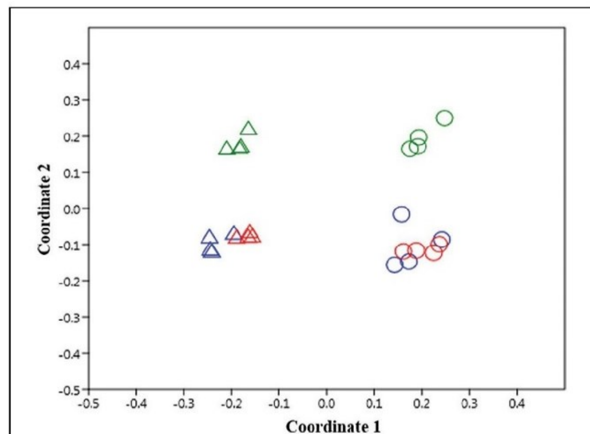

FIGURE 4 | Effect of soil treatments on the bacterial community composition according to data of operational taxonomic units (OTUs) at genus level as revealed by principal coordinate analysis using the Bray–Curtis distance metric, $n = 4$. Δ and ○ are for Kle and Alv soils, respectively. Colors in blue, red and green represent Con, H50 and Gamma treatment, respectively.

community diversity in Con soil was by trend higher than in the H50 and Gamma soils, but these differences were not significant (**Figure 3**, Supplementary Table S4).

Principal coordinate analysis considering the bacterial community composition at the genus level clearly separated Kle soil from Alv soil (**Figure 4**) (ANOSIM test, $R = 0.94$, $p < 0.001$). For both Kle and Alv soils, differences were also recorded between Con and H50 soil, but a more pronounced dissimilarity was observed for Gamma and Con as well as for Gamma and H50 soils (**Figure 4**). Overall, the ANOSIM tests showed that after soil treatments the bacterial community composition significantly shifted for both soils (R-values of 1.0 and 0.77 for Kle and Alv soils, respectively, and $p < 0.001$).

Taxonomic Composition of Bacteria in Replant Disease Soils

A total of 10,227 OTUs from 22 phyla, 62 classes, 98 orders, 185 families, and 342 genera were identified in both soils (Kle and Alv). Phyla with a relative abundance below 1% were considered rare (Supplementary Table S3 shows relative abundance of 13 phyla). The dominant phyla were *Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria, Bacteroidetes,* and *Gemmatimonadetes* (**Figure 5**) to which 96.3 and 96% of the sequences in Kle and Alv soils were affiliated, respectively. An average of 30.5 and 28.8% of the total sequences for Kle and Alv soils, respectively, were assigned to the *Proteobacteria* (**Figure 5**). Within the phylum *Proteobacteria*, *Alphaproteobacteria* were the most abundant followed by *Gammaproteobacteria, Betaproteobacteria,* and *Deltaproteobacteria*.

Although the gene copy numbers of 16S rRNA detected in TC-DNA of RD soils were comparable (KleCon vs. AlvCon) (**Figure 2**), the bacterial community composition differed (Supplementary Table S2). For instance, the relative abundance of *Acidobacteria* and *Gemmatimonadetes* was significantly lower in AlvCon compared to KleCon. At the genus level, members of the genera *Gp1, Gp2, Gp3, Gp6, Gp16, Arthrobacter, Bacillus, Paenibacillus, Clostridium sensu stricto, Gemmatimonas,* and *Sphingomonas* were dominant in both Con soils (**Table 3**). Many genera such as *Gp1, Gp2, Arthrobacter, Nocardioides, Paenibacillus, Phenylobacterium, Lysobacter,* and others (Supplementary Table S2) had a similar relative abundance in both soils. Other genera such as *Bacillus* were significantly higher in relative abundance in AlvCon soil (11.1 ± 4%) than in KleCon soil (Supplementary Table S2).

Treatment-Dependent Bacterial Responders

Even 8 weeks after apple rootstock growth, changes in the relative abundances of different phyla were recorded in the treated soils compared to the control. In H50 soil, a significant decrease in the relative abundance of the phylum *Acidobacteria* and an increase of the phylum *Firmicutes* was observed in both soils. In Gamma soil the relative abundances of the phyla *Actinobacteria* and *Acidobacteria* were significantly reduced, whereas the relative abundances of the *Proteobacteria* and the *Bacteroidetes* were significantly increased in both soils (**Figure 5**).

Genera with significantly higher or lower relative abundance in response to the treatment (so-called responders) were different in both soils. In Kle soil, both treatments (H50, Gamma) reduced the relative abundances of several genera belonging to the *Acidobacteria* (*Gp2, Gp3, Gp5, Gp6, Gp7,* and *Gp13*) as well as the genera *Ilumatobacter, Tuberibacillus,* and *Dokdonella*. The decrease in relative abundances of the acidobacterial genera was less pronounced in Alv soil and only *Gp2, Gp3,* and *Dokdonella* were significantly less abundant (H50, Gamma). *Pseudonocardia* and *Methylobacter* showed a significantly decreased relative abundance in both H50 and Gamma treatments of Alv soil, while a significantly decreased relative abundance of the genera *Mycobacterium, Nocardioides, Bacillus, Clostridium sensu stricto,*

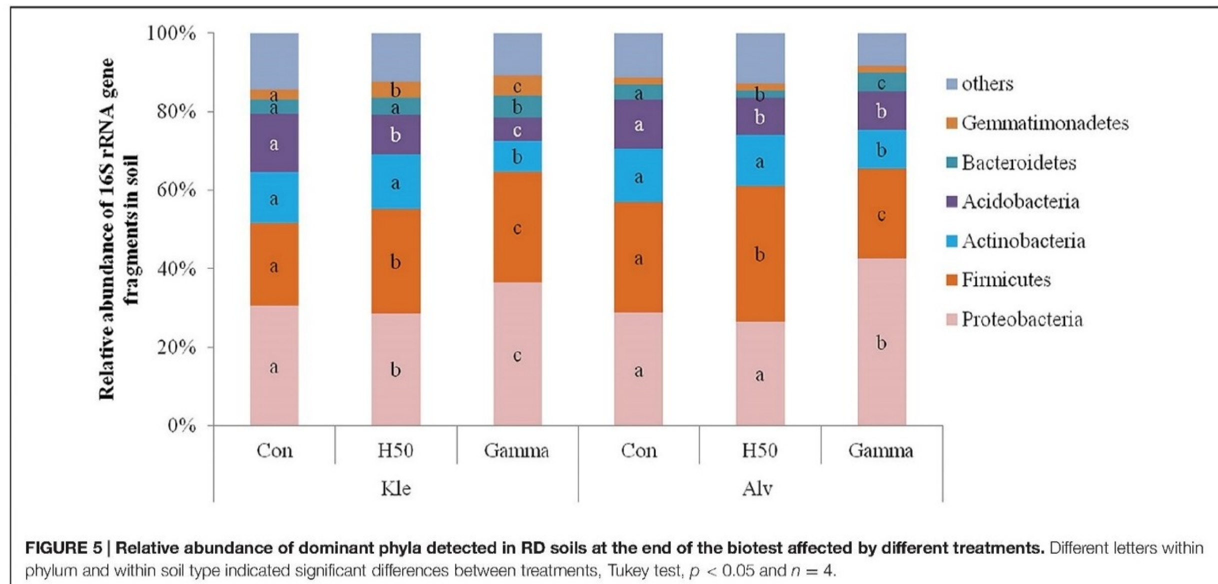

FIGURE 5 | Relative abundance of dominant phyla detected in RD soils at the end of the biotest affected by different treatments. Different letters within phylum and within soil type indicated significant differences between treatments, Tukey test, $p < 0.05$ and $n = 4$.

and *Clostridium III* were observed only in AlvGamma soil (**Table 3**).

Soil treatments and the M26 plants not only decreased, but also enriched the relative abundances of a wide range of different genera compared to the control soils. Significantly increased abundances in both KleH50 and KleGamma compared to Con soil were observed for the genera *Bacillus*, *Gemmatimonas*, *Phenylobacterium*, *Microvirga*, *Burkholderia* and *Ramlibacter*. In Alv soil the genera *Streptomyces* and *Paenibacillus* significantly increased in abundance in H50 soil, while *Granulicella*, *Arthrobacter*, *Mucilaginibacter, Devosia,* and *Rhodanobacter* showed a significantly higher relative abundance in Gamma soil. Besides soil type specific treatment responses, only a few genera were recorded as responders to the treatment in both soils. Remarkably, *Streptomyces*, *Bacillus*, *Paenibacillus,* and *Sphingomonas* showed a significantly increased abundance in the H50 soil of Alv and Kle soils, while *Mucilaginibacter*, *Devosia,* and *Rhodanobacter* were detected in higher relative abundance in the Gamma treatment of the two soils. Very few genera even showed the same response to the H50 and Gamma treatments in both soil types: while *Gp2*, *Gp3,* and *Dokdonella* showed a decrease in relative abundance, a significantly increased abundance was observed for the genus *Phenylobacterium*.

DISCUSSION

Biotest

Growth of apple rootstock M26 plants improved significantly in RD soils after H50 or Gamma treatments (**Table 1**). The enhanced plant growth was mainly observed aboveground while the treatments did not affect RDM. Significant increases of the shoot growth and biomass of apple M26 plants in heat-treated soil were also observed in the studies by St. Laurent et al. (2010) and Yim et al. (2013). The differences in growth of M26 plants in both RD soils were associated with differences in soil physicochemical properties and cropping histories. Among other functions, roots are important for water and nutrient uptake, release of exudates and production of cytokinins for the shoot growth (Gregory, 2006). Although there were no significant differences in the RDM of apple M26 plants in different RD soil treatments, damages in the root system of the plants in Con soils have resulted in higher root-to-shoot ratios (**Table 1**, Supplementary Figure S1). Since the roots were damaged in RD soil (Con), the plants might have invested energy in defense reactions of the root. Similarly, in the study of Yim et al. (2013), histological analyses of apple roots grown in RD soil revealed more lignin in vascular cells and a secondary protecting layer derived from the endodermis. The stronger lignifications might have resulted from oxidation of phenolic compounds that are known to play an important role in plant defense mechanisms. Several reviews have reported that under stress conditions biosynthesis of antimicrobial metabolites was enhanced as a defense mechanism of the plant (Sticher et al., 1997; Doornbos et al., 2012; Badri et al., 2013). The brownish roots of M26 grown in RD soil (Supplementary Figure S1) could have resulted from such a stress response of the plants. Phytochemicals were contained in, and released from roots in high quantities in response to biotic stress (Badri et al., 2013). Hofmann et al. (2009) have identified phloridzin (phloretin-2-β-D-glucoside) as the most abundant phenolic root exudate detected in apple seedlings (*Malus x domestica* Borkh.). Likewise, Emmett et al. (2014) reported that the production of phloridzin in roots of apple rootstock M26 plants in untreated RD soil was significantly higher than in pasteurized RD soil. Chizzali et al. (2012) detected phytoalexins including the biphenyls 3-hydroxy-5-methoxyaucuparin, aucuparin and others in the transition zone

TABLE 3 | Relative abundance of dominant genera detected at the end of the biotest in RD soils of Kle and Alv affected by soil treatments.

Phylum	Genus	Kle			Alv		
		Con	H50	Gamma	Con	H50	Gamma
Acidobacteria	*Gp1*	3.9 ± 0^{a}	3.5 ± 0^{a}	1.7 ± 0^{b}	3.4 ± 1	3.3 ± 1	2.9 ± 1
	Gp2	1.8 ± 0^{a}	0.9 ± 0^{b}	0.4 ± 0^{c}	1.8 ± 1^{a}	0.6 ± 0^{b}	0.7 ± 0^{b}
	Gp3	3.7 ± 0^{a}	2.2 ± 0^{b}	1.2 ± 0^{c}	3 ± 1^{a}	2.1 ± 1^{b}	1.3 ± 0^{c}
	Gp4	0.8 ± 0^{a}	0.6 ± 0^{ab}	0.4 ± 0^{b}	0.1 ± 0	0.2 ± 0	0 ± 0
	Gp5	0.3 ± 0^{a}	0 ± 0^{b}	0.1 ± 0^{b}	0.1 ± 0	0.1 ± 0	0 ± 0
	Gp6	1.1 ± 0^{a}	0.4 ± 0^{b}	0.5 ± 0^{b}	0.3 ± 0	0.2 ± 0	0.2 ± 0
	Gp7	0.5 ± 0^{a}	0.3 ± 0^{b}	0.1 ± 0^{b}	0.2 ± 0	0.2 ± 0	0.2 ± 0
	Gp13	0.1 ± 0^{a}	0 ± 0^{b}	0 ± 0^{b}	0.1 ± 0	0 ± 0	0 ± 0
	Gp14	0 ± 0^{a}	0.2 ± 0^{b}	0.1 ± 0^{ab}	0.5 ± 0	0.5 ± 0	0.4 ± 0
	Gp16	1.6 ± 0^{a}	1.4 ± 0^{a}	0.5 ± 0^{b}	0.8 ± 0^{a}	0.6 ± 0^{a}	0.3 ± 0^{b}
	Granulicella	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0^{a}	0 ± 0^{a}	0.4 ± 1^{b}
Actinobacteria	*Ilumatobacter*	0.4 ± 0^{a}	0.1 ± 0^{b}	0 ± 0^{b}	0.1 ± 0	0 ± 0	0 ± 0
	Blastococcus	0.2 ± 0^{a}	0.5 ± 0^{b}	0.1 ± 0^{a}	0.1 ± 0^{ab}	0.2 ± 0^{a}	0 ± 0^{b}
	Arthrobacter	1.1 ± 0	0.8 ± 0	0.9 ± 0	0.9 ± 1^{a}	1 ± 1^{a}	1.8 ± 0^{b}
	Mycobacterium	0 ± 0	0.1 ± 0	0 ± 0	0.3 ± 0^{a}	0.2 ± 0^{ab}	0.1 ± 0^{b}
	Nocardioides	0.9 ± 0	0.8 ± 0	0.8 ± 0	0.7 ± 0^{a}	0.6 ± 0^{a}	0.2 ± 0^{b}
	Pseudonocardia	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.3 ± 0^{a}	0.1 ± 0^{b}	0.1 ± 0^{b}
	Streptomyces	0.1 ± 0^{a}	0.6 ± 0^{b}	0.2 ± 0^{a}	0.2 ± 0^{a}	0.5 ± 0^{b}	0.2 ± 0^{a}
Bacteroidetes	*Niastella*	0 ± 0^{a}	0.3 ± 0^{b}	0 ± 0^{a}	0 ± 0	0 ± 0	0 ± 0
	Mucilaginibacter	0.1 ± 0^{a}	0.1 ± 0^{a}	0.3 ± 0^{b}	0.1 ± 0^{a}	0 ± 0^{a}	0.5 ± 0^{b}
Firmicutes	*Bacillus*	4.1 ± 1^{a}	5.1 ± 0^{b}	5.5 ± 0^{b}	11.1 ± 4^{a}	13.2 ± 3^{b}	7.6 ± 2^{c}
	Tuberibacillus	0.2 ± 0^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0	0 ± 0	0 ± 0
	Brevibacillus	0.1 ± 0^{a}	0.4 ± 0^{b}	0.1 ± 0^{a}	0.1 ± 0	0.1 ± 0	0.1 ± 0
	Paenibacillus	1.4 ± 0^{a}	2.1 ± 0^{b}	1.6 ± 0^{a}	1.4 ± 0^{a}	2.5 ± 1^{b}	1.7 ± 1^{a}
	Clostridium sensu stricto	0.9 ± 0	0.9 ± 0	1 ± 0	2 ± 1^{a}	2.4 ± 1^{a}	0.7 ± 0^{b}
	Clostridium XI	0.6 ± 0^{a}	0.5 ± 0^{a}	1 ± 0^{b}	0.5 ± 0	0.6 ± 0	0.5 ± 0
	Clostridium III	0.3 ± 0	0.3 ± 0	0.5 ± 0	0.3 ± 0^{a}	0.3 ± 0^{a}	0.1 ± 0^{b}
Gemmatimonadetes	*Gemmatimonas*	2.6 ± 0^{a}	4.1 ± 1^{b}	5.2 ± 1^{c}	1.7 ± 0	1.8 ± 1	1.7 ± 1
Proteobacteria (Alpha)	*Phenylobacterium*	0.4 ± 0^{a}	1.1 ± 0^{b}	0.6 ± 0^{c}	0.3 ± 0^{a}	0.6 ± 0^{b}	0.6 ± 0^{b}
	Devosia	0.3 ± 0^{a}	0.2 ± 0^{a}	0.8 ± 0^{b}	0.3 ± 0^{a}	0.1 ± 0^{a}	0.8 ± 0^{b}
	Microvirga	0 ± 0^{a}	0.2 ± 0^{b}	1.3 ± 0^{c}	0 ± 0	0.1 ± 0	0 ± 0
	Mesorhizobium	0.3 ± 0^{a}	0.1 ± 0^{b}	0.5 ± 0^{a}	0.1 ± 0^{ab}	0 ± 0^{a}	0.3 ± 0^{b}
	Acidocella	0 ± 0	0 ± 0	0 ± 0	0.6 ± 0^{a}	0.1 ± 0^{b}	0.9 ± 1^{a}
	Sphingomonas	1.7 ± 0^{a}	2.7 ± 0^{b}	1.8 ± 0^{a}	0.7 ± 1^{a}	2.6 ± 1^{b}	1.7 ± 0^{c}
Proteobacteria (Beta)	*Burkholderia*	0.1 ± 0^{a}	0.6 ± 0^{b}	1.3 ± 0^{c}	0.5 ± 0	0.8 ± 0	0.8 ± 0
	Ramlibacter	0 ± 0^{a}	0.2 ± 0^{b}	0.4 ± 0^{c}	0 ± 0	0 ± 0	0.1 ± 0
Proteobacteria (Delta)	*Geobacter*	0.2 ± 0^{a}	0 ± 0^{b}	0.2 ± 0^{a}	0.1 ± 0	0.1 ± 0	0 ± 0
Proteobacteria (Gamma)	*Methylobacter*	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.2 ± 0^{a}	0.1 ± 0^{b}	0 ± 0^{b}
	Arenimonas	0.1 ± 0^{a}	0 ± 0^{a}	0.6 ± 0^{b}	0 ± 0	0 ± 0	0 ± 0
	Dokdonella	0.5 ± 0^{a}	0.2 ± 0^{b}	0.1 ± 0^{b}	0.4 ± 0^{a}	0.1 ± 0^{b}	0.1 ± 0^{b}
	Dyella	0.2 ± 0^{a}	0 ± 0^{b}	0.2 ± 0^{a}	0.2 ± 0	0.2 ± 0	0 ± 0
	Lysobacter	0.2 ± 0^{a}	0.3 ± 0^{ab}	0.4 ± 0^{b}	0.1 ± 0^{ab}	0 ± 0^{a}	0.2 ± 0^{b}
	Rhodanobacter	0.2 ± 0^{a}	0.2 ± 0^{a}	1 ± 0^{b}	0.8 ± 1^{a}	0.8 ± 0^{a}	3.6 ± 1^{b}

Selected were genera of a relative abundance of $\geq$0.1% and significantly different within the soil type. Average relative abundances ± SD, significant differences between treatments within soil type at genus level were indicated by different letters after Tukey test, $p < 0.05$ and $n = 4$. Significant increases in abundance compared to Con are highlighted in green, while significant decreases are highlighted in orange.

of apple stems as a result of plant defense responses against fire blight caused by *Erwinia amylovora*.

The increased biosynthesis of phenylpropanoids in young apple leaves was shown to be negatively correlated with the shoot growth (Rühmann et al., 2002). Thus, we hypothesize that an inverse relationship between shoot growth and biosynthesis of phenolic compounds or antimicrobial metabolites could be the explanation for the reduced biomass of the apple M26 plants in RD soil observed in the present study.

Soil Bacterial Composition and Diversity in RD Soils

Although the copy numbers of 16S rRNA genes detected in TC-DNA of both RD soils (KleCon/AlvCon) at the end of the biotest revealed no differences (**Figure 2**), distinct bacterial community compositions (**Figure 4**) and diversity (**Figure 3**) were recorded in the present study (Supplementary Table S2). Differences in the bacterial community compositions were also shown in the two soils collected before the biotest (**Table 2**). Several other studies had shown that soil bacterial communities were strongly correlated to soil physicochemical properties (Janssen, 2006; Araujo et al., 2012; Schreiter et al., 2014). The soils used in the present study differed in their mineral composition, pH, organic matter content, cropping histories and horticultural management. Rose and apple rootstock plants were previously cultivated in Kle and Alv soils, respectively, and crop rotation was applied mainly in Kle soil. Plant species and soil type dependent diversity of bacterial communities was shown in different studies (Smalla et al., 2001; Badri et al., 2013; Bulgarelli et al., 2013) and thus the crop rotation might have contributed to the higher bacterial diversity found in Kle soil.

The relative abundance of common responders in both RD soils most likely was influenced by plant root exudates released by the apple rootstocks cultivated in these soils in 2012 (M4 planted in May. and M26 in November 2012), as also shown for other crops (Smalla et al., 2001; Berg and Smalla, 2009; Bakker et al., 2012; Berendsen et al., 2012). Soil type-dependent differences of the root exudate composition for the same plant species (lettuce) grown in different soil types were recently reported by Neumann et al. (2014). Apple rootstock exudates might have influenced the bacterial community composition contributing to the differences observed between the DGGE fingerprints of the RD soils before and after the biotest (KleT0 vs. KleCon, AlvT0 vs. AlvCon, **Table 2**).

Sun et al. (2014) have also studied the bacterial diversity associated with RD soils in apple orchards. Only a few genera such as *Lysobacter* and *Phenylobacterium* detected by Sun et al. (2014) were also identified in the present study. The relative abundances of the genera *Lysobacter* and *Phenylobacterium* were higher in RD soil than in healthy soil (Sun et al., 2014). In our experimental design healthy soil was not included as it was difficult to obtain soil with similar chemical and physical properties. In contrast, in the present study the relative abundance of the genus *Phenylobacterium* was significantly higher in RD soil with H50 and with Gamma treatment in both soils, while *Lysobacter* was enriched in the Gamma-treated Kle soil (**Table 3**).

Based on plant, soil, and soil bacterial community interaction, we hypothesized that soil bacterial community composition and diversity are site specific, influenced by different chemical and physical properties of the soil, as well as shaped by planting management practices. Also in other systems it was shown that the microbial community composition and the abundance of soilborne pathogens are influenced by the soil type, cropping history and weather conditions (Smalla et al., 2001; Badri and Vivanco, 2009; Berg and Smalla, 2009; Bakker et al., 2012; Berendsen et al., 2012; Badri et al., 2013).

Responses of the Bacterial Composition and Diversity in RD Soils to Different Treatments

The 16S rRNA gene copy numbers detected in soil TC-DNA showed a minor but still significant reduction only in Gamma-treated Kle soil 8 weeks after planting apple rootstock M26 plants (Con vs. Gamma) (**Figure 2**). Thus, recolonization of the soil must have taken place within this time span which was most likely influenced by the growing apple rootstock. The integration of an unplanted control would have allowed elucidating the effect of the plant growth and should be included in future experiments. In the study by McNamara et al. (2007) the bacterial counts decreased immediately after irradiation at a dose of 10 kGy, but 2 weeks later the cell counts rose to levels of up to 10^7 g^{-1} soil, which was even higher than in the untreated soil (10^6 g^{-1}). The soil analyzed in the present study loosely adhered to the root; a stronger influence of the plant would be expected if true rhizosphere soil was analyzed.

Both treatments of RD soils caused pronounced shifts in the bacterial community composition compared to the control which were detectable even 8 weeks after apple rootstock growth, with the effect of the Gamma treatment being more pronounced (**Figure 4**). Although the response of the acidobacterial populations was more striking in Kle soil, a decrease in the relative abundance of *Acidobacteria* was observed in response to the treatments in both soils (**Figure 5**). A significantly decreased relative abundance of the phylum *Acidobacteria* after treatment of maize RD soil with ethanol-free chloroform was recently reported by Domínguez-Mendoza et al. (2014). *Acidobacteria* were detected in apple RD soil as the dominant phylum, and their abundance was shown to be about 20% higher in soils in which the apple rootstock genotype M26 (considered susceptible to RD) was cultivated than in soils where the more tolerant genotype CG6210 was grown (St. Laurent et al., 2010). A significantly decreased relative abundance of *Acidobacteria* was also reported when treating soil with manure (Ding et al., 2014) or with mineral nutrients (Campbell et al., 2010). Therefore, the decreased relative abundance of *Acidobacteria* in treated RD soil observed in the present study might result from the release of nutrients from killed organisms due to the treatments and the proliferation of copiotrophic bacteria.

The genera *Nocardioides, Clostridium sensu strictu,* and *Clostridium III* were significantly reduced in Gamma-treated

Alv soil (**Table 3**). *Nocardioides* also significantly decreased in relative abundance by soil sterilization with ethanol-free chloroform in maize RD soil (Domínguez-Mendoza et al., 2014). Isolates belonging to the genus *Nocardioides* were reported as beneficial bacteria as they contributed to carbon cycling in soil via degrading alkanes (Hamamura et al., 2001) and via degrading pesticides (Topp et al., 2000).

In the present study, soil treatments did not only reduce but also strongly enrich the relative abundances of a wide range of bacterial genera. *Bacillus, Paenibacillus,* and *Sphingomonas* were shown to increase in relative abundance with H50 treatment of both soils (**Table 3**). Isolates belonging to the genera *Bacillus* and *Paenibacillus* were reported to be involved in the early stage of mineralization of decomposable organic materials derived from killed soil microorganisms (Domínguez-Mendoza et al., 2014). Bruce et al. (2010) have shown that most of the isolates from soil belonging to the genera *Bacillus* and *Paenibacillus* play a role in carbon cycling as they degrade cellulose and lignin. The genus *Bacillus* is known to contain plant growth promoting bacteria with most of the isolates being able to produce indole acetic acid (IAA), ammonia, siderophores, catalase (Joseph et al., 2007) and antibiotics against soilborne pathogenic fungi (Cazorla et al., 2007). Antagonistic activity of several *Sphingomonas* isolates from plants against pathogenic *Pseudomonas syringae* in *Arabidopsis thaliana* was revealed by Innerebner et al. (2011).

Members of the genera *Mucilaginibacter, Devosia,* and *Rhodanobacter* showed significantly increased relative abundance only in the Gamma treatments of both soils (**Table 3**). *Mucilaginibacter* species are heterotrophic bacteria capable to degrade pectin, xylan, laminarin and other polysaccharides (Pankratov et al., 2007). Isolates from the genus *Devosia* were also reported as plant growth promoting bacteria, e.g., *D. neptuniae* is capable to fix nitrogen in the roots of the aquatic legume plant *Neptunianatans* (Rivas et al., 2002). Isolates of the genus *Rhodanobacter* from subsurface area contaminated with uranium and nitric acid wastes were identified as denitrifying bacteria (Green et al., 2010).

The treatment-dependent enrichment of potentially beneficial or aromatic compound degrading bacteria in treated RD soil might have contributed to the enhanced growth of apple rootstock M26 plants in treated RD soils.

CONCLUSION

Apple rootstock M26 plants showed significant growth enhancement in treated RD soil after heat treatment at 50°C or gamma irradiation in a biotest. The DGGE and pyrosequencing analyses of 16S rRNA gene fragments amplified from TC-DNA of soil collected from M26 plant roots at the end of the biotest revealed distinct bacterial community compositions and diversity between the two RD soils. The pronounced differences in the relative abundance of soil bacteria were affected directly by soil treatments, by recolonization and proliferation after treatment, and by the plant root exudates. The 16S rRNA gene-based approaches can indicate changes in the relative abundance in response to treatments that might have contributed to the improved aboveground growth. However, conclusions concerning the potential activity and role of responders remain purely speculative. Thus, a polyphasic approach is urgently needed to shed more light on the phenomenon of RDs.

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SUPPLEMENTARY MATERIAL

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2.2 Effects of biofumigation using *Brassica juncea* and *Raphanus sativus* in comparison to disinfection using Basamid on apple plant growth and soil microbial communities at three field sites with replant disease

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REGULAR ARTICLE

Effects of biofumigation using *Brassica juncea* and *Raphanus sativus* in comparison to disinfection using Basamid on apple plant growth and soil microbial communities at three field sites with replant disease

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Abstract
Aims The effects of biofumigation with *Brassica juncea* 'Terra Plus' and *Raphanus sativus* 'Defender' in comparison to Basamid on apple plant growth and on soil microbial communities were studied at three sites affected by replant disease under field conditions.
Methods Apple rootstocks were planted on differently treated plots to evaluate the effect of the treatments on plant growth under field and greenhouse conditions. The glucosinolates in biofumigant plant organs and their breakdown products in soils were determined. Denaturing gradient gel electrophoresis fingerprints were performed with 16S rRNA gene and ITS fragments amplified from total community DNA extracted from different soils.
Results The highest glucosinolate concentrations were found in inflorescences of both biofumigant plant species with no differences between sites. The most abundant degradation product in soil biofumigated with *B. juncea* was 2-propenyl isothiocyanate, while in soil treated with *R. sativus* only 4-(methylthio)-3-butenyl isothiocyanate was detected. Effects of biofumigation were recorded to be stronger on fungi than on bacteria. Growth of apple rootstocks was positively affected by the treatments in a site-dependent manner.
Conclusions The effects of biofumigation evaluated by the apple plant growth were site-dependent and might result from suppression of soil-borne pests and pathogens, changes in soil microbial community compositions, and additional nutrients from the incorporated biomass.

Keywords Apple replant disease · Bacterial community composition · Biofumigation · DGGE · Fungal community composition · Glucosinolate · Indicator plant · Isothiocyanate · *Malus domestica*

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Introduction

Methods for soil disinfection before replanting crops at the same sites have become a major concern for growers since the phase-out of soil fumigants, such as methyl bromide in 2005 under the Montreal Protocol (Porter et al. 2010). Moreover, the EU directive EC 128/2009 aims at a reduced and more sustainable use of pesticides in agriculture. Therefore, alternative disinfection methods are needed. Biofumigation using products from Brassicaceae crops have been suggested as a means to mitigate effects of replant disease (RD) soils (Brown et al. 1991; Mazzola et al. 2007; Mattner et al. 2008). To suppress pests and pathogens, biofumigant materials can be applied both belowground and aboveground as (1) green manures from growing plants (Larkin and Griffin 2007; Norsworthy et al. 2007; Lazzeri et al. 2010), (2) pellets or meals from seeds or dry plants (Mazzola et al. 2001, 2007, 2015; Lazzeri et al. 2004; Cohen and Mazzola 2006; Fayzalla et al. 2009), and (3) as a liquid formulation through a drip irrigation (De Nicola et al. 2013) or a foliar spray system (Rongai et al. 2009).

The effect of biofumigation in soils results from the action of volatile substances which are degradation products from Brassicaceae plant secondary metabolites, particularly glucosinolates (GS) from a hydrolysis reaction catalyzed by plant enzymes called myrosinases (Brown et al. 1991; Kirkegaard and Sarwar 1998). The enzymatic GS breakdown products can be isothiocyanates (ITCs), nitriles, thiocyanates, epithionitriles, and oxazolidine-2-thiones. Among those, ITCs have been shown to be toxic to a wide range of pests and pathogens (Peterson et al. 1998; Matthiessen and Shackleton 2005; Bones and Rossiter 2006; Agerbirk and Olsen 2012). Several plant pathogenic nematodes such as *Pratylenchus penetrans*, *Globodera rostochiensis*, and *Meloidogyne incognita* or fungal plant pathogens such as *Helminthosporium solani* and *Verticillium dahliae* were suppressed by biofumigation (Olivier et al. 1999; Buskov et al. 2002; Mazzola et al. 2007; Zasada et al. 2009). The soil disinfection of multiple soil-borne pathogens in potatoes including *Rhizoctonia solani*, *Phytophthora erythroseptica*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, and *Fusarium sambucinum* by Brassicaceae crops as green manures was also reported by Larkin and Griffin (2007). Mattner et al. (2008) found the volatile ITCs from a mixture of *Brassica rapa* and *Brassica napus* to reduce the pathogens *Alternaria alternata*, *Colletotrichum dematium*, *Cylindrocarpon destructans*, *Fusarium oxysporum*, *Pythium ultimum*, *Phytophthora cactorum*, and *Rhizoctonia fragariae* as well as weeds in strawberry.

In apple or rose RD soils, the causal agents of the disease are not known and a pathogen complex is assumed to be involved. But more likely, microbial community shifts contribute to the disease symptoms such as reduced aboveground growth as well as changes in the root morphology and changes of the cortical root cells as previously described by Yim et al. (2013). However, several soil-borne pests and pathogens were previously suggested to be associated with the disease incidence in RD soils including actinomycetes, *Pythium* spp., *Cylindrocarpon* spp., *Phytophthora* spp., *R. solani*, and *P. penetrans* (Hoestra 1994; Mai et al. 1994; Mazzola 1998; Spethmann and Otto 2003; Kelderer et al. 2012; Emmett et al. 2014). To eliminate specific organisms related to the RD of apples or roses, we intended to study the effects of the biofumigation at three different sites with RD soils using two plant species of the Brassicaceae family under field conditions. These had been bred for high GS concentrations (*Brassica juncea* cv. Terra Plus and *Raphanus sativus*, var. *oleiformis* cv. Defender) by P. H. Petersen Saatzucht Lundsgaard GmbH, Germany. In three private nurseries producing apple and rose rootstocks, the biofumigation was tested in comparison with the commercial soil fumigant, Basamid® granules (Basamid or Dazomet). The study aimed at (1) identifying the GS profiles and concentrations in different organs of the plants, (2) determining the GS breakdown products in biofumigated and in Basamid treated soils, (3) analyzing the effects on the bacterial and fungal community composition by biofumigation and Basamid treatment, and (4) evaluating the effects of biofumigation on plant growth in biotests performed under greenhouse as well as under field conditions. The greenhouse biotest was developed by Yim et al. (2013) to determine the degree of apple RD in a given soil with different soil treatments. Thus, we hypothesized that the growth of apple rootstock plants used as indicators is significantly higher in biofumigated and Basamid than in non-biofumigated soils, both in the greenhouse and in the field. The differences in plant growth and

observed symptoms resulted from changes in the microbial community composition in RD soils after biofumigation treatments.

Biofumigant plants were sampled for GS determination before they were incorporated into soil. For ITC measurement in the soils, samples were taken 6 h after biofumigant tissue or Basamid incorporation. Analyses of bacterial and fungal community composition were done by denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal RNA (rRNA) gene or internal transcribed spacer (ITS) region fragments amplified from total community DNA (TC-DNA) extracted from different soil samples taken 4 weeks after the treatments.

Materials and methods

Experimental design

A 3-year project started in 2012 in collaboration with three family-owned nurseries: K (53° 41′ 58.51″ N, 9° 41′ 34.12″ E), A (53° 42′ 18.81″ N, 9° 48′ 16.74″ E), and M (53° 44′ 25.21″ N, 9° 46′ 55.18″ E) in the region of Ellerhoop, Pinneberg, Northern Germany. Each nursery contributed an area of 1000 m^2 with a severe problem after replanting apple or rose rootstock plants (RD soil, confirmed by a biotest experiment in November 2012, data not shown). At site K, rose rootstock plants had been intensively cultivated from 1980 until 2011. In 2004 and 2007, a planting rotation with *Tagetes* was integrated mainly to repel soil-borne parasitic nematodes. At site A, apple rootstock plants had been produced for several years until 2009. Then, *Prunus domestica* and *Cydonia oblonga* were grown in 2010 and 2011, respectively. Regarding site M, rose rootstocks had been planted until 2011. Since 1995, a crop rotation with cereals had been applied every 4 years, except that *Tagetes* was grown in 2010. The experimental area (Fig. 1) was established at each site, consisting of four treatments performed in three independent plots (45 m^2 per plot/replicate) based on a completely randomized design.

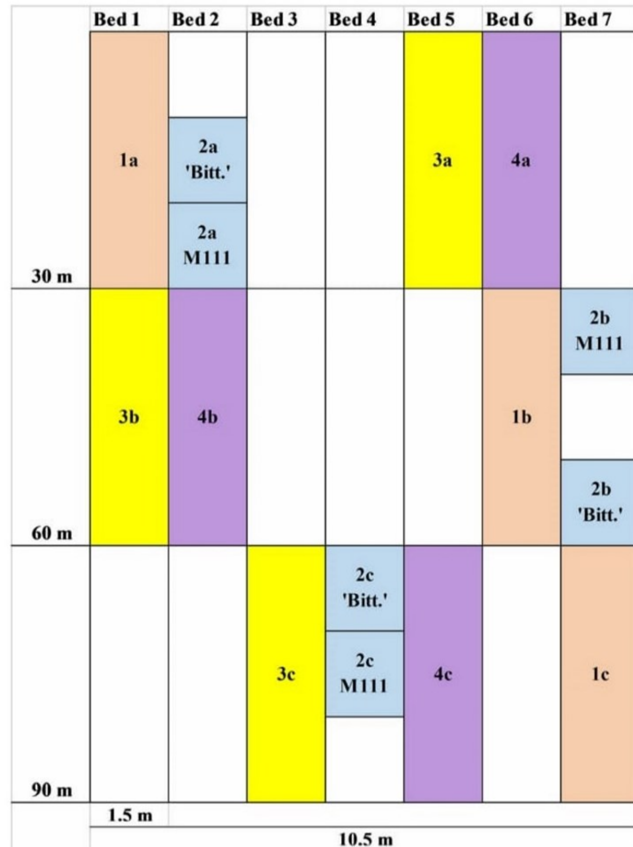

Fig. 1 Field experimental design showing different treatments in an area of 1000 m^2 per site. Treatments: *1*, Basamid; *2*, subplot pre-cultivated with apple rootstocks *M.* 'Bittenfelder' ('Bitt.') and M111 (2013); *3*, *B. juncea*, and *4*, *R. sativus*. Replicates: *a*, *b*, and *c*. Each replicate (plot) is 45 m^2 (1.5 m width and 30 m length). In treatment 2, each replicate was sub-divided into three sub-plots (15 m^2 per plot)

Treatment 1 Grass was grown in 2012 and 2013 to maintain the RD soil status since soil-borne pests and pathogens are persistent for several years (Klaus 1939). In August 2013, a commercial soil fumigant, Basamid, was incorporated at a dose of 400 kg ha^{-1} as recommended by the manufacturer (ProfiFlor GmbH, Stommeln, Germany). Soil layering was followed by soil sealing with plastic films.

Treatment 2 Apple rootstocks were cultivated to intensify the RD incidence. To compare the effect of different apple rootstock cultivars on the RD soils, each plot of the treatment 2 was divided into three sub-plots (15 m^2 per replicate). The rootstocks M4 and *Malus* 'Bittenfelder' were planted and sown, respectively, in May 2012 and were up-rooted in November 2012. In 2013, M4 was replaced by M111 due to availability of plants, while *M.* 'Bittenfelder' was sown again (both plant species were also harvested in November 2013). The sub-plot splitting of the treatment 2 was also carried out using a completely randomized design (Fig. 1).

Treatments 3 and 4 Two cultivars with high GS content, *Brassica juncea* 'Terra Plus' (3) and *Raphanus sativus*, var. *oleiformis* 'Defender' (4) were cultivated for biofumigation twice per year in 2012 and 2013 (sowing in April and June/July of each year). Sowing of both plant species was performed on the same day, 12 and 30 kg ha^{-1} for *B. juncea* and *R. sativus*, respectively, considering germination rates (P. H. Petersen Saatzucht Lundsgaard GmbH, Germany) using the machine SEMBDNER Rasenbaumaschinen RS 60 (SEMBDNER Maschinenbau GmbH, Fürstenfeldbruck, Germany). At full bloom, the plants (shoots and roots) were incorporated into the soil. The biofumigation was performed by mechanically cutting and chopping the plants into small pieces by Humus WM Flail mulchers (Humus®, Bermatingen, Germany) and incorporating them into the soil immediately, using a common rotary cultivator. Soil layering was applied with the rolls of the sowing machine (see above) to close the pores of the soil so that the active substances could remain in the soil as long as possible. Because of technical limitations, the biofumigated and Basamid plots were not treated with additional irrigation. But all treatments were carried out when the soil was moist and the sky was cloudy. About 2 weeks after the second biofumigation treatment (in October 2012 and September 2013), *Avena nuda* was sown as a cover crop (12 kg ha^{-1}).

In August 2013, one day before biofumigation, the aboveground shoot fresh mass (SFM) of the biofumigant plants was determined from 1 m^2 of each replicate ($n=3$).

In April 2014, each plot of the treatment (1, 3, and 4) was split into sub-plots which were then planted with M106 and *M.* 'Bittenfelder' to verify the effects of the different treatments on these two kinds of indicator plants. Only in treatment 2, the split plots remained during all 3 years. All apple rootstock plants were propagated vegetatively, only *M.* 'Bittenfelder' was sown directly.

Soil characteristics and management

In October 2012, the pH values for the soil from sites K, A, and M were 5.2, 4.8, and 5.7, respectively, determined according to the method of the VDLUFA-Methodenbuch Bd. 1/A5.1.1 (1991). Based on soil fraction analysis performed according to the method by Van Reeuwijk (2002), the soils from three sites were classified as two groups, sandy soil for site K and slightly loamy sand for sites A and M. Briefly, the soil from site A had a relatively low organic matter content and pH value, whereas the soil from site K differed in the proportions of sand, silt, and clay content from the soils from sites A and M (Table 1).

Every planting was fertilized with 30 g m^{-2} Kalimagnesia (30 % K_2O, 10 % MgO, 17 % S), 20 g m^{-2} magnesium sulfate (16 % MgO, 13 % S), and 20 g m^{-2} Novatec premium (15 % N, 3 % P_2O_5, 20 % K_2O) in April/May each year. In July and September, 8 g m^{-2} calcium ammonium nitrate with 27 % N was incorporated into the soils (www.compo-expert.com). In April 2013, plant available nutrient sulfur (S) was checked in soil treatments 3 and 4 from all three

Table 1 Characterization of replant disease soils used in the study

Site	pH	Clay (%)	Silt (%)	Sand (%)	% Organic matter
K	5.2	3.06	4.30	92.64	4.2
A	4.8	7.36	13.94	78.70	3.7
M	5.7	7.01	17.34	75.64	4.6

Soil properties from samples collected on 2 Oct 2012

sites (K, A, and M) using the protocol described by Combs et al. (2012). The detected plant available S was 4 mg kg^{-1} soil at site K (soil treatments 3 or 4), 6 mg kg^{-1} soil at site A (soil treatments 3 or 4), and 6 and 8 mg kg^{-1} soil at site M in soil treatments 3 and 4, respectively. To provide for optimal GS production in the biofumigant plants, additional fertilization containing sulfur with 5 g m^{-2} sulfur 90 (90 % sulfur, 10 % bentonite) was performed in June 2013 (before sowing the seed of *B. juncea* or *R. sativus*), as recommended by the manufacturer (www.compo-expert.com). In March 2014, soils from different treatments of all sites were again sampled for analysis of soil pH and mineral nutrients (N, P, and K) (Table S7).

Pest and pathogen management on aboveground growing shoots was carried out based on common horticultural practices (data not shown).

Determination of glucosinolates in *Brassica juncea* and *Raphanus sativus*

The GS profiles and concentrations were determined in different organs (inflorescence, leaf, stem, and root (Fig. S1)) from plants previously undergoing biofumigation treatment in summer 2012 and 2013 at the three sites (K, A, and M; treatments 3 and 4). In 2012, sowing of both plant species was on 31 July, and sampling for GS determination was on 24 September and 2 October (55 and 63 days after sowing) for *B. juncea* and *R. sativus*, respectively. In 2013, sowing for both species was performed on 26 June, and the plant samples were taken on 20 August (55 days after sowing). In general, plants were harvested at the onset of full bloom for GS determination. Twenty plants were selected randomly from three biological replicates for each species and were prepared to obtain three replicates per plant organ. Efforts were made to minimize tissue damage, and samples were transported on ice packs in cooling boxes. The samples were deep-frozen at −18 °C and freeze-dried prior to analysis. After freeze-drying of leaf and inflorescence for 3 days and of stem and root for 4 days (Christ ALPHA 1-4 LSC, Osterode, Germany), the samples were ground and stored at room temperature. The GS were analyzed by ultra-high-performance liquid chromatography with diode array detection (UHPLC-DAD) using a method according to DIN EN ISO 9167-1 described in Hanschen et al. (2015). Briefly, 20 mg of the plant samples were extracted twice using 70 % (*v*/*v*) methanol, and extracts were desulfated on DEAE-Sephadex A-25 columns with arylsulfatase. Desulfo-GS were then analyzed by UHPLC-DAD as described previously (Hanschen et al. 2015), and response factors used for calculation were according to DIN EN ISO 9167-1.

Determination of breakdown products in biofumigated or Basamid-treated soils

Soil samples were collected at a depth of 0–20 cm from Basamid- (treatment 1) and biofumigation-treated soils (treatments 3 and 4) using a 3.5-cm diameter core soil sampler. Sub-samples for each soil variant were prepared from 30 composites of the core soil sampler along the three biological replicates. Based on the results of a model experiment performed under laboratory conditions (10 min, 30 min, 1, 2, 4, 6, 16, and 24 h after tissue incorporation) (Hanschen et al. 2015) and under field conditions (1 day after biofumigation in October 2012) in all soils (Table S4) as well as 1, 6, and 24 h after tissue incorporation in soil A in June 2013 (Table S5), soil sampling in August 2013 was carried out 6 h after biofumigation or Basamid treatment from all soils (K, A, and M). The samples were stored in 50-mL centrifuge tubes at −50 °C prior to analysis.

The hydrolysis products were analyzed by gas chromatography-mass spectrometry (GC-MS) using the protocol as described by Hanschen et al. (2015). For soils treated with *B. juncea*, the analytes were quantified with the selected ion monitoring (SIM) method reported previously (Hanschen et al. 2015), except that 3-phenylpropanenitrile (2PE-CN) and 2-phenylethyl isothiocyanate (2PE-ITC) were quantified using m/z 91. The analytes in soils treated with *R. sativus* were quantified using a modified SIM method. In this case, the temperature gradient of the GC-MS system was identical to the full-scan mode (TIC) method reported recently (Hanschen et al. 2015), but the analytes were quantified in SIM mode using the m/z 45, 87, and 127 for 5-methylthio-4-pentenylnitrile (4MT3But-CN) and the m/z 45, 87, and 159 for 4-methylthio-3-butenyl isothiocyanate (4MT3But-ITC). Methyl isothiocyanate (Methyl-ITC) from soils treated with Basamid was quantified using a modified SIM method: The temperature gradient of the GC-MS system was initiated as described in (Hanschen et al. 2015) with a ramp up in temperature over 20 °C min^{-1} to 230 °C and increased further as reported by Hanschen et al. (2015). The Methyl-ITC was quantified using the m/z values of 45,

58, and 73. Each compound was calculated using the response factor (RF) in relation to the internal standard benzonitrile. The RF was experimentally determined for Allyl-ITC ($RF_{SIM}=3.07$), 3-butenenitrile (Allyl-CN, $RF_{SIM}=7.32$), Methyl-ITC ($RF_{SIM}=5.23$), 2PE-ITC ($RF_{SIM}=0.84$), 2PE-CN ($RF_{SIM}=0.73$) (all purchased from Sigma-Aldrich Chemie GmbH, München, Germany), and 4-(methylthio)butyl ITC (4MTB-ITC; $RF_{TIC}=0.76$, $RF_{SIM}=1.53$; Santa Cruz Biotechnology, Heidelberg, Germany). The RF of the degradation products of 4-methylthio-3-butenyl (4MT3But) GS in the SIM mode was calculated based on the assumption that the RF of 4MT3But breakdown products in TIC mode was identical to the RF of 4MTB-ITC as this compound is chemically most similar. To quantify 4MT3But degradation products in SIM mode, the RF_{SIM} was calculated by measuring the response of plant samples containing 4MT3But degradation products in both TIC and SIM mode. The ratio of the substance responses in both modes was used to calculate the $RF_{SIM(4MT3But\ ITC/CN)}$ out of the RF_{TIC} of 4MTB-ITC (Eq. 1).

$$RF_{4MT3But-BP(SIM)} = \frac{RF_{TIC(4MTB-ITC)}}{\frac{Area_{4MT3But-BP(SIM)}}{Area_{IST(SIM)}} / \frac{Area_{4MT3But-BP(TIC)}}{Area_{IST(TIC)}}} \quad (1)$$

Equation 1: Calculation of RFs for SIM (*R. sativus*) method. IST: internal standard, 4MT3But-BP = 4-methylthio-3-butenyl breakdown product.

Denaturing gradient gel electrophoresis fingerprints to reveal bacterial and fungal community changes in different soils

The DGGE fingerprints were generated from 16S rRNA gene fragments (bacteria) and ITS fragments (fungi) amplified from TC-DNA extracted from bulk soil samples taken from the three plots of the different treatments at all three sites.

In 2012, comparisons were made between soils from treatment 2 (pre-planted with apple rootstock M4), treatment 3 (biofumigated with *B. juncea*), and treatment 4 (biofumigated with *R. sativus*). In 2013, treatment 1 with Basamid was included in the analyses (with M111 plants in treatment 2). All soils were taken 4 weeks after biofumigant plant tissue incorporation or Basamid treatment using the soil core sampler (10 composites of core soil samplers per plot as explained in the section of breakdown products). As four replicates are required in order to apply the permutation test according to Kropf et al. (2004), an additional replicate from one plot (plot c, Fig. 1) was prepared to obtain four replicates per treatment for the analyses. The wet soil of each replicate weighed approximately 40.2 ± 7.5 g.

Five hundred milligrams of homogenized soil (sieved with a mesh size of 2 mm) of each replicate was submitted to TC-DNA extraction and purification applying FastDNA® SPIN Kit and GENECLEAN® SPIN Kit (Qbiogene) for soil, respectively, according to the manufacturer's protocol (MP Biomedicals, Santa Ana, CA, USA). Briefly, the TC-DNA isolation was accomplished by direct extraction from the soil sample by bead beating of the FastPrep® Instrument from mpbio (MP Biomedicals, Santa Ana, CA, USA) and, finally, the extracted nucleic acids were eluted in 50 μL GENECLEAN® SPIN elution solution.

16S rRNA gene fragments (GC-PCR) were amplified from soil TC-DNA for a total bacterial community analysis carried out as described previously (Yim et al. 2013), except that 0.5x PCR GoTaq® buffer, 3.75 mM $MgCl_2$, and 1.25 U GoTaq® (Promega GmbH, Mannheim, Germany) were used for the PCR reaction (25 μL). For the DGGE fingerprints of total fungal communities, a nested PCR approach of ITS regions was applied following the protocol of Weinert et al. (2009). The DGGE gradient solutions for both bacterial and fungal community analyses were prepared according to Weinert et al. (2009), and loading and staining of the DGGE gel was performed according to Heuer et al. (1997; 2001).

Evaluation of biofumigation and Basamid treatment on the growth of indicator plants

To check the effect of the biofumigation after 2 years of biofumigant plant tissue incorporation in RD soils (after treatments 3 and 4 in 2013), the observations on the apple rootstock plant growth (as indicators) were made both in the greenhouse and directly in the field in 2014.

Performance of the indicator plants in the greenhouse (biotest)

In March 2014, soil samples were collected at a depth of 0–25 cm and were mixed from the three plots within

each treatment. Five soil variants per site (K/A/M) were included in the comparisons: treatment 1 (fumigation with Basamid in 2013), treatment 2 (sub-plot pre-cultivated with *M*. 'Bittenfelder' in 2012/2013 and sub-plot pre-planted with M4 in 2012 and M111 in 2013), treatment 3 (biofumigation with *B. juncea* in 2012/2013), and treatment 4 (biofumigation with *R. sativus* in 2012/2013).

The soils from each treatment, totaling 30 L, were supplemented with 60 g Osmocote Exact® 3–4 M [16 + 9 + 12(+2)] (www.compo-expert.com) and mixed manually before being filled into 3-L pots for 10 replicates. Acclimatized in vitro apple rootstocks M26 (23 days old), used as model plants for the biotest (Yim et al. 2013), were planted as indicators. The mean daily temperature in the greenhouse chamber was adjusted to 20 ± 2 °C, and 16-h photoperiods were ensured by high-pressure sodium lamps (Philips Master Agro 400 W). Irrigation was carried out daily, and standard plant protection practices were applied weekly on aboveground plant parts to prevent the spread of insects or pathogens. Plant height (SL) was recorded weekly, and the plants were harvested for determination of SFM, shoot dry mass (SDM), and root dry mass (RDM) 8 weeks after planting.

Performance of the indicator plants in the field

The effects of the four soil treatments were compared by sowing and planting *M*. 'Bittenfelder' and M106, respectively, used as indicator plants, in April 2014. The plants, supposed to be ready for harvest in autumn 2014, were further cultivated until February 2015 since, due to environmental conditions, they had not shed their leaves in November 2014. To evaluate the performance of the indicator plants, the SL, the SFM, and the root fresh mass (RFM) of 100 *M*. 'Bittenfelder' and 57 ± 8 M106 plants per replicate were determined ($n = 3$).

Data analyses

The software R3.1.0 (http://www.r-project.org) was used for statistical analyses. Data of GS and their breakdown products (ITC and non-ITC) were log-transformed for variance homogeneity before submitting them to a Tukey test at $p < 0.05$ to check for significant differences between sites. The data of indicator plants (SL, SFM, SDM, RFM, and RDM) obtained from biotest and field experiments were analyzed using a least significant different test (LSD test) at $p < 0.05$ to demonstrate significant differences between different soil treatments within each site.

DGGE fingerprints were analyzed by the GelCompar II 6.5 software (Applied Math, Sint-Martens-Latern, Belgium). The analyses were based on Pearson correlation coefficients of pairwise similarity measure of two lanes from the relative intensity signal in each lane of a DGGE gel. A similarity matrix of the lanes was obtained from the unweighted pairwise grouping method using arithmetic means (UPGMA). For statistical tests, a permutation test at 10,000 times according to Kropf et al. (2004) was applied with the Pearson similarity matrices from the UPGMA. The statistics revealed differences (d-values) between the average of all correlation coefficients within one group (within treatment) and the average overall correlation coefficients of different groups (different treatments). Thus, the d-values indicated the differences between the DGGE fingerprints of different soil treatments.

Results

Glucosinolates in different plant organs of *Brassica juncea* and *Raphanus sativus*

The GS profiles and concentrations of *B. juncea* and *R. sativus* are presented for the samples grown in soil treatments 3 and 4, respectively (summer 2012 and 2013, Tables 2 and 3), at the three different sites.

Brassica juncea

In *B. juncea* plants, nine GS from the three main GS classes (aliphatic, indolic, and aromatic GS) were detected in different plant organs: 2-phenylethyl (2PE), 2-propenyl (Allyl), 1-methylpropyl (1MP), 3-butenyl (3But), 3-(methylsulfinyl)propyl (3MSOP), 4-methoxy-3-indolylmethyl (4MOI3M), 3-indolylmethyl (I3M), 1-methoxy-3-indolylmethyl (1MOI3M), and 4-hydroxy-3-indolylmethyl (4OHI3M) GS. The highest total GS concentrations were found in the inflorescences and leaves (in 2012/2013). The total GS concentrations ranged from 37.6 ± 9.1 to 54.6 ± 9.1 μmol g^{-1} dry mass (DM) of inflorescences in 2013 (Table 2). The total GS concentrations were higher in stems and roots of the 2012 samples than in those of 2013. However, the concentrations of GS in the inflorescences and leaves

Table 2 Glucosinolate profiles and concentrations in different organs of *B. juncea* in replant disease soils of three different sites and 2 years (μmol g^{-1} DM)

Plant organ	Time	Site	Aromatic	Aliphatic				Indolic				Total GS
			2PE	Allyl	1MP	3But	3MSOP	4MOI3M	I3M	1MOI3M	4OHI3M	
Inflores-cence	Summer 2012	K	0.35 ± 0.1ab	33.27 ± 10.8	0.53 ± 0.1	0.17 ± 0.0	n.d.	0.02 ± 0.0	0.16 ± 0.1	0.04 ± 0.0a	0.02 ± 0.0a	34.54 ± 11.2
		A	0.63 ± 0.2b	42.90 ± 8.8	0.97 ± 0.2	0.29 ± 0.1	n.d.	0.03 ± 0.0	0.30 ± 0.1	0.14 ± 0.0b	0.14 ± 0.1b	45.40 ± 9.5
		M	0.29 ± 0.1a	34.21 ± 13.6	0.56 ± 0.2	0.17 ± 0.1	n.d.	0.03 ± 0.0	0.18 ± 0.1	0.03 ± 0.0a	0.03 ± 0.0ab	35.49 ± 14.1
Leaf		K	0.32 ± 0.1	38.79 ± 2.0a	0.69 ± 0.0a	0.18 ± 0.0a	n.d.	0.12 ± 0.0	0.47 ± 0.1a	0.04 ± 0.0	n.d.	40.63 ± 2.0a
		A	0.41 ± 0.1	43.74 ± 0.2b	0.91 ± 0.1b	0.33 ± 0.1b	n.d.	0.14 ± 0.1	0.92 ± 0.3b	0.06 ± 0.0	n.d.	46.50 ± 0.5b
		M	0.25 ± 0.1	41.54 ± 2.5ab	0.73 ± 0.1a	0.20 ± 0.0a	n.d.	0.12±0.0	0.63 ± 0.1ab	0.07 ± 0.0	n.d.	43.54 ± 2.8ab
Stem		K	0.50 ± 0.1	5.76 ± 1.9	0.14 ± 0.1	n.d.	n.d.	0.16 ± 0.1a	0.12 ± 0.1	0.06 ± 0.0	0.03 ± 0.0a	6.76 ± 2.2
		A	0.63 ± 0.1	8.41 ± 2.7	0.19 ± 0.1	0.02 ± 0.0	n.d.	0.06 ± 0.0b	0.08 ± 0.0	0.09 ± 0.0	0.12 ± 0.0b	9.60 ± 2.7
		M	0.65 ± 0.1	5.82 ± 2.5	0.12 ± 0.1	n.d.	n.d.	0.13 ± 0.0a	0.09 ± 0.0	0.08 ± 0.0	0.04 ± 0.0a	6.95 ± 2.7
Root		K	5.41 ± 0.6	4.36 ± 0.8	0.05 ± 0.0	n.d.	n.d.	0.28 ± 0.1	0.24 ± 0.1	0.10 ± 0.0	0.02 ± 0.0	10.46 ± 1.5
		A	5.96 ± 1.0	4.83 ± 1.4	0.07 ± 0.1	n.d.	n.d.	0.50 ± 0.3	0.60 ± 0.4	0.20 ± 0.1	0.11 ± 0.2	12.26 ± 3.1
		M	5.42 ± 0.7	4.77 ± 1.3	0.06 ± 0.1	n.d.	n.d.	0.33 ± 0.1	0.35 ± 0.2	0.20 ± 0.1	0.00 ± 0.0	11.13 ± 2.1
Inflores-cence	Summer 2013	K	0.08±0.12	46.88 ± 11.9	0.38 ± 0.2	0.21 ± 0.1	0.12 ± 0.1	0.00 ± 0.0	0.15 ± 0.1	0.01 ± 0.0	n.d.	47.82 ± 12.4
		A	n.d.	36.77 ± 8.9	0.30 ± 0.1	0.19 ± 0.0	0.10 ± 0.0	0.05 ± 0.0	0.13 ± 0.1	0.02 ± 0.0	0.00 ± 0.0	37.56 ± 9.1
		M	n.d.	53.63 ± 8.9	0.45 ± 0.1	0.24 ± 0.1	0.13 ± 0.0	0.01 ± 0.0	0.16 ± 0.1	0.03 ± 0.0	n.d.	54.65 ± 9.1
Leaf		K	0.06 ± 0.1	43.03 ± 5.9a	0.64 ± 0.1	0.19 ± 0.0	n.d.	0.12 ± 0.0	0.86 ± 0.3	0.12 ± 0.0	n.d.	45.02 ± 5.8a
		A	n.d.	32.24 ± 2.7b	0.52 ± 0.0	0.15 ± 0.0	n.d.	0.16 ± 0.1	0.93 ± 0.1	0.11 ± 0.0	n.d.	34.11 ± 2.8b
		M	n.d.	36.51 ± 1.3ab	0.63 ± 0.0	0.16 ± 0.0	n.d.	0.12 ± 0.0	0.89 ± 0.1	0.10 ± 0.0	n.d.	38.40 ± 1.4ab
Stem		K	0.06 ± 0.1	0.82 ± 0.8	n.d.	n.d.	n.d.	0.08 ± 0.1	0.06 ± 0.0	0.02 ± 0.0ab	n.d.	1.03 ± 1.0
		A	0.04 ± 0.0	0.62 ± 0.4	0.01 ± 0.0	n.d.	n.d.	0.07 ± 0.0	0.06 ± 0.0	0.02 ± 0.0a	0.00 ± 0.0	0.82 ± 0.4
		M	0.01 ± 0.0	0.17 ± 0.1	n.d.	n.d.	n.d.	0.05 ± 0.0	0.02 ± 0.0	0.01 ± 0.0b	0.01 ± 0.0	0.26 ± 0.1
Root		K	0.12 ± 0.0	1.31 ± 0.7	n.d.	n.d.	n.d.	0.13 ± 0.1	0.07 ± 0.0	0.01±0.0	n.d.	1.64 ± 0.8
		A	0.35 ± 0.2	2.22 ± 1.2	n.d.	n.d.	n.d.	0.25 ± 0.1	0.2 ± 0.1	0.07±0.0	0.05 ± 0.1	3.16 ± 1.6
		M	0.18 ± 0.1	2.30 ± 2.1	n.d.	n.d.	n.d.	0.19 ± 0.2	0.25 ± 0.3	0.04±0.0	0.02 ± 0.0	2.98 ± 2.7

Mean ± SD, letters indicate significant differences of GS between sites (within the plant organ), Tukey test, $p < 0.05$, and $n = 3$, n.d. (not detectable). Colors: aromatic, aliphatic, and indolic GS are in light blue, light red, and light green, respectively

GS glucosinolate, *2PE* 2-phenylethyl, *Allyl* 2-propenyl, *1MP* 1-methylpropyl, *3But* 3-butenyl, *3MSOP* 3-(methylsulfinyl)propyl, *4MOI3M* 4-methoxy-3-indolylmethyl, *I3M* 3-indolylmethyl, *1MOI3M* 1-methoxy-3-indolylmethyl, *4OHI3M* 4-hydroxy-3-indolylmethyl

were comparable (Table 2). The differences in the GS concentrations between the three sites detected in the leaves in 2012 were not consistent in the following year (in 2013, the GS concentrations in leaves were significantly lower in soil of site A than of site K (Table 2)). For the other plant organs, there was no influence of the site on the total GS production.

The GS profiles differed between the plant organs, and 3MSOP GS was only detected in inflorescences in 2013. The aliphatic GS in both years was the most dominant class at all sites (Table 2). Among the aliphatic GS, Allyl-GS was most abundant in all plant organs, except for roots grown in 2012 (Fig. 2a).

Raphanus sativus

In *R. sativus* plants, 10 GS were identified: 2PE, Allyl, 4-(methylthio)-3-butenyl (4MT3But), 4-methylsulfinyl-3-butenyl (4MSO3But), 4-(methylsulfinyl)butyl (4MSOB), 4-(methylthio)butyl (4MTB), 4MOI3M, I3M, 1MOI3M, and 4OHI3M. Higher total GS concentrations in inflorescences compared to leaves, stems, and roots were detected in the samples collected in 2013. In 2012, due to delayed formation of inflorescences at all three sites, these could not be analyzed. The total GS content in the leaves was higher than in the stems and unexpectedly high in roots, especially in the samples from site A soil (Table 3). The total GS concentration ranged from 40.7 ± 4.2 to 52.6 ± 12.8 μmol g^{-1} DM for the inflorescences in 2013 (Table 3). Except for the differences of the total GS concentrations in the roots (2012) and the stems (2013), the soils at the different sites did not cause any significant differences in the total GS contents (Table 3).

Total GS concentrations and GS diversity were higher in samples harvested in 2013 compared to those harvested in 2012 (Table 3). The aliphatic 4MSO3But-GS and 4MT3But-GS were the most abundant GS detected in all organs of the plants in both years (Fig. 2b). In 2012, roots had a high content of aliphatic (4MT3But, 4MSO3But) and indolic (4MOI3M, I3M) GS in site A soil.

Overall, the GS profiles and concentrations differed between the two biofumigant plants, but both contained

Table 3 Glucosinolate profiles and concentrations in different organs of *R. sativus* in replant disease soils of three different sites and 2 years (μmol g^{-1} DM)

Plant organ	Time	Site	Aromatic	Aliphatic					Indolic				Total GS
			2PE	Allyl	4MT3But	4MSO3But	4MSOB	4MTB	4MOI3M	I3M	1MOI3M	4OHI3M	
Inflores-cence	Summer 2012	K, A, M	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Leaf		K	n.d.	n.d.	19.25 ± 7.1	7.49 ± 3.5	n.d.	n.d.	0.24 ± 0.1	3.75 ± 1.8	n.d.	0.02 ± 0.0	30.75 ± 8.5
		A	n.d.	n.d.	14.57 ± 2.4	5.18 ± 0.9	n.d.	n.d.	0.25 ± 0.0	3.92 ± 0.1	n.d.	0.01 ± 0.0	23.93 ± 1.6
		M	n.d.	n.d.	12.34 ± 4.2	7.69 ± 1.0	n.d.	n.d.	0.23 ± 0.1	2.90 ± 0.5	n.d.	n.d.	23.16 ± 4.6
Stem		K	n.d.	n.d.	16.47 ± 6.2	2.96 ± 1.4	n.d.	n.d.	0.28 ± 0.1	0.46 ± 0.1ab	n.d.	0.11 ± 0.0	20.28 ± 7.6
		A	n.d.	n.d.	14.09 ± 1.3	3.03 ± 08	n.d.	n.d.	0.20 ± 0.0	0.61 ± 0.1a	n.d.	0.09 ± 0.0	18.01 ± 2.0
		M	n.d.	n.d.	11.94 ± 2.1	2.42 ± 0.4	n.d.	n.d.	0.29 ± 0.1	0.39 ± 0.0b	n.d.	0.07 ± 0.0	15.11 ± 2.4
Root		K	n.d.	n.d.	13.85 ± 11.4ab	0.87 ± 0.8ab	n.d.	n.d.	0.22 ± 0.1ab	0.16 ± 0.2ab	0.08 ± 0.1	0.02 ± 0.0	15.19 ± 12.6ab
		A	n.d.	n.d.	27.13 ± 9.3a	1.55 ± 0.8a	n.d.	n.d.	0.43 ± 0.1a	0.71 ± 0.4a	0.27 ± 0.2	0.04 ± 0.0	30.12 ± 10.9a
		M	n.d.	n.d.	3.05 ± 1.5b	0.19 ± 0.1b	n.d.	n.d.	0.12 ± 0.0b	0.03 ± 0.0b	0.02 ± 0.0	n.d.	3.42 ± 1.5b
Inflores-cence	Summer 2013	K	n.d.	0.23 ± 0.0ab	4.22 ± 1.7	46.23 ± 11.3	1.23 ± 0.2	0.04 ± 0.0	0.06 ± 0.0	0.63 ± 0.1a	n.d.	0.01 ± 0.0	52.65 ± 12.8
		A	n.d.	0.13 ± 0.0a	6.91 ± 0.7	35.02 ± 7.8	0.89 ± 0.2	0.07 ± 0.0	0.08 ± 0.0	0.68 ± 0.1a	0.00 ± 0.0	n.d.	43.79 ± 7.5
		M	n.d.	0.27 ± 0.1b	6.00 ± .03	33.12 ± 4.2	0.87 ± 0.0	0.06 ± 0.1	0.05 ± 0.0	0.37 ± 0.0b	n.d.	n.d.	40.73 ± 4.2
Leaf		K	n.d.	0.06 ± 0.1	11.18 ± 7.5	9.95 ± 2.9	0.49 ± 0.2	0.10 ± 0.1	0.16 ± 0.0	1.00 ± 0.4	0.02 ± 0.0	n.d.	22.97 ± 11.1
		A	n.d.	0.06 ± 0.1	7.68 ± 7.2	2.98 ± 3.1	0.12 ± 0.2	0.06 ± 0.1	0.18 ± 0.0	0.76 ± 0.6	0.02 ± 0.0	n.d.	11.86 ± 11.1
		M	n.d.	n.d.	3.13 ± 2.1	3.26 ± 2.6	0.11 ± 0.14	n.d.	0.11 ± 0.1	0.52 ± 0.5	0.03 ± 0.0	n.d.	7.20 ± 5.0
Stem		K	n.d.	n.d.	6.83 ± 2.3a	3.19 ± 0.1a	0.05 ± 0.1	0.043 ± 0.0	0.10 ± 0.0	0.27 ± 0.1	n.d.	0.07 ± 0.0	10.56 ± 2.7a
		A	n.d.	n.d.	1.61 ± 1.6b	0.83 ± 0.9b	n.d.	n.d.	0.06 ± 0.1	0.08 ± 0.1	n.d.	0.02 ± 0.0	2.59 ± 2.6b
		M	n.d.	n.d.	1.89 ± 1.8b	0.63 ± 0.6b	n.d.	n.d.	0.06 ± 0.0	0.06 ± 0.1	n.d.	0.01 ± 0.0	2.65 ± 2.5b
Root		K	0.10 ± 0.0	n.d.	4.07 ± 4.7	0.24 ± 0.2	n.d.	0.03 ± 0.0	0.20 ± 0.2	0.16 ± 0.2	0.19 ± 0.3	n.d.	4.99 ± 5.6
		A	0.13 ± 0.0	n.d.	0.83 ± 0.5	0.07 ± 0.0	n.d.	n.d.	0.05 ± 0.0	0.02 ± 0.0	0.01 ± 0.0	n.d.	1.10 ± 0.5
		M	0.14 ± 0.0	n.d.	2.50 ± 1.1	0.11 ± 0.1	n.d.	n.d.	0.18 ± 0.1	0.06 ± 0.0	0.03 ± 0.0	n.d.	3.01 ± 1.4

Mean ± SD, letters indicate significant differences of GS between sites (within the plant organ), Tukey test, $p < 0.05$, and $n = 3$, n.d. (not detectable) and n.a. (not available). Colors: aromatic, aliphatic, and indolic GS are in light blue, light red, and light green, respectively

GS glucosinolate, *2PE* 2-phenylethyl, *Allyl* 2-propenyl, *4MT3But* 4-(methylthio)-3-butenyl, *4MSO3But* 4-(methylsulfinyl)-3-butenyl, *4MSOB* 4-(methylsulfinyl)butyl, *4MTB* 4-(methylthio)butyl, *4MOI3M* 4-methoxy-3-indolylmethyl, *I3M* 3-indolylmethyl, *1MOI3M* 1-methoxy-3-indolylmethyl, *4OHI3M* 4-hydroxy-3-indolylmethyl

mainly aliphatic GS and less indolic and aromatic GS at the flowering stage in 2013 (Tables 2 and 3). In 2012, this GS rank was true only for *R. sativus*, while in *B. juncea* aromatic GS were more abundant than indolic GS and occurred mainly in the roots (Table 2).

Glucosinolate breakdown products from biofumigation and ITC released from Basamid in different soils

The breakdown products from GS and Basamid were recorded for the treatments 1, 3, and 4 at the three different sites in autumn 2013. The *B. juncea* plants used for biofumigation produced significantly lower aboveground SFM (3.3 ± 0.5–4.5 ± 0.5 gm^{-2}) than *R. sativus* (6.6 ± 1.4–9.5 ± 1.5 gm^{-2}) (SFM in summer 2013, $n = 3$). Both plant species tended to have a higher biomass in soil K than in the other two soils (A/M) (Table S3).

In soil treatment 3, four liberated compounds were detected 6 h after biofumigation: two aliphatic (Allyl-CN and Allyl-ITC) and two aromatic (2PE-CN and 2PE-ITC) compounds. Allyl-ITC was the most abundant GS breakdown product detected in the soils of all three sites (>80 % of total liberated compounds), and in soil M the highest Allyl-ITC concentration was detected (15.0 ± 8.6 nmol g^{-1} dry soil). Allyl-ITC and 2PE-ITC were detected in significantly lower concentrations in soil A compared to the other soils (K/M). In contrast, a higher proportion of nitriles was observed in soil A compared to soils K and M (10.1 % Allyl-CN and 0.5 % 2PE-CN) (Table 4).

In soil treatment 4, only the aliphatic 4MT3But-ITC was detected in all soils 6 h after biofumigant tissue incorporation. The highest concentration was observed in soil K (2.274 ± 1.8 nmol g^{-1} dry soil). The 4MT3But-ITC concentration was significantly lower in soil A compared to soil K (Table 4).

Thus, more diverse GS breakdown products and higher concentrations were detected in soil treated with *B. juncea* than in soil treated with *R. sativus* which was not reflected in the biomass production (Tables 4, S3).

Six hours after treatment 1, a significantly higher Methyl-ITC concentration was recorded in soil K (278.16 ± 47.9 nmol g^{-1} dry soil, Table 4) than in the

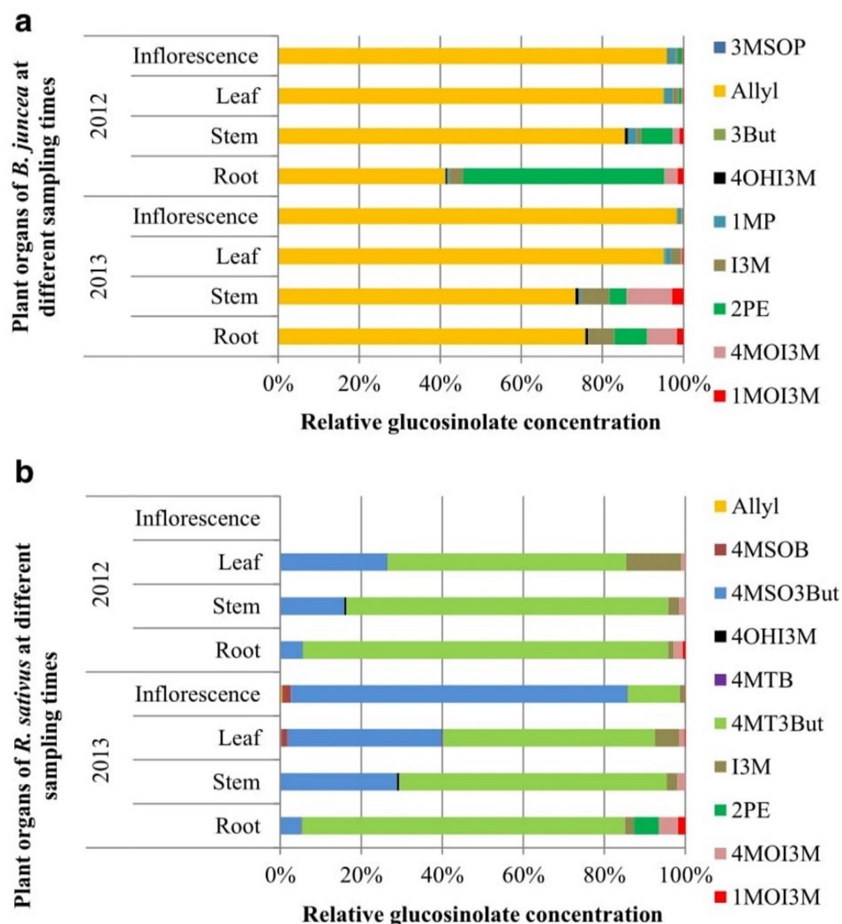

Fig. 2 Relative concentration of glucosinolates detected in different organs of biofumigant plants in summer 2012 and 2013: **a**, *B. juncea*, and **b**, *R. sativus* (data from means of three sites). *3MSOP*, 3-(methylsulfinyl)propyl; *Allyl*, 2-propenyl; *3But*, 3-butenyl; *4OHI3M*, 4-hydroxy-3-indolylmethyl; *1MP*, 1-methylpropyl; *I3M*, 3-indolylmethyl; *2PE*, 2-phenylethyl; *4MOI3M*, 4-methoxy-3-indolylmethyl; *1MOI3M*, 1-methoxy-3-indolylmethyl; *4MSOB*, 4-(methylsulfinyl)butyl; *4MSO3But*, 4-(methylsulfinyl)-3-butenyl; *4OHI3M*, 4-hydroxy-3-indolylmethyl; *4MTB*, 4-(methylthio)butyl; and *4MT3But*, 4-(methylthio)-3-butenyl

soils of the other two sites, although the same dose and technical procedure was applied. The ITC and nitrile concentrations released from both biofumigation treatments were much lower compared to the Methyl-ITC concentrations measured after Basamid treatment (Table 4).

Table 4 Breakdown products of glucosinolates in biofumigated soil and methyl-ITC released in Basamid-treated soil in replant disease soils of three different sites (nmol g^{-1} dry soil) and proportion (%) of liberated GS

Treatment	Site	Allyl-CN	%	Allyl-ITC	%	2PE-CN	%	2PE-ITC	%	4MT3But-ITC	Methyl-ITC
3	K	0.314 ± 0.4	4	**6.689 ± 3.0a**	84.4	**0.022 ± 0.0a**	0.3	**0.897 ± 1.0a**	11.3	n.d.	n.d.
	A	0.261 ± 0.5	10.1	**2.072 ± 1.4b**	80.6	**0.012 ± 0.0a**	0.5	**0.227 ± 0.1b**	8.8	n.d.	n.d.
	M	0.131 ± 0.1	0.7	**15.035 ± 8.6a**	83.8	**0.072 ± 0.1b**	0.4	**2.711 ± 3.6a**	15.1	n.d.	n.d.
4	K	n.d.		n.d.		n.d.		n.d.		**2.274 ± 1.8a**	n.d.
	A	n.d.		n.d.		n.d.		n.d.		**0.855 ± 0.6b**	n.d.
	M	n.d.		n.d.		n.d.		n.d.		**1.181 ± 0.5ab**	n.d.
1	K	n.d.		n.d.		n.d.		n.d.		n.d.	**278.160 ± 47.9a**
	A	n.d.		n.d.		n.d.		n.d.		n.d.	**168.407 ± 29.3b**
	M	n.d.		n.d.		n.d.		n.d.		n.d.	**206.822 ± 54.8b**

Mean ± SD, Tukey test, $p < 0.05$, $n = 10$, n.d. (not detectable), soil samples were taken 6 h after biofumigation with *B. juncea* (treatment 3) and *R. sativus* (treatment 4) and after Basamid treatment (treatment 1) in summer 2013. Colors: aromatic and aliphatic breakdown products are in light blue and light red, respectively

GS glucosinolate, *ITC* isothiocyanate, *CN* cyanide, *Allyl-CN* 3-butenenitrile, *2PE-CN* 3-phenylpropanenitrile, *2PE-ITC* 2-phenylethyl ITC, *4MT3But-ITC* 4-(methylthio)-3-butenyl ITC

Effect of biofumigation and Basamid treatment on bacterial and fungal community compositions in replant disease soils

To evaluate the effects of treatments 3 and 4 (1 and 2 years of biofumigation in summer 2012 and 2013, respectively) as well as the effects of the treatment 1 (Basamid treatment in summer 2013) on soil bacterial and fungal community composition in comparison to the treatment 2 (RD soil) at three different sites, DGGE fingerprints of 16S rRNA gene and ITS fragments amplified from soil TC-DNA (treatments 1, 2, 3, and 4) taken 4 weeks after the treatments were compared.

The DGGE fingerprints of bacteria and fungi revealed distinct changes of community compositions affected by both 1 and 2 years of biofumigation with *B. juncea* or *R. sativus* at all sites, except for the differences in bacterial communities in the site A soil treated with *R. sativus* in 2013 (d-values of 2 vs. 4, Table 5). The differences between the bacterial fingerprints for samples taken after 1 year were recorded higher than for the 2-year biofumigated soils with *B. juncea* or *R. sativus* (K, A, and M), while this was true for fungal community only in soil K. Remarkably, the d-values were higher for the fungal than for the bacterial community compositions in soils treated with *B. juncea* or *R. sativus* either for 1 or 2 years at all sites (Table 5).

The effects of the biofumigant plants in RD soils were dependent on the site and frequency of tissue incorporation (1 or 2 years of biofumigation). For instance, the d-value indicated that soil treatment 4 had a higher effect on bacterial and fungal community compositions than soil treatment 3 in site A soil (d-values of 2 vs. 4 > 2 vs. 3) (Table 5). The biofumigated soils (*B. juncea* or *R. sativus*) at site K led to a higher shift in the bacterial (2012 and 2013) and fungal (2012) community compositions than at sites A or M (Table 5).

In Basamid-treated RD plots (treatment 1), the d-values indicated that the effect on the soil bacterial community composition was higher than in treatments 3 and 4 in the soils of all three sites, but interestingly this stood in contrast to the effect on fungal community in soil of sites K and A (Table 5).

Table 5 Effect of 1 and 2 years of biofumigation and of Basamid treatment on total bacterial and fungal community compositions in soils of three replant disease sites (d-values from DGGE fingerprints of 16S rRNA gene fragments and of ITS regions)

Site	Comparison between treatments	Dissimilarity (% d-value)			
		Bacteria		Fungi	
		2012	2013	2012	2013
K	1 vs. 2	n.a.	21.7*	n.a.	32.3*
	1 vs. 3	n.a.	4.1*	n.a.	55.6*
	1 vs. 4	n.a.	9.3*	n.a.	69.7*
	2 vs. 3	43.2*	16.5*	67.5*	38.3*
	2 vs. 4	41.2*	8.7*	63.8*	42.6*
	3 vs. 4	5.9	5.2*	19.9*	10.5*
A	1 vs. 2	n.a.	9.2*	n.a.	22.8*
	1 vs. 3	n.a.	2.9*	n.a.	41.7*
	1 vs. 4	n.a.	4.6*	n.a.	47.7*
	2 vs. 3	11.8*	4.1*	27.1*	37.6*
	2 vs. 4	16.1*	7.3	34.8*	44.6*
	3 vs. 4	6.4*	4.8	2.8	5.01*
M	1 vs. 2	n.a.	12*	n.a.	34.3*
	1 vs. 3	n.a.	5.2*	n.a.	39.4*
	1 vs. 4	n.a.	7.8*	n.a.	22.8*
	2 vs. 3	12.9*	7.6*	26.0*	33.8*
	2 vs. 4	23.1*	3.8*	32.4*	25.1*
	3 vs. 4	5.5*	2.2*	6.8*	6.3*

Soil samples taken 4 weeks after biofumigant tissue incorporation (summer 2012 and 2013) and Basamid treatment (summer 2013). Treatments: 1, Basamid; 2, soil pre-cultivated with apple rootstock plant M4 (2012) and M111 (2013); 3, *B. juncea*; and 4, *R. sativus*. Asterisk "*" indicates significant differences between treatments, a permutation test, $p < 0.05$, $n = 4$ (Kropf et al. 2004), *$p < 0.05$, and n.a., not analyzed

Effect of biofumigation on the growth of indicator plants

Biotest with apple rootstock M26 in the greenhouse

The effects of the different soil treatments were tested by growing in vitro propagated apple rootstock M26 plants in 3-L pots filled with soil sampled from all three plots per treatment and site in March 2014 and after thoroughly mixing the replicates of each treatment. The growth of M26 plants was clearly influenced by the different soil treatments. Among four measured parameters, the plant RDM showed less pronounced effects in terms of statistical differences, especially in soils of sites A and M (Table 6). However, in soil treatments 1 (with Basamid) and 3 or 4 (biofumigation with *B. juncea* or *R. sativus*), the roots were healthier and noticeably less brown in coloration than the roots of M26 plants grown in the soil of treatment 2 (soils pre-cultivated with M111 or *M.* Bittenfelder) from all sites (Fig. S3 shows an example of the roots from M26 grown in the site K soil). In soil treatment 2, the growth of M26 plants was more reduced in soil pre-cultivated with M111 than in soil pre-planted with *M.* 'Bittenfelder' at site K (i.e., >100 % increase of SDM, Table 6). Regarding the plant growth, the strongest positive effects induced by the biofumigation were detected in the site K soil, followed by site A, whereas at site M the previous biofumigation treatment did not influence the plant growth in the greenhouse (Tables 6 and S6).

In site K soil, the effects of biofumigation by the two plant species were comparable: The SDM of the M26 plants significantly increased by more than 100 % in soil treatments 3 or 4 (biofumigation with *B. juncea* or *R. sativus*) compared to soil treatment 2 (soils pre-cultivated with M111) (Table 6). Interestingly, the biofumigation in site K soil resulted in a growth comparable to that of the Basamid treatment in terms of SL, SFM, and SDM (Table 6).

For site A soil, no significantly increased growth of the M26 plant in *B. juncea* biofumigated soil (treatment 3) was observed. Soil treatment 4, and to a lesser extent treatment 3, resulted in improved

Table 6 Effect of two-year biofumigation in three replant disease soils on the growth of in vitro propagated apple M26 in the greenhouse in summer 2014

Site	Treatment	SL (cm)	SFM (g plant^{-1})	SDM (g plant^{-1})	% increases of SDM compared to soil pre-cultivated with		RDM (g plant^{-1})
					M. 'Bittenfelder'	M111	
K	2 *M.* 'Bittenfelder'	20.8 ± 6.7ab	5.6 ± 1.9ab	1.8 ± 0.7ab	0	–	0.6 ± 0.1 bc
	2 M111	13.1 ± 4.7a	3.7 ± 1.2a	1.1 ± 0.4a	–	0	0.3 ± 0.1 a
	1	24.7 ± 5.4b	7.5 ± 1.4b	2.5 ± 0.5bc	37	125	0.4 ± 0.1 ab
	3	25.4 ± 6.5b	7.2 ± 1.8b	2.5 ± 0.7c	38	125	0.6 ± 0.1 bc
	4	22.3 ± 4.5b	6.3 ± 1.1b	2.4 ± 0.4bc	30	113	0.7 ± 0.2 c
A	2 *M.* 'Bittenfelder'	14.8 ± 2.3ab	4.7 ± 0.8a	1.7 ± 0.2a	0	–	0.4 ± 0.2 a
	2 M111	14.4 ± 4.8a	4.4 ± 1.3a	1.5 ± 0.3a	–	0	0.6 ± 0.1 ab
	1	19.4 ± 3.5bc	5.7 ± 1.2ab	1.7 ± 0.4a	4	14	0.5 ± 0.2 ab
	3	17.9 ± 3.5ac	5.4 ± 1.3ab	1.9 ± 0.5ab	11	22	0.5 ± 0.2 a
	4	20.7 ± 3.9c	6.3 ± 1.0b	2.3 ± 0.4b	37	51	0.7 ± 0.2 b
M	2 *M.* 'Bittenfelder'	17.8 ± 2.1a	4.0 ± 0.5a	1.8 ± 0.2a	0	–	0.7 ± 0.1 a
	2 M111	17.6 ± 2.9a	3.8 ± 0.8a	1.7 ± 0.3a	–	0	0.7 ± 0.1 a
	1	25.8 ± 2.8b	6.8 ± 0.8b	2.9 ± 0.3b	57	67	1.0 ± 0.2 b
	3	17.6 ± 3.4a	3.6 ± .0.7a	1.8 ± 0.3a	−4	3	0.8 ± 0.1 ab
	4	15.6 ± 4.8a	4.1 ± 1.2a	1.8 ± 0.4a	−5	2	0.6 ± 0.3 a

Mean ± SD, letters indicate significant differences between treatments (within same site), LSD test, $p < 0.05$, $n = 10$. Treatment 2, RD soil pre-cultivated with apple rootstock M111 or with *M.* 'Bittenfelder'; treatment 1, Basamid treatment; and treatments 3 and 4, biofumigation with *B. juncea* and with *R. sativus*, respectively. (−) data were not compared. Data were collected at the end of the biotest, eight weeks after planting

SL shoot length, *SFM* shoot fresh mass, *SDM* shoot dry mass, *RDM* root dry mass

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growth in comparison to treatment 2 indicated by significantly higher SL, SFM, and SDM of the M26 plants. Furthermore, the M26 plant growth in soil treatment 4 was even higher than treatment 1 (i.e., the SDM showed a significant increase by 51 % compared to the growth in treatment 2, soil pre-planted with M111) (Table 6). No effects of the biofumigation (treatments 3 or 4) were observed for site M soil. However, the plant growth was significantly improved (SL, SFM, SDM, and RDM) in site M soil after Basamid treatment.

Apart from the statistically significant differences between the SDM of M26 in the site A soil, no different effects between the two biofumigant species were observed in the present study. On the other hand, the comparable effects of Basamid to biofumigation treatments were revealed by the M26 plant growth at two sites, site K soil (SL, SFM, and SDM) and site A soil (SL, SFM, and RDM) (Table 6).

Performance of indicator plants M. 'Bittenfelder' and M106 in the field

To evaluate the effects of the 2-year biofumigation under field conditions, apple rootstocks *M.* 'Bittenfelder' and M106 were cultivated directly in the field in April 2014. In site K soil, due to high standard deviations, no significant differences in all measured parameters (SL, SFM, and RFM) were observed for the indicator plant *M.* 'Bittenfelder' grown in the different soil treatments. However, the lowest growth of the plants was recorded in treatment 2 (soil pre-planted with *M.* 'Bittenfelder'). Compared with the soil variants pre-cultivated with M111, significantly increased SFM by 148 and 165 % of the plant M106 was recorded after biofumigation treatments 3 and 4, respectively (Table 7). Based on the growth of both indicator plants (M106 and *M.* 'Bittenfelder'), no different effects between the biofumigation and Basamid treatments were revealed in site K soil (treatments 1 vs. 3 or 4, Table 7).

Table 7 Effect of 2-year biofumigation in replant disease soils of three different sites on indicator plants, *M.* 'Bittenfelder' and M106, in the field in summer 2014

Site	Treatment	Indicator plant *M.* 'Bittenfelder' in soil pre-cultivated with *M.* 'Bittenfelder'				Indicator plant M106 in soil pre-cultivated with M111			
		SL (cm)	SFM		RFM (g plant^{-1})	SL (cm)	SFM		RFM (g plant^{-1})
			SFM (g plant^{-1})	% Increase			SFM (g plant^{-1})	% Increase	
K	2	25.6 ± 11.6	3.5 ± 2.7	0	8.3 ± 4.1	51.1 ± 2.5b	30.5 ± 2.9b	0	61.6 ± 17.7b
	1	48.1 ± 13.7	10.4 ± 4.3	197	11.4 ± 1.8	78.9 ± 6.8a	77.8 ± 9.9a	155	83.5 ± 13.8a
	3	48.5 ± 13.8	9.5 ± 4.5	171	11.0 ± 2.7	85.6 ± 13.0a	75.5 ± 15.2a	148	70.6 ± 10.1ab
	4	44.4 ± 9.3	8.6 ± 2.9	146	10.2 ± 1.0	90.6 ± 4.5a	80.8 ± 4.2a	165	70.0 ± 7.3ab
A	2	18.4 ± 5.8	2.2 ± 1.0	0	5.6 ± 3.0	61.7 ± 3.0	34.1 ± 2.7	0	47.2 ± 6.7
	1	26.7 ± 12.9	4.0 ± 2.6	82	7.5 ± 3.3	67.5 ± 16.0	39.4 ± 13.5	16	56.3 ± 10.5
	3	16.1 ± 0.9	1.8 ± 0.4	−18	4.6 ± 0.6	63.6 ± 9.8	34.5 ± 9.6	1	44.3 ± 7.4
	4	18.1 ± 14.5	2.2 ± 2.6	0	5.2 ± 5.7	68.3 ± 11.8	37.6 ± 7.1	10	47.8 ± 4.0
M	2	37.4 ± 5.7b	7.6 ± 1.7b	0	11.9 ± 0.6b	74.2 ± 4.6b	44.1 ± 5.7b	0	51.4 ± 6.3
	1	68.0 ± 1.9a	16.5 ± 2.9a	117	14.5 ± 1.8ab	103.4 ± 4.9a	78.1 ± 16.6a	77	56.4 ± 6.5
	3	67.6 ± 4.3a	16.7 ± 1.9a	120	16.1 ± 2.3a	104.6 ± 10.2a	79.5 ± 9.3a	80	60.9 ± 17.9
	4	66.1 ± 9.8a	18.9 ± 3.8a	149	18.0 ± 2.3a	103.1 ± 13.9a	79.0 ± 17.2a	79	58.7 ± 7.6

Mean ± SD, letters indicate significant differences between treatments (within same site), LSD test, $p < 0.05$, $n = 3$. Treated RD soils: Basamid treatment (treatment 1) and biofumigation with *B. juncea* (treatment 3) and with *R. sativus* (treatment 4). Data were collected in February 2015

SL shoot length, *SFM* shoot fresh mass, *SDM* shoot dry mass, *RDM* root dry mass

In contrast to the biotest results, the effects of biofumigation were obvious in site M soil for both of the indicator plants (significantly higher SL, SFM, and RFM of *M*. 'Bittenfelder' and M106 in soil treatment 3 or 4 compared to soil treatment 2). There was no effect at all of the biofumigation and Basamid (treatment 1) treatments in site A soil (Table 7).

Different effects of the two biofumigant plant species were not detectable, and in the soils from all three sites biofumigation resulted in growth comparable to that of the Basamid treatment (Table 7).

Discussion

Glucosinolates in different organs of biofumigant plants

The total GS concentration detected in different organs of *B. juncea* and *R. sativus* plants in the summers of 2012 and 2013 was relatively high compared to those reported in other studies (Carlson et al. 1985; Kirkegaard and Sarwar 1998; Bellostas et al. 2007; Vervoort et al. 2014). The differences in the GS profiles and concentrations between the two plant species used in this study were in accordance with those reported by Carlson et al. (1985) and Kirkegaard and Sarwar (1998).

Overall, the development of *R. sativus* was slower than that of *B. juncea* at all sites (K, A, and M). Since both plant species had to be incorporated into soil on the same day, no inflorescences from *R. sativus* were available for analysis in 2012. In 2013, the samples for the GS analysis were taken when also the *R. sativus* plants had reached their flowering stage. GS profiles and contents in plants are influenced by environmental conditions (Zhang et al. 2008; Antonious et al. 2009). Thus, variability of the individual and the total GS concentration in the plant organs of the same plant species between the 2 years was recorded at all sites (Tables 2 and 3; 2012 vs. 2013). It was most likely caused by the differences in temperature, precipitation, and solar radiation between 2012 and 2013 (Tables S1a and S1b). Antonious et al. (2009) reported that ultraviolet radiation, temperature, and water availability significantly affected, through induction of stress responses, the GS production in Brassicaceae plants grown in different environments.

The lower total GS concentrations in stems and roots of *B. juncea* and *R. sativus* in 2013 compared to 2012 were likely due to the differences in the plant development stages observed at the sampling times in both years and at all sites. In 2013, but not in 2012, a setting of siliques of *B. juncea* was observed. Bellostas et al. (2007) reported that in Brassicaceae plants the GS concentration increased in their reproductive organs at the reproductive stage but decreased in roots and stems. The high total GS content in roots of *R. sativus* plants grown in 2012 at site A, compared to sites K and M, could also be explained by the different plant development stages sampled at the different sites (Table 3). The plants in site A soil had less biomass than those in soils from sites K and M (Table S3). Differences in the growth of the biofumigant plants between sites K, A, and M were also supported by the differences in soil types (Table 1) and climatic conditions of the respective site (Tables S1 and S2). Differences in the GS concentration in the biofumigant plant organs of the three sites might have affected the biofumigation effect on indicator plant growth and soil microbial community.

Breakdown products from biofumigation and Basamid treatment in different soils

Biofumigation with *B. juncea* (treatment 3 in summer 2013) released two major ITCs, Allyl-ITC and 2PE-ITC, and these compounds were also detected in previous studies (Olivier et al. 1999; Bangarwa et al. 2011). Only one compound, 4MT3But-ITC, was identified in soil biofumigated with *R. sativus* (treatment 4 in summer 2013) at all three sites.

Obviously, the Allyl-GS and 2PE-GS from *B. juncea* and 4MT3But-GS from *R. sativus* (Carlson et al. 1985; Bellostas et al. 2007; Bangarwa et al. 2011; Vervoort et al. 2014) were hydrolyzed enzymatically into the corresponding ITCs and nitriles (Allyl-CN, 2PE-CN). Due to the low abundance of other GS, their hydrolysis products could not be detected in the present study.

Although the total GS concentration in the whole plant or in the aboveground part of the biofumigant plants was not analyzed in this study, it was reported that the breakdown GS concentration is proportional to the GS incorporated into the soil (Warton et al. 2003). This can be seen in site A soil in which the concentration of GS breakdown products was lower compared to sites K and M, corresponding to the lower amount of incorporated plant biomass (Tables 4 and S3).

In contrast, the higher amount of plant biomass of *R. sativus* incorporated into soil compared to *B. juncea*

did not result in higher ITC concentration in the soils of all sites (Table 4). The lower ITC concentration in soil treatment 4 compared to treatment 3 could be due to the different peak times of hydrolysis reactions (Table S5) as well as a very low stability of 4MT3But-ITC in the soil (Hanschen et al. 2015).

Although the ITC concentrations detected in the soils 6 h after tissue incorporation were relatively low (Table 4), they were still higher than the approximately 1 nmol g^{-1} soil reported by Morra and Kirkegaard (2002). However, ITC concentration in soil was found to be variable, ranging between 1.2 and 100 nmol g^{-1} of soil depending on the optimal application under field or laboratory conditions (Gimsing and Kirkegaard 2009). Moreover, the concentrations of ITCs strongly depend on the incorporated plant material and the degree of tissue disruption (Gimsing and Kirkegaard 2009). For example, in a model experiment performed under optimal laboratory conditions (high water content as well as high plant biomass and maximal tissue disruption), the detected Allyl-ITC concentration ranged from 93.5 to 109.5 μmol g^{-1} soil 6 h after biofumigation with *B. juncea* (Hanschen et al. 2015). Other studies have also indicated that increasing tissue disruption of the biofumigant plant and increasing water content enhanced the release of the ITCs from the biofumigant tissues (Morra and Kirkegaard 2002; Cohen and Mazzola 2006; Mattner et al. 2008). In this study, the ITC concentration detected in the soils would have been higher if the three sites had been additionally irrigated. Lower precipitation at site A compared to K/M during the period of biofumigant tissue incorporation (19–26 August 2013) also might have an influence on the ITC concentration in the soils (Table S2).

The efficacy of biofumigation does not only depend on a high GS content in the plant tissues but also on the myrosinase in the plant tissue and in the soil (Al-Turki and Dick 2003; Gimsing et al. 2006). Plant- or microbe-derived myrosinases might have contributed to the hydrolysis reaction (Gimsing et al. 2006; Al-Turki and Dick 2003). Differences in bacterial community composition at least between the soils from sites K and A were recently reported by Yim et al. (2015). Hanschen et al. (2015) studied the degradation of the GS using the same soils as in the present study under laboratory conditions and found that the soils originating from different sites influenced the GS degradation.

Although the Basamid treatment (treatment 1) was standardized at all sites, a relatively low Methyl-ITC abundance was observed in soil from site A, which was in line with the biofumigation results (treatments 3 and 4) (Table 4). The lower Methyl-ITC concentration observed 6 h after treatment 1 might be attributed to differences in soil pH, humidity, soil fractions, organic matter content, or microbial communities, as reported previously (Dungan et al. 2003; Matthiessen et al. 2004; Zheng et al. 2006; Hanschen et al. 2015). On the other hand, the soil physicochemical properties and different historical management practices were also reported to be involved in the degradation of the ITCs (Brown et al. 1991; Brown and Morra 1993; Warton et al. 2003).

In conclusion, the present study showed that the detected breakdown products derived from biofumigation and Basamid treatment were different and site-dependent. In order to minimize the effects of environmental conditions influencing the production of GS in Brassicaceae plants, materials containing a higher GS concentration, e.g., commercially available seed meals or dried plant pellets, could be used as alternative in future studies (Lazzeri et al. 2013; Mazzola et al. 2015).

Effect of biofumigation and Basamid treatment on bacterial and fungal community compositions

The DGGE fingerprints of 16S rRNA gene fragments (for bacteria) and of ITS regions (for fungi) amplified from TC-DNA of different soil treatments revealed that the biofumigation (treatments 3 and 4 in 2012/2013) affected bacterial and fungal community compositions in RD soils from all three sites (Table 5). Hanschen et al. (2015) recently reported effects of GS on soil bacterial community composition by DGGE fingerprints under laboratory conditions. A significant reduction in the bacterial and fungal diversity and community composition in RD soils treated by biofumigation was also reported by Mazzola et al. (2015). The significantly stronger effect on fungal compared to bacterial community composition observed in the present study was in line with the observations reported by Hu et al. (2015). The DGGE bands which disappeared or were reduced in their intensity possibly indicated those populations affected either by a direct toxic effect of the biofumigation or the growth of other populations in response to the plant biomass incorporation (Fig. S2). Suppression of soil bacterial or fungal populations by biofumigation was reported in several earlier cultivation-based studies (Olivier et al. 1999; Mazzola et al. 2001, 2007; Cohen

and Mazzola 2006; Larkin and Griffin 2007; Mattner et al. 2008).

The present study also revealed that some soil bacterial and fungal populations increased in relative abundance in response to the biofumigation treatments as indicated by new bands or more intense bands compared to non-fumigated soil (Fig. S2). Several cultivation-based studies indicated the utilization of GS by various microbial taxa. Palop et al. (1995) found that *Lactobacillus agilis* R16 was not only able to degrade the GS in brown mustard seed extract by its intracellular myrosinase but also to detoxify Allyl-ITC. Moreover, *Bacillus subtilis* and *Pseudomonas aeruginosa* were found to be capable to consume sinigrin when grown on a mixture of glucose (0.2 %) and thioglucoside (0.2 %) (Reese et al. 1958). Some fungal isolates of the genera *Aspergillus* (i.e., *Aspergillus sydowii*) and *Penicillium* were also shown to consume sinigrin as a C-source (Reese et al. 1958). *Streptomyces* spp. were reported to proliferate in apple RD soil when the soil was amended with seed meals from *B. juncea* and *B. napus* (Cohen and Mazzola 2006; Mazzola et al. 2007). Therefore, repeating biofumigation might not only lead to changes in soil microbial composition and diversity but could also enrich several microbial taxa utilizing GS or the incorporated plant material. Thus, different effects between 1 and 2 years of biofumigation were proven in the present study. Plant root exudation was described in the review by Bertin et al. (2003) in which different plant species exuded different compounds containing C that led to different selection toward soil microbiota. Likewise, the biofumigant plants might have recruited specific bacterial and fungal community compositions which could be revealed by the existing dominant DGGE bands compared to treatment 2 (Fig. S2). The plant root exudation altering the soil microbial communities was also reported in several previous studies (Smalla et al. 2001; Berg and Smalla 2009; Bakker et al. 2012; Berendsen et al. 2012; Neumann et al. 2014; Schreiter et al. 2014). Biofumigation using green manures from growing plants involves a huge amount of biomass incorporated into the soil. In Brassicaceae, apart from GS, flavonoids and other phenolic compounds were also reported to be present in the plant tissues (Antonious et al. 2009; Cartea et al. 2011). The role of flavonoids influencing rhizosphere soil microbial communities was discussed by Weston and Mathesius (2013). Moreover, the biomass of the biofumigant plants can be later decomposed by soil organisms, resulting in a release of additional nutrients into the soils (Mazzola et al. 2001; Campbell et al. 2012). Thus, treating soil with manures (Ding et al. 2014) or with mineral nutrients (Campbell et al. 2010) also affects the soil microbial community composition and diversity. Furthermore, differences in soil pH and mineral nutrients after 2 years of biofumigation were documented in the present study (Table S7).

The higher effect of Basamid treatment compared to biofumigation, especially on soil bacterial community composition, corresponded with the higher ITCs detected in the soil (Tables 4 and 5). In future studies, more in-depth taxonomic information on specific bacterial and fungal responders to the biofumigation and Basamid treatment should be provided by amplicon sequencing.

Effect of biofumigation and Basamid treatment on indicator plant growth

Two-year biofumigation significantly increased the growth of indicator plants in biofumigated soils (treatments 3 and 4) compared to non-fumigated soils (treatment 2, the soils pre-cultivated with *M.* 'Bittenfelder' or with M111) under greenhouse and field conditions (treatment 2, the soils pre-cultivated with M111) only for site K (Tables 6 and 7), while the effects were more variable in the soils from sites A and M. The positive effect of the biofumigation in RD soils, especially in soil from site K (in the field and in the greenhouse) (Tables 6, 7, and S6), confirmed reports of other studies with different horticultural and agricultural crops (Lazzeri et al. 2003, 2010; Mazzola et al. 2007, 2015; Mattner et al. 2008; Fayzalla et al. 2009).

The lighter color of the roots and the higher RDM of M26 plants grown in treatments 3 and 4 (Tables 6 and 7; Fig. S3) showed that the changes in the microbial community composition in biofumigated soils most likely had a positive effect on the apple plants as previously also reported by others (Norsworthy et al. 2007; Mattner et al. 2008; Mazzola et al. 2009, 2015; Fayzalla et al. 2009; Zasada et al. 2009). As a consequence, the healthy roots enhanced plant growth whereas it was reduced by the infected roots in the RD soils (Gregory 2006; Yim et al. 2013; Emmett et al. 2014).

The observed improvement in plant growth might also result from the addition of nutrients by the biofumigation treatment (Mazzola et al. 2001; Lazzeri et al. 2010). Mazzola et al. (2001) reported that the mineral content in apple leaves (N, P, and S) at harvest

was influenced by the application rate of *Brassica* seed meals incorporated into the soil. In the present study, soil pH and mineral nutrients such as N, P, and K were analyzed. Among the analyzed soil nutrients, the extractable K_2O concentrations were significantly higher in biofumigated compared to non-biofumigated soils of all sites. The soil pH was significantly altered mainly in the soils from sites K and M, and the differences in mineral nutrients N and P_2O_5 in the soils were less pronounced and site-dependent (Table S7).

The soil treatment 2, pre-cultivated with M111 in the field, showed a stronger growth reduction of the indicator plants than the soil pre-cultivated with *M.* 'Bittenfelder' in both tests, in the greenhouse and in the field (Tables 6 and 7). On the one hand, this might be attributed to the respective plant genotypes which induced the RD to a distinct extent (Kviklys et al. 2008; St. Laurent et al. 2010). On the other hand, the soil pre-cultivated with M111 or *M.* 'Bittenfelder' probably influenced the soil microbial communities distinctly due to root exudates (St. Laurent et al. 2010). Moreover, *M.* 'Bittenfelder' seedlings were sown and had a lag phase in the beginning, whereas the vegetatively propagated rootstocks were bigger when planted. The growth increase of the indicator plants (M26, *M.* 'Bittenfelder', and M106) in site K soil (treatment 2 vs. treatments 3 and 4) was higher compared to the other two sites (A/M). This difference might be due to more biomass incorporated into the soil which led to the stronger shifts (higher d-values) in soil bacterial and fungal communities (Tables 6, 7, and S3).

Based on the indicator plant performances, it has to be concluded that the Basamid treatment (treatment 1) in site A soil was not effective which is in accordance with the lowest Methyl-ITC concentration observed in this soil (Tables 4, 6, and 7). In comparison with the recommended concentration of Methyl-ITC (517 - 1294 nmol g^{-1} soil) (Brown et al. 1991), in the present study, the detected Methyl-ITC concentration 6 h after the treatment was much lower (Table 4). However, the Methyl-ITC concentration in the soil was reported to decrease over time (Dungan et al. 2003; Matthiessen et al. 2004). Also, because of weeds established in the field at all sites, it cannot be ruled out that soil disinfection by Basamid was not optimal in the present study.

Despite the huge differences in detected ITCs between the biofumigation and the Basamid treatments, comparable effects on indicator plant growth were shown both in the greenhouse (sites K and A) and in the field (all sites). This might indicate that the biofumigation effect was caused by the direct toxicity of the ITCs on soil organisms, by changes in diversity and composition of the soil microbiome by the biofumigant plant root exudation, and by the nutrients incorporated with green manure.

Conclusion

The effects of the biofumigation, but also the Basamid treatments, were site-dependent. Soils from different sites have differences in soil physical and chemical properties and soil microbiome as well as climatic conditions, all of which supported the growth of the biofumigant plants differently. In consequence, variability in the GS breakdown products as well as the stability of the compounds varied between the sites.

Despite the huge differences between the ITCs detected in biofumigated and Basamid-treated soils, a comparable effect on the indicator plant growth, especially in the field at all sites, was observed pointing to the fact that the changes in the soil microbiome had a large impact. Yim et al. (2015) reported that treating RD soil by temperature or gamma irradiation did not only reduce the bacterial diversity in the soil but also boosted the abundance of taxa that potentially promote plant growth or suppress pathogens. Taxonomic information on specific bacterial and fungal responders to the biofumigation provided by amplicon sequencing will be needed to gain further insights.

From this study, we conclude that the biofumigation could be an alternative strategy to the previously used Basamid treatment for the growers, although further optimization of the process is needed.

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Compliance with ethical standards

Conflict of interest statement None declared.

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2.3 Effects of soil pre-treatment with Basamid® granules, *Brassica juncea*, *Raphanus sativus* and *Tagetes patula* on bacterial and fungal communities at two replant disease sites

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Abstract

Nurseries producing apple and rose rootstock plants, apple orchards as well as rose production often experience replanting problems after several cultivations on the same site when a chemical soil disinfectant is not applied. The etiology of apple and rose replanting problems is most likely caused by soil-borne pathogen complex, defined as ‘replant disease (RD)’. RD symptoms are typically reduced shoot and root growth, a smaller leaf area, a significant decrease in plant biomass, yield and fruit quality and a shorter life span. In our previous study, we showed that RD symptoms were reduced when apple rootstock M106 were grown in RD soils treated either with the soil fumigant Basamid or after biofumigation by incorporating *Brassica juncea* or *Raphanus sativus* or by growing *Tagetes* under field conditions compared to untreated control soil. The present study aimed at identifying potential bacterial and fungal taxa that were affected by different soil treatments and linking bacterial and fungal responders to plant performance. Miseq® Illumina® sequencing of 16S rRNA gene fragments (bacteria) and ITS regions (fungi) amplified from total community DNA extracted from soil samples taken four weeks after treatments were performed. Soil properties and culture history of the two RD sites influenced greatly soil microbiomes, with different capacities in RD development. Several bacterial genera were identified that significantly increased in treated soils such as *Arthrobacter* (*R. sativus*, both sites), *Curtobacterium* (Basamid, both sites), *Terrimonas* (Basamid and *R. sativus*, site A) and *Ferruginibacter* (*B. juncea*, site K and *R. sativus*, site A) that were also significantly and positively correlated with growth of apple M106 plants. Only few fungal genera, such as *Podospora*, *Monographella* and *Mucor*, were significantly promoted in soils treated with *B. juncea* and *R. sativus* (both sites). The least pronounced changes were recorded for bacterial as well as fungal communities in the RD soils planted with *Tagetes*. The detection of bacterial and fungal genera that were significantly increased in relative abundance in response to the treatments and that were positively correlated with plant growth suggests that management of the soil microbial community could contribute to overcome the apple RD encountered in affected sites.

Keywords: amplicon sequencing, apple replant disease, biofumigation, soil microbiome

2.3.1 Introduction

The soil microbiome is assumed to play a crucial role for plant growth and health in terms of acquiring water and nutrients, acting antagonistically against soil-borne plant pests and pathogens, as well as inducing plant defense responses against pathogens (Berendsen et al., 2012). Negative effects of the soil microbiome on plant growth and yield were also revealed, especially at sites with monocultures and with lack of sustainable management practices (Magarey, 1999; Seigies and Pritts, 2006; Wu et al., 2015; Zhao et al., 2016). This is likely due to a reduced microbial diversity because of the repeated monoculturing (Howe et al., 2014).

Apple plants cultivated repeatedly at the same site have often been reported to show reduced shoot and root growth. It is assumed that pathogenic microorganisms increased in abundance in response to plant root exudations of previous cultures (Badri and Vivanco, 2009; Mazzola and Manici, 2012; Yim et al., 2013; Nicola et al., 2017). This so-called apple replant disease (ARD) has severe consequences in terms of economic losses in tree nurseries and apple production worldwide.

A recent study employing transcriptomic analysis in roots of apple rootstock M26 plants grown in ARD soils compared to Gamma-sterilized soil discovered that the expression of plant genes associated with plant defense, i.e. phytoalexin production genes was increased while genes involved in the primary metabolism were less expressed (Weiß et al., 2017) indicating plant response to soil-borne pathogens. Possible ARD causing organisms identified from cultivation dependent approaches included actinomycetes (Otto et al., 1994), *Pythium* sp. (Hoestra, 1994; Emmett et al., 2014), *Cylindrocarpon* sp., *Phytophthora* sp., *Rhizoctonia solani* (Mazzola, 1998; Tewoldemedhin et al., 2011; Kelderer et al., 2012) and nematodes, e.g. the root endoparasitic nematode *Pratylenchus penetrans* (Mai et al., 1994). Several recent studies employed total community (TC-) DNA-based based approaches to identify these pathogens, but rather showed microbial community shifts in ARD soils after soil treatments that restored apple growth (Yim et al., 2013; Sun et al., 2014; Franke-Whittle et al., 2015; Nicola et al., 2017). Because the etiology of ARD is complex, conventional soil fumigants with a broad spectrum of biocides such as chloropicrin, 1.2 dichloropropane, 1.3 dichloropropene, methyl bromide and Basamid® granules were shown to be the most effective treatments against ARD (Mai and Abawi, 1978; Brown and Koutoulis, 2008; Yim et al., 2013; Nicola et al., 2017). However, those chemical substances were reported to be toxic, and their application no longer allowed in many countries (Ruzo, 2006; Porter et al., 2010).

For environmental friendly approaches, crop rotation or treating replant disease (RD) soil using several Brassicaceae species (biofumigation) or *Tagetes* (nematode repelling) demonstrated promising effects against disease-causing organisms in soils (Sarwar et al., 1998; Topp et al., 1998; Mattner et al., 2008; Marahatta et al., 2012; Pino et al., 2016), and subsequently reduced RD symptoms on plant growth (Seigies

and Pritts, 2006; Mazzola et al., 2015; Yim et al., 2016). Effects of biofumigation originate from plant secondary metabolites glucosinolates (GS) that are hydrolyzed mainly by plant myrosinase enzymes (reviewed by Halkier and Gershenzon 2006), subsequently releasing several compounds depending on soil properties (Halkier and Gershenzon, 2006), such as isothiocyanates (ITC), nitriles, thiocyanates, epithionitriles and oxazolidine-2-thiones (Brown et al., 1991; Kirkegaard and Sarwar, 1998). Among GS degraded products, volatile ITCs were shown to be responsible for suppression of weeds (Sarwar et al., 1998; Mattner et al., 2008; Malik et al., 2008), soil-borne plant pests and pathogens in different crop systems (Borek et al., 1998; Peterson et al., 1998; Matthiessen and Shackleton, 2005; Bones and Rossiter, 2006; Mazzola et al., 2007; Mattner et al., 2008; Agerbirk and Olsen, 2012; Aires et al., 2009; Neubauer et al., 2014). On the other hand, *Tagetes* plants are renowned to exhibit toxicity in soils due to their thiophene contents (Hooks et al., 2010; Saha et al., 2012). Highly suppressed growth of several soil-borne plant pathogenic fungi such as *Rhizoctonia solani* and *Fusarium solani* mediated by these biocidal compounds was demonstrated via *in vitro* evaluations (Saha et al., 2012).

In our previous field study, the effects of pre-treatments of RD soils with the soil fumigant Basamid, biofumigation with *B. juncea* and *R. sativus* and growing *Tagetes* plants at the two sites K and A on plant performance were investigated. Findings revealed that effects of the different treatments evaluated by field growth of apple rootstock M106 plants were site-dependent. At site K, shoot fresh mass (SFM) of the M106 plants significantly increased by 155, 148, 165 and 175% in treated soils with Basamid, *B. juncea*, *R. sativus* and *Tagetes*, respectively, relative to the corresponding RD soil. At site A, a moderate effect was observed only for the RD soil cropped with *Tagetes*, with 52 % increment in SFM (Yim et al., 2016). Changes in the bacterial and fungal community composition based on DGGE fingerprint analysis revealed a treatment- and site-dependent pattern (Yim et al., 2016), calling for deeper molecular investigations and characterization of these differences.

In the present study, a detailed analysis of the changes soil bacterial and fungal community composition in the two sites was performed, focusing on diversity and relative abundances at different taxonomic levels in response to the treatments by means of Miseq® Illumina® sequencing. This study identified soil bacterial and fungal taxa affected by the different soil treatments (Basamid, *B. juncea*, *R. sativus* and *Tagetes*) at the two sites under field conditions, and linked these microbial responders to ARD suppression.

2.3.2 Materials and methods

The two RD sites K (53° 41′ 58.51″ N, 9° 41′ 34.12″ E) and A (53° 42′ 18.81″ N, 9° 48′ 16.74″ E) that had been used for producing rose and apple rootstocks, respectively, were submitted to different treatments under field conditions during the years 2012 and 2013. The sites differ in soil chemical and physical properties as described in Yim et al. (2016). Briefly, site K (sandy soil) has a higher proportion in organic

matter and sand than site A (slightly loamy sand). Five treatments and three biological replicates (plots) per treatment were randomized in blocks on an area of 1000 m^2 per site (45 m^2 per replicate). Parcels replanted with apple rootstocks M4 and M111 in May 2012 and 2013, respectively, served as untreated RD soils. The rootstocks were harvested each year in November. For treatment with Brassicaceae plants, seeds from two species, *B. juncea* 'Terra Plus' (12 kg ha^{-1}) and *R. sativus* 'Defender' (30 kg ha^{-1}) were sown onto RD soils twice, in April/May and in June/July (2012 and 2013). The plants at full flowering, about 8 weeks after sowing were cut at the soil line, chopped and subsequently incorporated into the soils using Humus WM Flail mulchers (Humus®, Bermatingen, Germany) and a common rotary cultivator (Yim et al., 2016). For treatment with *Tagetes patula* 'Nemamix', 10 kg ha^{-1} seeds were sown once per year in 2012 and 2013, in April/May. In both years, the plants grew until November before they were ploughed. Seeds of *B. juncea*, *R. sativus* and *Tagetes* were supplied by P. H. Petersen Saatzucht Lundsgaard GmbH, Germany. A chemical soil fumigant treatment with Basamid® granules (97% Dazomet) was performed once in August 2013 at a dose of 400 kg ha^{-1} (ProfiFlor GmbH, Stommeln, Germany) applied when the second biofumigation was performed (end of August 2013).

Four weeks after the Basamid and biofumigation treatments, bulk soils were sampled the same day in September 2013 using a 3.5 cm diameter core soil sampler at 0-20 cm depth. The sampling schedule and procedures were the same as for the treatments with *Tagetes* and untreated RD. At the sampling date, the flowering *Tagetes* plants had not been incorporated into the soil. The homogenized and sieved (mesh sizes ≤ 2 mm) soil samples were submitted to TC-DNA extraction and purification as described in Yim et al. (2016).

Amplicon sequencing for bacteria and fungi was implemented via Miseq® Illumina® (Illumina, San Diego, CA, USA) sequencing. For the bacterial 16S rRNA gene fragments, an initial PCR amplification step was performed using a set of primer pairs 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACHVGGGTWTCTAAT) to flank the approximate 460 bp variable V3-V4 regions as described by Nunes et al. (2016). Regarding the ITS regions for fungi, primers gITS7 (GTGARTCATCGARTCTTTG) and ITS4 (TCCTCCGCTTATTGATATGC) were applied to obtain the fragments of interest (Ihrmark et al., 2012). Purification and size-selection of products of greater than 100 bp from a second amplification step using the same primers with attachment of adaptors and barcode tags was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. The samples were then pooled and adjusted to equimolar concentrations measured using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA), concentrated using the DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA, USA), and finally subjected to 2x250 bp paired-end high-throughput sequencing on an Illumina® MiSeq® platform.

Amplicon sequences were analysed using qiime_pipe (https://github.com/maasha/qiime_pipe) with default settings, which performs sample demultiplexing, quality-based sequence trimming, primer removal and paired-end reads assembly prior to annotation workflow (Caporaso et al., 2010). Annotation procedure for bacterial sequences is derived from previously described work (Nunes et al., 2016). Chimera check was done with UCHIME (Edgar et al., 2011) and Operational Taxonomic Units (OTUs) were picked at 97% sequence identity level. OTU representative sequences were selected by the highest abundance within the cluster and assigned to taxonomy using the RDP classifier, with a confidence threshold of 80%. Read contingency tables were exported at the species level in order to defined OTUs. For fungi, if a sequence had the same bit score to more than one species hypothesis (SH) in the UNITE version 7.0 database (Koljalg et al., 2013) of Megablast (Camacho et al., 2009), then it was assigned to the most abundant SH in the dataset. Selected OTU were based on the assigned sequences that were greater than 95% similarity to any SH or had greater than 100 bp alignment length. Illumina sequencing data were deposited at the NCBI sequence read archive under the accession number PRJNA352771.

Data Analyses

For subsequence analyses, three biological replicates were used for bacteria, and four replicates for fungi, except for the treatment with *Tagetes* for which only three replicates could be employed. The excluded replicates of the respective treatments were based on high variability of the sequence reads (two to three time differences). The effects of the different soil treatments on bacterial and fungal community compositions were analysed by a Principal Coordinate Analysis (PCoA) applying Bray-Curtis distance metrics and the analysis of similarity (ANOSIM) test by Past3 (3.02) (Hammer et al., 2001). Species richness and diversity index were evaluated using rarefied sequence data applying Tukey test adapted based on Herberich et al. (2010) at $p < 0.05$ with transformed data by sqrt(n/N * 100 +1) (n, the number of sequences for each OTU and N, the total number of sequences from the sample) to reveal significant differences in relative abundances of soil bacteria and fungi at phylum levels (software R 3.2.2). Any bacterial and fungal genera that presented significant differences in their relative abundances between the soil treatments, and those which were greater than 0.5% relative abundance were tested for correlation with shoot and root fresh mass of apple rootstock M106 plants grown in the field in 2014 using the Pearson correlation coefficient (r) by Past3 (3.02).

2.3.3 Results

2.3.3.1 Effects of treatments on soil bacterial community composition and diversity

The numbers of bacterial sequences detected ranged from 18,576 to 27,738 and from 21,267 to 40,089 in soils at sites K and A, respectively, with no significant differences between the treatments. However, a tendency for higher sequence counts was observed in untreated RD soils rather than in the other treatments at both sites (Table 2.3.1). Subsequent analyses using rarefied sequence data recorded more OTUs in soils treated with *B. juncea* (sites K, 347 and A, 302) and *R. sativus* (sites K, 353 and A, 340) than in soils subjected to the other treatments. Except that significantly higher species richness in *R. sativus*-treated soil at site A was observed, bacterial compositions and diversities were not significantly altered by the treatments in soils at both sites (numbers of OTUs, Chao1 and Shannon indices, Table 2.3.1) in comparison to untreated RD soils. The bacterial diversities were significantly lower in soils at site A than K, regardless of different soil treatments (Table S2.3.2; Figure S2.3.1). Analyses of similarity (ANOSIM) indicated significantly distinct bacterial community compositions between sites (R = 0.46, $p < 1E-4$, Table 2.3.2), irrespective of the treatment. Both PCoA and ANOSIM tests revealed that the bacterial community composition in soil of the *Tagetes* treatment at site A was less affected compared to the other treatments (Figure 2.3.1; Table 2.3.2). Overall, the soil treatments resulted in stronger alterations of the bacterial community composition at site A than at site K (R-values, Table 2.3.2; PCoA, Figure 2.3.1). In addition, for soil samples from the *R. sativus* treatments at site A, the highest R value (0.74) was recorded (Table 2.3.2).

Table 2.3.1: Bacterial community diversity based on operational taxonomic unit (OTUs) at 97% similarity in different soil treatments

Site	Treatment	Sequences per condition	Numbers of OTU (97%)	Chao1	Shannon
K	**K_RD**	27,738±2,755	332±16 ab	368±18 ab	4.18±0.12
	K_Basamid	18,576±3,728	311±5 a	350±7 a	4.30±0.02
	K_*B. juncea*	24,632±3,770	347±3 ab	395±14 b	4.36±0.02
	K_*R. sativus*	26,946±4,508	353±1 b	389±6 ab	4.29±0.05
	K_*Tagetes*	25,259±3,909	327±7 ab	362±7 ab	4.13±0.10
A	**A_RD**	40,089±7,422	284±13 a	317±18 a	3.69±0.11
	A_Basamid	32,016±2,551	274±20 a	308±18 a	3.74±0.17
	A_*B. juncea*	30,793±8,640	302±31 ab	360±15 ab	3.51±0.65
	A_*R. sativus*	21,267±3,228	340±6 b	383±14 b	4.14±0.05
	K_*Tagetes*	29,665±2,160	293±3 a	349±16 ab	3.84±0.04

Data is presented as mean±SEM. RD, replant disease soil. Letters indicate significant differences within site, Tukey test $p < 0.05$ and n = 3. Chao1, species richness. Increased and decreased bacterial richness and diversity in treated RD soils compared to untreated within site are highlighted in green and red, respectively.

Table 2.3.2: Analysis of similarity of the bacterial community composition detected in different soil treatments with respect to untreated replant disease soil based on OTUs of bacterial 16S rRNA gene fragments

Treatment	Site K		Site A	
	R-value	*p*-value	R-value	*p*-value
Basamid	0.48	0.2015	0.56	0.0948
B. juncea	0.22	0.4032	0.48	0.1016
R. sativus	0.30	0.2949	0.74	0.1003
Tagetes	-0.26	0.9056	0.07	0.5998

Sites K vs. A, R-value = 0.46 and $p < 0.0001$.

R- (-1 to 1) and p-values were obtained from ANOSIM test. R value closes to "1" suggests strong dissimilarity between the communities being compared, the value close to "0" represents an even distribution of the communities within and between treatments, whereas the value below "0" suggests dissimilarities are greater within than between treatments.

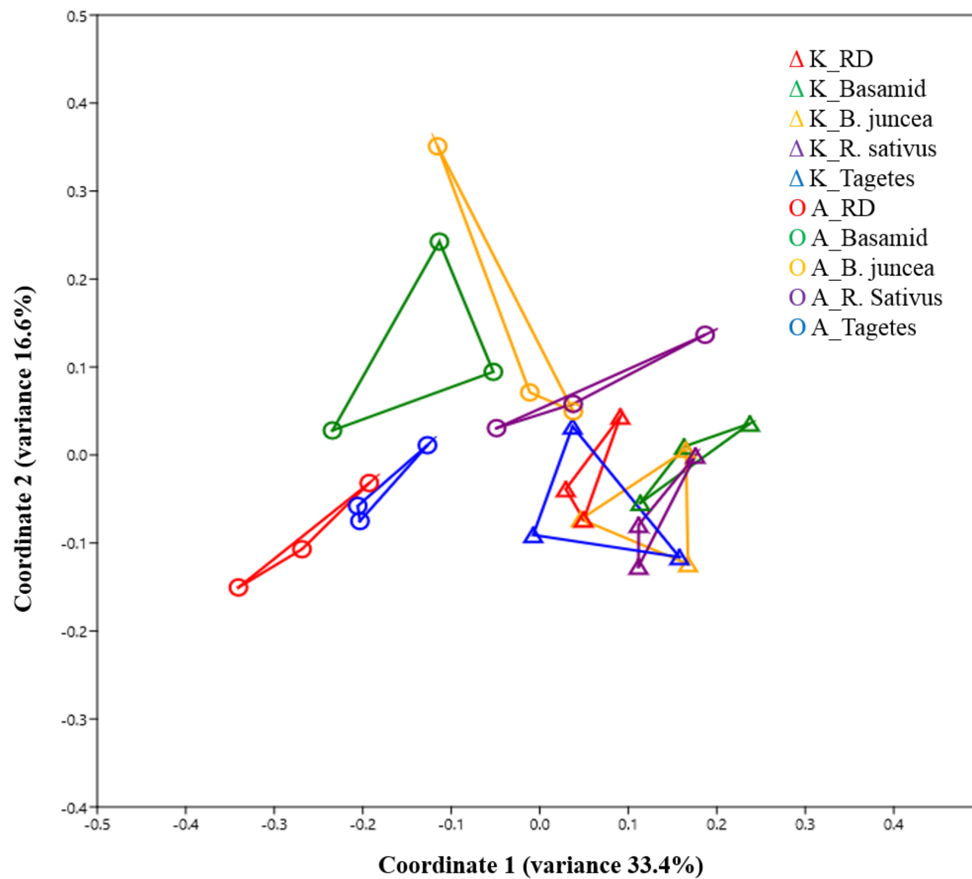

Figure 2.3.1: Effect of different treatments on soil bacterial community composition under field condition revealed by principal coordinate analysis (PCoA) using Bray-Curtis distance metric. Past3 and n = 3. Soil samples were taken four weeks after different treatments in September 2013.

Among the analysed samples, 12 bacterial phyla were identified, and *Firmicutes* were most dominant in relative abundance, followed by *Proteobacteria* and *Actinobacteria* in all soil treatments and at both sites

(Figure 2.3.2; Table S2.3.3). *Firmicutes* shared proportions of about 29-39 % in soils at site K, but higher abundances of approximately 40-52% at site A (Figure 2.3.2). Members of the bacterial phyla *Actinobacteria* and *Bacteroidetes* were observed in significantly higher relative abundances in soils treated with *R. sativus* compared with untreated RD soils at both sites, K and A. Site-dependent effects of the treatments on other bacterial phyla were detected. For instance, the relative abundance of *Proteobacteria* was significantly higher in *R. sativus* and *Tagetes* than in untreated RD soils only at site A (Figure 2.3.2). Another bacterial phylum, *Planctomycetes,* was significantly reduced only in soils at site A when the RD soil was treated with Basamid, *B. juncea*, *R. sativus* and *Tagetes*. At site K, treatments with Basamid and *Tagetes* did not significantly affect members of any bacterial phylum (Figure 2.3.2).

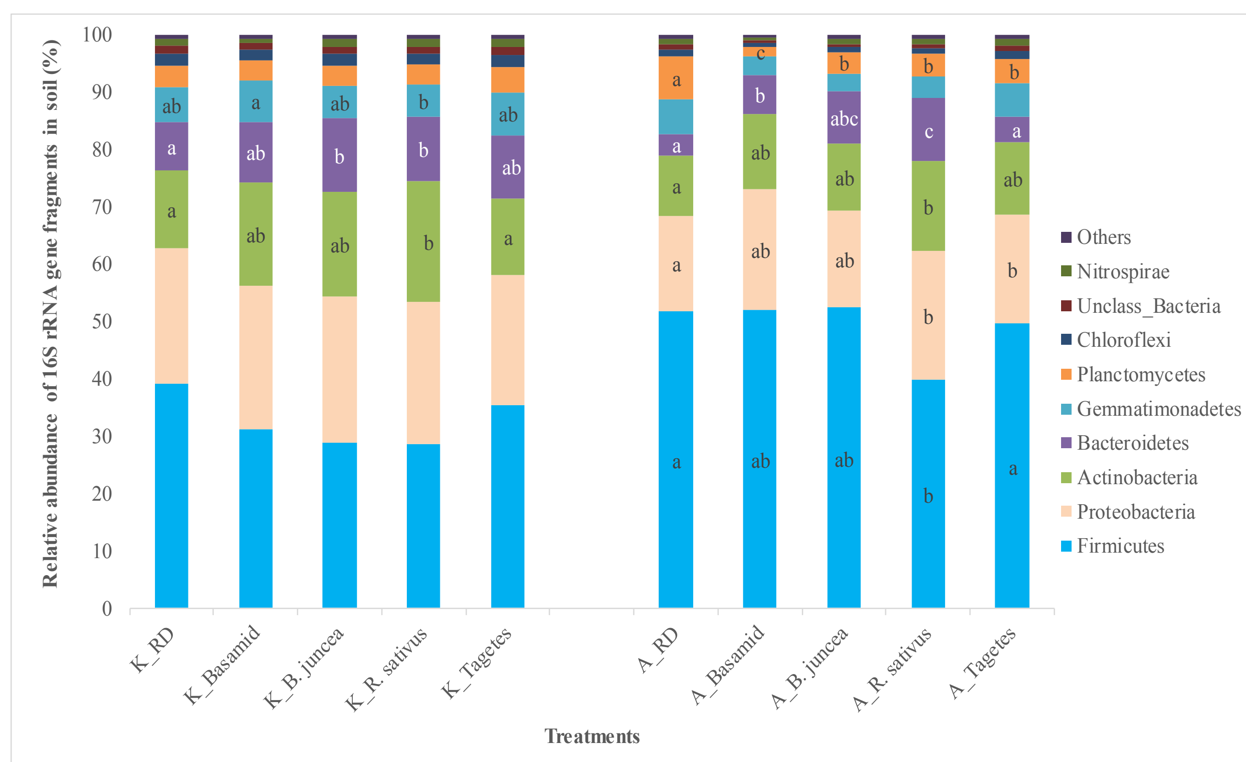

Figure 2.3.2: Relative abundance of dominant bacterial phyla in replant disease soils at two sites affected by different treatments. Different letters indicate significant differences in relative abundance of bacteria at phylum level affected by soil treatments, Tukey test, $p < 0.05$ and $n = 3$ (comparison within site).

At genus levels, soils fumigated with Basamid exhibited the following common increased responders in relative abundance: *Salinibacterium*, *Curtobacterium*, *Thiobacillus* and *Rhodanobacter* with the strongest response (33- and 23-fold increase at sites K and A, respectively) recorded for *Rhodanobacter*. Only the unclassified *Bacteroidales* related sequences significantly decreased in relative abundance in Basamid-treated soils at both sites (Table 2.3.3).

For soil treated with *B. juncea*, no common responders were discovered due to high standard deviations within the treatment (both sites). At site K, members of *Arthrobacter* were the most dominant in soil treated

with *B. juncea* (5.89%) and their relative abundances were about three times higher than those in untreated RD soil (Table 2.3.3).

Members of the bacterial genus *Arthrobacter* were recorded in significantly enhanced abundance in soils treated with *R. sativus* (8.61 and 4.33% for sites K and A, respectively) compared with untreated RD soils. Another bacterial genus *Terrabacter* was a common responder in soils treated with *R. sativus* being significantly enriched at both sites (Table 2.3.3).

For RD soils planted with *Tagetes*, because of site-dependent effects, no common responders were observed for bacteria at the genus levels. A less pronounced effect on the relative abundance of bacterial genera in *Tagetes*-treated soil compared with the other treatments corresponds to the results of the PCoA and the analysis of similarity (Tables 2.3.2, 2.3.3; Figure 2.3.1).

The bacterial genus *Streptomyces* was significantly reduced in relative abundance about 4- to 5-fold after all treatments at site K (Table 2.3.3). Irrespective of the soil treatment and the site, Pearson correlation coefficient analysis revealed several bacterial genera to be significantly and positively correlated with growth of apple rootstock M106 plants (SFM or RFM), such as *Arthrobacter*, *Curtobacterium*, *Terrimonas*, *Ferruginibacter* amongst others (Table 2.3.4). These bacteria showed higher relative abundances in treated RD soils at site K than A (Table 2.3.3).

Table 2.3.3: Relative abundance of bacterial genera detected in TC-DNAs extracted from bulk soils collected four weeks after different treatments of replant disease soils at two sites (only genera with relative abundance > 0.5 % are shown)

Phylum/ Family	**Genus**	**Site K**					**Site A**				
		K_RD	**K_Basamid**	**K_*B. juncea***	**K_*R. sativus***	**K_*Tagetes***	**A_RD**	**A_Basamid**	**A_*B. juncea***	**A_*R. sativus***	**A_*Tagetes***
Actinobacteria											
Micrococcaceae		2.04±0.53 a	4.02±0.79 ab	6.14±0.43 b	8.95±0.43 c	1.80±0.18 a	0.95±0.09 a	4.82±0.24 b	2.38±0.61 ab	4.49±1.14 bc	1.32±0.06 ac
	Arthrobacter	1.92±0.51 a	3.64±0.64 ab	5.89±0.34 b	8.61±0.41 c	1.70±0.18 a	0.92±0.09 a	2.50±0.80 ab	2.30±0.61 ab	4.33±1.06 b	1.31±0.07 a
Microbacteriaceae		0.16±0.02 a	1.12±0.23 b	0.24±0.03 a	0.30±0.01 a	0.25±0.05 a	0.07±0.02 a	0.79±0.25 b	0.11±0.03 a	0.16±0.01 a	0.09±0.01 a
	Salinibacterium	0.07±0.02 a	0.59±0.14 b	0.11±0.01 a	0.12±0.01 a	0.13±0.02 a	0.04±0.01 a	0.62±0.23 b	0.05±0.02 a	0.06±0.02 a	0.05±0.01 a
	Curtobacterium	0.08±0.01 a	0.54±0.09 b	0.13±0.02 a	0.17±0.01 a	0.11±0.02 a	0.03±0.01 a	0.17±0.03 b	0.06±0.02 ab	0.09±0.01 ab	0.04±0.01 a
Intrasporangiaceae		0.53±0.04 a	0.59±0.10 a	1.23±0.21 b	1.33±0.17 b	0.48±0.04 a	0.51±0.09 a	0.45±0.16 a	0.83±0.18 ab	1.29±0.10 b	0.69±0.12 ab
	Terrabacter	0.27±0.02 a	0.29±0.04 a	0.73±0.14 b	0.86±0.11 b	0.22±0.01 a	0.36±0.08 a	0.26±0.10 a	0.58±0.13 ab	0.92±0.07 b	0.49±0.07 a
Streptomycetaceae		1.01±0.13 a	0.67±0.09 ab	0.46±0.01 b	0.47±0.02 b	0.39±0.02 b	0.57±0.10 ab	0.39±0.02 a	0.56±0.16 ab	0.69±0.01 b	0.52±0.05 ab
	Streptomyces	0.60±0.14 a	0.14±0.02 b	0.14±0.03 b	0.13±0.02 b	0.12±0.00 b	0.05±0.02	0.04±0.00	0.07±0.02	0.08±0.01	0.03±0.00
Bacteroidetes											
Chitinophagaceae		6.04±0.20 a	8.22±0.86 ab	9.26±0.49 b	7.87±0.17 b	8.05±1.07 ab	2.09±0.14 a	5.38±0.23 b	7.68±2.33 abc	9.20±0.20 c	2.92±0.29 a
	Terrimonas	2.79±0.11	3.62±0.19	3.65±0.27	3.56±0.22	3.74±0.43	0.47±0.11 a	1.50±0.20 b	1.85±0.71 abc	2.49±0.13 c	0.95±0.09 ab
	Ferruginibacter	1.03±0.05 a	1.31±0.27 ab	1.91±0.04 b	1.38±0.09 a	1.23±0.09 a	0.25±0.03 a	1.14±0.36 ab	1.05±0.34 ab	1.33±0.11 b	0.43±0.09 a
	Flavitalea	0.24±0.03	0.28±0.04	0.33±0.02	0.27±0.05	0.41±0.13	0.34±0.02 a	0.55±0.14 ab	1.21±0.41 ab	1.20±0.30 b	0.54±0.04 b
Unclass_Bacteroidales	*Unclass_Bacteroidales*	0.85±0.26 a	0.27±0.01 b	1.40±0.47 a	0.99±0.08 a	0.89±0.14 a	0.88±0.00 a	0.14±0.03 b	0.48±0.10 ab	0.53±0.12 a	0.73±0.07 a
Flavobacteriaceae	*Unclass_Flavobacteriaceae*	0.34±0.05 a	1.35±0.16 b	0.61±0.18 ab	0.46±0.01 a	0.54±0.07 a	0.29±0.04	0.34±0.05	0.31±0.09	0.42±0.10	0.22±0.01
Planctomycetes											
Planctomycetaceae	*Unclass_Planctomycetaceae*	3.70±1.35	3.51±0.19	3.67±0.81	3.53±0.28	4.28±1.11	7.60±0.57 a	1.50±0.08 b	3.65±0.84 c	4.06±0.22 c	4.34±0.75 c
Alphaproteobacteria											
Rhizobiaceae		0.47±0.08 ab	0.17±0.03 a	0.63±0.02 b	0.77±0.12 b	0.36±0.07 ab	0.12±0.03 a	0.07±0.04 a	0.33±0.17 ab	0.49±0.09 b	0.16±0.01 a
	Rhizobium	0.38±0.12 ab	0.08±0.01 a	0.52±0.02 b	0.61±0.09 b	0.30±0.06 b	0.08±0.02 a	0.05±0.02 a	0.26±0.13 ab	0.36±0.07 b	0.14±0.01 ab
Sphingomonadaceae		2.52±0.24 ab	3.40±0.27 b	2.73±0.14 ab	2.43±0.12 ab	1.92±0.18 a	1.14±0.09	1.39±0.20	1.43±0.33	1.79±0.20	1.60±0.31
	Sphingomonas	0.05±0.02 a	0.51±0.12 b	0.03±0.01 a	0.03±0.00 a	0.03±0.01 a	0.03±0.01	0.22±0.13	0.01±0.00	0.03±0.01	0.00±0.00
Betaproteobacteria											
Oxalobacteraceae	*Massilia*	0.24±0.06 a	0.88±0.04 b	0.23±0.02 a	0.25±0.02 a	0.15±0.03 a	0.07±0.01	0.16±0.07	0.16±0.04	0.22±0.05	0.27±0.07
Hydrogenophilaceae	*Thiobacillus*	0.21±0.01 a	0.54±0.12 b	0.21±0.03 ab	0.24±0.02 ab	0.22±0.02 ab	0.11±0.02 a	0.86±0.12 b	0.25±0.09 ac	0.21±0.04 ac	0.25±0.01 c
Gammaproteobacteria											
Xanthomonadaceae		0.91±0.08 a	2.29±0.05 b	1.62±0.14 c	1.60±0.17 abc	1.09±0.13 ac	1.01±0.23	4.18±1.63	1.11±0.23	1.82±0.27	0.97±0.05
	Rhodanobacter	0.05±0.01 a	1.65±0.12 b	0.22±0.12 a	0.12±0.03 a	0.05±0.02 a	0.15±0.06 a	3.49±1.57 b	0.10±0.03 a	0.22±0.10 a	0.07±0.02 a
Pseudomonadaceae		1.93±0.25 ac	0.74±0.06 b	1.84±0.08 c	2.06±0.23 c	0.99±0.13 ab	0.98±0.04	3.18±2.68	0.90±0.25	1.27±0.16	0.88±0.06
	Pseudomonas	1.15±0.24 a	0.15±0.03 b	0.78±0.07 a	0.90±0.22 ac	0.26±0.08 bc	0.06±0.03 a	2.69±2.68 ab	0.21±0.09 ab	0.35±0.18 ab	0.28±0.04 b

Data is presented as mean±SEM. Significant differences in relative abundance due to different treatments were assessed by R3.2.2 applying Tukey test, $p < 0.05$ and n = 3. Increased and decreased relative abundance in treated replant disease (RD) soils compared to untreated within site are highlighted in green and red, respectively. Colored cells indicate those changes that were found at both sites.

Table 2.3.4: Pearson correlation coefficient (r) between bacterial relative abundance and growth of apple rootstock M106 plants grown under field conditions

Phylum	Genus	Relative abundance (%)	SFM		RFM	
			r	*p*-value	r	*p*-value
Actinobacteria	*Arthrobacter*	3.31±0.45	0.43	0.019	0.25	0.192
	Curtobacterium	0.14±0.03	0.46	0.010	0.56	0.001
Bacteroidetes	*Terrimonas*	2.46±0.23	0.66	0.000	0.63	0.000
	Ferruginibacter	1.11±0.10	0.47	0.009	0.43	0.017
	Unclass_Flavobacteriaceae	0.49±0.06	0.50	0.005	0.55	0.002
	Flavitalea	0.54±0.08	-0.40	0.028	-0.43	0.018
Betaproteobacteria	*Massilia*	0.26±0.04	0.35	0.062	0.45	0.012
Alphaproteobacteria	*Sphingomonas*	0.09±0.03	0.29	0.124	0.44	0.015

Relative abundance is presented as mean±SEM. SFM, shoot fresh mass and RFM, root fresh mass. Pearson correlation coefficient was evaluated by Past3 and n = 3.

2.3.3.2 Effects of treatments on soil fungal community composition and diversity

The fungal ITS sequence reads ranged from 24,479 to 34,494 and from 27,123 to 36,234 in soils at sites K and A, respectively, for the different treatments. By trend, higher numbers were displayed in Basamid-treated soils (sites K and A, Table 2.3.5). After rarefied sequence data, the OTU numbers and diversity indices were significantly lower in Basamid treated soil compared to untreated RD soil at site K. At site A, soils treated with *B. juncea* and *R. sativus* possessed significantly more species richness than untreated RD soil. However, the fungal diversity indices were not influenced by any of the treatments in relation to untreated RD soil (Shannon indices, Table 2.3.5). Regardless of different soil treatments, the fungal community compositions and diversity were significantly higher in soils at sites A than K (Table S2.3.4; Figure S2.3.2).

As also observed for soil bacteria, differences in fungal community composition between sites were demonstrated (R = 0.40 and p < 1E-04, Table 2.3.6; Figure 2.3.3). Effects of the different soil treatments on fungal community composition were clearly stronger compared to effects seen on the bacterial community composition (Tables 2.3.2, 2.3.6; Figures 2.3.1, 2.3.3), especially at site K. Significantly different soil fungal community compositions between untreated RD soils and all kinds of treatments were found, except for the soil from *Tagetes* treatment at site A (Table 2.3.6).

Table 2.3.5: Fungal community diversity based on operational taxonomic unit (OTUs) at 95% similarity in different soil treatments

Site	Soil treatment	Sequences per sample	Number OTU (97%)	Chao1	Shannon
K	**K_RD**	32,718±3,916	112±2 a	130±2	3.13±0.09 a
	K_Basamid	34,494±1,908	86±2 b	121±18	2.36±0.19 b
	K_*B. juncea*	28,665±3,258	105±1 ab	120±3	2.72±0.05 ab
	K_*R. sativus*	28,592±3,253	107±3 a	135±10	2.80±0.08 a
	K_*Tagetes*	24,479±5,631	112±10 a	123±14	2.94±0.09 a
A	**A_RD**	27,123±6,325	119±3 a	126±5 a	2.88±0.18
	A_Basamid	36,234±3,054	117±9 a	132±12 a	2.80±0.20
	A_*B. juncea*	28,425±3,014	151±8 b	179±15 b	3.21±0.09
	A_*R. sativus*	29,545±4,991	151±5 b	175±3 b	3.06±0.09
	A_*Tagetes*	31,643±980	128±10 ab	142±12 a	3.26±0.10

Data is presented as mean±SEM. RD, replant disease soil. Letters indicate significant differences within site, Tukey test $p < 0.05$ and n = 4, except that RD soil treated with *Tagetes*, n = 3. Increased and decreased fungal richness and diversity in treated RD soils compared to untreated within site are highlighted in green and red, respectively. Chao1, species richness.

Table 2.3.6: Analysis of similarity of fungal community composition in treated replant disease soils compared to untreated based on OTUs of fungal ITS regions

Treatment	Site K		Site A	
	R-value	*p*-value	R-value	*p*-value
Basamid	0.59	0.030	0.65	0.025
B. juncea	1.00	0.031	0.31	0.028
R. sativus	1.00	0.028	0.64	0.029
Tagetes	0.74	0.030	0.13	0.310

Sites K vs. A, R-value = 0.40 and $p < 0.0001$.
R- (-1 to 1) and *p*- values were obtained from ANOSIM test. R value closes to "1" suggests strong dissimilarity between the communities being compared, the value close to "0" represents an even distribution of the communities within and between treatments.

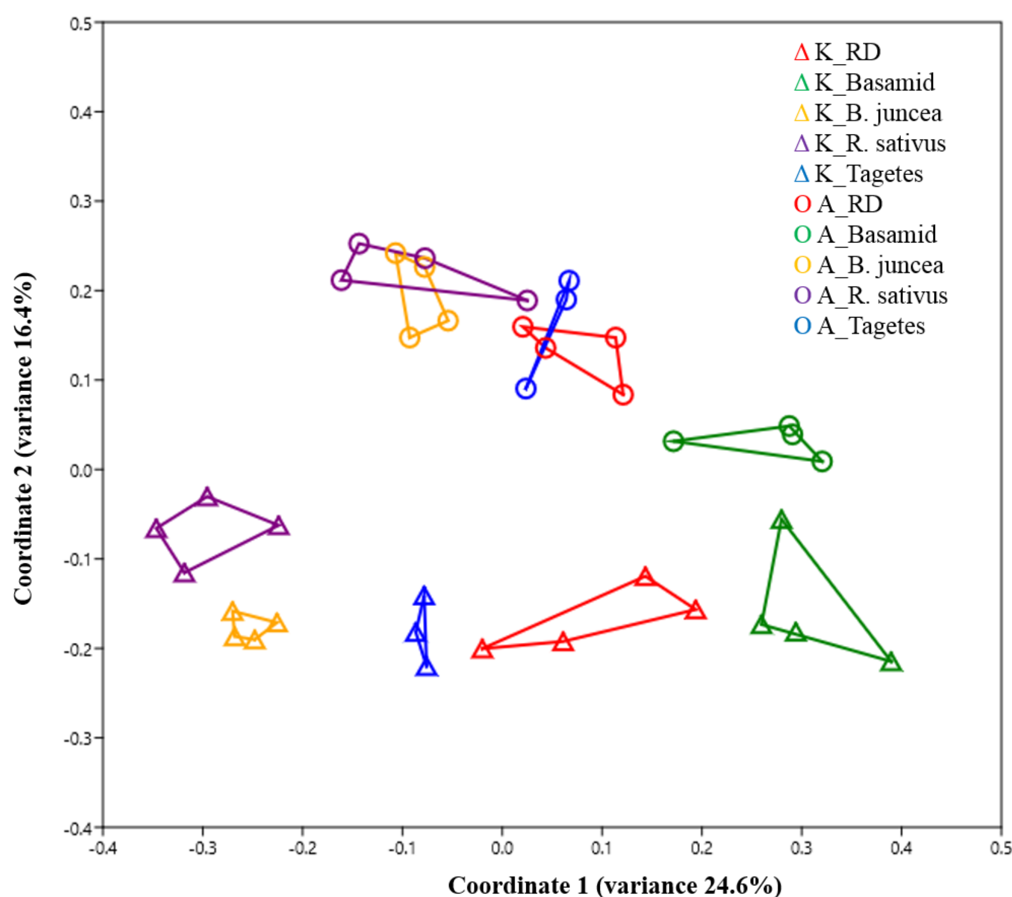

Figure 2.3.3: Effects of different treatments under field condition on soil fungal community composition revealed by principal coordinate analysis (PCoA) using Bray-Curtis distance metric. Past3 and n = 4, except that treatment with *Tagetes*, n = 3. Soil samples were taken four weeks after different treatments in September 2013.

The fungal phylum *Ascomycota* was most abundant in all soils and at all sites (Figure 2.3.4; Table S2.3.5). Relatively high proportion was observed for unclassified fungi, accounting for 11.03 and 19.43% in RD soils at sites K and A, respectively (Figure 2.3.4). The fungal phylum *Basidiomycota* was significantly

reduced in relative abundance by about 50% by Basamid treatment at both sites. Its members were found significantly increased (3.7-fold) by the *R. sativus* treatment at site K, but not significantly at site A. Here, high variation between the replicates was recorded and no significant effects of the treatments were detected, except for those mentioned for *Basidiomycota* (Figure 2.3.4).

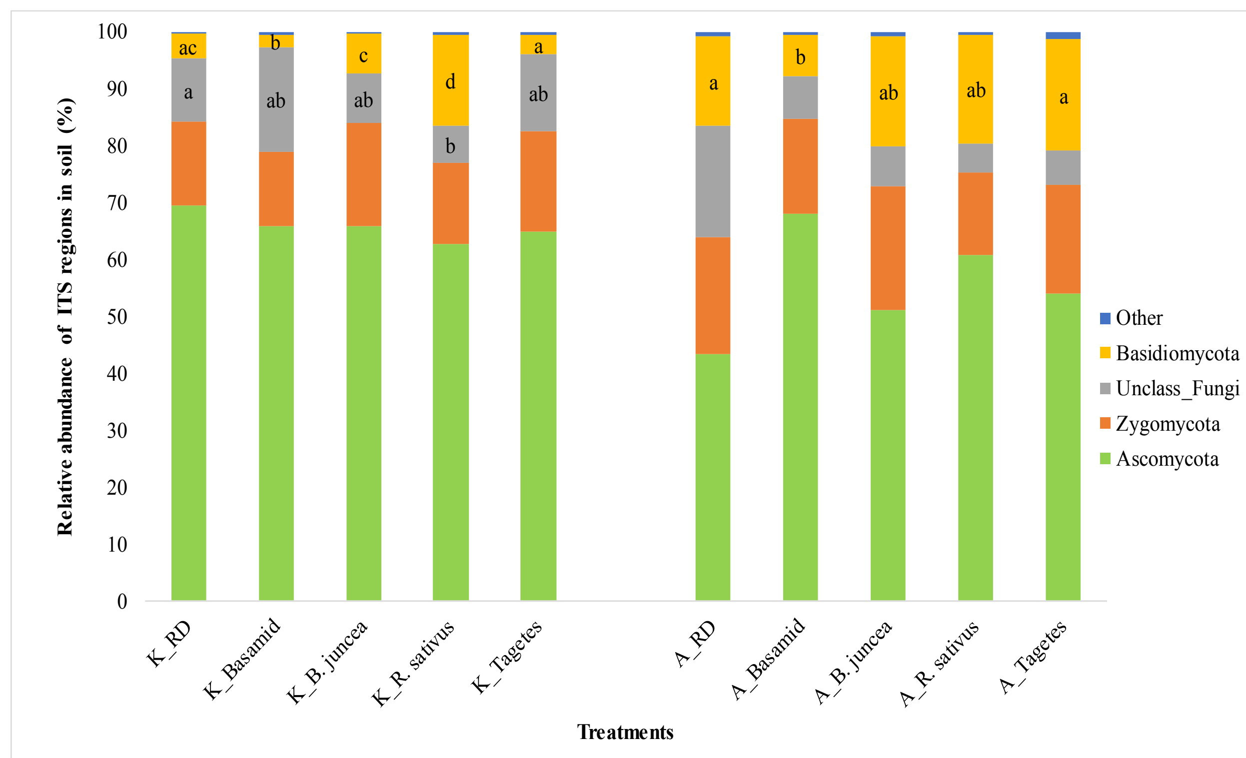

Figure 2.3.4: Relative abundance of dominant fungal phyla in replant disease soils at two sites affected by different treatments. Different letters indicate significant differences in relative abundance of fungi at phylum levels affected by soil treatments within site, Tukey test, $p < 0.05$ and n = 4, except that soil treated with *Tagetes*, n = 3.

Due to the high standard deviations, only fungal sequences affiliated to *Leotiomycetes (Incertae sedis),* were identified as common responder to the Basamid treatment with significantly higher relative abundance compared to untreated RD soils (Table 2.3.7). Similar responses in RD soil biofumigated with either *B. juncea* or *R. sativus* were obtained for the fungal genera *Podospora*, *Monographella* and *Mucor*, all of them significantly increasing in relative abundance, and for *Ypsilina* the proportions of which significantly decreased at both sites. Among them, the fungal genera *Podospora* (19.19%) and *Monographella* (16.52%) had the highest relative abundances in soil treatments with *B. juncea* at site K and *R. sativus* at site A, respectively (Table 2.3.7). Regarding soil treated with *Tagetes*, more pronounced effects were observed at site K compared with site A. Not only the analysis of similarity showed a significant higher R-value (0.74), but also several fungal genera were highly affected in their population compared to the untreated RD soil,

e.g. members of unclassified *Pleosporales*, *Tetracladium* and unclassified *Sordariomycetes* (site K, Tables 2.3.6, 2.3.7).

Irrespective of soil treatments and sites, members of unclassified *Pleosporales*, *Cryptococcus* and *Mucor* were negatively and significantly correlated with growth of apple rootstock M106 plants (shoot and root). Correspondingly, the relative abundance of unclassified *Pleosporales* was significantly reduced after treatments with *B. juncea*, *R. sativus* and *Tagetes* at site K (Tables 2.3.7, 2.3.8). The remarkably increased relative abundance of members of unclassified *Sordariomycetes* in *B. juncea* (11.64%), *R. sativus* (15.06%) and *Tagetes* (16.15%) soils at site K were positively and significantly correlated with the growth of M106 plants. Furthermore, a positive correlation to growth of the apple M106 plants was demonstrated for the fungal genera *Podospora* and unclassified *Sordariales* (Table 2.3.8).

Table 2.3.7: Relative abundance of fungal genera detected in TC-DNAs extracted from bulk soils collected four weeks after different treatments of replant disease soils at two sites (only genera with relative abundance > 0.5 % are shown)

Phylum/ Family	**Genus**	**Site K**					**Site A**				
		K_RD	**K_Basamid**	**K_*B. juncea***	**K_*R. sativus***	**K_*Tagetes***	**A_RD**	**A_Basamid**	**A_*B. juncea***	**A_*R. sativus***	**A_*Tagetes***
Ascomycota											
Unclass_Pleosporales	*Unclass_Pleosporales*	6.35±0.78 a	3.44±0.67 ac	1.17±0.08 b	0.80±0.07 b	2.10±0.24 c	5.22±1.46	3.36±1.20	4.51±0.78	2.91±0.39	5.25±1.27
Pleosporaceae		0.48±0.13	0.11±0.05	0.55±0.22	0.16±0.03	1.18±0.45	0.29±0.11 ab	0.06±0.04 a	0.83±0.20 b	0.37±0.07 b	0.45±0.11 ab
	Dendryphion	0.09±0.02 a	0.00±0.00 b	0.41±0.20 abc	0.11±0.01 a	1.14±0.46 c	0.20±0.10 ab	0.02±0.01 a	0.60±0.21 b	0.22±0.07 b	0.28±0.08 b
Trichocomaceae		0.47±0.08 a	6.82±2.05 b	0.77±0.22 a	1.84±1.14 ab	0.28±0.01 a	3.46±1.10 ab	5.24±0.90 b	1.89±0.22 a	4.61±1.63 ab	1.59±0.15 a
	Penicillium	0.34±0.10 a	6.67±2.05 b	0.70±0.19 a	1.76±1.15 ab	0.21±0.05 a	3.15±1.10 ab	3.59±0.45 a	1.23±0.23 b	4.23±1.51 ab	0.96±0.12 b
Incert_sed_Ascomycota		2.29±0.57 a	0.36±0.24 b	0.23±0.07 b	0.37±0.06 b	2.82±0.39 a	1.68±0.48 ac	0.20±0.08 b	0.44±0.07 ab	0.56±0.13 bc	0.84±0.07 c
	Ypsilina	1.61±0.38 ab	0.30±0.24 bc	0.04±0.03 c	0.07±0.01 c	2.29±0.38 a	1.49±0.39 a	0.07±0.04 b	0.16±0.01 b	0.18±0.01 b	0.74±0.03 a
Incert_sed_Helotiales		5.12±0.53 a	0.49±0.37 b	0.57±0.15 b	0.72±0.14 b	10.96±0.92 c	1.69±0.65 ab	1.15±0.64 ab	0.54±0.17 b	0.36±0.14 b	2.94±0.48 a
	Tetracladium	4.12±0.73 a	0.09±0.05 b	0.44±0.11 bc	0.67±0.13 c	10.41±0.99 d	1.08±0.34 ab	0.84±0.63 ab	0.29±0.10 a	0.28±0.10 a	2.66±0.43 b
Incert_sed_Leotiomycetes		0.39±0.12 a	4.17±1.17 b	0.09±0.04 a	0.11±0.04 a	0.22±0.04 a	0.42±0.07 a	5.61±1.94 b	0.30±0.07 a	0.20±0.04 a	0.38±0.09 a
	Incert_sed_Leotiomycetes	0.17±0.04 a	3.88±1.01 b	0.04±0.02 a	0.10±0.03 a	0.06±0.02 a	0.41±0.06 a	5.42±1.96 b	0.22±0.03 a	0.19±0.04 a	0.23±0.12 a
Myxotrichaceae	*Pseudogymnoascus*	6.02±2.13 ab	26.19±10.85 b	0.62±0.14 cd	0.38±0.06 c	1.20±0.13 ad	0.58±0.15 a	10.85±3.16 b	0.47±0.31 a	0.54±0.28 a	0.33±0.17 a
Ascobolaceae		5.68±2.02 ac	0.09±0.07 b	8.87±2.12 ac	16.83±5.59 c	2.46±0.53 a	0.58±0.26	0.09±0.06	0.39±0.18	1.09±0.49	1.20±0.64
	Ascobolus	5.68±2.02 ac	0.09±0.07 b	8.86±2.11 ac	16.83±5.59 c	2.44±0.53 a	0.55±0.26	0.01±0.00	0.37±0.17	1.04±0.50	1.13±0.58
Unclass_Sordariomycetes	*Unclass_Sordariomycetes*	2.09±0.87 a	1.09±0.12 a	11.64±1.78 b	15.06±0.38 b	16.15±5.84 b	3.19±0.95	3.17±1.02	5.08±1.32	4.52±0.84	3.28±1.29
Unclass_Sordariales	*Unclass_Sordariales*	1.51±0.40 a	0.82±0.28 a	6.99±1.14 b	1.12±0.19 a	3.16±1.21 ab	0.52±0.08 a	0.63±0.11 a	1.35±0.28 ab	1.54±0.68 ab	2.21±0.48 b
Chaetomiaceae		0.69±0.06 ab	0.36±0.10 a	0.47±0.10 a	1.30±0.21 b	0.53±0.13 ab	0.36±0.09 ab	0.19±0.06 a	1.11±0.24 b	1.12±0.22 b	0.51±0.11 ab
	Unclass_Chaetomiaceae	0.28±0.06 a	0.03±0.00 b	0.36±0.09 a	1.19±0.21 c	0.31±0.06 a	0.33±0.09 a	0.15±0.05 a	1.00±0.17 b	1.05±0.20 b	0.42±0.06 ab
Lasiosphaeriaceae		0.50±0.19 a	0.84±0.28 a	20.02±1.23 b	6.19±0.52 c	1.56±0.36 a	0.25±0.06 a	0.54±0.15 ab	2.16±0.55 b	1.51±0.60 ab	1.21±0.23 b
	Podospora	0.20±0.12 a	0.22±0.12 a	19.19±1.06 b	5.59±0.45 c	0.19±0.04 a	0.02±0.01 a	0.04±0.01 a	1.48±0.56 b	0.39±0.07 b	0.01±0.01 a
Incert_sed_Xylariales	*Monographella*	0.53±0.26 a	0.11±0.02 a	2.56±0.37 bc	4.21±0.53 c	1.92±0.21 b	0.22±0.10 a	0.13±0.03 a	7.47±1.08 b	16.52±4.46 b	0.60±0.24 a
Basidiomycota											
Incert_sed_Tremellales		2.20±0.61 ab	1.00±0.29 a	2.91±0.41 b	7.72±0.76 c	2.00±0.28 ab	11.44±1.75	2.60±0.58 b	10.10±0.61 a	12.64±2.70 ac	14.58±0.10 c
	Cryptococcus	2.17±0.59 ab	0.85±0.27 a	2.79±0.40 b	7.63±0.76 c	1.93±0.28 ab	11.28±1.72	2.49±0.60 b	9.89±0.60 a	12.51±2.70 ac	14.30±0.17 c
Trichosporonaceae	*Trichosporon*	0.18±0.07 a	0.15±0.09 a	3.39±0.84 b	6.43±1.83 b	0.06±0.01 a	0.66±0.26 a	0.21±0.09 a	7.61±4.31 ab	4.93±0.49 b	0.35±0.13 a
Zygomycota											
Mucoraceae	*Mucor*	0.30±0.05 a	0.16±0.08 a	0.85±0.06 b	2.00±0.37 c	1.00±0.68 abc	0.47±0.17 a	1.12±0.21 ab	3.33±0.95 b	2.89±0.50 b	0.61±0.16 a

Data is presented as mean±SEM. Significant differences in relative abundance due to different treatments were assessed by R3.2.2 applying Tukey test, $p < 0.05$ and n = 3. Increased and decreased relative abundance in treated replant disease (RD) soils compared to untreated within site are highlighted in green and red, respectively. Colored cells indicate those changes that were found at both sites.

Table 2.3.8: Pearson correlation coefficient (r) between fungal relative abundance and growth of apple rootstock M106 plants grown under field conditions

Phylum	Genus	Relative abundance (%)	SFM		RFM	
			r	*p*-value	r	*p*-value
Ascomycota	*Unclass_Pleosporales*	3.58±0.43	-0.57	0.001	-0.37	0.044
	Unclass_Sordariomycetes	6.57±1.11	0.54	0.002	0.39	0.035
	Unclass_Sordariales	1.98±0.39	0.44	0.016	0.23	0.218
	Podospora	2.76±1.08	0.38	0.036	0.17	0.364
Basidiomycota	*Cryptococcus*	6.54±0.99	-0.36	0.049	-0.54	0.002
Zygomycota	*Mucor*	1.26±0.23	-0.22	0.239	-0.40	0.027

Relative abundance is presented as mean±SEM. SFM, shoot fresh mass and RFM, root fresh mass. Past3 and n=4, except that treatment with *Tagetes*, n = 3

2.3.4 Discussion

Changes in bacterial and fungal community composition and relative abundances based on Illumina sequencing of 16S rRNA gene or ITS fragments amplified from TC-DNAs extracted from soils after treatments with Basamid, *B. juncea*, *R. sativus* and *Tagetes* were investigated via comparison to corresponding untreated RD soils at two sites in order to identify causes for the differential improved plant growth in treated soils.

The observed differences in soil bacterial and fungal community compositions between the two RD sites were in line with our previous findings (Yim et al., 2015; 2016). The two RD sites differed in soil type, soil physical and chemical properties and soil cultivation and management history (Yim et al., 2015; 2016). Different soil microbiomes with different capacities in RD development of the two studied sites were in line with previous observations of soil microbiomes being shaped by different plant species or genotypes (St. Laurent et al., 2010; Uroz et al., 2016), soil types and soil amendments like mineral nutrients (Bakker et al., 2015).

Also the soil treatments differed in their efficacy in a site dependent way (Figures 2.3.1, 2.3.3; Tables 2.3.3, 2.3.7). This is most likely due to the fact that ITCs, the toxic compounds released from the treatments with Basamid (methyl-ITC), *B. juncea* (allyl-ITC) and *R. sativus* (4-methylthio-3-butenyl-ITC) differed in their profiles and concentrations depending on the site (Yim et al., 2016). Variations in toxicity of different ITC compounds against tested pathogens were previously reported (Neubauer et al., 2014).

Microbial taxa associated with apple RD symptoms were not consistently detected in the recent TC-DNAs based studies in apple RD soils (Sun et al., 2014; Franke-Whittle et al., 2015; Yim et al., 2015; Nicola et al., 2017). For example, several bacterial genera such as *Gp5*, *Gp6*, *Gp9*, *Geobacter* (Nicola et al., 2017), *Gemmatimonas*, *Devosia*, *Sphingomonas* (Franke-Whittle et al., 2015), *Phenylobacterium* and *Lysobacter*

(Sun et al., 2014; Franke-Whittle et al., 2015) and the fungal genera *Cryptococcus*, *Mortierella* and *Tricharina* (Nicola et al., 2017) were not routinely identified to be linked with apple RD incidence in which their relative abundances were negatively correlated with growth of apple plants. In the present study, the bacterial genus *Flavitalea* and the fungal genera unclassified *Pleosporales*, *Cryptococcus* and *Mucor* could be associated with RD incidence with M106 plants as indicated by a negative correlation to the shoot or root growth (Tables 2.3.4, 2.3.8). In contrast, the bacterial genera *Arthrobacter*, *Curtobacterium*, *Terrimonas*, *Ferruginibacter* and the fungal genera unclassified *Sordariomycetes*, unclassified *Sordariales* and *Podospora* revealed a positive correlation to the shoot or root growth of M106 plants.

The positive and negative correlations of the fungal genera *Podospora* and *Cryptococcus*, respectively, to plant growth in the present study were in agreement with the observations by Franke-Whittle et al. (2015) who analysed microbial communities at different apple replant disease sites. The relative abundances of several bacterial genera, like *Arthrobacter*, *Terrimonas* and *Ferruginibacter* and fungal genera, for instance *Podospora* that were positively and significantly correlated with growth of the apple M106 plants (Tables 2.3.4, 2.3.8) were lower in RD soils treated with Basamid, *B. juncea*, *R. sativus* and *Tagetes* at site A than at site K (Tables 2.3.3, 2.3.7). These differences might contribute to explain the lower effectiveness of these treatments at site A revealed by the growth of M106 plants. Thus, knowing RD site specificities such as its local selected microbiomes influenced by soil properties, soil quality and pedoclimatic conditions is an important point before choosing the right RD management strategies. Such sequence approaches used in the present work are important in identifying potential bioindicators in the RD soils (Nunes et al., 2016; Schöler et al., 2017).

The effects of the *Tagetes* treatment on soil bacterial and fungal community composition (Tables 2.3.2, 2.3.6; Figures 2.3.1, 2.3.3) and relative abundances of different fungal and bacterial genera (Tables 2.3.3, 2.3.7) were lower than those resulting from *B. juncea* and *R. sativus* treatments. This could at least partially be due to the fact that samples were taken when *Tagetes* plants were still growing in 2013, thus only root exudates, but not ploughed plant biomass could contribute to the observed effects. Shifts in bacterial and fungal relative abundances in the *Tagetes*-treated soils would probably have been higher if the analysed samples had been taken four weeks after plant tissue incorporation. In 2012, however, the total plant biomass from *Tagetes* was incorporated into the soil. Therefore, several bacterial and fungal groups were significantly altered in abundance by this treatment, although site-dependently (Tables 2.3.3, 2.3.7). *Tagetes* are known as nematode-repellent plants due to their sulfur-containing heterocyclic compounds, thiophenes, produced by plant roots (Marotti et al., 2010; Marahatta et al., 2012; Saha et al., 2012). In the present study, soil-borne plant endoparasitic nematode *Pratylenchus* sp. which has previously been reported to be associated with apple RD soil (Mai et al., 1994) were strongly reduced in *Tagetes*-treated soil compared with the untreated RD soils, especially at site A (Table S2.3.6). Besides thiophenes, terpenoids including

dihydrotagetone, piperitone and α-terpineol were predominantly identified in leaves and flowers of *Tagetes* (Saha et al., 2012). The thiophenes and terpenoids showed highly suppressive potential for several soil-borne and foliar plant pathogenic fungi of several crops such as finger millet (*Pyricularia grisea*), French bean (*Rhizoctonia solani*, *Fusarium solani* and *Sclerotium rolfsii*), pea (*Fusarium oxysporum*) and tomato (*Alternaria solani*) in an *in vitro* study (Saha et al., 2012). Despite the less pronounced changes in soil bacterial and fungal community composition in soils cropped with *Tagetes* plants compared to other treatments (Tables 2.3.3, 2.3.7; Figures 2.3.1, 2.3.3), interestingly, the growth of the indicator plants, M106, showed comparable effects among all treatments at site K (Table S2.3.1). Therefore, soil-borne plant pathogenic nematodes were possibly one of the causal ARD agents in the analysed soils that were suppressed by the *Tagetes* treatment.

The stronger effect observed on fungal community compositions in RD soils treated with *B. juncea* and *R. sativus* compared to bacteria (Figures 2.3.1, 2.3.3; Tables 2.3.2, 2.3.6) confirmed the observations made in several other studies when the soils were submitted to products containing ITCs (Hollister et al., 2013; Hu et al., 2015). Interestingly, at site K, a higher effect on soil fungi and a lower effect on soil bacteria in RD soils treated with *B. juncea*, *R. sativus* and *Tagetes* (R values, Tables 2.3.2, 2.3.6) was found in line with the biomass of apple rootstock M106 plants being significantly higher only at this site as well (Table S2.3.1; Yim et al., 2016). This shows that soil at site K was more affected by RD, pointing to a more important role of fungi in RD incidences, as stated earlier by Mazzola (1998).

2.3.4.1 Bacterial responders to different treatments of replant disease soils

A pronounced and significant enrichment of the bacterial phylum *Actinobacteria* was observed in RD soils treated with *R. sativus* at sites K and A (Figure 2.3.2; Table S2.3.3). Members of this phylum are generally known as plant growth promoting (PGP) bacteria being involved in soil-borne disease suppression (Palaniyandi et al., 2013). A closer look at the genus levels of the responders belonging to this phylum revealed that *Arthrobacter* shared the highest proportion in the RD soils when they had been treated with *B. juncea* (at site K) or *R. sativus* (at both sites) (Table 2.3.3). *Arthrobacter* sp. were previously reported as PGP bacteria, as degraders of phenolic compounds in soil (Karigar et al., 2006; Unell et al., 2008) and releasing plant-available iron (Valencia-Cantero et al., 2007). Siddikee et al. (2010) identified traits of isolates affiliated to *Arthrobacter nicotianae* such as nitrogen fixation, indole acetic acid (IAA) production, thiosulfate oxidation, ammonia production and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity strengthening plants to tolerate salt stress conditions. The bacterial genus *Arthrobacter* was also significantly higher in relative abundance in RD soils treated with gamma irradiation and concomitantly, apple plant growth was significantly enhanced in irradiated soils (Yim et al., 2015). Hence, *Arthrobacter* species in biofumigated soils possibly contributed to enhanced growth of M106 plants.

Furthermore, other members of *Actinobacteria* such as *Salinibacterium* and *Curtobacterium* also responded to the Basamid treatments at sites K and A (Table 2.3.3). These bacterial groups were possibly involved in biodegradation of the Basamid remnant in the soil. The *Curtobacterium* sp. strain 114-2 was capable to degrade the toxic trichothecenes in culture medium (Ueno et al., 1983). Moreover, *Curtobacterium flaccumfaciens* strain ME1 that was discovered to promote the plant growth and to protect cucumber plants from leaf spot disease (Raupach and Kloepper, 2000). In addition, this strain was reported to have a comparable effect as the soil fumigant methyl bromide (Raupach and Kloepper, 2000). Other plant growth promoting traits such as solubilizing phosphate, producing IAA as well as catalase and ACC deaminase activity were reported for the *Curtobacterium* sp. strain S6 (Bulgari et al., 2014).

Members of the bacterial genus *Ferruginibacter* (phylum *Bacteroidetes*) which were identified in significantly higher abundance in *B. juncea* (site K) and *R. sativus* (site A) soils compared with untreated RD soil (Table 2.3.3) were demonstrated to be able to decompose cellulose (Lewin et al., 2016). Cellulose is the major component of the plant cell wall (Kögel-Knabner, 2002) and oomycetes (Mélida et al., 2013). Therefore, it cannot be excluded that these members (*Ferruginibacter*) play a role in carbon mineralization and oomycete cell wall degradation in the treated soil.

The enrichment of the genus *Rhodanobacter* in Basamid soil at sites K and A was in line with their detection in higher abundance in gamma-irradiated RD soil (Yim et al., 2015), and the apple plants were significantly increased in their biomass in this treated soil.

The significant increase in *Massilia* relative abundance in Basamid soil at site K and its positive correlation with plant growth (Tables 2.3.3, 2.3.4) suggest that it might be part of a beneficial soil bacterial group, as this genus contains species that are able to produce and secrete chitinase (Cretoiu et al., 2013). Activating chitin degraders in soils has been shown to be related with the suppression of plant pathogens containing chitin structures like fungal cell walls and the exoskeleton of invertebrates (Rinaudo, 2008; Hjort et al., 2009; Jacquiod et al., 2013). The bacterial genus *Massilia* was also reported to show a positive correlation to the shoot growth of apple plants grown in ARD soils in a recent TC-DNA based study (Nicola et al., 2017).

Although members of the genus *Pseudomonas* were significantly reduced in relative abundance in soils treated with Basamid and *Tagetes* at site K, their abundances were not negatively associated with the growth of apple M106 plants in the present investigation (Table 2.3.3). *Pseudomonas* sp. is known as a beneficial bacterium for plant growth since it enhances sulphate uptake (Behera et al., 2014) and acts as antagonists against soil pathogenic fungi (Zaccardelli et al., 2013). At the same time, the genus contains plant pathogens; therefore, an identification of the species would be needed to enable statements on their effects.

A significantly decreased relative abundance of *Streptomyces* in all treated soils at site K and an increase of relative abundances of *Arthrobacter* in *B. juncea* (site K) and *R. sativus* (sites K, A) soils observed in the

present study was also reported by Mazzola et al. (2015) when soils were treated with seed meal from *Brassica* crops.

2.3.4.2 Fungal responders to the different treatments of replant disease soils

In the present study, a huge amount of plant biomass from *B. juncea* and *R. sativus* was incorporated into soils for biofumigation, and thus enhanced fungal groups that are potentially able to degrade plant celluloses were recorded. Among identified responders, cellulose degraders were previously reported for isolates belonging to the fungal genera *Trichosporon* (Santos and Linardi, 2001; Štursová et al., 2012), *Mucor* (Mahmood et al., 2006) and *Podospora* (Couturier et al., 2016).

The fungal genus *Podospora* contains *Podospora anserina* as a coprophilous fungus which is efficient in degrading plant biomass due to its lignocellulose-acting enzymes (Couturier et al., 2016). Besides, the genus *Podospora* was also previously shown to enhance root growth of pea plants (Xu et al., 2012). Moreover, the positive correlation of the fungal genus *Podospora* to apple growth was also recorded by Franke-Whittle et al. (2015). Thus, the significantly increased relative abundance of *Podospora* in *B. juncea* and *R. sativus* treated soils at both sites in the present study (Table 2.3.7) might suggest that these taxa contributed to antagonism relationship with pathogenic microorganisms in apple RD soils.

A high relative abundance in soils treated with *B. juncea* or *R. sativus* (at both sites) and planted with *Tagetes* at site K was also recorded for the fungal genus *Monographella* (Table 2.3.7). Berg et al. (2005) reported that isolates of the genus *Monographella* from the rhizosphere of *Brassica napus* plants displayed antagonistic activity against *Verticillium dahliae* Kleb.

The significantly enriched members of *Penicillium* in Basamid-treated soil (site K) and *Trichosporon* in *B. juncea*- (site K) and *R. sativus*- (sites K, A) treated soils were in agreement with the study of Franke-Whittle et al. (2015) who assumed these genera to be beneficial for growth of apple rootstock plantlets.

Members of *Tetracladium* were significantly reduced by treatments with Basamid, *B. juncea* and *R. sativus* at site K (Table 2.3.7), which is in contrast to the finding that this fungal group was earlier shown to have a positive effect on growth of apple plants (Franke-Whittle et al., 2015). On the other hand, the relative abundance of members of *Tetracladium* was 2.5 times higher after *Tagetes* treatment than in untreated RD soils at site K (Table 2.3.7).

The unclassified fungal genus *Pleosporales* was recorded in a relatively high proportion in untreated RD soils (both sites), but significantly decreased in relative abundance after treatments with *B. juncea*, *R. sativus* and *Tagetes* at site K (Tables 2.3.7, 2.3.8). They are belonging to the order *Pleosporales* which contains several plant pathogens (Zhang et al., 2009). The genome analysis confirmed that the fungal order *Pleosporales* contained several enzymes that are associated with plant pathogenicity (Ohm et al., 2012) such as glycoside hydrolases, lipases and peptidases as well as small secrete protein to infect the plant cells.

In the present study, the detected relative abundance of the unclassified *Pleosporales* was negatively correlated with the growth of the apple M106 plants (Table 2.3.8). Thus, the suppression of their relative abundance in *B. juncea*-, *R. sativus*- and *Tagetes*-treated soils (site K, Table 2.3.7) might have positive effects on the plant growth due to possible reduction of specific microbial pathogenic groups. No obvious correlation between bacteria and fungi at the alpha and beta diversity levels could be detected (data not shown). The relative abundance of the fungal unclassified *Pleosporales* in the untreated RD soils was observed to be negatively correlated to several bacterial groups that were significantly enhanced in their relative abundances by the soil treatments (Figure S2.3.3). Thus, the interaction between different bacterial and fungal taxa should be studied in detail in further analyses.

The pathogenic oomycetes associated with apple RD incidence such as *Pythium* sp. (Hoestra 1994; Emmett et al. 2014) and *Phytophthora* sp. (Mazzola, 1998; Tewoldemedhin et al., 2011; Kelderer et al., 2012) were not detected in the present study due to the primer system used. Thus, primers specific for the oomycetes, Riit et al. (2016), should be included for future amplicon studies as well. For future studies, selected bacterial and fungal genera, which were positively and negatively correlated with the growth of the apple plants in the present work should be further investigated and isolated for their potential application in overcoming RD as promising microbial bioindicators in order to better refine our treatment procedures against RD affected soils.

2.3.5 Conclusion

Bacterial or fungal responders to the soil treatments applied in this study were treatment- and site-dependent. Altered soil microbiome is not only depending on the treatments, but also on soil types (Tkacz et al., 2015; Bakker et al., 2015). Most importantly pre-RD soil treatments improved apple growth as previously published (Yim et al., 2016). The positive and significant effects of the different RD soil treatments on growth of the M106 plants at site K were associated with alterations of both bacterial and fungal communities in the treated RD soils. Since more significant changes involved increased abundances of the respective genera, a certain number of beneficial bacterial and fungal genera is possibly required to enhance the plant growth and to counteract plant-pathogenic taxa. The enriched bacterial and fungal groups detected should be further studied with regard to their potential roles in overcoming RD. The negative correlation with growth of the M106 plants as well as the high relative abundance of the fungal order *Pleosporales* in the untreated RD soils was possible as an indication of a potential fungal pathogenic group in the analysed soils. Overall, the present study revealed shifts in the bacterial and even more pronounced in the fungal communities in response to the treatments of RD soils, and the relative abundance of numerous taxa that were positively correlated to apple plant growth were identified.

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Conflict of Interest Statement: None declared

2.3.6 Supplement

List of supplementary tables

Table S2.3.1: Increases (%) in shoot fresh mass (SFM) of apple M106 plants in treated replant disease soils compared to untreated

Treatment	SFM	
	Site K	Site A
RD	0	0
Basamid	155***	16
B. juncea	148***	1
R. sativus	165***	10
Tagetes	175***	52*

Evaluation was performed with SFM of M106 plants grown under field conditions in 2014. Significant codes: *, $p < 0.05$; **, $p < 0.001$ and ***, $p < 0.0001$. LSD test and n = 3.

Table S2.3.2: Bacterial community diversity based on operational taxonomic unit (OTUs) at 97% similarity in soils at two sites

Site	Number OTU	Chao1	Shannon
K	334±5 a	373±6 a	4.25±0.04 a
A	299±9 b	343±10 b	3.78±0.13 b

Data is presented as mean±SEM. Letters indicate significant differences, Tukey test $p < 0.05$ and n = 15. Chao1, species richness. Increased and decreased bacterial richness and diversity are highlighted in green and red, respectively.

Table S2.3.3: Relative abundance of bacteria at phylum levels in replant disease soils affected by different treatments at two sites

Phylum	Site K					Site A				
	K_RD	K_Basamid	K_*B. juncea*	K_*R. sativus*	K_*Tagetes*	A_RD	A_Basamid	A_*B. juncea*	A_*R. sativus*	A_*Tagetes*
Acidobacteria	0.03±0.02	0.03±0.01	0.04±0.02	0.03±0.00	0.04±0.01	0.10±0.01 a	0.11±0.07 ab	0.20±0.08 ab	0.27±0.03 b	0.10±0.02 a
Actinobacteria	13.47±0.77 a	18.01±1.97 ab	18.22±1.43 ab	21.13±0.98 b	13.55±1.39 a	10.51±0.52 a	13.15±0.84 ab	11.64±3.05 ab	15.74±0.88 b	12.65±0.41 ab
Bacteroidetes	8.38±0.36 a	10.67±0.88 ab	12.79±1.16 b	11.14±0.27 b	10.99±1.34 ab	3.77±0.14 a	6.70±0.36 b	9.08±2.71 abc	10.93±0.16 c	4.54±0.27 a
Chloroflexi	2.19±0.40	1.93±0.36	2.05±0.17	1.98±0.17	2.31±0.09	1.25±0.11 ab	0.82±0.33 ab	0.94±0.28 ab	0.87±0.06 a	1.41±0.09 b
Firmicutes	39.09±2.05	31.17±2.82	28.83±4.36	28.57±2.30	35.45±6.94	51.87±0.47 a	51.97±4.95 ab	52.39±12.63 ab	39.93±0.97 b	49.64±1.11 a
Gemmatimonadetes	6.19±0.80 ab	7.08±0.09 a	5.60±0.44 ab	5.59±0.35 b	7.44±0.80 ab	5.89±1.09	3.42±0.28	3.23±0.68	3.85±0.20	5.69±0.76
Ignavibacteriae	0.26±0.01 a	0.32±0.03 ab	0.35±0.01 b	0.30±0.02 ab	0.33±0.03 ab	0.12±0.02	0.14±0.04	0.20±0.07	0.17±0.01	0.16±0.03
Nitrospirae	1.29±0.18 ab	0.81±0.15 a	1.34±0.14 ab	1.34±0.14 ab	1.47±0.01 b	1.03±0.21 ab	0.49±0.14 a	0.90±0.28 ab	0.92±0.14 ab	1.20±0.06 b
Planctomycetes	3.70±1.35	3.51±0.19	3.67±0.81	3.53±0.28	4.28±1.11	7.60±0.57 a	1.50±0.07 c	3.65±0.84 b	4.06±0.22 b	4.34±0.75 b
Proteobacteria	23.79±1.05	25.05±0.54	25.59±0.80	24.83±0.97	22.58±2.35	16.62±0.41 a	21.11±3.42 ab	16.99±4.61 ab	22.29±1.28 b	19.00±0.38 b
Unclass_Bacteria	1.27±0.11	1.12±0.09	1.16±0.06	1.18±0.06	1.26±0.10	0.87±0.05 a	0.43±0.04 b	0.54±0.16 ab	0.68±0.08 ab	0.92±0.03 a
Verrucomicrobia	0.33±0.01	0.28±0.01	0.35±0.04	0.39±0.03	0.30±0.05	0.37±0.06 ab	0.16±0.02 a	0.25±0.09 ab	0.28±0.01 b	0.36±0.08 ab

Data is presented as mean±SEM. Different letters indicate significant differences between treatments within site, Tukey test, $p < 0.05$ and n = 3. Increased and decreased relative abundances in treated replant disease (RD) soils compared to untreated within site are highlighted in green and red, respectively. Colored cells indicate those changes that were found at both sites.

Table S2.3.4: Fungal community diversity based on operational taxonomic unit (OTUs) at 95% similarity in replant disease soils at two sites

Site	Number OTU	Chao1	Shannon
K	104±3 a	126±5 a	2.78±0.08 a
A	133±5 b	151±7 b	3.03±0.07 b

Data is presented as mean±SEM. Letters indicate significant differences, Tukey test $p < 0.05$ and n = 19.
Chao1, species richness. Increased and decreased fungal richness and diversity are highlighted in green and red, respectively.

Table S2.3.5: Relative abundance of fungi at phylum levels in replant disease soils affected by different treatments at two sites

Phylum	Site K					Site A				
	K_RD	**K_Basamid**	**K_*B. juncea***	**K_*R. sativus***	**K_*Tagetes***	**A_RD**	**A_Basamid**	**A_*B. juncea***	**A_*R. sativus***	**A_*Tagetes***
Ascomycota	69.55±0.69	66.01±6.45	66.02±0.94	62.86±3.16	64.82±3.65	43.38±6.14	68.14±6.76	51.19±3.50	60.74±2.15	53.95±0.49
Basidiomycota	4.38±0.55 ac	2.06±021 b	7.07±0.92 c	16.18±1.65 d	3.35±0.16 a	15.71±1.19 a	7.22±1.86 b	19.52±4.08 ab	19.06±2.95 ab	19.63±2.35 a
Chytridiomycota	0.15±0.05	0.13±0.04	0.08±0.02	0.11±0.05	0.20±0.05	0.46±0.16	0.31±0.11	0.35±0.10	0.22±0.06	0.41±0.07
Glomeromycota	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.09±0.04 b	0.02±0.01	0.01±0.01	0.00±0.00	0.00±0.00	0.02±0.01
Rozellomycota	0.16±0.04	0.44±0.16	0.17±0.03	0.25±0.09	0.26±0.07	0.33±0.12	0.23±0.04	0.27±0.07	0.19±0.08	0.84±0.46
Unclass_Fungi	11.03±0.93 a	18.39±4.41 ab	8.74±0.34 ab	6.53±0.80 b	13.54±2.53 ab	19.43±8.94	7.45±2.91	6.82±1.88	5.23±1.37	5.86±2.06
Zygomycota	14.72±1.94	12.97±4.64	17.92±0.90	14.08±2.96	17.74±1.86	20.66±3.90	16.64±9.88	21.85±2.27	14.55±1.84	19.29±1.63

Data is presented as mean±SEM. Different letters indicate significant differences between treatments within site, Tukey test, $p < 0.05$ and n = 4, except that soil treated with *Tagetes*, n = 3. Tukey test, $p < 0.05$ and n = 3. Increased and decreased relative abundance in treated replant disease (RD) soils compared to untreated within site are highlighted in green and red, respectively. Colored cells indicate those changes that were found at both sites.

Table S2.3.6: Selected nematode populations in analyzed soils sampled in October 2013 (per 100 ml soil)

Species	Site K					Site A				
	RD	**Basamid**	***B. juncea***	***R. sativus***	***Tagetes***	**RD**	**Basamid**	***B. juncea***	***R. sativus***	***Tagetes***
Pratylenchus sp.	10.3±6.8	n.d.	16.0±17.4	19.7±13.9	4.0	77.0±53.4	11.3±5.8	90.7±83.3	89.3±86.4	11.0±2.8

Statistical test was not applied due to heterogeneity of nematode population within the treatment. Data is presented as mean±SD, n = 3. n.d., not detected. The number without ±SD, meaning the detection was found only in one replicate. RD, replant disease soil.

List of supplementary figures

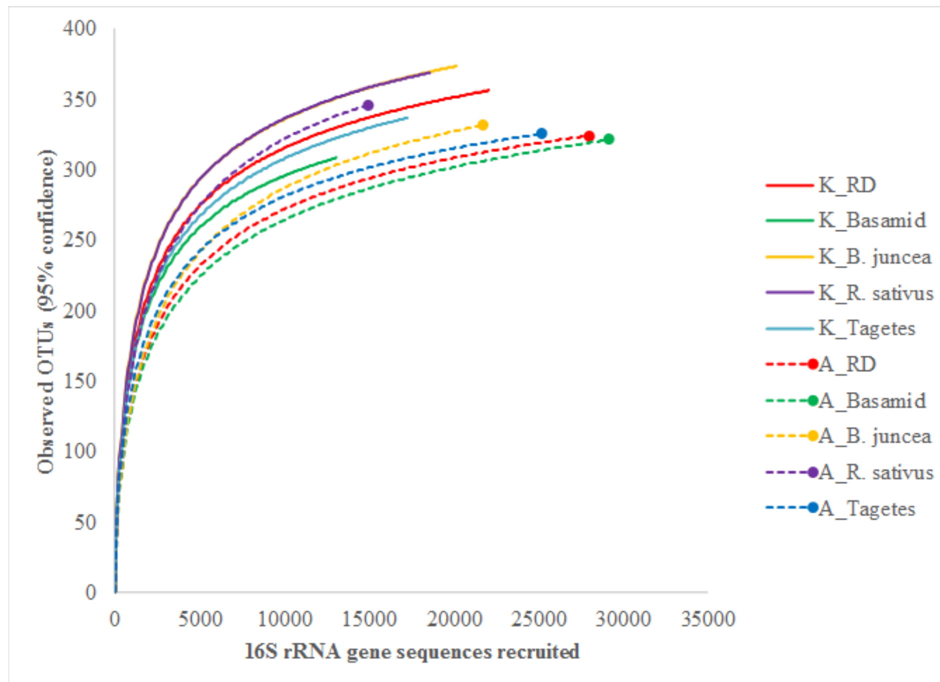

Figure S2.3.1: Rarefaction curves indicating the observed numbers of operational taxonomic unit (OTUs) of bacterial communities in TC-DNA extracted from different treatments of replant disease (RD) soils at sites K and A.

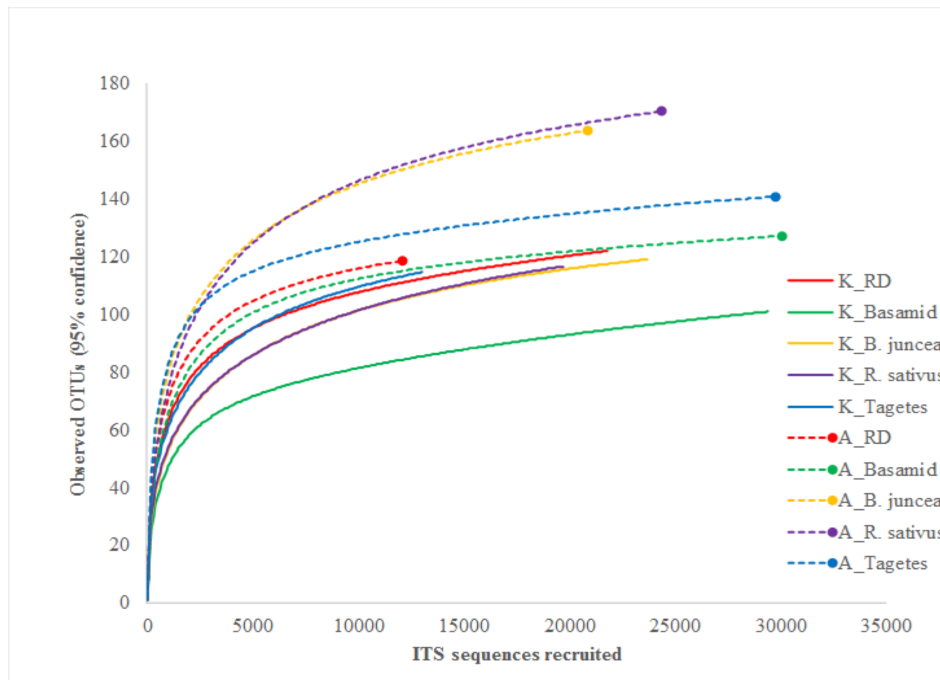

Figure S2.3.2: Rarefaction curves indicating the observed numbers of operational taxonomic unit (OTUs) of fungal communities in TC-DNA extracted from different treatments of replant disease soils at sites K and A.

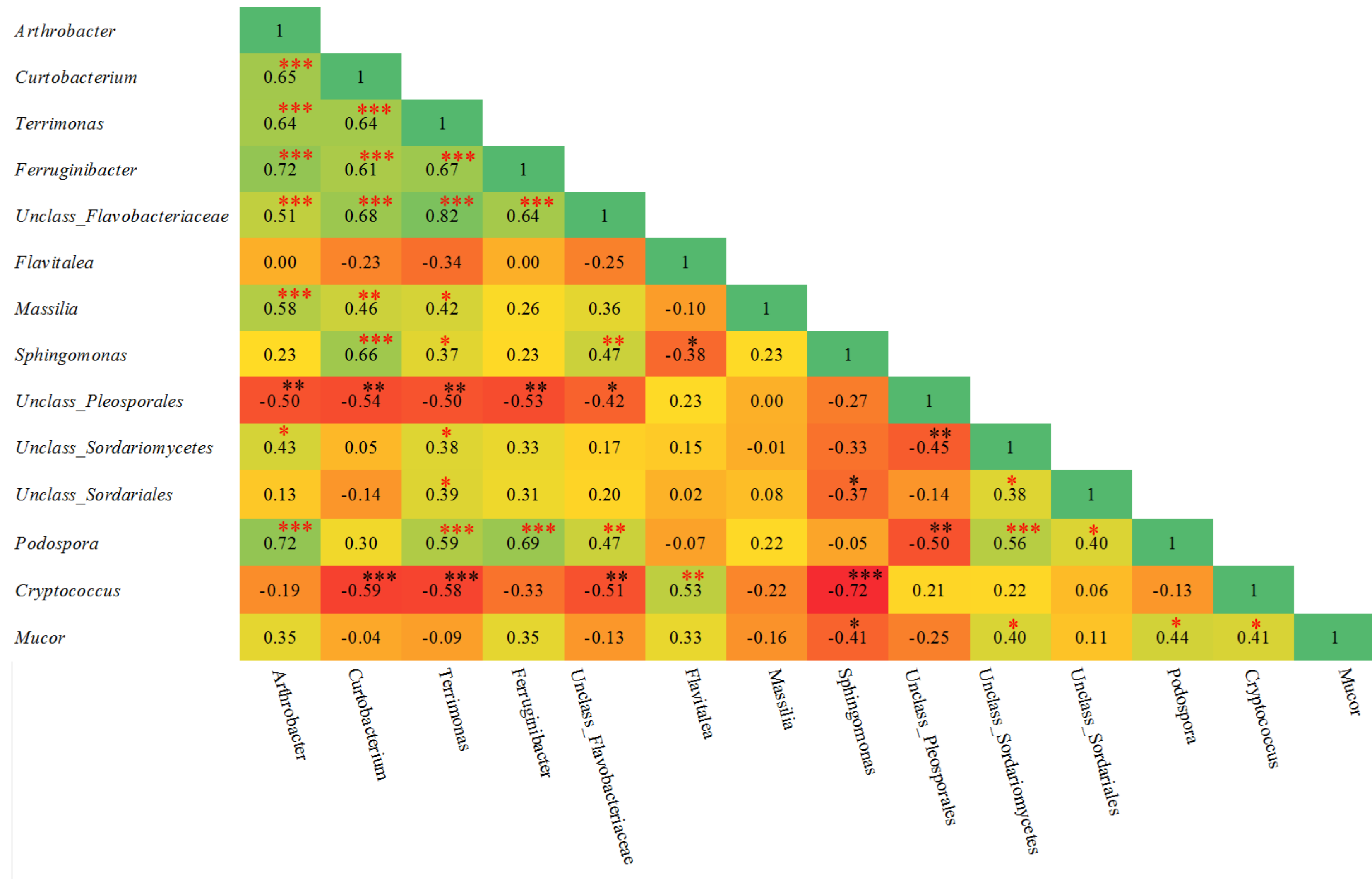

Figure S2.3.3: Spearman correlation coefficient (data in cells) between relative abundance of bacteria and fungi at genus levels, presented by heat map. Asterisk indicates significant correlations between the pairs: *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$. Past3 software.

3 Overall discussion

The study was initiated to develop alternative approaches to overcome replant problems with apple and rose rootstocks through ecologically and environment friendly methods. Biofumigation with *B. juncea* 'Terra Plus' and *R. sativus* 'Defender', treatment with the nematode repellent *Tagetes* 'Nemamix' and conventional soil fumigation with Basamid were included as soil treatments for comparing their effects at three RD sites K, A and M. The effects of the different soil treatments were evaluated based on the growth of indicator plants both in the greenhouse and in the field as well as the soil bacterial and fungal communities.

This section includes additional aspects to supplement the discussion of chapter 2 (sections of the manuscripts) that have only been partly or have not yet been addressed: consisting of (3.1) GSs in organs of *B. juncea* and *R. sativus* and their liberated products in amended soils, (3.2) soil bacterial and fungal communities affected by different treatments of RD soils, (3.3) effects of treatments of RD soils on growth of apple plants, (3.4) mode of action of biofumigation in RD soils and (3.5) mode of action of *Tagetes* treatment in RD soils.

3.1 Glucosinolates in organs of *B. juncea* and *R. sativus* and their degradation products in amended soils

In the present study, biofumigation was applied as a crop rotation using *B. juncea* and *R. sativus* plants, sown from seeds. After eight weeks, when the plants were in mid-flowering stage, the total aboveground shoots were cut off followed by mechanical chopping and crushing prior to incorporating into RD soils. Before the biofumigation treatment, aboveground biomass of the two plant species was determined (per m^2, n = 3 per site) and different plant organs (inflorescences, leaves, stems and roots) were sampled for GS determination.

The total aboveground biomass of *R. sativus* (6.61 – 9.50 kg m^{-2}) was higher than the biomass of *B. juncea* (3.31 – 4.45 kg m^{-2}) plants (Table A6.2). Due to differences in soil physical and chemical properties of the three sites (Yim et al. 2016), the aboveground biomass of *B. juncea* or *R. sativus* plants were lower at site A than those at sites K and M (Table A6.2).

Different GS profiles were detected for the two plant species. The most abundant aliphatic allyl- and 4MT3But-GS was identified in all organs and were highest in inflorescences of *B. juncea* (36.77 – 53.63 µmol g^{-1} dry mass, summer 2013) and *R. sativus* (33.12 – 46.23 µmol g^{-1} dry mass, summer 2013) plants, respectively (Yim et al. 2016). Overall, there was no site effect on the total GS production within plant organs of the respective plant species in the present study. Environmental conditions such as temperature, solar radiation and precipitation as well as plant developmental stages most likely influenced the GS profiles

and concentration detected in the *B. juncea* or *R. sativus* plant organs grown at different time points (analyzed samples in summer 2012 vs. summer 2013 vs. spring 2013) as previously reported (Bellostas et al. 2007; Zhang et al. 2008; Antonious et al. 2009).

Regarding the GS degradation products detected 6 h after biofumigation (plant tissue incorporation), a higher diversity of the detected compounds was recorded for the treatment with *B. juncea* (allyl-CN, allyl-ITC, 2PE-CN and 2PE-ITC) than with *R. sativus* (only 4MT3But-ITC). Differences in the GS degradation products in the two biofumigated soils (with *B. juncea* and *R. sativus*) were due to differences in the GS profiles of the two plant species (see Yim et al. 2016). The ITC concentration detected in treated soils (ally-ITC, 2.072 – 15.035 and 4MT3But-ITC, 0.855 – 2.274 nmol g^{-1} dry soil) of the present study was low although within the range of a previous report (Gimsing and Kirkegaard 2009), which pointed at optimization of the application procedures that needed to be improved, such as maximizing tissue disruption of the biofumigant plants before incorporation into the soil, watering the amended field after the treatments as well as covering the treated field with plastic film due to highly hold the volatile compounds (Morra and Kirkegaard 2002, Cohen and Mazzola 2006; Mattner et al. 2008; Hanschen et al. 2015). Defatted seed meals that are commercially available with higher GS contents, i.e. defatted seed meal from *B. juncea* with up to 303 µmol g^{-1} allyl-GS at 99% of the total GS (Mazzola et al. 2009) could be used for future studies to reach a higher ITC concentration in the soil.

3.2 Soil bacterial and fungal communities affected by different treatments of replant disease soils

3.2.1 Soil bacterial communities affected by heat and gamma irradiation treatments

After growing for eight weeks under greenhouse conditions, the bacterial community composition and diversity was investigated in TC-DNAs extracted from soils attached to roots of the apple M26 plants in untreated (Con) and treated at 50°C (H50) as well as gamma irradiation (Gamma) - at a minimal dose of 10kGy - RD soils from two sites K and A (Yim et al. 2015). At both sites, the treatments of H50 and Gamma in RD soils significantly increased SL, SFM and SDM of the M26 plants compared to those in Con soils after eight weeks of cultivation (Yim et al. 2015). Here, we would like to correlate the enhanced growth of the M26 plants with changes in the bacterial communities and responders.

The DGGE and 454-pyrosequencing of the bacterial 16S rRNA genes amplified from soil TC-DNAs revealed that the two soils (sites K and A) had distinct bacterial community compositions. The treatments H50 and Gamma significantly altered the bacterial community composition of the respective RD sites. The bacterial diversity was reduced by trend in the treatments H50 and Gamma compared to Con, at both sites. The bacterial phylum *Proteobacteria* (28.8 – 30.5 % of total sequences) was most dominant followed by *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Gemmatimonadetes* at both sites (Yim et al.

2015). Site- and treatment-dependent effects were revealed regarding the relative abundance of the bacterial communities both at phyla and genera levels.

The bacterial phylum *Acidobacteria* was significantly reduced in relative abundance by H50 and Gamma treatments compared to Con at both sites and this fact was discussed in Yim et al. (2015) as it possibly resulted from a higher nutrient release from killed organisms due to treatments and proliferation of copiotrophic bacteria. However, another study by Nicola et al. (2017), reported the significant association of the bacterial phylum *Acidobacteria* with RD incidence in apple tree cv. Fuji Fubrax grafted onto M9 rootstock (Spearmann's rank correlation coefficient p = -0.67, relative abundance vs. shoot growth). At genera level, the acidobacterial subgroups *Gp5* and *Gp6* were significantly suppressed in relative abundance by H50 and Gamma treatments at site K in the present study also corresponding to apple RD incidence (Nicola et al. 2017).

In other TC-DNAs based studies several bacterial genera such as *Geobacter* (Nicola et al. 2017), *Gemmatimonas*, *Devosia*, *Sphingomonas* (Franke-Whittle et al. 2015), *Phenylobacterium* and *Lysobacter* (Sun et al. 2014; Franke-Whittle et al. 2015) were linked to apple RD incidence (the relative abundance was negatively correlated to the growth of the apple plants). In contrast, in the present study, these bacterial groups (except for the bacterial genus *Geobacter*) were significantly enhanced in relative abundance H50 or Gamma treatments in which the growth of M26 plants (SL, SFM and SDM) was significantly increased (at site K or A or both sites, Yim et al. 2015). Along with the present finding, Nicola et al. (2017) found the bacterial genera *Phenylobacterium* and *Gemmatimonas* to be positively correlated with the shoot growth of apple trees. Thus, functional roles of these bacterial groups (mentioned above) need to be thoroughly investigated.

Total phenolic compounds (Henfrey et al. 2015) including phlorizin (Hofmann et al. 2009; Emmett et al. 2014; Yin et al. 2016) and phytoalexins 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzofuran, 2'-hydroxyaucuparin and noreriobofuran (Stefan Weiß, unpublished data, Leinbniz Universität Hannover) were detected in affected apple roots grown in untreated RD soils in higher concentrations compared to those cultivated in sterilized RD soils. Due to exudation by apple roots, phlorizin (Hofmann et al. 2009) and other phenolic compounds might be more abundant in apple RD soils. H50 and Gamma treatments of RD soils employed in the present study possibly enriched several bacterial groups that were involved in phenolic compound degradation as well as plant growth promotion and subsequently enhanced the growth of M26 plants.

In the present study, a significantly increased relative abundance via soil treatments was recorded for the bacterial genera *Arthrobacter* (Gamma, site A), *Bacillus* (H50, both sites and Gamma, site K) and *Sphingomonas* (H50, both sites and Gamma, site A). The bacteria *Sphingomonas chlorophenolica* spp. strain RA2 (Bielefeldt and Cort 2005), *Bacillus brevis* (Arutchelvan et al. 2006), *Bacillus cereus*, *Bacillus*

*licheniformi*s, *Bacillus pumilus* and *Arthrobacter* spp. (Karigar et al. 2006; Unell et al. 2008; Gayathri and Vasudevan 2010) were discovered to be able to degrade phenolic compounds in growth medium. Besides, other functional roles of the bacterial responders that were significantly increased in relative abundance in H50/Gamma treatments of RD soils were discussed as plant growth promoting bacteria in Yim et al. (2015) due to production of IAA, ammonia, siderophores and production of antibiotics against soil-borne pathogenic fungi. However, active roles of those bacterial groups (mentioned above) in analyzed soils were speculated in the present study. Hence, future studies on functional roles of the bacterial responders in reducing the RD incidence via H50/Gamma treatments should be considered.

3.2.2 Bacterial and fungal communities affected by soil treatments of replant disease soils with Basamid, *B. juncea*, *R. sativus* and *Tagetes*

At two RD sites K and A, bacterial and fungal communities were investigated in TC-DNAs extracted from bulk soils of different treatments, namely Basamid, two-year biofumigation (with *B. juncea* and *R. sativus*) and *Tagetes* under field conditions, in order to identify responders that caused the suppressed and improved growth of apple plants.

The DGGE fingerprints and Miseq® Illumina® sequencing approaches revealed distinct bacterial and fungal community compositions between the two sites as previously observed (Yim et al. 2015; 2016; Yim et al. 2017, under revision). Higher bacterial diversity was present at site K compared to site A which was in contrast to fungal diversity.

The relative abundance of all bacterial phyla and genera detected in Yim et al. (2017, under revision) were different from those reported in Yim et al. (2015). For instance, the most dominant bacterial phylum reported by Yim et al. (2017, under revision) was *Firmicutes* with 29 - 39 and 40 - 52% at sites K and A, respectively. Differences in relative abundance of the same bacterial phylum/ genus in soil of the same site in the two reports were due to analyzed soils: soil adhered to roots of M26 plants (Yim et al. 2015) vs. bulk soil (Yim et al. 2017, under revision). Differences in total bacterial community composition and relative abundance between rhizosphere and bulk soil were reported previously (Schreiter et al. 2014; Hu et al. 2016; Uroz et al. 2016).

Greater shifts in soil fungal compared to bacterial communities affected by all treatments (Basamid, *B. juncea*, *R. sativus* and *Tagetes*) of RD soils were demonstrated in the present study. Members of the bacterial phyla *Actinobacteria* and *Bacteroidetes* were significantly higher in relative abundance in *R. sativus* treated RD soils compared to those in untreated RD soils at both sites. The relative abundance of other bacterial phyla was site- and treatment-dependent (Yim et al. 2017, under revision).

In the present study, no bacteria genera (with the exception of the bacterial genus *Flavitalea*) were identified to associate with RD incidence. Instead, there was a positive correlation between higher relative abundance

of several bacterial groups and growth of apple M106 plants grown under field conditions. The relative abundance of the bacterial genera *Arthrobacter*, *Curtobacterium*, *Terrimonas*, *Ferruginibacter*, unclassified *Flavobacteriaceae*, *Massilia* and *Sphingomonas* was positively and significantly correlated to the shoot or root growth of M106 plants in the present study.

Several studies presented beneficial functions of the bacterial genus *Arthrobacter* to enhance plant growth as they contain species that are able to degrade phenolic compounds in soil (Karigar et al. 2006; Unell et al. 2008), to release plant-available iron (Valencia-Cantero et al. 2007), to produce IAA and ACC deaminase for promoting root growth and to increase abiotic stress tolerance of plants (Siddikee et al. 2010). The relative abundance of the bacterial genus *Arthrobacter* was also significantly higher in Gamma treated RD soil (Yim et al. 2015). Hence, the bacterial genus *Arthrobacter* in biofumigated soils with *B. juncea* (site K) and *R. sativus* (both sites) possibly contained species contributing to enhance growth of M106 plants.

The positive correlation of bacterial genus *Massilia* to the shoot growth of apple plants (Table 2.3.4) in the present study was in line with finding by Nicola et al. (2017). The significant increase in relative abundance of the bacterial genus *Massilia* in Basamid treated soil at site K (Table 2.3.3) and its positive correlation with the growth of M106 plants possibly resulted in suppression of plant pathogens containing chitin structures like fungal cell walls and the exoskeleton of invertebrates (Rinaudo 2008; Hjort et al. 2009; Jacquiod et al. 2013).

The bacterial genus *Curtobacterium* in Basamid treated soil was significantly higher in relative abundance at both sites. Interestingly, the genus contains the species *Curtobacterium flaccumfaciens* strain ME1 that was discovered to promote the plant growth and to protect cucumber plants from leaf spot disease which showed a comparable effect to soil fumigant methyl bromide (Raupach and Kloepper 2000). Plant growth promoting traits such as solubilizing phosphate, producing IAA as well as catalase and ACC deaminase activity were reported to belong to the bacterial *Curtobacterium* spp. strain S6 (Bulgari et al. 2014).

The bacterial genus *Favitalea* was significantly higher in relative abundance in RD soils treated with *R. sativus* and *Tagetes* at site A, but they showed a negative correlation to both shoot and root growth of M106 plants (Yim et al. 2017, under revision). No study has focused on the negative effect of this bacterial genus (*Flavitalea*) on plant growth so far. However, the isolates *Flavitalea populi* strain HY-50RT (Wang et al. 2011) and *Flavitalea gansuensis* strain JCN-23T (Zhang et al. 2013) did not show any plant growth promoting traits such as production of IAA, solubilizing phosphate, nitrate reduction and urease activity. Thus, functional roles of *Flavitalea* spp. in apple RD incidence should be further investigated in future studies.

Regarding fungi, the fungal phylum *Ascomycota* was most abundant at all sites. Due to high variation, only the fungal phylum *Basidiomycota* was significantly reduced in relative abundance about 50% via Basamid

treatment at both sites. In the present study, the fungal genera unclassified *Pleosporales*, *Cryptococcus* and *Mucor* were associated to RD incidence with M106 plants (as indicated by a negative correlation to the shoot or root growth of plants). In contrast, the fungal genera unclassified *Sordariomycetes*, unclassified *Sordariales* and *Podospora* revealed positive correlation to the shoot or root growth of M106 plants. The association of the fungal genus *Cryptococcus* to apple RD symptoms was previously reported (Franke-Whittle et al. 2015; Nicola et al. 2017).

The biofumigation involved a huge amount of plant biomass from *B. juncea* and *R. sativus* incorporation into soils, and thus enrichment of fungal groups that are able to degrade plant celluloses could have occurred. Among identified responders, cellulose degraders were previously reported for isolates belonging to the fungal genera *Trichosporon* (Santos and Linardi 2001; Štursová et al. 2012) and *Mucor* (Mahmood et al. 2006).

Another study reported that the fungal genus *Podospora* contains *Podospora anserina* as a coprophilous fungus which is efficient in degrading plant biomass due to its lignocellulose-acting enzymes (Couturier et al. 2016). The fungal genus *Podospora* was also previously shown to enhance root growth of pea plants (Xu et al. 2012). In addition, the positive correlation of the fungal genus *Podospora* to apple growth was recorded by Franke-Whittle et al. (2015). Thus, a beneficial effect of *Podospora* members in *B. juncea* and *R. sativus* treated soils at both sites might be due to combating pathogenic microorganisms in apple RD soils in the present study.

Members of *Tetracladium*, which were significantly enhanced in relative abundance in *Tagetes* treated RD soil at site K, were previously identified to show a positive correlation to apple plant growth (Franke-Whittle et al. 2015).

The fungal genus unclassified *Pleosporales* showed a negative correlation to M106 plant growth in the present study. It was also reported that the genus contains plant pathogens to several crops (Zhang et al. 2009; Ohm et al. 2012). Hence, the reduction in relative abundance of this fungal member at site K (treatments *B. juncea*, *R. sativus* and *Tagetes*) possibly contributed to enhance the growth of apple plants.

Overall, *Tagetes* treatment caused less changes in total bacterial and fungal community composition as well as responders compared to Basamid and two-year biofumigation treatments of RD soils at both sites (Yim et al. 2017, under revision). As discussed (Yim et al. 2017, under revision), analyzed soils were collected when *Tagetes* plants were still growing. Thus, for a better comparison, soil collected after total plant biomass incorporation should be used for future TC-DNAs based studies. Besides altering soil bacterial and fungal communities, the striking effect of *Tagetes* treatment against root lesion nematode associated with apple RD, such as *Pratylenchus* spp., was revealed and the effect was comparable to Basamid treatment, especially at site A (Yim et al. 2017, under revision). In contrast, *B. juncea* and *R. sativus* treatments were not efficient against nematodes, especially *Pratylenchus* spp., in both analyzed soils (data not shown).

Different sites with different cropping histories and soil amendment like adding mineral nutrients (Bakker et al. 2015) and different plant species (St. Laurent et al. 2010; Uroz et al. 2016) shaped soil microbiomes. Therefore, detected responders associated with apple RD symptoms were not routinely found among TC-DNAs based studies in apple RD soils (Sun et al. 2014; Franke-Whittle et al. 2015; Yim et al. 2015; Nicola et al. 2017; Yim et al. 2017, under revision). Functional roles of responders which were significantly enhanced and suppressed in their relative abundance due to treatments were not resolved in the present study. For future studies, selected bacterial and fungal genera, which were positively or negatively correlated with the growth of apple plants, should be further investigated for their potential application in overcoming RD incidence.

3.3 Effects of soil treatments of replant disease soils on growth of apple plants

Under greenhouse conditions, apple rootstock M26 plants were significantly increased in their aboveground growth in RD soils with 50°C, Gamma (sites K and A, Yim et al. 2015), biofumigation (with *B. juncea* or *R. sativus*), Basamid or *Tagetes* soil treatments, especially at site K (Yim et al. 2016) compared to those grown in untreated RD soils. Under field conditions, the growth of apple rootstock M106 plants was significantly enhanced in soils treated with *B. juncea* and *R. sativus*, Basamid and *Tagetes* compared to those grown in untreated RD soils (at sites K and M, Yim et al. 2016). The RDM of M26 plants measured at the end of the biotest showed relative increases by trend in treated compared to untreated soils. However, roots of M26 plants grown in all soil treatments (above) demonstrated brighter coloration (Yim et al. 2015; 2016) indicating healthier roots compared to those grown in untreated RD soils (darker in coloration).

The darker coloration of M26 roots in untreated RD soils was previously presumed to be caused by phenolic compounds (Yim et al. 2013) which were later discovered in high abundance in apple roots grown in untreated soil (Emmett et al. 2014; Henfrey et al. 2015; Nicola et al. 2016). One of the phenolic compounds, namely phlorizin, was typically detected in apple roots and exudates (Hofmann et al. 2009; Emmett et al. 2014), and it was recently shown to be associated with the apple RD incidence (Nicola et al. 2016) where apple seedlings growth was significantly reduced when cultivated on a soil mixed with ground apple roots. Similarly, 14 days after cultivation, phytoalexins were detected in 8.5-fold higher concentration in roots of M26 plants grown in RD soil compared to those grown in Gamma-sterilized RD soil (Stefan Weiß et al., unpublished data, Leibniz Universität Hannover). The higher phytoalexin production in M26 roots affected by RD incidence was also recently confirmed through significantly upregulated plant genes involved in phytoalexin production in RD soil compared to Gamma-sterilized RD soil (Weiß et al. 2017). Furthermore, genes involved in primary metabolism, e.g. cell, cell wall, photosynthesis and protein were of lower abundance in M26 roots grown in RD soils compared to Gamma-sterilized soil (Weiß et al. 2017). Thus, the inverse relationship between the production of primary and secondary metabolites in M26 plants as well

as potential autotoxicity resulting from highly abundant phenolic compounds in apple roots grown in untreated soil, could explain the growth reduction of apple plants cultivated in untreated RD soils.
Moreover, previous histological analyses of M26 roots grown in untreated soils (Yim et al. 2013), revealed strong damages, especially in cortical layers of roots. As roots are important for water and nutrient uptake as well as production of cytokinins (among other functions) for shoot growth (Gregory 2006), in the present study, the decline, especially of aboveground growth of M26 plants, was revealed in untreated RD soils compared to treated RD soils.
Root lesion nematodes, especially *Pratylenchus* spp., possibly contributed significantly in RD incidence at site A because the populations were not reduced after the two-year biofumigation with the two plant species (Table S2.3.6; Yim et al. 2017, under revision). The lower abundance of beneficial bacterial and fungal groups (as discussed above) at site A compared to site K showed the non-effect of the biofumgation at site A for both indicator plants M26 and M106 grown under greenhouse and field conditions, respectively. Mazzola et al. (2015) discovered that biofumigation with one of the seed meals from *B. juncea*, *B. napus* or *Sinapsis alba* did not control apple RD symptoms where the root endoparasitic nematode *Pratylenchus penetrans* contributed significantly to the disease development. However, combining *B. juncea* and *B. napus* seed meals (1:1), resulted in an effect for the biofumigation treatment comparable to conventional soil fumigant 1,3-dichloropropene-chloropicrin as revealed by apple tree growth (Mazzola et al. 2015). Thus, the biofumigation with a combination of different plant species either grown on site followed by tissue incorporation, or using formulated seed meals should be tested in future studies.

3.4 Mode of action of biofumigation in replant disease soils

In the present study, biofumigation was applied as a crop rotation using *B. juncea* and *R. sativus* plants, sown from seeds. After eight weeks, when plants were at mid-flowering stage, the total aboveground shoots were cut off followed by mechanical chopping and crushing prior to incorporation into RD soils. The ITC concentration detected in biofumigated soils was relatively low, i.e. the highest concentration was found for ally-ITC with 15.035 nmol g^{-1} dry soil (Yim et al. 2016). Allyl- and 4-methylthio-3-butenyl- ITC concentrations in soil amended with *B. juncea* and *R. sativus*, respectively, reported in other studies against soil-borne plant pests and pathogens was greater than 88 nmol g^{-1} soil resulting in a lethal rate of 90% against *Verticillium dahliae* (Neubauer et al. 2014). Thus, the effects of biofumigation in the present study, especially shown at site K, might have been complemented by several other mechanisms. Presumably, they resulted from combinations of improved soil structure, reduced phytotoxicity in soils via absorption of plant roots (phytoremediation), altering soil microbial community structure via ITCs and non-ITCs, especially boosting beneficial bacterial and fungal groups for plant growth and adding nutrients into treated soils which might affect both plant growth and microbial activities (Mazzola et al. 2001; Mattner et al. 2008;

Kapourchal et al. 2009; Antonious et al. 2009; Bhuiyan et al. 2011; Khan and Gaikwad 2013; Pino et al. 2016; Yim et al. 2016). Soil compactness can be reduced by plant root penetration (Passioura 1991; Stirzaker et al. 1996) and soil structure improved via organic amendments like green manure (Sultani et al. 2007). Reducing bulk soil density, increasing soil porosity as well as enhancing water availability was observed when soil was amended with green manure from legumes (Sultani et al. 2007).

Besides, *B. juncea* plants were reported to recruit plant growth promoting bacterial isolates like *Pseudomonas* spp. and *Stenotrophomonas* spp. which showed the ability to solubilize phosphorus, to fix nitrogen and to produce IAA (Pino et al. 2016). Several responders such as the bacterial genera *Arthrobacter*, *Terrimonas* and *Ferruginibacter* and the fungal genus *Podospora* were identified in significantly higher relative abundance in biofumigated soils compared to untreated RD soils, and were also shown to be significantly and positively correlated to apple plant growth in the present study. Strains of these genera were previously reported as plant growth promoting bacteria or fungi, e.g. *Arthrobacter* spp. (Karigar et al. 2006; Valencia-Cantero et al. 2007; Unell et al. 2008; Siddikee et al. 2010) and *Podospora* spp. (Xu et al. 2012).

Thus, all possible combinations resulting from growing *B. juncea* and *R. sativus* plants followed by incorporation of their biomass into RD soils most likely contributed to reduced apple RD symptoms in the present study.

3.5 Mode of action of the *Tagetes* treatment in replant disease soils

The growth of indicator plants showed comparable effects between *Tagetes*, biofumigation and Basamid treatments in RD soils (Yim et al. 2016). Although *Tagetes* are known as nematode repellent plants (Marotti et al. 2010; Marahatta et al. 2012; Saha et al. 2012), our results showed additional complementary effects such as changes in bacterial and fungal community composition and relative abundance of several responders (Yim et al. 2017, under revision). The bacterial genus *Thiobacillus* and the fungal genus *Tetracladium* as well as *Sordariales* were significantly enhanced in this treatment (Yim et al. 2017, under revision). Those bacterial and fungal groups were also shown to be positively correlated to apple plant growth (Franke-Whittle et al. 2015). Additional nutrients, especially K_2O in amended soils were also remarkably noted in this treatment compared to untreated RD soils (data not shown). Furthermore, root lesion nematodes, especially *Pratylenchus* spp. were effectively controlled by *Tagetes* treatment which was demonstrated at site A with a comparable effect to Basamid treatment (Table S2.3.6; Yim et al. 2017, under revision). In future studies, *Tagetes* plants should be further investigated for their potential roles in overcoming RD.

4 Outcomes of the study and future prospects

The total GS concentration in the different plant organs of *B. juncea* or *R. sativus* was comparable between sites and was highest in inflorescences followed by leaves. Differences in soil physical and chemical properties, soil microbiome as well as climatic conditions at the sites led to variability in GS breakdown products and methyl-ITC in biofumigated and Basamid treated soils, respectively.

It was clear that environmental conditions in spring and summer significantly affected growth and GS production of biofumigant plants as well as their liberated products in treated soils (Tables A6.2, A6.3; Figure A6.2). The biofumigant plants should be cultivated in summer to obtain a high efficacy of the treatment. The *Brassica* cultivars that have both the highest biomass production and GS concentrations in plant tissues should not be excluded, for instance *B. juncea* cv. Energy and *R. sativus* cv. Adagio have the highest GS in the plant tissues compared to other cultivars (Neubauer et al. 2014). Moreover, maximizing ITCs released from biofumigant tissue incorporation should be considered including an appropriate machinery that enables finer maceration and incorporation, a proper biofumigation schedule and tarping of amended soil with plastic films (Morra and Kirkegaard 2002; Cohen and Mazzola 2006; Mattner et al. 2008; Neubauer et al. 2014; Hanschen et al. 2015). Alternatively, to avoid seasonal effects on growth of biofumigant plants, the use of seed meal from *Brassica* spp. with higher GS concentrations, i.e. up to 300 $\mu mol\ g^{-1}$ defatted seed meal, and high ITC release efficiency could be an option as reported previously (Mazzola et al. 2009; Mazzola et al. 2015; Neubauer et al. 2015). Thus, maximizing the ITC release in RD soils using *Brassica* seed meals should be considered for future studies. Combining seed meals from different *Brassica* species containing *B. juncea* as a constituent to other seed meal, i.e. *B. napus* (1:1) demonstrated similar effects on reducing apple RD symptoms compared to the soil fumigant 1,3-dichloropropene-chloropicrin (Mazzola et al. 2015).

One year and two-year biofumigation treatments (with *B. juncea* or *R. sativus*) of RD soils showed the same effect as revealed by M26 plant growth at all sites K, A and M (Table A6.5). Repeated biofumigation might lead to a lower ITC release efficiency in treated soils due to proliferation of several microbial taxa that are able to consume GSs as a C-source (Reese et al. 1958; Palop et al. 1995; Mazzola et al. 2007). Moreover, Brassicaceae club roots caused by the fungus *Plasmodiophora brassicae* were commonly reported as a soil-borne fungal pathogens in cruciferous plants (Hwang et al. 2012). The club roots were observed mainly on roots of *B. juncea* and *R. sativus* at the 4th cultivation in the present study (data not shown). In addition, the DGGE fingerprints of bacterial and fungal community compositions showed greater shifts in one- than two-year biofumigated soils. Thus, the one-year treatment is advisable.

Site-dependent effects of biofumigation with *B. juncea* and *R. sativus* on M26 and M106 plant growth investigated under greenhouse and field conditions (Yim et al. 2016; Table A6.5), respectively, were

associated with differences in (1) total incorporated biomass of the biofumigant plants and subsequently the ITC release efficiency, (2) altering soil bacterial and fungal community compositions and (3) relative abundance of beneficial groups examined in the present study. Thus, more sites with differences in soil physical and chemical properties, cropping histories as well as biological properties should be included for future studies to get a better understanding of the treatment effects. For instance, Neubauer et al. (2014) reported that the toxicity of allyl-ITC against *Verticillium dahliae* in 22 naturally infested soils was negatively correlated to the organic content in soil.

The positive effects of biofumigation with *B. juncea* and *R. sativus* and the treatment with *Tagete*s was demonstrated at site K on M26 and M106 plant growth (the shoot dry mass increased > 100% in treated compared to untreated RD soil, Yim et al. 2016, Table A6.5), and suggested that the treatments can be used as alternatives to the soil fumigant Basamid for controlling apple RD, although further optimization is required (as mentioned above).

Functional roles of bacterial and fungal groups that showed positive (i.e. the bacterial *Arthrobacter* spp.) and negative correlation (i.e. the fungal genus unclassified *Pleosporales*) to shoot and root growth of apple plants should be further studied, i.e. in inoculation assays for their potential roles in overcoming RD.

The pathogenic oomycetes associated with apple RD incidence such as *Pythium* spp. (Hoestra 1994; Emmett et al. 2014) and *Phytophthora* spp. (Mazzola 1998; Tewoldemedhin et al. 2011; Kelderer et al. 2012) were not discovered as the primers used in the present study targeted only fungi. Thus, oomycetes should be included for future TC-DNAs based studies as well. Since rhizosphere soil, which adhered to roots after vigorous shaking, harbors higher bacterial and fungal abundance than bulk soil (Lugtenberg 2015; Hu et al. 2016; Uroz et al. 2016), using the true rhizosphere soil should be an alternative to identify potential responders in RD soils affected by treatments.

Combined approaches including metabolomics and metagenomics analyses of soil, transcriptomic analysis of plant roots, culture dependent identification and inoculation to plants may help identifying the complex causal agents of apple or rose RD and to overcome RD incidence. Furthermore, detailed identification of species/ strain levels and networks/ interactions between identified organisms (i.e. bacteria and fungi) should be taken into account for future studies.

Conclusions

The effects of biofumigation and treatments with Basamid and *Tagetes* in RD soils as revealed by indicator plant growth of M26 and M106 under greenhouse and field conditions, respectively, were site-dependent. Apple plant SDM increased more than 100% in biofumigated and *Tagetes* RD soils compared to untreated RD soils, especially at site K. Therefore, we concluded that the treatments (biofumigation and *Tagetes*) could be an alternative strategy, for growers, to the previously employed soil fumigant Basamid, although

further optimization of the processes are needed. The treatment effects of both biofumigation and *Tagetes* cultivation in RD soils possibly resulted from combinations of improving soil structure, reducing soil toxicity, suppressing soil-borne pests and pathogens, changes in soil microbial community composition and nutrient amendments (K_2O and P_2O_5) from the incorporated biomass resulting in higher microbial activities. Studying bacterial and fungal community composition, diversity and responders associated with RD soils by employing next generation sequencing has limitations to prove the causes related to RD incidence (Sun et al. 2014; Franke-Whittle et al. 2015; Yim et al. 2015; Nicola et al. 2017; Yim et al. 2017, under revision). This is also the case in the present study. However, at genera levels, several bacterial and fungal responders were identified to be positively and negatively correlated to apple plant growth. The inverse relationship between the production of primary and secondary metabolites in M26 plants (Weiß et al. 2017), the potential autotoxicity resulting from high amounts of phytoalexins (Weiß et al., unpublished data, Leibniz Universität Hannover) as well as damages in M26 roots grown in untreated soil (Yim et al. 2013) led to a reduction in growth of M26 plants grown in untreated RD soils. Findings of the present study revealed shifts in the bacterial and even more pronounced in the fungal communities in response to the treatments of RD soils and the relative abundance of numerous taxa that might have contributed to improved growth of indicator plants in treated RD soils were identified.

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6 Appendices

6.1 Replant disease incidence at trial sites

In the present study, the three trial sites K, A and M were claimed to be RD soils due to replanting rootstocks of rose (sites K and M) and apple (site A) according to the nursery owners. To evaluate RD incidence, a greenhouse biotest was performed in September 2012 using *in vitro* propagated rootstocks from apple M26, 20 days old, and *R. corymbifera* 'Laxa' seedlings, 40 days old, as described by Yim et al. (2015). Soils of three variants were taken from each site, including soils from the plots of treatment 1 (grass was growing aiming at maintaining the RD status in 2012) and the two sub-plots of treatment 2 (plots planted with apple rootstock M4 and *R.* 'Laxa' aiming at intensifying the RD incidence, see Figure 1.5, chapter 1).
Because SL and SFM showed similar reactions as the SDM of plants (Yim et al. 2013; 2015; 2016), only SDM and RDM are presented and discussed in this chapter. Differences between the SDM of M26 and *R.* 'Laxa' plants (eight weeks after planting) grown in Con (untreated) and H50 (50°C) or Con and Gamma (gamma irradiation) treatments indicated different levels of RD severity at the respective sites (Figure A6.1 and Table A6.1). Based on increases in the SDM of M26 and *R.* 'Laxa' plants in H50/Gamma compared to Con soils, RD incidence in plot of treatment 1 with grass was lower than in plots of treatment 2 with M4 (shown by both indicator plants, Con vs. Gamma) and *R.* 'Laxa' (indicated by *R.* 'Laxa', Con vs. Gamma) at site K (Table A6.1). For site A, stronger RD incidence was observed in the sub-plot with *R.* 'Laxa' shown by both indicator plants, i.e. the SDM of M26 plants significantly increased up to 313% in Gamma soils compared to Con soils (Table A6.1). Regarding site M, the severity of RD was lower compared to the other two sites (K and A) which was indicated by a lower increase in the SDM of indicator plants, especially M26 (Table A6.1).
Overall, the experimental sites were confirmed to show replanting problems by both indicator plants (Table A6.1). A stronger RD incidence through replanting M4 or *R.* 'Laxa' rootstocks was evident at all sites when compared to the grass plot (Table A6.1). Intensified RD soil incidence through repeated cultivation of the same plants or closely related species was reported previously by Spethmann and Otto (2003). The population of soil-borne plant pathogens was possibly enhanced by root exudates from M4 and *R.* 'Laxa' plants, and therefore, stronger effects were recorded in these plots compared to grass plots. The DGGE fingerprintings of bacterial and fungal community structures amplified from bulk soil TC-DNAs extracted from grass, M4 or *R.* 'Laxa' plots were also significantly distinct (Table A6.4). As reported by Yim et al. (2013; 2015; 2016) the changes in soil microbial communities strongly affected plant growth and likely vice versa. Thus, a variation in RD incidence was demonstrated on the different RD plots (grass, M4 and *R.* 'Laxa'). The RD intensity revealed by M26 and *R.* 'Laxa' plants (treatments Con vs. H50/Gamma, H50 vs. Gamma) was site specific (the lowest RD incidence was demonstrated at site M). The three sites had

differences in soil physical and chemical properties, cropping histories and soil managements practices as well as soil bacterial and fungal community composition and diversity (Yim et al. 2015; Yim et al. 2017, under revision), and therefore different RD intensity could be shown between sites. At site A, in overall, the SDM of both indicator plants (M26 and *R.* 'Laxa') grown in H50 and Gamma treatments was comparable (Table A6.1). The H50 treatment of RD soil controlled mainly nematodes and low heat sensitive organisms (Spethmann and Otto 2003; Yim et al. 2013). Therefore, nematode was probably one of the causal RD agents occurred at site A. More problematic with soil-borne plant parasitic nematodes at site A than sites K and M was discovered by our cooperating partner, i.e. numbers of root endoparasitic nematode *Pratylenchus* spp. were greater than 100 per 100 ml analyzed soil from site A collected at the same sampling period as for biotest (Dr. Andreas Wrede and Heike Nitt, the Chamber of Agriculture, Schleswig-Holstein).

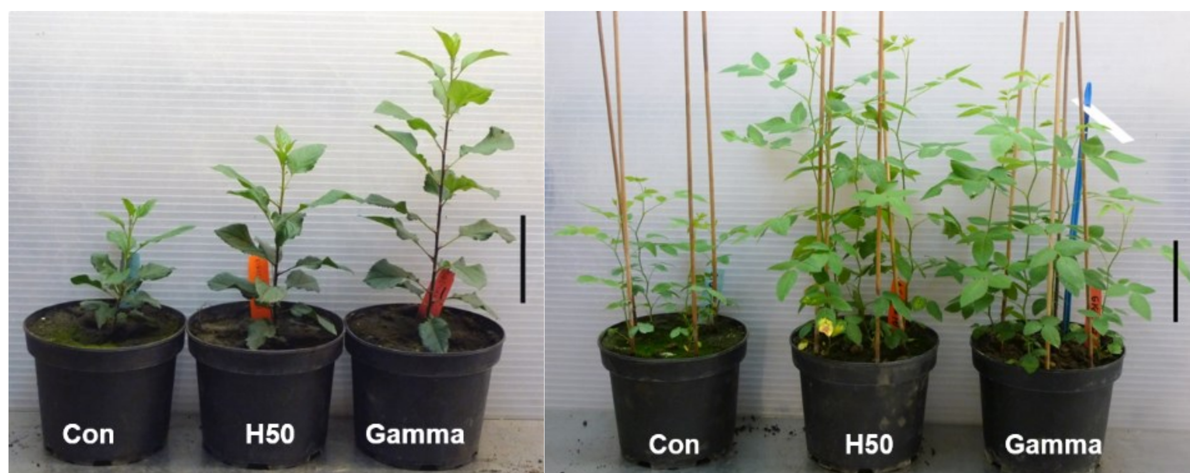

Figure A6.1: Rootstocks of apple M26 and *R. corymbifera* 'Laxa' plants grown in different soil treatments eight weeks after planting (in November 2012). Con, untreated RD soil; H50, temperature treatment at 50°C and Gamma, gamma irradiated treatment. Left, the soil from site K, sub-plot of treatment 2 with apple M4. Right, the soil from site A, sub-plot of treatment 2 with *R.* 'Laxa'. Bar is 10 cm.

Table A6.1: Effects of different replant disease (RD) soil treatments on biomass production of apple rootstock M26 and *R.* 'Laxa' plants eight weeks after planting in November 2012

Site	Sub-plot	Treatment (bioassay)	Indicator plant					
			Apple M26			*Rosa corymbifera* 'Laxa'		
			SDM (g plant^{-1})	% (fold) increases of SDM	RDM (g plant^{-1})	SDM (g plant^{-1})	% (fold) increases of SDM	RDM (g plant^{-1})
K, rose RD	1(Grass)	Con	3.4±0.5 a		2.8±0.4	1.3±0.2 a		0.4±0.1 a
		H50	2.9±1.3 a	-15 (0.9)	3.0±0.4	1.7±0.2 b	31 (1.3)	0.5±0.1 b
		Gamma	5.5±1.2 b	62 (1.6)	3.2±0.5	2.1±0.3 c	62 (1.6)	0.4±0.1 ab
	2 (*R.* 'Laxa')	Con	3.5±0.5 a		3.0±0.4	1.0±0.2 a		0.3±0.1 a
		H50	4.2±0.8 a	20 (1.2)	3.5±0.6	1.7±0.4 b	70 (1.7)	0.5±0.1 b
		Gamma	5.4±0.8 b	54 (1.5)	3.0±0.5	1.9±0.4 b	90 (1.9)	0.5±0.1 b
	2 (M4)	Con	3.1±0.4 a		2.7±0.6	1.3±0.2 a		0.5±0.1
		H50	4.5±0.3 b	45 (1.5)	3.0±0.5	1.9±0.1 b	46 (1.5)	0.5±0.1
		Gamma	5.7±0.4 c	84 (1.8)	3.1±0.5	2.2±0.2 c	69 (1.7)	0.5±0.1
A, apple RD	1 (Grass)	Con	1.6±0.4 a		3.7±0.4	0.8±0.1 a		0.3±0.0 a

		H50	2.7±0.8 b	69 (1.7)	3.6±0.2	1.4±0.3 b	75 (1.8)	0.4±0.1 b
		Gamma	2.4±0.5 b	50 (1.5)	3.8±0.4	1.5±0.3 b	88 (1.9)	0.5±0.1 b
	2 (*R.* 'Laxa')	Con	0.8±0.4 a		3.1±0.5	0.5±0.1 a		0.2±0.0 a
		T50	3.4±0.9 b	325 (4.3)	3.4±0.4	1.2±0.1 b	140 (2.4)	0.4±0.1 b
		Gamma	3.3±0.9 b	313 (4.1)	3.2±0.6	1.0±0.1 c	100 (2.0)	0.4±0.0 c
	2 (M4)	Con	1.5±0.4 a		3.3±0.1	0.6±0.1 a		0.3±0.0 a
		H50	2.6±0.8 b	73 (1.7)	3.4±0.2	1.4±0.2 b	133 (2.3)	0.5±0.1 b
		Gamma	2.3±0.6 b	53 (1.5)	3.2±0.3	1.1±0.2 c	83 (1.8)	0.4±0.1 c
M, rose RD	1 (Grass)	Con	2.6±0.5 a		4.0±0.2	1.5±0.1 a		0.6±0.1
		H50	2.8±0.4 a	8 (1.1)	4.2±0.2	1.6±0.2 a	7 (1.1)	0.6±0.1
		Gamma	3.4±0.4 b	31 (1.3)	4.2±0.2	2.0±0.2 b	33 (1.3)	0.7±0.1
	2 (*R.* 'Laxa')	Con	2.3±0.3 ab		3.7±0.2	0.7±0.1 a		0.4±0.0
		H50	2.3±0.2 a	0 (1.0)	3.8±0.1	0.8±0.1 a	14 (1.1)	0.4±0.1
		Gamma	2.7±0.5 b	17 (1.2)	3.8±0.1	1.0±0.2 b	43 (1.4)	0.4±0.1

	Con	2.2±0.4 a		4.0±0.2 a	1.2±0.2 a		0.4±0.1 a
2 (M4)	H50	2.2±0.6 a	0 (1.0)	4.1±0.2 a	1.3±0.2 a	8 (1.1)	0.5±0.1 a
	Gamma	3.1±0.9 b	41 (1.4)	3.7±0.2 b	2.3±0.2 b	92 (1.9)	0.7±0.1 b

Mean ± SD within soil variant followed by different letters indicate significant differences, Tukey test at $p < 0.05$ and n equals 10 and 5 for M26 and *R.* 'Laxa', respectively. SDM, shoot dry mass and RDM, root dry mass. Significant increases and decreases in shoot or root dry mass of plants are highlighted in green and red, respectively.

6.2 Seasonal effects on GS production and liberated products in amended soils

The biofumigant plants *B. juncea* 'Terra Plus' and *R. sativus* 'Defender' were planted in the treated plots 3, 4, 5 and 6 (Figure 1.5). The cultivation was carried out twice per year in spring and summer of 2012 and 2013 in treatments 3 and 4. For treatments 5 and 6, the plants were grown only in summer 2013. The GS profiles and concentrations detected in different plant organs (inflorescences, leaves, stems and roots) of the two *Brassica* species grown in summer 2012 and 2013 as well as their liberated products detected in the biofumigated soils were already presented and discussed in Yim et al. (2016, see chapter 2.2).
In this chapter, comparisons between GS production in different plant organs (treatments 3 and 4) and their degradation products (treatments 3, 4, 5 and 6) in soils that were affected by growing season in 2013 are presented and discussed.

6.2.1 Biomass production and GS concentration in organs of *B. juncea* and *R. sativus*

The aboveground biomass of *B. juncea* or *R. sativus* plants grown in summer was significantly higher compared to spring (Table A6.2, t-test, $p < 0.05$ and n = 3) shown at all sites. A similar trend was also obtained for GS concentrations, especially in inflorescences and leaves of plants (*B. juncea* and *R. sativus*) with lower GS concentrations detected in spring compared to summer samples (Figure A6.2). Obviously, environmental conditions had a major impact on plant growth and GS production in plants as observed previously (Zhang et al. 2008; Antonious et al. 2009; Yim et al. 2016). Thus, planting biofumigant plants in summer is recommended for a higher total GS production. Otherwise, further selected or bred *Brassica* species or cultivars that are suitable for spring should be considered for biofumigation.

Table A6.2: Biomass of *B. juncea* and *R. sativus* planted in spring and summer 2013 in treatments 3 and 4, respectively (kg m^{-2})

Site	*B. juncea*		*R. sativus*	
	Spring	Summer	Spring	Summer
K	0.95±0.5	4.45±0.5	1.15±0.3 a	9.50±1.5
A	0.43±0.2	3.31±0.5	0.48±0.1 b	6.61±1.4
M	1.16±0.7	3.70±0.6	1.89±0.4 a	6.81±0.6

Mean±SD followed by different letters at the same sampling time indicate significant differences, Tukey test, $p < 0.05$ and n = 3. The biomass of *B. juncea* or *R. sativus* in spring vs. summer was significantly different at all sites, t-test, $p < 0.05$ and n = 3.

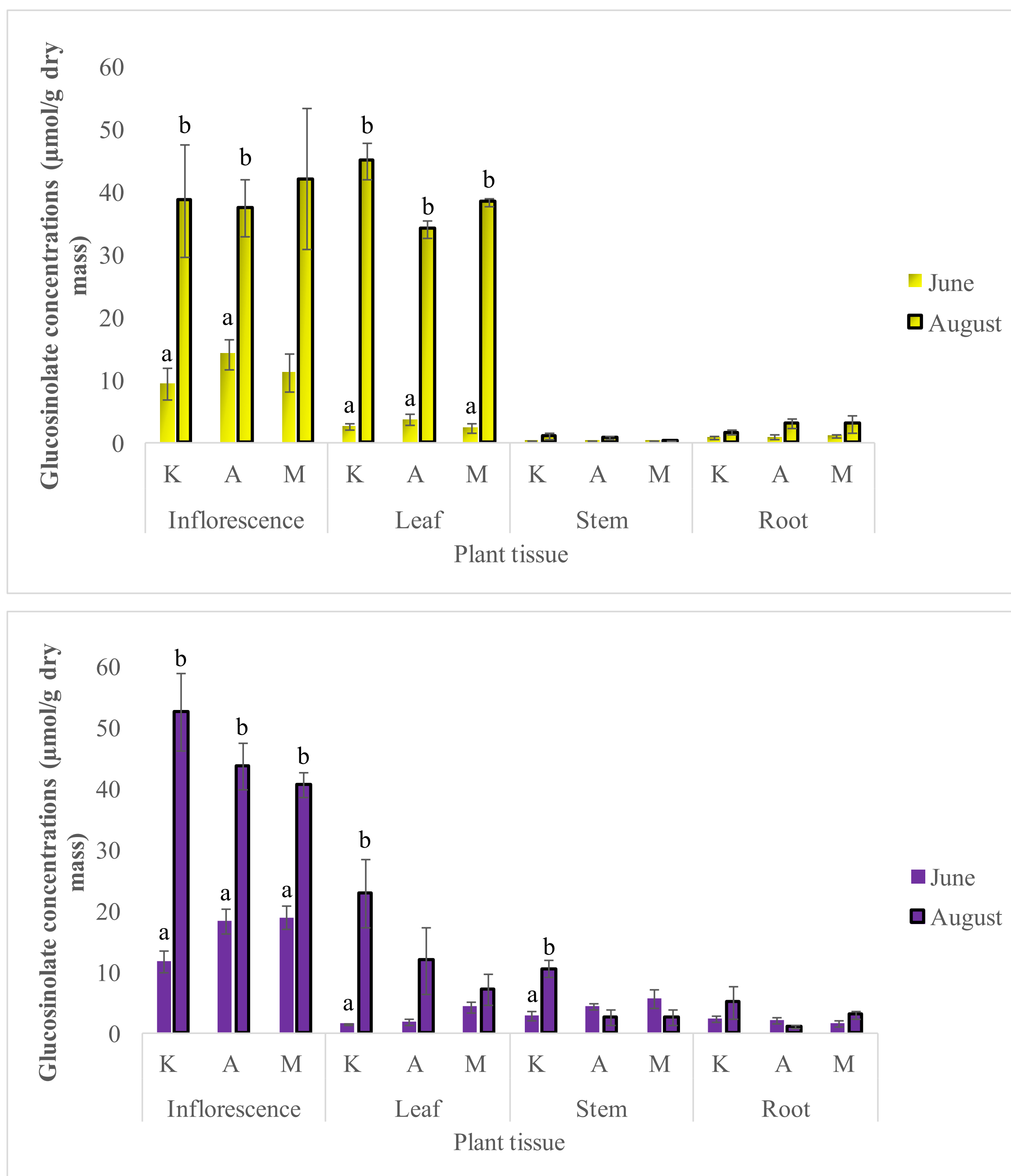

Figure A6.2: Glucosinolate (GS) concentration in different organs of *B. juncea* (above) and *R. sativus* (below) grown in spring and summer 2013 on plots of treatments 3 (*B. juncea*) and 4 (*R. sativus*). Bars indicate standard deviation. Different letters within site indicate significant differences between GS concentrations at the two time points (t-test, $p < 0.05$, n = 3). No sites effect was revealed for GS production in plant organs of *B. juncea* and *R. sativus* (Tukey test, $p < 0.05$ and n = 3).

6.2.2 Liberated glucosinolate degradation products

Four compounds, allyl-CN, allyl-ITC, 2-phenylethyl-CN and 2-phenylethyl-ITC were detected in biofumigated soil with *B. juncea* after 6 h of treatments 3 and 5 in summer at all sites (Table A6.3). The compound 2-phenylethyl-CN was below the detection level in analyzed soil samples taken in spring (treatment 3; sites K, A and M). For biofumigation with *R. sativus* (treatments 4 and 6), only 4-methylthio-3butenyl-ITC was identified in spring and summer.

The concentrations of the major degradation products allyl-ITC and 4-methylthio-3butenyl-ITC were significantly higher in treated soils in summer compared to spring, except for treatment with *B. juncea* at site M (Table A6.3). The ITC and non-ITC degradation products were proportional to the incorporated GS containing tissues into the soil (Yim et al. 2016). Therefore, the higher ITC concentrations were detected in soil samples taken in summer compared to spring (Table A6.3). The GS degradation products could have been enhanced due to higher temperature as well as earlier sampling time points (Hanschen et al. 2015).

Table A6.3: Glucosinolate degradation products detected 6 h after incorporation of total plant biomass of *B. juncea* and *R. sativus* in spring and summer 2013

Site	Species	Treatment_Season	Allyl-CN	Allyl-ITC	2-phenylethyl-CN	2-phenylethyl-ITC	4-methylthio-3butenyl-ITC
K	*B. juncea*	3_Spring	0.044±0.0	0.853±1.0 a	n.d.	0.272±0.2	
		5_Summer	0.235±0.1	6.138±3.2 b	0.022±0.02	0.716±0.7	
		3_Summer	0.314±0.4	6.689±3.0 b	0.022±0.02	0.897±1.0	
	R. sativus	4_Spring					0.316±0.3 a
		6_Summer					1.682±0.9 b
		4_Summer					2.274±1.8 b
A	*B. juncea*	3_Spring	0.094±0.0	0.094±0.2 a	n.d.	0.221±0.2	
		5_Summer	0.277±0.2	3.836±2.4 b	0.129±0.3	1.080±1.6	
		3_Summer	0.261±0.5	2.072±1.4 b	0.012±0.02	0.227±0.1	
	R. sativus	4_Spring					0.182±0.2 a
		6_Summer					1.022±0.6 b
		4_Summer					0.855±0.6 b
M	*B. juncea*	3_Spring	0.440±0.9	1.925±2.0 a	n.d.	0.599±0.5	
		5_Summer	0.155±0.1	4.126±3.4 a	0.013±0.02	0.670±0.8	
		3_Summer	0.131±0.1	15.035±8.6 b	0.072±0.1	2.711±3.6	
	R. sativus	4_Spring					0.684±0.6 a
		6_Summer					1.222±0.6 b
		4_Summer					1.181±0.5 b

Data is presented as Mean±SD. Different letters indicate significant differences between time points within site, Tukey test, $p < 0.05$ and n = 10. Treatments 3 and 4, a two-year biofumigation with *B. juncea* and *R. sativus*, respectively; 5 and 6, a one-year biofumigation with *B. juncea* and *R. sativus*, respectively.

Table A6.4: Treatment effects on soil bacterial and fungal community structures at three replant disease sites revealed by the dissimilarity percentage (d-value) in summer 2012

Comparison between treatments	Bacteria			Fungi		
	K	A	M	K	A	M
1 (Grass) vs. 2 (M4)	**8.36**	10.31*	29.6*	17.24*	29.28*	4.88*
1 (Grass) vs. 2 (*R.* 'Laxa')	**18.79**	7.93*	**5.99**	21.15*	18.16*	3.06*
2 (M4) vs. 2 (*R.* 'Laxa')	12.25*	10.57*	21.94*	18.21*	7.41*	2.5*
2 (*R.* 'Laxa') vs. 2 (*R.* 'Laxa' Rhi.)	9.46*	5.3*	7.2*	13.48*	**-0.11**	**1.63**
2 (M4) vs. 2 (M4 Rhi.)	**10.71**	10.55*	**3.17**	21.59*	**8.08**	**1.02**
1 (Grass) vs. 3	32.84*	24.57*	22.65*	56.01*	38.88*	31.72*
1 (Grass) vs. 4	**21.25**	27.69*	26.26*	51.58*	36.72*	37.94*
1 (Grass) vs. 7	25.55*	36.11*	11.75*	18.32*	26.32*	27.7*
2 (M4) vs. 3	43.25*	11.78*	12.89*	67.47*	27.07*	25.96*
2 (M4) vs. 4	41.18*	16.08*	23.07*	63.75*	34.8*	32.44*
2 (M4) vs. 7	46.58*	26.91*	21.23*	14.57*	24.75*	18.36*
2 (*R.* 'Laxa') vs. 3	55.01*	36.97*	12.72*	58.55*	28.46*	26.69*
2 (*R.* 'Laxa') vs. 4	47.4*	35.99*	20.59*	55.74*	37.19*	34.61*
2 (*R.* 'Laxa') vs. 7	48.44*	41.12*	12.08*	22.56*	19.72*	20.91*
3 vs. 4	**5.93**	6.36*	5.51*	19.91*	**2.83**	6.82*
3 vs. 7	27.33*	18.37*	9.62*	40.06*	21.44*	8.22*
4 vs. 7	16.59*	11.97*	8.8*	23.78*	24.54*	10.96*

D-value, average within-group pairwise Pearson's correlation – average between-group pairwise Pearson's correlation. * indicates significant differences between compared groups at $p < 0.05$ and n = 4 (Kropf et al. 2004). Treatments 1 (grass plot); 2 (sub-plots with apple M4 and *R.* 'Laxa' and Rhi., soil attached to roots of M4 or 'Laxa' plants); 3 and 4, biofumigation with *B. juncea* and *R. sativus*, respectively and 7, plot with *Tagetes* cultivation. Soil total community (TC-) DNA was extracted from bulk soil taken in summer 2012 at the same day like treatments 1, 2, 3, 4 and 7 (Figure 1.5). The sampling time was at four weeks after treatments 3 and 4. Regarding treatments 1, 2 and 7, the soils were sampled when plants were still growing.

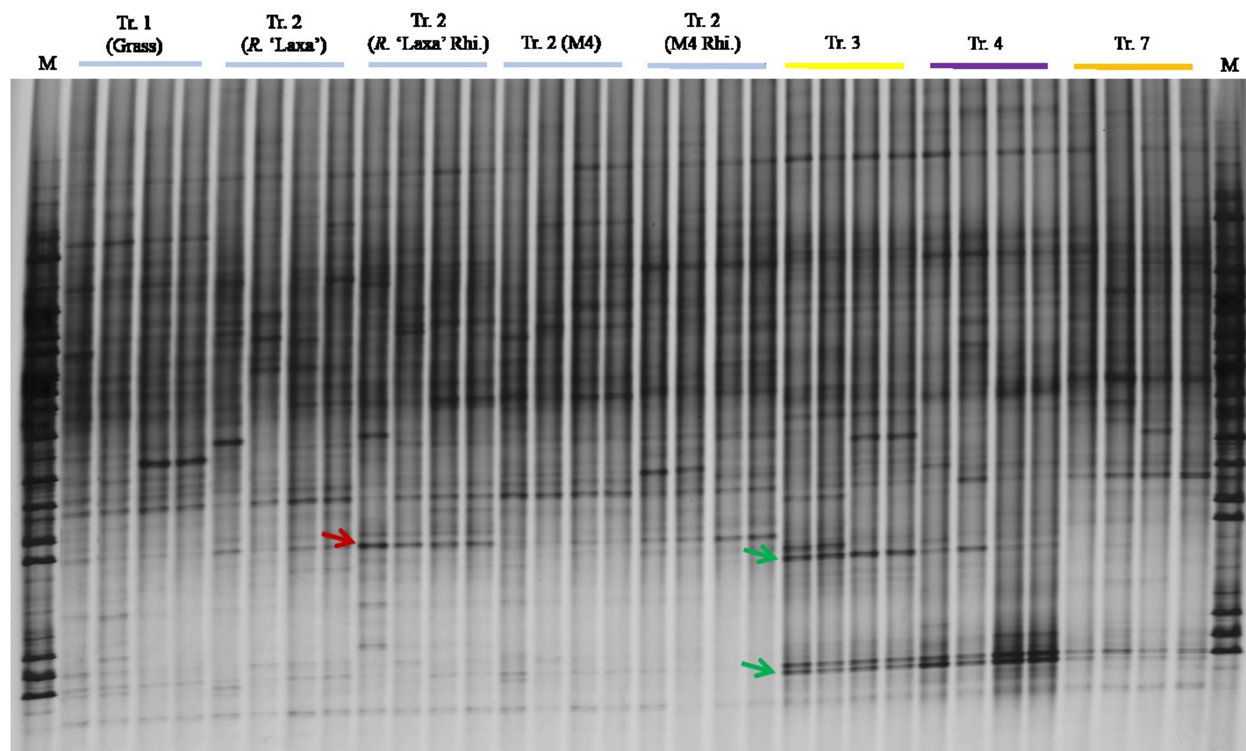

Figure A6.3: Denaturating gradient gel electrophoresis (DGGE) fingerprint of fungal community structure amplified from different soil TC-DNAs at site K. Treatments 1 (grass plot); 2 (sub-plots with apple M4 and *R.* 'Laxa' and Rhi., soil attached to roots of M4 or 'Laxa' plants); 3 and 4, biofumigation with *B. juncea* and *R. sativus*, respectively and 7, plot with *Tagetes* cultivation. Blue and red arrows indicate bands that were enhanced and decreased their intensity by treatments, respectively. M, marker. The band patterns for the DGGE fingerprint for bacteria showed similar pattern at all sites (data not shown).

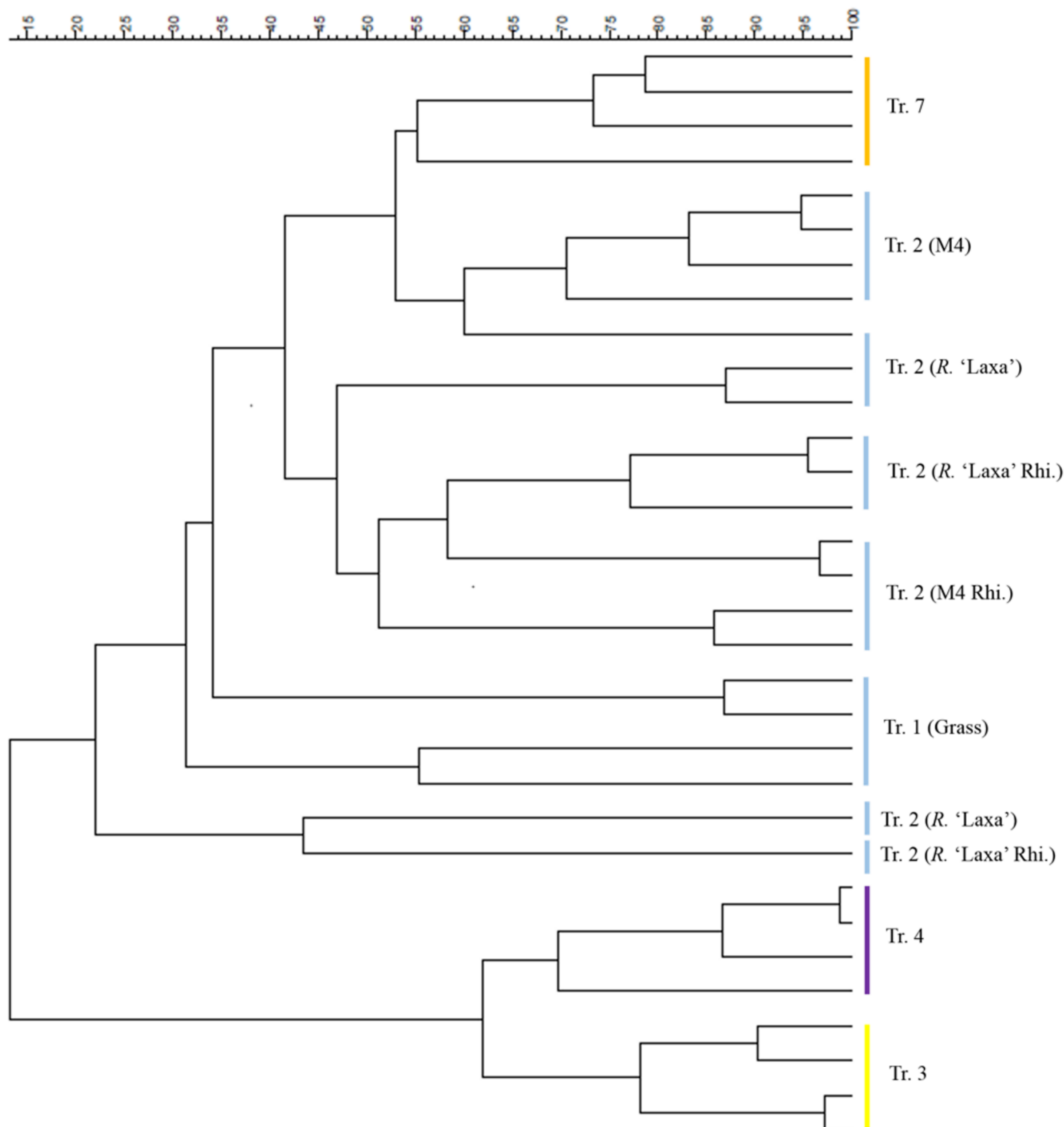

Figure A6.4: Dendrogram of fungal community structure amplified from different soil TC-DNAs at site K. Treatments 1 (grass plot); 2 (sub-plots with apple M4 and *R.* 'Laxa' and Rhi., soil attached to roots of M4 or 'Laxa' plants); 3 and 4, biofumigation with *B. juncea* and *R. sativus*, respectively and 7, plot with *Tagetes* cultivation. A clear clustering of the fungal community structures in biofumigated soils compared to other soil treatments was also revealed in soils of the other two sites A and M (data not shown).

6.3 Effects of soil treatments on growth of indicator plants

The effects of RD soil treatments 1 (Basamid in 2013), 3 and 4 (a two-year biofumigation *with B. juncea* and *R. sativus*, respectively) and 7 (*Tagetes*) on apple plant growth in 2014 were presented and discussed in Yim et al. (2016, chapter 2.2). Comparisons between effects of one-year (treatments 5 and 6) and two-year (treatments 3 and 4) biofumigation as well as RD soil sub-plots with apple rootstock *M.* 'Bittenfelder' and rose rootstock *R.* 'Laxa' on indicator plant growth have not been presented yet.

Nine soil variants were taken from treatments 1, 2 (sub-plots with rootstocks M111, *M.* 'Bittenfelder' and *R.* 'Laxa' in 2013), 3, 4, 5, 6 and 7 from each site (Figure 1.5). The procedures of the biotest experiment and data evaluation were describedby Yim et al. (2016, chapter 2.2).

Site- and treatment-dependent effects were revealed by the SDM of M26 plants at harvest (Table A6.5). Overall, the effects of different RD soil treatments were evident at site K, especially when comparing all soil treatments to the RD soil sub-plot with M111 plants. No differences in effects between one- and two-year biofumigation treatments as well as between both biofumigant plant species (*B. juncea* and *R. sativus*) on M26 plant growth under greenhouse conditions were observed at sites K, A and M (Tables A6.5).

Table A6.5: Effects of biofumigation on shoot dry mass (SDM, g plant^{-1}) of apple rootstock M26 plants, eight weeks after planting

Treatment	Plot with	Site		
		K	A	M
2	***M.* 'Bittenfelder'**	1.8±0.7 ab	1.7±0.2 ab	1.8±0.2 a
	M111	1.1±0.4 a	1.5±0.3 a	1.7±0.3 a
	***R.* 'Laxa'**	2.1±0.4 bc	1.7±0.4 ab	2.3±0.4 ab
1	**Basamid**	2.5±0.5 bc	1.7±0.4 ab	2.9±0.3 b
5	***B. juncea* (1)**	2.5±0.6 bc	2.0±0.6 ab	2.3±0.6 ab
3	***B. juncea* (2)**	2.5±0.7 bc	1.9±0.5 ab	1.8±0.3 a
6	***R. sativus* (1)**	2.3±0.4 bc	1.8±0.4 ab	1.9±0.6 a
4	***R. sativus* (2)**	2.4±0.4 bc	2.3±0.4 b	1.8±0.4 a
7	***Tagetes***	2.7±0.4 c	2.3±0.6 b	2.0±0.4 a

Mean±SD, letters indicate significant differences between treatments within one site, Tukey test, $p < 0.05$ and n = 10. (1) and (2), one- and two-year biofumigation; respectively.

Curriculum vitae

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2003 - 2009	Project assistant and project coordinator, at Cambodian Centre for Study and Development in Agriculture (CEDAC), Department of Agricultural Extension, Cambodia

List of publications

Yim B., Winkelmann T., Ding G-C. and Smalla K. (2015). Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis – contribution to improved aboveground apple plant growth? Front Microbiol 6: 1224. doi:10.3389/fmicb.2015.01224

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Conference presentations

Oral presentations

Yim B., Smalla K. and Winkelmann T. (2013). New approaches for apple replant problems – investigating rhizosphere microbial communities and establishing a bio-test. 48. Gartenbauwissenschaftliche Tagung, Bonn, Germany

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Poster presentations

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