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Michael Schloter and Kornelia Smalla
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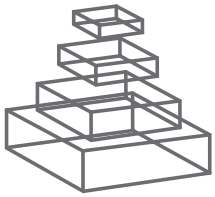

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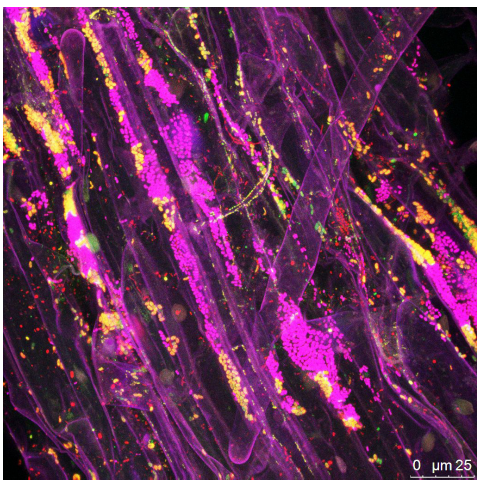
THE PLANT MICROBIOME AND ITS IMPORTANCE FOR PLANT AND HUMAN HEALTH

Topic Editors:
Gabriele Berg, Graz University of Technology, Austria
Martin Grube, Karl-Franzens-University Graz, Austria
Michael Schloter, Helmholtz Zentrum München, Germany
Kornelia Smalla, Julius Kühn-Institut(JKI), Federal Research Centre for Cultivated Plants, Germany



Plant-associated bacteria on lettuce roots visualized by Fluorescence In Situ Hybridization coupled with Confocal Laser Scanning Microscopy (FISH-CLSM). Gammaproteobacteria (yellow) and Betaproteobacteria (pink) form large colonies on the lateral roots of young lettuce plantlets. Micrograph provided by Armin Erlacher and Gabriele Berg (TU Graz).

The study of plant-microbe associations by new techniques has significantly improved our understanding of the structure and specificity of the plant microbiome. Yet, microbiome function and the importance of the plant's microbiome in the context of human and plant health are largely unexplored. Comparable with our human microbiome, millions of microbes inhabit plants, forming complex ecological communities that influence plant growth and health through its collective metabolic activities and host interactions. Viewing the microbiota from an ecological perspective can provide insight into how to promote plant health and stress tolerance of their hosts or how to adapt to a changing climate by targeting this microbial community. Moreover, the plant microbiome has a substantial impact on human health by influencing our gut microbiome by eating raw plants such as lettuce and herbs but also by influencing the microbiome of our environment through airflow. This research topic comprising reviews, original and opinion articles highlights the current knowledge regarding plant microbiomes, their specificity, diversity and function as well as all aspects studying the management of plant microbiomes to enhance plant growth, health quality and stress tolerance.

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The plant microbiome and its importance for plant and human health

Gabriele Berg[1]*, Martin Grube[2], Michael Schloter[3] and Kornelia Smalla[4]

[1] *Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Plant Sciences, University of Graz, Graz, Austria*
[3] *Environmental Genomics, Helmholtz Zentrum München, Neuherberg, Germany*
[4] *Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany*
**Correspondence: gabriele.berg@tugraz.at*

Edited by:
Gail Preston, University of Oxford, UK

Reviewed by:
Penny Hirsch, Rothamsted Research, UK

Keywords: plant microbiome, bacterial communities, endophytes, omics technologies, FISH technology, biocontrol, stress control, plant-microbe interaction

To study plant-associated microorganisms has a long history that reaches back to Lorenz Hiltner's definition of the rhizosphere in 1904 (Hartmann et al., 2008). Today, we know that microorganisms colonizing plant surfaces and inner tissues play an eminent role in shaping of our planet—from our natural vegetation to intense agricultural production systems up to human health. Plant-associated microorganisms have to be considered as key drivers for plant health, productivity, community composition, and ecosystem functioning.

For this e-book "The plant microbiome and its importance for plant and human health" we collected 18 articles, including reviews, original, and opinion articles that highlight the current knowledge regarding plant microbiomes, their specificity, diversity, and function as well as all aspects studying the management of plant microbiomes to improve plant performance and health. The contribution of the single articles of this research topic to these questions is discussed in detail in the mini-review and 1st chapter of the book by Berg et al. (2014a).

Overall the presented articles confirm that the plant-associated microbiome has greatly expanded the metabolic repertoire of plants and often increase resource uptake and provide novel nutritional and defense pathways. Thus, the plant microbiome has a direct impact on plant functional traits, such as leaf longevity, specific leaf area, leaf nutrient levels, and shoot/root ratio. By providing novel nutritional and defense pathways and by modifying biochemical pathways, the plant associated microbiome can enhance or decrease species coexistence and consequently influence not only a single plant but complete ecosystems. Thus, future breeding strategies may take the importance of plant-microbe interactions more into account than in the past, to obtain plants that generate high yields and are more tolerate to the constraints of global change.

Studies related to raw-eaten vegetables are a special show case in this e-book. Here the plant-associated microbiome does not only influence plant performance but strongly contributes to human health. As those microbes are also part of our diet they can either improve human health (Blaser et al., 2013) or cause heavy outbreaks of infectious diseases by transferring possible pathogens (Van Overbeek et al., 2014).

Interestingly, the gathered manuscripts indicate that microbiomes of different environments are not isolated but show interplay. For example, the microbiome of vegetables, humans as well as build environment such as hospitals seems to be well-connected (Berg et al., 2014b). Thus, maintaining microbial diversity in the different environments is an important issue to avoid pathogen outbreaks, which can be often explained by microbial imbalances and poorness (Van Elsas et al., 2012), confirming basic theories of ecology that a loss of native species enhances the probability of invasive species to colonize new environments. Therefore, to maintain and support microbial diversity is of interest to stabilize ecosystems and their resilience toward biotic and abiotic stressors. Biotechnological solutions like probiotics, prebiotics, and synbiotics for plants as well as humans can provide support for the indigenous microbiome (De Vrese and Schrezenmeir, 2008).

The most significant recent advances in plant microbiology involve interdisciplinary approaches that link different methodological approaches including omics-technologies. Due to the new methods available and interdisciplinary research cooperation we have the chance to solve many problems of a changing world, but also to address basic hypotheses and questions of microbial ecology and host microbe interactions. Integrating epigenetics in multi-omics techniques opens existing opportunities for new discoveries (Chen et al., 2014). Therefore, we think this comprehensive e-book especially the many reviews can contribute to hold the current knowledge in our hand. This is a very exciting but also challenging time for all researchers in this field. Major advances will come rapidly!

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Unraveling the plant microbiome: looking back and future perspectives

Gabriele Berg[1,2]*, Martin Grube[3], Michael Schloter[4] and Kornelia Smalla[5]

[1] *Austrian Centre of Industrial Biotechnology, Graz, Austria*
[2] *Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[3] *Institute of Plant Sciences, University of Graz, Graz, Austria*
[4] *Environmental Genomics, Helmholtz Zentrum München, Oberschleissheim, Germany*
[5] *Julius Kühn-Institute (JKI), Institute for Epidemiology and Pathogen Diagnostics, Federal Research Centre for Cultivated Plants, Braunschweig, Germany*

Edited by:
Johan Leveau, University of California at Davis, USA

Reviewed by:
Julia Vorholt, Swiss Federal Institute of Technology, Switzerland
Steven Earl Lindow, University of California at Berkeley, USA

****Correspondence:***
Gabriele Berg, Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria
e-mail: gabriele.berg@tugraz.at

Most eukaryotes develop close interactions with microorganisms that are essential for their performance and survival. Thus, eukaryotes and prokaryotes in nature can be considered as meta-organisms or holobionts. Consequently, microorganisms that colonize different plant compartments contain the plant's second genome. In this respect, many studies in the last decades have shown that plant-microbe interactions are not only crucial for better understanding plant growth and health, but also for sustainable crop production in a changing world. This mini-review acting as editorial presents retrospectives and future perspectives for plant microbiome studies as well as information gaps in this emerging research field. In addition, the contribution of this research topic to the solution of various issues is discussed.

Keywords: meta-organisms, plant microbiome, plant-microbe interaction, biocontrol, stress protection, plant growth promotion

INTRODUCTION AND RETROSPECT ON THE STUDY OF PLANT-ASSOCIATED MICROORGANISMS

Many studies on plant-associated microorganisms reflect the enormous interest in this topic and the full effect of ongoing research (Bulgarelli et al., 2013). Due to the importance of the soil habitat of plants, the majority of research focuses on the rhizosphere, even though microorganisms are also able to readily colonize most plant compartments. Several recent reviews addressed particular aspects of plant microbiome research. The current knowledge of rhizosphere inhabitants, their function, and their promising biotechnological potential was summarized by Hirsch and Mauchline (2012), Bakker et al. (2013), Mendes et al. (2013). Berendsen et al. (2012) reviewed more specifically the plant microbiome and plant health relationship, while Berg et al. (2005a) focused on the occurrence of potential human pathogenic bacteria in the rhizosphere. The important question about the factors contributing to selective enrichment of microorganisms from the soil into the rhizosphere was addressed by Bais et al. (2006), Doornbos et al. (2012). It now appears that in addition to carbohydrates and even amino acids which act as general chemical determinants in the rhizosphere (Moe, 2013), secondary metabolites such as plant-specific flavonoids were identified as key drivers in the development of plant-specific microbial communities in the rhizosphere (Weston and Mathesius, 2013).

While the well-studied rhizosphere presents the soil-plant interface, the phyllosphere forms the air-plant interface. This microhabitat is also of special interest due to its large and exposed surface area and its connection to the air microbiome, especially air-borne pathogens (rev. in Lindow and Brandl, 2003; Vorholt, 2012; Rastogi et al., 2013). However, in addition to the well-studied rhizo- and phyllospheres, each plant can be divided into more microenvironments, e.g., the endorhiza (root), the anthosphere (flower), the spermosphere (seeds), and the carposphere (fruit). Moreover, we generally differentiate between the endosphere (inner tissues) and ectosphere (outer surfaces; Ryan et al., 2008). All these microenvironments provide specific biotic and abiotic conditions for microbial life, which also have a correspondingly specific function for the host. The potential of these findings and the use of plant growth-promoting bacteria and biocontrol agents for the development of sustainable forms of agricultural management were discussed by Leveau (2007), Köberl et al. (2012), Berg et al. (2013).

The first section of this editorial focuses on several historical milestones in plant microbiome research. Despite the enormous progress already made, many challenges still exist. We address some information gaps in the second section of this editorial, and conclude with an overview of the present contributions. The papers in this special issue focus mainly on the bacterial dimension of the plant-associated microbiome, and we will show how they complement and extend the current research and how they will spur further questions.

THE RHIZOSPHERE WAS DEFINED MORE THAN A CENTURY AGO

Hiltner (1904) defined the "rhizosphere" as root-surrounding soil influenced by root exudates (Hartmann et al., 2008). In addition, he was the first to suggest the importance of microbial root inhabitants for plant growth and health. The rhizosphere is of central importance not only for plant nutrition, health, and quality. Today we are aware of microorganism-driven carbon sequestration in this ecological niche, which has an important role in ecosystem functioning and nutrient cycling in terrestrial ecosystems. In contrast to the other microenvironment of plants, the rhizosphere is characterized by high microbial abundances (Berg et al., 2005b)

and activities (Herron et al., 2013). Due to the densely colonized surface and surrounding soil (**Figure 1**), the rhizosphere was suggested as a protection shield against soil-borne pathogens (Weller et al., 2002).

THE ENDOSPHERE IS A HABITAT FOR INTIMATE INTERACTIONS

Although endophytes were ignored or considered contaminants for a long time, many endophytic inhabitants of plants are now often recognized as symbionts with a unique and intimate interaction with the plant (Ryan et al., 2008; Reinhold-Hurek and Hurek, 2011; Mitter et al., 2013). In these and other more recent studies, evidence of the occurrence of endophytes was assessed by cultivation-independent analyses, and via fluorescence *in situ* hybridization-confocal laser scanning microscopy (FISH-CLSM; an example for endophytes in the lettuce endorhiza is shown in **Figure 1**). After the first definition by De Bary (1866) as "any organism occurring within plant tissues," various researchers have defined endophytes in different ways, which were usually related to their own research context and perspective (Wilson, 1995; Hallmann et al., 1997; Bacon and White, 2000).

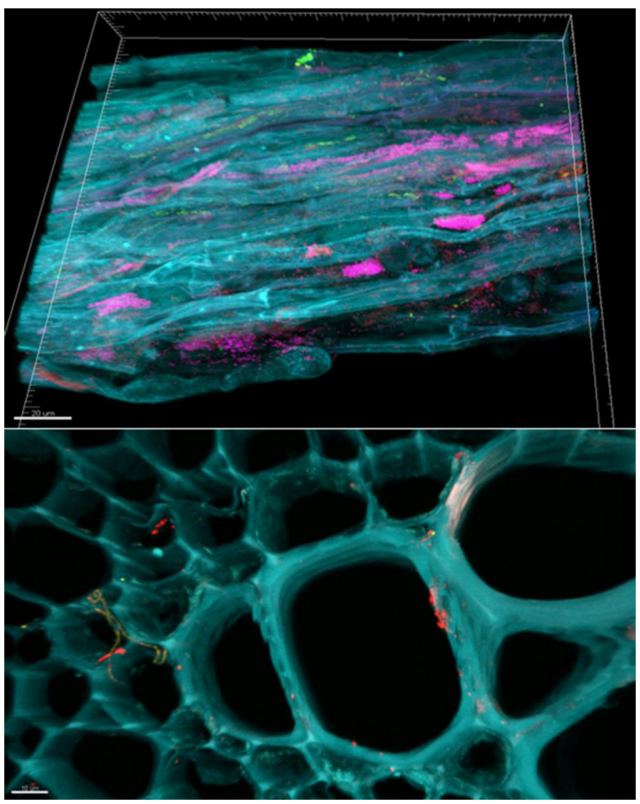

FIGURE 1 | Bacterial micro-colonies in the rhizosphere and endosphere visualized by fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy(CLSM). The rhizosphere microbiome of lettuce is dominated by Betaproteobacteria (purple) forming dense colonies on the root surface. The endosphere is shown as section of the main root of a lettuce plant. While unspecifically labeled bacteria are in red, Alphaproteobacteria (orange-green) are to be seen as colonies as well as filamentous forms.

THE FUNCTIONS OF THE PLANT MICROBIOME ARE ESSENTIAL FOR THE HOST

Plant-associated microorganisms can help plants to suppress diseases, to stimulate growth, to occupy space that would otherwise be available to pathogens, to promote stress resistance, and influence crop yield and quality by nutrient mobilization and transport (Lugtenberg and Kamilova, 2009; Yang et al., 2009). Therefore, the plant microbiome is one of the key determinants of plant health and productivity. Additional essential roles of the plant microbiome for phenotypic and epigenetic plasticity as well as the evolution of plants were suggested by Partida-Martínez and Heil (2011).

SPECIFIC ENRICHMENT OF MICROORGANISMS IN PLANT-ASSOCIATED COMMUNITIES EXISTS

So far, research on the specificity of plant-associated microbiomes focused on the rhizosphere, while only few other compartments have been studied in this respect (Vorholt, 2012). Although plant specific microbiomes in the rhizosphere have already been postulated via cultivation-based approaches (Germida and Siciliano, 2001), molecular fingerprints provided the first clear evidence for plant-dependent microbial community compositions (Smalla et al., 2001). Differences in plant root exudates play an important role as both chemo-attractants as well as repellents (Badri and Vivanco, 2009), to which bacteria are especially responsive (Costa et al., 2006, 2007; DeAngelis et al., 2009). In addition, plant defense signaling plays a role in this process as well (Doornbos et al., 2012). Haichar et al. (2008) used a stable isotope probing (SIP) approach to show that plant host habitat and root exudates shape the soil bacterial community structure. Thus, the plant is clearly able to select microorganisms for rhizosphere colonization primarily from the large pool living in the surrounding soil. Lundberg et al. (2012), Bulgarelli et al. (2012) revealed that only a subset of the bacterial community in the soil is present around the plant roots of *Arabidopsis thaliana* through amplicon sequencing of 16S rRNA gene fragments. Furthermore, the use of catalyzed reporter deposition and *in situ* hybridization or FISH was used to confirm the co-localization and dynamics of dominant taxa determined by 454 pyrosequencing (Bulgarelli et al., 2012; Lundberg et al., 2012; Ofek et al., 2012). While the use of FISH and catalyzed reported deposition-fluorescence in situ hybridization (CARD-FISH) helped to unravel the spatial distribution of dominant indigenous bacterial communities, the use of marker and reporter genes was employed in several studies to localize inoculated potential biocontrol strains and to measure distributions of nutrients, metals, and organic exudates along the roots on a microscale (Sørensen et al., 2009).

However, the plant (species, cultivar, age, health, and developmental stage) is not the only factor that influences microbial communities in the rhizosphere: a multitude of abiotic factors modulate the structural and functional diversity of the rhizosphere microbiome, including soil properties, nutrient status, and climatic conditions (rev. in Berg and Smalla, 2009). Moreover, large-scale agricultural management such as manure application has a clear impact on the microbiome composition (Jechalke et al., 2014).

THE ORIGIN OF PLANT-ASSOCIATED BACTERIA IS DIFFERENT

Plants are in constant contact with diverse microorganisms originating either through soil, wind, and air, or water via the water cycle. After initial exposure, some of these microorganisms are able to colonize the plant and survive (Rastogi et al., 2012). In some cases, microorganisms can even be transferred vertically from the parent plants to their progeny. Endophytes present in plant seeds may subsequently colonize the roots and the rhizosphere (Johnston-Monje and Raizada, 2011; Links et al., 2014). In addition, generative organs such as anther pockets, producing pollen (Fürnkranz et al., 2012), and moss sporophytes (Bragina et al., 2012) share a microbiome containing beneficials with their host plant.

Pseudomonas AND *Bacillus* ARE MODEL PLANT-ASSOCIATED BACTERIA

Although we now know that plant-associated bacteria are phylogenetically diverse, *Pseudomonas* and *Bacillus* have been studied as models for beneficial plant-microbe interaction (Emmert and Handelsman, 1999; Weller et al., 2002; Raaijmakers et al., 2010) for a long time. Interestingly, the importance of both genera on plants has been corroborated in many metagenomic studies. While *Pseudomonas* is abundant under humid conditions (Mendes et al., 2012), *Bacillus* dominates plant microbiomes under arid conditions such as in Egypt where *Pseudomonas* cannot survive (Köberl et al., 2011). The more detailed information obtained for *Pseudomonas*–plant interactions now help in understanding the bigger picture of *Pseudomonas* genome–plant interaction in its entirety as shown in the excellent review by Loper et al. (2012).

Antibiotic production by plant-associated microorganisms, with the rhizosphere and endosphere as a "hot spot" for potential producers, is a further aspect of research, for which both model organisms again play an important role. *Pseudomonas* is known for its versatile antibiotic production, which has also been shown *in situ* in the rhizosphere (Bonsall et al., 1997). Yet, a lot has still to be learned about the diffusion and action of small molecule antibiotics. Antibiotics are not only acting in solutes, some bioactive compounds act as volatiles, both in antibiosis against pathogens as well as in communication with plants (Ryu et al., 2003). According to recent reports, antibiotics and lipopeptides of bacteria are regulators and support biofilm formation, signaling, motility, and acquisition of micronutrients at sub-inhibitory concentrations (Raaijmakers et al., 2010; Raaijmakers and Mazzola, 2012). An interesting regulatory network was also detected for redox-active antibiotics such as phenazine, which is also involved in the reduction of Fe^{3+} (Raaijmakers and Mazzola, 2012). This high number of antibiotic producers associated with plants may have driven the evolutions of resistance genes as well (Allen et al., 2010).

Several studies, which focused primarily on *Pseudomonas* demonstrated bacterial intra- and interspecies communication in the plant-soil interface plant-microbe interaction via quorum sensing molecules such as *N*-acyl homoserine lactones (*N*-AHLs), or antibiotics at sub-inhibitory concentration (Steidle et al., 2001; DeAngelis et al., 2009; Hartmann and Schikora, 2012; Raaijmakers and Mazzola, 2012). Bacterial AHLs were demonstrated to change the plant transcriptome, modify root growth, and induce systemic resistance to phytopathogens (von Rad et al., 2008; Hartmann and Schikora, 2012; Raaijmakers and Mazzola, 2012); yet substantial differences were observed in the uptake, transport, and degradation of various AHLs for different plants (Götz et al., 2007).

HORIZONTAL GENE TRANSFER CONTRIBUTES TO PLASTICITY AND EVOLUTION OF PLANT-ASSOCIATED BACTERIA

Owing to the availability of various nutrients and surfaces, the plant-soil interface is also considered a hot spot for horizontal gene transfer processes via plasmids (Heuer and Smalla, 2012). The recent progress in microscopy tools has been extremely helpful in gaining further insight into the spatial distribution and dynamics of the plant-soil interface. Plant species-dependent differences were observed for the conjugation of a *gfp*-tagged IncP-1ε plasmid that did not express the *gfp* in its original host due to the presence of a *lac*-repressor (Mølbak et al., 2007). Through *in situ* visualization, these authors could demonstrate that both exudation patterns and root growth rates determined plasmid transfer in the pea and barley rhizospheres.

FUTURE PERSPECTIVES AND INFORMATION GAPS

Although the plant microbiome is recognized as an immense treasure trove of microbial diversity, numerous important crop species and their natural relatives have not yet been studied for their associated bacterial communities. With an approximate number of 500,000 plant species a lot of work lays ahead of plant microbiome research to explore new aspects about phylogenetic diversity of plant-associated microorganisms in the future. This might be particularly interesting with plants from extreme natural ecosystems or with unique life styles (carnivores, parasites, etc.).

Despite this enormous progress in the description of the plant microbiome, more fundamental and practical studies to address the processes leading to community assembly and function in and on plants are needed. Metagenomic analysis and comparison of plant-associated communities will lead to novel phylogenetic and functional insight. The first metagenomes, -proteomes, and -transcriptomes are currently published (Delmotte et al., 2009; Knief et al., 2012). An interesting example for a novel function is the detection of potential coexistence of microbial and plant photosynthesis on *Tamarix* leaves (Atamna-Ismaeel et al., 2012). Functional analysis will demonstrate whether the plants are able to benefit from the presence of certain microorganisms. In this context it should also be kept in mind that activation patterns and induction pathways can differ between ecotypes and strains.

Amplicon sequencing of 16S rRNA gene fragments provided valuable insight into the dominant colonizers, but too much emphasis on this locus may underdiagnose the potential biological variation. For example, biological functions provided from the mobilome (Eltlbany et al., 2012) do not correspond with 16S rRNA gene data. In addition, ribosomal gene amplicon quantities can depend on extraction methods, primer efficiency (Pinto and Raskin, 2012), and their copy-number variation (Kembel et al., 2012).

Although with the following articles in this special issue focus was given to the bacterial aspect of plant microbiomes we predict a future integration with fungal–bacterial interactions, specifically in the context of mycorrhiza (Bonfante and Anca, 2009; Song et al., 2010).

Plant microbiome discoveries could fuel advances in sustainable agriculture (Berg, 2009; Lugtenberg and Kamilova, 2009), such as the development of microbial inoculants as biofertilizers, biocontrol, or stress protection products (Berg, 2009; Berg et al., 2013).

In the future, the plant microbiome will have a greater importance for plant breeding and plant biotechnology. Until now, primarily plant pathogens were considered in these approaches. However, we suggest that the beneficial aspect of the entire microbiome should also be integrated as a biomarker.

A better understanding of the whole plant microbiome might be important to prevent outbreak of plant diseases or critical association of human pathogens with plants. We have learned that the human microbiome is much more involved in diseases than recently thought, and that pathogen outbreaks are associated with shifts in the entire community, including supporting pathogens (Blaser et al., 2013). While these processes are studied for human pathogens, much less is known about plant pathogens (Fürnkranz et al., 2012; Ottesen et al., 2013).

Furthermore, we envision the plant microbiome as an important source shaping other microbiomes. By the comparison of microbiome structures, a meaningful overlap of phylogenetic diversity can be recognized among microbiomes which are in some way linked to each other. This may also include the human habitat and plants. After we have received our first microbial inoculants by delivery and breast milk from our mother, our food becomes an important source not only of nutrients, but also of microorganisms (Blaser et al., 2013). Thus, digestive factors of plants and their microorganisms may modulate our own "second genome." Observations of domestic microbiomes suggest that they are significantly influenced by their human inhabitants and by the surrounding vegetation (Oberauner et al., 2013). These connections, which we conceive as links in a complex network among microbiomes, are still little understood and need further attention.

WHAT IS THE CONTRIBUTION OF THIS RESEARCH TOPIC?

This special issue will close some of the information gaps in plant microbiome ecology. It includes studies about the microbial diversity of yet unknown plants. In medicinal plants, the production of bioactive plant metabolites leads to a highly pronounced specificity in the microbiome structure (rev. in Köberl et al., 2014). Interestingly a correlation between the bioactive substances (drimane sesquiterpenes) and the endophytic community of roots was shown for the medical tree *Warburgia ugandensis* (Drage et al., 2014). Although it is known that plant secondary metabolites play an important role as drivers for microbial community structure, these studies show for the first time the importance with medicinal plants. Vice versa – Schmidt et al. (2014) could show that *Chamomile* plants treated with selected *Bacillus* strains produced more bioactive substances than untreated controls, thus microbes might be able to induce production of secondary metabolites of interest.

To better understand the significance of the plant-associated microbiome in prevention of pathogen outbreaks several studies focused on the lettuce microbiome and connected aspects of plant– and human health (Erlacher et al., 2014; Schreiter et al., 2014). Erlacher et al. (2014) showed that pathogens as well as beneficals induce a shift in the structure of the microbial community. To our knowledge, this is the first study analyzing this background effect, which can be important for plant protection strategies. However, also soil type was identified as important driver of the lettuce-associated community as well as the corresponding biocontrol effect (Schreiter et al., 2014). In addition, also for lettuce plants the impact of plant secondary metabolites exudated by roots in different soil types was pointed out (Neumann et al., 2014).

Another contribution presents evidence that *Escherichia coli* and *Salmonella enterica* infections occur due to consumption of vegetables, sprouts, and occasionally fruits (van Overbeek et al., 2014). The authors described a new transmission route of pathogens via plants or products derived from plants, and defined this process as "phytonosis".

The role of multitrophic interactions for plant diseases and the occurrence of the western corn rootworm were analyzed by Dematheis et al. (2014). In addition to biotic factors, the impact of abiotic factors on the plant microbiome was investigated. Elevated atmospheric O_3 changed the community structure of biocontrol active actinobacteria in the rhizosphere of European beech (Haesler et al., 2014).

Two studies suggest members of the plant-associated *Burkholderia* cluster as model to study plant-microbe interactions. Oxalate acts as carbon source and as determinant in colonization processes in lupins and maize (Kost et al., 2014), while nitrogen-fixing *Burkholderia* populations are highly abundant in *Sphagnum* bogs (Bragina et al., 2014).

Two mini-reviews focus on the interplay of microbiomes as well as the importance of the plant microbiome for others. The connection between plant and our built environment microbiome is discussed by Berg et al. (2014), and another one highlighted similarities between the gut and root microbiome and suggested to transplant "healthy microbiomes" to avoid or therapy plant diseases (Gopal et al., 2013). A step forward to understand the plant-microbe networking was presented in the review by Hartmann et al. (2014). They come to the conclusion that functional interaction studies of holobiotic plant systems, including the plant host and its associated microbes, may result in a more profound understanding of the complicated social network of basic innate immune responses with specific effector molecules, if quorum sensing compounds of endophytic bacteria are integrated.

Overall, this issue presents new results about (i) the role of plant secondary metabolites for the microbiome and *vice versa*, (ii) health issues related to the consumption of raw-eaten plants, (iii) the interplay of microbiomes as well as within them and (iv) the impact of biotic and abiotic factors on the structure and function of plant-associated microbial communities.

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Scanning a microhabitat: plant-microbe interactions revealed by confocal laser microscopy

Massimiliano Cardinale [1,2]*†

[1] Institute of Plant Sciences, University of Graz, Graz, Austria
[2] Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

Edited by:
Michael Schloter, Helmholtz Zentrum München, Germany

Reviewed by:
Rita Musetti, University of Udine, Italy
Rodrigo Costa, Centre of Marine Sciences, Portugal

***Correspondence:**
Massimiliano Cardinale, Institute of Plant Sciences, University of Graz, Holteigasse 6, 8010 Graz, Austria
e-mail: massimiliano.cardinale@uni-graz.at

†Present address:
Massimiliano Cardinale, Institute of Applied Microbiology, Justus-Liebig-University, Giessen, Germany
e-mail: massimiliano.cardinale@umwelt.uni-giessen.de

No plant or cryptogam exists in nature without microorganisms associated with its tissues. Plants as microbial hosts are puzzles of different microhabitats, each of them colonized by specifically adapted microbiomes. The interactions with such microorganisms have drastic effects on the host fitness. Since the last 20 years, the combination of microscopic tools and molecular approaches contributed to new insights into microbe-host interactions. Particularly, confocal laser scanning microscopy (CLSM) facilitated the exploration of microbial habitats and allowed the observation of host-associated microorganisms *in situ* with an unprecedented accuracy. Here I present an overview of the progresses made in the study of the interactions between microorganisms and plants or plant-like organisms, focusing on the role of CLSM for the understanding of their significance. I critically discuss risks of misinterpretation when procedures of CLSM are not properly optimized. I also review approaches for quantitative and statistical analyses of CLSM images, the combination with other molecular and microscopic methods, and suggest the re-evaluation of natural autofluorescence. In this review, technical aspects were coupled with scientific outcomes, to facilitate the readers in identifying possible CLSM applications in their research or to expand their existing potential. The scope of this review is to highlight the importance of confocal microscopy in the study of plant-microbe interactions and also to be an inspiration for integrating microscopy with molecular techniques in future researches of microbial ecology.

Keywords: plant-microbe interactions, cryptogams, confocal laser scanning microscopy (CLSM), endophyte, pathogen, GFP, DsRed, fluorescence *in situ* hybridization (FISH)

INTRODUCTION

Plant-microbe interaction studies, including plant colonization by microbes, have benefitted from the development of high-throughput molecular methods, such as metagenomics and metatranscriptomics (Kint et al., 2010; Röling et al., 2010; Zhang et al., 2010; Jansson et al., 2012). Consequently, studies of microbe-host associations have become a core theme in microbial ecology, as their role for the macroscopic hosts was increasingly recognized. Omics methodologies based on the extraction of molecules (such as nucleic acid or proteins) directly from environmental samples, incremented tremendously the detection limit, thus broadening the spectrum of potentially targeted organisms to include also the rare microbiome. On the other hand, such methods have the disadvantage to lose the spatial information, since microbial cells are physically removed from their original location. For these reasons, methods allowing localization and visualization of microbes in microbe-host systems have also progressed during the past two decades, parallel to molecular microbiology methods. One of the frequently used approaches includes confocal laser scanning microscopy (CLSM) (Pawley, 2006). Plants, plant-like organisms, or fungi, are structurally complex and intricately linked with their substrates. For analyses of their interactions with microbes, CLSM has come in the prime of life as one of the standard techniques used. In this review I highlight the progresses achieved in understanding microbial interactions with plants and plant-like organisms using CLSM and image analysis, focusing on fluorescence *in situ* hybridization (FISH) and labeling with fluorescent proteins as common methods to specifically detect target organisms. As a direct method to study microorganisms, microscopy avoids the PCR biases typical of molecular methods, thus is best suited to accurately quantitate environmental microbes when a statistical approach is applied to image acquisition. I critically discuss this aspect together with the use of natural autofluorescence. Confocal image series contain an exceptional amount of potential information, but suitable methods for image analysis are required to exploit this potential. Here I show how different visualization methods can influence outcomes and conclusions of CLSM observations. Finally, I discuss future perspectives with CLSM and related techniques, and how their integration with molecular microbiology methods can contribute to a better understanding of host-microbe systems ecology. As already recognized explicitly for biofilms (Lourenço et al., 2012) I suggest the integration of CLSM with omics techniques as the optimal approach also in host-microbe interaction studies, both for laboratory-scale systems as well as for environmental samples.

BASIC PRINCIPLES: IMAGE ACQUISITION, IMAGE ANALYSIS, AND DETECTION METHODS

CLSM is based on the detection of fluorescent light, but it differs from conventional epifluorescence microscopy by acquiring

the fluorescent signal(s) exclusively from the focal plane as a pinhole excludes out-of-focus light. In addition, consecutive optical slices along the Z-axis of an image series ("confocal stack") can be prepared for projections and three-dimensional reconstructions. Different signals can be acquired separately and then assigned to different colors for their discrimination in the images. Many CLSM instruments allow for addition of a (non-confocal) transmission light image to the confocal stacks. Confocal stacks can be analyzed in different ways, either by browsing the image series and selecting individual optical slices, or by sliding along any of the Euclidean axes to obtain X-, Y-, and Z-projections, respectively. Proprietary software tools can transform original fluorescent signals into artificial objects. Their surfaces are recognized by differences in fluorescence intensity ("isosurfaces") and spheres. Such three-dimensional models facilitate precise localization of signals and intimate associations of organisms. For presentation, so-called time series can be compiled as short video clips, e.g., to move the viewing perspective, or to zoom regions of interest (flythrough).

Several freeware tools are available for qualitative and quantitative analysis of CLSM stacks. Although ImageJ was initially established for analysis of medical images (Schneider et al., 2012; http://rsbweb.nih.gov/ij/), several plugins were since then developed and applied for CLSM analyses of microbial communities. In plants these helped to analyze rhizosphere and phyllosphere communities (Iverson and Maier, 2009; Downie et al., 2012; Lee et al., 2012). Image surfer was developed with the specific purpose of imaging confocal stacks and it is not open to plugin implementation (Feng et al., 2007; http://imagesurfer.cs.unc.edu/). Nevertheless, it includes sophisticated visualization tools which allow the analysis of complex systems such as host-microbes interactions in the rhizosphere (Zachow et al., 2010). DAIME is a tool for quantitative analysis of complex microbial communities, such as biofilms, and also includes procedures for evaluation of fluorescence *in situ* hybridization probes (Daims et al., 2006; www.microbial-ecology.net/daime/).

CLSM allows the detection of three kinds of objects: (1) molecules, cells and tissues stained with one or more fluorochromes; (2) genetically modified organisms (GMO) that express fluorescent proteins; (3) autofluorescent cells, tissues and substrates. As autofluorescence of biological and synthetic substrates is usually considered as a negative aspect of CLSM images, efforts often aim toward avoiding autofluorescent signals (Lo Piccolo et al., 2010). As will be shown, autofluorescence may actually be a useful phenomenon for interpretation of the confocal images at least in the context of plant-microbes interactions.

FISH is most frequently used for visualization of microbial colonization patterns and community composition (Moter and Gobel, 2000; Amann et al., 2001). Owing to the direct visualization of target cells, FISH-CLSM can provide useful estimates of bacterial numbers in certain habitats, also because it avoids any quantification biases associated with methods based on cultivation or PCR (Bulgarelli et al., 2012). FISH is based on the hybridization of DNA-probes labeled with fluorochromes with the complementary target sequence. In most cases these are characteristic signature sequences of rRNA genes. Since specificity of the probes is defined by their sequence, it is ideally possible to detect a specific taxonomic range. Cautious interpretation of data is required with some probes which are known to have a lower specificity than ideally expected. Such information is included in databases for FISH probes, such as probeBase (Loy et al., 2003, 2007; http://131.130.66.201/probebase/).

Detection of mRNA targets is interesting for addressing functional questions, such as to understand the molecular bases of the mechanism(s) of interactions between beneficial microbes or pathogens and their respective host. However, low numbers of targets may impair detection with fluorescent FISH probes. Eventually, the signal can be increased by double labeling of oligonucleotide probes (DOPE-FISH; Stoecker et al., 2010) and by enzymatic amplification of the signal production, or by amplification of the target via *in situ* PCR (Ruppel et al., 2006).

FISH usually requires a preliminary fixation. Hence, the confocal images represent snapshots of the dynamic biological system, taken at the time of fixation. As fixation kills all cells, FISH staining generally does not allow any live imaging of cells, and separate samples fixed at different biological stages do not represent "true" time-lapse experiment. For live imaging (4D microscopy), fluorescent proteins produced in host- associated microorganisms after genetic transformation offer an alternative detection possibility. With this approach, time-lapse experiments can track the effects of substrates, growth enhancers and inhibitors. Genes coding for fluorescent proteins are usually inserted in plasmids successively cloned into competent cells but they can be also integrated chromosomally (Morschhäuser et al., 1998). Such proteins include green fluorescent protein (GFP), yellow fluorescent protein (YFP), and DsRed protein (Leveau and Lindow, 2002; Larrainzar et al., 2005). The use of the plasmids allows the insertion of additional genes, such as antibiotic resistance genes, useful to maintain the strain under selective growth. In fact, one of the biggest disadvantages of using the fluorescent proteins is their instability. Moreover, only the tagged strain can be visualized, which explains why GFP-tagged strains are usually applied in gnotobiotic systems, or used in microcosms with only one or few different microorganisms. Other plasmid constructs can include promoters upstream of the *gfp* gene, allowing the investigation of gene regulation by external factors such as the presence/concentration of chemicals (Rothballer et al., 2005).

Recently a new protocol for FISH without prior sample fixation was presented (Yilmaz et al., 2010). This method offers new and exciting perspectives for enabling simultaneous detection of FISH-stained natural populations and fluorescent protein-tagged strains. FISH was often coupled with other staining techniques. Many protocols have been developed; among others, Raman-FISH (Read et al., 2009), catalyzed reporter deposition-FISH (CARD-FISH; Pernthaler et al., 2002) and enhanced element labeling-FISH (EL-FISH; Behrens et al., 2008) address one of the most critical points of microbial ecology: to link identity and function of members of the natural microbial communities. These hybrid methods have not been used yet to study plant-microbes interactions.

CLSM APPLIED TO HOST-MICROBE INTERACTIONS

PLANTS

CLSM in microbial ecology was first used by Schloter et al. (1993) to show the interactions between wheat roots and *Azospirillum brasilense* SP7, a plant growth promoting rhizobacterium (PGPR). In this case, the bacteria were stained with specific fluorescent labeled antibodies, and the authors pointed out the advantages of CLSM observations in comparison with those of traditional epifluorescence microscopy: they could precisely localize bacteria, root tissue and mucilaginous layer, and used XY or Z-scan images to show them. In this pioneering work, the authors could clearly show the great potential of CLSM in the field of microbial ecology. In the following 20 years, the number of scientific articles based on, or discussing, CLSM in plant-microbe interactions increased regularly, reached a plateau during the first decade of the new century of about 20 publications per year followed by a recent increase. This trend clearly reflects the technical development of new confocal systems. Applications of CLSM during these two decades ranged from studies of plant colonization to tracking the fate of inoculated strains. The studied hosts comprised vascular plants as well as cryptogams, such as mosses and lichens. A selection of relevant papers of the last 5 years is presented in Table 1 Supplementary material.

Plants provide a variety of microniches and surface types for bacterial colonization. Hence, the benefit of CLSM is to precisely localize the bacterial cells on plants. Bacteria were either detected on the rhizoplane, inside the root (endorhiza), in the apoplastic spaces, embedded in extracellular matrices, inside root cells, or inside the xylem vessels. Plant-associated bacteria not only use the microhabitats provided by the host as a house and eventually as substrate, but instead can actively shape them by modifying their development (Zamioudis et al., 2013).

Ahmed et al. (2010) described five distinct phases of root colonization by the Cyanobacterium *Leptolyngbya* within the same optical view (from root cell intrusion until total filling). As an alternative to multiple observations, this approach is only feasible when two prerequisites are met: (1) the target microorganism shows a stepwise colonization behavior with clearly discernible differences between the steps, and (2) its high density allows detecting different stages of colonization in close vicinity. CLSM offers the unique opportunity to elegantly show successive steps of microbial colonization as movies (Czymmek et al., 2007) or as image gallery (Prieto et al., 2011).

Zachow et al. (2010) studied interactions between fungal and bacterial beneficial strains in the root of sugar beet. The authors combined volume rendering and isosurface imaging to display the interactions between fungal hyphae and plant roots (Zachow et al., 2010), so providing an example of the CLSM versatility in imaging different organisms by mean of different visualization techniques. The results were interpreted in light of the microbial effects to the plant. It was concluded that neither endophytism nor direct contact with the pathogen was the discriminative feature of efficient biocontrol strains, so shedding light on their possible modes of action. Similarly, Maldonado-González et al. (2013) showed that, although not showing a direct contact with the pathogen, the biocontrol agent *Pseudomonas fluorescens* PICF7 was able to affect both the colonization patterns and the disease incidence of the tumor inducing *Pseudomonas savastanoi* NCPPB3335. Also in the phyllosphere of grapes, Gasser et al. (2012) showed that *Pantoea ananatis* BLBT1-08 efficiently controlled the plant pathogen *Botrytis cinerea*, although neither contact nor inhibition of conidia germination was observed.

Complementing CLSM with the identification of native beneficial bacteria in environmental samples sheds light onto the ecology of such strains in nature or under field conditions, as shown by Köberl et al. (2013) for *Bacillus* and *Streptomyces* in an arid ecosystem.

Fan et al. (2012) were able to show, in gnotobiotic systems, how the same rhizobacterium exhibited different colonization patterns on three different hosts, thus suggesting that every plant-microbe system is putatively unique and that it would be imprudent to draw general conclusions from results obtained with one system.

Bacterial-fungal interactions in the rhizosphere (such as mychorrizal systems) are ubiquitous and play an outstanding role for soil ecosystems, yet, they were not extensively studied by CLSM *in situ*. Mogge et al. (2000) studied the bacterial community on the ectomycorrhizal mantles of beech (*Fagus sylvatica*) and characterized its taxonomic composition by FISH. By using the fluorescence intensity as a quantitative reporter of metabolic activity, they demonstrated that incubation with nutrient sources such as yeast extract did not increase bacterial metabolism. In the rhizosphere of barley, intrahyphal occurrence of *Paenibacillus* and *Rhizobium* strains was proved with CLSM and correlated with their beneficial effect on the plant fitness (Sharma et al., 2008).

Rhizobia are unique among plant symbionts. They frequently induce development nodules as specific symbiont-hosting organs in certain plant lineages. Their infection process, elucidated at both phenotypic and molecular level, was also complemented by CLSM studies (Timmers et al., 1999; Haynes et al., 2004). *Burkholderia* strains (so called "β-rhizobia") have been isolated from root nodules of several plants in the past. Such non-rhizobial symbionts were shown by CLSM to actually nodulate *Cyclopia* ssp. as well as the promiscuous legume *Macroptilium atropurpureum* (Elliott et al., 2007), and *Mimosa pigra* (Chen et al., 2005). CLSM revealed more bacterial species to be able to colonize the internal parts of root nodules, such as *Paenibacillus polymyxa* (Annapurna et al., 2013).

Kamilova et al. (2007) observed substantial differences in the interactions between a pathogenic fungus and its biocontrol agent *in vivo* and *in vitro*. While analysis *in vitro* does not suggest significant effects of *Collimonas fungivorans*, this bacterial strain exerts antagonistic activity *in vivo*. The authors illustrated their finding by CLSM and found no direct interaction between bacterial cells and fungal hyphae at microscopic scales. Olivain et al. (2006) followed colonization patterns of two different strains of the ascomycete *Fusarium oxysporum* (a pathogenic one and its antagonistic strain) in tomato roots. The image segmentation demonstrated that the two strains co-occur in the same areas of the root, which suggests competition for nutrients rather than a competition for space. This example shows how CLSM can contribute to the understanding of ecological relationships between microbes, including biological control. *In situ* auxine (indole-3-acetic acid) production of two *Azospirillum brasilense* strains was compared by using a fusion construct where the

promoter of the gene *ipdC* (responsible for auxine synthesis) was integrated in a plasmid upstream of the *gfp* gene (Rothballer et al., 2005). Differences between the signal intensity of the two strains were then explained at molecular level by sequence analysis, which revealed the occurrence of a region exclusive for the most performing strain, probably involved in the regulation of expression. The same technique could be theoretically used for studying the *in situ* expression of any gene of interest.

Colonization of xylem vessels was shown, among others, for *Enterobacter gergoviae* (An et al., 2006), *Bacillus subtilis* (Ji et al., 2008), *Herbaspirillum frisingense* (Rothballer et al., 2008) and *Burkholderia terricola* (Gasser et al., 2011). Such observations are among the most challenging, because they are strongly dependent on the quality of the sectioning and the integrity of root anatomy. Clear identification of root tissues and preservation of the root anatomy provide the perfect background to investigate the localization of microorganisms in the rhizosphere or in the endorhiza. For example, cellulose autofluorescence revealed details of root anatomy, both with longitudinal (Maciá-Vicente et al., 2008) or transversal sections (An et al., 2006; Kutter et al., 2006).

Pathogenicity represents a special case within host-microbe interactions. The role of confocal microscopy can be relevant when complemented with molecular tools such as transformation and mutation. Mechanism(s) of interactions with the host can be understood and dynamics of infection processes elucidated. In the phyllosphere of lettuce the human pathogen *Salmonella enterica* intrudes the plant via the open stomata (Kroupitski et al., 2009). In this work, differential interference contrast images were overlapped with the confocal images, to visualize the inner tissues of the leaf, and co-occurrence of both bacterial signal (GFP) and chloroplasts (chlorophyll autofluorescence) revealed endophytically living bacteria in the plant's leaf tissue. Demonstrating the entry through the stomata is of critical importance, since this explains why conventional sanitation strategies based on soil treatment may fail to prevent pathogen infection of leafy vegetables. Li et al. (1999) demonstrated, by quantification of fluorescent signals derived by a promotorless GFP gene, that the expression level of *vir* genes in *Agrobacterium tumefaciens* varies during the infection process, also accompanied by changes in cell morphology. Newman et al. (2003) identified the vessel-to-vessel movement as the mechanism of infection responsible for the degenerative disease of *Vitis vinifera* induced by *Xylella fastidiosa* (an otherwise harmless endophyte).

Plant-microbes interactions in plant microbial fuel cells were studied by Timmers et al. (2012). In such devices, living plant roots provide electron donor for electricity generation in a mixed microbial community which generates electricity. The authors analyzed anode–rhizosphere bacterial communities of a *Glyceria maxima* (reed mannagrass) fuel cell. They found electrochemically active bacteria on the root surfaces, but at much lower abundance than on the graphite anode. As anaerobic cellulolytic bacteria neighbored the electrogenic bacteria, current production was enhanced by hydrolysis of cellulose.

CRYPTOGAMS: MOSSES AND LICHENS

In a study of bog mosses of the genus *Sphagnum*, Bragina et al. (2012) demonstrated by FISH-CLSM that the hyalocytes, i.e., dead moss cells which serve as water containers and are in direct contact with the external environment, are the preferred colonization sites. Further studies on the functions of such endophytes demonstrated their potential involvement on nitrogen fixation and methane degradation (Bragina et al., 2011, 2013). This suggests that these specific niches are not only water reservoirs. They might represent a sort of "micro-bioreactors" for nutrient production that supports the growth of the host, and may also exert direct ecosystem impact (Kip et al., 2010).

Lichens are traditionally considered as mutualistic symbioses of fungi and photoautotrophs (algae or cyanobacteria). Recent microscopic studies revealed high abundances of bacteria in these symbioses, comparable to those of rhizosphere soil and other microbial hot spots (Cardinale et al., 2008; Grube et al., 2009; Schneider et al., 2011). Counting of bacteria in confocal images of FISH-labeled bacteria helped to statistically evaluate the effect of environmental factors on the frequency of main bacterial phyla in different lichen species (Cardinale et al., 2012; this was one of the few cases in which data obtained with confocal microscopy were statistically assessed). Three-dimensional modeling of lichen microhabitats lead to reconsider the hypothesis of lichens as autonomous mini-ecosystems, this time including bacterial communities functionally adapted to the different thallus regions (Farrar, 1985; Grube et al., 2009; Cardinale et al., 2012).

CRITICAL ISSUES IN CONFOCAL MICROSCOPY OF PLANT-MICROBE INTERACTIONS

CLSM images are presented as either maximum projections or single optical slides. Therefore, it is necessary to know the thickness of the confocal stack as well as the Z-step dimension, in the case of maximum projections, for interpretating the images. In particular this is critical for studying endophytism, physical interactions and spatial arrangement of microbial populations. In the case of single optical slices, the thickness of sections should also be mentioned. If colonization is scant, low resolution may contribute to a misunderstanding of signals in the images unless critical interpretation confirms bacterial signal. Size and shape characters help to distinguish bacterial cells from autofluorescent objects in the same emission range. Conspecific microbial colonies are then recognized by discernible single cells with shared phenotype.

Visualization tools are available for sophisticated analyses and improved interpretation of image data. As an example, the colonization pattern and the endophytism of the PGPR *Burkholderia terricola* ZR2-12 (Gasser et al., 2011) in the root system of sugar beet are impossible to assess in the maximum projection (**Figure 1A**), but become apparent only in the volume rendering (**Figure 1B**), three-dimensional modeling, and its cutting plane (**Figures 1C,D**, respectively). Such operations are possible with freeware Image Surfer (Feng et al., 2007) or professional software such as Imaris (Bitplane, Switzerland) and Amira (TGS Inc., US).

Autofluorescence is a typical phenomenon of CLSM with plant material. Pretreatments of the samples may help to reduce autofluorescence and prevent blurring of target signals in FISH experiments. In observations of plant-microbes interactions, however, the genuine autofluorescence can also help in precisely locating the microorganisms. Multichannel confocal systems with

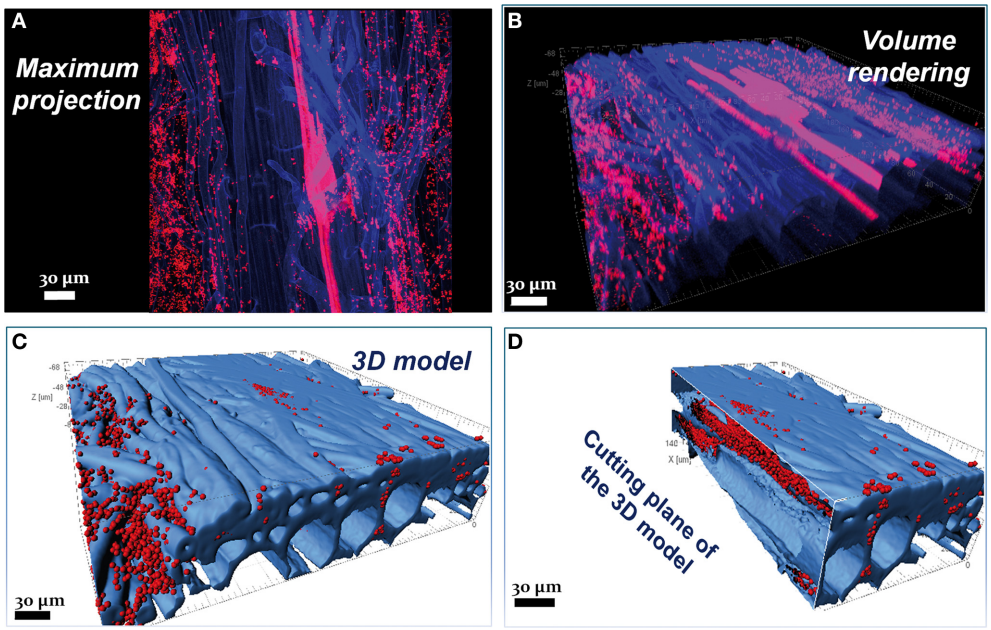

FIGURE 1 | Combination of FISH with autofluorescence. Confocal images showing root colonization by the PGPR *Burkholderia terricola ZR2-12*. **(A)** In the maximum projection it is not possible to assess the colonization pattern of *Burkholderia terricola ZR2-12* (red) on this 3 weeks-old sugar beet root (blue); it is impossible as well to discriminate endophytism from ectophytism. **(B)** The volume rendering of the same confocal stack shows the cells colonizing the internal root tissues but only in the three-dimensional models **(C,D)** it appears clear that the same bacterium shows a double colonization style: ectophytic at the sides of the root **(C)** and endophytic, following the apoplastic spaces **(D)**; furthermore, the data from the three-dimensional models (number of spots, volume, etc.) can be easily retrieved and treated with statistics. This confocal stack has a thickness of 70.16 µm and was acquired with a Leica TCS SPE (Leica Microsystems GmbH, Mannheim, Germany) using the oil immersion objective Leica ACS APO 40.0x1.15. Z-step was 0.8 µm. Three-dimensional models were created with the software Imaris 7.3 (Bitplane, Zurich, Switzerland). Figure was prepared with Adobe Creative Suite version 3 (Adobe Systems Inc., San Jose, CA, USA).

adjustment of detection ranges allow the dedication of one detection channel to the wavelength band of autofluorescence. This requires preliminary CLSM observations of unstained samples to find the lower and upper boundaries of autofluorescence absorption and emission (as well as its intensity). It is uncommon that a wide emission spectrum of the autofluorescence prevents application of suitable fluorochromes for staining of target microorganisms. It otherwise happens that autofluorescence is relatively weak. Signal accumulation during the image acquisition followed by a digital improvement by image post-processing can then help to suitably visualize an autofluorescent host matrix. **Figure 1** gives an example of how the autofluorescence of sugar beet roots can be exploited to image the microhabitat of microbes as a reliable three-dimensional model.

Autofluorescence was already used for structural analysis of biofilm (Muñoz-Egea et al., 2013); however in case of weak autofluorescence of the host structures, histochemical staining can be coupled with FISH to enhance the signal of the host tissues supporting the microbial communities. A suitable staining is the Calcofluor white, which stains α 1-4-glucans characterizing many plant- and fungal cell walls as well as certain components of microbial outer layers (**Figure 2**). This hybrid approach allows distinguishing between structurally different populations within the same taxon, as shown for Betaproteobacteria in **Figure 2**.

QUANTITATION OF CLSM DATA

Microscopy is applied often for qualitative description of both complex populations and single species (for example pathogens or PGPR) and their localization. Indeed, there is only a handful of scientific papers on plant-microbes associations where CLSM data were analyzed quantitatively (e.g., Pivato et al., 2008; Iverson and Maier, 2009; Cardinale et al., 2012), even though the direct *in situ* observations could complement the PCR-based approach and even reveal PCR biases (Bulgarelli et al., 2012; Cardinale et al., unpublished data). However, certain factors can strongly limit the possibility of statistical approaches with CLSM images, even after it has been verified that detected signals represent target objects and not artifacts. For example, strain specific variation for species rich communities cannot be resolved by CLSM. It is thus advisable to complement CLSM data by other approaches, such as deep 16S rRNA gene amplicon sequencing, or metagenome sequencing, which both deliver suitable information for assessment of community structures as well as for evaluation of alfa- and beta-diversity.

Microbial cells are unevenly distributed on their plant hosts. Therefore, the values of density of a certain host-associated microbial community does not inform about its actual dispersion across the host. **Figure 3** shows an example of how the bacterial community associated with lettuce root can be differentially dispersed: in the first example (**A–C**) the community

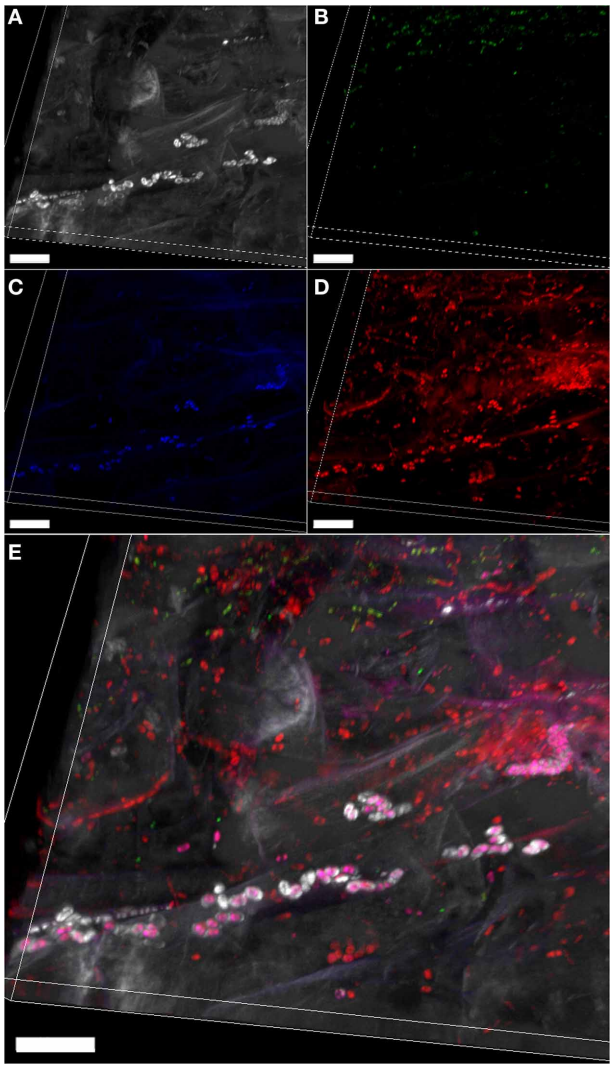

FIGURE 2 | Combination of FISH with histochemical staining. Volume rendering of a confocal stack showing the bacterial colonization of salad root (*Lactuca sativa*) by the native bacterial community, stained by FISH. Gammaproteobacterial **(A)**, betaproteobacterial **(B)**, and other bacterial cells **(C)** (green, blue, and red, respectively) stained with the FISH probes Gam42a (Cy5-labeled), Bet42a (ATTO488-labeled) and EUBMIX (Cy3-labeled), respectively. **(D)** Compounds and tissues stained with calcofluor white (0.15% in H_2O, 15 min incubation) appear gray. **(E)** Overlap of images **(A–D)**; yellow, Gammaproteobacteria; pink, Betaproteobacteria; red: other bacteria; gray: compounds surrounding some Betaproteobacteria stained by calcofluor white. Scale bars: 20 μm. Confocal stack has a thickness of 16.99 μm, and was acquired with a Leica TCS SPE (Leica Microsystems GmbH, Mannheim, Germany) using the oil immersion objective Leica ACS APO 40.0x1.15. The Z-step was 0.38 μm. Volume rendering was created with the software Imaris 7.3 (Bitplane, Switzerland). Figure was prepared with Adobe Creative Suite version 3 (Adobe Systems Inc., CA, USA).

spreads evenly over the root surface, but different populations (Betaproteobacteria and Gammaproteobacteria) are unevenly distributed since it is possible to find regions exclusively colonized by one or the other group; the second example (**D–F**) shows a more drastic situation: only a big colony of Betaproteobacteria, surrounded by unidentified bacteria was detected, while the Gammaproteobacteria appear as single cells evenly distributed over the root; some regions of the root are almost bacteria-free. Such features can hardly be assessed statistically. In theory, a dispersion coefficient can be calculated for any host-associated microbial community provided that a sufficient number of confocal stacks (randomly acquired throughout the specimen) are analyzed (Ford and Harvey, 2007). The variance of different bacterial taxa *in situ* can be due to different growth strategies or even death rates. The host actively participates to trigger bacterial communities in the rhizosphere by root exudation (reviewed by Dennis et al., 2010) and a role in shaping its genetic structure was also suggested (Mølbak et al., 2007).

Bianciotto et al. (2004) demonstrated the vertical transmission of a bacterial endophyte of the arbuscular michorrizal fungus *Gigaspora margarita* through 4 generations of axenic culture. A statistical approach using CLSM allowed proving that the density of intrasporal bacteria strongly diminished from Generation 0 to Generation 4. This approach was based on manual counts of bacterial cells within 100 × 100 μm squares on single 3 μm-thick optical slices. The total number of detected cells for all the 7 optical slides of each confocal stack represented the density expressed as bacteria*mm^{-3}. In different approaches the bacterial density is measured as colony forming units (CFUs, cultivation-dependent approaches) or as gene copy number (cultivation-independent approaches, q-PCR) per gram of host; thus a direct comparison with the CLSM results (volumetric values) is not possible. In order to directly compare CLSM data with data obtained by cultivation and q-PCR, it would be suitable to convert observed volumes into respective weights of sample. I and colleagues developed the "Delta-volume method" to express the density of bacteria detected by CLSM in lichen hosts as number of cells per gram of lichen thallus (Cardinale et al., 2012). To achive this, a subsample of the lichen specimens fixed for FISH-CLSM was immersed into a graduated tube partially filled with water: the difference in the volume was recorded (Delta-volume) and then the specimen was dried out and weighted. The ratio Delta-volume/weight was then used to convert the values expressed as bacteria*mm^{-3} (obtained by FISH-CLSM) into bacteria*g^{-1} lichen dw. This method might be applicable to every kind of environmental sample.

COMBINATION WITH OTHER MICROSCOPIC TECHNIQUES: CURRENT STATE AND PERSPECTIVES

The combination of CLSM with other microscopic methods could offer additional advantages. In fact, the resolution of confocal microscopy, although higher than conventional light microscopy, is constrained by the optical limitations of the light microscopy; coupling fluorescent microscopy with cryo-electron microscopy in a correlative approach offers a possibility to first localize regions of interest or target objects and then visualize them at nanometric resolution (Sartori et al., 2007; Jahn et al., 2012). Coupling CLSM with a scanning probe system (such as an atomic force microscope—AFM) is another correlative approach which has been used for medical sciences but not yet for plant-microbes interactions (Haupt et al., 2006). Although an efficient CLSM-AFM protocol could be difficult to optimize (due to the

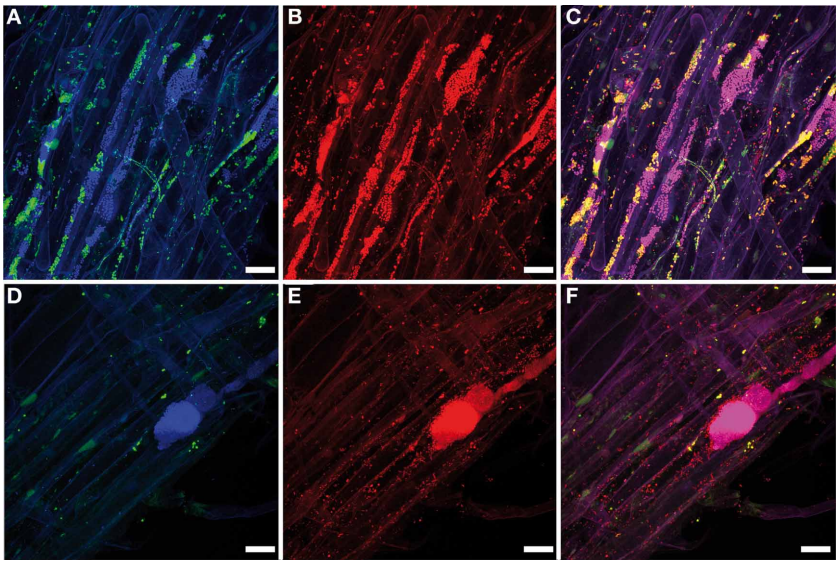

FIGURE 3 | Microbial interactions in the rhizosphere. Maximum projections of a confocal stack showing the colonization pattern of salad root (*Lactuca sativa*) by the native bacterial community stained by FISH. **(A,D)** Gammaproteobacterial and betaproteobacterial cells (green and blue, respectively) stained with the FISH probes Gam42a (Cy5-labeled) and Bet42a (ATTO488-labeled), respectively. **(B,E)** All bacterial cells (red) stained with the FISH probe EUB338-MIX (Cy3-labeled). **(C,F)** Overlap of images **(A,B,D,E)**, respectively; yellow, Gammaproteobacteria; pink, Betaproteobacteria; red: other bacteria. Different taxa do not share the habitats, but instead colonize microniches of the rhizoplane dominantly, excluding each other (see text for more explanations). Scale bars: 20 μm. Confocal stacks **(A,C,D,E)** have a thickness of 30.72 and 37.26 μm, respectively, and were acquired with a Leica TCS SPE (Leica Microsystems GmbH, Mannheim, Germany) using the oil immersion objective Leica ACS APO 40.0x1.15. The Z-step was 0.5 μm. Maximum projections were created with the software Imaris 7.3 (Bitplane, Zurich, Switzerland). Figure was prepared with Adobe Creative Suite version 3 (Adobe Systems Inc., San Jose, CA, USA).

fact that AFM works properly only with relatively flat specimens), it has anyway a potentiality to deliver deep structural information not available with CLSM only, such as interaction forces between beneficial microorganisms, pathogens and hosts. Correlative microscopy that combines FISH-CLSM with nanoSIMS could be particularly interesting, as this may ideally provide information on functional contributions of individual groups of bacteria. This has not yet been achieved and, until now, nanoSIMS has still rarely been used for studying plant-bacteria interactions. Clode et al. (2009) used this approach to visualize differential partitioning of $^{15}NH_4^+$ between plant roots and native soil microbial communities at the submicron scale.

CLSM ANALYSIS AS GUIDANCE FOR DOWNSTREAM EXPERIMENTS

CLSM has been used to complement studies with other methods, such as deep sequencing or quantitative-PCR. Yet, the analysis of the native microbial communities using CLSM can help in hypothesis development and testing, and for proper sample size estimation for subsequent experiments. This includes the role of environmental factors on colonization patterns (**Figure 4**). Otherwise FISH-CLSM could guide the focus to particular bacterial groups in subsequent culture-dependent or culture-independent studies: in the case of lettuce root colonization (**Figure 3**), different taxa do not share the same site, but instead dominate in microniches of the rhizoplane and exclude each other (**Figures 3A–C**), and hence form locally extremely dense colonies (**Figures 3D–F**). The following hypotheses could result from these observations: (**A**) Lettuce roots either release specific exudates at microscale resolution, or concentrate them at particular sites on the root surface; (**B**) Different bacterial species arrive at different times, and the initial colonies exclude the following; (**C**) Finally, in case these bacteria are simultaneous colonizers, some species are locally enriched by faster growth. These hypotheses can be tested with specifically designed inoculation experiments under controlled conditions of growth.

CONCLUSIONS AND PERSPECTIVES

Research of host-microbes systems requires a polyphasic approach to unravel the complexity of their interactions and ecological significance. Still, direct qualitative and quantitative information of bacterial colonization and its variation on the hosts' structures is only possible through direct visualization *in situ* and therefore CLSM serves as a central technology in such studies. The intrinsic variance of this information needs to be properly assessed by a statistical approach, to gain new and deeper insights into the stability and plasticity of host associated microbes in a changing environment.

Several other exciting microscopic techniques emerged over the past few years, e.g., Coherent Anti-Stokes Raman Scattering (CARS, Cheng et al., 2002), Multi-Isotope Imaging Mass Spectrometry (MIMS, McMahon et al., 2006), or Stimulated Emission Depletion Microscopy (STED, Westphal et al., 2008). However, these still depend on substantial infrastructure and their applicability to study a broader range of environmental samples has still to be shown. FISH-CLSM not only remains as a widely

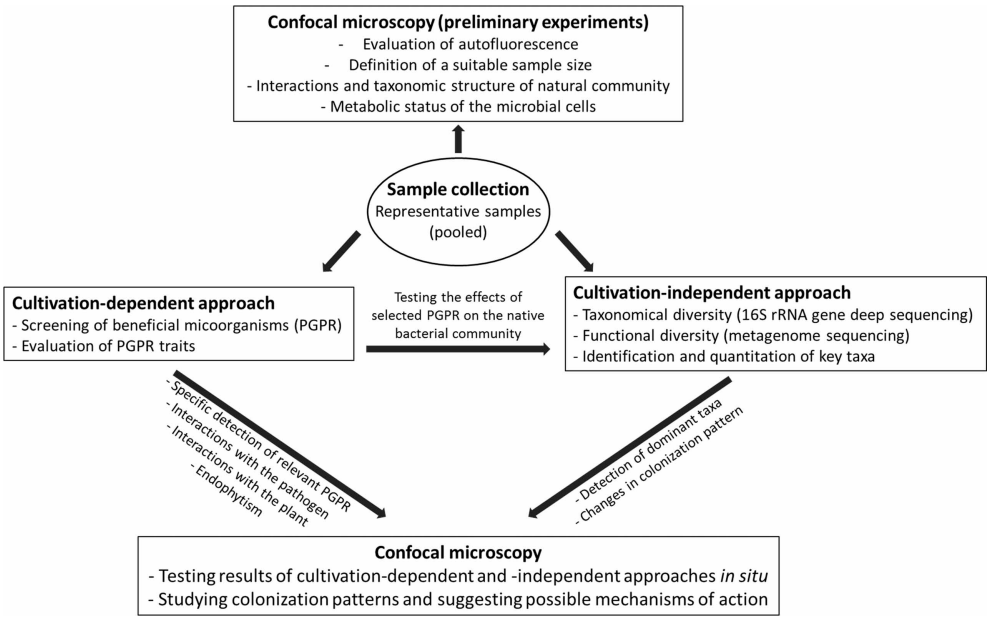

FIGURE 4 | Integration of CLSM with other techniques. The workflow shows the combination of CLSM with other methods for plant-microbes interactions studies. A case study of PGPR is used in this example.

applicable methodology for studying plant-microbe interactions, but can be extended and complemented by other microscopic techniques.

In the last few years, microbial ecology was revolutionized by the advent of the deep-sequencing as a tool affordable for every laboratory. This was somehow similar to what happened in the 90 years, when fingerprinting techniques allowed for the first time the study of total microbial communities, including uncultivated organisms. Once more, the effect was that the scientists' attention was focused on the sequence-based information delivered by the new techniques and microscopy was overshadowed. Here I showed the critical role that microscopy (especially CLSM) had in the understanding of the processes. Localization at microscale, colonization pattern or cell-cell interaction, are not detectable by cultivation, fingerprinting, or deep-sequencing analysis, yet being the basic processes of plant-microbe interactions. High-resolution microscopy, coupled with suitable visualization and statistical methods, still represent the optimal tool that can provide such information and represents the best suited method to validate the results of molecular analysis in microbial ecology studies.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00094/abstract

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The arable ecosystem as battleground for emergence of new human pathogens

Leonard S. van Overbeek[1]*, Joop van Doorn[2†], Jan H. Wichers[3], Aart van Amerongen[3], Herman J. W. van Roermund[4] and Peter T. J. Willemsen[4]

[1] Plant Research International, Wageningen University and Research Centre, Wageningen, Netherlands
[2] Applied Plant Research, Wageningen University and Research Centre, Lisse, Netherlands
[3] Food and Biobased Research, Wageningen University and Research Centre, Wageningen, Netherlands
[4] Central Veterinary Institute, Wageningen University and Research Centre, Lelystad, Netherlands

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Eelco Franz, Centre for Infectious Disease Control, Netherlands
Paolina Garbeva, Netherlands Institute of Ecology, Netherlands

***Correspondence:**
Leonard S. van Overbeek, Plant Research International, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB, Wageningen, Netherlands
e-mail: l.s.vanoverbeek@wur.nl

†Present address:
Joop van Doorn, Enza Zaden Seed Operations BV, Enhuizen, Netherlands

Disease incidences related to *Escherichia coli* and *Salmonella enterica* infections by consumption of (fresh) vegetables, sprouts, and occasionally fruits made clear that these pathogens are not only transmitted to humans via the "classical" routes of meat, eggs, and dairy products, but also can be transmitted to humans via plants or products derived from plants. Nowadays, it is of major concern that these human pathogens, especially the ones belonging to the taxonomical family of *Enterobacteriaceae*, become adapted to environmental habitats without losing their virulence to humans. Adaptation to the plant environment would lead to longer persistence in plants, increasing their chances on transmission to humans via consumption of plant-derived food. One of the mechanisms of adaptation to the plant environment in human pathogens, proposed in this paper, is horizontal transfer of genes from different microbial communities present in the arable ecosystem, like the ones originating from soil, animal digestive track systems (manure), water and plants themselves. Genes that would confer better adaptation to the phytosphere might be genes involved in plant colonization, stress resistance and nutrient acquisition and utilization. Because human pathogenic enterics often were prone to genetic exchanges via phages and conjugative plasmids, it was postulated that these genetic elements may be hold key responsible for horizontal gene transfers between human pathogens and indigenous microbes in agroproduction systems. In analogy to zoonosis, we coin the term phytonosis for a human pathogen that is transmitted via plants and not exclusively via animals.

Keywords: EHEC, emerging pathogens, phytonoses, gene transfer, rhizosphere, *Enterobacteriaceae*

INTRODUCTION

THE PLANT ENVIRONMENT AS A HABITAT FOR HUMAN PATHOGENS

Agricultural plants have become a source for human pathogens, especially the emerging ones belonging to the group of Shiga toxin-producing *Escherichia coli* (STEC) strains (Feng and Reddy, 2013). The threat of human pathogens in freshly consumable products of plant origin became apparent during the outbreak caused by *Escherichia coli* O104:H4 in Germany and France in 2011, where almost 4000 persons became infected leading to 54 casualties and over 900 incidences of hemolytic uremic syndrome (HUS; Karch et al., 2011; Beutin and Martin, 2012). The most likely transmission route of the pathogen to consumable products was remarkable as the source was fenugreek seeds that were transported from Egypt to Rotterdam harbor, the Netherlands, approximately 17 months before appearance of the first disease incidences in Hamburg and surrounding area (Karch et al., 2011). Although the pathogen was neither found in Fenugreek sprouts, nor in the seeds themselves, epidemiological facts revealed that the pathogen must have been associated with seeds over a relatively long period of time. The questions rise on how a human pathogen can persist as a viable entity in a hostile environment for such a long period and why similar observations had not been made before. Must this be considered as the first incidence of a disease outbreak caused by a human pathogenic bacterium that was adapted to the plant environment?

To address this question, it must be referred to the nature of the causative agent that is atypical for pathogenic *Escherichia coli* strains commonly occurring in Europe and the USA (Scheutz et al., 2011; Beutin and Martin, 2012). The *Escherichia coli* O104: H4 outbreak strain was an entero-aggregative *Escherichia coli* strain that does not have animals, which is often the case for other *Escherichia coli* O type strains, but instead only humans as major reservoir (Wieler et al., 2011). Outbreaks caused by this type of pathogen are rare in Western societies, whereas those caused by *Escherichia coli* O157:H7 and *Salmonella enterica* are more common (**Table 1**). This indicates that particular features in these human pathogens already exist, extending their life-time in the plant environment. The question is whether these features were intrinsic to particular subsets of human pathogens or were recently gained, e.g., via horizontal gene transfer. The strain causing the outbreak in the Hamburg area must be considered as a highly virulent pathogen and it must have acquired its virulence and antibiotic

Table 1 | Examples of large disease outbreaks resulting from contamination of vegetables and sprouts with human pathogenic bacteria.

Location	Pathogen	Food source	Reference
East Anglia (UK)	*E. coli* O157:H7	Potato tubers	Morgan et al. (1988)
Osaka (Japan)	*E. coli* O157:H7	Radish sprouts	Michino et al. (1999)
Connecticut (USA)	*E. coli* O157:H7	Mesclun lettuce	Hilborn et al. (1999)
Western USA, British Columbia (Canada)	*E. coli* O157:H7	Unpasteurized apple juice	Cody et al. (1999)
California (USA)	*E. coli* O157:H7, *S. enterica*	Alfalfa and clover sprouts	Mohle-Boetani et al. (2001)
Michigan (USA)	*E. coli* O157:H7	Alfalfa sprouts	Breuer et al. (2001)
Multistate outbreak USA	*E. coli* O157:H7	Packaged spinach	Wendel et al. (2009)
Hamburg (Germany)	*E. coli* O104:H4	Fenugreek sprouts	Bielaszewska et al. (2011)

resistance (extended spectrum beta-lactamase, ESBL) traits via horizontal gene transfer events like transduction (phage infection) and conjugation (plasmid transfer; Mellmann et al., 2011; Muniesa et al., 2012). Acquisition of new virulence traits is one aspect in the evolution of a new pathogen, but selection pressure is another, and the outbreak strain must have been evolved by increasing its virulence in humans side-by-side with improvement of its ecological competence in plants. The threat of this development is the emergence of new types of highly virulent human bacterial pathogens that are fully adapted to life near, or may be inside agricultural plants.

BACTERIA ASSOCIATED WITH MULTIPLE HABITATS

The agricultural plant environment is an environment where microbial communities of at least four different ecosystems may come together, namely those from soil, plants, farm animal digestive track systems (manure), and fresh water sources (irrigation). Agricultural plant production thus must be considered as a cross road of communities originating from these different sources. The microbial community compositions in these systems substantially differ from each other, but representatives of the different communities may, at least temporarily, coexist with micro-organisms typically associated with plants. However, much is still unknown about the microbial composition and functioning of these microbes in the different environments. Some remarkable correspondence in the taxonomy of human disease-causing bacteria can be found with typical soil/rhizosphere bacterial species. Clearest examples are the so called cross-domain pathogens belonging to the genera of *Burkhoderia* (e.g., *Burkhoderia cepacia*) and *Pseudomonas* (e.g., *Pseudomonas aeruginosa*; Barak and Schroeder, 2012; Kumar et al., 2013). These are opportunistic pathogens in humans and are commonly found in plant and soil habitats. Distinction of pathogenic from non-pathogenic types (of which some can be excellent plant growth promoters) of both genera is often difficult to accomplish. *Pseudomonas fluorescens* is a bacterial species that is commonly found near plant roots, however, representatives of this species were also shown to be associated with patients suffering from Crohn's disease (Wei et al., 2002; Eckburg and Relman, 2007). A representative of *Pseudomonas veronii*, another typical rhizosphere-borne bacterial species was found to be associated with human intestinal inflammatory pseudotumour formation (Cheuk et al., 2000). Typical plant pathogens belonging to the genus of *Pantoea (Pantoea ananatis* and *P. agglomerans*) and *Erwinia* (*Erwinia tasmaniensis*) were also demonstrated to cause diseases in humans (Cruz et al., 2007; Shin et al., 2008; Coutinho and Venter, 2009). Taxonomical commonalities exist between human pathogens and species known to thrive in soil habitats. Recently, a new taxa belonging to a hitherto uncultured group of bacteria named *Verrucomicrobia* subdivision 1 was shown to be competent in the rhizospheres of different plant species (Nunes da Rocha et al., 2011), whereas a representative of the same group (*Akkermansia muciniphila*) had been found as a commensal species living in the intestinal track system of humans (Derrien et al., 2004), where it deemed to play a role in degradation of mucus and health support of humans. This illustrates that the taxonomical diversity of cross domain species are high and up to date still rather unexplored. Important proposition in this paper is that the chance on genetic exchanges via horizontal transfer will be highest between cross-domain species commonly occurring in different ecosystems and coming into physical contact with each other near or within plants. Human pathogens may acquire genes from plant-associated bacteria (Szmolka and Nagy, 2013), leading to new phenotypes of increased persistence in plants or their propagative materials, higher resistance to stresses or broader spectra in acquisition or utilization of available nutrients. These traits will make human pathogens fitter for survival in the plant environment.

Especially, the rhizosphere and phyllosphere of plants must be considered as hot spots of microbial life because of the nutrients leaking from growing roots and leaves. Plant surfaces are important reservoirs for (enteric) opportunistic human pathogens (Berg et al., 2005; Holden et al., 2009). *Escherichia coli* belong to the *Enterobacteriaceae*, and species belonging to this taxonomical class can be found frequently as mutualistic, commensal, or plant pathogenic species in plants. Species belonging to the genera of *Enterobacter, Serratia, Klebsiella, Erwinia, Pantoea,* and *Pectobacterium* are typical plant-associated bacteria and if gene transfer events with plant-invasive human pathogens, like ones belonging to *Escherichia coli* and *S. enterica*, will occur then expectedly it will be highest with these species. Prophages and plasmids in plant-associated *Enterobacteriaceae* are extrachromosomal gene pools that, thus far, remained inaccessible for human pathogens. On the other hand harmless bacteria living as commensals in plant

environments may pick up mobile elements from plant-invasive human pathogenic species to become human pathogens (Szmolka and Nagy, 2013). The crucial difference between a relatively harmless bacterium and a dangerous human pathogen can rely on presence or absence of a single mobile genetic element (Frost et al., 2005). It, thus, might be possible that harmless bacterial species living as commensals in plants acquire traits making them virulent to humans although these type of incidences has not been reported to occur in plants to date.

Practices common in agriculture make it possible to bring gene pools from different ecosystems together and making these accessible for commensal and human pathogenic species, leading to acquisition of new traits and possibly to emergence of new human pathogens (**Figure 1**). Phytonoses (singular phytonosis), occurring from human infecting agents transmitted by plants (van der Riet, 1997), in analogy to zoonoses, which are diseases transmitted by animals, can be coined as a new term for the group of diseases caused by human pathogens, viz. *Escherichia coli* and *S. enterica*, that are transmitted via consumption of fresh produce.

THE ARABLE PLANT ENVIRONMENT AS CROSS ROAD OF DIFFERENT ECOSYSTEMS

THE ANIMAL DIGESTIVE TRACK SYSTEM AS MAJOR RESERVOIR FOR *Escherichia coli* O157:H7 AND OTHER HUMAN PATHOGENIC ENTERICS

Shiga toxin-producing *Escherichia coli* serotypes, the most commonly found human pathogens in plant-derived products, pose serious threats to human health. The question often raised in different studies is why these strains, and especially the ones of *Escherichia coli* O157:H7, are so often associated with plants (**Table 1**). *Escherichia coli* O157:H7 live as commensal species in the digestive track system of ruminants without causing any visible symptoms to the host (Wells et al., 1991; Chapman et al., 1993). The bacterium is excreted via feces and can survive in manure for over a long period of time. The most important STEC serotypes causing disease in humans are O157:H7, O26:H11, O103:H2, O145:H28, and O111:H8 (Pearce et al., 2004, 2006). In North America, Japan and Europe, O157:H7 is the type causing most disease incidences in humans and this is the reason why most research is focussed on this serotype. However, other serotypes like O26, O103, and O145 were regularly found in cattle from Scotland and USA (Wells et al., 1991; Wieler et al., 1996; Pearce et al., 2004, 2006; Shaw et al., 2004). Shiga toxin-producing O104:H4 or other entero-aggregative *Escherichia coli* strains were not found in cattle manure from Northern Germany during the Hamburg outbreak in 2011, indicating that cattle is not a major reservoir for the entero-aggregative *Escherichia coli* pathotype, to which the *Escherichia coli* O104:H4 outbreak strain belong to (Wieler et al., 2011).

The occurrence of *Escherichia coli* O157:H7 is more common in the intestinal track systems of cattle than in that of other ruminants and farm animals (Karmali et al., 2010; Ferens and Hovde, 2011 and references therein). The distribution of the pathogen in cattle feces is heterogeneous and occasionally numbers can be very high in excrements of particular individuals within the herd, so called super shedders (Ferens and Hovde, 2011). Epidemiological research on *Escherichia coli* O157:H7 infections in livestock in slaughterhouses in the Netherlands revealed prevalence of 10.6% ($n = 540$) in adult cattle, 0.5% ($n = 397$) in veal calves, 3.8%

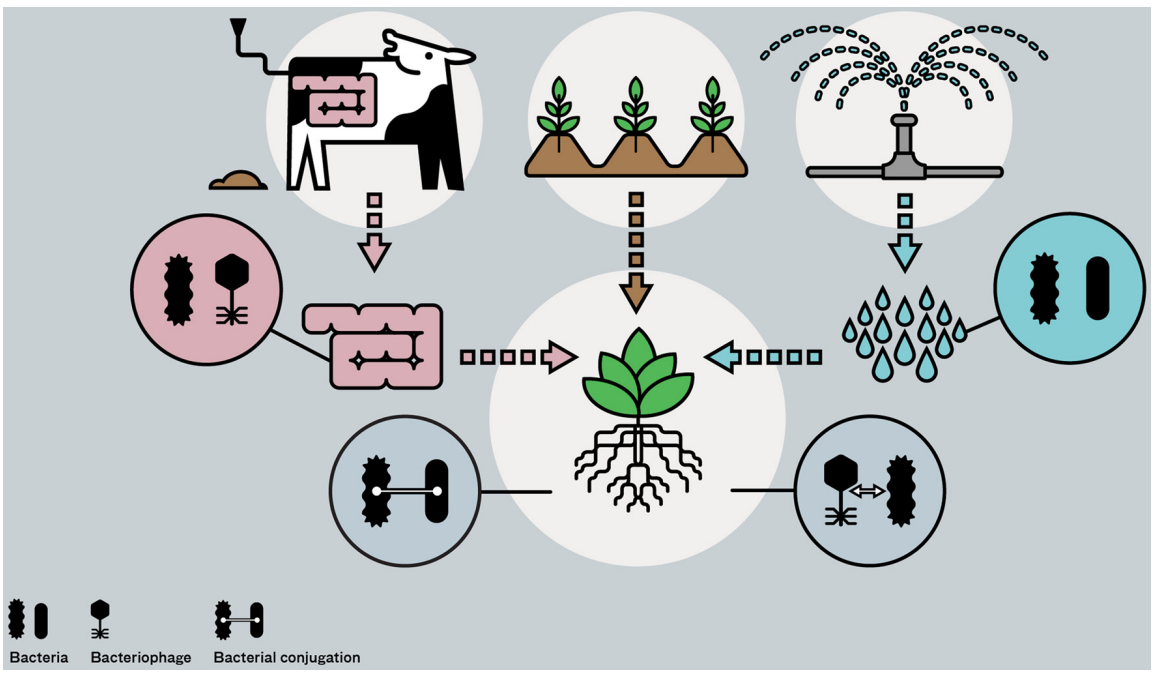

FIGURE 1 | Bacterial communities from different habitats, i.e., from cattle intestinal track system via manure, surface water via irrigation, and from soil and plants, all coming together at plant growth in arable production systems. Four magnifications in the figure depict origin of different bacterial groups and their phages, and the possible occurrences of gene transfer near or inside plants, either via conjugation or via transduction.

($n = 52$) in sheep, 4.1% ($n = 49$) in lambs, 1.4% ($n = 145$) in pigs, 0% ($n = 501$) in chicken and 1.3% ($n = 459$) in turkey (Heuvelink et al., 1998a, 1999). Highest incidences in serotype O157:H7 contamination was thus found in adult cattle, but this serotype was also present in carcasses of other animals, with the exception of chicken. Examination of 10 dairy farms in the Netherlands, of which five were demonstrated to be serotype O157:H7 positive in their cattle, resulted in seven farms where at least one serotype O157:H7-positive individual was identified to be present (Heuvelink et al., 1998a). Within-herd prevalence on these seven farms varied between 0.8 and 22.4%. Surveillance of *Escherichia coli* O157:H7 in pooled fecal samples over 1051 dairy herds in the years 1997–2005 revealed on average prevalence of 8.0% (variation between 6.4 and 9.6%; Berends et al., 2008). In the same study surveillance of two distinct types of veal herds (so called pink and white veal herds) revealed clear differences between both types, i.e., 107 positive of 269 examined pink herds (39.8%) and 10 positive of 661 examined white herds (1.5%; Berends et al., 2008). The reason for this difference in infection prevalence was not further investigated, but may be related to difference in age before slaughtering, which is 35 weeks for pink veal and 25 weeks for white veal. Interestingly, screening of Dutch dairy farms on the basis of presence of Shiga toxin genes revealed much higher prevalence (80%) than via screening on serotype O157:H7 determinants (Franz et al., 2007). This would indicate that Shiga toxin genes, which are considered to be major virulence determinants in enterohemorrhagic *Escherichia coli* (EHEC), are not only present in serotype O157:H7, but can also be present in other serotypes as well. One important observation in the longitudinal study done in the Netherlands was the fact that the number of serotype O157:H7 in positive cattle herds shifted during the season showing maximum peaks in the second half of the summer period, whereas this serotype was undetectable in samples collected in wintertime (Heuvelink et al., 1998b; Bouwknegt et al., 2004; Schouten et al., 2004, 2005; Valkenburgh et al., 2007). Such a fluctuation during the season was also observed in other countries (Hancock et al., 1994; Chapman et al., 1997; Conedera et al., 2001; Barkocy-Gallagher et al., 2003; Milnes et al., 2009). This would indicate that fluctuations in serotype O157:H7, and possibly also in other serotypes, exist in cattle manure, which has a consequence in the control of serotype O157:H7 contamination of vegetable plants grown in manure-amended soils. Depending on the period in the season, contamination with pathogenic *Escherichia coli* serotypes expectedly will vary.

It was hypothesized that *Escherichia coli* O157:H7 is better adapted to circumstances prevailing in habitats outside of the digestive track of cattle than other non-Shiga-toxin producing *Escherichia coli* serotypes (Durso et al., 2004). This study revealed that a higher incidence of competitive strains were found among *Escherichia coli* O157:H7 than over non-Shiga toxin producing serotypes, but the non-Shiga toxin types were able to utilize a broader spectrum of tested substrates than *Escherichia coli* O157:H7. There were no other factors that could explain better fitness of *Escherichia coli* O157:H7 over non-Shiga toxin producing serotypes in alternative habitats, in spite of their relatively larger genome sizes that might be indicative for better adaptation to multiple habitats. Other, unknown factors therefore must be held responsible for survival of *Escherichia coli* O157:H7 outside the intestinal track systems of cattle.

The most likely route of contamination from cattle to plants is via manure through soil to plant roots, but other routes may exist like irrigation water (Erickson et al., 2010), flies (Talley et al., 2009) and equipment used by field workers (Johnston et al., 2009). Different transmission routes of *Escherichia coli* O157:H7 to arable plants may exist and remarkable is the rather long-term persistence of these strains in plant and soil ecosystems. It seems that at least some of the STEC strains and strains of other human pathogens like *Salmonella, Campylobacter*, and *Listeria* are better adapted to the circumstances prevailing in plants and soils than ever thought before.

TRANSMISSION ROUTES OF HUMAN PATHOGENS IN PLANT PRODUCTION FIELDS

One of the first reported disease outbreaks caused by *Escherichia coli* O157:H7 and associated with consumption of vegetables was from potatoes in the UK (Morgan et al., 1988; **Table 1**). In this particular case, it was potato tubers that were suspected to be contaminated with *Escherichia coli* O157:H7 most likely originating from cattle manure and applied at potato production in the field. Because potatoes are not eaten raw, it was postulated that the transmission route of the pathogen was not via potato consumption itself, but rather from hand-to-mouth transfer at food preparation and handling of potatoes. From this case it became clear that the pathogen was able to persist over longer periods of time in soil near or at the surface of potato tubers. Potato lots containing the pathogen were not traceable anymore, so it is unknown at which densities and locations on, or may be inside tubers the human pathogen was present (Morgan et al., 1988). Concerning internalization, invasiveness of the pathogen would depend on specific enzymes like endoglucanases to create openings in plant cells, like plant pathogens do, or they would enter plants via natural openings (stomata and hydathodes) or woundings caused by feeding insects or nematodes or via co-infections with plant pathogens or soil-indigenous micro-organisms. The underground route of *Escherichia coli* O157:H7 and other human pathogenic enterics to plants will be discussed below as it is often hypothesized, but not always accepted as a realistic transmission route to plants.

Survival of human pathogens in manure-amended soils

The underground route of transmission of *Escherichia coli* O157:H7 to plants can be plausible if the survival time of the pathogen in manure-amended soil is long enough to allow successful colonization of plants. Under experimental circumstances, *Escherichia coli* O157:H7, introduced into autoclaved soils and mixed at different ratios with manure, was able to survive for more than 226 days at 15 and 21°C (Jiang et al., 2002). Lowering the temperature to 5°C led to a reduction in survival time to down to 35 days. Introduction into non-autoclaved manure-amended soil reduced survival to a maximal time of 193 days at 15 and 21°C, but there was no reduction in survival time at 5°C. This indicates that temperature is an important factor directly affecting survival time of the pathogen in soil, but also indirectly

by influencing the activities of microbial populations resident in soil that antagonizes the invasive human pathogen. Higher average temperatures and higher oscillation amplitudes resulted in faster decline of both populations (Semenov et al., 2007). However, lower temperatures, between 10 and 15°C, commonly occur in temperate regions where incidences with *Escherichia coli* O157:H7 outbreaks are highest. Soil horizons where plant roots grow are often more constant in temperature than topsoil horizons and therefore, invasion of human pathogens to deeper soil layers would favor longer-term persistence in soil and thus increasing their chances on contact with plant roots. The effect of manure type and application to soil on percolation to deeper soil layers of the same *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium strains were investigated in another study (Semenov et al., 2009). Both strains percolated to deeper layers (maximal studied depth was 40 cm) within a few hours after introduction, independent of manure type, and application of slurry resulted in percolation of both pathogens to greater depths than of solid manure, whereas the mode of application (spreading over soil surface versus injection into topsoil) had smaller effects on percolation depths of both pathogens. Percolation to deeper soil layers in fields may have two consequences for transmission to crop plants: one, that survival time may be increased because of the lower and more constant temperature present in deeper soil layers, and two, ground water may become contaminated which can be used for irrigation of crop plants (Brennan et al., 2010).

Available nutrients in manure and soil also were demonstrated to play important roles in survival of *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium strains (Franz and Van Bruggen, 2008). Survival of *Escherichia coli* O157:H7 across 36 different manure-amended soils was investigated and it was shown that survival increased when slurry or chemical fertilizer (relatively rich in available C and/or N) were applied to soils in comparison with applications of farmyard manure or compost (relatively poor in available C and N). Therefore, it was concluded that circumstances characterized to be oligotrophic for bacteria decreased *Escherichia coli* O157:H7 survival in manure-amended soils. Manure type itself is also another factor influencing survival of human pathogens in manure-amended soils. Manure type depends on the diets that cows had received and the effect of roughage of the diet on the fate of introduced *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium strains in manure was investigated (Franz et al., 2005). Population decline of introduced *Escherichia coli* O157:H7 was fastest in manure from cows fed with the roughest diet type (straw). The effect of roughage of the diet on *S. enterica* serovar Typhimurium survival in manure was less clear than for *Escherichia coli* O157:H7, although decline rates of both pathogens were fastest in manures of cows fed with the roughest diet. Fiber content and pH of manure were the best explanatory factors for decline rates of both pathogens.

The nutrient status in manure-amended soils is a factor that plays a direct role in survival of *Escherichia coli* O157:H7 and other human pathogens. However, indirectly, the nutrient level also may play a role in the diversity and evenness of microbial populations that are indigenous to manure-amended soil that might compete with invasive human pathogens (Semenov et al., 2008; Van Overbeek et al., 2010). Soil bacterial diversity as an experimental variable indeed was shown to be negatively correlated with longevity in survival of an introduced *Escherichia coli* O157:H7 strain, representing a species invasive to soil (van Elsas et al., 2012). These data all together illustrate the importance of high microbial diversity to reduce survival time of human pathogens as species invasive to soils.

Finally, survival time is dependent on strain differences, even within the same species. Remarkable differences in survival time between different *Escherichia coli* O157:H7 strains was observed among 18 different strains of which eight originated from animal, one from food, and nine from human sources (Franz et al., 2011). Survival time in manure-amended soils of the nine human strains were longer than of the other nine strains. Principle component analyses on metabolic profiles of all 18 strains revealed separate clustering of the human strains from the others and the ability of the nine human strains to oxidize propionic acid, α-ketobutyric acid and α-hydroxybutyric acid appeared to be discriminative for this particular environmental group. It was therefore concluded that phenotypic diversity found among different *Escherichia coli* O157:H7 strains may explain observed differences in survival time in manure-amended soils. Differences in survival time among different strains of *Escherichia coli* O157:H7 would indicate that some strains are better adapted to circumstances prevailing in natural habitats, like soils, than others. Presence or absence of important virulence factors in all 18 *Escherichia coli* O157:H7 strains appeared not to play any role in survival time in manure-amended soils. This is in line with observations made in a study done on different *stx*1, *stx*2, both *stx* and *eae* (intimin) gene deletion mutants made from *Escherichia coli* O157:H7 EDL933 strain in soils differing in texture (loamy sand, sandy loam, silty clay; Ma et al., 2011). There are thus no indications that virulence to humans has an effect on *Escherichia coli* O157:H7 survival time in (manure-amended) soils. However, mutation in an important gene involved in stress regulation, *rpo*S, had an effect on survival of *Escherichia coli* O157:H7, leading to reduced persistence in manure-amended soil (van Hoek et al., 2013).

Plant colonization by human pathogens

Presence of human pathogens in contaminated soil at high cell densities may lead to plant internalization. This was demonstrated in lettuce plants grown in soil amended with cow manure containing high doses (10^6 and 10^8 CFU/g soil) of a GFP-marked *Escherichia coli* O157:H7 strain (Solomon et al., 2002). The introduced strain was retrieved from lettuce seedlings even after 10 min treatments with $HgCl_2$, indicating that internalization of plant tissue by the pathogen had occurred. Confocal microscopy on lettuce leafs colonized by the GFP-marked *Escherichia coli* O157:H7 strain revealed the presence of fluorescent aggregates in intercellular spaces. There was no evidence for intracellular colonization by *Escherichia coli* O157:H7, but the presence of the pathogens inside plant tissue of the edible parts of the plant already indicate that there is a risk upon consumption of fresh produce, because cells cannot easily be removed or inactivated by washing and disinfection procedures. Adherence and internalization of plant tissue

are consequences when human pathogens come into contact with plants.

Longer-term survival of human pathogens in manure-amended soils ultimately will lead to lower inoculum levels in soils and thus to lower risks of crop plants to become contaminated. The question that arises is whether human pathogens are still capable to contaminate plants after longer periods of residence in soils. A mixture (1:1) of *S. enterica* (serovars Baildon and Enteritidis) strains was detectable to up to 6 weeks after introduction into soil (Barak and Liang, 2008). Sowing tomato seeds at weekly intervals after inoculation of this mixture revealed the presence of the pathogen mixture in the rhizoplane and phylosphere of plants for up to 7 weeks after inoculation. Inoculation of soils with *Escherichia coli* O157:H7 via three different composted manure types and irrigation water revealed the presence of the pathogen strain in lettuce and parsley plants for up to, respectively, 77 and 177 days after transplanting (Islam et al., 2004a). Using the same carriers, *S. enterica* serovar Typhimurium was detectable in radish and carrots after, respectively 84 and 203 days after sowing of seeds into soils (Islam et al., 2004b). No clear effects of the carrier types for the *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium inoculants were found in these studies, however, longevity of survival differed among different tested plant species, indicating that there was an effect of plant type on survival of both pathogen strains. *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium strains inoculated at levels of 10^7 CFU per g manure-amended soil were retrieved from the rhizosphere (Ongeng et al., 2011a) and internal compartments (Ongeng et al., 2011b) of transplanted cabbage (*Brassica oleracea*) plants grown under tropical field conditions. These two studies indicated that the presence of plant roots may extend survival time of human pathogens in soils and that internalization of crop plants may occur following successful rhizosphere colonization.

Long-term persistence in soil may lead to nutrient starvation of human pathogens in soil. These pathogens may gear to forms that are more resistant against stresses, although they also may become metabolically arrested, entering the so called viable-but non-culturable (VBNC) state as was demonstrated for another gammaproteobacterial species, *P. fluorescens* (Van Overbeek et al., 1995). Cells of the *Escherichia coli* O104:H4 Hamburg-outbreak strain (**Table 1**) imposed to copper and low temperature as stress factors became VBNC as demonstrated by viability staining of non-culturable cells (Aurass et al., 2011). However, non-culturability most likely did not affect virulence, as genes responsible for virulence appeared to remain intact. This to the contrary to a typical soil-borne plant pathogenic bacterium, *Ralstonia solanacearum* biovar 2, that also became VBNC upon treatment at low temperature, but these cells apparently lost their virulence upon injection into host (tomato) plants, where these cells still were capable to multiply, demonstrating that they were resuscitated from the VBNC state (van Overbeek et al., 2004). Exposure of *S. enterica* serovar Typhimurium LT2 to cold stress (5 h at 5°C) resulted in higher resistance to acid (pH 4.0) stress (Shah et al., 2013). Persistence of *S. enterica* serovar Typhimurium DT104 in soil and lettuce plants resulted in better survival in simulated gastric fluid than cells freshly grown in culture, however, these cells were less capable to attach and invade epithelial cells (Oliveira et al., 2011). Observed effects slightly differed between the two studied strains (one originating from pig carcass and the other from lettuce plants), but illustrates that physiological aspects in cells of human pathogens can play a role in adaptation to circumstances prevailing in the phytosphere, possibly leading to increased transmission to humans upon consumption.

Presence of plant roots in soil play an important role in the entrance of human pathogens into plants. During root colonization, human pathogens must successfully compete with rhizosphere-indigenous micro-organisms for available nutrients. Iron is an essential element for bacteria and a limiting factor for growth of bacteria in the rhizosphere. Mutations in a precursor in siderophore production, or in siderophore production itself, in *S. enterica* serovar Typhimurium resulted in a significant lower colonization of alfalfa roots (Hao et al., 2012). Siderophore production in *S. enterica* is essential for root colonization, as it is typical for rhizosphere bacteria like *P. fluorescens* and *R. solanacearum*. Siderophore production is a colonization fitness factor for *S. enterica* (Hao et al., 2012) and this would indicate that the pathogen is well-adapted to circumstances prevailing in the rhizosphere.

However, human pathogens contaminating vegetable plants do not necessarily originate from soil, but may originate from seeds, as seeds were supposed to be the main contamination route for the *Escherichia coli* O104:H4 outbreak strain to fenugreek sprouts at the Hamburg outbreak incidence in 2011. *S. enterica* and *Escherichia coli* O157:H7 were both shown to persist on lettuce seeds for 2 years and still capable to colonize young lettuce plants (van der Linden et al., 2013). Of both human pathogens, *S. enterica* was shown to be the best survivor on lettuce seeds. These examples show that contamination of arable plants can occur via different routes and that long-term persistence in soil or on dry seeds both can be important factors in successful colonization of plants.

Proposed mechanisms of plant internalization by human pathogens

Internalization of plants by human pathogens may result from passive or active processes. Cells of *Escherichia coli* O157:H7 "Sakai" strain, introduced to spinach and lettuce plants under experimental conditions using a high inoculum dose of 2×10^7 CFU per ml, were shown to be present inside root tissue of both plant species (Wright et al., 2013), indicating that this human pathogen potentially can enter these plants by itself. It may be assumed that human pathogens do not possess the same specific traits, like cell wall degrading (pectinolytic) enzymes present in plant pathogens that are required for efficient invasion of plants. The presence of plant pathogens at the same locations on the plant surface where human pathogens reside would facilitate entrance of human pathogens into plants as was proposed in Teplitski et al. (2009, 2011). Under practical circumstances in agronomic systems, other soil species including plant pathogens indeed appeared to play important roles in spread, colonization, and internalization of plants by human pathogens. The bacterivorous nematode *Caenorhabditis elegans* was demonstrated to transport *S. enterica* serovar Newport, initially introduced into manure, through manure-amended soil to lettuce, strawberry and carrot plants (Kenney et al., 2006). *Xanthomonas campestris* pathovar vesicatoria facilitated a mixture of *S. enterica* strains to

colonize the tomato phytosphere (Barak and Liang, 2008). Soft rot caused by *Pectobacterium chrysanthemi* in postharvest lettuce resulted in higher density levels of *Escherichia coli* O157:H7 in lettuce leafs (Brandl, 2008) and coinoculation of *S. enterica* serovar Typhimurium with the plant pathogen *Dickeya dadantii* under experimental conditions in lettuce and cilantro leaves resulted in higher densities of *S. enterica* serovar Typhimurium in leafs of both plant species than by inoculation with the human pathogenic strain alone (Goudeau et al., 2013). However, the presence of the root knot nematode *Meloidogyne hapla* in soil with bioluminescent-labeled *Escherichia coli* O157:H7 cells did not result in colonization of the areal parts of young spinach plants (Hora et al., 2005). Mechanic wounding of roots and coinoculation of leafs with the *Escherichia coli* O157:H7 strain and *Pseudomonas syringae* also did not result in colonization of the aerial parts of spinach plants, indicating that not all plant pathogens are facilitating entrance of human pathogens into, and/or spread through plants. Other species living in soil, like protozoa and fungi, may play an important role in the spread of *S. enterica* through soil and colonization of plants (Brandl et al., 2013) and it was demonstrated that *S. enterica* also can live inside protozoa cells (Jacobsen and Bech, 2012, and references therein). All together, these studies indicate that presence of plant pathogens and other species living in soil can play different roles in colonization and internalization of plants by human pathogens. Interesting within this aspect is that *Enterobacter* sp. 638, an endophyte in poplar tree, is capable to degrade pectate, facilitating this strain to colonize spaces between plant cells (Taghavi et al., 2010). Considering its close taxonomical relatedness with *Escherichia coli* and *S. enterica*, it emphasizes the fact that specific traits required for plant colonization must have been acquired by particular *Enterobacteriaceae* during evolution near or inside plants.

Enterobacteriaceae are common inhabitants of the phytosphere. To this taxonomical family belong important plant pathogens like *Erwinia, Pantoea,* and *Pectobacterium* species (Holden et al., 2009; Teplitski et al., 2009, 2011). Of more importance are genera whose species are beneficial to plants and pathogenic to animals, such as the ones belonging to *Serratia* and *Klebsiella* (Berg, 2000; Tan et al., 2001; Dong et al., 2003; Tyler and Triplett, 2008; Holden et al., 2009). Plant and animal-associated enteric species share genes that encode for important virulence traits such as attachment, plant colonization and internalization, biofilm formation and cell invasion (Tyler and Triplett, 2008; Holden et al., 2009; Teplitski et al., 2011). The taxonomic relatedness between human pathogens and plant-associated species may explain why at least some of the human pathogenic strains can colonize plants so well. Ancestors of both groups diversified along evolution, but parts of their genomes, so called cores, remained the same and possibly some of the orthologous genes shared by both groups may be involved in plant interactions (Holden et al., 2009).

Attachment to plant surfaces is an important feature related with settlement of human pathogens to, or inside plants. Constituents of the extracellular matrix of human pathogens like curli fibers, cellulose and lipopolysaccharide capsule (O antigen) were important for attachment to, and colonization of alfalfa sprouts by *S. enterica* serovar Newport (Barak et al., 2007) and spinach leafs by *Escherichia coli* O157:H7 (Macarisin et al., 2012). Possible mutations in regulatory genes responsible for curli and/or cellulose fiber production and leading to the non-rdar morphotype (defective in the formation of red dry and rough colonies on Congo red agar plates; Römling, 2005) resulted in better fitness of *S. enterica* serovar Typhimurium inside tomato fruits (Gu et al., 2011; Zaragoza et al., 2012). The same phenotype in *S. enterica* was less competitive in growth medium in comparison with its near isogenic wildtype strain (Zaragoza et al., 2012). Particular subsets of strains of human pathogens appear to possess features enabling them to attach to plant surfaces.

Entrance of human pathogens via aerial plant parts like woundings or natural openings such as hydathodes or stomata is realistic. A GFP-labeled *S. enterica* serovar Typhimurium strain was attracted to the stomatal openings of iceberg lettuce leafs under the influence of light, where it entered the stomatal cavity (Kroupitski et al., 2009). This strain was able to circumvent the stoma-based innate immune system. Internalization of *Escherichia coli* O157:H7 via stomata was also demonstrated in spinach leafs (Saldaña et al., 2011). Reduced cell numbers of a type 3 secretion defective mutant of this strain was found in the stomatal opening, in comparison with the wild type strain, indicating that type 3 secretion must play an important role in internal colonization of plants. Transfer of the locus of enterocyte effacement (LEE) pathogenicity island, containing type 3 secretion effector genes, of the *Escherichia coli* O157:H7 strain into a non-pathogenic *Escherichia coli* K12 strain resulted in improved colonization of derived strain. Both *S. enterica* and *Escherichia coli* O157:H7 possess mechanisms to actively enter the apoplast, thereby circumventing plant host immunity responses. Colonization of the interior parts of Roman lettuce by *S. enterica* serovar Dublin strain evoked an upshift in the expression of stress-related plant genes (Klerks et al., 2007b). Of the five different *S. enterica* serovars tested (Dublin, Typhimurium, Enteritidis, Newport and Montevideo), the strain of serovar Dublin was demonstrated to be the best colonizer of Roman lettuce, indicating the existence of differences in plant colonizing traits among different *S. enterica* serovars (Klerks et al., 2007a). *S. enterica* serovar Dublin strain was attracted to sugar-like carbon sources present in root exudates of lettuce and these compounds were responsible for induction of different genes amongst which genes that are regulators of the type 3 secretion system (Klerks et al., 2007a). *S. enterica* serovars actively colonize plants and expression of genes under type 3 secretion control also appeared to play an important role in the suppression of the plant immune system (Schikora et al., 2012).

Once inside plants, human pathogens may colonize plants locally, but also may systemically spread through plants by making use of the vascular tissues present in plants and needed for the transport of water and inorganic substances to leafs (xylem) or photosynthates to roots (phloem; McCully, 2001; Warriner et al., 2003). Five different *S. enterica* serovar strains, applied to plants via inoculation of the flowers, were later found to be present in tomato fruit by Guo et al. (2001). Systemic transport to aerial parts of tomato plants occurred upon growth in hydroponic solution containing the five different *S. enterica* serovar strains at levels of between 10^4 and 10^5 cell per ml (Guo et al., 2002). From the last study there are clear indications that transport inside tomato

plants to developing leafs and branches must have occurred. Presence of *S. enterica* serovar Newport and *Escherichia coli* O157:H7 strains in chaff and seed of Arabidopsis plants was demonstrated when plants were grown under gnotobiotic circumstances and in autoclaved and non-autoclaved soils (Cooley et al., 2003). All together, the last two studies are indicative for systemic transport of human pathogens through vascular tissue, but no conclusive data could be provided yet. Vascular transport may occur in low quantities, but numbers may be too low to be detectable with common technologies (Warriner and Namvar, 2010).

In conclusion, it is likely to assume that features in particular subsets of *Escherichia coli* and *S. enterica* groups exist, enabling them to persist near or inside plants over extended periods in time. Genes in human pathogens involved in persistence in plants may be orthologs, but it cannot be ruled out that at least some of these genes were also recently gained via horizontal gene transfer, as was demonstrated to be the case for the *Escherichia coli* O104:H4 outbreak strain in Hamburg and surrounding area in 2011. In modern agriculture, practices are applied to optimize crop yields by irrigation, fertilization, pest, and disease control and maintenance of soil quality. This requires input from different sources, and bacterial communities from plants themselves, soils, intestinal track systems of farm animals (manure), water reservoirs (for sprinkling and irrigation of plants) and agricultural runoff water (Jacobsen and Bech, 2012) all come together at plant production (**Figure 1**). Human pathogens or their phages (examples are provided later) were found to be present in all four ecosystems, potentially bringing these into contact with indigenous bacteria near or inside plants. The consequences of these contacts can be exchanges in genetic material and the likelihood on occurrence of genetic exchanges between human pathogens and plant-associated bacteria and potential risks resulting from these will be discussed in the following section.

HORIZONTAL GENE TRANSFER BETWEEN HUMAN PATHOGENS AND THEIR (PRO) PHAGES IN THE PHYTOSPHERE

HISTORICAL OCCURRENCES OF GENE EXCHANGES IN TWO MAJOR HUMAN PATHOGENS, *Escherichia coli* SEROTYPES O104:H4 AND O157:H7

Gene transfer can occur when bacterial cells physically meet within the same habitat (Toth et al., 2006). Proposed vehicles for transmission of genomic islands are plasmids, (pro) phages and conjugative transposons (Juhas et al., 2009). Acquisition of new phenotypic traits will occur when human pathogens reside outside the human host. The plant-soil ecosystem is such an environment where these species can occur and thus it is likely to assume that auxiliary traits will be acquired from the microbial communities indigenous to this ecosystem.

Escherichia coli strain O104:H4, the outbreak strain in Germany and France in 2011, substantially differed from *Escherichia coli* O157:H7 outbreak strains (Bielaszewska et al., 2011; Eppinger et al., 2011; Mellmann et al., 2011). Some characteristics of the outbreak strain were: (i) that it belonged to the pathotype of enteroaggregative *Escherichia coli* and not to that of enterohemorrhagic *Escherichia coli*, (ii) that it did not produce intimin (encoded by the *eae* gene located on the LEE) but instead Iha adhesin and (iii) that it only produced Shiga toxin 2. The strong adherence of the outbreak strain to the intestinal epithelium in humans in combination with high tolerance to acid (to survive passage through the stomach) and Shiga toxin production were believed to be the main responsible factors for the occurrence of high incidence in HUS in patients, stressing the risks for humans by blends of virulence factors that can occur among human pathogens (Bielaszewska et al., 2011). These differences can be explained by differences in the nature of both serotypes, but also in genomic changes resulting from gene insertions and deletions. The LEE in serotype O157:H7 strains is a conserved pathogenicity island containing genes that are coding for chaperone and effector proteins, belonging to the type 3 secretion system, and responsible for the attaching and effacing lesions in the human large intestine. Intimine is a protein that is involved in cellular attachment of O157:H7serotype strains and the gene coding for this protein (*eae*) was not present in serotype O104:H4 as well as in other entero-aggregative *Escherichia coli* strains.

Escherichia coli O157:H7 strains commonly possess a virulence plasmid (pO157) and a conjugal plasmid (pEC4115; Eppinger et al., 2011), whereas in both serotypes, O104:H4 and O157:H7, *stx* genes are located on lambdoid prophages integrated in the *wrb*A gene (Mellmann et al., 2011). The genome of the serotype O157 Sakai strain possessed a total of 18 prophages, of which two contained the Shiga toxin genes *stx*1 and *stx*2 (Asadulghani et al., 2009). Despite the fact that these prophages contained multiple mutations, they were still functional, indicating that these genetic elements were able to propagate and to recombine with other genetic elements. The roles of phages that did not carry *stx* genes were inferred from complete genome sequence and comparative genome analysis of *Escherichia coli* strains (Ogura et al., 2008; Iguchi et al., 2009). It revealed that virulence genes on exchangeable effector loci were present that code for non-LEE and LEE effectors belonging to the type 3 secretion system. Some of these genes harbor homology to effector proteins of plant pathogens, strengthening the idea of a common evolutionary plan for the type 3 secretion system.

An evolutionary model of the serotype O104:H4 outbreak strain was constructed, based on available whole genome sequence data from a proposed ancestor strain, another *Escherichia coli* O104:H4 strain from an outbreak in 2001 (Mellmann et al., 2011). Interesting to note is that the German *Escherichia coli* O104:H4 outbreak strain of 2011 must have acquired the plasmid containing the aggregative adherence fimbriae type I (AAF/I) locus, but lost a plasmid containing the AAF/III locus and gained a plasmid with the gene encoding CTX-M-15 ESBL.

Virulence traits were gained, likely via horizontal gene transfer, and it is still under debate whether plasmid or phage exchanges between human pathogens and commensal species will occur under changing environmental circumstances. In a model for genome evolution proposed by Dini-Andreote et al. (2012), it was postulated that bacterial genomes will tend to expand under situations of shifting environmental conditions and that genomes shrink under stable environmental conditions. Translating this model for our study, it means that genomes of pathogens that occupy single habitats, like inside human bodies, will shrink whereas the ones that are exposed to multiple habitats will tend to

increase in size, which will be the case for environmental strains. Proposed increase in genome size may occur by acquisition of new genes via gene transfer events. The "pan-genome" encompasses all genes that are present among strains originating from different environments, but still belonging the same taxonomic group (Dini-Andreote et al., 2012). It is characteristic for genomes of enterohemorrhagic species that stretches of housekeeping genes, forming the core genome, are punctuated by gene islands, forming the flexible genome (Mellmann et al., 2009). Genes belonging to the flexible genome most easily will be exchanged between bacteria of the same species, but also between bacteria of sometimes entirely different taxonomic groups. The flexible gene pool will consist of gene clusters, so called genomic islands that are responsible for auxiliary traits. The formation of genomic islands in bacterial genomes will facilitate the transfer of new phenotypic traits to recipients, resulting in "quantum leaps" in the adaptation of receiving strains to new environments (Juhas et al., 2009).

HORIZONTAL GENE TRANSFER BY BACTERIOPHAGES

Bacteriophages play a role in the virulence of EHEC strains by conferring genes involved in toxin production and type 3 secretion. Bacteriophages are recognized to be the main contributors to the transmission of virulence determinants, via transduction and lysogenic conversion, between bacterial strains. This process of lateral gene transfer is a significant factor in the evolution of bacteria. DNA of bacteriophage origin often comprises 10–20% of a bacterial genome, and approximately two-third of gammaproteobacterial and low-GC Gram-positive species harbor intact bacteriophages or their remnants in their genomes (Davidson et al., 1990; Saye et al., 1990; Kidambi et al., 1994; Boyd et al., 2000; Perna et al., 2001; Wagner and Waldor, 2002; Canchaya et al., 2003a,b; Casjens, 2003; Toth et al., 2003). A recent metagenomics survey on the virome of the bovine rumen showed that 64% of known virus genomes are from bacteriophages (Berg Miller et al., 2012). In particular, prophages were shown to originate from bacterial hosts within the taxonomical groups of *Firmicutes* ($68 \pm 1\%$), *Proteobacteria* ($18 \pm 1\%$) and *Bacteriodetes* ($8 \pm 1\%$). Comparison of the bovine rumen virome with a selected set of mobile elements from microbial genomes of the bovine rumen revealed a similarity of $80 \pm 19\%$. The vast majority ($79 \pm 19\%$) was similar to prophages, demonstrating the importance of this type of mobile element within this ecosystem.

Shiga toxins, encoded by *stx*1 and *stx* 2 genes, are located on temperate bacteriophages and low or avirulent forms of *Escherichia coli* can be converted to pathogenic strains upon infection with these phages. Bacterial conversion by phages from non-pathogenic to pathogenic forms occur more often, for example in *Corynebacterium diphtheria* and in different *Salmonella* species (Saunders et al., 2001). Bacterial phages are responsible for horizontal transfer of virulence determinants between different bacterial species. Bacteriophage $\Phi 24_B$, isolated from an enterohemorrhagic *Escherichia coli* O157 strain and carrying the *stx*2 gene, was able to infect commensal and pathogenic *Escherichia coli* and *Shigella* strains from different sources (James et al., 2001). This indicates that the host-range of this bacteriophage is broader, conferring new virulence traits toward different species among the group of *Enterobacteriaceae*. Expression of Shiga toxin genes are controlled by lambdoid prophage cI repressor (Mauro and Koudelka, 2011). Shiga toxin gene expression is stimulated upon cleavage of cI by RecA, and on its turn RecA expression can be stimulated by the presence of reactive oxygen species released from eukaryotic predators like *Tetrahymena thermophila* (Lainhart et al., 2009; Mauro and Koudelka, 2011). Shiga toxin production is a virulence determinant whose contribution to bacterial fitness is obscure. As discussed before, knock out mutants in Shiga toxin production did not survive better or worse in soils and plants. A question that still remains open is the role of Shiga toxin production in bacterial survival in the environment. Shiga toxin production may be a defense mechanism against eukaryotic unicellular predators, suggesting that humans may not be the prime targets for Shiga-toxin producing *Escherichia coli* strains.

A total of 15 phages carrying Shiga toxin *stx*2 genes, purified from environmental water samples, were shown to belong to three different virus families: *Podoviridae* (9), *Myoviridae* (6) and *Siphidoviridae*, the family to which phage lambda belong (2; Rooks et al., 2012). Environmental water thus can be a source of *stx*-carying phages and transduction with this type of phages may increase virulence levels in *Escherichia coli* strains. A *Siphidoviridae* lytic phage targeting the plant pathogenic bacterium *Pectobacterium carotovorum* subsp. *carotovorum*, My1, was recently sequenced (Lee et al., 2012). Although no *stx* genes were found in the genome of this phage, it illustrates the possibility that lytic phages released from plant-pathogenic bacteria can infect plant-invasive *Escherichia coli* strains. Broad-host range lytic phages have been characterized, like phage ΦOT8 from treated sewage effluent, that was able to infect and tranduce resistance and prodigiosin marker genes from *Serratia* sp. ATCC 39006 to *Pantoea agglomerans* (Evans et al., 2010). Phages isolated from soil and trees from Ontario, Canada (Gill et al., 2003) and from apple and pear orchard soils in Switzerland (Born et al., 2011) were able to infect the plant pathogenic bacterium *Erwinia amylovora*. All bacteriophages obtained (42 from the study of Gill et al., 2003 and 24 of the one of Born et al., 2011) belonged to *Podoviridae* or *Myoviridae*. Commonalities in genome sequence were found between two *Myoviridae* phages from both studies, ΦEa21-4 and M7, and both were considered to be broad-host range phages infecting three different *Erwinia* species and *Pantoea agglomerans* (Born et al., 2011). The same phage type (ΦEa21-4) showed a remarkable similarity at a protein level of 56% with *Salmonella* phage Felix O1 (Lehman et al., 2009). Major difference between phages ΦEa21-4 and Felix O1 was the presence of the *nad*V homolog encoding a nicotinamide-scavenging enzyme that may supplement nicotinic acid in *E. amylovora* (Lehman et al., 2009). Typical ΦEa21-4-like phages may be cosmopolites able to infect multiple species within the class of *Enterobacteriaceae*. In potential, this type of phages could also be able to horizontally transfer genes from and to human pathogens upon invasion into the phytosphere.

HORIZONTAL GENE TRANSFER BY CONJUGAL PLASMIDS

Virulence traits are present on different plasmids in *Escherichia coli* (Johnson and Nolan, 2009). Commonalities exist in pathogenicity determinants among plant and animal *Enterobacteriaceae* (Toth et al., 2006). Therefore, conjugal plasmids can play pivoting

roles in horizontal transmission of virulence factors located on pathogenicity islands in plant-soil ecosystems.

The Hamburg serotype O104:H4 outbreak strain contained TEM-1 and CTX-M-15 beta lactamase genes on an incompatibility group I (IncI) type plasmid (Mellmann et al., 2011). Extended spectrum beta lactamase phenotypes have been found in many enteric species over the world, and CTX-M15 was found in *Escherichia coli, Klebsiella pneumonia, Enterobacter aerogenes* and *S. marcescens* (Bonnet, 2004 and references therein). Further, CTX-M class of beta lactamase genes were found in *Erwinia persicina* (Vimont et al., 2002) and in *P. aeruginosa* and *S. maltophilia* (Naiemi et al., 2005). IncI-type plasmids are narrow host-range plasmids and were not transferrable between donor and recipient *Escherichia coli* strains in nutrient-amended soil (Pukall et al., 1996). In that study, broad-host range plasmids belonging to incompatibility groups IncN, IncP1, IncW3, and IncQ were transferred to the *Escherichia coli* recipient strain and it was hypothesized that size and flexibility of the pili covering the cellular surface of donor cells would play an important role in the frequency of plasmid transfer in soil (Pukall et al., 1996; Ghigo, 2001). IncP-type of plasmids are self-transmissible plasmids, whereas IncQ types are not, however, both were considered to be the major vectors in horizontal transfer of antibiotic resistances in natural environments (Heuer et al., 2012; Popowska and Krawczyk-Balska, 2013).

Virulence factors in human pathogens are often located on plasmids. Tellurite (TeO_3) resistance is a trait that is commonly found among different human pathogens including *Escherichia coli* O157:H7 and O46:H$^-$, and were located on a pathogenicity island (Taylor, 1999). Tellurite resistance genes in enteric bacteria, located on IncHI2 and IncHII plasmids, showed remarkable homology with tellurite resistance genes located on plasmid R478 from *S. marcescens* (Taylor et al., 2002). Tellurite resistance determinants were also found on a IncP2 plasmid from *P. aeruginosa*, but these genes were not related with the ones found in *Escherichia coli* O157:H7 (Hou and Taylor, 1994). Iron acquisition genes of *Yersinia* pathogens (*Y. pestis, Y. pseudotuberculosis, Y. enetrocolitica*) is located on the *Yersinia* high pathogenicity island (HPI) and this HPI was found among different species of *Enterobacteriaceae*, including *Escherichia coli*, *Citrobacter diversus*, different *Klebsiella* species, non-I serotypes of *S. enterica*, but not in *S. enterica* serovars Enteritidis and Typhimurium and in EHEC (Bach et al., 2000; Carniel, 2001). Mobility of the HPI among enteric species must occur, although in *Y. pseudotuberculosis* excision was shown to be a rare event (frequency of about 10^{-9}; Carniel, 2001). An HPI found in *Escherichia coli* showed strong (99%) identity with HPI present in *Y. pestis*, indicating that gene transfer between *Y. pestis* and *Escherichia coli* must have occurred in the past (Schubert et al., 2004). Mobilization of HPI in the bacterial chromosome is a phage-mediated process and P4-phage-related integrase and excision genes were found to be present in HPI (Benedek and Schubert, 2007). However, promiscuous mobile plasmids play important roles as shuttle vectors in the horizontal transfer of HPI (Antonenka et al., 2005; Schubert et al., 2009) and other pathogenicity islands in *Escherichia coli* (Schneider et al., 2011) and in environmental and other pathogenic micro-organisms (Dobrindt et al., 2004). Upon introduction of the self-transmissible plasmid RP4 into *Y. pseudotuberculosis*, a cointegrate between this plasmid and HPI was formed and transferred to a recipient strain (Antonenka et al., 2005). Trapping of HPI into the cointegrate was based on precise excision of the element from the chromosome and the frequency in occurrence of this event was estimated at 1 on 10^6 cells. Precise excision did not always occur, and occasionally regions flanking the HPI were also integrated and transferred to recipients (Schubert et al., 2009). *Salmonella* genomic island 1 (SGI1), conferring multiple antibiotic resistances to the bacterial host, was shown to be excised from the chromosome by a lambdoid integrase (Doublet et al., 2005). However, the circular form of SGI1 was not transferred to recipient strains by itself under experimental circumstances. A helper IncC plasmid (R55) was needed for the transfer of SGI1 from different *Salmonella* donor strains to *Escherichia coli* K12 as recipient (Doublet et al., 2005). A screening over 902 *S. enterica* serovar types from poultry revealed a higher incidence of a ColV type plasmid among a clonal type of serovar Kentucky than over other serovar types (Johnson et al., 2010). The ColV plasmid conferred virulence and fitness traits toward its host and strains carrying the plasmid better colonized the chicken cecum and successfully competed with the indigenous microbial community in there. This illustrates the ecological advantage that may be gained by human pathogens via transfer of genes located on mobile elements like plasmids. The genes present on plasmids in human pathogens may come from different sources as demonstrated by the full sequence of the large virulence plasmid, pO157, from *Escherichia coli* O157:H7 (Burland et al., 1998). A strong resemblance between genes located on this plasmid was found with ToxA and ToxB toxin protein genes from *Clostridium difficile*. Genes of archeal origin (presumably from *Methanobacterium thermoautotrophicum* and *Halobacterium* species) were proposed to be present in the genome of *Escherichia coli* O157:H7 strain EDL933 (Faguy, 2003). The last would indicate that cross-domain gene transfer events would have occurred in the past between *Escherichia coli* and *Archeae* species.

Besides transfer of genes belonging to the flexible genome (mostly located on genomic or pathogenicity islands), also genes of the core genome can be transferred like household and metabolic genes. Among these genes can be ones that improve fitness of the recipient strain under environmental circumstances like present in the rhizosphere. Transfer of metabolic genes involved in oxidation or fermentation of small carbohydrates like sugars, acids, and amino acids may improve fitness of receiving strains in the rhizosphere where these compounds are exuded by plant roots. Genes responsible for L-sorbose transport and metabolism in *Escherichia coli* was also found in *K. pneumonia* (Wehmeier et al., 1992) and genes involved in glucose fermentation were transferred via genetic elements among different *Salmonella* species (Wohlhieter et al., 1975; Bartlett and Trust, 1980). Only a few strains of *Salmonella* and *Escherichia coli* can ferment glucose and acquisition these genes can be important for adaptation to new environments. Transfer of sucrose fermenting genes was found to be facilitated by conjugative transposons (Pembroke et al., 2002).

Under experimental circumstances, transfer of an environmental (wastewater treatment plant) self-transmissible and broad-host-range (IncP1β) plasmid from *P. putida* to different *S. enterica*

serovar and *Escherichia coli* O157:H7 strains was demonstrated (Van Meervenne et al., 2012). Upon bacterial mating between donor and recipient strains, transconjugants were found at most, but not all occasions, indicating that human pathogens are not equally susceptible for horizontal gene transfer events under applied conditions. Bacterial gene transfer between introduced plasmid donor and/or recipient strains was demonstrated at different places in the phytosphere, such as in the rhizosphere (Lilley and Bailey, 1997), the phylloplane (Normander et al., 1998) and inside plants between endophytes (Taghavi et al., 2005). Transfer of two plasmids from *P. putida* recipients to indigenous bacteria associated with ready-to-eat alfalfa sprouts was demonstrated (Mølbak et al., 2003). In this study, transconjugants were found among different *Pseudomonas* and *Erwinia* species. Mobilizable plasmids from indigenous bacteria of piggery manure were obtained by introduction of *Escherichia coli* and *P. putida* strains, acting as recipients for exogenous plasmid isolation, in samples from different ecosystems (Smalla et al., 2000). Identities of plasmids selected from transconjugants revealed that they were all IncQ like. IncQ and IncP, IncN, and IncW type plasmids carrying gentamycin (Heuer et al., 2002) or streptomycin (van Overbeek et al., 2002) resistance determinants were obtained by exogenous plasmid isolations from bulk and rhizosphere soils, manure, sludge, and seawater samples. These data demonstrate the omnipresence of mobilisable (like IncQ type) and sometimes self-transmissible (like IncP type) plasmids in a wide variety of ecosystems, including the ones that are relevant for agriculture. Remarkable was the high abundance of IncQ-type of plasmids obtained by exogenous plasmid isolation, because these plasmids need a helper plasmid from another incompatibility group for transfer. Transfer of IncQ plasmids would require three parents for mating and it was believed that these type of matings would occur less frequently in soils (Pukall et al., 1996).

Cryptic plasmids can play important roles in horizontal transfer of IncQ plasmids in natural ecosystems. A cryptic plasmid, pIPO2, was found in the rhizosphere of wheat and this plasmid was able to mobilize IncQ plasmids to recipient strains (Van Elsas et al., 1998). PCR detection based on pIPO2 revealed that this plasmid was present in 7 of 10 tested rhizosphere DNA samples, in 2 of 12 bulk soil DNA samples, but not in any of the single DNA samples from manure, seawater and wastewater (Tauch et al., 2002). Sequence comparisons made between pIPO2 and plasmid pTER331 from the soil bacterium *Collimonas fungivorans* revealed a high similarity between both plasmids (Tauch et al., 2002; Mela et al., 2008). Cryptic plasmids belonging to the pIPO2-type thus are common in the rhizosphere where they are supposed to be responsible for the mobilization and retromobilization of plasmids. In principle, the roles of these type of plasmids in horizontal gene transmission in arable plant environments have not been explored so much.

Among the species that are typical for the plant-soil ecosystem are plant pathogenic and endophytic bacteria and different plasmid types were found among species belonging to alpha, beta and gammaproteobacteria (Vivian et al., 2001). Whole-genome sequencing of the poplar endophyte *Enterobacter* sp. 638 revealed the presence of important adhesion and colonization genes on plasmid pENT638-1 (Taghavi et al., 2010), explaining the excellent colonization of strain 638 in poplar trees. This plasmid is thus of ecological relevance and supportive for the endophytic lifestyle of this strain. In *E. amylovora*, a plant pathogen commonly causing diseases in a wide variety of (rosaceous) plants, plasmids were found in different strains of this species and DNA sequences of three plasmids were annotated. Plasmid pEA29 from *E. amylovora* is a non-self-transmissible plasmid that stably replicates within its host (McGhee and Jones, 2000). On this plasmid auxiliary virulence and streptomycin resistance genes were found. DNA sequences of two other plasmids from *E. amylovora* pEU30 and pEL60, respectively, revealed strong similarities with conjugal transfer genes found in *Pseudomonas syringae* pathovar *syringae* and with pCTX-M3 plasmid of *Citrobacter freundii* (Foster et al., 2004). Plasmid pEL60 was an IncL/M type of plasmid and contained genes that are responsible for DNA repair and was proposed to increase environmental fitness of the host. The close resemblance of this plasmid with a plasmid from an animal (mouse) pathogenic species revealed that animal and plant pathogenic enteric species might share the same gene pool (Foster et al., 2004). In two strains of another plant pathogenic bacterial species, *P. chrysanthemi*, a gene was found that was immunologically related with the intimin-encoding gene *eae* commonly found among EHEC strains (Duarté et al., 2000). The two strains were able to kill human colon carcinoma cells, whereas a mutant defective in type 3 secretion lost the capacity to kill these cells. The combination of type 3 secretion and intimin is important for attachment to human cells and demonstrates a high conservation of virulence genes among plant and human pathogens (Duarté et al., 2000). Among strains of *Klebsiella pneumoniae* are human pathogens and (commensal) endophytes. Genome comparisons between a human pathogenic and an endophytic strain of this species revealed that there was a higher abundance of genes supposed to be responsible for survival under circumstances present inside plants like polysaccharide degradation, transport, protection against oxidative agents and nitrogen fixation (Fouts et al., 2008). In the same study, the endophytic strain was virulent in a mouse model test system, although virulence was milder in comparisons with the human pathogenic strain. *Pseudomonas aeruginosa* is a bacterial species that harbors plant as well as (opportunistic) human pathogenic strains and it was found that virulence mechanisms necessary for infection of evolutionary different hosts (vertebrate and invertebrate animal and plant species) were conserved (Rahme et al., 2000). Mutations in the genes coding for exotoxin A (*tox*A), responsible for protein synthesis inhibition in mammalian cells, phospholipase C (*plc*C), phospholipid degradation in eukaryotic cells, and *gac*A, transcriptional activator of genes responsible for production of extracellular compounds, lead to reduced virulence both in Arabidopsis and mouse test systems. Other genes responsible for multihost pathogenesis were involved in synthesis of membrane derived oligosaccharides, quorum sensing, trans-membrane export of proteins, multidrug efflux, phenazine-1-carboxylate production, motility, and surface attachment (Rahme et al., 2000). A strain of the non-pathogenic species *Wolinella succinogenes*, originating from the intestinal track system of cattle, contained many homologs of known virulence genes (Baar et al., 2003). *W. succinogenes* is a epsilon proteobacterium closely related with

human pathogenic species *Campylobacter jejuni* and *Helicobacter pylori*. Next to pathogenicity gene homologs, genes proposed to be important for survival in soil and plants, including nitrogen fixation genes, were found (Baar et al., 2003). Conservation of genes that are known to be responsible for pathogenesis in humans thus are found among entirely different plant and soil bacterial species that have human pathogens as close relatives. This indicates that the plant-soil ecosystem is a source of genes that may increase fitness of many human pathogens besides *Escherichia coli* and *S. enterica* that can reside in the phytosphere.

IN CONCLUSION

Horizontal gene transfer, either mediated by phages or by conjugal plasmids, can play a role in the acquisition of new phenotypes in human pathogens present in one of the four ecosystems relevant for arable plant production, i.e., soil, plant, animal (cattle) gut, and fresh water systems (**Figure 1**). Acquired phenotypes are related to resistance, virulence and ecological competence. All human pathogens had been prone to gene transfer events in the past and examples of recent and multiple gene transfer events were demonstrated in *Escherichia coli* serotypes O104:H4 and O157:H7. Serotype O157:H7 and some of the other O serovar types are zoonotic pathogens, which is not the case for serotype O104:H4 (entero-aggregative *Escherichia coli*), which is believed to be transmitted via humans only. From epidemiological studies it became clear that the Hamburg *Escherichia coli* O104:H4 outbreak was not transmitted via humans, but most likely via seeds. It is clear that human pathogens passing these four ecosystems might acquire mobile genetic elements, supplying them additional virulence genes and/or making them better adapted to their new environment. The combination of increased resistance to antibiotics and higher virulence levels in commensal or low-pathogenic bacteria is a major threat for human health (Szmolka and Nagy, 2013). Another hotspot boosting horizontal gene transfer thus might be, next to the mammalian gut system (Stecher et al., 2012), the rhizosphere. Studies going on from the 80-ties of the last century revealed that gene transfer via conjugation occur in the rhizosphere. New pathogenic types thus may arise at agricultural practice that are adapted to life near or inside plants; the so called phytonic types.

Products derived from plants are considered to be healthy and microbiologically safe by consumers. The public is unaware of eventual microbiological risks related to consumption of fresh produce (Beutin and Martin, 2012) and therefore emergence of phytonic human pathogens deserves attention. Appropriate and timely detection of emerging pathogens in plants and food derived from plants is of importance, but will be complicated by the flexibility of their genomes. Appearance of new features, encrypted in genomes of STEC, *S. enterica* and other human pathogenic strains, that determine fitness in plants and virulence to humans, like genes from plasmids and bacteriophage sequences, should be monitored frequently (Mellmann et al., 2011; Noguera et al., 2011a,b; Laing et al., 2012). Novel, rapid, innovative high throughput detection technologies will facilitate screenings for these regions in genomes of human pathogenic isolates from clinical and environmental samples.

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Bacterial quorum sensing compounds are important modulators of microbe-plant interactions

Anton Hartmann[1]*, Michael Rothballer[1], Burkhard A. Hense[2] and Peter Schröder[1]

[1] Research Unit Microbe-Plant Interactions, Department of Environmental Sciences, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany
[2] Institute of Computational Biology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany
*Correspondence: anton.hartmann@helmholtz-muenchen.de

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Ingyu Hwang, Seoul National University, South Korea

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PLANT—MICROBIOME INTERACTIONS IN THE LIGHT OF THE HOLOBIONTIC CONCEPT

Higher organisms evolved in the omnipresence of microbes, which could be of pathogenic or symbiotic nature. A framework of response patterns evolved which is known as innate immunity. A major part of this response is the recognition of microbial-associated molecular patterns (MAMP) such as chitin or lipochitooligosaccharides, peptidoglycan, lipopolysaccharides or flagellum structures, and the initiation of efficient plant defence reactions (Janeway and Medzhitov, 2002; Jones and Dangl, 2006). However, there are many plant-associated endophytic bacteria known, which are living within plants without triggering persistent and apparent defence responses or visibly do not harm the plant. In some cases, even a stimulation of plant growth due to the presence of specific players within the plant microbiome was reported (Turner et al., 2013). It is now generally accepted, that plant performance and activities can only be characterized and understood completely, if the "holobiont," the plant plus the intimately associated microbiota, is considered (Zilber-Rosenberg and Rosenberg, 2008). The evolutionary advantage of an integrated holobiontic system is characterized by a much better adaptability and flexibility towards rapidly changing adverse environmental conditions. It is still mostly unknown, which particular plant genetic loci are controlling the interactions with the plant microbiome and which signals are steering this cooperativity. Mutualistic microbes are able to overcome or short-circuit plant defence responses to enable successful colonization of the host (Zamioudis and Pieterse, 2012; Alqueres et al., 2013). Beneficial associations with microbes other than mycorhiza or *Rhizobia* are also controlled by systemically regulated or autoregulated processes on top of the basic innate immunity response. The induction of systemic immunity responses like ISR (induced systemic resistance) by some beneficial rhizosphere bacteria or the SAR (systemic acquired resistance) response provoked by pathogens are results of multiple response cascades employed by the plant host to respond to microbial and other environmental interactions. However, the entire response network is by far not yet revealed. For example, bacteria-induced plant responses resulting in improved resistance towards pathogens can also be due to the perception of secondary metabolites, like the surfactin lipopeptide, produced by certain biocontrol Bacilli (Garcia-Gutiérrez et al., 2013) or volatile compounds of plant-associated microbes (Yi et al., 2010). The biocontrol activity of microbial inoculants is probably due to multiple effects of their secondary metabolites to achieve direct inhibition of the pathogenic counterpart as well as an increase of systemic resistance of the plant host.

BACTERIAL QUORUM SENSING MOLECULES LIKE *N*-acyl HOMOSERINE LACTONES MODULATE PLANT RESPONSES TOWARD CONTACT WITH BACTERIA

It is hypothesized, that eukaryotic organisms developed ways to sense microbes in addition to the recognition of their MAMPs by their diffusible small molecules. A very ancient and basic feature of unicellular bacteria is their way of environmental sensing and social communication. In many Gram-negative bacteria the synthesis of autoinducers of the *N*-acyl-homoserine lactone (AHL) type is tightly regulated in response to cell density or the cell "quorum" (Eberl, 1999). These metabolites are released into the cellular environment to sense the quality of the ecological niche in terms of diffusion space and the density and distribution of their own population. This environmental sensing mechanism helps to adapt the regulation of their gene expression to the given conditions in their habitat and thus optimizes the fitness of the population. Therefore, the generally known term "quorum sensing" (QS) was supplemented by the more broadly defined concept of "efficiency sensing" (Hense et al., 2007). Since this optimization of *in situ* gene expression is of very basic importance, autoinducer QS-molecules are widespread among bacteria and have quite different molecular structures. AHL are common in Gram-negative bacteria, while cyclic peptides as QS-signals are only to be found in Gram-positive bacteria. The detailed structure of the AHL-molecules can vary; the acyl side chain consists of 4–14 carbon atoms and may also contain double bonds. The C3-atom can be hydroxylated or oxidized to a carbonyl-carbon; thus, considerable information and quite different physicochemical properties can be present within these different AHL-structures. As is outlined below, also plants have obviously learned during their evolution to respond to these QS compounds in

Table 1 | Recent findings of direct AHL impact on different plants.

AHL type	Plant reaction	Plant species	References
Short chain length	Increased transpiration, stomatal conductance	*Phaseolus vulgaris*	Joseph and Phillips, 2003
C6	Primary root elongation	*A. thaliana*	von Rad et al., 2008
C6	Upregulation of metabolism, transport and transcriptional regulation	*A. thaliana*	von Rad et al., 2008
C6 (*Serratia liquefaciens*)	Upregulation of defense genes	*Lycopersicon esculentum*	Schuhegger et al., 2006
C6, C8, C10	Lactonase induction	*Pachyrhizus erosus*	Götz et al., 2007
Oxo-C6, oxo-C8	G-protein coupled receptors for root growth	*A. thaliana*	Jin et al., 2012; Liu et al., 2012
3-oxo-C6 (*Serratia plymuthica*)	Triggering plant immunity	*Cucumis sativa* *Lycopersicon esculentum*	Pang et al., 2009
C6, C8, C10	Root and shoot growth	*Hordeum vulgare*	Götz et al., 2007
3-O-C10	Adventitious root formation	*Vigna radiata*	Bai et al., 2012
C10	Lateral root formation	*A. thaliana*	Bai et al., 2012
C12	Root hair development	*A. thaliana*	Ortíz-Castro et al., 2008
3-oxo-C12 from *P. aeruginosa*	Defense and stress management genes, phytohormones, and metabolic regulation	*Medicago truncatula*	Mathesius et al., 2003
oxo-C12	Resistance induction	*A. thaliana*	Schikora et al., 2011
oxo-C14	Systemic resistance against *Golovinomyces orontii*	*A. thaliana*	Schikora et al., 2011
oxo-C14	Systemic resistance against *Blumeria graminis*	*Hordeum vulgare*	Schikora et al., 2011
3-oxo-C16 from (*Sinorhizobium meliloti*)	Defense and stress management genes, phytohormones and metabolic regulation	*Medicago truncatula*	Mathesius et al., 2003

different specific ways. We speculate, that QS-compounds are early signals indicating that pathogens are in the surroundings to gather themselves for attack or that mutualists are about to interact with roots.

The first demonstration of specific responses of a plant to bacterial AHLs was demonstrated for the legumes *Phaseolus vulgaris* (Joseph and Phillips, 2003) and *Medicago truncatula* (Mathesius et al., 2003) (**Table 1**). AHLs from symbiotic (*Sinorhizobium meliloti*) or pathogenic (*Pseudomonas aeruginosa*) bacteria provoked at concentrations as low as nano- to micromolar significant changes in the accumulation of over 150 proteins. Auxin-responsive and flavonoid synthesis proteins were induced and also a secretion of plant metabolites that mimic QS compounds were found, which may have the potential to disrupt QS signaling by associated bacteria. In tomato plants, a specific induction of systemic resistance proteins after inoculation of the roots with C4- and C6-side chain AHL-producing *Serratia liquefaciens* MG1 was discovered independently (Hartmann et al., 2004; Schuhegger et al., 2006). The fungal leaf pathogen *Alternaria alternata* was much less effective, when *S. liquefaciens* MG 1 wild type had been inoculated to roots of tomato plants as compared to the AHL-negative mutant. It could be shown, that salicylic acid was increased as well as SA- and ethylene-dependent defence genes (i.e., PR1a) in MG1-inoculated plants. Furthermore, *Serratia plymuthica* HRO-C48, producing C4-/C6- and OHC4-/OHC6-homoserine lactones, is able to induce ISR-like systemic protection of bean and tomato plants against the fungal leaf pathogen *Botrytis cinnera*; this response was greatly reduced with mutants impaired in AHL-production (Liu et al., 2007; Pang et al., 2009). In contrast, *Arabidopsis thaliana* responds to short (C4- and C6-) *N*-acyl AHL-compounds in a different manner: C4- and C6- homoserine lactones alter the expression of selected hormonal regulated genes which results in changes of the plant's hormone content, in particular an increased auxin/cytokinin ratio (von Rad et al., 2008). However, no systemic resistance response was found to be induced in *A. thaliana* when roots were stimulated with short side-chain AHLs. Ortíz-Castro et al. (2008) found that C10-homoserine lactone elicited developmental changes in the root system in *Arabidopsis* plants by altering the expression of cell division and differentiation-related genes. Furthermore, Liu et al. (2012) and Jin et al. (2012) demonstrated that the root stimulatory effect of C6- and C8- homoserine lactones in *Arabidopsis* plants is mediated through the G-protein coupled receptor encoded by *AtGPA1*. In mung bean, oxoC10-homoserine lactone activates auxin-induced adventitious root formation via H_2O_2- and NO-dependent cyclic GMP signaling (Bai et al., 2012). On the other hand, *N*-acyl-AHLs with C12- and C14- side chains induce systemic resistance to the obligate biotrophic fungus *Golovinomyces orontii* in *A. thaliana* and to *Blumeria graminis* f. sp. *hordei* in barley (*Hordeum vulgare*) (Schikora et al., 2011). This response is mediated through altered activation of *AtMPK6*. The mitogen-activated protein kinases AtMPK3 and AtMPK6 were stronger activated by the model elicitor flg22 in the presence of C12- or C14-AHL compounds which resulted in a higher expression of the defence-related transcription factors WRKY26 and WRKY29 as well as the PR1 gene (Schikora et al., 2011). Thus, AHLs with short and medium side chain lengths induce developmental effects on root architecture, while long side chain AHLs induce systemic resistance in *A. thaliana* (Schenk et al., 2012). Furthermore, it was shown, that better water soluble short side chain AHL-compounds are actively taken up into plant roots and transported along the roots into the shoot; in contrast, the

lipophilic long acyl side chain AHLs are not transported in barley and *A. thaliana*. (Götz et al., 2007; von Rad et al., 2008; Sieper et al., 2014). However, no uptake was detected in the legume yam bean (*Pachyrhizus erosus* (L.) Urban) (Götz et al., 2007). The latter finding corroborates the report of Delalande et al. (2005) that legumes like *Lotus corniculatus* produce lactonases which degrade AHLs and prevent their uptake and transport. In barley, it could further be demonstrated that C8- and C10-AHLs are taken up in a cell energy dependent manner by ABC-transporters into the root and transported via the central cylinder into the shoot (Sieper et al., 2014).

Interestingly, several plants have been demonstrated to produce AHL-mimic substances or to develop other activities influencing QS of plant associated bacteria (Gao et al., 2003; Bauer and Mathesius, 2004). Flavonoids released by legumes increase the expression of AHL synthesis genes in *Rhizobia* (Pérez-Montano et al., 2011). Indole acetic acid and cytokinin biosynthesis of *Gypsophila* was shown to influence QS, type III secretion system and gall formation activity by *Pantoea plantarum* (Chalupowicz et al., 2009). On the other hand, tobacco plants have been engineered to produce short- and long-side chain AHL-compounds which could be detected in substantial amounts at leaf and root surfaces as well as in soil (Scott et al., 2006). Constitutive expression of QS genes in transgenic tobacco plants leads to alteration in induced systemic resistance elicited by the rhizobacterium *Serratia marcescens* 90–166 (Ryu et al., 2013). Furthermore, transgenic tomato plants engineered to produce different AHL-compounds were demonstrated to alter the activity of plant growth promoting rhizobacteria and resulted, e.g., in increased salt tolerance (Barriuso et al., 2008). We hypothesize, that QS in a plant-microbe holobiont system should be regarded in a bidirectional way with influences from the plant and the microbial partners.

Uptake of AHL-compounds and specific perception of AHLs in animal cells were also studied intensively in recent years (Teplitski et al., 2011; Hartmann and Schikora, 2012). 3-oxo-C12-homoserine lactone (C12-AHL), the major AHL-compound of *Pseudomonas aeruginosa*, was shown to selectively impair the regulation of the nuclear transcription factor NF-κB which controls innate immune responses in mammalian cells (Kravchenko et al., 2008). C12-AHL also impaired human dendritic cell functions required for priming of T-cells (Bernatowicz et al., submitted). Since the response to AHL-compounds in mammalian systems is complicated due to the interferences with the adaptive immune system, plants provide an ideal model for the detailed interaction studies of basic innate immune responses and developmental processes with *N*-acylhomoserine lactones as modifying bacterial effector molecules.

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The microbiome of medicinal plants: diversity and importance for plant growth, quality, and health

***Martina Köberl*[1]*, *Ruth Schmidt*[1], *Elshahat M. Ramadan*[2], *Rudolf Bauer*[3] *and *Gabriele Berg*[1]**

[1] *Institute for Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Faculty of Agriculture, SEKEM, Heliopolis University, Ain Shams University, Cairo, Egypt*
[3] *Department of Pharmacognosy, Institute of Pharmaceutical Sciences, University of Graz, Graz, Austria*

Edited by:
Martin Grube,
Karl-Franzens-University Graz, Austria

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Franz Narberhaus, Ruhr University Bochum, Germany
Nai-Chun Lin, National Taiwan University, Taiwan

***Correspondence:**
Martina Köberl, Institute for Environmental Biotechnology, Graz University of Technology, Petersgasse 12/I, 8010 Graz, Austria
e-mail: martina.koeberl@tugraz.at

Past medicinal plant research primarily focused on bioactive phytochemicals, however, the focus is currently shifting due to the recognition that a significant number of phytotherapeutic compounds are actually produced by associated microbes or through interaction with their host. Medicinal plants provide an enormous bioresource of potential use in modern medicine and agriculture, yet their microbiome is largely unknown. The objective of this review is (i) to introduce novel insights into the plant microbiome with a focus on medicinal plants, (ii) to provide details about plant- and microbe-derived ingredients of medicinal plants, and (iii) to discuss possibilities for plant growth promotion and plant protection for commercial cultivation of medicinal plants. In addition, we also present a case study performed both to analyse the microbiome of three medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L., and *Solanum distichum* Schumach. and Thonn.) cultivated on organically managed Egyptian desert farm and to develop biological control strategies. The soil microbiome of the desert ecosystem was comprised of a high abundance of Gram-positive bacteria of prime importance for pathogen suppression under arid soil conditions. For all three plants, we observed a clearly plant-specific selection of the microbes as well as highly specific diazotrophic communities that overall identify plant species as important drivers in structural and functional diversity. Lastly, native *Bacillus* spec. div. strains were able to promote plant growth and elevate the plants' flavonoid production. These results underline the numerous links between the plant-associated microbiome and the plant metabolome.

Keywords: biocontrol, desert farming, medicinal plants, microbial communities, organic agriculture, soil-borne pathogens

THE PLANT-ASSOCIATED MICROBIOME: INTRODUCTION INTO STRUCTURE AND FUNCTION

All plant-associated microenvironments are colonized in high abundances by microorganisms, especially the nutrient-rich rhizosphere, the soil area influenced by plant roots, hosts a plethora of microbes that are of central importance for plant nutrition, health, and quality (Hiltner, 1904 in Hartmann et al., 2008; Berg, 2009; Mendes et al., 2011, 2013). The rhizosphere can host up to 10^{11} microbial cells per gram root with more than 30,000 different prokaryotic species (Berendsen et al., 2012). These rhizospheric microorganisms from a highly diverse reservoir of soil microbes are attracted by the rhizosphere's plant root secretions and other rhizodeposits (Compant et al., 2010), and driven via the compositional variability of these exudates (Bais et al., 2006; Doornbos et al., 2012). Each plant species harbors a specific rhizosphere microbiome dependent of the present soil community (Smalla et al., 2001). Besides plant species, the composition and diversity of microbial rhizosphere communities is shaped by soil type and pedoclimate, plant health and developmental stage, climate and season, pesticide treatments, grazers and animals, and several other biotic and abiotic factors (Singh and Mukerji, 2006; Berg and Smalla, 2009; Barnard et al., 2013). Some genera are ubiquitous and can be found distributed over the entire plant, such as the well-known plant-associated genera *Bacillus* and *Pseudomonas* (Berg et al., 2011). However, a high degree of specificity for each microenvironment was also observed via comparison of microbial colonization patterns of different microhabitats (Berg et al., 2005b; Fürnkranz et al., 2012; Köberl et al., 2013). Certain plant-associated microorganisms including beneficials and pathogens are also passed down from previous generations via the seed (Hardoim et al., 2012; Hirsch and Mauchline, 2012), and likewise a transmission between plants through pollen grains was recently observed (Fürnkranz et al., 2012). Interestingly, the phylogenetically oldest land plants, mosses, transfer a highly diverse core microbiome of primarily potential beneficial bacteria from the sporophyte to the gametophyte and *vice versa* (Bragina et al., 2012).

Medicinal plants harbor a distinctive microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms (Qi et al., 2012). The analyses of several Chinese medicinal plant microbiomes showed interesting results (*Ainsliaea henryi* Diels, *Dioscorea opposita*, *Potentilla discolor*

Bge, *Stellera chamaejasme* L., *Ophiopogon japonicus* (Thunb) Ker-Gawl., *Juncus effusus* L. var. *decipiens* Buchen., *Rhizoma arisaematis,* and others; Li et al., 2008; Zhao et al., 2011), as each of them hosted a specific actinobacterial community and showed a remarkably high and diverse rhizospheric and endophytic colonization with Actinobacteria featuring both antimicrobial and antitumor properties (Zhao et al., 2012). These Gram-positive and often spore-forming bacteria are promising biological control agents (BCAs), such as the genus *Streptomyces* that is a known and unique source of novel antibiotics (Goodfellow and Fiedler, 2010; Niraula et al., 2010; Nachtigall et al., 2011; Raaijmakers and Mazzola, 2012).

MEDICINAL PLANTS: PLANT- AND MICROBE-DERIVED INGREDIENTS

Plants contain numerous different biologically active compounds, and plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years. Traditional Chinese medicine (TCM), phytotherapeutic knowledge from the Mayans, the aboriginal medicine of Australia, and several other cultures comprise a huge spectrum of natural remedies that can be exploited as sources for new and effective therapeutic agents. Still widely practiced in the modern era, TCM supplies ethnopharmaceutical knowledge on over 5,000 plant species used for the treatment of numerous diseases and has already provided the basis for the discovery of many modern drugs, such as anticancer agents (Miller et al., 2012a,b). In general, natural products play a highly considerable role in the drug discovery and development process, as about 26% of the new chemical entities introduced into the market worldwide from 1981 to 2010 were either natural products or those derived directly therefrom, reaching a high of 50% in 2010 (Newman and Cragg, 2012). In the past, medicinal plant research focused primarily on their ingredients, however, recently the focus has shifted to include the structure and function of several medicinal plant microbiomes. Surprisingly, not only were the plants themselves able to produce substances with phytotherapeutic properties, but their associated microbes, in particular endophytes, could as well (**Table 1**). Currently, research continues to show that a significant number of natural products are actually produced by microbes and/or microbial interactions with the host from whence they were isolated (Gunatilaka, 2006), and for several medicinal plants it is presumed that the plant-associated microbiome, especially the complex community of the endomicrobiome, is directly or indirectly involved in the production of bioactive phytochemicals. Presently, however, only a small subset of potential microbial strains could be definitively attributed to phytotherapeutic properties (Strobel and Daisy, 2003; Strobel et al., 2004; Chandra, 2012; Miller et al., 2012a,b), and their relative contribution to the recognized valuable bioactivity of medicinal plants is not clear as of yet.

In regards to the alarming incidence of antibiotic resistance in bacteria with medical relevancy, medicinal plants with antibacterial properties are of central importance as bioresources for novel active metabolites (Palombo and Semple, 2001). Likewise, there is an increasing need for more and better antimycotics to treat those with weakened immune systems who are more prone to developing fungal infections, such as from the AIDS epidemic, cancer therapy, or organ transplants (Strobel and Daisy, 2003; Strobel et al., 2004). For centuries, several phytotherapeutics have also been known for their antiphlogistic features, yet despite the progress within medical research, chronic inflammatory diseases such as asthma, arthritis, and rheumatism remain one of the world's leading health problems (Li et al., 2003). Hypertension is another critical issue for human health and is a primary risk factor for stroke, heart disease, and renal failure. Many herbal remedies as well as foods, however, are known and effective folk medicines in the prevention and/or treatment of high blood pressure (Abdel-Aziz et al., 2011). Hence, nature must still harbor plenty of currently unknown active agents that may serve as leads and scaffolds for the development of desperately needed efficacious drugs for a multitude of diseases (Newman and Cragg, 2012). Today, globalization has also had an impact on the use of medicinal plants and has proven beneficial in allowing greater access to these medicines for people all across the globe. For example, TCM plants are very popular in Europe, whereas the traditional German chamomile is primarily produced in Egypt. Growth, quality, and health of the medicinal plants are highly influenced and controlled by their microbiota through microbial metabolisms and host interactions.

PLANT GROWTH PROMOTION AND BIOLOGICAL CONTROL FOR MEDICINAL PLANTS

Several rhizospheric microbes interact beneficially via different mechanisms with their host plant. They can have a direct plant growth promoting effect based on improved nutrient acquisition or hormonal stimulation, or indirectly affect the plant health by suppression of phytopathogens (Berg, 2009; Lugtenberg and Kamilova, 2009). Biofertilizers are microbes that supply the plant with nutrients, for example symbiotic root-nodulating rhizobia are the most prominent among the nitrogen-fixing microorganisms. Other microbial biofertilizers, such as mycorrhizal fungi and several rhizobacteria, are able to solubilise plant-available phosphate from either organic or inorganic bound phosphate (Lugtenberg et al., 2002). Microbes that hormonally promote plant growth are termed phytostimulators, and the phytohormone auxin, for instance, produced by fluorescent pseudomonads is one of the best understood examples (Kamilova et al., 2006; Khare and Arora, 2010). Various rhizobacteria, including for example *Burkholderia cepacia*, *Staphylococcus epidermidis,* and strains of the *Bacillus subtilis* group, stimulate plant growth by the emission of volatile organic compounds (VOCs; Ryu et al., 2003; Kai et al., 2007; Effmert et al., 2012; Bitas et al., 2013). VOCs are low molecular weight molecules (<300 Da) that have high vapor pressures and are therefore able to diffuse over long distances through the porous structure of the soil and through water-filled pores (Kai et al., 2007; Insam and Seewald, 2010). Indirectly, the plant growth can be promoted via biological control of phytopathogens. Pathogen growth can be inhibited by antibiotics or VOCs, toxins, biosurfactants, or extracellular cell wall-degrading enzymes, but microbial antagonism can also occur via degradation of pathogenicity factors like toxins, or simply by the competition for nutrients, minerals, or colonization sites (Berg, 2009). Another possible way to reduce the activity of pathogenic microorganisms is the activation of the plant defense mechanisms, or the so called induced

Table 1 | Examples for bioactive phytometabolites where microorganisms are involved in their production.

Bioactive compound	Therapeutic properties	Host plant	Producing microorganism	Reference
Munumbicins	Antibacterial, antimycotic, antiplasmodial	*Kennedia nigriscans*	*Streptomyces* sp.	Castillo et al. (2002)
Kakadumycins	Antibacterial, antiplasmodial	*Grevillea pteridifolia*	*Streptomyces* sp.	Castillo et al. (2003)
Coronamycins	Antimycotic, antiplasmodial	*Monstera* sp.	*Streptomyces* sp.	Ezra et al. (2004)
Oocydin A	Antimycotic (Oomycota)	*Rhyncholacis penicillata*	*Serratia marcescens*	Strobel et al. (1999a)
Cryptocandin	Antimycotic	*Tripterigeum wilfordii*	*Cryptosporiopsis quercina*	Strobel et al. (1999b)
Colletotric acid	Antibacterial, antimycotic	*Artemisia mongolica*	*Colletotrichum gloeosporioides*	Zou et al. (2000)
Artemisinin	Antiplasmodial	*Artemisia annua*	*Colletotrichum* sp.	Wang et al. (2001)
Cochliodinol	Antibacterial, antimycotic, anticancer	*Salvia officinalis*	*Chaetomium* sp.	Debbab et al. (2009)
Botryorhodines	Antimycotic, anticancer	*Bidens pilosa*	*Botryosphaeria rhodina*	Abdou et al. (2010)
Pestacin and Isopestacin	Antimycotic, antioxidant	*Terminalia morobensis*	*Pestalotiopsis microspora*	Strobel et al. (2002), Harper et al. (2003)
Phomol	Antiphlogistic, antibacterial, antimycotic, anticancer	*Erythrina crista-galli*	*Phomopsis* sp.	Weber et al. (2004)
Podophyllotoxin	Anticancer, antiphlogistic	*Podophyllum hexandrum* *Juniperus communis*	*Alternaria* sp. *Aspergillus fumigatus*	Yang et al. (2003), Kusari et al. (2009a)
Paclitaxel (Taxol)	Anticancer	*Taxus brevifolia* *Ginkgo biloba* *Aloe vera*	*Taxomyces andreanae* *Alternaria* sp. *Phoma* sp.	Wani et al. (1971), Stierle et al. (1993), Kim et al. (1999), Immaculate et al. (2011)
Camptothecin	Anticancer, antiviral (HIV)	*Nothapodytes foetida* *Camptotheca acuminate*	*Entrophospora infrequens* *Fusarium solani*	Puri et al. (2005), Amna et al. (2006), Kusari et al. (2009b)
Maytansine	Anticancer	*Putterlickia verrucosa*	*Actinosynnema pretiosum*	Wings et al. (2013)
Rohitukine	Antiphlogistic, anticancer, immunomodulatory	*Dysoxylum binectariferum*	*Fusarium proliferatum*	Mohana Kumara et al. (2012)
Subglutinols	Immunomodulatory	*Tripterigeum wilfordii*	*Fusarium subglutinans*	Lee et al. (1995)

systemic resistance (ISR) triggered by certain non-pathogenic rhizobacteria. Flagella, lipopolysaccharides, siderophores, VOCs, and several other bacterial components are thought to be involved in activating the non-pathogenic rhizobacteria-mediated ISR signaling pathway (van Loon et al., 1998; Lugtenberg and Kamilova, 2009).

Biological control of plant pathogens as well as plant growth promotion with microorganisms has been intensively studied over the past decades and is becoming a realistic alternative to chemical pesticides and fertilizers in sustainable agriculture (Weller, 2007). Several microbial inoculants have already been successfully commercialized (Berg, 2009, 2013), but a specific biological control strategy for medicinal plants, which are increasingly affected by different soil-borne phytopathogens, has not been available until now. While specific biocontrol agents for medicinal plants are needed, their associated microbiomes with outstanding metabolic activities also provide a promising source for novel BCAs.

MEDICINAL PLANTS AND (POTENTIAL) HUMAN PATHOGENS: OCCURRENCE AND POSSIBLE BIOCONTROL

Traditional medicinal plants are often consumed raw, such as berries or other edible fruits, or in dried form as herbal brews or teas. Therefore, it is of crucial importance that any potentially harmful effect of associated microorganisms or of an applied biocontrol agent on human health be avoided completely. Recently, for instance, bacterial strains closely related to *Stenotrophomonas maltophilia* and *Rhodococcus* sp. were isolated from the roots of oregano (*Origanum vulgare* L.) cultivated in a sub-Himalayan region (Bafana, 2013). Similarly, *Ochrobactrum* and *Rhodococcus* were also detected on the studied medicinal plants in Egypt (*Matricaria chamomilla* L., *Calendula officinalis* L., and *Solanum distichum* Schumach. and Thonn.; Köberl et al., 2011). Among several others, these bacterial genera are known for their ambiguous interactions with eukaryotic hosts whereby the mechanisms responsible for plant growth promotion are similar to those also responsible for opportunistic infections in humans

and animals (Berg et al., 2005a). In addition to the suppression of phytopathogens, antagonistic activity against potentially harmful human pathogens should also be considered in the biocontrol strategy.

Conversely, ethanolic extracts from the Chinese medicinal plants *Mallotus yunnanensis* Pax et. Hoffm., *Schima sinensis* (Hemsl. et. Wils) Airy-Shaw., *Garcinia morella* Desr., *Evodia daneillii* (Benn) Hemsl., *Meliosma squamulata* Hance., *Skimmia arborescens* Anders., and *Brandisia hancei* Hook. f. were determined as highly active against the clinical pathogens *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* which corresponds to their traditional applications in skin and other infections (Zuo et al., 2012). Promising antimicrobial activities against human multi-drug-resistant pathogens have been observed for Mexican medicinal plants as well (Jacobo-Salcedo Mdel et al., 2011). As previously discussed for phytotherapeutic properties, the suppression of human pathogens can also be frequently attributed to medicinal plant-associated microbes and their secondary metabolites (Miller et al., 2012b; Mousa and Raizada, 2013).

In conclusion, medicinal plants should be considered as meta-organisms that comprise both the plant themselves and their microbiome. As meta-organisms, they are a largely untapped and enormous bioresource for bioactive compounds and microorganisms of potential use in modern medicine, agriculture, and pharmaceutical industry. As such, more research is necessary to exploit this immense reservoir for mankind.

A CASE STUDY: THE MICROBIOME OF MEDICINAL PLANTS GROWN ON A DESERT FARM UNDER ORGANIC MANAGEMENT

In comparison to soils of humid areas, the soil microbiome of the Egyptian desert farm Sekem was comprised of a high abundance of Gram-positive, spore-forming bacteria primarily of the Firmicutes branch with 37% of the total bacterial soil community as revealed through a pyrosequencing-based amplicon sequencing approach (Köberl et al., 2011). However, a global soil community analysis including 32 libraries of 16S rRNA and 16S rRNA gene libraries from a variety of soils reported Firmicutes contribute a mean of only 2% in the total bacterial soil community (Janssen, 2006). *Bacillus* and *Paenibacillus* play the key role in explaining this remarkably high abundance of Firmicutes in the investigated desert agro-ecosystem. These drought-resistant genera are of prime importance for pathogen suppression under arid conditions as nearly all isolated antagonists with activity against soil-borne phytopathogenic fungi could be affiliated to this taxonomic group. This is in direct contrast to humid soils, where primarily Gram-negative bacteria like *Pseudomonas* are responsible for the indigenous antagonistic potential (Berg et al., 2005b; Haas and Défago, 2005; Costa et al., 2006; Zachow et al., 2008). A significantly higher proportion of Firmicutes and antifungal isolates were observed in field soil from the Egyptian farm than in the surrounding desert soil uninfluenced by human activities. In general, the total bacterial soil microbiome of the anthropogenic ecosystem exhibited a higher diversity and better ecosystem function for plant health in comparison to the natural desert soil (**Figure 1**). Due to the long-term agricultural use of the desert and the associated increasing occurrence of plant pathogens, the indigenous antagonistic potential in soil was almost twice as high as in the uncultivated desert soil. However, the diversity of antagonistic bacteria was lower and highly dominated by isolates of the *Bacillus subtilis* group. The most efficient antagonists from the native desert soil belonged to *Streptomyces*, and *Bacillus* and *Paenibacillus* species were the most frequently isolated antagonists from all investigated arid habitats including both desert and agriculturally used soil, as well as from the rhizosphere and endorhiza of three different species of medicinal plants cultivated on the desert farm (*Matricaria chamomilla* L., *Calendula officinalis* L., and *Solanum distichum* Schumach. and Thonn.). None of the plants are native to Egypt, and therefore were exposed to a previously unencountered microbiome. Interestingly, despite a clearly plant-specific selection of the associated bacterial microbiome, indigenous *Bacillus* and *Paenibacillus* strains of native desert soil with promising antagonistic properties against a wide range of soil-borne phytopathogens were enriched in all investigated plant roots. Conversely, several extremophilic bacterial groups, such as *Acidimicrobium*, *Rubellimicrobium*, and *Deinococcus–Thermus* decreased or completely disappeared from soil after agricultural use (Köberl et al., 2011).

Nitrogen is an essential macronutrient for plants and one of the most yield-limiting factors in agricultural production systems throughout the world (Bhattacharjee et al., 2008; Orr et al., 2011). To gain insight into the indigenous community of diazotrophic plant growth promoting microorganisms that inhabit desert agro-ecosystems, community profiles of the *nifH* gene encoding the nitrogenase reductase subunit were assessed. A broad diversity and high abundance of diazotrophs were detected in all investigated habitats, thus underlining their importance in native and agricultural desert ecosystems. Due to watering and cultivation of desert soil, a considerable shift toward a higher abundance and diversity was also observed for the nitrogen-fixing community. Phylogenetic analyses distinguishing between the major *nifH* gene types (Zehr et al., 2003; Gaby and Buckley, 2012) revealed that all NifH sequences from soil libraries were affiliated with the canonical *nifH* clusters I (conventional molybdenum nitrogenases) and III (molybdenum nitrogenases from anaerobes), while no sequences of alternative nitrogenases (cluster II) and *nifH* paralogs (clusters IV and V) were found. In general, the diazotrophic soil microbiota was highly dominated by NifH sequences related to Alphaproteobacteria. Each investigated medicinal plant cultivated on the desert farm harbored a specific root-associated diazotrophic microbiome. The rhizosphere inhabitants of *Matricaria chamomilla* (**Figure 2**) and *Calendula officinalis* were similar and both dominated by potential root-nodulating rhizobia acquired mainly from soil. Conversely, the rhizosphere of *Solanum distichum* was colonized in higher abundances by free-living nitrogen fixers most likely transmitted between plants as they were undetectable in soils. Although well-known for taxonomic community structure (Berg and Smalla, 2009; Bulgarelli et al., 2012), this high degree of plant-specificity identified plants as important drivers for functional diversity as well (Köberl, 2013). The total bacterial and fungal communities also revealed similar colonization patterns between the medicinal plants *Matricaria chamomilla* and *Calendula officinalis*

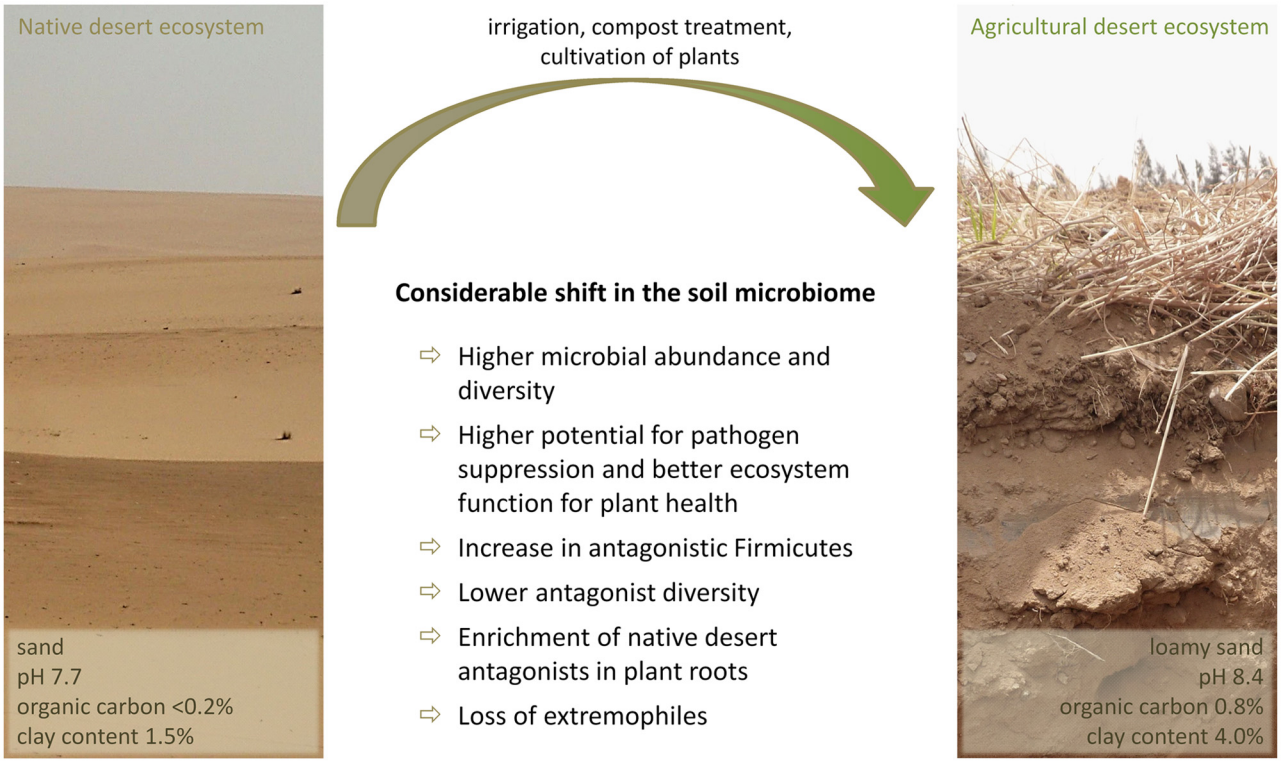

FIGURE 1 | Community shift in the soil microbiome after long-term agricultural use of the desert under organic management.

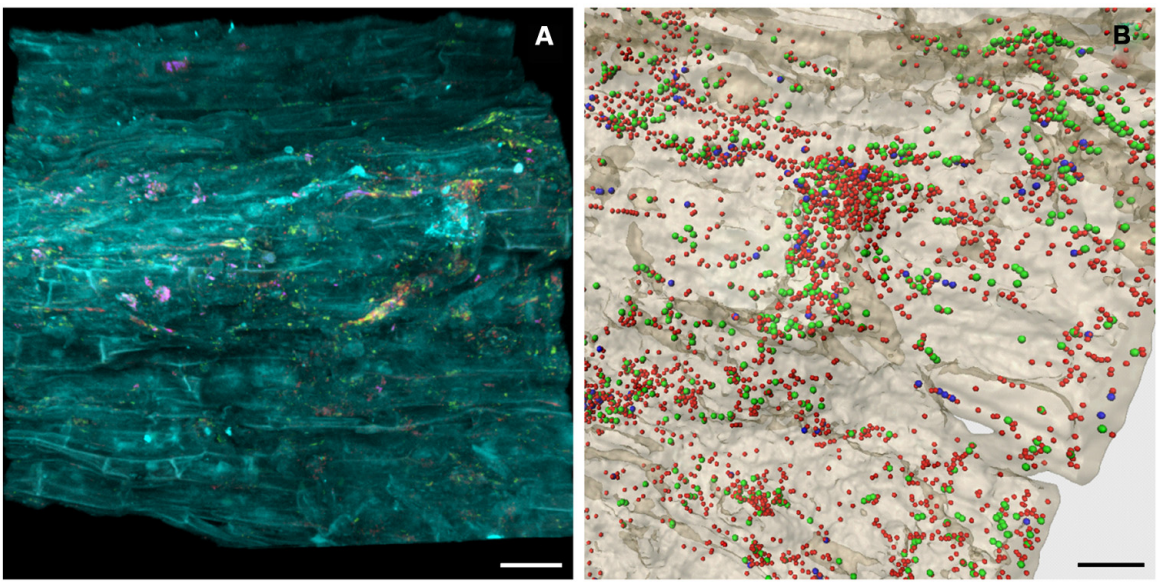

FIGURE 2 | *In situ* visualization of the bacterial root colonization of *Matricaria chamomilla*. Volume rendering **(A)** and three-dimensional reconstruction model **(B)** of confocal laser scanning microscopy stacks. **(A)** yellow = Alphaproteobacteria, pink = Betaproteobacteria, red = other eubacteria, cyan = root tissue, scale bar = 30 μm. **(B)** green = Alphaproteobacteria, blue = Betaproteobacteria, red = other eubacteria, beige = root tissue, scale bar = 15 μm.

compared to *Solanum distichum* (Köberl et al., 2013). This effect may have been intensified as a result of the close relationship between *Matricaria chamomilla* and *Calendula officinalis* who both belong to the Asteraceae family and therefore produce more similar bioactive metabolites. Furthermore, both *Matricaria chamomilla* and *Calendula officinalis* are annual herbal medicinal plants, while *Solanum distichum* is a perennial plant thus providing a longer timeframe to specifically select a stable associated microbiome.

In contrast to the highly specific bacterial communities associated with cultivated medicinal plants, fungal communities were less discriminative and characterized primarily by potential pathogens. Phytopathogenic species *Fusarium*, *Verticillium*, and several others were frequently identified, and, apart from *Rhizoctonia*, were the main soil-borne pathogens on the investigated desert farm that caused high yield losses on a wide host range of economically important crops, including the medicinal plants. To biologically control these soil-borne diseases, different desert habitats were screened for potential BCAs adapted to the unique and arid conditions of desert farming. Due to this high content of potential plant pathogens in the fungal community, the selection of antagonists was focused on the indigenous bacterial microbiome. An *in vitro* screening of 1,212 bacterial isolates linked with the comprehensive ecological data resulted in an antagonist collection of 45 genotypically different antifungal strains. In a hierarchical evaluation including their antifungal properties against *Verticillium dahliae*, *Rhizoctonia solani*, and *Fusarium culmorum* in addition to their antagonistic activity against the soil-borne plant pathogenic bacterium *Ralstonia solanacearum* and the nematode *Meloidogyne incognita*, three promising drought- and heat-resistant biocontrol candidates were selected: *Streptomyces subrutilus* Wb2n-11 isolated from desert soil in Sinai, *Bacillus subtilis* subsp. *subtilis* Co1-6 obtained from the rhizosphere of *Calendula officinalis*, and *Paenibacillus polymyxa* Mc5Re-14 isolated from the endorhiza of *Matricaria chamomilla*. Each belongs to risk group 1 and poses no risk for humans or the environment. These three potential BCAs have already shown promising *in vitro* plant growth promoting activities and stress tolerances; *Bacillus subtilis* Co1-6 exhibited high drought and salt resistance, protease and glucanase activity, and the production of siderophores, *Paenibacillus polymyxa* Mc5Re-14 had a lower tolerance to abiotic stresses in comparison to the *Bacillus* strain, but also tested positive for siderophores and glucanase activity, and the desert bacterium *Streptomyces subrutilus* Wb2n-11 showed hydrolytic degradation of chitin and glucan. All of them produced antibiotics against the nematode *Meloidogyne incognita*, however, their antibacterial activities were highly specific. While *Bacillus subtilis* and *Streptomyces subrutilus* exhibited antagonistic suppression of the plant pathogen *Ralstonia solanacearum*, only the *Paenibacillus* isolate was active against the opportunistic human pathogen *Escherichia coli* (Köberl et al., 2013).

These three autochthonous Gram-positive strains were selected for *ad planta* evaluation in the field under desert farming conditions in comparison to three allochthonous Gram-negative strains already known for their beneficial plant–microbe interactions in humid soils: *Pseudomonas fluorescens* L13-6-12 isolated from the rhizosphere of potato (*Solanum tuberosum*), *Stenotrophomonas rhizophila* P69 from oilseed rape (*Brassica napus*) rhizosphere, and *Serratia plymuthica* 3Re4-18 from the endorhiza of potato (Lottmann and Berg, 2001; Wolf et al., 2002; Kai et al., 2007; Zachow et al., 2010; Alavi et al., 2013). The first results revealed that priming chamomile seedlings with the autochthonous strains not only showed a stabilizing effect on plant performance, but *Bacillus subtilis* Co1-6 and *Paenibacillus polymyxa* Mc5Re-14 were also able to further elevate the plants' flavonoid production. Higher contents of the bioactive compounds apigenin-7-*O*-glucoside and apigenin, which belong to the major flavonoids of chamomile florets (Kato et al., 2008; Srivastava and Gupta, 2009), were measured in blossoms of plants treated with the two Bacillales strains compared to blossoms of other treatments and uninoculated control plants (Schmidt et al., 2013). These findings demonstrate that a targeted bacterial treatment could influence the metabolic activity of the plant, and therefore represent one of the many poorly understood links between the structure and metabolic profile of the plant-associated microbiome and the plant metabolome.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Gabriele Berg, Rudolf Bauer, Elshahat M. Ramadan and Martina Köberl. Performed the experiments: Martina Köberl and Ruth Schmidt. Analyzed the data: Martina Köberl, Ruth Schmidt and Gabriele Berg. Contributed reagents/materials/analysis tools: Gabriele Berg. Wrote the paper: Martina Köberl and Gabriele Berg.

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Antimicrobial drimane sesquiterpenes and their effect on endophyte communities in the medical tree *Warburgia ugandensis*

Sigrid Drage[1†], Birgit Mitter[2*†], Christina Tröls[2], Alice Muchugi[3], Ramni H. Jamnadass[3], Angela Sessitsch[2] and Franz Hadacek[1,4]

[1] Division of Terrestrial Ecosystem Research, Department of Microbiology and Ecosystem Science, Faculty of Life Sciences, University of Vienna, Vienna, Austria
[2] Bioresources Unit, Health & Environment Department, AIT Austrian Institute of Technology GmbH, Tulln, Austria
[3] World Agroforestry Centre (ICRAF), Nairobi, Kenya
[4] Department for Plant Biochemistry, Albrecht-von-Haller-Institute, Georg August Univeristät, Göttingen, Germany

Edited by:
Martin Grube, Karl-Franzens-Universität Graz, Austria

Reviewed by:
Alison Bennett, James Hutton Institute, UK
Nai-Chun Lin, National Taiwan University, Taiwan

***Correspondence:**
Birgit Mitter, Bioresources Unit, Health & Environment Department, AIT Austrian Institute of Technology GmbH, Konrad Lorenz Strasse 24, 3430 Tulln, Austria
e-mail: birgit.mitter@ait.ac.at
† These authors have contributed equally to this work.

Metabolite profiles (GC–MS), drimane sesquiterpenes, sugars and sugar alcohols, were compared with bacterial and fungal endophyte communities (T-RFLP, DNA clones, qPCR) in leaves and roots of the pepper bark tree, *Warburgia ugandensis* (*Canellaceae*). Ten individuals each were assessed from two locations east and west of the Great Rift Valley, Kenya, Africa, which differed in humidity and vegetation, closed forest versus open savannah. Despite organ- and partially site-specific variation of drimane sesquiterpenes, no clear effects on bacterial and fungal endophyte communities could be detected. The former were dominated by gram-negative *Gammaproteobacteria*, *Pseudomonadaceae* and *Enterobacteriaceae*, as well as gram-positive *Firmicutes*; the fungal endophyte communities were more diverse but no specific groups dominated. Despite initial expectations, the endophyte community of the pepper bark tree did not differ from other trees that much.

Keywords: endophytes, warburgia ugandensis, drimane sesquiterpene, bacteria diversity, fungi diversity

INTRODUCTION

Warburgia ugandensis Sprague [=*W. salutaris* (Bertol.f.) Chiov], the pepper bark tree belongs to the *Canellaceae*, a small family of tropical trees, all of them aromatic and most with medicinal properties. *W. ugandesis* has a restricted distribution in evergreen forests and woodland ravines of northern KwaZulu-Natal, Swaziland, Mpumalanga, Uganda, and Kenya. This species is widely used in traditional medicine within local communities in Eastern Africa, known to cure several ailments such as stomach-ache, constipation, toothache, common cold, cough, fever, muscle pains, weak points, measles, and malaria (Beentje and Adamson, 1994; Kokwaro, 2009). Previous phytochemical studies led to the isolation of a series of unique drimane sesquiterpenes. The biological activity of the drimane sesquiterpenoids is well documented and includes antimicrobial, antifungal, insect antifeedant, cytotoxic, molluscicidal, plant growth regulation, and skin irritant effects (Jansen and De Groot, 2004). Water extracts of *W. ugandensis* elicited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and antifungal activity against *Candida albicans* (Oilila, 2001). Preliminary phytochemical analysis revealed qualitative as well as quantitative differences in the drimane sesquiterpene profiles of individual trees grown at the same location as well as of the different organs of one tree. Consequently, the pepper bark tree represents an interesting model to explore relationships between microbial endophytes and host plant secondary metabolites, not only in terms of obtaining insights on how those interactions affect biodiversity and community composition, but also in terms of how the content of active constituents in plants that are used in traditional medicine—drimane sesquiterpenes from *Warburgia* are even considered as anti-malaria drugs (Were et al., 2010; Wube et al., 2010)—can be affected by colonization with endophytic microbes.

For this study, two populations of *W. ugandensis*, one located west (Kitale) and one east (Rumuruti) of the Great Rift Valley, Kenya, Africa, were chosen. Kitale forest is a tropical area in western Kenya situated between Mount Elgon and the Cherengani Hills at an elevation of around 2000 m above sea level. The Rumuruti forest is a dry upland forest at an elevation of 1700–2000 m above sea level. Both differ in their humidity as documented by annual rainfall amounts. Based on AFLP comparisons, the two *Warburgia* populations were recently suggested to constitute two different species as a consequence of allopatric speciation, which also occurs for species of other genera that occur both west and east of the Great Rift Valley (Muchugi et al., 2008). At both locations, leaves and roots of ten individuals were sampled. Fruits only were available at the Rumuruti site and also included into the study. We performed a polyphasic approach combining a concomitant chemical analysis of secondary metabolites in *W. ugandensis* and cultivation-independent analysis of microbes colonizing this tree.

The literature suggests that interactions between endophytes and *Warburgia* secondary metabolites should to be expected as not neutral (Carter et al., 1999; Schulz and Boyle, 2005; Saunders and Kohn, 2009). Following those assumptions we predict that:

1. Bacterial and fungal communities will resemble each other in both localities due to the selection of resistant and dominating genotypes.
2. Specific drimane sesquiterpene patterns will correlate with the presence of specific members of the endophytic microbial community, either due to their tolerance against host plant drimane sesquiterpenes or involvement in their biotransformation.
3. Drimane sesquiterpene diversity will correlate with microbial community diversity.

If, by contrast, the interactions are of more stochastic nature, we predict that

1. Bacterial and fungal communities will vary between individuals and localities with no recognizable clustering in terms of plant organ and study site.
2. No correlations will exist between the presence of specific strains in the microbial assemblage and the dominance of specific drimane sesquiterpenes in the profile.
3. Drimane sesquiterpene diversity will not correlate with microbial community diversity.

MATERIALS AND METHODS

PLANT MATERIAL

Leaves and roots, and, if available, fruits of pepper bark trees, *Warburgia ugandensis* Sprague, from 10 different individuals, were accessed from two distinct sites in Kenya, the first one west of the Great Rift Valley near the village Rumuruti, Marnanet North forest (1845 m. a. s. l., 0°16′ N/36°31″ E, 29.09.2007), the second one east of the Great Rift Valley in Kitale forest near the town Kitale (1900 m. a. s. l., 01°00′ N/35°01′ E, 2.10.2007), west of the Great Rift Valley. Kitale is located in a moist savannah in western Kenya situated between Mount Elgon and the Cherengani Hills at an elevation of around 2000 m. a. s. l. and has an average annual precipitation of 1269 mm; Rumuruti is located in a savannah with *W. ugandensis* mostly growing in or close to moist ravines embedded in semi-arid land at an elevation of 1700–2000 m. a. s. l. in the Laikipia district situated northwest of Mount Kenya with 739 mm average annual precipitation (http://www.climatedata.eu) (Kindt et al., 2005). Both sites are natural forests, which are managed by the Forest Department of Kenya. Voucher specimens are deposited in the University of Göttingen Herbarium (GOET), encoded K1–K10 (Kitale) and R1–R10 (Rumuruti) respectively.

Roots and leaves dedicated for DNA analysis were surface-sterilized (Sessitsch et al., 2002) and embedded in 1.5% (w/v) agar supplemented with mineral salt as used for plant tissue cultures (Murashige and Skoog, 1962). This step was performed in attempts to preserve the plant material during the transport to Austria. Roots and leaves dedicated to chemical analyses were packed into paper bags and dried in an incubator at 40°C to prevent rot during transport.

METABOLITE EXTRACTION AND ANALYSIS

Three grams dried and pulverized plant tissue (leaves, fruits, and roots) were extracted with 80 ml methanol for 24 h at ambient temperature. The extract was filtered (MN 615; Macherey-Nagel, Düren, Germany) and concentrated under vacuum. Two hundred mg crude extract were fractioned over Amberlite XAD-1180 (Fluka, Buchs, Switzerland). Glass columns (15 mm diameter) were filled with 20 g resin and prepared according to the manufacturer's guidelines. Two 50 ml fractions were eluted, one with water, one with absolute ethanol. The evaporated eluates were dissolved in 10 ml methanol and stored at −20°C until further use. All used solvents were at least p.a. quality. This procedure was performed for all extracts to obtain fractions that could be analyzed in terms of drimane sesquiterpene composition. The crude extracts contained high quantities of sugar alcohols, specifically mannitol. Metabolite quantitation only was performed with the ethanol fraction, in which the drimanes were accumulated but which still contained notable amounts of sugars and sugar alcohols. A clean separation proved impossible. Consequently, the ethanol fraction represented the only fraction that provided a dataset allowing a relative comparison of drimane sesquiterpenes, fatty acids and sugar alcohols, assuming that lower sugar alcohol or sugar amounts present in the ethanol fraction represent a lower ethanol—drimane sesquiterpene ratio in the crude extract.

For GC–MS measurements, 100 µg of the dried ethanolic eluate (Amberlite XAD fractionation were dissolved in 100 µl N-methyl-N-TMS-trifluoroacetamide (MSTFA, Thermo Scientific, Waltham, MS, USA) for derivatisation into trimethylsilyl ethers. One µl of this solution was injected into an AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MS, USA) in the splitless mode, the injector temperature was 250°C. The column was a Zebron 5 ms column (18 m × 0.18 mm, 0.18 µm film thickness; Phenomenex, Torrance, CA, USA), the helium follow rate 0.8 ml/min. The temperature gradient started at 70°C and, after 3 min, rose to 300°C at a rate of 3°C/min. The gas chromatograph was linked to a TurboMass™ quadrupole mass analyzer (Perkin Elmer, Waltham, MS, USA); the transfer line temperature was set to 280°C, the ion source to 200°C, the filament current to 70 eV. The mass spectrometer was run in the TIC mode from 40–620 amu. The obtained chromatograms were integrated with TurboMass 4.1.1 (Perkin Elmer, Waltham, MS) and the peak areas were expressed as relative amounts of the total peak area (100%). The majority of drimane structures were identified on basis of a tentative fragmentation pattern analysis of the silylated derivatives. Published structures, both from natural sources and from synthesis, served as templates for spectrum interpretation.

DNA EXTRACTION

Prior to isolation of microbial community DNA, microbial cells were dislodged from plant tissue as previously described (Reiter and Sessitsch, 2006). Therefore, leaves and roots were pulled out carefully of the agar and the remaining agar was removed thoroughly in sterile conditions. Surface disinfected fruits were cut open and the pulp was removed with a sterile spoon. DNA was isolated using the Fast DNA SPIN for Soil Kit (MP Biomedicals, Solon, OH,) as described by the manufacturer with the following modifications. Bacterial pellets were re-suspended in Na_2PO_4

buffer, MT buffer was added and everything was transferred to the lysing matrix E tube followed by 30 s bead-beating with a bead beater (FastPrep FP 120, Bio101, Savant Instruments, Inc., Holbrook, NY).

T-RFLP ANALYSIS

Bacterial and fungal endophyte community profiles were examined by T-RFLP. Endophytic 16S rRNA genes were PCR-amplified using the primers 799F (5′-AAC(AC)GGATTAGATACCC(GT)-3′) (Chelius and Triplett, 2001) and 1520R (5′-AAGGAGGTG ATCCAGCCGCA-3′) (Edwards et al., 1989), which was labeled with 6-carboxyfluorescein at the 5′ end. Partial fungal rRNA genes were PCR-amplified using the primers ITS1F (5′- CTTGGTCA TTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993), which was labeled with 6-carboxyfluorescein at the 5′ end and ITS4 (5′- CGCCGTTACTGGGGCAATCCC -3′) (White et al., 1990). For detailed description of PCR conditions see supplementary information.

T-RFLP and data collection has been done as described by Szukics et al. (2010). The analysis of the T-RFLP profiles (identification of peaks and binning of the different fragments lengths) was done by making use of the R functions available at http://www.ibest.uidaho.edu/tools/trflp_stats/index.php (Abdo et al., 2006).

DNA CLONE LIBRARIES

Ribosomal DNA libraries were constructed from a pool of aliquots of all DNA samples as well as selected plant samples making use of the Strata Clone PCR Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) and the StrataClone SoloPack *E. coli* competent cells (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. For a more detailed description of the cloning procedure see supplementary information. Clones have been sequenced with the primer M13f and/or M13r making use of the sequencing service of LGC Genomics (Berlin, Germany). Retrieved sequences were visualized and vector sequences were removed with sequence alignment editor package of BioEdit (Ibis Biosciences, Carlsbad, CA, USA). For identification sequences were subjected to the Basic Local Alignment Search Tool (BLAST) analysis with the National Center for Biotechnology Information (NCBI) database.

REAL-TIME PCR

Pseudomonadaceae-, *Enterobacteriaceae*- and *Firmicutes*-specific 16S rRNA genes within selected plant samples were analyzed in more detail by real-time PCR. Following primers were as used: *Pseudomonadaceae*; 8f: 5′-AGAGTTTGATCCTGGCTCAG-3′ (White et al., 1990) and PSMgX: 5′-CCTTCCTCCCAACTT-3′ (Braun-Howland et al., 1993); *Enterobacteriaceae*; En-Isu-3F: 5′-TGCCGTAACTTCGGGAGAAGGCA-3′ and En-Isu-3R: 5′-TCAAGGACCAGTGTTCAGTGTC-3′ (Matsuda et al., 2009) and *Firmicutes* 5′-CAGCAGTAGGGAATCTTC-3′ and 5′-CCGCGGT AATACGTAGGT-3′ (Pfeiffer et al., 2014). Automated analysis of PCR amplicon quantities was performed using the iCycler Optical System Software Version 3.1 (Bio-Rad Laboratories). A more detailed description is given in supplementary information.

STATISTICS

Metabolite and endophytic T-RFLP patterns were analyzed by multidimensional scaling analyses (MDS) employing Bray–Curtis similarity as resemblance measure; similarity boundaries were determined by group average clustering of the Bray–Curtis similarity matrix. SIMPER analyses were performed to identify variables that contribute to similarities and dissimilarities of defined sample groups (Clarke, 1993). Diversity indices were calculated by using Fisher's alpha diversity (Fisher et al., 1943). All these analyses and their respective visualizations were performed with Primer 6 (Primer-E Ltd., Plymouth, UK). For further multivariate analyses, PCA and PLS regression, SIMCA-P 11 (Umetrics AB, Umeå, Sweden), was employed.

Parametric analyses of variance (ANOVA), provided normal distribution and variance homogeneity was given, or non-parametric Kruskal–Wallis analysis of variance, in case the above mentioned criteria failed, were carried out with respective multiple range tests (Scheffe, Bonferroni). These and simple linear regression analyses (minimum $n = 3$) were performed with Statgraphics Centurion XV (Statpoint Technologies, Inc., Warrenton, VA, USA).

NUCLEOTIDE SEQUENCE NUMBERS

The nucleotide sequences determined in this study have been deposited in the GenBank database; accession numbers will be provided as soon as possible.

RESULTS

METABOLITES, SPECIFICALLY DRIMANE SESQUITERPENES, CHARACTERIZE ORGANS

GC–MS analyses identified 173 analytes in the extracts from 10 root, 10 leaf, and 5 fruit accessions, the latter originating only from the locality Rumuruti (R1, R3, R4, R6, R10), of which 141 were assigned as terpenoids on basis of their fragmentation patterns, *m/z* of 91, 93, 103, 105, 109, 115, 117, 119, 120, 122, 129, 131, 133, and 135, indicating the presence of a largely oxygen-free saturated ring system. These fragments do not occur in this combination in spectra of other metabolites that are usually detected by GC–MS profiling of trimethylsilylated plant metabolites, such as mono-, di-, and trisaccharides (13), sugar alcohols (4), fatty acids (6) and glycerol, which also were detected. Only one triterpene was detected, β-sitosterol.

A multivariate analysis, MDS (non-metric multidimensional scaling) of a Bray–Curtis resemblance matrix, revealed an organ-specific clustering (**Figure 1A**) that was more pronounced when only drimane sesquiterpenes were included in the analysis (**Figures 1C**, **2D** stress improving from 0.17 to 0.11). The two localities only differed in their leaf profiles; the root profiles overlapped and fruits also showed different patterns but unfortunately were available only from one locality. Metabolite diversity in each accession, Fisher's α accounting not only for the number but also the abundance of each analyte, varied considerably (**Figure 1B**). Again, data set limitation to drimane sesquiterpenes increased differentiation between the accession groups (**Figure 1D**, see levels of significance); fruits showed the lowest diversity but highest dissimilarity (**Table 1**). On average, only half or less of the metabolites were shared by similar organ accessions from the

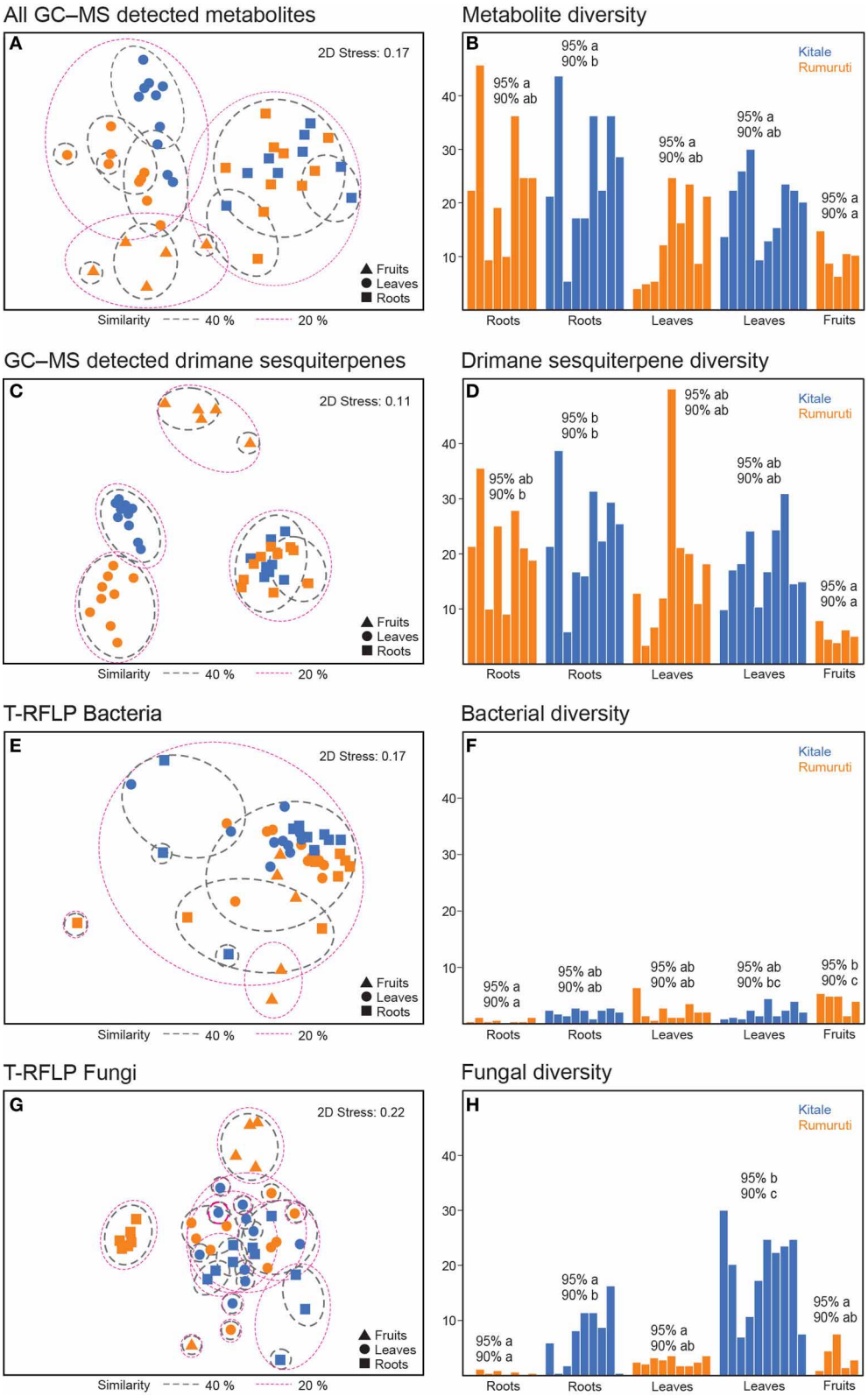

FIGURE 1 | Similarity and diversity of metabolites and endophyte communities of *Warburgia ugandensis*. All GC-MS detectable metabolites: MDS **(A)**, Fishers's α **(B)**, drimane sesquiterpes: MDS **(C)**, Fishers's α **(D)**, bacterial T-RFLP (16S rRNA): MDS **(E)**, Fishers's α **(F)**, fungal T-RFLP (ITS1, ITS4): MDS **(G)**, Fishers's α **(H)**, accessions from two localities: Kitale (blue), Rumuruti (orange); levels of significance: 95% Bonferroni; leaves and roots ($n = 10$), fruits ($n = 5$).

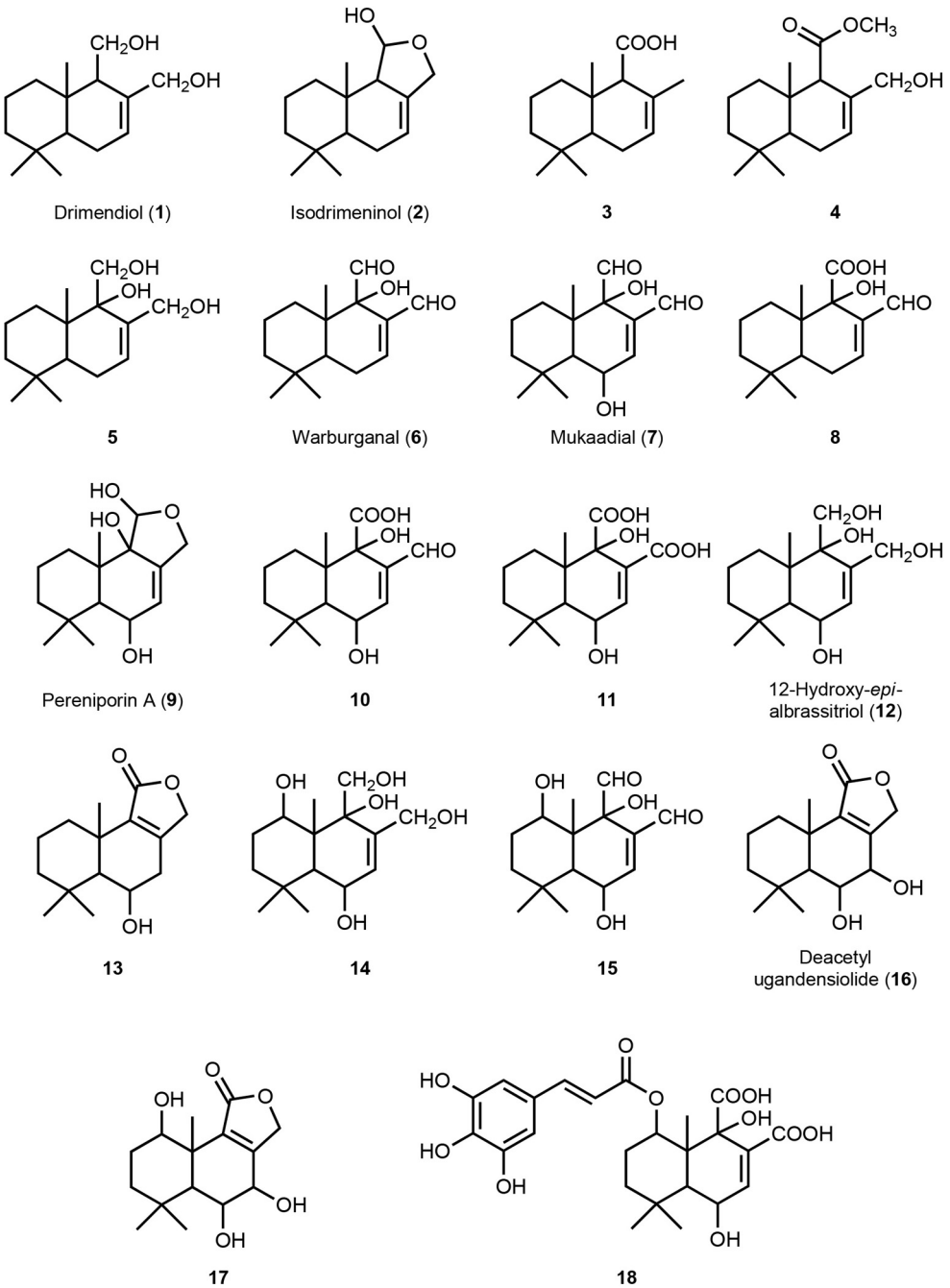

FIGURE 2 | Drimane sesquiterpene structures. All structures are identified on basis of retention time comparison and MS fragment interpretation obtained in the GC-MS analysis (for details see Supplementary Data S1).

same locality, the five fruit accessions only shared 34% of all analytes. The considerable metabolite variation was reflected in the low average similarity within and the high dissimilarity between the organ accessions from the two localities (**Table 1**). Only root profiles showed some similarity.

Besides fatty acids, sugars, and sugar alcohols—by far, mannitol was the most prominent metabolite in all samples, which had to be specifically fractioned to facilitate analysis of the drimane sesquiterpenes. The latter represented the characteristic secondary metabolites that were detected by GC–MS. Peaks that were identified contributing to similarity and dissimilarity of the accessions were subjected to a tentative structure elucidation by comparative fragment analysis. The classic phytochemical literature does not provide MS spectral information for silylated

Table 1 | Similarity and dissimilarity between metabolite patterns, bacterial and fungal ebdophyte communities.

			Kitale		Rumuruti		
			Leaves	Roots	Fruits	Leaves	Roots
BACTERIA (TRFs)							
Average similarity (%)			51	41	47	51	42
Average dissimilarity (%)	**Kitale**	Leaves	–	61	64	52	65
		Roots	61	–	69	58	65
	Rumuruti	Fruits	64	69	–	61	69
		Leaves	52	58	61	–	54
		Roots	65	65	69	54	–
BACTERIA (qPCR representing TRF 135)							
Average similarity (%)			65	39	12	32	22
Average dissimilarity (%)	**Kitale**	Leaves	–	62	91	57	73
		Roots	62	–	85	68	74
	Rumuruti	Fruits	91	85	–	86	
		Leaves	57	68	86	–	81
		Roots	73	74	92	81	–
FUNGI							
Average similarity (%)			12	17	31	26	74
Average dissimilarity (%)	**Kitale**	Leaves	–	91	98	93	98
		Roots	91	–	100	91	100
	Rumuruti	Fruits	98	100	–	99	100
		Leaves	93	91	99	–	97
		Roots	98	100	100	97	–
METABOLITES							
Average similarity (%)			50	45	34	47	40
Average dissimilarity (%)	**Kitale**	Leaves	–	90	87	75	88
		Roots	90	–	91	90	59
	Rumuruti	Fruits	87	91	–	85	90
		Leaves	75	90	85	–	87
		Roots	88	59	90	87	–

Average similarities (within accession) and dissimilarities (between accessions) of bacterial and fungal TRFs, qPCR (Enterobacteriaceae, Pseudomonadaceae, Firmicutes, representing the bacterial TRF 135) and GC–MS metabolite profiles (roots, leaves, n = 10; fruits, n = 5) from two Warburgia ugandensis localities from Kenya, Africa.

sesquiterpenes, in contrast to the majority of primary metabolites (Kopka et al., 2005). Usually, only MS spectra of the underivatized metabolites are available. The structures of all thus tentatively identified drimane sesquiterpenes are illustrated in **Figure 2**, numbered from **1** to **18**. Their MS spectra and the tentative interpretation of the fragmentation pattern are presented in datasheet S1: **1**, drimendiol, was originally isolated from *Drymis winteri*, also a member of the *Canellaceae* (Brown, 1994); **2**, isodrimeninol, was discovered as metabolite of the moss *Porella aboris-vitae* (Asakawa et al., 1979) and the angiosperm *Polygonum hydropiper* (Asakawa et al., 1979); **3** is known as intermediate of lanosterol synthesis (Van Tamelen et al., 1982); **4** is a synthetic precursor of the drimane sesquiterpene polygodial (Jallali-Naini et al., 1981); **5** is a precursor in the chemical synthesis of warburganal (**6**) (Nakata et al., 1980); **6**, warburganal, was first isolated from the bark of *W. ugandensis*, the tree under investigation in this study (Kubo et al., 1976); **7**, mukaadial, was also isolated from the same source as **6** (Kubo et al., 1983); **8** is unknown so far; **9**, pereniporin A, was isolated from the basidiomycete *Perenniporia medullaepanis* (Kida et al., 1986); **10** is unknown; **11** is unknown; **12**, 12-Hydroxy-6-*epi*-albrassitriol, was isolated from an *Aspergillus* strain culture (Grabley et al., 1996); **13** was isolated from the fern *Protowoodsia manchuriensis* (Tanaka et al., 1980); **14** is unknown; **15** is unknown; **16**, deacetylugandensiolide, was first isolated from the heartwood of *W. ugandensis* (Brooks and Draffan, 1969); **17** is unknown; **18** is unknown.

Root accessions from both localities, Kitale and Rumuruti, showed the lowest percentage of dissimilarity, but similarity within them also was low, 40 and 45%, respectively (**Table 1**). The roots were characterized by the highly oxygenated drimane sesquiterpene alcohols **12** and **14**, the dialdehyde **15**, and the lactone deacetylugandensiolide (**16**); the single amounts varied considerably and this also contributed to dissimilarity (**Table 2**). The leaves differed not only from the roots, but also among each other. Kitale leaves were characterized by the drimane sesquiterpene dialcohol drimendiol, the acid **3**, and the hemiacetal pereniporin A (**9**). By contrast, Rumuruti leaves showed the dialdehyde mukaadial (**7**) and the acids **10** and **11**. In fruits, which were only available from some individuals at Rumuruti, again other drimane sesquiterpenes contributed to similarity: triol **5** and

Table 2 | Relevant Metabolites in the GC–MS profile.

Bacteria						
LEAVES						
Similarity:		**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Kitale						
	Drimendiol (**1**)	28	20	2.2	39	39
	Mannitol	11	4	0.6	7	46
	Palmitic acid	5	3	1.9	7	53
	9	4	2	1.7	5	58
	Fructose	7	2	0.8	4	62
	3	3	2	1.6	3	65
Rumuruti						
	Palmitic acid	15	11	2.0	23	23
	Mannitol	17	8	0.9	18	41
	Mukaadial (**7**)	11	5	1.4	11	52
	Glycerol	9	5	0.8	10	62
	10	3	2	2.8	5	67
	11	3	2	1.7	4	71
	Fructose	4	1	0.7	3	74
Dissimilarity:			**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
	Drimendiol (**1**)		13	1.9	18	18
	Mannitol		8	1.2	11	29
	Mukaadial (**7**)		5	1.0	7	36
	Palmitic acid		5	1.4	7	43
	Glycerol		4	1.3	6	49
	Fructose		4	1.9	5	54
FRUITS						
Similarity:		**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Rumuruti						
	Warburganal (**6**)	14	7	1.5	19	19
	5	9	6	3.6	17	36
	Mannitol	9	4	1.3	11	47
	Palmitic acid	6	4	2.2	9	56
	myo-Inositol	6	3	0.7	7	63
Dissimilarity:			**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
Fruits and leaves Rumuruti						
	Mannitol		7	1.2	9	9
	Warburganal (**6**)		7	1.1	9	18
	Mukaadial (**7**)		5	1.0	7	25
	Palmitic acid		5	1.4	6	31
	Glycerol		4	1.3	5	36
	5		4	2.3	5	41

(Continued)

Table 2 | Continued

Bacteria					
ROOTS					
Similarity:	**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Kitale					
14	15	10	1.4	22	22
15	6	4	3.1	11	33
12-Hydroxy-6-*epi*-albrassitriol (**12**)	8	4	1.0	9	42
Mannitol	12	3	0.5	6	48
17	5	2	1.5	5	53
Deacetylugandensiolide (**16**)	4	2	0.8	5	58
Raffinose	3	2	1.1	4	62
13	4	2	1.1	4	66
Rumuruti	**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
15	14	6	0.9	16	16
14	11	6	1.3	15	31
Mannitol	18	6	0.8	14	45
12-Hydroxy-6-*epi*-albrassitriol (**12**)	5	2	0.8	6	51
Deacetylugandensiolide (**16**)	4	2	0.8	5	56
Dissimilarity:		**Av. diss.**	**diss. /*SD***	**Contr. %**	**% cum.**
Mannitol		10	0.9	17	17
15		6	1.0	10	27
14		5	1.4	9	36
12-Hydroxy-6-*epi*-albrassitriol (**12**)		3	1.3	6	42
18		2	0.8	4	46

*Contributions (Contr.) of specific metabolites (numbers 1–18 represent drimane sesquiterpenes whose structures are shown in **Figure 2**) of Warburgia ugandensis accessions from two localities in Kenya, Africa, to similarity (simil.) and dissimilarity (diss.) (SD, standard deviation,(roots, leaves, n = 10; fruits, n = 5; Av. abund., average abundance).*

the corresponding dialdehyde warburganal (**6**). By its presence, the sugar alcohol mannitol contributed to similarity in all analyzed organs; its detected concentration fluctuations, however, also added to the dissimilarity. In fruits, another sugar alcohol, was prominent, *myo*-inositol; in roots, it was the monosaccharide fructose. In aerial organs, palmitic acid was more prominent than in roots. These facts (**Table 2**) also contribute to the clustering shown in **Figures 1A,C**.

BACTERIAL ENDOPHYTES SHOW LOW DIVERSITY AND STOCHASTIC DISTRIBUTION

Cultivation-independent analyses (T-RFLP, bacterial 16S rDNA) revealed a low complexity for all accessions; only three peaks were prominent (**Table 3**). One peak at 153 bp was present in all accessions with a generally higher intensity in roots than in leaves and an average relative abundance of 46%. One peak (300 bp) was found in twelve root samples, mainly from Kitale, as well as in two leaf samples with an average relative abundance of 7%. Finally, another peak (142 bp) was present in all but eleven samples showing an average intensity of 14%. Consequently, Bray–Curtis similarity analysis revealed no clustering of the bacteria assemblages, both in terms of locality and plant organ (**Figure 1E**). Fisher's alpha was low in general and varied between 0.2 and 6.4 (**Figure 1F**). One-Way ANOVA and Scheffe's multiple range test indicated significant differences for Rumuruti fruits, which showed the highest diversity; the lowest was found in the roots. In Kitale, the bacterial diversity in roots and leaves was more similar. SIMPER analysis identified the peak at bp 153 as the most responsible for the similarities within the bacterial communities, and its quantitative variation contributed most to the dissimilarity of the accessions (**Table 3**).

In order to identify the dominant genera in the bacterial assemblages we constructed a 16S rRNA gene clone library from a pool of aliquots of all DNA samples. Among 53 ribotypes five chimeric sequences, two chloroplast sequences and one mitochondrial sequence were found and excluded from further analysis. Two thirds of the clearly bacterial sequences showed at least 97% similarities to known 16S rRNA genes in the NCBI database, and 30% of the clones were distantly (92–96%) related to known species (datasheet S2). The majority (90%) of the sequences belonged to the *Gammaproteobacteria*, with 52% of the clones being *Pseudomonadaceae*, predominantely the genus *Pseudomonas*, and the rest *Enterobacteriaceae*. The remaining

Table 3 | Relevant bacterial T-RFs (T-RF 153 bp was resolved further by qPCR).

Bacteria						
LEAVES						
Similarity:		**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Kitale						
	153	35	34	1.7	66	66
	Pseudomonadaceae				82	
	Enterobacteriaceae				18	
	145	12	8	0.9	16	82
	147	8	3	0.6	7	89
	144	7	2	0.6	4	9
Rumuruti						
	153	52	45	2.0	89	89
	Pseudomonadaceae				50	
	Enterobacteriaceae				44	
	145	5	1	0.3	3	92
Dissimilarity:			**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
	153		9	1.3	13	13
	Pseudomonadaceae				49	
	Enterobacteriaceae				41	
	115		8	0.9	12	25
	145		8	1.2	12	37
	141		5	0.9	8	45
	147		5	0.9	7	52
FRUITS						
Similarity:		**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Rumuruti						
	153	31	27	2.0	58	58
	Enterobacteriaceae				52	
	Firmicutes				48	
	115	15	4	0.4	10	68
	141	7	2	0.3	4	72
Dissimilarity:			**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
Fruits and leaves Rumuruti						
	153		16	1.6	26	26
	115		8	0.9	13	39
	Pseudomonadaceae				44	
	Enterobacteriaceae				33	
	Firmicutes				10	
	141		5	0.9	8	47
	145		3	0.7	5	50
	72		3	0.8	5	53

(Continued)

Table 3 | Continued

Bacteria						
ROOTS						
Similarity:		**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Kitale						
	153	37	28	1.3	70	70
	Pseudomonadaceae				39	
	Enterobacteriaceae				31	
	Firmicutes				30	
	300 (Paenibacillaceae)	15	8	1.0	19	89
	145	4	1	0.5	3	92
Rumuruti						
	153	59	40	1.3	95	95
	Pseudomonadaceae				62	
	Firmicutes				35	
Dissimilarity:			**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
	153		20	1.6	31	31
	Pseudomonadaceae				46	
	Enterobacteriaceae				40	
	Firmicutes				14	
	300 (Paenibacillaceae)		7	1.1	11	42
	298		5	0.4	8	50

SIMPER analyses (Bray Curtis similarity): Contributions (Contr.) of bacterial T-RFs (bp) and qPCR (Enterobacteriaceae, Pseudomonadaceae, Firmicutes, the former two γ-Proteobacteria and the latter Bacilli, which more or less represent the T-RF at 153 bp) of Warburgia ugandensis accessions from two localities in Kenya, Africa, to similarity (simil.) and dissimilarity (diss.) (SD, standard deviation, roots, leaves, n = 10; fruits, n = 5; Av. abund., average abundance).

clones belonged to the divisions of *Actinobacteria* (5%) and *Firmicutes* (5%).

To gain further insights into the bacterial community in individual trees, 16S rRNA gene clone libraries from eight selected accessions, two leaf and root accessions from each locality, were constructed. About 96 clones of each cloning experiment were analyzed by RFLP profiling and sequencing. In all libraries, the majority of clones belonged to *Gammaproteobacteria* and *Bacillus* group (data not shown). Altogether five families were identified, *Pseudomonadaceae, Enterobacteriaceae, Bacillaceae, Paenibacillaceae,* and *Staphylococcaceae*, with the latter being present only in Kitale leaf accession L1. The main difference between the individual clone libraries was the varying *Pseudomonadaceae–Enterobacteriaceae* abundance ratio. Quantitative PCR detection of 16S rDNA genes specific for the taxa *Firmicutes*, *Enterobacteriaceae* and *Pseudomonadaceae* confirmed the clone library data. Conversely, some accessions contained mainly *Pseudomonadaceae* and hardly any *Enterobacteriaceae* or *Frimicutes* and again other accessions contained no *Pseudomonadaceae* but were dominated by *Enterobacteriaceae* or *Firmicutes*, respectively (**Figure 3**). In assumptions that T-RFs, which are found in a profile and in a DNA sequence, are identical if they do not differ more than in 2 bp, the T-RF at 153 bp could be assigned to the *Gammaproteobacteria* and some *Bacillaceae*. The peak at 300 bp was most probably derived from *Paenibacillaceae*. No sequence was found corresponding to the T-RF at 142 bp. The cloning and qPCR data corroborate the low complexity and relative uniformity of the bacterial T-RFLP profiles, but also indicate strong individual variation in the bacterial assemblages within individual plant tissues that is hidden within the 153 bp peak in the T-RFLP profile.

LOCALITY AFFECTS DIVERSITY OF DISSIMILAR FUNGAL COMMUNITIES

Cultivation-independent analysis of fungal communities (T-RFLP, ITS1) in *W. ugandensis* detected 178 TRFs ranging from 35 to 500 bp in all analyzed accessions. In contrast to bacterial assemblages, fungal T-RFLP profiles varied strongly; no specific TRFs dominated the profiles, resulting in low average similarity and high dissimilarity (**Table 1**). Only a few common peaks were found in T-RFLPs from both sites. The Bray–Curtis similarity analysis (**Figure 1G**) revealed some tendencies for organ-specific clustering within roots, leaves and fruits of Rumuruti trees, but not for Kitale trees. Fungal diversity was generally higher in all Kitale accessions (**Figure 1H**). Root T-RFLPs showed no common peak that contributed to the similarity of accessions from the same location. Notably, Rumuruti roots were characterized by a peak at 72 bp. This TRF was found with an average abundance of

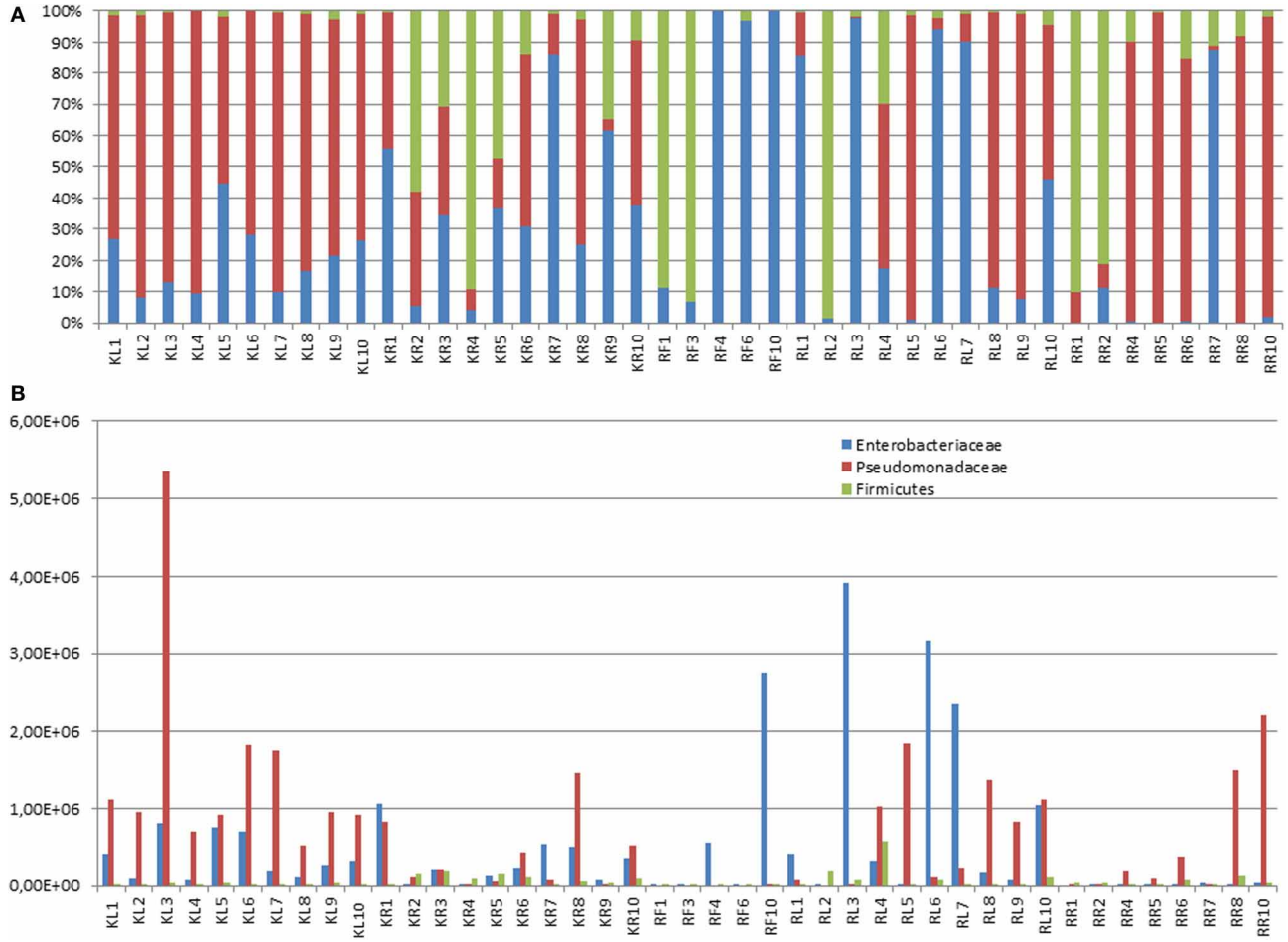

FIGURE 3 | Distribution of bacterial families in leaves and roots of *Warburgia ugandensis*. Quantiative determination of Bacili and γ-Proteobacteria (Pseudomonadaceae and Enterobacteriaceae) was carried out on basis of taxa specific quantitative PCR for all individuals (L, leaf; R, root) from each of the two accessed localities, Kitale and Rumuruti. **(A)** Relative distribution of Bacili, Pseudomonadaceae and Enterobacteriaceae describing the composition of peak at bp 153 in the 16S rDNA TRFLP analysis. **(B)** Occurrence of 16S rDNA gene copies of Bacili, Pseudomonadaceae and Enterobacteriaceae (copy numbers per ng DNA).

83% in the accessions and not present in those from Kitale (**Table 4**).

In order to identify the dominant species in the fungal assemblages in *W. ugandensis* trees, we constructed an ITS region clone library from a pool of aliquots of all DNA samples. In total, 96 clones were analyzed and assigned by RFLP analysis to 47 ribotypes for which sequences were determined (datasheet S3). Three % were chimeric sequences and excluded from further analysis. The clearly non-chimeric sequences belonged to *Ascomycota* (81%) and *Basidiomycota* (16%). The two biggest classes of fungi were *Dothidiomycetes* (28%) and *Sordariomycetes* (27%), followed by *Microbotryomycetes* with 16%. The remaining sequences could be assigned to the classes of *Saccharomycetes* (12%), *Leotiomycetes* (12%), *Pezizomycetes* (2%), *Eurotiomycets* (2%), and *Tremellomycetes* (1%). The library comprised a total number of 20 fungal species; the basidiomycete *Sporidobolus ruinae* was the most abundant, representing 16% of the clones in the library. None of the identified clones unambiguously correlated with the peak at bp 72.

LOW OR NO CORRELATION WAS FOUND BETWEEN HOST METABOLITES AND ENDOPHYTE COMMUNITIES

Explorative PLS regression (not shown) indicated only a few correlations between *Warburgia* metabolites and predominance of specific microbial community member groups, which were explored further by simple regression analyses. The identification of correlations in the data was hampered by the fact that similarity within accession groups was rather low, in many cases less than 50%. This not only applied to drimane sesquiterpenes but also to bacterial and fungal endophyte communities. Thus we defined the following criteria for acceptable correlations: (1) the correlation had to be supported by at least three cases ($n \geq 3$); (2) reproducibility was to be at least 90% ($p \leq 0.1$), and (3) at least 50% of all cases should support the correlation ($r^2 \geq 0.5$). **Table 5** summarizes the results. Accordingly, the more consistently occurring metabolites, such as palmitic acid and the sugar alcohol mannitol, show higher correlation than the more variable drimane sesquiterpenes. In roots, the bacterial T-RFs and the fungal TRFs at 141 and 157 bp both

Table 4 | Relevant fungal T-RFs.

LEAVES					
Similarity:	**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Kitale					
422	8	2	0.4	17	17
133	6	2	0.4	13	30
139	6	2	0.4	11	41
146	5	1	0.3	8	49
149	3	1	0.5	5	54
433	4	0	0.4	3	57
41	3	0	0.3	3	60
129	2	0	0.4	3	63
407	3	0	0.5	3	66
408	3	0	0.5	3	69
Rumuruti					
76	13	7	1.0	29	29
79	12	7	1.1	28	57
469	13	4	0.4	15	72
149	9	3	0.5	10	82
432	2	1	0.9	4	86
Dissimilarity:		**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
469		6	0.8	7	7
76		6	1.4	7	14
79		6	1.5	6	20
467		5	0.4	6	26
149		5	1.0	5	31
422		4	0.9	5	36
419		4	0.3	5	41
133		3	0.7	3	44
139		3	0.6	3	47
129		3	0.7	3	50
FRUITS					
Similarity:	**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Rumuruti					
179	27	16	1.1	52	52
171	25	10	0.8	33	85
47	7	3	1.1	8	94
Dissimilarity:		**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
Fruits and leaves Rumuruti					
179		14	1.7	14	14
171		12	1.2	12	26
76		6	1.4	7	33
469		6	0.8	6	39
79		6	1.6	6	45
149		5	0.8	5	50
ROOTS					
Similarity:	**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
Kitale					
73	8	2	0.6	15	15
149	12	2	0.2	11	26
75	14	2	0.2	9	35
133	8	1	0.3	9	44
76	6	1	0.4	8	52
423	4	1	0.7	8	60
140	5	1	0.3	5	65
129	3	1	0.6	5	70
141	5	1	0.3	5	75
131	2	0	0.4	3	78
Rumuruti	**Av. abund.**	**Av. simil.**	**Simil. /*SD***	**Contr. %**	**% cum.**
72	83	72	4.6	98	98
Dissimilarity:		**Av. diss.**	**Diss. /*SD***	**Contr. %**	**% cum.**
72		41	5.0	41	41
75		7	0.5	7	48
149		6	0.6	6	54
133		4	0.6	4	58

SIMPER analyses (Bray Curtis similarity): Contributions (Contr.) of fungal T-RFs (bp) of Warburgia ugandensis accessions from two localities in Kenya, Africa, to similarity (simil.) and dissimilarity (diss.) (SD, standard deviation, roots, leaves, n = 10; fruits, n = 5; Av. abund., average abundance).

correlated with palmitic acid and mannitol. Leaf endophytes, by contrast, did not correlate with palmitic acid, but with the sugars glucose and fructose and the sugar alcohol quercitol. Without exception, all correlations were positive (**Table 5**). On the contrary, drimane sesquiterpenes showed much fewer correlations; the majority was positive, but also three negative correlations were found, the ester **4** with the fungal TRF 141, isodrimenol (**2**) and deacetylugandensiolide (**16**) with *Firmicutes* rDNA copy numbers. The same drimanes, however, **2** and **16**, also were positively correlated, **2** with bacterial TRF 165, similarly as 12-hydroxy-*epi*-albrassitriol (**12**), and **16** with root-occurring *Enterobacteriaceae* rDNA copy numbers. The latter phenomenon was detected in both localities, Kitale and Rumuruti. Further positive correlations comprised the alcohol **5** and its oxidized derivative **8** with *Pseudomonadaceae* rRNA gene copy numbers. Moreover the metabolite diversity in *W. ugandensis* trees did not correlated.

With bacterial and fungal endophyte diversity, at least on basis of GC–MS and T-RFLP results (**Table 6**).

DISCUSSION

Drimane sesquiterpenes diversity turned out to be higher than that of bacterial and fungal endophyte communities, both in the leaves and roots the two accessed sites. The T-RFLP patterns of all assessed accessions did not form any specific clusters in terms of collection site and plant organ. By contrast, metabolite patterns, especially if only drimane sesquiterpenes were considered, formed organ-specific clusters. Leaf profiles differed between Kitale and Rumuruti, but root patterns were similar (**Figures 1A,C**). The Rumuruti leaves contained the dialdehyde mukaadial (**7**) and the corresponding acids **10** and **11**; the Kitale leaves showed drimendiol (**1**) as major compound, which represents the most reduced

Table 5 | Correlations of metabolites with microbial community components.

	4	5	8	2	12	16	Palmitic acid	Fructose	Glucose	Mannitol	Quercitol
KITALE											
Leaves (%)	1.6	1.0	0.4				22.4	29.5	2.5	45.6	
Roots (%)	0.7		0.6	3.8	8.4	4.6	12.5	3.5	2.2	81.8	
Leaves											
Bacteria				165[A] 0.77* (0.59)[b]	165[A] 0.78* (0.62)[b]						
Fungi	141[A] −0.92** (0.85)[b]									132[A] 0.89** (0.79)[c]	
Roots											
Bacteria					Enterobact.[B] 0.94** (0.88)[c]		300[A] 0.70* (0.49)[d]			300[A] 0.73* (0.53)[d]	
		Pseudomon.[B] 0.90** (0.80)[c]	Pseudomon.[B] 0.99** (0.98)[c]		Firmicutes[B] −0.95** (0.91)[c]						
Fungi							141[A] 0.90* (0.82)[b] 157[A] 0.96* (0.91)[a]			141[A] 0.99** (0.97)[b] 157[A] 0.99** (0.99)[a]	
RUMURUTI											
Fruits			1.1		1.4		19.0	21.8	8.1	33.0	18.0
Leaves	1.4	0.1	0.4				13.9	1.4	2.2	45.7	
Roots		1.9	1.3	3.4	5.3	4.1	14.8	11.4		83.3	
Fruits											
Bacteria	–									170[A] 0.99* (0.97)[a]	
Fungi								46[A] 0.99** (0.99)[b]	46[A] 0.99** (0.99)[b]		46[A] 0.99** (0.98)[b]
Leaves											
Bacteria									Firmicutes[B] 0.85** (0.73)[d]	Firmicutes[B] 0.76* (0.57)[d]	
Fungi										79[A] 0.84** (0.71)[e]	
Roots											
Bacteria			Firmicutes[B] −0.99* (0.99)[a]		Enterobact.[B] 0.97** (0.94)[b]						

*Numbers represent drimane sesquiterpene structures shown in **Figure 2**. Occurrence (tissues and accessions, % total peak area, mean, n = 10).*

[A] T-RF.

[B] qPCR, linear model y = a + bx, correlation coefficient [r^2].

[a] n = 3.

[b] n = 4.

[c] n = 5.

[d] n = 6.

**p ≤ 0.10.*

***p ≤ 0.05; Enterobact., Enterobacteriaceae; Pseudomon., Pseudomonadaceae; TRFs: bacteria: 165, not specified; 300, Paenibacillaceae; fungi: 46, not specified; 79, Penicillium sp.; 132, Cordyceps sinensis, Fimetariella rabenhorstii, Colletotrichum truncatum or Fusarium sp.; 141 and 157, not specified; negative correlations are marked in red).*

Table 6 | Correlation of metabolite with bacterial and fungal endophyte diversity.

		p	R^2
KITALE			
Leaves			
	Bacteria	0.83	0.64
	Fungi	0.15	24.26
Roots			
	Bacteria	0.54	4.94
	Fungi	0.18	21.09
RUMURUTI			
Leaves			
	Bacteria	0.32	12.31
	Fungi	0.15	23.85
Roots			
	Bacteria	0.34	11.19
	Fungi	0.19	20.82
ALL			
	Bacteria	0.74	0.32
	Fungi	0.78	0.22

Fisher's a coefficients were determined for metabolite and bacterial and fungal endophyte diversity for 10 individual each from two population of Warburgia ugandensis in Kenya (Africa); ALL, correlation of all assessed data from both localities; metabolite diversity was determined by GC–MS and bacterial and fungal by T-RFLP (linear model, $y = a + bx$).

derivative. The co-occurring hemiacetal **7** and the simple acid **3** somehow suggest a lower degree of oxidation in Kitale leaf tissues than in those of Rumuruti. Oxidation reactions on drimane sesquiterpene structural diversity will be discussed in detail later in this text. If the more humid climate of the Kitale site, which was suggested by more intensive colonization by epiphytic lichens and ferns, caused this phenomenon can only be clarified after more study sites have been studied with a focus on this aspect.

Bacterial endophyte diversity was low in the investigated *Warburgia ugandensis* trees, and the abundant genera *Pseudomonas*, *Pantoea, Bacillus/Paenibacillus* represented both endophyte species frequently encountered in other plant species (Rosenblueth and Martinez-Romero, 2006). A recent review on the diversity of endophytic bacteria in forest trees (Izumi, 2011) pointed out that *Gammaproteobacteria* belong to the most prominent gram-negative bacterial endophytes, but also mentioned *Alphaproteobacteria* and *Betaproteobacteria*, which we did not detect as endophytes in *Warburgia.* The genus *Pantoea* is less often reported from tropical trees, but has been identified as endophyte in other tree genera including *Conzattia*, *Eucalyptus* and *Populus* (Wang et al., 2006; Izumi, 2011). The analysis of clone libraries suggested that bacterial colonization may be more a stochastic process. The high observed variability of *Pseudomonadaceae* and *Enterobacteriaceae* between the single accessions (**Figure 3**) that does not correlate with the composition of host secondary metabolites provides some support for this hypothesis.

In contrast to bacterial endophytes, fungi were more diverse, at least in Kitale. The T-RFLP profiles did not reveal any dominating bands in any accessed organs at any site, except perhaps for Kitale roots, which, however, were colonized by a very species-poor endophytic fungal community. The majority of the detected genera are known to occur as endophytes: *Cladosporium* (Guo et al., 2000), *Epicoccum* (Jumpponen and Jones, 2009), *Cryptococcus* (Schweigkofler and Prillinger, 1999), *Sporomiella* (Suryanarayanan et al., 1998), *Penicillium* (Narisawa et al., 2002), *Kabatiella* (Butin, 1992), *Lecythophora* (de Errasti et al., 2010), *Coniochatea* (anamorph *Lecythophora*) (Weber, 2002), *Nigrospora* (Soca-Chafre et al., 2011), *Cordyceps* (Rubini et al., 2005), *Fimetariella* (Martin-Garcia et al., 2012), *Fusarium* (Verma et al., 2011), *Neurospora* (Qi et al., 2009). Others have so far not been detected as endophytes: *Gloetinia temulenta* is a grass pathogen (Hardison, 1962); *Pseudaleuria* is a soil fungus (Xu et al., 2012); *Zopfiella latipes* is a marine fungus, but can colonize *Phragmites* (Poon and Hyde, 1998).

Contrary to initial expectations, the endophyte communities of the pepper bark tree do not differ from literature reports from other trees that much. The variation of drimane sesquiterpene profiles that was hinted in preliminary investigations was confirmed, but no substantial correlations were found with endophyte community composition. This suggests that any potential antimicrobial metabolites—drimane sesquiterpenes reportedly possess this activity (Wube et al., 2005)—do not affect the formation of an endophytic lifestyle in case of the *Warburgia* colonizers.

We may only speculate that the endophytic strains colonizing the pepper bark tree have evolved strategies to avert the toxic effects of host drimane sesquiterpenes with which they might come into contact in some stage of their life history. Due to potential hydroxyl radical formation following reduction of molecular oxygen in the presence of ferrous iron catalysts, drimane sesquiterpenes may cause oxidative stress in the affected plant tissue. A study exploring the metagenome of the rice root endophytic community identified the expression of glutathione synthase genes, a metabolite that is known to mitigate oxidative stress, as consequence of endophyte colonization amongst others (Sessitsch et al., 2012). Tolerance against oxidative stress may represent a widespread trait in soil microbes; their nutrition depends on the decomposition of organic polymers, an oxidative process that also may involve the formation of hydroxyl radicals in the Fenton reaction or the activity of oxidative enzymes (ten Have and Teunissen, 2001). The survival and growth of bacteria in a Fenton reaction milieu was demonstrated at least *in vitro* (Howsawkeng et al., 2001). Consequently, evolved tolerance to exposure to oxidative stress might help soil microbes to colonize plant tissues and help endophytes in tolerating toxic secondary metabolites of the host plant in general and in establishing populations in tissue of *W. ugandensis* trees in particular. This study, however, does not provide any information on the susceptibility of the endophytic strains against drimane sesquiterpenes. Isolation and functional characterization of endophytes of *Warburgia ugandensis* will give more insights into the strategies that have been evolved by microbes to avert the toxic effects of host drimane sesquiterpenes.

Conversely, although drimane sesquiterpenes constitute an efficient chemical defense, they may pose a threat to the producer itself by causing autotoxic effects. This is illustrated by a study showing that high amounts of acccumulated cyanogenic glycosides may cause severe autotoxic effects during strong frost periods which severe the tissue and in particular the storage compartments of the secondary metabolites that become activated by contact with sugar-cleaving enzymes (Daday, 1965). *Warburgia* tissues contain comparatively large amounts of the sugar alcohol mannitol; sugar alcohols have been shown to protect against hydroxyl radicals (Smirnoff and Cumbes, 1989). It is quite feasible assuming that the high amounts of mannitol in the pepper bark tissue are linked to the drimane sesquiterpenes and aim to keep the oxidative effects of drimanes from destroyed compartments at a tolerable level. This potential incurring of protective costs might explain why drimane sesquiterpenes are only utilized by few organisms despite their wide distribution in secondary-metabolite-producing organisms (Jansen and De Groot, 2004). Another aspect merits consideration: the microbial communities that colonize *Warburgia* tissues do not differ substantially from those reported to colonize other trees. In diverse plant communities, plants with extreme chemical defenses most probably have low effects on the assemblage of possible microbial colonization candidates. Those, which stochastically colonize *Warburgia* tissues, may be doomed, but others, which colonize also other plants, will survive, propagate and build assemblages of microbes that are able to colonize *Warburgia*. Deterministic and stochastic factors both can shape the specific composition of these assemblages in a complex and difficult-to-elucidate fashion. This also complicates and unambiguous the decision if endophytes affect the biosynthesis of and the yield of secondary metabolites in their host plants.

Warburgia ugandensis is a tree species with high ethnopharmaceutical relevance. In traditional local medicine the powdered bark is usually taken orally as aqueous infusion, smoked, or mixed with fat and applied externally as an ointment for treatment of a broad range of human diseases including measles and malaria (Beentje and Adamson, 1994; Kokwaro, 2009). The existence of this tree species in its natural environment is however under severe threat. Deforestation and unsustainable use (harvest of roots and barks) results in drastic loss of these trees. Knowledge of the factors determining the variation in the patterns of drimane sesquiterpenes could help to identify individuals with high yield production traits for drimane sesquiterpenes in order to identify suitable genotypes or cultivation practices for plantations of this tree. This would substantially increase the value of this tree species for local farmers and facilitate preservation programs. The genetic background of drimane sesquiterpene biosynthesis in *Warburgia ugandensis* is still poorly understood. Muge and colleagues isolated and characterized a partial gene encoding for a sesquiterpene synthase (Muge, 2008). Variations in the drimane sesquiterpene content in different plant organs of the tree may be explained partly by plant tissue specific expression of genes for secondary metabolites (Kombrink and Somssich, 1995). However the actual reasons for the strong individual variations in the drimane sesquiterpene pattern in the pepper bark tree still remain obscure.

In conclusion, our study revealed that (1) the endophyte community of the tropical tree *Warburgia ugandensis* resembles at the genus level that of trees in temperate climates; (2) the endophyte community is not shaped by host drimane sesquiterpenes; (3) the diversity of the endophytic microflora in *Warburgia ugendensis* does not correlate with that of host drimane sesquiterpenes**;** and (4) other factors rather than endophytic microbes might be responsible for the high variations in the content and composition of drimane sesquiterpenes in the pepper bark tree.

Further studies including also other tropical trees are required to explore if the conclusions from this paper can be confirmed on a broader scale. Ideally, such studies should consider the effect of climatic stress such as drought or high light exposure for the individual trees, if possible, to facilitate a more insightful assessment of the implications of variation in the analyzed microbial assemblages in geographically different host populations. Endophytes can affect the metabolism and health of their hosts in some cases, but they may fail to do so in other cases (Porras-Alfaro and Bayman, 2011). Amensalistic, commensalistic and mutualistic relations can occur. The unbiased assessment of endophytes (that report positive, negative, and null relationships) will be required to obtain insights into the potential effects of microbial endophytes on the development and metabolism of the host plant as well as contributions to its resistance against various forms of abiotic and biotic stress.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00013/abstract

Supplementary Data S1 | Tentative Structures of all Drimane Sesquiterpene Analytes from *Warburgia ugandensis*. This file provides information on which the tentative structure identification of drimane sesquiterpenes analytes is based in this study. Each analyte is presented on three pages: Page 1 contains the tentative structure with the analysis retention time, page 2 presents the MS spectrum together with the structure of the derivatized analyte, and page 3 illustrates structure fragments corresponding to specific fragments in the EI–MS spectrum. The tentative structure assignment is based on these data for each analyte respectively. The numbering corresponds to that presented in **Figure 2**.

Table S2 | Bacterial endophytes from leaves, fruits and roots of Warburgia ugandensis.

Table S3 | Endophytic fungi from leaves, fruits and roots of Warburgia ugandensis.

Material and Methods. Supplementary Information.

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Effects of bacterial inoculants on the indigenous microbiome and secondary metabolites of chamomile plants

Ruth Schmidt[1], Martina Köberl[1], Amr Mostafa[2], Elshahat M. Ramadan[2], Marlene Monschein[3], Kenneth B. Jensen[4], Rudolf Bauer[3] and Gabriele Berg[1]*

[1] *Institute for Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Faculty of Agriculture, SEKEM, Heliopolis University, Ain Shams University, Cairo, Egypt*
[3] *Department of Pharmacognosy, Institute of Pharmaceutical Sciences, University of Graz, Graz, Austria*
[4] *Institute of Chemistry, University of Graz, Graz, Austria*

Edited by:
Martin Grube, Karl-Franzens-University Graz, Austria

Reviewed by:
Igor Kovalchuk, University of Lethbridge, Canada
Don Cipollini, Wright State University, USA

****Correspondence:***
Gabriele Berg, Institute for Environmental Biotechnology, Graz University of Technology, Petersgasse 12/I, 8010 Graz, Austria
e-mail: gabriele.berg@tugraz.at

Plant-associated bacteria fulfill important functions for plant growth and health. However, our knowledge about the impact of bacterial treatments on the host's microbiome and physiology is limited. The present study was conducted to assess the impact of bacterial inoculants on the microbiome of chamomile plants *Chamomilla recutita* (L.) Rauschert grown in a field under organic management in Egypt. Chamomile seedlings were inoculated with three indigenous Gram-positive strains (*Streptomyces subrutilus* Wbn2-11, *Bacillus subtilis* Co1-6, *Paenibacillus polymyxa* Mc5Re-14) from Egypt and three European Gram-negative strains (*Pseudomonas fluorescens* L13-6-12, *Stenotrophomonas rhizophila* P69, *Serratia plymuthica* 3Re4-18) already known for their beneficial plant-microbe interaction. Molecular fingerprints of 16S rRNA gene as well as real-time PCR analyses did not show statistically significant differences for all applied bacterial antagonists compared to the control. In contrast, a pyrosequencing analysis of the 16S rRNA gene libraries revealed significant differences in the community structure of bacteria between the treatments. These differences could be clearly shown by a shift within the community structure and corresponding beta-diversity indices. Moreover, *B. subtilis* Co1-6 and *P. polymyxa* Mc5Re-14 showed an enhancement of the bioactive secondary metabolite apigenin-7-*O*-glucoside. This indicates a possible new function of bacterial inoculants: to interact with the plant microbiome as well as to influence the plant metabolome.

Keywords: bioactive secondary metabolites, biological control agents, chamomile, microbial communities, soil-borne pathogens

INTRODUCTION

Whereas large-scale efforts have rapidly advanced the understanding of plant genomes, the impact and importance of the plant's microbiome is largely unexplored. Comparable to the human microbiome, millions of microbes inhabit plants, forming a complex ecological community that influences plant growth and health through its collective metabolic activities and host interactions (Berg, 2009; Lugtenberg and Kamilova, 2009). Currently, the many studies on plant-associated microorganisms reflect the full effect of ongoing research and the enormous interest in this topic (Mendes et al., 2011, 2013; Berendsen et al., 2012; Bakker et al., 2013). Viewing the microbiota from an ecological perspective could provide insight into how to promote health and stress tolerance of their hosts or how to adapt to a changing climate by targeting this microbial community. Furthermore, new functions of the plant microbiome can be detected. Several studies revealed that rhizobacteria have an effect on the aroma of fruits, e.g., strawberry and grapes (Pirlak and Köse, 2009; Verginer et al., 2010). Moreover, induced systemic resistance has been triggered in several crops by plant growth promoting bacteria (Murphy et al., 2003; Kloepper et al., 2004; Ryu et al., 2004a,b). Interestingly, certain plant growth-promoting rhizobacteria (PGPR) elicit induced systemic resistance and plant growth promotion in the absence of physical contact with plants via volatile organic compound (VOC) emissions (Farag et al., 2013). In addition, selected PGPR strains have been shown to reduce disease in plant parts through the induction of defense compounds, and especially members of the endomicrobiome have been shown to be involved in the production of bioactive compounds (Pimentel et al., 2011; Gutierrez et al., 2012). For several medicinal plants, bacteria and fungi were detected as producers of their active ingredients. Paclitaxel and maytansine, known as important anticancer lead molecules, were detected to be produced by endophytes (Chandra, 2012; Wings et al., 2013). Traditional Chinese Medicine is using indigenous medicinal plants integrated in an ancient healing system originating almost 4500 ago. For several of those plants it was shown that they are active due to its microbial endophytes (Miller et al., 2012; Zhao et al., 2012). However, these discoveries are only the beginning of understanding the complex interactions between plants and microbes, and new omics technologies will promote this.

Promotion of plant health or biological control is one of the well-studied functions of the plant microbiome. It is based on naturally occurring antagonists and offers sustainable solutions for plant protection (Weller, 2007; Berg, 2009; Raaijmakers et al., 2009). Gram-negative bacteria, especially those from the genus *Pseudomonas*, were identified as the dominant members of the indigenous antagonistic communities under humid conditions (Berg et al., 2005a; Haas and Defago, 2005; Costa et al., 2006; Zachow et al., 2008). In addition, these strains were also identified as a major group of disease-suppressive bacteria through pyrosequencing (Mendes et al., 2011). In contrast, under arid conditions, we found mainly Gram-positive bacteria as antagonistic counterparts (Köberl et al., 2011). To verify this finding as well as to find out which bacterial strains—indigenous Gram-positive strains in comparison with allochthonous Gram-negative strains—support plant growth under arid conditions in desert farming, different bacterial inoculants were developed and tested under field conditions.

The overall aim of this study was to evaluate the impact of several bacterial inoculants on the indigenous microbial community of chamomile plants and the production of bioactive secondary metabolites. We selected three Gram-positive strains isolated in Egypt and three European Gram-negative strains already known for their beneficial plant-microbe interactions and used as biocontrol agents (Lottmann and Berg, 2001; Zachow et al., 2010). Bacterial inoculants were applied to chamomile plants [*Chamomilla recutita* (L.) Rauschert] grown under field conditions with organic (biodynamic) management on Sekem farms in Egypt, and the impact of the treatment on the indigenous microbial communities was monitored. This is important to understand the potential risk of biocontrol but also to understand the mode of action of bacterial inoculants, which can be mediated by the plant microbiome as well (Scherwinski et al., 2008).

RESULTS

CHEMICAL ANALYSIS OF CHAMOMILE SECONDARY METABOLITES

HPLC-MS experiments of the flower extracts yielded slightly different contents of apigenin-7-*O*-glucoside and apigenin between the different treatments (**Figure 1**). For apigenin-7-*O*-glucoside, statistically significant higher contents than for the non-inoculated control plants (0.86 ± 0.04) were observed for the treatments with the indigenous Gram-positive strains *Bacillus subtilis* Co1-6 ($1.06\% \pm 0.07$) and *Paenibacillus polymyxa* Mc5Re-14 ($1.04\% \pm 0.06$). The autochthonous *Streptomyces subrutilus* Wb2n-11 ($0.87\% \pm 0.09$) and all three allochthonous Gram-negative strains *Pseudomonas fluorescens* L13-6-12 ($0.79\% \pm 0.06$), *Stenotrophomonas rhizophila* P69 ($0.78\% \pm 0.15$), and *Serratia plymuthica* 3Re4-18 ($0.82\% \pm 0.06$) showed no elevation of apigenin-7-*O*-glucoside content (Table S1). Highest contents of apigenin were obtained for treatments with the Gram-positive strains *B. subtilis* Co1-6 ($0.95\% \pm 0.14$) and *P. polymyxa* Mc5Re-14 ($0.95\% \pm 0.10$) as well, however without significant difference to the control ($0.94\% \pm 0.10$). Also *P. fluorescens* L13-6-12 ($0.84\% \pm 0.07$), *S. rhizophila* P69 ($0.77\% \pm 0.12$), *S. plymuthica* 3Re4-18 ($0.91\% \pm 0.10$), and *S. subrutilus* Wb2n-11 ($0.78\% \pm 0.08$) did not show an alteration (Table S2). Investigation of peaks which arose in the treatments with the two autochthonous Bacillales strains (m/z = 327 and 329 for $[M\text{-}H]^-$) resulted in the possible identification of $[C_{18}H_{32}O_5 - H^+]^-$ (Rt: 13.15 min) and $[C_{18}H_{34}O_5 - H^+]^-$ (Rt: 14.8 min), respectively.

MOLECULAR FINGERPRINTING OF THE CHAMOMILE-ASSOCIATED BACTERIAL COMMUNITIES

In the molecular fingerprinting approach, universal primers were used to get a first overview about the whole bacterial community associated with *Chamomilla recutita* (L.) Rauschert as well as *Pseudomonas*- and *Firmicutes*-specific primers. Community composition was determined by image analysis of the band profiles generated by single-stranded conformational polymorphism (SSCP) analysis, and differences in the bacterial community composition based on these band patterns were calculated using the Pearson's correlation index. Cluster analysis of the microbial profiles resulted in grouping of rhizosphere samples at different sampling times at about 10% similarity (**Figure 2**). These differences between the two sampling times were confirmed by a principal component analysis (**Figure 3**). Within each cluster,

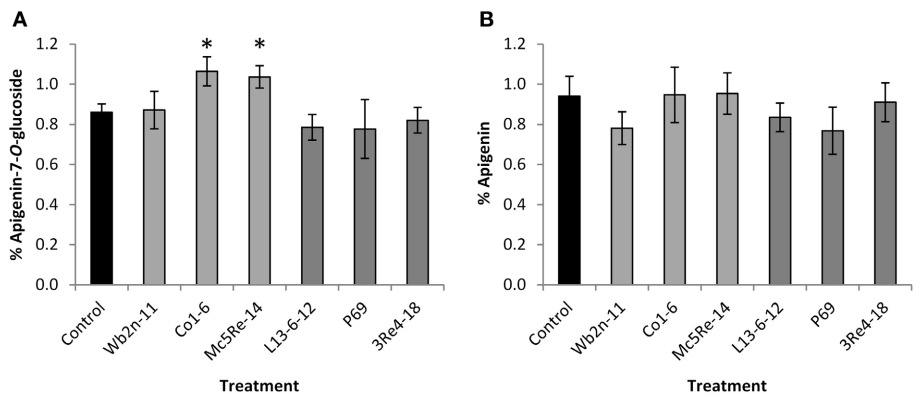

FIGURE 1 | Content (%) of apigenin-7-*O*-glucoside (A) and apigenin (B) in *Chamomilla recutita* (L.) Rauschert samples. Averages of individual HPLC-MS measurements and confidences are shown. Significant differences ($p < 0.05$) of bacterial treatments (*Streptomyces subrutilus* Wb2n-11, *Bacillus subtilis* Co1-6, *Paenibacillus polymyxa* Mc5Re-14, *Pseudomonas fluorescens* L13-6-12, *Stenotrophomonas rhizophila* P69, *Serratia plymuthica* 3Re4-18) to the control are indicated by asterisks.

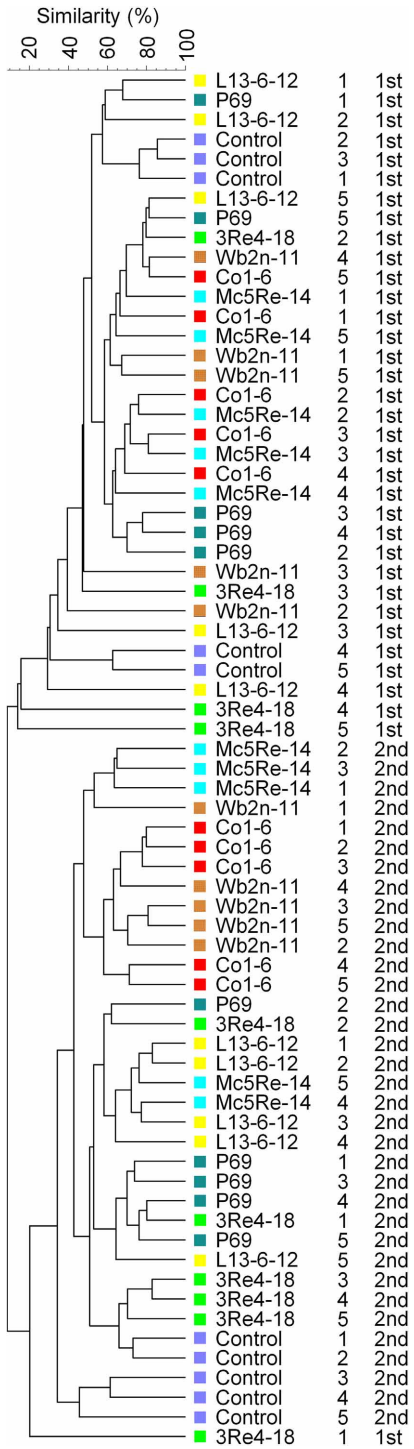

FIGURE 2 | Cluster analysis of eubacterial community fingerprints. Similarities between SSCP fingerprints were calculated using the curve-based Pearson correlation coefficient and grouped according to their similarity using the hierarchical UPGMA. Treatments (*Streptomyces subrutilus* Wb2n-11, *Bacillus subtilis* Co1-6, *Paenibacillus polymyxa* Mc5Re-14, *Pseudomonas fluorescens* L13-6-12, *Stenotrophomonas rhizophila* P69, *Serratia plymuthica* 3Re4-18, and water control) and sampling times (1st) after 4 weeks and (2nd) after 8 weeks are indicated. Numbers 1–5 mark the five independent replicates per treatment.

only several of the samples from the different treatments were grouped together. Generally, samples from different treatments were found to be more similar to each other than the samples of the five replicates of each specific treatment, suggesting no significant differences arising from the bacterial inoculants. Moreover, no cluster group was found containing exclusively SSCP patterns of one specific treatment.

The bacterial community associated with *C. recutita* (L.) Rauschert showed a high abundance of *Bacillus* spp. (Table S3). In the *Pseudomonas* community two dominant bands could be detected, which were abundant in both sampling times. These two bands were identified by partial 16S rRNA gene sequence analysis as *Pseudomonas* sp. (closest database match *Pseudomonas* sp. MOC14, 100% similarity to JX122114.1) (Table S4). In the *Firmicutes* community *Bacillus* spp. were found in all samples, whereas *Bacillus* sp. BMR7, 99–100% similarity to JX434152.1 and *Bacillus* sp. DV9-6, 99% similarity to GQ407151.1, represented the most abundant species (Table S5).

PYROSEQUENCING-BASED 16S rRNA PROFILING OF CHAMOMILE-ASSOCIATED BACTERIAL COMMUNITIES

To deeply investigate the diversity and the composition of the bacterial communities associated with *C. recutita* (L.) Rauschert, a pyrosequencing approach was employed. Rarefaction analysis was performed to an extent of diversity coverage (Figure S1). Assessment of richness revealed that pyrosequencing effort attained 35.8-46.5% of estimated richness at a genetic similarity of 97% (**Table 1**). At the genetic similarity levels of 95% and 80%, amplicon libraries covered 41.7–49.7% and 56.6–88.8% of estimated richness, respectively (**Table 1**). Taxonomic composition of bacterial communities was similar at phylum level, comprising *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* as the most dominant phyla (**Figure 4**). However, the phylum *Verrucomicrobia* was only present in the sample from the treatment with *S. rhizophila* P69, considering only taxa covering more than 1% of quality sequences. *Acidobacteria* were observed in samples treated with *B. subtilis* Co1-6, *S. rhizophila* P69, and *S. plymuthica* 3Re4-18. At genus level, *Rhizobium* (phylum Proteobacteria), *Pseudoxanthomonas* (phylum Proteobacteria), *Pseudomonas* (phylum Proteobacteria), *Flavobacterium* (phylum *Bacteroidetes*), and *Arthrobacter* (phylum *Actinobacteria*) represented the most abundant genera, showing a different composition according to the different treatments. Alpha-diversity of the amplicon libraries was characterized by Shannon index (H′) for 97, 95, and 80% similarity levels. Slight differences between treatments where revealed by the comparison of the index values (**Table 1**). Jackknifed weighted UniFrac two-dimensional (**Figure 5**) and three-dimensional (**Figure 6**) principal coordinates analysis (PCoA) biplots were constructed in order to visualize relationships among samples based on differences in taxonomic diversity. Weighted biplots showed that the samples were clearly separated, implying a difference in bacterial community composition according to the treatments.

QUANTITATIVE ANALYSIS OF BACTERIAL ABUNDANCES

The bacterial abundances of total bacteria and *Firmicutes* were determined by quantitative PCR. For total bacteria, abundances

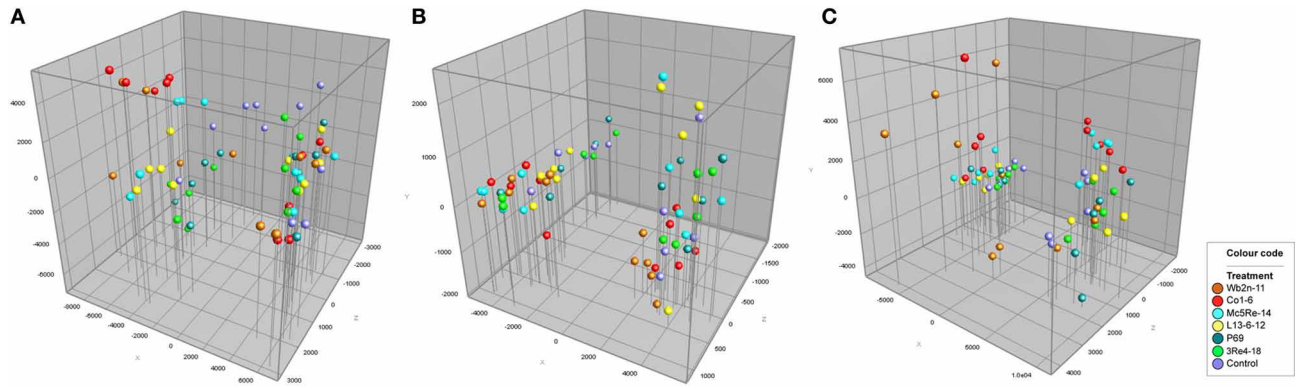

FIGURE 3 | Principal component analysis (PCA) of eubacterial (A), *Pseudomonas* (B) and *Firmicutes* (C) community fingerprints. Treatments (*Streptomyces subrutilus* Wb2n-11, *Bacillus subtilis* Co1-6, *Paenibacillus polymyxa* Mc5Re-14, *Pseudomonas fluorescens* L13-6-12, *Stenotrophomonas rhizophila* P69, *Serratia plymuthica* 3Re4-18, and water control) are indicated by colors. Sampling time (1st) after 4 weeks is shown on the left side and (2nd) after 8 weeks is shown on the right side. PCA was calculated based on relative positions and intensity of DNA bands.

Table 1 | Richness estimates and diversity indices for amplicon libraries of rhizosphere samples[a].

Genetic similarity[b]	Sample[c]	No. of OTUs	Chao1	Coverage (%)	H'[d]
97%	Wb2n-11	557	1443	38.6	5.14
	Co1-6	596	1280	46.5	5.32
	Mc5Re-14	729	1928	37.8	5.66
	P69	559	1209	46.2	5.23
	3Re4-18	546	1525	35.8	5.37
95%	Wb2n-11	433	871	49.7	4.80
	Co1-6	477	998	47.8	5.00
	Mc5Re-14	560	1342	41.7	5.26
	P69	435	930	46.8	4.90
	3Re4-18	419	973	43.1	5.01
80%	Wb2n-11	81	143	56.6	2.85
	Co1-6	76	86	88.8	3.05
	Mc5Re-14	90	134	67.4	2.87
	P69	92	120	76.9	3.11
	3Re4-18	89	100	88.6	3.20

[a] *The number of sequences of each sample was normalized to 1858.*

[b] *Genetic similarities represent the taxonomic levels of species (97%), genera (95%), and phyla (80%).*

[c] *Abbreviations correspond to the treatments with Streptomyces subrutilus (Wb2n-11), Bacillus subtilis (Co1-6), Paenibacillus polymyxa (Mc5Re-14), Stenotrophomonas rhizophila (P69), and Serratia plymuthica (3Re4-18).*

[d] *Shannon diversity indices.*

ranged from 7.45 $\log_{10}$ copies per g root fresh weight (fw) for the treatment with *S. subrutilus* Wb2n-11 to 8.03 $\log_{10}$ copies per g fw for the treatment with *P. polymyxa* Mc5Re-14 (Figure S2). Abundances for *Firmicutes* ranged from 6.97 $\log_{10}$ copies per g fw for the treatment with *S. subrutilus* Wb2n-11 to 7.54 $\log_{10}$ copies per g fw for the treatment with *P. polymyxa* Mc5Re-14 (Figure S2). However, no statistically significant differences between the treatments could be detected.

The colonization pattern of the labeled bacterial (DsRed) strain *S. plymuthica* 3Re4-18 was monitored with confocal laser scanning microscopy. For chamomile seedlings cultivated in a gnotobiotic system, cells of *S. plymuthica* 3Re4-18 were able to colonize the rhizosphere. (**Figure 7A**). Two different colonization patterns could be observed: single cells, covering the root surface and forming a dense network. Furthermore, cells were often found as surrounding clouds (**Figure 7B**), where they were loosely arranged around the root surface.

DISCUSSION

In this study, we examined the impact of six bacterial inoculants on the native bacterial community of *C. recutita* (L.) Rauschert as well as on the production of flavonoid compounds. We found an impact of the treatments on both, host's microbiome and physiology.

The impact of bacterial inoculants was analyzed by three methods. No impact was found at quantitative level: all abundances were highly similar and showed no statistically significant differences. Quantitative insight into the microbial communities in the rhizosphere showed that total bacteria (up to 8.03 $\log_{10}$ copies per g root fw for the treatment with *P. polymyxa* Mc5Re-14) as well as *Firmicutes* (up to 7.54 $\log_{10}$ copies per g fw for the treatment with *P. polymyxa* Mc5Re-14) resulted in a high abundance in the rhizosphere. The phenomenon that the number of microorganisms in the rhizosphere is enhanced as a result of exudation of compounds by the root was described as the rhizosphere effect by Lynch (1990). However, no clear influence on the abundance of microorganisms by bacterial inoculants could be detected, even though results give a hint that Gram-positive bacteria are more dominant in the rhizosphere than introduced Gram-negative strains. The high abundance of Gram-positive bacteria in bulk soil was already described by Smalla et al. (2001).

At qualitative level, the applied methods resulted in different conclusions. All molecular fingerprints obtained with

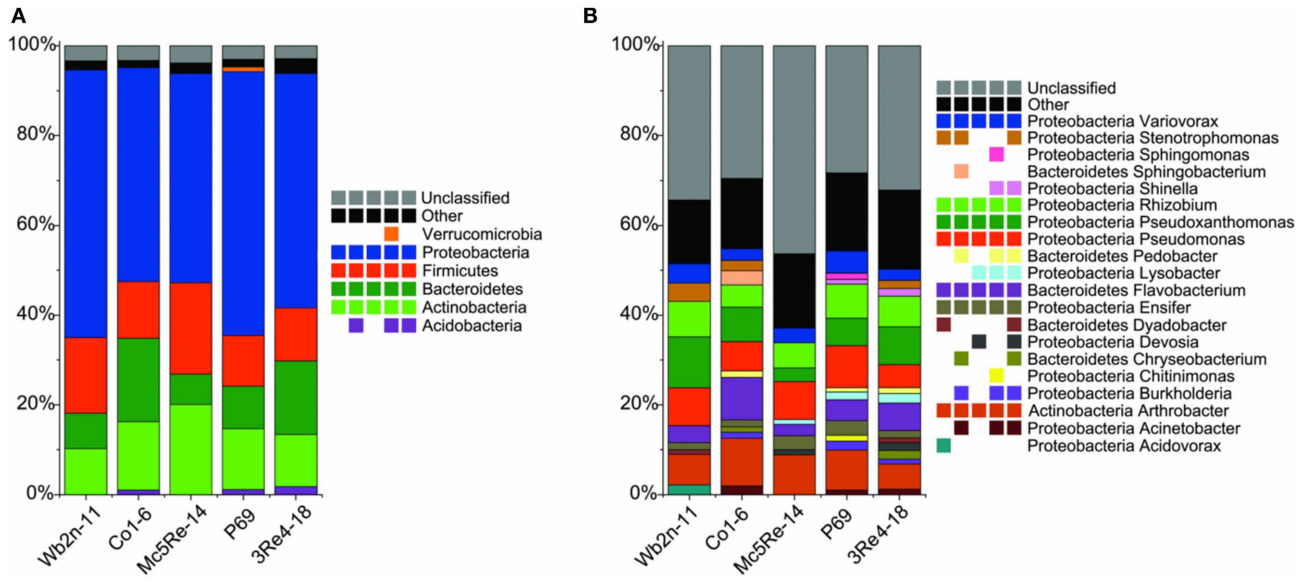

FIGURE 4 | Classification of bacterial communities associated with *Chamomilla recutita* (L.) Rauschert. Pyrosequencing reads were classified at phylum **(A)** and genus **(B)** level against RDP core set within QIIME pipeline with an 80% confidence threshold. Taxa below 1% of relative abundance are included in "Other." Multi-colored charts at the legend are shown for each sample correspondingly.

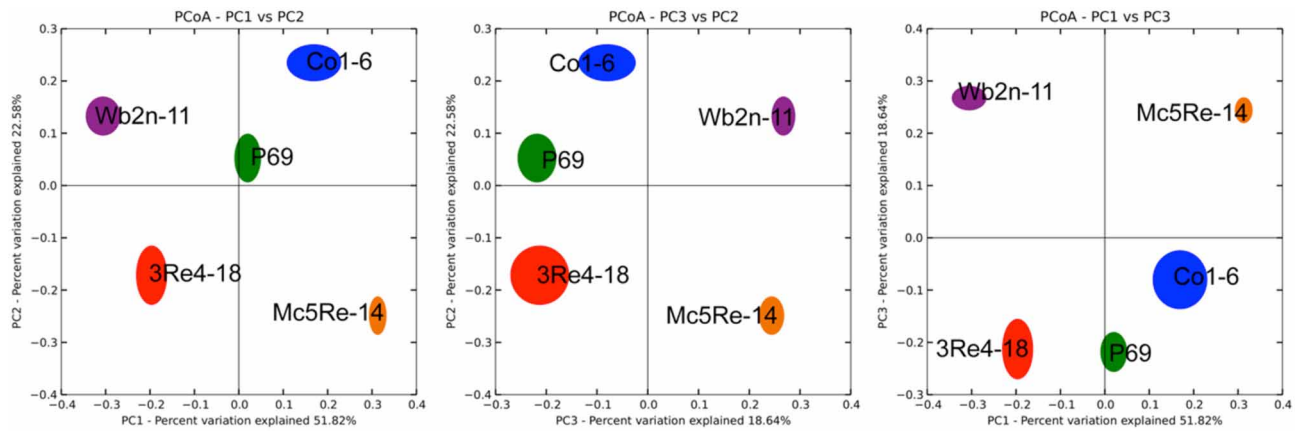

FIGURE 5 | Comparison of the microbial communities of *Chamomilla recutita* (L.) Rauschert rhizosphere by jackknifed principal coordinate analysis. The 2D-plot illustrates the compositional similarity between samples based on weighted UniFrac. The positions of the points are the averages for the jackknifed replicates generated by QIIME and are shown with ellipses representing the interquartile range (IQR) in each axis. Larger ellipses represent more diverse communities. Colors correspond to the different treatments.

universal and group-specific primers revealed high similarity of microbial community composition and were strongly related to the sampling times, showing about 10% similarity. In both sampling times, the bacterial rhizosphere community did not differ between the treatments and the control. The rhizosphere of the young chamomile plant was shown to be colonized mainly by *Bacillus* spp. and *Pseudomonas* spp. The high abundance of *Bacillus* species in the field soil at Sekem farms was already described by Köberl et al. (2011). Pseudomonads represent important members of the rhizosphere microbial community due to their aggressive colonization (Scherwinski et al., 2006). Their plant growth promoting ability was described in several studies (Bloemberg and Lugtenberg, 2001; Weller, 2007). Generally, the species composition differed slightly between the two sampling times. However, due to the limited number of identified strains, these results should be interpreted carefully. The SSCP analysis showed that there was no clear influence by the bacterial inoculants on the diversity of the naturally occurring bacterial populations in the rhizosphere of *C. recutita* (L.) Rauschert. Next-generation sequencing allows a deeper insight into microbial community composition, and therefore answers to the following questions can be provided: (i) how are microbial communities composed on different taxonomic levels? (ii) how does the structure of communities look like

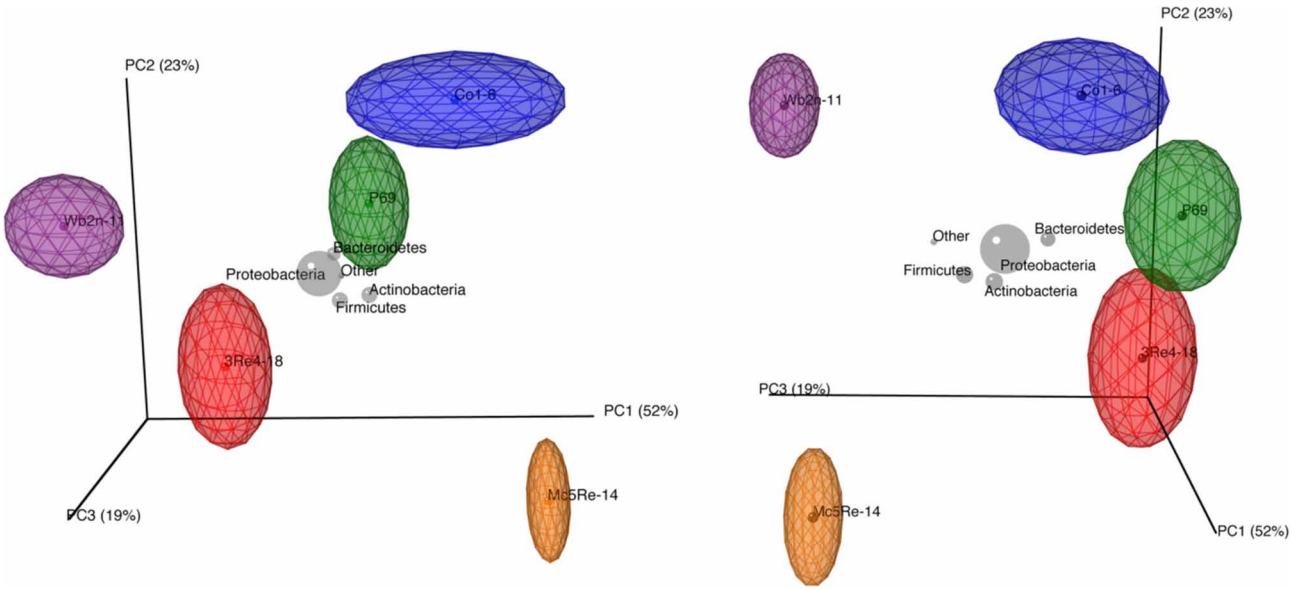

FIGURE 6 | Comparison of the microbial communities of *Chamomilla recutita* (L.) Rauschert rhizosphere by jackknifed principal coordinate analysis. The biplot illustrates the compositional similarity between samples based on weighted UniFrac. Taxa coordinates are indicated by gray orbs with size, as a function of relative abundance. To confine the biplot, the number of the displayed taxa was restricted to 5. The positions of the points are the averages for the jackknifed replicates generated by QIIME and are shown with ellipses representing the interquartile range (IQR) in each axis. Larger ellipses represent more diverse communities. Colors correspond to the different treatments.

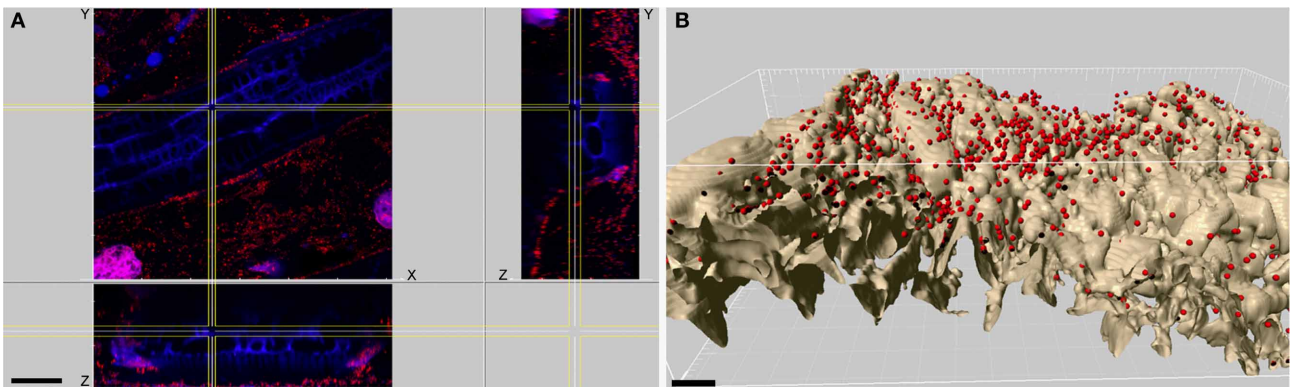

FIGURE 7 | Root epidermis of a chamomile seedling 14 days after inoculation. Visualization of DsRed marked bacteria with confocal laser scanning microscopy. **(A)** xy, xz, and yz maximum projections showing a colonizing of small colonies and single cells of *Serratia plymuthica* 3Re4-18 of the surrounding area of the root. Red: bacterial cells, blue: root, scale bar: 30 μm. **(B)** Surface model of **(A)** shows the root-surface localization of *Serratia plymuthica* 3Re4-18 in the three-dimensional space. Red: bacterial cells, brown: root, scale bar: 10 μm.

between different samples? (iii) how do communities change across treatments? To address these questions, samples from different treatments with bacterial inoculants were investigated and compared among each other. At first, the pyrosequencing approach showed similar patterns of bacterial diversity between the different treatments, whereas *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Bacteroidetes*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes* represented the most abundant phyla in all analyzed samples (>1% of all sequences). Janssen (2006) already described members of these identified phyla as the most abundant soil bacteria. The phylum *Verrucomicrobia* was only present in the sample from the treatment with *S. rhizophila* P69 (=DSM14405^T). In other *ad planta* studies, this strain was found to have an indirect positive interaction with their host plants by altering fungal communities (Schmidt et al., 2012). When bacteria were analyzed at the genus level, the relative abundances of different genera belonging to phyla *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* varied among the samples under different treatments. These results showed that it is of crucial importance to be aware of using the appropriate levels of taxonomy for different investigations. Therefore, this study suggests that for the detection of differences

within microbial communities deriving from bacterial inoculants the use of genus or even lower levels of taxonomy yield the greatest benefits. Unlike alpha diversity measurements, such as species richness and rarefaction curves, beta diversity measures the degree of similarity, e.g., phylogenetic relatedness between pairs of communities (Caporaso et al., 2010). Therefore, phylogenetic beta diversity metrics were used to investigate the structure of communities between the samples. PCoA plots showed that samples differed according to the treatments. Despite the SSCP results, pyrosequencing results suggest that the introduction of antagonistic bacterial strains leads to changes in the native bacterial community structure.

The impact on the physiology of the host was found for the treatments with the two Gram-positive strains *B. subtilis* Co1-6 and *P. polymyxa* Mc5Re-14, yielding a significantly higher content of apigenin-7-*O*-glucoside compared to the treatments with Gram-negative strains. Apigenin was only found in traces and may have been produced postharvest by hydrolysis from apigenin-7-*O*-glucoside (Bauer and Wagner, 1991; Maier et al., 1993). Likewise, previous studies have isolated and identified the presence of both mono- and di-acetylated apigenin-7-*O*-glucoside, which are known to undergo rapid ester hydrolysis leading to the formation of apigenin-7-*O*-glucoside (Redaelli et al., 1981; Svehlikova et al., 2004). Moreover, apigenin-7-*O*-glucoside is highly susceptible for the hydrolysis to its corresponding aglycone in presence of acid or hydrolytic enzymes. As a result, the formation or degradation of apigenin-7-*O*-glucoside may lead to a falsification of quantification. To date, only few studies have considered the influence of rhizobacteria on the secondary metabolites of plants (Zuanazzi et al., 1998; Singh et al., 2002; Orhan et al., 2006). Therefore, little is known about the mechanisms behind positive influence of rhizobacterial secondary metabolites on the plants secondary metabolite production. Plants have the ability to acquire enhanced level of resistance to pathogenic microorganisms, a mechanism first described by Van Peer et al. (1991) and Wei et al. (1991) as induced systemic resistance (ISR). However, ISR can also be induced by various non-pathogenic microorganisms that may activate inducible defense mechanisms in the plant in a similar way to pathogenic microorganisms (Loon, 2007). Activation of defense mechanisms by plants suggests that even a beneficial rhizobacterium may be recognized as a potential threat, leading to the production of resistance compounds (Kloepper et al., 2004; Ongena et al., 2005; Schuhegger et al., 2006; Arkhipova et al., 2007; Vleesschauwer and Höfte, 2007; Lehr et al., 2008; Choudhary and Johri, 2009; Lal and Tabacchioni, 2009).

Next-generation sequencing allows a deeper insight into plant-associated microbial communities. This was already shown for suppressive soils (Mendes et al., 2011) and for the analysis of core microbiomes (Lundberg et al., 2012). In our study we show that they also improve our understanding of the mode of interaction of bacterial inoculants. The latter are promising for a sustainable agriculture and the challenges for crop production in the context of climate change. To understand the effects and interactions of biocontrol agents will allow the improvement of biocontrol products.

MATERIALS AND METHODS

The bacterial strains used in this study are summarized in **Table 2**. For the preparation of inoculums, several colonies of each bacterial strain were inoculated in 500 ml liquid LB medium (Roth, Karlsruhe, Germany) and grown at 30°C and 150 rpm for 24 h (Co1-6, L13-6-12, P69, and 3Re4-18) and for 48 h (Wb2n-11 and Mc5Re-14). To harvest the cells, bacterial suspensions were centrifuged at 13,500 g for 20 min. Pellets were dissolved in 2 ml sucrose solution (1%), serving as cryoprotectant agent, and frozen to −70°C for 5 h. Tubes containing the frozen bacterial suspension were put into ampules and connected to a freeze-dryer (Labconco FreeZone 4.5 Liter Benchtop, USA) for 12 h under vacuum at <0.1 Pa.

FIELD EXPERIMENT AND SAMPLING PROCEDURE

Prior to planting, one-month old field-grown *C. recutita* (L.) Rauschert seedlings were root-dipped in suspensions of the bacteria for 15 min. The non-treated control seedlings were dipped in tap water and planted. Five replicates of each treatment were performed in a randomized block design (1 × 2 m per plot) at a field at Adleya farm/Sekem (30°22′88″N; 31°39′41″E). During the growth stage, the field was irrigated with water (2607 l m^{-3} on average per year) coming from the Nile or from local groundwater drillings—drip irrigation systems were used. Composite soil samples were collected from each plot at a surface depth of 0–5 cm, and the soil-texture was classified as sandy silt with a pH of 8.48, 254.75 µS/m electrical conductivity, 1.44% organic matter, 0.82% organic carbon, 0.15% total nitrogen, 0.01% total phosphorous, and 0.07% total potassium. Rhizosphere samples were taken at 4 and 8 weeks after planting, each with five replicates per treatment. A sample consisted of 5 g roots with adhering soil. In the end of the growing season (March 2012), chamomile flowers were harvested, each with five replicates per treatment.

Table 2 | Bacterial strains used in this study.

Bacterial strain	Origin	References
Streptomyces subrutilus Wb2n-11	Desert soil in Sinai, Gram-positive	Köberl et al., 2011
Bacillus subtilis subsp. subtilis Co1-6	Rhizosphere of *Calendula officinalis* (L.), Gram-positive	Köberl et al., 2011
Paenibacillus polymyxa Mc5Re-14	Endorhiza of *Chamomilla recutita* (L.) Rauschert, Gram-positive	Köberl et al., 2011
Pseudomonas fluorescens L13-6-12	Rhizosphere of *Solanum tuberosum* (L.), Gram-negative	Lottmann and Berg, 2001
Stenotrophomonas rhizophila P69 (= DSM14405^T)	Rhizosphere of *Brassica napus* (L.), Gram-negative	Wolf et al., 2002
Serratia plymuthica 3Re4-18	Endorhiza of *Solanum tuberosum* (L.), Gram-negative	Berg et al., 2005b; Grosch et al., 2005

DETERMINATION OF FLAVONOIDS

Fresh chamomile flowers were dried in a forced-convection oven at 40°C and milled to a fine powder using a universal IKA M 20 grinding mill (IKA-Werke, Staufen, Germany). The extraction of chamomile flower heads was performed using an accelerated solvent extractor, ASE 200 (Dionex Corporation, Sunnyvale, CA, USA). Extraction was carried out at a temperature of 68°C, with a constant pressure of 69 bar and a static time of 5 min using 100% methanol (v/v) as extraction solvent. Based on preliminary experiments, conditions of ASE 200 were set as follows: no preheating period, heating time of 5 min, flush volume at 30% of the extraction cell volume, three extraction cycles, nitrogen purge time of 60 s. The extracts were filtered (Whatman No. 42) and stored at 4°C in darkness until the chromatographic analysis. HPLC analysis was performed on an UltiMate 3000 RS chromatographic system (Dionex, Sunnyvale, CA, USA). A LiChrospher 100 RP-18 (125x4 mm, 5 μm) (Merck, Darmstadt, Germany) was employed for the separation. The binary mobile phase consisted of solvents A (water) and B (acetonitrile) according to the following profile: 0–17 min, 15–40% B; 17–18 min, 40–75% B; 18–29 min, 75% B. The flow rate was 1.0 ml/min and the injection volume was 5 μl. Chromatograms were recorded at a wavelength of 340 nm. For the mass analysis, a linear ion trap quadrupole (LTQ) XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) with an electrospray interface (ESI) was used. ESI negative ion mode conditions were set as follows: source voltage 3.0 kV, sheath gas flow rate 50 au, auxiliary gas flow rate 10 au, source current 100.0 μA, capillary voltage −45.0 kV, and capillary temperature 330°C. The screening was performed in full scan, covering the range from m/z 50 up to 2000. Calibration curves of apigenin-7-O-glucoside and apigenin were obtained by the external standard method. Apigenin-7-O-glucoside and apigenin were quantified using Xcalibur Quan Browser (Version 2.0, Thermo Fisher, San Jose, CA, USA). Chromatograms of each treatment were overlaid with the control using MZmine (Version 2.8, Katajamaa et al., 2006) in order to identify new peaks arising from the treatments. Identification of new peaks was performed using a Dionex Ultimate 3000 UHPLC focused LC system coupled via a heated electrospray source HESI2 to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). HPLC conditions were set as described above. HESI conditions were set as follows: spray voltage: −3 kV, capillary temp: 450°C, sheath and auxiliary gas: N2, flow: 75 & 20 instrument units, gas temp heater: 350°C. The MS instrument was operated in negative mode, externally mass calibrated, and with the resolving power sat to 140000 (FWHM), scanned between m/z 133-2000. With an exclusion time of 10 s, the five most intense ions were selected for collision induced fragmentation. The selection window was 0.4 Dalton, and the fragmentation energy NCE sat to 30 ± 20% instrument units. MS2 spectra were obtained with the resolving power sat to 35000. Putative compounds were identified using the online METLIN metabolite database (http://metlin.scripps.edu).

RHIZOSPHERE SAMPLING AND TOTAL COMMUNITY DNA ISOLATION

The bacterial fraction associated with *C. recutita* (L.) Rauschert was extracted using the protocol adapted from Opelt and Berg (2004). In brief, for each rhizosphere sample, 5 g of roots with adhering soil were mixed with 45 ml NaCl solution (0.85%) and vortexed for 5 min. A total volume of 4 ml of the suspension was centrifuged at 16,000 g at 4°C for 20 min, and the pellet was used for isolation of the total community DNA. For mechanical lysis, the cells were homogenized in a FastPrep FP120 Instrument (MP Biomedicals, Solon, OH, USA) for 40 s at speed 6.0. The obtained DNA was purified using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. Final aliquots of the total community DNA were further used for PCR-SSCP, 454 pyrosequencing, and qPCR.

MICROBIAL FINGERPRINTING BY PCR-SSCP

Fingerprinting of microbial communities was carried out by PCR-based SSCP described by Schwieger and Tebbe (1998). Bacterial 16S rRNA gene sequences were amplified by PCR using the universal eubacterial primer pair Unibac-II-515f/Unibac-II-927r^{P} (Zachow et al., 2008). The 60 μl reaction mixture contained 1×Taq-&Go Ready-to-use PCR Mix (MP Biomedicals), 3 mM $MgCl_2$, 0.2 μM of each primer, and 1 μl DNA template (95°C, 5 min; 32 cycles of 95°C, 20 s; 54°C, 15 s; 72°C, 30 s; and elongation at 72°C, 10 min). For the analysis of the Pseudomonas community, a nested PCR was performed. In the first PCR, the Pseudomonas specific primer pair F311Ps/1459rPs^{P} (Milling et al., 2005) was used in a 20 μl reaction mixture containing 1×Taq-&Go Ready-to-use PCR Mix, 3 mM $MgCl_2$, 0.5 mg/ml BSA, 1.5% DMSO, 0.2 μM of each primer, and 1 μl DNA template (94°C, 7 min; 30 cycles of 94°C, 45 s; 56°C, 2 min; 72°C, 2 min; and elongation at 72°C, 10 min). Samples served as templates for the second PCR using the primer pair Unibac-II-515f/Unibac-II-927r^{P}. For the analysis of the Firmicutes community, the universal eubacterial primer pair 27f/1492r (Lane, 1991) was used in a 20 μl reaction mixture containing 1×Taq-&Go Ready-to-use PCR Mix, 3 mM $MgCl_2$, 0.2 μM of each primer, and 1 μl DNA template (95°C, 5 min; 30 cycles of 95°C, 30 s; 57°C, 30 s; 72°C, 90 s; and elongation at 72°C, 5 min). In the second PCR, the Firmicutes specific primer pair BLS342f/BACr833r^{P} (Blackwood et al., 2005) was used in a 60 μl reaction mixture containing 1×Taq-&Go Ready-to-use PCR Mix, 0.2 μM of each primer, and 3 μl of the product from the first PCR (95°C, 5 min; 30 cycles of 95°C, 45 s; 57°C, 60 s; 72°C, 45 s; and elongation at 72°C, 10 min). The obtained amplicons were separated using the INGENY phorU system (INGENY International BV, Goes, Netherlands) at 400 V and 26°C followed by silver staining. Dominant bands were excised from SSCP gels as described by Schwieger and Tebbe (1998). Extracted DNA fragments were re-amplified by PCR and sequenced. For phylogenetic analysis and identification of related sequences, the obtained sequences were aligned with reference gene sequences from GenBank using BLAST algorithm.

AMPLICON SEQUENCING OF BACTERIAL COMMUNITIES

To characterize the rhizosphere bacterial communities associated with *C. recutita* (L.) Rauschert, the V4-V5 hypervariable region of the bacterial 16S rRNA gene (*Escherichia coli* positions 515 to 927) was chosen for the amplification and subsequent pyrosequencing of the PCR products. Due to lack of funds, only samples from the treatments Wb2n-11, Co1-6, Mc5Re-14, P69, and 3Re4-18 from the first sampling time were sequenced. The V4-V5 region was

amplified using the primer pair Unibac-II-515f/Unibac-II-927r. The 20 µl reaction mixture contained 1×Taq-&Go Ready-to-use PCR Mix, 3 mM $MgCl_2$, 0.5 µM of each primer, and 1 µl of template DNA (95°C, 2 min; 34 cycles of 95°C, 20 s; 65°C, 15 s; 72°C, 29 s; and elongation at 72°C, 10 min). PCR products from four samples of the same treatment were purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Amplicons of each treatment were pooled together in an equimolar ratio and subjected to pyrosequencing using a Roche 454 GS-FLX+ Titanium platform executed by Eurofins MWG (Ebersberg, Germany).

PROCESSING OF PYROSEQUENCING DATA

Raw sequencing reads were demultiplexed, quality and length filtered using ribosomal database project's (RDP) pyrosequencing pipeline (Cole et al., 2009). Primers were cropped and all sequence reads shorter than 150 bp—with a minimum average quality score <20 and with any ambiguous characters were discarded. Data were normalized to the same number of sequences using an in-house developed Perl script (10 times random re-samplings followed by subset formation (Bragina et al., 2013). A further downstream analysis of normalized data was achieved using the QIIME toolkit (Caporaso et al., 2010). Bacterial sequences were clustered into OTUs using 3, 5, and 20% dissimilarity thresholds with UCLAST (Edgar, 2010), and the most abundant sequence from each OTU was selected as a representative sequence for that OTU. Taxonomy was assigned by using a QIIME-based wrapper of the RDP classifier program (Wang et al., 2007) against the RDP core set (Cole et al., 2009) using an 80% confidence threshold for taxonomic assignment. Rarefaction analysis and estimation of alpha-diversity was performed using Chao1, Shannon, and observed OTU metrics at 3, 5, and 20% dissimilarity. Beta-diversity was examined using weighted UniFrac distances (Lozupone and Knight, 2005) between samples sub-sampled 20 times, with replacement, at a depth of 100 sequences per sample. This method takes phylogenetic relationships between community members in account, incorporating the abundances of phylotypes into the pairwise community comparisons (Eilers et al., 2010). The compositional similarity of all samples was visualized in a three-dimensional principal coordinate system (PCoA) based on previously calculated jackknifed principal coordinates. To reveal the most abundant taxa in different areas of the PCoA plot, taxonomic classification of 20% genetic distance was included.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

The same region of the 16S rRNA gene was amplified by quantitative PCR to determine the total bacterial as well as the Firmicutes abundances in the rhizosphere of *C. recutita* (L.) Rauschert. For the total bacteria, the universal eubacterial primer pair Unibac-II-515f/Unibac-II-927r was used, while the Firmicutes specific primer pair BLS342f/BACr833r was used for Firmicutes. To estimate bacterial gene abundances, standard curves were generated using 10-fold serial dilutions of plasmid DNA containing a full-length copy of either the *P. polymyxa* PB71 16S rRNA gene or the *B. subtilis* Sd3-12 16S rRNA gene (Köberl et al., 2011). For the total bacteria, the qPCR 10 µl reaction mixture contained 1x KAPA SYBR FAST qPCR MasterMix Universal (PEQLAB, Polling, Austria), 0.25 µM of each primer, and 1 µl of the standard and DNA template (95°C, 5 min; 35 cycles of 95°C, 20 s; 54°C, 15 s; 72°C, 30 s; and melt from 72 to 95°C). For Firmicutes, the 10 µl qPCR reaction mixture contained 1x KAPA SYBR FAST qPCR MasterMix Universal, 0.25 µM of each primer, and 1 µl standard and DNA template (95°C, 5 min; 30 cycles of 95°C, 45 s; 56°C, 60 s; 72°C, 45 s; and melt from 72 to 95°C). qPCR was performed in duplicate for each sample using the Rotor-Gene 6000 real-time rotary analyser (Corbett Research, Sydney, Australia). The melting curve analysis of the PCR products was performed immediately after the amplification. Bacterial copy numbers for each reaction were generated from the standard curves and calculated to copy number per g root fresh weight (fw). Each replicate was analyzed two times in two independent runs.

CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

DsRed2-labeled bacteria (3Re4-18) were grown in 5 ml nutrient broth (NB) supplemented with tetracycline (40 µg ml^{-1}) at 30°C and 120 rpm for 24 h. Bacterial cells were collected by centrifugation at 13,500 g for 5 min and resuspended in fresh NB medium without addition of antibiotics. The cell suspension was adjusted to an optical density corresponding to a cell count of 10^9 cells ml^{-1}. Seeds were mixed with 1 ml of the cell suspension in 1.5 ml Eppendorf tubes and incubated at room temperature for 15 min, followed by a washing step with sterile NaCl solution (0.85%). Seeds were placed on a filter paper in moist chambers which were kept at 22°C for 5 days (16/8 h day/night). Seeds incubated with sterile water served as control. Roots of chamomile were examined on a Leica TCS SPE confocal scanning microscope (Leica Microsystems GmbH, Wetzler, Germany).

STATISTICS

Computer-assisted comparisons of SSCP generated community profiles were performed using GelComparII (version 5.1, Applied Maths, Kortrijk, Belgium). For the cluster analysis, similarity matrices based on Pearson's correlation coefficients were constructed, and a dendrogram using the unweighted paired group means algorithm (UPGMA) was created. Relative positions and intensity of DNA bands were used for a principal component analysis (PCA). Significances in the difference between the treatments were calculated using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). First, data were checked for normal distribution and homogeneity of variance. Second, an one-way ANOVA analysis was performed with data that follow a normal distribution, and a *post-hoc* test was applied depending on the homogeneity of the variance index using the Tukey honestly significant difference (HSD) analysis for qPCR data and the Tamhane T2 test for HPLC-MS data.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Ruth Schmidt, Martina Köberl, Marlene Monschein, Gabriele Berg, Rudolf Bauer, and Elshahat M. Ramadan. Performed the experiments: Ruth Schmidt, Amr Mostafa, Martina Köberl, and Marlene Monschein. Analyzed the data: Ruth Schmidt, Martina Köberl, Marlene Monschein, and Kenneth B. Jensen. Contributed reagents/materials/analysis tools: Gabriele Berg. Wrote the paper: Ruth Schmidt and Gabriele Berg.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00064/abstract

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Root exudation and root development of lettuce (*Lactuca sativa* L. cv. Tizian) as affected by different soils

G. Neumann[1]*, S. Bott[1], M. A. Ohler[1], H.-P. Mock[2], R. Lippmann[2], R. Grosch[3] and K. Smalla[4]

[1] Department of Nutritional Crop Physiology, Institute of Crop Science (340h), University of Hohenheim, Stuttgart, Germany
[2] Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany
[3] Department Plant Health, Leibniz Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V., Großbeeren, Germany
[4] Julius Kühn-Institut Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Kemal Kazan, Commonwealth Scientific and Industrial Research Organization, Australia
Nai-Chun Lin, National Taiwan University, Taiwan

***Correspondence:**
G. Neumann, Department of Nutritional Crop Physiology, Institute of Crop Science (340h), University of Hohenheim, 70593 Stuttgart, Germany
e-mail: gd.neumann@t-online.de

Development and activity of plant roots exhibit high adaptive variability. Although it is well-documented, that physicochemical soil properties can strongly influence root morphology and root exudation, particularly under field conditions, a comparative assessment is complicated by the impact of additional factors, such as climate and cropping history. To overcome these limitations, in this study, field soils originating from an unique experimental plot system with three different soil types, which were stored at the same field site for 10 years and exposed to the same agricultural management practice, were used for an investigation on effects of soil type on root development and root exudation. Lettuce (*Lactuca sativa* L. cv. Tizian) was grown as a model plant under controlled environmental conditions in a minirhizotrone system equipped with root observation windows (rhizoboxes). Root exudates were collected by placing sorption filters onto the root surface followed by subsequent extraction and GC-MS profiling of the trapped compounds. Surprisingly, even in absence of external stress factors with known impact on root exudation, such as pH extremes, water and nutrient limitations/toxicities or soil structure effects (use of sieved soils), root growth characteristics (root length, fine root development) as well as profiles of root exudates were strongly influenced by the soil type used for plant cultivation. The results coincided well with differences in rhizosphere bacterial communities, detected in field-grown lettuce plants cultivated on the same soils (Schreiter et al., this issue). The findings suggest that the observed differences may be the result of plant interactions with the soil-specific microbiomes.

Keywords: lettuce, root exudates, root morphology, soil effects

INTRODUCTION

Root exudates of higher plants with nutritional, signaling, and antibiotic functions are shaping rhizosphere-microbial communities, which in turn can exert stimulatory or inhibitory effects on plant growth and development. Already Hiltner (1904) postulated that specific patterns of root exudation in different plant species may recruit a specific rhizosphere microflora, which may comprise beneficial partners but also pathogens as uninvited guests. He also pointed out that a more detailed knowledge of these interactions may open perspectives for practical applications in agriculture and plant protection. Nowadays, the availability of novel techniques for the characterization of microbial communities by high throughput sequencing approaches, metabolomics and the development of non-destructive, localized exudate sampling techniques (Neumann et al., 2009; Bakker et al., 2012; Chaparro et al., 2013) opens the way for a more detailed and comprehensive look on the interactions between rhizosphere microbiomes and roots of their host plants. During the last two decades enormous progress was achieved in the characterization of factors determining root exudation, which exhibits high variability within different plant species and even cultivars, within different root zones and developmental stages of individual plants and in response to various biotic and abiotic stress factors (Neumann, 2007; Neumann and Römheld, 2007; Badri and Vivanco, 2009). An increasing number of investigations meanwhile also address the interactions between root exudates and corresponding changes of the microflora in the rhizosphere (Marschner et al., 2002; Weisskopf et al., 2006; Bakker et al., 2012).

In the present study, we hypothesized that different soil types with different physicochemical properties will influence root growth patterns and root exudation, which in turn may have an impact on composition and function of rhizosphere-microbial communities (see Schreiter et al., this issue). The enormous plasticity of root growth and root exudation in response to different soil conditions and particularly to stress factors, such as nutrient limitation, mineral toxicities, and extremes in soil moisture and soil structure, is a well-described phenomenon (Neumann and Römheld, 2002, 2007; Neumann, 2007). In our study, we used field soils originating from a unique experimental plot system with three different soil types, which were stored at the same field site for 10 years under the same agricultural management and used in parallel by Schreiter et al. (this issue) to characterize the influence of soil type on rhizosphere-bacterial communities under field conditions. This approach offered the opportunity to study the influence of

soil properties independent from cropping history or climatic factors.

Lettuce plants (cv. Tizian) were grown in minirhizotrons, equipped with root observation windows (Neumann, 2006a), which allowed root growth monitoring and localized collection of root exudates and rhizosphere soil solution from defined root zones by use of sorption filters (Neumann, 2006b; Haase et al., 2007) with subsequent re-extraction and GC-MS profiling of the exudate patterns (Lippmann et al., 2009). Due to the limited available field plot size, unfortunately direct collection of root exudates under field conditions by use of root windows (Neumann et al., 2009) was not possible.

MATERIALS AND METHODS

PLANT CULTIVATION

Lactuca sativa L. cv. Tizian seedlings were pre-cultivated until the 2-leaf stage (BBCH 12) in peat culture substrate sand mixture (7:3 w/w; TKS1-Anzuchtsubstrat, Floragard, Germany). Thereafter, the seedlings were transferred to minirhizotrons (rhizoboxes) with transparent root observation windows (36 × 11.5 × 2.5 cm; Neumann, 2006a) filled each with 1 kg of the selected test soils (**Figure 1**).

The experimental system included three soils of different origin: Arenic-Luvisol with less silty sand and 5.5% clay [diluvial sand (DS)], Gleyic-Fluvisol with heavy sandy loam and 27.5% clay [alluvial loam (AL)], and Luvic-Phaeozem with medium content of clayey silt and 17.2% clay [loess loam (LL)]. Soil properties are summarized in **Table 1**.

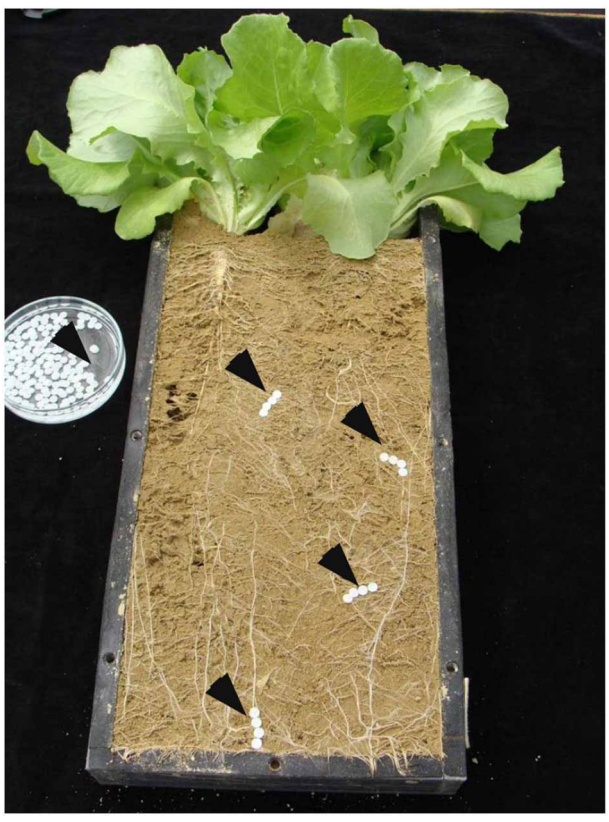

FIGURE 1 | Lettuce plants *Lactuca sativa* L. cv. Tizian (BBCH 19) grown on loess loam. Root observation window of a minirhizotron (rhizobox) prepared for exudate collection with sorption filters (indicated by black arrows).

To exclude nutrient limitation, prior to transplanting, the soils received a basal macronutrient fertilization of N, P, K, and Mg. Nitrogen was applied as $CaNO_3$ at 100 mg N kg^{-1} soil, P as $Ca(H_2PO_4)_2$ at 100 mg P kg^{-1} soil, K as K_2SO_4 at 150 mg K kg^{-1} soil and Mg as $MgSO_4$ at 50 mg Mg kg^{-1} soil. Final soil moisture level was adjusted to 18–20% w/w and controlled gravimetrically during the culture period.

Rhizoboxes were fixed at an angle of 45° to promote root development along the observation windows and plants were cultivated until BBCH 19 in a growth chamber with a 16 h light period (200 µmol m^{-2} s^{-1}), 60% rel. humidity and a day/night temperature of 25°/23° C.

FINAL HARVEST AND EXUDATE SAMPLING

Prior to final harvest (BBCH 19; 5 weeks after transplanting), the root observation windows were opened and root exudates were collected by placing sorption filters onto the surface of 2 cm sub-apical root zones (**Figure 1**) according to the method described by Haase et al. (2007). The time point for exudate sampling was selected since root growth, shoot to root carbohydrate partition and root exudation usually is particularly intense during vegetative growth and declines after entering the generative phase (Marschner, 1995). The collection period was 4 h and the filters were subsequently stored at −20°C until further analysis. Thereafter, roots were washed out of the soil using sieves with 1 and 0.5 mm mesh size. Roots were dried on filter paper and fresh biomass was recorded. Thereafter, the root samples were stored in 30% (v/v) ethanol until further analysis. Biomass of the shoot material was recorded and dried at 60° C for mineral nutrient analysis. Root analysis was performed using the WinRhizo system (Regent Instruments, Quebec, Canada).

MINERAL NUTRIENT ANALYSIS

For determination of the plant-nutritional status, 500 mg of dried leaf material was ashed in a muffle furnace at 500°C for 5 h. After cooling, the samples were extracted twice with 2 mL of 3.4 M HNO_3 until dryness to precipitate SiO_2. The ash was dissolved in 2 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min. After addition of 0.1 mL Cs/La buffer to 4.9 mL ash solution, Fe, Mn, and Zn concentrations

Table 1 | Characteristics of the field soils used for the experiments.

Soil type	pH	Ct [%]	TRD [g/cm^3]	FAT [%]	P CAL. [mg/100 g]	K CAL. [mg/100 g]
Loess loam	7.3	1.90	1.36	23.6	26.1	30.0
Diluvial sand	6.1	0.80	1.46	7.7	25.4	12.3
Alluvial loam	6.7	1.80	1.31	34.9	46.0	26.0

Ct, total carbon content; TRD, bulk density; FAT, fine grained particles/particles <6.3 µm.

were measured by atomic absorption spectrometry (UNICAM 939, Offenbach/Main, Germany).

EXUDATE PROFILING

The extraction of the sorption filters containing root exudates and rhizosphere soil solution was conducted with 80% methanol. Filters were removed by centrifugation, and the supernatant was evaporated to dryness at 30°C using a speed vac concentrator (Savant, Farmington, USA) and stored at −80°C until further analysis. For analysis, supernatants were re-dissolved in 200 µL methanol, transferred into GC-MS glass vials and evaporated to dryness at 30°C. Derivatisation was performed online directly prior to injection using a MPS Autosampler (Gerstel, Mühlheim a. d. R., Germany) by adding 25 uL methoxyhydroxymethylamine (20 mg mL^{-1} in pyridine) and incubation for 2 h at 37°C, 350 rpm. Thereafter, 50 µL MSTFA including standard alcanes from Sigma C7–C30 (0.1% v/v) were added and incubated for 30 min at 37°C, 350 rpm.

One µL aliquots were analyzed by an Agilent 7890 gas chromatograph (Agilent, Santa Clara, CA, USA) in the splitless mode, coupled to a TOF mass spectrometer GCT Premier (Waters Corporation, Eschborn, Germany). Separation was performed on a Rxi®5Sil MS Integra column (Restek) with 0.25 mm inner diameter and 0.25 µm film thickness, including a 5 m guard column according to Lippmann et al. (2009). Injection temperature was 240°C. The temperature program for GC separation was: 3 min 80°C isothermal followed by a ramp of 5°C min^{-1} to 300°C for 5 min. Mass spectroscopical (MS) data were recorded with Mass Lynx 4.1 (Waters Corporation) at a rate of 10 spectra s^{-1} in a range of 50–700 m/z. Metabolites were identified automatically with the internal software ChromaLynx using the NIST 5 library and interesting components were verified manually by comparison with reference spectra. For principal component analysis (PCA) MarkerLynx (Waters Corporation) was used with following settings: 20 masses per retention time in the range of 100–330 m/z and a tolerance of 0.05 Da were isolated at a threshold at 5% of base peak intensity. Pareto algorithm was used for visualization.

RESULTS

ROOT GROWTH CHARACTERISTICS

Biomass production and particularly root length were significantly influenced by the selected soil types (**Table 2**). Root growth characteristics of lettuce plants grown on DS and LL were significantly different from AL. Total root length on AL, mainly represented by fine roots (diameter < 0.4 mm, 70%) was 2.5–4 fold increased as compared with DS and LL, respectively. This was also reflected in a significantly higher (fine) root length, root biomass and a lower average root diameter of lettuce plants grown on AL. However, differences in root growth were not associated with corresponding differences in shoot biomass production and surprisingly, the highest shoot biomass was detected in lettuce plants grown on LL with development of the smallest root system.

ROOT EXUDATES

At BBCH 19 (5 weeks after transplanting), root development along the root observation window was sufficiently expressed to enable the collection of root exudates and rhizosphere soil solution from defined sub-apical root zones in young actively growing roots (**Figure 1**), which have been identified in many earlier studies as the sites of the most intense expression of root exudation, nutrient uptake and root-induced rhizosphere-chemical changes (for review see Neumann and Römheld, 2007).

In total 33 compounds were identified by GC-MS profiling in the soil solutions collected with sorption filters from the root surface and rhizosphere of 2 cm sub-apical root zones, comprising 17 amino acids and amides, 8 sugars, and sugar alcohols, 5 organic acids as well as ornithine, urea, and phosphate (**Table 3**). Benzoic and lauric acids were detected as exudate compounds with allelopathic and antibiotic properties (Walters et al., 2003; Lee et al., 2006; Yoon et al., 2012).

PCA analysis revealed clear differences in the root exudate profiles collected from lettuce plants grown on the different soils (**Figure 2**), with DS separated from AL. However, with the exception of several amino acids (beta-alanine, glutamate, 4-amino butyric acid), the differences were rather quantitative than qualitative (**Table 3**).

Interesting quantitative differences were observed for low molecular weight sugars, such as glucose, fructose, mannose, maltose, sucrose, and trehalose, which were much less abundant in samples collected from plants grown on AL as compared with DS or LL soil (**Table 2**). This is also reflected in the cumulative quantification of all sugars based on peak areas (**Table 4**), where the detected amounts increased in the order AL < DS < LL. The same trend was detectable for the cumulative quantification of amino acids but not for organic acids (**Table 4**).

Urea was detected in similar amounts in soil solutions collected from the rhizosphere and from the bulk soil, while phosphate was present exclusively in rhizosphere soil solutions (data not shown).

The plants did not show any macronutrient disorders, since all soils received a standard N, P, K, Mg fertilization prior to

Table 2 | Biomass production and root characteristics of *Lactuca sativa* L. cv. Tizian (BBCH19), grown on three different soils.

Soil type	Shoot fresh weight [g $plant^{-1}$]	Root fresh weight [g $plant^{-1}$]	Total root length [cm $plant^{-1}$]	Fine root length [cm $plant^{-1}$] 0–0.4 mm diameter	Average root diameter [mm]
Alluvial loam	6.67 ± 1.07a	0.84 ± 0.18a	562.2 ± 92.2a	391.0 ± 58.3a	0.35 ± 0.01a
Loess loam	9.07 ± 1.02a	0.30 ± 0.04b	126.8 ± 14.9b	87.3 ± 12.5b	0.49 ± 0.02b
Diluvial sand	5.32 ± 0.60a	0.24 ± 0.05b	197.1 ± 27.5b	150.9 ± 18.2b	0.43 ± 0.01b

Means ± SE of four independent replicates. In each column different characters indicate significant differences (One-way ANOVA, Holm-Sidak test, p = 0.05).

Table 3 | List of low molecular-weight compounds detected by GC-MS root exudate profiling from roots of *Lactuca sativa* cv Tizian (BBCH19) grown on three different soils.

Chemical group	Compound	Loess loam	Alluvial loam	Diluvial sand
Amino acids and amines	Alanine	+	+	++
	beta-Alanine	+	−	+
	Aspartate	+	+	+
	Glutamate	−	−	+
	Glutamine	+	+	+
	Glycine	+++	++	++
	Leucine	++	+	+
	Isoleucine	+	+	+
	Proline	+	+	+
	4-Hydroxyproline	+	+	+
	Pyroglutamate	++	+	+
	Serine	++	++	+
	Threonine	++	+	+
	Valine	++	+	++
	beta-Aminobutyric acid	+	+	+
	4-Aminobutyric acid	+	−	+
	Putrescine	+	+	+
Sugars and sugar alcohols	Glucose	+++	+	++
	Fructose	+++	+	++
	Mannose	+	−	−
	Maltose	+++	+	+++
	Trehalose	+++	+	+++
	Sucrose	+++	++	++
	Glycerol	+++	+++	+++
	Inositol	+++	+	+
Organic acids	Malate	+	+	+
	Fumarate	+	+	+
	Succinate	++	+++	++
	Lauric acid	++	+	+
	Benzoic acid	++	++	++
Others	Urea	+++	++	++
	Phosphate	+	+	+
	Ornithine	+	+	+

(−, absent; +, low; ++, medium; +++, high).

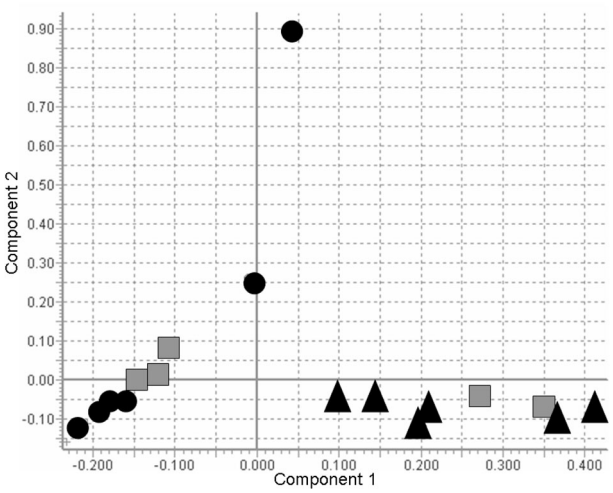

FIGURE 2 | Principal component analysis (PCA) of the GC-MS root exudate profiles collected from 2 cm-subapical root zones of *Lactuca sativa* L. cv. Tizian BBCH19 grown on three different soils (Loess loam ■; Alluvial loam ▲; Diluvial sand ●).

Table 4 | Amounts of sugars, amino acids, and aliphatic organic acids in root exudate samples collected from 2-cm sub-apical root zones of lettuce, *Lactuca sativa* L. cv. Tizian (BBCH 19) grown on three different soils (calculations based on cumulative peak area after subtraction of background levels in bulk soil samples).

Class of compounds	Alluvial loam	Diluvial sand	Loess loam
Sugars	2.95	103.60	427.60
Amino acids	1.39	6.05	22.66
Organic acids	2.83	0.05	4.44

Table 5 | Micronutrient concentrations in leaf dry matter (DM) of young lettuce *Lactuca sativa* L. cv Tizian (BBCH 19), grown on three different soils.

Soil type	Fe [mg kg^{-1} DM]	Mn [mg kg^{-1} DM]	Zn [mg kg^{-1} DM]
Loess loam (LL)	180.2 (8.6)	97.1 (4.2)	47.8 (0.6)
Diluvial sand (DS)	308.5 (8.5)	81.9 (16.2)	42.8 (2.1)
Alluvial loam (AL)	74.3 (5.3)	35.0 (3.2)	34.5 (4.2)

Means and SEs (in brackets) of four independent replicates.

the onset of the experiment. Also micronutrient concentrations (**Table 5**) were above the critical levels reported for lettuce by Bergmann (1988). No toxicities, apparent symptoms of diseases or pH extremes were observed and the soil pH ranged between 6.6 and 7.6. The soil moisture level was adjusted gravimetrically in regular intervals between 18–20% (w/w), equivalent to 70% of the water holding capacity, which is considered as optimal for plant growth.

DISCUSSION

Higher plants exhibit an enormous adaptive plasticity of root growth and morphology in response to external biotic and abiotic stress factors. Root growth stimulation particularly of fine root structures is frequently induced by moderate limitations of P, N, Fe, and water but lateral root development and root hair proliferation can be also stimulated by localized patches of high P, NO_3^-, and NH_4^+ supply. By contrast, root growth inhibition is a typical response to extreme limitations of water and nutrients, toxicities, and increased mechanical impedance induced by extreme drought or soil compaction (Neumann and Römheld, 2002).

However, in the present study, high variability observed in root growth of lettuce on three different field soils cannot be

attributed to macronutrient disorders since all soils received a full N, P, K, and Mg fertilization prior to the onset of the experiment. Mineral nutrient analysis revealed soil-specific differences in the plant nutritional status but no apparent nutrient deficiencies or toxicities. Cropping history of the soils during the last 10 years was identical and no symptoms of plant diseases were visible. The largest differences in root length development were detected between the two similarly structured loamy soils (AL and LL), suggesting that also potential effects of soil structure on root growth are not the major cause for the observed variability in root growth. Moreover, the influence of soil structure was further minimized by homogenous sieving of all soils with 2 mm mesh size prior to the experiment. Therefore, the huge differences observed in root growth and morphology on the three investigated soils were unexpected.

Interestingly, Schreiter et al. (this issue) reported the highest number of bacterial rhizosphere responders in the rhizophere of lettuce grown on AL followed by DS and finally LL soils, which exactly reflects the order of root length development in the different soils (**Table 2**). Root growth stimulation of host plants by bacterial production of phytohormones (e.g. auxins) is a well-documented mechanism for plant-growth promotion by rhizosphere bacteria (Berg, 2009) and various bacterial genera, such as *Rhizobium* and *Pseudomonas* with known potential for root growth stimulation by auxin production (Biswas et al., 2000; Iqbal and Hasnain, 2013) were among the rhizosphere responders detected with the highest abundance in AL soil (Schreiter et al., this issue). This raises the question whether the observed differences in root growth and fine root production of lettuce on the different soils may be the result of differences in the abundance of root growth-promoting rhizobacteria. More fine-root development, resulting in a larger root surface area would in turn also provide increased space for root colonisation by rhizosphere responders.

ROOT EXUDATES

Apart from variability in root development, also huge quantitative differences particularly for sugars and amino acids were detected in the root exudate samples collected from lettuce plants, grown on the three different soils (**Table 5**). Comparable variations in root exudation over 1–3 orders of magnitude are characteristic in some plant species for the adaptive release of specific compounds, such as carboxylates or phytosiderophores involved in the mobilization of sparingly soluble mineral nutrients but also for detoxification of toxic elements or in response to membrane damage due to severe nutrient limitations or drought stress (Neumann and Römheld, 2007). However, the presence of nutrient deficiencies/toxicities or of other stress factors, limiting water and nutrient uptake was not indicated by soil and plant analysis (**Tables 1**, **5**) and cannot explain the huge quantitative differences in root exudation. The fact that the samples collected from different soils exhibit mainly quantitative differences, while the qualitative composition was very similar (**Table 3**) suggests, that the detected compounds represent mainly root exudates and not microbial metabolites. In the latter case, a much higher qualitative diversity would have been expected due to the large variation of microbial populations in the different soils (Schreiter et al., this issue) which would consequently be reflected in a high diversity of microbial metabolites released into the rhizosphere.

However, root exudate sampling was performed over a time period of 4 h to increase the chance of collecting also less polar aromatic compounds and other secondary metabolites, usually less abundant in root exudates (Neumann, 2006b). On the other hand, longer sampling periods are associated with a risk of losses of readily soluble exudate compounds, such as sugars, amino acids, and organic acids, which are easily used as carbon and N sources by rhizosphere microorganisms (Neumann, 2006b), resulting in half life times of only several hours in rhizosphere soil solutions (Jones et al., 1996). Drastically reduced levels of sugars and amino acids in exudate samples collected from AL as compared with DS and LL soils, may therefore reflect a particularly intense microbial consumption of these compounds as a consequence of a higher abundance of bacterial rhizosphere responders detected by Schreiter et al. (this issue) in the rhizosphere of AL-grown plants. Differences in the root exudate profiles collected from lettuce plants grown on the different soils were further confirmed by PCA (**Figure 2**).

Another interesting finding is the detection of benzoic and lauric acids in the root exudate samples. Both compounds have been previously reported as constituents of root exudates of lettuce in hydroponic culture with (auto-)allelopathic (Lee et al., 2006) and antifungal properties (Walters et al., 2003; Yoon et al., 2012). Moreover, the rhizosphere responders (*Sphingomonas*, *Pseudomonas*, and *Variovora)* detected by Schreiter et al. (this issue) in the rhizosphere of lettuce grown on the same soils exhibit a high potential for degradation of aromatic hydrocarbons, such as benzoic acid and De la Fuente et al. (2009) reported the ability of various *Pseudomonas* strains to utilize trehalose and benzoic acids as sole carbon sources, both detected in the root exudates of lettuce in this study.

Urea detected at similar levels, both in bulk soil and root exudate samples (data not shown) cannot be attributed to N fertilization since nitrate was applied as a mineral N source. The presence of urea in all analyzed samples may reflect a background level of microbial urea production, which has been previously confirmed by isotope studies also for other agricultural soils (Nielsen et al., 1998). By contrast, phosphate, detected only in root exudate samples may indicate preferential mobilization of weakly adsorbed phosphate in the rhizosphere, mediated by plant roots or rhizosphere microorganisms (Neumann and Römheld, 2002).

In summary, the present study suggests that even under controlled conditions on well-fertilized soils, excluding the influence of cropping history and stress factors with impact on root growth and activity, mutual interactions between plant roots, and soil-specific microbiomes seem to be important determinants for shaping root architecture, root exudation and thereby the establishment of rhizosphere-microbial communities.

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Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce

Susanne Schreiter[1,2], Guo-Chun Ding[1,3], Holger Heuer[1], Günter Neumann[4], Martin Sandmann[2], Rita Grosch[2], Siegfried Kropf[5] and Kornelia Smalla[1]*

[1] Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut, Braunschweig, Germany
[2] Department of Plant Health, Leibniz Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V., Großbeeren, Germany
[3] College of Resources and Environmental Sciences, China Agricultural University, Beijing, China
[4] Institute of Crop Science (340h), Hohenheim University, Stuttgart, Germany
[5] Department for Biometrics und Medical Informatics, Otto von Guericke University, Magdeburg, Germany

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Armin Erlacher, Graz University of Technology, Austria
Anja Bettina Dohrmann, Johann Heinrich von Thünen Institute, Germany

***Correspondence:**
Kornelia Smalla, Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Messeweg 11-12, D-38104 Braunschweig, Germany
e-mail: kornelia.smalla@jki.bund.de

The complex and enormous diversity of microorganisms associated with plant roots is important for plant health and growth and is shaped by numerous factors. This study aimed to unravel the effects of the soil type on bacterial communities in the rhizosphere of field-grown lettuce. We used an experimental plot system with three different soil types that were stored at the same site for 10 years under the same agricultural management to reveal differences directly linked to the soil type and not influenced by other factors such as climate or cropping history. Bulk soil and rhizosphere samples were collected 3 and 7 weeks after planting. The analysis of 16S rRNA gene fragments amplified from total community DNA by denaturing gradient gel electrophoresis and pyrosequencing revealed soil type dependent differences in the bacterial community structure of the bulk soils and the corresponding rhizospheres. The rhizosphere effect differed depending on the soil type and the plant growth developmental stage. Despite the soil type dependent differences in the bacterial community composition several genera such as *Sphingomonas, Rhizobium, Pseudomonas,* and *Variovorax* were significantly increased in the rhizosphere of lettuce grown in all three soils. The number of rhizosphere responders was highest 3 weeks after planting. Interestingly, in the soil with the highest numbers of responders the highest shoot dry weights were observed. Heatmap analysis revealed that many dominant operational taxonomic units were shared among rhizosphere samples of lettuce grown in diluvial sand, alluvial loam, and loess loam and that only a subset was increased in relative abundance in the rhizosphere compared to the corresponding bulk soil. The findings of the study provide insights into the effect of soil types on the rhizosphere microbiome of lettuce.

Keywords: ***Lactuca sativa,*** **bacterial communities, 16S rRNA gene analysis, DGGE, pyrosequencing, rhizosphere responders**

INTRODUCTION

Plants influence soil microorganisms in the vicinity of their roots through their root architecture, exudates, and mucilage (Bais et al., 2006; Badri and Vivanco, 2009). The so-called rhizosphere effect was already recognized in the beginning of the 20th century by Hiltner (1904). Not only the available nutrients released by the plant but also changes in the pH and redox gradients are assumed to shape the composition of microbial communities in the rhizosphere (Schmidt et al., 2011). Microorganisms which profit from the chemical changes in the vicinity of the roots and utilize these compounds increase in abundance and typically show enhanced metabolic activity. Obstacles to study rhizosphere microbial communities are manifold ranging from sampling the rhizosphere to limitations of traditional cultivation-based methods and resolution level of 16S rRNA gene-based methods. The rhizosphere effect was comprehensively studied by means of molecular fingerprints based on 16S rRNA gene fragments amplified from total community (TC) DNA of bulk soil and rhizosphere (reviewed by Berg and Smalla, 2009). While bulk soils were typically characterized by a high number of faint bands indicating a high evenness of many equally abundant populations, the rhizosphere fingerprints display several intense bands indicating populations with increased abundance in the vicinity of the roots (Smalla et al., 2001; Costa et al., 2006a). When different plant species or cultivars were grown at the same field sites, different fingerprint methods revealed a plant species or even a cultivar-dependent composition of the bacterial communities in the rhizosphere (Smalla et al., 2001; Schmalenberger and Tebbe, 2002; Weinert et al., 2009, 2011). The latter was typically less pronounced. Although it was clear that the soil from which plants select their microbiome must play an important role, the evaluation of the extent to which the soil type influences the microbial community was difficult to assess under field conditions as not only the soil characteristics but also the climate, the cropping history or the agricultural management are assumed to influence the soil microbiome (Costa et al., 2006b, 2007). Therefore, studies

investigating the same crops grown at different locations could only report on the effect of the sites. Costa et al. (2006b, 2007) could show that the site was the overriding factor although for *Actinobacteria* or *Pseudomonas* similar populations were enriched in the rhizosphere of strawberries at different locations. Through pyrosequencing of 16S rRNA gene fragments amplified from TC-DNA of *Arabidopsis thaliana* grown in different soils under greenhouse conditions both Bulgarelli et al. (2012) and Lundberg et al. (2012) provided insights into the effects of the soil type on the bacterial community composition in the rhizosphere. However, the effect of the soil type on the microbial community composition in bulk soil and in the rhizosphere has never been studied under field conditions. The importance of the plant microbiome has only recently been recognized and was proposed as the second genome of plants (Berendsen et al., 2012). The fact that many recent studies revealed that the plant microbiome is of great importance for plant growth and health triggered the idea to include the microbiome as an essential part in plant breeding programs (Berendsen et al., 2012). In the present study, we hypothesized that different soil types characterized by different physicochemical properties harbor different microbial communities. The soil type dependent microbial community composition as well as the root exudation patterns and architecture studied by Neumann et al. (2014) might determine the composition of microbial communities in the rhizosphere. We used an experimental plot system with three soil types stored at the same site for 10 years under the same agricultural management to reveal differences directly linked to the soil type and not influenced by other factors like local climate or cropping history under field conditions. In order to investigate to what extent the soil type or the plant determine the bacterial community composition in the rhizosphere, lettuce plants were grown in four replicate plots per soil type. Bulk soil and lettuce rhizosphere were sampled at two time points 3 and 7 weeks after planting (3WAP and 7WAP) and analyzed by denaturing gradient gel electrophoresis (DGGE) and pyrosequencing of 16S rRNA gene fragments amplified from TC-DNA.

MATERIALS AND METHODS

FIELD EXPERIMENT

Experimental design

The field experiment was performed in a unique experimental plot system at the Leibniz Institute of Vegetable and Ornamental Crops (Großbeeren, Germany, 52° 33′ N, 13° 22′ E) to evaluate the effect of soil types on bacterial communities in the rhizosphere and in bulk soil. The experimental system included three soils of different origin in separate blocks: Arenic-Luvisol with less silty sand and 5.5% clay (diluvial sand, DS), Gleyic-Fluvisol with heavy sandy loam and 27.5% clay (alluvial loam, AL), and Luvic-Phaeozem with medium content of clayey silt and 17.2% clay (loess loam, LL) (Rühlmann and Ruppel, 2005). Each block consisted of 24 plots of 2 × 2 m in size and 0.75 m depth (Table S1). Previous crops from 2000 to 2009 were pumpkin, nasturtium, pumpkin, amaranth, wheat, wheat, pumpkin, nasturtium, wheat, and wheat. Lettuce (cultivar "Tizian") was selected as a model plant in our experiment. Seeds were germinated in a seedling tray containing the respective soil types at 12°C for 48 h and further cultivated under greenhouse conditions at approximately 20/15°C (day/night). All seedlings were watered daily to maintain the soil moisture and fertilized weekly (0.2% Wuxal TOP N, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany). Lettuce seedlings pregrown in the same soils were planted at the three- to four-leaf stage (BBCH 13-14) in the experimental system. Each plot included six rows with a within-row and intra-row distance of 30 cm between lettuce plants (36 plants per plot). Four replicate plots were established for each treatment and soil type. Overhead irrigation was applied based on the irrigation computer program "BEREST" (Gutezeit et al., 1993). Input variables for the irrigation program were the daily soil water content in the rooted soil layer using the water holding capacity of the soil, the plant growth stage, and the potential evapotranspiration (Table S2). Irrigation decisions were made on the basis of the calculated soil water content and the expected evapotranspiration and precipitation of the next five days. The temperature (reflectometer PT100b1/3 DIN, Messtechnik Geraberg GmbH, Germany) and the matric potential (tensiometer T22968, transmitter ES 1075, bambach GbR Tensio-Technik, Geisenheim, Germany) were recorded in 10 cm soil depth during the field experiment in four replicates for each soil type (Table S3, Figures S1, S2). One day before planting fertilizer was added based on a chemical analysis of each soil type (Table S1). Each soil type was adjusted to 168 mg/100 g N by fertilizer (Kalkamon, 27% N, TDG mbH Lommatzsch, Germany) to exclude effects by different N contents on lettuce growth. Soil samples for chemical analysis and characterization of soil parameters were taken one week before planting (three random replicates per soil type). Soil analysis was done by the Agricultural Tests and Research Institutions Association (VdLUFA, Germany) according to standard protocols. Lettuce plants were finally harvested by hand 7WAP (BBCH 49) to obtain lettuce shoot dry weight measured for each plant at harvest. The data of lettuce dry weight were parametrically analyzed after ANOVA using Dunnett's procedure with $P \leq 0.05$ with the STATISTICA program (StatSoft Inc., Tulsa, OK, USA).

Sampling and DNA extraction

Bulk soil and rhizosphere samples were collected before planting lettuce into the field as well as 3WAP and 7WAP. Ten cores (10 cm of top soil; 2 cm core diameter) of bulk soil were randomly taken from each plot and mixed by sieving (mesh size 2 mm). From these approximately 200 g soil a subset of approximately 2 g was collected in a 2 ml Eppendorf tube and stored at −80°C until DNA extraction. For the rhizosphere samples the complete root systems of three plants per plot were combined as a composite sample after removing loosely adhering soil by vigorous shaking. Microbial cells were extracted from the samples as follows: plant roots were cut into pieces of approximately 1 cm length using scissors, carefully mixed and treated by a Stomacher 400 Circulator (Seward Ltd, Worthing, UK) for 30 s at high speed after adding 15 ml sterile 0.3% NaCl to 5 g root pieces. After centrifugation at 500 *g* for 2 min the supernatant was collected and the resulting pellet was re-suspended, transferred to the Stomacher bag with root pieces and exposed to another Stomacher treatment after adding 15 ml sterile 0.3% NaCl. This step was repeated one more time and the combined supernatants of three Stomacher blending

steps (45 ml) were centrifuged at 10.000 *g* for 30 min to obtain the microbial pellet. The supernatant of this processing step was discarded and the pellet was re-suspended in the remaining solution, transferred to a 2 ml reaction tube and centrifuged at 14.000 *g* for 20 min. The pellets were stored at −80°C.

TC-DNA was extracted from 0.5 g of bulk soil or the microbial pellets obtained from 5 g of roots with tightly adhering soil using the FastDNA SPIN Kit for Soil® (MP Biomedicals, Heidelberg, Germany) after a harsh lysis step as described by the manufacturer. The TC-DNA was purified with GENECLEAN SPIN Kit® (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions and was 1:10 diluted with 10 mM Tris HCl pH 8.0 before use.

DGGE analysis of 16S rRNA gene fragments amplified from TC-DNA

16S rRNA gene fragments were PCR-amplified from TC-DNA of bulk soil and rhizosphere samples using the bacterial primers F984-GC and R1378 as described by Heuer et al. (1997). The PCR products were analyzed by DGGE. The gradient of the DGGE gel was performed as described in Weinert et al. (2009) and the electrophoresis conditions as well as the silver staining procedure were done according to Heuer et al. (2001).

Analysis of the DGGE fingerprints

Bacterial DGGE community fingerprints were evaluated with GELCOMPAR II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). The gel images were normalized and the background was subtracted according to the spectral analysis of each gel. For establishing the similarity matrix a curve based method was chosen. The fingerprints were grouped according to their similarity using the hierarchical cluster method UPGMA (unweighted pairwise grouping method using arithmetic means) based on Pearson correlation coefficient for each pair of lanes. The Pearson similarity matrices were analyzed by means of the permutation test calculating the *d*-value from the average overall correlation coefficients within the groups minus the average overall correlation coefficients between samples from different groups as suggested by Kropf et al. (2004) to test the significant differences in community composition between the soil types, rhizosphere, and bulk soil at two sampling times.

Pyrosequencing and statistical analysis

16S rRNA gene fragments amplified from TC-DNA of rhizosphere and bulk soil samples collected 3WAP and 7WAP were analyzed by barcoded pyrosequencing for all replicates. The PCR reaction and the sequencing of the hypervariable V3-V4 region of the 16S rRNA gene was performed at the Biotechnology Innovation Center (BIOCANT, Cantanhede, Portugal) using the primers 338F and 802R (Huse et al., 2008; Vaz-Moreira et al., 2011) which were fused to the 454 A and B adaptors, respectively. Sequencing was performed on a 454 Genome Sequencer FLX platform according to standard protocols (Roche—454 Life Sciences, Branford, CT, USA).

The analysis of the pyrosequencing data was done according to Ding et al. (2012a). Briefly, only those sequences matching the barcode and primer were selected for BLASTN analysis against a SILVA 16S rRNA gene database to truncate the unpaired regions for each sequence. Low quality sequences or chimera resulted in a short alignment which was subsequently filtered out. Only those sequences with a length of more than 200 bp were included in the analysis. Operational taxonomic units (OTUs) were generated with the following steps: sequences were assigned to OTUs (defined at 97% sequences similarity) with the program Mothur 1.21. software (Schloss et al., 2009) and the Naïve Bayesian Classifier (Wang et al., 2007) was used to classify the sequences. The OTU assignment and the classification of each sequence were loaded into a MySQL-data base for producing the taxonomic OTU report. Statistical analysis of the OTU report was done with the Tukey's honest significance test and visualization of the result was performed with R 2.15 (http://www.r-project.org). The Pearson similarity matrices based on relative abundance of the OTUs were analyzed by means of the permutation test calculating the *d*-value as described for DGGE by Kropf et al. (2004).

For the comparison of the community composition between samples the number n of sequences for each OTU was divided by the total number of sequences N from the sample and transformed by $\log(n/N * 1000 + 1)$. The transformed data were used to analyze the effect of soil type, habitat (rhizosphere or bulk soil), and their interaction by a modified principal components test according to Ding et al. (2012b) in the rotation test version.

Pyrosequencing data were deposited at the NCBI Sequence Read Archive under the study accession number SRP029944.

RESULTS

SOIL CHARACTERISTICS, CULTIVATION CONDITIONS, AND LETTUCE GROWTH

The three soil types displayed striking differences not only in the mineral composition but also in pH, total C, N, P and their content in metals and trace elements (Table S1). Furthermore, the average temperature in the 10 cm top soil measured during the vegetation of lettuce was significantly different for AL and LL (LSD test, $P \leq 0.05$). On average a temperature of 16.3°C (range between 9.6 and 26.9°C) was recorded in AL and of 15.9°C (9.1–26.4°C) in LL. No significant differences were detected between average soil temperature of 16.2°C (range between 9.1 and 26.4°C) in DS and the other soil types. The highest day/night temperatures were measured within the first two weeks of lettuce growth in all three soils (Figure S1). In contrast the volumetric soil water content (VWC) varied significantly between all soils (Table S3). The lowest percent VWC was recorded in DS whereas the highest VWC was observed in AL (Figure S2, Tables S2, S3). On average a percent VWC of 15.7% was calculated in DS, of 29.4% in AL, and of 24.3% in LL soil. Furthermore, a mean daily global radiation of 3.28 kWh m^{-2} was measured during the growth period of lettuce.

A total of 120 lettuce plants per soil type grown in four plots with 30 plants per plot were harvested 7WAP. Lettuce plants grown in AL showed with 31.8 g per plant the highest shoot dry weight on average compared to lettuce plants grown in DS (24.4 g/plant) and in LL soil (20.9 g/plant). The dry weight of lettuce harvested from AL soil was significantly higher compared to the shoot dry weight of plants grown in the other two soil types according to Dunnett's procedure ($P \leq 0.05$) while no significant differences were observed for lettuce grown in DS or LL soil.

DGGE ANALYSIS OF 16S rRNA GENE FRAGMENTS AMPLIFIED FROM TOTAL COMMUNITY DNA REVEALED

Soil type dependent bacterial community composition in bulk soil

Bacterial community DGGE fingerprints of bulk soil samples taken 3WAP and 7WAP from all three soil types displayed that some of the bands were soil type specific, while most of the bands were shared among all soil types (Figures S3, S4). The permutation test of the bacterial community fingerprints revealed statistically significant differences between the three bulk soils (DS-AL; DS-LL; AL-LL) at both sampling times (**Table 1**). Higher dissimilarities (*d*-values) between bacterial community fingerprints, in particular of DS-AL and DS-LL were observed 3WAP compared to 7WAP. The lowest differences were observed between AL and LL soil fingerprints at both sampling times (*d*-value 3WAP 22.4, 7WAP 20.1) (**Table 1**) indicating that the bacterial community composition of AL and LL bulk soil were more similar to each other compared to DS bulk soil.

Soil type dependent rhizosphere effect

Compared to the corresponding bulk soil fingerprints a number of bands with stronger intensity was typically detected in the lettuce rhizosphere fingerprints (Figures S3, S4), indicating that some populations were enriched in the rhizosphere. Significant differences in the rhizosphere and the corresponding bulk soil bacterial community fingerprints were detected for all soils but the extent of this rhizosphere effect differed depending on the soil type and the sampling time. Measures for the extent of the rhizosphere effect were the *d*-values obtained after permutation test analysis. At both sampling times the highest *d*-values were observed for AL soil (*d*-values: 3WAP 28.1, 7WAP 30.2) indicating that the strongest rhizosphere effect was observed in AL soil. The lowest *d*-values were observed for DS soil 3WAP and 7WAP for LL soil (**Table 1**).

Table 1 | Soil type dependent differences of bacterial communities in bulk soil and rhizosphere.

Method	Figure	Sampling time	Differences in the bulk soil		
			DS-AL	DS-LL	AL-LL
DGGE	S3	3WAP	41.8*	44.9*	22.4*
	S4	7WAP	30.4*	28.8*	20.1*
Pyrosequencing		3WAP	28.3*	38.2*	17.3*
		7WAP	23.5*	29.9*	16.7*
			Differences between bulk soil and rhizosphere		
			DS	**AL**	**LL**
DGGE	S3	3WAP	18.1*	28.1*	23.7*
	S4	7WAP	23.1*	30.2*	20.3*
Pyrosequencing		3WAP	37.9*	42.2*	32.7*
		7WAP	42.6*	30.5*	32.3*
			Differences in the rhizosphere		
			DS-AL	**DS-LL**	**AL-LL**
DGGE	S3	3WAP	22.8*	29.2*	29.0*
	S4	7WAP	19.8*	32.3*	26.7*
Pyrosequencing		3WAP	22.0*	33.0*	29.2*
		7WAP	20.2*	46.5*	30.8*

Percent dissimilarity (d-value) based on DGGE fingerprints and pyrosequncing of Bacteria from bulk soil and rhizosphere of lettuce (cv. "Tizian") grown in three soils (DS, diluvial sand; AL, alluvial loam; LL, loess loam) of an experimental unit at the same field site. Samples were collected 3 and 7 weeks after planting (3WAP, 7WAP). The asterisks indicate significant differences ($P \leq 0.05$) between the soil types determined by a permutation test.

Soil type dependent rhizosphere community composition

Cluster analysis based on the Pearson correlation indices showed that the bulk and rhizosphere fingerprints formed separate clusters for each of the soil types. Interestingly, at both time points the rhizosphere fingerprints of AL clustered with the DS rhizosphere, once again indicating a strong shift of bacterial community in the rhizosphere of lettuce grown in AL soil (Figures S5, S6). Statistical analysis confirmed that the bacterial communities in the rhizosphere of lettuce grown in the three soil types were significantly different at both time points. Indeed the lowest *d*-values were obtained for DS-AL at both time points.

Shifts in the bacterial community composition with plant development

Bacterial community fingerprints of rhizosphere samples taken before transplanting, 3WAP and 7WAP from DS, AL, and LL indicated changes in the bacterial community composition over time (Figure S7). While many bands were detected at all time points, some bands with changes in band intensity depending on the plant growth developmental stage were identified. Typically these changes were seen for all four replicates analyzed.

PYROSEQUENCING

The 16S rRNA gene amplicons from TC-DNA of 47 samples (the same TC-DNA used for DGGE analysis) were sequenced and altogether 249,350 sequences with a sequence length of more than 200 bp were obtained. The sequences from DS rhizosphere replicate b taken 7WAP had to be excluded from the analysis because the number of obtained sequences was unusually low and treated as an outlier.

A total of 23 phyla, 49 classes, 55 orders, 145 families, 421 genera, and 28,650 OTUs were obtained. Dominant phyla were defined as phyla with more than 1% relative abundance. The phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Acidobacteria*, and *Bacteroidetes* were dominant in bulk soil as well as in the rhizosphere of all three soil types (**Table 2**). In the bulk soil 3WAP the highest relative abundance was observed for *Proteobacteria*, followed by the *Actinobacteria*, *Firmicutes*, *Acidobacteria*, and *Bacteroidetes* in all three soil types (**Table 2**). Compared to bulk soil, the relative abundance of *Proteobacteria* was significantly enhanced in the rhizosphere of lettuce grown in all three soil types at both sampling times. The strongest increase in relative abundance was observed for DS and AL soil. Especially *Gammaproteobacteria* were enriched in DS soil,

Table 2 | Relative abundance of phyla in bulk soil and rhizosphere.

Sampling time	Phylum	Class	DS		AL		LL	
			Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere
3WAP	*Proteobacteria*		29.7 ± 1	50.6 ± 1*	29.0 ± 1	45.7 ± 1*	32.1 ± 1	44.4 ± 3*
7WAP	*Proteobacteria*		30.7 ± 1	50.3 ± 2*	30.2 ± 1	42.7 ± 3*	33.0 ± 4	44.3 ± 10*
3WAP	*Proteobacteria*	*Alphaproteobacteria*	15.8 ± 1	30.7 ± 2*	15.7 ± 1	27.0 ± 1*	17.4 ± 1	27.1 ± 3*
7WAP	*Proteobacteria*	*Alphaproteobacteria*	17.9 ± 2	33.3 ± 3*	17.8 ± 1	21.8 ± 1*	17.9 ± 1	28.4 ± 8*
3WAP	*Proteobacteria*	*Betaproteobacteria*	5.3 ± 0	10.0 ± 1*	3.6 ± 0	8.2 ± 1*	3.7 ± 0	6.1 ± 0*
7WAP	*Proteobacteria*	*Betaproteobacteria*	4.3 ± 2	8.4 ± 1*	2.3 ± 0	10.1 ± 4*	2.8 ± 1	7.5 ± 3*
3WAP	*Proteobacteria*	*Deltaproteobacteria*	4.6 ± 0*	2.2 ± 0	4.6 ± 1*	2.5 ± 0	4.2 ± 0*	2.7 ± 0
7WAP	*Proteobacteria*	*Deltaproteobacteria*	4.5 ± 1*	2.1 ± 0	5.1 ± 1*	2.9 ± 0	4.5 ± 0*	2.6 ± 1
3WAP	*Proteobacteria*	*Gammaproteobacteria*	2.6 ± 0	5.9 ± 1*	3.6 ± 0	6.6 ± 1*	4.7 ± 0	6.5 ± 1*
7WAP	*Proteobacteria*	*Gammaproteobacteria*	2.4 ± 0	5.1 ± 0*	3.6 ± 0	6.6 ± 2*	6.0 ± 3*	4.0 ± 1
3WAP	*Actinobacteria*		26.4 ± 1*	12.7 ± 1	29.7 ± 2*	11.7 ± 1	29.7 ± 1*	16.7 ± 3
7WAP	*Actinobacteria*		19.0 ± 3*	11.7 ± 1	23.9 ± 2*	15.1 ± 2	22.8 ± 2*	16.1 ± 3
3WAP	*Firmicutes*		13.5 ± 1*	7.3 ± 1	11.8 ± 1*	6.9 ± 0	10.6 ± 1	12.0 ± 3*
7WAP	*Firmicutes*		16.3 ± 3*	7.7 ± 0	12.0 ± 1*	7.8 ± 3	12.0 ± 3	14.2 ± 6*
3WAP	*Acidobacteria*		10.3 ± 0*	7.0 ± 0	9.4 ± 1	9.1 ± 1	7.6 ± 1*	5.6 ± 2
7WAP	*Acidobacteria*		12.4 ± 1*	6.8 ± 1	11.3 ± 1*	7.7 ± 1	8.7 ± 1*	6.6 ± 0
3WAP	*Bacteroidetes*		4.7 ± 1	8.7 ± 1*	5.2 ± 1	12.9 ± 1*	5.6 ± 0	8.4 ± 1*
7WAP	*Bacteroidetes*		5.5 ± 1	6.8 ± 1	6.6 ± 1	10.2 ± 3*	7.7 ± 2*	5.7 ± 2

Relative abundance [%] of phyla in the rhizosphere of lettuce (cv. "Tizian") compared to the bulk soil 3 and 7 weeks after planting (3WAP, 7WAP) based on pyrosequencing data. Lettuce plants were grown in three soils (diluvial sand, DS; alluvial loam, AL and loess loam, LL) in an experimental unit at the same field site. The asterisks indicate significant differences ($P \leq 0.05$) between the bulk soil and corresponding rhizosphere samples identified by Tukey's honest test under a generalized linear model via logistic function for binomial data.

whereas *Betaproteobacteria* were enriched in AL and LL soil with up to a four times increase in AL 7WAP. In contrast, in comparison to the corresponding bulk soil the relative abundance of *Actinobacteria* was significantly decreased in all rhizospheres. The relative abundance of *Firmicutes* was lower in the rhizosphere of lettuce grown in AL and DS compared to the corresponding bulk soil but remained nearly unchanged for LL soil (**Table 2**). The relative abundance of the *Acidobacteria* decreased in the rhizosphere of lettuce compared to the corresponding bulk soil (except for AL 3WAP), while the relative abundance of *Bacteroidetes* increased in the rhizosphere compared to the corresponding bulk soil with the strongest increase in the rhizosphere of lettuce grown in AL of up to two and a half times higher at 3WAP. This strong enrichment could not be detected 7WAP. In the LL soil even a decrease in the rhizosphere compared to the corresponding bulk soil was observed (**Table 2**).

SOIL TYPE-DEPENDENT BACTERIAL COMMUNITY COMPOSITION IN THE RHIZOSPHERE AND IN BULK SOIL

UPGMA analysis was based on relative abundance of all bacterial OTUs ($\geq$ 97% sequence identity) obtained for bulk soil and rhizosphere samples from both sampling times (**Figure 1**). Two main clusters were obtained clearly separating the fingerprints bulk soil and rhizosphere samples. Within the bulk soil cluster three soil type dependent clusters were formed (**Figure 1**). Samples from both sampling times clustered together for each soil type with a trend to sub-clusters for samples from 3WAP and 7WAP. Typically a higher similarity of all four replicates per soil type was observed 3WAP compared to 7WAP. The AL and LL bulk soils clustered, indicating that the bacterial communities in the two loamy soils showed a higher similarity to each other compared to the bacterial community of DS bulk soil which formed a separate cluster. This was confirmed by the *d*-values which were lowest for the AL-LL soil comparison (**Table 1**). However, in the rhizosphere the bacterial communities of DS and AL became more similar and formed a joint cluster with sub-clusters according to sampling time and soil type (**Figure 1**). This was supported by the lowest *d*-value for the comparison of DS-AL rhizosphere. The rhizosphere of lettuce grown in LL soil clustered separately with nearly no influence of the sampling time. The lowest *d*-value was observed 3WAP for the comparison $LL_{bulk\ soil}$-$LL_{rhizosphere}$. The *d*-values for the comparison between bulk soil and rhizosphere were in the range of the differences between bulk soils and were highest for AL 3WAP and DS 7WAP, respectively (**Table 1**). Two-factorial multivariate analysis of variance by a modified principal components test revealed highly significant effects of the soil type and of the habitat (rhizosphere or bulk soil) on the community composition for both sampling times ($P < 0.001$). Also the interaction effect was significant ($P = 0.0001$) which means that the rhizosphere effect was soil type dependent (**Figure 2**). The sampling time had an effect on the community composition, but the trends were the same for both samplings (**Figure 2**). Rhizosphere and bulk soil communities of each soil type were clearly separated in principal component analysis, and the communities of both habitats were separated according to the soil type. Communities from soils AL and LL were more similar to each other than to DS for rhizosphere

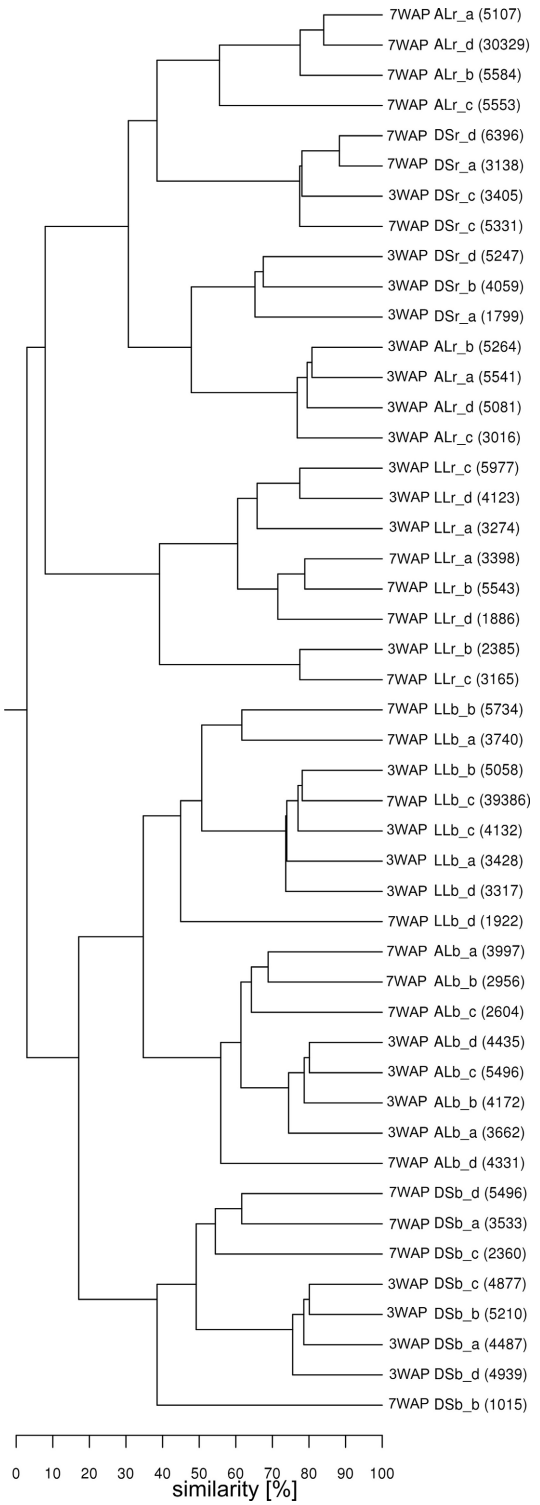

FIGURE 1 | Cluster analysis of *bacterial* communities from rhizosphere and bulk soil samples. Samples from rhizosphere of lettuce and the corresponding bulk soil of DS, AL, and LL soil were taken at 3 and 7 weeks after planting (3WAP, 7WAP). Similarities between samples were calculated as the Pearson correlation of the relative abundance of OTUs based on pyrosequencing data. Numbers in brackets indicate the number of sequences for the sample; r: rhizosphere samples; b: bulk soil samples; a–d: replicates.

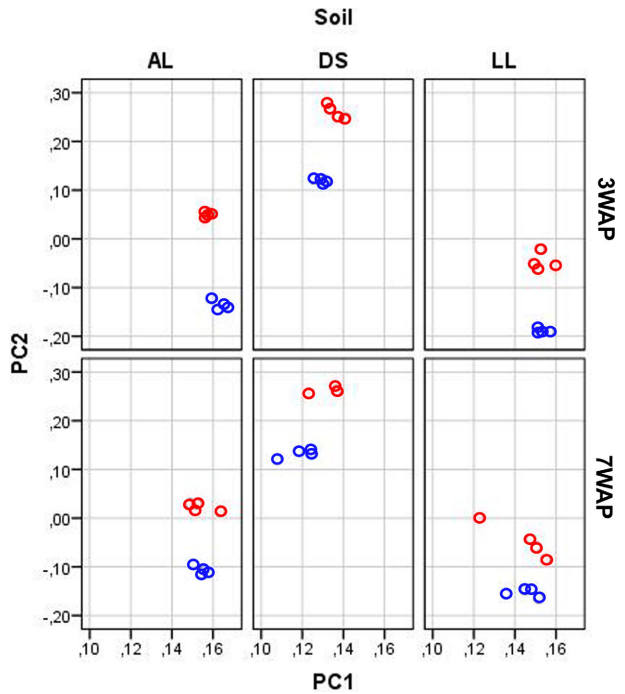

FIGURE 2 | Principal component analysis of the bacterial community composition according to pyrosequencing data. The relative abundance of OTUs (log transformed) in samples from rhizosphere (red circles) and bulk soil (blue circles) taken 3 and 7 weeks after planting (3WAP, 7WAP) of lettuce in soils DS, AL, and LL were compared. The first and second principal components are shown which explained 47 and 9% of the total variance, respectively. The data were analyzed together, but for clarity separated plots for each soil type and sampling are shown.

and bulk soil at both samplings. In soil LL the communities from rhizosphere and bulk soil were more closely related than in the other two soils (**Figure 2**).

SOIL TYPE DEPENDENT AND INDEPENDENT RESPONDERS IN THE LETTUCE RHIZOSPHERE

The discriminative taxa between the soil types were identified by Tukey's honest significance test under a generalized linear model via logistic function for binomial data.

Twenty-four genera were significantly increased in abundance in the rhizosphere compared to the corresponding bulk soil at least in one of the soils 3WAP (**Table 3**). The highest number of rhizosphere responders was detected in AL (15) followed by DS (13) and LL (11) soils. The relative abundances of the genera *Sphingomonas, Rhizobium, Pseudomonas, Variovorax,* and *Flavobacterium* were enriched in the rhizosphere of lettuce grown in all three soils 3WAP. At this sampling time, the genus *Rhizobium* showed the strongest relative enrichment in the rhizosphere in DS and AL soil, in the LL soil *Rhizobium* was not detected in the bulk soil but increased to 1.2% in the rhizosphere. Several rhizosphere responders were soil type specific. Furthermore, 3WAP the highest number of soil type specific rhizosphere responders was observed in AL (*Adheribacter*, *Burkholderia*, *Chitinophaga*, *Flavisolibacter*, *Pedobacter, Phenylobacterium*, *Pontibacter,* TM7) followed by

Table 3 | Enriched genera in the rhizosphere of lettuce 3 weeks after planting.

Genus	DS		AL		LL	
	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere
Acidovorax	0.0 ± 0	1.5 ± 0*	0.0 ± 0	0.2 ± 0	0.0 ± 0	0.6 ± 0*
Acinetobacter	0.0 ± 0	0.2 ± 0	0.0 ± 0	0.7 ± 1*	0.0 ± 0	0.7 ± 1*
Adhaeribacter	0.2 ± 0	0.2 ± 0	0.6 ± 0	1 ± 0*	0.5 ± 0	0.5 ± 0
Brevundimonas	0.1 ± 0	0.3 ± 0*	0.0 ± 0	0.1 ± 0	0.0 ± 0	0.2 ± 0
Burkholderia	0.0 ± 0	0.1 ± 0	0.0 ± 0	0.8 ± 0*	0.0 ± 0	0.0 ± 0
Chitinophaga	0.1 ± 0	0.0 ± 0	0.0 ± 0	0.3 ± 0*	0.0 ± 0	0.1 ± 0
Devosia	0.2 ± 0	0.6 ± 0*	0.0 ± 0	0.3 ± 0	0.0 ± 0	0.5 ± 0*
Dyadobacter	0.0 ± 0	0.4 ± 0*	0.0 ± 0	0.1 ± 0	0.0 ± 0	0.3 ± 0
Flavisolibacter	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.5 ± 0*	0.2 ± 0	0.1 ± 0
Flavobacterium	0.0 ± 0	0.3 ± 0*	0.1 ± 0	0.3 ± 0*	0.1 ± 0	0.5 ± 0*
Hydrogenophaga	0.0 ± 0	0.5 ± 0*	0.0 ± 0	0.2 ± 0	0 ± 0	0.3 ± 0
Methylibium	0.1 ± 0	0.5 ± 0*	0.2 ± 0	0.4 ± 0	0.2 ± 0	0.1 ± 0
Novosphingobium	0.0 ± 0	0.1 ± 0	0.1 ± 0	0.4 ± 0*	0.1 ± 0	0.4 ± 0*
Paenibacillus	2.1 ± 0	1.8 ± 0	1.7 ± 0	1.5 ± 0	1.9 ± 0	3.6 ± 1*
Pedobacter	0.2 ± 0	0.3 ± 0	0.0 ± 0	0.4 ± 0*	0.2 ± 0	0.3 ± 0
Phenylobacterium	0.2 ± 0	0.5 ± 0	0.2 ± 0	0.4 ± 0*	0.1 ± 0	0.3 ± 0
Pontibacter	0.0 ± 0	0.0 ± 0	0.3 ± 0	0.7 ± 0*	1 ± 0	1.4 ± 0
Pseudomonas	0.1 ± 0	0.4 ± 0*	0.1 ± 0	0.7 ± 0*	0.2 ± 0	0.8 ± 0*
Ramlibacter	0.1 ± 0	0.5 ± 0*	0.1 ± 0	0.3 ± 0	0.1 ± 0	0.3 ± 0*
Rhizobium	0.1 ± 0	1.5 ± 0*	0.1 ± 0	2.0 ± 0*	0.0 ± 0	1.2 ± 0*
Rubellimicrobium	0.2 ± 0	0.7 ± 0*	0.1 ± 0	0.1 ± 0	0.0 ± 0	0.1 ± 0
Sphingomonas	2.2 ± 0	8.2 ± 1*	2.2 ± 0	8.9 ± 1*	1.7 ± 0	4.7 ± 1*
TM7_genera_incertae_sedis	0.1 ± 0	0.3 ± 0	0.1 ± 0	0.4 ± 0*	0.1 ± 0	0.4 ± 0
Variovorax	0.0 ± 0	0.3 ± 0*	0.1 ± 0	0.6 ± 0*	0.0 ± 0	0.5 ± 0*

The relative abundance of genera in the rhizosphere of lettuce, grown in the three soil types (diluvial sand, DS; alluvial loam, AL and loess loam, LL), was compared with the corresponding bulk soil. Percent abundance of genera ±SD is shown. The asterisks indicate significantly enriched genera in the rhizosphere identified by Tukey's honest test under a generalized linear model via logistic function for binomial data ($P \leq 0.05$). Genera in bold letters indicate enrichment in all three soil types.

DS (*Brevundimonas, Dyadobacter, Hydrogenophaga, Methylibium*, *Rubellimicrobium*) and LL soil (*Paenibacillus*). Several rhizosphere responders were only detected in two of the three soils. Thus, *Acidovorax*, *Devosia,* and *Ramlibacter* were only significantly enriched in DS and LL rhizospheres and *Acinetobacter* and *Novosphingobium* in AL and LL rhizospheres (**Table 3**). The number of rhizosphere responders identified 7WAP was lower compared to 3WAP as only 12 genera were found to be significantly increased in abundance in the rhizosphere of lettuce compared to the corresponding bulk soil (**Table 4**). The highest number of rhizosphere responders was detected in AL (9) and LL (8) followed by DS rhizosphere (7). Again, *Sphingomonas, Rhizobium,* and *Variovorax* were enriched in all three soil types. In addition *Acidovorax* and *Methylophilus* were enriched in the rhizosphere of lettuce grown in all three soil types (**Table 4**). *Pseudomonas* was only enriched in the DS and AL soils 7WAP, while *Burkholderia* was detected as rhizosphere responders in AL and LL soils. *Flavobacterium* and *Acinetobacter* were specific rhizosphere responders for AL soils, *Mesorhizobium* was specific for DS soil while *Caulobacter* and *Devosia* were rhizosphere responders in LL soil.

ONLY A SUBSET OF DOMINANT OTUs WAS ENRICHED IN THE RHIZOSPHERE OF LETTUCE

For analysis at the OTU level the OTUs with an abundance of more than 0.5% were selected (**Figures 3**, **4**). Thirty-four of the dominant OTUs in the rhizosphere taken 3WAP were affiliated to *Proteobacteria* (34/50) with the vast majority being *Alphaproteobacteria* (28/34) followed by the phyla *Actinobacteria* (9/50), *Firmicutes* (4/50), and *Bacteriodetes* (3/50). At the later sampling time (7WAP) 32 of the 45 most dominant OTUs were affiliated also to the *Proteobacteria* (32/45) with the majority being *Alphaproteobacteria* (23/32) followed by the phyla *Firmicutes* (7/45) and *Actinobacteria* (4/45). The relative abundances of 27 of the 50 dominant OTUs were significantly enriched in the rhizosphere 3WAP whereas 7WAP less OTUs were enriched.

Strong rhizosphere responders 3WAP displayed a high sequence identity with isolates *Sphingomonas* sp. (OTU 1894), *Sphingomonas suberifaciens* (OTU 413), *Alkanindiges hongkonggensis* (OTU 3170), *Rhizobium radiobacter* (OTU 1642), and *Sphingobium* sp. (OTU 23993) (**Figure 3**). Strong responders at the later sampling time (7WAP) were *Sphingomonas* sp. (OTU 25625), *Burkholderia* sp. (OTU 22450), *Novosphingobium* sp.

Table 4 | Enriched genera in the rhizosphere of lettuce 7 weeks after planting.

Genus	DS		AL		LL	
	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere
Acidovorax	0.0 ± 0	1.0 ± 0*	0.0 ± 0	0.6 ± 0*	0.0 ± 0	0.5 ± 0*
Acinetobacter	0.1 ± 0	0.1 ± 0	0.0 ± 0	0.9 ± 1*	0.3 ± 0	0.0 ± 0
Burkholderia	0.0 ± 0	0.1 ± 0	0.0 ± 0	2.0 ± 1*	0.0 ± 0	0.6 ± 0*
Caulobacter	0.0 ± 0	0.4 ± 0	0.0 ± 0	0.2 ± 0	0.0 ± 0	0.2 ± 0*
Devosia	0.1 ± 0	0.3 ± 0	0.1 ± 0	0.1 ± 0	0.0 ± 0	0.4 ± 0*
Flavobacterium	0.1 ± 0	0.2 ± 0	0.3 ± 0	1.1 ± 1*	0.6 ± 0	0.5 ± 0
Mesorhizobium	0.1 ± 0	0.6 ± 0*	0.2 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0
Methylophilus	0.0 ± 0	0.4 ± 0*	0.0 ± 0	0.6 ± 0*	0.0 ± 0	0.4 ± 0*
Pseudomonas	0.3 ± 1	0.7 ± 0*	0.3 ± 0	1.7 ± 1*	2.0 ± 2	0.5 ± 0
Rhizobium	0.1 ± 0	1.4 ± 0*	0.1 ± 0	0.7 ± 0*	0.2 ± 0	0.9 ± 0*
Sphingomonas	3.2 ± 1	14.0 ± 3*	3.0 ± 0	5.8 ± 0*	2.2 ± 0	4.1 ± 0*
Variovorax	0.0 ± 0	0.6 ± 0*	0.1 ± 0	0.5 ± 0*	0.2 ± 0	0.6 ± 1*

The relative abundance of genera in the rhizosphere of lettuce, grown in the three soil types (diluvial sand, DS; alluvial loam, AL and loess loam, LL), was compared with the corresponding bulk soil. Percent abundance of genera ±SD is shown. The asterisks indicate significantly enriched genera in the rhizosphere identified by Tukey's honest test under a generalized linear model via logistic function for binomial data ($P \leq 0.05$). Genera in bold letters indicate enrichment in all three soil types.

(OTU 13685), *Alkanindiges hongkonggensis* (OTU 16000), and *Sphingobium* sp. (OTU 5735) (**Figure 4**).

Several OTUs showed a soil type specific occurrence 3WAP, e.g., *Rubrobacteridae* (OTU 17580), *Nocardioides* sp. (OTU 15453), *Bacillus* sp. (OTU 7878), and *Paenibacillus* (OTU 2139) were less abundant in the DS soil compared to AL and LL soil (**Figure 3**). OTU 13568 which displayed 100% sequence identity to *Marmoricola* sp. was detected only in DS rhizosphere and bulk soil. The OTU 637 identified as *Acidovorax facilis* was only detected in DS rhizosphere samples (**Figure 3**).

Several soil type specific OTUs were detected among the dominant OTUs 7WAP, e.g., *Pelobacter* (OTU 12696) which was less abundant in DS soil and *Ramlibacter* sp. (OTU 4780) which was only detected in DS soil (**Figure 4**).

DISCUSSION

Our study showed that different soil types exposed to identical cropping history and agricultural management for about 10 years and identical climatic conditions for more than 30 years still displayed a soil type dependent bacterial diversity. Although the soil type was identified as a major factor shaping composition of microbial communities in the rhizosphere in many previous studies, this study showed the importance of the soil type under field conditions. The same main dominant phyla were detected in the three soils, but significant differences in the bacterial community composition among the soils were detected by both DGGE and pyrosequencing analyses of 16S rRNA gene fragments amplified from TC-DNA. UPGMA analysis of DGGE fingerprints and of OTUs as well as the principal component analysis indicated a higher similarity of AL and LL soils compared to DS soil which corresponded to the more similar soil characteristics of the AL and LL soils for many parameters including pH, electric conductivity, fine grained particles, total, and organic C, N, P, and all metal ions measured (Table S1). All these parameters were lower in the DS soil. Recently, Kuramae et al. (2012) reported that several soil bacterial taxa were strongly correlated to physicochemical soil characteristics.

The experimental set-up allowed for the first time to determine the effect of the soil type on the lettuce rhizosphere bacterial community composition under field conditions. However, we could not pinpoint specific soil properties responsible for these differences as the soil properties were determined before the field experiment. The statistical analysis of both DGGE and pyrosequencing data sets revealed significant differences in the bacterial community composition between the rhizosphere of the three soils. In particular, for AL and DS the bacterial community composition became more similar in the rhizosphere compared to bulk soil (**Table 1**, **Figure 1**, Figure S3), while for soil LL the rhizosphere community was more similar to that of the corresponding bulk soil than to the rhizosphere communities of AL and DS (**Figure 2**). The data indicate that depending on the soil type the rhizosphere was differently shaped by lettuce growth. Soil type dependent composition of bacterial communities in the rhizosphere of *Arabidopsis thaliana* grown under greenhouse conditions in different soils was also observed by Bulgarelli et al. (2012) and Lundberg et al. (2012). Although the same phyla were reported in the rhizosphere of different plants (Uroz et al., 2010; Bulgarelli et al., 2012; Lundberg et al., 2012; Dohrmann et al., 2013) their relative abundances substantially differed.

Remarkably, in all three soil types similar shifts in the relative abundance of the major phyla were detected in response to lettuce growth which might be attributed to the root exudates and deposits of lettuce. Root exudates were investigated in parallel with the same lettuce cultivar "Tizian" planted in DS, AL, or LL soil in minirhizotron systems which were equipped with root observation windows (Neumann et al., 2014). The GC-MS analysis revealed qualitatively similar root exudates which quantitatively differed depending on the soil type. Thus, the

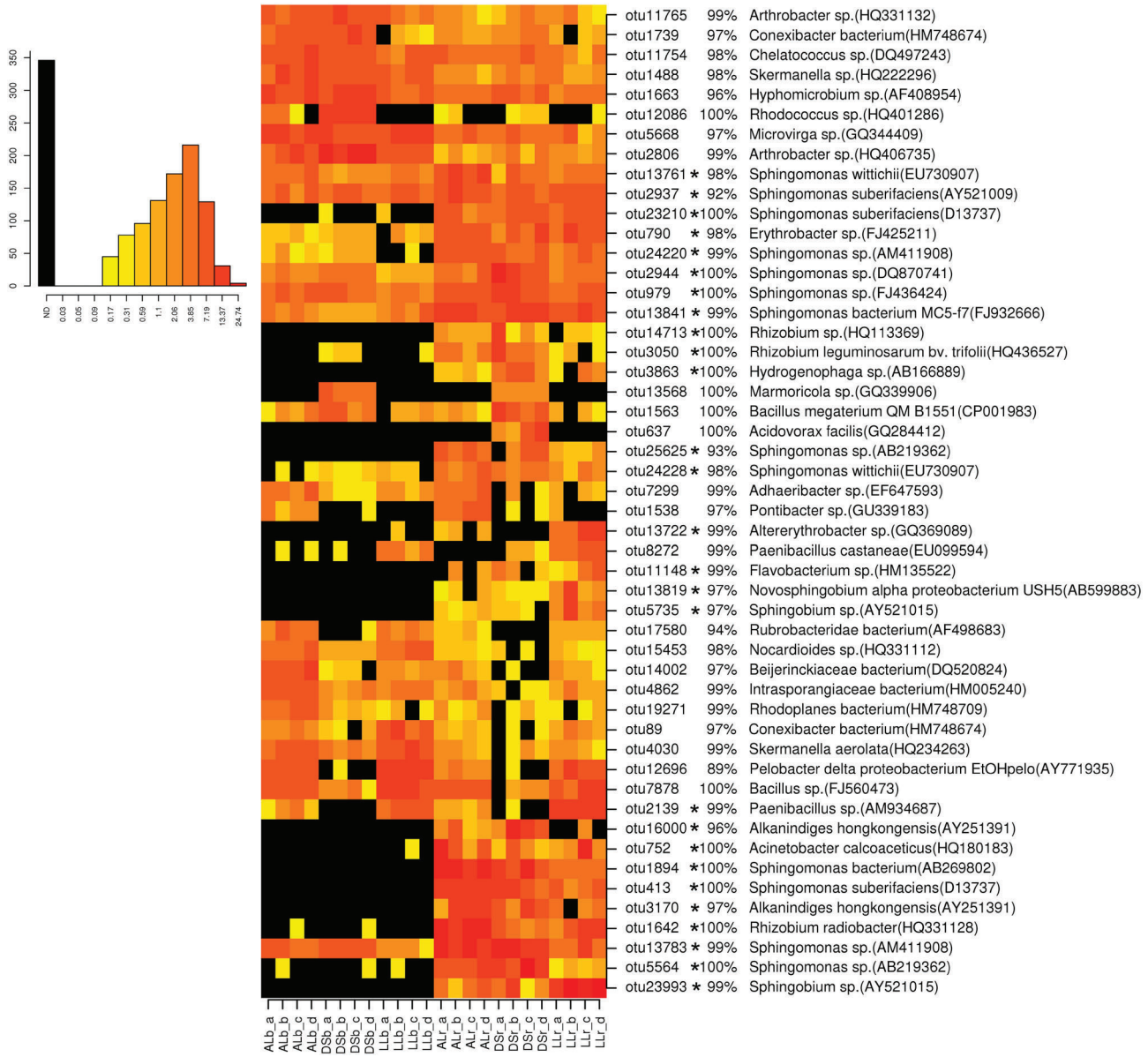

FIGURE 3 | Relative abundance of the most dominant OTUs detected 3 weeks after planting. The heatmap indicates differences in the relative abundances of OTUs in the bulk soil and rhizosphere from lettuce, and between soil types DS, AL, and LL. The vertical columns represent one sample, horizontal rows depict OTUs. The color code grades from black (not detected) over yellow (low abundance), orange (medium abundance) to red (high abundance). Numbers in brackets indicate the number of the NCBI GenBank accession that was most similar to the OTU representative sequence. A strong increase in abundance was indicated by not detecting the OTU in the bulk soil (black) or only in one or two samples present (yellow), raising to a high abundance in the rhizosphere which was indicated by orange to red color. Asterisks indicate a significantly increased abundance of that OTU in the rhizosphere compared to bulk soil.

relative abundance of OTUs affiliated to *Proteobacteria* almost doubled in DS, AL, and LL soil at both sampling times indicating that bacterial populations with copiotrophic lifestyle belonging to the *Proteobacteria* were able to utilize the exudates and deposits provided by lettuce roots. The proportion of most proteobacterial classes was increased in the rhizosphere in all three soil types except for *Deltaproteobacteria*. An increased abundance of *Proteobacteria* in the rhizosphere compared to bulk soils was reported in several recent studies based on 16S rRNA amplicon sequencing (Bulgarelli et al., 2012; Lundberg et al., 2012). The proportion of *Actinobacteria* and *Firmicutes* in the rhizosphere of lettuce decreased in all three soil types. Several OTUs that belonged to these phyla were similarly abundant in both rhizosphere and bulk soil, while an enrichment in the rhizosphere was not observed (**Figures 3**, **4**). For a long time it has been assumed that microbial community composition and function

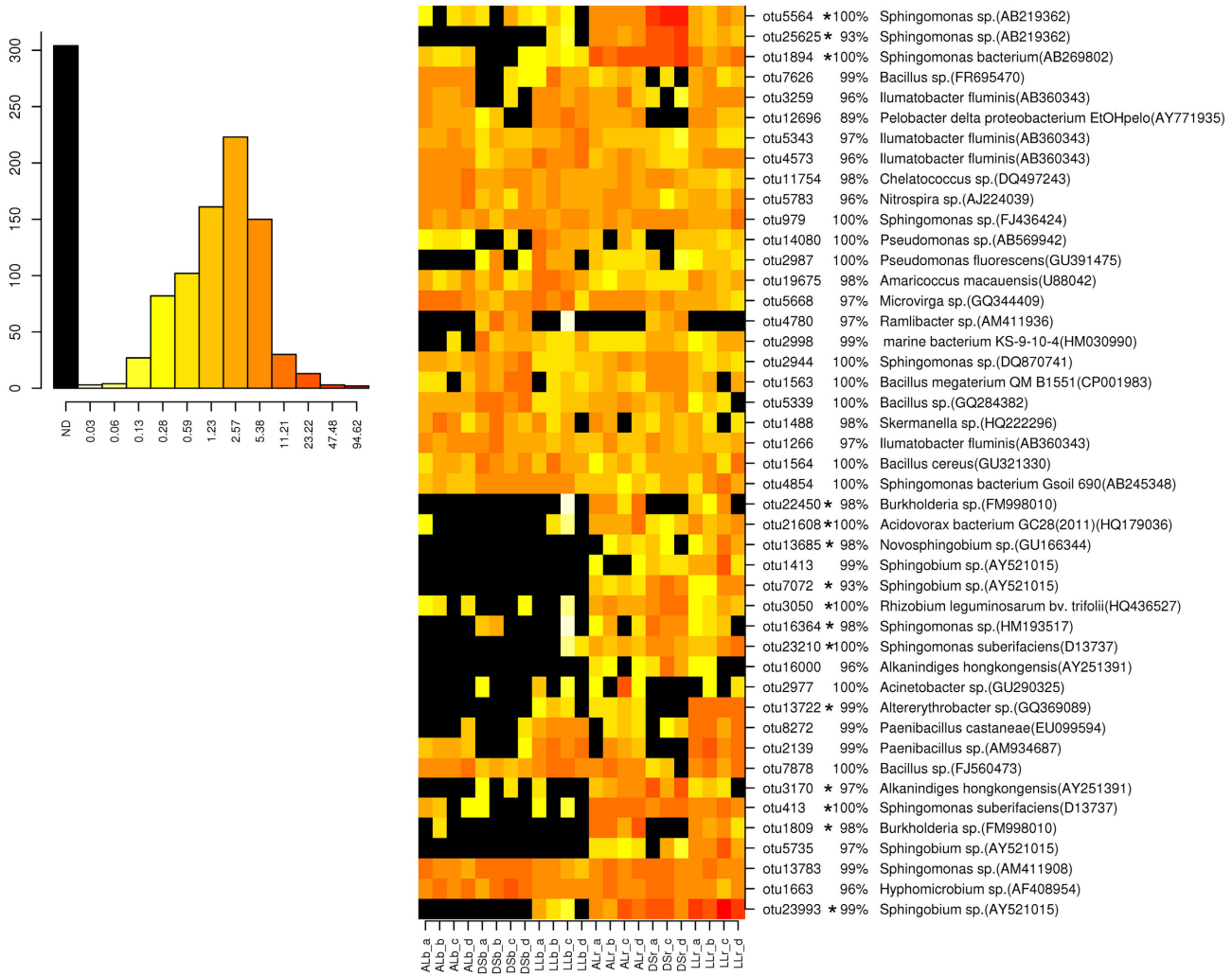

FIGURE 4 | Relative abundance of the most dominant OTUs detected 7 weeks after planting. The heatmap indicates differences in the relative abundances of OTUs in the bulk soil and rhizosphere from lettuce, and between soil types DS, AL, and LL. The vertical columns represent one sample, horizontal rows depict OTU. The color code grades from black (not detected) over yellow (low abundance), orange (medium abundance) to red (high abundance). Numbers in brackets indicate the number of the NCBI GenBank accession that was most similar to the OTU representative sequence. A strong increase in abundance was indicated by not detecting the OTU in the bulk soil (black) or only in one or two samples present (yellow), raising to a high abundance in the rhizosphere which was indicated by orange to red color. Asterisks indicate a significantly increased abundance of that OTU in the rhizosphere compared to bulk soil.

in the rhizosphere are tightly linked to the root exudation patterns (Brimecombe et al., 2001) and that changes in the exudates composition result in dramatic changes of the soil microbial community composition. Root exudation patterns were assumed to be affected by the plant growth developmental stage (Baudoin et al., 2002) and several biotic and abiotic factors. Root secretion of some proteins was shown to be changed with plant development or when challenged with pathogenic or symbiotic bacteria. But only recently, Chaparro et al. (2013) could show how root-secreted primary and secondary plant metabolites change during plant growth development, and pyrosequencing of mRNA revealed a tight link with microbial functions involved in metabolism of the root exudates. In the present study changes in the bacterial community composition likely related to plant development were observed by both methods employed for 16S rRNA amplicon analysis. Shifts in the bacterial community composition in lettuce rhizosphere during plant growth were previously also reported by Adesina et al. (2009) and Chowdhury et al. (2013). Both the *d*-values obtained from the statistical analyses of DGGE fingerprints and pyrosequencing data set as well as the number of so-called responders to the lettuce root exudates which was higher 3WAP compared to 7WAP indicated that the rhizosphere effect was stronger at the earlier time point. Although the root length and exudation patterns of lettuce plants (cultivar "Tizian") grown in DS, AL, and LL soil under rhizotron conditions differed significantly (Neumann et al., 2014), similar genera were selected in the rhizosphere of lettuce plants of all soil types. Several genera which were significantly enriched in

the rhizosphere of lettuce grown in all three soils (**Tables 3**, **4**) were previously reported as degraders of aromatic hydrocarbons or pesticides with aromatic ring structures such as *Sphingomonas*, *Pseudomonas,* and *Variovorax* (Bers et al., 2011; Ding et al., 2012a). Although the abundance of these genera significantly increased in the rhizosphere of lettuce grown in all three soils, the extent of enrichment seemed to be different in particular for the genus *Sphingomonas* that showed a remarkably increased abundance in DS soil followed by AL and LL. The comparative analysis of the relative abundance of the most dominant OTUs in the rhizosphere and in bulk soil showed that enrichment in the rhizosphere seemed to be species- or even strain-dependent as only some of the OTUs affiliated to *Sphingomonas* were strongly enriched (**Figure 3**). We could previously also demonstrate an enrichment of IncP-1 plasmids in the rhizosphere of lettuce in all three soils which was particularly pronounced for DS soil (Jechalke et al., 2014). IncP-1 plasmids carry frequently genes encoding degradative functions. Thus, it is tempting to speculate that *Sphingomonas* might be the host of these IncP-1 plasmids. Also strains of other genera such as *Burkholderia*, *Novosphingobium*, and *Acinetobacter* that are possibly involved in the degradation of aromatic ring structures were identified as rhizosphere responders 7WAP (**Figure 4**). This nicely correlated with the detection of benzoic acid in the root exudates collected from lettuce plants grown in DS, AL, and LL soil under rhizotron conditions (Neumann et al., 2014). Benzoic acid was also previously reported in root exudates of lettuce grown in hydroponics (Lee et al., 2006). In the rhizotron experiment performed by Neumann et al. (2014) with lettuce grown in DS, AL, and LL soils, a strong effect of the soil type was detected not only on the quantitative composition of root exudates but also on root biomass production and root length. Total root length in AL, mainly represented by fine roots of 0–0.4 mm diameter (70% of total root length) was about two and a half times higher as compared with DS, and even four times higher than in LL soil. In general, mainly quantitative differences in the exudate profiles were detected (Neumann et al., 2014) which might explain that several similar responders to lettuce growth were identified based on the pyrosequencing data set (**Tables 3**, **4**; **Figures 3**, **4**). Another member of the *Alphaproteobacteria*, the genus *Rhizobium,* known for specific interactions with host plants was found to be enriched in the rhizosphere of lettuce independent from the soil type and at both sampling times. Interestingly, the genus *Acidovorax* was found to be enriched in the rhizosphere independently from the soil type (**Tables 3**, **4**). Several species belonging to the genus *Acidovorax* are known plant pathogens (Thiele et al., 2012). However, the differentiation between *Variovorax,* often involved in the degradation of aromatic compounds, and *Acidovorax* is complicated based on 16S rRNA gene sequence analysis (Bers et al., 2011). The identification of similar but also soil type specific responders in different soils likely provides insights into populations triggered by root exudates. Whether lettuce plant exudates deliberately increase the abundance of particular populations or whether the shifts in the bacterial community composition are merely due to the nutrients provided remains to be shown. Our data clearly demonstrated the strong effects of lettuce growth on the bacterial community composition in the rhizosphere in all three soils as revealed by analyzing DGGE and pyrosequencing data. Pyrosequencing confirmed the reduced diversity in the rhizosphere as previously assumed based on DGGE fingerprints and provided insights into the taxonomic affiliation of rhizosphere responders which were shared by all soils or which were specific to particular soil types. DGGE and pyrosequencing data indicated that lettuce grown in AL soil overall had the strongest rhizosphere effect which correlated with the highest root biomass observed in the study by Neumann et al. (2014) and to the highest shoot dry mass observed under field conditions. Remarkably, many dominant OTUs (**Figures 3**, **4**) were detected in all three soil types and differed mainly in their relative abundance which nicely corresponds to the high number of bands shared among the bacterial community fingerprints of all three soils (Figures S3, S4). The heatmap analysis also indicated that several OTUs which were not detected at all in the bulk soil became detectable after their enrichment in the rhizosphere.

Although 16S rRNA gene sequences-based conclusions on potential functions are problematic (Eltlbany et al., 2012), we have noticed that many genera and OTUs were enriched in the rhizosphere known for their involvement in the degradation of aromatic compounds. Similar responders were observed in response to phenanthrene pollution from two soil types as revealed by pyrosequencing of 16S rRNA gene fragments from soil TC-DNA (Ding et al., 2012a). In conclusion, the present study revealed that three different soil types exposed for more than 10 years to the same climatic conditions and cropping history still displayed distinct bacterial community compositions. Pyrosequencing analysis of 16S rRNA gene amplicons largely confirmed the DGGE data but provided more quantitative data and taxonomic information on the bacterial community composition and information on main responders to the lettuce growth at two time points of plant development. The present study showed under field conditions that both the plant as well as the soil type shape the bacterial community composition in the rhizosphere. Several rhizosphere responders were detected independently from the soil type indicating taxa which are likely enriched in the rhizosphere and thus might allow predictions on the soil type dependent rhizosphere competence of inoculant strains.

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SUPPLEMENTARY MATERIAL

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Effect of the strain *Bacillus amyloliquefaciens* FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing

Magdalena Kröber[1], Daniel Wibberg[1], Rita Grosch[2], Felix Eikmeyer[1], Bart Verwaaijen[1], Soumitra P. Chowdhury[3], Anton Hartmann[3], Alfred Pühler[1] and Andreas Schlüter[1]*

[1] Center for Biotechnology, Institute for Genome Research and Systems Biology, Bielefeld University, Bielefeld, Germany
[2] Leibniz-Institute of Vegetable and Ornamental Crops, Großbeeren, Germany
[3] Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany

Edited by:
Michael Schloter, Helmholtz Zentrum München, Germany

Reviewed by:
Leo Van Overbeek, W-UR Plant Research International, Netherlands
David John Studholme, University of Exeter, UK
Anja Bettina Dohrmann, Johann Heinrich von Thünen Institute, Germany

***Correspondence:**
Andreas Schlüter, Center for Biotechnology, Institute for Genome Research and Systems Biology, Universität Bielefeld, Universitätsstr. 27, 33615 Bielefeld, Germany
e-mail: aschluet@cebitec.uni-bielefeld.de

Application of the plant associated bacterium *Bacillus amyloliquefaciens* FZB42 on lettuce (*Lactuca sativa*) confirmed its capability to promote plant growth and health by reducing disease severity (DS) caused by the phytopathogenic fungus *Rhizoctonia solani*. Therefore this strain is commercially applied as an eco-friendly plant protective agent. It is able to produce cyclic lipopeptides (CLP) and polyketides featuring antifungal and antibacterial properties. Production of these secondary metabolites led to the question of a possible impact of strain FZB42 on the composition of microbial rhizosphere communities after its application. Rating of DS and lettuce growth during a field trial confirmed the positive impact of strain FZB42 on the health of the host plant. To verify *B. amyloliquefaciens* as an environmentally compatible plant protective agent, its effect on the indigenous rhizosphere community was analyzed by metagenome sequencing. Rhizosphere microbial communities of lettuce treated with *B. amyloliquefaciens* FZB42 and non-treated plants were profiled by high-throughput metagenome sequencing of whole community DNA. Fragment recruitments of metagenome sequence reads on the genome sequence of *B. amyloliquefaciens* FZB42 proved the presence of the strain in the rhizosphere over 5 weeks of the field trial. Comparison of taxonomic community profiles only revealed marginal changes after application of strain FZB42. The orders *Burkholderiales, Actinomycetales* and *Rhizobiales* were most abundant in all samples. Depending on plant age a general shift within the composition of the microbial communities that was independent of the application of strain FZB42 was observed. In addition to the taxonomic profiling, functional analysis of annotated sequences revealed no major differences between samples regarding application of the inoculant strain.

Keywords: metagenome sequencing, taxonomic profiling, *B. amyloliquefaciens* FZB42, lettuce, rhizosphere, fragment recruitment

INTRODUCTION

Phytopathogenic organisms are a serious threat to food production and storage affecting crop plants and vegetables. Crop losses of approximately 20% and more were caused by plant pathogens worldwide depending on the particular crop (Oerke, 2006). Crop rotation, breeding of resistant cultivars and the use of chemical pesticides are the preferred approaches to reduce the effects of phytopathogens. However, these strategies are insufficient, especially to control root diseases caused by soil-borne plant pathogens of economically important crops (Johri et al., 2003). Additionally, intensive crop management practices increase the problems concerning accumulation of pathogens in the soil (Chellemi, 2002). The facts that excessive use of chemical pesticides caused several environmental problems like soil pollution (Asaka and Shoda, 1996) and that society is becoming more conscious about the environment support the development of alternative control strategies worldwide (Leistra and Matser, 2004; Wang et al., 2004; Alabouvette et al., 2006). Currently, it is well documented that the treatment of plants with plant-associated microorganisms can be effective to decrease the negative impact brought about by infections of the plants by pests (Kazempour, 2004; Scherwinski et al., 2008; Andrews et al., 2012).

Several products based on the application of plant-associated bacteria such as *Bacillus* spp. are commercially available (Choudhary and Johri, 2009). The ability of members of the Gram-positive genus *Bacillus* to form spores is advantageous for preparation of bacterial formulations, storage and product shelf life (Tiago et al., 2004). The effectiveness of these strains relies on different mechanisms. Efficient strains colonize the root system of

the host plant, promote plant growth and are able to activate their defense system. This phenomenon of triggering the systemic resistance reaction of the plant has been termed rhizobacteria-induced systemic resistance (ISR) (Arguelles-Arias et al., 2009). Another mechanism that contributes to plant protection is the secretion of different secondary metabolites with antibacterial or antifungal impact on phytopathogenic organisms. In addition, the competition for nutrients and especially iron between biocontrol strains and pathogens is an important factor in the protection of the plant (Arguelles-Arias et al., 2009).

Bacillus amyloliquefaciens FZB42 is known for its plant growth promoting properties and its ability to suppress different plant pathogens (Grosch et al., 1999; Idriss et al., 2004; Koumoutsi et al., 2007). Pot and field experiments demonstrated that the strain FZB42 is able to effectively colonize the rhizosphere of lettuce during host plant cultivation and promotes significant suppression of bottom rot disease caused by *Rhizoctonia solani* (Chowdhury et al., 2013). High rhizosphere competence referring to the survival and rhizosphere colonization potential (Bulgarelli et al., 2013) is a key factor for a successful improvement of plant traits and control of plant pathogens by plant-protective agents (Lugtenberg and Kamilova, 2009). Although the commercially available strain FZB42 is a beneficial bacterium, its application may have a negative impact on the indigenous microbial rhizosphere community and associated important ecological functions (Winding et al., 2004). Genome analyses of *B. amyloliquefaciens* revealed that the organism harbors eight different gene clusters comprising genes associated with the production of secondary metabolites providing it with its biocontrol properties (Chen et al., 2007). These secondary metabolites can be classified into three different categories: the polyketides macrolactin (*mln*), bacillaene (*bae*), and difficidin (*dfn*) featuring antibacterial properties (Chen et al., 2006), the cyclic lipopeptides surfactin (*srf*), fengycin (*fen*), and bacillomycin (*bmy*) providing mainly antifungal properties (Koumoutsi et al., 2004) and the third category including the iron-siderophore bacillibactin (*bac*) and a putative siderophore the synthesis of which is encoded by the *nrs* operon.

Due to the high potential of producing secondary metabolites and their possible impact on other microorganisms within the microbial community in the rhizosphere of host plants, the complex interaction between an inoculant strain and the indigenous microbial rhizosphere community has to be analyzed. Knowledge regarding the microbial ecology of the target habitat "rhizosphere" is required for reasonable risk assessment studies related to the application of beneficial plant associated bacterial strains.

To increase the knowledge about the interaction of the strain FZB42 with indigenous rhizosphere community members of lettuce, field trials were carried out at natural occurring pathogen pressure in the field and at higher pathogen pressure achieved by inoculation of the bottom rot pathogen *R. solani* at the same field site (Chowdhury et al., 2013). During the growth period of lettuce whole community DNA samples were isolated from the rhizospheres of lettuce that either were treated with the strain FZB42 (1 week before planting and 4 days after planting) or not treated. Samples from both experiments were collected at planting, 2 weeks and 5 weeks after planting. Analysis of DNA samples from these field trials after 2 and 5 weeks of cultivation of the host plants by 16S rRNA gene amplicon terminal restriction fragment length polymorphism (T-RFLP) revealed no major impact on the general composition of the microbial community regarding the application of the inoculant strain. However, a temporal shift independent from the application of strain FZB42 was observed (Chowdhury et al., 2013).

In contrast to T-RFLP analyses, metagenome studies by means of whole community DNA extraction, high-throughput sequencing and various kinds of sequence analyses applying bioinformatic tools are exceptionally convenient strategies to provide an extended insight into the whole microbial community within a habitat e.g., the rhizosphere. MG-RAST is an established platform to analyze and compare metagenome sequence data in several approaches (Meyer et al., 2008). Within MG-RAST, taxonomic profiling of the community based on metagenome sequences is performed using the NCBI GenBank database comprising sequences of more than 380,000 species. It therefore provides an exceptionally suitable basis for the annotation of metagenome sequences (Benson et al., 2011). Additionally to taxonomic classifications, the MG-RAST software platform provides the possibility to compare functional subsystems based on the SEED resource (http://www.theSEED.org), (Aziz et al., 2008; Overbeek et al., 2014).

To obtain deeper and more refined insights into microbial rhizosphere communities of lettuce, the same DNA samples as for the T-RFLP analysis described above (Chowdhury et al., 2013) were used in a comparative whole metagenome sequencing approach to follow up changes in the taxonomic community profiles during the growth period of lettuce and to identify dominant rhizosphere microorganisms. In addition to the samples used in the 16S rRNA T-RFLP analysis, DNA samples isolated from the rhizosphere at planting 1 week after the first application of strain FZB42 were analyzed by metagenome sequencing in order to reveal possible shifts in the microbial community within the first 2 weeks of cultivation in the field. Previous field trials showed that during this time infections with the phytopathogen *R. solani* are most likely to occur (Grosch et al., 2004).

Another objective of this study was to elucidate the competitiveness and survivability of the inoculant strain FZB42 within the bacterial community in the course of host plant cultivation. The ability of *B. amyloliquefaciens* FZB42 to successfully colonize the rhizosphere is an assumption for reducing disease severity (DS) caused by phytopathogens (Lugtenberg and Kamilova, 2009; Barret et al., 2011; Ghirardi et al., 2012). Expression of genes conferring antifungal activity often is regulated in a cell-density dependent manner (Steidle et al., 2002) and hence corresponding microorganisms should be highly competent in rhizosphere colonization to exert antifungal activity. Proving the establishment of this strain in the rhizosphere of lettuce therefore is the prerequisite for the following analyses addressing alterations in the community profiles between with FZB42 treated and non-treated samples. Therefore, fragment recruitments were performed in which metagenome sequence reads were mapped to the known genome sequence of *B. amyloliquefaciens* FZB42 under strict

settings in order to track the occurrence of this strain in the rhizosphere.

In addition to taxonomic profiling, whole metagenome sequencing also allows functional characterization of the rhizosphere community. Studies of microbial rhizosphere communities of different plant species showed that their roots are colonized by only a few dominating phyla (Bulgarelli et al., 2013; Chaparro et al., 2013). These show a common set of metabolic functions connected to genes involved in carbohydrate, nitrogen and amino acid metabolism. This observation has led to the assumption that specific functions are essential for a successful colonization of this habitat during the growth of the plants. The treatment of plants with *B. amyloliquefaciens* FZB42 and its secretion of secondary metabolites with antimicrobial properties potentially may lead to shifts within rhizosphere microbial functional subsystems affecting e.g., availability of nutrients for the plant and other important properties in the rhizosphere. Furthermore, the functional subsystem of virulence and defense mechanisms was analyzed since alterations within this category may be caused by application of the inoculant strain FZB42.

MATERIALS AND METHODS

FIELD TRIAL AND PREPARATION OF DNA SAMPLES

A field trial was carried out at the Institute of Vegetable and Ornamental Crops (Golzow, Germany, 52° 34′ N, 14° 30′ E) to determine the ability of *B. amyloliquefaciens* FZB42 to colonize the rhizosphere of lettuce plants (cv. Tizian, Syngenta, Bad Salzuflen, Germany) and the potential impact of the treatment with this strain on the composition of the microbial community within this habitat. Prior to the cultivation in the field the young plants were grown in peat blocks at 20/15°C (16/8 h, day/night cycle) and were then transferred in the 3–4 leaf state to the field with alluvial loam (total N 112; P 32.3; K 17.4; and Mg 9.1 mg/100 g soil; pH 6.5). Lettuce plants were first treated with FZB42 in the 2–3-leaf stage 1 week before planting into the field beds. Each seedling tray with 150 plants was watered with 1.74 l spore suspension (10^7 CFU/ml) of FZB42. Four days after planting, the lettuce plants at the 3–4-leaf stage were treated with a spore suspension (10^7 CFU/ml) of FZB42 for a second time. An amount of 0.5 l spore suspension was applied by hand sprayer to each bed with the size of 6.75 m^2 with 11 plants per m^2. The experiment comprised non-treated (control) and treated (inoculated) plants at planting (t0), 2 weeks (t1) and 5 weeks (t2) after planting of lettuce into the field. Total community DNAs were isolated from the rhizosphere of three lettuce plants per replicate and combined prior to DNA isolation. Samples were taken from four replicates randomly arranged in the experimental field. This approach resulted in the analysis of 12 plants per treatment and time point. The experimental design and sampling regime of this study is outlined in detail in a previous publication (Chowdhury et al., 2013).

HIGH-THROUGHPUT SEQUENCING OF METAGENOMIC DNA

Whole metagenome shotgun libraries were constructed based on the metagenomic DNA preparations extracted from the rhizosphere of lettuce. Prior to library preparation 3.3 ng DNA from three replicates (six plants each) were pooled per treatment and sampling time point. Accordingly, each metagenomic library comprises rhizosphere total community DNA preparations from 12 plants per treatment. Sequencing of these libraries was performed on the MiSeq system (Illumina) according to protocols provided by Illumina.

BIOINFORMATIC ANALYSIS OF METAGENOME SEQUENCE DATA

Metagenome reads were processed and analyzed within the MG-RAST software package (Meyer et al., 2008; Wilke et al., 2013). Sequence data are available from the MG-RAST platform under the following IDs: t0 control: http://metagenomics.anl.gov/linkin.cgi?metagenome=4535147.3, t0 inoculated: http://metagenomics.anl.gov/linkin.cgi?metagenome=4535148.3, t1 control: http://metagenomics.anl.gov/linkin.cgi?metagenome=4535149.3, t1 inoculated http://metagenomics.anl.gov/linkin.cgi?metagenome=4535150.3, t2 control: http://metagenomics.anl.gov/linkin.cgi?metagenome=4535151.3, t2 inoculated: http://metagenomics.anl.gov/linkin.cgi?metagenome=4535152.3.

Taxonomic profiling and determination of α-diversity was performed within MG-RAST applying the "Reprehensive Hit Classification" option using the GenBank database as reference with the following settings: maximum e-value cut off of $1*10^{-5}$, minimum identity of 80%, and minimum alignment length of 15 measured in amino acids for proteins and base pairs for RNA databases. Taxonomic information was inferred from the best BLAST hit applying the "Representative Hit" option within MG-RAST. Compositions of microbial communities were compared based on normalized ratios for each taxon identified within a specific dataset. Ratios of identified taxa were calculated by referring them to the total amount of classified sequences within each dataset. The deduced taxonomic profiles were visualized by means of Microsoft Excel (2008). Additionally, MA-plots were calculated. These plots visualize the distribution of annotated genera within control and inoculated sample sets by plotting the logarithmic ratios between the numbers of annotated sequences (*M*-value) against the logarithmic mean average of annotated sequences (*A*-value) of each genus.

ANNOTATION OF FUNCTIONAL SUBSYSTEMS

Analysis of the relevant functional subgroups was performed using the SEED subsystems technology within the MG-RAST software package with default settings. Proteins deduced from metagenome reads were compared to the FIGfam protein family collection within MG-RAST and then classified according to the predefined subsystems applying the SEED technology implemented in MG-RAST (Aziz et al., 2008; Meyer et al., 2008; Overbeek et al., 2014). All metagenome sequences matching a specific subsystem were counted and this value was then divided by the number of sequences that were assigned to any subsystem to give fractions and therefore normalized, comparable values (Meyer et al., 2008).

MAPPING OF METAGENOME SEQUENCE READS TO THE *B. AMYLOLIQUEFACIENS* FZB42 GENOME

Metagenome sequence reads of the six datasets were mapped to the *B. amyloliquefaciens* FZB42 reference genome (GenBank

Accession: CP000560) by means of the gsMapper program (Roche Genome Analyzer Data Analysis Software Package, version 2.8) in order to follow the occurrence of the inoculant strain in the rhizosphere. Strict settings of the gsMapper (98% sequence identity, minimum 225 bp length, minimum 90% overlap) were used for this fragment recruitment to specifically map reads originating from *B. amyloliquefaciens* FZB42. Multiple contigs and corresponding consensus sequences were generated from the mapped reads. To visualize the positions of the mapped reads in the *B. amyloliquefaciens* FZB42 genome, a Circos plot of the resulting contigs for each time point was calculated (Krzywinski et al., 2009). For the determination of the traceable amounts of strain FZB42 in the course of the cultivation, the normalized numbers of metagenome reads only matching specific *B. amyloliquefaciens* FZB42 genes were used.

RESULTS

METAGENOME SEQUENCING OF LETTUCE RHIZOSPHERE MICROBIAL COMMUNITIES

To determine the capability of *B. amyloliquefaciens* FZB42 to colonize the lettuce rhizosphere, taxonomic and functional profiles of microbial rhizosphere communities were deduced from corresponding metagenome sequence data. Since in the previous study (Chowdhury et al., 2013) comparison of T-RFLP patterns obtained for replicates of rhizosphere DNA preparations revealed consistent results over the replicates per sample, it was decided to pool the rhizosphere samples per treatment and sampling time point in the metagenome study described here. **Table 1** shows the results of the metagenome sequencing approach of six different DNA-libraries from a field trial that schematically is represented in **Figure 1**.

Table 1 | Statistics of metagenome sequencing of DNA preparations from the rhizosphere of *Lactuca sativa* treated with *B. amyloliquefaciens* FZB42 (+FZB42) and corresponding controls.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Sequences	1,909,294	2,500,586	799,247	1,510,665	1,590,425	995,370
High quality sequences	1,869,056	2,446,824	783,195	1,470,887	1,550,845	971,074
Mean sequence length (bp)	218 ± 52	218 ± 53	220 ± 52	213 ± 55	215 ± 54	216 ± 54
Mean GC content (%)	58 ± 12	58 ± 11	62 ± 10	62 ± 10	61 ± 11	60 ± 12
Annotated sequences	624,429	706,259	169,574	286,817	344,059	203,447
Annotated *Bacteria*	614,382	695,861	166,878	281,151	338,012	198,840

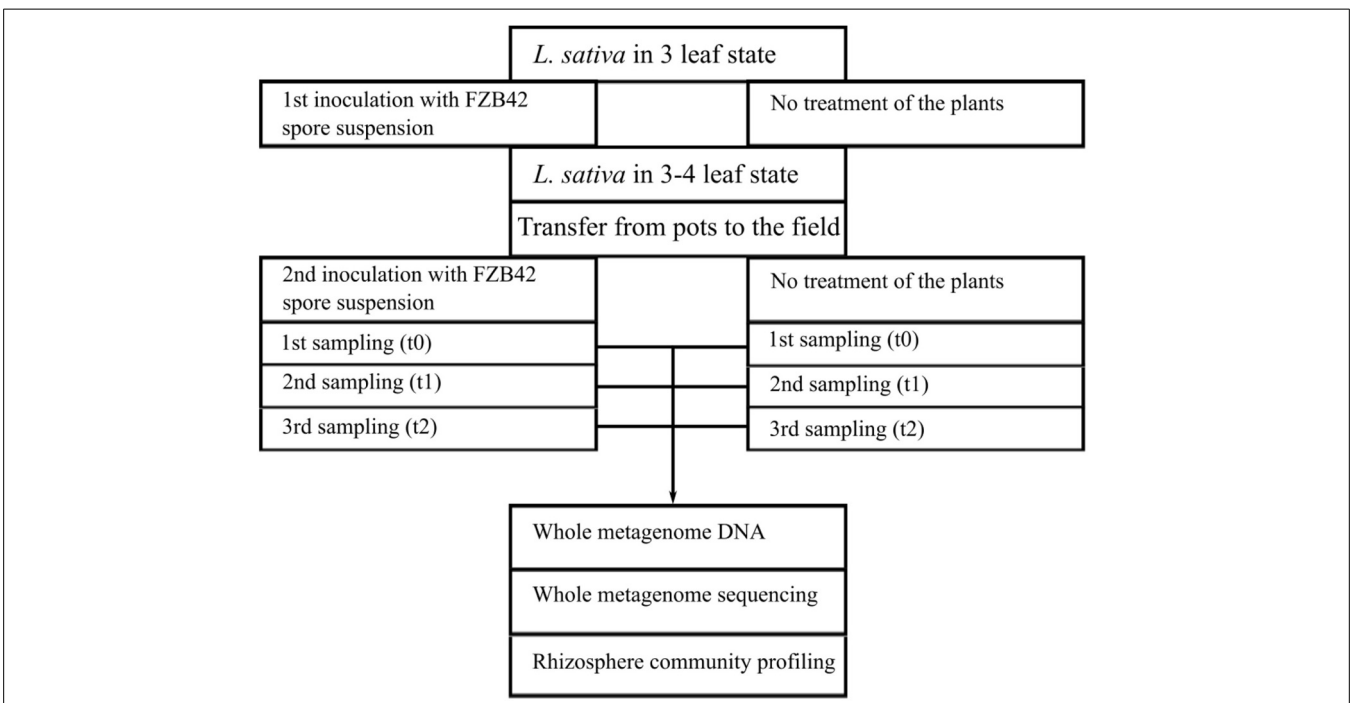

FIGURE 1 | Scheme of the lettuce field cultivation experiment. Lettuce was either inoculated with *B. amyloliquefaciens* FZB42 or not treated. Samples of both experiments were drawn at planting (t0), 2 weeks (t1), and 5 weeks (t2) of cultivation from the rhizosphere of the inoculated and non-treated plants. Rhizosphere samples were used for whole community DNA extraction and subsequently for metagenome sequencing. Annotated metagenome reads were used for taxonomic and functional profiling of the microbial communities within the lettuce rhizospheres.

Yields of the sequencing runs were determined to be between 783,195 and 2,446,824 reads after quality control (**Table 1**). Taxonomic annotation with the implemented "Representative Hit Classification" of MG-RAST against the GenBank database resulted in the annotation of between 169,574 and 706,259 sequence reads considering all taxonomic domains. Classified reads represent between 19.5 and 33.4% of all sequences. Among all annotated taxonomic domains 98.2% of the reads were assigned to the domain *Bacteria*. (**Table 1**).

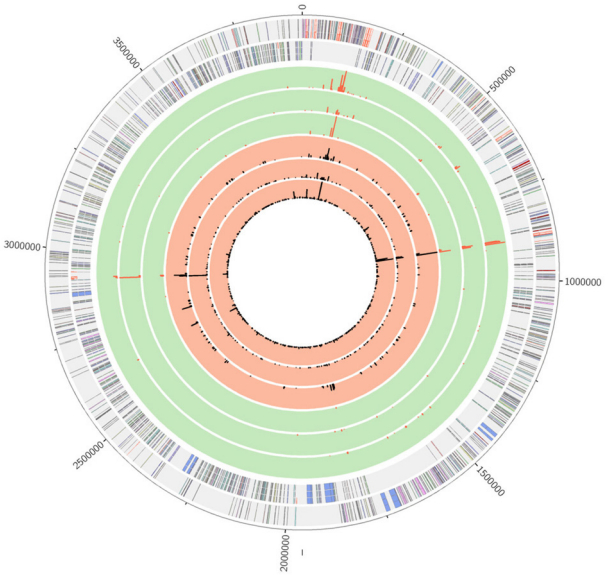

FIGURE 2 | Mapping of metagenome sequence reads to the *B. amyloliquefaciens* FZB42 genome. The circles represent from inner to outer most: (i) t0 inoculated sample; (ii) t1 inoculated sample; (iii) t2 inoculated sample; (iv) t0 control; (v) t1 control; (vi) t2 control; (vii) annotated coding sequences in both strands; (viii) scale in bp. The replication initiation gene *dnaA* was chosen as first gene of the chromosome. Coding sequences are colorized depending on their gene function according to COG (Clusters of Orthologous Groups of proteins) categories. The scale for mapped reads represents the relative abundance of reads per base normalized to the maximum value of mapped reads per base.

ESTIMATION OF LETTUCE RHIZOSPHERE COLONIZATION BY *B. AMYLOLIQUEFACIENS* FZB42 BASED ON FRAGMENT RECRUITMENT ANALYSES

Prior to taxonomic profiling and comparison of community profiles, metagenome sequences were used to estimate rhizosphere colonization of the inoculant strain in order to relate potential changes within the taxonomic and functional profiles to the application of *B. amyloliquefaciens* FZB42. Mapping of the metagenome sequences to the reference genome of *B. amyloliquefaciens* FZB42 revealed that the strain is able to colonize the rhizosphere of lettuce over the course of the whole growth period (**Figure 2** and **Table 2**).

Comparison of the amount of mapped reads and the resulting assembled contigs between samples taken at the different time points showed that for the first sample (t0) more reads from inoculated rhizospheres were mapped to the reference genome. **Figure 2** clearly shows that within the first inoculated sample a wide set of genes of FZB42 is covered by 10,143 metagenome sequences adding up to a contig size of 203,115 bp. In the course of the experiment, the number of sequences specifically matching the *B. amyloliquefaciens* FZB42 genome decreased considerably to 5539 in the t1 and 3478 in the t2 inoculated sample, but still a part of the reference genome is covered by metagenome sequences. In contrast, metagenome sequence reads from non-inoculated samples mapping to the reference genome accumulate in very small sections of the genome.

In addition to the number of mapped reads to the reference genome, the corresponding annotated genes were determined. The number of FZB42 genes covered by metagenome reads was considerably higher in all inoculated samples compared to the control samples and are distributed over the whole genome (**Table 2**). Sequence reads from control samples that were mapped to the reference genome mainly represent genes encoding rRNAs and mobile genetic elements, which most probably are widely spread in species of the genus *Bacillus*. Among the completely covered genes for these samples, only 19 were already annotated and either corresponded to rRNA genes or other conserved housekeeping genes or have no assigned function yet. Accordingly, reads recruited to the *B. amyloliquefaciens* FZB42 genome from the metagenome data sets of the control samples most probably originate from indigenous *Bacillus* species that share particular genes

Table 2 | Mapping of reads, contigs and genes from the metagenome datasets to the *B. amyloliquefaciens* FZB42 reference genome.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Mapped reads[a]	7,859	10,143	2,808	5,539	5,251	3,478
Mapped reads (%)[b]	0.21	0.20	0.18	0.18	0.17	0.18
Number of contigs[c]	28	619	48	256	75	155
Contig size [bp][d]	5,957	203,115	9,038	80,095	13,421	44,859
Covered genes[e]	25	598	58	266	74	162

[a] *Number of metagenome sequence reads mapped to the FZB42 reference genome.*

[b] *Ratio of mapped reads within all high quality metagenome sequence reads (see **Table 1**).*

[c] *Number of contigs assembled from mapped reads.*

[d] *Resulting contig size of the assembled contigs.*

[e] *Number of genes hit by metagenome sequence reads.*

or DNA elements with *B. amyloliquefaciens* FZB42. However, 835 *B. amyloliquefaciens* FZB42 genes featured recruited metagenome reads originating only from the inoculated samples. These genes found no matching reads in any control sample. The 835 specific genes were used to trace occurrence of the inoculant strain during the growth period of lettuce. Metagenome sequence reads hitting any of these specific genes with high stringency (settings are given in Material and Methods) were counted for the inoculated samples for the sampling time points t0, t1, and t2 after normalization of datasets (Supplementary table 1). As mentioned above, none of the reads from the control samples matched any gene determined to be specific for *B. amyloliquefaciens* FZB42. It appeared that recruited read counts from with FZB42 treated samples decreased from 100 (t0) to 64% (t1) to 55% at the end of the experiment (t2). In summary, fragment recruitments proved occurrence of the inoculant strain *B. amyloliquefaciens* FZB42 in the rhizosphere of lettuce. However, abundance of the strain declines in the course of the experiment.

COMPARATIVE TAXONOMIC CHARACTERIZATION OF THE MICROBIAL COMMUNITIES IN THE LETTUCE RHIZOSPHERES WITH AND WITHOUT APPLICATION OF *B. AMYLOLIQUEFACIENS* FZB42 BY WHOLE METAGENOME SEQUENCE ANALYSES

Determination of the α-diversities summarizing the mean species diversity in habitats represented by the six metagenome data sets showed a clear increase from 332.82 in the control sample and 349.23 in the with FZB42 treated sample taken after at planting to 430.81 in the control sample and 447.81 in the with FZB42 treated sample taken 2 weeks after planting (**Table 3**). After 5 weeks, the α-diversities of 412.71 in the control and 437.21 in the inoculated sample were measured.

On average, the reads of the sequenced metagenome libraries were assigned to 27 phyla, 41 classes, 92 orders, 211 families, and 596 genera within the superkingdom *Bacteria* (**Table 4**). Considering the six samples for different treatments and the three sampling time points, a total of 27 phyla, 41 classes, 95 orders, 217 families, and 689 genera were detected. Deduced taxonomic profiles clearly overlap at higher taxonomic levels and in a slightly decreasing manner at lower levels for the different samples. These results indicate that the general compositions of the analyzed microbial communities within the lettuce rhizospheres are similar.

Analysis of the most abundant phyla within the sample sets taken at different time points revealed a high overlap resulting in only 11 phyla representing the 10 most prominent phyla within the single sample sets. The most abundant phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes,* and *Firmicutes* represented 95.0 to 96.7% of all assigned sequences (**Table 5a** and **Figure 3**). Within the metagenome datasets derived from the control samples and samples from inoculated rhizospheres, only minor changes are noticeable, whereas a more distinct shift between relative frequencies of assigned community taxa in the course of the experiment can be observed. The most obvious changes were detectable within the mentioned three most prominent phyla of the microbial communities after 2 weeks of cultivation in the field. The amount of annotated *Proteobacteria* decreased from over 74% to approximately 59% in the t1 samples to 64% in the t2 samples, and *Bacteroidetes* decreased from around 8% (t0) to about 4.5% (t1) to 6.6% (t2). The ratios of annotated *Actinobacteria* increased from between 9% in the control sample and 12% in the with FZB42 treated sample from t0 to around 24% within the samples taken after 2 and 5 weeks. Additionally the ratio of *Firmicutes* increased from around 0.4% in the t0 samples to around 4% within the t1 and then decreased again to around 2.7% in the t2 samples. The remaining frequencies of annotated phyla varied only slightly between all samples.

The 10 most abundant classes of the single data sets add up to 12 different classes represented by around 97.4% of all annotated bacterial sequences. The four most prominent classes *Alphaproteobacteria, Betaproteobacteria*, *Actinobacteria*, and *Gammaproteobacteria* were represented by 84.6–88.2% of all annotated sequences (**Table 5b** and **Figure 4**). Distinct changes between samples taken at different time points were detected within the first 2 weeks of cultivation, whereas the microbial rhizosphere communities were relatively stable in the last 3 weeks of cultivation. The relative abundance of annotated *Betaproteobacteria* that was around 30% at t0, decreased to 22% at t1 and around 20% in the t2 samples. The amount

Table 3 | α diversities within the metagenome datasets.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
α diversity	332.82	349.23	430.81	447.14	412.71	437.21

Table 4 | Numbers of different taxa assigned to the superkingdom *Bacteria* detected within the metagenome sequences as analyzed by the MG-RAST software package.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated	sum[a]
Phylum	27	27	27	27	27	27	27
Class	41	41	41	41	41	41	41
Order	92	93	91	92	92	91	95
Family	210	213	211	214	210	210	217
Genus	602	613	588	601	598	573	689

[a] *Combined number of different taxa within all samples.*

Table 5 | Relative abundances of the dominant bacterial phyla (a), classes (b), orders (c), and genera (d) within the rhizosphere of *L. sativa* calculated for all classified sequences within the bacterial domain.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
(a) PHYLUM						
Proteobacteria	79.35	74.23	59.31	60.27	63.74	64.33
Actinobacteria	9.03	12.21	26.74	25.98	23.95	22.12
Bacteroidetes	7.88	8.92	4.65	5.26	5.04	6.62
Firmicutes	0.39	0.56	4.27	4.07	2.92	2.54
Acidobacteria	1.90	2.37	1.63	1.34	1.28	1.42
Verrucomicrobia	0.82	0.98	1.20	1.13	1.65	1.31
Chloroflexi	0.08	0.09	0.73	0.64	0.38	0.55
Planctomycetes	0.18	0.22	0.53	0.43	0.36	0.37
Cyanobacteria	0.11	0.13	0.30	0.25	0.20	0.25
Deinococcus-Thermus	0.06	0.08	0.22	0.19	0.12	0.14
Chlorobi	0.07	0.09	0.10	0.13	0.10	0.11
Others	0.12	0.14	0.33	0.33	0.26	0.25
(b) CLASS						
Alphaproteobacteria	24.49	27.14	25.38	26.11	31.61	31.73
Betaproteobacteria	30.61	29.62	20.63	23.88	20.42	20.54
Actinobacteria	9.07	12.27	26.99	26.18	24.10	22.26
Gammaproteobacteria	23.99	17.10	11.63	8.49	10.70	10.86
Sphingobacteria	4.26	5.15	2.25	2.28	3.27	2.96
Bacilli	0.19	0.35	3.55	3.39	2.46	2.03
Flavobacteria	2.05	1.91	1.37	1.92	0.88	2.65
Deltaproteobacteria	0.59	0.70	2.11	2.12	1.33	1.52
Cytophagia	1.35	1.61	0.81	0.86	0.70	0.78
Acidobacteria	1.47	1.87	0.47	0.39	0.70	0.66
Opitutae	0.43	0.55	0.37	0.35	0.84	0.65
Clostridia	0.18	0.19	0.71	0.65	0.43	0.49
Others	1.33	1.55	3.74	3.39	2.57	2.86
(c) ORDER						
Burkholderiales	28.87	27.98	19.25	22.53	17.85	17.63
Actinomycetales	8.63	11.84	25.25	24.66	23.00	21.13
Rhizobiales	12.20	14.15	14.29	14.27	19.02	17.58
Pseudomonadales	14.54	8.29	5.97	3.28	5.94	5.98
Caulobacterales	8.43	8.76	3.94	3.80	5.68	5.82
Sphingomonadales	1.84	1.93	5.62	6.41	5.24	6.54
Xanthomonadales	4.94	5.17	2.38	2.69	2.65	2.60
Sphingobacteriales	4.30	5.21	2.27	2.30	3.32	3.00
Bacillales	0.16	0.32	3.51	3.34	2.45	1.99
Flavobacteriales	2.03	1.90	1.36	1.91	0.87	2.65
Enterobacteriales	3.05	1.94	2.00	1.14	0.75	0.90
Methylophilales	1.11	1.07	0.65	0.56	2.08	2.22
Others	9.89	11.44	13.50	13.11	11.16	11.95
(d) GENUS						
Mycobacterium	3.89	7.42	3.82	3.80	5.67	4.42
Pseudomonas	5.55	5.04	3.70	2.20	5.53	5.39
Burkholderia	8.07	6.86	2.71	2.66	3.13	2.73
Caulobacter	5.10	5.18	2.64	2.37	4.17	4.43
Acidovorax	3.28	3.51	2.98	4.09	2.32	2.81
Variovorax	1.77	1.82	3.65	4.56	3.19	3.00
Streptomyces	0.39	0.47	3.36	3.37	4.47	3.80
Arthrobacter	0.69	0.56	5.45	5.44	1.66	1.58
Rhodopseudomonas	1.85	2.42	2.22	2.34	3.23	3.19

(Continued)

Table 5 | Continued

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Acinetobacter	8.47	2.73	2.11	0.94	0.16	0.36
Polaromonas	2.81	3.25	1.98	2.45	2.14	2.12
Nocardioides	1.02	0.76	2.77	2.61	2.87	2.93
Sphingomonas	0.61	0.64	2.44	2.78	2.24	2.99
Xanthomonas	2.45	2.59	1.18	1.37	1.32	1.37
Pedobacter	2.19	2.57	0.93	0.93	1.19	1.22
Bacillus	0.05	0.16	2.57	2.48	1.95	1.54
Others	51.82	54.02	55.49	55.60	54.76	56.12

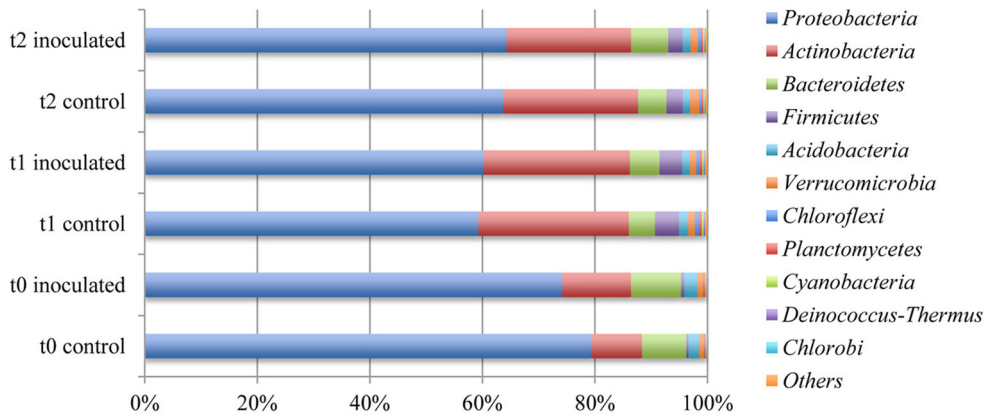

FIGURE 3 | Comparison of the ten most abundant phyla within the rhizosphere of lettuce. Abundances were calculated by means of comparison of metagenome sequence reads against the GenBank database within the MG-RAST software package.

of annotated *Actinobacteria* increased noticeably from between 9 and 12% to around 24.8% in average in the samples drawn after 2 and 5 weeks. The relative abundances of annotated *Gammaproteobacteria* varied between the t0 samples between 24% in the control and 17% in the inoculated samples. Within the samples taken after 2 and 5 weeks of cultivation in the field the ratio of *Gammaproteobacteria* was around 10%.

Analysis of the most abundant orders revealed that *Burkholderiales, Actinomycetales, Rhizobiales*, and *Pseudomonadales* represented between 62% and almost 66% of the microbial communities in all six samples that where either inoculated or not treated (**Table 5c** and **Figure 5**). A decrease from around 28.5% annotated *Burkholderiales* in the t0 sample to an average of 19.3% in the other samples was noticeable. The ratios of annotated *Actinomycetales* increased from around 10% in the first samples to over 20% after 2 and 5 weeks of cultivation. The differences between samples collected at different time points were more distinct as compared to samples for different treatments.

The most obvious change probably caused by the application of strain FZB42 was the relative abundance of annotated *Pseudomonadales* varying between 14.5 and 8.3% in the samples taken at planting. The amount decreases to 6.0% in the control and 3.3% in the with FZB42 treated sample after 2 weeks and around 6% in both samples drawn after 5 weeks.

The 10 most prominent genera within the samples taken from different time points add up to 16 genera representing around 45% of all annotated sequences. *Mycobacterium, Pseudomonas, Burkholderia*, and *Caulobacter* represented the most abundant genera within the microbial rhizosphere communities (**Table 5d** and **Figure 6**). Among the ratios for *Mycobacteria*, an increase from 3.9 to 7.4% was detectable for the control and the with FZB42 treated samples of t0, whereas the ratios were almost stable among the remaining samples that were either treated with FZB42 or not treated. Relative abundances for other genera revealed more pronounced changes between the samples that were taken at different time points than between the differentially treated samples. M/A plots of the annotated metagenome sequences on genus level visualized the distributions of detected genera clearly (**Figures 7–9**). The dense clouds around zero on the y-axis of these plots indicated a low fold-change in the abundances of annotated genera between the control and the inoculated samples. Higher variations were only noticeable within the genera that were annotated at very low abundances and were therefore of minor interest, because they do not contribute to the main share of the microbial communities. Within the t0 control sample a higher number of sequences were classified as *Acinetobacter*, *Enterobacter*, and *Citrobacter* in comparison to the inoculated sample. In the inoculated sample from this time point, the number of annotated *Bacillus, Pantoea*, and

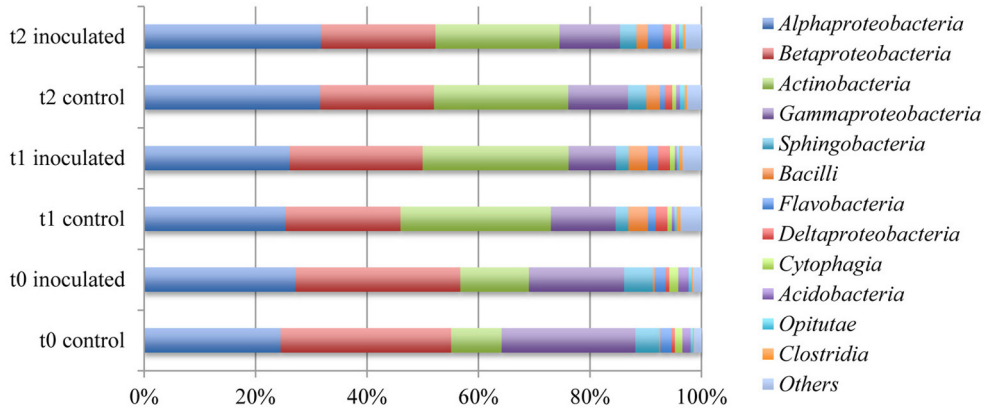

FIGURE 4 | Comparison of the ten most abundant classes within the rhizosphere of lettuce. Abundances were calculated by means of comparison of metagenome sequence reads against the GenBank database within the MG-RAST software package.

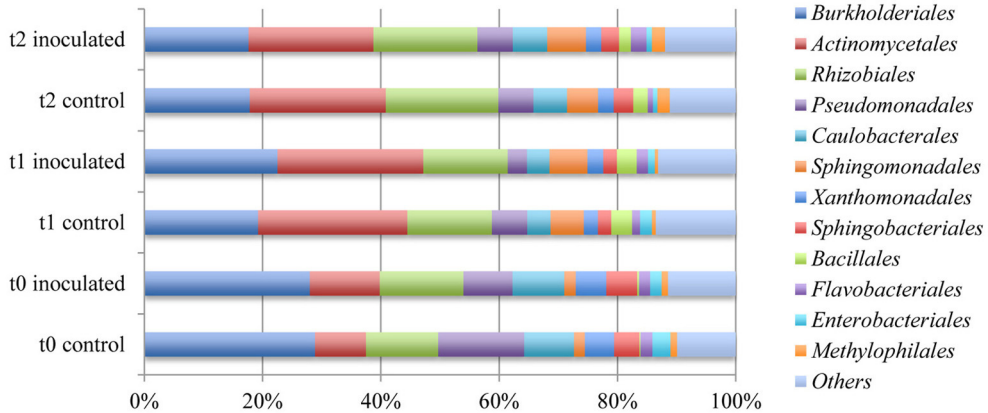

FIGURE 5 | Comparison of the ten most abundant orders within the rhizosphere of lettuce. Abundances were calculated by means of comparison of metagenome sequence reads against the GenBank database within the MG-RAST software package.

Mycobacterium was increased (**Figure 7**). The samples taken after 2 weeks of cultivation in the field showed even less noticeable differences (**Figure 8**). Only the abundances of *Acinetobacter* and *Enterobacter* were slightly increased in the control samples. Within the samples of t2 only the genus *Flavobacterium* was detected in a higher abundance in the inoculated sample (**Figure 9**).

Accordingly, application of *B. amyloliquefaciens* FZB42 appeared to have almost no effect on composition of the lettuce rhizosphere communities, whereas the growth states of the analyzed plants seem to alter the composition of the communities in a more noticeable manner.

ANALYSIS OF THE POTENTIAL IMPACT OF *B. AMYLOLIQUEFACIENS* FZB42 ON ANNOTATED FUNCTIONAL SUBSYSTEMS OF MICROBIAL COMMUNITY MEMBERS IN THE LETTUCE RHIZOSPHERES

In order to gain further insights into community changes at the functional level, classifications of annotated genes according to functional subsystems as defined within MG-RAST of all samples were compared. The ratios of sequences assigned to any subsystem decreased from around 74.6% in the t0 samples to around 62% within the control samples and 57% within the inoculated samples taken after 2 and 5 weeks of cultivation in the field (**Table 6**). The main focus was put on the carbohydrate, nitrogen and amino acid metabolic pathways, since these subsystems are important for utilization and metabolism of plant root exudates.

Examination of the relative amounts of metagenome sequences assigned to specific subsystems for different samples did not show any pronounced changes neither between with FZB42 treated or non-treated samples nor over the duration of the cultivation (see **Table 7**). Closer examination of the annotated genes within these mentioned functional subsystems of the samples also revealed very similar sets of assigned genes.

Within the functional subclass of carbohydrate metabolism, some minor changes were detected between the samples taken at different time points (**Table 8**). Frequencies of detected genes associated with the metabolism of di-, oligo-, and polysaccharides increased slightly in the samples taken after 2 and 5 weeks of cultivation. Additionally, the frequencies of annotated genes involved in the metabolism of different organic acids decrease slightly from

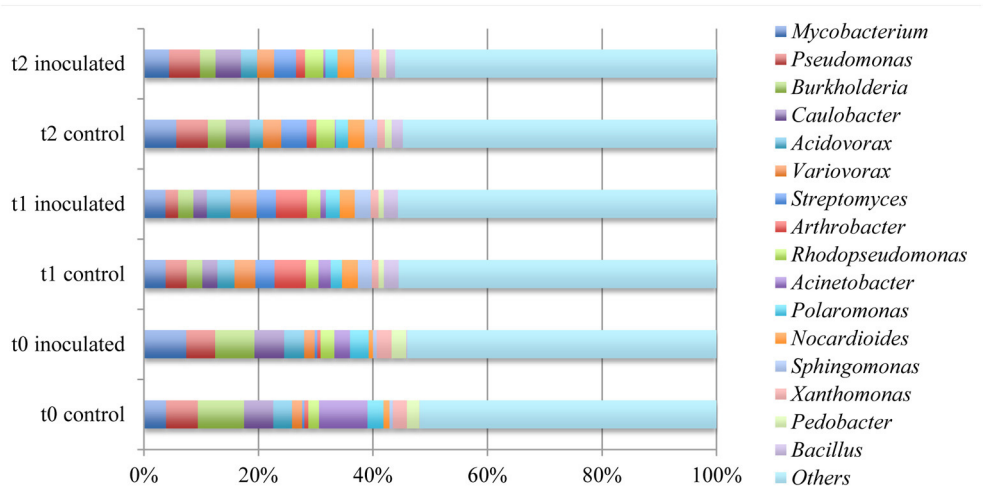

FIGURE 6 | Comparison of the ten most abundant genera within the rhizosphere of lettuce. Abundances were calculated by means of comparison of metagenome sequence reads against the GenBank database within the MG-RAST software package.

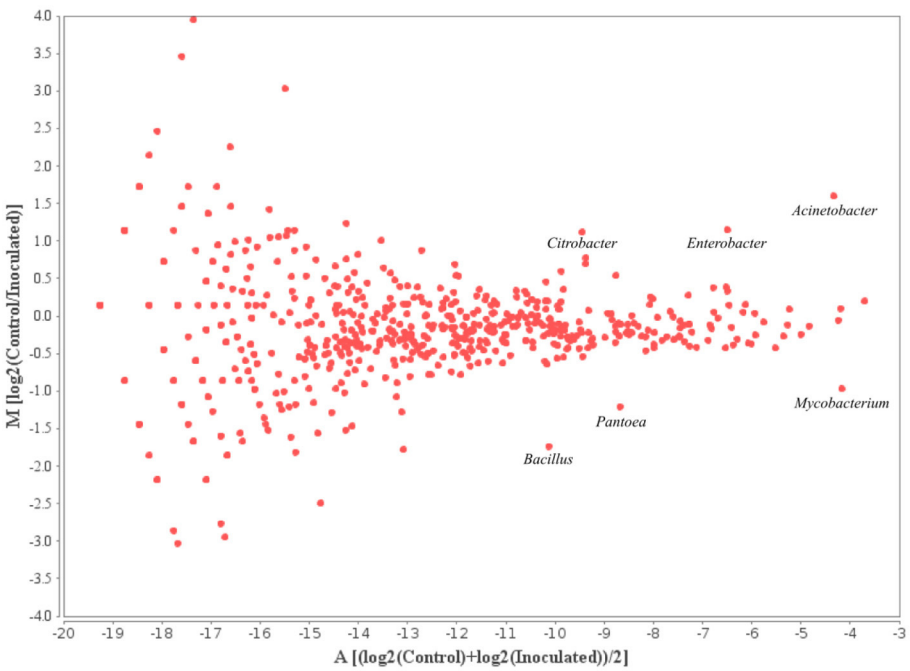

FIGURE 7 | MA-Plot visualizing the values of annotated genera within the t0 samples. Log2(Control/Inoculated) indicates the fold-changes in the abundances of annotated genera between the two samples; (log2(Control)+log2(Inoculated))/2 shows the average abundance of an annotated genus.

around 6.1% within the samples taken after 2 days to 5.5% for the samples taken after 2 and 5 weeks.

Frequencies of several annotated genes within the functional subclass of nitrogen metabolism are probably influenced by either the growth state of the plants or the transfer of plants into the field (**Table 9**). Genes involved in ammonia assimilation are more prominent within the first sample. Their frequencies decrease from around 47% at planting to values between 44 and 45% after 2 and 5 weeks. The ratios of genes within the annotated subsystem of nitrosative stress decreased in the course of the cultivation from 5.7% to around 5% and 4.4%. In contrast, frequencies of genes within the subsystem of nitrate and nitrite ammonification increased slightly during the course of the cultivation. Additionally, genes involved in nitrogen fixation increased in a similar manner.

Within the functional subsystem of amino acid and amino acid derivative metabolism, the calculated frequencies of annotated subsystems almost showed no changes (**Table 10**). The most

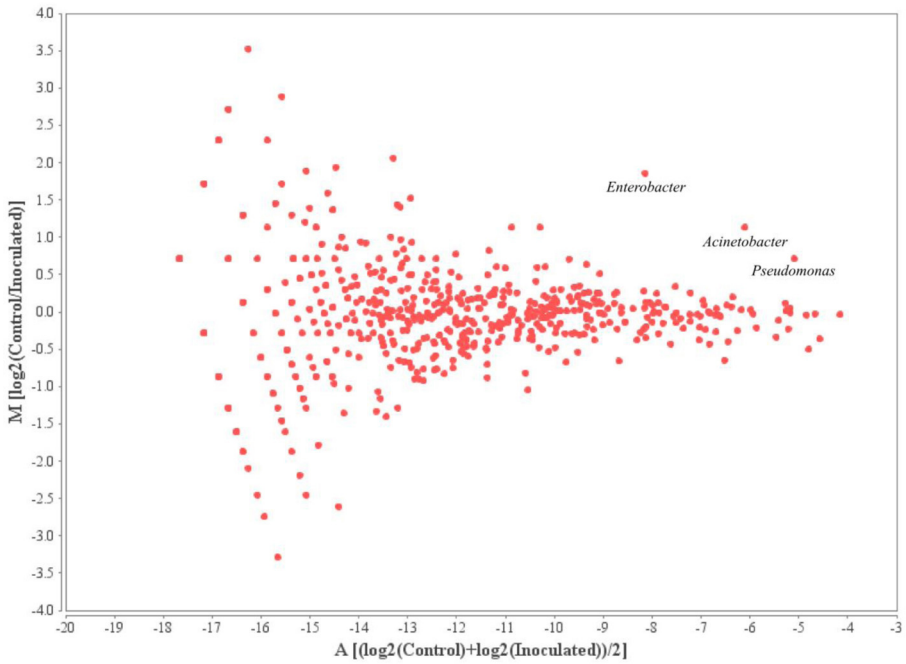

FIGURE 8 | MA-Plot visualizing the values of annotated genera within the t1 samples. Log2(Control/Inoculated) indicates the fold-changes in the abundances of annotated genera between the two samples; (log2(Control)+log2(Inoculated))/2 shows the average abundance of an annotated genus.

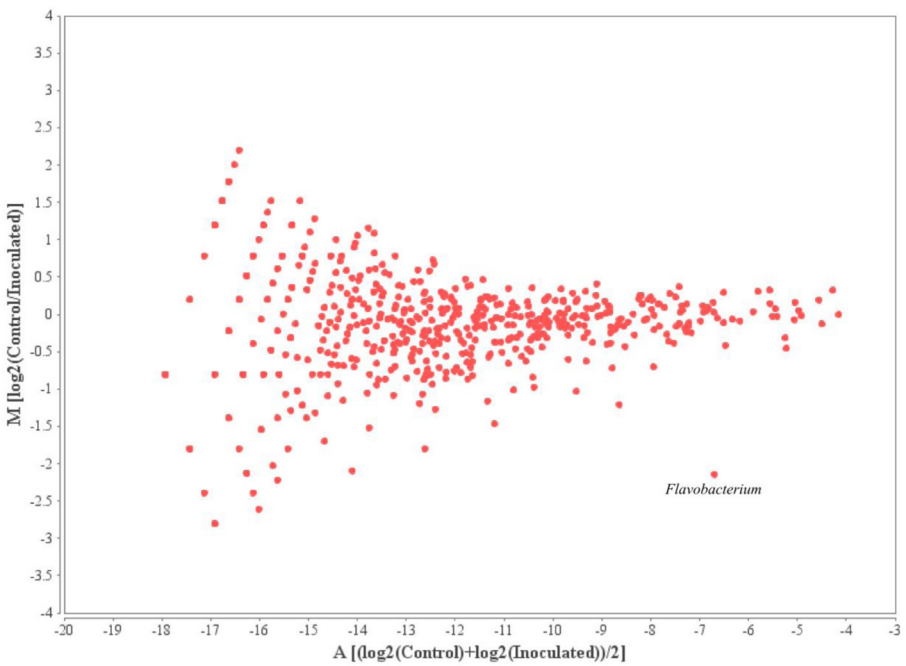

FIGURE 9 | MA-Plot visualizing the values of annotated genera within the t2 samples. Log2(Control/Inoculated) indicates the fold-changes in the abundances of annotated genera between the two samples; (log2(Control)+log2(Inoculated))/2 shows the average abundance of an annotated genus.

noticeable changes occurred in the subsystem of glutamine, glutamate, aspartate, asparagine and ammonia assimilation with frequencies increasing from around 10.3% to values around 11% in the samples taken after 2 and 5 weeks.

Additionally, metagenome sequences assigned to the functional context "defense mechanisms" such as antibiotic resistances and transport systems for toxic compounds that may be indicative for microorganisms resistant to secondary metabolites produced

Table 6 | Sequences assigned to all functional subclasses within the metagenome data sets calculated within the MG RAST analysis software with default settings.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t1 inoculated
HQ sequences	1,869,056	2,446,824	783,195	1,470,887	1,550,845	971,074
Assigned sequences	1,419,743	1,791,952	485,868	837,363	967,685	557,903
	75.96%	73.24%	62.04%	56.93%	62.40%	57.45%

Table 7 | Relative distributions of functional subsystems calculated within the MG RAST analysis software with default settings.

Functional subsystems	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Amino acids and derivatives	8.6	8.6	9.0	9.0	8.8	8.9
Carbohydrates	10.2	10.1	10.8	11.0	10.7	10.9
Cell division and cell cycle	1.3	1.3	1.4	1.3	1.4	1.3
Cell wall and capsule	3.8	3.7	3.5	3.4	3.5	3.5
Clustering-based subsystems	14.7	14.8	15.2	15.1	15.1	15.0
Cofactors, vitamins, prosthetic groups, pigments	6.6	6.7	6.8	6.8	6.9	6.9
DNA metabolism	4.2	4.3	4.1	4.1	4.1	4.1
Dormancy and sporulation	0.2	0.2	0.2	0.2	0.2	0.2
Fatty acids, lipids, and isoprenoids	3.1	3.2	3.2	3.1	3.2	3.2
Iron acquisition and metabolism	1.2	1.1	0.7	0.7	0.8	0.8
Membrane transport	3.5	3.4	3.2	3.1	3.2	3.2
Metabolism of aromatic compounds	2.1	2.0	2.0	2.0	2.0	2.1
Miscellaneous	8.4	8.4	8.4	8.3	8.3	8.3
Motility and chemotaxis	1.2	1.1	0.8	0.8	0.9	0.9
Nitrogen metabolism	1.2	1.2	1.2	1.2	1.3	1.3
Nucleosides and nucleotides	2.8	2.8	3.0	3.0	2.9	2.9
Phages, prophages, plasmids, transposable elements	1.5	1.6	1.5	1.5	1.4	1.4
Phosphorus metabolism	0.9	0.9	0.9	0.9	0.8	0.8
Photosynthesis	0.1	0.1	0.1	0.1	0.1	0.1
Potassium metabolism	0.5	0.5	0.4	0.4	0.5	0.4
Protein metabolism	7.6	7.7	7.5	7.7	7.6	7.5
RNA metabolism	4.1	4.1	3.9	3.9	3.9	3.9
Regulation and cell signaling	1.5	1.5	1.5	1.5	1.5	1.5
Respiration	3.4	3.4	3.6	3.5	3.5	3.6
Secondary metabolism	0.4	0.4	0.4	0.5	0.5	0.4
Stress response	2.7	2.7	2.6	2.7	2.7	2.7
Sulfur metabolism	1.3	1.3	1.3	1.3	1.3	1.3
Virulence, disease, and defense	3.0	3.0	2.8	2.8	2.8	2.8

Table 8 | Relative abundances of annotated subsystems within the annotated functional system of carbohydrate metabolism.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Central carbohydrate metabolism	29.7	30.1	30.1	30.4	30.0	30.3
Fermentation	12.8	13.4	13.1	12.6	12.8	12.7
One-carbon metabolism	11.6	11.8	11.9	12.1	11.8	11.7
Monosaccharides	11.2	10.5	10.0	9.8	10.3	10.3
Miscellaneous	8.8	8.5	8.5	8.5	8.6	8.5
Di- and oligosaccharides	8.1	7.8	8.5	8.7	8.5	8.8
Organic acids	6.2	6.0	5.5	5.6	5.5	5.5
CO_2 Fixation	5.6	5.7	5.5	5.5	5.6	5.5
Sugar alcohols	2.9	2.8	3.3	3.3	3.2	3.1
Aminosugars	1.6	1.7	1.4	1.4	1.6	1.5
Polysaccharides	1.4	1.4	1.9	1.9	1.9	1.9
Predicted carbohydrate hydrolases	0.3	0.3	0.3	0.3	0.3	0.3

by *B. amyloliquefaciens* FZB42 were analyzed (**Table 11**). The relative abundances of sequences corresponding to this subsystem varied between 2.8 and 3% of all assigned sequences within each sample. Examination of the subgroups of the subsystem "defense mechanisms" substantiates the conclusion that application of *B. amyloliquefaciens* FZB42 does not affect rhizosphere microorganisms featuring functions in the context of defense.

In summary, comparison of the relative abundances of sequences assigned to sub-systems between samples neither showed pronounced differences between the inoculated and non-treated samples nor between the samples taken over the course of the field trial. Therefore, no major impact of the inoculant strain FZB42 on functional community profiles could be affirmed.

DISCUSSION

B. AMYLOLIQUEFACIENS FZB42 IS ABLE TO SUCCESSFULLY COLONIZE THE LETTUCE RHIZOSPHERE IN THE COURSE OF THE FIELD EXPERIMENT

Tracking of the inoculant strain *B. amyloliquefaciens* FZB42 in the lettuce rhizosphere in the course of the experiment was of crucial importance to estimate its competitiveness in this habitat and was achieved by mapping of metagenome sequences to the published reference genome of *B. amyloliquefaciens* FZB42. Corresponding fragment recruitments proved persistence of strain FZB42 in the rhizosphere. However, the number of mapped reads (contigs) and the number of covered *B. amyloliquefaciens* FZB42 genes decreased in the course of the plant cultivation indicating that the

Table 9 | Relative abundances of annotated subsystems within the annotated functional system of nitrogen metabolism.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Ammonia assimilation	47.2	47.1	44.1	43.3	44.7	45.3
Nitrate and nitrite ammonification	21.0	19.8	22.1	21.4	22.3	22.4
Denitrification	8.5	8.4	7.4	8.2	6.9	7.1
Allatoin utilization	5.7	5.5	7.1	6.1	5.4	5.2
Nitrosative stress	5.7	5.7	5.1	5.0	4.6	4.2
Nitric oxide synthase	4.7	5.8	6.5	6.5	7.6	7.3
Dissimilatory nitrite reductase	3.2	3.3	3.0	3.7	3.5	3.5
Cyanite hydrolysis	3.2	3.4	3.1	3.5	3.6	3.2
Nitrogen fixation	0.4	0.7	1.2	1.7	1.2	1.4
Amidase cluster	0.3	0.3	0.2	0.3	0.3	0.4
Nitrilase	0.0	0.1	0.1	0.1	0.0	0.1

Table 10 | Relative abundances of annotated subsystems within the annotated functional system of amino acids and derivatives.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Branched-chain amino acids	28.7	29.9	28.3	29.1	28.8	29.5
Lysine, threonine, methionine, and cysteine	21.4	21.7	21.2	21.4	21.3	21.4
Aromatic amino acids and derivatives	11.5	11.2	10.9	10.5	10.7	10.9
Arginine; urea cycle, polyamines	11.0	10.5	11.2	11.0	11.1	11.0
Glutamine, glutamate, aspartate, asparagine; ammonia assimilation	10.4	10.2	11.4	11.2	11.1	10.7
Alanine, serine, and glycine	9.8	9.6	10.2	10.0	10.1	10.0
Proline and 4-hydroxyproline	3.7	3.5	3.1	3.0	3.1	3.0
Histidine metabolism	2.9	2.7	2.8	2.7	2.7	2.7
Miscellaneous	0.7	0.7	1.1	1.1	1.1	1.0

Table 11 | Relative abundances of annotated subsystems within the annotated functional system of Virulence, disease, and defense.

	t0 control	t0 inoculated	t1 control	t1 inoculated	t2 control	t2 inoculated
Resistance to antibiotics and toxic compounds	80.7	81.4	78.9	78.8	79.7	79.8
–	9.7	9.1	10.3	10.2	10.4	10.2
Multi locus sequence typing	7.2	7.2	8.2	8.4	7.5	7.4
Adhesion	1.4	1.4	1.5	1.5	1.5	1.5
Bacteriocins, ribosomally synthesized antibacterial peptides	0.4	0.5	0.6	0.5	0.4	0.6
Invasion and intracellular resistance	0.4	0.3	0.3	0.3	0.3	0.3
Toxins and superantigens	0.1	0.1	0.2	0.2	0.2	0.1

inoculant strain was less competitive compared to other community members. At the end of the experiment, strain FZB42 could still be traced at a clear level. Corrected and normalized values of the read numbers matching specific *B. amyloliquefaciens* FZB42 genes revealed that after 2 weeks 64% and after 5 weeks about 55% of the inoculant FZB42 was still traceable within the rhizosphere. These values are in accordance with previous results from reference experiments using a FZB42-derivative conferring rifampicin (Rif) resistance as inoculum. The CFUs per gram of soil of this strain decreased to 59% after 2 weeks and to 14% after 5 weeks of field cultivation (Chowdhury et al., 2013). The relatively high rate of recruited metagenome sequences from the non-inoculated samples is most probably due to the fact that sequences of rRNA genes and mobile genetic elements are very similar or even identical between different *Bacillus* species (Edwards et al., 2012). Accordingly, increasing numbers of mapped reads from the non-inoculated samples can be explained by the presence of other indigenous *Bacillus* species in the plant rhizosphere.

INOCULATION WITH *B. AMYLOLIQUEFACIENS* FZB42 HAS NO MAJOR IMPACT ON THE MICROBIAL COMMUNITIES IN THE LETTUCE RHIZOSPHERE AND ON THE FUNCTIONAL SUBSYSTEM ASSIGNMENTS OF COMMUNITY MEMBERS

Although competitiveness of *B. amyloliquefaciens* FZB42 in the lettuce rhizosphere is limited, application of the strain may affect composition of the indigenous microbial rhizosphere community, especially in early stages of plant development. Metagenomic sequencing and the subsequent comparison of deduced taxonomic profiles combining samples from several equally treated plant rhizospheres indicated that application of *B. amyloliquefaciens* FZB42 did not result in pronounced effects on the composition of the microbial communities of the lettuce rhizosphere. Microbial community changes seem more distinct between the samples drawn at different time points. Most obvious, changes in the composition of the microbial communities occur within the first 2 weeks of plant cultivation. It is known that plant root exudates have a major impact on the composition of microbial rhizosphere communities (Hartmann et al., 2009). Root exudation patterns change quantitatively and qualitatively during the growth of the plant and higher amounts of exudates were released into the rhizosphere usually at the initial growth phase as given after planting (Baudoin et al., 2002). Hence, changes in root exudate patterns may be responsible for a shift in microbial community structure in the course of plant growth. Similar studies showed that shifts within microbial rhizosphere communities are mainly due to different developmental stages of the plant (Inceoglu et al., 2013) and root exudates produced by the plants (Chaparro et al., 2013). In the study described here, community changes were most pronounced at the onset of cultivation whereas comparison of the taxonomic profiles between the samples after 2 and 5 weeks of plant growth revealed only minor differences in the ratios of identified taxa suggesting that environmental conditions in this time period are almost constant and therefore enable the establishment of an adapted bacterial community in the rhizosphere.

Comparison of the taxonomic profiles within the lettuce rhizosphere with other soil microbial communities shows similar compositions. Several studies on related habitats revealed the same five most abundant phyla *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Proteobacteria* with *Firmicutes* and *Proteobacteria* being the most prominent taxa in these microbial communities. Referenced studies include the analysis of the rhizosphere of *Lotus japonicus* (Unno and Shinano, 2013), the rhizosphere of sugarcane plants (Pisa et al., 2011) and the analysis of several soil metagenome samples *via* 16S rRNA gene amplicon sequencing (Vasileiadis et al., 2012).

Evaluation of assignments to functional subsystems of community members supported the results of the taxonomic classification. Similar to the taxonomic profiles, a shift over the course of the cultivation but not between the control and the with FZB42 treated samples was observed. Most obvious was the decrease within the ratio of sequences that could generally be assigned to any subsystem over the course of the study.

Changes within the functional subgroups that are associated with the metabolism of different saccharides, organic acids or amino acids are probably due to changes within the composition of root exudates from the lettuce plants (Baudoin et al., 2002). An additional factor for the slight decrease in the relative number of genes associated with the metabolism of organic acids after 2 and 5 weeks of cultivation probably was the transfer of plants into the field. The plants were grown in peat blocks that probably contained a higher amount of organic acids such as humic acids.

The detected changes corresponding to genes associated with nitrogen metabolism can probably be traced back to reactions of the lettuce plants to several abiotic stress factors (Molassiotis and Fotopoulos, 2011) such as reactive nitrogen species (RNS) including NO and other derivatives. The increase in the number of annotated nitrogen fixation genes may be ascribed to limited amounts of nitrogen in the soil and therefore the need to utilize atmospheric dinitrogen as nitrogen source. Additionally, nitrogen fixation has been proven to be a factor in the promotion of plant growth (Bulgarelli et al., 2013).

Although metagenome analysis is a very convenient method to determine the composition and functional profile of microbial rhizosphere communities only very few published studies applied this approach. Most of the earlier studies are based on T-RFLP and 16S rRNA gene amplicon sequencing which complicates direct comparison of the results obtained in this study. In addition to the inadequacy of comparing results obtained by applying different techniques, most studies only provide taxonomic profiles down to the level of orders. Therefore comparisons of microbial communities within different rhizospheres down to lower taxonomic levels are a relatively challenging task and demonstrate the need to perform further metagenome studies to get deeper insights in microbial communities associated with plants.

CONCLUDING REMARKS

Metagenome analyses of lettuce rhizosphere samples inoculated with *B. amyloliquefaciens* FZB42 vs. non-treated samples revealed that the inoculant strain only had a minor impact on the community structure within this habitat and on specific functional subsystems of community members. The fact that the number

of sequences specifically matching the *B. amyloliquefaciens* FZB42 genome decreased during the growth period of lettuce indicated that the inoculant strain did not overgrow other relevant species within the lettuce rhizosphere.

In previously published studies, the described set of secondary metabolites produced by strain FZB42 was proven to mediate antibacterial and antifungal properties (Koumoutsi et al., 2004; Chen et al., 2006). These metabolites were suspected to induce changes within the microbial rhizosphere community, noticeable i.a., by shifts within the annotated functional subsystem of defense and resistance mechanisms. However, sequence analysis revealed only marginal changes within this subsystem so that it is likely that secondary metabolites do not have a major impact on phytopathogens in the rhizosphere and thus probably are not the key factor in the protection of the plant from pathogenic microorganisms. It should also be mentioned here that *B. amyloliquefaciens* FZB42 probably had not reached a critical density in the lettuce rhizosphere to exert its antibacterial and antifungal properties. Hence, other properties of the inoculant strain such as affecting the plant's ISR may have a more important impact. Studies addressing corresponding mechanisms should be performed to elucidate the mode of action of plant-growth-promoting rhizobacteria on the plant leading to prevention of infections by pathogens.

The results of this metagenome study suggest that the application of the commercially available inoculant strain FZB42 can be considered as a safe method to promote the health of the economically important lettuce plant and reduce severity of infections by phytopathogens like *R. solani*.

AUTHOR CONTRIBUTIONS

The authors Magdalena Kröber, Daniel Wibberg, Rita Grosch, Soumitra P. Chowdhury, Felix Eikmeyer, and Bart Verwaaijen contributed to this work with either relevant experimental work, data analysis or writing, and revising the article critically for important intellectual content. The work was supervised by Andreas Schlüter, Anton Hartmann, and Alfred Pühler. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00252/abstract

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The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome

Armin Erlacher[1,2], Massimiliano Cardinale[1,2], Rita Grosch[3], Martin Grube[2] and Gabriele Berg[1]*

[1] *Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Institute of Plant Sciences, University of Graz, Graz, Austria*
[3] *Leibniz-Institute of Vegetable and Ornamental Crops, Grossbeeren, Germany*

Edited by:
Kornelia Smalla, Julius Kühn-Institut, Germany

Reviewed by:
Zuhua He, Chinese Academy of Sciences, China
Franz Narberhaus, Ruhr University Bochum, Germany
Kornelia Smalla, Julius Kühn-Institut, Germany
Monica Höfte, Ghent University, Belgium

***Correspondence:**
Gabriele Berg, Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, Graz 8010, Austria
e-mail: gabriele.berg@tugraz.at

Lettuce belongs to the most commonly raw eaten food worldwide and its microbiome plays an important role for both human and plant health. Yet, little is known about the impact of potentially occurring pathogens and beneficial inoculants of the indigenous microorganisms associated with lettuce. To address this question we studied the impact of the phytopathogenic fungus *Rhizoctonia solani* and the biological control agent *Bacillus amyloliquefaciens* FZB42 on the indigenous rhizosphere and phyllosphere community of greenhouse-grown lettuce at two plant stages. The rhizosphere and phyllosphere gammaproteobacterial microbiomes of lettuce plants showed clear differences in their overall and core microbiome composition as well as in corresponding diversity indices. The rhizosphere was dominated by Xanthomonadaceae (48%) and Pseudomonadaceae (37%) with *Rhodanobacter, Pseudoxanthomonas, Dokdonella, Luteimonas, Steroidobacter, Thermomonas* as core inhabitants, while the dominating taxa associated to phyllosphere were Pseudomonadaceae (54%), Moraxellaceae (16%) and Enterobacteriaceae (25%) with *Alkanindiges*, *Pantoea* and a group of Enterobacteriaceae unclassified at genus level. The preferential occurrence of enterics in the phyllosphere was the most significant difference between both habitats. Additional enhancement of enterics on the phyllosphere was observed in bottom rot diseased lettuce plants, while *Acinetobacter* and *Alkanindiges* were identified as indicators of healthy plants. Interestingly, the microbial diversity was enhanced by treatment with both the pathogen, and the co-inoculated biological control agent. The highest impact and bacterial diversity was found by *Rhizoctonia* inoculation, but FZB42 lowered the impact of *Rhizoctonia* on the microbiome. This study shows that the indigenous microbiome shifts as a consequence to pathogen attack but FZB42 can compensate these effects, which supports their role as biocontrol agent and suggests a novel mode of action.

Keywords: lettuce microbiome, Gammaproteobacteria, soil-borne pathogens, 16S rRNA gene pyrosequencing, phyllosphere, rhizosphere, Lactuca sativa

INTRODUCTION

Plants host a broad range of ubiquitous but also highly adapted and specific bacterial communities that colonize their epi- and endophytic compartments (Berg and Smalla, 2009; Berendsen et al., 2012; Bulgarelli et al., 2012). Due to their complexity, specific morphology, and production of secondary metabolites, the structure and function of plant-associated microbial communities are specific in these habitats but also at plant species and cultivar levels (Smalla et al., 2001; Berg et al., 2002; Berg and Smalla, 2009; Raaijmakers et al., 2009). The rhizosphere has already been investigated as a microbial habitat for more than one century (Hartmann et al., 2008), while the phyllosphere microbiome is only partly understood. Recent work suggests that the long-term colonization of phyllosphere is preferred by specific bacteria, while short-time colonization comprises many ubiquitous bacteria (Vorholt, 2012). All plant-associated habitats contain a high proportion of plant-beneficial microorganisms such as antagonists, diazotrophs, and plant growth promoting bacteria (PGPB) but also plant pathogens as well as potential human pathogens (Berg et al., 2005; Mendes et al., 2013). While the modes of action are often understood for single beneficial as well as pathogenic strains and species, less is known about the microbial community impact of single strains. Risk assessment and colonization studies for specific biocontrol agents showed minor and only transient effects on the rhizosphere community (Scherwinski et al., 2007; Adesina et al., 2009; Chowdhury et al., 2013; Schmidt et al., 2014), while impacts of pathogens on the indigenous microbiome are severely underexplored.

Soil-borne plant pathogens cause crucial damage to crops. The phytopathogenic fungus *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk; basidiomycetes] is

subdivided into anastomosis groups (AGs) according to their hyphal anastomosis reactions (Carling et al., 2002). The fungus causes a wide range of commercially significant plant diseases, such as Brown patch, damping off in seedlings, root rot and belly rot. *R. solani* strains are characterized by a distinct degree of host specificity as well as by different virulence levels to their plant host. *Rhizoctonia* strains occur almost ubiquitously in soils but isolates AG1-IB (Germany), AG2-1 (UK, the Netherlands) and AG4 (the Netherlands, UK, USA) have been isolated from diseased lettuce plants (Grosch et al., 2004). Strains belonging to AG1-IB were responsible for up to 70% yield loss of field-grown lettuce (Davis et al., 1997; Wolf and Verreet, 1999). One strain 7/3/14 of the supposed diploid and heterokaryotic *R. solani* AG1-IB, which was already sequenced, shows a large genome with many unique and unknown features in comparison with other *Rhizoctonia* strains and phylogenetically related fungi (Wibberg et al., 2013). Due to the low degree of host specificity, *Rhizoctonia* strains of different AGs can lower the general fitness of the plant during colonization, which results in higher sensitivity for additional pathogens such as spoilage enterobacteria (Berg et al., 2005). Interestingly, Adesina et al. (2009) could demonstrate direct changes caused by *R. solani* to the fungal and bacterial community patterns using molecular fingerprinting. All *Rhizoctonia* diseases, and subsequent secondary infections, in plants are difficult to control. In the past, only methyl bromide (MeBr) was effectively used. However, this fumigant has been banned for its ozone-depleting and toxic effects (UNEP, 1999). Alternative and environmentally friendly methods to suppress *Rhizoctonia* comprise naturally occurring antagonists such as *Serratia plymuthica* and *Pseudomonas jessenii* (Faltin et al., 2004; Grosch et al., 2005; Scherwinski et al., 2007; Adesina et al., 2009). In addition, *Bacillus amyloliquefaciens* FZB42, a long-time established plant strengthener was successfully applied to suppress *R. solani* on lettuce (Chowdhury et al., 2013). Genome sequencing of FZB42 revealed a high capacity of metabolite production with antimicrobial and antifungal activity, which suggested direct antifungal effects (Chowdhury et al., 2013). However, some reports suggest additional impacts of individual strains on the microbial community (Scherwinski et al., 2007; Schmidt et al., 2012). We therefore hypothesize that both beneficials as well as pathogens can cause significant shifts in the plant-associated microbiome.

The aim of this study was to identify the impact of the phytopathogenic fungus *R. solani* and the biological control agent *B. amyloliquefaciens* FZB42 on the indigenous rhizosphere and phyllosphere community of lettuce, cultivated under controlled conditions in a growth chamber. In our study we focus on the human health relevant group of Gammaproteobacteria, which was studied by analyzing specific amplicon libraries together with corresponding bioinformatic and statistical analysis. Gammaproteobacteria belong to the plant microbiome in general (Brandl, 2006), and are especially a substantial fraction of the lettuce-associated microbiome (Rastogi et al., 2012, 2013). However, they also comprise several species which were frequently identified to cause severe foodborne outbreaks (Teplitski et al., 2011).

MATERIAL AND METHODS

INOCULANTS USED IN THIS STUDY

The effect of *R. solani* and *B. amyloliquefaciens* FZB42 on lettuce growth and health was evaluated in this study. All experiments were performed with the product Rhizovital® 42 liquid (ABiTEP GmbH, Berlin, Germany), which is based on vital spores of FZB42 (Chowdhury et al., 2013). The bottom rot pathogen *R. solani* AG1-IB (isolate 7/3) was obtained from the strain collection of the Leibniz Institute of Vegetable and Ornamental Crops (Großbeeren, Germany) (Grosch et al., 2004).

EXPERIMENTAL DESIGN OF POT EXPERIMENTS

The effect of FZB42 and the pathogen *R. solani* on the microbial community of lettuce was studied by 454-amplicon sequencing analysis. Seeds (cv. Tizian, Syngenta, Bad Salzuflen, Germany) were germinated at 18°C in a seedling tray (92 holes) filled with a non-sterile mixture of quartz sand and substrate [Fruhstorfer Einheitserde Typ P, Vechta, Germany; chemical analysis (mg per l): N = 120, P = 120, K = 170, Mg = 120, S = 100, KCl = 1, organic substance = 167, peat = 309; pH 5.9] at a 1:1 ratio (v/v). The seedlings were further cultivated at 20/15°C until planting in a growth chamber (York, Mannheim, Germany; 16 h/8 h day/night cycle, 500 µmol m^{-2} s^{-1}, 60/80% relative humidity). Lettuce was planted at two-leaf stage into pots (500 ml) filled with the same substrate sand mixture as mentioned above inoculated and non-inoculated with the pathogen *R. solani* AG1-IB and grown at 22/15°C for 4 weeks. In the treatments with pathogen inoculation, the substrate mixture was inoculated with 10 *R. solani*-infested barley kernels and incubated at 25°C for 1 week until planting of lettuce into the pots.

For application of the inoculant FZB42 each lettuce plant was drenched with 20 ml spore solution (10^7 spores ml^{-1}) 3 days before and at planting time respectively. The pots were watered lightly each day to maintain the substrate moisture, and fertilized weekly (0.2% Wuxal TOP N, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany). All pot experiments were done at the Leibniz Institute of Vegetable and Ornamental Crops.

An overview about the sampling design is presented in **Table 1**. Here, abbreviations for compartments and treatments used throughout the manuscript were explained: P, phyllosphere; R, rhizosphere; Y, young; M, mature; RS, *R. solani*; C, untreated (control); FZB42RS, FZB42 and *R. solani* co-inoculation; G, healthy; K, diseased.

SAMPLE COLLECTION AND DNA ISOLATION

Sampling was carried out 2 weeks after planting (young plants) for treatments with and without FZB42 application, followed by a second sampling 4 weeks after planting (mature plants) for control and co-inoculated treatments with FZB42 and *R. solani*. The total community DNA was extracted per treatment, and habitat from two young plants and three mature plants (two independent DNA extractions were performed for each plant and the DNA was pooled prior to PCR), according to Bragina et al. (2011). Briefly, 5 g of plant material were physically disrupted with sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl. Two ml of suspension were centrifuged (16,500 × *g*, 20 min, 4°C) and the obtained pellets were used for isolation of the total-community

Table 1 | Sample design description.

ID	Habitat	Age (weeks after planting)	Treatment	Disease condition	Plant replicates	PCR replicates[b]	Sequencing
PYC	Phyllosphere	Young (2)	Untreated (Control)	not determinable	2	2	MWG, Eurofins
PYRS	Phyllosphere	Young (2)	*R. solani* inoculated	not determinable	2	2	MWG, Eurofins
PYfzb42RS	Phyllosphere	Young (2)	*R. solani* and FZB42 inoculated	not determinable	2	2	MWG, Eurofins
PMG	Phyllosphere	Mature (4)	Untreated (Control)	healthy	3 (6[a])	2	MWG, Eurofins
PMK	Phyllosphere	Mature (4)	*R. solani* and FZB42 inoculated	bottom rot	3 (6[a])	2	MWG, Eurofins
RYC	Rhizosphere	Young (2)	Untreated (Control)	not determinable	2	2	Macrogen, Korea
RYRS	Rhizosphere	Young (2)	*R. solani* inoculated	not determinable	2	2	Macrogen, Korea
RYfzb42RS	Rhizosphere	Young (2)	*R. solani* and FZB42 inoculated	not determinable	2	2	Macrogen, Korea
RMG	Rhizosphere	Mature (4)	Untreated (Control)	healthy	3 (6[a])	2	Macrogen, Korea
RMK	Rhizosphere	Mature (4)	*R. solani* and FZB42 inoculated	bottom rot	3 (6[a])	2	Macrogen, Korea

[a] *Two Independent DNA extractions per plant were pooled prior to PCR.*

[b] *PCR was carried out twice for each DNA sample and each PCR step and pooled prior to sequencing.*

DNA with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenized twice in a FastPrep® FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30 s at a speed of 5.0 m s^{-1} and treated according to the manufacturer's protocol.

BARCODED DEEP 454-PYROSEQUENING OF 16S rRNA GENE AMPLICON

The 16S rRNA genes of 24 samples (details are provided in **Table 1**) were amplified (two technical replicates for each sample) in a nested PCR approach with the Gammaproteobacteria primer set 395f (5′-CMA TGC CGC GTG TGT GAA-3′) and 871r (5′-ACT CCC CAG GCG GTC DAC TTA-3′) (Mühling et al., 2008). The PCR reaction mixture (20 µl) contained 5 × Taq-&GO Ready-to-use PCR Mix (MP Biomedicals, Germany), 0.25 µM of each primer, 25 mM $MgCl_2$ and 1 µl of template DNA (96°C, 4 min; 32 cycles of 96°C, 1 min; 57°C, 1 min; 74°C, 1 min; and final elongation at 74°C, 10 min). In a second PCR, 1 µl of the amplicon (1:10 diluted phyllosphere and 1:100 diluted rhizosphere derived PCR products) was used. 16S rRNA gene sequences were amplified by using the forward primer Unibac-II-515f (5′-GTG CCA GCA GCC GC-3′) containing the 454-pyrosequencing adaptors and the reverse primer Gamma871r_454 (5′-CTA TGC GCC TTG CCA GCC CGC TCA GAC TCC CCA GGC GGT CDA CTT A-3′). The reaction mixture for the second PCR (30 µl) contained 5 × Taq-&GO Ready-to-use PCR Mix, 0.25 µM of each primer (96°C, 4 min; 32 cycles of 96°C, 1 min; 66°C, 1 min; 74°C, 1 min; and final elongation at 74°C, 10 min). PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). The technical replicates per sample were pooled and the partial 16S rRNA gene fragments were sequenced using 454 Roche GS FLX (MWG Eurofins, Germany) and 454 Roche GS FLX Titanium (Macrogen Korea, South Korea) pyrosequencer. The nucleotide sequences obtained in this work were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) and are available under the accession number PRJEB6022.

DNA SEQUENCE ANALYSIS AND TAXONOMICAL IDENTIFICATION

Sequences were analyzed with the Qiime software version 6.0 (Caporaso et al., 2010). Replicates from sequencing of each treatment and habitat were bioinformatically pooled during the Qiime analysis for data evaluation. MID-, primer and adapter sequences were removed, length filtered (≥350 nt), quality filtered (score: 50), denoised, chloroplast removed and singletons adjusted. The cut-off level was set to 97% sequence identity. Chimeras were detected with Chimera Slayer and then removed. To compute alpha and beta diversity, the dataset was normalized to 5920 reads per sample. Ring-charts were created using the Krona software package version 2.2 (Ondov et al., 2011) and the profile network was constructed using Cytoscape version 3.0.2 (Shannon et al., 2003). Statistical tests based on the operational taxonomic units (OTUs) table were performed with the nonparametric ANOVA Kruskal Wallis test. This test is functionally an expansion of ANOVA to cases where the sample means are unequal and the distribution is not normal.

RESULTS

THE GAMMAPROTEOBACTERIAL MICROBIOME OF LETTUCE

Two sequential batches of 16S rRNA gene amplicon sequencing resulted in a total of 242,022 reads. After removing chimeras, singletons, and chloroplast sequences, 8233 quality mean reads per sample remained with a median absolute deviation of 1842.5 sequence reads. We analyzed the gammaproteobacterial fraction subjected to different treatments, separately for the phyllosphere and rhizosphere (Figure S1). Twenty-four samples (12 from per habitat) yielded in a total of 4.909 distinct OTUs, 1.102 were statistically different (Nonparametric ANOVA Kruskal Wallis Test, $p \leq 0.05$) between both habitats. The gammaproteobacterial microbiome from whole lettuce plants contained mainly taxa from Pseudomonadales, followed by Xanthomonadales, Enterobacteriales and Legionellales (**Figure 1**). The rhizosphere was dominated by Xanthomonadaceae (48%) and Pseudomonadaceae (37%) while the dominating taxa associated to phyllosphere were Pseudomonadaceae (54%), Moraxellaceae (16%) and Enterobacteriaceae (25%). The genus *Pseudomonas* was almost exclusively assigned to the family Pseudomonadaceae (98%) associated to foliage. The most abundant genus of the root associated microbiome was *Rhodanobacter* (27%) followed by *Pseudomonas* (24%), while 8% of the rhizosphere associated reads could not be taxonomically assigned (**Figure 1**). In addition,

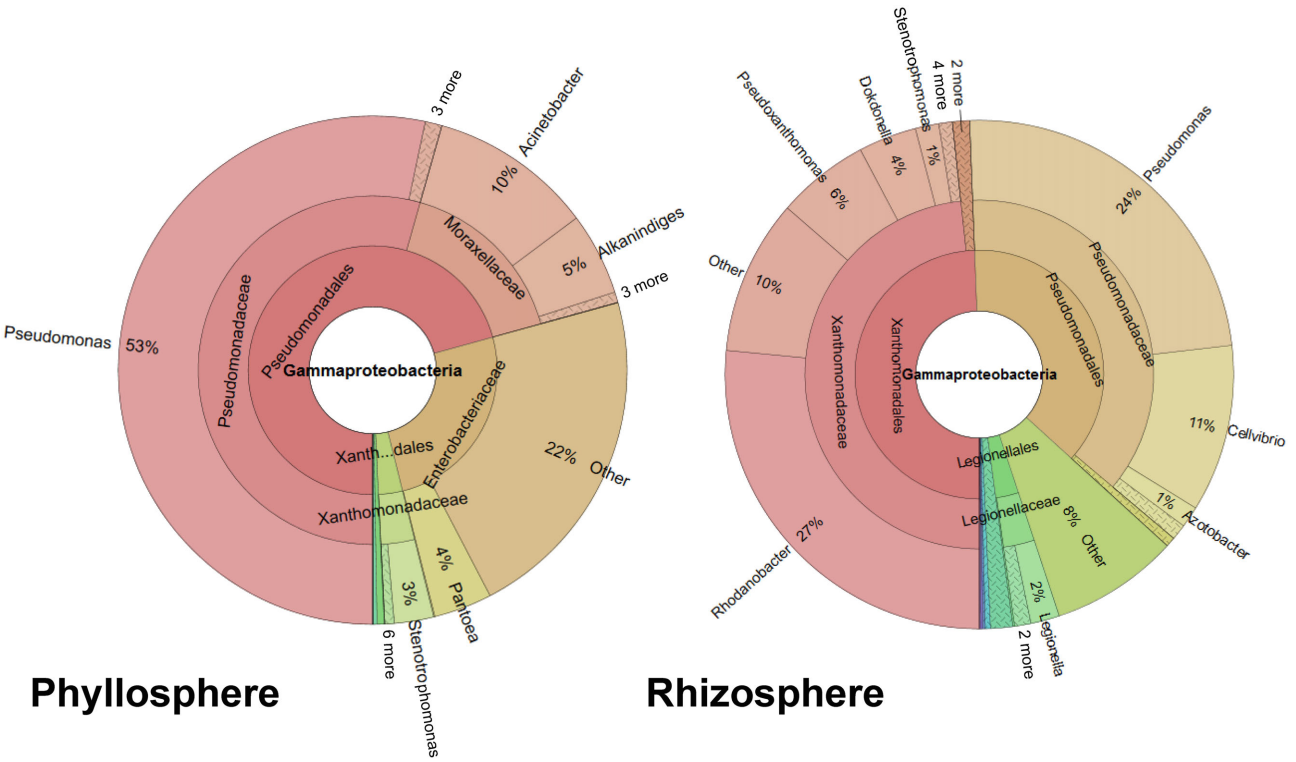

FIGURE 1 | Ring-charts showing the gammaproteobacterial community structure associated with investigated lettuce habitats. The RDP classifier analysis is shown from the mean of 12 samples per habitat. The rings represent different taxonomic ranks (order, family, and genus), and the columns represent distinct taxa. Minor taxa are not specified.

differences were found between both plant development stages as well as between the different treatments (Figure S1). Interestingly, comparing only the phyllosphere samples of mature plants with bottom rot disease against the untreated control, we found 371 distinct OTUs with 99 statistically distinct differences.

THE CORE GAMMAPROTEOBACTERIAL COMMUNITY AND ITS DIVERSITY

A profile clustering network based on all 454-amplicon Gammaproteobacteria libraries was applied to visualize relationships of lettuce-associated bacterial genera and RDP-classified conjoined taxa. The network presented in **Figure 2** can be used to identify the core microbiome of lettuce but also to connect specific taxa with different treatments, plant stages or with healthy and diseased plants. Our study revealed that plants belonging to different growth stages harbor several shared genera (at least 0.1% abundant in all samples compared). From 46 taxonomical assignments, 19 taxa could be assigned to a particular core microbiome structure of lettuce. Only *Alkanindiges*, *Pantoea,* and a lineage of Enterobacteriaceae (not classified at genus level) were exclusively assigned to the phyllosphere; they are indicated as phyllosphere core microbiome (**Figure 2**, surrounded by green color). For the rhizosphere a more diverse core microbiome was identified (red color): core taxa were assigned to *Rhodanobacter, Pseudoxanthomonas, Dokdonella, Luteimonas, Steroidobacter, Thermomonas, Legionella,* and *Haliea*. Additional OTUs were identified at family level as Xanthomonadaceae, Legionellaceae, Pseudomonadales, and Gammaproteobacteria. The highly abundant *Pseudomonas*, *Acinetobacter,* and *Stenotrophomonas* occurred in the core of both habitats (blue color). There are also other taxa found only associated to a specific habitat but did not account to any core group, due to low abundance or not comparable occurrence in the different treatments. In addition, detailed differences in the composition between the particular treatments are displayed by the network structure. For example, *Cellvibrio* was found preferentially in higher abundances in the rhizosphere of *R. solani* treated young plants and decreased in the rhizosphere of mature plants of the same treatment. Comparing all samples which were inoculated with *R. solani* (RS: PMK, PYRS, PYfzb42RS, $n = 7$) with the non-inoculated (C: PMG, PYC, $n = 5$), the highly abundant group of Enterobacteriaceae was dominantly retrieved from phyllosphere samples inoculated with *R. solani* (RS: 34.5% to C: 12.2% mean abundance), while Moraxellaceae including *Acinetobacter* and *Alkanindiges* were more abundant on the foliage of healthy plants (C: 30% to RS: 5.7% mean abundance). Similar gammaproteobacterial patterns could be observed on young plants inoculated only with *R. solani* compared to plantlets inoculated additionally with FZB42.

Beta diversity (pairwise sample dissimilarity) indices based on weighted UniFrac distances revealed clear differences between the habitats, but also between young and mature plants infected by *R. solani* with bottom rot disease compared to non-inoculated healthy young and mature plants (**Figure 3**, Table S1). A general

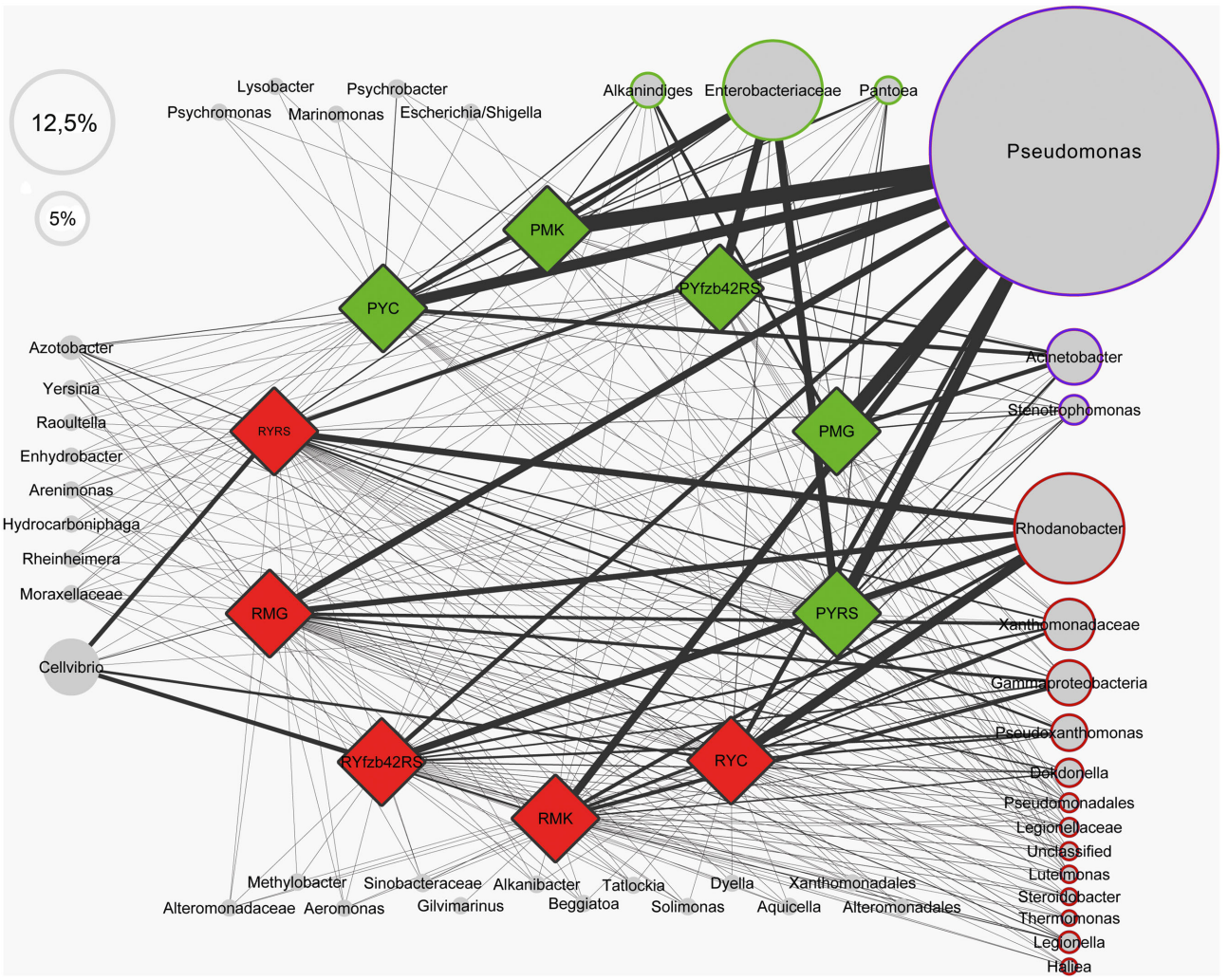

FIGURE 2 | Profile clustering network visualizing the investigated groups of lettuce (var. Tizian) and conjoined taxa. Red diamonds indicate samples derived from the roots and green diamonds from the lettuce foliage, respectively. Nodes represent taxa derived from the RDP classifier and the node sizes correspond to the mean relative abundance of all samples. The abundance of a certain taxa correlated to a particular investigated group is visualized trough the line width of the corresponding connection. The frame color represents the affiliation to the observed core taxa (green—phyllosphere, red—rhizosphere, blue—occurrence in both habitats and all samples, white—not a core taxon). Taxa less abundant than mean ≤0.5% are displayed without size-correlation. Abbreviations: P, phyllosphere; R, rhizosphere; Y, young; M, mature; RS, *R. solani*; C, untreated (control); FZB42RS, FZB42 and *R. solani* co-inoculation; G, healthy; K, diseased.

higher variation was observed among samples derived from the phyllosphere. Alpha diversity indices (Table S1) based on the observed species metric showed a higher diversity of Gammaproteobacteria associated with plants inoculated with *R. solani*, but the co-inoculation with FZB42 seems to reduce this effect (**Figure 4**). This was observed across both investigated habitats, and additionally a slight increase of the overall diversity correlated with the maturity state.

DISCUSSION

Our study gave new insights into the general structure of the lettuce microbiome as well as showed the impact of the plant pathogen *R. solani* AG1-IB and its antagonistic counterpart *B. amyloliquefaciens* FZB42. Sequencing of 16S rRNA gene amplicons provided especially a deeper look into the fraction of often health relevant Gammaproteobacteria in the lettuce-associated microbiome, down to the taxonomic rank of genera. While *R. solani* is a serious lettuce pathogen (Wolf and Verreet, 1999), Chowdhury et al. (2013) demonstrated that FZB42 is an efficient biocontrol agent. FZB42 was able to effectively reduce the disease severity of bottom rot caused by *R. solani* in pot and field experiments. In our study we showed that both microorganisms have not only a significant impact on plant health, they also significantly influence the structure of the plant-associated microbiome.

The rhizosphere and phyllosphere gammaproteobacterial microbiomes of healthy lettuce plants showed significant differences in their overall composition, their core, and diversity

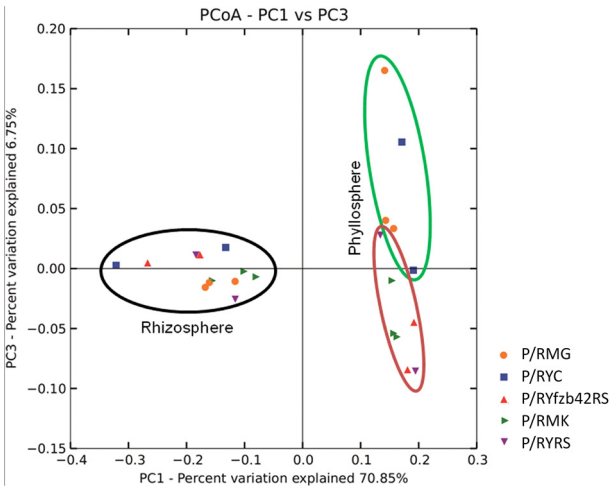

FIGURE 3 | Beta diversity metrics of bacterial 16S rRNA genes reveal distinctly clustered Gammaproteobacteria communities structured between healthy plants and plants affect by *Rhizoctonia solani*. Beta diversity community clustering is observed for phylogenetic beta diversity metrics (weighted UniFrac). In the panel, each point corresponds to a sample from either the lettuce rhizosphere (black) or the phyllosphere (green and red). Red—samples inoculated with *R. solani;* green—untreated control group. The percentage of variation explained by the plotted principal coordinates is indicated on the axes.

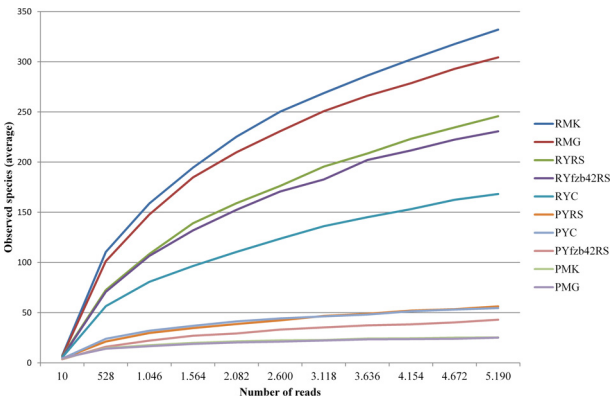

FIGURE 4 | Rarefaction analysis comparing overall diversity of the indigenous microbiota of the investigated lettuce samples (var. Tizian). Prior to rarefaction analysis, rhizosphere, and phyllosphere libraries were pairwise combined corresponding to the particular treatments. Rarefaction curves show saturation of the combined datasets that were clustered at 97% sequence similarity. The curves are supported by 95% confidence intervals. The overall diversity was higher affected in the rhizosphere. In both habitats plants treated with *Rhizoctonia solani* showed higher diversity than plants treated additionally with FZB42 or untreated plants. Abbreviations: P, phyllosphere; R, rhizosphere; Y, young; M, mature; RS, *R. solani*; C, untreated (control); FZB42RS, FZB42 and *R. solani* co-inoculation; G, healthy; K, diseased.

indices. This can be explained by the completely different abiotic conditions in both plant habitats (Raaijmakers et al., 2009; Vorholt, 2012). Here, the most significant and surprising difference we found was the preferential occurrence of enterics in the phyllosphere. Our results are in accordance with the principal findings of Rastogi et al. (2012), who analyzed spatiotemporal variation in bacterial community composition on field-grown lettuce in California. The general composition of phyllosphere bacteria was similar, and Enterobacteriacaea were a substantial fraction also in this study. In general, plant microhabitats are a reservoir for Enterobacteriaceae including potentially human pathogenic bacteria such as human enteric pathogens (Brandl, 2006). Especially after intermediate disturbances such as plant diseases, their abundance was enhanced (Erlacher et al., unpublished data). Due to their impact on human health as either pathogens or immunostimulants, this is an observation that could be of considerable importance for health concerns. Hanski et al. (2012) could show correlation between bacterial diversity and atopy, suggesting significant interactions with Gammaproteobacteria. These authors further showed a positive association between the abundance of *Acinetobacter,* found abundantly in healthy lettuce in our study and Interleukin-10 expression in peripheral blood mononuclear cells in healthy human individuals. Interleukin-10 is an anti-inflammatory cytokine and plays a central role in maintaining immunologic tolerance to harmless substances (Eskdale et al., 1997; Lloyd and Hawrylowicz, 2009).

In general, members of the genus *Pseudomonas* play a prominent role in the lettuce gammaproteobacterial microbiome. They were the dominant inhabitants of the phyllosphere; at family level pseudomonads present 54% and at genus level 53% of the microbiome. Also in the rhizosphere they represent 37% of the OTUs at family level. Altogether, *Pseudomonas* was the most dominant member of the lettuce core microbiome. *Pseudomonas* is a model organism to study beneficial plant-microbe interactions (Haas and Défago, 2005). Interestingly, in our study, *Pseudomonas* was not only related to healthy plants, there was also strong connection to diseased plants. The fact is not surprising because pathogenic pseudomonads are well-known but shows the limitation of the applied method. Using amplicon sequencing we can identify the genus or species but not their functional traits. For functional analysis metagenomic/transcriptomic techniques are required.

Plant-microbe interactions are highly complex and changes in the abundance of individual strains, either pathogens or beneficials can result in non-linear alterations of the entire microbiome composition. Such alterations may lead to negative effects to plants and humans as consumers (Berg et al., 2005). However, microbiome shifts can hardly be predicted and must be traced by thorough screening using culture-independent and sequencing-based approaches. Adesina et al. (2009) used DGGE fingerprint to study microbiome shifts and showed that *R. solani* AG1-IB inoculation severely affected the bacterial and fungal community structure in the rhizosphere of lettuce and that these effects were much less pronounced in the presence of the antagonistic counterpart *P. jessenii* RU47. In our study we used amplicon sequencing, which allowed a deep insight into the composition of Gammaproteobacteria. The human health relevant group of Enterobacteriaceae was affected by the *R. solani*; we found a significant increase in gammaproteobacterial diversity due to the pathogen outbreak. The overall enhancement of diversity after biotic stimulation by a pathogen agrees well with the intermediate

disturbance hypothesis known to ecology from studies of higher plants or coral reefs (Connell, 1978). However, together with FZB42 this increase was less distinct. Until today, the mechanisms described for biocontrol agents focus on direct antagonistic effects against a pathogen or an interaction via the plant's immune system (Doornbos et al., 2012). In this study we showed a selective compensation of the impact of a pathogen on the indigenous plant-associated microbiome by the biocontrol agent, which is an interesting effect of the beneficial aspect of the inoculant.

Biocontrol of plant pathogens is a promising solution for sustainable agriculture. Molecular techniques, which allowed a deeper insight into the crop-associated microbiome, can also be applied to develop new biocontrol strategies (Berg et al., 2013). Using a profile clustering network in our study, *Acinetobacter* and *Alkanindiges* were identified as indicators of healthy lettuce plants. Therefore, they could be promising biocontrol agents. An endophytic *Acinetobacter* strain isolated from healthy stems of the plant *Cinnamomum camphora* was already used as biocontrol strain against fungal diseases (Liu et al., 2007) but nothing is known about any biocontrol activity of *Alkanindiges*. In contrast, in the study of Rastogi et al. (2012), the foliar presence of *Xanthomonas campestris* pv. vitians, which is the causal agent of bacterial leaf spot of lettuce, correlated positively with the relative representation of bacteria from the genus *Alkanindiges*. Here, more research is needed for understanding bacterial networking on plants, which is an essential step toward predictable biocontrol. In addition, beneficial *Pseudomonas* strains could be other interesting candidates for biocontrol because we found a strong connection to healthy lettuce plants. This was shown already successfully for *P. jessenii* RU47 by Adesina et al. (2009). Altogether, new results favorite diverse bacterial cocktails to control plant diseases (Berg et al., 2013); for lettuce they could contain *Acinetobacter, Bacillus*, *Pseudomonas,* and *Serratia* strains as well.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00175/abstract

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Oxalotrophy, a widespread trait of plant-associated *Burkholderia* species, is involved in successful root colonization of lupin and maize by *Burkholderia phytofirmans*

Thomas Kost[1†], Nejc Stopnisek[1,2], Kirsty Agnoli[1], Leo Eberl[1] and Laure Weisskopf[1,2]*

[1] Laboratory of Microbiology, Institute of Plant Biology, University of Zurich, Zurich, Switzerland
[2] Ecology of Noxious and Beneficial Organisms, Institute of Sustainability Sciences, Agroscope, Zurich, Switzerland

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Anastasia Bragina, Graz University of Technology, Austria
Vittorio Venturi, International Centre for Genetic Engineering and Biotechnology, Italy

***Correspondence:**
Laure Weisskopf, Institute of Sustainability Sciences, Agroscope, Reckenholzstrasse 191, CH-8046 Zurich, Switzerland
e-mail: laure.weisskopf@agroscope.admin.ch

†Present address:
Thomas Kost, Plant Pathology Group, ETH Zurich, Zurich, Switzerland

Plant roots and shoots harbor complex bacterial communities. Early seed and plantlet colonization plays a key role in determining which bacterial populations will successfully invade plant tissues, yet the mechanisms enabling plants to select for beneficial rather than harmful populations are largely unknown. In this study, we demonstrate a role of oxalate as a determinant in this selection process, using members of the genus *Burkholderia* as model organisms. Oxalotrophy, i.e., the ability to use oxalate as a carbon source, was found to be a property strictly associated with plant-beneficial species of the *Burkholderia* genus, while plant pathogenic (*B. glumae, B. plantarii*) or human opportunistic pathogens (*Burkholderia cepacia* complex strains) were unable to degrade oxalate. We further show that oxalotrophy is required for successful plant colonization by the broad host endophyte *Burkholderia phytofirmans* PsJN: an engineered Δ*oxc* mutant, which lost the ability to grow on oxalate, was significantly impaired in early colonization of both lupin and maize compared with the wild-type. This work suggests that in addition to the role of oxalate in heavy metal tolerance of plants and in virulence of phytopathogenic fungi, it is also involved in specifically recruiting plant-beneficial members from complex bacterial communities.

Keywords: oxalate, root colonization, *Burkholderia*, PGPR, oxalate decarboxylase

INTRODUCTION

In the rhizosphere, most bacteria rely on root exudates as a source of carbon and energy. Exudates are of highly diverse chemical nature, from small carboxylates to complex phenolic compounds, and their secretion depends mostly on plant species and growth conditions. In nutrient-limited as well as in heavy-metal contaminated soils, exudation of organic acids is increased. This differential exudation of specific compounds has been shown to influence bacterial community structure (Weisskopf et al., 2005, 2008; Badri et al., 2009; Doornbos et al., 2012; Chaparro et al., 2013). Carboxylates such as citrate and malate are a major source of carbon for rhizosphere bacteria, and malate has even been postulated to act as a signal to recruit beneficial microorganisms (Rudrappa et al., 2008). In contrast, using oxalate as carbon source, a phenotype referred to as "oxalotrophy," is a rare trait of bacteria, although it occurs across a wide range of phylogenetically distant groups (Sahin, 2003; Khammar et al., 2009). In addition to citrate and malate, which are common components of root exudates, oxalate has also been shown to be a major root exudate of soil-grown plants (Dessureault-Rompre et al., 2007). However, neither the function of oxalate in recruiting specific microbes nor the relevance of oxalotrophy for bacterial rhizosphere competence has so far been investigated.

Members of the *Burkholderia* genus are frequently retrieved in plant microbiome surveys and seem to play a substantial role in direct plant growth promotion or in protection against soil-borne fungi (Mendes et al., 2007; Opelt et al., 2007; Compant et al., 2008; Li et al., 2008; Hardoim et al., 2011; Ikeda et al., 2013). Yet, beside plant beneficial members of the genus (e.g., *B. phytofirmans, B. phymatum*), others represent a threat to human health, such as the opportunistic pathogens of the *Burkholderia cepacia* complex (Mahenthiralingam et al., 2005). In an effort to characterize the bacterial communities living in and on the roots of white lupin, we have recently shown by both culture-independent and culture-dependent approaches that *Burkholderia* species are predominant members of the bacterial community inhabiting the cluster roots (Weisskopf et al., 2011). In addition to their ability to grow on citrate or malate, almost all isolated *Burkholderia* strains were able to use plant-secreted oxalate as a carbon source: 98% of the *Burkholderia* strains were oxalotrophic, compared with only 2% of the non *Burkholderia* strains isolated from the same environment. Moreover, *Burkholderia* sequences and strains almost exclusively belonged to the plant beneficial species and not to the opportunistic pathogenic ones (Weisskopf et al., 2011). These results led us to hypothesize that the capacity to utilize plant-exuded oxalate might explain why the roots of white lupin are strongly enriched for *Burkholderia* species. To test this hypothesis, we determined the capacity to utilize oxalate among a wide range of *Burkholderia* strains that belong either to plant beneficial or to opportunistic pathogenic species. In addition, we mutated

the oxalotrophy pathway in the plant beneficial endophytic *B. phytofirmans* and monitored seed and root colonization of the mutant and the wild-type strains in white lupin and in maize.

MATERIALS AND METHODS

STRAINS, PLASMIDS AND CULTURE MEDIA

Strains and plasmids used in this study are listed in **Table S1**. For long-term storage, bacterial strains were kept at −80°C in 50% glycerol. Chemicals were purchased from Sigma Aldrich if not specified otherwise. Bacteria were routinely grown on Luria-Bertani (LB) medium (20 g LB powder (Difco) per liter) and 18 g agar, *Pseudomonas* Isolation Agar (PIA) medium (45 g *Pseudomonas* Isolation Agar (Difco), 5 g additional agar, 20 ml glycerol per liter), or Mueller-Hinton agar (21 g Mueller Hinton Broth (Difco) and 15 g agar per liter). For oxalate degradation assay, AB minimal medium was used with (per liter) 2 g $(NH_4)_2SO4$, 6 g Na_2HPO_4, 6 g Na_2HPO_4, 3 g NaCl, 2 mM $MgCl_2 \times 6\ H_2O$, 100 μM $CaCl_2 \times 6\ H_2O$, 3 μM $FeCl_3 \times H_2O$ and 40 μl oligoelement solution (10 mg $ZnSO_4 \times 7\ H_2O$, 13 mg $MnCl_2 \times 4\ H_2O$, 3 mg $Na_2MoO_4 \times 2\ H_2O$, 30 mg H_3Bo_3, 20 mg $CoCl_2 \times 6\ H_2O$, 1 mg $CuCl_2 \times H_2O$, 2 mg $NiCl_2 \times 6\ H_2O$). pH was adjusted to 7. This medium, supplemented with 18 g agar per liter, was used as the first layer of the oxalate degradation medium. A second layer, which contained 7 g calcium oxalate $\times\ H_2O$ and 12 g agar per liter was freshly stirred and added on the first layer. MS medium contained 2.2 g Murashige and Skoog medium (Sigma-Aldrich) and 5 g agar per liter. pH was adjusted to 5.7 prior to autoclaving.

OXALATE DEGRADATION ASSAY

Strains were grown overnight in 5 ml of AB minimal medium with 5 g l^{-1} glucose as carbon source. 2 ml of the overnight culture were centrifuged at 4000 rpm for 5 min and the pellet was washed twice and resuspended in 1 ml 0.9% NaCl solution. OD_{600} was measured and all samples were diluted with 0.9% NaCl to OD_{600} of 0.2. 50 μl of diluted cell suspension were pipetted onto the double layer oxalate-medium and incubated for at least 2 days at 30°C. The formation of a transparent halo revealed the ability to degrade oxalate.

CONSTRUCTION OF A MUTANT IMPAIRED IN OXALATE DEGRADATION AND FLUORESCENT TAGGING

In *B. phytofirmans* PsJN, the oxalate degradation cluster is located on chromosome 2 and consisted of three genes encoding (i) the oxalate/formate antiporter (Bphyt_6739), (ii) the oxalate decarboxylase (*oxc*, Bphyt_6740), and the formyl-CoA transferase (*frc*, Bphyt_6741) (Weilharter et al., 2011). Unlike the antiporter and the formyl-CoA transferase, the oxalate decarboxylase was present as single copy in the genome, and was thus chosen as a target for mutagenesis. A 1650 bp region spanning Bphyt_6740 (*oxc*) was amplified using XhoI and BglII restriction site-containing primers 5′-GCGCCTCGAGCTGAACGACATCAAAACCAT-3′ and 5′-GCGCAGATCTGATTACTTTTTCATTGCCGC-3′, which were designed using the CLC workbench software and purchased from Microsynth, Balgach, Switzerland. The PCR reaction was performed as follows: 1 cycle of 2 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 48°C, and 100 s at 72°C, and a final extension at 72°C for 5 min. The resulting amplicon was purified using Qiagen PCR purification kit, digested with BglII and XhoI and ligated overnight at room temperature with the vector pSHAFT2 (4552 bp) previously digested with the same enzymes. The ligation product was transformed into *E. coli* CC118λpir cells followed by selection for chloramphenicol resistant clones on LB plates. The resulting plasmid (pSHAFT2 carrying *oxc*) was then isolated and digested with NcoI, a restriction site located in the middle of *oxc*, dephosphorylated and purified. In parallel a trimethoprim resistance cassette was amplified by PCR using NcoI containing primers 5′-GCGCCCATGGCAGTTGACATAAGCCTGTTC-3′ and 5′-GCGCCCATGGTTAGGCCACACGTTCAAGTG-3′, which were designed using the CLC workbench software and purchased from Microsynth, Balgach, Switzerland. The PCR reaction was performed by 1 cycle of 2 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 100 s at 72°C, and a final extension at 72°C for 5 min. The resulting amplicon was digested with NcoI and purified. Ligation was performed overnight and the ligation product was transformed into CC118λpir cells. Clones were selected on Mueller-Hinton plates supplemented with trimethoprim and correct insertion in the isolated plasmids was verified by restriction with NcoI or XhoI and BglII. This strain carrying the interrupted *oxc* gene was used as donor strain for triparental mating with *E. coli* MM294 strain as a helper and *B. phytofirmans* PsJN as a recipient. 2 ml of overnight culture (5 ml LB medium with appropriate antibiotic) was centrifuged and washed twice in 0.9% NaCl solution. Then the cells were resuspended in 0.5 ml LB media. 100 μl of the helper culture; 100 μl of the donor strain culture were mixed and kept at room temperature (RT) for 20 min and then 100 μl of recipient strain were added. Afterwards 150 μl of the mixed culture were pipetted in drops of about 50 μl on a LB plate and incubated for 6 h at 30°C. Then the cells were harvested, resuspended in 1.5 ml 0.9% NaCl solution, diluted and spread on PIA plates supplemented with trimethoprim. Loss of chloramphenicol resistance was used to select clones where double crossing-over recombination had occurred (see **Figure S1** for a diagram of the cloning procedure). Fluorescent tagging of *B. phytofirmans* wild-type and Δ*oxc* mutant was carried out by triparental mating as described above. The donor strains were *E. coli* carrying either the plasmid pBBR1MCS-2-gfpmut3 (GFP, kanamycin resistance) or the plasmid pIN62 carrying the dsRED encoding gene and a chloramphenicol resistance cassette (see **Table S1**). Transformants were selected on PIA plates with kanamycin or chloramphenicol.

PLANT COLONIZATION EXPERIMENTS

Early colonization

Two plant species were used as models for the colonization assays of *B. phytofirmans* wild-type and Δ*oxc*-mutant: white lupin (*Lupinus albus* L., cv. Amiga) and maize (*Zea mays* subsp. *mays*, cv. Birko). Seeds were sterilized by vigorous shaking (200 rpm) in 2.5% NaHClO solution 0.2‰ (v:v) Triton X for 5 min, followed by rinsing twice in sterile water and drying under the sterile bench. Seeds were bacterized with *B. phytofirmans* strains using the following procedure: dsRED- or GFP-tagged derivatives of the wild-type strain and the Δ*oxc* mutant, were grown

overnight in LB broth. The dsRED-tagged strains were used for single inoculation experiments due to the higher signal intensity compared to the GFP-tagged cells. Cells were harvested by centrifuging for 5 min at 6000 rpm, washed twice in NaCl 0.9% and resuspended in 20 ml NaCl solution to adjust the OD_{600} to 0.25 (corresponding to approximately 10^7 cells/ml). For mixed inoculations (GFP-tagged wild-type (wt): dsRED-tagged Δ*oxc*, dsRED-tagged wt: GPF-tagged Δ*oxc*), the two strains were mixed after cell washing in a 1:1 ratio (OD_{600} of 0.125 for each strain). 20 surface-sterilized seeds of maize or 20 seeds of lupin were dipped in 10 ml of the respective bacterial suspension and incubated in a Falcon tube for 1 h at room temperature. Control seeds were incubated in NaCl solution. Thereafter, bacterized seeds were washed in NaCl to remove non-attached cells and sterilely transferred to Petri dishes with 1/2 MS medium. Plates were incubated for 3 days at room temperature in the dark to allow seed germination. After 3 days, selected germinated seeds were examined with a Leica M165FC fluorescent microscope for colonization pattern while other seeds from the same batch were used for colony forming unit (CFU) determination. For the latter, germinated seeds were placed in a 15 ml Falcon tube filled with 10 ml NaCl 0.9% and gently detached by 15 min incubation in a sonication water bath (Memmert WB 14, Germany). Thereafter, the cell suspensions were serially diluted and plated on PIA medium. Colonies were counted after four day incubation at 30°C. To verify statistical significance student's *t*-test was performed. For dual inoculation, colonies were counted under the binocular (to verify green fluorescence, which was not visible by eye on the plate unlike the red color originating from dsRED-tagging). After 7 days of incubation in the Petri dish that contained 1/2 MS medium, new seedlings were harvested and examined for early colonization pattern using a NightOWL LB 983 NC100 (Berthold technologies, Germany).

Persistence in planta

Seeds of maize and lupin were bacterized using the procedure described above. After 3 days of germination, four seeds for each treatment were transferred to 50 ml Falcon tubes filled with vermiculite (one seed per tube). Plants were transferred to a greenhouse with natural light, approximately 25°C and 70% humidity. 7 days later, a second inoculation step was carried out on these vermiculite microcosms by adding 4 ml of a cell suspension adjusted to an OD_{600} of 0.25 to each Falcon (NaCl for the control microcosms). Plants were fertilized once a week with MIOPLANT fertilizer (Migros, Switzerland) using half the concentration recommended by the manufacturer and watered twice a week. They were harvested after 28 days. To determine CFUs, roots were gently ground in NaCl 0.9% and ground tissues serially diluted and plated on PIA plates (see above, Early colonization).

OXALATE MEASUREMENTS IN PLANT TISSUES

Oxalate measurements in lupin and maize root tissues were performed after 3 and 28 days with an enzymatic kit from LIBIOS (France). Prior to analysis, washed roots were weighted and ground in liquid N_2. The resulting powder was extracted in twice its weight of water for 30 min under continuous shaking. Thereafter, the extract was centrifuged at 13,000 rpm for 5 min and 10 µl of the supernatant was used for oxalate quantification according to the manufacturer's protocol.

CONSTRUCTION OF PHYLOGENETIC TREE OF *BURKHOLDERIA* SPECIES

Forty one *Burkholderia* 16S rRNA gene sequences were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). 1130 bp long sequences were aligned using ClustalW (Thompson et al., 1994) in MEGA5.05 software (Tamura et al., 2011). Phylogenetic trees were obtained by applying the Neighbor-Joining (NJ) method in the MEGA 5.05 software. The tree topology was inferred with a Kimura 2-parameter correction model (Kimura, 1980) and with 1000 bootstrap replications. 16S rRNA gene sequence of *Ralstonia solanacearum* LMG 2299 was used as an outgroup.

RESULTS

OXALOTROPHY IS WIDESPREAD IN PLANT-ASSOCIATED *BURKHOLDERIA* SPECIES BUT ABSENT FROM OPPORTUNISTIC PATHOGENIC SPECIES

Fifty eight strains, which belong to 41 different species were tested for their ability to utilize oxalate as a sole carbon source. None of the strains from the *Burkholderia cepacia* complex species could grow on oxalate (**Table 1**, **Figure 1**). Likewise, all plant pathogenic *Burkholderia*, including strains of *B. glumae, B. plantarii*, and *B. gladioli* were unable to do so. In contrast, all *Burkholderia* strains that belonged to the "plant beneficial cluster" (Suarez-Moreno et al., 2012) were oxalotrophic, with the exception of *B. phenazinium*, which could not grow on oxalate (**Table 1**) and from which the *frc* gene [formyl-CoA transferase, catalyzing the first step of oxalate catabolism (Khammar et al., 2009)] could not be amplified (data not shown). The ability or inability to degrade oxalate was conserved within the same species, as shown for diverse examples (**Table 1**). The almost universal trait of plant-associated *Burkholderia* to utilize oxalate and the incapacity of all tested plant or human opportunistic pathogens to do so led us to hypothesize that oxalotrophy might be involved in the establishment of mutualistic interactions between bacteria and plants.

OXALOTROPHY IS INVOLVED IN SUCCESSFUL PLANT COLONIZATION BY *B. PHYTOFIRMANS*

To evaluate the role of oxalotrophy in plant colonization, the oxalate decarboxylase gene *oxc* was inactivated in the broad-host endophytic bacterium *B. phytofirmans* PsJN (Sessitsch et al., 2005). The *oxc* gene is the second gene in a putative oxalate catabolism gene cluster, which contains the putative oxalate/formate antiporter Bphyt_6739, *oxc*, and the formyl-coA transferase gene *frc* (Bphyt_6741) (**Figure S1**). As expected, oxalotrophy was abolished in the mutant strain (**Figure 2**). The wild-type and the Δ*oxc* mutant were marked with either GFP or dsRED to allow monitoring of their plant colonization abilities (see Materials and Methods for details). The marked strains exhibited the same growth behavior in LB medium in single as well as in mixed inoculation experiments, indicating that the marker genes (GFP, dsRED) did not affect the results (**Figure S2**).

Sterilized seeds of lupin and maize were inoculated with (i) the wild-type, (ii) the Δ*oxc* mutant, and (iii) both strains

Table 1 | Oxalate degradation ability (OX) in various species of the *Burkholderia* genus.

Species	Strain	OX	Species	Strain	OX
Plant beneficial environ. *Burkhoderia* sp.			***Burkholderia cepacia* complex sp.**		
B. caledonica	LMG19076	+	*B. ambifaria*	LMG17828	–
B. caribensis	LMG18531	+	*B. anthina*	LMG21821	–
B. caryophylli	LMG2155	+	*B. arboris*	LMG24066	–
B. bryophila	LMG23646	+	*B. cenocepacia*	R–6274	–
B. fungorum	LMG16225	+	*B. cepacia*	ATCC25416	–
B. graminis	LMG18924	+	*B. contaminans*	LMG23361	–
B. hospita	LMG20598	+	*B. diffusa*	LMG24065	–
	Isolate NS11	+	*B. dolosa*	LMG18941	–
	Isolate NS7	+	*B. lata*	LMG22485	–
B. kururiensis	LMG19447	+	*B. latens*	LMG24064	–
B. phenoliruptrix	LMG22037	+	*B. metallica*	LMG24068	–
B. phymatum	LMG21445	+	*B. multivorans*	LMG18825	–
B. phytofirmans	LMG22487	+	*B. pyrrocinia*	LMG14191	–
B. sacchari	LMG19450	+		LMG21822	–
B. terricola	FN313521	+		LMG21823	–
	LMG20594	+	*B. seminalis*	LMG24067	–
B. tropica	LMG22274	+	*B. stabilis*	Isolate R6270	–
B. tuberum	LMG21444	+		LMG14294	–
B. xenovorans	LMG21463	+	*B. ubonensis*	LMG20358	–
B. phenazinium	LMG2247	–	*B. vietnamiensis*	LMG18835	–
	Isolate S1	–	**Plant pathogenic *Burkholderia* sp.**		
	Isolate S7	–	*B. gladioli*	LMG2216	–
	Isolate S18	–		LMG11626	–
	Isolate 1S9	–		LMG18157	–
Unclassified *Burkholderia* sp.			*B. glumae*	LMG2196	–
				ATCC33617	–
B. glathei	LMG14190	+		AU6208	–
B. sordidicola	LMG22029	+		ATCC43733	–
B. thailandensis	LMG20219	–	*B. plantarii*	Isolate TT	–
B. andropogonis	LMG2129	–		Isolate VV	–
				LMG9035	–

Oxalate degradation for pure Burkholderia cultures was revealed by a halo surrounding growing colonies when inoculated on a minimal medium with calcium oxalate as a sole carbon source (see Materials and Methods for details).

in equal cell densities (approximately 10^7 cells/ml of inoculation solution). For single inoculation studies, the dsRED-tagged strains were used, as the signal was brighter than in the GFP-tagged strains. For dual inoculations, both combinations were used (GFP-tagged wild-type and dsRED-tagged Δ*oxc*, or dsRED-tagged wild-type and GFP-tagged Δ*oxc*) to avoid any bias due to fluorescent marker genes. When inoculated as single strains, a significant decrease in root colonization capacity was observed in the mutant relative to the wild-type on both lupin and maize (**Figure 3A**). This difference, which was confirmed by microscopic inspection (**Figure 4**), was more pronounced at early stages of colonization than after one month of cultivation, especially for maize. In lupin, about a million cells/g root fresh weight could be detected for the wild-type in all three plants after 28 days, yet the mutant was only detectable in one of three plants and present at a much lower population density (100-fold decreased relative to the wild-type). In maize, the difference was less pronounced after one month of cultivation when compared to the beginning of colonization (just below significance level, $P = 0.055$). This difference might be explained by the fact that lupins produced much more oxalate than maize (30 nmol vs. 6 nmol per g root fresh weight after 3 days and 60 nmol vs. 30 nmol after 28 days). When inoculated together with the wild-type, the colonization defect of the Δ*oxc* mutant was restored (**Figure 3B**), that was confirmed by visual inspection of 7 day-old seedlings (**Figure 5**). While the Δ*oxc* mutant was not able to spread from the seeds to the roots when inoculated as a pure culture (**Figure 5C**), this phenotype was partially rescued in the presence of the wild-type strain (**Figure 5D**).

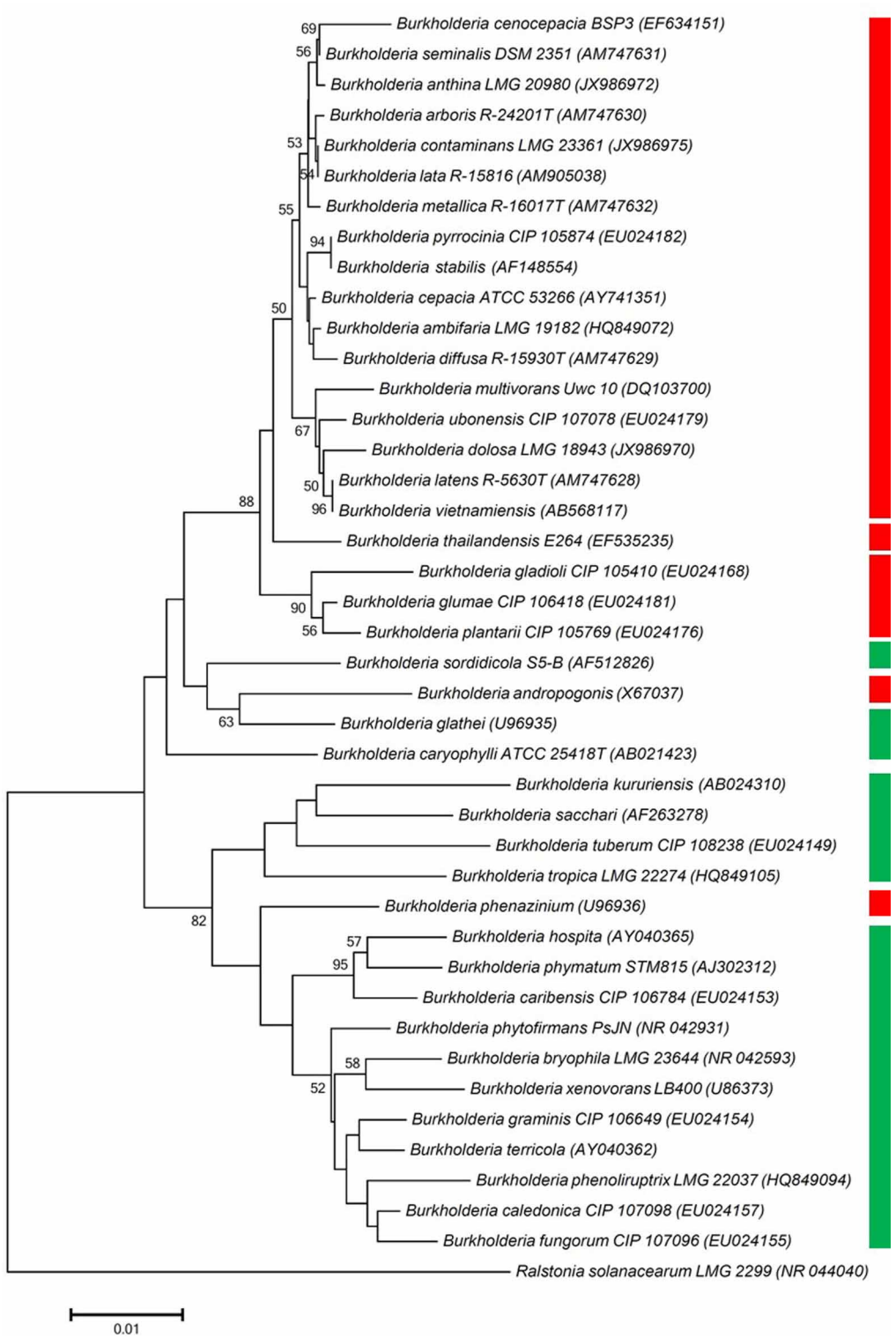

FIGURE 1 | Phylogenetic tree, constructed using one representative 16S rRNA gene sequence per *Burkholderia* species included in the oxalotrophy assay. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Only bootstrap values exceeding 50% are labeled. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Green bar indicates oxalotrophy of the tested strains of a given species, red bar indicates inability to degrade oxalate (see also **Table 1** for detailed results).

DISCUSSION

One major source of oxalate in natural ecosystems is fungal production, e.g., in wood-rotting fungi, where it is involved in lignin degradation or in some phytopathogenic fungi, e.g., *Sclerotinia* or *Botrytis* species, where it acts as virulence factor (Dutton and Evans, 1996; Criscitiello et al., 2013; Heller and Witt-Geiges, 2013). High quantities of oxalate are toxic to animals and humans, due to the formation of calcium- or magnesium oxalate crystals, which can lead to depletion in essential cations or to kidney stone formation (Coe et al., 2010). However, oxalate is also an important metabolite of many plant species, where it is thought to be important for calcium storage and for repelling herbivores (Franceschi and Nakata, 2005). Moreover, oxalate secretion is involved in tolerance to heavy metals including aluminum, as demonstrated e.g., in buckwheat (Klug and Horst, 2010) or in rice (Yang et al., 2000).

When plants grow in situations where nutrients such as phosphate or iron are limited, or when heavy metals are abundant, excretion of organic acids is increased (Meyer et al., 2010). This enhanced secretion of citrate, malate or oxalate enriches the rhizosphere in organic carbon, which can be used by certain microorganisms as a nutritional source. Consequently, those members of the community that possess the metabolic means to catabolize those exudates will be enriched. In a previous study, we observed an overrepresentation of *Burkholderia* species in various development stages of white lupin cluster roots (Weisskopf et al., 2011). This enrichment might be linked to the acidic environment that prevails around mature cluster roots and to the preference of *Burkholderia* species to exist in acidic soils (Stopnisek et al., 2013). Given that most of the *Burkholderia* strains isolated from white lupin were able to utilize oxalate as a carbon source, we asked whether this property is, like acid tolerance, a genus-wide property or is restricted to species that are predominantly associated with plants and/or fungi. By testing strains that belong to 41 different species, we observed that the ability to grow on oxalate as a sole carbon source is restricted to members of the plant-beneficial environmental cluster (Suarez-Moreno et al., 2012) (**Figure 1**) and absent in pathogenic species, including the human pathogen *B. pseudomallei*, plant pathogens such as *B. plantari* or *B. glumae* and opportunistic pathogens, which belong to the Bcc cluster. Interestingly, virulent strains of *B. glumae*, an important pathogen of rice, have been shown to produce oxalate, while non-virulent ones were not oxalogenic (Li et al., 1999), suggesting that oxalate production might be important for virulence, as it is the case with fungal pathogens. Beyond its role as a virulence factor, oxalate has been postulated to be a common good of pathogenic *Burkholderia* species, including *B. glumae, B. pseudomallei and*

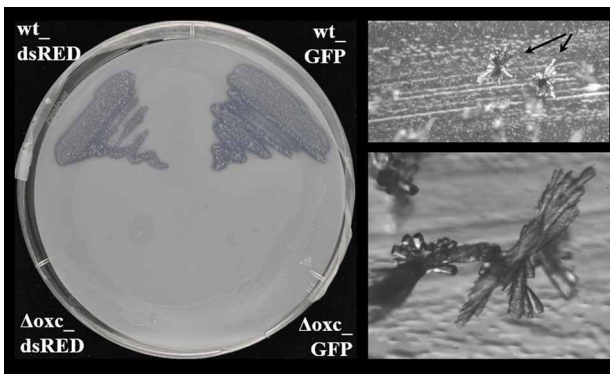

FIGURE 2 | Assessment of oxalotrophy in the dsRed and GFP-tagged wild-type (wt_dsRed respectively wt_GFP) and the accordingly tagged (Δ*oxc*_dsRED respectively Δ*oxc*_GFP) Δ*oxc* mutants of *B. phytofirmans* (left) strain. Both tagged wild-type strains (upper half of the Petri dish) showed a cleared halo around the grown colonies, which indicates degradation of the Ca-oxalate present in the upper layer of the minimal medium. Both tagged Δ*oxc* mutant strains (lower half of the Petri dish) were unable to grow on the minimal medium with oxalate as a sole carbon source. Picture was taken after 10 days of growth at room temperature. In the oxalate degrading strains, characteristic crystal structures (most probably $CaCO_3$) were formed above the agar surface (right, arrows, and zoomed view below).

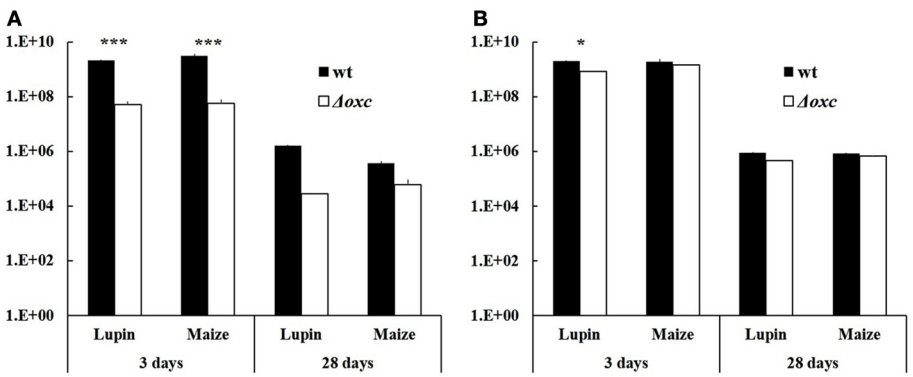

FIGURE 3 | Abundance of *B. phytofirmans* PsJN wild-type and Δ*oxc* mutant after seed germination (3 days) and after one-month microcosm cultivation in vermiculite (28 days). Seeds were either bacterized with one strain (single inoculation, **A**) or with a 1:1 mixture of wt and Δ*oxc* mutant (dual inoculation, **B**). Results are expressed as CFU per seed after 3 days and as CFU per g root fresh weight after 28 days. Average of 2–3 replicates (seeds/plants) are shown, with the exception of one case, where no mutant cells were retrieved in two out of three replicate plants (**A**, lupin, 28 days, Δ*oxc*). Stars indicate significant differences between wild-type and mutant (Student's *t*-test, $n = 2$–4, $^{*}P < 0.05$, $^{***}P < 0.001$).

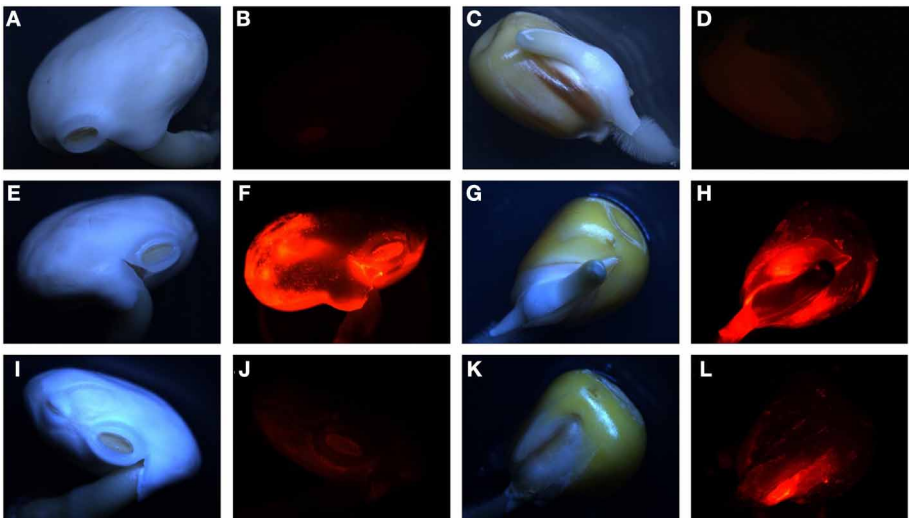

FIGURE 4 | Representative pictures of seed colonization of lupin (A,B,E,F,I,J) and maize (C,D,G,H,K,L) by dsRED-tagged wild-type (E–H) or Δ*oxc* mutant (I–L) after 3 days. A-D: non inoculated seeds. Pictures were taken using a Leica M165FC fluorescent microscope, under normal light **(A,C,E,G,I,K)** or dsRED fluorescent filter **(B,D,F,H,J,L)** with 0.4 s. exposure in all cases.

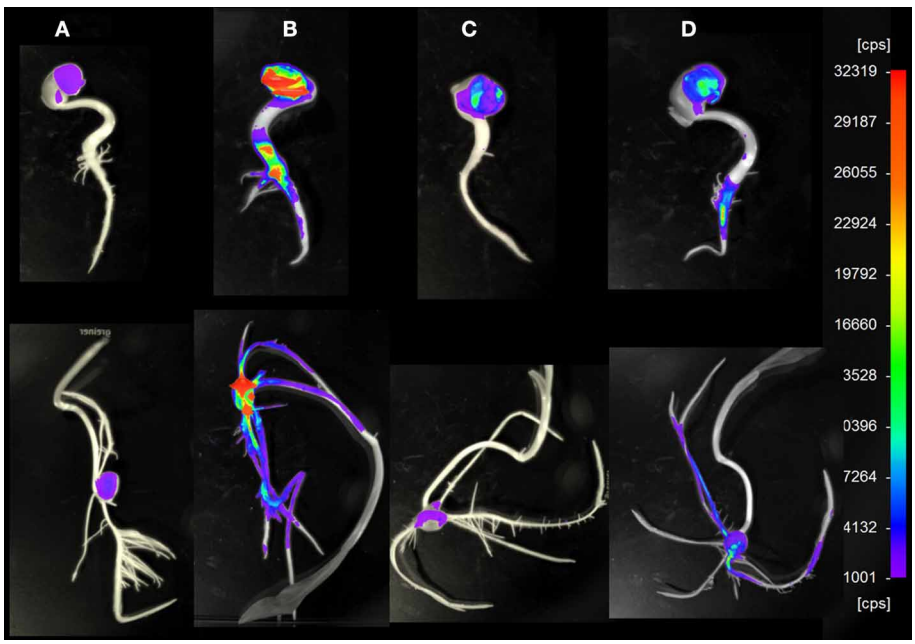

FIGURE 5 | Representative pictures of lupin (upper row) and maize (lower row) 7 day old seedlings colonized by *B. phytofirmans* PsJN wild-type or Δ*oxc* mutant in single or combined inoculation. For imaging, a NightOWL LB 983 NC100 was used, under conditions where dsRED-tagged cells are visible. **(A)**: non inoculated control, **(B)**: inoculated with dsRED-tagged wild-type, **(C)**: inoculated with dsRED-tagged Δ*oxc*, **(D)**: inoculated with GFP-tagged wild-type and dsRED-tagged Δ*oxc* mutant. cps: counts per second.

B. thailandensis (Goo et al., 2012). Oxalate production in these species is controlled by quorum-sensing and was shown to neutralize the alkalinization of the medium caused by the emission of NH_3 in the late stationary phase, thereby ensuring that the pH remains at a physiological level (Goo et al., 2012).

Oxalate degradation by plant-beneficial *Burkholderia* might be considered a plant-protecting feature, as lowering the oxalate levels on plant surfaces might alleviate the infection potential of oxalate-producing phytopathogenic fungi or bacteria. This was shown in the case of *Cupriavidus campinensis*, which could significantly reduce disease symptoms caused by the oxalogenic fungi *Botrytis cinerea* or *Sclerotinia sclerotiorum* on *Arabidopsis*, grapevine and tomato plants, while a mutant strain impaired in oxalate degradation showed only reduced protecting potential

(Schoonbeek et al., 2007). In order to investigate whether oxalate degradation might provide an advantage in plant colonization, a mutant, in which oxalotrophy is abolished, was generated (**Figure 2**). The broad-host endophyte *B. phytofirmans* PsJN (Sessitsch et al., 2005) served as a model organism in this study. The colonization behavior of the wild-type and the mutant on plants with moderate (white lupin) or low (maize) oxalate secretion was compared. When inoculated alone, the mutant suffered a drastic disadvantage both in early colonization steps (3 days) and in persistence on the plants (**Figures 3A**, **4**, **5**). Similar differences between the wild-type and the mutant were observed for lupin and maize at the early stage of colonization; however, after one month of cultivation the effects were much more dramatic on lupins, where only in one out of three plants mutant cells could be recovered, than on maize, for which the difference between wild-type and mutant was not significant. Surprisingly, when the mutant and the wild-type were inoculated in a 1:1 ratio, the mutant recovered most of its lost capacity to colonize the plants (**Figures 3B**, **5**). This suggests that oxalate might act as a toxic compound for the strains that cannot degrade it. The presence of the wild-type would then alleviate this toxic effect by lowering the levels of free oxalate through oxalotrophy. When grown in glucose-supplemented minimal medium, the mutant's growth was only very marginally reduced upon addition of oxalate, which indicates that oxalate is not toxic under laboratory conditions. However, this does not exclude a putative toxicity of oxalate in the seed or plant environment. Moreover, the better colonization performance of the mutant when co-inoculated with the wild-type might also be explained by the utilization of degradation products resulting from oxalate catabolism of the wild-type.

Roots are the entry point for most endophytic bacteria, which then can spread to above-ground plant tissues. Understanding how plants select for beneficial root and shoot inhabitants and/or against plant pathogenic species is obviously very important for plant health. This work sheds light on the so far overlooked role of oxalotrophy in root colonization, which in the case of *Burkholderia* species selects for plant beneficial bacteria over colonization by plant and even animal pathogens.

AUTHOR CONTRIBUTIONS

Laure Weisskopf designed the research, Thomas Kost, Kirsty Agnoli and Laure Weisskopf performed experiments, Thomas Kost, Nejc Stopnisek, Kirsty Agnoli and Laure Weisskopf analyzed the data, Laure Weisskopf wrote the MS with help from Kirsty Agnoli, Nejc Stopnisek, Thomas Kost, and Leo Eberl.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Journal/10.3389/fmicb.2013.00421/abstract

Table S1 | Strains and plasmids used in this study.

Figure S1 | Structure of the gene cluster involved in oxalate degradation and construction of a Δ*oxc* mutant in *B. phytofirmans* PsJN.

Figure S2 | Growth curves and *in vitro* competition experiment of *B. phytofirmans* PsJN wt and Δ*oxc* mutant.

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Vertical transmission explains the specific *Burkholderia* pattern in *Sphagnum* mosses at multi-geographic scale

***Anastasia Bragina*[1], *Massimiliano Cardinale*[1,2], *Christian Berg*[2] *and Gabriele Berg*[1]*

[1] *Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Institute of Plant Sciences, Karl-Franzens University of Graz, Graz, Austria*

Edited by:
Michael Schloter, Helmholtz Zentrum München, Germany

Reviewed by:
David J. Studholme, University of Exeter, UK
Michael Schmid, Helmholtz Zentrum München, Germany

***Correspondence:**
Gabriele Berg, Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 10-12/I, Graz, 8010, Austria
e-mail: gabriele.berg@tugraz.at

The betaproteobacterial genus *Burkholderia* is known for its versatile interactions with its hosts that can range from beneficial to pathogenic. A plant-beneficial-environmental (PBE) *Burkholderia* cluster was recently separated from the pathogen cluster, yet still little is known about burkholderial diversity, distribution, colonization, and transmission patterns on plants. In our study, we applied a combination of high-throughput molecular and microscopic methods to examine the aforementioned factors for *Burkholderia* communities associated with *Sphagnum* mosses – model plants for long-term associations – in Austrian and Russian bogs. Analysis of 16S rRNA gene amplicons libraries revealed that most of the *Burkholderia* are part of the PBE group, but a minor fraction was closely related to *B. glathei* and *B. andropogonis* from the pathogen cluster. Notably, *Burkholderia* showed highly similar composition patterns for each moss species independent of the geographic region, and *Burkholderia*-specific fluorescent *in situ* hybridization of *Sphagnum* gametophytes exhibited similar colonization patterns in different *Sphagnum* species at multi-geographic scales. To explain these patterns, we compared the compositions of the surrounding water, gametophyte-, and sporophyte-associated microbiome at genus level and discovered that *Burkholderia* were present in the *Sphagnum* sporophyte and gametophyte, but were absent in the flark water. Therefore, *Burkholderia* is a part of the core microbiome transmitted from the moss sporophyte to the gametophyte. This suggests a vertical transmission of *Burkholderia* strains, and thus underlines their importance for the plants themselves.

Keywords: *Sphagnum fallax*, *Sphagnum magellanicum*, *Burkholderia* communities, amplicon pyrosequencing, FISH-CLSM

INTRODUCTION

The genus *Burkholderia*, which was described by Yabuuchi et al. (1992), encompasses a diverse group of Betaproteobacteria with currently more than 60 validly described species. *Burkholderia* species are known for their beneficial as well as pathogenic interaction with plants, animals, and humans (Coenye and Vandamme, 2003). In the past, most studies focused on the pathogenic species for their enormous clinical importance (Mahenthiralingam et al., 2005). Recently, a specific plant-beneficial-environmental (PBE) *Burkholderia* cluster that contains non-pathogenic species was divided from the cluster that comprises human, animal and plant pathogens (Caballero-Mellado et al., 2007; Suárez-Moreno et al., 2010, 2012). However, there is no clear border between both groups especially within the plant-associated species; for example *B. glathei* was suggested to be transfered from the pathogenic to the PBE group (Verstraete et al., 2013). Many PBE members belong to *Burkholderia* species symbiotic to tropical plants; each nodulating plant species is colonized by a single unique endophytic *Burkholderia* species (Van Oevelen et al., 2002; Lemaire et al., 2011). Several species from the PBE cluster share characteristics that are of use in association with plants, such as quorum sensing systems, the presence of nitrogen fixation and/or nodulation genes, and the ability to degrade aromatic compounds (Suárez-Moreno et al., 2012), and many of them are characterized by an endophytic lifestyle (Sessitsch et al., 2005; Gasser et al., 2009; Mitter et al., 2013). While single strains of the PBE cluster are already well-characterized, little is known about the ecology and colonization pattern of *Burkholderia* species on plants.

Plants have been recognized as meta-organisms due to their close symbiotic relationship with their microbiome that fulfills important host functions (Berg, 2009; Bulgarelli et al., 2012; Hirsch and Mauchline, 2012; Lundberg et al., 2012; Berg et al., 2013). These advances were driven by both "omic"-technologies guided by next-generation sequencing (NGS) and microscopic insights (Berendsen et al., 2012; Jansson et al., 2012). Mosses belong to the phylogenetically oldest group of land plants on Earth, and their long-term intense relationship with their associated microbes has contributed to the co-evolution of a highly specific microbiome (Opelt and Berg, 2004; Opelt et al., 2007c; Bragina et al., 2012). Therefore, mosses are important models in studying plant-microbe interactions and the ecology of plant-associated bacteria. The genus *Sphagnum* is among the most abundant and cosmopolitan of bog vegetation in the Northern hemisphere, and greatly contributes to both global carbon turnover and global climate (Raghoebarsing et al., 2005; Dise, 2009). The ecological significance of bogs is directly related to the physical, morphological, and chemical characteristics of

Sphagnum peat mosses which set *Sphagnum* apart from other mosses in practically every stage of the life cycle (Daniels and Eddy, 1985). *Burkholderia* species play an important role for *Sphagnum* mosses and peatland ecosystem (Opelt et al., 2007a,b), and new *Burkholderia* species, which belong to the PBE cluster, have recently been isolated from these mosses (Vandamme et al., 2007). However, their composition and occurrence on *Sphagnum* at various geographical scales—ranging from the moss gametophyte and sporophyte up to continental level—is not yet understood. We hypothesize that *Sphagnum* species are colonized by specific *Burkholderia* from the PBE cluster independent from the geographic region.

To study this hypothesis and understand the ecological role, composition, colonization, as well as distribution pattern on plants, we studied *Burkholderia* communities on two *Sphagnum* species (*S. magellanicum* and *S. fallax*) associated with different a-biotic parameters from different bogs in Austria and Russia. We used an assortment of methods combining the analysis of *Burkholderia*-specific 16S rRNA gene pyrosequencing libraries with FISH-CLSM analysis. Furthermore, we compared the compositions of water, gametophyte-, and sporophyte-associated microbiomes to understand the transmission and distribution patterns of the *Burkholderia* communities.

MATERIALS AND METHODS

SAMPLING DESIGN

To analyze the diversity and distribution pattern of the *Sphagnum*-associated *Burkholderia* community, *S. magellanicum* BRID. (section *Sphagnum*) and *S. fallax* H. KLINGGR. (section *Cuspidata*) were selected. Both bryophytes are members of the typical and cosmopolitan vegetation in peat bogs (Daniels and Eddy, 1985). Adult gametophytes of mosses were sampled in three acidic peat bogs in Austria and in three acidic peat bogs in Russia in September 2009 and July 2010, respectively (**Table S1**). Four single replicates per *Sphagnum* species (15–20 plantlets) were collected in each of the investigated bogs at a minimum distance of about 40 m from each other. The plant samples were placed into sterile plastic bags and transported to the laboratory. In addition, two sporophyte samples of *S. fallax* consisting of enclosed spore capsules, and one water sample from a small wet depression (flark) were collected into sterile screw cap tubes and processed separately.

TOTAL-COMMUNITY DNA ISOLATION

The microbial fractions associated with moss gametophytes and sporophytes were extracted as previously described (Bragina et al., 2012). In short, 5 g of plant material were physically disrupted and resuspended in 10 ml of 0.85% NaCl. 2 ml of suspension were centrifuged at 13,000 r.p.m. for 20 min at 4°C and the supernatant was discarded. For extraction of the sporophyte-associated microbial community, 10 enclosed spore capsules per sample were surface-sterilized and ground with 1.5 ml of 0.85% NaCl. The ground suspension was centrifuged at 13,000 r.p.m. for 20 min at 4°C and the supernatant was discarded. The pellet from the flark water sample was obtained through several rounds of centrifugation at 10,000 r.p.m. for 15 min at 4°C until a constant pellet size was obtained. The resulting cell pellets were applied for isolation of the total-community DNA using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). Final aliquots of the total-community DNA were further used for a deep sequencing-approach.

454-PYROSEQUENCING AND BIOINFORMATIC PROCESSING

The diversity of the *Sphagnum*-associated microbiome with a special focus on the genus *Burkholderia* was investigated using a barcoded pyrosequencing technology. For this purpose, 16S rDNA amplicons were generated using Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, OH, USA). The total-community DNA of gametophyte samples was selectively amplified with *Burkholderia*-specific primers BKH143Fw/BKH1434Rw (Schönmann et al., 2009) followed by amplification with universal bacterial primers Unibac-II-515f/Unibac-II-927r (Lieber et al., 2003). In addition, the total-community DNA of *S. fallax* gametophyte samples from the bog Pürgschachen Moor (**Table S1**) and flark water sample was amplified with universal bacterial primers Unibac-II-515f/Unibac-II-927r. The total-community DNA of sporophyte samples was amplified with universal bacterial primers 799f/1492r (Lane, 1991; Chelius and Triplett, 2001) because application of the Unibac-II-515f/Unibac-II-927r achieved mostly plant-derived sequences (data of preliminary experiments). Primer sequences are listed in **Table 1**. Duplicate PCR products from all templates were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Amplicons derived from the same *Sphagnum* sp. and sampling site were pooled in equimolar ratios and subjected to pyrosequencing using the Roche 454 GS FLX and FLX+ Titanium platforms performed by LGC Genomics (Berlin, Germany) and Eurofins MWG (Ebersberg, Germany), respectively. In total, we produced 12 pyrosequencing libraries specific for *Burkholderia* and four general bacterial pyrosequencing libraries.

The 16S rDNA pyrosequencing libraries were processed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME) version 1.6.0 (Caporaso et al., 2010) with default parameters. The raw datasets were de-multiplexed, the primer sequences were truncated, and the datasets were filtered by removing sequences of low-quality (quality score, <25), short sequences (<200 bp), and sequences containing ambiguous characters and/or homopolymers (>6 bp). The quality-filtered datasets were de-noised and chimeras were removed if present. Sequences were clustered into operational taxonomic units (OTUs) using the UCLUST algorithm with a 97% similarity cut-off (Schloss and Handelsman, 2006; Edgar, 2010). The most abundant sequence within each OTU was taxonomically assigned using the Ribosomal Database Project (RDP) classifier with 80% confidence threshold (Wang et al., 2007). To refine the analysis, generated OTU-tables were filtered based on taxonomic metadata: OTUs classified to genera other than *Burkholderia* and OTUs containing chloroplast-derived sequences were removed from the burkholderial and general bacterial OTU-tables, correspondingly. Rarefaction analysis was performed for the complete datasets, while richness and diversity estimations were performed by calculating Chao1 and Shannon (H′) indices for the datasets normalized to the same number of sequences. For the general bacterial datasets, the occurrence of bacterial taxa was analyzed using the

Table 1 | Nucleotide probes used for the PCR and FISH.

Name	Sequence (5′–3′)	Specificity	References	Formamide concentration (%)[a]	Fluorescent dye
PCR PRIMERS					
Unibac-II-515f	GTGCCAGCAGCCGC	Most bacteria	Lieber et al., 2003	-	-
Unibac-II-927r	CCCGTCAATTYMTTTGAGTT	Most bacteria	Lieber et al., 2003	-	-
799f	AACMGGATTAGATACCCKG	Most bacteria	Chelius and Triplett, 2001	-	-
1492r	ACCTTGTTACGACTT	Most bacteria	Lane, 1991	-	-
BKH143Fw	TGGGGGATAGCYCGGCG	*Burkholderia* spp.	Schönmann et al., 2009	-	-
BKH1434Rw	TGCGGTTAGRCTASCYACT	*Burkholderia* spp.	Schönmann et al., 2009	-	-
FISH PROBES					
EUB338[b]	GCTGCCTCCCGTAGGAGT	Most bacteria	Amann et al., 1990	15	Cy3
EUB338II[b]	GCAGCCACCCGTAGGTGT	Planctomycetales	Daims et al., 1999	15	Cy3
EUB338III[b]	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	Daims et al., 1999	15	Cy3
Burkho	ACCCTCTGTTCCGACCAT	*Burkholderia* spp.	Hogardt et al., 2000	40	Cy5
NONEUB	ACTCCTACGGGAGGCAGC	-	Amann et al., 1990	-[c]	-[d]

[a] *The stringency conditions for hybridization at 41° C.*

[b] *The probes were applied together in equimolar ratio.*

[c] *The probe used for negative control at the same stringency conditions applied for positive FISH probe.*

[d] *The probe used for negative control was labeled with the same fluorescent dye as corresponding positive FISH probe.*

normalized datasets. Beta-diversity of the burkholderial datasets was analyzed using weighted UniFrac distance metric (Lozupone et al., 2010) and jackknife re-sampling (1,781 sequences per sample × 100 times). Statistical analysis was performed for the normalized datasets using the adonis test with 999 permutations (http://qiime.org/tutorials/category_comparison.html).

Representative sequences of the burkholderial OTUs were aligned with reference sequences from the non-redundant nucleotide database (nt) of the NCBI server using the BLASTN algorithm. A bootstrapped neighbor-joining phylogenetic tree of the representative sequences and the closest database matches was constructed using software packages ClustalX version 2.0.12 (Larkin et al., 2007), Phylip version 3.69 (Felsenstein, 1989), and MEGA version 4.0 (Tamura et al., 2007) as previously described (Bragina et al., 2012).

SEQUENCE ACCESSION NUMBERS

The raw pyrosequencing data was deposited in the European Nucleotide Archive (ENA) under the project number PRJEB4660 with the accession numbers ERR361316–ERR361331.

FLUORESCENT *in situ* HYBRIDIZATION AND CONFOCAL LASER SCANNING MICROSCOPY

Single plants of *S. magellanicum* and *S. fallax* were fixed with 4% paraformaldehyde/phosphate buffered salt (3:1, v/v) and stained by in-tube FISH (Grube et al., 2009). The samples were hybridized with rRNA-targeting probes (genXpress, Wiener Neudorf, Austria) specific for *Burkholderia* and with a set of universal bacterial probes. Hybridization was carried out at 41°C. The probes and corresponding stringency conditions are listed in **Table 1**. Confocal laser scanning microscopy (CLSM) was performed with a Leica TCS SPE confocal microscope (Leica Microsystems, Mannheim, Germany) as previously described (Bragina et al., 2012) followed by volume rendering of confocal stacks using the software Imaris 7.3 (Bitplane, Zurich, Switzerland).

RESULTS

SPHAGNUM MOSSES ARE PREFERENTIALLY COLONIZED BY BURKHOLDERIAL STRAINS FROM THE PBE CLUSTER AND A MINOR COMMUNITY FRACTION BELONGS TO THE PLANT-PATHOGENIC CLUSTER

High-throughput analysis of the *Burkholderia* community was achieved via an amplicon pyrosequencing approach targeting the V4–V5 region of the 16S rRNA gene. The pyrosequencing of 12 amplicon libraries of *S. fallax* and *S. magellanicum* samples from Austria and Russia retrieved 149,024 raw sequences (**Table 2**). After initial processing, 87,917 quality sequences (average length, 405 bp) specific for *Burkholderia* genus were subjected to a detailed investigation. Rarefaction analysis of the pyrosequencing libraries, which were clustered with 97% sequence similarity, resulted in similar saturation profiles for all *Sphagnum* samples (**Figure S1**). Richness estimation of the normalized datasets revealed that the current pyrosequencing survey attained 81.1-100% of the estimated richness (**Table 2**). Low values of the Shannon diversity index (0.21–0.90, **Table 2**) indicated that the retrieved burkholderial communities contained a low number of highly abundant phylotypes. Through the use of automatic classification of the representative sequences, these phylotypes were assigned to *B. bryophila*, *B. andropogonis*, and *B. glathei* with several of them remaining unclassified at species level (**Figure 1**). According to the division of the genus *Burkholderia* sensu Suárez-Moreno et al. (2012), the most abundant *B. bryophila* species belongs to the plant-beneficial cluster, while minor *B. andropogonis* and *B. glathei* species are within the plant-pathogenic cluster.

To achieve a deeper insight into burkholderial diversity, we performed a phylogenetic analysis of the partial 16S rRNA

Table 2 | Description and alpha-diversity estimation of the 16S rDNA pyrosequencing libraries of *Sphagnum* samples[a].

Library[b]	Habitat	Country	Bog	No. of raw seq.	No. of filtered seq.	No. of OTUs (97%)	Chao1	Coverage (%)	Shannon, H′
16S rDNA LIBRARIES SPECIFIC FOR *Burkholderia*[c]									
AM1	*S. magellanicum*	Austria	Rotmoos	12,740	9,663	3.58	3.63	98.6	0.23
AM2	*S. magellanicum*	Austria	Wasenmoos	11,750	7,393	3.57	3.72	96.0	0.21
AM3	*S. magellanicum*	Austria	Pürgschachen Moor	13,271	9,612	4.45	4.53	98.2	0.24
AF1	*S. fallax*	Austria	Rotmoos	13,189	11,016	5.64	6.25	90.2	0.75
AF2	*S. fallax*	Austria	Wasenmoos	11,987	7,870	5.18	5.37	96.5	0.46
AF3	*S. fallax*	Austria	Pürgschachen Moor	13,843	7,595	4.25	4.27	99.5	0.38
RM1	*S. magellanicum*	Russia	Polesje	12,566	9,256	4.89	4.97	98.4	0.27
RM2	*S. magellanicum*	Russia	Polewoi mys	10,213	6,632	5.04	5.38	93.8	0.31
RM3	*S. magellanicum*	Russia	Oblojni moch	12,831	2,130	5.00	5.00	100.0	0.90
RF1	*S. fallax*	Russia	Polesje	13,051	1,788	3.00	3.00	100.0	0.79
RF2	*S. fallax*	Russia	Polewoi mys	10,279	7,637	4.40	4.64	94.9	0.26
RF3	*S. fallax*	Russia	Oblojni moch	13,304	7,325	5.22	6.44	81.1	0.33
16S rDNA LIBRARIES OF BACTERIA									
RW	Flark water	Russia	Oblojni moch	4,296	3,934	173.00	343.26	50.4	5.65
AFG	*S. fallax* gametophyte	Austria	Pürgschachen Moor	5,399	2,869	252.00	591.94	42.6	6.47
AFS	*S. fallax* sporophyte	Austria	Rotmoos	1,665	1,051	159.00	325.14	48.9	4.93
RFS	*S. fallax* sporophyte	Russia	Polewoi mys	1,869	1,299	83.00	131.00	63.4	4.23

[a] *Richness estimates and diversity indices were calculated for the datasets normalized to the same number of sequences per library: 1,781 (Burkholderia), 1,051 (Bacteria).*

[b] *Abbreviations specify the sampling sites and habitats: A, Austria; R, Russia; F, S. fallax; M, S. magellanicum; W, flark water; G, gametophyte; S, sporophyte. Arabic numerals specify different bogs in Austria and Russia.*

[c] *16S rDNA pyrosequencing libraries specific for Burkholderia were obtained from gametophyte samples of Sphagnum mosses.*

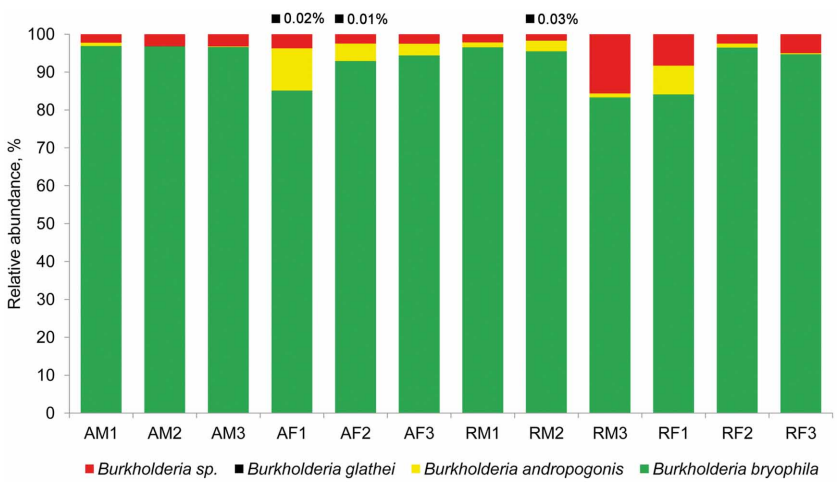

FIGURE 1 | Taxonomic classification of burkholderial communities associated with *Sphagnum* mosses. Bar charts represent the composition of *Burkholderia*-specific 16S rDNA pyrosequencing libraries classified using RDP-classifier with a confidence threshold of 80%. The burkholderial sequences that remained unclassified at the species level are shown as *Burkholderia* sp. (red). Black squares and percentage values above the bar charts show occurrence and abundance of *B. glathei*. Abbreviations: A, Austria; R, Russia; F, *S. fallax*; M, *S. magellanicum*. Arabic numerals specify different bogs in Austria and Russia.

gene sequences from pyrosequencing libraries and closely related environmental strains (**Figure 2**). The closest database matches showed ≥96% of sequence identity to representative burkholderial sequences from pyrosequencing libraries. Clustering of the representative and reference sequences on the phylogenetic tree reflected several ecological traits of the examined burkholderial community. Specifically, cluster 2 was formed from representative sequences (this study) and the *B. bryophila* strain LMG 23648, a plant growth-promoting and antagonistic bacterium that was originally isolated from mosses in a nature reserve bog

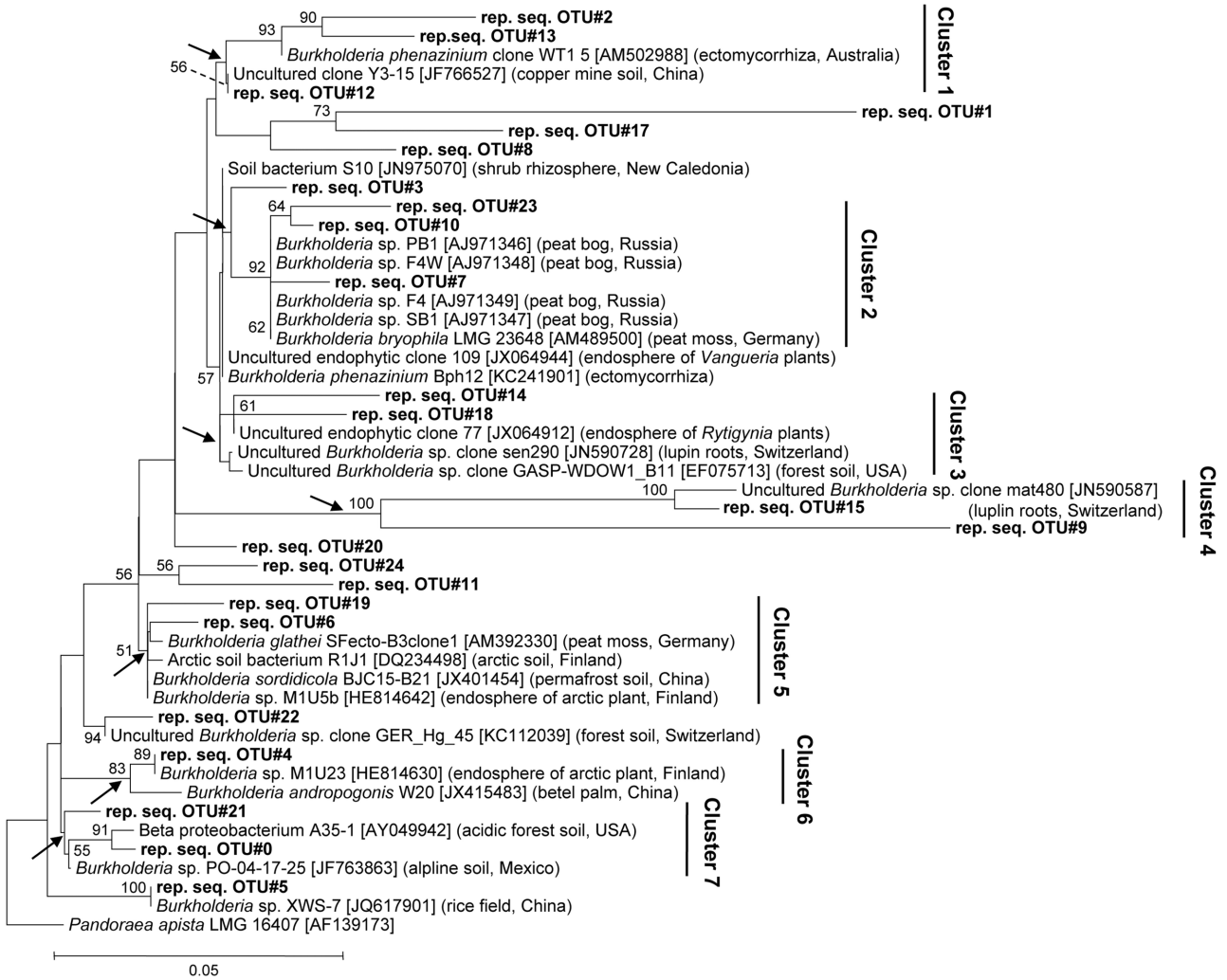

FIGURE 2 | Neighbor-joining tree of *Sphagnum*-associated burkholderial communities. Phylogenetic relationships are shown for 16S rDNA sequence representing burkholderial OTUs (97%) (bold) and the nearest ecologically related reference sequences. The isolation sources and accession numbers of reference sequences are shown in brackets. Partial 16S rDNA sequence of *Pandoraea apista* strain LMG 16407 (AF139173) was used as an out-group. Numbers at nodes indicate bootstrap values out of 100 data re-samplings exceeding 50%. Arrows show monophyletic branches for cluster designation. Distance bar: 0.05 substitutions per site.

in Germany (Vandamme et al., 2007). This cluster also contained burkholderial strains PB1, F4W, F4, and SB1 which were isolated from acidic peat bogs in Russia (Belova et al., 2006). The phylogenetic clusters 1, 3, 4, 5, and 6 were represented by various endophytic and rhizospheric bacteria. These bacteria included the endophytic mycorrhizal *B. phenazinium* clone WT1 5, burkholderial clones sen290 and mat480 associated with lupin cluster roots (Weisskopf et al., 2011), and burkholderial endophytes M1U5b and M1U23 that were isolated from the arctic plants (Nissinen et al., 2012). Within the clusters 5 and 6, we detected *B. andropogonis* strain W20, a causative agent of the leaf spot in betel palm, and SFecto-B3clone1 clone of *B. glathei* species, a free-living or moss-associated bacterium that is not considered a member of the PBE cluster (Opelt et al., 2007a; Suárez-Moreno et al., 2012). Interestingly, the representative sequence in these clusters showed higher sequence similarity (98–100%) with the harmless burkholderial strains M1U5b and M1U23 than with *B. andropogonis* and *B. glathei* sequences. Furthermore, cluster 7 contained bacteria from acidic and alpine soils. Overall, the phylogenetic analysis revealed that *Sphagnum*-associated *Burkholderia* are phylogenetically closely related to plant-beneficial and non-pathogenic *Burkholderia* from various acidic habitats, especially peat bogs, but also potential plant pathogens.

BURKHOLDERIA COMMUNITIES OF SPHAGNA EXHIBIT SIMILAR DISTRIBUTION AND COLONIZATION PATTERNS INDEPENDENT OF THE GEOGRAPHIC REGION

Biogeographical distribution of the *Burkholderia* communities was examined for the peat mosses *S. fallax* and *S. magellanicum* collected from Austrian and Russian bogs

(**Table S1**). *Burkholderia* showed highly similar distribution patterns for the analyzed moss species independent of the geographic region (**Figure 3**). An average weighted UniFrac distance was 0.47% with a maximum value of 1.08% for *S. magellanicum*-associated communities in Russian bogs (**Table S2**). The statistical analysis using an adonis test confirmed that neither *Sphagnum* species ($P = 0.261$) nor geographic position ($P = 0.363$) had a significant influence on the beta-diversity of burkholderial communities.

The general distribution of *Burkholderia* was confirmed through fluorescent *in situ* hybridization (FISH) of *Sphagnum* gametophytes with genus-specific and universal bacterial probes (**Figure 4**). *Sphagnum* mosses are characterized by a unique morphology (Daniels and Eddy, 1985) which makes them easily accessible for microbial colonization (Bragina et al., 2012). CLSM observation of hybridized plants showed that the *Burkholderia* community inhabited the leaves, but not the stem tissues of mosses. Straight and slightly curved rods of *Burkholderia* were detected in hyalocyte cells of leaves being likely attached to their cell walls. Inside the hyalocytes, *Burkholderia* remained as individual cells or formed microcolonies composed of few cells. Burkholderial cells were also found in association with other bacteria of unidentified taxonomy as shown in **Figure 4A**. Analysis of FISH-CLSM data showed that *Burkholderia* communities established similar colonization patterns in different *Sphagnum* species across the examined geographic scales.

BURKHOLDERIA ARE VERTICALLY TRANSMITTED WITHIN THE CORE MICROBIOME OVER ENTIRE LIFE CYCLE OF THE HOST PLANTS

The 16S rDNA pyrosequencing libraries from the moss sporophyte, gametophyte, and flark water samples were compared to reveal potential transmission mechanisms of *Sphagnum*-associated bacteria with a special focus on the genus *Burkholderia*. The libraries were rarefied as shown in **Figure S1**. The pyrosequencing survey achieved 42.6–63.4% of total richness as estimated by the Chao1 index (**Table 2**). Classification of the normalized datasets revealed the occurrence of certain bacterial taxa in *S. fallax* and water microhabitats (**Figure 5**). Thus, Proteobacteria, Bacteroidetes, and Acidobacteria were among the most abundant phyla in all examined microhabitats. At class level, Alphaproteobacteria (within the phylum Proteobacteria) comprised the dominant portion of the plant-associated microbiome, while the water microbiome was dominated by Sphingobacteria (Bacteroidetes). Furthermore, a comparison of microbiome structure at family level revealed several different occurrence patterns. For instance, *Acidobactriaceae* (within the class Acidobacteria) were ubiquitously distributed unlike the family *Xanthomonadaceae* (Gammaproteobacteria) that specifically colonized the moss-associated microhabitats, gametophyte, and sporophyte. Moreover, several bacterial taxa, namely *Methylocystaceae* (Alphaproteobacteria), inhabited moss gametophytes and flark water.

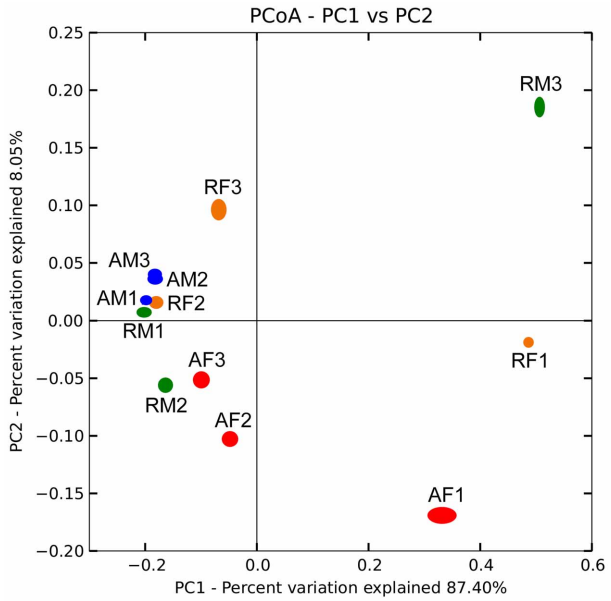

FIGURE 3 | Comparison of burkholderial communities on *Sphagnum* mosses in Austrian and Russian bogs by principal coordinate analysis (PCoA). PCoA biplot is based on the weighted UniFrac distance matrix of the 16S rDNA pyrosequencing libraries specific for *Burkholderia* and supported by 100 jackknife data re-samplings using 1781 sequences per library. The single libraries are shown by colored ellipses: blue, *S. magellanicum* (Austria); red, *S. fallax* (Austria); green, *S. magellanicum* (Russia); orange, *S. fallax* (Russia). Letters and arabic numerals specify library names. Variation explained by each principal coordinate (PC) is defined on the biplot, respectively.

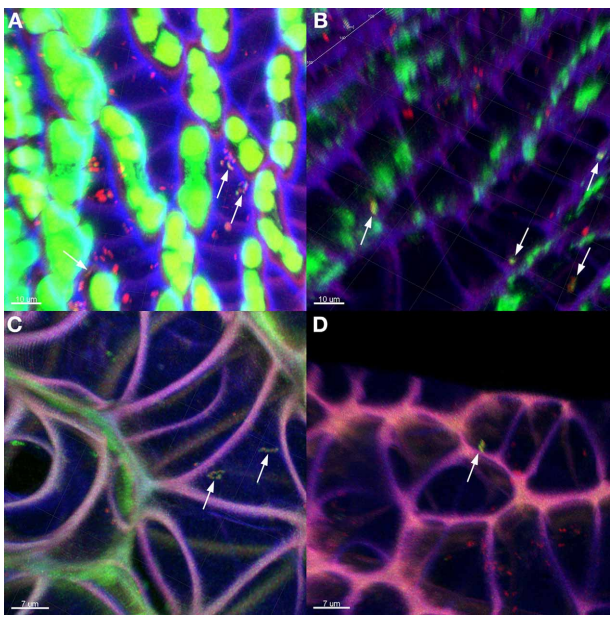

FIGURE 4 | Detection of *Burkholderia* in *Sphagnum* plants by FISH-CLSM visualization. Branch leaves of *S. fallax* **(A,B)** and *S. magellanicum* **(C,D)** from Austrian **(A,C)** and Russian **(B,D)** bogs hybridized with *Burkholderia*-specific and universal bacterial probes. Yellow: *Burkholderia* spp. indicated by arrows; red: other bacteria; green: algae, *Sphagnum* chlorocytes; violet: moss cell walls. Scale bar = 10 μm **(A,B)**; 7 μm **(C,D)**.

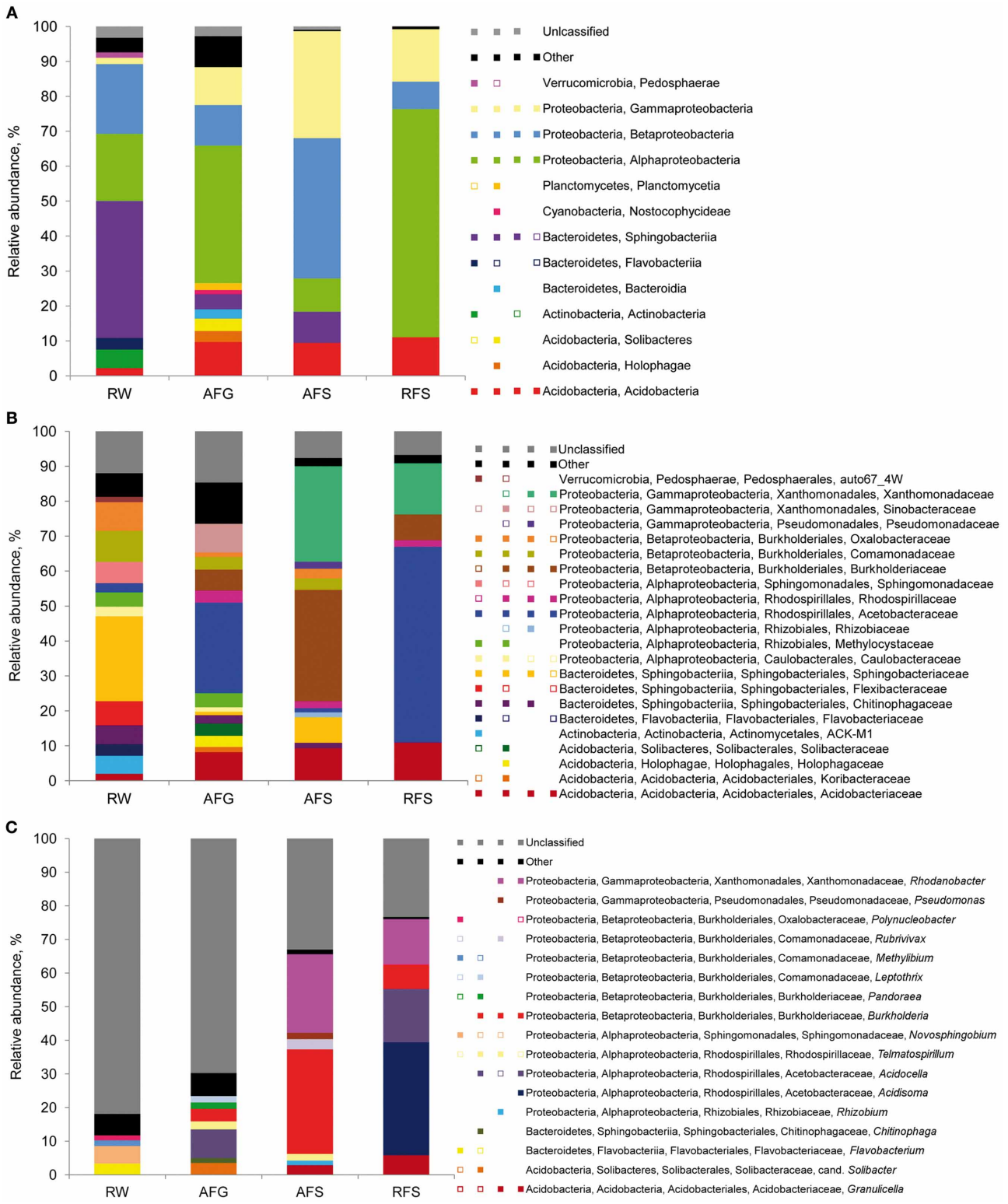

FIGURE 5 | Taxonomic classification of bacterial communities of *Sphagnum* mosses and flark water. Bar charts represent the composition of 16S rDNA pyrosequencing libraries of Bacteria classified at class **(A)**, family **(B)** and genus **(C)** level using RDP-classifier with a confidence threshold of 80%. Multi-colored charts in the legend represent occurrence of each taxon in each library correspondingly. Taxons below 1% of relative abundance are included in 'Other' and depicted as empty squares on the multi-colored charts. Abbreviations: A, Austria; R, Russia; W, flark water; F, *S. fallax*; G, gametophyte, S, sporophyte.

To study the occurrence of *Burkholderia* in various microhabitats, we compared the compositions of water, gametophyte-, and sporophyte-associated microbiomes at genus level. Consequently, *Burkholderia* were detected in the *Sphagnum* sporophyte and gametophyte, but were absent in the flark water. To ensure that normalization did not influence the *Burkholderia* occurrence pattern, the non-normalized pyrosequencing libraries were checked for the presence of this genus. The occurrence pattern of *Burkholderia* coincided between the normalized and non-normalized datasets (data not shown). However, pyrosequencing of the flark water microbiome achieved partial coverage of the estimated diversity (**Table 2**) and therefore additional experiments would be required to confirm this finding. Altogether, the obtained results indicated that the moss microbiome exhibits potential for both water-mediated and host-mediated transmission and that *Sphagnum*-associated *Burkholderia* are potentially transmitted over the entire life cycle of the host plants.

DISCUSSION

The genus *Burkholderia* is very important for plant and human health (Coenye and Vandamme, 2003; Suárez-Moreno et al., 2012; Mitter et al., 2013). We found that the microbiome of our model *Sphagnum* plant is preferentially enriched by the plant-beneficial and non-pathogenic *Burkholderia* from the PBE cluster, but also contains minor fraction of potential plant pathogens. We have provided new ecological insights into these important plant inhabitants including their composition, distribution, colonization, and transmission pattern.

Our hypothesis that *Sphagnum* species are colonized by specific *Burkholderia* from the PBE cluster has to be slightly revised. Although the most abundant *B. bryophila* species belongs to the plant-beneficial cluster, a minor fraction of *B. andropogonis* and *B. glathei* species are within the pathogen cluster (Suárez-Moreno et al., 2012). However, the phylogenetic analysis was crucial for elucidating their intra-specific diversity and ecological background (**Figure 2**). Amazingly, the resolved phylogenetic clusters contained plant-beneficial and non-pathogenic burkholderial strain as well as environmental clone sequences. This fact led us to the conclusion that *Burkholderia* members from the PBE cluster are of a great importance for the health and growth of *Sphagnum* plants. Our conclusion was supported by the isolation of *B. bryophila* and *B. phenazinium* beneficial strains from *Sphagnum* mosses at the same sampling sites by Shcherbakov et al. (2013). The minor fraction of the burkholderial community was formed by *Burkholderia* spp. from the plant-pathogenic cluster sensu Suárez-Moreno et al. (2012). However, the collected *Sphagnum* plants did not exhibit any disease symptoms. Therefore, we support the transfer of *B. glathei* the PBE cluster as recently suggested by Verstraete et al. (2013), who identified the species as common endosymbiont in plants of the *Rubiaceae* family. In contrast, *Burkholderia andropogonis* is the causal agent of numerous plant diseases affecting a wide range of monocot and dicot plants, e.g., sweet and field corn, blueberry, sorghum, carnation, coffee, statice, rye, and clover. Bacterial leaf stripe is one of the three major bacterial diseases of sorghum, and strict quarantine regulations against importation of *B. andropogonis*-infested sorghum feed grains and seeds are imposed by numerous countries (Ramundo and Claflin, 2005). *Sphagnum* mosses seem to be a natural reservoir for this plant pathogen. This is important because dry *Sphagnum* is often used for orchid and ornamental cultivation and transferred world-wide. On the other side, there are also hints that saprophytic *B. andropogonis* exists (Estrada-de los Santos et al., 2013), and many disease outbreaks depend on the abundance of pathogens and the diversity of the indigenous microbiome. At last, it is impossible to predict any pathogenic or beneficial effect from 16S rDNA analysis, and additional studies would be required to prove or contradict the pathogenicity of *Sphagnum*-associated *B. andropogonis*.

In this study, we discovered similar distribution patterns of *Sphagnum*-associated burkholderial communities independent of the geographic region, which well-confirmed our hypothesis. To elucidate this distribution pattern, we aimed to answer the question—what factors shape this community? In terrestrial habitats, pH serves as both a primary driver of microbiome structure as well as a specific determinant of the genus *Burkholderia* as it is known to exhibit pH tolerance as a general phenotypic trait (Lauber et al., 2009; Stopnisek et al., 2013). In our previous study, the same sampling sites in Austria were characterized as extremely to moderately acidic by means of Ellenberg's indicator values for pH (expressed as soil reaction) (Bragina et al., 2012). For sampling sites in Russia, the Ellenberg's values for pH varied at the same range (data not shown) and therefore all examined sites possessed favorable a-biotic conditions for the burkholderial colonization. Apart from the a-biotic factors, we previously demonstrated that various *Sphagnum* species determine the microbiome composition to different extents (Bragina et al., 2011, 2012). Through statistical analysis, we showed that neither geographic location nor *Sphagnum* species had a significant influence on the distribution of *Burkholderia*. Moreover, the similar colonization patterns of the moss-associated *Burkholderia* were verified using FISH-CLSM in a semi-quantitative way.

For a better understanding of the distribution and colonization pattern revealed for *Sphagnum*-associated *Burkholderia*, we addressed the issue of bacterial transmission in the peat bog ecosystems. Recently, Putkinen et al. (2012) described a water dispersal of methane-oxidizing bacteria in the peat bogs. Moreover, our previous study revealed that nitrogen-fixing bacteria were transferred within the moss sporophyte (Bragina et al., 2013) As a result, we hypothesized that either host-mediated or water-mediated transmission is possible for *Sphagnum*-associated *Burkholderia*. Through the comparison of microbiome composition in various bog microhabitats, we found that burkholderial communities are potentially transmitted by the host plants. The violent spore discharge and wind dispersal of the *Sphagnum* spores would enable associated bacteria to migrate over the long distances and support spore germination at a new site (Szövényi et al., 2008; Sundberg, 2010). Altogether, the detected host-mediated transmission underlines the importance of *Burkholderia* for *Sphagnum* mosses themselves and defines their distribution pattern.

In recent decades, burkholderial community was considered a typical and well-adapted component of acidic peat bogs (Belova et al., 2006). In this study, we demonstrated that *Burkholderia* associated with the main vegetation of peat bogs,

Sphagnum mosses, contain both plant-beneficial but also potentially pathogenic *Burkholderia* that are transmitted by the host plants over their life cycle. However, global warming and human disturbance may significantly shift the environmental conditions in the peat bog ecosystems and lead to the elimination or substitution of the beneficial microbes (Dise, 2009). The obtained data supports our knowledge on native plant microbiomes and can help for the maintenance of climate-relevant bog ecosystems.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Journal/10.3389/fmicb.2013.00394/abstract

Table S1 | Sampling sites.

Table S2 | Weighted UniFrac distance matrix of 16S rDNA pyrosequencing libraries specific for *Burkholderia*.

Figure S1 | Rarefaction curves for 16S rDNA amplicon libraries of *Sphagnum* samples.

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Differences between the rhizosphere microbiome of *Beta vulgaris* ssp. *maritima*—ancestor of all beet crops—and modern sugar beets

Christin Zachow[1,2]*, Henry Müller[2], Ralf Tilcher[3] and Gabriele Berg[2]

[1] Austrian Center of Industrial Biotechnology (ACIB GmbH), Graz, Austria
[2] Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria
[3] KWS SAAT AG, Einbeck, Germany

Edited by:
Kornelia Smalla, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Germany

Reviewed by:
Kornelia Smalla, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Germany
Weixing Shan, Northwest A&F University, China
Yusuke Saijo, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**
Christin Zachow, Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12/1, Graz 8010, Austria
e-mail: christin.zachow@tugraz.at

The structure and function of the plant microbiome is driven by plant species and prevailing environmental conditions. Effectuated by breeding efforts, modern crops diverge genetically and phenotypically from their wild relatives but little is known about consequences for the associated microbiota. Therefore, we studied bacterial rhizosphere communities associated with the wild beet *B. vulgaris* ssp. *maritima* grown in their natural habitat soil from coastal drift lines (CS) and modern sugar beets (*Beta vulgaris* ssp. *vulgaris*) cultivated in CS and potting soil (PS) under greenhouse conditions. Analysis of 16S rRNA gene fingerprints and pyrosequencing-based amplicon libraries revealed plant genotype- and soil-specific microbiomes. Wild beet plants harbor distinct operational taxonomic units (OTUs) and a more diverse bacterial community than the domesticated sugar beet plants. Although the rhizospheres of both plant genotypes were dominated by *Proteobacteria* and *Planctomycetes*, 37.5% of dominant OTUs were additionally detected in the wild beet rhizosphere. Analysis of the cultivable fraction confirmed these plant genotype-specific differences at functional level. The proportion of isolates displayed *in vitro* activity against phytopathogens was lower for wild beet (≤45.8%) than for sugar beet (≤57.5%). Conversely, active isolates from the wild beet exhibited stronger ability to cope with abiotic stresses. From all samples, active isolates of *Stenotrophomonas rhizophila* were frequently identified. In addition, soil type-specific impacts on the composition of bacterial communities were found: *Acidobacteria*, *Chloroflexi*, and *Planctomycetes* were only detected in plants cultivated in CS; whereas *Bacteroidetes* and *Proteobacteria* dominated in PS. Overall, in comparison to modern sugar beets, wild beets were associated with taxonomically and functionally distinct microbiomes.

Keywords: wild beet, sea beet, sugar beet, rhizosphere, antagonistic bacteria, stress protecting bacteria

INTRODUCTION

The domestication of plants began with the identification of wild plant species exploitable for food, animal feed, or other domestic purposes. Selective crop breeding programs are mainly oriented toward increasing food production and high-yielding varieties, percentage of usable plant parts, and resistance against diseases (Akhalkatsi et al., 2012). The genetic background of sugar beet is narrower compared to other crops because the sugar beet breeding had started in the late eighteenth century when lines accumulating sugar in the storage root were selected from crosses made with chard and fodder beet (Fischer, 1989). In contrast to many other crops, the ancestor of domesticated sugar beets (*Beta vulgaris* ssp. *vulgaris* L.) is known and still distributed throughout Europe, especially in the Mediterranean region along coastal drift lines. The genome of the wild form *B. vulgaris* L. ssp. *maritima* (L.) Arcang. (trivial name: wild or sea beet) differs significantly from domesticated sugar beet lines (Dohm et al., 2014). For example, it shows high variation at the vernalization *B*-locus, and therefore most of the plants are perennials in contrast to all cultivated lines, which have a biennial life cycle enabling seed production (Dohm et al., 2014). Today, modern sugar beet cultivars that accumulate enormous amount of sugar are of importance, not only for commercial sugar production, but also as renewable resource, e.g., for bioethanol production (Dodić et al., 2009). Although several studies analyzing sugar beet-associated bacterial communities already exist (Zachow et al., 2008; Mendes et al., 2011; Shi et al., 2014), little is known about the relation of the microbiome to existing cultivars and their wild relatives.

Plants have recently been recognized as meta-organisms, due to a close symbiotic relationship with their microbiome. Comparable to humans and other eukaryotic hosts, plants also harbor a "second genome" that fulfills important host functions including protection against biotic and abiotic stress (Berendsen et al., 2012; Berg et al., 2013). Interestingly, plant-associated

bacteria derived from different origins. Microorganisms can be transmitted by pollen and seeds (Fürnkranz et al., 2012); the latter was also shown for sugar beet (Dent et al., 2004). However, specific microorganisms are enriched from the surrounding environment as well, e.g., attracted by root exudates containing carbohydrates, proteins, and vitamins (Chaparro et al., 2013). In result of these processes, each plant harbors to a certain degree specific microbes (rev. in Berg and Smalla, 2009; Bulgarelli et al., 2012). This specificity was also shown for the plant-associated microbiome at cultivar level, e.g., for rice (Engelhard et al., 2000; Hardoim et al., 2011) as well as for maize (Haichar et al., 2008; Philippot et al., 2013). The evolutionary relationship in wheat–microorganism interactions was revealed already by Germida and Siciliano (2001); ancient wheat cultivars were colonized by phylogenetically diverse rhizobacterial isolates, whereas the rhizosphere of modern cultivars was dominated by fast-growing *Proteobacteria*. Therefore, we developed the hypothesis that the microbiome of the wild beet harbors a high degree of specific microorganisms in comparison to the sugar beet crop. We expected this degree of specificity also for the functions of the associated bacteria. The wild beet plants commonly grow in salinated dune soil of coastal drift lines. Thus, protection against abiotic stress by the microbiome should be more important than against biotic stress because plant diseases are not known for these populations (Biancardi et al., 2012). As reason for the specific microbiome of the wild beet, we assumed not only the genetic background of the wild ancestor; we also hypothesized an impact of the microbiome of the coastal drift line soil.

The objective of this study was: to determine key players in the rhizosphere microbiomes of (i) wild beet plants grown in coastal drift line soil (WB-CS), and (ii) domesticated sugar beets cv. BERETTA (SB) in dependence of the soil type (coastal drift line soil, CS, and potting soil, PS), and (iii) to determine the potential of isolates to cope with biotic and abiotic stresses in successive screenings.

MATERIALS AND METHODS

SAMPLING AND EXPERIMENTAL DESIGN

Wild beet plants (WB) with the synonymous common name sea beet [*B. vulgaris* ssp. *maritima* (L.) Arcang.] were sampled from the drift line at the Mediterranean Sea coast in Slovenia (N 45.590725, E 13.719469). Along a 200 m coastal drift line, four independent samples were taken. On average, plants were 20 cm high. Leaves were unfolded and separately spread on the beet heads without flower buds or seed development. Additionally, we collected coastal drift line soil (CS, pH 9.5, electric conductance $S = 1728\,\mu S\ cm^{-1}$) from the layer of 0–10 cm depth. Samples were placed into sterile plastic bags and transported to the laboratory at 4°C. Commercial sugar beet (SB) seeds cv. BERETTA KWS were provided by KWS SAAT AG (Einbeck, Germany). Seeds (10 per pot) were planted in a potting bulk soil (PS, soil:sand:vermiculite mixture 3:1:1, potting soil basis: "Gramoflor Profi-Substrat-Topfpikier M+Ton+Fe," GBC, Kalsdorf, Austria, pH ($CaCl_2$) 5.8, electric conductance $S = 1330\,\mu S\ cm^{-1}$, containing white and black peat with 90 kg m^{-3} moist clay, 1.0 kg m^{-3} PG-Mix (Greenworld, Wels, Austria), 50 g m^{-3} Radigen® (Terraflor, Iserlohn, Germany); sand: "Maxs Spielsand®," Scherf GmbH & Co KG, Hartberg, Austria; vermiculite: "Verm 3–6 mm," Ratioform, Vienna, Austria) as well as in coastal bulk soil from the drift line (CS). For SB-PS, five replicates, and for CS—due to the limited amount of available soil and plant material—three (SB-CS) replicates were incubated under greenhouse conditions (12 h light/dark at 18°C, 60% humidity) for 2 weeks. To isolate microorganisms, 20 g of roots with adhering soil were mixed with 50 ml sterile 0.85% NaCl for 3 min in a laboratory blender (BagMixer, Interscience, Mourjou, France). Suspensions were used for cultivation-independent and -dependent analyses. Samples were labeled as follows: CS—coastal drift line soil, PS—potting soil, SB-CS—sugar beet plants cultivated in coastal drift line soil, SB-PS—sugar beet plants cultivated in potting soil, WB-CS—and wild beet plants grown in coastal drift line soil.

MOLECULAR FINGERPRINTS OF 16S rRNA GENES

For cultivation-independent analyses, 8 ml of the above mentioned suspension were centrifuged at high speed (16,000 × g, 4°C) for 20 min and the resulting microbial pellets were stored at −70°C until further processing. Total DNA of the rhizosphere communities were extracted by mechanical disruption and homogenization of the pellet using FastDNA Spin Kit for Soil and a FastPrep Instrument (MP Biomedicals, Illkirch, France) for 30 s at 5.0 m s^{-1}. DNA was additionally purified by the GeneClean Turbo Kit (MP Biomedicals, Illkirch, France) containing guanidine thiocyanate to remove humic acids. Extracted DNA was treated with RNase (0.02 ng μl^{-1}) for 5 min at 65°C to obtain the template for PCR amplification of 16S rRNA genes from total community DNA.

Single strand conformation polymorphism (SSCP) analysis was carried out according to Schwieger and Tebbe (1998) using a 8.0% polyacrylamide gel running 26 h for bacterial community analysis at 400 V. SSCP-PCR was performed according to Köberl et al. (2011). Gels were transmissively scanned (Epson perfection 4990 Photo, Nagano, Japan) to obtain digitized gel images. Normalization and cluster analysis of band patterns, evaluated on band intensity, were carried out with the GelCompar II program (Applied Maths, Sint-Martens-Latem, Belgium). The cluster analysis was performed using the following settings: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); curve based similarity coefficient: Pearson correlation; optimization 4%, and position tolerances 1%. Background correction was applied for each track. The Pearson correlation index for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints.

AMPLICON PYROSEQUENCING AND BIOINFORMATIC ANALYSIS

The hypervariable V4-V5 region of the 16S rRNA gene (*Escherichia coli* positions 515–927) was amplified in a nested PCR approach for 454 pyrosequencing to analyze the taxonomic composition of the bacterial rhizosphere community. The first PCR was conducted with the primer pair 27f/1492r (Lane, 1991), while the second PCR targeted the V4-V5 region with the primer set that contained the 454 pyrosequencing adaptors and sample-specific tags (Table S1). The reaction mixture for the first PCR (10 µl) contained 1 × Taq&Go (MP Biomedicals, Eschwege,

Germany), 0.1 μM of each primer and 1 μl of template DNA (95°C for 5 min, 30 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 90 s, and elongation at 72°C for 5 min). The second PCR (60 μl) was performed by using 1 × Taq&Go, 1.5 mM $MgCl_2$, 0.1 μM of each primer and 3 μl of first PCR template (95°C for 5 min, 32 cycles of 95°C for 20 s, 54°C for 15 s, 72°C for 30 s, and elongation at 72°C for 10 min). PCR products of four replicates per samples of the same habitat were pooled in equal molarities and purified with the Wizard™ SV Gel and PCR Clean-Up System (Promega, Madison, USA). 16S rRNA gene amplicons were pyrosequenced with Roche 454 FLX GS conducted by MWG Biotech (Ebersberg, Germany). De-multiplexed raw sequences were processed using the open source software package QIIME (release 1.8.0; Caporaso et al., 2010). Prior to denoising (Reeder and Knight, 2010) sequences were quality (minimum average quality score in reads: 25) and length filtered (430–450 bp). Chimeric sequences were detected via ChimeraSlayer and subsequently removed. Chimera check was followed by excluding plant-originated plastid sequences using BLASTn algorithm (Altschul et al., 1990). Remaining sequences were clustered at 97% similarity using the UCLUST algorithm (Edgar, 2010) and taxonomic assignment of representative sequences were performed using the RDP naïve Bayesian rRNA classifier (Wang et al., 2007) based on the reference database Greengenes release 13_5 (De Santis et al., 2006). Read number per sample was normalized to 5578. Rarefaction analysis (species level at 97%), richness estimates, and diversity indices were calculated; Shannon (1997) and Chao1 (Chao and Bunge, 2002) indices were calculated based on the complete linkage clustering data. For OTU-based analysis, only OTUs accounting for at least 1% of total reads were considered. Classifications of the reads (one representative per OTU) were performed by manual alignment of representative sequences with 16S rRNA reference gene sequences from NCBI database using BLASTn algorithm. Raw pyrosequencing data were deposited at the National Center for Biotechnology Information under the BioProject number PRJNA233435 with the SRA accession numbers SRX652486 (WB-CS), SRX652836 (CS), SRX652838 (SB-CS), SRX652839 (SB-PS), SRX652840 (PS).

ISOLATION AND CHARACTERIZATION OF BACTERIA

The homogenized suspensions of wild beet rhizosphere replicates were used for dilution and plating on R2A (Roth, Karlsruhe, Germany), and Kings B amended with ampicillin (50 μg ml^{-1}), novobiocin (45 μg ml^{-1}), and cycloheximide (50 μg ml^{-1}). Each dilution was plated in duplicates. Plates were incubated for 5 days at room temperature (RT) and colony forming units (CFU) were counted to calculate the means of colonies (log_{10} CFU) based on fresh weight (fw). To obtain the microbial communities of commercial sugar beet plants (SB-CS, SB-PS), 2 weeks after germination the culturable fraction of the rhizospheres were harvested, suspended, diluted, and plated as described above. If possible, 24 bacterial isolates were selected randomly for each replicate and subsequently cultured on nutrient agar (NA). Due to the limited amount of CS, fewer replicates were available and therefore, a lower number of bacteria were isolated. The isolates were purified and then stored at −70°C in nutrient broth II (NB II) (Sifin, Berlin, Germany) stocks containing 15% (v/v) glycerol. Isolates were encoded using a combination of letters and numbers indicating: (1) plant/soil type (WB—wild beet plants grown in coastal drift line soil WB-CS, VN—sugar beet plants cultivated in coastal drift line soil, VS—sugar beet plants cultivated in potting soil), (2) replicate (1–4), (3) consecutive number of the isolate per replicate, and (4) origin of the medium (no further indication—bacteria from R2A, Ps—*Pseudomonadaceae* from Kings B). Bacteria with the highest antagonistic activity and stress tolerance were identified by sequencing partially the 16S rRNA gene using the primer pair 27f/1492r (Lane, 1991). Purified fragments were sequenced using LGC Genomics GmbH sequencing service (Berlin, Germany) and identified as described. Sequences obtained were submitted to EMBL Nucleotide Sequence Database under accession numbers KJ024636 to KJ024701. Co-occurrence of 16S rRNA genes from isolates and OTUs obtained by pyrosequencing was determined by aligning corresponding regions using BLASTn algorithm.

SCREENING FOR BACTERIA ANTAGONISTIC TOWARD PLANT PATHOGENS

The antagonistic potential of randomly selected isolates was assessed by *in vitro* inhibition of sugar beet-pathogenic fungi *Alternaria alternata* Nees, *Botrytis cinerea* Persoon, *Rhizoctonia solani* Kühn AG2-2IIIB, *Sclerotinia sclerotiorum* Fuckel, and *Verticillium dahliae* Klebahn V25 according to Berg et al. (2002). Zones of inhibition were measured 3–7 days after incubation at 25°C.

SCREENING FOR STRESS TOLERATING BACTERIA

Overnight cultures grown in 10 ml NBII were used as inoculum (5 μl) for all bacterial assays. For desiccation assays, 20 μl of culture fluids were dried under sterile conditions. After 3, 6, 9, 16, 21, and 56 days, bacteria were re-suspended with the same volume NBII and dropped on NA. Re-cultivated cells were registered in a positive/negative response. For osmolarity stress, bacteria were cultivated in a 96-well plate filled with 145 μl per well modified Luria Bertani (per liter: peptone 3 g, meat extract 5 g) with various sodium chloride concentrations (1% steps, 0–20%) and incubated at 30°C for 48 h under agitation. In reactive oxygen species stress assays, bacteria were cultivated in NBII amended with different tellurite (1, 3, 5, 7, and 9 mg ml^{-1}) and H_2O_2 (0.1, 0.3, 0.5, 0.7, and 0.9 mM) concentrations. The assays were performed in 96-well-plates filled with 195 μl medium per well and 5 μl 1:100 diluted overnight incubated culture. Optical density (OD) was measured for all 96-well plates at 600 nm. Bacterial growth was positively evaluated when OD_{600} was higher than 0.2 (that is 5-fold higher than the untreated control) after 24 h.

STATISTICS

Significant differences of 16S rRNA gene based fingerprints between all samples were calculated with permutation analysis of pairwise similarities using permtest package for R statistics (The R Foundation for Statistical Computing Version 2.1.1) (Kropf et al., 2004). Other significant differences were calculated using ANOVA with *post-hoc* test Scheffé in SPSS-PASW Statistics v.18.

RESULTS

MOLECULAR FINGERPRINTS OF 16S rRNA GENES OF THE BACTERIAL COMMUNITIES

Molecular fingerprints were performed by SSCP analysis of 16S rRNA genes amplified from DNA obtained from all samples to gain first insight into the bacterial communities. Bacterial fingerprints of potting soil samples (SB-PS and PS) comprised fewer but more dominant bands compared to the SSCP profiles from WB-CS, SB-CS, and CS, where a higher number of SSCP bands were detected (Figure S1). According to cluster analyses, bacterial communities from SB-CS and CS significantly differed by 78% from SB-PS and PS ($P \leq 0.001$), which clearly indicated the high impact of soil type and the composition of bacterial fingerprints. In addition, the rhizosphere effect was highly pronounced: SB-CS and SB-PS were significantly different from their respective bulk soils CS and PS by 72% ($P \leq 0.029$) and 52% ($P \leq 0.028$), respectively, (**Figure 1**). Differences between both investigated plant genotypes were also identified: the WB-CS rhizosphere was separated from all others and showed only 16% similarity with the SB-CS rhizosphere. Noticeably, due to different life cycles not only the genotype but also the plant developmental stage were different and could account for observed effects.

PYROSEQUENCING-BASED 16S rRNA PROFILING OF THE BACTERIAL COMMUNITIES

A pyrosequencing-based analysis of partial 16S rRNA gene sequences was employed to survey the diversity and composition of the bacterial rhizosphere communities in WB-CS, SB-CS and SB-PS, and the respective bulk soils CS and PS. In all samples, we recovered between 5578 and 9567 quality sequences with read lengths ranging from 430 to 450 bp. Prior to further analysis, read numbers were normalized to 5578 for each sample. To calculate rarefaction curves and to perform taxonomic assignments, reads were clustered in operational taxonomic units (OTUs) at sequence divergences of 3% (species level). The rarefaction curves of the bacterial communities of WB-CS, SB-CS, SB-PS, and their respective bulk soils CS and PS are shown in Figure S2. At a dissimilarity level of 3%, the curves of WB-CS and PS samples generally showed low slopes, but did not reach saturation. Accordingly, the number of observed OTUs covers only 31.4 and 34.5% of the estimated taxonomic richness by the Chao1 richness estimator (**Table 1**). That indicates evenly contributing species and a low number of very common or very rare species. The computed Shannon indices of diversity (H′) were much higher for the wild beet plants grown in coastal drift line soil (WB-CS) and the sugar beets grown therein SB-CS, and CS (8.7, 8.1, 8.2) than for SB-PS and PS (6.0, 4.3).

Altogether, 98.8% of the OTUs (total number: 5163) were affiliated to 10 different phyla representing at least 1% of reads (**Figure 2**): *Actinobacteria* and *Proteobacteria* were found in all samples. Only in WB-CS, SB-CS, and CS *Acidobacteria* (8.7, 13.0, 17.1%), *Chloroflexi* (4.3, 7.0, 6.0%), *Gemmatimonadetes* (1.6, 4.7, 3.7%), and *Planctomycetes* (26.9, 39.3, 36.3%) were found. *Bacteroidetes* were found exclusively in rhizospheres of WB-CS (2.7%) and SB-PS (14.1%). Low percentages of *Fibrobacteres* were found exclusively in the rhizosphere of SB-CS

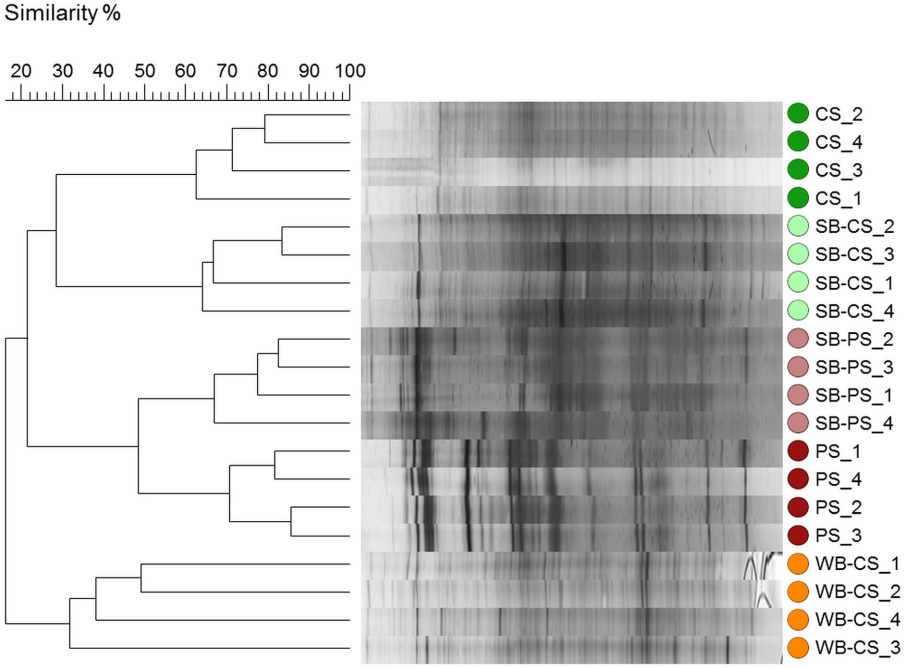

FIGURE 1 | Dendrogram of 16S rRNA gene-based SSCP profiles of bacterial communities in association with wild beet (WB-CS) and sugar beet rhizosphere grown in coastal drift line (SB-CS) and potting soil (SB-PS) and their respective bulk soils (CS, PS) with four repetitions per sample. Following settings were used: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: curve based, Pearson correlation; optimization: 4%, position tolerance: 1%.

(1.0%) and *Verrucomicrobia* in the rhizosphere of WB-CS (1.7%).

SOIL TYPE- AND PLANT GENOTYPE-SPECIFIC OTUs

A more detailed analysis of the dominant soil and plant genotype-specific OTUs was performed on the base of a manual taxonomic assignment by BLASTn alignment using NCBIs 16S rRNA gene reference database. In total, 48 OTUs were obtained when a cut-off level of 1% was applied (**Table 2**). The dominant genus within the phylum *Proteobacteria* was *Pseudomonas* comprising four species (OTU1726, 2094, 2281, 4102) with the relative abundance of 18.8% detected in the SB-PS rhizosphere, 1.8% WB-CS rhizosphere, 0.2% in the SB-CS rhizosphere, and 16.9 and 0.1% in PS and CS, respectively. The second major genus assigned to *Stenotrophomonas* comprised only one OTU (3731), and was detected in the SB-PS (1.8%) and WB-CS (0.4%) rhizosphere, and in PS (21.1%). Most of the *Firmicutes* sequences belonged to the genus *Paenibacillus* comprising three species (OTU1033, 2251, 3065) in SB-PS (0.2%) and PS (8.3%), followed by *Bacillus* (OTU379) in the SB-PS (0.2%) and WB-CS rhizosphere (0.1%), and in PS (6.7%). *Blastopirellula,* the main genus within the phylum *Planctomycetes*, occurred in approximately equal abundances in the SB-CS (4.9%) and WB-CS (4.0%) rhizosphere, and in CS (5.2%). The majority of *Bacteroidetes* reads belonged to *Flavobacterium* (OTU254, 3902) and *Pedobacter* (OTU172, 3215) detected in the SB-PS and WB-CS rhizosphere. Within the *Actinobacteria*, *Thermoleophilum* occurred as the only genus (OTU745) in the SB-CS (3.1%) and WB-CS rhizosphere (0.6%), and CS (2.5%). Within the *Acidobacteria* the OTU945 assigned to *Candidatus Solibacter* occurred with 0.7% in SB-CS, 0.2% WB-CS, and 1.3% in CS.

Comparing wild beet and sugar beet cultivated in CS, 20 out of 38 dominant OTUs (41.7%) were detected in both rhizospheres, whereas 18 OTUs (37.5%) accounting for 2.3% of total reads occurred exclusively in WB-CS, i.e., OTU172, 254, 1122, 1155, 2094, 2281, 2797, 3190, 3215, 3262, 3731, 3889, 3902, 3915, 3972, 4395, 5140, 5143 (**Table 2**). The WB-CS-specific OTU with the highest percentage of reads (0.5%) was OTU2094 assigned to *Pseudomonas grimontii*. Remarkably, none of the OTUs was found to be specific for SB-CS. In the soil type comparison of sugar beet plants cultivated in CS and PS, 12 specific OTUs (25.1% of total reads) were found in SB-CS and 25 in SB-PS (33.7%). In SB-CS, the most dominant and specific OTU (5.6%) was assigned to *Angiococcus disciformis* (OTU1264) and in SB-PS to *Enterobacter* sp. with 6.1% (OTU3915). Altogether, a high impact of soil type and also of plant genotype on the rhizosphere communities was observed.

Table 1 | Species richness (normalized at 5578 sequences per sample) estimates obtained at 3% genetic dissimilarity from 454 pyrosequencing-derived sequences of DNA extracted from SB-CS—Sugar beet plants cultivated in coastal drift line soil, SB-PS—Sugar beet plants cultivated in potting soil, WB-CS—Wild beet plants grown in coastal drift line soil, CS—Coastal drift line soil, PS—Potting bulk soil.

Sample ID	Sample	Shannon index[a] (H′)	Rarefaction[b] (no. of OTUs)	Chao1[c] (no. of OTUs)	Coverage (%)
MID1	WB-CS	8.7	1369.9	4356.9	40.6
MID2	CS	8.2	1156.1	3153.2	36.7
MID3	SB-CS	8.1	1190.7	2930.2	36.7
MID4	SB-PS	6.0	361.5	986.2	34.5
MID5	PS	4.3	121.3	351.1	31.4

[a] higher number indicates more diversity; [b] results from the rarefaction analyses; [c] non-parametric richness estimator based on the distribution of singletons and doubletons.

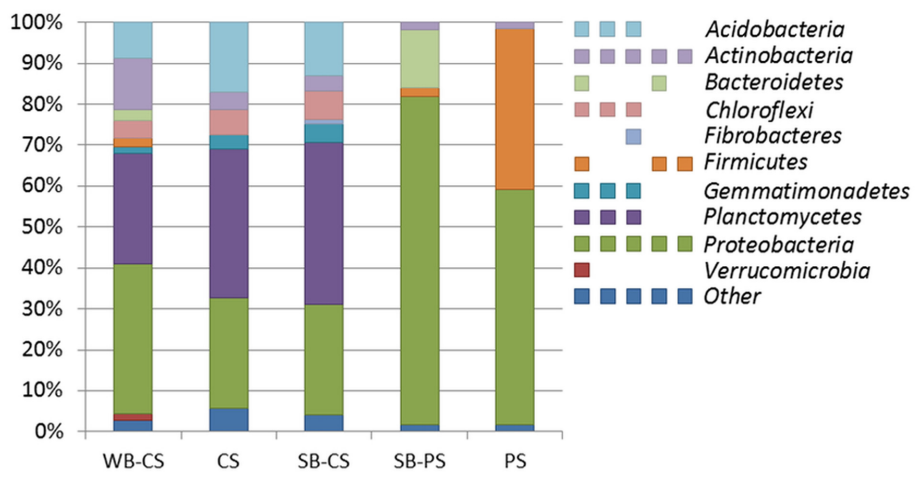

FIGURE 2 | The structure of bacterial communities at phylum level of wild beet rhizosphere (WB-CS), coastal drift line bulk soil (CS), sugar beet plants cultivated in coastal drift line soil (SB-CS), sugar beet plants cultivated in potting soil (SB-PS), and potting bulk soil (PS). Relative composition of major phyla (>1% of total reads) was determined by 454 amplicon pyrosequencing of 16S rRNA extracted from all samples. Multi-colored charts at the legend are shown for each phylum and sample correspondingly. Phyla to which <1% of reads were assigned were summarized as "other."

Table 2 | Taxonomic classification and relative abundance of dominant OTUs (cut-off level: 1%) from rhizosphere and soil samples.

OTU	Phylum	Genus	Species	Accession	SI	WB-CS	CS	SB-CS	SB-PS	PS
945	*Acidobacteria*	*Candidatus Solibacter*	*Candidatus Solibacter usitatus* Ellin6076	NR_074351.1	90	0.19	1.27	0.71	0.00	0.00
745	*Actinobacteria*	*Thermoleophilum*	*Thermoleophilum minutum*	NR_036932.1	84	0.62	2.49	3.05	0.00	0.00
3902	*Bacteroidetes*	*Flavobacterium*	*Flavobacterium cutihirudinis*	NR_109728.1	98	0.01	0.00	0.00	1.11	0.00
254			*Flavobacterium myungsuense*	NR_108537.1	98	0.27	0.00	0.00	1.08	0.00
3215		*Pedobacter*	*Pedobacter borealis*	NR_044381.1	99	0.02	0.00	0.00	2.03	0.00
172			*Pedobacter nyackensis*	NR_044380.1	99	0.03	0.00	0.00	2.10	0.00
3911	*Firmicutes*	*Alicyclobacillus*	*Alicyclobacillus pomorum*	NR_024801.1	99	0.00	0.00	0.00	0.00	1.17
379		*Bacillus*	*Bacillus ginsengihumi*	NR_041378.1	99	0.09	0.00	0.02	0.24	6.68
4794		*Brevibacillus*	*Brevibacillus reuszeri*	NR_040982.1	99	0.00	0.00	0.00	0.02	2.28
3224		*Cohnella*	*Cohnella formosensis*	NR_109515.1	99	0.00	0.00	0.00	0.03	1.54
1531		*Oxalophagus*	*Oxalophagus oxalicus*	NR_036979.1	98	0.00	0.00	0.00	0.01	1.13
1033		*Paenibacillus*	*Paenibacillus koleovorans*	NR_024752.1	96	0.00	0.00	0.00	0.02	1.75
2251			*Paenibacillus sediminis*	NR_108601.1	99	0.00	0.00	0.00	0.18	3.73
3065			*Paenibacillus terrigena*	NR_041398.1	97	0.00	0.01	0.00	0.00	2.81
3895		*Paenisporosarcina*	*Paenisporosarcina indica*	NR_108473.1	99	0.16	0.03	0.02	0.11	2.49
2595		*Tumebacillus*	*Tumebacillus permanentifrigoris*	NR_043849.1	97	0.00	0.00	0.00	0.00	1.66
616	*Planctomycetes*	*Blastopirellula*	*Blastopirellula marina*	NR_029226.1	88	0.96	1.05	0.89	0.00	0.00
2186			*Blastopirellula marina*	NR_029226.1	90	1.44	2.09	1.59	0.00	0.00
2807			*Blastopirellula marina*	NR_029226.1	90	1.65	2.11	2.43	0.00	0.00
2164	*Proteobacteria*	*Achromobacter*	*Achromobacter spanius*	NR_025686.1	99	0.00	0.01	0.00	0.16	7.15
5140		*Aeromonas*	*Aeromonas veronii*	NR_102789.1	99	0.03	0.00	0.00	3.87	0.00
3190		*Agrobacterium*	*Agrobacterium tumefaciens*	NR_041396.1	99	0.33	0.01	0.00	1.36	0.33
4395		*Brevundimonas*	*Brevundimonas bullata*	NR_025831.1	99	0.05	0.00	0.00	1.37	0.00
3816		*Cellvibrio*	*Cellvibrio fulvus*	NR_025210.1	99	0.30	0.00	0.09	1.42	0.00
1264		*Cystobacter*	*Angiococcus disciformis*	NR_104864.1	85	1.09	6.71	5.63	0.00	0.00
2092			*Angiococcus disciformis*	NR_104864.1	85	5.80	2.60	3.70	0.00	0.00
4604		*Desulfobacca*	*Desulfobacca acetoxidans*	NR_074955.1	85	0.47	1.22	1.48	0.00	0.00
3651		*Desulfomonile*	*Desulfomonile limimaris*	NR_025079.1	87	1.10	1.42	1.18	0.01	0.00
3799		*Desulfonema*	*Desulfonema ishimotonii*	NR_025991.1	87	1.13	0.80	0.71	0.00	0.00
4187		*Desulfotalea*	*Desulfotalea arctica*	NR_024949.1	84	0.29	1.10	1.30	0.00	0.00

(Continued)

Table 2 | Continued

OTU	Phylum	Genus	Species	Accession	SI	WB-CS	CS	SB-CS	SB-PS	PS
187		*Devosia*	*Devosia crocina*	NR_044213.1	98	0.07	0.00	0.02	1.27	0.00
3262		*Duganella*	*Duganella phyllosphaerae*	NR_108529.1	99	0.03	0.00	0.00	1.17	0.00
3915		*Enterobacter*	*Enterobacter* sp.	NR_074777.1	99	0.14	0.00	0.00	6.06	0.02
276		*Geobacter*	*Geobacter grbiciae*	NR_041826.1	84	1.08	2.25	2.41	0.00	0.00
5143		*Janthinobacterium*	*Janthinobacterium agaricidamnosum*	NR_026364.1	99	0.01	0.00	0.00	1.86	0.00
3972		*Methylophilus*	*Methylophilus flavus*	NR_104519.1	99	0.04	0.00	0.00	1.10	0.00
281		*Novosphingobium*	*Novosphingobium resinovorum*	NR_044045.1	99	0.41	0.01	0.02	1.62	0.00
3889		*Paracoccus*	*Paracoccus limosus*	NR_109093.1	99	0.15	0.00	0.00	1.00	0.00
3620		*Pelobacter*	*Pelobacter acetylenicus*	NR_029238.1	86	0.13	0.98	1.18	0.00	0.00
2797		*Phenylobacterium*	*Phenylobacterium haematophilum*	NR_041991.1	99	0.03	0.00	0.00	1.91	0.20
2281		*Pseudomonas*	*Pseudomonas brassicacearum* subsp. *brassicacearum*	NR_074834.1	98	0.12	0.01	0.00	1.01	0.00
1726			*Pseudomonas entomophila*	NR_102854.1	99	1.11	0.12	0.18	14.04	16.91
2094			*Pseudomonas grimontii*	NR_025102.1	99	0.52	0.01	0.00	2.52	0.01
4102			*Pseudomonas poae*	NR_102514.1	98	0.00	0.00	0.00	1.20	0.00
1122		*Rhodopseudomonas*	*Rhodopseudomonas palustris*	NR_036771.1	98	0.03	0.00	0.00	0.23	1.85
1155		*Sphingopyxis*	*Sphingopyxis italica*	NR_108877.1	99	0.05	0.00	0.00	0.50	4.44
3731		*Stenotrophomonas*	*Stenotrophomonas maltophilia*	NR_074875.1	99	0.39	0.00	0.00	1.82	21.08
3680		*Variovorax*	*Variovorax paradoxus*	NR_074646.1	99	0.32	0.00	0.14	1.82	0.10

Relative abundances (%) of OTUs in the rhizosphere and bulk soil based on pyrosequencing data. Accession numbers of the NCBI GenBank accession was indicated for the closest representative sequence to the OTUs with the corresponding similarity index SI in %. Relative abundances are indicated by colors ranging from low abundances (yellow) raising to a high abundance (red). Non-detected OTUs in respective samples are white colored.

COMPARATIVE ANALYSES OF RHIZOSPHERE AND BULK SOIL RESPONDERS

Taxonomic comparisons were done at genus level for representative sequences of all OTUs obtained by clustering at 97% similarity. Classification was performed using the RDP naïve Bayesian rRNA classifier and Greengenes 16S rRNA database. Overall, 37.4% of the sequences could be assigned to 70 genera with a confidence score of ≥80% (Table S2). By comparing CS and WB-CS, 44 taxa assigned to eight phyla were detected (including *Other*). In general, 31 taxa responded positively in the rhizosphere with increased ratio higher than 2, where the highest ratio was found for *Novosphingobium* sp. (74.4) followed by *Pseudomonas* sp. (50.8). Comparing CS and SB-CS, only six taxa (ratio > 2) were positive rhizosphere responders with the highest ratio found for *Cellvibrio* sp. (6.1) followed by *Aquimonas* sp. (4.3); whereas 32 taxa were not detected at all in CS. By comparing PS and SB-PS seven taxa (ratio > 2) were detected with the highest ratio of 116.0 found in the rhizosphere and assigned to *Sphingomonas* sp. followed by *Pseudomonas* sp. (90.6). Fifty-two taxa were not detected in PS. For wild beet plants in CS and sugar beet plants cultivated in CS and PS, the rhizosphere effect could be confirmed. In general, a stronger rhizosphere effect was observed for sugar beet plants in potting soil compared to plants cultivated in coastal drift line soil.

BACTERIAL ANTAGONISTS AND THEIR STRESS RESPONSE

In total, 576 bacteria were isolated; 192 isolates were selected from WB-CS, 144 from SB-CS, and 312 from SB-PS rhizospheres. The lower number of isolates from WB-CS and SB-CS resulted from the limited amount of sample material. All bacteria were tested in

dual culture assays for their *in vitro* antagonistic activity against various sugar beet phytopathogens. In total, 357 bacteria were positive against at least one of the phytopathogens. The highest proportion of antagonistic bacteria originated from Kings B agar compared to R2A (**Table 3**).

The highest percentage of antagonists were found for bacteria isolated from SB-PS (R2A 15.0 ± 9.7, Kings B 57.5 ± 11.6) against *Botrytis cinerea* independently from the selection medium. Within isolates from R2A, a higher percentage of active bacteria were found from SB-CS compared to WB-CS except for *Sclerotinia sclerotiorum*. Similarly, for bacteria isolated from Kings B, a higher percentage of active bacteria were isolated from SB-CS compared to WB-CS against *A. alternata*, *S. sclerotiorum*, and *V. dahliae*, except for *B. cinerea* and *R. solani*. When cultivated in different soil types, isolates from SB-PS (Kings B) showed a higher antagonistic percentage against *B. cinerea* and *R. solani* and less activity against *A. alternata* compared to SB-CS.

All positively evaluated bacterial isolates (357) were further tested for their ability to tolerate stress including desiccation, salt, and reactive oxygen species caused by hydrogen peroxide and tellurite (**Figure 3**). In desiccation assays, the survival of the cells over several days was tested. In general, slightly more isolates obtained from the Kings B medium could be re-cultivated after 3, 6, and 16 days when compared to R2A. After 56 days, most of the isolates were not able to be re-cultivated after desiccation (**Figure 3A**). In salt (NaCl) stress assays, a high percentage of isolates from WB-CS and sugar beet plants cultivated in the same soil (SB-CS) were able to grow in the presence of up to 6% salt. The majority of bacteria from all samples could deal with a maximal salt concentration of 6%; with the exception of SB-PS, where most of the isolates were unable to grow above 2% NaCl (**Figure 3B**). In the presence of reactive oxygen species (ROS) induced by hydrogen peroxide and tellurite, 68.0 and 28.2%, respectively, of the antagonists were able to cope with the lowest concentration, and 1.1 and 4.5%, respectively, with the highest concentration. The highest percentage of isolates able to deal with high ROS concentrations caused by hydrogen peroxide were determined for isolates from WB-CS (0.1 mM: 91.7%; 0.3 mM: 53.3%; 0.5 mM: 16.7%) selected on Kings B (**Figure 3C**). The maximum concentration of hydrogen peroxide at which isolates were able to grow was 0.7 mM. None of the isolates from SB-CS and from Kings B were able to grow in the presence of H_2O_2. Among all samples, only a few isolates were able to tolerate high ROS concentrations from tellurite. These isolates were obtained from SB-CS. The maximum concentration of tellurite at which isolates were able to grow was 9 mg ml^{-1} (**Figure 3D**).

Bacterial isolates with the highest antagonistic activity and stress tolerance were identified (**Table 4**). From all samples, isolates of *Pseudomonas* and *Stenotrophomonas* species were identified. Isolates from the sugar beet rhizospheres (SB-CS, SB-PS) were mainly identified as *Staphylococcus* and *Stenotrophomonas* species. In contrast, from WB rhizospheres more diverse bacterial species were isolated, i.e., *Curtobacterium* sp., *Erwinia* sp., *Microbacterium* sp., *Micrococcus* sp., *Streptomyces* sp., *Sphingobacterium* sp., *Agrobacterium* sp., *Luteimonas* sp., *Pseudomonas* sp., *Rheinheimera* sp., and *Stenotrophomonas* sp. Based on their 16S rRNA gene similarity, the abundance of bacterial isolates in the amplicon libraries was calculated. In all samples, 16S rRNA sequences were detected of species to which the active isolates were affiliated. Their relative abundances ranged from 0.01 to 2.94% with the highest abundances of *Pseudomonas* and *Stenotrophomonas*.

DISCUSSION

In this study, we found differences within the microbiome composition of the sugar beet rhizosphere and its wild ancestor. At phylum level, the rhizosphere microbiome of wild beet and domesticated sugar beet plants (WB-CS, SB-CS) resembled each other independently from the climate and plant development, whereas, at a higher taxonomic resolution plant genotype-specific patterns were identified. This plant genotype-specific effect was confirmed for functional traits. The wild beet rhizosphere (WB-CS) was colonized by a low number of bacteria with antagonistic activity against pathogens but their antagonists showed a high potential to cope with abiotic stresses. Conversely, the sugar beet cultivar was able to enrich a high antagonistic potential from both soil types but harbored less antagonists with high stress resistance. In addition, an impact of both soil types (CS, PS) on the bacterial composition was found. However, the differences in structure and function of the microbiomes of ancient and modern beets underline the relationship between the plant genotypes and their associated bacteria and confirmed our hypotheses.

Table 3 | Antagonistic potential toward various plant pathogens.

Origin	Cultivation medium	No. of isolates[a]	*A. a.* (%)	*B. c.* (%)	*R. s.* (%)	*S. s.* (%)	*V. d.* (%)
WB-CS	R2A	96	7.3 ± 10.2 a	4.2 ± 7.1 a	0.0 ± 0.0 a	2.1 ± 3.5 a	2.1 ± 3.5 a
SB-CS	R2A	72	12.5 ± 3.3 a	10.4 ± 8.9 a	2.1 ± 3.5 a	2.1 ± 3.5 a	12.5 ± 16.8 b
SB-PS	R2A	120	1.7 ± 2.1 a	15.0 ± 9.7 a	0.8 ± 1.5 a	1.7 ± 2.1 a	n.d.
WB-CS	Kings B	96	17.7 ± 15.6 a	45.8 ± 20.2 ab	10.4 ± 8.9 a	1.0 ± 1.8 a	4.2 ± 2.9 a
SB-CS	Kings B	72	20.8 ± 8.2 a	27.1 ± 3.5 a	4.2 ± 7.1 a	16.7 ± 11.5 b	20.8 ± 7.1 b
SB-PS	Kings B	120	10.1 ± 5.5 a	57.5 ± 11.6 b	4.6 ± 2.5 a	n.d.	n.d.

Legend: SB-CS—Sugar beet plants cultivated in coastal drift line soil, SB-PS—Sugar beet plants cultivated in potting soil, WB-CS—Wild beet plants grown in coastal drift line soil. Inhibition of typical and new emerging sugar beet phytopathogenic fungi A. a.—Alternaria alternata Nees, B. c.—Botrytis cinerea Pers., R. s.—Rhizoctonia solani Kühn AG2-2IIIB, S. s.—Sclerotinia sclerotiorum Fuckel, and V. d.—Verticillium dahliae V25. n.d.—not determined. [a]The lower number of isolates from WB-CS and SB-CS resulted from the limited amount of sample material.

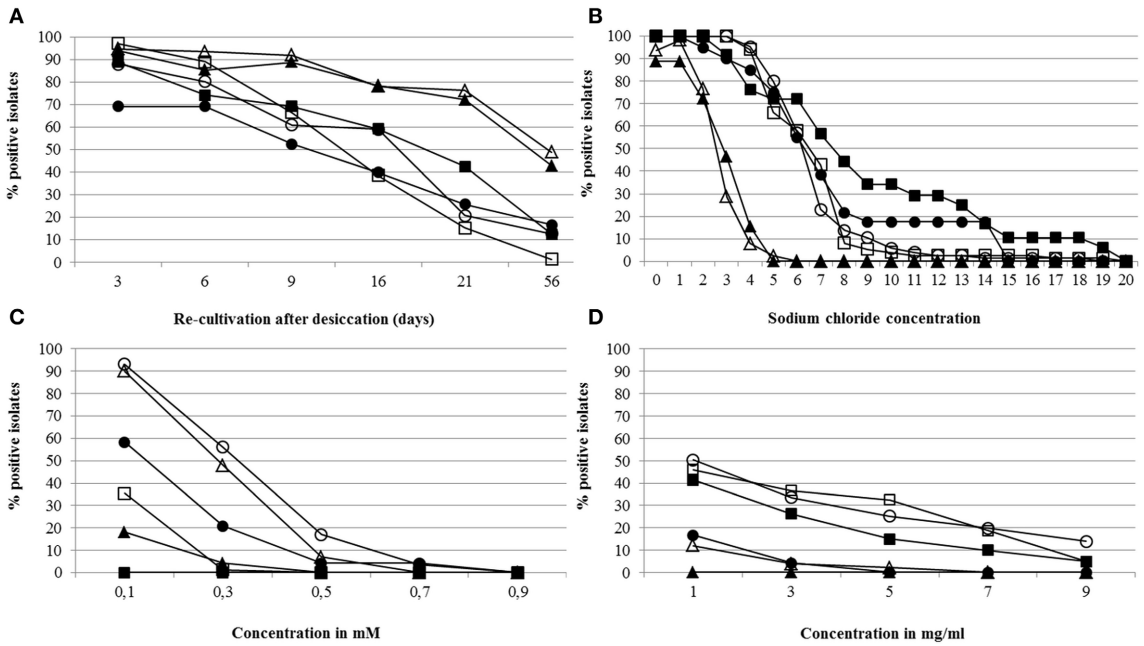

FIGURE 3 | Stress tolerance assays. Bacterial isolates able to be re-cultivated after desiccation **(A)**, grown in presence of different sodium chloride concentrations **(B)**, and cultivated in presence of reactive oxygen species caused by hydrogen peroxide **(C)** and tellurite **(D)**. In total, 357 antagonistic bacteria, previously selected from R2A or Kings B medium, were tested. Legend: bacteria isolated from R2A—closed symbols, and from Kings B—open symbols; wild beet rhizosphere (WB-CS)—circle, rhizosphere of sugar beet plants cultivated in coastal drift line soil (SB-CS)—square, rhizosphere of sugar beet plants cultivated in potting soil (SB-PS)—triangle. Further statistics were indicated in Tables S3A–D.

The composition of the microbiome of both plant genotypes, when grown in CS, was comparable at phylum level. However, resolved at OTU level, based on results from molecular fingerprint analysis and amplicon pyrosequencing of 16S rRNA genes, plant genotype-specific OTUs were detected. The wild beet (WB-CS) showed the highest number of unique bands in bacterial fingerprints but the bacterial communities in the rhizosphere of sugar beet (SB-CS) and in bulk soil (CS) were similar to each other. Based on amplicon libraries, the WB-CS rhizosphere consisted of 18 unique out of 38 detected OTUs including *Flavobacterium*, *Pedobacter*, and *Pseudomonas* spp. In contrast, no specific OTUs could be identified for SB-CS. The low rhizosphere effect in SB-CS might be a result of the shorter growth period in addition to the different climate. The majority of the dominant OTUs (20) was shared by SB-CS and WB-CS and included genera of the phyla *Actinobacteria* (*Thermoleophilum*), *Proteobacteria* (*Cystobacter*, *Desulfomonile*), and *Planctomycetes* (*Blastopirellula*). In all rhizospheres (CS), the myxobacterium *Angiococcus disciformis* was found in highest relative abundances. The more diverse spectrum of OTUs associated with wild beet plants compared to SB-CS was confirmed by the elevated Shannon index, which was 8.7 for WB-CS and 8.1 for SB-CS. The impact of the plant genotype on the associated microbiota, which was clearly shown in our study, was dependent on the applied method and their taxonomic resolution. This genotype-specific impact was also shown or other crops such as maize (Peiffer et al., 2013), potato (Weinert et al., 2009, 2011), and rice (Engelhard et al., 2000; Hardoim et al., 2011).

In addition to the plant genotype-specific community structures, we found differences in *in vitro* functions of the isolates as well. The proportion of strains with antagonistic *in vitro* activity against phytopathogens was lower in the rhizosphere WB-CS in comparison to the domesticated sugar beet SB-CS and SB-PS. The lower antagonistic activity of wild beet-associated bacteria can be traced back to the fact that pathogen pressure barely exists in coastal drift-line soil as it is influenced by seawater spray, tidal flow, and storms (Biancardi et al., 2012). In contrast, modern sugar beets are threatened by many diseases including fungal pathogens which were involved in this study (Khan, 2013). Under agricultural conditions, the pathogen pressure is often much higher than in natural habitats; one reason for this can be the naturally occurring biodiversity which protects ecosystems from spread of diseases (Latz et al., 2012). *B. maritima* grows under extreme saltwater conditions, and can tolerate both high salt concentrations in soil and severe drought (Shaw et al., 2002). Wild beet-associated bacterial isolates showed a higher extend of stress tolerance than isolates of domesticated sugar beet plants with *in vitro* antagonistic activity. These isolates were identified mainly as *Pseudomonas* and *Stenotrophomonas* species; members of both are well-known for their ability to cope with biotic and abiotic stress (Alavi et al., 2013; Zachow et al., 2013). Within the amplicon libraries their relative abundances was lower than 1%. However, the protective functions can be fulfilled by a minor fraction, which may contribute to important ecosystem functions (Pester et al., 2010).

Table 4 | Taxonomic classification of the most active antagonists isolated from R2A (universal bacteria) and Kings B (selective for *Pseudomonadaceae*) by sequencing partial 16S rRNA genes.

Antagonist	Identification				Antagonistic activity					Stress tolerance[c]			
	Closest BLAST database match (reference RNA sequences)	SI in %[a]	Accession no.	Relative abundances (%)[b]	*A. a.*	*B. c.*	*R. s.*	*S. s.*	*V. d.*	Dry-off	NaCl	H_2O_2 [μmol]	Tellurit [mg/ml]
WILD BEET PLANTS GROWN IN COASTAL DRIFT LINE SOIL—WB-CS (R2A)													
ZR1-24	*Micrococcus luteus*	99	NR_075062.1	No match	−	−	−	−	+	56	7	300	0
ZR3-3	*Rheinheimera aquimaris*	98	NR_044068.1	0.38	−	+	−	−	−	1	8		
ZR3-4	*Agrobacterium fabrum*	97	NR_074266.1	No match	−	−	−	+	−	21	5	100	0
ZR3-9	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	99	NR_025467.1	0.01	+	−	−	−	−	56	7	700	0
ZR3-18	*Sphingobacterium faecium*	99	NR_025537.1	No match	−	+	−	−	−	16	5	100	0
ZR4-2	*Luteimonas aestuarii*	98	NR_044343.1	0.56	+	−	−	−	n. d.	9	15	0	0
ZR4-6	*Streptomyces phaeochromogenes*	98	NR_041200.1	No match	+	−	−	−	−	1	5	0	0
ZR4-9	*Microbacterium aoyamense*	99	NR_041332.1	No match	+	−	−	−	−	21	3	0	0
WILD BEET PLANTS GROWN IN COASTAL DRIFT LINE SOIL—WB-CS (KINGS B)													
ZR1-2_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.04	−	+	−	−	−	6	19	0	0
ZR1-10_Ps	*Pseudomonas poae*	99	NR_102514.1	0.54	−	−	+	−	−	56	7	300	0
ZR2-5_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.04	−	+	−	−	−	16	14	100	1
ZR2-9_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.04	−	+	−	−	−	21	9	100	0
ZR3-2_Ps	*Erwinia rhapontici*	99	NR_041976.1	0.14	−	+	−	−	−	56	11	300	0
ZR3-5_Ps	*Pseudomonas brassicacearum* subsp. *brassicacearum*	99	NR_074834.1	0.03	−	+	−	−	−	56	7	300	9
ZR3-8_Ps	*Erwinia rhapontici*	99	NR_041976.1	0.14	−	+	−	−	−	56	9	300	0
ZR4-7_Ps	*Pseudomonas poae*	99	NR_102514.1	0.54	+	+	+	−	−	56	5	700	1
ZR4-10_Ps	*Stenotrophomonas maltophilia*	99	NR_074875.1	0.39	+	−	−	−	−	56	8	300	0
ZR4-23_Ps	*Pseudomonas libanensis*	99	NR_024901.1	0.54	+	+	−	+	+	16	8	100	1
SUGAR BEET PLANTS CULTIVATED IN COASTAL DRIFT LINE SOIL—SB-CS (R2A)													
VN1/1-7	*Staphylococcus epidermidis*	99	NR_036904.1	No match	+	+	−	+	−	16	20	0	3
VN1/2-9	*Staphylococcus epidermidis*	99	NR_036904.1	No match	−	+	−	−	−	3	14	0	0
VN1/2-11	*Microbacterium paraoxydans*	98	NR_025548.1	No match	+	−	−	−	−	16	8	0	0
VN2/1-12	*Stenotrophomonas rhizophila*	99	NR_028930.1	No match	+	+	−	−	−	9	11	0	0
VN3/1-8	*Micrococcus luteus*	99	NR_075062.1	No match	−	+	−	−	−	21	5	0	1
SUGAR BEET PLANTS CULTIVATED IN COASTAL DRIFT LINE SOIL—SB-CS (KINGS B)													
VN1/1-2_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	No match	−	−	−	−	+	56	10	0	0
VN1/1-5_Ps	*Staphylococcus epidermidis*	99	NR_036904.1	No match	+	−	−	+	+	21	11	300	3
VN1/2-9_Ps	*Stenotrophomonas maltophilia*	98	NR_074875.1	No match	−	−	−	−	+	21	6	0	0
VN1/2-11_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	No match	−	−	−	+	+	16	7	0	0
VN1/2-12_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	No match	−	−	−	+	+	21	7	0	0
VN3/1-1_Ps	*Pseudomonas brassicacearum* subsp. *brassicacearum*	99	NR_074834.1	No match	+	+	−	+	+	6	8	100	7

(Continued)

Table 4 | Continued

Antagonist	Identification				Antagonistic activity					Stress tolerance[c]			
	Closest BLAST database match (reference RNA sequences)	SI in %[a]	Accession no.	Relative abundances (%)[b]	*A. a.*	*B. c.*	*R. s.*	*S. s.*	*V. d.*	Dry-off	NaCl	H_2O_2 [μmol]	Tellurit [mg/ml]
VN3/1-8_Ps	*Pseudomonas brassicacearum* subsp. *brassicacearum*	99	NR_074834.1	No match	−	+	−	+	+	16	8	100	7
VN4/1-1_Ps	*Pseudomonas mosselii*	99	NR_024924.1	0.18	+	+	+	+	+	9	8	100	5
VN4/1-2_Ps	*Pseudomonas gessardii*	99	NR_024928.1	No match	+	+	+	+	+	9	8	100	7
SUGAR BEET PLANTS CULTIVATED IN POTTING SOIL—SB-PS (R2A)													
VS2/3-9	*Arthrobacter ilicis*	99	NR_104950.1	0.21	−	+	−	−	n. d.	56	4	0	0
VS2/3-10	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	−	n. d.	56	5	100	0
VS2/4-8	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.38	−	+	−	−	n. d.	56	4	0	0
VS3/1-7	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	−	n. d.	56	5	0	0
VS3/2-2	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	−	n. d.	56	5	0	0
VS3/2-9	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	−	n. d.	56	4	0	0
SUGAR BEET PLANTS CULTIVATED IN POTTING SOIL—SB-PS (KINGS B)													
VS1/1-2_Ps	*Pseudomonas veronii*	99	NR_028706.1	2.82	−	+	−	n. d.	n. d.	56	5	300	1
VS1/1-9_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	3	500	0
VS1/2-8_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	3	300	0
VS1/3-5_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	4	100	0
VS1/3-7_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	No match	−	+	−	n. d.	n. d.	56	4	100	0
VS1/4-5_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	4	100	0
VS2/1-7_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	4	100	0
VS2/2-6_Ps	*Yersinia kristensenii*	99	NR_025159.1	0.01	−	−	+	n. d.	n. d.	56	5	300	0
VS2/4-4_Ps	*Pseudomonas oryzihabitans*	99	NR_025881.1	0.21	−	+	−	n. d.	n. d.	56	5	100	0
VS2/4-7_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	3	300	0
VS2/4-8_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.38	−	+	−	n. d.	n. d.	56	3	300	0
VS2/4-9_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	3	300	0
VS3/1-5_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.38	−	+	+	n. d.	n. d.	56	3	100	0
VS3/2-3_Ps	*Pseudomonas brenneri*	99	NR_025103.1	2.70	−	+	−	n. d.	n. d.	9	5	500	1
VS3/3-4_Ps	*Pseudomonas putida*	99	NR_074739.1	0.11	−	+	-	n. d.	n. d.	9	6	500	1
VS3/3-9_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	3	300	0
VS3/4-1_Ps	*Pseudomonas lurida*	100	NR_042199.1	2.94	+	+	+	n. d.	n. d.	21	5	500	3
VS4/1-5_Ps	*Stenotrophomonas rhizophila*	99	NR_028930.1	0.43	−	+	−	n. d.	n. d.	56	3	300	0
VS4/3-2_Ps	*Pseudomonas costantinii*	99	NR_025164.1	2.94	−	−	+	n. d.	n. d.	56	4	300	1
VS4/3-10_Ps	*Stenotrophomonas maltophilia*	99	NR_074875.1	0.01	−	+	−	n. d.	n. d.	56	4	300	0
VS4/4-5_Ps	*Stenotrophomonas maltophilia*	99	NR_074875.1	0.01	−	+	−	n. d.	n. d.	21	3	500	0
VS4/4-10_Ps	*Pseudomonas veronii*	99	NR_028706.1	2.82	−	+	−	n. d.	n. d.	21	4	500	1

Sequences from the isolates were aligned with representative sequences of OTUs from amplicon sequencing data set. [a] *Similarity index (SI) for isolates identified by partial 16S rRNA gene sequencing.* [b] *Relative abundances (%) of OTUs with at least 99% similarity were considered and indicated for OTUs in corresponding samples.* [c] *Maximum concentration of bacterial isolates able to be re-cultivated after desiccation (dry-off), to grow at maximum concentration of sodium chloride concentrations (NaCl), hydrogen peroxide (H_2O_2) and tellurite. n. d.—not determined.*

The community composition of both sugar beet genotypes showed clear soil type-specific effects. In bacterial fingerprints, SB-CS showed only 36% similarity to that of SB-PS. Based on the pyrosequencing approach, the phyla *Actinobacteria* and *Planctomycetes* were pin-pointed as determinants for these differences. These taxa were found exclusively in WB-CS and SB-CS. *Planctomycetes* are known as soil oligotrophes and were enriched in the bulk soil compared to the maize rhizosphere (Peiffer et al., 2013). *Bacteroidetes* and *Proteobacteria* were important members of the rhizosphere community. At OTU level the most dominant OTUs belong to the genus *Pseudomonas*. Sugar beets were previously reported as highly colonized by *Pseudomonas* species using a broad variety of techniques (Lambert et al., 1990; Thrane et al., 2000; Zachow et al., 2008; Mendes et al., 2011). In the current study, *Pseudomonas* species were detected within all bacterial communities using fingerprint and pyrosequencing analysis. In bacterial fingerprints, *Pseudomonas* species were mainly detected in samples grown in PS and were less dominant in the rhizosphere samples cultivated CS. Interestingly, pseudomonads appears to have a minor relevance for the investigated *Beta* genotypes grown or cultivated in their natural habitat.

In this study, we compared the microbiome of both ancestral and domesticated beet rhizospheres and linked functions to particular isolates within the bacterial community. These results support the suggestion by Wissuwa et al. (2009) to apply the knowledge of plant genotype-specific traits of associated microorganisms for breeding strategies. Moreover, wild types of crops as well as soil from their original distribution area are important sources for isolates with antagonistic activity against plant pathogens or with stress protecting activity for their hosts.

AUTHOR CONTRIBUTIONS

Christin Zachow, Henry Müller, Gabriele Berg conceived and guided the research, and wrote the manuscript. Ralf Tilcher provided experimental suggestions. Christin Zachow, Henry Müller performed the laboratory experiments and bioinformatoric analyses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00415/abstract

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Multitrophic interactions among Western Corn Rootworm, *Glomus intraradices* and microbial communities in the rhizosphere and endorhiza of maize

Flavia Dematheis[1], Benedikt Kurtz[2], Stefan Vidal[2] and Kornelia Smalla[1]*

[1] *Julius Kühn-Institut - Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany*
[2] *Department of Crop Science, Agricultural Entomology, Georg-August University Göttingen, Göttingen, Germany*

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Rita Grosch, Leibniz Institute of Vegetable and Ornamental Crops, Germany
Anant V. Patel, FH Bielefeld-University of Applied Sciences, Germany

***Correspondence:**
Kornelia Smalla, Julius Kühn-Institut, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, D-38104 Braunschweig, Germany
e-mail: kornelia.smalla@jki.bund.de

The complex interactions among the maize pest Western Corn Rootworm (WCR), *Glomus intraradices* (*GI*—recently renamed *Rhizophagus intraradices*) and the microbial communities in both rhizosphere and endorhiza of maize have been investigated in view of new pest control strategies. In a greenhouse experiment, different maize treatments were established: C (control plants), W (plants inoculated with WCR), G (plants inoculated with *GI*), GW (plants inoculated with *GI* and WCR). After 20 days of WCR root feeding, larval fitness was measured. Dominant arbuscular mycorrhizal fungi (AMF) in soil and maize endorhiza were analyzed by cloning of 18S rRNA gene fragments of AMF, restriction fragment length polymorphism and sequencing. Bacterial and fungal communities in the rhizosphere and endorhiza were investigated by denaturing gradient gel electrophoresis of 16S rRNA gene and ITS fragments, PCR amplified from total community DNA, respectively. *GI* reduced significantly WCR larval development and affected the naturally occurring endorhiza AMF and bacteria. WCR root feeding influenced the endorhiza bacteria as well. *GI* can be used in integrated pest management programs, rendering WCR larvae more susceptible to predation by natural enemies. The mechanisms behind the interaction between *GI* and WCR remain unknown. However, our data suggested that *GI* might act indirectly via plant-mediated mechanisms influencing the endorhiza microbial communities.

Keywords: Western Corn Rootworm, *Glomus intraradices*, rhizosphere, endorhiza, ITS, 16S rRNA gene, denaturing gradient gel electrophoresis, 18S rRNA gene restriction fragment length polymorphism

INTRODUCTION

The Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is an invasive maize pest in North America and in Europe (Wesseler and Fall, 2010). WCR larvae feed on maize root tissues causing bent stalks (goose necking) and lodging. Economic losses are mainly due to difficulties in mechanical harvesting of injured maize plants.

For large-scale farming operations the main options in controlling the WCR include chemical control, the use of transgenic plants and crop rotation. Unfortunately, the repeated use of pesticides can provide high selective pressure, which can lead to chemical resistance in the WCR populations, resulting in poor control of the pest, increasing insecticide application rate and control costs (Meinke et al., 1998; Siegfried et al., 2004). With the crop biotechnology *Diabrotica*-resistant transgenic maize expressing the *cry(3Bb1)* gene from the bacterium *Bacillus thuringiensis kumamotoensis* (Bt maize) has been introduced already in 2003 (Vaughn et al., 2005; Hellmich et al., 2008). The concentration of *cry(3Bb1)* expressed in Bt maize is not considered a high dose for WCR (Al-Deeb and Wilde, 2005; Oyediran et al., 2007), and resistance was reported to build up within three generations of selection on Bt maize in greenhouse experiments (Meihls et al., 2008; Gassmann et al., 2011). Another strategy, widely used in the past in the United States (U.S.) for managing the WCRs is the crop rotation. Corn rotated annually with soybeans was, in fact, not susceptible to rootworm larval damage as WCR adults laid eggs exclusively in cornfields and larvae hatched in soybeans starved to death. Unexpectedly, the intensive annual rotation of corn with soybeans caused in the U.S. the selection of a WCR variant with reduced egg-laying fidelity to maize field (Onstad et al., 2001; Levine et al., 2002; Spencer et al., 2009). As a consequence of rotation resistance, farmers have experienced, since 1995, economic losses caused by WCR larval injury to first-year maize. In Europe, where only the WCR wild type is present, the best management option remains, up to now, the crop rotation. However, it is clear that due to the development in the WCR populations of resistances against the main WCR pest control options described above, new and long-term resistance management strategies need to be developed. An improved knowledge of the ecology of this soil-dwelling insect and its multitrophic interactions in the rhizosphere and endorhiza are important prerequisites to achieve this goal.

The rhizosphere and endorhiza are dynamic environments in which plant, fungi, bacteria, viruses, nematodes and herbivore insects interact with each other influencing the agro-ecosystem functionality, and thus the sustainability of the crop production

(Weller and Thomashow, 1994; Berg and Smalla, 2009). Beneficial rhizosphere microorganisms promote plant growth and health by nutrient solubilization, nitrogen fixation and plant hormone production (Hayat et al., 2010). Microbial endophytes influence plant fitness as well, affecting plant-microbe-arthropod interactions (Finkes et al., 2006; Rudgers et al., 2007). Within the endophytes, the arbuscular mycorrhizal fungi (AMF) are well known to improve plant survival in harsh environments by enhancing several plant functions (Newsham et al., 1995; Smith and Read, 2008) including drought resistance (Davies et al., 2002), tolerance to heavy metal contaminations (Gildon and Tinker, 1983), protection against pathogens through microbial antagonism and increased plant defensive capacity (Newsham et al., 1995). Furthermore, AMF are prominent through their well-established ability to affect insect-herbivore-plant interactions (Gehring and Bennett, 2009). Several reports showed that AMF can affect the behavior, development and insect performance (Gange et al., 1994; Wardle, 2002; Davet, 2004; Bezemer and van Dam, 2005; Hartley and Gange, 2009; Koricheva et al., 2009), either changing the nutritional status of the plant or triggering plant defense responses (Goverde et al., 2000; Nishida et al., 2010). Bennett and Bever (2007) showed that plant feeders tend to be negatively or positively influenced by the AMF species which the plant is associated with. In particular, the mycorrhizal fungus *Glomus* white does not alter the response of the narrow-leaved plantain (*Plantago lanceolata*) to the specialist lepidopteran herbivore, *Junonia coenia*; the plant association with the AMF *Archaeospora trappei* leads to tolerance to herbivore in the form of an increased plant growth rate; the association with the fungus *Scutellospora calospora* reduces plant tolerance to the herbivores. It must be noticed that, due to monitoring difficulties, belowground herbivore insects have been seldom examined. However, Gange et al. (1994) showed the effect of the AMF, *Glomus mosseae*, on the reduction of black vine weevil (*Otiorhynchus sulcatus Fabricius*) larval growth.

It has been shown that AMF may influence directly or indirectly the activity and the community structure of the rhizosphere- and root-associated microorganisms either through the release of hyphal compounds or through changes in the plant root exudation patterns (Marschner and Baumann, 2003; Wamberg et al., 2003; reviewed by Jones et al., 2004; Offre et al., 2007). The microbial community assembly can be affected also by belowground insect attackers (Denton et al., 1998; Grayston et al., 2001; Dawson et al., 2004; Currie et al., 2006). Upon insect attacks, changes in the plant transcriptome, in the production of volatiles or root exudates have been often detected (Köllner et al., 2008; Dicke et al., 2009). Root feeding effects of WCR larvae on the bacterial and fungal community composition in the maize rhizosphere were recently observed (Dematheis et al., 2012). However, effects of WCR larval feeding on the indigenous microbial communities inhabiting the maize endorhiza remained up to now unexplored. In addition, no studies on the effect of *Glomus intraradices* (GI), recently renamed *Rhizophagus intraradices* (Schüßler and Walker, 2010), on WCR larval fitness and on both rhizosphere and endorhiza microbes of maize have been reported yet.

The present study aimed to investigate the multitrophic interaction among WCR, *GI* and the microbial communities in the rhizosphere and endorhiza of maize. We specifically addressed the following questions: (1) Does *GI* mycorrhization of maize roots affect the WCR larval fitness measured as larval number/survival, developmental stage and root feeding? (2) Does *GI* mycorrhization affect the composition of microbial populations in the rhizosphere and endorhiza of maize? (3) Does the feeding of WCR larvae alter the microbial communities in the endorhiza and rhizosphere of mycorrhized and unmycorrhized maize plants?

In the present study AMF, total fungal and bacterial communities were investigated. AMF communities naturally occurring in the soil and colonizing the maize endorhiza were studied by PCR-RFLP analysis and sequencing of AMF-specific 18S rRNA gene fragments, PCR amplified from total community (TC) DNA. The total fungal and bacterial communities in both rhizosphere and endorhiza of maize were analyzed by means of denaturing gradient gel electrophoresis (DGGE) of ITS and 16S rRNA gene fragments, PCR amplified from TC-DNA.

MATERIALS AND METHODS

EXPERIMENTAL SETUP

A greenhouse experiment was performed under quarantine conditions. The maize variety used in this study was KWS13, an early maturing Northern European flint x dent maize breeding line developed by the seed company KWS (Einbeck, Germany). Maize seeds were sterilized according to Benziri et al. (1994) and pre-germinated at room temperature in Petri dishes containing sterile wet filter paper. The seedlings were pre-grown singly in pots (13 cm diameter) containing Haplic Chernozem inoculated or not with *GI* for 6 weeks. The maize growing conditions were 40% relative humidity, 24°C mean temperature and 16 h of additional illumination with sodium lamps (400W, HS2000, Hortilux Schréder, Monster, The Netherlands). Plants were placed into the same tray that was moved twice a week in the greenhouse to randomize the growing conditions. Every 14 days of growth, each plant was fertilized with 20 µl 0.2% Wuxal top N (Manna, Düsseldorf, Germany) by watering.

After six weeks of plant growth (plant growth stage V7) four plant replicates per treatments with and without *GI* were harvested in order to quantify by real-time PCR (qPCR) the *GI*-root colonization. The remaining plants were used to assess the following treatments: C (control plant grown in Haplic Chernozem), W (maize plants inoculated with ∼200 eggs), G (maize plants mycorrhized by *GI*) and GW (maize plants mycorrhized by *GI* and inoculated with ∼200 non-diapausing WCR eggs). Because of logistic constraints only four independent replicates (one replicate = one plant) per treatment were established. Three weeks later (plant growth stage VT) the larvae were collected from the treatments W and GW to evaluate the total number of larvae per plant and the development of the larval instars (L1, L2, and L3). In parallel, the plants were harvested, and the fresh weight of the roots was recorded. After the rhizosphere isolation the roots were surface sterilized. TC-DNA was extracted from soil, rhizosphere and surface sterilized roots in order to determine (a) the 18S/ITS rRNA gene copy numbers of *GI* in the roots by

qPCR; (b) the AMF community structure in soil and roots by cloning of 18S rRNA gene fragments of AMF, restriction fragment length polymorphism and sequencing, and (c) the bacterial and fungal community assembly in the rhizosphere and endorhiza by DGGE analysis of PCR-amplified 16S rRNA gene and ITS fragments.

SOIL TYPE AND SAMPLING METHOD

The soil used in this study was Haplic Chernozem, collected in 2008 nearby Göttingen (geographic coordinates, 51°30′29.44 N and 9°55′38.26 E). 400 kg were taken from four different spots, five meters apart from each other, along a transect to a depth of 25 cm. In order to avoid any alteration of the microbial content, the soil samples were immediately transported to the laboratory and homogenized by a soil crusher machine (Unifix 300, Möschle, Ortenberg, Germany) and sieved through a 10 mm mesh to remove stones and plant residues. Fresh soil was used for the experiment described here.

GLOMUS INTRARADICES INOCULUM AND SOIL APPLICATION

The arbuscular mycorrhizal *Glomus intraradices* (Glomeromycota) was provided by Dr. Henning von Alten (Isolate n° 501, Institute of Plant Disease and Plant Protection, University of Hannover, Germany) as expanded clay material contains a high level of *GI* spores. The inoculum was mixed as 5% of the total volume of soil estimated for the whole experiment (Dehne and Backhaus, 1986).

WCR EGG INOCULUM AND APPLICATION

Non-diapausing WCR eggs were provided by USDA-ARS (Northern Grain Insect Research Laboratory, Brookings, USA) and stored at 8°C until their use. In order to stimulate the larval development, the eggs were incubated at 26°C, 60% relative humidity in dark conditions for 12 days and checked for visible larvae presence using a dissecting microscope. Afterwards the eggs were washed in a sieve (Ø 250 μm) and the collected eggs were suspended in 0.15% agar solution. 0.5 ml of egg suspension were applied on a sterile humid filter paper and incubated at the same conditions as described for larval development, and checked daily to assess the hatch time (HT) and the hatch rate (HR). HT and HR mean values were two days and 72%, respectively. Approx. 200 eggs were applied into the soil, at 5 cm depth close to the stem of the plants for the establishment of the treatments W and GW.

WCR LARVAL EXTRACTION FROM THE SOIL, LARVAL DEVELOPMENT ANALYSIS, ROOT FEEDING EVALUATION AND STATISTICS

Larvae were extracted from the soil of plants inoculated with WCR eggs (treatments W and GW) using a high gradient Kempson extraction system (Kempson et al., 1968). The larvae extracted from each plant were counted and classified into larval stages (L1, L2, and L3) by measuring head capsule width as described by Hammack et al. (2003). The WCR root feeding was evaluated based on the root fresh weight of four plant replicates for each treatment.

The root weight values and total numbers of larvae per plant were analyzed with One-Way ANOVA combined with Tukey's HSD test to evaluate statistical differences among treatments. The analysis of the composition of larval stages was performed using a Tukey's HSD test under a generalized linear model via a logistic function for binomial data. The program used was R add-on package multicomp.

TOTAL COMMUNITY (TC) DNA EXTRACTION FROM RHIZOSPHERE AND ROOT SAMPLES

Maize plants at the growth stages V7 and VT were taken out from the soil and shaken vigorously. The soil tightly adhering to the roots was considered as rhizosphere and collected using a Stomacher blender (Stomacher 400, Seward, England) as described by Costa et al. (2006). The microbial pellet was obtained from the cell suspensions by centrifugation at 10,000 g at 4°C for 30 min. The microbial pellet of each root was homogenized with a spatula and 0.5 g were used for the TC-DNA extraction.

Fresh root material was prewashed under running tap water and surface sterilized as described by Götz et al. (2006). Afterwards, each root was cut into 1 cm-segments and mixed to randomize the selection of different root areas. 0.4 g of root pieces per plant were used for the TC-DNA extraction.

The TC-DNA was extracted from 0.5 g of rhizosphere pellet and from 0.4 g of surface sterilized root pieces using the FastDNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer's protocol. The treatment of the root material required the following additional initial step, root fragments were placed into bead tubes containing a mixture of ceramic and silica particles (included in the kit), frozen by immersion into liquid nitrogen and subsequently processed twice for 1 min at speed 5.5 ms^{-1} in a FastPrep bead beating system (Bio-101, Vista, CA, USA). All TC-DNA samples were purified with the GENECLEAN Spin Kit (Q-Biogene, Heidelberg, Germany) according to the manufacturer's protocol. DNA concentrations were estimated visually by 0.8% agarose gel electrophoresis using the quantitative marker High DNA Mass Ladder (*Invitrogen*). TC-DNA from both rhizosphere and root samples were diluted in MilliQ sterilized water to obtain ca. 20 ng/μl for use as a PCR template.

DETECTION AND QUANTIFICATION OF GI BY QUANTITATIVE REAL-TIME PCR (qPCR)

The abundance of *GI* in the maize roots of all treatments was determined by means of qPCR using the primer pair VC-F/VC-R targeting in a specific manner the ITS1+18SrRNA gene fragments of the mycorrhizal fungus (Alkan et al., 2006). The qPCR was carried out in the CFX96 Real Time PCR System (Biorad, Hercules, California). The reaction mixture and cycling program were performed as described by Alkan et al. (2006) with few modifications, 25 μl aliquot of reaction mixture contained 1 μl DNA template and 2X SYBR Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany).

The qPCR was calibrated with the cloned ITS1+18SrRNA fragment of the *GI* strain used in this study. From the standard calibration curves, the amount of *GI* in 1 g of plant root was calculated.

The standard for the qPCR was prepared as follows: TC-DNA of roots colonized by *GI* was amplified as described above by Alkan et al. (2006). Amplicons, 110 bp length, were ligated in the pGEM-T vector system (Promega) and transformed into

Escherichia coli (JM109 Competent Cells, Promega) according to the manufacturer's instructions. Positive transformants were re-amplified in a Tgradient thermal cycler (Biometra, Göttingen, Germany) with the primers SP6 and T7, purified with the "MinElute PCR purification Kit" (Qiagen GmbH, Hilden, Germany) and sequenced. The BLAST analysis of DNA sequences at NCBI site showed 100% identity with *GI* (accession no. JN83667-JN836670). The PCR products from single clones amplified with SP6 and T7 were quantified with the *NanoDrop* Spectrophotometer ND-1000 (Peqlab, Erlangen, Germany) and serial dilutions 10^{-4} to 10^{-10} were used as a standard for the detection and quantification of *GI* in the root samples.

CLONING OF 18S rRNA GENE FRAGMENTS OF AMF, RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND SEQUENCING

To investigate the AMF communities, the partial 18S rRNA gene fragments (550 bp) were amplified from TC-DNA extracted from a composite soil sample and four root samples from each treatment. The PCR was performed with the primer pair NS31/AM1 according to Vallino et al. (2006) with the following modifications, no bovine serum albumin (BSA) was added to the PCR reaction mixture and 2 U of Taq DNA polymerase (AmpliTaqGold with GeneAmp, Applied Biosystems, USA) and 10 pmol of each primer were used. Moreover, the PCR extension temperature was increased to 62°C. PCR modifications were made to optimize the AMF amplification in the soil. Due to a multiple pattern obtained from the soil sample, 550 bp length amplicons were cut out from the agarose gel and purified by "QIAEXII gel extraction kit" (Qiagen GmbH, Hilden, Germany).

Amplicons of 550 bp length from soil and roots were ligated in the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer's instructions. Positive transformants were amplified with the primer pair NS31/AM1 to select the clones carrying the insert with the right size. The PCR conditions were optimized for the cloned target sequence as follows, 95°C for 10 min, 30 cycles at 94°C for 35 s, 63°C for 35 s, 72°C for 45 s, and final step at 72°C for 10 min. Positive clones (180 clones obtained from a soil composite sample and 140–155 clones obtained from root samples for each treatment) were tested for RFLP type by independent digestion with the enzymes *HinfI* and *Hin1II* (Fermentas), as recommended by the manufacturer and analyzed on 3% agarose gel electrophoresis. For an appropriate identification of the size of restricted fragments, the Molecular weight marker IX (Boehringer Mannheim GmbH, Germany) was used as a standard. Each clone was identified as RFLP type according to Vallino et al. (2006). Representative clones for each RFLP type were re-amplified with the primers SP6 and T7, purified with the "MinElute PCR purification Kit" (Qiagen GmbH, Hilden, Germany) and sequenced. The DNA sequences were analyzed by BLAST-n program at the NCBI site for multiple sequence alignment.

PCR AMPLIFICATION OF THE INTERNAL TRANSCRIBED SPACER (ITS) REGIONS AND 16S rRNA GENE FRAGMENTS FOR DGGE FINGERPRINTING

ITS fragments of the fungal communities in the endorhiza and rhizosphere of maize were amplified from TC-DNA extracted from plants of the treatments C, W, G, and GW. The ITS amplification was performed using a nested PCR approach with the primer pair ITS1F/ITS 4 and ITS 2/ITS1F-GC according to Weinert et al. (2009).

The 16S rRNA gene fragments of complex bacterial populations contained in the same set of samples were amplified by direct PCR performed with the primer pair F984GC/R1378 (Heuer et al., 1997). PCR conditions were applied as described by Costa et al. (2006).

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AND DATA ANALYSIS

The DGGE analyses of the fungal and bacterial communities were carried out in the PhorU2 machine (Ingeny, Goes, The Netherlands). DGGE gels were prepared as described by Weinert et al. (2009). Gels were silver-stained and air-dried according to Heuer et al. (2001). Digitalized DGGE gel images were analyzed with the software package GELCOMPAR II program, version 4.5 (Applied Math, Kortrijk, Belgium) as described by Rademaker et al. (1999). Background was subtracted and lanes were normalized as described by Gomes et al. (2003). Cluster analysis based on the Pearson correlation coefficient (UPGMA) was performed to evaluate the percentage of similarities among samples.

Pairwise statistical analysis (Permutation test) was applied on the values of the similarity matrix according to Kropf et al. (2004) to evaluate if the differences (*D*-values) observed were statistically supported. *P*-values and *D*-values were always reported.

IDENTIFICATION OF SPECIFIC ENDORHIZA FUNGI BY DGGE FINGERPRINTS OF ITS FRAGMENTS BAND SEQUENCING

Four bands of the fungal DGGE fingerprints of ITS fragments bands which occur exclusively in the roots of plants treated with *GI* (treatments G and GW) were excised from the acrylamide gel. DNA was eluted during overnight incubation of the gel slices at 4°C in sterile TE buffer, pH 8. After centrifugation at 11,000× *g* for 60 s, the supernatant was transferred to a new tube and 1 μl of it was used as a template in the second PCR amplification described for DGGE fingerprints of ITS fragments analysis, except for the use of primers without GC clamp (ITS1F/ITS2). PCR products were ligated into the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer's instructions. Positive clones were re-amplified with the primers ITS1F-GC/ITS2 and the electrophoretic mobility of the cloned fragments was checked by DGGE gel. To identify different ribotypes co-migrating on acrylamide gel, four to five clones per excised DGGE band were sequenced. The DNA sequences were analyzed with BLAST-n program at NCBI site for multiple sequence alignments with sequences available in the database.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

Nucleotide sequences determined in this study were deposited in the GenBank database under the accession numbers JN836634-JN836670.

RESULTS

GI DETECTION AND QUANTIFICATION IN MAIZE ROOTS

In order to assess *GI* abundance in the endorhiza of maize before WCR egg inoculation, a qPCR was performed on TC-DNA

extracted from roots of maize at the growth stage V7 with and without *GI* inoculant (C, G). The qPCR revealed a specific *GI*-signal exclusively in the roots of plants grown in the soil inoculated with *GI* (G) with a mean of 9.5×10^5 copy numbers of 18S/ITS fragments per g root.

The roots of maize at the growth stage VT of each treatment (C, W, G, and GW) were analyzed by qPCR as well, in order to study the treatment effect on *GI* root colonization. A specific qPCR signal was detected only in the roots of plants grown in soil inoculated with *GI* in presence and in absence of WCR larvae (G, GW). The *GI* mean value was about 1.8×10^6 (s.d. 0.2) and 2×10^6 (s.d. 0.3) copies of 18S/ITS fragments per g root in the treatments G and GW, respectively. No significant differences were observed between these treatments ($P = 0.8$) indicating that WCR larval feeding did not influence the abundance of *GI* in the roots. Differences in the *GI* abundance were observed instead between plants at the growth stages V7 and VT ($P < 0.05$), indicating that the mycorrhization increased during the 9 weeks of plant growth.

GI-SOIL INOCULATION AFFECTS LARVAL DEVELOPMENT

In order to evaluate the effect of *GI* on the root biomass and on the WCR root feeding, the root fresh weight of plants at the growth stage VT from the treatments C, W, G, and GW was determined. Significant differences of the root fresh weight between the treatments with and without larvae ($P < 0.01$) indicated a clear larval effect on the root biomass with ∼20% reduction of the root weight for the treatments W and GW. No significant differences of root biomass were observed between the treatments with and without *GI* soil inoculation (C/G and W/GW), indicating that *GI* mycorrhization did not improve the belowground plant development and did not affect the root larval feeding.

The numbers of WCR larvae determined for the treatments W and GW did not significantly differ from each other indicating that *GI* mycorrhization did not affect the viability of the WCR eggs or the larval survival. However, the analysis of the larval instars composition in the treatments W and GW revealed a significant reduction of the WCR larval development in presence of the *GI* (**Figure 1**) with the relative number of 3rd larval instars being significantly lower in the GW than in the W treatment ($P = 2e^{-16}$).

AMF COMPOSITION IN SOIL AND ROOT SAMPLES

In order to assess (i) the AMF community structure in the soil, (ii) the AMF populations naturally occurring in the maize roots and (iii) the effect of both *GI*-soil inoculation and WCR larval feeding on the endorhiza AMF communities, a PCR-RFLP analysis was performed on the TC-DNA extracted from one composite soil sample and from four root samples per treatment (C, W, G and GW). The PCR-RFLP analysis of 180 clones carrying the 18S rRNA gene fragments of AMF obtained from the soil sample, revealed five different RFLP patterns including RFLP types 1, 2, 3, 6, 8, and several (ca. 32%) unclassified RFLP types. Among the unclassified RFLP profiles, one occurred more often and was termed as RFLP X. The dominant AMF in the soil belonged to the RFLP types 8 and 1. The percentage of clones carrying 18S rRNA gene fragments of AMF on the total number of clones investigated by means of RFLP method

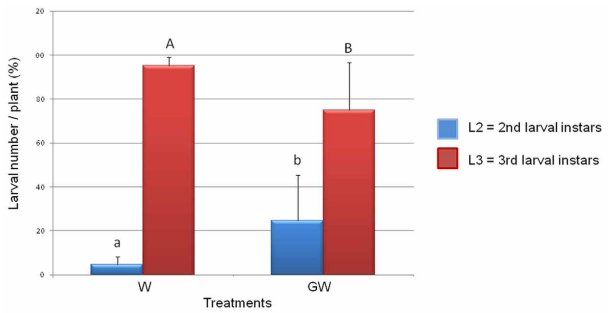

FIGURE 1 | Effect of *Glomus intraradices* on WCR larval development. The number of 3rd larval instars (L3) was significantly lower in the *Glomus*-treated plants (treatment GW) than in untreated control plants (treatment W). Four biological replicates per treatment were used. The error bars represent standard deviations. Lowercase letters above columns indicate significance differences between the number of L2 larval instars, while uppercase letters indicate significance differences between L3 larval numbers ($P = 2e^{-16}$).

Table 1 | RFLP types and their relative abundance in Haplic Chernozem and in root samples from the treatments C, W, G, and GW grown in the same soil type.

RFLP type	Relative abundance of RFLP types in soil and maize roots				
	Soil	Treatment C	Treatment W	Treatment G	Treatment GW
RFLP 1	14.4	0	0	0	0
RFLP 2	10	5.8	3.5	0	0
RFLP 3	2.2	18.7	25.3	0	0
RFLP 6	1.1	2.5	1.4	0	0
RFLP 8	40	62	60	7.5	5
RFLP 11	0	0	0	93	94.3
RFLP X	6.7	0	0	0	0
Unclassified RFLP profiles	25.5	11	9.8	0	0

C, maize plant grown in Haplic Chernozem, natural source of different mycorrhizal species; W, maize plants characterized by 3 weeks root feeding of WCR larvae; G, maize plants mycorrhized by GI; GW, maize plants mycorrhized by GI and characterized by 3 weeks root feeding of WCR larvae. The relative abundance of the RFLP types found in soil and roots was calculated as percentage of clones carrying the insert of a certain RFLP type on the total number of clones digested with HinfI and Hin1II per soil or plant treatment.

are reported in **Table 1**. The RFLP analyses of 140–155 cloned 18S rRNA gene fragments obtained from root samples per treatment revealed that the AMF colonizing the maize roots from the treatments C and W belonged to the RFLP types 2, 3, 6, and 8. In these roots, the RFLP types 8 and 3 were dominant. Differently, in the roots of plants from the treatments G and GW the RFLP analysis showed a significant reduction of the AMF evenness and almost all clones were assigned to the RFLP type 11. Cloned 18S rRNA gene fragments representative of each RFLP type were sequenced and virtually digested with the

enzymes *Hinf1* and *Hin1II* in order to obtain clear information about the restriction fragment lengths characterizing each RFLP type. Database searches of 18S rRNA gene sequences representative of each RFLP type allowed the identification of different AMF species from the genera *Scutellospora* (RFLP type 6) and *Glomus* (RFLP types 1, 2, 3, 8, 11, and X). In **Table 2** the RFLP types found in both soil (Haplic Chernozem) and plant roots, the source of isolation, the corresponding accession number, the species with highest sequence identity found in the GenBank, and the exact coordinates and restriction fragment lengths are reported.

FUNGAL COMMUNITIES IN THE ENDORHIZA AND RHIZOSPHERE OF MAIZE

Comparative analysis of DGGE fingerprints of ITS fragments showed highly similar fungal community structure between the treatments C and W, and between the treatments G and GW in the endorhiza of maize. Four dominant differentiating bands appeared exclusively in the endorhiza fungal fingerprints of *GI* mycorrhized plants (bands 1, 2, 3, and 4, **Figure 2**). Cluster analysis of DGGE fingerprints of ITS fragments profiles showed that the treatments G and GW grouped together as well as the treatments C and W, with just one exception (**Figure 3**). However, differences ($P = 0.03$) in the fungal community composition observed between the treatments with and without *GI* (**Table 3**) indicated a clear effect of *GI* soil inoculation on the fungal populations in the endorhiza of maize. Differently, no effect of WCR larval feeding on the composition of the endorhiza fungal communities was observed.

The DGGE fingerprints of the fungal communities in the maize rhizosphere showed high similarity among all treatments. A mixed cluster of samples from all treatments was obtained (**Figure 3**). Statistical analysis did not reveal significant differences between treatments with and without *GI*, indicating that *GI* soil inoculation did not affect the fungal communities in the rhizosphere. No significant differences were observed between treatments with and without WCR, except between G and GW with $P = 0.03$ and $D = 2.1$ (**Table 3**).

Table 2 | RFLP types found in the soil Haplic Chernozem and in plant roots from the treatments C (control plants), W (maize plants characterized by 3 weeks root feeding of WCR larvae), G (maize plants mycorrhized by *GI*) and GW (maize plants mycorrhized by *GI* and characterized by 3 weeks root feeding of WCR larvae); accession numbers; sequence identity; AMF-18S rRNA gene fragment coordinates and restriction fragment lengths obtained with the enzymes *Hinf1* and *Hin1II* by virtual digestion at BioLabs web site.

RFLP type	Source	Access. no	Identity sequence (ID)	*Hinf 1*		*Hin1II*	
				Coordinates	Length (bp)	Coordinates	Length (bp)
RFLP1	Soil	JN836649	*G. etunicatum* (99% ID)	268–552	285	1–297	297
				1–267	267	388–552	165
						298–387	90
RFLP2	Soil	JN836650	*Uncultured Glomus* (99% ID)	280–523	244	258–548	291
	Root C	JN836641		1–189	189	1–257	257
	Root W	JN836645		190–279	90		
				524–548	25		
RFLP3	Soil	JN836651	*Uncultured Glomus* (98% ID)	280–523	244	258–548	291
	Root C	JN836642		1–189	289	1–164	164
	Root W	JN836646		190–279	90	165–257	93
				524–548	25		
RFLP 6	Soil	JN836652	*Scutellospora calospora* (99% ID)	1–301	301	260–547	288
	Root C	JN836643		302–522	221	1–169	169
	Root W	JN836647		523–547	25	170–259	90
RFLP8	Soil	JN836653	*G. mosseae* (100% ID)	267–550	284	1–295	295
	Root C	JN836644		23–266	244	296–438	143
	Root W	JN836648		1–22	22	439–550	112
	Root G	JN836636-37					
	Root GW	JN836640					
RFLPX	Soil	JN836654	*G. aurantium* (99% ID)	283–550	268	260–550	291
				1–141	141	1–169	169
				142–282	141	170–259	90
RFLP 11	Root G	JN836634-35	*G. intraradices* (99% ID)	142–524	383	259–549	291
	Root GW	JN836638-39		1–141	141	117–258	142
				525–549	25	1–116	116

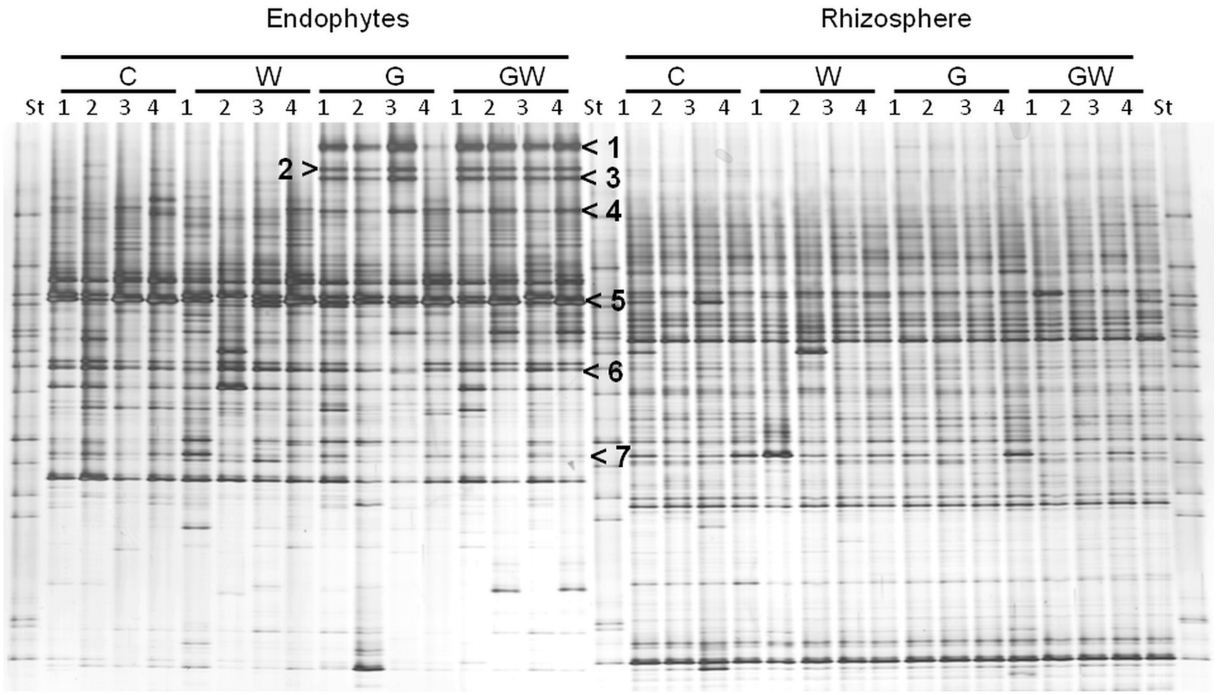

FIGURE 2 | DGGE fingerprints of ITS fragments showing the endorhiza and rhizosphere fungal communities of maize plants from the treatments C, W, G, and GW. C, maize plant grown in Haplic Chernozem, natural source of different mycorrhizal species; W, maize plants characterized by 3 weeks root feeding by WCR larvae; G, maize plants with *GI* inoculum added before sowing; GW, maize plants mycorrhized by *GI* and characterized by 3 weeks WCR larval feeding on the roots; St, ITS standard. The fingerprinting was generated by separation of ITS fragments amplified from TC-DNA extracted from root and rhizosphere. Arrows indicate bands which were sequenced, except for band 4. Band 1, 2, and 3: *Glomus* sp.; band 5: *Microdochium bolleyi*; band 6: *Tetracladium* sp.; band 7: *Periconia macrospinosa*.

The BLAST analysis of the ITS-sequences obtained by cloning of bands 1, 2, and 3 (**Figure 2**) matched against the same type of *Glomus* sp., although with different percentage of similarity (96–100% identity) (accession no. JN36655–JN836661). No clones carrying an insert with the electrophoretic mobility of band 4 were obtained. Although this study focused on the identification of the four differentiating bands occurring only in *GI*-treated plants, other bands (bands 5, 6, and 7 in **Figure 2**) were also sequenced to obtain information on dominant fungal population in the endorhiza of maize. Band 5 was affiliated to *Microdochium bolleyi* with 99% sequence identity (accession no. JN836662 and JN8366623). Band 6 sequences showed 99% sequence identity with *Tetracladium* sp. (accession no. JN836664 and JN836665). The sequencing of band 7 revealed *Periconia macrospinosa* (98% sequence identity, accession no. JN836666).

BACTERIAL COMMUNITIES IN THE ENDORHIZA AND RHIZOSPHERE OF MAIZE

In order to elucidate the interactions among WCR larval feeding, *GI* and bacterial populations inhabiting the rhizosphere and endorhiza of maize, a comparative analysis of DGGE fingerprints of 16S rRNA gene fragments was performed.

The bacterial DGGE fingerprints in the maize endorhiza showed high variability among replicates. Differences in the relative abundance of two bacterial populations upon WCR larval feeding or of *GI*-soil inoculation were observed (bands 1 and 2, **Figure 4**). Statistical analysis based on the Pearson correlation indices revealed significant differences in the endorhiza bacterial composition between the treatment C and the treatments W, G and GW ($P = 0.03$) indicating a clear effect of both *GI*-soil inoculation and WCR larval feeding on the endorhiza bacteria in maize roots. Although a differentiating band (band 2, **Figure 4**) in the treatments with *GI*-soil inoculation was displayed, no significant differences were observed between the treatments W, G, and GW (**Table 3**).

The DGGE patterns of the bacterial communities in the rhizosphere of maize showed pronounced shifts due to the WCR larval feeding independently of the *GI*-soil inoculation, while no shifts were observed in response to *GI*-soil inoculation (**Figure 4**). No treatment dependent clustering was observed (**Figure 5**). However, statistical tests revealed significant differences between all of them with *D*-values of >7.1 (**Table 3**).

DISCUSSION

The present study provided new insights into the interaction among WCR larval feeding, *GI* and microorganisms living in the rhizosphere and in the endorhiza of maize. An inhibitory effect of the WCR larvae growth caused by the *GI* root mycorrhization was observed in the present study for the first time. Our findings are in agreement with Boucher (2001) who reported a reduction in head capsule diameter of emerging WCR beetles from *GI*-treated

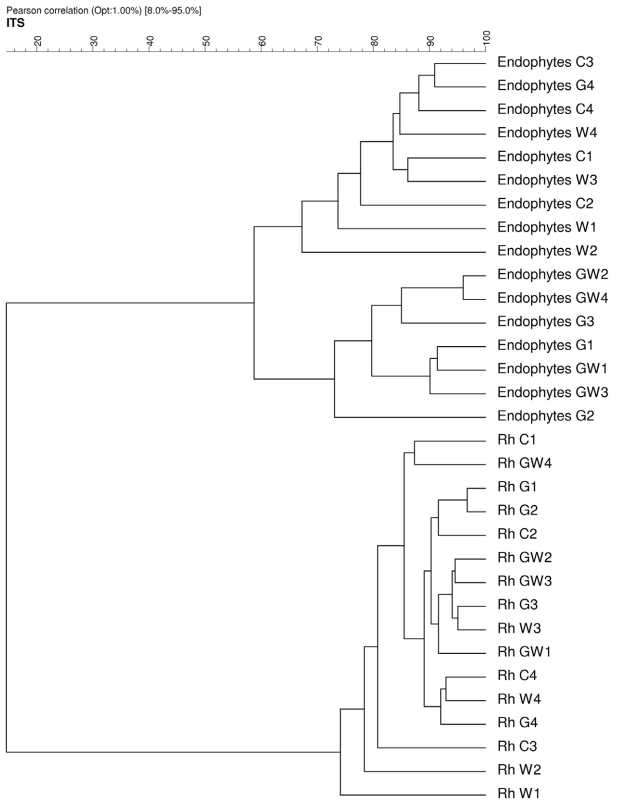

FIGURE 3 | Dendrogram constructed with the fungal communities fingerprints in the endorhiza and rhizosphere of maize reported in Figure 2. The differences between the profiles are indicated by percentage of similarity. The dendrogram was based on the Pearson correlation indices and cluster analysis by the unweighted pair group method using arithmetic averages.

Table 3 | Significant values (*P*-values) and *D*-values of pairwise comparisons between treatments (C, G, W, and GW) of fungal and bacterial communities fingerprints in the endorhiza and in the rhizosphere of KWS13 cultivar grown in Haplic Chernozem.

	Fungi				Bacteria			
	Endorhiza		Rhizosphere		Endorhiza		Rhizosphere	
	P	*D*	*P*	*D*	*P*	*D*	*P*	*D*
C/W	0.3	0.8	0.5	0.3	**0.03**	8.2	**0.03**	7.8
C/G	**0.03**	8.7	0.06	2.9	**0.03**	5.2	**0.03**	7.1
C/GW	**0.03**	12.4	0.17	1.2	**0.03**	8.4	**0.03**	22.1
W/G	**0.03**	14.7	0.3	1.3	0.06	5.2	**0.03**	12.4
W/GW	**0.03**	23	0.08	1.3	0.1	5.1	**0.03**	13.4
G/GW	0.2	3.6	**0.03**	2.1	0.06	2.4	**0.03**	14.4

C, maize plant grown in Haplic Chernozem, natural source of different mycorrhizal species; W, maize plants characterized by 3 weeks root feeding of WCR larvae; G, maize plants mycorrhized by GI; GW, maize plants mycorrhized by GI and characterized by 3 weeks root feeding of WCR larvae. Values of $P < 0.05$ indicate significant differences between rhizosphere samples of different maize genotypes grown in the same soil type. Bold values show significant differences. Simulations: 10.000.

plants vs. control plants. According to the slow-growth-high-mortality hypothesis developed by Benrey and Denno (1997), the prolonged time of early larval instars renders WCR larvae more susceptible to predation by natural enemies. Therefore, *GI* can be proposed as a biocontrol microorganism for integrated pest management programs against WCR larval damages.

The mechanisms of the interaction between WCR larvae and *GI* remain unknown. However, the reduction of larval growth might be due to either a direct interaction between *GI* and WCR larvae or to plant-mediated interaction resulting, for instance, in root exudate changes. Maize secondary metabolites such as hydroxamic acids (Xie, 1991) or protease inhibitors and phenolic compounds (Karban and Baldwin, 1997) might have a toxic activity toward WCR larvae or limit the insect assimilation of plant nutrients and thus delay herbivore growth, respectively.

Several papers demonstrated the effect of the root exudation on shaping the rhizosphere and root-associated microbial communities and *vice versa* (Rettenmaier and Lingens, 1985; Bröckling et al., 2008; Berg and Smalla, 2009; Meier et al., 2012). The present study showed shifts in the indigenous endorhiza populations of AMF, fungi and bacteria in the *GI* treatments. Thus, we speculated that *GI* might act indirectly on the WCR larval growth via plant-mediated response to the presence of *GI* rather than directly affect specific compounds.

PCR-RFLP comparative analysis and sequencing of AMF-18S rRNA gene fragments amplified from DNA from soil and maize roots of plants grown in absence of *GI* showed significant differences in the AMF composition between soil and root samples, indicating a selective interaction between maize plants and the AMF populations naturally occurring in the soil. In particular, *G. mosseae*, uncultured *Glomus* species and *Scutellospora calospora* were positively selected by the plant most likely through the release of specific compounds (e.g., plant secreted proteins) mediating the process of signaling and recognition between compatible and incompatible plant-microbe interactions (De la Peña et al., 2008). PCR-RFLP analysis and sequencing of AMF-18S rRNA gene fragments in DNA from the roots of plants of treatments C and G revealed that *GI*-soil inoculation reduced the AMF richness in the maize roots to almost exclusively the RFLP type 11 identified by sequencing as *GI* itself. The dominance of *GI* in the roots indicated the preferential establishment of a mutualistic symbiosis between maize plants and *GI* rather than *G. mosseae*, uncultured *Glomus* species and *Scutellospora calospora*. As reviewed by Parniske (2008), the host preference reflects different fungal strategies and levels of functional compatibility.

Effects of *GI* inoculated into soil on the fungal and bacterial communities in the endorhiza and in the rhizosphere of maize were revealed. *GI* strongly affected the fungal community composition in particular in the endorhiza of maize (**Figure 2**). However, the effects were mainly caused by the appearance of bands in the fingerprints of endorhiza fungal communities in *GI*-treated plants that were all affiliated to *Glomus* sp. Thus, both qPCR and DGGE fingerprint data suggest that the inoculant belonged to the dominant endorhiza fungal populations associated with the maize. Some studies showed that ITS sequences

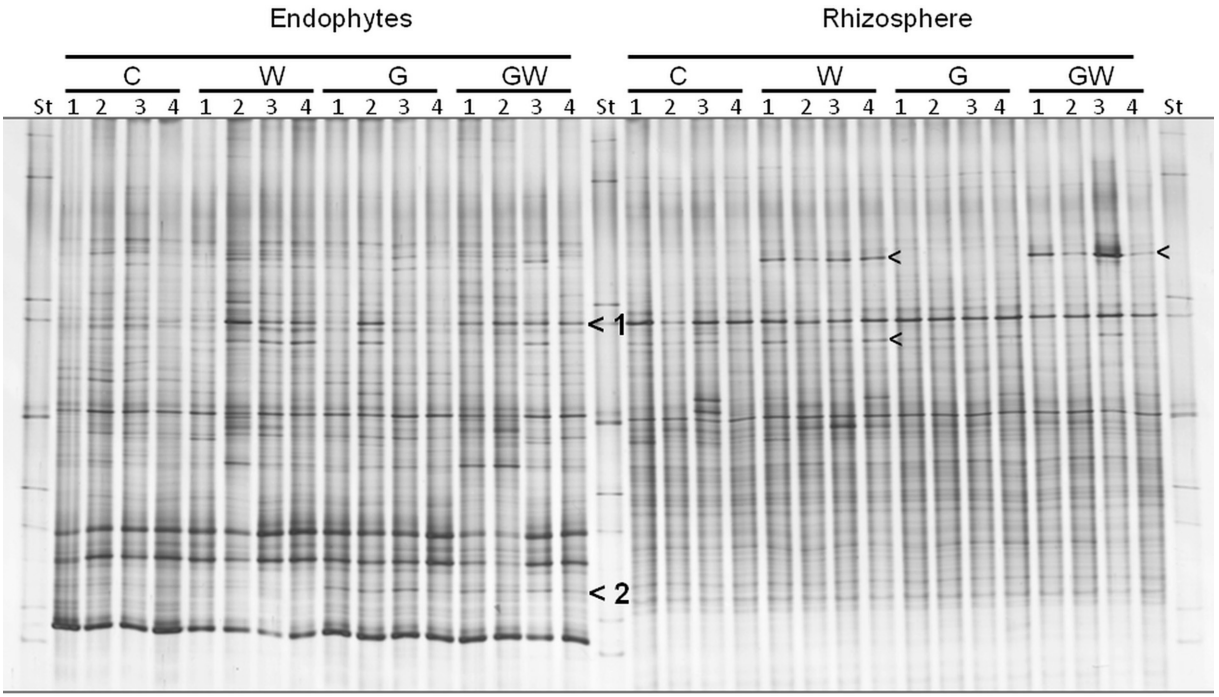

FIGURE 4 | DGGE fingerprints of 16S rRNA gene fragments showing the endorhiza and rhizosphere bacterial communities of maize plants from the treatments C, W, G, and GW. C, maize plant grown in Haplic Chernozem, natural source of different mycorrhizal species; W, maize plants characterized by 3 weeks root feeding by WCR larvae; G, maize plants with *GI* inoculum added before sowing; GW, maize plants mycorrhized by *GI* and characterized by 3 weeks WCR larval feeding on the roots; St, ITS standard. The fingerprinting was generated by separation of bacterial 16S rRNA gene standard fragments amplified from TC-DNA extracted from root and rhizosphere. Arrows show treatment dependent bands.

were rarely recovered twice from a single spore (Lanfranco et al., 1999; Antoniolli et al., 2000), most likely due to the multiple and polymorphic genome of the AMF (Hijiri and Sanders, 2005). Furthermore, these data might indicate that the ITS region alone has a too low resolution power to differentiate AMF at the species level. *GI* inoculation affected significantly also the bacterial community composition in the endorhiza of maize, although to a much lesser extent (**Figure 4**). In the rhizosphere no clear differentiating bands on the DGGE fingerprints of fungal and bacterial communities were observed between the treatments with and without *GI*. However, permutation testing still indicated significant effects of *GI* treatment on the bacterial communities in the maize rhizosphere (**Table 2**). *GI* effects on the microbial communities in the rhizosphere and endorhiza of plants were reported also in other studies. Filion et al. (1999) showed that soluble substances released by the extraradical mycelium of *GI* induced differential growth of soil microorganisms. Marschner and Baumann (2003) showed that mycorrhizal colonization by *GI* changed the bacterial community structure in the soil and on the surface of maize roots.

WCR larval feeding on maize roots was found to strongly alter the bacterial community composition in the endorhiza and rhizosphere of maize in *GI*-treated and untreated plants. While WCR larval feeding did not affect the diversity of AMF and total endorhiza fungal communities in the maize endorhiza, the endorhiza bacterial communities of the treatments without *GI* were significantly affected. Interestingly, WCR feeding did not affect the bacterial communities in presence of *GI*. However, the absence of statistically significant differences could have been caused also by the high variability in the DGGE profiles between replicates. Confirming previous results by Dematheis et al. (2012), no change in the fungal community composition in the maize rhizosphere in response to WCR larval feeding was observed in the present study. However, larval feeding strongly affected bacterial communities in the rhizosphere of *GI*-treated and untreated maize plants. Thus WCR feeding influenced mainly the bacterial populations colonizing the rhizosphere and, to a lesser extent, those living in the endorhiza. One of the dominant bacterial populations occurring, upon larval feeding, in the maize rhizosphere of KWS13 maize, was recently shown by Dematheis et al. (2012) to share 100% sequence identity of the 16S rRNA gene with a phenol degrading *Acinetobacter calcoaceticus* strain. The identification of dominant bacterial populations responding to larval feeding in the maize endorhiza could be the subject of further investigations.

In conclusion, the present study provided new insights into the complex interaction among WCR larval feeding, *GI* and the microbial communities in both rhizosphere and endorhiza of maize. The most relevant result concerned the inhibitory effects of *GI* on the WCR larval development. The mechanisms of

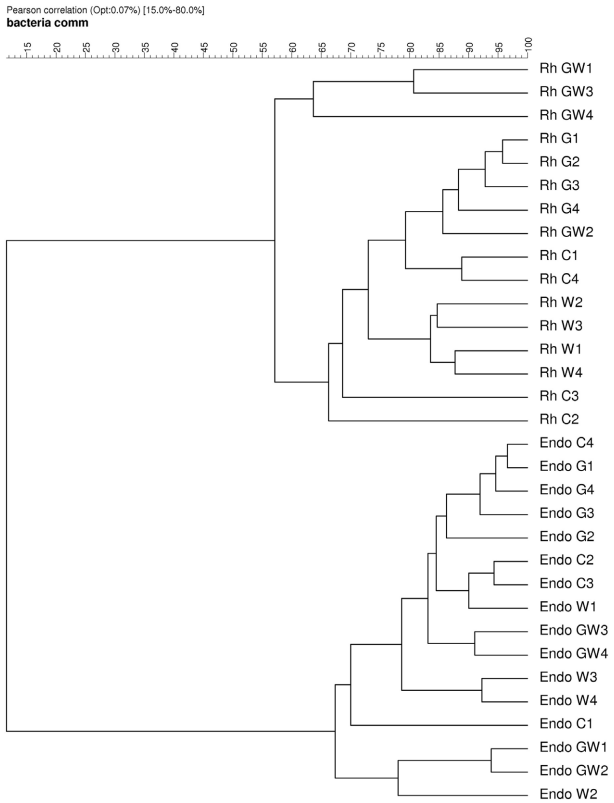

FIGURE 5 | Dendrogram constructed with the bacterial communities fingerprints in the endorhiza and rhizosphere of maize reported in Figure 4. The differences between the profiles are indicated by percentage of similarity. The dendrogram was based on the Pearson correlation indices and cluster analysis by the unweighted pair group method using arithmetic averages.

the interaction between WCR larvae and *GI* remain unknown, although our data suggested an indirect plant-mediated mechanism resulting in a shift of the microbial communities. However, our findings revealed for the first time a biocontrol activity of *GI* against WCR larvae which could be used in integrated pest management programs.

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Impact of elevated atmospheric O_3 on the actinobacterial community structure and function in the rhizosphere of European beech (*Fagus sylvatica* L.)

Felix Haesler, Alexandra Hagn, Marion Engel and Michael Schloter*

Research Unit for Environmental Genomics, Helmholtz Zentrum München - German Research Centre for Environmental Health, Neuherberg, Germany

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Gábor M. Kovács, Eötvös Loránd University, Hungary
Andrea Squartini, University of Padova, Italy

***Correspondence:**
Michael Schloter, Research Unit for Environmental Genomics, Helmholtz Zentrum München - German Research Centre for Environmental Health, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
e-mail: schloter@helmholtz-muenchen.de

Many bacteria belonging to the phylum of Actinobacteria are known as antagonists against phytpathogenic microbes. This study aimed to analyze the effect of ozone on the actinobacterial community of the rhizosphere of four years old European beech (*Fagus sylvatica* L.) trees during different time points of the vegetation period. Effects of ozone on the total community structure of Actinobacteria were studied based on the analysis of 16S rRNA gene amplicons. In addition effects of the ozone treatment on the diversity of potential biocontrol active Actionobacteria being able to produce antibiotics were characterized by using the type II polyketide synthases (PKS) genes as marker. Season as well as ozone treatments had a significant effect on parts of the actinobacterial rhizosphere community of European beech. However on the basis of the performed analysis, the diversity of Actinobacteria possessing type II PKS genes is neither affected by seasonal changes nor by the ozone treatments, indicating no influence of the investigated treatments on the biocontrol active part of the actinobacterial community.

Keywords: European beech, Actinobacteria, biocontrol, polyketide synthase gene, ozone

INTRODUCTION

Background ozone concentrations in the troposphere have doubled in the Northern hemisphere since the industrial revolution and are predicted to remain at high levels in the near future (The Royal Society, 2008). Ozone is a major secondary air pollutant, produced by a complex series of photochemical reactions from primary precursor emissions of nitrogen oxides (NOx) and volatile organic compounds (Ashmore, 2005). Deleterious effects on trees include chronic oxidative plant stress resulting in reduced photosynthesis, foliar injuries and premature leaf loss (Matyssek and Innes, 1999). This leads to an impairment of many metabolic pathways (Olbrich et al., 2005) and reduced primary production due to stomatal closure and damage to leaf mesophyll (King et al., 2005).

Plant derived carbon is the main energy source for soil biological processes and therefore a key factor influencing the community composition of the rhizosphere (Andersen, 2003). The quantity and quality of substances released into the rhizosphere by the plant are known to influence the structure and function of soil-borne microbial communities (e.g., Bais et al., 2006). Since ozone stress may affect carbon input into soils through changes in root physiology and altered root exudation, reduced carbon allocation to the roots due to a reduced photosynthesis could provide less carbon to organisms in the rhizosphere (Andersen et al., 1997), while possible qualitative changes of root exudates might alter nutrient conditions favoring specific rhizosphere organisms. Studying the effect of ozone on microbial groups in the rhizosphere exhibiting specific functions in soil will thus lead to a better understanding about the impact of ozone on microbes and relevant processes in the rhizosphere.

In this respect, plant growth promoting rhizobacteria (PGPR) are of special interest. PGPRs have the potential to stimulate plant growth and/or to actively inhibit performance of plant pathogens and thereby reduce the impact of plant diseases (Compant et al., 2005). Conditions favoring PGPRs in soil therefore have the potential of increasing plant health by reducing the success of pathogens (Janvier et al., 2007). A major group of PGPRs are various members of the phylum Actinobacteria. These microorganisms belong to the Gram-positive bacteria with a high G + C content in their DNA, which are usually able to form branching hyphae at some stage of their development (Goodfellow and Williams, 1983). Members of this group are well known for their ability to produce a wide range of secondary metabolites including commercially important antibiotics such as tetracyclines or cycloheximide (Haesler et al., 2008). The ability of Actinobacteria to inhibit diverse groups of phytopathogens *in vitro* and *in vivo* is a well-documented phenomenon (Paulitz and Belanger, 2001) and their active role in plant rhizospheres has been demonstrated in many studies (Smalla et al., 2001; Billings and Ziegler, 2005; Hjort et al., 2007). A first indication that groups of Actinobacteria might be sensitive to ozone was provided by Dohrmann and Tebbe (2005); they could show a clear change in Actinobacterial community structure in the rhizosphere of the ozone sensitive composite *Sonchus asper*, however no effect could be observed for other plants in the same study. In general, studies on the effect of ozone on the microbial community in rhizosphere of plants are scarce and no analysis has been performed focusing on Actinobacteria in the rhizosphere of woody plants.

A relevant example in this respect is European beech (*Fagus sylvatica* L.). European beech is of great economical importance

since it is the most frequently planted deciduous tree species in central European forests (Jung et al., 2005) and also partly detrimental in urban areas, where besides an increase in tropospheric ozone levels also near ground ozone concentrations have increased significantly in the last decades. With respect to ozone, European beech can be considered a sensitive species (Skärby et al., 1998). Yet, interestingly there has been evidence that even though beech trees show a clear reduction of belowground competitiveness under enhanced O_3, it apparently does not reduce the defense capacity against root pathogens like the oomycetous *Phytophthora citricola*. Luedemann et al. (2005) even postulated an increase in the resistance of European beech trees toward this pathogen in response to increased ozone concentrations in the atmosphere. Even though it is known that O_3 is capable of eliciting plant responses typically associated with pathogen defense (Matyssek and Innes, 1999), it can also be hypothesized that this stable defense capacity against selected plant pathogens of European beech in the face of other stressors is partly related to a very stable antagonistic biocontrol active microflora in the rhizosphere which inhibits growth and activity of *P. citricola*.

Molecular studies on antagonistic microbial communities often focus on structural rRNA gene analysis of the population rather than on functional antagonistic traits of the organisms. Yet, while phylogeny and expression of phenotypic traits are often closely connected (Berg, 2000; Oda et al., 2003), a lack of correspondence has also been demonstrated for a variety of soil organisms including antibiotics producing streptomycetes (Davelos et al., 2004). Therefore, analyzing changes within groups of possible plant pathogen inhibiting genes, e.g., encoding for antibiotics production, in response to changing environmental factors might be a more conclusive approach to describe the antagonistic population in soil. A large number of antibiotics produced by Actinobacteria are synthesized via type II polyketide synthases (PKS) (Hertweck et al., 2007) making this group of genes an interesting target for culture independent analysis. Among the produced antibiotics and anti-cancer drugs are tetracyclines, anthracyclines, aureolic acids and many more. Wawrik et al. (2005) developed primers for terminal restriction fragment length polymorphism (T-RFLP) analysis of these genes in soil and conducted an ecological study comparing soils from New Jersey to Uzbekistan (Wawrik et al., 2007). However, the applicability of these primers for studies within one ecosystem, where less variability is to be expected, has not been tested so far.

This study aimed to analyze the effect of ozone on the actinobacterial community of the rhizosphere of four-year old European beeches during different times of one year (spring, summer and autumn). Effects of ozone on Actinobacteria were studied on the structural level of the community (16S rRNA gene), as well as on the functional level using primers targeting type II PKS genes by fingerprinting and clone library analysis.

MATERIALS AND METHODS

EXPERIMENTAL SETUP AND SAMPLING

In 2006, 36 pots (14 L) were planted with three four-year old saplings of European beech each and incubated outside. Beech seedlings were obtained from a nursery (Bayr. Staatsforsten, Laufen, Germany). The soil was taken from the Ah-B horizont of mixed beech/spruce stand in the "Eurasburger Forst" near Augsburg, Germany (11° 5′ E and 48°18′ N). This soil has been characterized as a podsolic para-brown soil (orthic luvisol). Soil pH (H_2O) was 3.9 and C/N analyses result in 6.4% of total carbon, 0.3% of total nitrogen and a C/N ratio of 19.9. The soil texture was: 41% sand, 36% silt and 23% clay. (Kreutzer et al., 1991).

In spring 2009 before the start of the vegetation period the pots were transferred in climate controlled greenhouse chambers at the Helmholtz Zentrum München. Half of the pots were exposed to ambient O_3 conditions (natural O_3 levels outside the greenhouse ranging from 20 to 80 ppb), the other half to twice ambient O_3 conditions (restricted to <150 ppb). Therefore pots were placed into two tents volume about 7000 l) built of transparent plastic foil (ethylene-tetrafluorethylene ETFE, film thickness 80 mm, Koch Membranen GmbH, Rimsting, Germany), to separate the plants from the outer greenhouse atmosphere and incubated under the respective ozone concentrations. Ozone concentrations were measured continuously using an O_3-analyser (Columbia Scientific Industries, Austin, USA). Fumigation was performed with an O_3-generator (Fischer, Meckenheim, Germany) via a compressor. Ozone concentrations were adjusted automatically every hour. Relative humidity was adjusted in all pots to outside conditions and only natural light was used. Irrigation was carried out automatically, starting out with 200 mL of demineralized water every 56 h in spring and adjusting regularly to changing water demands due to increased temperature and plant performance. 150 mL of double strength Hoagland solution (Hoagland and Arnon, 1950) were applied as fertilizer in April, June and August.

Sampling took place at three different time points throughout of the vegetation period (bud break in May, full leaf development in July and senescence in September. At each time point six pots for each of the two treatment combinations ($1 \times O_3$; $2 \times O_3$) were harvested and treated as true replicates. Roots were cleaned from loosely adhering soil by hand. Soil tightly attached on the root surface was rinsed using phosphate buffer solution, and centrifuged at 8000 × g for 10 min. The pellet was collected as rhizosphere soil. Samples from one pot (three plants) were pooled to minimize the effect of genetic variation between different beech trees, resulting in approximately 10 g of rhizosphere soil per pot and stored in plastic bags at −80°C until further processed.

DNA EXTRACTION

Environmental DNA was extracted using the Fast Spin DNA Extraction Kit for Soil (MP Biomedicals, Eschwege, Germany) according to the manufacturer's instructions with modifications. 0.5 g of homogenized soil was used. The original protocol was modified by adding two washing steps of the silica binding matrix using 5.5 M guanidine thiocyanate solution to remove inhibitory substances. DNA extractions from soil samples were performed in duplicates. The amount of DNA was estimated by Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

PCR AMPLIFICATION

To evaluate the diversity of Actinobacteria in beech rhizosphere the following Actinobacteria specific 16S rRNA gene primers were used: as forward primer S-C-Act-235a-S-20, 5′-CGCGGCCTATCAGCTTGTTG-3′ (Stach et al., 2003) and as reverse primer Act-1360, 5′-CTGATCTGCGATTACTAGCGACTCC-3′ (McVeigh et al., 1996). Amplification was performed as follows: each 50 μL reaction contained 1x buffer (Gibco BRL, Karlsruhe, Germany), 0.2 μM of each primer (Thermo Hybaid, Ulm, Germany), 2 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 5% DMSO, 0.3% BSA, 20 ng of template DNA and 2.5 U *Taq* polymerase (Gibco BRL, Karlsruhe, Germany). A hot start was applied with a denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 45 s, 72°C for 1 min and 45 s and a final elongation at 72°C for 5 min.

For a culture independent functional analysis of microbial communities the type II PKS specific primer pair 540f, 5′-GGXTGCACSTCXGGXMTSGAC-3′, and 1100r, 5′-CCGATSGCXCCSAGXGAGTG-3′, (Wawrik et al., 2005) was used (X = inosine). For amplification, a 50 μL PCR reaction consisted of 1x buffer (Gibco BRL, Karlsruhe, Germany), 0.4 μM of each primer (Thermo Hybaid, Ulm, Germany), 2 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.2 mM of each dNTP (MBI Fermentas, St. Leon- Rot, Germany), 5% DMSO, 0.3% BSA, 20 ng of template DNA and 5 U Taq polymerase (Gibco BRL, Karlsruhe, Germany). The cycle program was initiated with a hot start for 5 min at 95°C followed by 35 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 45 s and a final elongation at 72°C for 10 min.

TRFLP ANALYSIS

PCR amplification was performed with primers which were Cy5 fluorescently labeled at the 5′-terminal end. For 16S rRNA gene amplification primer S-C-Act-235a-S-20 and for PKS type II gene amplification primer 540f was labeled. For each DNA extract PCRs were repeated three times, the products were subsequently pooled and purified with PCR Purification Kit (Qiagen, Hilden, Germany). A double digestion with enzymes *MboI* (New England Biolabs, Frankfurt am Main, Germany) and *Fau*I (SibEnzyme, Zweibrücken, Germany) was performed for actinobacterial 16S rRNA gene TRFLP analysis. For the first digestion reaction, a total volume of 10 μL contained 1x NEBuffer 1 (New England Biolabs, Frankfurt am Main, Germany), 2.5 U of *Mbo*I and 100 ng of pooled PCR products. The digestion mixture was incubated for 16 h at 37°C. Then, 10 μL of *Fau*I solution containing 1 U of enzyme in 1x NEBuffer 1 was added. This reaction mixture was incubated at 55°C for 16 h, subsequently heated to 65°C for 20 min to inactivate the enzymes and cleaned using the Minelute PCR Purification Kit (Qiagen, Hilden, Germany). For the analysis of PKS type II genes, 100 ng of the PCR products were digested with 20 U *Hha*I (New England Biolabs, Frankfurt am Main, Germany) in a total of 20 μL of 1x NEBuffer 4 (New England Biolabs, Frankfurt am Main, Germany) supplemented with 2 μg BSA. DNA was digested for 18 h at 37°C followed by an enzyme inactivation at 65°C for 20 min. The reaction mix was purified using the Minelute PCR Purification Kit (Qiagen, Hilden, Germany).

For detection of labeled fragments 2.5 μL of the purified digestion reaction was mixed with 0.25 μL GenomeLab DNA Size Standard 600 (Beckman Coulter GmbH, Krefeld, Germany) and 27.25 μL SLS buffer (Beckman Coulter GmbH, Krefeld, Germany). Separation of the fragments was conducted using a CEQ 2000 XL sequencer (Beckman Coulter GmbH, Krefeld, Germany). Each reaction was run three times on different capillaries to minimize capillary effects. One representative profile was taken for each sample for further analysis. To analyze peak profiles the CEQ 8000 Genetic Analysis System software version 8.0.52 (Beckman Coulter GmbH, Unterschleißheim, Germany) was used. Peak recognition was checked and edited manually to include all peaks within a profile. Peak heights were expressed relative to total peak height within a sample and all peaks below 0.5% of the total peak height within a sample were excluded from the analysis. Mean values were calculated for each peak from the duplicate DNA extractions for each soil sample.

CLONE LIBRARY ANALYSIS

To identify major peaks of the TRFLP profiles a reference data basis was generated for 16S rRNA and PKS type II genes based on clone libraries using DNA extracted from the 1 × O_3 treatment taken during full leaf development in July, where the most pronounced influence of the plant on the rhizosphere microflora was expected due to highest assimilation and exudation rates. The PCR products were cloned into a pCR® 2.1-TOPO® vector with the TA Cloning® Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Selection of positive clones was done by standard blue-white screening (Sambrook et al., 1989). Colonies were grown over night in 2–5 mL LB broth containing 50 μg/mL kanamycin and were used for plasmid extraction according to Bimboim & Doly (11). Plasmids containing inserts of the correct size were selected after digestion with EcoRI (MBI Fermentas, St. Leon-Rot, Germany) and sequenced. For the 16S rRNA gene library 56 clones and for the PKS type II library 54 clones were analyzed respectively. Sequencing was carried out using the BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, USA) and reactions were performed according to manufacturer's instructions on an ABI 3730 sequencer (Applied Biosystems, Foster City, USA).

16S rRNA gene sequences of the clone banks were classified using the higher-order bacterial taxonomy implemented in the Ribosomal Database Project II Release 9.54 (Cole et al., 2005) naïve Bayesian rRNA classifier (http://rdp.cme.msu.edu/) (Wang et al., 2007). For PKS type II sequences, a phylogenetic tree was calculated from protein sequences using the maximum-likelihood algorithm implemented in ARB (http://www.arb-home.de) after aligning the sequences with the ARB Fast Aligner tool. For testing the robustness of tree topology, trees were additionally reconstructed using parsimony (phylip protein sequence parsimony method—ProtPars, implemented in ARB) and neighbor joining methods (correction: PAM matrix) implemented in ARB. Actinobacterial 16S rRNA gene sequences were submitted to GenBank database with the accession numbers

EU138966-EU139021 and PKS type II gene sequences with the number EU138915-EU138965.

STATISTICAL ANALYSIS

Two indices, a moving window analysis (MWA) of the actinobacterial community based on a % change value matrix (calculated from a Pearson product-moment correlation coefficient matrix) expressed as the rate of change between two consecutive harvests (Δ_t), and a Pareto-Lorenz (PL) curve as introduced by Marzorati et al. (2008), were calculated to describe the T-RFLP profiles. To do this, the respective peaks are ranked from high abundance to low abundance, based on their peak heights. The cumulative normalized number of bands can be used as *x*-axis, and their respective cumulative normalized intensities represent the *y*-axis. The more the PL curve deviates from the 45° diagonal (the theoretical perfect evenness line), the less evenness can be observed in the structure of the studied community. To compare curves from different samples with each other, the *y*-axis projection of their respective intercepts with the vertical 20% *x*-axis line (PL20) can be scored (Wittebolle et al., 2008).

While MWAΔ_t describes the rate of change of a population, PL20 can be interpreted as an indication of the dominance of the most common groups of a population and therefore the evenness observed in the community structure. Univariate ANOVA was used to compare the values of different treatments utilizing S-Plus package Version 6.2 (Insightful Corp., Seattle, USA).

Non-metric multidimensional scaling (NMS) on the basis of Euclidean distance measure was used as an unconstrained ordination method to visualize patterns for multivariate data sets utilizing PC-ORD version 5.0 (MjM Software, Gleneden Beach, USA). The best dimensionality for the data sets was assessed by comparing stress values of 250 runs performed for 1-D to 6-D solutions. Additional dimensions were considered useful if they reduced the final stress by five or more. For all data sets 2-D solutions fitted this criterion. To evaluate whether NMS extracted stronger axes than expected by chance this procedure was repeated with randomized versions of the data sets and compared with the real data (Monte Carlo test). For all solutions the *p*-value was $p < 0.01$. For final solutions a maximum of 500 iterations was set using a stability criterion of <0.0000001 for the last 10 iterations (McCune and Grace, 2002).

To test for differences in composition and relative abundance of the multivariate data between samples from different treatments or groups non-parametric multivariate analysis of variance (PerMANOVA) was used (Anderson, 2001). For each term in the analysis, 4999 permutations of raw data units were done to obtain *p*-values. Individual pair-wise multiple comparisons by permutation were performed for factors showing significant differences (4999 permutations). In cases where there were not enough permutations possible to get a reasonable test 4999 Monte Carlo samples were drawn from the theoretical asymptotic permutation distribution Analyses were carried out using the FORTRAN program PerMANOVA (http://www.stat.auckland.ac.nz/~mja/Programs.htm).

To contrast the abundance of T-RFs across different groups of samples, indicator species analysis was performed according to Dufrêne and Legendre (1997) as implemented in PC-ORD version 5.0 (MjM Software, Gleneden Beach, USA).

RESULTS

PLANT RESPONSE TO THE OZONE TREATMENT

Whereas a significant increase of the plant biomass over the vegetation period was visible the ozone treatment did not influence the development of the above ground plant biomass. For the below ground biomass in contrast besides the effect of the sampling time point during the vegetation period, also an influence of the ozone was visible, resulting in a higher root biomass mainly in July in the pots treated with double ozone concentrations compared to the pots where only ambient ozone had been applied (data not shown). This indicates at least for the belowground biomass pronounced changes in the ecophysiology of the plants in response to the ozone application.

INFLUENCE OF INCREASED OZONE LEVELS ON ACTINOBACTERIAL COMMUNITY STRUCTURE IN THE RHIZOSPHERE

Changes in diversity of Actinobacteria over the vegetation period and in response to ozone

Partial actinobacterial 16S rRNA genes were successfully amplified from all soil samples and reproducible T-RFLP profiles could be obtained from environmental DNA. In most cases profiles of replicates for each treatment produced very similar patterns of peak intensities (coefficient of variation of ~6.5% for the major peaks). Thus, the observed differences in the profiles of treatments can be attributed to effects of the factors season and ozone elevation.

From the 36 samples included in the analysis of actinobacterial 16S rRNA genes, 39 different T-RFs were identified after relativization and removal of background noise. When comparing the average peak intensities for each treatment combination, the actinobacterial rhizosphere community was dominated by few major groups. The mean PL20 for all samples was 87.2 ($\pm$1.6) and did neither differ significantly between ambient (87.6 $\pm$ 0.8) and ozone treated samples (86.8 $\pm$ 2.1) nor between spring (88.4 $\pm$ 1.3), summer (86.8 $\pm$ 1.7) and autumn (86.4 $\pm$ 1.3) samples. Even though the relative evenness of the samples was not influenced by any of the factors, the quality of the profiles changed over time. The dissimilarity between spring and summer samples ($\Delta_{t(\text{spring/summer})} = 13.4 \pm 3.1$ for ambient and 11.7 $\pm$ 4.4 for ozone treated plants) was higher than between summer and autumn ($\Delta_{t(\text{summer/autumn})} = 4.2 \pm 1.5$ for ambient and 5.7 $\pm$ 2.1 for ozone treated plants). This difference was statistically significant ($p < 0.0001$) for the different sampling times and not significantly different between the two treatments.

For 16S rRNA gene profiles a two dimensional plot captured most of the variance in the T-RFLP profiles, with the first two dimensions containing 63.7 and 35.1% of the information in the analytical data set respectively (cumulative = 98.8%). The most dominant effect seen in the NMS plot was for samples collected in spring (upright triangles, **Figure 1**); this effect was independent of the ozone treatment. Separation of summer and autumn samples was not as pronounced with the exception of ozone treated summer samples which formed a distinct cluster.

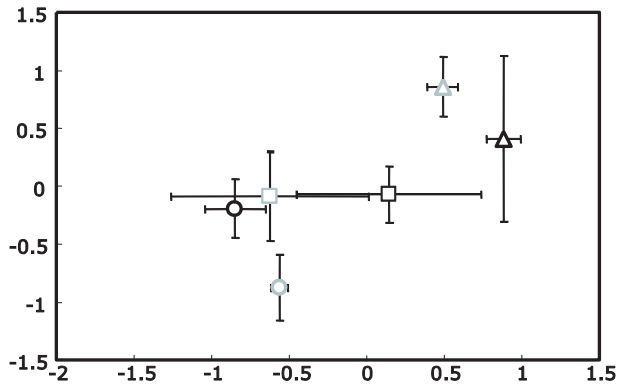

FIGURE 1 | Non-metric Multidimensional Scaling (NMS) plot of actinobacterial 16S rRNA gene T-RFLP profiles for rhizosphere DNA. Upright triangles indicate spring, circles summer and squares autumn samples. Ozone treated samples are shown in gray, while controls are black. Stress value was 4.48 given according to Kruskal's stress formula 1 multiplied by 100.

These observations were in agreement with the results of a non-parametric multivariate analysis of variance (PerMANOVA) and "a posterior" performed multiple pair-wise comparisons. With PerMANOVA, the effects of factors season and treatment were statistically significant (with $P = 0.0002$ and $P = 0.0288$ respectively). When performing a multiple pair-wise comparison between the three levels of the factor season, it was apparent that all seasons were significantly different from each other (spring vs. summer $P = 0.0002$, spring vs. autumn $P = 0.0006$ and summer vs. autumn $P = 0.0042$). Since there was a significant interaction between factors season and treatment ($P = 0.0238$), the effect of the treatment was analyzed separately within each level of the factor season, indicating that the separation of rhizosphere samples from ozone treated plants was only statistically significant in summer ($P = 0.0026$).

Identification of main responders

In order to find T-RFs responsible for the separation of different groups in the NMS plots of actinobacterial 16S rRNA genes, an indicator species analysis was performed. Groups chosen for a detailed analysis were, first, spring samples vs. autumn and summer samples and, secondly, a contrasting of the ozone treatment for samples harvested in summer. T-RFs with a significant result in the indicator species analysis were further analyzed by means of a permutation based univariate ANOVA with "a posterior" multiple pair-wise comparison (**Table 1**).

Of the T-RFs tested positive for an indication of spring samples, T-RF 102 stands out as the most dominant peak (**Figure 2**). It can be considered as negative indicator for spring since the average relative peak height of this T-RF almost doubled from 12.3% in spring to 22.4% in summer, followed by a small reduction toward autumn to 18.1%. These differences were statistically significant between all groups. Other major T-RFs like peaks 69, 162, 226, and 380 were positive indicators of spring. Yet, the observed differences in relative abundance for those peaks were generally rather small and should therefore not be over interpreted. Still it was obvious that a change in the overall composition of the actinobacterial rhizosphere community took place throughout the year.

When we tried to figure out the T-RFs which were responsible for the separation of T-RFLP profiles in response to the ozone fumigation in summer, most of the T-RFs identified were only very minor peaks, with the exception of T-RF 579. The phyla represented by this peak were less abundant in the rhizosphere of ozone treated plants. The ozone effect was statistically significant, but could only be seen at the summer harvest (**Table 1B**). Therefore, the separation of ozone treated samples in the NMS plot during summer was very likely due to a change in the abundance of this peak. T-RF 411 was a second good, yet only minor, indicator showing significant influence of the treatment.

Phylogeny main responders

The established actinobacterial 16S rRNA gene clone bank confirmed the high specificity of the chosen primer pair S-C-Act-235-a-S-20/Act1360. Out of the 56 clones all could be assigned to the phylum Actinobacteria, 21 of which had to be categorized as unclassified (showing less than 95% probability of belonging to a genus). The remaining clones were dominated by sequences belonging to the family Catenulisporaceae (19 clones of the genus *Actinospica* and 4 of the genus *Catenulispora*). Another large fraction of the clones was related to *Mycobacterium*; for each of the genera *Nocardioides*, *Pseudonocardia*, *Rhodococcus* and *Terrabacter* one clone was detected

When comparing the fragment sizes of the indicator T-RFs with peaks obtained from the clone library, it was possible to identify actinobacterial genera which were influenced by the sampling time point. Organisms belonging to the family Catenulisporaceae (genera *Actinospica* and *Catenulispora*) formed were very likely to be responsible for the T-RF with the fragment size of 102 bp. While all clones belonging to the genus *Actinospica* had the same size, the four *Catenulispora* clones produced three different T-RF fragment sizes (**Table 2**).

None of the classified clones from the 16S rRNA gene library corresponded to either T-RF 579 or 411, which showed a clear response the ozone fumigation. Yet, a group of unclassified clones were shown to have a T-RF of 578 bp (data not shown). Also, T-RFs from actinobacterial isolates from the same soil belonging to the genera *Kitasatospora* and *Streptomyces* gave signals at 411 and 409 bp respectively (**Table 2**) and were therefore might represented by this peak in the profiles.

INFLUENCE OF INCREASED OZONE LEVELS ON ACTINOBACTERIAL COMMUNITY FUNCTION IN THE RHIZOSPHERE

Changes in diversity of Actinobacteria harboring the PKS II gene over the vegetation period and in response to ozone

Like for 16S rRNA genes also for PKS type II genes DNA from all samples was successfully amplified and the obtained TRFLP profiles from replicates were highly comparable. Overall the T-RFLP profiles were less diverse compared to actinobacterial 16S rDNA profiles, as was expected, and were also strongly dominated by a few major peaks. An overall analysis of all samples based on NMS plots did not show any response of the PKS II harboring Actinobacteria to the treatments season and ozone (**Figure 3**).

Table 1 | Results of indicator species analysis in combination with univariate ANOVAs based on selected t-RFs of the 16S rRNA amplicon profiles.

(A) Indicator species analysis contrasting spring vs. summer and autumn samples

T-RF	IG	Mean relative peak height within groups (%)						PerMANOVA	Pair-wise comparisons		
		sp	SD	*su*	SD	*au*	SD	[*P*-value]	*sp* vs. *su*	*sp* vs. *au*	*su* vs. *au*
69	sp	10.9	1.5	8.1	1.0	8.0	1.2	0.0002	0.0002	0.0002	ns
102	**su**	**12.3**	**3.4**	**22.4**	**2.9**	**18.1**	**3.9**	**0.0002**	**0.0002**	**0.0010**	**0.0064**
162	sp	14.6	1.6	12.2	1.6	13.0	1.5	0.0016	0.0022	0.0170	ns
226	sp	3.1	0.4	2.3	0.6	2.1	0.9	0.0014	0.0004	0.0010	ns
367	sp	2.6	0.4	1.9	0.5	1.9	0.4	0.0002	0.0006	0.0004	ns
378	sp	1.0	0.3	0.7	0.2	0.9	0.2	0.0022	0.0002	ns	0.0180
380	sp	6.6	0.8	4.3	0.6	5.3	1.1	0.0002	0.0002	0.0026	0.0136

(B) Indicator species analysis contrasting ambient vs. O3 samples in summer.

T-RF	IG	Mean relative peak height within groups (%)				PerMANOVA
		am	SD	O_3	SD	[*P*-value]
160	O3	1.0	0.1	1.4	0.1	0.0040
226	O3	1.9	0.3	2.7	0.5	0.0100
411	am	1.3	0.3	0.5	0.2	0.0040
579	**am**	**14.8**	**2.5**	**8.9**	**1.8**	**0.0010**

Values in bold refer to dominant T-RFs. Selected indicator peaks are shown. Values in bold highlight major peaks that were most likely to be responsible for the separation of the groups. P-values for univariate ANOVA were obtained by 4999 permutations for each peak. P-values in italics were obtained using 4999 Monte Carlo samples from the asymptotic permutation distribution. IG, contrast group indicating the group with the highest abundance of the peak; sp, spring; su, summer; au, autumn; am, ambient; SD, standard deviation.

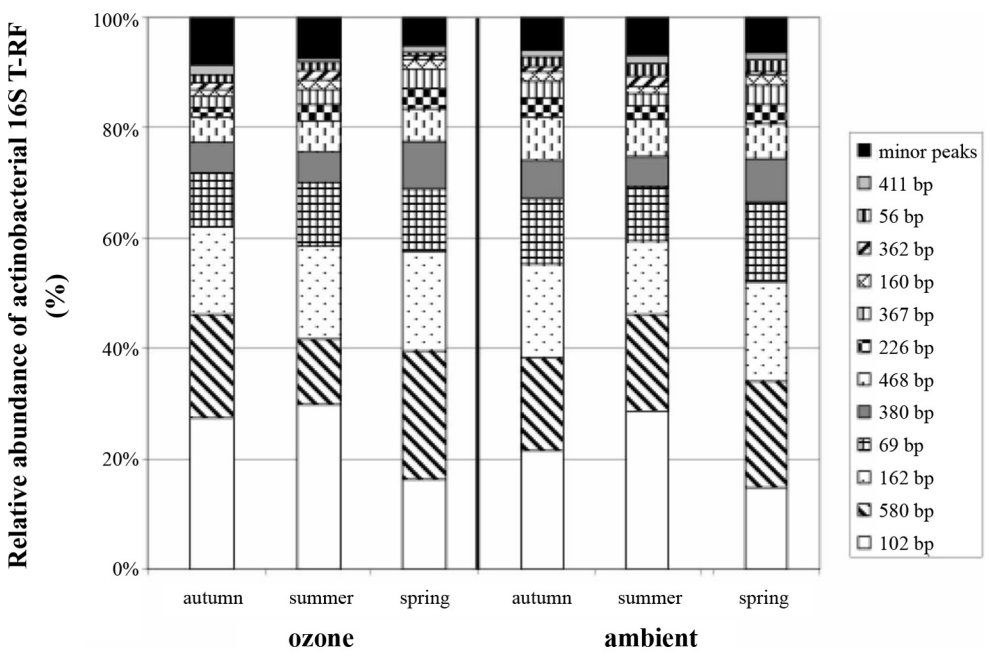

FIGURE 2 | Average relative abundance of actinobacterial 16S rRNA gene T-RFs from replicate soil samples. Peak size is given in base pairs, and relative abundance of T-RFs is given as percentage of total peak height.

Table 2 | T-RF sizes of the amplicons of 16S rRNA gene fragments from clones and pure cultures after double digest (*MboI/FauI*).

Clone no./ strain*	Genus	Genus	Enzyme	Expected fragment size [bp]	Actual fragment size [bp]
A15	*Actinospica*	Catenulisporaceae	*FauI*	106	102
A7	*Catenulispora*	Catenulisporaceae	*FauI*	106	102
A19	*Catenulispora*	Catenulisporaceae	*FauI*	366	362
A37	*Catenulispora*	Catenulisporaceae	*FauI*	388	381
A2	*Mycobacterium*	Mycobacteriaceae	*FauI*	230	226
A6	*Mycobacterium*	Mycobacteriaceae	*FauI*	378	371
A27	*Mycobacterium*	Mycobacteriaceae	*FauI*	375	367
A49	*Nocardioides*	Nocardioidaceae	*MboI*	154	149
A13	*Pseudonocardia*	Pseudonocardineae	*FauI*	476	471
A17	*Rhodococcus*	Nocardiaceae	*FauI*	473	464
A39	*Terrabacter*	Intrasporangiaceae	*MboI*	596	596
PT-1	*Kitasatospora*	Streptomycetaceae	*MboI*	415	411
PT-7	*Streptomyces*	Streptomycetaceae	–	–	409

*A, clone from actinobacterial 16S rRNA gene amplicon library; PT, 16S rRNA gene of actinobacterial isolates (see Haesler et al., 2008).

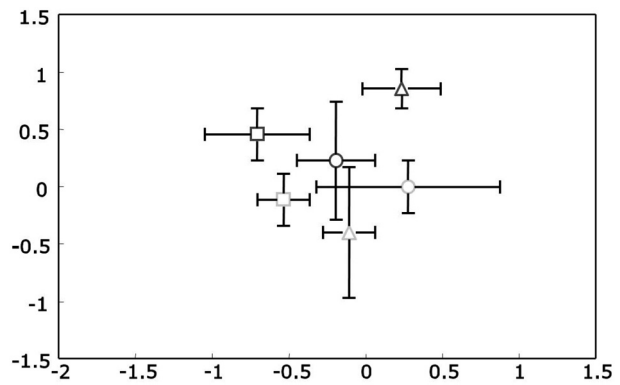

FIGURE 3 | Non-metric Multidimensional Scaling (NMS) plots of PKS Type II t-RFLP profiles for rhizosphere DNA. Upright triangles indicate spring, circles summer and squares autumn samples. Ozone treated samples are shown in gray, while controls are black. Stress value was 4.96 given according to Kruskal's stress formula 1 multiplied by 100.

Therefore to study changes in the diversity of these antibiotic producing genes two separate analyses were performed. One analysis was done from all ambient treated samples for each season (a total of 18 samples). The other analysis was performed with the 12 summer samples to analyze the effects of the ozone treatment. The summer harvest was chosen based on the observations that the clearest separation of actinobacterial 16S rRNA gene T-RFLP profiles was seen for this season. After relativization and removal of background noise 26 (for seasonal analyses) and 28 (for treatment analyses) T-RFs were included in the matrices respectively.

The mean PL20 for all samples was even higher than for the 16S rDNA profiles at 92.2 ± 3.8. There were no statistically significant differences neither for the factor season nor the factor ozone (when analyzed for the summer sampling). The dissimilarity between ambient spring and summer samples ($\Delta_{t(\text{spring/summer})} = 7.2 \pm 1.6$) did not differ significantly from the dissimilarity between summer and autumn ($\Delta_{t(\text{summer/autumn})} = 7.0 \pm 3.2$). Additionally, dissimilarity between ambient and ozone treated summer samples was in the same range at $\Delta_{t(\text{ambient/ozone})} = 7.5 \pm 2.3$ indicating no influence of any of the factors. These observations were verified with PerMANOVA and for all factors, season, and ozone, no statistically significant differences were observed.

Diversity of Actinobacteria harboring the PKS II gene

Although no clear response of the Actinobacteria harboring the PKS II gene in response to the factors season and ozone was visible, a clone library was constructed to do a phylogenetic identification of major groups of Actinobacteria harboring the PKS II gene. Out of the 54 selected clones 51 sequences had polyketide type II KS-domains as closest hits when submitted to a database search (blastx). The remaining three did not show any homology to known proteins. The nucleic acid codes of all obtained sequences were translated into protein sequences and aligned. Upon translation it was discovered that two of the sequences included internal stop codons. These sequences were considered to be pseudogenes and were therefore excluded from the phylogenetic analysis (accession numbers: EU138920 and EU138948). A maximum-likelihood tree was calculated for the remaining clones and 32 reference protein sequences (**Figure 4**). References were obtained from GenBank database and included sequences of known actinobacterial PKS type II KS-domains. Two outgroups were included, *fabB* (beta-ketoacyl-ACP synthase I) from *Escherichia coli* involved in fatty acid synthesis and a PKS type II from *Photorhabdus luminescens* TTO1 (γ-Proteobacteria). When looking at the phylogenetic tree eleven groups could be differentiated on the basis of 95% similarity of the protein sequences. Sequences obtained from the clone library were very diverse as seen by the wide distribution of the different groups throughout the tree. Eight of the eleven groups clustered alone or close to known antibiotics producing PKS type II KS-domains (groups 1–7 and 11), while three groups clustered in close vicinity to known spore pigment producing KS-domains (groups 8, 9 and 10).

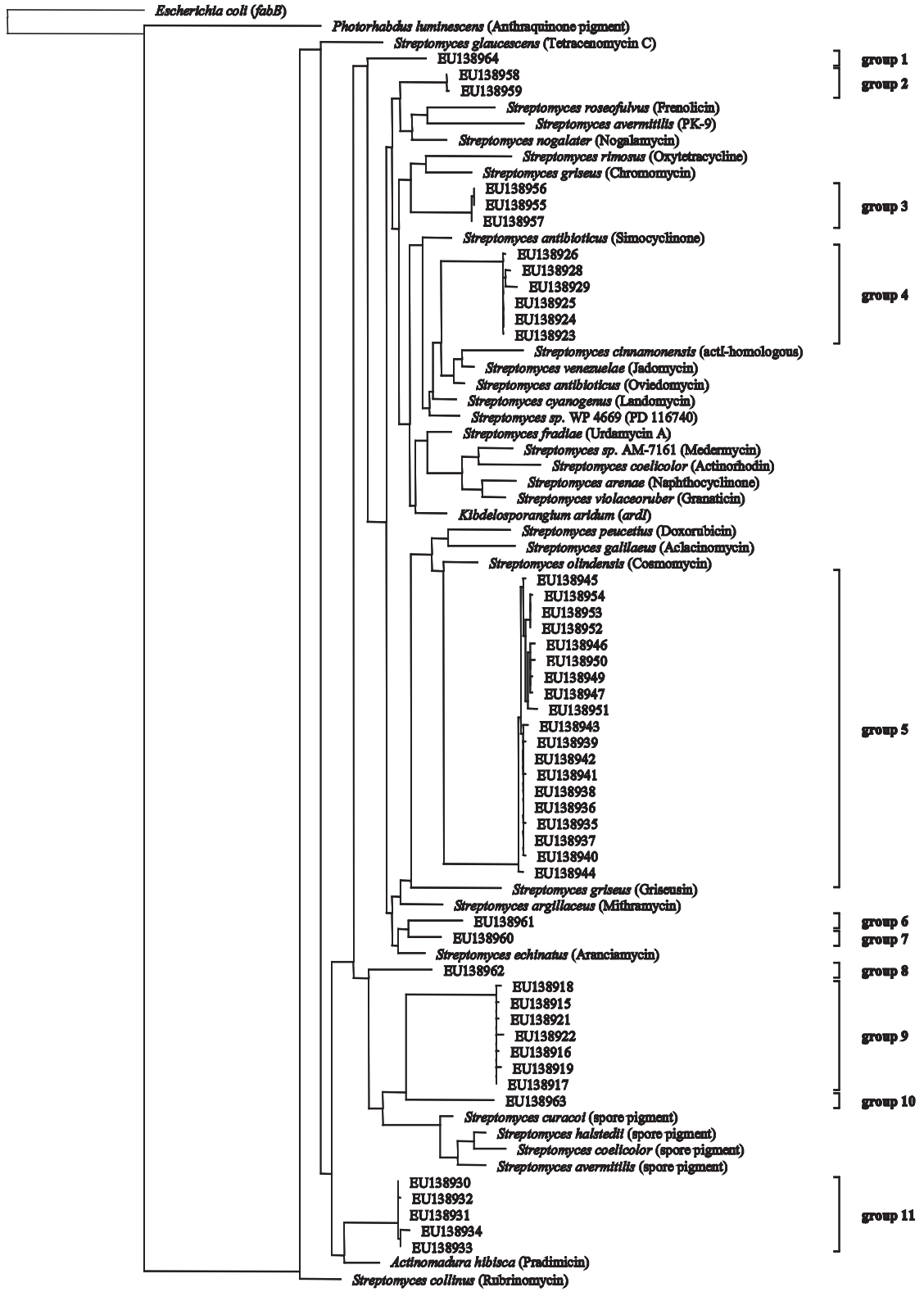

FIGURE 4 | Maximum-likelihood tree based on partial PKS type II protein sequences (185 amino acid positions) from cultured polyketide producers with known PKS Type II sequences (NCBI database) and sequences from beech rhizosphere clone library (accession numbers indicated). Tree topology was supported by parsimony and neighbor-joining methods (data not shown). Products of the reference PKS systems are given in parentheses. Groups are assigned on the basis of 95% similarity of the aminoacid sequences. The scale bar indicates 10% dissimilarity in aminoacid sequences.

DISCUSSION

STRUCTURAL DIVERSITY OF ACTINOBACTERIAL COMMUNITIES IN THE RHIZOSPHERE OF BEECH

Using T-RFLP analysis of 16S rRNA gene fragments as a culture independent method to monitor changes in the actinobacterial beech rhizosphere community, the overall variability observed between different samples was very low. For all major peaks detected, the differences observed were merely on the level of peak intensities and no differences could be seen based on the presence or absence of these peaks. It can therefore be concluded that none of the applied factors (O_3 and season) had the capability of qualitatively changing the actinobacterial community concerning its major components and that the general influence of the factors applied could be considered small. Nevertheless quantitative differences between samples for some T-RFs could be observed and clearly assigned to the influence of certain factors.

The clearest separation of samples was detected to be caused by seasonal shifts. Unique profiles were observed for spring samples and the major T-RF 102 bp responsible for this separation could be assigned to represent genera from the suborder Catenulisporinae based on comparisons with the clone library. This suborder contains mycelium-forming Actinobacteria, which are globally distributed and often isolated from acidic soils (Busti et al., 2006a,b; Tamura et al., 2008). Interestingly, no study documenting details of their ecology has been published so far and therefore the seasonal variability and dominance of this group observed in this study is a first indication of their active role for nutrient mobilization in forest soils. However, since the same peak was observed for two different genera (e.g., *Catenulispora* and *Actinospica* for T-RF 102 bp) the observed shifts cannot be assigned to a certain species. The phenomenon that several species or even genera are represented by the same peak in T-RFLP and other fingerprinting techniques has been reported in many studies (e.g., Smalla et al., 2007), thus interpretations of the results have to be done in general cautiously.

The drastic increase of T-RF 102 bp from spring to summer was statistically significant and similar for ozone treated and control plants. Seasonal shifts of microbial rhizosphere communities have been demonstrated in several studies (Smalla et al., 2001; Thirup et al., 2001) and in some cases these responses were associated specifically with Actinobacteria. Smalla et al. (2001) recorded a strong seasonal shift at the beginning of the vegetation period for rhizosphere communities of strawberries, oil seed rape and potato plants. Based on DGGE analysis of the rhizosphere communities they found indications that the abundances of bacterial high G + C populations were different during the developmental stages of all plants studied. Successional changes have also been described for plant associated Actinobacteria based on CFU counts and quantitative PCR methods (Thirup et al., 2001). The authors could show that at later time points in the season the abundance of Actinobacteria in the vicinity of barley roots increased significantly. They concluded that Actinobacteria are persistent during microbial succession beyond the early stages of root growth in annual plants due to their capability to penetrate and solubilize dead root litter (Thirup et al., 2001). Additionally, the active role of Actinobacteria in rhizospheres of diverse plants has been demonstrated in numerous studies (Billings and Ziegler, 2005). In the case of European beech the process of decomposition of old roots is likely to be more constant throughout the growing season compared to annual plants. The seasonal effect observed can thus not be assigned to increasing decomposition processes throughout the year. Additionally, the T-RF in question (T-RF 102 bp) peaked in summer and exhibited a slight decrease toward the end of the growing season. If this part of the bacterial population was mostly dependent on dead root litter as opposed to root exudates, it would rather be expected to peak in autumn, when the balance between new fine root production and older dead roots is largely in favor of the latter (Hertel and Leuschner, 2002).

A second statistically significant separation was observed in summer, where ozone samples showed unique profiles due to the relative reduction of one major T-RF (579 bp). Yet, the effect was very subtle and in this case transient, since autumn harvest of ambient and O_3 treatments could not be differentiated any more. Clones from the library possibly representing this peak could not be identified by the RDP classifier. Therefore, the identity and ecology of this putative O_3 responsive group would be of great interest and efforts are being made to isolate corresponding organisms from the studied soil. Only then, their functionality within the soil and possible implications on suppression of soil borne diseases can be investigated.

Responses of the microbial community to elevation of tropospheric ozone have been shown in other studies, but as observed in this study, the effects were relatively small in most cases. Dohrmann and Tebbe (2005) reported that from neither general Bacteria nor group specific SSCP profiles an ozone effect could be seen for the rhizosphere communities of different ozone-sensitive and insensitive herbaceous plants. The only exception in this study was the ozone sensitive composite *Sonchus asper* L., where changes were observed exclusively for the Actinobacteria specific profiles under elevated O_3. In other studies effects on the fungal rhizosphere community were shown. Chung et al. (2006) demonstrated that elevation of O_3 significantly altered the fungal community composition in a free-air enrichment experiment under three deciduous tree species utilizing DGGE. They also observed an increase in fungal relative abundance by PLFA analysis. In contrast to these result, Phillips et al. (2002) observed a decrease in fungal PLFAs while the relative proportion of Gram-positive and Gram-negative indicator PLFAs were not affected.

PKS TYPE II

This is the first study performed to investigate the effect of elevated ozone on genes potentially responsible for antibiotics production in the rhizosphere. PKS type II systems are of great interest when investigating the antibiotics production potential of actinobacterial soil populations (Wawrik et al., 2007). This especially holds true since a large portion of the actinobacterial community in the studied soil belongs to the newly described suborder Catenulisporinae. For this new lineage of Actinobacteria, Busti et al. (2006a,b) described a high potential to produce secondary metabolites with a polyketide scaffold. This is a feature they share with members of Streptomyces and related genera. All strains analyzed by Busti et al. (2006a,b) belonging to this

group yielded distinct bands when checked with specific primers for PKS type I and II. For one of the strains, the production of a bioactive molecule similar to the well-studied antibiotic actinorhodin was demonstrated. This antibiotic is synthesized by a PKS type II system (Hopwood, 1997). However, when analyzing the diversity of PKS type II genes, an effect was discovered for neither season, nor an influence of the ozone treatment.

Since PKS type II genes do not follow the trend observed for 16S rRNA genes, it can be concluded that phylotypes responsible for the observed changes (e.g., T-RF 102 bp) do either not contain similar PKS genes or they do not possess PKS type II genes at all, which is very unlikely for members of the Catenulisporinae but cannot be excluded. Anyhow it can be stated that in this case no correlation could be seen between phylogenetic trends and the genotypical trait PKS type II. This is in line with the findings of Metsä-Ketelä et al. (2002) who observed that the phylogenies of 16S rRNA genes and PKS genes in Actinobacteria soil isolates were not congruent. They concluded therefore that the phylogenetic grouping of Actinobacteria is an inadequate predictor for the type of secondary metabolites they produce.

However, another explanation for a lack of a significant separation of PKS type II genes might be the high background noise of these fingerprints.. Almost 10% of all clones in the PKS type II clone library have to be considered "junk" DNA (e.g., pseudogenes and sequences with no homology). Their distribution is likely to be random since no selective pressure takes effect on them. Previous studies using this primer pair used presence-absence data rather than relative peak intensities (Wawrik et al., 2005). Yet, it is very likely that this will only increase the influence of minor peaks, since they would have the same impact on multivariate statistical analysis as major, well-reproducible peaks. Furthermore, an additional nine sequences (16.7%) were closely related to spore pigment producing genes. The necessity to differentiate between antibiotics and spore pigments producing PKS systems has been stressed by Metsä-Ketelä et al. (1999) due to different ecological functions of the resulting molecules. While the property of a molecule to act as a pigment does not yield any information about its chemical properties concerning bioactivity, a major difference is that spore pigments are presumably covalently attached to macromolecular components of spores (Lee et al., 2005). Furthermore precursors of pigments e.g., monomers could well act as antibiotics or have other functions, while their massive production leads to incorporation in pigment macromolecules (Kämpfer, 2006). Therefore, they might act as protective against grazing by microfauna of the soil, but are not very likely to be involved in antibiosis against competing microorganisms.

CONCLUSION

On the basis of the performed analysis, the diversity of Actinobacteria possessing type II PKS genes was only slightly affected by seasonal changes. The applied ozone treatment did not have any effect on the distribution of these genes in the rhizosphere of beeches, although the ecophysiology of the plant was changed in response to the increased ozone levels in the atmosphere. Also the dynamics of the total phylum of Actinobacteria which were monitored in the presence study based on 16S rRNA gene fingerprints, was lower toward changes in the rhizosphere compared to other phylogenetic groups of bacteria, which showed significant changes in response to changes in the plant performance (e.g., bacteria of the genus Pseudomonas). This the large flexibility of Actinobacteria might be related their relatively large genome size and the possibility of this group of bacteria maybe to adapt faster and better to changing environmental conditions. Vice versa for the plant this indicates a huge stability of one important functional group of bacteria at the plant soil interface even if plant performance is changed. However, it has to be kept in mind, that changes were analyzed exclusively on DNA and not RNA levels. A transcriptomic approach might therefore yield further insights into the active part of the actinobacterial population. Furthermore the observed response pattern of Actinobacteria might change if beech trees of different age classes are studied or other stressors are investigated like increased drought periods or lack of nutrients.

AUTHOR CONTRIBUTION

Felix Haesler, Alexandra Hagn, Marion Engel, and Michael Schloter were involved in the development of this study, in the planning of the experimental setup, data analysis and manuscript writing. Felix Haesler performed all experiments described in this study.

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Bespoke microbiome therapy to manage plant diseases

Murali Gopal*, Alka Gupta and George V. Thomas

Microbiology Section, Central Plantation Crops Research Institute, Kudlu, Kasaragod, India

Edited by:
Gabriele Berg, Graz University of Technology, Austria

Reviewed by:
Gabriele Berg, Graz University of Technology, Austria
Henry Mueller, Graz University of Technology, Austria

****Correspondence:***
Murali Gopal, Microbiology Section, Central Plantation Crops Research Institute, Kudlu, Kasaragod 671 124, India
e-mail: mgcpcri@yahoo.co.in

Information gathered with advanced nucleotide sequencing technologies, small molecule detection systems and computational biology is revealing that a community of microbes and their genes, now termed "the microbiome," located in gut and rhizosphere, is responsible for maintaining the health of human beings and plants, respectively. Within the complete microbiome a "core-microbiome" exists that plays the pivotal role in well being of humans and plants. Recent studies in medicine have shown that an artificial mixture of bacteria representing the core gut microbiome of healthy person when transferred into gut of diseased person results in re-establishment of normal microflora in the latter leading to alleviation from diseased condition. In agriculture, though not exactly in similar manner as in medicine, success in plant disease management has been achieved through transfer of microbiome by mixing disease suppressive soils with disease conducive soils. A study more similar to artificial gut microbiome transfer in medical field has been recently reported in agriculture, in which transfer of microbiome via soil solutions (filtered and unfiltered) has shown ability to alleviate drought stress in *Arabidopsis thaliana*. However, the exact practice of transferring artificially cultivated core-microbiome as in medicine has not thus far been attempted in plant disease management. Nonetheless, as the gut and rhizosphere microbiome are known to share many common traits, there exists a good scope for accomplishing similar studies in agriculture. Based upon the information drawn from all recent works in microbiome studies of gut and rhizosphere, we propose that tailor-made core-microbiome transfer therapy can be a success in agriculture too and it could become a viable strategy for management of plant diseases in future.

Keywords: microbiome, core-microbiome, plant, disease management, soil, rhizosphere, root, gut

MICROBIOME IN RELATION TO HUMAN AND PLANT HEALTH

The power of next generation sequencing technology is transforming today's biology (Mardis, 2008; Schuster, 2008). Combined with bioinformatics (Lee et al., 2012), it is prising open the microbial "dark matter" and revealing the diversity and functions of microbiome at resolutions unknown hitherto (Forde and O'Toole, 2013; Rinke et al., 2013). It is shedding new light on the role played by the gut microbiome in governing the human health (Turnbaugh et al., 2007; Kinross et al., 2011; Cho and Blaser, 2012; Ottman et al., 2012; Norris et al., 2013), reviving the Metchnikoffian paradigm: colonizing the gut with beneficial microflora could lengthen the human life. The gut microbiota is not only limiting its influence on the human health by its functions in the intestine, it also is impacting the human brain and behavior (Heitz et al., 2011; Cryan and Dinan, 2012; Mulle et al., 2013) as well as social development evidenced by studies in mice (Desbonnet et al., 2013). Remarkably, similar train of evidences is being uncovered in plant world; root microbiome is observed to be tightly linked with the health of the plants (Friesen et al., 2011; Chaparro et al., 2012; Bulgarelli et al., 2013; Gaiero et al., 2013; Mendes et al., 2013). In insects, too, the same story is unfolding (Engel and Moran, 2013). The microbial diversity associated within these ecosystems is being referred to as the "second genome" that is easily 10 times more in scale than the host genome (Grice and Segre, 2012; Turner et al., 2013) and its impact on regulating human and plant health is becoming more apparent.

CORE MICROBIOME

From among the multitude microbial communities inhabiting the gut and root, there appears to be a clutch of them which constitute the core microbiome (Tschöp et al., 2009). Core microbome contains organisms common across the microbiome hypothesized to play a key role in ecosystem function within a habitat (Lederberg and McCray, 2001). Core microbiome of human gut (Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009; Huse et al., 2012; Petrof et al., 2013a,b) and plant (Bulgarelli et al., 2012; Lundberg et al., 2012; Peiffer and Ley, 2013) have been determined at Operational Taxonomic Unit (OTU) levels with small subunit ribosomal RNA genes or random sequencing of all genes. Any changes in the core-microbiome composition or function leads to debilitative or destructive diseases in humans as well as plants (Kinross et al., 2011).

DISEASE SUPPRESSIVE SOILS AND THEIR MICROBIOME

It is well known that farmers moved soil from one field to another to take advantage of its disease suppression abilities endowed by the soil microbial populations harbored in it (Weller et al., 2002). Soil microbial studies mainly based on cultivation dependent methods lead to the finding of several bacteria

termed plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978), particularly the genus *Pseudomonas* spp., in imparting the disease suppressive ability to such soils (Schroth and Hancock, 1982; Haas and Defago, 2005; Mendes et al., 2013). Today, with advanced technologies, studies are generating evidence that it is not an individual or couple of microbes, rather it is "microbiome" (Forde and O'Toole, 2013; Rinke et al., 2013), the complete assemblage of microbial communities of a habitat and their functions, in rhizosphere that is determining plant health (Berendsen et al., 2012; Mendes et al., 2013; Rout and Southworth, 2013). In insects too, the same phenomenon is being observed (Hussa and Goodrich-Blair, 2013). Rhizosphere/core microbiome of *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012), desert shrubs *Zygophyllum dumosum* (Zygophyllaceae) and *Atriplex halimus* (Kaplan et al., 2013), and maize (Peiffer and Ley, 2013) have been deciphered and reported to be stable (Lozupone et al., 2012; Lundberg et al., 2012; Li et al., 2013), inheritable (Peiffer et al., 2013) and tightly linked to host tissues (Lee et al., 2013).

ROOT MICROBIOME TRANSFER TO MANAGE PLANT DISEASE

In plant disease management, a simple method of transferring complete microbiome by mixing disease suppressive soils with disease conducive one is practiced. Mendes and colleagues (2011) showed that when soils suppressive to *Rhizoctonia solani*, an important fungal pathogen, is mixed with disease conducive soils at 1:9 ratio (w/w), it successfully suppressed the infection in sugar beet. Metagenomic analysis of the soils using PhyloChip revealed consistent involvement of 17 bacterial communities belonging to Proteobacteria, Firmicutes, and Actinobacteria, considered as core-microbiome, in disease suppression. Other works too, similarly point to the involvement of core-microbiome in soils suppressive to potato common scab (Rosenzweig et al., 2012) and tobacco black root rot (Kyselkova et al., 2009). In all the above mentioned works, Pseudomonadaceae group of bacteria has been suggested as a key player in disease suppressiveness within the core microbiome.

GUT MICROBIOME TRANSFER TO MANAGE HUMAN HEALTH

As with rhizosphere microbiome of plants in agriculture, in medical studies too, the gut microbiome has been found to control the health of the human beings (Turnbaugh et al., 2007; Cho and Blaser, 2012) with a core mainly involved (Tschöp et al., 2009; Turnbaugh et al., 2009; Huse et al., 2012). "Stool transplant" therapy (de Vos, 2013) is one of the several medical practices that is adopted wherein stool taken from healthy person is transferred to diseased person resulting in suppression of many important gastro-intestinal diseases. The principle here is to re-establish normal gut bacteria in the gut of diseased person and bring about positive changes in their health. However, the "stool transplant" therapy is not widely followed since the method is not acceptable to many patients, besides the apprehension that it can transfer pathogenic microbes too. Two recent studies (Petrof et al., 2013a; Ridaura et al., 2013) have found a way to overcome "stool transplant therapy" by using "stool substitute" in which a culturable consortium representing core microbiome is transferred and found to transmit the phenotype expression aimed for. Petrof and colleagues' (2013a) work was first of its sort successfully demonstrating that patients suffering from *Clostridium difficile* infection, a debilitative disease of intestine, can be cured when administered with stool substitute mixture comprising a multi-species community of bacteria (RePOOpulate sample) of a healthy individual exhibiting resistance to the disease. Post-treatment metagenomic analysis of the cured patients revealed that the OTU reads from their guts were similar to that of the RePOOPulate sample until six months after its administration even though the microbiota profiles were different. This work was quickly followed by Ridaura et al. (2013) in which they transplanted intact uncultured or cultured human fecal microbiota from each member of a discordant twin pair (one lean and other obese) into separate groups of recipient germ-free mice and found that the obese twin's fecal microbiota significantly increased the body biomass and adiposity in the germ free mice. It will not, therefore be, contrary to consider that the "stool substitute" transfer consisting of the core-microbiome is an extension of the "stool therapy" and is able to reproduce the expected microbial ecology with desired results. Such successful scientific endeavors are spurring development of new disease management paradigm termed MET: Microbial Ecosystem Therapy (Petrof et al., 2013b).

ROOT CAN FOLLOW THE GUT

In agriculture, a system of manipulating the root environment by artificially inoculating plant and soil beneficial microbes has been followed for long time for improving crop yields. The PGPRs and other plant beneficial microbes (nitrogen fixing and phosphate solubilizing bacteria, *Trichoderma* spp., arbuscular mycorrhizae fungi etc.) isolated from rhizospheres were mass multiplied and artificially inoculated, either singly or in combination of twos, for disease management in plants (Berg, 2009; Lugtenberg and Kamilova, 2009; Chaparro et al., 2012; Qiu et al., 2013). Though this approach has been widely adopted, its success in field conditions have been limited (Bakker et al., 2012). With unequivocal reports coming out indicating that it is not a single taxon, but a consortium of microorganism that is responsible for bringing about diseases suppression in plants (Mendes et al., 2011; Rosenzweig et al., 2012; Trivedi et al., 2012; Klein et al., 2013), the stage is now set for the root to follow gut by adopting the strategy of using "stool substitute" for disease management. Transferring disease suppressive soils has been the only alternate method for transferring the complete rhizosphere/core microbiome in plant protection strategy. Coming closer to "stool substitute" therapy, transfer of microbiome via soil solutions (filtered and unfiltered) has shown ability to alleviate drought stress in *Arabidopsis thaliana*. Pyrosequencing analysis of soils revealed a core microbiome (*Burkholderia, Phormidium, Bacillus, Aminobacter, Acidiphilum* among others) involved in alleviating the abiotic stress (Zolla et al., 2013). However, the exact transfer of artificially cultivated core-microbiome as performed by Petrof et al. (2013a) and Ridaura et al. (2013) with gut environment is yet to be attempted in root environment. The possibilities of achieving success is high as there exists a striking similarity between the gut and root microbiota (Berendsen et al., 2012; Ramírez-Puebla et al., 2013). Also, the fact that soil type

plays significant role in the selection and election of microbiome of rhizosphere and root compartment (Berg and Smalla, 2009; Bulgarelli et al., 2012; Lundberg et al., 2012), chances of success for the "rhizosphere substitute" is significantly augmented.

ROOT MICROBIOME CULTURING

In an important meeting convened on topic "culturing a plant-microbiome community" in Rhodes, Greece in 2012, a long-term future research strategy became apparent in which it was suggested that after an initial culture-independent survey of the plant microbiota, the corresponding community members are isolated in collections of pure cultures (Lebeis et al., 2012). Today, by converging information deduced on microbial diversity and functions using next-generation sequencing technologies and multi-species transcriptome analysis (Schenk et al., 2012), molecules/volatile involved in plant-microbe interaction using mass-spectral investigations (Watrous et al., 2012; Badri et al., 2013) combined with power of bioinformatics (Lee et al., 2012), it has become very much possible to culture the appropriate core-microbiome and apply it successfully (Ridaura et al., 2013). To assemble a robust core microbiome of an ecosystem not limited to OTU records alone, Shade and Handelsman (2012) and Lozupone and colleagues' (2012) suggested collecting the data on (i) OTU membership/α diversity, (ii) OTU composition/β diversity, (iii) OTU persistence across time and space and (iv) communication/metabolic networking among the OTUs. Determination of models, particularly of root environment, in which the plants favor the recruitment of antibiotic-producing (and -resistant) bacteria by stimulating interference competition through production of abundant resources, can help improve establishment of the artificially introduced microbiomes(Scheuring and Yu, 2013).

BESPOKE MICROBIOME THERAPY FOR PLANT DISEASE MANAGEMENT

Artificial core-microbiome transfers can decrease the noise intrinsic to any complex communities and are step in right direction in disease management, both for plants and humans, built upon the principles of binary plant/human-microbe interaction in an ecological perspective.

The similarities between the gut and rhizosphere microbiota is striking in many aspects which can encourage emulating experiments carried out in gut with root environment and vice-versa. Based on the increasingly available body of evidences discussed in this article, we propose the model of transfer of bespoke core-microbiome, rather than individual species, as a viable strategy for management of plant diseases in future.

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Beneficial effects of plant-associated microbes on indoor microbiomes and human health?

Gabriele Berg[1]*, Alexander Mahnert[1] and Christine Moissl-Eichinger[2]

[1] *Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Institute for Microbiology and Archaea Center, University of Regensburg, Regensburg, Germany*
**Correspondence: gabriele.berg@tugraz.at*

Edited by:
Martin Grube, Karl-Franzens-University Graz, Austria

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PLANT MICROBIOMES—AN INTRODUCTION

Just like humans, plants have recently been recognized as meta-organisms, possessing a distinct microbiome and revealing close symbiotic relationships with their associated microorganisms (Berg et al., 2013; Mendes et al., 2013). Each plant harbor specific species to a certain degree but also cosmopolitan and ubiquitous microbial strains; the majority of them fulfill important host as well as ecosystem functions (rev. in Berg and Smalla, 2009). In addition to the microbe-rich rhizosphere, which has been studied extensively, the phyllosphere is of special interest for the study of indoor microbiomes due to its large and exposed surface area and its remarkable microbial diversity (Lindow and Leveau, 2002; Lindow and Brandl, 2003; Redford et al., 2010; Meyer and Leveau, 2012; Vorholt, 2012; Rastogi et al., 2013). In addition to the majority of beneficial and neutral inhabitants, all plant-associated microbiomes contain plant as well as human pathogens (Berg et al., 2005; Mendes et al., 2013). A broad spectrum of plant pathogens is well-known from disease outbreaks. Human pathogens belong mainly to the so called opportunistic or facultative human pathogens such as *Burkholderia cepacia*, *Pseudomonas aeruginosa* or *Stenotrophomonas maltophilia*, which cause diseases only in patients with predisposition or in hospital (Berg et al., 2005; Ryan et al., 2009).

Microbiomes of humans and plants are currently intensively studied using the same methods and addressing similar scientific questions (Ramírez-Puebla et al., 2013). However, knowledge about the microbiomes' interaction, microbial dynamics and exchange in a certain biotope or even indoor environment is very much limited. Although the composition and function of plant microbiomes is well-studied, there is still little to no information regarding their overlap, interaction with -and impact on other microbiomes or the microbiome-harboring hosts. Information is available about the connection of soil and rhizosphere microbial diversity, which share a selective sub-set (Smalla et al., 2001). The root-soil interface is the selection site for plant-associated bacteria by root exudates, which acts as chemo-attractants as well as repellents to which bacteria respond (Badri and Vivanco, 2009). In addition, plant defense signaling play a role in this process (Doornbos et al., 2012). For the phyllosphere we know that there is only a part of residents, while a substantial part of bacteria is shared with the air microbiome (Lindow and Brandl, 2003). Based on these data, a strong interaction and exchange of rhizosphere and phyllosphere microbiomes with other microbiomes is obvious. However, this opinion paper focuses on the question, if there is also a connection from plant–to indoor microbiomes as well as an impact on human health.

INDOOR MICROBIOMES—IMPORTANCE AND ORIGIN

Despite the fact that the majority of our lifetime is spent in indoor environments such as home, work place, or public buildings, our knowledge of microbial diversity inside buildings is limited. We are not alone in these indoor environments: they provide new habitats and residence to numerous microbial communities comprising possibly hundreds of individual bacterial, archaeal and fungal species including diverse viruses. Recent studies analyzed potentially pathogenic and allergenic indoor microorganisms with mainly cultivation-based methods (Täubel et al., 2009; Yamamoto et al., 2011). Since the fraction of cultivable microbes on one specific medium is extremely low, information about specifically-adapted microorganisms, or those with special needs, remains inaccessible by standard cultivation assays. Recently, however, the application of molecular methods, including next generation sequencing (NGS) techniques has provided new insights into indoor microbial communities, revealing a generally high prokaryotic diversity including diverse bacterial, archaeal and fungal phyla (Flores et al., 2011, 2013; Moissl-Eichinger, 2011; Hewitt et al., 2012, 2013; Kembel et al., 2012; Dunn et al., 2013; Kelley and Gilbert, 2013; Meadow et al., 2013).

Indoor microbial communities are an important component of everyday human health (Arundel et al., 1986; Lee et al., 2007; Kembel et al., 2012). Due to human activity and high emission rate of up to 10^6 bacteria per person-hour as measured via 16S rRNA gene quantification from aerosols (Qian et al., 2012), indoor environments are strongly influenced by typically human-associated bacteria (Fierer et al., 2008). Hence, built environments like hospitals are more easily colonized to a large extent by patient-associated microbes (Oberauner

et al., 2013). As a result, many patients in hospitals and especially in intensive care units (ICUs) develop hospital-acquired "nosocomial infections" that compound their underlying severe disease (Vincent et al., 1995; Plowman, 2000). Moreover, these nosocomial infections remain among the leading causes of death in developed country hospitals. The risk to get nosocomial infections for patients in European ICUs was reported as 45% (Plowman, 2000). Hospital surfaces are often overlooked reservoirs for these bacteria (Hota, 2004; Gastmeier et al., 2005; Kramer et al., 2006). Apart from hospitals, indoor microorganisms affect human health as allergenic agents as well (Hanski et al., 2012). Indoor microorganisms are also involved in the development of the Sick Building Syndrome (SBS), which causes symptoms such as sensory irritation of the eyes, nose, and throat, neurotoxic or general health problems, skin irritation, non-specific hypersensitivity reactions, and odor and taste sensations (Godish, 2001).

Indoor microbiomes originate primarily from human skin, pets, or the outside air (Flores et al., 2011; Kembel et al., 2012; Meadow et al., 2013). Plants as a source of indoor microbes are so far less considered. However, air-borne microbes as substantial part—bacteria, fungi or microscopic algae—are scattered and can travel long distances such as in the wind or in clouds before returning to ground-level (Hamilton and Lenton, 1998). They have received more attention because they can serve as nuclei for condensation and as such influence our world climate as rain-making bacteria. Interestingly, cloud and hailstone studies indicated plant-surface bacteria as the dominant source of these rain-making microbes (Morris et al., 2008; Šantl-Temkiv et al., 2013). In addition, little is known about the impact of houseplants and its microbes, although older studies indicate indoor plants as important source (Burge et al., 1982).

Comparing indoor with plant microbiomes, it is our opinion that both outside and inside plants are of importance for our indoor microbiome. Plants provide beneficial bacteria for indoor rooms and therefore can positively influence human health. The following facts support our opinion about the importance of plants as source for a beneficial microbial biodiversity:

1. Empirically the positive effects of houseplants and flowers are well-known, but there is also evidence for psychological effects such as stress reduction and creative task performance (Fjeld et al., 1998; Shibata and Suzuki, 2004; Chang and Chen, 2005; Bringslimark et al., 2007; Dijkstra et al., 2008). In addition houseplants feature a remarkable capacity to improve indoor air quality (Orwell et al., 2004). This melioration of indoor air is not only due to the filtering capacity of plant leaves, but also by the degrading effects of their root associated microbes (Pegas et al., 2012 up to 90% formaldehyde removal during night according to Kim et al., 2008).
2. Plant DNA as frequently detected as chloroplast 16S rRNA gene sequences in amplicon surveys is a substantial part of all indoor microbiomes, but mainly filtered out for the presentation of data (Oberauner et al., 2013). This emphasizes, that pollen and seeds of plants, which are densely colonized by bacteria (Fürnkranz et al., 2012) are dispersed into the indoor environment and thus provide excellent shuttles for microbiome exchange.
3. Typical and often dominant plant-associated bacteria are members of the indoor microbiome. A relationship of bacteria genera occurring on plants and indoors is given in **Figure 1**. There are many ways for plant microbes to enter the built environment; as already mentioned on pollen, seeds, fog, soil on shoes, flowers, fruits and vegetables as well as transmitted by animals and other visitors.
4. At species level, no differentiation was possible for clinical and plant-associated isolates. This was studied for *Burkholderia cepacia*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* (Ryan et al., 2009; Martins et al., 2013). Unfortunately, these plant-associated bacteria can infect immuno-compromised patients with high predisposition in hospitals. On the one hand this is an evidence for the interplay of the plant and indoor microbiome, but on the other hand it highlights the beneficial balance, which is necessary between microorganisms and hosts.
5. Interestingly, Thaumarchaeota, originally described to be associated with ammonia-oxidation in soil and the rhizosphere of plants, have been found on human skin (Probst et al., 2013). Currently it is unknown, whether the human skin archaea have positive or negative effect on human health and whether they have different genomic capabilities compared to their soil-relatives. However, it becomes clear, that closely related microorganisms can exist in different microbiomes, based on a dynamic exchange or distribution and subsequent development of adaptation strategies.

Based on these facts, we speculate the following:

Enclosed environments and their microbiomes—like private/public buildings, hospitals, and clean rooms, which are more or less separated from outside, are especially shaped by human influence and human associated microbes (Hospodsky et al., 2012; Dunn et al., 2013). Hence, microbial diversity is altered and partially reduced compared to the outdoor environment. A reduction in microbial diversity is well known to facilitate dominant proliferations of certain strains, which might bear the risk to have a negative effect toward our health. To increase microbial diversity in an indoor environment we could simply open our windows instead of using air-condition (Hanski et al., 2012; Kembel et al., 2012; Meadow et al., 2013). Alternatively, we could use potted houseplants in built environments as a source of microbial biodiversity and possibly beneficial microorganisms.

Microbes, which live in close vicinity to human beings, are adapted to us as symbionts, commensals, or pathogens, whereas these life-styles are changeable dependent on the host-microbe balance. Indoors we share these microbes, which might get deposited on various surfaces by one person and afterwards get collected by another. Human-associated microbes e.g., skin associated, are confronted with totally new biotic and abiotic factors in the built environment. Here they have to adapt to new surface materials, compete with

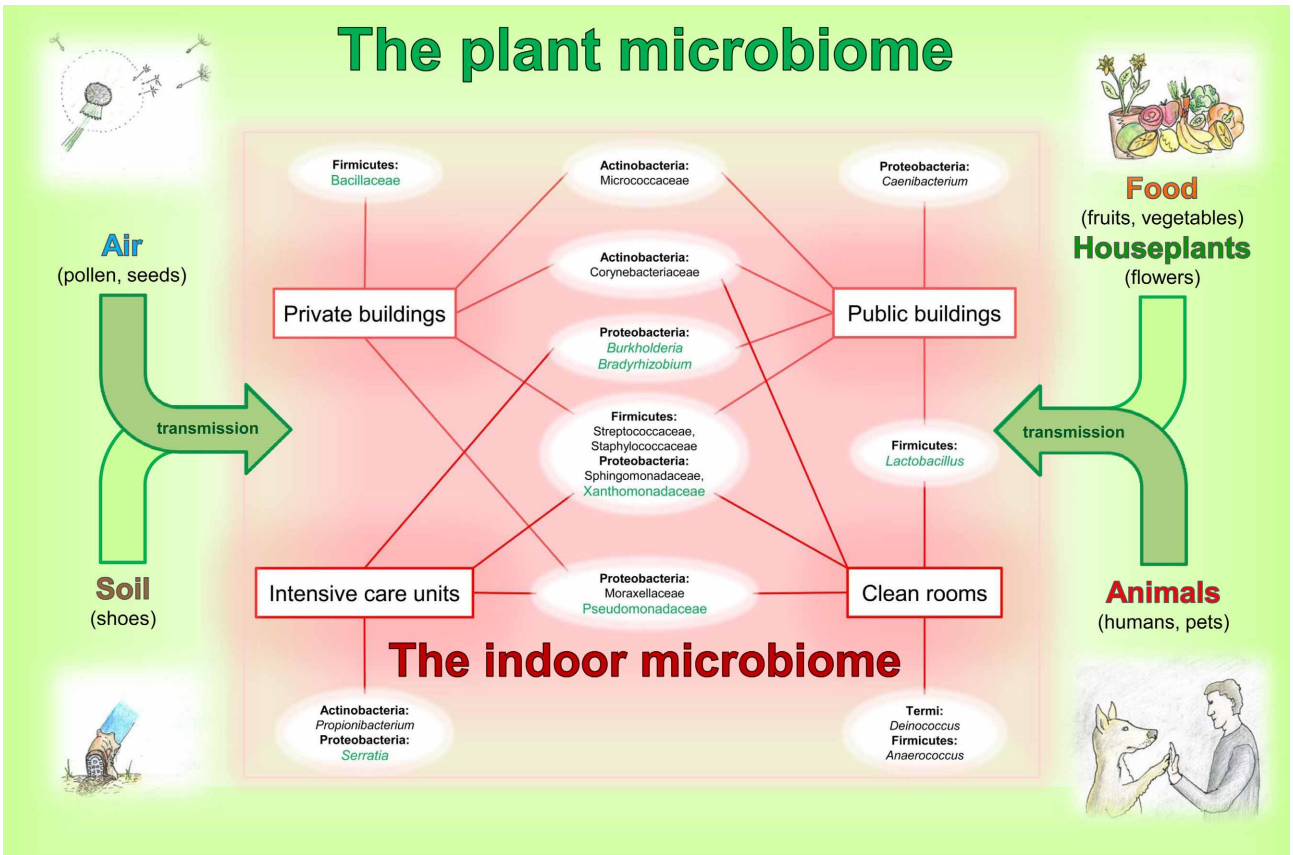

FIGURE 1 | Relationships between the plant and indoor microbiome. The indoor microbiomes, influenced by transmissions via air, soil, food, houseplants and animals from plant microbiomes, presents an overview on typical and dominant bacterial groups occurring in the built environment. Schematic chart represents occurrence of the bacterial inhabitants indoors. Bacterial families and genera (white ellipses) are arranged according to their phylum affiliation (bold) and are connected to certain types of the built environments (red squares). Taxa highlighted in green are typical phyla detectable in plant microbiomes. This image has no demand of being complete.

others for scarce nutrients and withstand stresses associated to cleaning reagents etc. However, in the case of houseplants we allow them to proliferate in a protected environment. Plant associated microbes stay on the leave or stem surface, where they have adapted to and are sheltered from cleaning procedures. Although these phyllosphere communities are confronted with an absence of direct sun light and rain as well as other changed meteorological parameters like air/dust turbulences, their rhizosphere and surrounding soil communities stay in their natural habitat. Hence, these well balanced plant communities, which we bring inside, have the potential to balance an indoor microbiome, by increasing its diversity and filter airborne microbes.

CONCLUSION

Members of the plant microbiome are an important source for indoor microbiomes. Both, plants from inside and outside can contribute to the microflora. Plant-associated bacteria could act as counterparts against pathogens within the microbial ecosystems. They stabilize the ecosystem, enhance biodiversity and avoid outbreaks of pathogens. However, more research is necessary to understand the microbiology of indoor environments. Currently used cleaning and hygiene strategies in built environments especially in hospitals and ICUs often promote multi-resistant pathogens instead of supporting beneficials. In future, it is important to re-think our understanding of necessary sterility and our relationship to our surrounding microbiomes. This "paradigm shift in ecology" is not only required for plants, humans (Jones, 2013) but also for our environment. Fortunately, "omics"-technologies guided by next-generation sequencing and microscopic techniques allow us now a much better assessment of them. Moreover, we can develop management strategies for beneficial interactions.

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