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Understanding the structure and function of tomato plant endophytes across generations

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TABLE OF CONTENTS

ABSTRACT

Plant-associated microbial communities represent the result of a long process of adaptation and coevolution. Within the multitude of microorganisms that inhabit plants, endophytes can asymptomatically reside within plant tissues and represent a tool of outstanding biotechnological potential for agricultural applications. To improve the efficacy of endophytes applications for the promotion of plant growth and resistance, the main objective of this thesis was the characterisation of the structure and dynamics of endophytic communities. Moreover, an additional aim was the biotechnological exploitation of less investigated areas of the plant microbiome, with a focus on the seed microbial community.

A combination of next-generation sequencing-based methods and bioinformatic analyses was used to visualize functional networks and identify key players in the microbiome of *Solanum lycopersicum*. The reconstruction of the bacterial assemblages associated with different tomato plant compartments by 16S rRNA gene metabarcoding revealed the structure of the tomato plant bacterial community. By coupling this information with the characterisation of bacterial isolates, it was possible to study distinct community patterns with specific plant growth promoting bacteria (PGPB) in each plant compartment. Several PGPB were tracked in the seed microbiome that was found to represent the main vehicle for the transmission of beneficial microorganisms to subsequent plant generations.

At the same time, the study revealed that the tomato plant bacterial community is strongly influenced by the soil composition, regardless from the plant genotype. Conversely to what was observed among bacteria, the dynamics of archaeal communities in tomato plants showed a remarkable influence of the plant genotype. Nevertheless, archaea were observed to play only a marginal role in seeds. Finally, studying the modifications induced by abiotic and biotic stress on the plant microbiome, it was observed that different stressors could modify the plant microbial assemblage to different degrees. While major modifications were observed following high salinity and *Verticillium dahliae* induced stress, the modifications induced by drought and *Fusarium oxysporum* were observed to have a minor impact on the plant bacterial community.

This improved understanding of the fundaments of transmission and dynamics of plant endophytes represents a step forward for their employment in agriculture. In a world challenged by growing population and climate change, the employment of efficient biological control agents represents a valuable tool for increasing crop yield while reducing the use of agrochemicals.

INTRODUCTION

Microbial Endophytes

The plant holobiont

Microbial communities represent a stable component of nearly every environment and macro-organism. In recent years it is becoming clear that these complex microbial assemblages influence virtually every aspect of the development, growth and health of the host organisms (Foster at al., 2017; Simon et al.,2019). In light of this, the neo-Darwinistic hologenome theory of evolution bases its roots on the concept that the unit of selection in evolution is represented by the host plus all its symbiont microbiota: the holobiont (Margulis, 1991). In other words, the host and its contiguous microorganisms are so co-dependent that their evolution cannot be considered self-standing but rather as an interconnected evolutionary path. In this framework, after a long process of adaptation and coevolution, the plant microbiota represents as a key determinant for plant health and productivity (Philippot et al., 2013; Berg et al., 2016; Yan et al., 2017). These microbial communities, that inhabit all plant compartments, have the ability to contribute multiple aspects to the functioning of the plant holobiont during the entire vegetative cycle of the plant (Vandenkoornhuyse et al., 2015). At the same time, plants can "select" their microbiome in order to have beneficial bacterial colonizers, including those living within the plant tissues (Hardoim et al., 2008; Marasco et al., 2012; Santoyo et al., 2016) . Plants cohabit with archaea, bacteria, fungi, and protists that inhabit all its compartments (Hassani et al., 2018) and especially the rhizosphere that represent a hotspot for microorganisms (Buée et al., 2009).

Endophytic microorganisms

Within the multitude of microorganisms that surround and populate habitats related to plants, the term "endophyte" generally refers to those microorganisms that can asymptomatically reside within plant tissues for at least a part of their life cycle (Hardoim et al., 2015). They were described at first in 1866 by De Bary (De Bary, 1866) as "any organism occurring within plant tissues" but, back then, only considered as contaminants. In more recent times, the definition of these microorganisms is still controversial and a series of definitions is concurrent due to the broad inclusivity of the term. The definition, that for its simplicity is the most applicable, has been proposed by Hallman and colleagues in 1997 and defines the term "endophyte" as encompassing "those bacteria that can be isolated from surface-disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant" (Hallmann et al., 1997).

While the molecular foundations of the endophytic life-style are not yet fully understood, since no clear evidence of host-specificity in these interactions has yet been discovered, it is thought that different endophytes might use similar genes to colonise their hosts. Endophytes can penetrate plant tissues using different mechanisms and through different entry points. The colonisation can occur as summarized by Hallmann and colleagues (1997) through: natural openings as stomata and lenticels; tissues discontinuities as wounds and root cracks; specific tissues such as germinating radicles and root hair cells (Hallmann et al., 1997). In general, the penetration through the root tissues is believed to represent the main colonisation stream. At the same time, some bacteria can actively penetrate the plant tissue with the production of cell wall degradative enzymes (Quadt-Hallmann et al., 1997). Ultimately, as discussed in this dissertation, an alternative way for the establishment of the endophytic relationship is represented by vertical inheritance. Microorganisms have in fact been observed to be transmitted to the offspring plant by the seed (Bergna et al., 2018 - Publication II).

In a study published in 2014, Ali and colleagues identified a total of 40 putative genes potentially involved in these interactions (Ali et al., 2014). The functions of these genes have been summarised in the following categories:

- Transporter related proteins. With these molecules, endophytes are able to transport plant-synthesized nutrients (mainly carbohydrates and amino acids) present in the plant endosphere or that have been exuded into the rhizosphere.
- Plant polymer degradation/modification. While these genes are constantly associated to bacterial and fungal phytopathogens for the degradation of cell walls, in non-pathogenic bacteria these enzymes seem to be connected with the exploitation of different sugar substrates.
- Transcriptional regulator. These genetic elements interact with the plant cellular metabolism in an interconnected way. In fact, transcriptional regulators could be involved in the activation of several systems for host defence. This can allow the bacterium to effectively colonize the plant tissues.
- Detoxification and redox potential maintenance. These genes are thought to relate to the necessity to withstand the plant environment after the colonisation.
- Secretion and delivery system. These proteins are predicted to be involved in the endophyte transition from free-living (in the soil) to the endophytic life style (within the plant). Interestingly, many of these proteins could play active roles in infection, virulence, and pathogenicity (Reinhold-Hurek & Hurek, 2011). While certain proteins

are present and characteristic for several bacterial endophytes other classes of secretion and delivery system proteins are specific to pathogenic bacteria and absent in endophytes (Downie, 2010).

While the experimental characterisation of these genes for endophytic colonisation is still undergoing, it is clear that endophytes and plant pathogens share a high degree of their genetic composition. For this reason it is suggested that, from a genetical point of view, bacterial endophytes can be considered as "disarmed pathogens" (Krause et al., 2006). They possess all the genetic machineries necessary to invade the host, exactly as for a pathogen, but they don't trigger any pathogenic state due to a lack of compatibility with the host plant or for the lack of genes encoding virulence factors (Kogel et al., 2006; Brader et al., 2017).

At the same time, the colonisation of the plant root is as well mediated by the plant itself. The plant mediates the colonisation with the help of root exudates and plant hormones such as ethylene, salicylic acid, and jasmonic acid. Root exudates can interplay with microorganisms by overexpressing rhizosphere competence genes and promote the colonisation (Shidore et al., 2012). At the same time, phytohormones part of the plant immune system can act to maintain microbial homeostasis and to control the abundance of commensal microorganisms within plant tissues. Interestingly, the variations in the quality and quantity of these effectors that distinguish plant genotypes have been found potentially determinant for the colonization of specific taxa (Long et al., 2010; Carvalhais et al., 2015; French et al., 2019).

Beneficial effects of bacterial endophytes

Endophytic bacteria have the ability to positively influence the growth of the plant host**.** This beneficial influence, that is not restricted to only this category of microorganisms, can be operated with both direct and indirect mechanisms (Berg et al., 2016). On the one hand, bacterial endophytes can directly support the acquisition of essential nutrients such as nitrogen, phosphorus and iron. At the same time, as previously seen in the description of the genes that characterise the endophytic lifestyle, bacterial endophytes possess the ability to modulate the expression of key plant genes. The main target of these genes' modulators are the levels of the following key phytohormones: auxin, cytokinin, gibberellin (all upregulated) and ethylene. These capacities play a crucial role in the host, especially during critical stages (as seed germination) and can confer resistance against abiotic stressors. On the other hand, bacterial endophytes can indirectly promote plant growth by protecting the plant against pathogenic

infections. These microorganisms can outplay phytopathogens by competing for the substrate or with the production of bioactive metabolites contrasting the phytopathogens. Other mechanisms for the indirect plant growth promotion entail: the production of cell wall-degrading enzymes, the reduction of plant ethylene levels, the induction of the plant systemic resistance (ISR) (Whipps, 2001; Compant et al., 2005; Berg, 2009; Santoyo et al., 2016).

Agricultural applications of plant endophytes

Modern agricultural practices primarily employ chemical based fertilizers and agrochemicals for the control of pathogens and pests. With the increasing agricultural demand, the research for greener alternatives has seen a large increment in the demand of microorganisms with plant growth–promoting (PGP) traits employable on a large scale (Berendsen et al., 2012; Santoyo et al., 2016). These microorganisms were initially addressed as mainly soil- and rhizosphere-derived that predominantly colonise the plant hypogeal compartments (10^5–10^7 CFU per gram of fresh root). Due to their greater abundance in the plant root system, most cultivation dependent and independent researches focused on the isolation and characterisation of plant growth promoting rhizobacteria (Spaepen et al., 2009; Bulgarelli et al., 2013). At the same time, in comparison to bacteria that exclusively bind to plant roots, the more intimate interaction that endophytes establish with the host results in superior plant growth promotion effects under a wide range of conditions (Ali et al., 2012). For this reason, the interest in these microorganisms as biotechnological solutions for agriculture is profound and increasing since the end of the 20th century (Berg et al., 2013; 2016).

On the other hand, while several endophytes showed enhanced plant growth promoting properties against biotic and abiotic stresses under laboratory conditions (Kate et al., 2016), their performance decreased when subjected to the uncontrollable variations of field conditions (Serfling et al., 2007).

Years of research for competent and applicable endophytes gave rise to the genotypical and phenotypical characterisation of these microorganism on a large scale. The most promising bacterial endophytes isolated from inner plant tissues with plant growth promoting traits belong to three major phyla: *Actinobacteria*, *Proteobacteria* and *Firmicutes*. At lower taxonomical level, as discussed by Malfanova (2013), the most studied endophytes with PGP traits account members of the following taxa: *Acetobacter* (renamed as *Gluconobacter*) (Bertalan et al., 2009), *Azoarcus* (Krause et al., 2006), *Bacillus* (Deng et al., 2011), *Burkholderia* (Weilharter et al., 2011), *Enterobacter* (Safiyh Taghavi et al., 2010), *Herbaspirillum* (Pedrosa et al., 2011),

Pseudomonas (Taghavi et al., 2009), *Serratia* (Taghavi et al., 2009), *Streptomyces* (Suzuki et al., 2007) and *Stenotrophomonas* (Ryan et al., 2009).

The holobiont concept and the plant microbiome

Endophytes as a central part of the plant holobiont

Endophytic microorganisms form complex microbial communities within the plant endosphere (Lindow & Brandl, 2003; Buée et al., 2009). Although biotic and abiotic factors can alter most microbiomes, endophytic communities of different plants often share similar traits. In 2015 Hardoim and colleagues performed an in-depth taxonomical analysis of endophytic 16S rRNA gene sequences present in peer-reviewed journals (Hardoim et al., 2015). Thanks to this analysis it has been possible to infer the composition of the plant bacterial endophytic community among plants. This bacterial assemblage shows a predominance of *Proteobacteria* (mainly *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*), *Actinobacteria*, *Firmicutes* (mainly represented by the *Bacilli* class) and *Bacteroidetes*. In addition, several other taxa are constantly present in the community but in low abundances (19 taxa with less than 1 %). Similarly, *Archaea* represent only a small fraction of the microbial community. In order to provide a clearer idea of the most important taxa within endophytic communities, the candidate proposes a connection between the abundance of main taxa found by Hardoim and colleagues and the information of dominant taxa retrieved for major plants employed for agricultural and research purposes (Table 1): *Oryza sativa* (Sessitsch et al., 2012), *Triticum aestivum* (Robinson et al., 2016), , *Solanum lycopersicum* L. (Bergna et al. 2018), *Saccharum* sp. (De Souza et al., 2016), *Zea mays* (Correa-Galeote et al., 2018), *Solanum tuberosum* (Manter et al., 2010), *Arabidopsis thaliana* (Lundberg et al., 2012).

Table 1. Reconstruction of the microbial endophytic community across plant hosts as reported by Hardoim and colleagues (2015). This information related to the ability of these microorganisms to dominate the root endosphere of major crops. Legend: DT = indicate the "Dominant Taxa" for each specific host (vertical). The letters link the study in which the taxa dominance has been observed for each plant: a (Sessitsch et al., 2012), *b* (Robinson et al., 2016), c (Bergna et al. 2018), d (De Souza et al., 2016), e (Correa-Galeote et al., 2018), f (Manter et al., 2010), g (Lundberg et al., 2012).

Domain	Phylum	Class	%	*Oryza sativa*	*Triticum aestivum*	*Solanum lycopersicum*	*Saccharum* sp.	*Zea mays*	*Solanum tuberosum*	*Arabidopsis thaliana*
Bacteria	*Acidobacteria*		0.72%							DT
	Actinobacteria		**19.88%**		DT	DT		DT	DT	DT
	Armatimonadetes		0.08%							
	Bacteroidetes		**6.29%**		DT	DT	DT	DT	DT	DT
	GOUTA4c		0.01%							
	ODc		0.08%							
	TM7c		0.03%							
	Chlamydiae		0.11%							
	Chlorobi		0.07%							
	Chloroflexi		0.04%							
	Cyanobacteria		1.39%							
	Deinococcus-Thermus		0.10%							
	Elusimicrobia		0.01%							
	Firmicutes	*Bacilli*	**15.41%**	DT	DT	DT		DT		
		Clostridia	0.93%							
	Fusobacteria		0.04%							
	Nitrospirae		0.04%							
	Planctomycetes		0.07%							
	Proteobacteria	*Alpha-*	**18.20%**	DT	DT	DT	DT	DT	DT	DT
		Beta-	**10.02%**							
		Delta-	0.35%							
		Epsilon-	0.04%							
		Gamma-	**25.56%**							
	Spirochaetae		0.04%							
	Tenericutes		0.03%							
	Verrucomicrobia		0.08%							
Archaea	*Euryarchaeota*		0.31%							
	Thaumarcheota		0.08%							
				a	b	c	d	e	f	g

The tomato plant as a model organism

The tomato plant (*Solanum lycopersicum* L.) represents one of the most important vegetables for the global market with an estimated production of 177 million tons per year (FAOSTAT 2016). The consumption of this products, as raw or industrially processed, has over the years been linked to a plethora of benefits for human health (He et al., 2006), mainly due to the presence of numerous bioactive compounds (e.g. folate, ascorbate, polyphenols, carotenoids) (Tommonaro et al., 2013). While the demand for this vegetable is increasing, both its production and its processing are associated with enormous financial losses that can reach up to 45% (FAO 2015). For what concerns the production on the field, the major factor that significantly lower the yields of this crop is represented by microbial pathogens as *Fusarium* sp., *Rhizoctonia* sp., and *Verticillium* sp. (Oerke, 2006).

In addition to its economical relevance and to its relative ease of use under laboratory conditions, the susceptibility of this plant to phytopathogens has contributed to its extensive employment in studies focusing on disease resistance. For this reason, the tomato plant is one of the main model plants for the study of the biological control of these diseases (The Tomato Genome Consortium, 2012; Kwak et al., 2018). Due to a limited understanding of the plant microbiome and of its potentiality, the study for the biological control of these diseases started only recently. In fact, the selection of resistant cultivars represented for decades, aside from the employment of agrochemicals, the main means of protection against phytopathogens (Huet, 2014). Only with modern investigative methods and with extensive studies of the microorganism-mediated plant-growth-promotion, the biological control of these diseases has started to be taken into consideration (Mendes et al., 2011; Scherwinski et al., 2008).

Due to accumulating evidence that the underground plant compartments represent the core of the plant resistance, the isolation and characterization of plant growth-promoting bacteria first, and the study of the plant microbiome later, have been mainly focusing on the hypogeal compartments. While this practice allowed the isolation and biotechnological employment of highly valuable plant growth promoting rhizobacteria (PGPR) (Yan et al., 2003; Upreti & Thomas, 2015; Liu et al., 2017), for decades the above ground plant compartments, the plant endosphere and seeds have been constantly ignored.

In the last decade, studies on tomato plants started to define these plant compartments as habitats, in which the microbiota is less exposed to external effects (*e.g*. soil) (Ottesen et al., 2013). For this reason, the resulting microbial assemblage is represented by microorganisms

recruited and filtered by the plant (Truyens et al., 2014) that are able to establish an intimate relationship with the host.

The meta-omics revolution in microbial ecology

The study of microbial communities has always been characterised by technological hurdles. In fact, the objective of characterising every microorganism present in a microhabitat represents a great technological task. With the advances in high-throughput sequencing techniques (Next Generation Sequencing: NGS) and the increasing number of microbial culture libraries that characterised the last decade, it is now possible to map the microbial community of interest in a fast and cost-effective way (Mendes et al., 2011; Bulgarelli et al., 2012; Rybakova et al., 2016; Hugerth & Andersson, 2017).

As of today, the most employed applications for the investigation of plant microbial communities are DNA metabarcoding and shotgun metagenomics. DNA metabarcoding relies on a combined mass PCR amplification and sequencing of a single marker gene (DNA barcode). These applications represent powerful tools for the in-depth characterisation of microbial communities and for the detection of indicator species and community shifts. On the other hand, shotgun metagenomics relies on the random fragmentation and sequencing of genomic DNA (shotgun metagenomics). By reconstructing the set of genes of the microbial community, metagenomics based applications allow to study both composition and the functional capabilities.

While the advantages connected with these applications facilitated our understanding of the composition and dynamics of plant associated microbiomes, it is also important to underline the limitations that these technologies have. The reliance on in-silico data alone represent the main disadvantage for NGS-based methodologies since they can only infer phenotypical characteristics. For this reason, an approach that combines both cultivation dependent and independent techniques is indispensable for a complete understanding of the ecological role of these microorganisms and, most of all, for their biotechnological applicability.

Discovery of novel niches: the seed microbiome and biotechnological prospects (Publication I)

Among microorganisms residing within the plant tissues, a subgroup has shown to be able to also colonise the seeds of the plant and to be vertically transmitted to the successive plant generation (Vannier et al., 2018). These microorganisms represent the result of a plant mediated

mechanism of selection that takes in consideration abiotic and abiotic factors. The true importance of these microorganisms is manifested by their crucial role for the development of the offspring plant where they represent the starter microbiome (Puente et al., 2009; Johnston-Monje & Raizada, 2011; Hardoim et al., 2012; Adam et al., 2018). Because of their crucial role in the developing plant and their high applicability in agriculture and horticulture, the study of these microorganisms is attracting attention and funding (Berg & Raaijmakers, 2018). While the characterization of the endophytes of this valuable plant compartment is progressing very rapidly, due to the singular nature of this plant compartment several technological setbacks are limiting its study.

The complexity of this subject, with a focus on plant seed endophytes, is hereby illustrated via a book chapter contribution to: "Endophyte Biotechnology: Potential for Agriculture and Pharmacology" (Publication I) authored by the candidate and colleagues. This book chapter is entitled: "Analysing seed endophytes for biotechnology" and provides a detailed review of the main methodologies that are employed as of today and that will represent the fundaments for the future of endophytes research and application.

AIM AND SUMMARY OF THE THESIS

The aim of this thesis is the characterization of the plant microbiome for its biotechnological exploitation for the promotion of plant growth and resistance. As previously described in the introduction of this dissertation, the endophytic microbiome is a complex assemblage of microorganisms and many are the factors that can modify its composition. The understanding of the key components of the microbiome, of its dynamics and of the nature of its modulators, represents the starting point for the successful application of these microorganisms. At the same time, the study of seed endophytes present in this dissertation represents one of the first attempts for a deepening characterisation of this valuable micro-niche and revealed several biotechnological prospects.

The characterisation of the tomato plant bacteriome and of its hidden niches (Publication II)

The tomato plant bacteriome and its modulators

To study the dynamics of these communities the first aim was the reconstruction of the bacterial assemblages associated with different plant compartments (using the tomato plant as model organism). The bacterial community of each plant habitat was simultaneously analysed by amplicon sequencing of the 16S rRNA gene and by the extensive isolation of plant associated bacteria. With a first analysis at low phylogenetic resolution (phylum level), we found high cross-habitat similarities, with *Proteobacteria* being the dominant phylum across the whole plant system, as characteristic for plant endophytic communities.

This reconstruction was also used to study the impact that potential microbiome modulators have on the bacterial communities. We therefore studied how the plant bacterial communities are structured among different cultivars and in presence of different soil substrates. Interestingly, we observed that the composition of the bacterial community of the soil plays a strong role in the shaping of the bacterial community of hypogeal plant compartments (rhizosphere, root endosphere). What is more, this behaviour was observed in both tomato cultivars where it was clear that the influence of the soil played a stronger role compared to the plant genotype. However, a gradient was also observed in this effect. In fact, this influence progressively lowered from the rhizosphere to the root endosphere and finally to the seeds.

Tracking beneficial endophytes across the tomato plant

By merging the information from the community reconstruction and the isolation of PGPB, we were able to track those bacterial taxa that showed PGP traits within the plant bacterial communities. As a result, we observed that each plant compartment was characterised by distinct community fingerprints and harboured different PGPB. While the community of soil, rhizosphere and root endosphere were characterised by high diversity, the plant seeds represented a selective community and a major vehicle of transmission for PGPB across generations. For this reason, these findings introduce the concept of the necessity to shift the attention from the soil to the plant, more specifically to the seed, as a novel PGPB isolation strategy.

The seed microbiome: its capacities and its evolutionary relevance

This plant compartment was characterised by taxa that, across the plant-system, have been detected only in this habitat. These bacteria (e.g. *Acidobacteria*) were mostly associated with plant-growth-promoting traits connected to the regulation of seed germination and of roots and shoots biomass: the seed represents a plant structure that activates in contact with the soil. For this reason, although the underlying mechanisms are currently unknown, the seed microbiota could represent a soil specific element specifically shaped under influence of the plant.

In a broader context, microorganisms able to also colonise the plant seeds and to be vertically transmitted to the successive plant generation represent an already selected portion of the plant microbiome to the developing seedling. This phenomenon of vertical inheritance of the microbiome is not novel, in fact it was already shown that mammals can also pass a part of their microbiome containing beneficial microorganisms to their offspring during birth.

From an evolutionary point of view, the vertical transmission of selected beneficial microorganisms represents a “continuity of partnership” between the host and its symbionts (Vandenkoornhuyse et al., 2015; Vannier et al., 2018). The idea that the plant holobiont has the capacity to select and vertically transmit beneficial endophytes to the offspring represents the essence of the hologenome theory of evolution. In fact, thanks to these aspects of Lamarckism (the capacity to acquire and transmit new characteristics), the selection and vertical transmission of seed endophytes also represent the bridging of the strict genotype-phenotype dichotomy of neo-Darwinism underlining the role of both cooperation and competition within the holobiont and with other holobionts (Rosenberg et al., 2009).

The role of *Archaea* in the tomato plant microbiome

(Manuscript I)

The tomato plant archaeal community

In a study characterised by an experimental setup similar to the previously described study (Publication II), we reconstructed the tomato plant archaeal community. With this study we aimed to understand the role that these microorganisms play in the plant and to understand their potential role in confined niches such as the seed.

In this study we observed that the soil quality is a main determinant for the archaeal community structure in bulk soil. Marked differences were found in both the archaeal diversity and in the abundance of dominant archaeal taxa with relevant activities for soil ecology. For example, ammonia oxidizing archaea (AOA), belonging to the phylum of *Thaumarchaeota*, and members of the phylum *Euryarchaeota* (consists for the most part of methanogens such as *Methanomicrobia*) were found linked to different soil qualities properties.

In contrast with what we observed within the bacterial community, no instances of soil driven effects were detected in rhizospheric archaeal communities. On the contrary, we found a remarkable effect connected by the plant genotype highlighting a great difference of behavior between bacteria and archaea on the rhizoplane. The explanation of this phenomenon is related with the whole process of rhizosphere formation. The establishment of rhizospheric communities represent a highly dynamic process in which bacteria represent the main actors from the beginning to the end. Due to their necessity of residing in biochemically stable environments and due to their high level of syntropy with bacteria, these microorganisms appear only in the late stages of plant growth and can be addressed as “rhizosphere late colonisers”. For this reason, archaeal taxa in the rhizosphere appear to be more bound to their interactions with bacterial communities rather than to their ability to adapt to soil characteristics. In this context, our finding of high differences in the rhizosphere effect among plant genotypes represents a proof of the high impact that the adjacent plant root system plays on archaeal communities. At the same time, we cannot yet determine whether this effect is direct or not: plant root exudates and plant phytohormones could directly act on archaea, or they could act on the bacterial or fungal communities at first and indirectly modify the archaeal community setup.

The role of archaea in tomato plant seeds

As previously shown, the seed can represent a key vehicle for the vertical transmission of beneficial bacteria across generations. Due to the important role that archaea play in soil nutrient cycles, we initially hypothesised that, as for bacteria, archaea would have been

conveyed by the plant to the seed where they could have helped the germination and development of the offspring plant. In contradiction with this initial hypothesis, while archaea have been found to be present in the seed, no archaeal patterns have been detected for supporting the idea of the vehiculation of these microorganisms from a generation to the next.

Within the previously described context of the forming rhizosphere, it is crucial that the plant transmit, within this confined environment, only those microorganisms that are crucial for the plant germination and initial rhizosphere formation. As we saw that archaea have no important roles in the formation of the rhizosphere, it is logical that the plant does not transmit microorganisms that cannot help the seedling in the initial developmental stages. The presence of archaea detected within the seed is therefore regarded as of bystander microorganisms that possibly based on syntrophic relationships with bacteria.

The influence of biotic and abiotic stressors on the plant microbiome
(Manuscript II)

In order to better understand how plant microbial communities are shaped by microbiome modulators, we studied the effects of both abiotic and biotic factors on the tomato plant microbiome. Two different pathosystems, represented by major phytopathogens (*Verticillium dahliae* and *Fusarium oxysporum*), have been studied in combination with abiotic stressors: drought and hyper salinity (all conditions under which plants have been grown are reported in the Figure 1).

Each of these abiotic and biotic factors, all responsible for microbiome modifications, have a small or high impact on the microbiome. At the same time, when applied together, it is clear how the modifications induced by the most severe stressor can prevail. As possible to see in the visualisation of these microbiome shifts (Figure 1) drought stress only mildly modified the composition of the plant microbiome. Similarly, the influence of *Fusarium oxysporum* on the microbiome resulted in a partially altered microbiome that did not uniformly responded to this condition (as represented by the scattered distribution in the PCoA visualisation). On the other hand, plant affected by salt stress or *Verticillium dahliae* were inhabited by microbiomes that were deeply modified in a very characteristic way when compared to control samples. Each of these abiotic and abiotic factors determined a very specific microbiome rearrangement as a clear response to the stress. Even in combination with other stressors, as drought and *Fusarium oxysporum*, the resulting microbiome assemblage was aligned with the response to these major stressors. In addition, a similar hierarchy of microbiome modifications was observed also

between these two major biotic and abiotic stressors: plants affected by both these stresses had a microbial assemblage characteristic for high salinity.

With these results we demonstrated how different factors can modify the plant microbial assemblage to different degrees. Furthermore, the impact of these stressors on the plant microbiome follow a strict hierarchy of influence that determines the nature of the modifications of the microbiome.

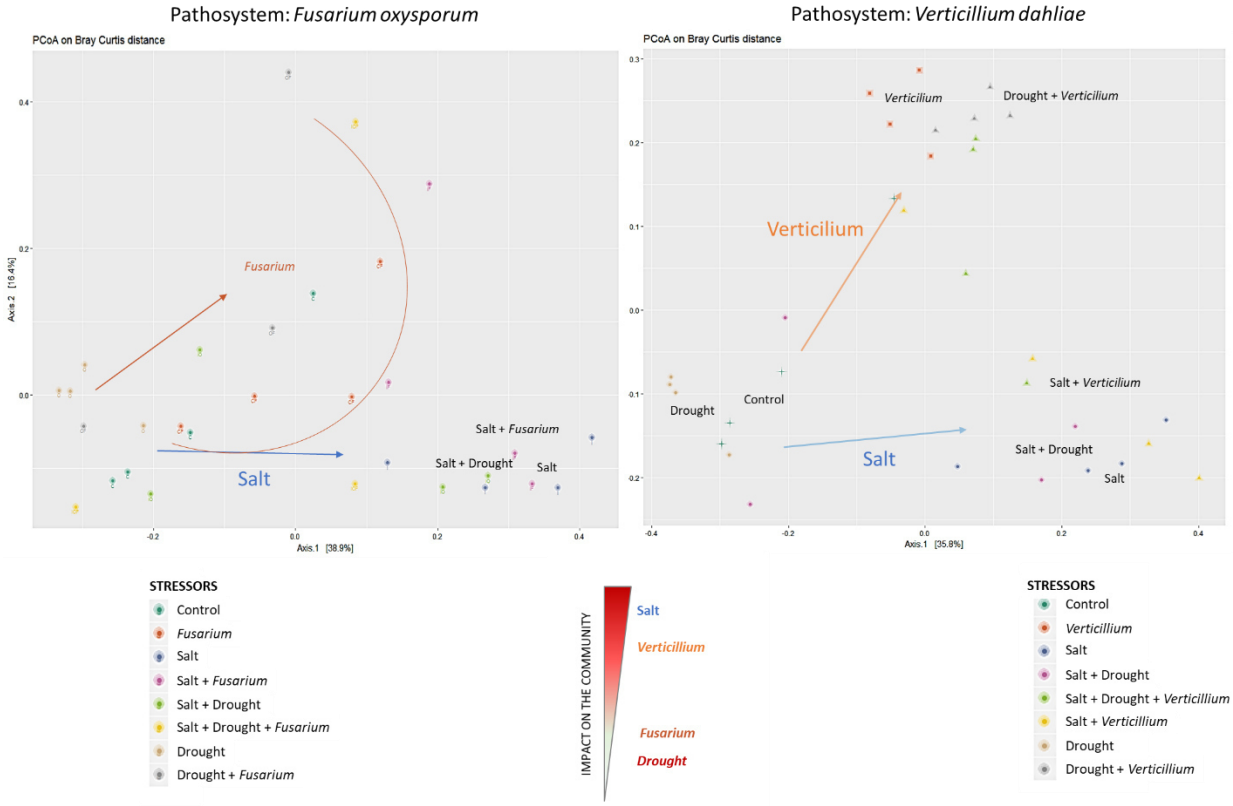

Figure 1. Bacterial community modifications between control and stressed plants visualized with a principal coordinate analysis (PCoA). These modifications have been summarised by dividing the two different studied pathosystems: *Fusarium oxysporum* and *Verticillium dahliae*. All the here displayed factors have also been arranged in a ranking system based on their impact on the plant microbiome and on the hierarchy among these modifications.

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Publication I

Analysing Seed Endophytes for Biotechnology

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Abstract

Seed endophytes play a crucial role during the entire life cycle of plants due to their ability to promote germination and plant growth and provide defence against biotic and abiotic stress. The increasing interest related to these microorganisms for applications in sustainable agriculture requires the use of a wide spectrum of techniques to investigate their ecological role and to exploit their biotechnological potential. While the isolation of microorganisms is the most straightforward method to characterize and select microorganisms, molecular techniques represent an advantageous option for the discovery and tracking of uncultivable microbial species. This chapter shows that the concomitant employment of cultivation-dependent and cultivation-independent techniques represents the most sophisticated approach for the study of endophytic communities. In addition to a general assessment of developments in this field, the most frequently used tools are described in detail. Moreover, their possible integration as shown in various studies targeting seed endophytes is highlighted. We expect that novel products for biotechnology will become more feasible in the future due to the recent technological and methodological developments.

Introduction

Endophytes are defined as microorganisms that are able to asymptomatically reside within plant tissues for at least a part of their life cycle (Hardoim *et al.*, 2015). While these microorganisms are primarily known for their ability to enhance plant growth and defence, they also represent a significant source of natural metabolites and bioactive compounds of biotechnological interest (Tan and Zou, 2001; Gunatilaka, 2006; Aly *et al.*, 2013; Martinez-Klimova *et al.*, 2017; Gao *et al.*, 2018). Among endophytes, a subgroup of microorganisms able to reside in plant seeds and to be vertically transmitted to the successive plant generation represent a crucial starting inoculum of beneficial microbes for

improved plant development and health (Puente *et al.*, 2009; Johnston-Monje and Raizada, 2011; Hardoim *et al.*, 2012). A better understanding of these microorganisms and of their vertical transmission will enhance the opportunities to exploit beneficial microbe-plant interactions in agriculture and horticulture (Berg and Raaijmakers, 2018).

The understanding of the plant microbiome, including endophytic communities, was revolutionized by the technological advancements in DNA sequencing and computational technologies of the last decade (Mendes *et al.*, 2011; Bulgarelli *et al.*, 2012; Rybakova *et al.*, 2016). However, the study of seed microbiomes can be considered to be still in its infancy as research efforts for their characterization are relatively recent (Barret *et al.*, 2015; Klaedtke *et al.*, 2016). In the past, seed microbiology was focused on seed-borne pathogens and their detection and control (Munkvold, 2009). Due to the dormant phase of many microorganisms inside of seeds, knowledge about seed endophytes obtained by cultivation was limited for a long time. Now it is possible to map the microbial community of interest with fast and cost-effective solutions by next-generation sequencing (NGS) technologies (Hugerth and Andersson, 2017), but it is important to emphasize that also such technologies have limitations when studying endophytes. For this reason, several techniques are potentially employable to identify and characterize seed endophytes. All have advantages and disadvantages, but the combined results are indispensable for a holistic understanding of the ecological role of these microorganisms and their biotechnological applicability. In this chapter we provide a summary of the main methodologies that are employed today and that represent the fundaments for the future of seed endophytes research. A schematic representation of the most commonly used approaches is provided in Fig. 4.1 and includes advantages and disadvantages connected with each strategy. In addition, an overview is provided and includes a selection of publications (Table 4.1) related to seed endophytes as well as methodologies that have been employed in these studies. The following sections include the most frequently applied approaches to study seed endophytes. Integrative approaches that combine different methodologies can more likely deliver deepening insights into the ecology and functioning of seed endophytes.

Table 4.1. Overview of current studies on the seed microbiome with the main endophytes-related methodologies employed.

Plant species/cultivar	Reference	Technologies employed
Maize (Zea mays)	Johnston-Monje and Raizada, 2011	Isolation and phenotyping, Cloning, TRFLP.
Rice (Oryza sativa)	Hardoim et al. 2012	Isolation of seed endophytes, PCR-DGGE.
Brassica and *Triticum* species	Links et al. 2014	*cpn60* metabarcoding, qPCR quantification of microorganisms of interest, isolation of seed endophytes and characterisation.
Bean (Phaseolus vulgaris)	Klaedtke et al. 2015	16S and ITS metabarcoding.
Radish (Raphanus sativus)	Rezki et al. 2016	Isolation of seed endophytes, qPCR, 16S, *gyr*B and ITS1 metabarcoding.
Pumpkin (*Cucurbita pepo)*	Adam et al. 2016	16S Metabarcoding.
Maize *(Zea mays)*	Johnston-Monje et al. 2016	TRFLP and 16S metabarcoding.
Sueda salsa	Qin et al., 2016	ITS1 and ITS2 metabarcoding, fungal isolation and *in-planta* characterisation.
Quinoa	Pitzschke, 2016	Microscopy, isolation of seed endophytes.
Cucurbitaceae species	Khalaf and Raizada, 2016	Isolation of seed endophytes and 16S rRNA gene fingerprinting.
Pepper (*Capsicum annuum*), Soybean (*Glycine max*), *Triticum aestivum*	Mitter et al. 2017	qPCR, 16S metabarcoding.
Malvaceae species	Irizarry and White, 2017	Isolation of seed endophytes and *in planta* characterisation.
Oilseed rape (Brassica napus)	Rybakova et al. 2017	16S metabarcoding, qPCR, isolation of seed endophytes and characterisation, FISH-CLSM microscopy.
Soybean (Glycine max)	(Huang et al., 2018)	ITS1 and ITS2 metabarcoding, bacterial isolation and *in-planta* characterisation.
Rice (Oryza sativa)	Walitang et al. 2017	Isolation of seed endophytes and functional and genetical characterisation of isolates.

Radish (Raphanus sativus)	Rezki et al. 2018	*gyr*B and ITS metabarcoding.
Muskmelon (Cucumis melo)	Glassner et al. 2018	Scanning electron microscopy (SEM) and CLSM DOPE-FISH.
Browntop millet (Brachiaria sp.)	Verma and White, 2018	Isolation of seed bacterial endophytes and *in vitro* characterisation.
Cucurbitaceae species	Khalaf and Raizada, 2018	Isolation of seed bacterial endophytes and *in vitro* characterisation.
Bean (Phaseolus vulgaris)	Malinich et al. 2018	Cloning, isolation of seed bacterial endophytes, qPCR.
Bean (Phaseolus vulgaris), Radish (Raphanus sativus)	(Torres-Cortés et al., 2018)	Shotgun Metagenomics, bacterial isolation and *in-planta* characterisation.
Barley (Hordeum vulgare)	Rahman et al. 2018	Isolation of seed endophytes, 16S metabarcoding, FISH-CLSM.
Crotalaria pumila	Sánchez-López et al. 2018	16S metabarcoding.
Phragmites australis	White et al. 2018	Isolation of seed bacterial endophytes and *in planta* characterisation.
Salvia miltiorrhiza	Chen et al. 2018	16S and ITS metabarcoding.
Ground-ivy *(Glechoma hederacea)*	Vannier et al. 2018	16S and 18S metabarcoding targeting bacteria/archaea and fungi.

Isolation of Seed Endophytes

Seed surface sterilization

In order to study or extract seed endophytes from non-endophytic microorganisms, surface sterilization is required for removing microbes on the seed surface (Fig. 4.1). This initial and yet crucial step can be problematic, as the sterilization should be achieved without destructive effects on the host tissue. The procedure normally entails two washing steps: shaking in sodium hypochlorite and a final soaking in ethanol. Concentrations of sodium hypochlorite and ethanol and washing/shaking time vary

based on the seed texture: we report valuable examples of seed surface sterilization methodologies for prominent crops.

- Maize (*Zea mays* L.) seeds can be subjected to two consecutive washing steps with 3% sodium hypochlorite for 10 min followed by 10 min washing in 95% ethanol for 10 min (Johnston-Monje and Raizada, 2011).
- Rice (*Oryza sativa* L.) seeds are surface-sterilized by washing for 25 min in a saline solution containing 0.12% sodium hypochlorite (NaClO) and 0.15% sodium hydroxide followed by a washing step with 2% sodium thiosulfate to remove surface-adhered NaClO (Hardoim *et al.*, 2012).
- Quinoa (*Chenopodium quinoa* Willd.) seeds are surface-sterilized by two consecutive incubations in 70% ethanol for 5 min followed by three washes in distilled water (Pitzschke, 2016).
- *Cucurbitaceae* seeds are surface-sterilized by washing them twice for 5 min in a sodium hypochlorite solution with a specific concentration (2.5–3.5%) based on seed coating texture for 5 min. After rinsing with autoclaved distilled water, the seeds were again washed with 95% ethanol for 5 min (Khalaf and Raizada, 2016).
- Bean (*Phaseolus vulgaris* L.) seeds are surface-sterilized by immersion in 95% ethanol for 3 min, followed by immersion in 20% NaClO for 20 min, and rinsed with sterile distilled water three times (Malinich and Bauer, 2018).

Seed activation and extraction of endophytes

The most straightforward approach to study the biotechnological capacities of endophytic microorganisms is the isolation and cultivation of the living strains (Fig. 4.1). This approach has the goal of extracting the microorganisms alive and growing them under laboratory conditions to study their metabolic properties and capacities. From a technical point of view, the isolation of endophytes from seeds is more complicated than from other plant compartments due to the dormant state of this structure.

For this reason, most of the current techniques for isolating microorganism from seeds rely on an initial seed activation step under gnotobiotic conditions. To soften seeds and revive endophytic populations, washing and gently shaking the seeds is necessary; the liquid phase is usually distilled water or 0.85% NaCl. The soaking time, as for the surface sterilization, depends on the seed texture: 4 h is employed for rapeseeds (*Brassica napus*) (Rybakova *et al.*, 2017) while 48 h is necessary for maize seeds (Johnston-Monje and Raizada, 2011). With this process the seed switches from a dry and quiescent state to a hydrated and active state (Dekkers *et al.*, 2013) that enables endophytes to overcome dormancy and improves cultivation ratios and extraction by softening the seed. In order to extract the endophytic microorganisms from inner tissues of seeds, maceration is the preferred methodology. The softened activated seeds are grinded with autoclaved mortar and pestle and a liquid phase (buffer) is added (Berg *et al.*, 2013). This suspension is therefore plated in serial dilutions on the selected medium. Commonly employed media for isolating bacteria from plant tissues are tryptic soya agar (TSA), R2A and nutrient broth–yeast extract (Gardner *et al.*, 1982). For what concerns the isolation of fungi, standard media include PDA (potato dextrose agar), malt extract–peptone–yeast extract and biomalt agar (Philipson and Blair, 1957; Schulz *et al.*, 1995; Hallmann *et al.*, 2006).

Assessment of yet uncultivable microorganisms

A second limitation for the isolation of seed endophytes is the high portion of 'so far uncultivable' microorganisms. The presence of dormant cells and insufficiently optimized cultivation media represents the main impediments for the isolation of endophytes from plant and seed tissues (Torsvik and Øvreås, 2002; Eevers *et al.*, 2015). Even if molecular and cultivation-independent techniques have undisputable higher screening power, in order to deeply understand the physiology of an endophyte, the cultivation of the microorganism in the laboratory is still required (Stewart, 2012). For this reason, even if time-consuming and expensive, attempts in the cultivation of 'so far uncultivable' microorganisms of biotechnological relevance are being carried out. Since the limitations for cultivation of these microorganisms are sometimes due to their reliance on the interaction with other beneficial microorganisms or with the biochemical surrounding, co-cultivation with helper strains and the

recreation of the environment in the laboratory can sometimes result in their successful culturing (Ohno *et al.*, 2000; Nichols *et al.*, 2008).

Phenotyping

The main advantage of extracting living microorganisms from seeds lies in the possibility to directly test their properties in the laboratory. Several screenings have been developed in the last decade and can be adapted to the characteristics of the microorganism or of the plant. Shahzad *et al.* (2018) provided an almost complete summary of the functional attributes tested for both bacteria and fungi isolated from different seeds linking it to plant host and reference methodology. The assessment of microbial functioning can be tested *in vitro*, *in vivo* or *in situ* to assess the behaviour of the microorganism, respectively, without the host, inside the host tissues (also referred as *in planta*) or in a specific structure of the host. Specific assessments, e.g. the production of bioactive volatile compounds (Cernava *et al.*, 2015), can be integrated in deepening screenings based on the specific research question. These approaches represent the ground floor for the biotechnological employment of seed endophytes in several fields of agriculture. Endophytes are employable as:

- biofertilizers, for their ability to promote the acquisition of essential nutrients by, for example, solubilizing phosphorus, fixating nitrogen and producing siderophores that enhance iron uptake (Chhabra and Dowling, 2017);
- plant biostimultants, for the production of phytohormones and spermidine against abiotic stress (Berg, 2009); and
- biopesticides, for their ability to produce lytic enzymes, antibiotics, antimicrobial volatiles (Rybakova *et al.*, 2016).

From the biotechnological standpoint, it is evident that the cultivation of seed endophytes has great advantages, but also undeniable limitations connected with the impossibility to isolate the greatest portion of the seed endophytic community. For this reason, more investments are crucial for enhancing the cultivation efficiency from this promising plant compartment.

Molecular Techniques for the Analysis of Seed Endophytes

Since most endophytic microorganisms are uncultivable, studies of seed endophytes mostly rely on culture-independent techniques for their detection and identification (Liaqat and Eltem, 2016). Cultivation-independent techniques are primarily based on the extraction and analysis of target molecules (DNA, RNA, proteins, metabolites). The resulting sequences and information can be compared with microbial databanks to identify the microorganism's taxonomy or specific genes in its genome. In a similar way, the extraction and analysis of endophytic RNA and proteins can be used to identify active genes from living cells. While older techniques such as fingerprinting (e.g. terminal restriction fragment length polymorphism – TRFLP) and qPCR cannot provide deep and accurate insights at community level, NGS-based omics techniques are used to characterize highly complex microbial communities. Even if molecular techniques can represent powerful tools for the description of seed endophytes, they become powerless if used in poorly designed experiments. In fact, good experimental design, replication and appropriate methodology selection are essential for a precise interpretation of molecular data (Hallmann *et al.*, 1997; Prosser, 2010) regardless of the resolution power of the tool employed.

DNA extraction

In order to extract microbial genetic material from the seed endosphere, an initial surface sterilization is required. Similarly to the procedure that allows the isolation of endophytes, according to the protocol described by Bragina *et al.* (2012), seeds can be homogenized with mortar and pestle and suspended in 0.85% NaCl. In contrast to isolation approaches, homogenization can be also done by using liquid nitrogen as no living cells are required. Pellets containing seed endophytes are collected by centrifugation and then used for total community DNA isolation, e.g. using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). This specific kit can be employed for the isolation of endophytic DNA (Compant *et al.*, 2011; Rybakova *et al.*, 2017; Adam *et al.*, 2018; Bergna *et al.*, 2018) as it contains a lysing matrix that allows treating complex tissues. It is important to specify that regardless of the intensity of pretreatments, plant genetic material will represent most of the

DNA extracted with this and any other extraction procedures. Specific methodologies to target endophytic DNA are described in the following paragraphs.

Differentiation between living and dead cells

Since DNA can persist in the environment for relatively long periods after cell death (Josephson, 1993; Nocker *et al.*, 2007), surface sterilization alone would lead to the overestimation of the number of living cells. The use of propidium monoazide (PMA) after seed surface sterilization and homogenization leads to covalent binding of the photoreactive dye to accessible DNA from dead cells. This blocks the PCR amplification of genetic material belonging to dead microbial and damaged plant cells. Even if not yet broadly employed in the study of endophytes, this procedure enhances the probability to specifically amplify endophytic DNA (Mckinnon, 2016).

DNA metabarcoding

DNA metabarcoding or amplicon sequencing is a rapid method for biodiversity assessment of highly diverse microbial communities (Fig. 4.1). Advances in sequencing technologies made this technique a fast and cost-effective solution that can now be considered as a routine assessment for endophytic microbial communities (Barret *et al.*, 2015; Berg *et al.*, 2015; Vandenkoornhuyse *et al.*, 2015; Rybakova *et al.*, 2017; Adam *et al.*, 2018). The approach relies on two main steps: (i) a mass PCR amplification of a single marker gene (DNA barcode) from environmental DNA followed by (ii) the sequencing of the amplicons with a high-throughput sequencing platform.

Primers and PCR reaction

The ribosomal operon is broadly accepted as 'golden standard' for diversity assessment for its broad presence in all organisms and its favourable topology (Amann *et al.*, 1995). In fact, the alternating presence of conserved and highly variable regions is ideal for the construction of PCR primers, recognizing highly conserved regions for the amplification of the neighbouring variable regions (Sanschagrin and Yergeau, 2014). The genes employed for 16S, ITS, or 18S are to describe the composition of, respectively, bacterial, archaeal, fungal (Lindahl *et al.*, 2013), and micro-eukaryote communities (Lentendu *et al.*, 2014). The central role that this operon has been playing in microbial

diversity assessments brought to the construction of databases of unmatched size that is perfect for this methodology. Due to the difficulty to design universal primers, primers for amplicon sequencing are in continuous evolution. The reference project is the Earth Microbiome Project (EMP, www.earthmicrobiome.org), providing primers designed for sequencing on Illumina platforms extensively used for the study of plant-associated microorganisms. Here we provide a short description of the key primers used for amplicon sequencing and of alternatives for the study of endophytes.

16S rRNA

This gene is extensively used for bacteria and archaea. The most commonly used primer pair in combination with metabarcoding approaches is provided by the EMP (www.earthmicrobiome.org/) and named 515F-806R (Caporaso *et al.*, 2012). It targets the V4 region of the 16S SSU rRNA gene. In addition to this primer pair, also primer pairs targeting the V2 region or V4–V5 and V5–V7 are applicable for these types of studies (Beckers *et al.*, 2016). As a microbial genome can host multiple copies of the 16S rRNA gene with intragenomic variability, alternative target single-copy housekeeping genes have also been successfully employed on seed endophytic communities (Links *et al.*, 2014; Rezki *et al.*, 2016, 2018).

- *gyr*B is an example of an alternative bacterial marker developed by Barrett *et al.* in 2015 to overcome the low sequence divergence among related bacterial taxa of 16S rRNA gene (Větrovský and Baldrian, 2013) and to provide valuable insights into the taxonomic composition of the seed microbiota (Barret *et al.*, 2015). This DNA barcode is based on a portion of *gyr*B, a gene encoding the subunit of the DNA gyrase, frequently employed as a phylogenetic marker for many bacterial genera (Yamamoto and Harayama, 1995; Větrovský and Baldrian, 2013).
- In cases where resolution beyond the genus level and the confident identification of potentially novel taxa is desirable, cpn60 metabarcoding could represent a solution for *de novo* assembly of sequence data. This DNA barcode has the great advantage to

simultaneously target bacteria and fungi with a unique primer providing a unified cross-domain view of the microbial community (Links *et al.*, 2012).

Although universal primers have been designed and tested for the cpn60 gene (Schellenberg *et al.*, 2011), the size of sequence databases of 16S rRNA gene greatly exceeds those of other bacterial genes. For this reason, the employment of 16S remains still the target of choice for studies in bacterial ecology and seed endophytes (Větrovský and Baldrian, 2013).

As already mentioned, when total community DNA (tcDNA) is extracted from seeds, microbial genetic material composes only a minor fraction of the obtained DNA. Since bacterial 16S rDNA primer pairs exhibit high affinity to plastid and mitochondrial DNA, a high proportion of NGS reads would include host-derived sequences. In order to facilitate PCR amplification of endophyte DNA, it is crucial to exclude non-target tcDNA from the PCR reaction. Two solutions are possible.

1. The employment of primer pairs (799F-1391R) targeting the V5–V7 hypervariable regions of the 16S rDNA. Primers targeting this region have been shown to produce very low amplification rate of non-target DNA across all plant compartments (Beckers *et al.*, 2016).
2. The employment of the peptide nucleic acid (PNA)-PCR clamping technique. This technique uses PNA oligomers with complementary sequences to mitochondria and plastid SSU rRNA genes. Their overlapping with the region in the 1492r primer-binding site suppresses the amplification of the two organelles (Sakai and Ikenaga, 2013).

Internal transcribed spacer

The internal transcribed spacer (ITS) region is extensively employed to identify fungal lineages and is formally the DNA barcode for fungi due to the presence of a rich and up-to-date database (Schoch *et al.*, 2012; Bates *et al.*, 2013). While microbiome projects utilize and endorse ITS1 subregion as a target using ITS1F (Gardes and Bruns, 1993) and ITS2 as primers (De Filippis *et al.*, 2017), recent studies

propose to test different target genes simultaneously, recommending the use of ITS2 or the whole ITS region for metabarcoding. Unlike the ability of 16S universal primer to cover the bacterial domain almost completely, primers constructed on the ITS cannot be considered as phylogenetically inclusive for fungal genomes. In fact, the employment of this gene for amplicon sequencing can be biased by the preferential amplification of specific taxa. This is due to the length variability of the ITS1-2 region among different fungal genera and species (Esteve-Zarzoso *et al.*, 1999). For this reason, in the use of ITS subregions for amplicon sequencing there is no consistency in the choice of primer pairs (Tedersoo *et al.*, 2016). Finally, ITS primers could also be selected based on the relative proportion of fungal DNA and the expected dominant groups (Tedersoo *et al.*, 2015) or by using 'mock communities' that allow the evaluation of the reliability of each primer pair (Tessler *et al.*, 2017).

High-throughput sequencing platforms

Current NGS platforms are not optimized for the production of reads long enough to cover a whole marker gene in combination with a low error rate and high sequencing depth. Since using a single marker gene for inferring whole genome differences, sequencing quality has become a crucial factor. For these reasons, the choice of the sequencing platform comes together with the choice of the primer pair and is made in order to sequence the target region with the higher quality and depth. Due to a preferred utilization of primers provided by the EMP, studies focusing on seed endophytes mainly use Illumina sequencing technologies (MiSeq or HiSeq platforms). For a good understanding of the sequencing platforms and their use, we recommend the clear summary table provided by Tessler *et al.* (2017).

Computational data analysis for microbial community reconstruction

The inference of endophytic diversity using sequencing data from a single marker gene involves the clustering of sequencing reads into operational taxonomic units (OTUs). In this computational step, similar marker gene sequences are clustered as considered belonging to the same taxon (Edgar, 2013). The taxonomy assignment of the OTU is retrieved by the comparison of a representative sequence of the OTU with a database. The main databases employed for this step are RDP (Cole *et al.*, 2014), SILVA (Quast *et al.*, 2013) and Greengenes (McDonald *et al.*, 2012). Various pipelines for such analyses are

employable; however, the most recognized and broadly used is QIIME (Caporaso *et al.*, 2010; https://qiime2.org).

As a conclusion, DNA metabarcoding is a technique that allows characterization of the complex structure of microbial communities. Its reliance on a single non-functional gene does not allow the study of the functional potentiality of microorganisms. In addition, it should be taken into account that the application of this methodology on endophytic communities can be challenging during DNA extraction, PCR and data analysis steps. Nevertheless, this technique has to be accounted as a powerful tool to routinely study microbial communities with the possibility to detect indicator species and community shifts.

Omics technologies

In order to answer important ecological questions on functional roles of seed endophytic microbiomes, metagenomic and metatranscriptomic approaches are often applied (Alibrandi *et al.*, 2018). It is important to highlight that although the sequencing depth that can be reached with NGS instruments is steadily increasing, complex microhabitats (e.g. seed endosphere) still cannot be completely assessed with these methods (Myrold *et al.*, 2014).

Metagenomics

Metagenomics comprise the study of the genomic content within complex microbial communities (Wooley *et al.*, 2010; Fig. 4.1). This technique relies on the random fragmentation and sequencing of genomic DNA isolated (shotgun metagenomics) and allows reconstruction of the gene set of the microbial communities residing in a specific environment with no cultivability restriction. In addition, the possibility of mapping discovered genes to known microbial genomes allows the simultaneous study of both the composition and the functional capabilities of the community in a single experiment (Kurokawa *et al.*, 2007; Arumugam *et al.*, 2011). An example is the study conducted by Torres-Cortés *et al.* (2018) that, having both ecological and functional insights into the community of germinating seeds (bean and radish), was able to determine the functional traits connected with the modification and selection of the microbiome.

Metatranscriptomics

The same principle of metagenomics has been successfully applied to the study of mRNA. In fact, the mRNA is converted to cDNA and sequenced on an NGS platform (Fig. 4.1). The possibility to map activated microbial genes inside plant tissues is essential to understand the endophytic phenomenon (Kaul *et al.*, 2016) and the role of seed-associated microbes in plant growth and development. Even if several studies showed how beneficial seed-borne endophytes could defend the plant from stress (Truyens *et al.*, 2014; Khamchatra *et al.*, 2016; Shahzad *et al.*, 2016, 2017) or produce compounds that inhibit pathogen growth or strengthen plant resistance (Bonos *et al.*, 2005; Tayung *et al.*, 2012; Shahzad *et al.*, 2017), the employment of metatranscriptomics on seed endophytes has not yet been accomplished.

In order to select the most suitable NGS-based approach to study seed endophytes, it is crucial to evaluate the methodology in a broader context. While the ability of metagenomics to analyse the genomic content in a complex mixture of microorganisms avoiding PCR biases is of undoubtable importance (Wooley *et al.*, 2010), its ability to assess biodiversity and community ecology analyses is highly dependent on additional factors. Difficulties of this approach can be represented by the choice of sequencing depth and length. Sequencing depth is an important factor for shotgun metagenomics, as it determines its ability to discover new genes. Similarly, longer reads are more likely to cover full protein domains, and therefore allow to distinguish between closely related genes from different organisms. In fact, short reads are frequently misaligned leading to an inflation of both species count and diversity estimates (Caporaso *et al.*, 2012; Schulze-Schweifing *et al.*, 2014; Clooney *et al.*, 2016). From another point of view, the analysis of shotgun metagenomics data can be performed with different strategies with a variety of tools that can be employed for every computational step (Breitwieser *et al.*, 2017). In addition, the absence of recognized and unified pipelines, as QIIME (Caporaso *et al.*, 2010) is for DNA metabarcoding studies, makes the analysis more complicated and require specialized training. However, well-curated databases, as those of major projects (as in the human microbiome), can lead shotgun metagenomics to have even more precise detection of species and diversity compared to DNA metabarcoding. Conversely, for environmental samples shotgun metagenomics-based assessment of

diversity allows identification of only half of the phyla and only 30% of the families when compared to DNA metabarcoding (Tessler *et al.*, 2017). This is mainly due to the lack of specific databases.

Metaproteomics

While proteomics is defined as the study of the different proteins expressed by an organism (Wilkins *et al.*, 1996), metaproteomics involves identification of the functional expression and metabolic activities within a microbial community (Siggins *et al.*, 2012; Fig. 4.1). From a technical point of view, proteomics is based on the employment of high-performance mass spectrometry (MS) to characterize the complete assemblage of proteins expressed by a microbial community. Similar to DNA- and RNA-based NGS approaches, the obtained data sets must be processed by bioinformatics, and peptide sequences must be aligned with specific database entries.

Metabolomics

This methodology relies on the assessment of all metabolites found in a specified sample. The technique is based on the ability of mass spectrometry to identify a large number of molecules by their specific masses and high accuracy. Based on the sensitivity and selectivity of both metabolite recovery and identification, a metabolomics approach can scan the whole set of metabolites present in the given environment (untargeted metabolomics) or focus on specific classes of metabolites (targeted metabolomics). Commonly these two approaches are coupled. At first, untargeted metabolomics is used to scan the whole spectrum of metabolites and, after studying which functions are present, targeted studies allows one to quantify specific pathways and functions (Johnson *et al.*, 2016). Even if the technical complexity and the difficult interpretation of data limit the accessibility to this technique, the possibility to directly identify metabolites in complex samples makes this technique one of the most promising for the study of symbiotic relationship, as it happens within endophytic communities (Kaul *et al.*, 2016).

Multi-omics approaches

Different omics techniques can be combined with each other in order to increase their informative value. For example, proteome-based studies are often incomplete without genomic information and the

accuracy of assignments can be substantially increased when these data are added. For this reason, the ideal concept is the parallel bioinformatics assessment of several omics strategies with a multi-omics approach allowing microbial communities to be analysed from different points of view. Since these technologies are in continuous evolution, advantages and disadvantages of omics technologies are difficult to evaluate. As of today, even if the continuous improvement and expansion of databases will gradually resolve most of the problems connected with these technologies, the bottleneck for their application remains the cost that relegates their employment only to big projects in advanced stages.

Microscopic Visualization of Seed Endophytes

Since omics methodologies are based on the extraction of nucleic acids or proteins, they cannot provide useful information on the microorganism localization at microscale level. This is the reason why microscopy is still valuable for complementing molecular microbiology tools as means for the visualization of the microbe-host systems (Cardinale, 2014). We here report the explanation of the main microscopy technique used for the study of seed endophytes.

Confocal laser scanning microscopy

The most frequently used microscopy approach to study plant and seed endophytes is confocal laser scanning microscopy (CLSM) (Cardinale, 2014; Pawley, 2006; Fig. 4.1). This is a widely applicable optical imaging technique for an accurate study for plant-microbe interactions. CLSM captures multiple two-dimensional images at different depths in a sample, allowing the *in situ* observation of host-associated microorganisms with an unprecedented accuracy.

In order to detect specific microorganisms, it is possible to employ the fluorescent *in situ* hybridization (FISH): a molecular cytogenetic technique that allows the identification and localization of cells in their microenvironment (Moter and Göbel, 2000). It uses the hybridization of designable DNA-probes labelled with fluorochromes able to bind with the complementary target sequence of choice. This technique is most frequently used for visualization of microbial colonization patterns (Moter and Göbel, 2000; Rudolf Amann *et al.*, 2001; Rybakova *et al.*, 2017), providing estimates of

microbial abundance while avoiding quantification biases associated with cultivation or PCR (Bulgarelli *et al.*, 2012). The possibility to employ DNA-probes targeting specific taxonomic ranges allows a qualitative-quantitative study of microbial populations. While its resolution power in assessing diversity is not remotely comparable with that of molecular techniques, this approach represents an optimal tool for the validation of molecular analysis results while studying basic processes of plant-microbe interactions such as microbial localization, colonization pattern and cell-cell interaction (Cardinale, 2014). An example of the usage of this microscopy technique is provided in the study by Rybakova *et al.* (2017; Fig. 4.2). The authors used CLSM visualizations to investigate microbial colonization patterns in oilseed rape.

Scanning electron microscopy

Other microscopy-based techniques are less frequently applied for the study of seed endophytes; one example is scanning electron microscopy. This microscopy technique relies on the use of a focused beam of electrons scanning the surface of the sample. The remarkable magnification power of this microscope allows the visualization of the morphological features of cells by producing micrographs with unmatched three-dimensional quality on natural surfaces. Nevertheless, this technique provides less specific information than CLSM when studying sections of biological samples. Even though this technique provides clear pictures at high magnification levels, it is not possible to obtain information on the taxonomy simultaneously. Therefore, it is of limited use for the study of plant-microbe and cell-cell interactions, which reduces its employment in the study of seed endophytes (Golding *et al.*, 2016).

Concluding Remarks

Different methods can be employed for the study of seed endophytes. Integrative approaches currently provide the most suitable strategies to describe these microorganisms due to various limitations of single methodologies (Fig. 4.3). While microbial cell culturing seemingly represents the most suitable way to exploit the biotechnological potential of seed endophytes, the low applicability and time requirements for microbial isolation and cultivation are setting boundaries to this approach. Holistic descriptions of seed endophytic communities require the application of molecular techniques for the

analysis of community structures and microbial functioning therein. Nevertheless, also molecular techniques have specific limitations. For example, the detection of genetic material from two microorganisms in the same niche could suggest specific interactions between them. However, the validation of their co-localization within the seed can be obtained only using microscopy techniques.

<COMP: Insert Fig. 4.3 here>

The investigation of seed-borne endophytes is in continuous evolution as are the methodologies. While the endophytic population in seeds has not yet been fully explored, the great applicability of these microorganisms for sustainable agriculture is attracting attention and funding. For this reason, it is feasible to believe that the characterization of this valuable plant compartment will progress very rapidly in the next decade.

As in a mosaic composed by precisely drawn tiles, integrating one methodology to the other shows that a combination of accurate methods is still the best solution to increase our understanding of the ecological role of microorganisms within these essential plant structures.

Fig. 4.1. Graphical representation of different methodologies employed in studies focusing on seed endophytes.

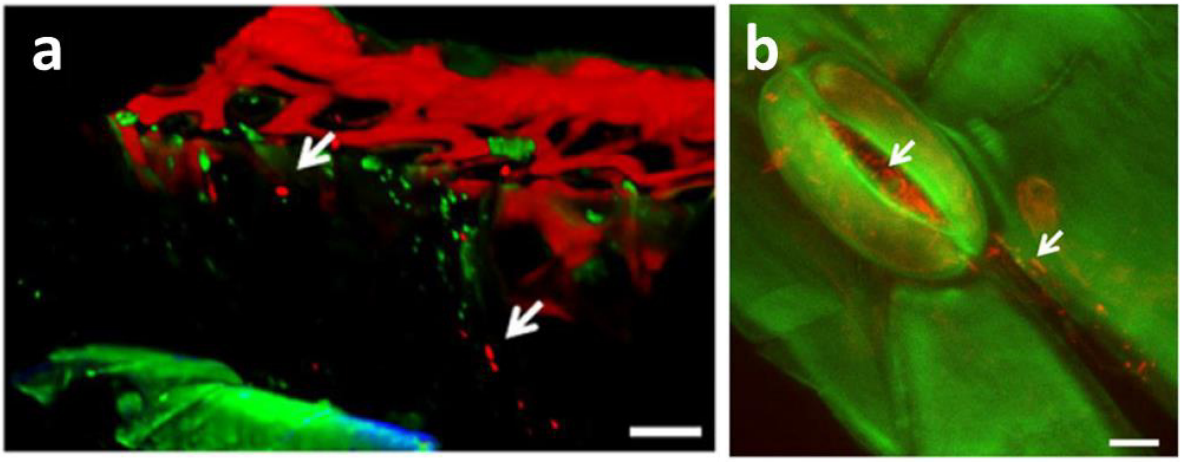

Fig. 4.2. Visualization of bacterial colonization patterns in oilseed rape (A) and seedlings (B). In the first visualization (A), differential BacLight LIVE/DEAD staining was used to visualize living (green) and dead (red) *Serratia plymuthica* cells. In the second micrograph (B), *Gammaproteobacteria* were localized in oilseed rape seedlings. White arrows highlight bacterial colonies (Pictures from Rybakova *et al.*, 2017.)

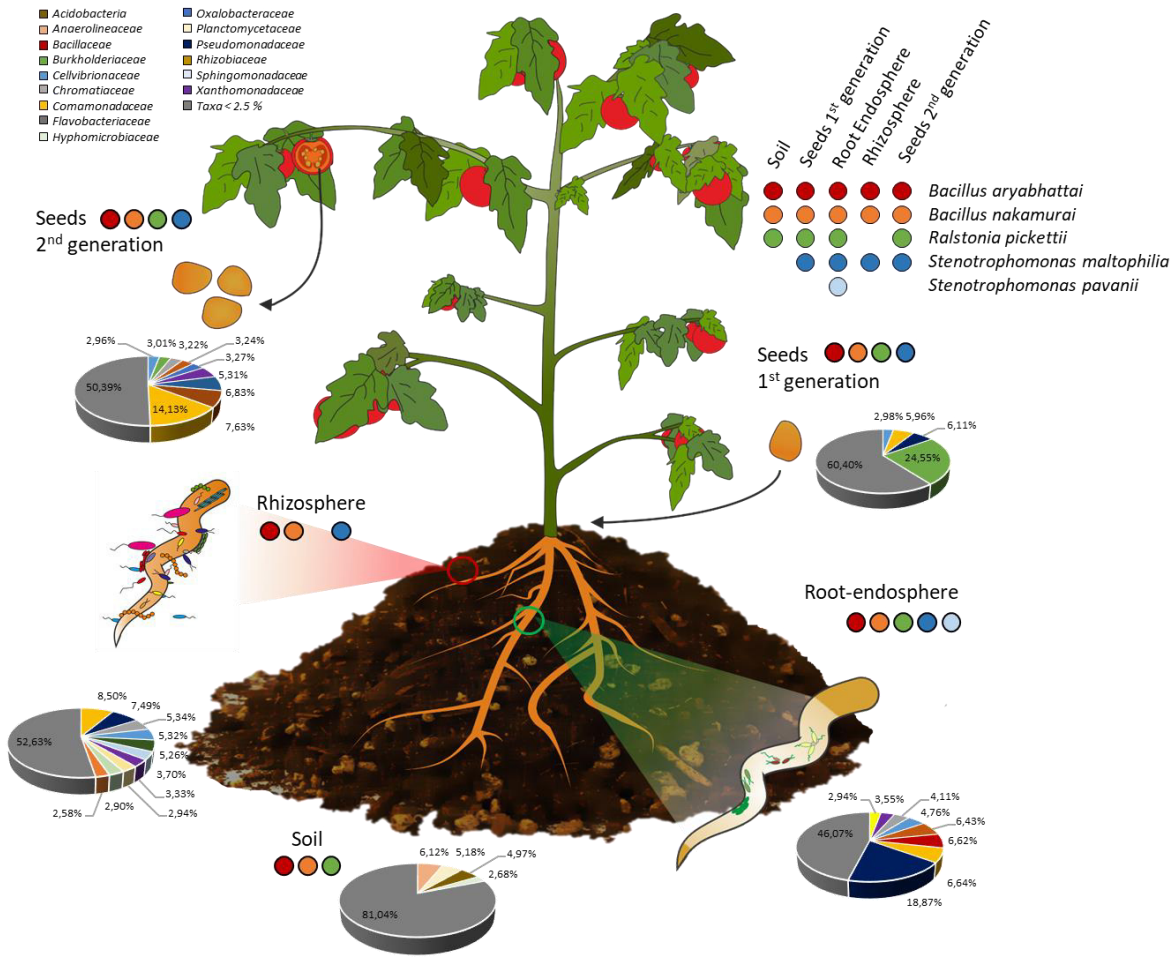

Fig. 4.3. Tracking of endophytes across a plant system. The integration of cultivation-dependent and cultivation-independent techniques provided insights into the allocation of beneficial bacteria across tomato plants. In a recent study (Bergna *et al.*, 2018), bacterial isolation was coupled with phenotyping and 16S metabarcoding to, respectively, detect plant beneficial bacteria and reconstruct the bacterial community of the tomato plant system. By merging these data, it was possible to reconstruct the association of beneficial bacteria to specific plant compartments. The identified key players are indicated for the specific plant compartments.

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Publication II

Tomato Seeds Preferably Transmit Plant Beneficial Endophytes

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RESEARCH *e-Xtra**

Tomato Seeds Preferably Transmit Plant Beneficial Endophytes

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ABSTRACT

Endophytes with plant growth-promoting activity can improve the health and development of plants during all life stages. However, less is known about their stability and transmission across plant genotypes, habitats, and generations. By combining community and isolate analyses, we found that each plant habitat and genotype harbored distinct bacterial communities and plant growth-promoting bacteria (PGPB). Soil, root endosphere, and rhizosphere were the habitats with the highest bacterial diversity, while seeds hosted more selective communities. Seeds generated under field conditions showed traces of a bacterial community composition connected to the suppression of plant pathogens. In contrast, seeds of the successive generation grown in a pathogen-free and low-nutrient environment showed a predominance of bacteria that facilitate the uptake of nutrients. These modifications of the microbiome can be explained by an adaptation to prevalent environmental conditions. Cultivation approaches revealed microhabitat-specific PGPB that were assigned to various species of *Bacillus*, *Stenotrophomonas*, and *Ralstonia*. Tracking down these bacteria among the whole tomato plant allowed us to identify the seed as a primary vehicle of PGPB transmission. This previously undescribed vertical transmission of PGPB represents a strategy to maintain plant beneficial bacteria over generations and has an impact for the design of seed treatments.

Additional keywords: seed microbiota, *Solanum lycopersicum*.

The plant microbiota has been known to be one of the key determinants of plant health and productivity for more than a century (Philippot et al. 2013; Yan et al. 2017). It has the ability to contribute to multiple aspects in the functioning of the plant holobiont (Vandenkoornhuyse et al. 2015), such as (i) seed germination and growth support, (ii) nutrient supply, (iii) resistance against biotic stress factors (pathogen defense), (iv) resistance against abiotic factors, and (v) production of bioactive metabolites (Berg et al. 2015). Due to this importance, the factors that shaped the plant microbiota have been studied for a long time. Following a long debate, it is accepted that the plant genotype and soil quality are the crucial factors influencing the composition of the rhizosphere microbiota (Berg and Smalla 2009). Both have an impact, but the extent depends on many factors (plant's morphology and secondary metabolism and soil type) and is triggered by plant root exudates and signaling (Badri and Vivanco 2009; Doornbos et al. 2012). The same question, namely whether the soil quality or the plant genotype determines the microbiota composition of the seeds, arose. For a long time, it was assumed that the emerging seedling is colonized by microorganisms from its surrounding environment, with soil being the main source, controlled by the plant through different strategies, such as the specific profile of root exudates and its immune system (Sánchez-Cañizares et al. 2017; Shade et al. 2017; Truyens et al. 2014). Recently, an impact of the plant genotype on the microbial seed composition was identified (Adam et al. 2018; Rezki et al. 2018; Rybakova et al. 2017). This indicates that possibly both factors are involved, which was also used as a first hypothesis for this study. Moreover, there are many knowledge gaps; especially regarding the transmission and stability of the seed microbiota (Berg and Raaijmakers 2018). Vertical transmission would thus permit a "continuity of partnership" between the plant and its beneficial bacteria (Vannier et al. 2018), which leads us to our second hypothesis that the beneficial partners are transmitted from seed to seed.

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To evaluate our hypotheses, we have selected tomato as a model plant. Tomato (*Solanum lycopersicum* L.) is one of the most important vegetables; 177 million tons are harvested each year (FAOSTAT 2016). Tomato and other vegetables are an important part of a healthy diet, they can lower blood pressure, reduce risk of heart disease and stroke, prevent some types of cancer, lower risk of eye and digestive problems, and have a positive effect upon blood sugar (He et al. 2006). Although new findings recommend an increased intake of fruits and vegetables, the production and processing is associated with enormous losses up to 45% (FAO 2015). Soilborne pathogens, e.g., *Fusarium*, *Rhizoctonia*, and *Verticillium*, are one of the major factors that significantly limit crop yields (Oerke 2006). They are difficult to suppress, but recent advances in our understanding of the plant microbiota and of the mechanisms responsible for plant growth promotion and biological control of pathogens are opening the way to control them biologically (Mendes et al. 2011; Scherwinski et al. 2008). The microbiome of tomato has been widely characterized in its below ground compartment, mainly focusing on its capability to host beneficial bacterial endophytes conferring resistance to biotic and abiotic stresses (Liu et al. 2017; Upreti and Thomas 2015; Yan et al. 2003). What these approaches are missing, is the characterization of the microbiome of the aboveground plant parts, where the microbiota is less exposed to the effect of the soil (Ottesen et al. 2013) and thus the plant plays a stronger role in the selection of bacteria (Truyens et al. 2014), as well as a link to the seed microbiome.

The objective of this work was to unlock the tomato microbiome and understand (i) which are the main drivers of the microbiome composition, (ii) what is the impact of soil quality on the seed microbiome, and (iii) how the transmission of PGPB in the seed across generations works. The experimental design was based on greenhouse experiments and included samples analyzed from bulk soil, rhizosphere, root endosphere, seeds (first generation) as well as seeds harvested from the grown tomato plants at maturity (second generation) from two cultivars (Moneymaker and Hildares F1). An integrative approach based on amplicon sequencing and a bacterial strain collection was linked with bioinformatic analyses. It allowed the reconstruction of the tomato plant system's bacteriome and the location of beneficial bacteria among plant microhabitats.

MATERIALS AND METHODS

Experimental design. Microbiome-related analyses were performed using the tomato (*Solanum lycopersicum*) cultivars Moneymaker (Austrosaat AG, Austria) and Hildares F1 (Hild Samen GmbH, Germany) grown under greenhouse conditions (approximately 24/20°C day/night temperature) at Graz Botanical Garden (Graz, Austria). Surface-sterilized seeds (first generation) were sown in pots (one seed per pot; 8 liters), filled with a mixture of sterile quartz-sand and diluvial sand (Rühlmann and Ruppel 2005) or commercial loamy soil (Ökohum GmbH, Herbertingen, Switzerland) in a proportion of 10:1. The substrate mixtures are considered as nutrient poor systems. Each tomato cultivar included 50 plants arranged in a randomized design. The seedlings were watered and fertilized once a week with a nutrient solution (100 ml/plant, the complete composition of the solution is reported in Supplementary Table S1) (Hoagland and Arnon 1950). For the analysis of the bacterial community in each quartz sand/soil mixture, eight pots without plants were watered and maintained under the same nutrient and greenhouse conditions as planted pots.

Sample collection and DNA isolation. Sampling of soil and plants was carried out 85 days after sowing (late flowering stage) followed by a second sampling at the ripening of fruits of Moneymaker plants (Supplementary Table S2). Soil samples were obtained from pots containing soil-sand mixture only. After removing the top layer (2 to 3 cm) of soil with sterile tools, soil samples from the central section of each pot was collected and stored in sterile bags. Rhizospheric soil was obtained by shaking the root compartment and collecting the falling off material. Root and soil samples from each pot were stored in separated sterile polyethylene bags at 4°C until further processing on the following day. Tomato seeds from the second plant generation were extracted from the ripened fruits. Seeds of the second generation were harvested from 10 Moneymaker plants. For extracting the total microbial community DNA, both soil and rhizosphere material were initially suspended in 0.85% sodium chloride solution (NaCl) and shaken for 30 min. Root material was surface sterilized and processed according to the protocol described by Bragina et al. 2012). Briefly, 5 g of roots was washed, surface sterilized in a 3% sodium hypochlorite solution (NaClO) for 5 min (manual shaking) and then again washed in sterile water three times. The plant material was imprinted on NA agar plates as a sterility check. Similarly, seeds of Moneymaker and Hildares F1 were washed in sterile water, divided in plastic vials (20 seeds per vial) with 4 ml of 0.85% NaCl and gently shaken for 4 h. All plant samples (root and seed) were homogenized with mortar and pestle and suspended in 0.85% NaCl. Bacteria-containing pellets from both plant and soil specimens were collected by centrifugation (20 min at 16.750 g) and stored at −70°C.

DNA isolation, 16S rDNA PCR amplification, and sequencing. The aforementioned pellets were used for the total community DNA isolations. DNA was isolated with the FastDNA SPIN Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. Final aliquots of the total community DNA were further quantified and used as template for PCR amplifications (thermal cycler by Biometra GmbH, Jena, Germany) using Taq-&GO Ready-to-use PCR Mix (MP Biomedicals) and the universal bacterial primer set 515f/806r (515f: 5′-GTGYCAGCMGCCGCGGTAA-3′; 806r: 5′-GGACTACNVGGGTWTCTAAT-3′) targeting the 16S rDNA hypervariable region 4 with the suggested PCR program (94°C for 3 min to denature the DNA, 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; 10 min at 72°C for final extension) (Caporaso et al. 2011). Barcode sequences for multiplexing of the data were used as provided by the earth microbiome project (earthmicrobiome.org/). In addition, peptide nucleic acid PCR clamps were used to block the amplification of plastid and mitochondrial 16S rRNA gene sequences of plants during the PCR amplification. The amplicons were purified by using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). A total of 86 barcoded samples were pooled equimolarly and sent for paired-end Illumina MiSeq sequencing (GATC Biotech, Germany) performed in two sequential batches. The 16S rRNA Illumina libraries obtained from the sequencing company were deposited at the European Nucleotide Archive (ENA) under the project number PRJEB27033.

Data analysis of 16S rDNA amplicons for determination of microbial community structure. Acquired 16S rRNA gene sequences went through an initial quality check. Only forward reads were selected for further analyses due to substantially higher read quality when compared with reverse reads. Demultiplexing followed by quality filtering with QIIME (Quantitative Insights into Microbial Ecology, version 1.9.0; Caporaso et al. 2010) default parameters (Bokulich et al. 2013) was conducted for the whole dataset. High quality reads were dereplicated and clustered with a similarity threshold of 97% via VSEARCH (version 2.4.3). After creating a set of representative sequences, chimeras were filtered via both de novo reference-based approaches while mapping high quality sequences (vsearch) (Rognes et al. 2016). The taxonomical assignment was obtained by employing QIIME environment RDP

(default parameters) in combination with the SILVA 16S database (release 128) (Pruesse et al. 2007). Unassigned operational taxonomic units (OTUs) and nonbacterial contaminants were filtered from the resulting OTU table. OTUs abundances have been rarefied via subsampling in QIIME environment to allow comparisons between samples. A consensus-table was obtained by averaging the subsampled tables. The description of the bacterial community structure was performed using a QIIME summarized table at phylum and family levels with samples belonging to the same microhabitat merged together. Graphical rendering of the community structure at phylum level was done with the open-source software Circos (Krzywinski et al. 2009) (Fig. 1). A more detailed description at family level was rendered with bar charts; the employment of analysis of variance (ANOVA) with false discovery rate correction allowed to identify bacterial families with significantly different abundances among tested sample groups. Alpha diversity was calculated and rendered at OTU level in the R environment with the Phyloseq package (McMurdie and Holmes 2013) using observed species, Chao 1, Shannon, and inverse Simpson measures. The PCoA plot was also generated with Phyloseq on an OTU table summarized at family level in QIIME. Selected OTUs were studied at more resolved taxonomic levels with the online nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/).

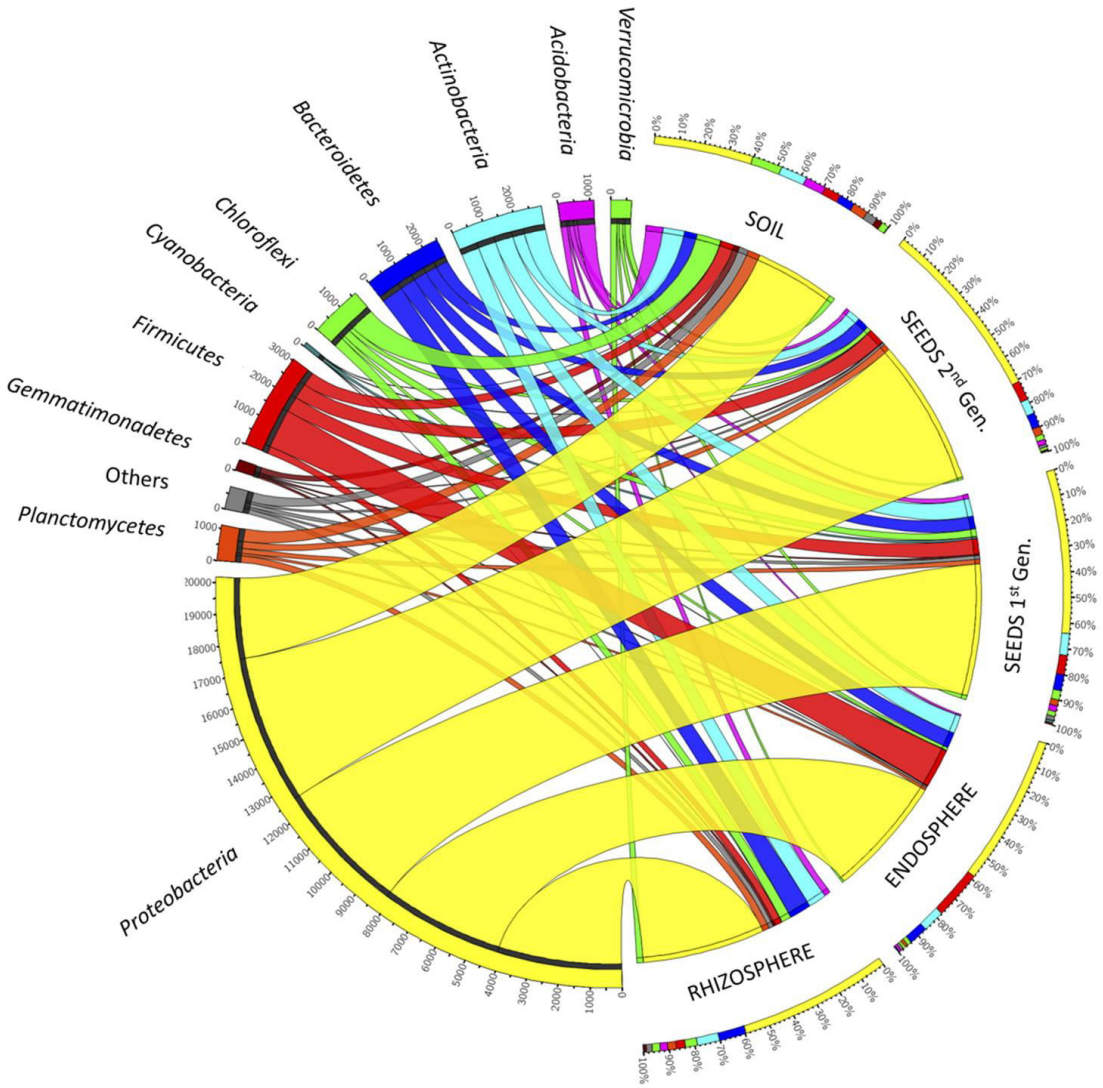

Fig. 1. Circular representation of the bacterial community structure (at phylum level) in different microhabitats associated with the tomato plant. Taxa with a proportion lower than 1% in all habitats are summarized as 'Others'. Values in the inner circle indicate the normalized number of reads assigned to the respective phylum. The visualization was rendered using the open-source software Circos (Krzywinski et al. 2009).

Bacterial community structure and plant beneficial bacteria. To analyze and visualize how OTUs are partitioned between microhabitat an OTU network was generated in QIIME and subsequently rendered with the software Cytoscape v. 3.5.1 (Shannon et al. 2003). The network was constructed using an OTU table rarefied at 7,000 reads per sample after removal of singletons and doubletons. In order to increase the representativeness of the subset table, random subsampling was repeated 10 times and average values have been considered. In the resulting network, lines connect OTUs (circles) to the microhabitats they are related to. Each OTU circle was rendered with a radius correlated to the OTU abundance among all studied samples. OTUs with taxonomical assignments matching with the characterized isolates were as well subjected to manual BLAST searches (representative sequences) in order to identify overlaps (using the same algorithm and parameters). OTUs were represented with a circle proportional to the abundance and those found to be beneficial in the conducted assays were noted with their respective taxonomical identification.

Isolation and characterization of bacteria with plant growth-promoting traits. Bacterial strains were isolated from the tomato plant system (soil, rhizosphere, root endosphere, and seeds of both generations) using the protocol published by Bragina et al. (2012). The obtained homogeneous sample suspensions were plated on 10% tryptic soy agar (pH = 7.3 ± 0.2, Sigma-Aldrich; Taufkirchen, Germany), water agar (pH = 6.8 ± 0.2), and R2A (pH = 7.2 ± 0.2, Sifin; Berlin, Germany). The selection of bacterial endophytes was carried out with a systematically randomized approach: solid media plates were divided in six equal parts and colonies of one of the six parts were collected, purified, and preserved. The ability of isolated strains to produce metabolites that, in other strains, have been associated with plant growth promoting abilities was assessed by the production of indol-3-acetic acid, siderophores as described in Berg et al. (2002), ACC-deaminase (Dworkin and Foster 1958), and acetoin (Murray and Baron 2007). Their ability to inhibit the growth of plant-pathogenic fungi was indirectly assessed based on chitinase production (Nagpure and Gupta 2013), and in dual-culture experiments using the pathogenic *Fusarium oxysporum* Fol 007 strain (Berg et al. 2002). In addition to classic antagonism tests, isolates were screened for the emission of growth-inhibiting bioactive volatile compounds (Cernava et al. 2015) with *F. oxysporum* Fol

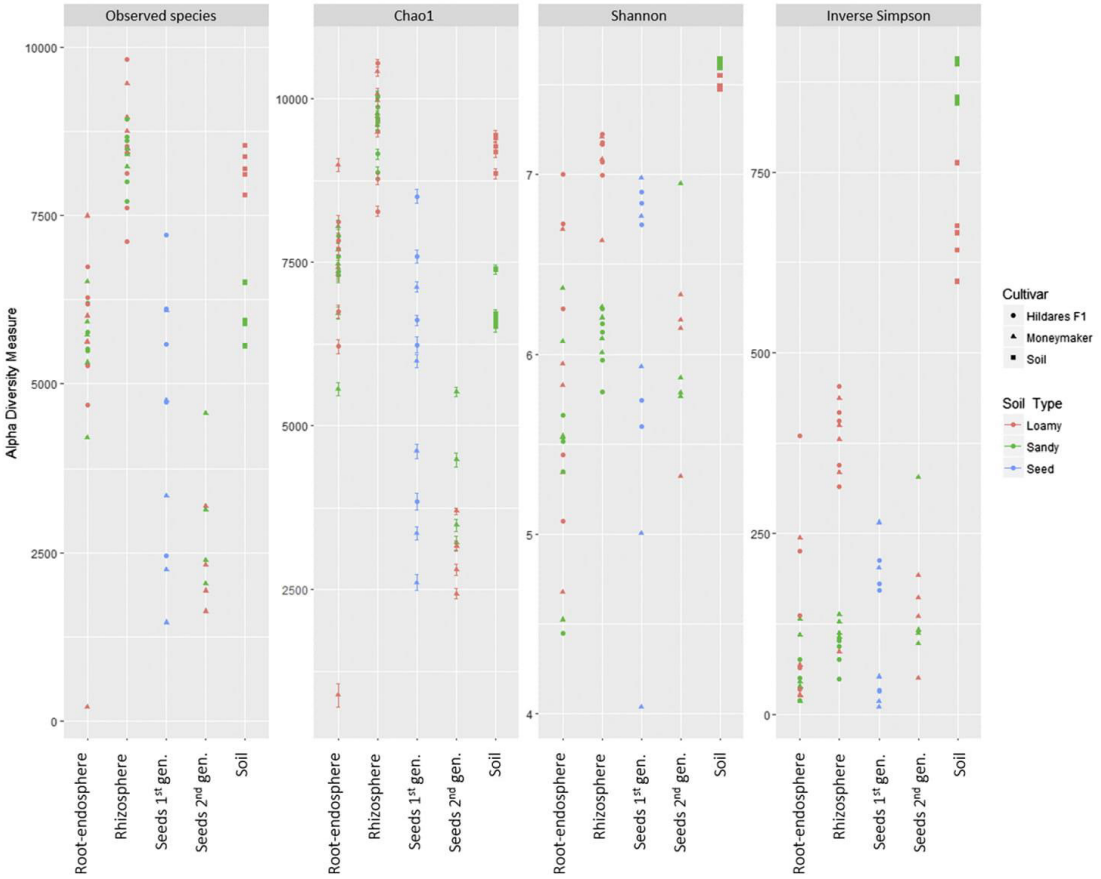

Fig. 2. Diversity assessment across all included samples and four different diversity measures: observed Species, Chao1, Shannon, and inverse Simpson. The combination of measures sensitive to rare operational taxonomic units (OTUs) (observed species and Chao1) and to dominant OTUs (inverse Simpson's index) provides a comprehensive assessment of bacterial diversity in the plant system. Samples are colored according to the soil type employed while the shape refers to the respective tomato cultivar.

007 as model pathogen. Moreover, salt/drought tolerance and phosphate-solubilization capacity were assessed in additional experiments (Naik et al. 2008; Pikovskaya 1948) together with in planta assays for plant growth promotion (climate chamber conditions: temperature (day/night) = 25/20°C, photoperiod: 16 h, light intensity: 400 µmol m^{-2} s^{-1}, humidity 70%). Isolates with plant growth-promoting traits and other beneficial functions underwent taxonomical identification by 16S rRNA gene sequencing and manual BLAST searches (https://blast.ncbi.nlm.nih.gov/) (Supplementary Table S3).

RESULTS

General structure of tomato-associated bacterial communities and diversity analyses. The DNA sequencing of marker genes from bacterial communities of two tomato cultivars (Moneymaker and Hildares F1) grown in two mixtures of quartz sand with two different soils (diluvial sand and loamy soil) resulted in a total of 32,411,312 high quality reads. After discarding chimeras, singletons, chloroplast and non-bacterial reads, 21,369,607 reads remained with an average abundance of 318,949 reads per sample and a standard deviation of 276,419 reads. The bioinformatic reconstruction of the bacterial community identified a total of 13,928 distinct OTUs.

Proteobacteria were predominant in all microhabitats (Fig. 1). In the first-generation seeds, second-generation seeds, root endosphere, and rhizosphere (64, 71, 69, and 60%, respectively) this phylum covered more than half of the bacterial community while in soil it was less representative (40%). Other representative phyla were *Firmicutes* (up to 19% in the endosphere), *Actinobacteria*, and *Bacteroidetes*. All three were equally distributed among microhabitats, averaging 9, 8, and 7%, respectively. *Chloroflexi*, *Acidobacteria*, and *Planctomycetes* were mostly found in soil samples (respectively 12, 8, and 6% in soil). Among all plant compartments (seeds of both first and second generation and root endosphere), *Pseudomonadaceae* and *Comamonadaceae* were the most occurring bacterial families. Considering both cultivars, the seeds of the first generation were characterized by a selective bacterial community, where only few taxa were dominant: *Burkholderiaceae* (19%), *Pseudomonadaceae* (7%), and *Comamonadaceae* (6%). Conversely, the soil was characterized by the high abundance of *Anaerolineaceae* (6%) and *Planctomycetaceae* (5%). In the rhizosphere, *Comamonadaceae* (8%), *Pseudomonadaceae* (7%), and *Flavobacteriaceae* (5%) represented the most abundant families, while the root endosphere was dominated by the family *Pseudomonadaceae* (18%) followed by *Comamonadaceae* (6%), *Bacillaceae* (6%), and *Rhizobiaceae* (6%). Even if decreased, the family *Burkholderiaceae* was assigned almost exclusively to seed specimens (19% in the first-generation seeds and 3% in the second-generation seeds).

The diversity among microhabitats was evaluated with alpha diversity metrics (Fig. 2). To better describe the bacterial community composition, we selected four different measures sensitive to rare OTUs (observed species and Chao1), to dominant OTUs (inverse Simpson's index) and incorporating both richness and evenness (Shannon). Values for observed species varied between 7,505 and 13,725, while Chao1 values between 8,662 and 13,785 were observed and inverse Simpson indices between 72 and 1,070 (Supplementary Table S4). Soil, rhizosphere, and root endosphere were the microhabitats with the highest diversity while seeds hosted more selective communities in both generations. Soil diversity varied among loamy and sandy soil: the loamy soil was characterized by a higher number of rare OTUs and lower of dominant OTUs when compared with sandy soil. Conversely,

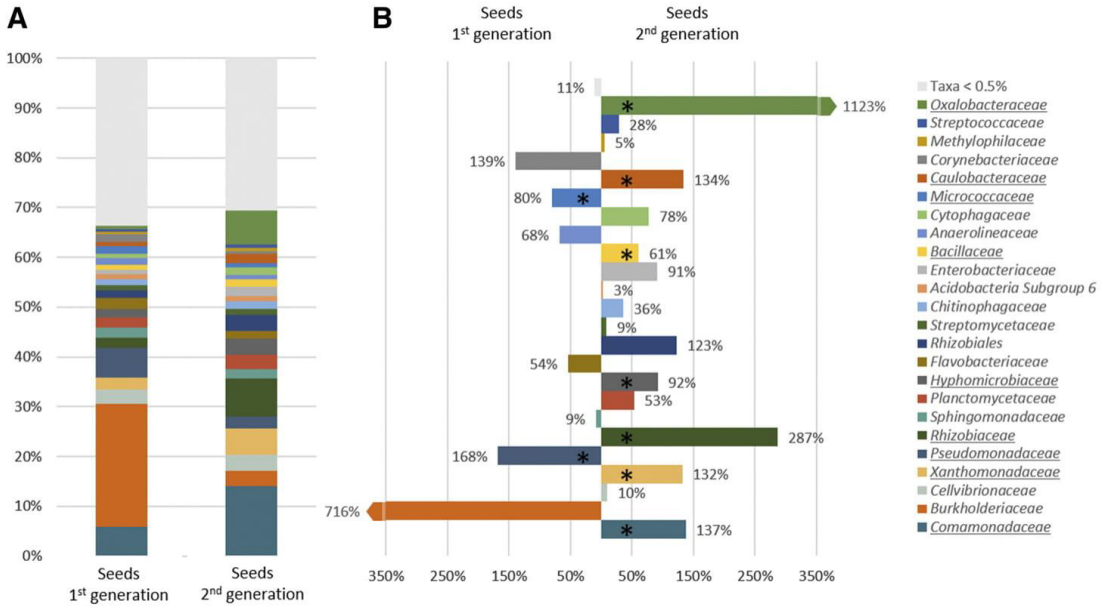

Fig. 3. Bacterial community composition of the tomato seeds across two generations. **A,** Bar charts represent the composition of the bacterial community (only key taxa were included, >0.5%) among two generations: seeds employed for generating the plants (left) and harvested seeds (right). **B,** Horizontal plots represent the relative variation of key taxa among generation types (statistically significant differences are marked with an asterisk (*) and corresponding taxa names are underlined).

rhizosphere and root endosphere hosted a comparable number of rare OTUs, but a lower number of dominant OTUs in plants grown on sandy soil.

Bacterial community structure of tomato seeds across generations. After harvesting root tissues and soil, a total of 10 Moneymaker plants were left in the nonacclimated greenhouse environment to harvest their seeds upon ripening. The comparison between all bacterial communities analyzed saw a similar pattern in key taxa composition (Fig. 3A). Significant differences were attributable to taxa that dominated the community of this cultivar: the family *Burkholderiaceae* dominated the first generation (25%) and drastically decreased in the second generation (3%) with *Pseudomonadaceae* also decreasing at the same time (from 6 to 2%). Conversely, families *Comamonadaceae* (14%), *Rhizobiaceae* (8%), and *Oxalobacteraceae* (7%) dominated the bacterial community of the second generation after a substantial increment over the studied generation span (these taxa represented, respectively, 6, 2, and 0.5% of the bacterial community of the first generation) (Fig. 3B). The variation in the abundance of nine bacterial families was found to be significant by ANOVA after false discovery rate correction. Manual BLAST of the 10 single OTUs assigned to the family *Burkholderiaceae* resulted in the identification of 19 potential taxonomical assignments at species level (best-hit results are shown in Supplementary Table S5). Additionally, the OTU composition of the family *Oxalobacteraceae* was similarly investigated by manual BLAST of representative sequences and revealed *Massilia* sp. to be the most frequent assignment.

Cultivar-driven variations in the bacterial communities of tomato microhabitats. Different plant compartments hosted distinctive bacterial communities depending on the cultivar or soil employed. Among different seed cultivars, even if characterized by a comparable diversity, prevailing bacteria (abundance >0.5% in each considered microhabitat) were demarked by a higher presence of taxa belonging to *Rhodobacteraceae* in Hildares F1 seeds (from 0.5 to 1%) and of *Cyanobacteria* ML635J-21, *Staphylococcaceae*, and *Burkholderiaceae* in Moneymaker first-generation seeds (Supplementary Fig. S1). In a similar way, root endosphere bacterial communities showed an increment of *Streptomycetaceae*, *Bacillaceae*, *Cellvibrionaceae* in Hildares F1, and of *Xanthomonadaceae*, *Rhizobiales*, and *Nocardioidaceae* in Moneymaker (Supplementary Fig. S2). PCoA plotting of the beta diversity (pairwise sample dissimilarity; Fig. 4) showed dissimilarities among analyzed samples. Major variations were visible among the two generations of Moneymaker seeds: while in the first generation

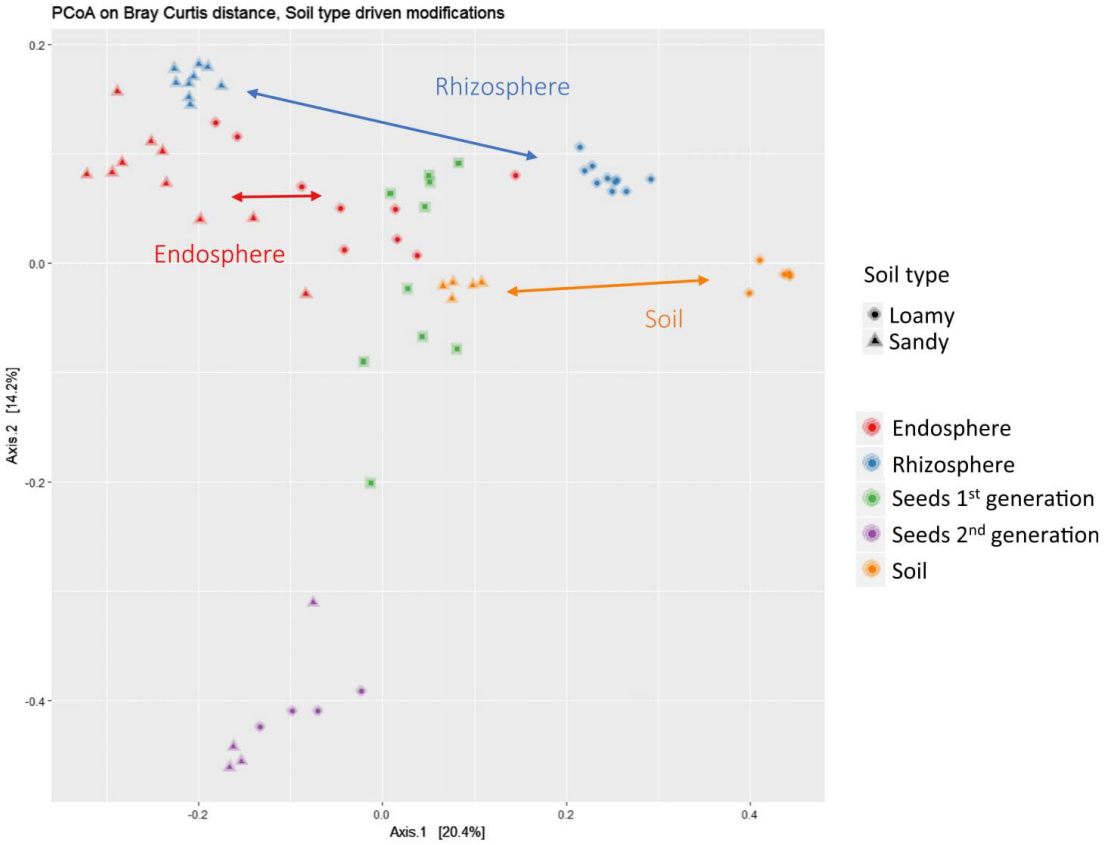

Fig. 4. PCoA plot with distances among samples based on the bacterial community composition. Samples are colored according to the different microhabitats. The soil types that were used to grow the tomato plants are denoted with different shapes. Seed bacteriome analyses with the second plant generation were only conducted with tomato cultivar Moneymaker.

the community resulted similar to other plant compartments (adjacent in the plot), the shift induced in the second generation showed a substantial modification in the community. Differences among soil sources were also visible in the plot: loamy and sandy bulk soils clearly hosted different communities as rhizosphere and, to a lower extent, root endosphere (Supplementary Fig. S3).

Tracking down beneficial bacteria inside plant compartments and across seed generations. To understand which PGPB are distributed across the plant system, an OTU network was created by graphically linking OTUs to the corresponding habitat (Fig. 5). With the organization of OTUs in compartments-specific clusters, the abundance and taxonomical diversity of shared and exclusive OTUs was assessed.

The bacterial community of soil was characterized by the highest taxonomical diversity and number of OTUs exclusive for a single compartment. Accounting the whole plant system, OTUs attributed to *Acidobacteria* were present almost exclusively in soil and rhizosphere-connected clusters but with a detectable presence in seeds. OTUs assigned to *Proteobacteria* composed the majority in clusters linked to plant compartments. *Firmicutes* OTUs were specifically allocated in root endosphere as peculiar for this habitat or as shared with the soil.

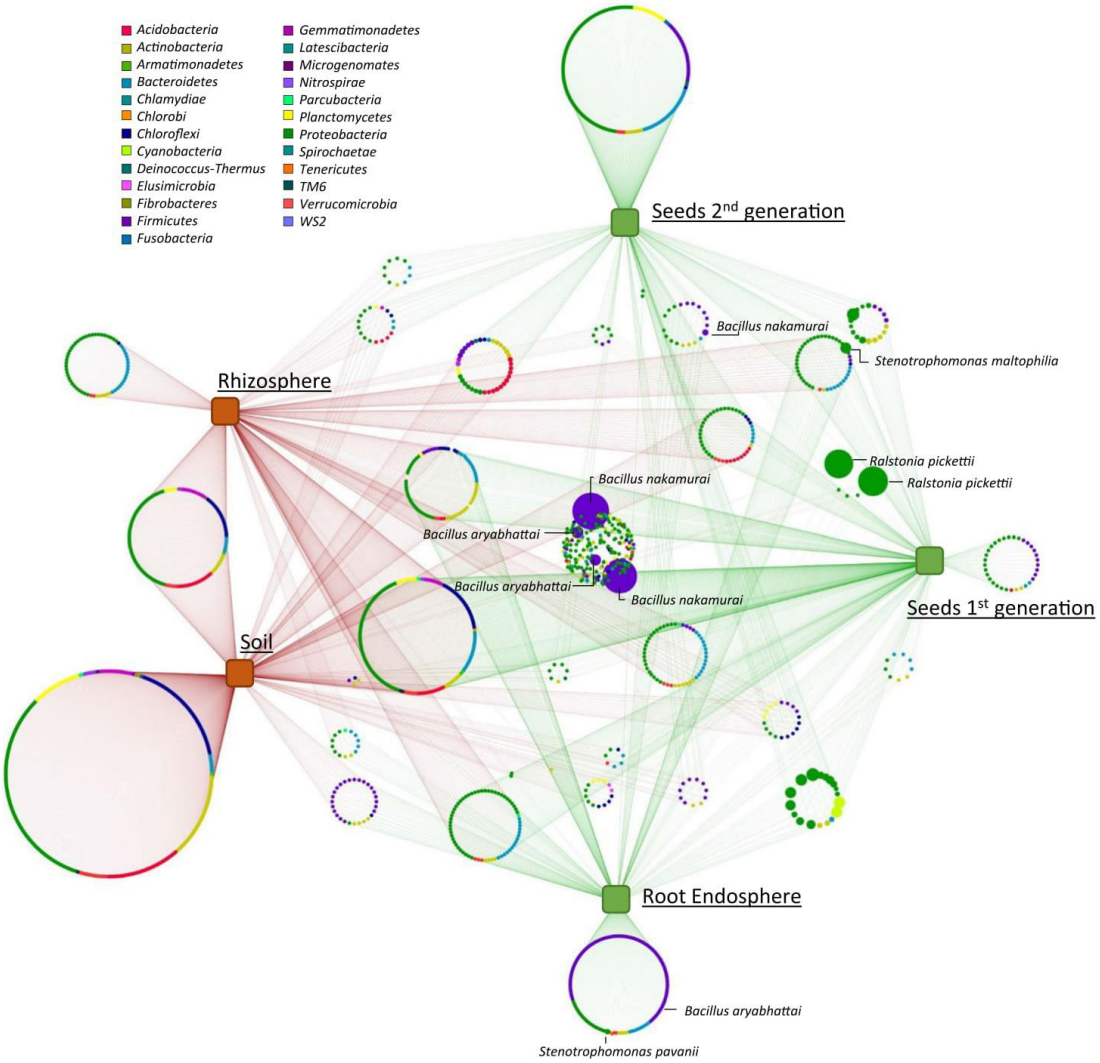

Fig. 5. Operational taxonomic unit (OTU) network of the tomato plant bacterial community structure. Dots represent single OTUs while labeled squares represent samples. Edges connecting samples with OTUs are colored to discern between soil (brown) and plant (green) habitats. Nodes are colored according to taxonomic assignments (phylum level). OTUs that correspond to isolated bacteria are highlighted with a node size proportional to their abundance. Labels highlight all plant growth-promoting bacteria in the network.

A total number of 5,153 bacteria was isolated from the tomato plant system. Isolates were characterized and selected for PGP properties and antagonistic effects against *F. oxysporum*. Following the screening, 19 isolates showed multiple positive traits and were thus considered for further analyses (Supplementary Table S6). Most of these bacterial strains showed the ability to produce siderophores, ACC-deaminase, and salt/drought tolerance. Moreover, in vivo tests showed the ability of *Bacillus aryabhattai* C6b and *Microbacterium flavescens* C7 to promote plant growth in tomato plant in absence of stressors.

Five of these beneficial phenotypes were also found in the amplicon data reconstruction of the tomato plant bacterial community by 16S rRNA gene comparison (*Bacillus aryabhattai*, *Bacillus nakamurai*, *Ralstonia pickettii*, *Stenotrophomonas maltophilia*, and *Stenotrophomonas pavanii*). OTUs belonging to these five plant beneficial taxa have been highlighted in the network analysis (Fig. 5). Both members of *Bacillus* spp. were found, in the core of the studied plant microbial communities with a single instance of OTU assigned to *Bacillus nakamurai* being shared among the root endosphere and second-generation seeds. *Ralstonia pickettii* was in both soil and first-generation seeds and has been found in the endosphere and in the seeds of the second generation (Fig. 6). While *Stenotrophomonas pavanii* was detected only in the root endosphere, *Stenotrophomonas maltophilia* was present in the seeds of the first generation and colonized the endosphere, the seeds of the second generation, and the rhizosphere.

DISCUSSION

Our results showed that soil bacterial community composition has a high impact on the bacterial community of below ground compartments (rhizosphere, root endosphere) in both tomato cultivars. However, the effect is progressively lowering from the rhizosphere to the root endosphere and finally to the seeds. At the same time, the seed bacterial community analysis across a generation identified a continuous turnover of the seed bacterial assemblage that might be triggered by environmental conditions. Interestingly, tracking down beneficial bacteria inside the whole plant system allowed us to identify plant seeds as a primary vehicle for transmission of PGPB.

Habitat specificity and key taxa in the tomato plant bacteriome. Plant seeds constitute the basis for each new plant generation and thus are essential determinants for the plant's early development. In the present study we found that the bacterial community of each plant habitat showed distinct fingerprints and harbored different PGPB. However, we found high cross-habitat similarity at phylum level; *Proteobacteria* were the most representative phylum within the whole plant system confirming previous findings (Bulgarelli et al. 2013). Soil, rhizosphere, and root endosphere were the microhabitats with the highest diversity, while seeds hosted more selective communities in both generations. As example, *Acidobacteria* were found to be under the detection threshold in the root endosphere as already described in a related

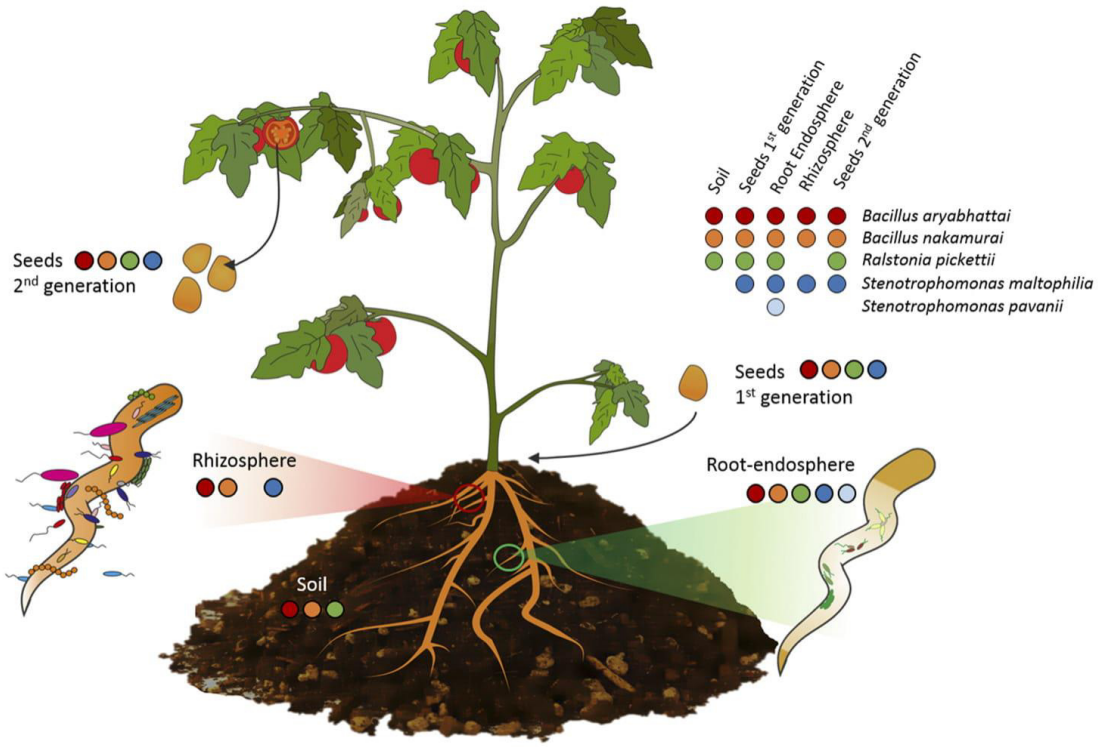

Fig. 6. Graphical representation of the plant system including microhabitat associations of analyzed isolates from the tomato endosphere. All identified bacteria with plant growth-promoting effects were linked to the microhabitats they were found to be associated with by colored dots. Both seed generations are included in the model.

study (Santoyo et al. 2016); however, seeds of both generations have been found enriched with members of this phylum, thus the plant endosphere creates a bridge between soil and the seeds. Members of this phylum have been recently described as possessing growth-promoting effects associated to auxin production (Kielak et al. 2016) and are therefore connected to the regulation of seed germination, increment in roots and shoots biomass as well as morphological changes in root system (Shu et al. 2016). Although being an aboveground-located structure in tomato plants, the seed is a quiescent structure designed to be biochemically active only when in contact with the soil. For this reason, it is in the interest of the plant to allocate and enrich this compartment with soil-compatible bacteria using the rhizosphere and the root endosphere as a roadway inside the plant.

Plant genotype and soil bacterial community–both factors shaped the seed bacteriome. In the rhizosphere we found a strong impact of the soil microbiota comparable to other studies (De Ridder-Duine et al. 2005; Inceoğlu et al. 2012), and even if less evident, this signature was still visible in root endosphere. Interestingly, we found no instance of this phenomenon in the bacterial community of second-generation seeds grown in different soil types. Although the underlying mechanisms are currently unknown, the seed microbiota could be a feature of below ground plant compartments that is specifically shaped under influence of the plant. The sequencing data shows that seeds of the first and second generation hosted a different bacterial community with the latter also diverging from the other studied plant microhabitats. Interestingly, the difference among seeds was not ascribable to a completely different structure of the bacterial community but to the modification of dominant taxa belonging to *Burkholderiaceae* in the first generation and *Oxalobacteraceae* in the second generation. Members of *Burkholderiaceae* are known in biological control primarily for the outstanding ability to produce various antifungal compounds (Eberl and Vandamme 2016). *Ralstonia piketii*, that was found preeminent among *Burkholderiaceae*, represents a niche-competing microorganism that could occupy the ecological niche of pathogenic *Ralstonia* spp. For these reasons, the consistent decrement over a generation of this bacterial species when passing from field condition (seed producer) to a pathogen-free environment (controlled greenhouse) could be indicative for the connection of the seed bacterial assemblage to environmental conditions. In the same way, we found evidence of environment-driven modifications in the seed microbiome of the second generation. *Massilia* (*Oxalobacteraceae*) has already been described as exhibiting attributes related to plant growth in vitro (Hrynkiewicz et al. 2010; Weinert et al. 2010) and as highly variating across medium modification stages (Ofek et al. 2012). Both these findings support our findings related to the plant's transitioning from a nutrient rich substrate (field) to a nutrient-poor substrate (90% sand). Similarly, also the plant-beneficial bacterium *Stenotrophomonas maltophilia* was found to be increased in the second generation of the seed. Bacteria belonging to *Stenotrophomonas* spp. have been extensively used in agriculture as biocontrol agents for their ability to promote plant growth and produce antimicrobial compounds that protect plants (Ryan et al. 2009), therefore also confirming our hypothesis.

The distribution of PGPB across the tomato plant system. Our reconstruction of the bacterial community included tracing of beneficial bacteria that were isolated and characterized from tomato plants. We propose a model (Fig. 6) where, despite the large bacterial diversity harbored by the soil, the seed represents a major vehicle of transmission for PGPB. This finding introduces the idea that new isolation strategies for plant-growth beneficial bacteria should focus less on the high biodiversity of the soil and more on the plant with emphasis on the seed. Most of the microorganisms within seeds are in a dormant stage, therefore it is difficult to isolate them in a traditional procedure. However, isolation after a certain period after germination and growth under gnotobiotic conditions is more promising (Adam et al. 2018) and allows the exploit of this important bioresource for novel seed treatment strategies.

Cross-kingdom similarities can be suggested for vertical microbiota transmission. Mendes and Raaijmakers (2015) already described cross-kingdom similarities in microbiota functions. In the present study, we found certain parallels between the plant's seed microbiome and the human placenta microbiome. Aagaard et al. (2014) described the placenta's microbiome as unique in the human body, with the next closest microhabitat with a similar composition being the mouth cavity. Both microbiotas harbor a unique microbial community that substantially differs from that of spatially close microhabitats. It was also shown that mammals equip their offspring with beneficial microorganisms during birth (Blaser 2006). Likewise, plants can transfer a selected community to the next generation over their seeds or vegetative organs (Vannier et al. 2018). A similar phenomenon was also observed for mosses as well as lichens, which equip their propagules with a beneficial, bacterial community (Aschenbrenner et al. 2014; Bragina et al. 2012). This vertical transmission allows "continuity of partnership" between the plant and its symbionts within the holobiont concept (Vandenkoornhuyse et al. 2015; Vannier et al. 2018).

With this study, we intended to understand how PGPB are distributed across the plant system while studying how they are influenced by plant genotype and soil quality. We reconstructed the microbiome of the tomato plant considering four key microenvironments: the soil, the seeds, the rhizosphere, the root endosphere, and the seeds harvested at plant maturity. Our results showed that the plant genotype has no substantial influence on the bacterial community of below ground plant compartments that are instead tightly connected to soil quality. Deepening studies of the microbial composition of seeds across generations provided instances of how this compartment represents an important vehicle for the transmission of PGPB. The overall findings suggest that plant seeds have a key role as carriers of PGPB and are hotspots for their isolation. Detailed mechanisms on how environmental conditions can influence the selection process for seed endophytes and adjust the bacterial assemblage to a new, potentially advantageous composition remain to be explored.

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Manuscript I

Cultivar-specific Archaea in the tomato rhizosphere mainly originate from soil

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ABSTRACT

Archaea have been recently identified as substantial members of the plant microbiome. They were shown to be enriched in the rhizosphere but less is known about their assembly, composition and transmission. Therefore, we investigated archaeal communities in the rhizosphere of tomato (*Solanum lycopersicum* L., cv. Moneymaker and Hildares) over two generations of seeds and in different bulk soil. The plant genotype strongly influenced the abundance of the archaeal rhizosphere community. Conversely to what has been observed within rhizospheric bacterial communities, these modifications were so distinct to mask any possible effect determined by the different composition of soil. No indications were found that

support the hypothesis of a plant-mediated vertical transmission of these microorganisms to offspring plants. In connection with the limited role that these microorganisms play in the early formation of the rhizosphere, it is plausible that archaea represent only bystander microorganisms in the seed.

INTRODUCTION

The plant microbiome was identified as key for the next green revolution towards sustainable agriculture (Bender et al. 2016). The focus of plant microbiome research is mainly on bacteria and fungi, whereas archaea are often overseen. So far, archaea have been found as part of numerous microbiomes, adapted to a great variety of conditions, colonizing soil, plants and animals, humans, and especially ruminants and termites (Janssen & Kirs, 2008; Leininger et al., 2006; Moissl-Eichinger et al., 2018; Samuel et al., 2007; Shi et al., 2015; Taffner et al., 2018). In soils both archaeal abundance and community structure can differ, as they are mainly shaped by the soil type and layer (Azziz et al., 2016; Chen et al., 2010). In plants, including domesticated plants such as arugula, olive trees, and maize, archaea have been found at high abundances in the rhizosphere and endosphere (exclusively in nutrient-rich hotspots like rotten roots), and with lower abundances in the phyllosphere (Chelius & Triplett, 2001; Müller et al., 2015; Taffner et al., 2019). Several abiotic and biotic factors have been found to influence the archaeal fraction of plants, reshaping community structure and abundance. Using metagenomic analyses, Archaea have been found to potentially interact with the plant holobiont by three different mode of actions: i) competition and support (syntrophic interaction) with bacteria and fungi, ii) nutrient supply for plants, and iii) plant growth promotion through auxin biosynthesis (Stams & Plugge, 2009, Taffner et al., 2019, Taffner et al., 2018). Different archaeal communities were found in plant microhabitats, e.g., a were enriched in the rhizosphere but less is known about their assembly and transmission (Taffner et al., 2018).

Tomato (*Solanum lycopersicum* L.) was selected as a model organism to unveil the composition and structure of archaeal communities. Tomatoes are important vegetable crop for human consumption with up to 177 million tons per year harvested (FAOSTAT, 2016). Together with other vegetables, tomatoes represent a significant part of a healthy diet linked to a reduced risk of heart disease and stroke, lower blood pressure, cancer prevention and other numerous beneficial effects for human health (He et al., 2006). As of today, the production and processing of this crop is commonly associated with conspicuous losses that reach up to 45% (FAO 2018). Soil-borne pathogens, e.g. fungi from the genera *Fusarium*, *Rhizoctonia* and *Verticillium*, are among the major microbiological threats for this crop that significantly limit its yields (Oerke, 2006). For both its relevance and the problems connected with tomato production, the microbiome of the tomato plant has been widely characterized. Most studies mainly focus on the hypogeal resistance-associated plant compartments and on the plant capability to host beneficial bacterial endophytes conferring resistance to biotic and abiotic stresses (Liu et al., 2017; Upreti & Thomas, 2015; Yan et al., 2003). Recently Bergna et al., (2018) identified tomato seeds as a carrier for microbiota of key relevance: beneficial microorganisms are vertically transmitted to successive generations and represent a significant portion of the plant microbiome in early developmental stages. Less is known about archaea in tomato but it was shown that root exudates of tomato lead to an enrichment of archaea in this habitat (Simon et al., 2005). These insights increase our understanding related to (i) factors shape the community structure of archaea in tomato plants, (ii) archaeal taxa that are transmitted from one generation to the next, and (iii) their potential role in subsequent plant generations.

For this reason, the objective of this study was to describe the tomato plant archaeal community while investigating for the first time the archaeal abundance and community structure in tomato seeds. To achieve this, we combined qPCR and next-generation sequencing to quantify and

describe the archaeal community of the tomato plant with a focus on the rhizosphere and two generations of seeds along with the soil in which plants were generated.

MATERIALS AND METHODS

Experimental design. Surface-sterilized seeds (1st generation) of tomato plants (*Solanum lycopersicum* L.) of the cultivars *Moneymaker* (Austrosaat AG, Austria) and *Hildares* F1 (Hild Samen GmbH, Germany) were sown in 8 L pots (one seed per pot). Each pot contained a defined soil mixture obtained by adding sterile quartz sand and commercial loamy soil (Ökohum GmbH, Herbertingen, Switzerland) or diluvial sand (Rühlmann and Ruppel 2005) (proportion 10:1). A total of 100 planted pots were kept in a non-acclimated greenhouse (approximately 24/20 °C day/ night temperature) of the Graz Botanical Garden (Graz, Austria) together with unplanted pots containing only the soil mixture.

Sample collection. At the late flowering stage of the plants (85 days post planting) soil and plants specimens were collected followed by a second sampling at the ripening of fruits of Moneymaker plants. Soil samples were collected from the central section of the pots containing the soil-sand mixture by only removing the top layer of soil (2-3 cm) with sterile tools. Rhizospheric soil has been obtained by shaking the root compartment and by collecting in sterile bags the material that was adhering to the roots. At ripening of tomato fruits, the seeds of the 2nd generation were collected from 10 Moneymaker plants and cleaned from leftover fruit tissues using sterile tools.

Samples were processed using a modified protocol of Bragina and colleagues (2012) following sample processing as described in the work of Bergna and colleagues (Bergna et al., 2018). Briefly, collected specimens of both soil and rhizosphere were suspended in 0.85% sodium chloride solution (NaCl) and shaken for 30 minutes. After this first step, the liquid phase was

extracted with laboratory pipettes and the microorganisms-containing pellets were obtained by centrifugation (20 min at 16,750 g) and stored at -70 °C. Seeds (I and II generation) of Moneymaker and Hildares F1 were washed in sterile water, divided in plastic vials (20 seeds per vial) with 4 ml of 0.85% NaCl and gently shaken for 4 hours. The seeds were then homogenized with mortar and pestle and suspended in 0.85% NaCl. After centrifugation (20 min at 16,750 g) microorganisms-containing pellets were stored at -70 °C.

DNA extraction and preparation of the 16S rRNA gene fragment amplicons. The so obtained pellets were used for the isolation of total community DNA employing the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. Extracted community DNA samples were used for PCR-based barcoding. In order to strictly amplify the archaeal 16S rRNA gene alone, we performed a nested PCR using the archaea-specific primers 344f and 915r in the first PCR and modified primer pair S-D-Arch-0349-a-S-17/S-D-Arch-0519-a-A-16 (here 349f/519r (Klindworth et al., 2013) with an additional 10 bp primer-pad (TATGGTAATT/AGTCAGCCAG) and linker (GT/GG) in the subsequent PCR, as previously described by protocols of the Earth Microbiome Project (Walters et al., 2016). In a third PCR the Golay barcodes were annealed. The PCR reactions were conducted as previously described (Taffner et al., 2019). All PCR reactions were conducted as triplicates, purified with the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI), and pooled in equimolar concentrations prior to sequencing. The Sequencing was then conducted using an Illumina HiSeq Personal Sequencer (GATC Biotech AG, Konstanz, Germany).

Quantitative real-time PCR with archaea-specific primers

The quantification of archaeal DNA in soil, rhizosphere, and two generations of seeds was conducted employing the aforementioned primer pairs 344aF and 517uR (Probst, Auerbach, & Moissl-Eichinger, 2013). For the qPCR reaction 1 µl of DNA was used for each 10 µl reaction

mix containing 5 µl KAPA SYBR Green 2X MM Mix (BIO-RAD, Hercules, CA, USA), 0.5µl forward and reverse primers (344aF and 517uR - 10 pmol/µl) and 3 µl of PCR grade water. *Haloferax denitrificans* 16S rRNA gene standards were employed. PCR amplifications were conducted in triplicates using a Rotor-Gene 6000 series (Corbett Research) thermal cycler using the following program settings: 95°C / 5 min, 95°C / 15 sec, annealing 60°C / 30 sec, extension 72°C / 30 sec; amplification steps were repeated 39 times. Final elongation was done from 72°C - 96°C.

Data analysis of 16S rRNA gene amplicons for determination of archaeal community structure. 16S rRNA gene paired-end sequences were joined (SeqPrep), reoriented and demultiplexed in the Qiime 1 environment (version 1.9.0) (Quantitative Insights into Microbial Ecology, version 1.9.0; (Caporaso et al., 2010). Sequences were then denoised, dereplicated and clustered using the DADA2 pipeline integrated within Qiime2 environment (version 2019.4 release). Chimeras were identified and filtered. The features' taxonomy assignment was conducted using a fitted classifier (Scikit-learn) (Pedregosa et al., 2011) and the Silva 16S (349af - 519ar 99 OTUs) Archaeal database (version: 128). Unassigned and non-archaeal features and contaminants were filtered from the resulting feature table.

A graphical rendering of the archaeal community structure at class level was produced using the open-source software Circos (Krzywinski, 2009) (Fig. 1). In order to display a more reliable differential abundance among samples, the number of reads of each sample has been normalised with the concentration value obtained with the qPCR. In this way we coupled the superior quantification accuracy of qPCR with the capacity of amplicon sequencing to describe complex microbial communities. Alpha diversity distances were calculated and rendered at feature level in the with the Phyloseq package (McMurdie & Holmes, 2013) within R environment using Observed Species, Chao 1, Shannon and Inverse Simpson measures. Phyloseq package (McMurdie & Holmes, 2013) was also employed for gerneration the PCoA plot. The statistical

analysis for sample difference significance was performed using the package vegan v. 2.5.5 (Oksanen et al., 2019) with the Adonis test (999 permutations).

RESULTS

Quantification of archaeal population density in tomato plants. Using a quantitative polymerase chain reaction (qPCR) with specific archaeal primers, targeting the 16S ribosomal RNA gene, we quantified the archaeal population of two tomato cultivars (Moneymaker and Hildares F1) grown in two soil mixtures. Archaeal rRNA gene abundances spanned between 2.9×10^9 and 2.3×10^{13} copies per g of sample (Figure 1). The highest archaeal abundance was found in the rhizosphere of Moneymaker plants. Irrespective of the soil mixture in which these plants were grown, archaea were significantly ($p < 0.05$) more abundant in the rhizosphere of this cultivar than in the rhizosphere of Hildares F1, showing archaeal abundances of 1.9-2.0 $\times$ 10^{12} copies per g sample. The composition and texture of the two soil mixtures did not result in significantly different archaeal abundance ($p > 0.05$) that has been quantified in 5.9×10^{11} and 2.9×10^{12} copies per g in loamy and sandy soil mixtures respectively. However, there was a distinct difference in the number of archaeal gene copies among the two analysed generations of Moneymaker seeds: we found the latter (second generation) characterised by a lower abundance of archaea with 2.9×10^9 to 7.1×10^9 copies per g. In contrast, the archaeal abundance in the seeds of the first generation was higher with 2.1×10^{12} copies per g, but with a high standard deviation. Additional statistical analysis with Kruskal-Wallis test did not show a significant result for the difference of the seed generations ($p > 0.05$).

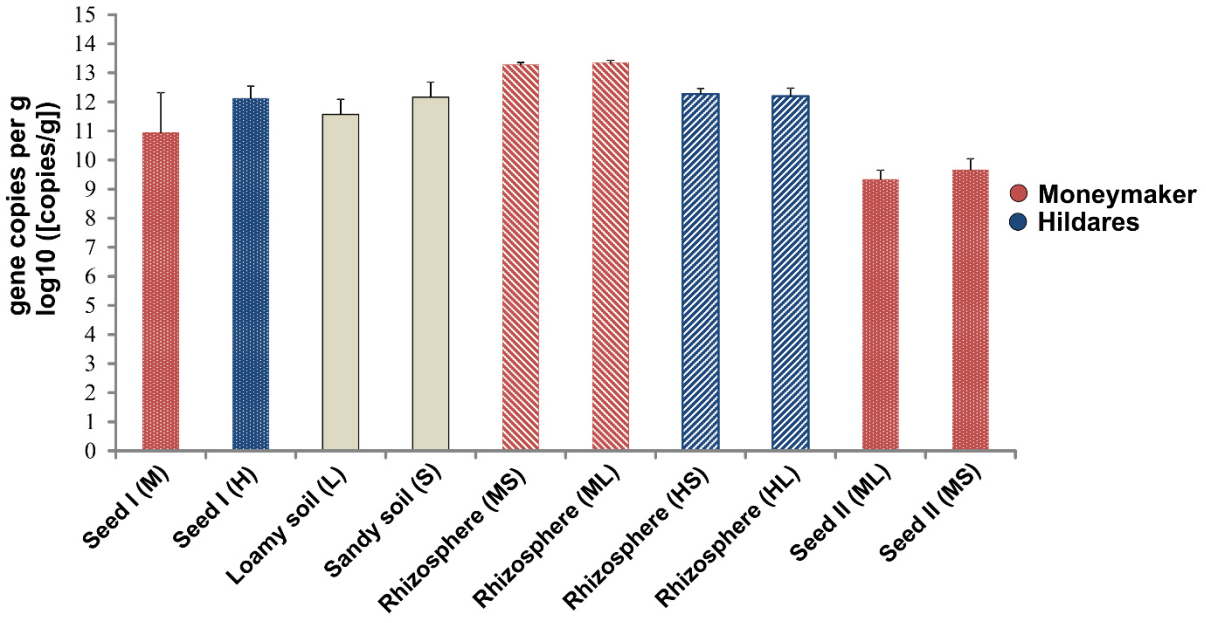

Figure 1: qPCR-based quantitative analysis of archaea in different samples. Archaeal abundances were determined for the habitats: seeds from the first (Seed I) and the second generation (Seed II), and the rhizosphere of the cultivars Moneymaker (M) and Hildares F1 (H). Abundances in the loamy (C) and sandy soil (G) were measured as well.

Structure of archaeal communities associated with tomato plants and diversity analyses. High throughput sequencing of 16S rRNA gene fragments of two soil mixtures, rhizosphere and seeds (1st and 2nd generation) of two tomato cultivars (Moneymaker and Hildares F1) yielded in a total of 748,221 high quality archaeal reads that have been clustered in a total of 1133 distinct features.

The composition of the tomato plant archaeal community was dominated by two main phyla: *Thaumarchaeota* and *Euryarchaeota* (Fig. 2). In all analysed microenvironments, members of these two phyla accounted for more than 80% of the whole community. More precisely, *Thaumarchaeota,* consistently accounted for more than the 60% of the archaeal community and lower abundances of this phylum have been found in the Hildares seeds used for generating the plants (1st generation) as well as in the loamy soil. The low abundance of *Thaumarchaeota* (46%) in the Hildares F1 seeds was accompanied by a high abundance of *Euryarchaeota* (34%) and unassigned reads (20%). On the other hand, the archaeal community composition of the

commercial loamy soil mixture represented a more equal distribution with *Euryarchaeota* representing 49% of the community and *Thaumarchaeota* 48%. At the same time, among the two soil types a great difference in the abundance of these phyla was observed. While *Thaumarchaeota* represented 79% of the archaeal community in sandy soil, in commercial loamy soil their abundance was only of 51%. The abundance of *Euryarchaeota*, on the other hand, varied between 11% in sandy soil to 48% in loamy soil.

At class level both community-dominating phyla were mostly represented by a single class; the *Soil Crenarchaeotic Group* for *Thaumarchaeota* and *Methanomicrobia* for *Euryarchaeota*. Other *Euryarchaeota* classes which were relevant for the archaeal community were identified as *Thermoplasmata* and *Methanobacteria* that found to be specific for the sandy soil (respectively 2 and 1 %) and representing up to 4 and 8 % in the rhizosphere. A more complete description of the archaeal community is provided in Table S2 (supplementary material).

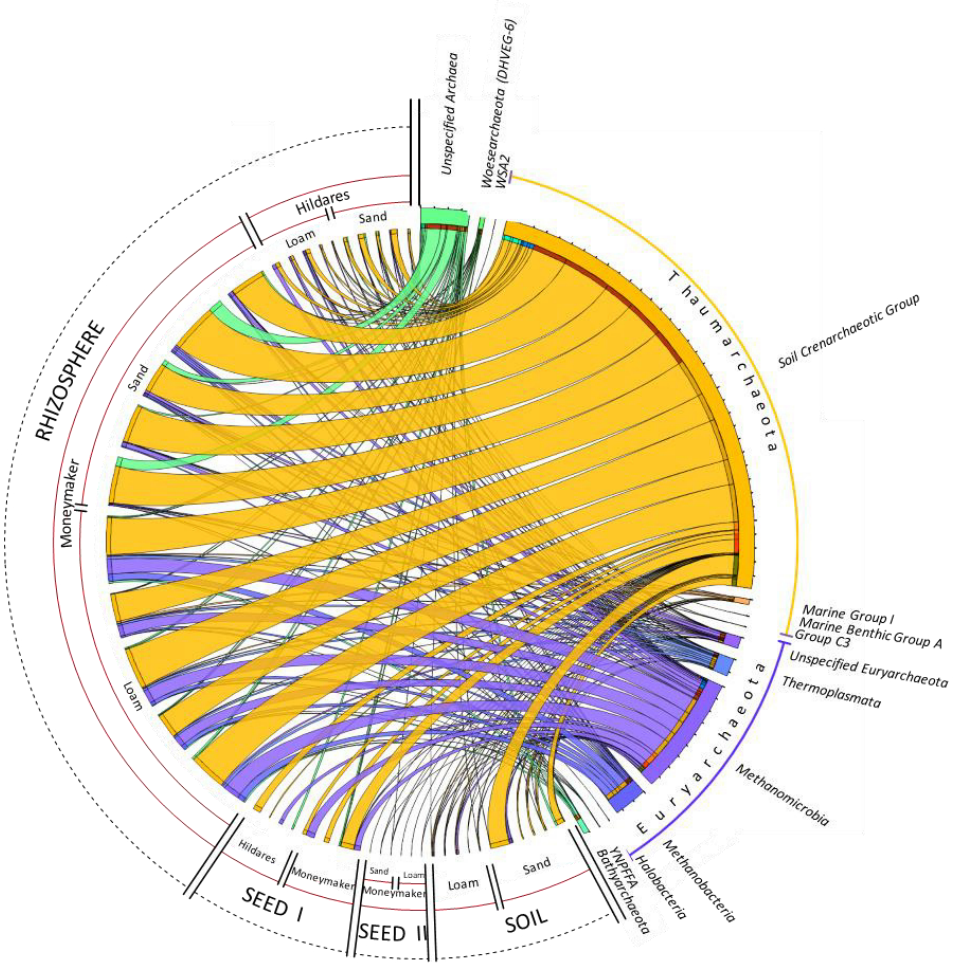

Figure 2. Archaeal communities of two soil-sand mixtures, seeds, and the rhizosphere of two tomato plant cultivars (Moneymaker and Hildares F1). Total abundances of the respective archaeal populations were adjusted with a qPCR-based quantification. The graph was obtained using the open-source software Circos (http://circos.ca).

Soil and cultivar driven variabilities. The diversity within archaeal communities (Table S2) was evaluated with metrics sensitive to dominant taxa (Inverse Simpson's index), to rare taxa (Chao1 and observed species) and incorporating both evenness and richness (Shannon). While alpha diversity values were not inferable for seed samples due to their reduced number of reads (low abundance across samples, as confirmed by qPCR results), the analysis showed a consistently higher diversity in the rhizosphere of Moneymaker plants regardless the soil mixture employed (Fig 3). The comparison of diversity levels of soil mixtures indicated that the sandy soil mixture had a higher archaeal diversity when compared

to the commercial loam mixture. Interestingly, the rhizosphere of plants grown in these two substrates were not determined by the archaeal community characteristics of the soil. The rhizosphere of Moneymaker plants was found to harbor an increased diversity, which was 3-fold higher than soil and the Hildares F1 rhizosphere (Observed and Chao1 indices).

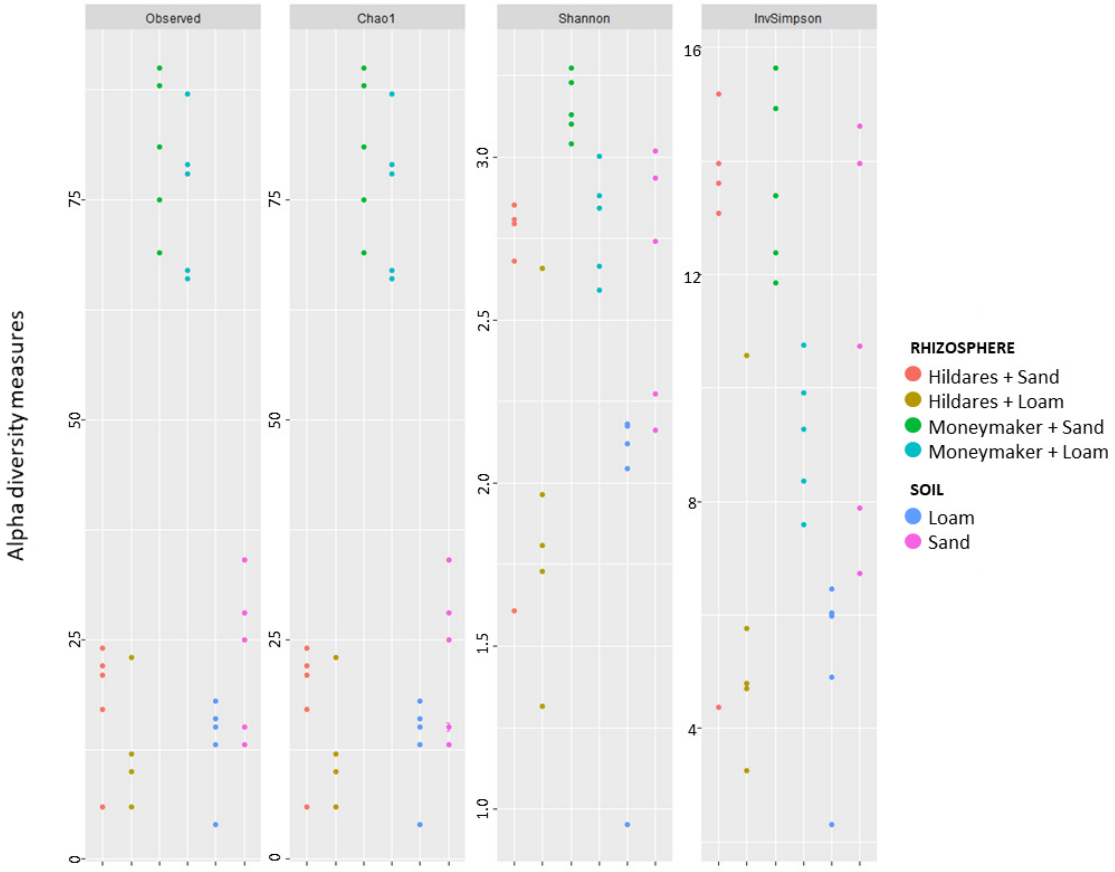

Figure 3. Assessment of alpha diversity across the rhizosphere and soil samples. Four different diversity measures were used: Observed Species, Chao1, Shannon and Inverse Simspson. A combination of measures sensitive to rare taxa (Observed species and Chao1) and to dominant taxa (Inverse Simpson's index) was performed in order to provide a comprehensive assessment of bacterial diversity.

In order to analyse similarities and dissimilarities among the archaeal communities of different samples, the beta diversity analysis has been graphically rendered with a principal coordinate analysis (PCoA) using the Bray Curtis distance (Fig. 4). Complementary to the alpha-diversity analysis, the beta-diversity analysis highlighted the unique nature of the rhizospheric communities of Moneymaker plants in this system. Rhizosphere archaeal communities of

Moneymaker plants showed slightly different archaeal communities (R^2 = 0. 69006, Pr (>F) = 0.01) among soil qualities. At the same time, regardless of the soil quality, the archaeal community of this sample group significantly differed to all other samples (Hildares F1 rhizosphere, 1st and 2nd generation seeds, and both soil mixtures) (R^2 = 0.49868, Pr (>F) = 0.001)). At the same time, in the PCoA no inter-samples similarities linked to the soil mixture quality were observed.

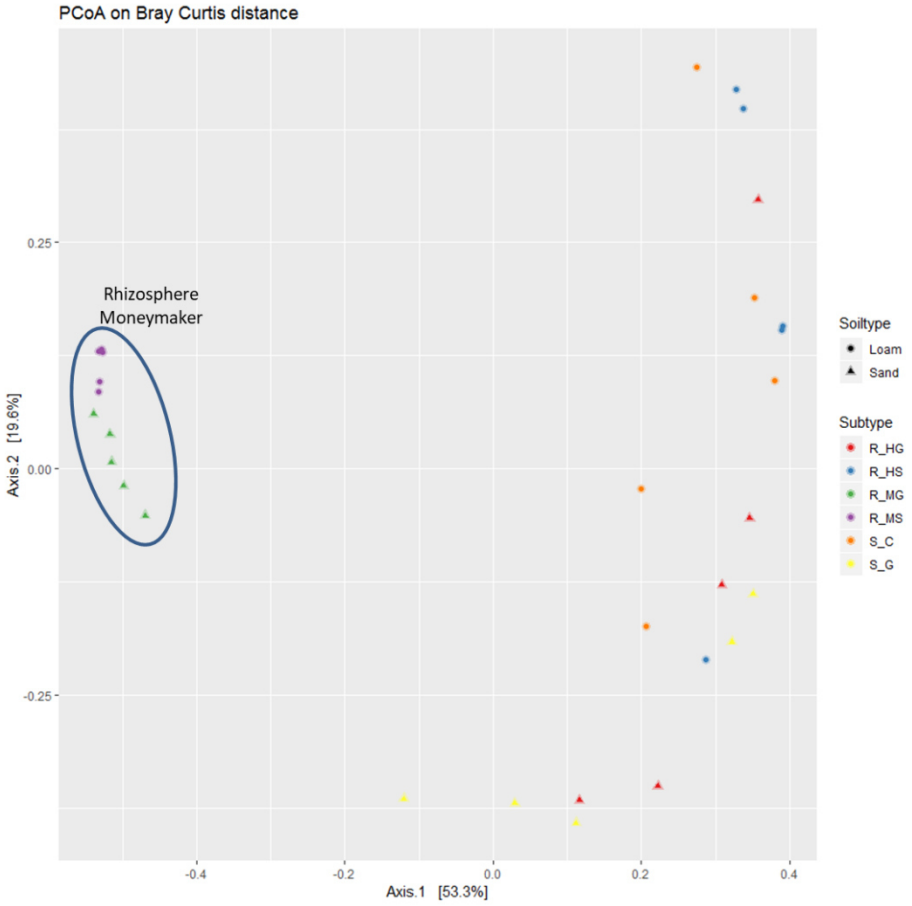

Figure 4. PCoA plot calculated using Bray Curtis metrics plotting the similarities/dissimilarities among samples based on their archaeal community composition. Samples are coloured according to the different microhabitats and the shape refers to the soil mixture employed.

Composition of the archaeal community in tomato plants. In tomato plants the archaeal core community (Figure 5) consisted of species assigned to the *Soil Crenarchaeotic Group* (*Thaumarchaeota*; 60.7%), *Methanosarcina* (*Euryarchaeota*; 12.6%), *Methanoculleus* (*Euryarchaeota*; 3.4 %), and unassigned archaeal species (7.2%), which were shared among all

habitats, including the seeds. Further, the seeds of Moneymaker harboured archaea of the genus *Candidatus Nitrososphaera* and several *Euryarchaeota* genera. Overall, a higher archaeal diversity was found associated to the cultivar Moneymaker, than in Hildares F1. In the rhizosphere of Moneymaker, several archaeal taxa specific for this habitat were found. Furthermore, the minor phylum of *Bathyarchaeota* was exclusively found associated to Moneymaker and loamy soil.

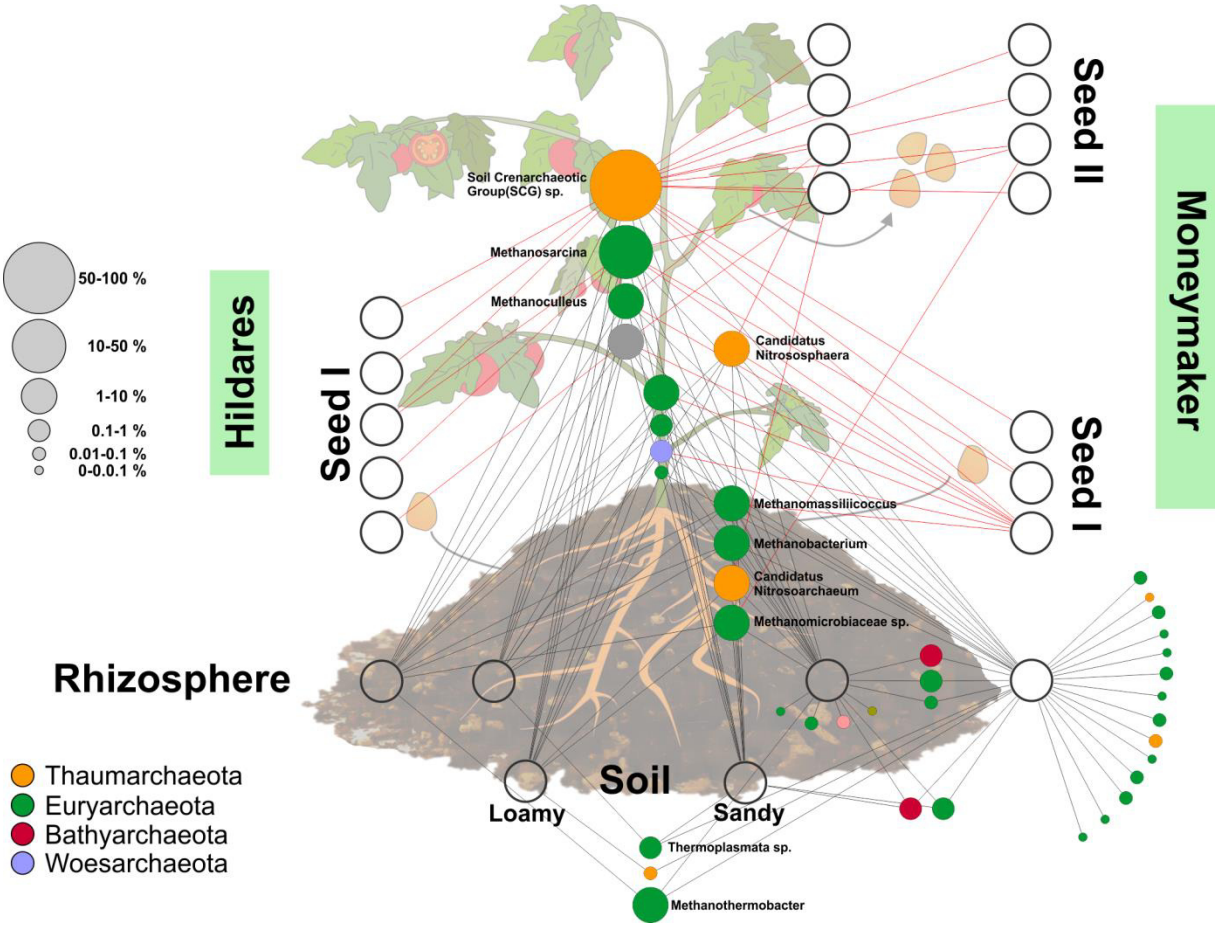

Figure 5. Feature network of the archaeal communities at the genus level, based on 16S rRNA gene fragment datasets. The datasets were obtained from the habitats soil, rhizosphere, and first and second generation of seeds of tomato plants of the cultivars Moneymaker and Hildares F1. Archaeal phyla are indicated by coloured bubbles: *Thaumarchaeota* in orange; *Euryarchaeota* in green; *Bathyarchaeota* in red; and *Woesearchaeota* in purple; unassigned taxa are shown in grey. Taxa found in the seeds are highlighted by red lines connecting the associated sample. The size of the bubble represents the relative abundance of the archaeal taxa throughout all habitats.

DISCUSSION

Habitat specificity of archaeal communities in tomato plants. In the present study, the abundance of archaea across the tomato plant was found to be highly habitat-specific and showed a strong so called rhizosphere effect (Buée et al., 2009). This was already shown for soil Crenarchaeota by Simon et al. (2000). As for bacteria, the enrichment of archaea in the rhizosphere is induced by the presence of nutrient-rich root exudates (Simon et al., 2005). On the other hand, the high archaeal abundance found in this habitat could be connected to a specific bacterial community setup that favouring archaeal colonisation.

Soil quality is a main driver of the archaeal community in soil. The archaeal community in the two soil qualities selected for this study (loamy and sandy) showed a difference in their diversity and in the abundance of specific dominant archaeal taxa. Our findings confirm that soil quality is a main determinant for the soil archaeal community structure (Chen et al., 2010; Di et al., 2010), and that it selects for archaeal taxa with specific characteristics (Azziz et al., 2016). An example is the increased abundance of ammonia oxidizing archaea (AOA) in sandy soil. AOA are part of the phylum *Thaumarchaeota*, which composed most of the archaeal community structure associated to the tomato plants in this study. The second most abundant phylum was assigned to *Euryarchaeota*. This phylum consists for the most part of methanogens such as *Methanomicrobia*, which represented the most abundant taxa of *Euryarchaeota* found. These anaerobic methanogens are usually part of microbiomes of crops, such as maize or arugula, mainly located in anoxic niches in the rhizosphere of the plants (Chelius & Triplett, 2001; Taffner et al., 2019).

The impact of soil quality on archaeal community in the rhizosphere. In contrast to the differences observed in bulk soils, we could not detect nor infer any significant soil quality-related effect on the abundance of the archaeal populations in the rhizosphere. On the one hand, it is possible that the absence of the effect might be due to the experimental design of this study

since the substantial dilution of both loamy and sandy soils with sterile sand might have mitigated the effects of soil texture on the rhizosphere. On the other hand, a previous analysis with an identical experimental setup (Bergna et al., 2018) observed that the differences in the bacterial communities of these two specific soils were highly conserved for the bacterial community of the rhizosphere of tomato plants. This different sensitivity of *archaea* and *bacteria* to soil quality is not new, but still not fully understood. While it is known that these microorganisms respond differently to soil depth, where the ratio of archaea to bacteria increases (Leininger et al., 2006b), archaea inhabit a far more restricted ecological niche in soils compared to bacteria (Bates et al., 2011). In addition, a recent study defined the process of rhizosphere formation as a dynamic and bacterial-exclusive process that does not include archaea until the last stages of plant development (Edwards et al., 2018). For these reasons, it is possible to hypothesise that, in contrast to what applies for bulk soil, archaeal rhizosphere communities are much less affected by soil quality compared to bacteria. Instead, archaea in the rhizosphere might be deeply influenced by the coexistence with bacterial communities and by the adjacent plant root system (Kang et al., 2019).

The plant genotype is a main driver for archaeal community in the rhizosphere. The archaeal diversity and abundance observed in the rhizosphere of tomato plants was consistently higher compared to both bulk soils employed. This rhizosphere effect is likely to be connected with the production of root exudates that represent a constant source of nutrients (Mendes et al.,2011) that attracts and allows the colonisation of archaea. Interestingly, the rhizospheric diversity shift was observed to be even clearer in Moneymaker plants that hosted a three times more diverse archaeal community than Hildares F1 plants. This is the first time that a plant-genotype effect of this magnitude has been observed on archaeal communities in the rhizosphere of agricultural plants. So far, a similar plant-genotype driven effect has been reported only for archaeal methanogens in the rhizosphere of rice in an aquatic environment

(Wu et al., 2009). This highly specific effect might not only be explained by the differences in the quality and quantity of root exudates, but also by different nutrient-uptake strategies of each plant cultivar (Grayston et al., 1997). It is in fact known that specific archaea, and notably AOA, are known to highly accumulate in N-demanding plants (Thion et al., 2016). Further, another explanation of this effect can be found in the inter-domain interactions that archaea can establish (Taffner et al., 2019). In this framework, the effects were shown that different root exudates have on bacterial and fungal microorganisms, they can modify the presence of metabolites in the rhizosphere and the soil-plant interface. Similarly, archaeal abundance is known to correlate with mycorrhizal abundance (Grayston et al., 1997); for this reason it is valuable to also integrate fungal communities in the analysis of archaea-plant interactions.

The potential ecological role of archaea in tomato seeds. A recent study described how the seed can represent an important vehicle for the vertical transmission of beneficial bacteria across generations (Bergna et al., 2018). Since archaea play relevant roles for plant nutrient cycling in the soil and the rhizosphere (Erkel et al., 2006; Leininger et al., 2006; Mendes et al., 2013), we initially hypothesized that archaea would have been conveyed by the plant to the seed where, as for bacteria (Bergna et al., 2018), they might support the germination and development of the offspring plant. The archaeal abundance registered by qPCR in tomato seeds was in the range of 10^9 gene copies/g in seeds, which follows the recent observations of Wassermann and colleagues (2019) in alpine seeds. However, the analysis of the archaeal community of tomato seeds did not provide any evidence that could indicate a selection of archaea for the offspring plant. As previously discussed, the recent work of Edwards and colleagues (2018) described the high dynamism of the process of rhizosphere formation during the vegetative phase of plant growth and how it compositionally stabilises only for the remainder of the life cycle. Only in this latter phase, archaea are found colonising the rhizosphere and are for this reason indicated as “late colonisers”. Therefore we suggest that

these microorganisms, that appear to be non-essential for the first stages of plant development, are not found in the seed (structures that represents the primary vehicle of beneficial microorganisms for the early stages of plant development). Moreover, it is more likely that archaea might have developed as bystander microorganisms in seeds, possibly based on syntrophic relationships with bacteria (Morris et al., 2013).

Unassigned archaeal features in tomato plants. The bioinformatic reconstruction of the archaeal community associated with the plant micro-niches was performed using an up-to-date established pipeline. This approach resulted in a well-defined archaeal community structure that was though not exempt from several taxonomically unassigned features. Features without taxonomical assignment represented 15% of total features found in these habitats, but can represent up to 20% at class level in seed samples. This is a well-known limitation for the characterisation of novel habitats, especially for archaea. In fact, the low frequency of archaeal community investigations resulted in the use of smaller and often incomplete taxonomy databases. The relatively low ratio of unassigned features of this analysis excludes the presence of significant PCR off-target effects or low read length. On the other hand, the high ratio of unassigned taxa in a low characterised habitat as the seed indicates that this problem is seemingly due to still poorly defined reference databases that can be increased only with further investigation of the archaeal domain.

Conclusion.

Archaea are substantial components of the plant microbiota with specific compositions in soil, rhizosphere and endosphere. Soil quality has a strong impact of archaeal soil communities but not on the rhizosphere composition. Here, the plant genotype (tomato cultivars) was identified as main driver that shaped the diversity and increased abundance. Moreover, we here showed for the first time a transmission of archaeal microorganisms to the offspring plant. At the same time, we found no indication for a specific selection. For this reason, we hypothesise that

archaea may represent bystanders microorganisms in seed based on syntrophic relationships with bacteria.

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ADDITIONAL MATERIAL

Table S2: feature table reporting the relative abundance of archaeal taxa across the tomato plant.

	Rhizosphere				Seeds 1st gen.		Seeds 2nd gen.		Soil	
	Hildares		Moneymaker		Moneymaker		Moneymaker			
	Loam	Sand	Loam	Sand	Hildares		Loam	Sand	Loam	Sand
Phylum *Bathyarchaeota*	0.00%	0.00%	1.47%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%
Crenarchaeota (class: *YNPFFA*)	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Euryarchaeota (class: *Halobacteria)*	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Euryarchaeota (class: *Methanobacteria*)	3.90%	1.40%	8.04%	2.20%	0.00%	0.00%	0.00%	0.00%	0.00%	1.11%
Euryarchaeota (class: *Methanomicrobia*)	38.46%	6.96%	22.76%	5.45%	34.02%	18.58%	16.29%	11.28%	49.82%	7.92%
Euryarchaeota (class: *Thermoplasmata*)	0.95%	0.48%	3.76%	1.54%	0.00%	0.78%	0.00%	0.00%	0.00%	2.09%
Unspecified *Euryarchaeota*	0.56%	2.18%	1.34%	2.32%	0.00%	0.20%	0.00%	0.00%	0.90%	0.56%
Thaumarchaeota (class: *Group C3*)	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thaumarchaeota (class: *Marine Benthic Group A*)	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thaumarchaeota (class: *Marine Group I*)	0.00%	0.00%	1.35%	0.00%	0.00%	0.00%	0.00%	2.54%	12.40%	0.32%
Thaumarchaeota (class: *Soil Crenarchaeotic Group*)	53.88%	71.83%	58.31%	76.18%	45.98%	77.17%	83.71%	70.62%	36.26%	82.28%
Phylum *WSA2*	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Woesearchaeota (*DHVEG-6*)	0.57%	0.03%	1.18%	0.10%	0.00%	1.14%	0.00%	0.00%	0.00%	0.00%
Unspecified *Archaea*	1.68%	17.12%	1.60%	11.84%	20.00%	2.13%	0.00%	15.56%	0.61%	5.48%

Manuscript II

Tomato microbiota and biotic and abiotic stress

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Tomato microbiota and biotic and abiotic stress

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Results

We carried out 16S rRNA amplicon sequencing (Illumina MiSeq, 2*300 bp) of the microbiota associated with roots of tomatoes subjected to two abiotic stresses (osmotic and ionic stress) and one biotic stress (*Verticillium dahliae*) and to all combinations thereof. In total we obtained sequences for 32 samples with 4 samples for each treatment. After quality control and singleton removal we were left with $2.74x10^5$ sequences per sample on average.

Infection with *V. dahliae* has a strong effect on the root-associated microbiota of tomato

We analyzed changes in microbial α-diversity using species richness and effective number of species (ENS) based on the Shannon-Wiener and Simpson indices. Treatment had a significant effect on α-diversity for all three measures of diversity ($P < .01$, Kruskal-Wallis test). Ionic treatment significantly decreased bacterial α-diversity compared to controls and to *V. dahliae* and osmotic treatment. Combined treatment with ionic stress and *V. dahliae* significantly decreased α-diversity compared to controls and treatment with *V. dahliae* alone ($P < .05$, Dunn's multiple comparison test with correction for multiple testing after Benjamini and Hochberg[Benjamini and Hochberg, n.d.] (Figure 1).

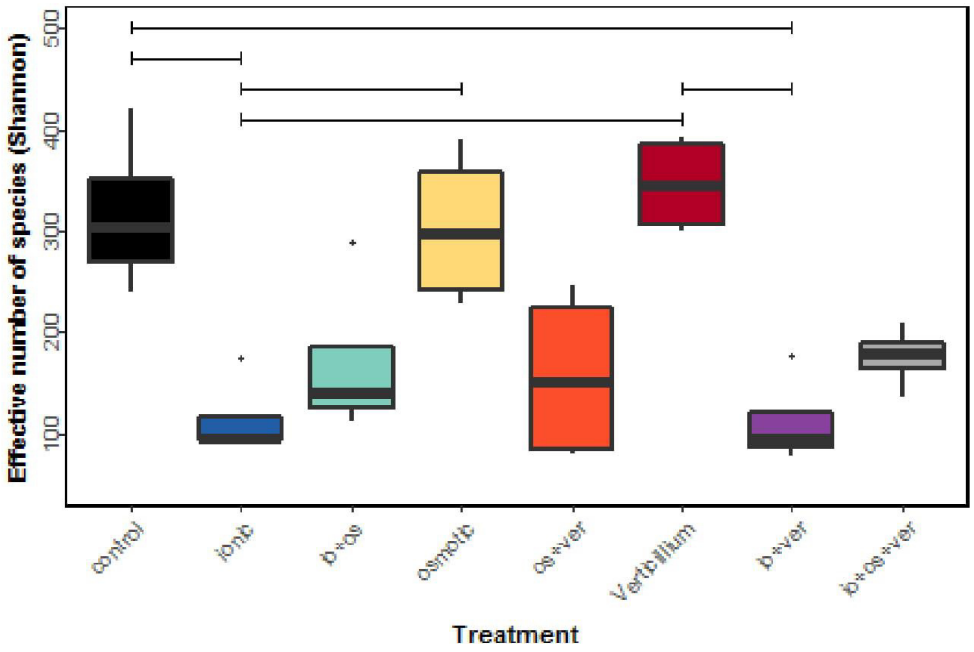


Figure 1: Abiotic and biotic stress has a significant effect on the α-diversity ($P < .01$, Kruskal-Wallis rank sum test). Shown are boxplots of the ENS based on the Shannon-Wiener index. Significant pairwise differences (all $P < .05$, Dunn's multiple comparison test) are indicated by bars. ENS: Effective number of species.

Principal coordinate analysis of the unweighted UniFrac distance matrix revealed that the treatments also had a significant effect on root-associated β-diversity (PERMANOVA, Pr(>F) < .001, Figure 2; for other distance metrics see Supplementary Figure 2). We also tested for differential abundance of bacterial OTUs upon treatment with *V. dahliae* and ionic and osmotic stress compared to non-treated controls using DESeq2 and detected 896, 454 and 250 differentially abundant OTUs, respectively. While most of the OTUs were only statistically significantly different under one stress regime (Figure 3), some

OTUs were either increased or decreased in two or even three conditions, indicating a putative general stress response.

Interestingyl, while ionic treatment had the strongest effect on bacterial α-diversity and resulted in a sharp decrease in both species richness and ENS (Figure 1), bacterial abundance was most strongly modulated by infection with *V. dahliae* as shown by the high number of differentially abundant OTUs (896). Strikingly, the overall microbiota of plants both treated with NaCl (i.e. ionic stress) and infected with *V. dahliae* was more similar to the microbiota of plants treated with NaCl alone than to the microbiota only infected with *V. dahliae* (Figure 2). Contrastingly, combined osmotic treatment and infection with *V. dahliae* led to a microbiota more similar to that of plants treated with *V. dahliae* alone. Thus ionic treatment has a loss-of-species effect on the tomato-root microbiota which dominates the modulating effect from treatment with osmotic stress or infection with *V. dahliae*.

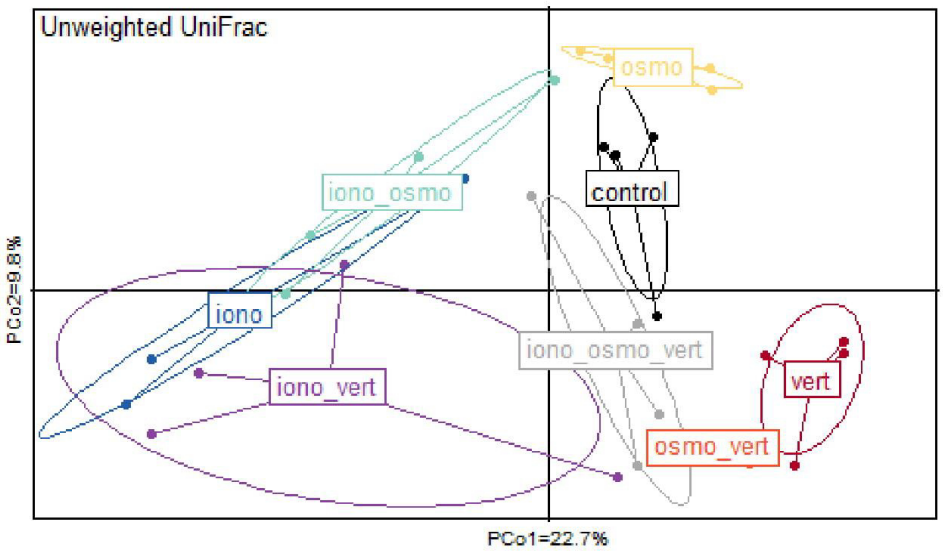


Figure 2: Abiotic and biotic stress has a significant effect on the microbiota composition of tomato roots (Pr(>F) < .001; PERMANOVA). Shown is the PCoA of the unweighted UniFrac distance.

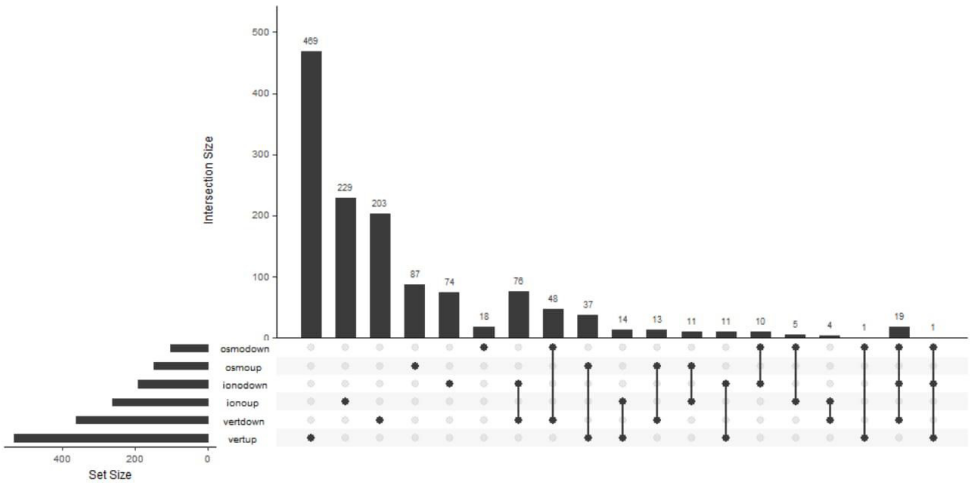
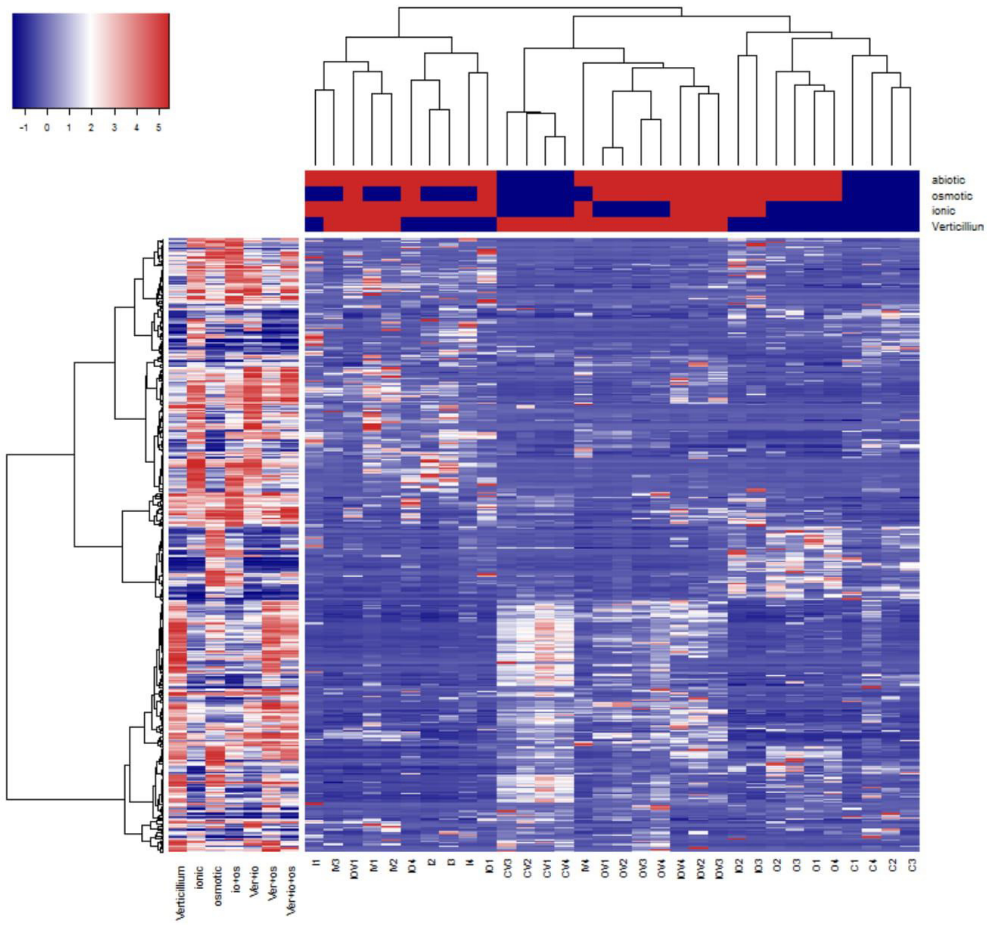


Figure 3: Most OTUs were only statistically different in one condition. Shown is the upset plot for the two abiotic stresses (ionic and osmotic stress) and the biotic stress (*V. dahliae*).



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56 **Figure 4:** Heatmap.

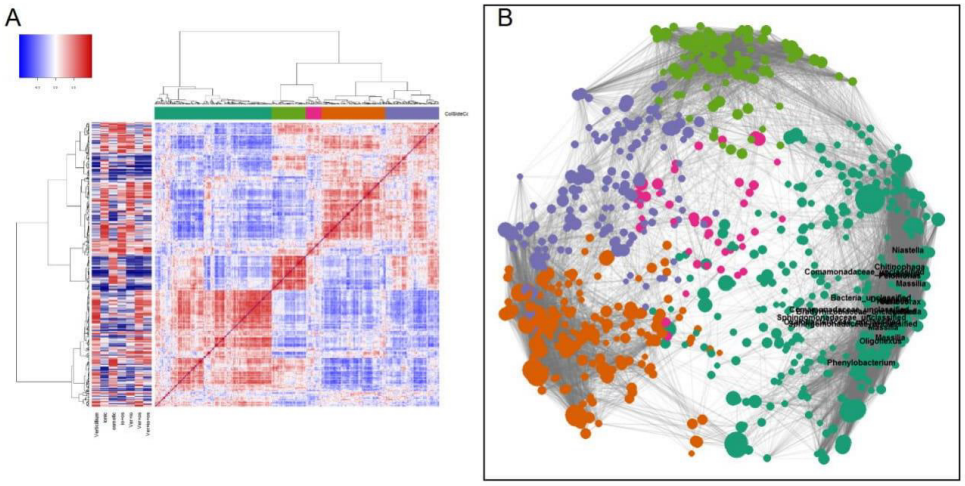
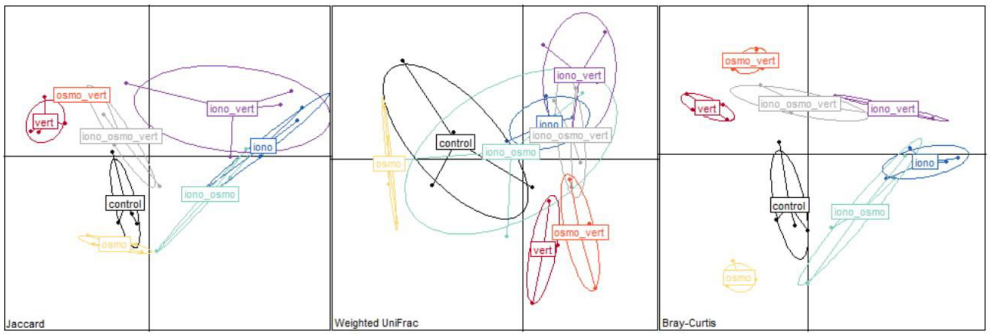


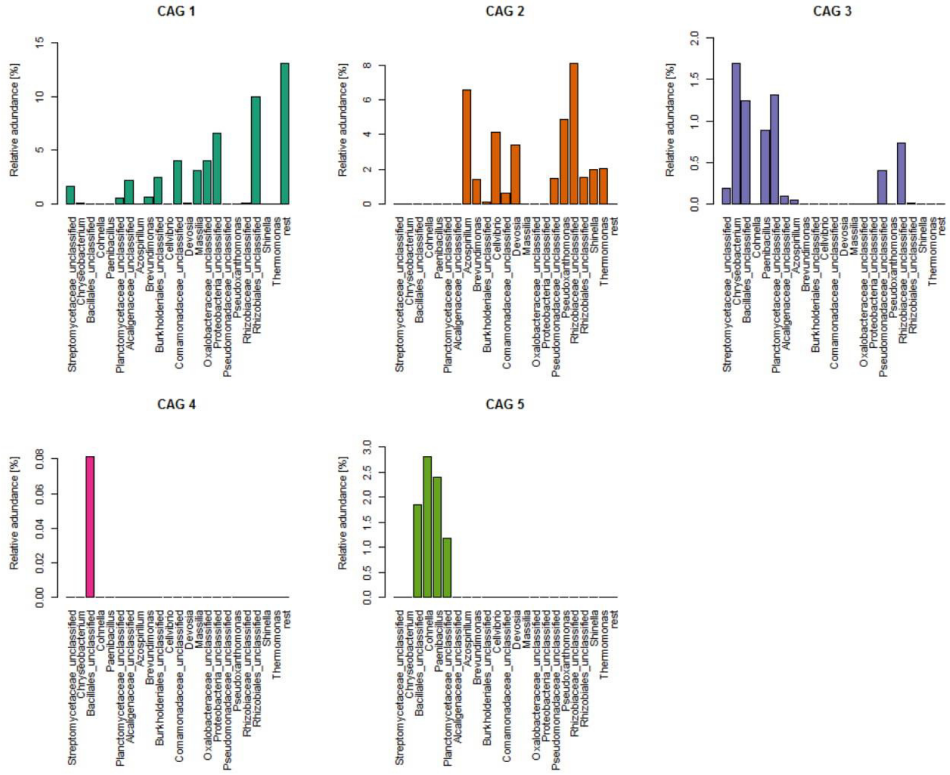
Figure 5: CAG-plot and network plot.

Supplementary Figures

PCoAs of other distance metrics



63 **CAG Plot: Abundance of phyla and genera per CAG**



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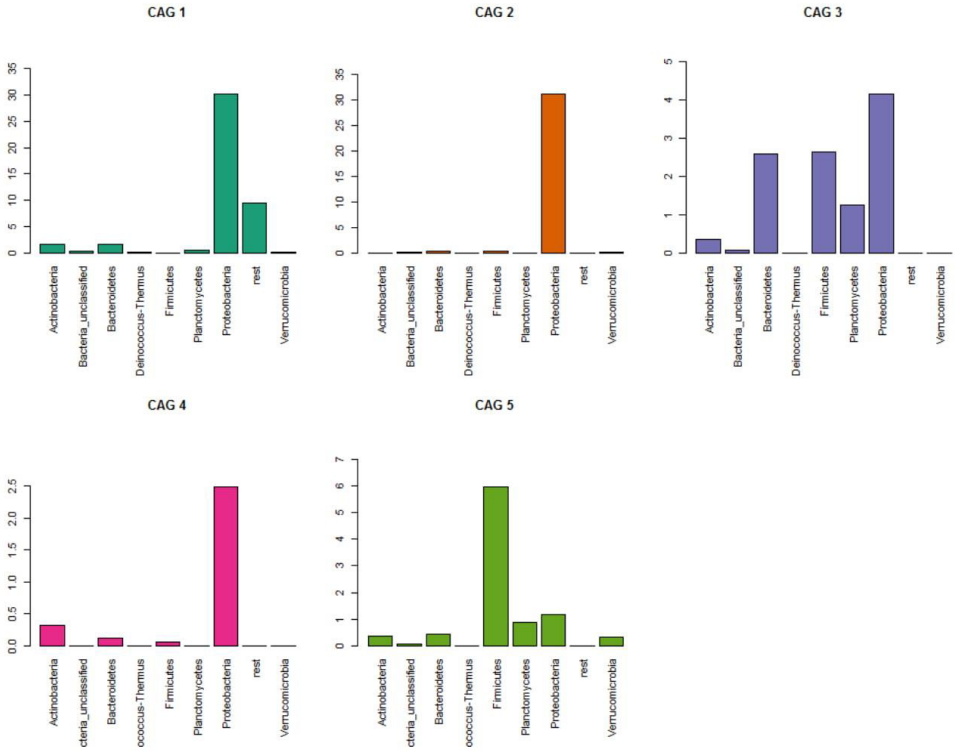

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Additional publication I

Microbiota associated with sclerotia of soil-borne fungal pathogens - a novel source of biocontrol agents producing bioactive volatiles

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RESEARCH e-Xtra*

Microbiota Associated with Sclerotia of Soilborne Fungal Pathogens – A Novel Source of Biocontrol Agents Producing Bioactive Volatiles

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ABSTRACT

Soilborne plant pathogens are an increasing problem in modern agriculture, and their ability to survive long periods in soil as persistent sclerotia makes control and treatment particularly challenging. To develop new control strategies, we explored bacteria associated with sclerotia of *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, two soilborne fungi causing high yield losses. We combined different methodological approaches to get insights into the indigenous microbiota of sclerotia, to compare it to bacterial communities of the surrounding environment, and to identify novel biocontrol agents and antifungal volatiles. Analysis of 16S rRNA gene fragment amplicons revealed significant compositional differences in the bacterial microbiomes of *Rhizoctonia* sclerotia, the unaffected tuber surface and surrounding soil. Moreover, distinctive bacterial lineages were associated with specific sample types. *Flavobacteriaceae* and *Caulobacteraceae* were primarily found in unaffected areas, while *Phyllobacteriaceae* and *Bradyrhizobiaceae* were associated with sclerotia of *R. solani*. In parallel, we studied a strain collection isolated from sclerotia of the pathogens for emission of bioactive volatile compounds. Isolates of *Bacillus*, *Pseudomonas*, and *Buttiauxella* exhibited high antagonistic activity toward both soilborne pathogens and were shown to produce novel, not yet described volatiles. Differential imaging showed that volatiles emitted by the antagonists altered the melanized sclerotia surface of *S. sclerotiorum*. Interestingly, combinations of bacterial antagonists increased inhibition of mycelial growth up to 60% when compared with single isolates. Our study showed that fungal survival structures are associated with a specific microbiome, which is also a reservoir for new biocontrol agents.

Keywords: agriculture, biocontrol consortia, ecology, microbial volatile organic compounds, microbiome, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Solanum tuberosum*

Soilborne pathogens are often limiting factors for crop yield and quality in various agricultural ecosystems, and therefore, a major issue worldwide (Allan et al. 2010; Raaijmakers et al. 2009; Weller et al. 2002). Fungi, oomycetes, and nematodes play important roles when underground plant parts are affected, and often act together in infection and disease (Raaijmakers et al. 2009). Soilborne fungi, which represent a major group of these pathogens, are characterized by a parasitic life cycle in their host plants, but they are also capable to endure in soil as saprophytes on plant residues or as survival structures from several weeks up to many years (Haas and Défago 2005; Lucas 2006). Examples for important soilborne fungi, which cause serious yield losses in a broad crop range, are *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* [Frank] Donk) and *Sclerotinia sclerotiorum* (Lib.) de Bary (1884). Both produce persistent sclerotia as survival structures, which makes outbreak predictions difficult and aggravates their control (Davis et al. 1994; Koike et al. 2003). In addition to the broad host range of *R. solani*, including

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The author(s) declare no conflict of interest.

multiple economically important crops (Ogoshi 1987; Sneh et al. 1996), its saprophytic lifestyle is an aggravating challenge in crop farming (Grosch et al. 2005). In the last years, several broad-spectrum as well as specific fungicides were developed to control *Rhizoctonia*, but the negative effects of various chemicals on the ecological balance of soil microorganisms, rising resistances, and groundwater pollution make them nonsustainable solutions for future crop protection (Mnif et al. 2016; Scherwinski et al. 2008). Traditionally, specific crop rotations were implemented to manage soilborne pathogens and avoid their accumulation in soil. In contrast, intensive management practices come along with negative consequences related to these pathogens, because they accumulate in the soil and simultaneously enhance disease pressure on crops over time (Weller et al. 2002). Currently, *Rhizoctonia* and *Sclerotinia* belong to the most important soilborne fungal pathogens; thus, novel approaches are needed to suppress their abundance and survival in order to avoid disease outbreaks and yield losses (Mendes et al. 2011).

Phytobiome studies have revealed that plant-associated microorganisms offer natural solutions for the suppression of soilborne pathogens, because one of their main functions is to protect plants against biotic and abiotic stresses (Berendsen et al. 2012; Berg et al. 2016; Vandenkoornhuyse et al. 2015). Biological control of plant pathogens by naturally occurring antagonists was identified as a sustainable and environmentally friendly plant protection approach a long time ago (Whipps 2001; Weller et al. 2002); however, due to the substantial progress in our understanding of the functioning of the plant microbiome, it recently became feasible to develop knowledge-based and predictable protection approaches (Mendes et al. 2011). Moreover, there is now the possibility to exploit the plant microbiota as a whole as well as other environmental microbiota for advanced plant protection strategies (Berg et al. 2013). While the rhizosphere was often the main target to identify successful biocontrol agents (Burr et al. 1978; Kloepper 1981; Weller et al. 2002), fungal structures were also identified as distinct habitat for antagonistic bacteria (Frey-Klett et al. 2011; Schmidt et al. 2016). Interestingly, fungal symbionts can also interact with the pathogens themselves as shown in a previous study, where *Enterobacter* was shown to enhance virulence of *R. solani* AG2-2IIIB (Obasa et al. 2017). However, there is still a lack of knowledge related to sclerotia-associated microbiota, especially when addressed with cultivation-independent tools (Zachow et al. 2011).

The objective of this study was to investigate bacterial communities associated with fungal survival structures, in order to (i) identify potential, bacterial biomarkers in *R. solani*-infected potatoes, (ii) isolate novel biocontrol agents, and (iii) provide evidence for improved efficacy of biocontrol consortia via complementation. In our experimental design, analyses of microbial volatile organic compounds (mVOCs) were included, because of their importance as communication molecules and powerful weapons for the suppression of pathogens (Kanchiswamy et al. 2015; Ryu et al. 2003; Wenke et al. 2012). Moreover, they can be involved in plant growth stimulation and restriction of fungal growth without physical contact, but are still understudied (Cernava et al. 2015; Ryu 2004; Schmidt et al. 2015). We have included them in our study to (i) reveal the impact of mVOCs on sclerotia viability, and (ii) describe the volatilomes of distinct antagonistic bacteria. Our poly-phasic approach included amplicon sequencing of 16S rRNA gene fragments combined with bioinformatic analyses, confocal laser scanning microscopy (CLSM), as well as isolate-based analyses to assess antagonistic effects toward pathogens and characterize their mVOCs with gas chromatography-mass spectrometry (GC-MS) experiments. Two sclerotia-forming model organisms were employed in order to make use of their specific morphology and lifestyles. While *R. solani* is an ideal model for three-way interactions (fungus, host plant, microbiome), the minor size of its sclerotia and attachment to the host aggravates in vitro experiments and observations related to morphological changes. Therefore, the significantly larger sclerotia of *S. sclerotiorum* were chosen to visualize the effects of mVOCs on their viability.

MATERIALS AND METHODS

Isolation of potential biocontrol agents from fungal sclerotia. Bacteria were isolated from *R. solani* sclerotia-infected potato tubers and a bait system with sclerotia from *S. sclerotiorum*. For the first approach naturally infected tubers were obtained from a field in Sanitz (Germany, latitude 54.07 | longitude 12.35). Visual inspection of the tubers (*Solanum tuberosum* L. 'Gala') resulted in a classification into three sample types on each tuber: tuber surface without sclerotia, surface area with microsclerotia and surface area with macrosclerotia on tubers (10 samples per group). Adherent soil (five samples) was obtained for comparisons with the tuber material. The dissected samples were homogenized in 1.5 ml of 0.85% NaCl and serial dilutions were plated on Reasoner's 2A-Agar (R2A-Agar; Carl Roth GmbH Co. KG) with and without cycloheximide at 20 µg/ml. In total, 400 bacteria (100 per sample type: macrosclerotia [MS], microsclerotia [mS], tuber surface [Ts], surrounding soil [So]) were isolated by randomized selection of bacterial colony forming units (CFU). In an additional approach a bait system was employed for the isolation of antagonistic bacteria from sclerotia as already described by Zachow et al. (2011). For this purpose, sclerotia of *S. sclerotiorum* Goa11 (strain collection of the Institute of Environmental Biotechnology–Graz University of Technology) were cultivated on potato-dextrose agar (PDA) (Carl Roth, Germany) in Petri dishes and packed in sealed sachets made of sterilized nylon. Sachets containing 20 sclerotia were buried in a depth of 10 to 15 cm in four different soil types (sand [S], peat [T], compost [K], and beech forest [B]) to attract bacteria from these soils. Three sclerotia bait systems per soil type remained underground for the enrichment of potential soilborne bacterial antagonists for 7 days (02/06/2016 to 09/06/2016) at the botanical garden of the University of Graz (Austria, latitude 47.08 | longitude 15.46). The recovery of sclerotia was followed by a homogenization in 0.85% NaCl solution and plating on Reasoner's 2A-Agar (R2A-Agar; Carl Roth). Cycloheximide (20 µg/ml) was added to a subset of R2A-Agar plates to prevent fungal growth. The isolated 160 bacterial isolates were designated according to the originated soil type (S, T, K, and B) and the usage of cycloheximide (C) during the isolation.

Extraction of total community DNA and barcoded primer 16S rDNA PCR. The DNA extraction from the same *R. solani* sclerotia samples that were used for the isolation of antagonistic bacteria was carried out by using the MP FastDNA Kit for soil (MP Biomedicals, U.S.A.). After following the manufacturer's protocol, the extracted total community DNA from *R. solani*-infected potato samples served as template for the 16S rDNA fragment amplification. The universal bacterial primers 515f/806r were used to target the hypervariable V4 region of 16S rRNA (Caporaso et al. 2011). Distinctive barcodes were utilized to label each sample. Purification of pooled DNA fragments was performed with the Promega (Madison, U.S.A.) Wizard SV GeL and PCR Clean-Up System Kit. Paired-end Illumina HiSeq sequencing of barcoded samples was conducted at GATC Biotech (Konstanz, Germany).

Data analysis of 16S rDNA amplicons for microbial community analyses. The 16S rRNA gene fragment library went through an initial quality check, where only forward reads were selected, which subsequently underwent demultiplexing and quality

(Wickham 2016). In addition, Dunnett's test and ANOVA analysis was performed in R environment using the packages DescTools (Signorell 2015) and multcompView (Graves et al. 2015) to assess significance of treatment efficiency in volatile assays.

RESULTS

Bacterial communities associated with sclerotia of *R. solani*. The community composition of macrosclerotia (MS) and microsclerotia (mS) of *R. solani* as well as potato tuber surface (Ts) and of surrounding soil (So) was analyzed with Illumina-based high-throughput sequencing. Analysis of the 16S ribosomal RNA gene fragment amplicons revealed a high bacterial diversity across the samples. A total of 8,226 OTUs were identified at species level (97% sequences identity) in a library that consisted of 14,328,349 quality filtered reads. Altogether, 37 bacterial families were detected with an average abundance of at least 0.5% among all samples (Supplementary Fig. S1). The predominant bacterial families across all samples were *Sphingomonadaceae* (macrosclerotia: 3.4 to 5.5%, microsclerotia: 2.1 to 10.9%, tuber surface: 4.2 to 15.6%, and surrounding soil: 6.9 to 8.1%) followed by *Streptomycetaceae* (macrosclerotia: 1.2 to 13.3%, microsclerotia: 2.2 to 9.1%, tuber surface: 1.3 to 6.2%, and surrounding soil: 4.8 to 6.1%), and *Pseudonocardiaceae* (macrosclerotia: 0.9 to 26.8%, microsclerotia: 1.2 to 15.4%, tuber surface: 0.6 to 12.8%, and surrounding soil: 3.1 to 5.6%). Network construction and Cytoscape-based (Shannon et al. 2003) rendering was used to represent the proportional abundance of OTUs in investigated habitats (Fig. 1). When the OTU distribution among samples was assessed, the highest number of unique OTUs was found in tuber surface samples (33 unique OTUs) followed by soil samples (12). One third (four OTUs) of the unique signatures in soil samples were assigned to the genus *Bacillus*. Different trends of the proportionate composition were detected at family level. *Caulobacteraceae* and *Flavobacteriaceae* were primarily found in tuber surface samples (51.8 and 52.4% of reads, respectively), whereas *Bacillaceae* showed higher relative abundance in surrounding soil samples (57.9% of reads). Moreover, *Micromonosporaceae*, *Methylophilaceae*, and *Anaerolineaceae* were primarily represented in tuber surface samples (51.1, 52.5, and 55.9%). Furthermore, *Phyllobacteriaceae*, *Bradyrhizobiacae*, *Intrasporangiaceae*, and *Gaiellaceae* were overrepresented in samples of macro- and microsclerotia (approximately 70% of reads). A complementary analysis of differential abundance of OTUs with the DESeq2 algorithm resulted in the identification of various taxa that were either prevalent in sclerotia or the tuber surface (Supplementary Fig. S2). The genera *Nonomuraea*, *Kribbella*, and *Taibaiella* revealed a higher relative abundance in sclerotia (macro- and microsclerotia) in comparison with the potato tuber surface. *Caulobacter* and *Flavobacterium* were significantly more abundant in tuber surface samples. Habitat-specific alpha diversity analysis, based on a nonparametric two-sample *t* test, revealed significant differences regarding species richness among habitats tuber surface and surrounding soil ($P = 0.036$) and tuber surface and macrosclerotia ($P = 0.006$) (Supplementary Fig. S3). Beta diversity analysis showed significant differences among the four sample types ($R^2 = 0.26484$, Pr (>F) = 0.001) and a similarity of tuber surface samples with soil samples when visualized in a PCoA plot (Supplementary Fig. S4). Additionally, a PERMANOVA analysis was employed to weight the significance of intersample differences. It revealed that the tuber surface and the surrounding soil harbor distinct bacterial microbiomes (Ts versus So, $P = 0.003$; Ts versus MS, $P = 0.001$; Ts versus mS, $P = 0.001$; So versus MS, $P = 0.002$, So versus mS, $P = 0.009$). In contrast, no significant differences in the community structure were found for samples derived from micro- and macrosclerotia ($P = 0.351$).

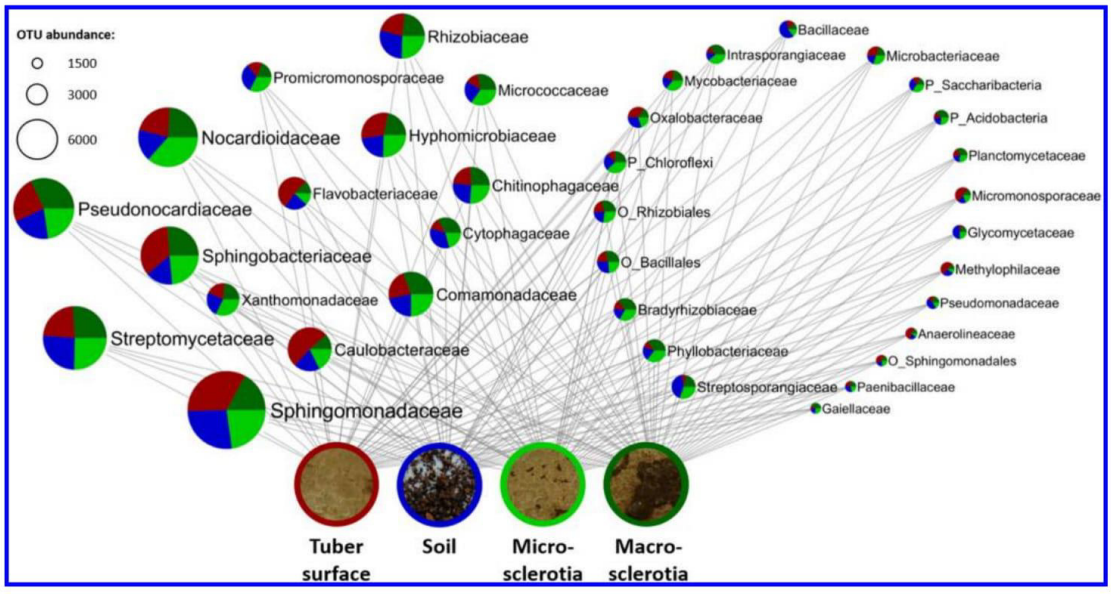

Fig. 1. Bacterial community composition on family level of different habitats obtained from *Rhizoctonia solani*-infected potatoes. A network was constructed in order to identify habitat-specific signatures. The node size represents the total amount of reads of the respective operational taxonomic unit (OTU). Pie charts indicate the relative amount of reads for each of the four habitats. Only OTUs with a relative abundance of at least 0.5% across all samples are considered.

Screening for potential antagonists – combination of volatile-producing bacteria to develop biocontrol consortia. An initial screening for antifungal traits of 560 sclerotia-associated bacterial strains resulted in a collection of 47 antagonistic isolates (a detailed overview of the conducted experiments is shown in Supplementary Fig. S5). These bacteria were able to inhibit the mycelial growth of *S. sclerotiorum* and/or *R. solani* in dual culture assays. Less than one third (13/47) of these preselected antagonistic isolates originated from the initial 400 bacterial strains obtained from *R. solani*-infected potato tubers. The majority of the 13 bacterial antagonists was isolated from macrosclerotia (Rs-MS14, Rs-MS28, Rs-MS53, Rs-MS84, and Rs-MS87), only one from microsclerotia (Rs-mS115), four from the unaffected tuber surface (Rs-Ts222, Rs-Ts259, Rs-Ts269, and Rs-Ts276), and the remaining from the surrounding soil (Rs-So360, Rs-So365, and Rs-So386). In parallel, 160 bacterial strains were isolated via the *S. sclerotiorum* bait system. This led to the identification of 34 (21.25% of all bait system isolates) isolates with antagonistic properties (Supplementary Table S2). The largest proportion of these 34 bacterial isolates associated with *Sclerotinia sclerotiorum* originated from sclerotia buried in compost (12), followed by sclerotia from beech forest soil (8), and sclerotia buried in sandy soil and peat (7 each). Almost all cultivated antagonistic isolates (41 of 47) were assigned to the genus *Bacillus*. The genera *Buttiauxella*, *Enterobacter*, and *Pseudomonas* were represented by two isolates each.

The antagonistic isolates from both isolation sources (47 isolates) were further tested in terms of their potential to suppress mycelial growth of both fungi via the production of bioactive volatiles. In the conducted mVOC assays, *S. sclerotiorum* was considerably more vulnerable to volatiles from isolates of both approaches: the 19 most effective antagonists revealed a growth inhibition of 49.6 to 96.2%, while the mycelium of *R. solani* was reduced no more than 33.6% (Figs. 2 and 3).

Isolates revealing the antagonistic effects via volatile production were randomly combined to augment the effect on each of the two

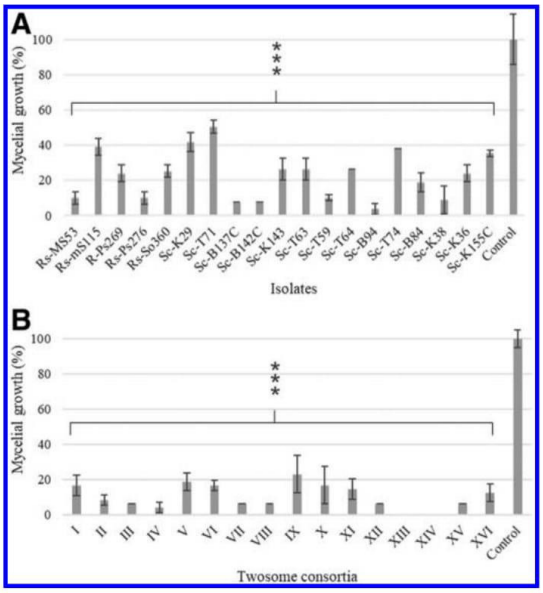

Fig. 2. Inhibition of mycelial growth of *Sclerotinia sclerotiorum* of different isolates via microbial volatile organic compounds (mVOCs). Effects of mVOCs emitted by antagonistic bacteria on the growth of plant-pathogenic fungi were evaluated with Petri dish VOCs assay (PDVA) experiments. The control (PDA plate with mycelium; NA without bacteria) was set to 100%. **A,** Application of single bacteria. All isolates were assigned to the genus *Bacillus* with the exception of the isolates Sc-T74 (genus *Buttiauxella*) and Sc-B94 (genus *Pseudomonas*). **B,** Combination of two isolates as bacterial consortia. Dunnett's test confirmed a highly significant decrease of mycelial growth in comparison with the control (* = $P \leq 0.05$; ** = $P \leq 0.01$; and *** = $P \leq 0.001$).

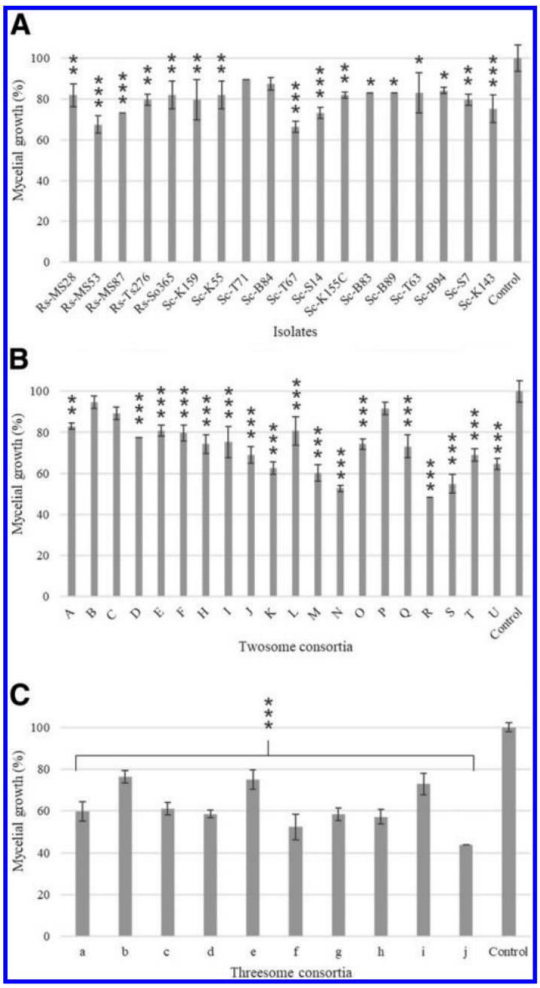

Fig. 3. Inhibition of mycelial growth of *Rhizoctonia solani* of different isolates via microbial volatile organic compounds (mVOCs). The growth reduction of the plant pathogenic fungus was evaluated with Petri dish VOCs assay (PDVA) experiments. The control (PDA plate with mycelium; NA without bacteria) was set to 100%. **A,** Application of single bacteria. All isolates were assigned to the genus *Bacillus* with the exception of isolate Sc-B94 (genus *Pseudomonas*). **B,** Combination of two isolates as bacterial consortia. **C,** Combination of three bacteria as consortia. Dunnett's test confirmed a significant decrease of mycelial growth in comparison with the control (* = $P \leq 0.05$; ** = $P \leq 0.01$; and *** = $P \leq 0.001$).

phytopathogenic fungi used in the assays (Supplementary Table S3). Assembled consortia with different isolates enhanced the antifungal activity significantly. Distinct combinations of antagonists resulted in a complete inhibition of mycelial growth of *S. sclerotiorum* and an increased impact on *R. solani* with a 51.6% reduction in twosome and 56.2% in threesome consortia (Figs. 2 and 3). *R. solani* revealed to be more sensitive to mVOCs of *Bacillus* isolates. The strongest inhibition in single isolate assays was caused by *B. cereus* Sc-T67. Twosome and threesome consortia of *Bacillus* spp. (consortium R [*B. cereus* Rs-MS53 + *B. amyloliquefaciens* Sc-K143]; consortium j [*B. cereus* Rs-MS53 + *B. aerius* Rs-So365 + *B. amyloliquefaciens* Sc-K55]) had the highest impact on the mycelium of *R. solani*. On the contrary, volatiles emitted by *Pseudomonas helmanticensis* Sc-B94 restrained mycelial growth of *S. sclerotiorum* most effectively (96.2%). The antagonistic activity peaked in total growth inhibition caused by the consortium XIV consisting of the *Pseudomonas* strain in combination with *B. cereus* Rs-MS53.

mVOCs alter the morphology of exposed sclerotia. Treatments of sclerotia of *S. sclerotiorum* with mVOCs over a period of 27 days resulted in a visible alteration of the morphology and an increased production of clear aqueous fluids. The preselected bacterial antagonists of *S. sclerotiorum* altered the hard, resilient texture of the survival structures substantially. Volatiles of *Buttiauxella warmboldiae*, *P. helmanticensis*, and *Bacillus* consortia intenerated the sclerotial tissue. The substantial effects were visualized with cross-sections mVOCs-treated sclerotia (Fig. 4). While controls of untreated sclerotia and treatments with *E. coli* K-12 revealed the typical light gray inner texture of compact fungal mycelium, treatments with *Bacillus* consortia, *P. helmanticensis* Sc-B94 and *Buttiauxella warmboldiae* Sc-T74 resulted in discolorations of the inner sclerotial tissue. When the treated sclerotia were incubated on PDA plates, no significant effect on their germination ability was observed (data not shown).

Confocal laser scanning microscopy of long-term treated sclerotia. After identification of morphological changes of sclerotia following the treatment with bacterial volatiles, the viability of the hyphae was analyzed. Confocal laser scanning microscopy combined with differential staining revealed layers of damaged and intact hyphae. Cross-sections of controls of untreated sclerotia and treatments with *E. coli* K-12 featured an outside layer, commonly filled with melanin, of nonviable hyphae with a diameter of 40 to 60 µm (Fig. 5). Bacterial volatiles produced by *P. helmanticensis* Sc-B94 and *Buttiauxella warmboldiae* Sc-T74 increased this layer to >200 and >130 µm, respectively. Nevertheless, the core of the medulla, the interior portion of sclerotia, remained viable after the treatment.

Mass-spectrometric identification of bioactive mVOCs. The volatilome of several potential biocontrol agents was analyzed for a possible association of compounds to the inhibitory effects on *R. solani* and *S. sclerotiorum*. *B. amyloliquefaciens* Sc-K143, *B. cereus* Rs-MS53, *B. amyloliquefaciens* Sc-K55, *B. cereus* Rs-MS53, *B. aerius* Rs-So365, and *P. helmanticensis* Sc-B94 were selected due to their emergence as compelling antagonists. A total of 41 different microbial volatiles were identified and matched to substances in the NIST MS Database 14 after sampling the headspace with two SPME fibers with defined coatings. A total of 22 compounds were identified in headspace analyses with both fibers, while 16 were solely detectable with a fiber coating consisting of DVB/CAR/PDMS and 3 that were exclusively detected with the PDMS/DVB coating (Table 1). A high proportion of volatile alcohols, alkanes, ketones organic acids, and sulfides were detected. Ketones were identified as volatiles with the highest frequency among the different isolates. Alkylated pyrazines were less common and mainly produced by *B. cereus* Rs-MS53 and *B. aerius* Rs-So365. The emission of dimethyl sulfide and 1-undecene differentiated *P. helmanticensis* Sc-B94 from other isolates assigned to the genus *Bacillus*. There was no mVOC shared among all five strains while several substances were produced by only one bacterial strain. A considerable amount of bacterial VOCs could not be classified due to missing database matches in the utilized NIST 14 MS database.

Alkylpyrazines affect the germination of sclerotia of *S. sclerotiorum*. The exposure of sclerotia to mVOCs of the bacterial isolates in confrontation assays was not followed by significant reductions of their viability. Therefore, an identified compound group that was prevalent in the mVOC profiles was selected to explore the potential of analogous synthetic compounds to inhibit the highly resistive structures. Sclerotia of *S. sclerotiorum* were exposed to the volatile alkylpyrazines 2-ethylpyrazine (EP) and 2,3-dimethyl-5-isobutylpyrazine (DMIBP) as single substances and in a combined treatment with both pyrazines (2P). When EP and 2P were applied, a significant reduction of the sclerotial germination rates was observed after 96 h of incubation on PDA plates

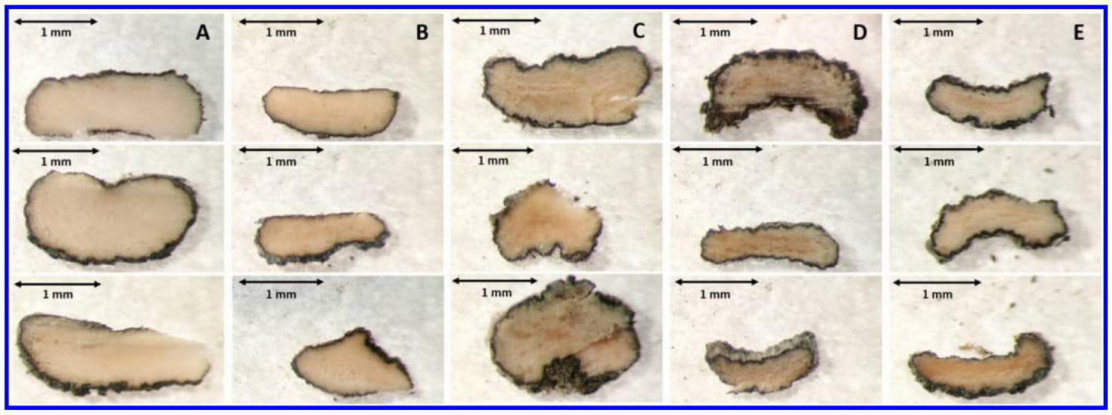

Fig. 4. Macrographs of sclerotia of *Sclerotinia sclerotiorum*. Cross-sections of sclerotia that were exposed to microbial volatile organic compounds for 27 days were obtained for comparative assessments. **A,** Untreated sclerotia. Treatments of sclerotia with **B,** *Escherichia coli* K-12; **C,** *Bacillus cereus* Rs-MS53/*Bacillus aerius* Rs-So360; **D,** *Pseudomonas helmanticensis* Sc-B94; and **E,** *Buttiauxella warmboldiae* Sc-T74.

(Supplementary Fig. S6). Both of the two alkylpyrazines, as well as the combination, significantly decreased the germination rate in an approach with a second application after 7 days.

DISCUSSION

Fungal survival structures were shown to harbor specific microbial fingerprints that differentiate them from microbial communities in their surroundings. Moreover, we found that they are associated with specific antagonists, which can substantially suppress mycelium growth of the fungal pathogens. The production of mVOCs by bacterial antagonists, especially by combinations of them, was identified as a major mechanism of antagonism. Application of an identified compound class that was so far not known for bioactive effects against sclerotia confirmed the mVOCs-based inhibition potential. Bacterial antagonists which affected fungal growth, sclerotia formation and survival, provide a promising basis for the development of biocontrol agents to control soilborne fungal pathogens under field conditions.

The assessment of the sclerotia microbiome and its differences to the unaffected tuber surface of potatoes led to the identification of bacteria that potentially interact with *R. solani*. Our observations primarily reflect habitat-specific enrichments of distinct bacterial taxa that are driven by differing occurrence of *R. solani*, because it was likely also present in its mycelial form in the seemingly unaffected samples. The dominant phylum in all four sample types

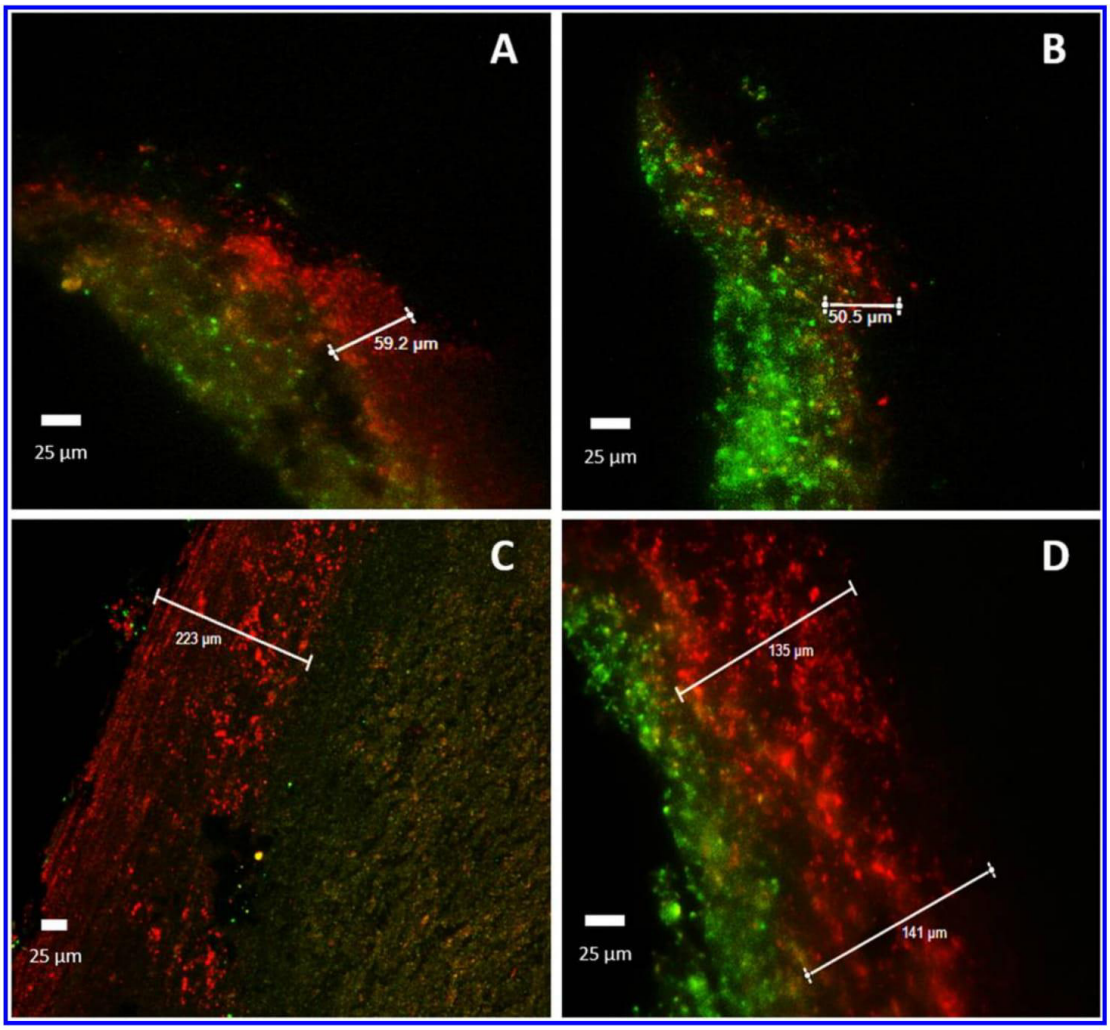

Fig. 5. Micrographs of differentially stained sclerotia cross-sections. The effects of microbial volatile organic compound exposure on the viability of sclerotia of *Sclerotinia sclerotiorum* were evaluated in a microscopy-based approach. Green indicates intact hyphae; and red indicates damaged hyphae. **A and B,** Untreated sclerotia; **C,** *Sclerotia* treated with *Pseudomonas helmanticensis* Sc-B94; and **D,** *Sclerotia* treated with *Buttiauxella warmboldiae* Sc-T74.

was assigned to *Proteobacteria* as previously shown with other potato cultivars (Weinert et al. 2011). In another related study, *Alphaproteobacteria* were together with *Actinobacteria* the most common bacterial colonizers of the potato rhizosphere (Inceoğlu et al. 2011; Weinert et al. 2011). This predominant class *Proteobacteria* comprises the bacterial family *Caulobacteraceae*, which were, along with *Flavobacteriaceae*, enriched on the sclerotia-free tuber surface. Species of *Caulobacteraceae* and *Flavobacteriaceae* such as *Brevundimonas diminuta* NBRI012 and *Flavobacterium* were shown to possess plant-beneficial properties (Singh et al. 2015; Soltani et al. 2010). Further families that had a higher relative abundance in samples of tuber surface were *Micromonosporaceae*, *Methylophilaceae*, and *Anaerolineaceae*, which so far could not be linked to any plant-beneficial effects. The augmented abundance of these potential biomarkers for unaffected tuber surfaces could correlate with the prevention of sclerotia formation. In contrast to the other habitats, *Bradyrhizobiaceae*, *Phyllobacteriaceae*, *Intrasporangiaceae*, and *Gaiellaceae* were located primarily in macro- and microsclerotia. Donn et al. (2014) showed a high abundance of *Bradyrhizobiaceae* in soil to be conductive to the proliferation of *R. solani* AG-8. These possible bacterial biomarkers of the AG-8 sclerotial microbiome could interrelate with a beginning *R. solani* infection and the cohering formation of sclerotia. The implications of most of these bacterial families in disease development is yet unexplained and modes of interactions of the microbiome of the geocaulosphere, the rhizosphere, and the bulk soil require further

TABLE 1
Volatiles produced by different bacterial isolates[a]

Compound	Kovats index	Bacterial isolates					Biological function	Reference
		Rs-MS53	Rs-So365	Sc-B94	Sc-K143	Sc-K55		
Methanethiol[b]	401	▲		▲	≤	▲	n.a.	
Isoprene[b]	510				—	—	Protection against heat stress, stabilizes cell membranes in response to heat stress, bacterial interactions	Tyc et al. 2017; Wilkins 1996
Dimethyl sulfide[b]	518			▲			n.a.	
2-Methylfuran[b]	611		—				Bacterial interactions	Tyc et al. 2017
2,3-Butanedione[b]	651			≤	▲	≤	n.a.	
2,3,4-Trimethyloxetane	655		▲				n.a.	
1-Chlorobutane	656	▲		—			n.a.	
2-Pentanone[b]	684			—	—	—	Nematicidal activity	Xu et al. 2015
Methylthioacetate	697	▲		▲			n.a.	
Acetoin[a]	708	≤	≤		—	—	Mediates plant-beneficial effects such as growth promotion and systemic resistance (ISR) in model plants and crops under in vitro and in situ conditions	Hahm et al. 2012; Han et al. 2006; Ryu et al., 2003
3-Methylbutanenitrile	722	—					n.a.	
3-Methyl-1-butanol[b]	725	—			▲	▲	Complete inhibition of mycelial growth of *Phyllosticta citricarpa*	Toffano et al. 2017
1-Chloropentane	725		—				n.a.	
2-Methyl-1-butanol[b]	728		—				Weakly active against wood-decaying fungi	Schulz and Dickschat, 2007
1-(3-Methyloxiranyl)-ethanone	728					—	n.a.	
Dimethyl disulfide[b]	733	▲		▲	▲	▲	Antagonistic effect against sapstain fungi, nematicidal activity, and reduces mycelium growth and sclerotia germination of *Sclerotinia sclerotiorum*	Giorgio et al. 2015; Schulz and Dickschat, 2007; Xu et al. 2015
Dimethyl sulfone[b]	733		—		—	—	n.a.	
3-Methyl-2-pentanone[b]	741		—		▲	▲	n.a.	
3-Methoxy-3-methyl-2-butanone	758	—	—			—	n.a.	

(*Continued on next page*)

[a] Identification was performed via headspace-solid phase microextraction (HS-SPME) gas chromatography-mass spectrometry (GC-MS), matching to the NIST Mass Spectral Database and confirmation by the Kovats index.
[b] Assignment as microbial volatile organic compounds (mVOCs) refers to the presence in the mVOC database (Lemfack et al. 2018); — = solely identified with fiber divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS); ≤ = only identified with fiber polydimethylsiloxane/divinylbenzene (PDMS/DVB); and ▲ = identified with both fibers.

research. The unaffected tuber surface harbored the lowest bacterial diversity, implying that potato plants gather a specific microbiome.

In addition, the sclerotia-associated bacterial microbiome was shown to be a potential source for biocontrol agents which, in specific combinations revealed exceeding capability. The bait system described by Zachow et al. (2011) provides a well-functioning accumulation method of antagonistic bacteria with 21.25% of all isolates exhibiting antifungal traits in comparison with only 3% by sampling infected potato tubers. The important role of interspecies interactions of bacteria in the accumulation of antibiotics has been emphasized in previous studies (Garbeva et al. 2011; Seyedsayamdost et al. 2012). Garbeva et al. (2011) observed that the assembly of antimicrobial metabolites of *P. fluorescens* Pf0-1 was enhanced by *Brevundimonas* and *Pedobacter*, but not *Bacillus*. In the present study, experiments of volatile confrontation exhibited an increased antagonistic effect against *S. sclerotiorum* when combining *B. cereus* Rs-MS53 and *P. helmanticensis* Sc-B94. This is most likely due to strain-specific compatibility. As already observed by Zachow et al. (2011), the majority of isolates with antagonistic properties toward sclerotia-forming fungi were assigned to the genus *Bacillus*. Biological control mechanisms of *Bacillus* spp. and *Pseudomonas* spp. have been investigated in a vast number of surveys (Ait-Lahsen et al. 2001; Asari et al. 2016; Hong and Meng 2003; Lim et al. 1991; Peighami-Ashnaei et al.

TABLE 1 (*Continued from previous page*)

Compound	Kovats index	Bacterial isolates					Biological function	Reference
		Rs-MS53	Rs-So365	Sc-B94	Sc-K143	Sc-K55		
2,3-Butanediol[b]	769					—	Induce growth promotion, ISR, and regulate auxin homeostasis in *Arabidopsis thaliana*	Ryu et al. 2003
S-Methyl propanethioate[b]	784	▲		▲			n.a.	
3-Hexen-2-one[b]	785			—	—	—	n.a.	
S-Methyl 2-methylpropanethioate[b]	837	▲					n.a.	
5-Methyl-2-hexanone	844				≤	▲	n.a.	
Acetone[b]	847		▲	≤	▲	▲	Promotion of performance of *Chlorella sorokiniana* Shih	Amavizca et al. 2017
2-Methylbutanoic acid[b]	855		—				n.a.	
S-methyl butanethioate[b]	878	—					Nematicidal activity	Xu et al. 2015
2-Heptanone[b]	881				▲	▲	Nematicidal activity	Xu et al. 2015
S-Methyl pentanethioate[b]	932	—					n.a.	
6-Methyl-2-heptanone[b]	948				▲	▲	n.a.	
5-Methyl-2-heptanone[b]	958				▲	▲	n.a.	
2-Methyl-5-(1-methylethyl)pyrazine[b]	1,046	≤	≤				n.a.	
1-Undecene[b]	1,086			▲			Inhibits the mycelial growth of *P. infestans* and changes its sporulation behavior	Hunziker et al. 2015
2-Isobutyl-3-methylpyrazine	1,130	≤	▲				n.a.	
2-Methoxy-3-(2-methylpropyl)-pyrazine	1,175	≤	▲				n.a.	
2,6-Dimethyl-3-sec-butylpyrazine	1,208	≤	▲				n.a.	
2,3-Dimethyl-5-(2-methylpropyl)-pyrazine	1,216	≤	▲				n.a.	
2,5-Dimethyl-3-(2-methylpropyl)-pyrazine	1,217	—					n.a.	
2-Nonanone[b]	1,350				≤	≤	Reduces mycelial growth and sclerotia germination of *Sclerotinia sclerotiorum*; inhibits growth of *Agrobacterium tumefaciens*, *Synechococcus* sp. PCC 7942, and *Rhizoctonia solani*, kills *Caenorhabditis elegans*	Giorgio et al. 2015; Popova et al. 2014
9-Hydroxy-2-nonanone	1,351					—	n.a.	
Hexadecane[b]	1,689		≤				n.a.	

2009) and the potential of consortia for biological control was predicted many years ago (Dowling and O'Gara 1994). However, this study is, to our knowledge, the first to demonstrate the augmented effect of mVOCs of bacterial consortia. The composition of bacterial consortia plays a crucial role in terms of the observed effects and requires a detailed assessment of the possible combinations. Similar to observations of Tyc et al. (2014) concerning nonvolatile secondary metabolites, combinations of bacteria could result in both increasing and decreasing of antimicrobial volatile activity. Disturbance of signal transduction pathways, e.g., quorum sensing, was stated as source for the decline of antimicrobial activity in the quorum sensing system (Christensen et al. 2013; Gonzalez and Keshavan 2006). Mechanisms of enhanced sensitivity of phytopathogenic fungi to mVOCs are still unexplained and require additional research. Further observations could facilitate the establishing of microbial consortia to form a "minimal microbiome" for specific ecosystem services such as the biological control of soilborne diseases (Mendes et al. 2013). Moreover, a better understanding of the plant microbiome appears to be crucial to prevent the outbreak of plant diseases; thus, the importance plant-associated microorganisms will increase in the fields of plant breeding and biotechnology (Berg et al. 2014).

Microbial volatiles proved to be essential mediators for antagonistic interactions and local defense in various microniches (Cernava et al. 2015) as well as for cross-kingdom interactions with fungi, plants, and nematodes (Létoffé et al. 2014). Schulz-Bohm et al. (2018) emphasized the significance of VOCs in belowground interactions by revealing that plants, bacteria, and pathogenic fungi can communicate over long distances in soil. Thereby, they also showed that various volatiles are able to disperse over more than 12 cm in soil. According to the mVOC database (Lemfack et al. 2018), 13 of 41 compounds released by *B. amyloliquefaciens* Sc-K143, *B. cereus* Rs-MS53, *B. amyloliquefaciens* Sc-K55, *B. cereus* Rs-MS53, *B. aerius* Rs-So365, and *P. helmanticensis* Sc-B94 have not been identified as microbial volatiles before and the biological function is only known for 13 out of 28 previously described mVOCs. Two of the most prominent bacterial volatile substances known for a plant beneficial effect are acetoin and 2,3-butanediol, which enhance growth and trigger induced systemic resistance of *Arabidopsis thaliana* against *P. syringae* DC3000 (Rudrappa et al. 2010; Ryu 2004; Ryu et al. 2003). It has been shown that dimethyl disulfide has a role in plant protection and inhibits the growth of plant-pathogenic fungi and nematodes (Coosemans 2005; Kai et al. 2009). Similarly, 3-methyl-1-butanol is able to completely suppress the mycelial growth, germination, and appressorium formation by conidia of *Phyllosticta citricarpa* (Toffano et al. 2017). CLSM micrographs obtained after long-term exposure to bacterial VOCs revealed a damage of sclerotial hyphae in outer layers while medullar mycelium remained intact. This is in accordance to the study of Giorgio et al. (2015), where fungistatic effects of mVOCs on sclerotia were observed. Nevertheless, observations of Cernava et al. (2015), Minerdi et al. (2009), and Spence et al. (2014) that mVOCs can significantly decrease mycelial viability of fungal plant pathogens were confirmed. In contrast to volatiles produced by the antagonistic strains in the confrontation assay, the alkylpyrazines 2-ethylpyrazine and 2,3-dimethyl-5-isobutylpyrazine, when applied as pure compounds, significantly inhibited the germination rate of the exposed sclerotia of *S. sclerotiorum*. This could be explained by an insufficient amount of naturally produced bacterial volatiles to completely inactivate the viability of sclerotia under the tested cultivation conditions. Bacteria were shown to produce antifungal volatiles, which make them less susceptible to mycoparasites such as *Coniothyrium minitans* and *Trichoderma* spp. (Jones et al. 2014). However, the emitted concentrations might not be sufficient to cause further damage than required for their protection. Understanding the complex interactions mediated by mVOCs will be the key to finding suitable tools for crop protection to aim for sustainable agriculture (Kanchiswamy et al. 2015).

In conclusion, *R. solani* proved to be an ideal model to extend present knowledge about sclerotia-associated bacteria that potentially correlate with the formation of these survival structures on potatoes. Complementary experiments with the larger sclerotia of *S. sclerotiorum* revealed that volatiles can alter the morphology of sclerotia and increase the layer of nonviable hyphae. We provided evidence for a boosted antifungal effect of bacterial consortia via mVOCs and identified novel microbial volatiles. We suggest further research to gather deeper insights on the role of bacteria in preventing or assisting phytopathogenic fungal infections and the investigation of bacterial consortia as advanced biocontrol agents.

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