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Contents

Chapter 1

General introduction

The soil ecosystem consists of the largest reservoir of biodiversity on Earth (Zak et al., 2003; Decaëns, 2010; Bardgett and Van der Putten, 2014). Microbial communities are unseen drivers in soil ecosystems, and they play an important role in determining a wide variety of soil processes in terrestrial ecosystems (Van der Heijden, et al., 2008; Fester et al., 2014; Delgado-Baquerizo et al., 2016; Singh and Gupta, 2018). Soil microbial communities can influence plant performance and can drive plant species composition on a particular soil. Soil microbes are associated with an extensive range of ecosystem processes, such as nitrogen (N) and carbon (C) cycling, organic matter decomposition, soil structural formation and stability and these processes, in turn, affect plant growth (Beare et al., 1992; Ehrenfeld et al., 2005; Schimel and Schaeffer, 2012; Bardgett et al., 2014). While these processes can benefit plant growth, the soil microbial community also harbors microbes that compete with plants for nutrients or are pathogenic and impair plant growth. This leads to the question if plants can manipulate the composition of the soil microbial community to their advantage.

In agriculture, the physical structure of the soil is often altered to improve crop production and this modifies biological components and microbial properties of the soil (Mousavi and Eskandari, 2011; Van der Heijden et al., 2013; Van der Putten, et al., 2013). Moreover, green crop management technologies, such as regulation of soil microbial biodiversity, application of beneficial microbial agents and induction of plant hormonal resistance, are regarded as promising approaches against pests and microbial pathogens (Chung et al., 1988; Kennedy and Smith, 1995; Neher, 1999; Sturz and Christie, 2003). Although many experiments have shown that activation of hormonal signaling pathways can boost a plant's immunity against pathogenic microbial attacks, whether and how these hormonal signaling pathways affect the soil microbial community and consequently plant growth is still poorly understood (Berendsen et al., 2012; Graham, et al., 2016). Therefore, to better understand the roles of soil microbial communities at both taxonomic and functional level, studies from a plant defensive perspective are timely and needed, and this is the main focus of this thesis.

1. The relationship between plants and soil microbial communities

In the early middle ages, under Charles the Great, cropping and fallow rotations were already applied in Europe (van der Putten et al., 2013). Chinese historical books record that cropping and fallow rotations in China began even in the ancient Warring States period, which ran from 475 BC to 221 BC (Zhang and Yu, 2006). In agriculture, all these ancient practical actions aimed to overcome the same problem: a soil becomes less suitable for a crop if this crop is grown in that soil repeatedly.

We have since become aware that the negative impact of soil on plant growth is dependent on the role of soil microbes. Particularly, in the early 2000s, a large number of studies began to emphasize that the relationship between plants and soil microbial communities is bidirectional, rather than unidirectional. Plants can affect the microbial communities in the soil, and in turn, soil microbial communities also influence plant growth. Nowadays it is clear that the interactions between plants and soil microbial communities are extremely complicated (Van der Heijden, et al., 2008; Fester et al., 2014; Singh and Gupta, 2018).

1.1 Effects of soil microbial communities on plants

Generally speaking, plant-microbial interactions can be broadly subdivided into three basic groups of effects. Firstly, in many cases plants and specific microbes do not affect each other strongly. Secondly, there are negative effects on plants through root-associated organisms that form pathogenic relationships with plants. These pathogenic organisms in the rhizosphere include parasitic nematodes, fungi, Archaea, bacteria and invertebrate herbivores. Soil pathogens can reduce plant productivity, thus impacting ecosystem processes. Ecologists are long aware that soil microbes can cause serious reductions in plant growth (Nijjer et al., 2007). Among a myriad of soil-borne microbial pathogens, *Phytopathora, Pythium, Fusarium* and *Verticillium* are well-known genera and they have been widely reported to have negative effects on the production of many crops and economically important tree species, such as potato, wheat, radish, pea and oaks (Harman et al., 1980; Nirenberg, 1981; Jung et al., 1999).

Thirdly, root-associated organisms can have positive effects on plant growth. For example, several plant growth-promoting *rhizobacteria* (PGPR), like *Pseudomonas* and *Burkholderia*, residing in the rhizosphere may repress the growth and activity of

soil-borne pathogens and other attackers (Bhattacharyya and Jha, 2012). PGPR commonly reside in the rhizosphere, where they are important regulators involved in numerous biological processes affecting host plants, e.g. solubilizing phosphate, fixing available soil nitrogen, producing siderophores, phytohormones, producing volatile organic compounds (VOCs), inducing host systemic resistance and systemic acquired resistance and stimulating antifungal compounds (Wei et al., 1991; Nelson, 2004; Esitken et al., 2010; Bhattacharyya and Jha, (2012). Arbuscular mycorrhizal fungi (AMF) can act as a natural extension of the host root system, to increase the possibility of plants to obtain resources from the soil, and plants provide carbon (C) to the AMF in exchange (Azcón-Aguilar et al., 1992; Barea, 2000). Moreover, some proteobacteria, such as legume-nodulating *Burkholderia* strains, and species of the genus *Azoarcus* and *Sinorhizobium meliloti*, are well known for their functions in fixing soil atmospheric nitrogen, which also benefits host plants (Reinhold-Hurek et al., 1993; Chen et al., 2003; Hayat et al., 2010; Schlüter et al., 2010). Additionally, root endophytes can play an important role in enhancing both biotic and abiotic stress tolerance in plants (Dimkpa et al., 2009), while some rhizosphere bacteria produce antibiotic compounds or protective biofilms that prevent the plant from attack by pathogenic soil bacteria.

Many studies report that the overall net effect of soil microbial communities on plant performance is negative (Nijjer et al., 2007). Inoculation of soil-borne microbial communities into sterilized soil often causes a reduction in plant growth. Negative effects of the soil microbial community on plant growth can be due to nutrient competition between plants and microbes and due to soil-borne plant-pathogenic microbes. Soil microbes, such as AMF, phosphorus-solubilizing bacteria, proteases and nitrogen-fixing bacteria can assist plants in taking up more nutrients from the surrounding soil. However, plants and microbes also depend largely on the same inorganic nutrients and therefore compete for these nutrients.

Plant-soil feedback studies, mostly show that inoculation of sterilized soil with microbial communities collected from underneath conspecific plants has a stronger negative effect on plant growth than inoculation with microbial communities from other plant species, suggesting that plant species-specific pathogenic or plant growth-

inhibiting microorganisms build up in the rhizosphere (Pendergast et al., 2013; Dawson et al., 2017; Howard et al., 2020). Studies with pure "home soil" and "away soil" also generally report negative effects of "home soil" on plant growth (Manning et al., 2008; Ayres et al., 2009). All these studies suggest that pathogenic effects of micro-organisms play an important role in the interaction between plants and the soil microbial community. These studies furthermore suggest that many of the interactions are species-specific and that plants affect the microbial community in a species-specific way and vice versa. Moreover, it is worth noticing that soil microorganisms can also affect the above and belowground defense system of a plant (Huberty et al., 2020). For instance, soil-borne microorganisms affect the composition of pyrrolizidine alkaloids (PAs) and the total PA concentration in the plant Jacobaea vulgaris (Joosten et al., 2009; Kostenko et al., 2012; Kos et al., 2015). Studies that explore the mechanisms behind the interactions between plants and the soil microbial community nowadays are boosted by the molecular tools that make it possible to study the composition and functions of microbial communities. In this thesis, I will concentrate on the effects of the microbial communities on plant growth.

1.2 Effects of plants on rhizosphere microbial communities

The term rhizosphere was introduced by Hiltner in 1904 to describe the layer of soil that was influenced by the root of a plant (Hiltner, 1904). In comparison to root-free soil, the rhizosphere is an area where plant roots and soil microorganisms are mutually interacting. Soil properties (pH, humidity, chemical composition, texture and structure) play an important role in the modulation of rhizosphere microbial communities (Börner, 1960; Bach et al., 2010). However, plants also greatly influence the structure and function and diversity of microbial communities, especially in the rhizosphere (Grayston et al., 1998; Girvan et al., 2003; Nunan et al., 2005; Berg and Smalla, 2009; Dennis et al., 2010). Plant species differ in their effect, and in the strength of this effect, on microbial communities (Zak et al., 2003; Mangan et al., 2010). Other studies showed that bacterial communities in the soil of grass and forb species differ (Hannula et al., 2019) and that the structure and function of soil microbial communities of exotic plant species are different from those of native species (Kourtev et al., 2003). A prediction of how specific crops will influence the

soil microbial community may help to reduce risks and yield losses in agriculture, but, so far, this is poorly understood and remains a long-term challenge.

Plants synthesize a vast array of secondary metabolites (SMs) and more than 100,000 are reported (Dixon, 2001; Quiroga et al., 2001; Bartwal et al., 2013). Many studies have demonstrated that these compounds are involved in the chemical defense of plants against pathogenic microbes (Van Loon, 2007; Boller and He, 2009). In particular, root exudates are key drivers of microbial diversity and composition in the rhizosphere. For example, sugars, organic acids and amino acids are well-known nutrients for microbes (Canarini, et al., 2019). The composition and concentration of these metabolites in the rhizosphere depend upon the plant species and overall environmental conditions (Broeckling et al., 2008; Zahar et al., 2008).

Plant chemical defenses play an important role in plant-pathogenic microbe interactions. It has been suggested that the diversity of defensive compounds has evolved as a result of an evolutionary arms race between the plants and their potential attackers (Ehrlich and Raven, 1964). In particular, those defenses based on molecules with low molecular weight, and long-distance communicating molecules, such as volatile organic compounds (VOCs) (Insam and Seewald, 2010; Frankenberger and Arshad, 2020). Moreover, SMs, such as citronellal, berberine and pyrazines are also functioning in plant defense against pathogenic microbes (Wink, 1988; Tyc et al., 2017).

In addition, plants can regulate the production of protease inhibitors to defend themselves (Lawrence and Koundal, 2002; Habib and Fazili, 2007). Microbial pathogens can secrete extracellular protease enzymes, and those enzymes can digest some proteins in the tissues of plants (Ryan, 1990). Plants can defend themselves from protease-related pathogens through expressing protease inhibitors and also regulate them to accurate and strict concentrations. Researchers have generated some transgenic plants with high expression of protease inhibitors, such as transgenic rice, potato, soybean, and these plants exhibit increased resistance against various pathogens (Cowgill et al., 2002; Rahbé et al., 2003).

Moreover, induced defense responses in the plant influence the chemical composition of root exudates and through that the bacterial community structure in the soil. Salicylic acid (SA), ethylene (ET), abscisic acid (ABA) and methyl jasmonate (MeJA) hormonal signaling pathways can alter the bacterial community composition in the soil (Carvalhais et al., 2015; Lebeis et al., 2015). Van der Meij et al. (2018) showed that application of SA to endophytic actinobacteria stimulates antibiotic production. Altogether these findings suggest that activating hormonal signaling may not only boosts the plant's defense system directly but also can affect the microbial composition on the soil thereby potentially mitigating the negative effects of the soil microbial community on plant growth.

2. Plant hormonal induced defense against soil-borne pathogens

To counteract the effects of microbial pathogens, plants have evolved a broad range of defensive mechanisms, which are partly regulated via hormonal signaling pathways (Fujita et al., 2006). Defense, as an essential and effective strategy for terrestrial plant species against pests and pathogens has been broadly developed in plants (Wesson and Wesson, 1993; Bronstein, 1998; Agrawal, 2011; Turley et al., 2013; Zhang et al., 2015). Induced defenses are defenses that are activated after infection occurs and enhance plant fitness (Boots and Best, 2018). Phytohormones are a group of natural plant compounds with low molecular weight that play an important role in the regulation of plant growth and development and induced plant resistance against pests and pathogens. SA, ET, abscisic ABA, MeJA, auxin, cytokinins (CKs), gibberellic acid (GA) and brassinosteroids (GAs) are commonly studied phytohormones. Besides these, there are also several other compounds (e.g. karrikins, triacontanol and nitric oxide) that can be involved in induced plant defense, but their functions are still under debate.

Each phytohormone has clear functions, however, they can also exhibit strong interactive effects. For instance, JA and SA are well-known for their negative cross-talk (Munné-Bosch and Müller, 2013). Upregulating the SA signaling can lead to downregulation of the JA signaling and vice versa. Such cross talk is one of the mechanisms that can explain why plant pathogens in the soil can e.g. affect herbivory above ground (Aljbory and Chen, 2018). Although most hormones have been

implicated to be involved in defense pathways, the key regulator against pathogens and pests, in particular, to defend plants against biotrophic, necrotrophic pathogenes and herbivores, are the phytohormones JA and SA (Bari and Jones, 2009). In the following sections, JA and SA induced resistance in plants against soil-borne pathogens is described separately.

2.1 JA-induced resistance

JA is associated with several biological processes in plants. Specifically, JA can stimulate the germination of seeds, negatively impacts root growth, and invokes tuber formation and fruit ripening. Apart from these functions, JA is well-known for being involved in induced resistance against herbivores and for being a regulator of the activation of induced systemic resistance (ISR) of plants against necrotrophic microbial pathogens.

The biosynthesis of JA has been mostly studied in the model plant species *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum*) (Ruan et al., 2019). Both biotic and abiotic stresses can induce the synthesis of JA. In plant tissues JA can be converted into JA-isoleucine conjugate (JA-Ile) by JAR1 (an auxin-induced gene), JA-Ile is a bioactive state of JA. MYC is a family of regulator genes that code for transcription factors and JA-Ile activates the MYC transcription factors by directly binding to the jasmonate zim-domain (JAZ) and a *coronatine insensitive1* (COI1) protein, which results in the degradation of JAZ transcriptional repressor proteins through the proteasome pathway. These processes result in the activation of transcription factors and the regulation of JA-responsive genes (e.g. MYC2, ERF1 and ORA59), which are associated with plant responses against environmental stresses from pathogens, wounding, and insect herbivory, biosynthesis of secondary metabolites, and with plant growth and development. For example, Carvalhais et al. (2015) demonstrated that the JA signaling pathway affects the composition of root exudates by enhancing the production of ornithine and that ornithine can be used by plant growth-promoting bacteria such as *Pseudomonas fluorescens*, which in turn has a positive effect on plant growth.

Due to the effectivity of JA-induced resistance against herbivores, in agriculture, foliar application of JA or MeJA has been considered as an alternative approach to control pests rather than using chemical pesticides. This theory has been tested in several crop plant species, including corn, tomato and wheat (Mandal et al., 2006; War et al., 2011; Chen et al., 2018).

In addition, to increased resistance to pests, JA-mediated defense also regulates the plant's response to necrotrophic microbial pathogens, e.g. *Pseudomonas syringae, Fusarium oxysporum and Hyaloperonospora parasitica* (Antico et al., 2012; Wasternack and Strnad, 2018; Li et al., 2019). The activation of JA-signaling pathways in the plant can result in changes in the composition of bacteria in the rhizosphere as was shown for the plant *A. thaliana* (Carvakhais et al., 2013). The mechanisms behind this are not yet fully resolved. JA signaling may directly affect microbial species or through the interaction with SA signaling.

2.2 SA-induced resistance

SA is another well-studied hormonal compound, which plays an important role in the activation of SA-induced resistance against biotrophic microbial pathogens. Hypersensitive response (HR) is a primary manifestation of a plant to pathogenic attack, e.g. due to cell death of the tissues surrounding the infection, to control the spread of pathogens. Cultivars that are highly sensitive to SA are often more tolerant of microbial pathogens (Seskar et al., 1998).

Infection of plant tissues with biotrophic pathogens leads to the accumulation of SA, as well as monomerization of NPR1 via SA-mediated redox changes in the cell. Later, monomeric NPR1 is relocated into the nucleus, at which the monomeric NPR1 interacts with TGA transcription factors, as a result, SA-responsive genes are activated. A large number of WRKY genes are induced by SA, among which some can regulate SA-responsive gene expression (Van der Does et al., 2013).

SAR is associated with the expression of pathogenesis-related proteins (PRPs). PRPs include proteins like β-1, 3-glucanase and chitinases (Van Loon and Van Strien, 1999). Commonly, both chitinases and glucanases show antimicrobial activities. Once PRPs

are induced by SA, they take actions in several ways to assist plants against pathogens. These actions can be direct and indirect (Edreva, 2005). PRPs can direct breakdown or damage pathogens based on their antifungal and antibacterial activities. In addition, PRPs can indirectly boost host defensive abilities through hydrolytic released compounds of fungal cell walls, e.g. ochitin and glucan fragments. These released oligosaccharides could further stimulate a series of defensive responses in host plants (Lawrence et al., 2000; Edreva, 2005).

SA induced resistance can interact with beneficial bacteria and fungi, such as *Pseudomonas*, plant growth-promoting bacteria (PGPR), arbuscular mycorrhiza fungi (AMF). Those beneficial microbes interact with SA-induced resistance through species-specific microbe-associated molecular patterns (MAMPs) (Bittel and Robatzek, 2007; Choi and Klessig, 2016). MAMPs are special components on the surface of general microbes, such as, *Trichoderma*, *Bacillus* and *Pseudomonas* (Pieterse et al., 2014). Once a host plant recognizes these MAMPs, the plant will activate its innate systemic defensive system to be ready to cope with future pathogens.

Exogenous application of SA or Methyl SA to activate SAR is a way to control microbial pathogenic diseases. In many crops, like tomato, pepper and pea exogenous application of SA results in a suppressing effect on microbial pathogens (Esmailzadeh et al., 2008; Barilli et al., 2010; Choi and Hwang, 2011). Overall, in agriculture, improving SA-mediated resistance has become a promising strategy to control microbial pathogens and viruses.

3. Adaptation of microbes to plant defenses

Plants are not the only organisms that can produce hormonal compounds, which stimulate the activation of signaling pathways in the plant. For example, plant growth-promoting rhizobacteria can produce and/or degrade phytohormones, and in this way interfere with the regulation of plant growth (Dodd et al., 2010). Not only beneficial bacteria but also pathogenic microbes can produce hormones or compounds that impact plant growth.

Some microbial pathogens can mimic the production of plant hormones and are able to highjack the plant immune system (Cui et al., 2005; Laurie-Berry et al., 2006; Navarro et al., 2008). Cui et al. (2005), for example, reported that the bacterial pathogen (*Pseudomonas syringae*) activated induced systemic resistance in *A. thaliana* by producing coronatine (COR). This compound can mimic the function of the JA hormone, thereafter induce the JA-related signaling pathway in host plants. Besides, Laurie-Berry et al. (2006) also demonstrated that *P. syringae* could utilize COR to upregulate the JA pathway in host plants and suppress the SA-mediated signaling pathway in tomato plants, making *P. syringae* even more virulent.

Some bacteria can synthesize SA by converting their chorismate through isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Chen et al., 2009; Dempsey et al., 2011). This has been reported for several bacteria species, like *Pseudomonas aeruginosa* and *P. fluorescens* (Mercado-Blancoet al., 2001; Kerbarh et al., 2005; AI-Mustafaet al., 2009). The production of SA by bacteria can increase the plant's resistance against pathogens. For example, the production of SA by *P. aeruginosa* can enhance the resistance of plants against the bacterial pathogen *Botrytis cinerea*, which causes leaf diseases on bean (De Meyer and Höfte, 1997); *P. fluorescens* enhances plant defense in chickpea against *Fusarium* wilt (Saikia et al., 2003).

4. Contradictory observations of SA signaling on the soil microbial community

Many studies investigated the effects of hormonally induced defenses on single pathogens (El-Khallal 2007; Abo-Elyousr et al., 2009; Mandal et al., 2009). However, our knowledge about the impact of activating plant hormonal signaling pathways on the composition of the soil microbial community is still limited and contradictory (reviewed in Hacquard et al., 2017).

Lebeis et al. (2015) examined the effect of SA on isogenic *A. thaliana* mutants with altered immune systems and found that plants with an altered SA signaling pathway contained rhizospheres that differed in the relative abundance of specific bacterial families as compared to wild type plants. Kniskern et al. (2007) using *A. thaliana* mutants found that activation of SA signaling pathways reduced endophytic bacterial

community diversity, whereas plants that were deficient in JA-mediated defenses experienced greater epiphytic bacterial diversity. When a plant is exposed to microbial pathogens or herbivory, the plant changes the composition of the primary and secondary metabolites that are produced, and this can impact the soil microbial community, and may result in a feedback to the plant (Rolfe et al., 2019).

Several other studies showed that there was no effect of activation of SA signaling on the soil microbial community. For instance, Wang et al. (2015) found that higher concentrations of exogenously added SA inhibited the growth of grape plants, however, there was no direct correlation between the inhibitory effects of SA on plant growth and the diversity of the soil bacterial or fungal community. Similarly, Doornbos et al. (2011) found that chemical activation of JA- or SA-induced resistance did not significantly affect the composition and diversity of the rhizosphere bacterial community in *A. thaliana*. Hein et al. (2008) compared the effect of SA-induced resistance on the diversity of rhizosphere bacterial communities in several *Arabidopsis* mutants and found that changes in microbial composition were not caused by the induction of the SA signaling pathway. Even though the previous mentioned study showed that activation of SA-dependent defenses did not change the composition of soil microbial community in *A. thaliana* and the SA-independent defense was not induced by foliar application of SA, this still provides a great value to understand the interplay of activating SA-signaling pathways and microbial composition (Sonnemann et al., 2002; Doornbos, et al., 2012; Moccia and Lebeis, 2019).

All taken together, the role of induced resistance on the soil microbial community is still being debated, and more research on the impact of upregulated hormonal signaling in plants on the composition and functionality of the soil microbial community is necessary.

5. Characterizing microbial communities

Microbial communities can be characterized using metagenomics tools. Metagenomics aims at determining the microorganisms as a whole and allows us to extract the biological information of all the microbes from the environment directly

(Hugenholtz and Tyson, 2008). Up to date, metagenomics has been widely applied in various environments to investigate microbial communities ranging from soils, water, ocean and human gut (Handelsman, 2004; Daniel, 2005). However, the lack of reference sequences and genomes is a major drawback of metagenomics (Krehenwinkel et al., 2019).

The microbial community can be characterized on basis of the species present or on the basis on the genes and their functions that are expressed in the microbial community. While the first is highly relevant to understand the diversity and the dynamics of microbial populations and communities, the second is of great importance if we want to understand the mechanisms behind plant-microbial interactions and how a microbial community adapts to the environment. In other words: taxonomic information helps to answer the most primary question for soil microbial-ecologists: Who is there? Analysis of gene expression helps to understand what they are doing. Amplification of 16S rRNA barcode markers is commonly used to determine bacterial microbial communities and the costs of characterizing part of the genome are much lower than sequencing the whole genome. Pipelines for 16S rRNA gene sequencing and identifying operational taxonomic units (OTUs) by aligning the reads against known public databases (e.g. NCBI, EzBioCloud 16S database and MBGD) are available. Nowadays, high-throughput sequencing has become a vital and cost-effective tool for profiling functions of soil microbial communities. It can generate a high volume of data and long read lengths. Illumina short reads sequencing (up to 250 bp) has a high output and low read errors. In this thesis, the Illumina sequencing platform is used to examine the microbial composition and functional genes of rhizosphere soil microbial communities.

Also to process metatranscriptomics data existing pipelines can be used. These pipelines can be modified and applied to different experimental designs. For example, the IMP pipeline incorporates robust read preprocessing and is suitable for analyzing metagenomic and metatranscriptomics as it provides information on both microbial structure and functional genes (Narayanasamy et al., 2016). MetaTrans is an open-source pipeline developed for a paired-end RNA-Seq analysis (Martinez et al., 2016) while the functional mapping and analysis pipeline (FMAP) provides alignment, gene

family abundance calculations and open-level statistical analysis (Kim et al., 2016). SAMSA2 is a standalone metatranscriptome analysis pipeline and is used on a supercomputing cluster, which is more flexible and reproductive in processing a large volume of sequence data (Ni et al., 2016). In this thesis I used a modified pipeline that can run on a regular computer and is easily customized. The pipeline that was used assists with the transcriptomic tools Trinity and Trinotate. Transcripts generated by Trinity can be annotated with Trinotate and Trinotate allows users to perform functional annotation with several selective methods, such as homology search, protein domain search, or protein peptide domain search. The combination of these two bioinformatic tools enabled us to explore the structure and the functionality of microbial communities (Haas et al., 2013).

6. Research questions

A number of studies have shown that the overall effect of the soil microbial community on the growth of *J. vulgaris* seedlings is negative (Bezemer and van Dam, 2005; Van de Voorde et al., 2012; Wang et al., 2019). In this project, we asked whether this negative effect of the soil microbial community could be mitigated by the activation of Me-JA and SA signaling pathways through an effect on the composition of the soil microbial community.

First, I studied in four plant species (*J. vulgaris*, *Cirsium vulgare*, *Trifolium repens* and *Daucus carota*) how the growth of these plants was affected by the presence of a live microbial community in the soil. Then, I examined, for *J. vulgaris* that grew less well in live soil than in sterilized soil and for which the negative effect of the live soil on plant growth was mitigated by exogenous application of SA on leaves of the plant, how the application of SA alters the soil microbial community on both taxonomic and functional levels through a multi-generational experiment where I analyzed mRNA of the soil. In addition, I studied for how long during the plant's life the negative effect of a live soil on plant growth is maintained.

Specifically, the following research questions are addressed in this thesis:

(1) Do the effects of the soil microbial community on plant growth differ among four plant species that occur in the same habitat? Does the foliar application of JA and SA alter the effects of the soil microbial community on plant growth of these four plant species? Does the negative effect of the soil microbial community increase or decrease over successive generations of plant growth in *J. vulgaris* inoculated with the soil of the previous generation, and how is this influenced by SA application?

(2) How does the application of SA on *J. vulgaris* affect the composition of the microbial community in the rhizosphere? How does the soil microbial composition change over plant generations, when in each generation sterilized soil is inoculated with soil from the previous generation for plants that are treated with SA and untreated control plants?

(3) Does the application of SA on *J. vulgaris* alter the gene expression in the rhizosphere? Does the application of SA impact microbial gene expression over generations? Which groups of genes are influenced by SA-treated soil samples compared to control over generations?

(4) How long does the effect of inoculum of 10% soil containing a natural microbial community on plant growth last? Does the timing of inoculation change the effect of soil microbial communities on plant growth in *J. vulgaris*?

7. Thesis outline

Many plant species grow better in sterilized soil than in soil that contains a live microbial community, this could be due to an overall net pathogenic effect of soil microbial communities. To find out if an overall negative effect on plant growth is a common phenomenon in nature, in **Chapter 2**, four plant species were grown in either sterilized soil or sterilized soil containing 10% of live soil. In addition, I exposed plant leaves to two hormonal treatments (jasmonic acid and salicylic acid) to examine if hormonal defense pathways can influence the microbial effects on plants.

In Chapter 3, I sequenced and analyzed the microbial communities from the experiment of **Chapter 2**, to investigate if SA-induced defense had an impact on the

taxonomic composition of the microbial community in rhizosphere samples using Illumina sequencing. Since the application of SA mitigated the negative effect of soil microbial communities on the growth of *J. vulgaris*, I used this species to study the changes in the composition of the microbial community in response to SA application for four generations of plant growth.

As described in **Chapter 2** and **3**, certain groups of microbial species responded differently to the exogenous application of SA on plant leaves. However, the functions of those microbial species in the rhizosphere are largely unknown. Therefore, **in Chapter 4** I used metatranscriptomics to study the functional genes and clusters in the rhizosphere microbiome of both SA-treated and control samples. The changes in microbial functional genes over four generations were analyzed and compared.

Studies on plant-soil-interactions often address the soil microbial effect with measurements on plant absolute biomass. However, even if there is no difference in the relative growth rate (RGR), the absolute difference in plant growth can still increase. In **Chapter 5,** I studied for *J. vulgaris* how long the negative effect of live soil on plant growth is maintained. Also, I studied if the timing of inoculation affected the RGR of this species. Finally, the results described in this thesis and their implications are discussed in **Chapter 6**.

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Chapter 2

Activation of hormone-associated plant defense pathways alters the effects of live soils on plant performance

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Abstract

Many plant species grow better in sterilized soil than in soil that contains a live microbial community. One hypothesis to explain this phenomenon is that the overall net pathogenic effect of soil microbial communities reduces plant performance. Induced plant defenses triggered by the application of the plant hormones jasmonic acid (JA) and salicylic acid (SA) may help to mitigate this pathogenic effect of live soil. However, little is known about how such hormonal application to the plant affects the soil and how this, in turn, impacts plant growth. We grew four plant species in sterilized and inoculated live soil and exposed their leaves to two hormonal treatments (JA and SA). Two species (*Jacobaea vulgaris* and *Cirsium vulgare*) were negatively affected by soil inoculation. In these two species foliar application of SA led to higher plant growth in live soil but not in sterilized soil. Two other species (*Trifolium repens* and *Daucus carota*) were not affected by soil inoculation and for these two species foliar application of SA reduced plant growth in both the sterilized and live soil. Application of JA reduced plant growth in both soils for all species. We subsequently carried out a multiple generation experiment for one of the plant species, *J. vulgaris.* In each generation, the live soil was a mixture of 10% soil from the previous generation and 90% sterilized soil and the same hormonal treatments were applied. The negative effects of live soil on plant growth were similar in all four generations, and this negative effect was mitigated by the application of SA. Our research suggests that the application of SA can mitigate the negative effects of live soil on plant growth. However, although the inoculum of soil containing a natural live soil microbial community had a strong negative effect on the growth of *J. vulgaris*, we found no evidence for an increase in the negative plant-soil feedback in either the control or the SA treated plants as plant performance did not decrease consistently with succeeding generations.

Keywords

Plant-soil interactions, Plant-soil feedback, Induced resistance, Rhizosphere soil, Salicylic acid, Jasmonic acid

Introduction

The interactions between plants and soil microorganisms have long been recognized for their importance in terrestrial ecological systems (Bever 1994; van der Heijden et al., 2008). Although the effects may vary depending on the plant species and the soils tested, in the majority of cases the soil microbial community has a negative effect on plant growth (Kulmatiski et al., 2008). Plants also affect the composition of the soil microbial community, which, in turn, will impact plant growth. The process is called plant-soil feedback (Bever et al., 1997; Van Breemen and Finzi, 1998). Most plant species exhibit negative conspecific soil feedbacks. This means that they grow worse in soil, in which the same species has been grown than in soil where other species have grown (Kulmatiski et al., 2008). From natural situations and agriculture, it is well-known that soil can become less suitable for a species if this species is grown in the same soil for multiple generations. This negative effect is thought to be caused by soil pathogens or root herbivores, allelopathy, nutrient immobilization or nutrient depletion (Miki, 2012). In some cases, plants also cause positive plant-soil feedbacks and these can be mediated by plant promoting rhizobacteria, mycorrhizal fungi or other unknown mechanisms (Revillini et al., 2016; van der Putten 2017).

Plant-induced resistance has been regarded as a promising defense strategy against pathogens or herbivores (Haney and Ausubel, 2015; Lebeis et al., 2015; Yang et al., 2015). In nature, plants are exposed to complex selection pressures, involving both abiotic and biotic stresses. Plants are under constant attack by a myriad of pathogens and pests and have to compete with neighboring plants. As a result, plants have evolved a wide range of responses to cope with biotic stresses. The abilities of plants to respond to different biotic stresses are regulated through sophisticated interacting hormonal signaling networks (Bezemer and van Dam, 2005; Fujita et al., 2006; Arnaud and Hwang, 2015). Phytohormones are a group of natural plant compounds with low molecular weights. Salicylic acid (SA), Jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), methyl jasmonate (MeJA), auxin, cytokinins (CKs), gibberellins and brassinosteroids are commonly studied phytohormones. Plant hormones regulate many developmental and signaling networks. Although most hormones have been implicated to be involved in defense pathways, the key regulator against pathogens and pests, are the phytohormones JA and SA (Bari and Jones, 2009). Experimental

evidence indicates that application of SA to plant leaves, activates systemic acquired resistance (SAR) against pathogens (Reymond and Farmer, 1998; Mandal et al., 2009). JA, in turn, activates induced defenses against herbivores and necrotrophic pathogens (Nahar et al., 2011). Although to some extent, the SA or JA-induced hormonal signaling pathway could interact with other phytohormones, such as CKs, ET, ABA and auxins, they do show clear effects on the plant's defense system when applied as single hormones (Fujita et al., 2006; Yang et al., 2015; Berens et al., 2019). The crosstalk between SA or JA and other hormones is still not fully understood.

A still uncharted territory is how plant hormone-activated signaling pathways impact soil microbial communities and how these, in turn, affect plant growth. Here we restricted ourselves to two prime hormones involved in activating defense pathways, SA and JA. We aimed to quantify the effect of induced SA or JA resistance on the soil microbial communities that affect plant growth. If the negative effect of the soil containing a live soil microbial community on plant growth is caused by an overall pathogenic effect we expect that activating SA signaling by exogenous application mitigates these negative effects. As a result, we expect that the effect of SA application on plant growth differs between plants in sterile soil and in live soil. Exogenous application of JA typically induces resistance against herbivores and necrotrophic pathogens (Pieterse et al., 2009; van Dam and Oomen, 2014; Carvalhais et al., 2017; Palmer et al., 2017). JA signaling can exhibit negative crosstalk with SA signaling (Leon-Reyes et al., 2010). One can therefore hypothesize that activating JA signaling will reduce the ability of plants to cope with pathogens (which causes induction of the SA pathway in the plant) and thus will increase the overall negative effect of the soil microbial community on plant growth. The responses of plants, after activating hormonal defense pathways, to an inoculum containing a live soil microbial community are, as yet, not well studied and understood. Moreover, the evidence for the existence of such effects is contradictory. Activation of JA and SA signaling pathways did not affect the resident soil microflora in several studies (Doornbos et al., 2012; Berendsen et al., 2012; Rashid and Chung, 2017), but a more recent study showed that SA modulates colonization of the root microbiome by specific bacterial taxa (Lebeis et al., 2015).

If the induction of signaling pathways in the plant leads to changes in the composition of the soil microbial community, its effect is likely to extend over time or plant generations. Potentially this could lead to the selection of more beneficial soil microbial communities either by suppressing pathogens or by promoting beneficial microbes. As far as we are aware, the effects of plant hormones through plants on soils containing a live microbial community over multiple generations have not been studied so far, despite its potential to select for more beneficial soils containing plant growth-promoting microbial communities in agriculture.

In a preliminary experiment, we found strong evidence for negative effects of soil that consisted of a mixture of 90% sterilized soil and 10% live soil on the growth of common ragwort (*Jacobaea vulgaris*), compared to sterilized soil. After treating plants with SA, this negative effect diminished. Based on these findings, we grew four different plant species individually in both sterilized soil and live soil. For *J. vulgaris*, the species which showed the strongest negative effect towards the live soil, and for which this negative effect was mitigated by foliar application of SA, we grew plants for three more generations. For each generation, sterilized soil was inoculated with live soil from the previous generation from the same treatment. We addressed four questions: (1) Do the effects of live soil on plant growth differ among plant species? (2) Does the foliar application of JA and SA alter the effects of the live soil on plant growth for those species that were negatively affected by the live soil? (3) Does the negative effect of live soil change in four successive generations of *J. vulgaris* for control plants and plants treated with SA or JA.

Materials and methods

Plant material and seeds germination

Jacobaea vulgaris (common ragwort), *Cirsium vulgare* (bull thistle), *Trifolium repens* (white clover) and *Daucus carota (wild carrot)*, were chosen because they are common native species at the dune area where we collected soil. We collected seeds at the dunes for *J. vulgaris*, *C. vulgare*, and *D. carota. T. repens* seeds were bought from Cruydt-Hoeck a seed company that sells seeds of wild plant species (Nijeberkoop, The Netherlands). Prior to seed germination, all seeds were shaken for

2 min in 70% ethanol, then washed with sterilized water, put for 12 min in 2% bleach, and finally rinsed four times with sterilized water to minimize influences of seed-borne microbes.

Soil material

The soil was collected at Meijendel, a calcareous sandy area from a coastal dune area north of The Hague, The Netherlands (52°11´N, 4°31´E). The topsoil was collected to a depth of 15 cm after removing the grassland vegetation and the organic layer of the surface. The soil was sieved using a 5 mm sized mesh, homogenized with a concrete mixer, and then stored into 20-liter plastic bags (Nasco Whirl-Pak Sample Bag). Bags were either sterilized by 35-K Gray gamma-irradiation (Synergy Health Company, Ede, The Netherlands) or kept at 4°C for inoculation.

Plant growth and foliar application of hormones

Surface sterilized seeds of the four species (*J. vulgaris, C. vulgare*, *T. repens* and *D. carota*) were germinated in sterile Petri dishes on filter paper. After one week, 60 seedlings per species were planted individually in 500 ml pots containing either sterilized soil or inoculated live soil. The live soil consisted of a mixture of 90% sterilized soil and 10% live soil. Nutrient availability often increases after sterilization of the soil, and we therefore inoculated the sterilized soil rather than using pure live soil, to enable comparison of the two types of soil. Sterilized soil and live soil were kept in bags and left in the climate room for 14 days to enable the establishment of microbial communities in the inoculated soil before potting. Before potting, the soil in each bag was mixed. After planting the seedlings, pots were randomly distributed over a climate room (relative humidity 70%, light 16h at 20°C, dark 8h at 20°C). Plants were watered regularly with Milli-Q water. Five ml Steiner nutrient solution was added per plant on day seven. Ten ml Steiner nutrient solution was added on day 13, and 20 ml Steiner nutrient solution was added on days 19, 28, 37, 42. The Steiner nutrient solution (Steiner, 1980) was prepared from seven different stock solutions (106.2 g $Ca(NO_3)_2 \cdot 4H_2O$, 29.3 g KNO_3, 13.6 g KH_2PO_4, 49.2 g $MgSO_4 \cdot 7H_2O$, 25.2 g K_2SO_4 , 2.24 g KOH and 3.29 g Fe-EDTA added to 1 liter demineralized water, and a stock solution with micro elements (a mixed solution of 0.181 g $MnCl_2 \cdot 4H_2O$, 0.286

g H_3BO_3, 0.022 g $ZnSO_4 \cdot 7H_2O$, 0.0078 g $CuSO_4 \cdot 5H_2O$ and 0.0126 g $NaMoO_4 \cdot 2H_2O$ added to 1 liter demineralized water). Ten ml of each stock solution was diluted in 1 liter of demineralized water before use.

The pots for each species were divided over six treatments: two soil treatments (sterilized soil and live soil) and three hormonal treatments (JA, SA and control (only solvent)). Each treatment was replicated 10 times. The experiment, therefore, consisted of 240 pots (4 species × 2 soil treatments × 3 hormonal treatments × 10 replicates). The plant hormones JA and SA were applied through foliar application three times a week for four consecutive weeks. The first application was given when plants were 14 days old. Either 0.75 ± 0.05 ml of 100 µM JA or SA was sprayed on the leaves while carefully avoiding spillover to the soil. One week later the treatment was repeated with 1.50 ± 0.05 ml of 100 µM JA or SA. In the next week, the treatment was repeated with 2.25 ± 0.05 ml of 100 µM JA or SA. The JA-solution was prepared by adding 105.135 µl JA stock solution into Milli-Q water until a final volume of 500 ml. The JA stock solution was prepared by adding 500 mg JA to 5 ml ethanol. JA was purchased from Cayman Chemical Company (product number: 88300). SA (purchased from Sigma-Aldrich, ≥ 99.0%) was made by dissolving 6.9055 mg in 69.055 µl of ethanol to which Milli-Q water was added until a final volume of 500 ml. Control plants were sprayed with sterile water with the same solvent (85 µl ethanol in 500 ml Milli-Q water).

Harvesting plants and soil samples

Fifty-four days after planting, all plants were harvested, except for *C. vulgare*. *C. vulgare* plants were considerably larger than the other species and were therefore harvested after 45 days to prevent pot size becoming limit growth. Plants were gently removed from the pots. Shoots were separated from roots with a scissor just above the root crown, and roots were rinsed with water and then put into paper bags. Harvested plant parts were oven-dried at 60°C for approximately one week. The dry weight of roots and shoots was determined until the nearest 0.1 mg. The rhizosphere soil was harvested individually from each pot by gently shaking the roots and soil three times to remove the loosely adhering soil, after which rhizosphere soil samples were collected onto a sterile filter paper by removing the remnant soil from the roots

with a fine sterilized brush. Finally, all the labeled soil samples were transferred to a 4°C room and stored for the multiple generation experiment.

Multi-generation experiment

J. vulgaris was chosen for the multiple generation selection experiment to examine if the observed effect on plant biomass of the first generation would increase further over later generations. For *J. vulgaris* we grew the plants from each of the six treatments (sterilized and live soils, two hormone treatments and control) for another three generations under the same conditions as described for the first generation. The only difference being that each time, the soil inoculate was derived from the previous generation from the same treatment, 100% sterilized soil was used as control. A schematic drawing of the experiment is presented in Fig. 1. Fourteen days after mixing the sterilized and live soil, a single *J. vulgaris* seedling was planted into each pot. All replicate rhizosphere soils from a single treatment were mixed before inoculation to avoid a selection of particular microbial species in individual pots. All treatments were carried out as described above.

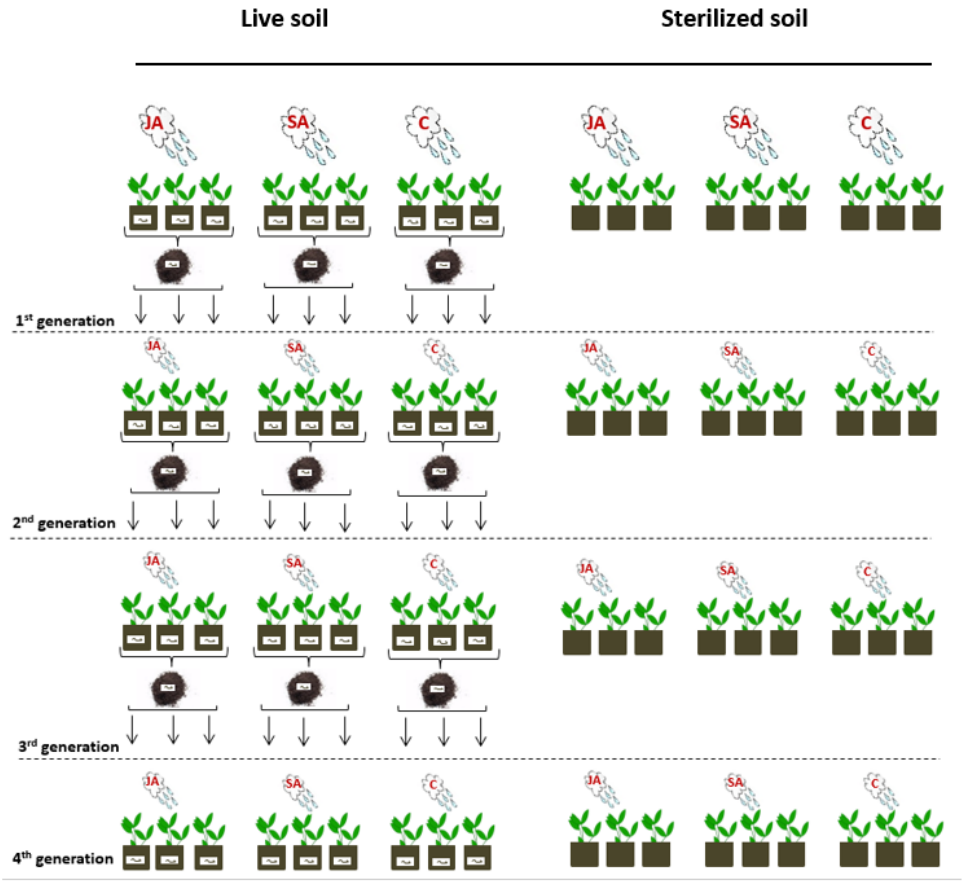

Fig. 1 Experimental design of the multigeneration experiment with *J. vulgaris*. Soil used for the 1st generation was a mixture of 90% sterilized soil and 10% live soil both collected from the dunes. Soil used for the 2nd, 3rd and 4th generations was a mixture of 10% rhizosphere soil collected from the previous generation from the same treatment and 90% sterilized soil collected from the dunes. In each generation we tested two hormonal treatments in inoculated and 100% sterilized soil. JA denotes foliar application of jasmonic acid, SA denotes foliar application of salicylic acid and

C denotes control. In each treatment 10 replicates were used even though only three are depicted.

Statistical analysis

Data were first checked for homogeneity of variance and normal distribution of errors and data were transformed when necessary. To test whether the effect of the live soil was different among the four species we performed a three-way ANOVA on the total data set of the first experiment with soil (sterilized and live, 2 levels)", hormones (3 levels) and species (4 levels) as fixed factors, Relative plant dry mass was used as a dependent variable and was arcsine square-root transformed prior to analysis. Relative plant dry mass was calculated as 100 times the dry mass of a plant divided by the average dry mass of the control plants from the same species in the sterilized soil. In this way, the average dry mass of the control plants in the sterilized soil was set at 100 for each of the four species. By doing so we removed species-specific size differences enabling to make the data more comparable among species. This analysis showed a significant soil × species interaction (see results section). On basis of this we divided the data set in two groups. One group for the two species that were negatively affected by the lives soil and one group for the two species that were not. We did this because we expected the effect of the hormonal treatments to be only present for the species that were affected by the live soil. To answer the question if the effect of the live soil was affected by foliar application of hormones, we performed four three-way ANOVAs (for the two groups of species and the two hormonal treatments) with plant mass as dependent variable and species (2 levels), soil (2 levels) and hormonal treatment (2 levels) as fixed factors. Usually, the negative effects of live soils on plant biomass are stronger in the roots than the shoots, thus we also carried out three-way ANOVA analysis for shoot-root ratios of the four plant species.

To answer the question whether the negative effect of live soil changes in four successive generations of *J. vulgaris* for control plants and plants treated with SA or JA we used a three-way ANOVA with soil (2 levels), generation (4 levels) and hormones (3 levels) as a fixed factor, and log-transformed plant dry mass or shoot-root ratio as dependent variables. We furthermore compared the effects of the two hormones separately using a three-way ANOVA with log-transformed plant dry mass

as a dependent variable and soil (2 levels), hormone (2 levels) and generation (4 levels) as fixed factors. Differences between treatments were tested with a Tukey post-hoc test.

We used a linear regression model to estimate the effects of SA and JA on the growth of *J. vulgaris* over four consecutive generations in both sterilized and live soil. In the regression model, the dry mass of plants of the SA or JA treatment divided by dry mass of control plants was the dependent variable and generation was the independent variable. Since we could not pair the pots (SA or JA/control) and we only had 10 replicates for each treatment, we used a Monte-Carlo simulation to test if the linear regression model differed from y =1. Each time we randomly paired one plant of the hormone treatment and one plant of the control to calculate the ratio of the dry mass of treated and dry mass of control. Then we repeated this procedure 1000 times, to obtain 1000 ratios of each generation per soil. Then we took the mean of 1000 ratios per generation to fit linear regression models for the two soils, respectively. To test whether the linear regressions in sterilized and live soils differed from y =1, we calculated the 95% confidence intervals (CFI) of the slopes for both soils. We also tested whether the two linear regression models differed between sterilized and live soils with ANCOVA (analysis of covariance) analysis. All analyses were performed in IBM SPSS Statistics 25.

Results

Do the effects of live soil on plant growth differ among plant species?

For *J. vulgaris* and *C. vulgare*, biomass in live soils was about half that in sterilized soils. This negative effect of the live soil was present irrespective of the hormonal treatment. For the other two species (*T. repens* and *D. carota*) biomass was not significantly different in live and sterilized soils (Fig. 2, Table 1). The difference in response to live soils among the four species is reflected by the highly significant interaction term (species × soil) in the ANOVA (Table 1)

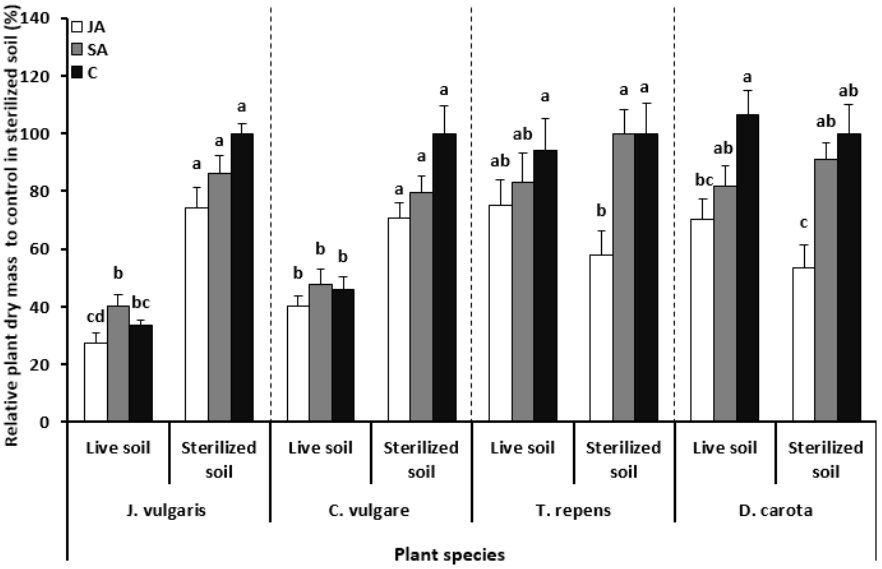

Fig. 2 Mean (+ SE) relative plant dry mass (%) of *J. vulgaris, C. vulgare, T. repens* and *D. carota* plants treated with JA and SA in sterilized soil and live soil. C represents the control treatment. Note: within species different letters above bars indicate significant differences between treatments based on a Tukey post-hoc test for each single species. N=10.

Table 1 Three-way ANOVA of arcsine square-root transformed relative plant dry mass of *J. vulgaris*, *C. vulgare*, *T. repens* and *D. carota* in live and sterilized soil for plants treated with JA or SA and for control plants. *df* = degrees of freedom.

Source of variations	*df*	F-value	*P*
species	3, 239	53.67	***
soil	1, 239	147.78	***
hormone	2, 239	27.17	***
species × soil	3, 239	59.81	***
species × hormone	6, 239	0.45	ns
soil × hormone	2, 239	4.75	**
species × soil × hormone	6, 239	1.48	ns

** $P < 0.01$, *** $P < 0.001$, ns not significant.

Does the foliar application of JA and SA alter the effects of live soil on plant growth for those species that were negatively affected by live soil?

Salicylic acid.

For the two species (*J. vulgaris and C. vulgare*) that were negatively affected by the live soil, foliar application of SA reduced the biomass for plants grown in the sterilized soil while it increased the biomass for plants grown in the live soil (Fig. 2). As a result, the main effect of SA in the ANOVA was not significant (Table 2). Although by itself the differences between the SA treatment and the control were not significant (Fig. 2), the effect of the SA treatment, as we hypothesized, depended strongly on soil type as is reflected by the significant soil × hormone interaction term in the ANOVA (Table 2). For the two species (*T. repens*, *D. carota*) that were not negatively affected by the live soil foliar application of SA reduced plant biomass in both soils, although this effect was not significant (Fig. 2, Table 2).

Jasmonic acid

Foliar application of JA decreased plant mass in all plant species in both sterilized and live soils. For the two species that were negatively affected by live soil the negative effect of JA was stronger in sterilized soils than in live soil (Fig. 2). This difference in response between plants grown in the two soils was significant as reflected by the soil × hormone interaction term in the ANOVA (Table 2). For the two species that did not grew less well in the live soils, such a difference in the response to JA application in the two soils was not found (Table 2).

Table 2 Three-way ANOVAs of arcsine square-root transformed relative plant dry mass for species that grew less well in live soil compared to sterilized soil (upper part) and for species that were not negatively affected by the live soil (lower part). Left: hormonal treatment is foliar application of SA. Right: hormonal treatment is foliar application of JA. Species, soil (live or sterilized), and hormone treatment were used as fixed factors. *df* = degrees of freedom.

		SA treatment			**JA treatment**		
Species respond to soil effect	Source of variations	*df*	F-value	*P*	*df*	F-value	*P*
Yes (*J. vulgaris* *C. vulgare*)	species	1, 79	10.00	*	1, 79	5.25	*
	soil	1, 79	190.26	**	1, 79	191.88	***
	hormone	1, 79	2.87	ns	1, 79	21.04	***
	species × soil	1, 79	11.17	**	1, 79	12.57	**
	species × hormone	1, 79	0.35	ns	1, 79	0.08	ns
	soil × hormone	1, 79	8.20	**	1, 79	8.49	**
	species × soil × hormone	1, 79	0.05	ns	1, 79	0.00	ns
No (*T. repens* *D. carota*)	species	1, 79	7.56	**	1, 79	5.74	*
	soil	1, 79	0.92	ns	1, 79	1.97	ns
	hormone	1, 79	0.35	ns	1, 79	32.94	***
	species × soil	1, 79	0.48	ns	1, 79	0.36	ns
	species × hormone	1, 79	1.10	ns	1, 79	1.86	ns
	soil × hormone	1, 79	1.21	ns	1, 79	1.58	ns
	species × soil × hormone	1, 79	0.08	ns	1, 79	1.44	ns

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns not significant.

The shoot-root ratio of plants differed among species soils and hormone treatments (Table S1). Except for *T. repens*, JA application increased the shoot-root ratio. We found no significant effects of SA application on the shoot-root ratio. The effects of hormone application on the shoot-root ratio varied among species and soils. In all species, the shoot-root ratio was on average higher in live soils (Fig. S1).

Does the negative effect of live soil change in four successive generations of *J. vulgaris* for control plants and plants treated with SA or JA?

The effect of the live soil across generations.

As in generation 1, in all three subsequent generations plants grew less well in the live soil than in the sterilized soil. Although the strength of this effect varied among generations there was no clear trend across subsequent generations (Fig. 3, Table 3).

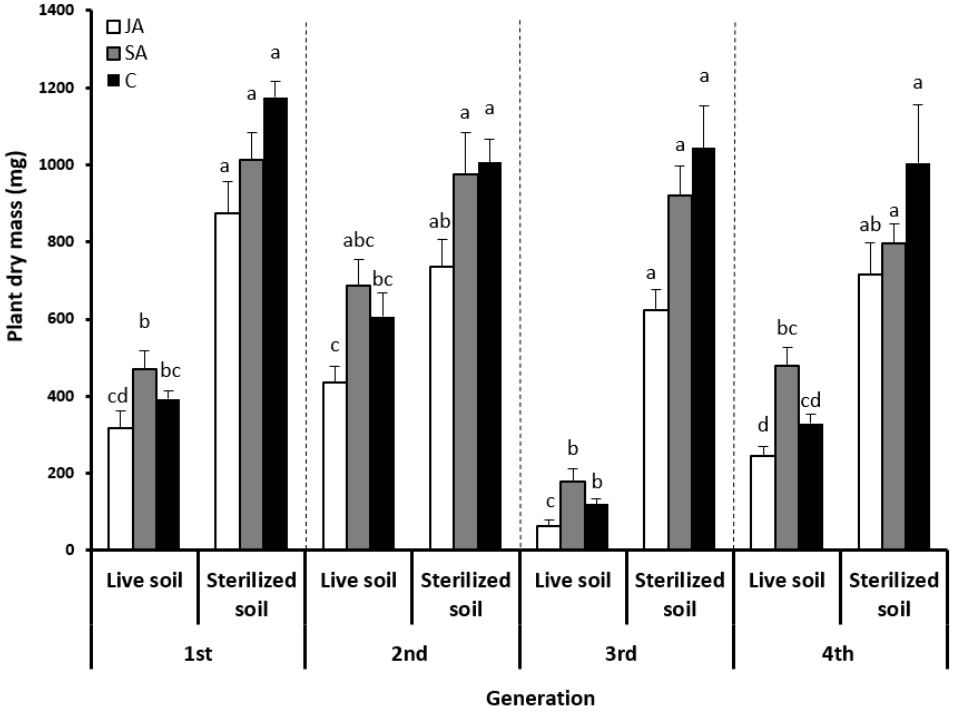

Fig. 3 Mean (+ SE) plant dry mass of *J. vulgaris* during four successive generations treated with JA and SA in sterilized soil and live soil. C represents the control treatment. For each generation soil from the previous generation and originating from the same treatment was used as an inoculum. Within each generation, different letters above bars indicate significant differences between treatment groups based on a Tukey post-hoc test. N=10.

Table 3 Three-way ANOVA of log-transformed plant dry mass of *J. vulgaris* during four generations in live and sterilized soils after JA, SA or control treatment. *df* = degrees of freedom.

Source of variation	*df*	F-value	*P*
soil	1, 250	569.88	***
hormone	2, 250	39.83	***
generation	3, 250	68.36	***
soil × hormone	2, 250	8.17	***
soil × generation	3, 250	57.96	***
hormone × generation	6, 250	1.88	ns
soil × hormone × generation	6, 250	0.68	ns

*** $P < 0.001$, ns not significant

The effect of foliar application of SA across generations

Again, as in generation 1, in all three subsequent generations foliar application of SA reduced plant biomass in sterilized soil and increased plant biomass in live soils (Fig. 3, Table 4). Although within generations and soils these differences were not significant, plants responded clearly different to the SA treatment in the two soils as is reflected by the significant soil x hormone interaction term in the ANOVA (Table 4, left part). The effect of foliar SA application did not differ among generations as was reflected by the non-significant interaction term in the ANOVA (Table 4). To examine if the effect of hormone application in live and sterilized soils showed a trend over generations in more detail, we regressed the ratio between the dry mass of SA-treated and control plants in both sterilized and live soils against generations (Fig. 4). This ratio was higher than 1 for all generations in live soils while it was close to 1 in sterilized soils. This difference between the two soils was significant (ANCOVA $df = (1, 7)$, $F = 20.18$, $P < 0.01$, Fig. 4A). The slopes of the regressions for both sterilized and live soils did not significantly differ from 0 (for sterilized soil the lower and upper 95% CFIs are -0.15 and 0.19; for live soil the lower and upper 95% CFIs are -0.13 and 0.33) The latter results indicate that there is no significant trend in the effect of foliar application of SA over generations.

The effect of foliar application of JA across generations

As in generation 1, in all three subsequent generations foliar application of JA reduced plant biomass in both sterilized and live soil (Fig. 3, Table 4). This reduction was less strong in live soils, as is reflected by the significant soil × hormone interaction term in the ANOVA (Table 4, right part). The effect of foliar JA application did not differ among generations as was reflected by the non-significant interaction term in the ANOVA (Table 4, right part). To examine if the effect of JA application in live and sterilized soils showed a trend over generations in more detail, we regressed the ratio between the dry mass of JA-treated and control plants in both sterilized and live soils against generations (Fig. 4). This ratio was lower than 1 for all generations in both live soils and sterilized soils. The ratios did not differ between the two soils (ANCOVA df = (1, 7), F = 0.01, $P > 0.05$, Fig. 4B). The latter result is somewhat surprising given the significant interaction we found between the effects of JA application and soil type in Table 4. The slopes of the regressions for both sterilized and live soils did not significantly differ from 0 (for sterilized soil the 95% CFI is -0.31 to 0.21; for live soil the 95% CFI is -1.8 to 0.24). The latter results indicate that there is no significant trend in the effect of foliar application of JA over generations.

Table 4 Three-way ANOVAs of plant dry mass of *J. vulgaris* during four generations in live and sterilized soils with soil (live and sterilized soils) generation, hormone (control and SA or JA) as fixed factors. df = degrees of freedom.

	SA treatment			**JA treatment**		
Source of variations	*df*	F-value	*P*	*df*	F-value	*P*
soil	1, 164	241.79	***	1, 170	307.05	***
hormone	1, 164	0.28	ns	1, 170	39.11	***
generation	3, 164	8.98	***	3, 170	11.07	***
soil × hormone	1, 164	8.75	**	1, 170	11.36	**
soil × generation	3, 164	7.98	***	3, 170	7.12	***
hormone × generation	3, 164	0.17	ns	3, 170	0.14	ns
soil × hormone × generation	3, 164	0.50	ns	3, 170	0.70	ns

** $P < 0.01$, *** $P < 0.001$, ns not significant.

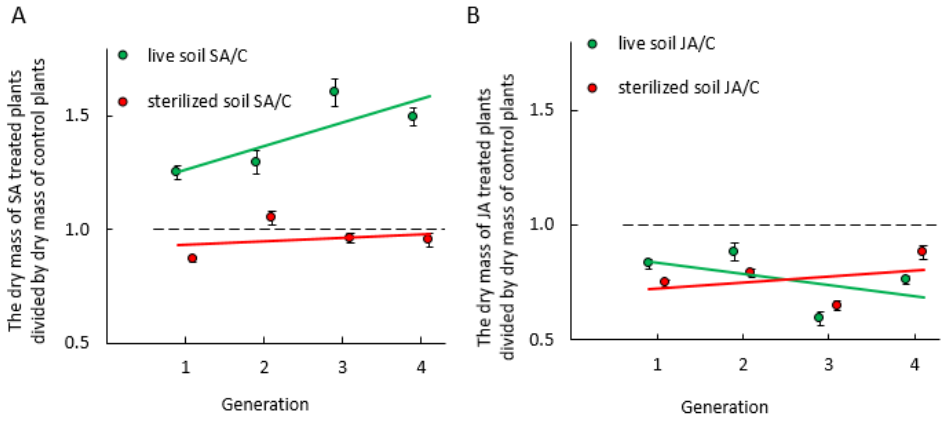

Fig. 4 The ratio of dry mass of hormone treated *J. vulgaris* plants divided by control plants in both sterilized and live soil for four generations. (A) SA treated plants (B) JA treated plants. C represents control treatment. The dashed line indicates y = 1. The data points are the average of 1000 ratios of dry mass of SA or JA and dry mass of control for each generation, the error bars represent the 95% confidence intervals of 1000 ratios for each generation (see material and methods for details).

The shoot-root ratio of *J. vulgaris* differed among generations and was affected by soil and hormone treatments (Table S2). While the effects of hormone application on the shoot-root ratio did not vary among generations, the effects of the hormone treatments differed among soils. In general, in both sterilized and live soils, application of JA increased shoot-root ratios relative to the control and the SA treatments except for the third generation in live soil (Fig. S2). Application of SA did not affect the shoot-root ratio across generations in the sterilized and live soils.

Discussion

In this study, we examined how exogenous application of the plant signaling hormones SA and JA interacts with the effects of inoculation of soil on plant growth of different plant species and how those effects altered plant performance during multiple consecutive generations. In two of the four species, plant biomass was lower in live soil than in sterilized soil. We found that foliar application of SA mitigated the

negative effects of the live soil on plant performance for these species. We then grew *J. vulgaris* for three additional generations and found that SA application mitigated the negative effect of live soil on plant growth in all four generations, and that this overall effect was significant.

Our results show that the effect of the live soil on plant growth strongly varied among plant species although all plants received the same soil inoculum containing a natural soil microbial community and the growth conditions were identical for all four species. Species-specific effects have also been found in other plant-soil feedback experiments that showed that *J. vulgaris* and *C. vulgare* responded negatively to soil conditioning by conspecifics, while this is not the case for *T. repens* and *D. carota* (Klironomos, 2002; Joosten et al., 2009; Harrison and Bardgett, 2010; Wang et al., 2019). Together these results show that the responses of plant species to live soil are highly species-specific. Other studies have suggested that net positive or negative plant-soil feedback effects are related to the capacity of plants to cope with biotic or abiotic stresses, to influence soil nutrients, or to the way they impact soil microbial communities (Bezemer et al., 2006; van der Heijden et al., 2008; Eisenhauer et al., 2011). The soil microbial community present in live soil might have pathogenic effects on plant growth. This is in line with previous studies that indicate that soil sterilization enhanced plant growth by killing soil-borne pathogens in crops (Li et al., 2019).

The effect of SA application also varied among plant species. Interestingly, a positive effect of SA on plant growth in live soils occurred in *J. vulgaris* and *C. vulgare*, the two species that responded negatively to exposure to the live soil, and not in the other two species, *T. repens* and *D. carota* that were unresponsive to the soil with a live soil inoculum. These results strongly suggest that the negative impact of the live soil on plant growth is driven by pathogens. The difference in response between the four species can have different non-exclusive causes. The pathogenic effect of the live soils itself may differ among plant species due to specificity of the soil microbial species in the live soil inoculum, or due to inherent plant characteristics. We started with our hormone application when seedlings were 14 days old. In retrospect, we should have started earlier. The negative effects of the live soil on plant growth are most apparent during the first few weeks of plant growth (Jing et al., Chapter 5; Bezemer et al., 2018). If we would have applied the exogenous SA earlier, effects may therefore have been

stronger because plants in our study may have outgrown the negative effects of the live soil e.g. by upregulating their defense system (Vernooij et al., 1994; Métrauxs, 2001). In addition, in this paper, we used only one concentration of the phytohormones. Plant species may have a different sensitivity to the foliar application of these hormones, and the species that did not show a response may have responded to higher concentrations. It is important to note that, in this paper, we performed experiments with two phytohormones. Other plant hormones like auxins and cytokinins have been reported to play a role in fighting off the potentially pathogenic bacteria in the live soil via changing physiological and morphological features of plants (Hamill, 1993; Clarke et al., 2000). They may interact with JA or SA signaling pathways; however, this is still not fully understood. Applying combinations of different phytohormones would present a next logical step. To find a clear effect of SA and JA on plant growth against the pathogenic effect caused by live soils is the base for carrying out more extensive experiments. For example, in further tests, different plant hormones and their crosstalk effects could be tested.

Importantly, application of SA mitigated the negative effects of the live soil on the growth of *J. vulgaris* in all four generations. Sterilization of the soil resulted in higher plant growth, indicating an overall pathogenic effect due to soil-borne pathogens, and SA-induced resistance may help to mitigate this pathogenic effect caused by soil pathogens. Activation of SA-dependent signaling pathway leads to the expression of pathogenesis-related proteins (PRP) contributing to resistance, by limiting pathogen growth, the access of pathogens to water and nutrients in the plant, or by changing the composition of the cell wall of the plant (O'Donnell, et al., 2001; Heil, 2002; Glazebrook, 2005; Spoel et al., 2007). All this can result in higher plant mass in SA-treated plants than in control plants in live soil. In addition, activation of SA pathway regulates a myriad of compounds and enzymes, for example, peroxidase (POD), polyphenol oxidase (PPO), superoxide dismutase (SOD) etc., and those compounds play an important role in plant SA-induced defense against biotic stresses caused by pathogens (Achuo et al., 2004; War et al., 2011; Bakker et al., 2018). The effects of SA in the first generation were similar to those observed in the second or later generations indicating that SA application did not result in selection for more beneficial soil microbial communities over time. In part, this may be an artefact of the

experimental set-up. For each generation, we used an inoculum, which means that we placed a subset of the microbial community in a sterile background. This may have led to selection for microbes with similar particular characteristics in each of the four generations. However, we urge not to overemphasize the conclusion that application of SA results in a change in the effects of live soils on plant growth over generations (see chapters 3 and 4). Future studies should also include a comparison between the growth of SA-treated plants and control plants grown in soils that are conditioned by either SA-treated plants and control plants in a full factorial design.

JA-induced defenses are activated in response to herbivore attack, the infection of necrotrophic organisms or nematodes (Pieterse et al., 2009; Nahar, et al., 2011; van Dam and Oomen, 2014; Carvalhais et al., 2017; Palmer et al., 2017). In our study, the foliar JA application did not clearly mitigate the negative effects of the live soil on plant performance. Instead, it led to a significant negative effect on plant growth. This exemplifies that hormonal signaling is costly for plants (Baldwin, 1998; Vos et al., 2013).

In conclusion, our study suggests that negative effects in live soil on plant growth can be mitigated with foliar applications of SA. Sterilization benefited plant growth for two of the four species we investigated, suggesting the microbial community in live soils contains pathogens. For *J. vulgaris*, the plant species that responded most strongly to SA application, we did not observe an increasingly stronger effect on plant growth over further plant generations, but instead, the effect was stable over time. To better understand what caused the positive effect of SA application on plant growth in live soil, we examined changes in the diversity and functional role of the soil microbial community in live soil in Chapters 3 and 4.

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Supplementary data

Table S1 Three-way ANOVA of shoot to root ratio of *J. vulgaris*, *C. vulgare*, *T. repens* and *D. carota* plants in live and sterilized soils after JA, SA application and control plants. Degrees of freedom, F- and P values are shown. *df* = degrees of freedom.

Source of variations	*df*	F-value	*P*
species	3, 239	35.82	***
soil	1, 239	5.16	*
hormone	2, 239	20.02	***
species × soil	3, 239	1.88	ns
species × hormone	6, 239	9.28	***
soil × hormone	2, 239	4.28	*
species × soil × hormone	6, 239	2.67	*

* $P < 0.05$, *** $P < 0.001$, ns not significant.

Table S2 Three-way ANOVA of shoot-root ratios of *J. vulgaris* grown in live and sterilized soil after JA, SA application and control plants over four generations. Degrees of freedom, F- and P values are shown. *df* = degrees of freedom.

Source of variations	*df*	F-value	*P*
soil	1, 250	18.88	***
hormone	2, 250	20.95	***
generation	3, 250	37.10	***
soil × hormone	2, 250	1.41	*
soil × generation	3, 250	5.34	ns
hormone × generation	6, 250	1.27	ns
soil × hormone × generation	6, 250	1.70	ns

* $P < 0.05$, *** $P < 0.001$, ns not significant.

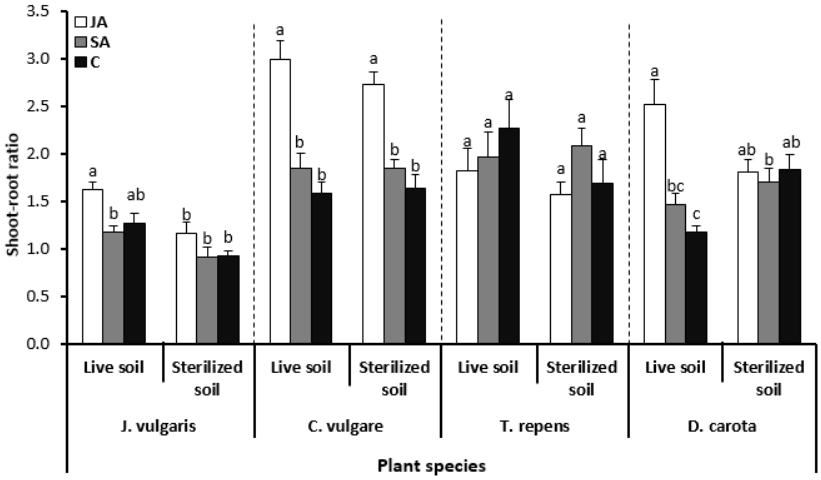

Fig. S1 Shoot-root ratio (+ SE) of *J. vulgaris, C. vulgare, T. repens* and *D. carota* plants treated with JA and SA in sterilized soil and live soil. C represents control treatment. Within species different letters indicate significant differences between treatments based on a Tukey post-hoc test for each species separately.

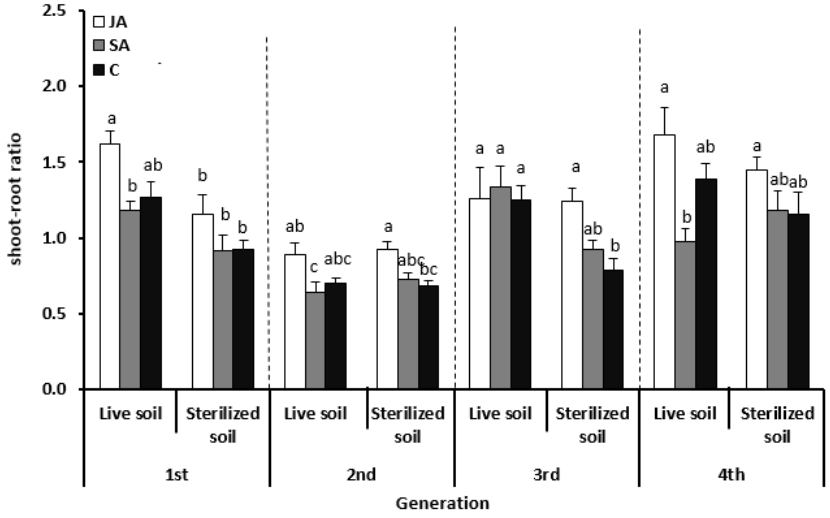

Fig. S2. Shoot-root ratio (+ SE) over four generations of *J. vulgaris* plants foliar treated with SA or JA and control plants grown in sterilized soil and live soil. C represents the control treatment. Within generations different letters indicate significant differences between treatment based on a Tukey post-hoc test for each generation separately.

Chapter 3

Activation of the SA-associated plant defense pathway alters the composition of soil bacterial communities

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Abstract

Many plant species grow better in sterilized than in live soil. Foliar application of SA mitigated this negative effect of live soil on the growth of the plant *Jacobaea vulgaris*, as described in Chapter 2. This "SA-effect" on plant growth in live soils did not change over further cycles (generations), neither did the negative effect of live soils. To examine what causes the positive effect of SA application on plant growth in live soils, in this chapter we analyzed the effects of SA application on the composition of active rhizosphere bacteria in the live soil and how this change over time using RNA sequencing of the microbial communities in the rhizosphere of *Jacobaea vulgaris*. Our study shows that the composition of the rhizosphere bacterial communities of *J. vulgaris* greatly differed among generations. Application of SA resulted in both increases and decreases in a number of bacterial genera in the rhizosphere soil, but the genera that were affected by the treatment differed among generations. In the first generation, there were no genera that were significantly affected by the SA treatment, indicating that induction of the SA defense pathway in plants does not lead to immediate changes in the soil microbial community. 89 species out of the total 270 (32.4%) were present in all generations in all soils of SA-treated and control plants suggesting that these make up the "core" microbiome. On average in each generation, 72.9% of all genera were present in both soils. Application of SA to plants significantly up-regulated genera of *Caballeronia*, unclassified *Cytophagaceae, Crinalium* and *Candidatus Thermofonsia Clade* 2, and down-regulated genera of *Thermomicrobiales*, unclassified *Rhodobacterales*, *Paracoccus* and *Flavihumibacter*. While the functions of many of these bacteria are poorly understood, bacteria of the genus *Caballeronia* play an important role in fixing nitrogen and promoting plant growth, and hence this suggests that activation of the SA signaling pathway in *J. vulgaris* plants may select for bacterial genera that are beneficial to the plant. Further studies should examine how activation of the SA signaling pathway in the plant changes the functional genes of the rhizosphere soil bacterial community. Overall, our study shows that aboveground activation of defenses in the plant affects soil

microbial communities and as soil microbes can greatly influence plant performance, this implies that induction of plant defenses, can lead to complex above-belowground feedbacks.

Keywords

Metatranscriptomics, Soil microbial community, Taxonomy, Plant-soil interactions, Microbial diversity, Rhizosphere soil, Salicylic acid

Introduction

Plants encounter a myriad of threats from the surrounding environment, including both abiotic and biotic stresses (Suzuki et al., 2014). Biotic stresses are mostly due to herbivory and pathogen infestation both below- and above-ground (Pieterse and Dicke, 2007; Adair and Douglas, 2017). Microbes in the soil can have a beneficial, pathogenic or neutral effect on the host plant. For example, soil bacteria such as *Rhizoctonia species*, often strongly negative affect plant growth and survival (Issac et al., 1971). On the other hand, plant growth-promoting rhizobacteria (PGPR), such as *Pseudomonas* and *Burkholderia* species are beneficial for the plant, e.g. via suppressing the growth of soil-borne pathogens or increasing nutrient availability (Bhattacharyya and Jha, 2012). However, the overall net effect of soil microbial communities on plant growth is often negative (Nijjer et al., 2007). Most plant species grow less well in soils that contain a natural microbial community than in sterilized soils. This might be due e.g. competition between plants and microbes for available nutrients or due to soil-borne plant pathogens (Callaway et al., 2004; Berendsen et al., 2012; Mazzoleni et al., 2015; Cesarano et al., 2017).

Systemic acquired resistance (SAR) is one of the most common defensive strategies of plants against biotrophic pathogenic microbes. Foliar application of salicylic acid to plant tissues can activate SAR and boost the innate immune system of a plant (Reymond and Farmer, 1998). Cultivars with a higher sensitivity to SA are often better defended against the pathogens. For example, in tomato, exogenous application of SA can be effective against the pathogens *Oidium neolycopersici* and *Botrytis cinerea*, which cause powdery mildew and grey mould diseases (Seskar et al., 1998; Achuo et al., 2004). In agriculture, application of SA is now used to suppress pathogenic microbial effects in e.g. tomato, pepper and pea crops (Esmailzadeh et al., 2008; Barilli et al., 2010; Choi and Hwang, 2011). How SA application to the plant affects the microbial community in the soil is not fully uncovered.

Because plants alter the composition of the microbial community in the soil in which they grow, and SAR protects plants against pathogens, an important question is how activation of SAR alters the plant's effect on the soil microbial community. Several studies have demonstrated that the activation of SAR indeed altered the composition

of soil microbial community and that SA can play a key role as a regulator in shaping root bacterial communities (Kniskern et al., 2007; Hein et al., 2008; Lebeis et al., 2015). However, several other studies reported that foliar application of SA did not affect the bacterial composition in the soil (Doornbos et al., 2011; Wang et al., 2015). These studies on the effects of SAR on soil bacterial composition were mostly limited to the model plant species *Arabidopsis.* As plant species differ greatly in the way and magnitude in which they influence the soil bacterial community (Wubs and Bezemer, 2018; Hannula et al., 2019; Pineda et al., 2020), we may expect that the effects of SA application on the soil microbial community also differ among plant species.

Several studies have shown that the composition of the soil bacterial microbial community varies greatly over time (e.g. Hannula et al., 2019). In a study on temporal variation in three land-use types, the number of taxa present in the soil showed strong temporal variability, and these changes over time were considerably larger than the variation associated with land-use types (Lauder et al., 2013). In contrast, Shade et al. (2012) demonstrated that soil microbial communities have clear successional trajectories. If generally true this would imply that application of SA to plants could also cause directed changes in the soil microbial community over time. An important question is therefore how activation of SAR will alter the soil microbial community over time.

In Chapter 2, we showed that inoculation of a sterilized soil with natural, live soil, reduced plant growth in comparison with that in sterilized soil for the plant species *Jacobaea vulgaris*. Interestingly, applying SA to the leaves mitigated these negative effects. This implies that activation of SA-induced resistance may potentially suppress microbial pathogens present in live soil. If this is the case, an important question is whether the repeated foliar application of SA during consecutive generations of plant growth will increase this effect and hence, whether there is a selection for a more beneficial bacterial community. Conceptually, the temporal dynamics of foliar application of SA can follow different trajectories (Fig. 1). First, it is possible that both foliar application of SA and the effect of different generations do not alter the soil bacterial composition (Fig. 1-i). Second, foliar application of SA may lead to different bacterial communities independent of time (Fig. 1-ii). Third, bacterial communities may differ among generations but are not influenced by the SA

application (Fig. 1-iii). Fourth, foliar application of SA may influence bacterial communities but these effects may differ among generations (Fig. 1-iv).

In this study, we sequenced the mRNA from rhizosphere soil samples of both SA-treated and control plants during four consecutive generations of growth of *J. vulgaris*. In each consecutive generation soil from the previous plant growth period was used. Using mRNA instead of DNA or rRNA enabled us to focus on the active soil microbial community (Gilbert et al., 2008). In this study, we focus on the bacterial community. Twenty-four rhizosphere soils were sequenced with an Illumina sequencing platform. The goal of this study is to answer the following questions: (1) How does the foliar application of SA in *J. vulgaris* affect the bacterial composition in the rhizosphere and is there a time effect or an interactive time x SA effect on the bacterial community? (2) What is the "core" bacterial community in the soils of plants exposed to the SA treatment and of control plants? (3) How does the application of SA influence the bacterial community in each generation? Are the SA effects consistent over time?

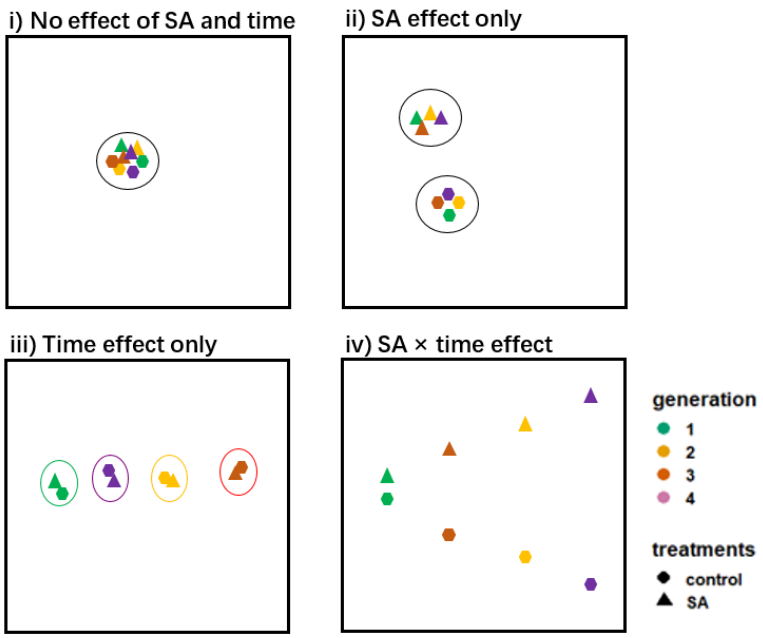

Fig. 1 Conceptual figure showing the potential effects of SA application on *J. vulgaris* over four generations.
i) No effect of SA and time. The bacterial community does not differ between SA-treated plants and control, and does not differ over time. ii) SA effect only. The bacterial community is affected by SA application but the effect does not differ over time. iii) Time effect only. The bacterial community changes over time, but is not affected by the SA treatment. iv) SA × time effect. The bacterial community is affected by SA-application but these effects differ among generations.

Materials and methods

Multi-generational plant growth experiment

In Chapter 2, we report the effects of foliar application of SA on plant growth in inoculated and sterilized soils. The current chapter focuses on the effect of foliar SA application on the composition of the bacterial community in the rhizosphere in the inoculated soil. Details of the experiment are described below.

J. vulgaris (common ragwort) seeds were collected at the dunes of Meijendel (a calcareous sandy area from a coastal dune area north of The Hague, The Netherlands, 52°11´N, 4°31´E). Prior to germination, all seeds were surface sterilized (shaken for 2 min in 70% ethanol, then rinsed with sterilized water, put for 12 min in 2% bleach, and then rinsed again four times with sterilized water to minimize influences of seed-borne microbes (Bakker et al., 2015). The soil was also collected at Meijendel. The topsoil was collected to a depth of 15 cm after removing the grassland vegetation and the organic layer of the surface. The soil was sieved using a 5 mm sized mesh, homogenized with a concrete mixer, and then stored into 20-liter plastic bags (Nasco Whirl-Pak Sample Bag). Bags were either sterilized by 35-K Gray gamma-irradiation (Synergy Health Company, Ede, The Netherlands) or kept at 4°C for inoculation.

Surface sterilized seeds were germinated in sterile Petri dishes on filter paper. After one week, seedlings were randomly transferred individually to 500 ml pots consisting of a mixture of 90% sterilized soil and 10% live soil. Prior to potting but after mixing, the soil was kept in bags and left in the climate room for 14 days so that the mixed

soil could settle and microbial communities could colonize the sterilized soil. After potting the seedlings, pots were randomly distributed over a climate room (relative humidity 70%, light 16h at 20°C, dark 8h at 20°C). Plants were watered regularly with Milli-Q and 5 ml Steiner nutrient solution was added per plant on day 7 after planting, 10 ml Steiner nutrient solution (Steiner, 1979) was added on day 13, and 20 ml Steiner nutrient solution was added on days 19, 28, 37, 42. The Steiner nutrient solution was prepared from 7 different stock solutions (106.2 g $Ca(NO_3)_2 \cdot 4H_2O$, 29.3 g KNO_3, 13.6 g KH_2PO_4, 49.2 g $MgSO_4 \cdot 7H_2O$, 25.2 g K_2SO_4 and 2.24 g KOH, 3.29 g Fe-EDTA added to 1 liter demineralized water, and a stock solution with micro elements (a mixed solution of 0.181 g $MnCl_2 \cdot 4H_2O$, 0.286 g H_3BO_3, 0.022 g $ZnSO_4 \cdot 7H_2O$, 0.0078 g $CuSO_4 \cdot 5H_2O$ and 0.0126 g $NaMoO_4 \cdot 2H_2O$ added to 1 liter demineralized water). Ten ml of each stock solution was diluted in 1 liter of demineralized water before use.

Plants were allocated to either a hormonal treatment (SA) or served as control (only solvent). Both treatments were replicated 10 times. During plant growth, the phytohormone SA was applied through foliar application three times a week for four consecutive weeks. The first application was given when plants were 14 days old. About 0.75 ml of 100 µM SA was sprayed on the leaves while carefully avoiding spillover to the soil. One week later the treatment was repeated with 1.50 ml of SA. In the next week, the treatment was repeated with 2.25 ml of SA. SA solvent (purchased from Sigma-Aldrich, ≥99.0%) was made by dissolving 6.91 mg in 69.10 µl of ethanol. Milli-Q water was then added until a final volume of 500 ml. Control plants were sprayed with sterile water with the same solvent (ethanol in Milli-Q water).

After six weeks, plants were gently removed from the pots. The rhizosphere soil for each treatment was harvested for each pot individually by gently shaking three times to remove the loosely adhering soil, after which rhizosphere soil samples were collected onto a sterile filter paper by removing the remnant soil with a fine sterilized brush. Rhizosphere soil samples were put in a 2 ml Eppendorf tube and stored at -80°C for further RNA extraction. The remaining rhizosphere soil and adhering soil of the ten pots were mixed and used as inoculum (live soil) for the next generational of plant growth. The inoculum soil (10%) was mixed with 90% sterilized soil.

The set-up was repeated for another three generations under the same conditions as described above so that there were four generations of plant growth. For the second, third, and fourth generation, the soil inoculum was derived from the previous generation from the same treatment and was a mixture of rhizosphere soil and the loosen adhering soil surrounding the roots. Again, after mixing, the soil was kept in bags and left in the climate room for 14 days. Hereafter, pots were filled with soil and a *J. vulgaris* seedling was planted into each pot. All replicate soils from the SA or control treatment were mixed before the inoculation. The SA treatment was carried out as described above in each generation. Fifty-four days after planting, all plants were harvested each time.

RNA extraction and metatranscriptomic sequencing

For each treatment, the three successively labeled samples (No. 1, 2, 3, No. 4, 5, 6 and No. 7, 8, 9) were mixed and used as one composed replicate, Hence, three replicates were generated and used for RNA extraction for each treatment in each generation and a total of 24 soil samples were used for RNA extraction (3 replicates x 2 treatments x 4 generations). Total RNA was extracted with the RNeasy PowerSoil Total RNA kit (Qiagen). RNA concentration and quality were assessed by running 1µl of the extracted raw RNA on the 4200 TapeStation (Agilent). Subsequently, unwanted DNA, salts and buffers were removed with the RNeasy minElute Cleanup Kit (Qiagen). Later, the Ribo-Zero Magnetic kit for bacteria (Illumina) was used for mRNA enrichment. In the end, a RNA Clean & Concentrator kit (Zymoresearch) was used to clean additional buffers and proteins of the rRNA-depleted RNA. All the steps in extracting and cleaning RNA were according to the supplier's instructions. Double-stranded cDNA was generated from the cleaned RNA obtained in the final step. Library preparation (Illumina Nextera XT DNA library), processing and sequencing were performed by FG Technologies (Leiden, The Netherlands) with paired-end (PE) 150 bp templates. Twenty-four metatranscriptomic libraries were generated, the size of each library was indicated in Table S1 and Fig. S1.

Bioinformatics processing

Trimmomatic 0.39 was used for the removal of adapters of paired-end raw reads (Bolger et al., 2014). FastQC was applied to check the qualities, the bases with a threshold lower than 30 were cut off with Trimmomatic (Andrews, 2010). Ribosomal RNAs of all 24 metatranscriptomic libraries were filtered with SortMeRNA (Sorting ribosomal RNA) (Kopylova et al., 2012). Eight rRNA representative databases (silva-bac-16s-id90, silva-arc-16s-id95, silva-euk-18s-id95, silva-bac-23s-id98, silva-arc-23s-id98, silva-euk-28s-id98 rfam-5s-id98, rfam-5.8s-id98) were derived from the SILVA SSU and LSU databases (release 119) and the RFAM databases with HMMER 3.1b1 and SumaClust v1.0.00 were used for fast filtering of rRNA from eukaryote, prokaryote and archaea. Then, all reads of the 24 metatranscriptomic libraries were combined into one set, which was the input of a de novo assembly. Trinity with default parameters was used for the metatranscriptomic assembly (Haas et al., 2013). Later, the quality of assembled contigs was assessed with Trinity scripts. The CD-HIT-EST algorithm was used to remove the duplicates of each transcript and reads with shorter than 300 bps were removed with a homemade script (Li and Godzik, 2006), after which reads of each library were mapped back to transcriptome with Bowtie2 (Langmead and Salzberg, 2012). The isoform IDs per sample were extracted with Seqkit (Shen et al., 2016). Contigs of each sample were generated and then aligned against the NCBI NR (non-redundant) database by DIAMOND with a cut off e-value at 1e-5 (Buchfink et al., 2015). The closest match with an identity higher than 80% was kept for mapping. The output file of Blastx was further analyzed with the lowest common ancestor (LCA) algorithm in MEGAN (version 6.0) with all default parameters (Camon et al., 2005; Huson et al., 2016). MEGAN helped to compute and explore our data at different taxonomic levels and in this process NCBI taxonomy was employed for summarizing and outputting results, the detailed workflow is referred to Huson et al. (2007). A count table of microbial species was obtained with read counts assigned directly to taxon for the 24 samples. The number of assigned reads per taxa was extracted at species, genus, family and phylum levels respectively. The number of identified phyla, families, genera and species were counted, and the composition and the percentage of reads used for each classification level were calculated.

Statistical analyses

Differences in the numbers of the total reads and the numbers of the non-rRNA reads over four generations were presented as mean ± SD. A Heinrich's triangle figure was generated to visualize microbial composition at different phylogenetic levels of all the identified microbes from the 24 rhizosphere soil samples. Log10 transformed hit numbers of each genus were plotted as a function of ranked genus abundance numbers including all species and a cut-off was performed with an abundance larger than 0.01% of the total reads. A Shapiro-Wilk test was used to test for differences between the distribution of abundance between the SA and control treatment.

The Shannon-diversity index was calculated for the 24 samples and differences between the Shannon-diversity of soils of SA treated plants and soils of control plants were tested with a student t-test. Subsequently, abundance at genus level was used for to construct NMDS (nonmetric multidimensional scaling), PCA (Principal component analysis), OPLS-DA (orthogonal projection to latent structures discriminant analysis), and Venn diagrams, and Pearson distance and the Ward clustering algorithm statistical analysis was calculated since most of the reads were identified at the genus level.

Two-factor Venn diagrams were constructed to illustrate the numbers of unique and common genera in soil samples within each generation for the SA and control treatments, and a four-factor Venn diagram including all generations was performed for the SA and the control treatment separately (Heberle et al., 2015).

PCA and OPLS-DA were performed with SIMACA 15.0 using relative abundance at genus level. The relative abundance was calculated using the absolute abundance number of one genus divided by the total abundance of all bacterial genera in the sample. Before performing OPLS-DA analysis, we checked that our data fitted the model with a cross-validated residual (CV)-ANOVA significance testing ($n = 270$, $P < 0.02$).

To visualize the compositional changes among different treatment and time categories, a NMDS using the Bray-Curtis index as a measure of dissimilarity was generated

using relative abundances. To verify changes in composition due to the SA treatment and time effect, a PERMANOVA test was performed using the *Adonis* function (number of permutations = 999) in R within the "vegan" package.

Local "immigration" and "extinction" in the rhizosphere soil of SA-treated or control plants over generations at genus level was calculated and the numbers were presented in Venn diagram. A Student's t-test was used to identify bacterial genera that were significantly enriched in soil samples of SA-treated or control plants. *P* values were adjusted for false discovery rates (FDR).

Spearman's rank correlation without multiple comparison tests were performed to identify the genera that were significantly positively or negatively correlated with generation within the SA or control treatment. Genera with *P* values smaller than 0.05 were selected to create a heatmap for all the 24 samples. Hierarchical clustering analysis was done for the 24 samples together, based on the relative abundance to show the similarity. The row-centered relative abundance of each genus was used to construct the color key (Chong et al., 2018). Heatmaps for only SA and only control treatments were generated in addition.

Results

Metatranscriptomic sequence data

A total of 898.4 million raw sequence reads were obtained from the 24 metatranscriptomic libraries, the smallest and largest library contained 25.0 and 52.0 million raw sequence reads, respectively (supplementary data Table S1). 846.9 million reads were kept after removing adapters and quality filtering control with FastQC. In total, 775.3 million reads were removed with the SortMeRNA program as ribosomal RNA (rRNA) reads when aligning them against eight rRNA representative databases (silva-bac-16s-id90, silva-arc-16s-id95, silva-euk-18s-id95, silva-bac-23s-id98, silva-arc-23s-id98, silva-euk-28s-id98 rfam-5s-id98, rfam-5.8s-id98), and 71.6 million reads were used as non-rRNA reads for further de novo assembly with Trinity (Fig. S1), of which the smallest library contained 1.5 million reads and the largest library 5.9 million reads. Reads for de novo assembly were normalized with Trinity

in silico normalization algorithm. The average guanine-cytosine (GC) content for the 24 libraries was 60.10%. After assembly, 0.99 million contigs were removed because their length was shorter than 300 bps. A total of 1.3 million unique contigs were identified after removing duplicates with CD-HIT-EST. In total, 392.4 million bases were assembled. After we checked the quality of the contigs in all samples by realigning all contigs back to the assemblies using Bowtie2, the average mapping rate for proper pairs was 45.41%.

Overview of the assigned reads at differential microbial classification levels

When we aligned the 1.3 million unique contigs against the NR (non-redundant) database with DIAMOND and MEGAN 6.0, 0.39 million contigs were taxonomically classified, while the others did provide a match with the available taxonomic information. Based on the analysis in MEGAN, the identified contigs were assigned at different classification levels. 22 different bacterial phyla were identified, 283 families and 382 bacterial genera and 1081 bacterial species (Fig. S2). At the phylum, family, genus and species level 23.4%, 23.4%, 20.4% and 14.9% of the total number of contigs were assigned, respectively. Bacteria were the most prevalent in the microbial community taking up 98.3 % of the total number of reads (Fig. S3a). Eukaryotes, with algae taking the largest proportion, were the second dominant, but Eukaryotes only covered 1.5% of the total number of reads (Fig. S3b).

SA application and time effects on bacterial community diversity and composition

From the total of 408 bacterial genera, 270 genera were included in the analysis (contigs with more than 0.01% of the total number of reads Fig. S5). The genera in both soils showed significantly different abundance curves (Shapiro-Wilk test, df = 407, $P < 0.0001$; Fig. S5), the abundance curve in the SA soil is lower than that in the control soil. Application of SA did not significantly increase or decrease the Shannon diversity at genus level within each generation (t-test for the 1st generation: t = -0.63, df = 5, P = 0.27; 2nd generation: t = 0.07, df = 5, P = 0.47; 3rd generation: t = 0.67, df = 5, P = 0.26; 4th generation: t = 0.50, df = 5, P = 0.31).

The NMDS plot showed that the bacterial communities of the same generation clustered together (Fig. 2a), PERMANOVA R^2 = 0.30, P = 0.001). The SA and control separated in the NMDS plot (Fig. S6) but this was not significant (PERMANOVA R^2 = 0.05, P = 0.18). Similar patterns were observed in a principal component analysis (PCA; Fig. S7). The OPLS-DA analysis showed clusters for replicates within each generation, and clear separation for the SA effect but only in the 2nd, 3rd and 4th generation (Fig. 2b). However, the generation effect was more evident than the SA effect.

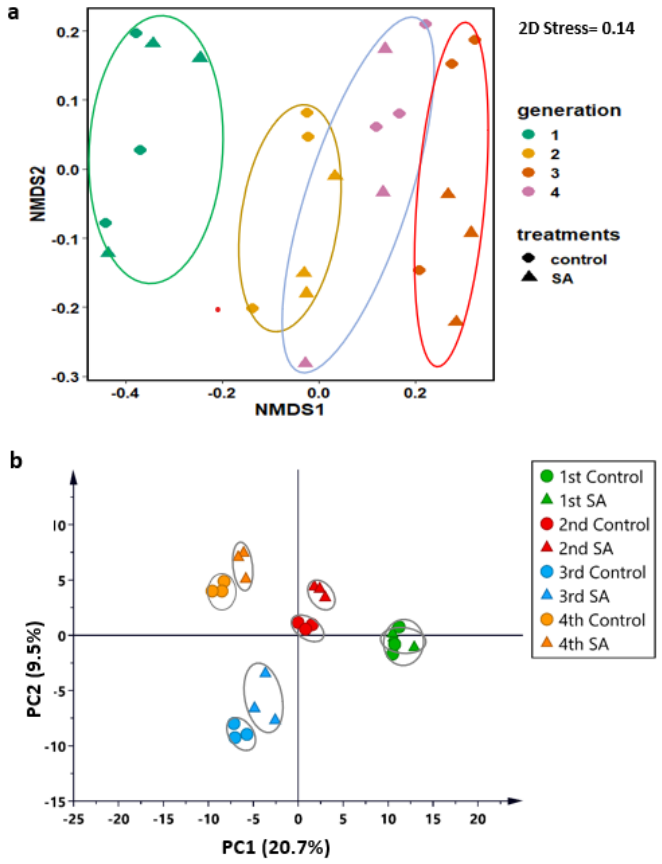

Fig. 2 Multivariate analysis of the bacterial community in soil samples from SA-treated and control plants grown in four generations. Shown are sample scores from a Nonmetric Multi-Dimensional Scaling (NMDS) plot (a) and an Orthogonal

Projections to Latent Structures Discriminant analysis (OPLS-DA) plot (b) from the 24 rhizosphere soil samples.

Core bacterial community

89 species out of the total of 270 (32.4%) were present in all generations in at least two out of the three replicates of the soils of SA-treated and control plants suggesting that these make up the "core" microbiome (Fig. 3a). On average in each generation, 72.9% of all the genera were present in both soils (Fig. 3b). In the first generation, both soils shared about 74.2% of the genera while 7.7% only occurred in the SA-treatment and 18.0% only in the control (Fig. 3b-1). The percentage of shared genera by the two soils in the 2nd, 3rd and 4th generation was 67.6%, 72.9% and 76.8% (Fig. 3b-2, 3, 4). For soils of the control treatment, 49.5% of the genera were shared over all four generations; while 45.1% of genera were shared in soils of the SA treated plants over four generations (Fig. S2c; Table S2). Immigration was somewhat higher in the SA treatment (on average 42 new genera) than in the control (on average 34 new genera) while the opposite was true for extinction rates (on average 31 genera in the SA treatment and 33 in the control treatment; Fig. 4). The information of Archaea, virus and eukaryote is listed in supplementary Fig. S4.

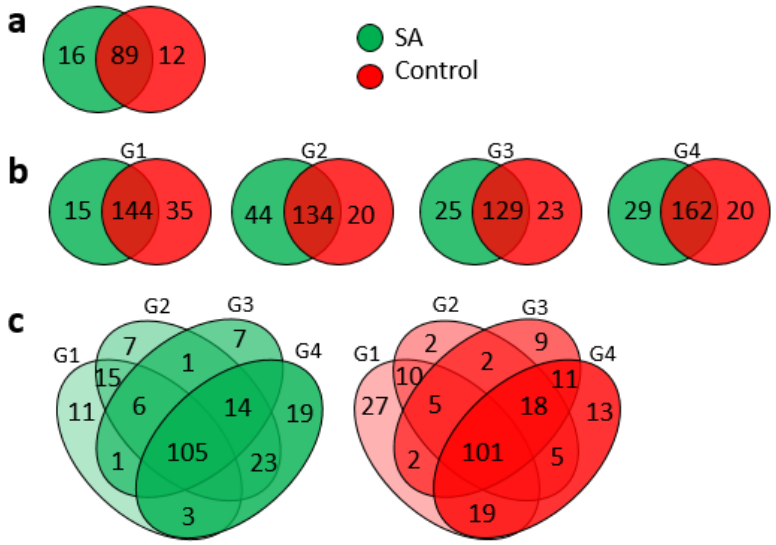

Fig. 3 Venn diagrams showing the unique and shared genera of bacteria in the rhizosphere soil samples of SA-treated and control *J. vulgaris* plants. The diagram in (a) is based on an analysis of genera that occur in all growth generations of the SA treatment, in (b) each generation is analyzed separately, (c) shows the diagram for all generations combined for the SA and control treatment.

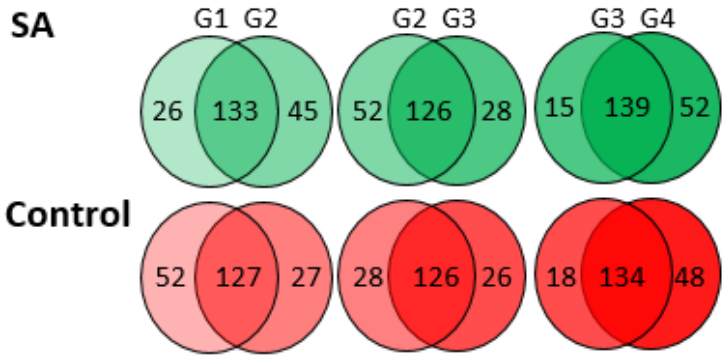

Fig. 4 Local "immigration" and "extinction" of bacterial genera in the rhizosphere soil of SA-treated and control plants over time. For each two consecutive generations, shown are the number of genera present only in the first of those generations (i.e., representing genera that go extinct), present in both generations, and present only in the second of those generations (i.e., representing generate that immigrate). Genera were considered present in a treatment when present in at least two of the tree replicates. 1, 2, 3 and 4 represent the 1st, 2nd, 3rd and 4th generation.

SA selection of soil bacteria

When analyzed per generation, in total eight genera differed among the SA treatment and control (Fig. 5). No genus was significantly affected in more than one generation and no genera were significantly affected in the first generation. Most of the significant genera were only present in either the control or SA treatment. A Spearman's rank correlation showed that 41 (out of 240) genera in the rhizosphere soil of SA-treated plants were significantly increasing or 31 genera were decreasing over generations. For the control soils these numbers were 47 and 27, respectively out of a total of 239 genera (Table S3). The heatmap including all 24 samples showed a clear generation effect, but no clear SA effect (Fig. 6). A heatmap representing the patterns of all identified genera in the 12 rhizosphere soils of SA-treated plants showed that replicates within a generation clustered and that the 2nd, 3rd and 4th generation showed a higher similarity than the 1st generation (Fig S8a). For the control plants, the samples from the 1st generation differed from the three other generations (Fig S8b).

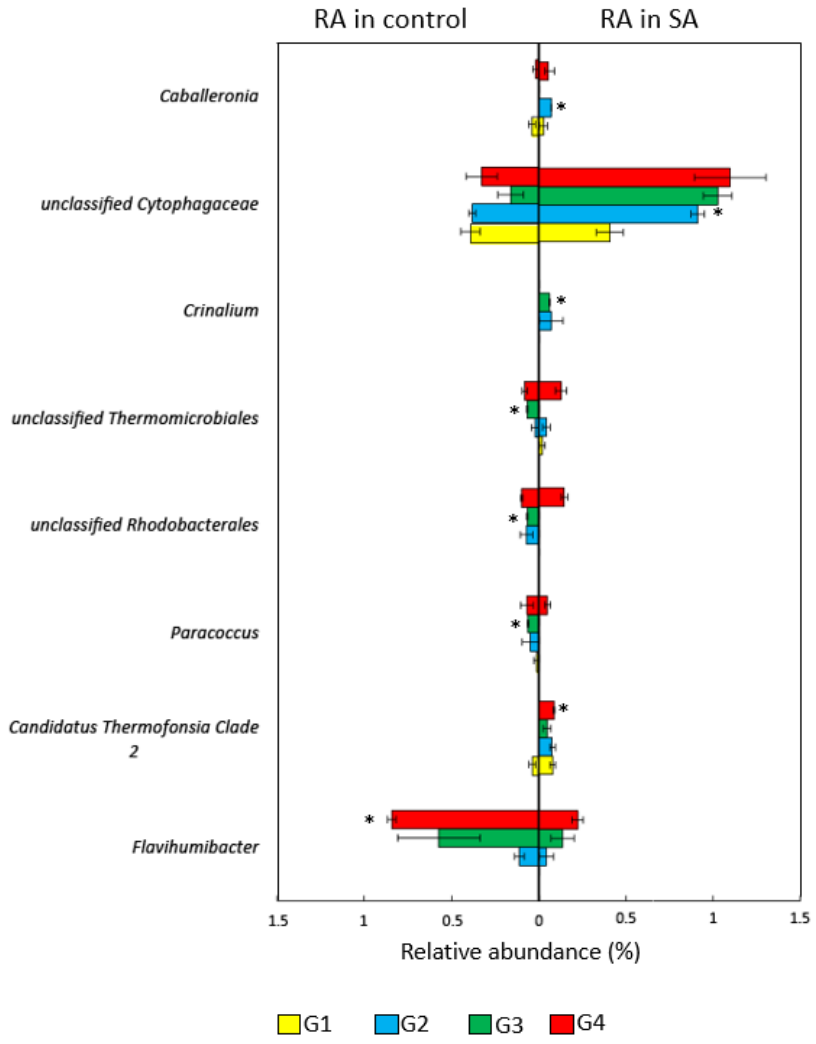

Fig. 5 Bar chart showing relative abundance (%) (mean ± SE) of the significant up or down regulated genera in the rhizosphere soils by SA-treated *J. vulgaris* plants. The significance is based on a student t-test with a false discovery rate (FDR) adjusted *P* values (< 0.05).

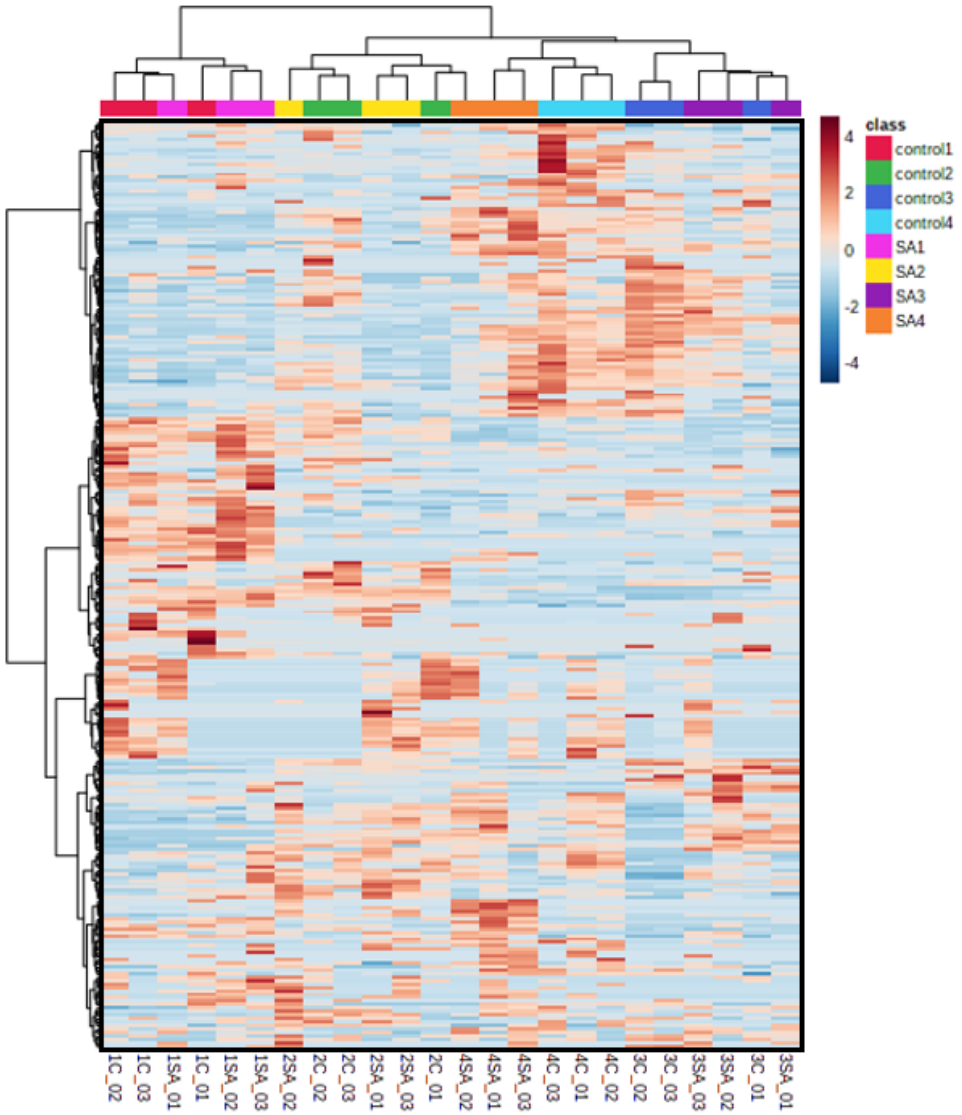

Fig. 6 Heatmap with a hierarchical clustering analysis of all the bacterial genera of rhizosphere soil of SA-treated *J. vulgaris* plants and control plants in the 24 samples. The hierarchical clustering was calculated with Pearson distance and the Ward clustering algorithm based on the relative abundance of the reads of each genus. The color code represents the row-centered relative abundance. SA1, SA2, SA3 and SA4 represent SA treatments from the 1st generation, 2nd generation of plant growth, 3rd generation and 4th generation. Control 1, control2, control3 and control4 represent

control treatments from the 1st generation, 2nd generation, 3rd generation and 4th generation.

Discussion

In this study, we examined how the activation of SA-induced resistance in the plant impacts the microbial composition in the rhizosphere, and how this change over generations of plant growth. Our study shows that the composition of rhizosphere bacteria communities of *J. vulgaris* changed significantly over generations, *but* that neither the effects of activation of SA-associated plant defense pathways nor the interaction between generation number and SA on the bacterial composition was significant. Within generations the application of SA selected for different bacterial genera in the rhizosphere soil, but these selected genera differed from generation to generation. There were no SA-mediated changes in active bacterial genera in the first generation, suggesting that there are no immediate effects of activation of the SA defense pathway on the soil microbial composition. The majority (76.1%) of the bacterial genera that we detected was present in all soils and represents the "core" bacterial microbiome.

Our study showed that aboveground activation of SA-associated plant defense pathways influenced different bacterial genera in the second, third and fourth generations. Effects of SA-induced resistance on the soil microbial community have been reported in several other studies. For example, Hein et al. (2008) compared the effect of SA application on the composition of rhizosphere bacterial communities in several *Arabidopsis* mutants with terminal restriction fragment length polymorphism (T-RFLP) analysis. They found that SA-induced resistance changed the structure of bacterial communities in the rhizosphere. In addition, Lebeis et al. (2015) demonstrated that SA application modulates colonization of the root microbiome by specific bacterial taxa. SA in plants is associated with the expression of pathogenesis-related proteins (PRPs). These PRPs possess antimicrobial activities resulting in suppression of microbial pathogens, consequently changing the microbial composition (Yalpani et al., 1991; Van Loon and Van Strien, 1999). Alternatively, hormonal-induced resistance in the plant may promote beneficial bacteria and fungi.

However, the impact of SA-induced resistance on soil microbial communities is still debated. For instance, Wang et al. (2015) and Doornbos et al. (2011) both demonstrated that activation of SA-induced resistance did not significantly affect the composition and diversity of the rhizosphere bacterial community.

Even though the experimental conditions and plant genotypes remained the same throughout the experiment, the effects of SA application on the bacterial community differed among generations. In this context, it is important to note that for each generation we used an inoculum, which means that we placed a subset of the microbial community in a sterile background. This may explain why we saw so much variation temporally as in each generation a different subset of the microbial community may have been activated. It is also possible that the composition of the bacterial community is variable over time within each generation and as a consequence also among generations (Gilbert et al., 2009; Hickey et al., 2013; Lauber et al., 2013; Hannula et al., 2019).

Of the four potential models, our data confirmed the third hypothesis (Fig. 1-iii), showing that the bacterial communities did differed among generations but were not strongly influenced by SA application. This is line with studies showing that the composition of the soil bacterial microbial community exhibits large fluctuations over time (Hannula et al., 2019; Lauder et al., 2013). Moreover, our data also shows that the application of SA selects for different bacterial genera in the rhizosphere soil but that these selected genera differ from generation to generation. This suggests that the effects of SA application to plants on the soil microbial community are not consistent over time and that it will be difficult to predict the effects of activation of plant defenses on soil microbes, and ultimately how this will influence the interactions between plants and microbes in the rhizosphere.

Interestingly, in soils of SA-treated plants, we found an increase of *Caballeronia*, unclassified *Cytophagaceae*, *Crinalium* and *Candidatus Thermofonsia Clade* 2. The *Caballeronia* genus is often reported as playing an important role in fixing nitrogen and promoting plant growth. Species in this genus are predominantly endophytic diazotrophic bacteria and N-fixing bacteria (Padda et al., 2018; Puri et al., 2018; Puri et al., 2020). This suggests that activation of SA signaling pathways in *J. vulgaris*

plants benefited bacteria that were more beneficial to plant growth, but further studies are needed to confirm this. The functions of the other species of which their abundance differentially increases are poorly understood. It is noteworthy though that *Crinalium* is a genus that is often isolated from sandy dune soils so it not surprising that we detected this genus as we used dune soils in our experiment. Further studies should extract the information of these detected genera at the species level.

In conclusion, we provide evidence that the composition of bacterial communities in the rhizosphere significantly differed between plant cycles (generation), but we found no evidence that application of SA altered this pattern. However, application of SA influenced different bacterial genera in the rhizosphere, but the responsive genera varied between generations. No bacterial genera were detected that responded to SA application in the first generation suggesting that there are no immediate responses of bacteria in the rhizosphere to SA application to plants. This would question the so-called 'cry for help" hypothesis (Biere and Bennett, 2013; Rasmann et al., 2017; Pineda et al., 2013), but further studies are required before we can make firm conclusions about this. Our results provide a new perspective on the effects of plant hormones on temporal changes in the soil microbial community.

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Supplementary data

Table S1. Summary of Illumina sequencing of the 24 libraries. Total RNA was extracted from 24 soil samples collected from *J. vulgaris* rhizospheres (2 treatments (control/SA application) × 4 generations × 3 replicates). The mapped reads and the percentage of properly paired reads that mapped back to the assembled metatranscriptome by Bowie2 are presented.

Gene.	Treatment (replicate)	No. Raw reads	No. filtered reads (percentage)	No. rRNA reads (percentage)	No. Non rRNA reads (percentage)	Mapped reads (percentage)
1st	Con-1	33,319,926	31,883,498 (95.6)	29,931,407 (93.9)	1,952,091 (6.1)	961,492 (49.3)
	Con-2	47,464,976	45,550,842 (96.0)	43,003,695 (94.4)	2,547,147 (5.6)	1,260,216 (49.5)
	Con-3	38,074,824	35,977,802 (94.5)	33,611,532 (93.4)	2,366,270 (6.6)	1,072,164 (45.3)
	Sa-1	36,891,926	35,557,542 (96.4)	33,148,711 (93.2)	2,408,831 (6.8)	1,046,220 (43.4)
	Sa-2	29,685,378	28,640,038 (96.5)	27,053,138 (94.5)	1,586,900 (5.5)	644,792 (40.6)
	Sa-3	41,699,758	39,325,110 (94.3)	36,398,366 (92.6)	2,926,744 (7.4)	1,445,924 (49.4)
2nd	Con-1	51,523,968	46,908,512 (91.0)	43,467,604 (92.7)	3,440,908 (7.3)	1,682,552 (48.9)
	Con-2	36,922,306	33,800,864 (91.6)	30,217,009 (89.4)	3,583,855 (10.6)	1,198,916 (33.5)
	Con-3	36,098,466	34,487,094 (95.5)	31,687,996 (91.9)	2,799,098 (8.1)	1,360,320 (48.6)
	Sa-1	34,498,252	33,458,636 (97.0)	30,528,949 (91.2)	2,929,687 (8.8)	1,122,652 (38.3)
	Sa-2	30,828,960	29,287,310 (95.0)	25,812,484 (88.1)	3,474,826 (11.9)	1,215,556 (35.0)
	Sa-3	30,241,370	29,244,564 (96.7)	27,187,397 (92.9)	2,057,167 (7.0)	724,424 (35.2)
3rd	Con-1	32,336,630	31,153,526 (96.3)	28,690,779 (92.1)	2,462,747 (7.9)	1,652,708 (67.1)
	Con-2	32,877,082	31,698,262 (96.4)	25,792,802 (81.4)	5,905,460 (18.6)	2,090,760 (35.4)
	Con-3	31,458,902	30,142,612 (95.8)	24,823,022 (82.4)	5,319,590 (17.7)	2,182,264 (41.0)
	Sa-1	25,035,684	24,001,844 (95.9)	22,402,014 (93.3)	1,599,830 (6.7)	866,692 (54.2)
	Sa-2	29,313,124	27,093,298 (92.4)	24,721,628 (91.2)	2,371,670 (8.8)	1,293,100 (54.5)
	Sa-3	52,080,670	48,472,384	43,560,075	4,912,309	2,907,372

			(93.1)	(89.9)	(10.1)	(59.2)
4th	Con-1	40,952,998	37,197,048 (90.8)	33,471,054 (90.0)	3,725,994 (10.0)	1,727,084 (46.4)
	Con-2	40,803,576	37,914,816 (92.9)	34,101,981 (89.9)	3,812,835 (10.1)	1,641,020 (43.0)
	Con-3	46,249,750	42,340,292 (91.6)	39,136,920 (92.4)	3,203,372 (7.6)	1,100,132 (34.3)
	Sa-1	39,495,864	36,597,750 (92.7)	34,868,480 (95.3)	1,729,270 (4.8)	814,560 (47.1)
	Sa-2	41,678,588	39,339,896 (94.4)	36,363,915 (92.4)	2,975,981 (7.6)	1,552,592 (52.2)
	Sa-3	38,834,084	36,852,408 (94.9)	35,344,564 (95.9)	1,507,844 (4.1)	580,832 (38.5)

Table S2. A list of all bacterial genera presents in different generations in the SA and control treatment. "+" represents present and "-" absent. When a genus is present in at least two out of three replicates within a treatment it is recorded as "+".

	1SA	2SA	3SA	4SA	1C	2C	3C	4C
Acidovorax	+	+	+	+	+	+	+	+
Acinetobacter	+	+	+	+	+	+	+	+
Aeromicrobium	+	+	+	+	+	+	+	+
Afipia	+	+	+	+	+	+	+	+
Aminobacter	+	+	+	+	+	+	+	+
Aquabacterium	+	+	+	+	+	+	+	+
Arenimonas	+	+	+	+	+	+	+	+
Arthrobacter	+	+	+	+	+	+	+	+
Azohydromonas	+	+	+	+	+	+	+	+
Bosea	+	+	+	+	+	+	+	+
Bradyrhizobium	+	+	+	+	+	+	+	+
Caenimonas	+	+	+	+	+	+	+	+
Cellvibrio	+	+	+	+	+	+	+	+
Chryseolinea	+	+	+	+	+	+	+	+
Cupriavidus	+	+	+	+	+	+	+	+
Curvibacter	+	+	+	+	+	+	+	+
Devosia	+	+	+	+	+	+	+	+
Dongia	+	+	+	+	+	+	+	+
Ensifer	+	+	+	+	+	+	+	+
environmental Bacteria	+	+	+	+	+	+	+	+
Flavobacterium	+	+	+	+	+	+	+	+
Fluviicola	+	+	+	+	+	+	+	+
Gemmatimonas	+	+	+	+	+	+	+	+
Herbaspirillum	+	+	+	+	+	+	+	+
Herminiimonas	+	+	+	+	+	+	+	+
Hydrogenophaga	+	+	+	+	+	+	+	+
Ideonella	+	+	+	+	+	+	+	+
Janthinobacterium	+	+	+	+	+	+	+	+
Lacibacter	+	+	+	+	+	+	+	+
Lacunisphaera	+	+	+	+	+	+	+	+
Luteimonas	+	+	+	+	+	+	+	+
Lysobacter	+	+	+	+	+	+	+	+
Marmoricola	+	+	+	+	+	+	+	+
Massilia	+	+	+	+	+	+	+	+
Mesorhizobium	+	+	+	+	+	+	+	+
Methylibium	+	+	+	+	+	+	+	+
Methylotenera	+	+	+	+	+	+	+	+
Microbacterium	+	+	+	+	+	+	+	+
Mycobacterium	+	+	+	+	+	+	+	+
Niastella	+	+	+	+	+	+	+	+
Nitrospira	+	+	+	+	+	+	+	+
Nocardioides	+	+	+	+	+	+	+	+
Noviherbaspirillum	+	+	+	+	+	+	+	+
Opitutus	+	+	+	+	+	+	+	+
Pelomonas	+	+	+	+	+	+	+	+
Phenylobacterium	+	+	+	+	+	+	+	+

Phycicoccus	+	+	+	+	+	+	+	+
Polaromonas	+	+	+	+	+	+	+	+
Pseudarthrobacter	+	+	+	+	+	+	+	+
Pseudomonas	+	+	+	+	+	+	+	+
Pseudoxanthomonas	+	+	+	+	+	+	+	+
Ramlibacter	+	+	+	+	+	+	+	+
Reyranella	+	+	+	+	+	+	+	+
Rhizobacter	+	+	+	+	+	+	+	+
Rhizobium	+	+	+	+	+	+	+	+
Rhodoferax	+	+	+	+	+	+	+	+
Rivibacter	+	+	+	+	+	+	+	+
Rubrivivax	+	+	+	+	+	+	+	+
Solimonas	+	+	+	+	+	+	+	+
Sphingomonas	+	+	+	+	+	+	+	+
Sphingopyxis	+	+	+	+	+	+	+	+
Sporichthya	+	+	+	+	+	+	+	+
Staphylococcus	+	+	+	+	+	+	+	+
Streptomyces	+	+	+	+	+	+	+	+
unclassified *Acidobacteria*	+	+	+	+	+	+	+	+
unclassified *Actinobacteria (class) (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Alphaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Bacteroidetes (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Betaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Burkholderiaceae*	+	+	+	+	+	+	+	+
unclassified *Burkholderiales (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Chitinophagaceae*	+	+	+	+	+	+	+	+
unclassified *Chloroflexi (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Comamonadaceae*	+	+	+	+	+	+	+	+
unclassified *Cytophagaceae*	+	+	+	+	+	+	+	+
unclassified *Deltaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Gammaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Gemmatimonadales*	+	+	+	+	+	+	+	+
unclassified *Gemmatimonadetes*	+	+	+	+	+	+	+	+
unclassified *Myxococcales (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Planctomycetes*	+	+	+	+	+	+	+	+
unclassified *Proteobacteria*	+	+	+	+	+	+	+	+
unclassified *Rhizobiales (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Sphingobacteriaceae*	+	+	+	+	+	+	+	+
unclassified *Sphingobacteriales*	+	+	+	+	+	+	+	+
unclassified *Verrucomicrobia (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Xanthomonadaceae*	+	+	+	+	+	+	+	+
Variovorax	+	+	+	+	+	+	+	+
Vulcaniibacterium	+	+	+	+	+	+	+	+
Azospira	+	+	+	+	-	+	+	+
Candidatus Thermofonsia Clade 2	+	+	+	+	-	+	+	+
Clostridioides	+	+	+	+	-	+	+	+
Collimonas	+	+	+	+	-	+	+	+
Escherichia	+	+	+	+	-	-	+	+
Hassallia	+	+	+	+	+	+	-	+
Ohtaekwangia	+	+	+	+	+	+	-	+
Pedobacter	+	+	+	+	+	+	-	+
Pedosphaera	+	+	+	+	+	+	-	+
Ralstonia	+	+	+	+	+	+	-	+
Rhodobacter	+	+	+	+	-	+	-	+

Shigella	+	+	+	+	+	-	-	+
Sinorhizobium	+	+	+	+	+	-	-	+
Tabrizicola	+	+	+	+	+	+	+	-
unclassified *Acidimicrobiaceae*	+	+	+	+	+	+	+	-
unclassified *Flavobacteriales (miscellaneous)*	+	+	+	+	+	-	+	-
unclassified *Oxalobacteraceae*	+	+	+	+	+	-	+	-
unclassified *Sphingomonadales*	+	+	+	+	+	-	-	-
Acidobacterium	-	+	+	+	+	+	+	+
Algoriphagus	-	+	+	+	+	+	+	+
Altererythrobacter	-	+	+	+	+	+	+	+
Paeniglutamicibacter	+	-	+	+	+	+	+	+
Piscinibacter	+	-	+	+	+	+	+	+
Rhodococcus	+	-	+	+	+	+	+	+
Rhodoplanes	+	+	-	+	+	+	+	+
Sphingobium	+	+	+	-	+	+	+	+
unclassified *Anaerolineaceae*	+	+	+	-	+	+	+	+
unclassified *Opitutae*	+	+	-	-	+	+	+	+
unclassified *Sinobacteraceae*	+	+	-	-	+	+	+	+
unclassified *Xanthomonadales*	+	-	-	-	+	+	+	+
Actinomycetales	+	-	+	+	-	+	+	+
Actinoplanes	+	-	+	+	-	+	+	+
Agromyces	+	-	+	+	+	-	+	+
Alistipes	+	-	+	+	+	-	+	-
Anaerobutyricum	+	-	+	+	+	-	-	-
Anaerostipes	+	-	+	+	+	-	-	-
Aquimonas	+	-	+	+	+	-	-	-
Aquincola	+	-	+	+	+	-	-	-
Azotobacter	+	+	-	+	+	+	-	+
Bacteriovorax	+	+	-	+	+	+	-	+
bacterium	+	+	-	+	+	+	-	+
Bacteroides	+	+	-	+	+	+	-	-
Batrachochytrium	+	+	-	+	-	-	-	-
Bdellovibrio	+	+	-	+	-	-	-	-
Bifidobacterium	+	-	-	+	+	+	-	+
Blautia	+	-	-	+	+	-	-	+
Brevundimonas	+	-	-	+	+	-	-	-
Bryobacter	+	+	+	-	-	-	+	+
Burkholderia	+	+	+	-	-	+	-	+
Caballeronia	+	+	+	-	+	+	+	-
Candidatus Kaiserbacteria	+	+	+	-	+	+	-	-
Candidatus Kapabacteria	+	-	+	-	+	+	-	-
Candidatus Nitrosocosmicus	+	-	+	-	-	-	-	-
Candidatus Rokubacteria	+	+	-	-	+	+	-	+
Catellatospora	+	+	-	-	+	+	-	+
Chitinophaga	+	+	-	-	+	+	-	+
Clostridium	+	+	-	-	+	+	+	-
Collinsella	+	+	-	-	+	+	-	-
Comamonas	+	+	-	-	+	-	-	-
Coprococcus	+	+	-	-	+	-	-	-
Crinalium	+	+	-	-	+	-	-	-
Crocinitomix	+	+	-	-	+	-	-	-
Cutibacterium	+	+	-	-	-	-	-	-
Dorea	+	+	-	-	-	-	-	-
Duganella	+	+	-	-	+	-	-	-

Dyadobacter	+	-	-	-	-	-	+	-
Dyella	+	-	-	-	+	+	-	-
environmental samples <bacteria,*phylum*								
Gemmatimonadetes>	+	-	-	-	+	+	-	-
environmental samples <*crenarchaeotes,phylum*								
Crenarchaeota>	+	-	-	-	+	+	-	-
environmental samples <*firmicutes,phylum*								
Firmicutes>	+	-	-	-	+	-	-	-
environmental samples <*GNS bacteria,phylum*								
Chloroflexi>	+	-	-	-	+	-	-	-
Erythrobacter	+	-	-	-	+	-	-	-
Eubacterium	+	-	-	-	+	-	-	-
Faecalibacterium	+	-	-	-	+	-	-	-
Flavihumibacter	+	-	-	-	-	-	-	-
Fontimonas	+	-	-	-	-	-	-	-
Gemmatirosa	+	-	-	-	-	-	-	-
Gemmobacter	-	+	+	+	-	+	+	+
Glaciecola	-	+	+	+	-	+	+	+
Herpetosiphon	-	+	+	+	-	+	+	+
Holdemanella	-	+	+	+	-	+	+	+
Hylemonella	-	+	+	+	-	+	+	+
Hyphomicrobium	-	+	+	+	-	-	+	+
Ilumatobacter	-	+	+	+	-	+	-	+
Inhella	-	+	+	+	-	+	-	+
Klebsiella	-	+	+	+	-	-	-	+
Knoellia	-	-	+	+	-	+	+	+
Kouleothrix	-	-	+	+	-	+	+	+
Kribbella	-	-	+	+	-	+	+	+
Lachnoclostridium	-	-	+	+	+	-	+	+
Leptolyngbya	-	-	+	+	-	-	+	+
Leptothrix	-	-	+	+	-	-	+	+
Leptothrix	-	-	+	+	-	-	-	+
Limnobacter	-	-	+	+	-	+	+	-
Limnohabitans	-	-	+	+	-	-	+	-
Listeria	-	+	-	+	-	-	+	+
Longispora	-	+	-	+	-	-	+	+
Methylobacteriaceae	-	+	-	+	+	+	-	+
Microcoleus	-	+	-	+	-	+	-	+
Micromonospora	-	+	-	+	+	-	-	+
Nitrobacter	-	+	-	+	+	-	-	+
Nitrosomonas	-	+	-	+	+	-	-	+
Nitrososphaera	-	+	-	+	+	-	-	+
Novosphingobium	-	+	-	+	+	-	-	+
Oscillochloris	-	+	-	+	+	-	-	+
Paenarthrobacter	-	+	-	+	+	-	-	+
Panacagrimonas	-	+	-	+	+	-	-	+
Parabacteroides	-	+	-	+	+	-	-	+
Paracoccus	-	+	-	+	+	-	-	+
Paucibacter	-	+	-	+	+	-	-	+
Paucimonas	-	+	-	+	+	-	-	+
Phormidium	-	+	-	+	+	-	-	+
Phyllobacterium	-	+	-	+	+	-	-	+
Pirellula	-	+	-	+	+	-	-	+
Planomicrobium	-	+	-	+	-	-	-	+

Prevotella	-	+	-	+	-	-	-	+
Pseudolabrys	-	+	-	+	-	+	+	-
Pseudonocardia	-	+	-	+	+	-	-	-
Pseudorhodobacter	-	+	-	+	+	-	-	-
Rheinheimera	-	+	-	+	-	-	-	-
Rickettsia	-	-	-	+	-	+	+	+
Roseateles	-	-	-	+	-	+	+	+
Roseburia	-	-	-	+	+	-	+	+
Roseiflexus	-	-	-	+	-	-	+	+
Ruminococcus	-	-	-	+	-	-	+	+
Sandaracinus	-	-	-	+	-	-	+	+
Sediminibacterium	-	-	-	+	-	-	-	+
Simplicispira	-	-	-	+	-	-	-	+
Sinimarinibacterium	-	-	-	+	-	-	-	+
Sphingorhabdus	-	-	-	+	-	-	-	+
Sporocytophaga	-	-	-	+	-	-	+	-
Stella	-	-	-	+	-	-	+	-
Stenotrophomonas	-	-	-	+	+	-	-	-
Streptococcus	-	-	-	+	-	-	-	-
Streptosporangiaceae	-	-	-	+	-	-	-	-
Subdoligranulum	-	-	-	+	-	-	-	-
Thermomonas	-	-	-	+	-	-	-	-
Thermomonosporaceae	-	-	-	+	-	-	-	-
unclassified *Acidobacteriia*	-	-	-	+	-	-	-	-
unclassified *Actinobacteria*	-	+	+	-	-	-	-	-
unclassified *Anaerolineae*	-	+	+	-	-	-	-	-
unclassified *Bradyrhizobiaceae*	-	-	+	-	-	+	+	+
unclassified *Caulobacteraceae*	-	-	+	-	+	-	+	+
unclassified *Clostridiales (miscellaneous)*	-	-	+	-	-	-	+	+
unclassified *Crocinitomicaceae*	-	-	+	-	+	+	+	-
unclassified *Cyanobacteria (miscellaneous)*	-	-	+	-	-	-	-	-
unclassified *Cyclobacteriaceae*	-	-	+	-	-	-	-	-
unclassified *Firmicutes sensu stricto (miscellaneous)*	-	+	-	-	-	-	-	+
unclassified *Frankiales (miscellaneous)*	-	+	-	-	+	+	-	-
unclassified *Hyphomicrobiaceae*	-	+	-	-	-	+	-	-
unclassified *Ignavibacteriae*	-	+	-	-	-	-	-	-
unclassified *Lachnospiraceae*	-	-	-	-	-	-	-	+
unclassified *Nitrosomonadales*	-	-	-	-	-	-	-	+
unclassified *Nitrosopumilales*	-	-	-	-	-	-	-	+
unclassified *Parcubacteria group*	-	-	-	-	-	-	-	+
unclassified *Phyllobacteriaceae*	-	-	-	-	-	--	+	-
unclassified *Pseudomonadales*	-	-	-	-	-	-	+	-
unclassified *Rhodobacteraceae*	-	-	-	-	-	-	+	-
unclassified *Rhodobacterales*	-	-	-	-	-	-	+	-
unclassified *Rhodospirillaceae*	-	-	-	-	-	-	+	-
unclassified *Rhodospirillales (miscellaneous)*	-	-	-	-	-	-	+	-
unclassified *Ruminococcaceae*	-	-	-	-	-	+	-	-
unclassified *Sphingomonadaceae*	-	-	-	-	-	+	-	-
unclassified *Thaumarchaeota (miscellaneous)*	-	-	-	-	+	-	-	-
unclassified *Thermomicrobiales*	-	-	-	-	+	-	-	-
unclassified *Verrucomicrobia subdivision 3*	-	-	-	-	+	-	-	-
unclassified *Verrucomicrobiaceae*	-	-	-	-	+	-	-	-
Undibacterium	-	-	-	-	+	-	-	-

Xanthomonas	-	-	-	-	+	-	-	-
Xenophilus	-	-	-	-	+	-	-	-

Table S3. Genera, of which the relative abundance was significantly positively or negatively correlated with generation number within the SA or control treatment. The correlation is based on a Spearman's rank correlation test. R_s represents Spearman's rank correlation coefficient. *P* values less than 0.05 were selected.

Genera Name	SA		Control	
	R_s value	*P*	R_s value	*P*
Agromyces	0.85	***	-0.68	*
Arthrobacter	0.93	***	-0.84	**
Candidatus Kaiserbacteria	-0.74	**	0.59	*
Crocinitomix	0.81	**	-0.83	**
Ensifer	0.89	***	-0.62	*
environmental samples <*crenarchaeotes, Crenarchaeota*>	0.77	**	-0.82	*
Erythrobacter	-0.83	**	0.70	*
Flavihumibacter	-0.81	**	0.86	***
Flavobacterium	0.73	**	-0.64	*
Gemmobacter	-0.83	**	0.69	*
Lacibacter	-0.60	*	0.68	*
Leptothrix	0.77	**	-0.72	*
Listeria	0.60	*	-0.82	**
Lysobacter	-0.76	**	0.64	*
Mycobacterium	-0.74	**	0.74	**
Opitutus	0.84	**	-0.82	**
Phyllobacterium	0.75	**	-0.62	*
Piscinibacter	0.95	***	-0.81	**
Polaromonas	-0.76	**	0.62	*
Pseudorhodobacter	-0.63	*	0.90	***
Pseudoxanthomonas	-0.73	**	0.67	*
Ramlibacter	0.81	**	-0.77	**
Rheinheimera	-0.87	***	0.62	*
Rhodobacter	0.82	**	-0.85	***
Rhodococcus	0.78	**	-0.69	*
Rhodoferax	0.60	*	-0.82	**
Rickettsia	-0.70	*	0.76	**
Sporichthya	-0.89	***	0.66	*

Tabrizicola	-0.60	*	0.59	*
unclassified *Acidobacteria*	-0.73	**	0.80	***
unclassified *Alphaproteobacteria (miscellaneous)*	-0.78	**	0.72	*
unclassified *Betaproteobacteria (miscellaneous)*	-0.71	*	0.73	*
unclassified *Chloroflexi (miscellaneous)*	-0.95	***	0.76	***
unclassified *Deltaproteobacteria (miscellaneous)*	-0.63	*	0.79	**
unclassified *Gemmatimonadales*	-0.70	*	0.64	*
unclassified *Myxococcales (miscellaneous)*	-0.81	**	0.80	**
unclassified *Parcubacteria group*	-0.77	**	0.72	*
unclassified *Rhodobacteraceae*	0.63	*	-0.72	*
unclassified *Rhodobacterales*	-0.69	*	0.69	*
Undibacterium	-0.69	*	0.66	*
Vulcaniibacterium	0.67	*	-0.61	*
Afipia	-0.82	**	0.57	ns
Aquabacterium	0.82	**	-0.42	ns
Arenimonas	-0.81	**	0.55	ns
Azotobacter	-0.80	**	0.59	ns
Bosea	-0.78	**	0.58	ns
Bradyrhizobium	0.78	**	-0.31	ns
Bryobacter	0.73	**	-0.40	ns
Caenimonas	0.72	*	-0.40	ns
Dyella	-0.72	*	0.54	ns
environmental Bacteria	-0.60	*	-0.02	ns
Gemmataceae	-0.71	*	-0.08	ns
Hassallia	0.78	**	-0.31	ns
Herpetosiphon	0.67	*	-0.36	ns
Inhella	-0.69	*	-0.36	ns
Limnobacter	0.67	*	-0.36	ns
Luteimonas	-0.65	*	0.55	ns
Methylotenera	-0.65	*	0.48	ns
Novosphingobium	0.65	*	-0.32	ns
Oscillochloris	0.64	*	-0.53	ns
Paracoccus	0.63	*	-0.54	ns
Pirellula	-0.60	*	0.35	ns
Pseudarthrobacter	-0.60	*	0.32	ns
Rhizobium	-0.60	*	0.29	ns

Rhodoplanes	-0.60	*	0.13	ns
Roseiflexus	-0.60	*	0.40	ns
Sandaracinus	0.95	***	-0.59	ns
Sphingobium	0.87	***	-0.50	ns
unclassified *Actinobacteria*	0.86	***	-0.43	ns
unclassified *Cytophagaceae*	-0.60	*	0.25	ns
unclassified *Flavobacteriales (miscellaneous)*	0.60	*	-0.14	ns
unclassified *Rhodospirillaceae*	-0.60	*	-0.02	ns
unclassified *Xanthomonadaceae*	0.61	*	-0.50	ns
Variovorax	-0.60	*	0.08	ns
Acidovorax	-0.59	ns	0.88	***
Algoriphagus	0.36	ns	-0.85	***
Azohydromonas	0.19	ns	-0.83	**
Bdellovibrio	0.10	ns	-0.82	**
Brevundimonas	-0.36	ns	-0.82	**
Burkholderia	-0.41	ns	0.82	**
Candidatus Nitrosocosmicus	0.02	ns	0.81	**
Candidatus Thermofonsia Clade 2	0.56	ns	0.80	**
Collimonas	-0.48	ns	0.78	**
Cupriavidus	-0.41	ns	0.78	**
Dongia	-0.37	ns	0.78	**
Herbaspirillum	-0.07	ns	-0.77	**
Hyphomicrobium	-0.37	ns	0.76	**
Klebsiella	-0.50	ns	0.76	**
Mesorhizobium	0.30	ns	-0.75	**
Microbacterium	-0.52	ns	0.73	**
Nitrososphaera	-0.23	ns	-0.72	*
Noviherbaspirillum	-0.04	ns	-0.72	*
Paeniglutamicibacter	-0.36	ns	0.71	*
Parabacteroides	-0.17	ns	0.69	*
Pedosphaera	-0.24	ns	0.69	*
Pelomonas	-0.29	ns	0.68	*
Planomicrobium	0.35	ns	-0.67	*
Sediminibacterium	-0.21	ns	0.67	*
Sinorhizobium	0.40	ns	-0.67	*
Stenotrophomonas	-0.58	ns	0.66	*
Streptomyces	-0.45	ns	0.65	*

unclassified *Anaerolineae*	0.32	ns	-0.65	*
unclassified *Caulobacteraceae*	-0.47	ns	0.65	*
unclassified *Comamonadaceae*	-0.52	ns	0.64	*
unclassified *Gemmatimonadetes*	-0.16	ns	0.63	*
unclassified *Nitrosopumilales*	-0.17	ns	-0.62	*
unclassified *Opitutae*	0.17	ns	-0.61	*
unclassified *Oxalobacteraceae*	-0.41	ns	0.61	*
unclassified *Phyllobacteriaceae*	0.45	ns	-0.61	*
unclassified *Planctomycetes*	-0.38	ns	0.61	*
unclassified *Sphingomonadales*	0.26	ns	-0.61	*
unclassified *Thaumarchaeota (miscellaneous)*	-0.26	ns	0.60	*
unclassified *Thermomicrobiales*	0.39	ns	0.60	*
unclassified *Verrucomicrobia (miscellaneous)*	-0.57	ns	0.59	*
unclassified *Verrucomicrobiaceae*	0.00	ns	-0.59	*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns not significant. n=3.

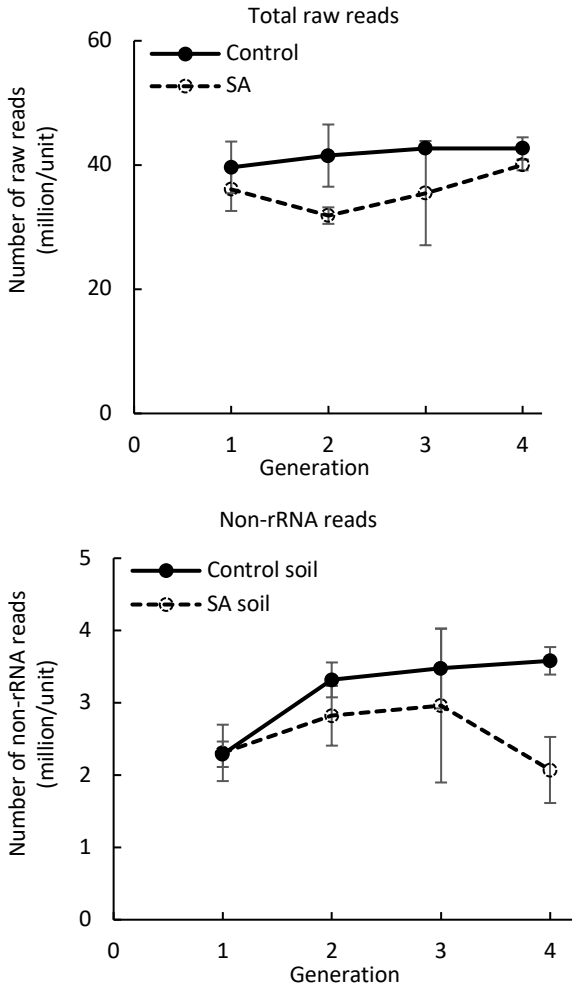


Fig. S1 Number of total raw reads (mean ± SE) (left) and non-rRNA reads (mean ± SE) (right) in the metatranscriptomic datasets over four generations in SA treatments and control. N=3.

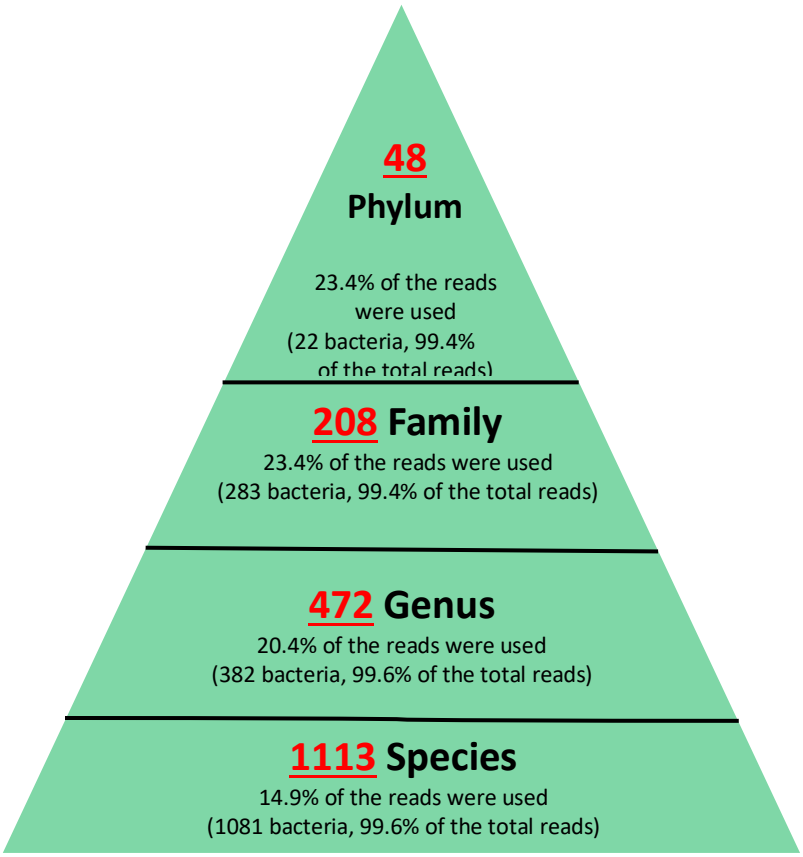


Fig. S2 Heinrich's triangle visualizing information on microbial composition at different classification levels of all 24 rhizosphere soil samples identified with MEGAN against NCBI. The numbers of identified phyla, families, genera and species are shown and the percentage of reads used for each classification level is noted. At phylum level, 2 Archaea, 22 bacteria, 22 eukaryotes and 2 viruses were found; at family level, 3 Archaea, 134 bacteria, 69 eukaryote (only fungi were counted) and 2 viruses were found; at genus level, 4 Archaea, 382 bacteria, 83 eukaryotes (9 fungi, 27 nematodes, 27 algae and 20 others) and 3 viruses were found; at species level, 12 Archaea, 1086 bacteria, 12 eukaryotes and 3 viruses were found. In the Heinrich's triangle, only the information of bacteria is listed, this includes numbers of bacteria and their read percentage against all the reads in that taxonomic level.

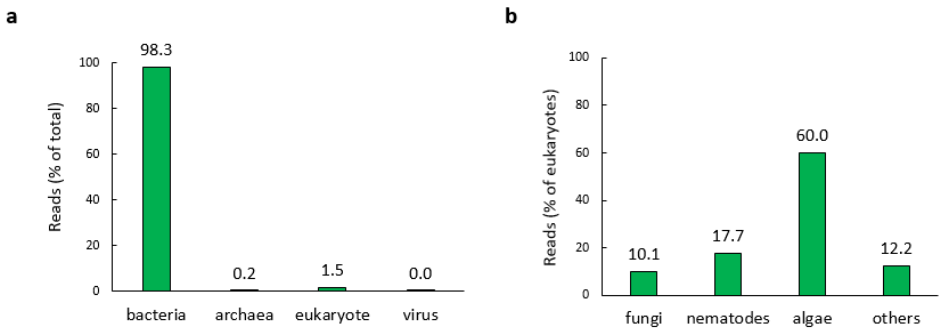


Fig. S3 The percentage of reads at different taxonomic levels. (a) The percentage of reads mapping to bacteria, archaea, eukaryote and virus reads of the total mapped reads. (b) The percentage of reads mapping to fungi, nematodes, algae and others in the total eukaryote reads.

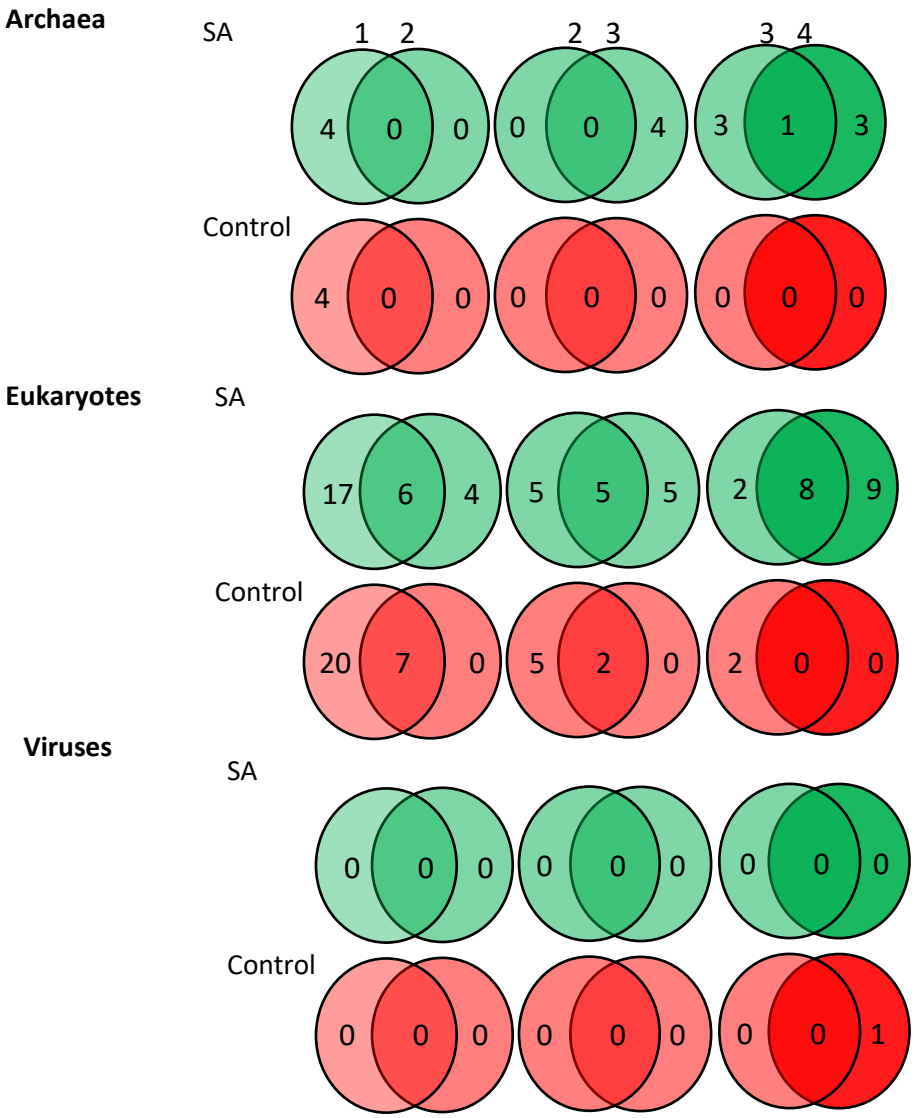


Fig. S4 Venn diagram showing the immigration and extinction numbers at genus level for Archaea, eukaryotes and viruses at each generation in the rhizosphere soil samples of SA-treated or control *J. vulgaris* plants.

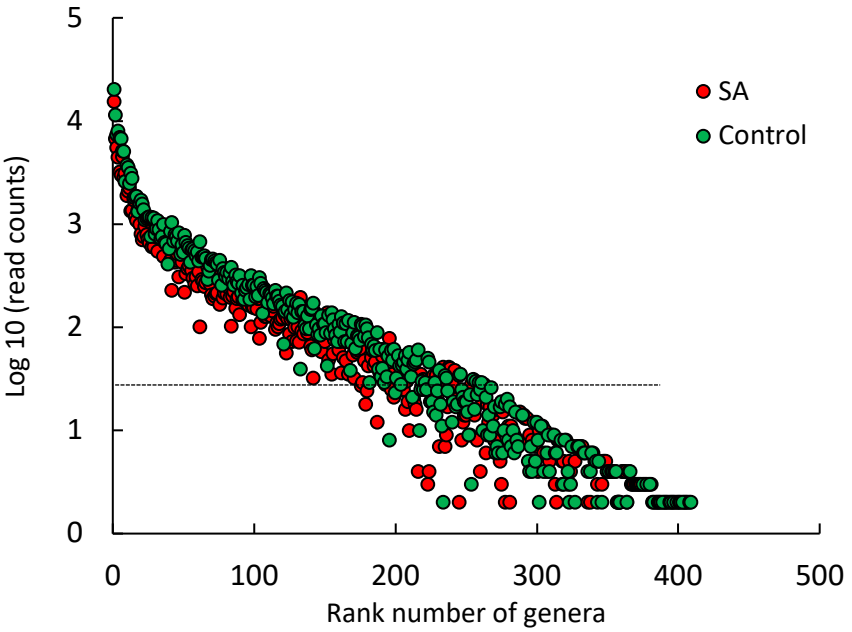


Fig. S5 Scatter plot showing log $_{10}$ transformed read counts of all 408 genera in rhizosphere soil samples of SA-treated and control plants against genus abundance. Rank number was based on the values of total read counts of all genera from the sum of the reads in SA and control treatments. The genus with highest abundance corresponds to the rank number 1. The cut-off line is based on < 0.01% of the total reads.

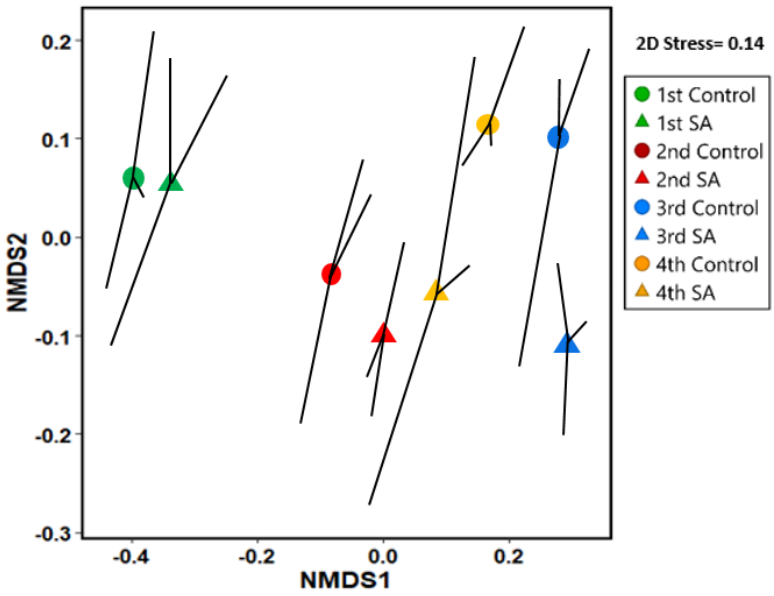


Fig. S6 Nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distance representing the taxonomic information from the bacterial genera of 24 rhizosphere soil samples. For each treatment combination, the centroid is connected to the three replicates.

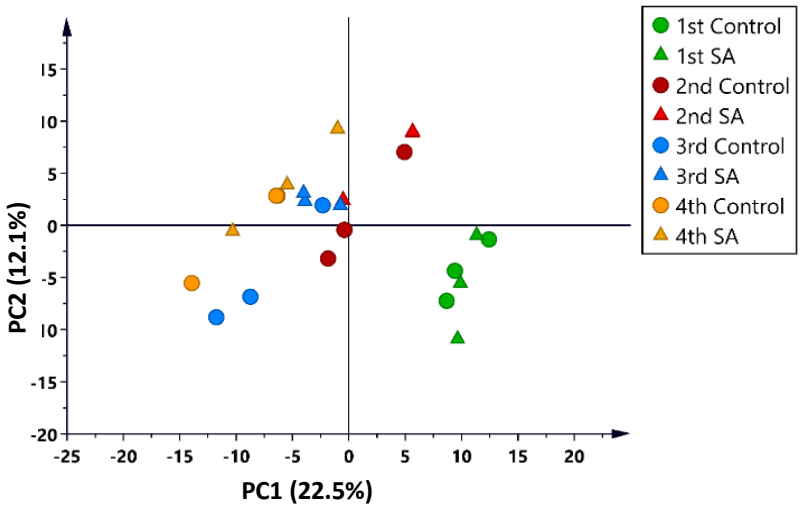
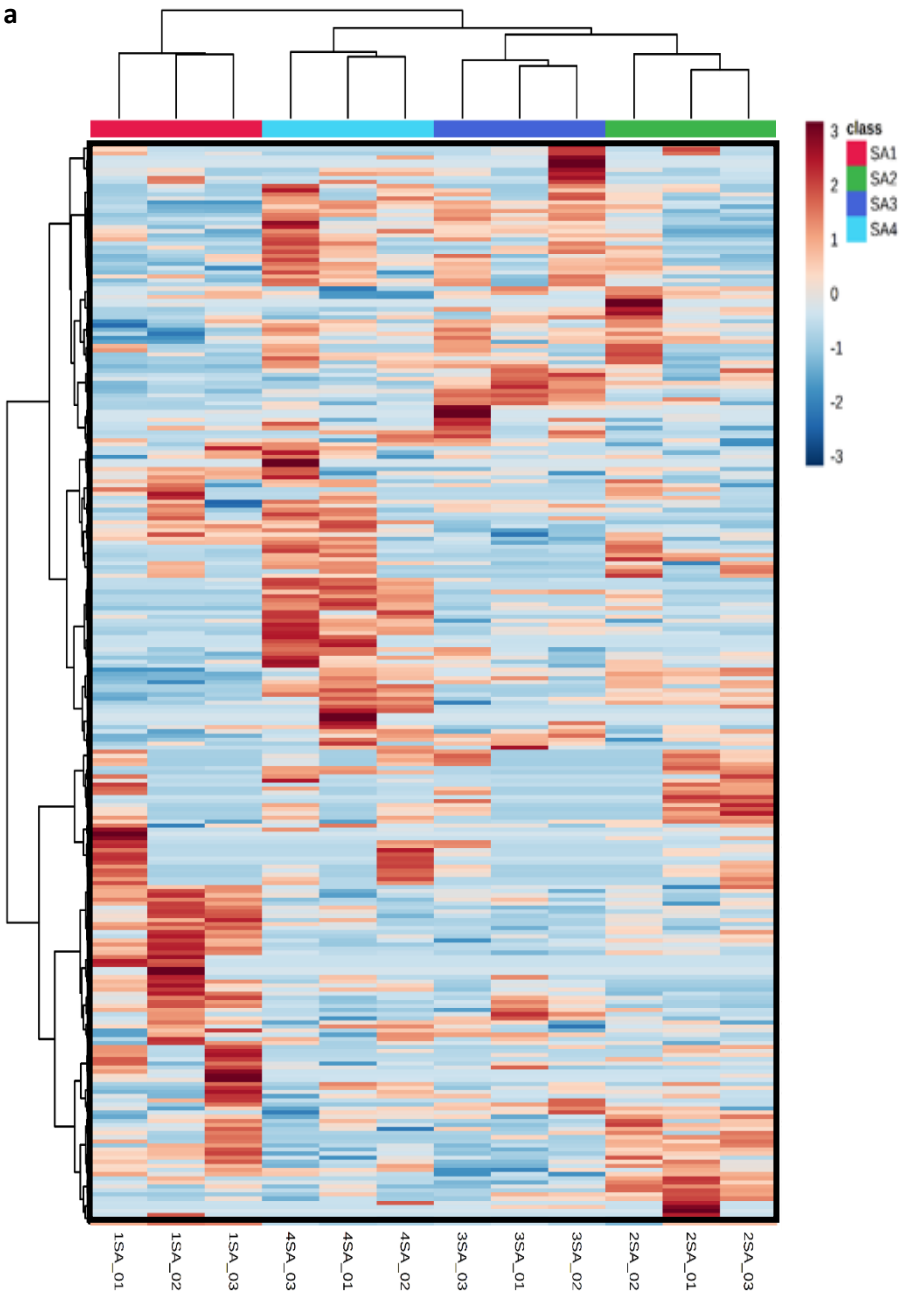


Fig. S7 Principal component analysis (PCA) representing the taxonomic information on the genera of 24 rhizosphere soil samples of SA-treated and control *J. vulgaris* plants. PCA scores are based on relative abundance at genus level.


a
class
SA1
SA2
SA3
SA4
3
2
1
0
-1
-2
-3
1SA_01
1SA_02
1SA_03
4SA_03
4SA_01
4SA_02
3SA_03
3SA_01
3SA_02
2SA_02
2SA_01
2SA_03

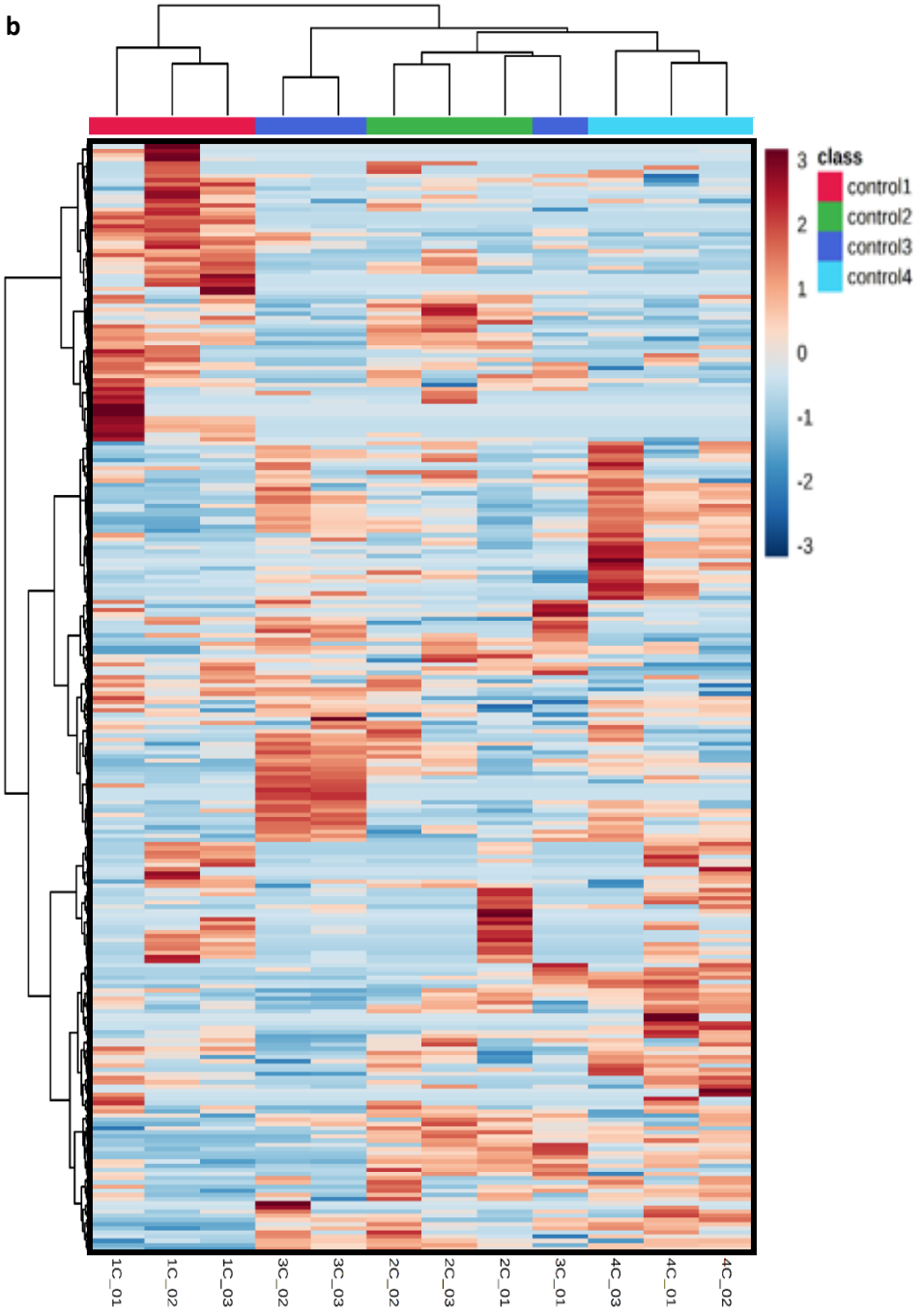

b
class
control1
control2
control3
control4
3
2
1
0
-1
-2
-3
1C_01
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Fig. S8 Heatmap with a hierarchical clustering analysis of 264 genera of rhizosphere soil of SA-treated plants (a) and 270 genera of soil samples of control plants (b). The hierarchical clustering analysis was calculated with Pearson distance and the Ward clustering algorithm based on the relative hit numbers of each genus. The color code represents the values of log2 transformed row-centered relative hit numbers. SA1, SA2, SA3 and SA4 represent SA treatments from the 1st generation, 2nd generation, 3rd generation and 4th generation. Control 1, control2, control3 and control4 represent control treatments from the 1st generation, 2nd generation, 3rd generation and 4th generation. Each treatment in each generation is represented by three replicates indicated as 01, 02 and 03 respectively. E.g. the code 1C_01 represent the first generation control's first replicate.

Chapter 4

Activation of the SA-associated plant defense pathway alters the functions of soil microbial communities in four sequential generations

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Abstract

Systemic acquired resistance (SAR) is an immune response of plants that regulates plant hormonal signaling pathways and strengthens the ability of the plant to withstand pathogenic microbes. Aboveground application of salicylic acid (SA) to the plant can induce SAR and we showed that it mitigates negative effects of the soil microbial community on the performance of the plant *Jacobaea vulgaris*. How SA-induced resistance affects the expression of functional genes and gene ontology in the rhizosphere and how this phenomenon extends over multiple generations is not well studied. In this study, a meta-transcriptomics approach was used to characterize gene expression profiles of microbial communities in 24 soil samples of SA-treated and control plants over 4 generations. 71.6 million reads were used for de-novo assembly of the microbial transcriptome, after which a total of 1.3 million unique contigs (genes) were identified. Multivariate analysis revealed that the SA treatment, generation and the interaction between these two affected the functional genes of the rhizosphere microbial communities of *J. vulgaris*. In general, the effect of the SA treatment on microbial gene expression was lowest in the first generation and strongest in the fourth generation. Microbes in soil samples of SA-treated and control plants showed 1663 differentially expressed genes. In the first generation only two genes differed significantly in gene expression between microbes from soils of SA treated and control plants while in the fourth generation 361 genes were differentially expressed between microbes from soils of SA treated and control plants. None of the significantly expressed SA-downregulated genes were present in all four generations, while only one SA-upregulated gene was observed in all four generations. Gene ontology (GO) analysis showed that soil microbial communities in rhizosphere soil of SA-treated plants increased the expression of thirteen GO terms in the second, third and fourth generation. These increased GO terms were mostly related to viral RNA genome replication, to interactions with host cells, to organelles of the host cells and to RNA polymerase activities. There were six GO terms of which the expression decreased in the second, third and fourth generation, and these were associated with processing nitrogen and macromolecules. Overall, our results show that aboveground activation of defenses in the plant affects the expression of functional genes in the soil microbial communities belowground. This suggests that plants may recruit functional

rhizosphere microbiomes that improve plant health and crop production in agriculture.

Keywords

Meta-transcriptomics, Soil microbial community, Functional genes, Plant-soil interactions, Induced resistance, Rhizosphere soil, Salicylic acid

Introduction

Plants can alter the microbiome of the soil in which they grow, and in turn, microorganisms can influence plant performance. The rhizosphere microbiome, defined as the microbial community established near or on plant roots, can have negative, positive and neutral effects on the growth of a host plant (Van Wees et al., 2008; Raaijmakers, et al., 2009; Berendsen et al., 2012). Microbes such as plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) are typically characterized as plant beneficial, because of their contribution to plant health and nutrient uptake (Jeffries et al., 2003; Compant et al., 2010). In contrast, pathogenic microbes typically reduce plant growth and trigger defense mechanisms in the plant (Pieterse et al., 2001). However, the overall net effect of soil microbial communities on plant growth is often negative (Nijjer et al., 2007; Wardle et al., 2011). This might be due to e.g. competition between plants and microbes for available nutrients or soil pathogens (Berendse, 1994; Callaway et al., 2004; Mazzoleni et al., 2015; Cesarano et al., 2017). In response, plants have evolved hormone-driven defensive strategies to suppress these pathogenic impacts, such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Bruce and Pickett, 2007; Berendsen et al., 2012; Huang et al., 2014; Ökmen and Doehlemann, 2014).

Systemic acquired resistance (SAR) is a distinct transduction pathway, which is involved in the biological processes that enhance the plant's immune system and defense against microbial pathogens (Reymond and Farmer, 1998; Walters and Heil, 2007; Pieterse et al., 2014; Haney and Ausubel, 2015). An infection caused by a pathogenic microbe can induce SAR, in which plants enhance their immune system by expressing genes coding for pathogenic-proteins (PR) in infested and uninfected tissues (Kachroo and Robin, 2013; Shah and Zeier, 2013; Gao et al., 2015). Apart from local induction by pathogenic microbes, SAR can also be induced by foliar sprays of the phytohormone salicylic acid (SA) (Reymond and Farmer, 1998). Applying a low concentration of SA directly to leaf tissues results in the activation of SA signaling pathways and this has been considered an effective way to activate defense signals in many plant species (Reymond and Farmer, 1998; Pozo and Azcón-Aguilar, 2007; Vlot et al., 2009).

In Chapter 2, we showed that the application of SA mitigates the negative effects of soil microbes on the growth of *J. vulgaris* although this effect did not increase further in subsequent generations of plant growth. A number of studies have examined the expression of functional genes in soil microbial communities. For example, Xue et al. (2016) showed that changing the temperature of soil significantly altered the gene expression in soil microbial communities and these genes were related to maintaining carbon and nitrogen stability in the soil, resulting in higher plant growth. Moreover, Castro et al. (2019) recently demonstrated that plants can change the expression of functional genes (i.e., carbon metabolic genes) in the soil microbial community in response to environmental changes such as drought. Here we hypothesize that application of SA to plants can also cause changes in the expression of functional genes in the soil microbial community and we hypothesize that the altered gene expression is related to the suppression of soil microbial pathogens of plants (Maurhofer et al., 1998; Verberne et al., 2000; Tanaka et al., 2015). Moreover, we expect, that the gene expression difference in the rhizosphere microbial community of control and SA treated plants will increased over generations of plant growth.

Previously, we analyzed the changes in the composition of the microbial community in the rhizosphere soil upon foliar application with SA and showed that the composition of rhizosphere bacterial communities differed among four plant generations of *J. vulgaris* and between soils from SA treated and control plants. However, the composition differed strongly among generations (Chapter 3). Functions of the soil microbial community can be performed by different microbial taxa (Burke et al., 2011; Liu et al., 2018; Liu et al., 2020) and hence we expect that there is functional redundancy in the soil microbial community and a consistent effect of SA application on gene expression in the microbial community.

In this study we ask the following questions: (1) Does the application of SA on leaves of *J. vulgaris* significantly alter the gene expression of the microbial community in the rhizosphere? (2) Does the effect differ between generations or is there an interaction between the SA treatment and generation on the gene expression in the microbial communities? (3) Which groups of genes or gene ontology pathways in the rhizosphere microbiome are influenced by SA-application over generations?

Materials, methods and bioinformatics processing

The multi-generation growth experiment with *J. vulgaris* has been described in Chapter 3. In short, *J. vulgaris* plants were grown for four generations on soils inoculated with soil from the previous generation from the same treatment with a foliar SA application treatment and a control treatment. Each treatment had 10 replicates. For each treatment, the three successively labeled replicates (No. 1, 2, 3, No. 4, 5, 6 and No. 7, 8, 9) were mixed and used as one pooled replicate, Hence, the three pooled replicates were used for RNA extraction for each treatment in each generation and a total of 24 soil samples were used for RNA extraction (3 replicates x 2 treatments x 4 generations). RNAseq was carried out using the Illumina platform.

Processing of the data included quality control of raw reads (FastQC), data trimming (Trimmomatic 0.39), filtering out ribosomal RNAs (SortMeRNA), de novo assembly of reads (Trinity), remove duplicates (CD-HIT-EST algorithm), mapping back to the transcriptome (Bowtie2). For a detailed description see Chapter 3. Gene ontology enrichment was performed using Trinotate and Goseq against the SwissProt, NR (non-redundant) and Pfam databases (Bryant et al., 2017; Bateman, 2019; El-Gebali et al., 2019).

Statistical analyses

Prior to analysis, the raw data were normalized. TMM (trimmed mean of M-values) normalization was used for read counts among all 24 samples (Robinson and Oshlack, 2010). A principal component analysis (PCA) was employed using the normalized number of genes to examine the composition of rhizosphere soil samples of SA-treated and control plants for the four generations. A PERMANOVA test was performed using the *adonis* function (number of permutations = 999) in R within the "vegan" package to verify the effects of the SA treatment and time on the composition of all expressed genes. To compare similarities among samples of treatment SA and control over four generations, a Pearson correlation for pairwise sample comparison based on the normalized raw read counts of all replicates in the control and SA treatments was performed in R and a heatmap was produced.

Differential gene expression (DE) analysis was performed for all possible combinations of replicates of sets of 8 samples (2 treatments x 4 generations) with EdgeR with raw read counts as input. EdgeR normalizes the data to TMM before further processing. After DE analysis in EdgeR, for all differentially expressed genes of the 8 samples Volcano plots were made for the contrast between SA-treated and control samples per generation. Log2 (FC) values were used as x-variable and -log10 (FDR) for the y-variable to produce a volcano plot of differentially expressed genes between control and SA-treated soil samples per generation. Genes that were significantly differentially expressed between SA-treated and control soil samples that could be annotated were listed. A clustered heatmap based on Euclidean distances (Danielsson, 1980) of gene expression derived from EdgeR per treatment after Z-scored transformation was generated in R using the package "pheatmap" (Kolde and Kolde, 2015).

To visualize the gene expression changes among different hormonal treatments and time categories, an NMDS (nonmetric multidimensional scaling) plot using the Bray-Curtis index as a measure of dissimilarity was generated using TMM normalized read counts. To verify changes in the composition of the 1663 expressed genes due to the SA treatment and time effect, a PERMANOVA test was performed using the adonis function (number of permutations = 999) in R within the "vegan" package.

Gene ontology (GO) enrichment was performed with "GoSeq" for each generation separately. Gene functional classification was determined for three categories: biological processes, cellular components and molecular functions. GO terms affiliated to Eukaryotes (e.g. mitochondria) were removed. The rich factor was calculated as the number of differentially expressed genes in the ontology divided by the number of all genes that were used as a background gene list.

Results

Comparing read counts between generations and treatments

A total of 898,4 million raw sequencing reads were obtained from the 24 metatranscriptomic libraries. The details of the library size and basic information

about read quality were described in Chapter 3. A principal component analysis (PCA) using log2 transformed normalized CPM showed that the read counts of contigs in the microbial community of rhizosphere soil of the *J. vulgaris* samples among generations were well separated (Fig. 1), this was in line with the permutation test (PERMANOVA $R^2 = 0.22$, F = 19.6, $df_1 = 3$, $df_2 > 999$, $p < 0.01$). In addition, the effect of SA application was significant (PERMANOVA $R^2 = 0.07$, F = 6.3, $df_1 = 1$, $df_2 > 999$, $p < 0.05$). Gene expression patterns of SA-treated *J. vulgaris* and control samples were better separated in the third and fourth generation than in the first and second generation (Fig. 1). In the correlation matrix for all sample replicates generated with PtR (a tool for comparing sample replicates in Trinity) (Fig. 1), samples within treatments were positively correlated with each other and also there was a positive correlation between samples within generations especially for the first generation. The heatmap showed clear clustering of treatments within generations except for generation 1. The separation between the SA and the control treatment became more distinct over generations (Fig. 2).

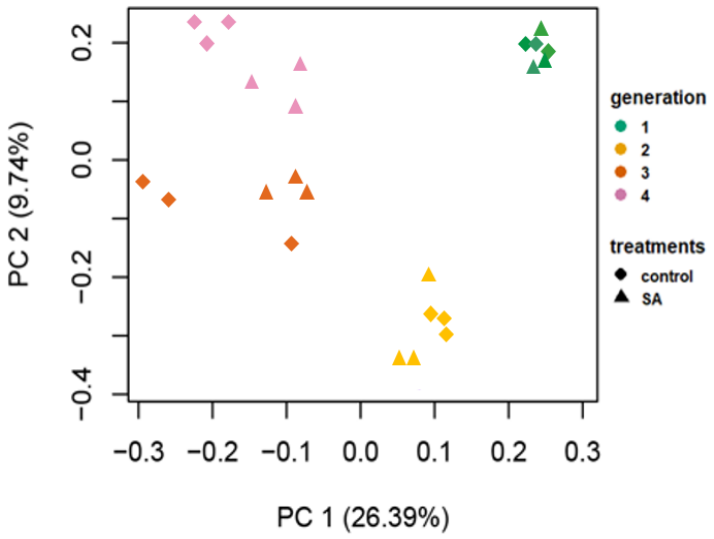

Fig. 1 Scatter plot from a principal component analysis (PCA) of TMM normalized CPM representing the overall gene expression patterns of different rhizosphere soil

samples of SA-treated and control *J. vulgaris* plants over generations. Shapes represent the treatments and colors represent generations.

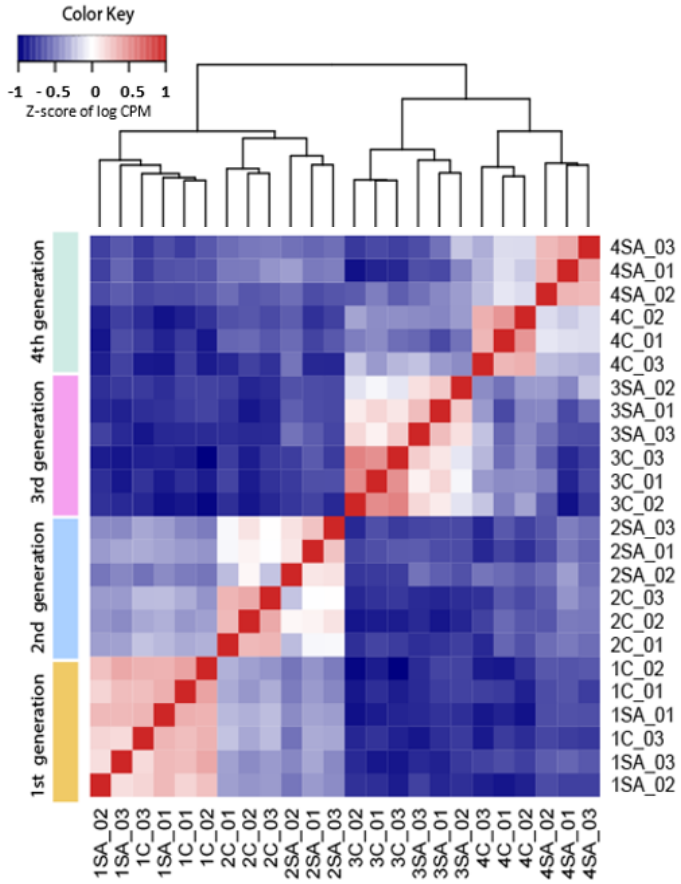

Fig. 2 Clustered heatmap visualizing the Pearson correlation matrix for pairwise sample comparisons based on TMM normalized read counts per million. The heat map shows the correlation in microbial gene expression in all paired replicates between rhizosphere soil samples of SA-treated and control *J. vulgaris* plants over four generations. The dendrogram illustrates the relationship-distance between samples and is calculated based on a Pearson correlation coefficient. The color key represents the z-score of log2 CPM. The legends on the sides represent: Generation (1-4), treatment (SA/Control) and replicate number (01-03).

Differential gene expression

In total, 0.36 million genes were detected. Of those genes, 1663 were differentially expressed between all possible combinations of replicates of sets of 8 samples (2 treatments x 4 generations). Hierarchical clustering on CPM for 1663 differentially expressed genes was performed to explore the patterns of gene expression of the microbial communities between all pairwise combinations of all the samples among SA and control treatments over four generations (Fig. 3). Except for the first generation, SA and control samples were separated from each other in different clusters (Fig. 3). However, among generations, different clusters of genes were differentially grouped. Differences were most pronounced between on the one hand, the first and second generation, and on the other hand, the third and fourth generation.

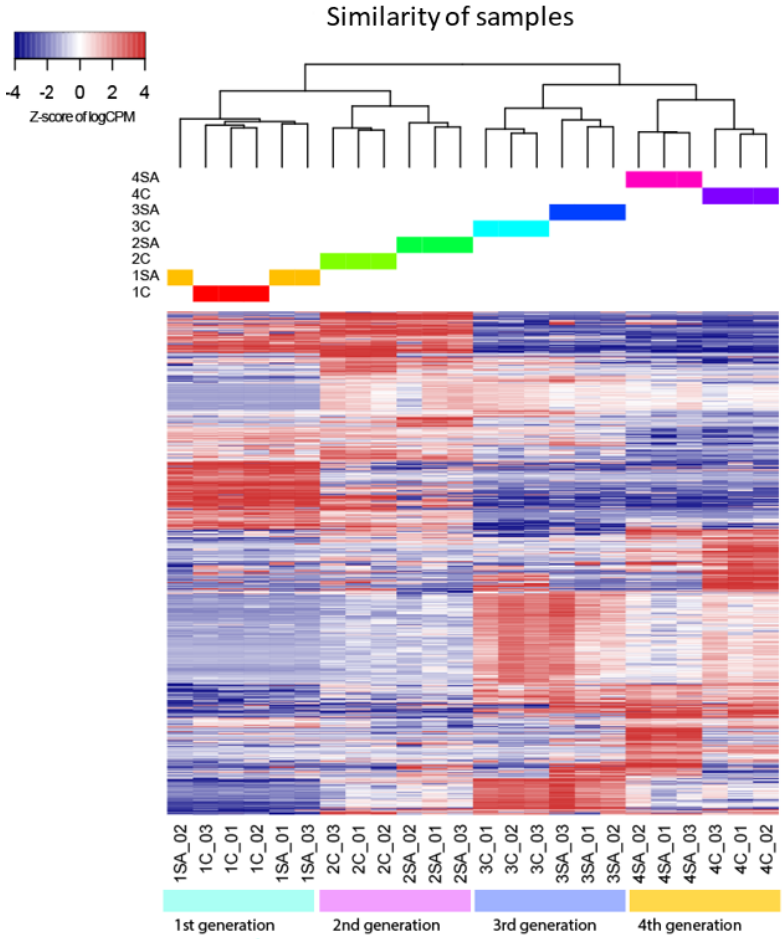

Fig. 3 Heatmap showing 1663 differentially expressed genes (FDR < 0.05 and fold change ≥ 2) between all possible combinations of replicates of rhizosphere soil samples of SA-treated and control *J. vulgaris* plants over four generations based on TMM normalized CPMs. The color key represents the z-score of log2 CPM. The dendrogram on the x-axis illustrates the hierarchal clustering of relationship-distance between replicates using TMM normalized log2-transformed CPM. The legend on the bottom represents: generation (1-4), treatment (SA/control) and replicate number (01-03).

The NMDS plot showed that the 1663 differentially expressed microbial genes detected with EdgeR were differentially expressed in the different generations

(Fig. 4, PERMANOVA $R^2 = 0.63$, F = 21.8, $df_1 = 3$, $df_2 = 1662$, $p < 0.01$) and also that genes were differently expressed between the SA treatment and the control (PERMANOVA $R^2 = 0.07$, F = 7.0, $df_1 = 1$, $df_2 = 1662$, $p < 0.01$). The effect of the SA treatment was not the same in each generation as indicated by the significant interaction (PERMANOVA $R^2 = 0.15$, F = 5.2, $df_1 = 3$, $df_2 = 1662$, $p < 0.001$).

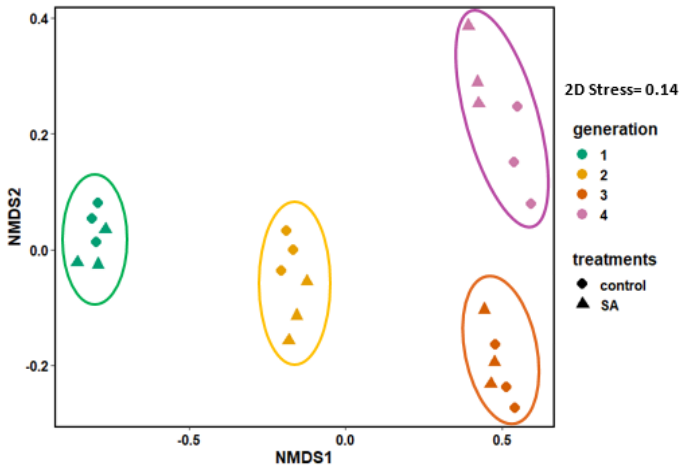

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Fig. 4 Multivariate analysis of 1663 differentially expressed microbial genes between all replicates in rhizosphere samples from SA-treated and control *J. vulgaris* plants grown in four generations. Shown are sample scores from a nonmetric multidimensional scaling (NMDS) plot.

To identify the numbers of significantly down- or up-regulated genes in the SA treatment in each generation in the rhizosphere microbial community, volcano plots were made (Fig. 5). In the first generation, no downregulated genes were observed and only two upregulated genes were detected (Fig. 5a). This increased to 59 and 76 in the second, 89 and 26 in the third, and 187 and 174 in the fourth generation, respectively (Fig. 5b, c, d). Among all the significant differentially expressed genes, no genes were found that were downregulated after SA application in all four generations, while only one gene was observed that was upregulated in SA in all four generations (Fig. 6). Circa 90% of the genes that were significantly altered by the SA

treatment could not be annotated. Among all the annotated genes, only two of the significant differentially expressed microbial genes were detected in three generations and eight genes were detected in two generations (Fig. 6). Not all the genes could be matched with a function in the database. Detailed information of successfully annotated genes was listed in Table S1.

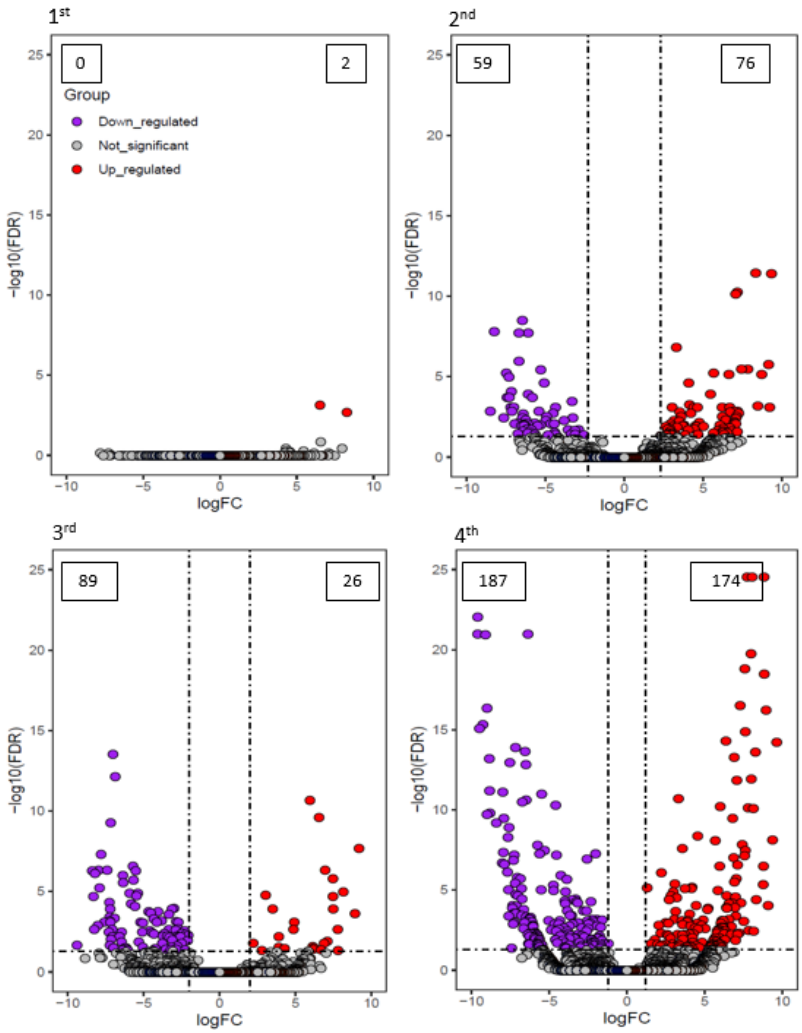


Fig. 5 Volcano plots of 1663 differentially expressed genes of the microbial community in rhizosphere samples of SA-treated and control *J. vulgaris* plants per generation. The x-axes show log2 fold changes of read counts of the genes of the SA treatment compared to thecontrol, and the y-axes show the -log10 adjusted for FDR

values. SA upregulated genes are presented in purple, and SA downregulated genes are displayed in red, while non-significant genes are shown as light grey dots. 1st, 2nd, 3rd and 4th represent the different generations. The numbers inside each box represent the number of significantly up/down-regulated genes. The two vertical dashed lines represent the positive or negative log2 fold changes in the number of readcounts in the SA treatment compared to the control in the generation when -log10(FDR) is 2 as presented by the horizontal dashed lines.

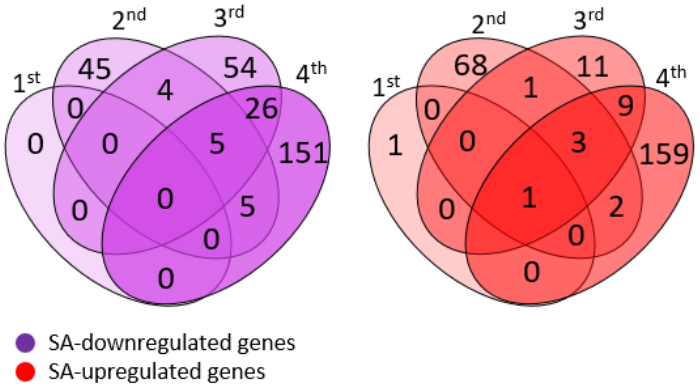


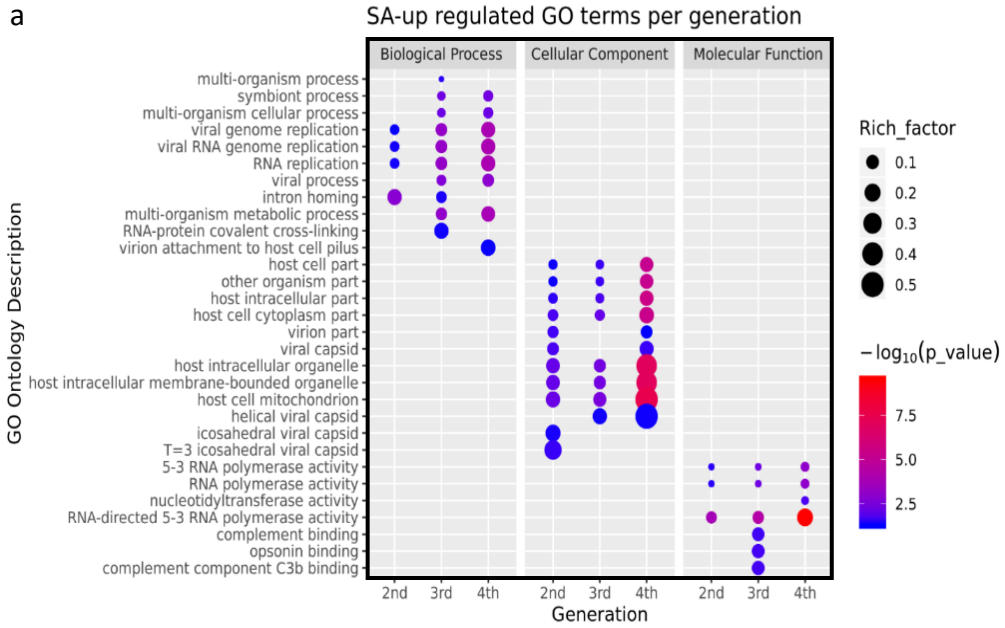
Fig. 6 Venn diagrams showing the number of shared and unique up and down-regulated microbial genes over generations in the rhizosphere of *J. vulgaris*. The numbers represent the significantly differently expressed genes from the volcano plot (Fig. 5). 1st, 2nd, 3rd and 4th represent the different generations.

Gene ontology (GO) analysis

To profile differentially expressed pathways, we performed a gene ontology (GO) analysis for the soil samples of SA-treated and control plants for each generation (Fig. 7, Table S2). No significantly upregulated or downregulated GO terms were observed in the first generation (Fig. 7a, 7b). In the second, third and fourth generations, genes from classes of the GO categories "biological processes", "cellular components" and "molecular functions" were differentially expressed (Fig. 7a). 13 GO terms were upregulated in the SA treatment in three generations, while 18 GO terms were upregulated in one or two generations (Fig. 7a). Of the 13 GO terms upregulated in

three generations three belonged to the GO category “biological processes”, and these GO terms were all related to viral RNA genome replication, seven belonged to the GO category “cellular components” and these GO terms were related to interactions with host cells and to organelles of the host cells and finally three belonged to the GO category “molecular function” and these GO terms were all related to RNA polymerase activity.

Only six GO terms were downregulated in the second, third and fourth generation in the rhizosphere of SA treated plants, while 58 GO terms were downregulated in one or two generations only (Fig. 7b). The six GO terms downregulated in three generations fell all in the GO category “biological processes” and the GO terms were related the localization of processes, to nitrogen processing and to processes involving macromolecules. None of GO terms involved in cellular components and molecular functions were present in these three generations.



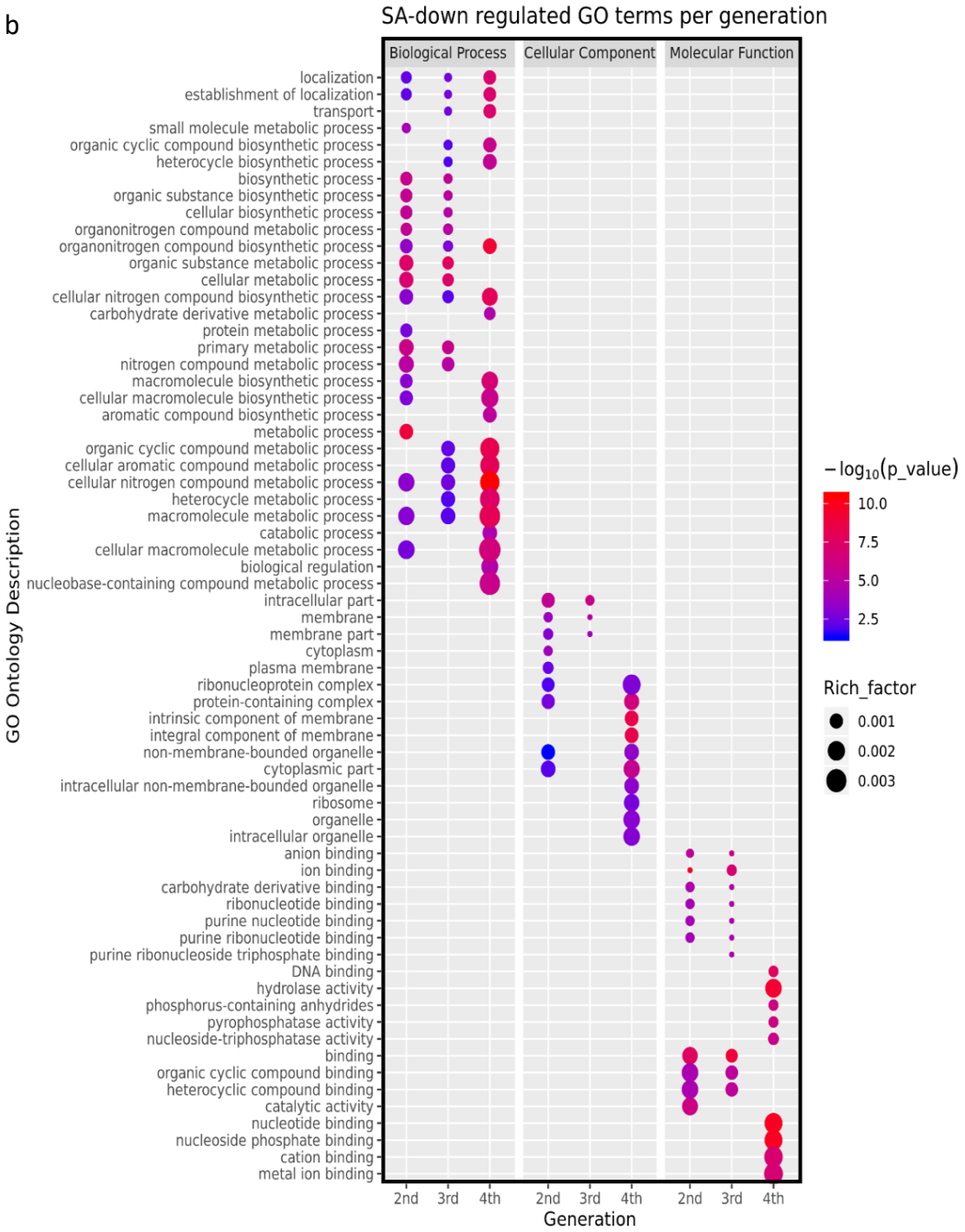


Fig. 7 Gene ontology (GO) enrichment analysis of significantly differentially expressed genes in the microbial community in the rhizosphere. A bubble chart shows

enrichment of differentially expressed GO terms. The Y-axis label lists the GO terms, the size of the bubbles represents the rich factor (= amount of differentially expressed genes enriched in the ontology/total amount of all genes in the background gene set) in different generations. Gene classification of the annotated GO terms was grouped in three categories. Colors of the bubbles represent the significance level of enrichment as calculated with Goseq. a and b represent up and down-regulated GO terms in the SA treatment, respectively. Note: in the first generation, there were no up- or down-regulated GO terms.

Discussion

In this study, a high-throughput metatranscriptomic sequencing approach was used to examine how the aboveground application of SA to the plant impacts the functional gene expression of the microbial communities in the rhizosphere over four subsequent generations of plant growth. Our study shows that the activation of the SA-associated plant defense pathways significantly affected the gene expression of the microbial communities in the rhizosphere, but this effect differed over four generations. Notably, the numbers of differentially expressed genes increased over generations, and there was almost no overlap of in the genes that were significantly expressed in the four generations. Moreover, foliar application of SA caused upregulation of genes of the microbial community related to GO terms associated to viral RNA genome replication, to interactions with host cells, to organelles of the host cells and to RNA polymerase activities, while downregulated GO terms of the microbial community were associated biosynthetic processes involving nitrogen and metabolic processes.

Our study shows that application of SA to plants changed the functional gene expression in the rhizosphere microbial community. This complements previous studies, which report that effects of different abiotic factors alter the expression of functional genes in the soil community (Xue et al., 2016; Castro et al., 2019). Interestingly, in our study, the highest number of significantly expressed genes was recorded in the fourth generation, which suggests that the effect of SA on gene expression becomes more pronounced over time. We did not find a selection-effect of SA on the rhizosphere bacterial community over multiple generations (results in Chapter 3). Hence, we cannot conclude that the increase in the number of significant

expressed genes in our study was due to a specific rhizosphere bacterial community that became increasingly active.

Our finding that the expression of functional genes differed strongly among generations is in line with the previous findings that different taxonomic groups are present in the rhizosphere of SA treated *J. vulgaris* plants in each generation (Chapter 3). However, this clearly contrasts our prediction that there will be functional redundancy in the microbial community. In the same experiment also plant biomass was measured (Chapter 2) and SA treated plants in all generations did better than the control plants showing that from the plant's perspective different microbial taxa with different gene expressions in the rhizosphere provided similar functions. Our findings are in contrast to studies (e.g. Burke et al., 2011; Liu et al., 2018; Liu et al., 2020) that mention that particular functions of the soil microbial community are often distributed across multiple microbial taxa and more closely resemble other studies that show that environmental changes can cause selection of both different taxa and functions in the soil microbial communities (Haggerty and Dinsdale, 2017). It is important to note that in our study, in each generation we placed a subset of the microbial community in a sterile background. This may have led to selection for microbes and consequently different functions in each generation.

At the gene ontology level, we mapped 13 SA-upregulated and six SA-downregulated GO terms that were expressed in the second, third and fourth generation. The proportion of significantly expressed GO terms was high, compared to the proportion of significantly expressed genes. This is because most of the functional genes in this study could not be annotated, while at the ontology level more reads were matched with a function. As the taxa significantly selected by SA differed strongly from generation to generation, it is notable that there we detected many significant GO terms that were found in multiple generations.

Our results show that activating SA resistance in the plant drives gene expression in the rhizosphere microbiome. However, whether SA application to the plant suppressed soil pathogenic microbes remains unproven in our study. SA induced resistance is often reported to play an important role in resistance to a broad range of microbial pathogens, such as bacteria, fungi and viruses (Murphy et al., 1999;

Gilliland et al., 2003; Mayers et al., 2005; Kundu et al., 2011; Li et al., 2019; Yuan et al., 2019). Interestingly, at the ontology level, we found up-regulated GO terms that were involved in viral (RNA) genome replication and viral processes, and these GO terms increased in importance over generations. These results indicate that viruses in the soil may play a role in SA-induced resistance of host plants against soil microbes. It is well known that the soil contains bacteriophages as well as virus controlling microbial pathogens (Duckworth and Gulig, 2002; Svircev et al., 2018; Jamal et al., 2019; Kortright et al., 2019; Rehman et al., 2019). However, their exact role in the rhizosphere microbiome is still poorly understood and further studies should examine these virus-microbe-plant interactions in more detail.

In conclusion, our study shows that application of SA to the plant *J. vulgaris* causes differential gene expression in the rhizosphere microbial community. However, our data also shows that these effects vary among plant generations. Plant-defense-soil microbe interactions may be regulated by viruses or viral phages.

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Supplementary data

Table S1 Log2 (FC) of 70 differentially expressed annotated genes, the expression of which are significantly altered by SA treatments in at least one generation in the rhizosphere of *J. vulgaris* plants in four generations. When Log2 (FC) is > 0, the gene is up-regulated in the SA treatment and when Log2 (FC) is < 0, the gene is down-regulated in the SA treatment. '-' indicates that the gene was not detected in the treatment; 'ns' represents the gene is not significantly altered by the SA treatment, but it is present. 1,2,3 and 4 represent the four generations.

Isoform ID	Functional gene name	1	2	3	4	SA down-/up-regulation	Go category	GO term	Homologous organisms	Function	Literature
TRINITY_DN187408_c0_g1_i1	PF05150.12^Legionella_OMP	-	-7.5	-	-	down	Biological process	adhesion of symbiont to host cell	bacteria	Major outer membrane protein; The attachment of a symbiont to a host cell via adhesion molecules, general stickiness etc., either directly or indirectly.	Jung et al., 2016; Hoppe et al., 2017; Younas et al., 2018.
TRINITY_DN237954_c0_g1_i1	PF05150.12^Legionella_OMP	-	-6.3	-	-	down	Biological process	adhesion of symbiont to host cell	bacteria	Major outer membrane protein; The attachment of a symbiont to a host cell via adhesion molecules, general stickiness etc., either directly or indirectly.	Jung et al., 2016; Hoppe et al., 2017; Younas et al., 2018.
TRINITY_DN2161_c0_g4_i1	PF00687.21^Ribosomal_L1	ns	ns	-5.1	-4.7	down	Biological process	cellular metabolic process	bacteria	Ribosomal protein L1 is the largest protein from the large ribosomal subunit, also can involve moleclar function of RNA binding and cellular component of large ribosomal subunit.	
TRINITY_DN116491_c0_g1_i1	PF00729.18^Viral_coat	-	-6.7	-	-	down	Biological process	virual process	virus	Involing with multi-organism process in which a virus is a participant. The other participant is the host. Includes infection of a host cell, replication of the viral genome, and assembly of progeny virus particles. In some cases the viral genetic material may integrate into the host genome and only subsequently, under particular circumstances, 'complete' its life cycle. Viral genome integration into host DNA.	Nandhagopal et al., 2002.
TRINITY_DN221013_c0_g1_i5	PF03863.13^Phage_mat-A	-	-7.7	-	ns	down	Biological process or cellular component	virion	bacteriophage	The process by which a virion attaches to a host cell by binding to a pilus on the host cell surface. The complete fully infectious extracellular virus particle.	Witherell et al., 1991.
TRINITY_DN342313_c0_g1_i2	PF03863.13^Phage_mat-A	ns	-	ns	-7.9	down	Biological process or cellular component	virion	bacteriophage	The process by which a virion attaches to a host cell by binding to a pilus on the host cell surface. The complete fully infectious extracellular virus particle.	Witherell et al., 1991.
TRINITY_DN497_c0_g1_i6	PF03863.13^Phage_mat-A	ns	ns	ns	-2.8	down	Biological process or cellular component	virion	bacteriophage	The process by which a virion attaches to a host cell by binding to a pilus on the host cell surface. The complete fully infectious extracellular virus particle.	Witherell et al., 1991.

TRINITY_DN2233_c0_g1_i1	PF03863.13^Phage_mat-A	ns	ns	ns	-1.5	down	Biological process or cellular component	virion	bacteriophage	The process by which a virion attaches to a host cell by binding to a pilus on the host cell surface. The complete fully infectious extracellular virus particle.	Witherell et al., 1991.
TRINITY_DN418_c0_g1_i14	PF00460.20^Flg_bb_rod	ns	ns	ns	-4.6	down	Cellular component	bacterial-type flagellum basal body, rod	bacteria	The central portion of the bacterial-type flagellar basal body, which spans the periplasm and threads through the rings.	
TRINITY_DN4152_c3_g1_i1	PF05736.11^OprF	-	ns	-4.3	ns	down	Cellular component	cell outer membrane	bacteria	This domain represents the presumed membrane-spanning region of the OprF proteins. This region is involved in channel formation and is thought to form an 8-stranded beta-barrel.	De Mot et al., 1994.
TRINITY_DN5762_c0_g1_i2	PF11645.8^PDDEXK_5	ns	ns	-	-3.7	down	Cellular component	integral component of membrane	bacteria	This family of endonucleases includes a group I intron-encoded endonuclease. This family belongs to the PD (D/E)XK superfamily	Bonocora and Shub, 2001.
TRINITY_DN21112_c0_g1_i1	PF03863.13^Phage_mat-A	-	-	ns	-5.6	down	Cellular component	membrane	bacteria	Flagellins polymerise to form bacterial flagella. This family includes flagellins and hook associated protein 3. Structurally this family forms an extended helix that interacts with PF00700.	Felix et al., 1999 ; Ramos et al., 2004; Beatson et al., 2006.
TRINITY_DN1559755_c0_g1_i1	PF00564.24^PB1	-	-	-6.1	ns	down	Cellular component	membrane		Phox and Bem1 (PB1) domains contain approximately 80 amino acids and are found in a number of cytoplasmic signaling proteins. A PB1 domain may form heterodimers with a paired PB1 domain, although not all PB1 domains will associate with one another. A highly conserved internal sequence known as OPR, PC or AID motifs is necessary for PB1 domain function. Regions outside the OPR, PC and AID help confer specificity for binding.	Ito et al., 2001; Moscat et al., 2006; Sumimoto et al., 2007.
TRINITY_DN4077_c1_g2_i1	PF00573.22^Ribosomal_L4	-	-4.7	ns	ns	down	Cellular component	ribosome	eubacteria	This family includes Ribosomal L4/L1 from eukaryotes and archaebacteria and L4 from eubacteria. L4 from yeast has been shown to bind rRNA.	Yeh and Lee, 1998.
TRINITY_DN4472_c0_g1_i5	PF00281.19^Ribosomal_L5	-	ns	ns	-6.8	down	Cellular component	ribosome			

TRINITY_DN164781_c0_g2_i2	PF03725.15^RNase_PH_C	-	ns	ns	-5.8	down	Molecular function	5'-3' exoribonuclease activity	bacteria	This family includes 3'-5' exoribonucleases. Ribonuclease PH contains a single copy of this domain, and removes nucleotide residues following the -CCA terminus of tRNA. Polyribonucleotide nucleotidyltransferase (PNPase) contains two tandem copies of the domain. PNPase is involved in mRNA degradation in a 3'-5' direction. The exosome is a 3'-5' exoribonuclease complex that is required for 3' processing of the 5.8S rRNA.	Mitchell et al., 1997.
TRINITY_DN41896_c0_g1_i1	PF05221.17^AdoHcyase	-	ns	-	-5.5	down	Molecular function	adenosylhomocysteinase activity		AdoHcyase is an enzyme of the activated methyl cycle, responsible for the reversible hydration of S-adenosyl-L-homocysteine into adenosine and homocysteine.	Sganga et al., 1992.
TRINITY_DN30386_c0_g2_i1	PF12081.8^GldM_N	-	-	ns	-5.7	down	Molecular function	ATP binding	bacteria	This domain is found in bacteria at the N-terminus of the GldM protein. This domain is typically between 169 to 182 amino acids in length. This domain has two completely conserved residues (Y and N) that may be functionally important. GldM is named for the member from *Cytophaga johnsonae* (*Flavobacterium johnsoniae*), which is required for a type of rapid gliding motility found in certain members of the *Bacteriodetes*.	Braun et al., 2005.
TRINITY_DN24155_c0_g2_i1	PF01471.18^PG_binding_1	-	ns	-	-6.0	down	Molecular function	binding	bacetria	Putative peptidoglycan binding domain. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation	Krogh et al., 1998; Steen et al., 2003; Briers et al., 2009.
TRINITY_DN1498_c0_g1_i2	PF01471.18^PG_binding_1	-	-	-	-3.2	down	Molecular function	binding	bacetria	Putative peptidoglycan binding domain. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation.	Krogh et al., 1998; Steen et al., 2003; Briers et al., 2009.
TRINITY_DN332299_c0_g1_i1	PF13620.6^CarboxypepD_reg	-	ns	ns	-5.9	down	Molecular function	carboxypeptidase activity			

TRINITY_DN664942_c0_g2_i1	PF00270.29^DEAD	-	-	-	-5.4	down	Molecular function	DEAD/H-box RNA helicase binding		The DEAD/DEAH box helicases are a family of proteins whose purpose is to unwind nucleic acids. The DEAD box helicases are involved in various aspects of RNA metabolism, including nuclear transcription, pre mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay and organellar gene expression.	Johnson and McKay, 1999.
TRINITY_DN16479_c0_g1_i8	PF01541.24^GIY-YIG	ns	-7.0	-7.1	-7.1	down	Molecular function	endonuclease activity	bacteriophage	It is involved in degradation of host DNA, permitting scavenging of host-derived nucleotides for phage DNA synthesis; in enzymes involved in DNA repair and recombination.	Sharma et al., 1992; Dunin-Horkawicz et al., 2006; Andersson et al., 2010.
TRINITY_DN4160_c0_g2_i3	PF01541.24^GIY-YIG	-	-6.4	ns	ns	down	Molecular function	endonuclease activity	bacteriophage	It is involved in degradation of host DNA, permitting scavenging of host-derived nucleotides for phage DNA synthesis; in enzymes involved in DNA repair and recombination.	Sharma et al., 1992; Dunin-Horkawicz et al., 2006; Andersson et al., 2010.
TRINITY_DN2301_c0_g4_i1	PF00006.25^ATP-synt_ab	-	-5.1	-	-	down	Molecular function	hydrolase activity or RNA binding	bacteria, archaea eukaryotes	Catalysis of the hydrolysis of various bonds, e.g. C-O, C-N, C-C, phosphoric anhydride bonds, etc. Hydrolase is the systematic name for any enzyme of EC class 3 or Interacting selectively and non-covalently with an RNA molecule or a portion thereof.	Wilkens et al., 2005.
TRINITY_DN1070_c1_g1_i1	PF13609.6^Porin_4	-	-5.7	-4.6	-4.0	down	Molecular function	porin activity	bacteria	Enables the transfer of substances, sized less than 1000 Da, from one side of a membrane to the other. The transmembrane portions of porins consist exclusively of beta-strands, which form a beta-barrel. They are found in the outer membranes of Gram-negative bacteria, mitochondria, plastids and possibly acid-fast Gram-positive bacteria.	Saier, 2000.
TRINITY_DN6228_c0_g1_i3	PF00989.25^PAS	-	-	-6.5	-5.9	down	Molecular function	protein binding	bacteria	L13are positioned at the amino terminus of signaling proteins such as sensor histidine kinases, cyclic-di-GMP synthases and hydrolases, and methyl-accepting chemotaxis proteins.	Henry and Crosson, 2011; Liu et al., 2015.

TRINITY_DN135_c0_g1_i7	PF13360.6^PQQ_2	ns	ns	ns	-2.5	down	Molecular function	pyrroloquinoline quinone binding		Interacting selectively and non-covalently with pyrroloquinoline quinone, PQQ, the coenzyme or the prosthetic group of certain alcohol dehydrogenases and glucose dehydrogenases.	
TRINITY_DN20104_c0_g1_i6	PF05919.11^Mitovir_RNA_pol	ns	-7.0	ns	-6.8	down	Molecular function	RNA-directed 5'-3' RNA polymerase activity	virus	Mitovirus RNA-dependent RNA polymerase proteins. The family also contains fragment matches in the mitochondria of *Arabidopsis thaliana*.	Hong et al., 1998; Yao et al., 2020.
TRINITY_DN264317_c0_g1_i1	PF03431.13^RNA_replicase_B	-	ns	-8.0	ns	down	Molecular function	RNA-directed 5'-3' RNA polymerase activity	virus	This family is of Leviviridae RNA replicases. The replicase is also known as RNA dependent RNA polymerase.	Fernando et al., 2020.
TRINITY_DN109372_c0_g1_i2	PF05919.11^Mitovir_RNA_pol	ns	-5.5	ns	-7.2	down	Molecular function	RNA-directed 5'-3' RNA polymerase activity	virus	Mitovirus RNA-dependent RNA polymerase proteins. The family also contains fragment matches in the mitochondria of *Arabidopsis thaliana*.	Hong et al., 1998; Yao et al., 2020.
TRINITY_DN5435_c0_g1_i4	PF05919.11^Mitovir_RNA_pol	-	-5.0	-	-7.3	down	Molecular function	RNA-directed 5'-3' RNA polymerase activity	virus	Mitovirus RNA-dependent RNA polymerase proteins. The family also contains fragment matches in the mitochondria of *Arabidopsis thaliana*.	Hong et al., 1998; Yao et al., 2020.
TRINITY_DN34032_c0_g1_i1	PF05919.11^Mitovir_RNA_pol	-	ns	-7.2	-6.6	down	Molecular function	RNA-directed 5'-3' RNA polymerase activity	virus	Mitovirus RNA-dependent RNA polymerase proteins. The family also contains fragment matches in the mitochondria of *Arabidopsis thaliana*.	Hong et al., 1998; Yao et al., 2020.
TRINITY_DN19010_c0_g4_i3	PF00978.21^RdRP_2	ns	-6.3	-	-	down	Molecular function	viral replication or RNA-directed RNA polymerase activity	virus	RNA-dependent RNA polymerase or RNA replicase is an enzyme that catalyzes the replication of RNA from an RNA template. Specifically, it catalyses synthesis of the RNA strand complementary to a given RNA template.	. Hardy et al., 1979; Boonrod et al., 2004; Schwan et al., 2005.
TRINITY_DN243032_c0_g1_i2	PF05919.11^Mitovir_RNA_pol	-	-	-	-6.3	down	Biological process	RNA-directed 5'-3' RNA polymerase activity	virus	Mitovirus RNA-dependent RNA polymerase proteins. The family also contains fragment matches in the mitochondria of *Arabidopsis thaliana*.	Hong et al., 1998; Yao et al., 2020.

TRINITY_DN2369_c0_g1_i4	PF00669.20^Flagellin_N	ns	ns	ns	-1.7	down	Cellular component	membrane	bacteria	Flagellins polymerise to form bacterial flagella. This family includes flagellins and hook associated protein 3. Structurally this family forms an extended helix that interacts with PF00700.	Felix et al., 1999; Ramos et al., 2004; Beatson et al., 2006.
TRINITY_DN306_c0_g2_i5	COG1278^Cold shock protein	ns	3.8	ns	ns	up	Biological process	Many biological processes like regulation of transcription, translation,		Response to stress, any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a cold stimulus, a temperature stimulus below the optimal temperature for that organism. Cold shock proteins are multifunctional RNA/DNA binding proteins, characterized by the presence of one or more cold shock domains. I	D'Amico et al., 2006; Williams et al., 2010; Lindquist and Mertens, 2018.
TRINITY_DN1057_c0_g1_i4	COG1278^Cold shock protein	ns	4.4	ns	-	up	Biological process	Many biological processes like regulation of transcription, translation,		Response to stress, any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a cold stimulus, a temperature stimulus below the optimal temperature for that organism. Cold shock proteins are multifunctional RNA/DNA binding proteins, characterized by the presence of one or more cold shock domains.	D'Amico et al., 2006; Williams et al., 2010; Lindquist and Mertens, 2018.
TRINITY_DN473649_c0_g1_i3	PF05551.11^zf-His_Me_endon	-	ns	-	5.8	up	Biological process	nucleic acid phosphodiester bond hydrolysis		Zinc-binding loop region of homing endonuclease. This domain is the short zinc-binding loops region of a number of much longer chain homing endonucleases.	Krishna et al., 2003.
TRINITY_DN3358_c0_g1_i2	PF05551.11^F25:F73n	-	-	-	5.9	up	Biological process	nucleic acid phosphodiester bond hydrolysis		Zinc-binding loop region of homing endonuclease. This domain is the short zinc-binding loops region of a number of much longer chain homing endonucleases. This domain is the short zinc-binding loops region of a number of much longer chain homing endonucleases.	Krishna et al., 2003.

TRINITY_DN422335_c0_g1_i1	PF01618.16^MotA_ExbB	-	ns	ns	3.3	up	Biological process	protein transport	bacteria	This family groups together integral membrane proteins that appear to be involved translocation of proteins across a membrane. These proteins are probably proton channels. MotA is an essential component of the flageller motor that uses a proton gradient to generate rotational motion in the flageller. ExbB is part of the TonB-dependent transduction complex. The TonB complex uses the proton gradient across the inner bacterial membrane to transport large molecules across the outer bacterial membrane	Braun et al., 1999.
TRINITY_DN1442_c0_g1_i12	PF13860.6^FlgD_ig	ns	ns	ns	2.5	up	Biological process	proteolysis	bacteria	This domain has an immunoglobulin like beta sandwich fold. It is found in the FlgD protein the flagellar hook capping protein. THe structure for this domain shows that it is inserted within a TUDOR like beta barrel domain	Kuo et al., 2008.
TRINITY_DN220624_c0_g1_i6	PF00072.24^Response_reg	-	-	ns	5.4	up	Biological Process	signal transduction		Response regulator receiver domain. This domain receives the signal from the sensor partner in bacterial two-component systems. It is usually found N-terminal to a DNA binding effector domain.	Pao and Saier, 1995.
TRINITY_DN6829_c0_g2_i1	PF03863.13^Phage_mat-A	-	ns	ns	2.2	up	Biological process or cellular component	virion	bacteriophage	The process by which a virion attaches to a host cell by binding to a pilus on the host cell surface. Or The complete fully infectious extracellular virus particle.	Witherell et al., 1991.
TRINITY_DN924_c0_g3_i1	PF00189.20^Ribosomal_S3_C	-	4.6	ns	-	up	Biological process or molecular function	cellular metabolic process or structural constituent of ribosome		The cellular metabolic process in which a protein is formed, using the sequence of a mature mRNA or circRNA molecule to specify the sequence of amino acids in a polypeptide chain. Translation is mediated by the ribosome, and begins with the formation of a ternary complex between aminoacylated initiator methionine tRNA, GTP, and initiation factor 2, which subsequently associates with the small subunit of the ribosome and an mRNA or circRNA. Translation ends with the release of a polypeptide chain from the ribosome or The action of a molecule that contributes to the structural integrity of the ribosome.	Burd and Dreyfuss, 1994.

TRINITY_DN5169_c0_g1_i4	PF12771.7^SusD-like_2	-	-	ns	2.4	up	Cellular component	cell outer membrane		SusD is a secreted starch-binding protein with an N-terminal lipid tail that allows it to associate with the outer membrane.	
TRINITY_DN520_c0_g2_i1	PF13715.6^CarbopepD_reg_2	-	ns	ns	2.7	up	Cellular component	integral component of membrane	bacteria, archaea or eukaryotes	This domain family is found in bacteria, archaea and eukaryotes, and is approximately 90 amino acids in length. The family is found in association with PF07715 and PF00593.	Cho and Salyers, 2001.
TRINITY_DN297806_c0_g2_i3	PF00669.20^Flagellin_N	ns	3.9	ns	ns	up	Cellular component	membrane	bacteria	Flagellins polymerise to form bacterial flagella. This family includes flagellins and hook associated protein 3. Structurally this family forms an extended helix that interacts with PF00700.	Felix et al., 1999; Ramos et al., 2004; Beatson et al., 2006.
TRINITY_DN429_c0_g2_i2	PF03401.14^TctC	-	-	-	2.2	up	Cellular component	membrane	bacteria	These probable extra-cytoplasmic solute receptors are strongly overrepresented in several beta-proteobacteria. This family, formerly known as Bug - Bordetella uptake gene (bug) product - is a family of bacterial tripartite tricarboxylate receptors of the extracytoplasmic solute binding receptor-dependent transporter group of families, distinct from the ABC and TRAP-T families	Antoine et al., 2003.
TRINITY_DN3530_c0_g1_i1	PF03458.13^UPF0126	-	-	ns	5.6	up	Cellular component	membrane	bacteria	This domain contains three transmembrane helices, with conserved glycine, which are suggestive of an ion channel. Proteins containing this domain have since been shown to be important in glycine utilizaion, and thus are predicted to be glycine transporters. This domain is always found as pair in such sequences.	Price et al., 2018.
TRINITY_DN1598_c0_g1_i1	PF07963.12^N_methyl	-	-	3.9	ns	up	Cellular component	membrane or integral component of membrane	prokaryotes	Prokaryotic N-terminal methylation motif. This short motif directs methylation of the conserved phenylalanine residue. It is most often found at the N-terminus of pilins and other proteins involved in secretion.	
TRINITY_DN653_c7_g1_i1	PF16320.5^Ribosomal_L12_N	-	ns	ns	2.9	up	Cellular component	ribosome		Catalysis of the hydrolysis of the terminal or penultimate peptide bond at the C-terminal end of a peptide or polypeptide.	
TRINITY_DN4687_c0_g1_i2	PF00466.20^Ribosomal_L10	-	ns	ns	4.5	up	Cellular component	ribosome		Catalysis of the hydrolysis of the terminal or penultimate peptide bond at the C-terminal end of a peptide or polypeptide.	

TRINITY_DN2568_c0_g1_i1	PF00466.20^Ribosomal_L10	-	-	-	5.4	up	Cellular component	ribosome		Catalysis of the hydrolysis of the terminal or penultimate peptide bond at the C-terminal end of a peptide or polypeptide.	
TRINITY_DN3585_c0_g1_i4	COG0402^deaminase	-	-	ns	3.8	up	Molecular function	deaminase activity		Catalysis of the removal of an amino group from a substrate, producing ammonia	
TRINITY_DN3461_c0_g3_i1	PF01471.18^PG_binding_1	-	3.5	-	5.7	up	Molecular function	binding	bacetria	Putative peptidoglycan binding domain. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation	Krogh et al., 1998; Steen et al., 2003; Briers et al., 2009.
TRINITY_DN3461_c0_g1_i1	PF01471.18^PG_binding_1	-	7.2	6.6	ns	up	Molecular function	binding	bacetria	Putative peptidoglycan binding domain. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation	Krogh et al., 1998; Steen et al., 2003; Briers et al., 2009.
TRINITY_DN64210_c0_g2_i1	PF01471.18^PG_binding_1	-	-	6.2	-	up	Molecular function	binding	bacetria	Putative peptidoglycan binding domain. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation	Krogh et al., 1998; Steen et al., 2003; Briers et al., 2009.
TRINITY_DN35_c0_g1_i1	PF01471.18^PG_binding_1	-	ns	ns	5.7	up	Molecular function	binding	bacetria	Putative peptidoglycan binding domain. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation	Krogh et al., 1998; Steen et al., 2003; Briers et al., 2009.
TRINITY_DN12161_c0_g4_i3	PF01193.24^RNA_pol_L	-	-	-	5.4	up	Molecular function	DNA-directed 5'-3' RNA polymerase activity or protein dimerization activity		The prokaryotic equivalent of the Rpb3/Rpb11 platform is the alpha-alpha dimer. The dimerisation domain of the alpha subunit/Rpb3 is interrupted by an insert domain (PF01000). Some of the alpha subunits also contain iron-sulphur binding domains	Zhang and Darst, 1998.
TRINITY_DN6187_c0_g1_i1	COG0302^GTP cyclohydrolase i	-	-	ns	3.4	up	Molecular function	GTP cyclohydrolase I activity	bacteria	This family includes GTP cyclohydrolase enzymes and a family of related bacterial proteins	Nar et al., 1995.
TRINITY_DN193314_c0_g1_i1	PF00557.24^Peptidase_M24	-	-	-	5.7	up	Molecular function	hydrolase activity	bacteria	This family contains metallopeptidases. It also contains non-peptidase homologues such as the N terminal domain of Spt16, which is a histone H3-H4 binding module.	Stuwe et al., 2008.
TRINITY_DN1632_c0_g1_i1	PF13328.6^HD_4	-	ns	ns	2.2	up	Molecular function	hydrolase activity		HD domains are metal dependent phosphohydrolases.	Aravind and Koonin, 1998.

TRINITY_DN2812_c0_g1_i4	COG0791^NLPP60 protein	-	-	ns	3.6	up	Molecular function	hydrolase activity		The function of this domain is unknown. It is found in several lipoproteins.	Anantharaman and Aravind, 2003.
TRINITY_DN9091_c0_g1_i1	PF02874.23^ATP-synt_ab	ns	ns	3.9	ns	up	Molecular function	hydrolase activity or RNA binding	bacteria, archaea eukaryotes	Catalysis of the hydrolysis of various bonds, e.g. C-O, C-N, C-C, phosphoric anhydride bonds, etc. Hydrolase is the systematic name for any enzyme of EC class 3 or Interacting selectively and non-covalently with an RNA molecule.	Wilkens et al., 2005.
TRINITY_DN1905_c1_g2_i1	PF03431.13^RNA_replicase_B	-	ns	8.2	-	up	Molecular function	RNA-directed 5'-3' RNA polymerase activity	virus	This family is of Leviviridae RNA replicases. The replicase is also known as RNA dependent RNA polymerase.	Fernando et al., 2020.
TRINITY_DN1761_c0_g2_i1	PF01819.17^Levi_coat	-	ns	ns	3.1	up	Molecular function	viral capsid	bacteriophage	The Levivirus coat protein forms the bacteriophage coat that encapsidates the viral RNA. 180 copies of this protein form the virion shell. The MS2 bacteriophage coat protein controls two distinct processes: sequence-specific RNA encapsidation and repression of replicase translation-by binding to an RNA stem-loop structure of 19 nucleotides containing the initiation codon of the replicase gene. The binding of a coat protein dimer to this hairpin shuts off synthesis of the viral replicase, switching the viral replication cycle to virion assembly rather than continued replication	Convery et al., 1998.
TRINITY_DN17868_c1_g1_i9	PF09984.9^DUF2222	-	-	ns	3.3	up				Domain of unknown function	
TRINITY_DN8253_c0_g1_i9	PF01595.20^DUF21	-	ns	ns	3.4	up				Domain of unknown function	

Table S2 The definition of GO terms in Fig. 7.

GO ID	GO term	SA regulation	Catergory	Definition
GO:0051704	multi-organism cellular process	up	Biological process	Interaction between organisms | physiological interaction between organisms | physiological interaction with another organism
GO:0044403	symbiotic process	up	Biological process	A process carried out by gene products in an organism that enable the organism to engage in a symbiotic relationship, a more or less intimate association, with another organism. Microscopic symbionts are often referred to as endosymbionts.
GO:0044764	multi-organism cellular process	up	Biological process	Any process that is carried out at the cellular level, which involves another organism of the same or different species.
GO:0019079	viral genome replication	up	Biological process	Any process involved directly in viral genome replication, including viral nucleotide metabolism.
GO:0039694	viral RNA genome replication	up	Biological process	The replication of a viral RNA genome.
GO:0039703	RNA replication	up	Biological process	The cellular metabolic process in which a cell duplicates one or more molecules of RNA.
GO:0016032	viral process	up	Biological process	A multi-organism process in which a virus is a participant. The other participant is the host. Includes infection of a host cell, replication of the viral genome, and assembly of progeny virus particles. In some cases, the viral genetic material may integrate into the host genome and only subsequently, under particular circumstances, 'complete' its life cycle.
GO:0006314	intron homing	up	Biological process	Lateral transfer of an intron to a homologous allele that lacks the intron, mediated by a site-specific endonuclease encoded within the mobile intron. It involves with cellular macromolecule metabolic and nucleic acid metabolic process
GO:0044033	multi-organism metabolic process	up	Biological process	A metabolic process - chemical reactions and pathways, including anabolism and catabolism, by which living organisms transform chemical substances, which involves more than one organism.
GO:0018144	RNA-protein covalent cross-linking	up	Biological process	The formation of a covalent cross-link between RNA and a protein. It involves in cellular protein modification.
GO:0039666	virion attachment to host cell pilus	up	Biological process	The process by which a virion attaches to a host cell by binding to a pilus on the host cell surface. Pili are retractile filaments that protrude from gram-negative bacteria. Filamentous viruses can attach to the pilus tip, whereas icosahedral viruses can attach to the pilus side.
GO:0033643	host cell part	up	Cellular Component	Any constituent part of a host cell. The host is defined as the larger of the organisms involved in a symbiotic interaction.
GO:0044217	other organism part	up	Cellular Component	Any constituent part of a secondary organism with which the first organism is interacting.
GO:0033646	host intracellular part	up	Cellular Component	Any constituent part of the living contents of a host cell; the matter contained within (but not including) the plasma membrane, usually taken to exclude large vacuoles and masses of secretory or ingested material. In eukaryotes it includes the nucleus and cytoplasm. The host is defined as the larger of the organisms involved in a symbiotic interaction.
GO:0033655	host cell cytoplasm part	up	Cellular Component	Any constituent part of the host cell cytoplasm, all of the contents of a cell excluding the plasma membrane and nucleus, but including other subcellular structures. The host is defined as the larger of the organisms involved in a symbiotic interaction.
GO:0044423	virion part	up	Cellular Component	Any constituent part of a virion, a complete fully infectious extracellular virus particle.
GO:0019028	viral capsid	up	Cellular Component	The protein coat that surrounds the infective nucleic acid in some virus particles. It comprises numerous regularly arranged subunits, or capsomeres.
GO:0033647	host intracellular organelle	up	Cellular Component	Organized structure of distinctive morphology and function, occurring within the host cell. Includes the nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes and the cytoskeleton. Excludes the plasma membrane. The host is

				defined as the larger of the organisms involved in a symbiotic interaction.
GO:0033648	host intracellular membrane-bounded organelle	up	Cellular Component	Organized structure of distinctive morphology and function, as found in host cells, bounded by a single or double lipid bilayer membrane and occurring within the cell. Includes the nucleus, mitochondria, plastids, vacuoles, and vesicles. Excludes the plasma membrane. The host is defined as the larger of the organisms involved in a symbiotic interaction.
GO:0033650	host cell mitochondrion	up	Cellular Component	A semiautonomous, self-replicating organelle as found in host cells that occur in varying numbers, shapes, and sizes in the cell cytoplasm. The host is defined as the larger of the organisms involved in a symbiotic interaction.
GO:0019029	helical viral capsid	up	Cellular Component	The protein coat that surrounds the infective nucleic acid in some virus particles; the subunits are arranged to form a protein helix with the genetic material contained within. Tobacco mosaic virus has such a capsid structure.
GO:0019030	icosahedral viral capsid	up	Molecular function	The protein coat that surrounds the infective nucleic acid in some virus particles; the subunits are arranged to form an icosahedron, a solid with 20 faces and 12 vertices. Icosahedral capsids have 12 pentamers plus 10(T-1) hexamers, where T is the triangulation number. Tobacco satellite necrosis virus has such a capsid structure.
GO:0039617	T=3 icosahedral viral capsid	up	Molecular function	The protein coat that surrounds the infective nucleic acid in some virus particles where the subunits (capsomeres) are arranged to form an icosahedron with T=3 symmetry. The T=3 capsid is composed of 12 pentameric and 20 hexameric capsomeres.
GO:0034062	5'-3' RNA polymerase activity	up	Molecular function	Catalysis of the reaction: nucleoside triphosphate + RNA (n) = diphosphate + RNA (n+1); the synthesis of RNA from ribonucleotide triphosphates in the presence of a nucleic acid template, via extension of the 3'-end.
GO:0097747	RNA polymerase activity	up	Molecular function	Catalysis of the reaction: nucleoside triphosphate + RNA (n) = diphosphate + RNA (n+1); the synthesis of RNA from ribonucleotide triphosphates in the presence of a nucleic acid template.
GO:0016779	nucleotidyltransferase activity	up	Molecular function	Catalysis of the transfer of a nucleotidyl group to a reactant. The upper group belongs to trasnfeerase activety.
GO:0003968	RNA-directed 5'-3' RNA polymerase activity	up	Molecular function	Catalysis of the reaction: nucleoside triphosphate + RNA (n) = diphosphate + RNA (n+1); uses an RNA template, i.e., the catalysis of RNA-template-directed extension of the 3'-end of an RNA strand by one nucleotide at a time.
GO:0001848	complement binding	up	Molecular function	Interacting selectively and non-covalently with any component or product of the complement cascade.
GO:0001846	opsonin binding	up	Molecular function	Interacting selectively and non-covalently with an opsonin, such as a complement component or antibody, deposited on the surface of a bacteria, virus, immune complex, or other particulate material.
GO:0001851	complement component C3b binding	up	Molecular function	Interacting selectively and non-covalently with the C3b product of the complement cascade.
GO:0051179	localization	down	Biological process	Any process, in which a cell, a substance, or a cellular entity, such as a protein complex or organelle, is transported, tethered to or otherwise maintained in a specific location. In the case of substances, localization may also be achieved via selective degradation.
GO:0051234	establishment of localization	down	Biological process	Any process that localizes a substance or cellular component. This may occur via movement, tethering or selective degradation.
GO:0006810	transport	down	Biological process	The directed movement of substances (such as macromolecules, small molecules, ions) or cellular components (such as complexes and organelles) into, out of or within a cell, or between cells, or within a multicellular organism by means of some agent such as a transporter, pore or motor protein.
GO:0044281	small molecule metabolic process	down	Biological process	The chemical reactions and pathways involving small molecules, any low molecular weight, monomeric, non-encoded molecule.

GO:1901362	organic cyclic compound biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of organic cyclic compound.
GO:0018130	heterocycle biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of heterocyclic compounds, those with a cyclic molecular structure and at least two different atoms in the ring (or rings).
GO:0009058	biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of substances; typically, the energy-requiring part of metabolism in which simpler substances are transformed into more complex ones.
GO:1901576	organic substance biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of an organic substance, any molecular entity containing carbon.
GO:0044249	cellular biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of substances, carried out by individual cells.
GO:1901564	organonitrogen compound metabolic process	down	Biological process	The chemical reactions and pathways involving organonitrogen compound.
GO:1901566	organonitrogen compound biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of organonitrogen compound.
GO:0071704	organic substance metabolic process	down	Biological process	The chemical reactions and pathways involving an organic substance, any molecular entity containing carbon.
GO:0044237	cellular metabolic process	down	Biological process	The chemical reactions and pathways by which individual cells transform chemical substances.
GO:0044271	cellular nitrogen compound biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of organic and inorganic nitrogenous compounds.
GO:1901135	carbohydrate derivative metabolic process	down	Biological process	The chemical reactions and pathways involving carbohydrate derivative.
GO:0019538	protein metabolic process	down	Biological process	The chemical reactions and pathways involving a protein. Includes protein modification.
GO:0044238	primary metabolic process	down	Biological process	The chemical reactions and pathways involving those compounds, which are formed as a part of the normal anabolic and catabolic processes. These processes take place in most, if not all, cells of the organism.
GO:0006807	nitrogen compound metabolic process	down	Biological process	The chemical reactions and pathways involving organic or inorganic compounds that contain nitrogen.
GO:0009059	macromolecule biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of a macromolecule, any molecule of high relative molecular mass, the structure of which essentially comprises the multiple repetitions of units derived, actually or conceptually, from molecules of low relative molecular mass.
GO:0034645	cellular macromolecule biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of a macromolecule, any molecule of high relative molecular mass, the structure of which essentially comprises the multiple repetition of units derived, actually or conceptually, from molecules of low relative molecular mass, carried out by individual cells.
GO:0019438	aromatic compound biosynthetic process	down	Biological process	The chemical reactions and pathways resulting in the formation of aromatic compounds, any substance containing an aromatic carbon ring.
GO:0008152	metabolic process	down	Biological process	Metabolic process resulting in cell growth | metabolism | metabolism resulting in cell growth | multicellular organism metabolic process | single-organism metabolic process
GO:1901360	organic cyclic compound metabolic process	down	Biological process	The chemical reactions and pathways involving organic cyclic compound.
GO:0006725	cellular aromatic compound metabolic process	down	Biological process	The chemical reactions and pathways involving aromatic compounds, any organic compound characterized by one or more planar rings, each of which contains conjugated double bonds and delocalized pi electrons, as carried out by individual cells.

GO:0034641	cellular nitrogen compound metabolic process	down	Biological process	The chemical reactions and pathways involving various organic and inorganic nitrogenous compounds, as carried out by individual cells.
GO:0046483	heterocycle metabolic process	down	Biological process	The chemical reactions and pathways involving heterocyclic compounds, those with a cyclic molecular structure and at least two different atoms in the ring (or rings).
GO:0043170	macromolecule metabolic process	down	Biological process	The chemical reactions and pathways involving macromolecules, any molecule of high relative molecular mass, the structure of which essentially comprises the multiple repetitions of units derived, actually or conceptually, from molecules of low relative molecular mass.
GO:0009056	catabolic process	down	Biological process	The chemical reactions and pathways resulting in the breakdown of substances, including the breakdown of carbon compounds with the liberation of energy for use by the cell or organism.
GO:0044260	cellular macromolecule metabolic process	down	Biological process	The chemical reactions and pathways involving macromolecules, any molecule of high relative molecular mass, the structure of which essentially comprises the multiple repetition of units derived, actually or conceptually, from molecules of low relative molecular mass, as carried out by individual cells.
GO:0065007	biological regulation	down	Biological process	Any process that modulates a measurable attribute of any biological process, quality or function.
GO:0006139	nucleobase-containing compound metabolic process	down	Biological process	Any cellular metabolic process involving nucleobases, nucleosides, nucleotides and nucleic acids.
GO:0033646	intracellular part	down	Cellular Component	Any constituent part of the living contents of a host cell; the matter contained within (but not including) the plasma membrane, usually taken to exclude large vacuoles and masses of secretory or ingested material. In eukaryotes it includes the nucleus and cytoplasm. The host is defined as the larger of the organisms involved in a symbiotic interaction.
GO:0016020	membrane	down	Cellular Component	A lipid bilayer along with all the proteins and protein complexes embedded in it an attached to it.
GO:0005737	cytoplasm	down	Cellular Component	All of the contents of a cell excluding the plasma membrane and nucleus, but including other subcellular structures.
GO:0005886	plasma membrane	down	Cellular Component	The membrane surrounding a cell that separates the cell from its external environment. It consists of a phospholipid bilayer and associated proteins.
GO:1990904	ribonucleoprotein complex	down	Cellular Component	A macromolecular complex containing both protein and RNA molecules.
GO:0032991	protein-containing complex	down	Cellular Component	A stable assembly of two or more macromolecules, i.e., proteins, nucleic acids, carbohydrates or lipids, in which at least one component is a protein and the constituent parts function together.
GO:0031224	intrinsic component of membrane	down	Cellular Component	The component of a membrane consisting of the gene products having some covalently attached portion, for example part of a peptide sequence or some other covalently attached group such as a GPI anchor, which spans or is embedded in one or both leaflets of the membrane.
GO:0016021	integral component of membrane	down	Cellular Component	The component of a membrane consisting of the gene products and protein complexes having at least some part of their peptide sequence embedded in the hydrophobic region of the membrane.
GO:0043228	non-membrane-bounded organelle	down	Cellular Component	Organized structure of distinctive morphology and function, not bounded by a lipid bilayer membrane. Includes ribosomes, the cytoskeleton and chromosomes.
GO:0005737	cytoplasmic part	down	Cellular Component	All of the contents of a cell excluding the plasma membrane and nucleus, but including other subcellular structures.
GO:0043228	non-membrane-bounded organelle	down	Cellular Component	Organized structure of distinctive morphology and function, not bounded by a lipid bilayer membrane. Includes ribosomes, the cytoskeleton and chromosomes.
GO:0005840	ribosome	down	Cellular Component	An intracellular organelle, about 200 A in diameter, consisting of RNA and protein. It is the site of protein biosynthesis resulting from translation of messenger RNA (mRNA). It

				consists of two subunits, one large and one small, each containing only protein and RNA.
GO:0043226	organelle	down	Cellular Component	Organized structure of distinctive morphology and function. Includes the nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes and the cytoskeleton, and prokaryotic structures such as anammoxosomes and pirellulosomes. Excludes the plasma membrane.
GO:0043229	intracellular organelle	down	Cellular Component	Organized structure of distinctive morphology and function, occurring within the cell. Includes the nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes and the cytoskeleton. Excludes the plasma membrane.
GO:0043168	anion binding	down	Molecular function	Interacting selectively and non-covalently with anions, charged atoms or groups of atoms with a net negative charge.
GO:0043167	ion binding	down	Molecular function	Interacting selectively and non-covalently with ions, charged atoms or groups of atoms.
GO:0097367	carbohydrate derivative binding	down	Molecular function	Interacting selectively and non-covalently with a carbohydrate derivative.
GO:0032553	ribonucleotide binding	down	Molecular function	Interacting selectively and non-covalently with a ribonucleotide, any compound consisting of a ribonucleoside that is esterified with (ortho) phosphate or an oligophosphate at any hydroxyl group on the ribose moiety.
GO:0032555	purine ribonucleotide binding	down	Molecular function	Interacting selectively and non-covalently with a purine ribonucleotide, any compound consisting of a purine ribonucleoside that is esterified with (ortho) phosphate or an oligophosphate at any hydroxyl group on the ribose moiety.
GO:0032557	pyrimidine ribonucleotide binding	down	Molecular function	Interacting selectively and non-covalently with a pyrimidine ribonucleotide, any compound consisting of a pyrimidine ribonucleoside that is esterified with (ortho) phosphate or an oligophosphate at any hydroxyl group on the ribose moiety.
GO:0035639	purine ribonucleoside triphosphate binding	down	Molecular function	Interacting selectively and non-covalently with a purine ribonucleoside triphosphate, a compound consisting of a purine base linked to a ribose sugar esterified with triphosphate on the sugar.
GO:0003677	DNA binding	down	Molecular function	Any molecular function by which a gene product interacts selectively and non-covalently with DNA (deoxyribonucleic acid).
GO:0016787	hydrolase activity	down	Molecular function	Catalysis of the hydrolysis of various bonds, e.g., C-O, C-N, C-C, phosphoric anhydride bonds, etc. Hydrolase is the systematic name for any enzyme of EC class 3.
GO:0016818	phosphorus-containing anhydrides	down	Molecular function	Catalysis of the hydrolysis of any acid anhydride, which contains phosphorus.
GO:0016462	pyrophosphatase activity	down	Molecular function	Catalysis of the hydrolysis of a pyrophosphate bond between two phosphate groups, leaving one phosphate on each of the two fragments.
GO:0017111	nucleoside-triphosphatase activity	down	Molecular function	Catalysis of the reaction: a nucleoside triphosphate + H2O = nucleoside diphosphate + phosphate.
GO:0005488	binding	down	Molecular function	The selective, non-covalent, often stoichiometric, interaction of a molecule with one or more specific sites on another molecule.
GO:0097159	organic cyclic compound binding	down	Molecular function	Interacting selectively and non-covalently with an organic cyclic compound, any molecular entity that contains carbon arranged in a cyclic molecular structure.
GO:1901363	heterocyclic compound binding	down	Molecular function	Interacting selectively and non-covalently with heterocyclic compound.
GO:0003824	catalytic activity	down	Molecular function	Catalysis of a biochemical reaction at physiological temperatures. In biologically catalyzed reactions, the reactants are known as substrates, and the catalysts are naturally occurring macromolecular substances known as enzymes. Enzymes possess specific binding sites for substrates, and are usually composed wholly or largely of protein, but RNA that has catalytic activity (ribozyme) is often also regarded as enzymatic.
GO:0000166	nucleotide binding	down	Molecular function	Interacting selectively and non-covalently with a nucleotide, any compound consisting of a nucleoside that is esterified with (ortho) phosphate or an oligophosphate at any hydroxyl group on the ribose or deoxyribose.

GO:1901265	nucleoside phosphate binding	down	Molecular function	Interacting selectively and non-covalently with nucleoside phosphate.
GO:0043169	cation binding	down	Molecular function	Interacting selectively and non-covalently with cations, charged atoms or groups of atoms with a net positive charge.
GO:0046872	metal ion binding	down	Molecular function	Interacting selectively and non-covalently with any metal ion.

Chapter 5

The negative effect of soil microorganisms on plant growth only extends to the first weeks

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Abstract

Soil biotic communities can strongly impact plant performance. Many plant species grow worse in live soil than in sterilized soil. So far, most studies on plant-soil-interactions have estimated the effect of the soil microbial community on plant mass after a fixed duration of plant growth. However, these interactions may change over time and several studies have argued that plant-soil interactions are more important for young seedlings than for older plants. In this paper we ask the question: how long-lasting is the effect of the soil microbial community on plant growth and we focus on relative plant growth rates at three stages: early growth (0-21 days), mid growth (22 to 42 days) and late growth (43 to 63 days). This is important as a plant with a reduced relative growth rate early in life, due to negative effects of the soil microbial community, may increase less in biomass for a much longer period, even though the relative growth rates do not differ anymore. We performed two growth experiments with *Jacobaea vulgaris* lasting 49 and 63 days. Plants were grown in sterilized soil or in sterilized soil inoculated with natural dune soil. In both experiments, differences in biomass of plants grown in sterilized soil and inoculated soil (live soil) increased throughout the experiment. Interestingly, linear regression models testing the relationship between *ln* transformed dry weight and time for younger plants and for older plants in sterilized soil and live soil, respectively, showed that the relative growth rate of plants in the sterilized soil was only significantly higher than that of plants in live soil in the first 2-3 weeks. After that period, there was no negative effect of live soil on plant relative growth rate anymore. In a third experiment, we examined the effect of the timing of soil inoculation prior to planting on the relative growth rate of *J. vulgaris* plants with four different timing treatments. Plant biomass was reduced in all inoculated soils compared to the sterilized soil. With increasing time between inoculation and planting, plant biomass decreased. Again, in all inoculated soils the negative effect of the soil microbial community on plant growth disappeared during the first weeks after planting. Overall, our results show that plants grow less well in live soil than in sterilized soil. The negative effects of soil inoculation on plant mass appear to extend over the whole growth period but arise from the negative effects on relative growth rates that occur in the first weeks after planting when plants have only less than 5% of the mass they obtained after 42 days. Our study highlights the

importance of examining relative growth rates rather than final biomass to estimate the effects of soil microbial communities on plants.

Keywords

Dry plant biomass, Growth analysis, *Jacobaea vulgaris,* Live soil, Plant performance, Plant-soil interactions, Pathogenic soil microbial community, Relative growth rate, Sterilized soil

Introduction

Interactions between plants and soil microbial communities are vital in mediating the balance and functioning of terrestrial ecosystems (Bever, 1994; Churchland and Grayston, 2014; Teste et al., 2017; Erktan et al., 2018). The soil microbiome is an important driver of plant performance. Soil microbial species e.g. pathogenic organisms, plant-growth-promoting rhizobacteria (PGPR, such as *Pseudomonas* and *Burkholderia*) and arbuscular mycorrhizal fungi (AMF), play an active role in modifying the development of plants (Johnson et al., 1997; Arora and Mishra, 2016; Artursson et al., 2016; Gil-Martinez et al., 2018). The effect of the soil microbial community on plant growth in laboratory experiments is often negative (Mangan et al., 2010; van de Voorde et al., 2012; Cortois et al., 2016).

One potential explanation for the negative effect of soil microbes on plant performance is that microbes and plants compete for nutrients. Alternatively, pathogens may accumulate in the soil over time, eventually resulting in a negative overall effect on plant performance (Dobson and Crawley, 1994; Wardle et al., 2004; Raaijmakers et al., 2009; Mordecai, 2011; van der Putten et al., 2013; Jacoby et al., 2017). In the previous chapters, we showed in experiments with ample nutrient supply that the negative effect of the soil microbial community on plant growth is mitigated if the plant's defense system is activated by foliar application of salicylic acid. This led us to hypothesize that the negative effect of the soil microbial community on plant growth in our system is due to an overall pathogenic effect of the soil microbial community. Although this effect was consistent, we did not find this effect to increase over several generations of plant growth (Chapter 2). An important question is therefore how long the negative effects of the soil microbial community on plant growth lasts.

So far, most studies on the effect of the soil microbial community on plant growth are conducted in pots (Hodge and Fitter, 2013). In such experiments, the negative effects of any treatment on plant mass often decline after some period of plant growth (typically six to eight weeks) (Bezemer et al., 2018; Dudenhöffer et al., 2018). This is often attributed to restricted root growth due to limitations in pot size, or to a decline in nutrient availability, and therefore considered an artefact of the experimental design

(Smith and Reynolds, 2012; Van de Voorde et al., 2012; Jing et al., 2015). It is also possible, however, that the pathogenic effect of the soil microbial community only last for a short period because (1) only seedlings are susceptible or (2) because over time plants alter the composition of the microbial community in the soil in which they grow so that it becomes less harmful (Bezemer et al., 2018; Dudenhöffer et al., 2018).

Previous studies on plant-soil-interactions typically focus on the effect of the soil microbial community on final plant biomass (van de Voorde et al., 2012; Bezemer et al., 2013; Anacker et al., 2014). It is important to note, however, that the effects of the soil microbial community on plant growth depend on the life stages of the plant (Arrigoni et al., 2018; Bezemer et al., 2018; Dudenhöffer et al., 2018). Seedlings are often highly vulnerable and susceptible to pathogenic microbes in the soil (Packer and Clay, 2000). In contrast, older plants with a more developed root system are typically less vulnerable (Kardol et al., 2013; Bezemer et al., 2018).

Effects on plant growth that occur during early life stages can still affect plant size and plant phenology in late life stages. When plants after some period grow with a similar relative growth rate, differences in absolute plant mass will still continue to increase. In Fig. 1 it is assumed that plants in sterilized soil grow with a constant relative growth rate (red line). Plants in live soil either grow with a constant relative growth rate lower than that of the plants in the live soil (green line) or they first grow with a lower relative growth rate but after an initial period (t_1) their relative growth rate becomes similar to that of plants in the sterilized soil (blue line). In the latter case, although the effect of the soil microbial community only is present until t_1, differences in plant mass still continue to increase (Fig. 1b). Hence, to study the effect of soil microbes on plants, it is important to analyze relative growth rates.

In this study, we used linear regression models and *ln* transformed biomass data from repeated harvests to estimate relative growth rates in sterilized and live soil. We hypothesized that i) relative growth rates of plants are smaller in live soil than in sterilized soils ii) the negative effect on relative growth rates lasts only for a short period during the early plant life stages; and iii) the differences in plant mass between

plants grown in live soils and sterilized soils will continue to increase during the experiment.

We used *Jacobaea vulgaris* to test these hypotheses. *J. vulgaris* is native to The Netherlands. In a former experiment, we found that the plant mass of *J. vulgaris* growing in soil containing a live microbial community was 66% lower than when plants were grown in sterilized soil (Jing et al., Chapter 2). This negative effect of live soil on plant growth is in line with previous findings (e.g. van de Voorde et al., 2012; Kos et al., 2015; Wang et al., 2019). In the present study, to avoid nutrient limitation during the growth of *J. vulgaris*, nutrients were supplied regularly according to estimates of nutrient demand obtained from previous experiments (Steiner, 1980; Joosten et al., 2009). We carried out growth experiments with multiple harvesting points to estimate changes in relative growth rates in live and sterilized soils. Additionally, we grew *J. vulgaris* plants in soil that had been inoculated with live soil at varying time points before planting to manipulate the abundance of the microbial community in the soil. With the latter experiment we aimed to examine how the timing of inoculation into sterilized soil impacts the growth of *J. vulgaris*.

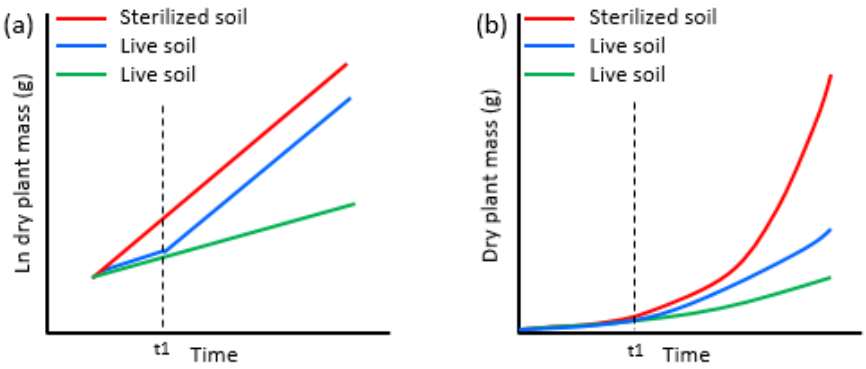

Fig. 1 Conceptual figures showing relative growth rate and plant mass of *J. vulgaris* in both sterilized soil and live soil over time. (a) The relative growth rate in sterilized soil (red line) is higher than that in live soil (green line) (hypothesis 1) and this difference is maintained during the entire plant growth period. The blue line indicates

an initial lower relative growth rate of plants in the live soil but at t_1 these plants obtain an equal relative growth rate as plants in the live soil (hypothesis 2). In Fig. 1a the y-axis denotes *ln* transformed plant mass. In Fig. 1b the y-axis denotes absolute plant mass. Note that even when relative growth rates become equal after an initial difference in relative growth rate in the early stage of life (the red line and blue line in Fig. 1a) the difference in absolute biomass continues to increase after that period (the red line and blue line in Fig. 1b).

Materials and methods

J. vulgaris (common ragwort) was used as plant species. We chose this species because it is a common species in The Netherlands that is strongly affected by plant-soil interactions (van de Voorde et al., 2011; van de Voorde et al., 2012; Bezemer et al., 2013). Seeds and soil were collected from Meijendel, a calcareous sandy dune area north of The Hague, The Netherlands (52°11´N, 4°31´E).

Seeds

Before seed germination, all seeds were shaken for 2 min in 70% ethanol, then washed with sterilized water, put for 12 min in 2% bleach, and finally rinsed four times with sterilized water to avoid influences of seed-borne microbes. The surface-sterilized seeds were then placed in standard Petri dishes containing filter paper, which was moistened with Milli-Q water. Afterwards, all Petri dishes containing seeds were placed in plastic zip-lock bags and stored in a climate room (relative humidity 70%, light 16 h at 20°C, dark 8 h at 20°C) for the duration of germination.

Soil

At Meijendel, the topsoil was collected to a depth of 15 cm after removing the grassland vegetation and the organic layer of the surface. The soil was sieved using a 5 mm sized mesh to remove plant roots and various soil fauna, homogenized with a concrete mixer and then stored into 20-liter plastic bags (Nasco Whirl-Pak Sample Bag). Bags were either sterilized by 35-K Gray gamma-irradiation (Synergy Health Company, Ede, The Netherlands) or kept at 4°C for inoculation. Potting soil

(Slingerland potgrond, Zoeterwoude, The Netherlands) was also sterilized by 35-K Gray gamma-irradiation.

Plant growth

After germination, seedlings were randomly transferred individually to 500 ml pots containing either "sterilized soil" or "live soil". The live soil treatment consisted of a mixture of 87.5% sterilized dune soil, 2.5% sterilized potting soil and 10% live soil. The sterilized soil treatment contained 97.5% of sterilized dune soil and 2.5% of sterilized potting soil. Sterilized potting soil was added to all pots to increase the organic matter content of the soil. Sterilized soil and live soil were kept in bags and left in the climate room for 14 days (relative humidity 70%, light 16h at 20°C, dark 8h at 20°C) to enable the establishment of microbial communities in the inoculated soil before potting. Before filling the pots, the soil in each bag was mixed. After filling, pots were randomly distributed over the climate room. Plants were watered regularly with Milli-Q water and 5 ml Steiner nutrient solution was added per plant on day 7, 10 ml Steiner nutrient solution was added on day 13, and 20 ml Steiner nutrient solution was added on days 19, 28, 37, and 42. The Steiner nutrient solution (Steiner, 1980) was prepared from 7 different stock solutions (106.2 g $Ca(NO_3)_2 \cdot 4H_2O$, 29.3 g KNO_3, 13.6 g KH_2PO_4, 49.2 g $MgSO_4 \cdot 7H_2O$, 25.2 g K_2SO_4 and 2.24 g KOH, 3.29 g Fe-EDTA added to 1 liter demineralized water, and a stock solution with micro elements (a mixed solution of 0.181 g $MnCl_2 \cdot 4H_2O$, 0.286 g H_3BO_3, 0.022 g $ZnSO_4 \cdot 7H_2O$, 0.0078 g $CuSO_4 \cdot 5H_2O$ and 0.0126 g $NaMoO_4 \cdot 2H_2O$ added to 1 liter demineralized water). Ten ml of each stock solution was diluted in 1 liter of demineralized water before use.

The effect of live soil on the growth of *J. vulgaris*

Experiment 1: An experiment to measure the growth of *J. vulgaris* overtime was performed starting with 1-week-old seedlings, two soil treatments and eight harvesting time points over seven weeks. The harvests were on days 0 (1-week-old seedlings), 7, 14, 21, 28, 35, 42 and 49 after planting. Pots were randomly labeled and allocated to the different harvests. Ten replicates were used for each treatment resulting in 2 treatments × 8 harvesting points × 10 replicates = 160 plants. Harvested

plants (shoots and roots) were oven-dried at 60°C for approximately one week and dry mass was determined.

Experiment 2: The growth experiment was repeated using the same soil treatments, but with more harvests during the first 3 weeks. In this experiment, plants were harvested at day 0, 3, 6, 9, 12, 15, 18, 21, 28, 35, 42, 49, 56 and 63 after planting. Ten plants per soil treatment were harvested at each harvesting time point thus resulting in 2 treatments × 14 harvest points × 10 replicates = 280 plants. In this experiment, at each harvest, the plants were gently removed from the pot. Shoots were separated from roots with a pair of scissors just above the root crown, and roots were cleaned with water and then put into aluminum foil. Then, all the harvested plant parts were freeze-dried for approximately one week, and dry mass was determined.

The effect of time of inoculation on the growth of *J. vulgaris*

To examine the effect of the timing of soil inoculation on the relative growth rate of *J. vulgaris* plants, sterilized soils were inoculated at different time points prior to planting the seedlings. In this experiment, 1-week-old seedlings were planted into 500 ml pots containing either “sterilized soil” or four different “live soil” treatments. For these four treatments, a mixture of 10% of live soil was mixed with 90% sterilized soil, and then the mixed soil was kept in the climate room for 0, 1, 2 and 4 weeks (relative humidity 70%, light 16h at 20°C, dark 8h at 20°C) to enable different build-up times for the microbial community in the soil at the time of planting. The live soil treatments were labeled as “live-0”, “live-1”, “live-2” and “live-4” respectively. Seedlings were randomly distributed over the five soil treatments and nine harvests over six weeks. Plants were harvested on days 0 (as seedlings), 4, 8, 12, 16, 20, 28, 35 and 42. Eight replicates were used per treatment combination, resulting in 5 treatments × 9 harvests × 8 replicates totaling 360 plants. Fresh weight was recorded, because leaves were frozen immediately as we intended to quantify the levels of SA in the plant material. However, due time limitations these data have not been collected.

Calculations and statistical analyses

Biomass was plotted against time for plants grown in sterilized and live soil. A student t-test was then performed to test for differences between dry plant mass in sterilized and live soils at each time point. *Ln* transformed biomass was also plotted against time. Plant growth was divided into three stages: early growth (0-21 days), mid growth (22 to 42 days) and late growth (43 to 63 days). For each experiment, a separate line was then fitted through the dry plant mass data for these different periods. Late growth was only measured in experiment 2. Because this division in two time periods is somewhat arbitrary, we backed this analyses up with a sequential backward regression approach for the entire growth period for each experiment. We started this analysis with the two latest harvesting points and then sequentially added the previous data point. In this way we could test for which time periods differences in relative growth rate were significant. For each regression the slope and standard error (SE) of the slope were determined and differences between the slopes for the linear regression models in sterilized and live soil were then tested with a *t*-test in Excel, $t = \frac{\text{Slope1}-\text{Slope2}}{\text{SQRT}\,(SE1^2+SE2^2)}$.

Results

The effect of live soil on the growth of *J. vulgaris*

Experiment 1: Soil inoculation had a strong negative effect on plant dry mass throughout the experiment (Fig. 2a). The difference in plant dry mass between the sterilized and live soil treatments increased during the entire experiment. From day 21 onward, the dry plant mass of *J. vulgaris* in sterilized soil was significantly larger than the dry mass of plants grown in live soil (Fig. 2). For young plants (0-21 days) the relative growth rate (slope in Fig. 2b, c) in sterilized soil was significantly larger than that for live soil while relative growth rates did not differ for mid-aged plants (22-49 days, Fig. 2b, c). This result was backed up by the sequential backward regression that showed that the relative growth rates were not significantly different for the periods between 22 and 49 days (Table S1).

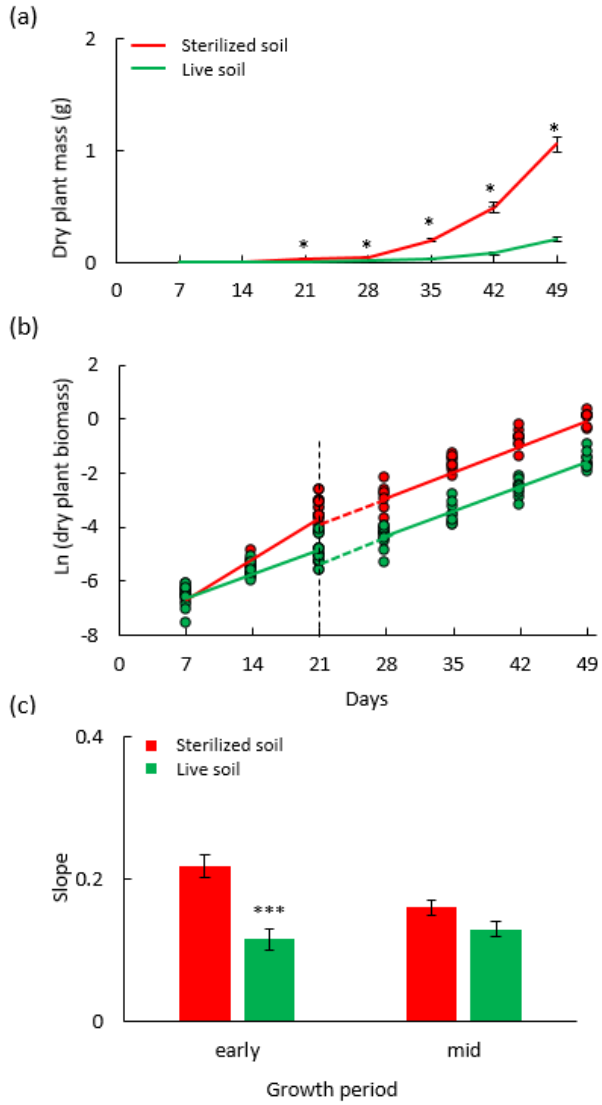

Fig. 2 Experiment 1. (a) Mean (± SE) biomass of *J. vulgaris* in sterilized and live soil over 49 days. For each time point, differences between the biomass of the plants in the two soils were tested for significance with a t-test, * indicates a significant difference ($P < 0.05$). (b) Two linear regression models (early: day 7-21, and mid: day 28-49) of *ln* transformed biomass of *J. vulgaris* in both sterilized and live soil. The extrapolated dashed parts of the lines are based on the linear regression models for day 28-49. (c) Slopes (mean± SE) of the regression lines in (b). Differences between

the slopes for live-soil and control soil were tested for significance with a-test. *** indicates $P < 0.001$.

Experiment 2: The first experiment was repeated with more harvesting points during the first 21 days and an extended growth period. Again, the effect of live soil on plant growth was negative (Fig. 3a). The difference in absolute plant biomass increased until day 56. Young plants (0-21 days) had significantly higher relative growth rates in sterilized soil, mid-aged plants (22-42 days) had similar relative growth rates; while for older plants (49-63 days) the relative growth rates were even higher in live soil (Fig. 3b, c). Backward regression showed that the relative growth rate was higher for the plants in live soil for the period 63-28 days. If younger ages are included in the analysis, differences are no longer significant (Table S2).

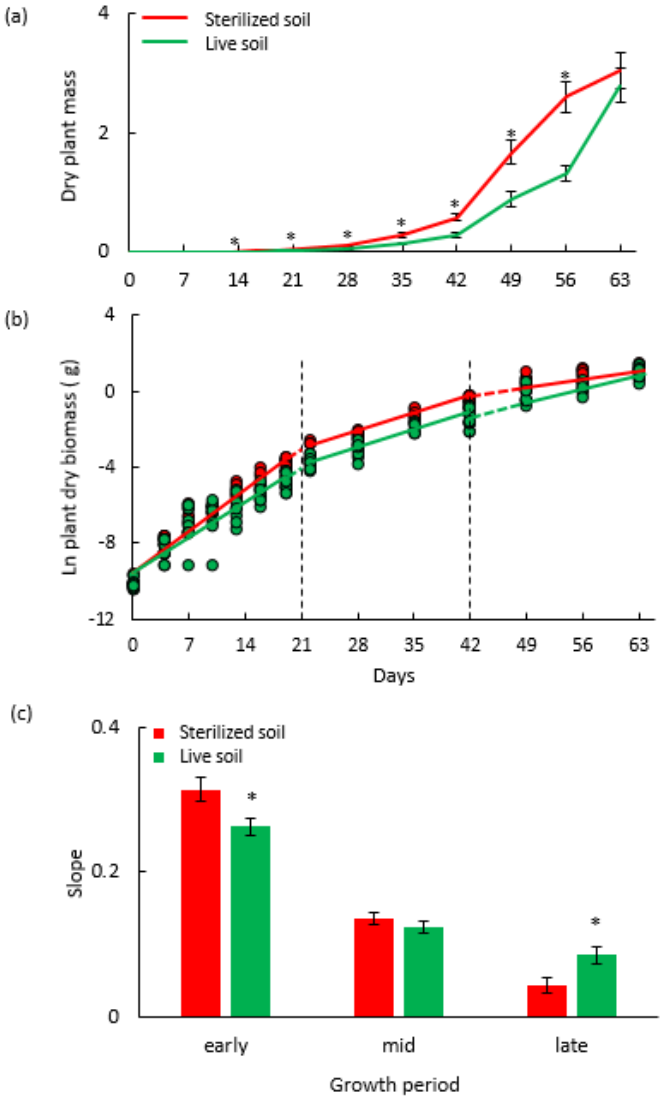

Fig. 3 Experiment 2. (a) Mean (± SE) of dry plant mass of *J. vulgaris* in sterilized and live soil over 63 days. For each time point, differences between the biomass of the plants in the two soils were tested for significance with a t-test, * indicates a significant difference at $P < 0.05$. (b) Three linear regression models (early: day 0-19, mid: 22-42, late: 49-63) of *ln* transformed biomass of *J. vulgaris* in both sterilized and live soil. The two wztrapolated dashed parts of the lines are based on the linear regression models for day 0-19 and 49-63 (c) Mean slope (± SE) of the linear

regression lines in (b). Differences between the slopes for live soil and sterilized soil were tested for significance with a t-test. * indicates $P < 0.05$.

The effect of time of inoculation before planting on the growth of *J. vulgaris*

Experiment 3: Plants produced less biomass in inoculated soils than in sterilized soil (Fig. 4a). For young plants (0-21 days) the relative growth rate in sterilized soil was significantly larger than that for live-0, live-1, live-2 or live-4 soil. Relative growth rates did not differ for mid-aged plants between live-0, live-1 and live-2 soil. Interestingly, relative growth rates of plants from live-4 soil for the mid-aged period were significantly higher than the relative growth rate of plants in sterilized soil (Fig. 3c; Table S3). Timing of the inoculation did affect the relative growth rates of plants in the early phase (0-21 days). The longer the time between inoculation and planting the lower the relative growth rate of young plants was ($R^2 = 0.99$, $P < 0.05$, $df = 3$). This was no longer true for the mid-aged period ($R^2 = 0.71$, $P = 0.15$, $df = 3$). These results were largely backed up by the backward sequential regression, which showed that relative growth rates were only higher for plants grown in the sterilized soil if very young plant ages were included. Especially for the live-4 soil the relative growth rate was even higher for plants grown in live soil when only older plants were included (Table S3).

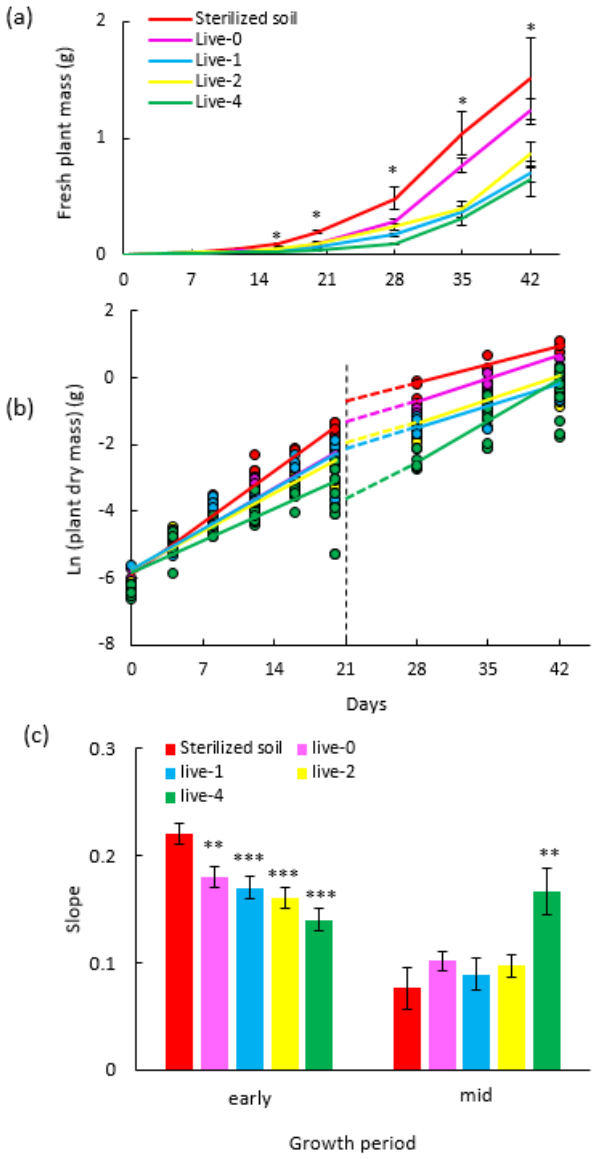

Fig. 4 Experiment 3. Plant growth of *J. vulgaris* in sterilized soil and in live soil 0, 1, 2 or 4 weeks before planting (live-0, live-1, live-2 and live-4). (a) Mean (± SE) fresh biomass of *J. vulgaris* in sterilized and live soil over 42 days. For each time point differences between the biomass of the plants in the sterilized soil and overall live soil (combining four live soils as an overall live soil treatment) were tested for significance with a t-test, * indicates a significant difference at $P < 0.05$. (b) Two linear regression

models (early: 0-20, mid: 28-42) of *ln* transformed fresh biomass of *J. vulgaris* in sterilized soil and four live soils. The extrapolated dashed parts of the lines are based on the linear regression models of day 28-42. (c) Mean slope (± SE) of linear lines in (b). Differences between the slopes for live-soil and sterilized soil were tested for significance with a t-test. *** indicates significant difference at $P < 0.001$; ** indicates significant difference at $P < 0.01$.

Discussion

In this study, we report the results of three experiments in which we measured the growth of *J. vulgaris* to test how the effects of soil microbial communities on plant growth change over time. We found a consistent negative effect of the soil microbial community in all three experiments. Biomass was larger in sterilized soil than in live soil. However, analyses of the *ln* transformed data, show that the relative growth rates were significantly higher in sterilized soil than in live soil only for young plants, and not for mid-aged plants. Moreover, in experiment 2, which was continued for a longer period, older plants even had a higher relative growth rate in the live soil. Hence, all data sets showed that the negative effects of soil inoculation on plant mass appear to extend during a long period, but arise from the negative effects that occur in the first weeks after planting when plants have only obtained less than 5% of the mass they obtain after 42 days.

We observed a consistent negative effect of live soil containing a natural soil microbial community in all three experiments. It is plausible that this was due to a net pathogenic effect of the soil microbial community on plant growth (Klironomos, 2002; Joosten et al., 2009; Harrison and Bardgett, 2010; Cortois et al., 2015). This hypothesis has been widely verified in other studies. For example, bacterial microbes such as *Ralstonia solanacearum, Agrobacterium tumefaciens*, *Erwinia amylovora and Streptomyces scabies* have been frequently isolated from natural soils (Curl et al., 1998; Michel et al., 1998; Gómez et al., 2017; Sharifazizi et al., 2017). These pathogenic microbes can adversely affect plant health and production (Huang et al., 2013; Cesarano et al., 2017). Several studies have indicated that soil microbes

compete with plants for available nutrients in the soil, and this could also result in negative effects on plant growth in inoculated soil (Bardgett et al., 2003; Fontaine et al., 2003; Dunn et al., 2006). However, in our study, we grew plants in a nutrient-rich environment by supplying a nutrient solution, and hence we argue that it is unlikely that the negative effect of live soil on plant growth was due to plant-microbe competition for nutrients. In Chapter 2, application of SA mitigated the negative effects of the live soil on the growth of *J. vulgaris*, in combination with the fact that activation of SA-dependent signaling pathways leads to the expression of pathogenesis-related proteins (PRP) contributing to resistance (Glazebrook, 2005; Spoel et al., 2007), this together suggests that the negative soil effect on plant growth was due to microbial pathogens.

Our study exemplifies that the negative effects of soil inoculation on plant mass can extend over the entire growth period, even though the differences are due to negative effects that occur during the first weeks after planting. There are several explanations for the observation that older plants do not exhibit a negative response to live soils. First, younger plants or seedlings may be more vulnerable and susceptible to pathogenic microbes in the soil than older plants with well-developed root systems (Packer and Clay, 2000). Root development plays an important role for plants in suppressing soil-borne pathogens (Watt et al., 2006; Emmett et al., 2014), and is correlated with soil abiotic or biotic characteristics (Kardol et al., 2013; Arrigoni et al., 2018; Bezemer et al., 2018). Herms and Mattson (1992) demonstrated that plants have to invest in their roots first before they can defend themselves against biotic stress. Hence, it may take a while for plants to build-up their defense systems (Raaijmakers et al., 2009; Hayat et al., 2010). Alternatively, it is well established that plants influence the soil microbial community during growth and hence, it is also possible that the differences in the response of younger and older plants to live soil is due to changes that have occurred in the soil microbial community. Previous work with the same plant species, *J. vulgaris*, where seedlings were planted in soil in which plants of the same species had been grown first, showed that the differences between responses of young and old plants are likely related to the sensitivity of plant stages and not due to changes in the soil community. Young *J. vulgaris* exhibited a strong

negative conspecific feedback, but this effect diminished over time and became neutral in older plants (Bezemer et al., 2018).

Interestingly we observed that the longer ago the soil was inoculated the stronger the negative effect of the inoculum on plant growth. This also indicates that the negative effects of live soil on plant growth that are commonly observed for this plant species are mediated by the soil microbial community and the variation that is typically observed in plant growth experiments may result from the different densities of soil-borne microbes. We expect that the oldest inoculated live soil contained the highest density of pathogenic microbes, leading to a stronger negative effect on plant growth (Pernilla et al., 2010; Dudenhöffer, et al., 2018). However, in this study, we did neither quantify the microbial density in the soil nor measure plant defense-related compounds such as salicylic acid, or pyrrolizidine alkaloids, and we suggest future work should focus on these two aspects.

In conclusion, our results indicate that live soil negatively affected plant growth. In most cases the difference between plant biomass of plants grown in sterilized soil and live soil increased during the entire experiment. However, the relative growth rates of plants in the sterilized soil and live soil only differed for young plants. Moreover, there was a negative correlation between the time of soil inoculation before planting and the relative growth rate of *J. vulgaris* plants, but for all incubation periods the negative effects were only present for young plants. Hence, our results suggest that young plants (≤ 21 days) or seedlings are most sensitive to soil pathogens while older plants (≥ 22 days) are no longer affected. Our study highlights the importance of examining relative growth rates rather than final biomass to estimate the effects of soil microbial communities on plants.

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Supplementary data

Table S1. Sequential regression analysis of *ln* transformed dry plant mass of *J. vulgaris* in experiment 1 in sterilized and live soil. The slopes were calculated backward sequentially. Slope1 and slope2 represent slopes in sterilized soil and live soil, respectively. SE indicates standard error, *df* means degrees of freedom. A t-test was used to determine significance between the two slopes. *** represents $P < 0.001$.

	Day range	slope1	SE1	slope2	SE2	*df*	*t*-value	*P*
Slopes for sequential backward regression	42-49	0.11	0.02	0.16	0.03	36	1.60	ns
	35-49	0.12	0.01	0.13	0.01	53	1.31	ns
	28-49	0.16	0.01	0.13	0.01	73	-2.86	ns
	21-49	0.14	0.01	0.12	0.01	93	-1.55	ns
	14-49	0.15	0.01	0.12	0.01	113	-4.54	***
	7-49	0.16	0.00	0.12	0.00	131	-6.85	***

Table S2. Sequential regression analysis of *ln* transformed dry plant mass of *J. vulgaris* in experiment 2 in sterilized and live soil. The slopes were calculated backward sequentially. Slope1 and slope2 represent slopes in sterilized soil and live soil. SE indicates standard error, *df* means degrees of freedom. A t-test was used to determine significance between the two slopes. * represents $P < 0.05$.

	Day range	slope1	SE1	slope2	SE2	*df*	*t*-value	*P*
Slopes for sequential backward regression	56-63	0.02	0.02	0.11	0.02	28	-2.61	*
	49-63	0.04	0.01	0.09	0.01	44	0.01	*
	42-63	0.08	0.01	0.11	0.01	60	-2.32	*
	35-63	0.09	0.01	0.11	0.01	76	-1.99	*
	28-63	0.10	0.00	0.12	0.00	92	-2.05	*
	22-63	0.11	0.00	0.12	0.00	108	-1.20	ns
	19-63	0.12	0.00	0.13	0.00	124	-1.62	ns
	16-63	0.13	0.00	0.13	0.00	140	-1.34	ns
	13-63	0.13	0.00	0.14	0.00	155	1.59	ns
	10-63	0.14	0.00	0.14	0.00	171	1.02	ns
	7-63	0.14	0.00	0.15	0.00	187	0.52	ns
	4-63	0.15	0.00	0.15	0.00	205	-0.19	ns
	0-63	0.17	0.00	0.17	0.00	223	-0.59	ns

Table S3. Sequential regression analysis of *ln* transformed fresh plant mass of *J. vulgaris* in experiment 3 in sterilized and inoculated soil 0, 1, 2 or 4 weeks before planting (live-0, live-1, live-2 and live-4). The slopes were calculated backward sequentially. Slope1 and slope2 represent slopes in sterilized soil and live soil respectively. SE indicates standard error, *df* degrees of freedom. A t-test was used to determine significance between the two slopes for each combination of sterilized and live soil. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$. Note that the slopes for the sterilized soil are used for comparison with the slopes of the live-0, live-1, live-2 and live-4 and are thus represented in the table 4 times.

Backward sequential slope calculation

	Day range	slope1	SE1	slope2	SE2	*df*	*t*-value	*P*
	35-42	0.04	0.04	0.07	0.02	28	0.48	ns
	28-42	0.08	0.02	0.10	0.01	44	1.09	ns
	20-42	0.09	0.01	0.12	0.01	60	2.77	*
	16-42	0.10	0.01	0.13	0.01	76	2.64	*
	12-42	0.11	0.01	0.12	0.01	92	1.57	ns
	8-42	0.12	0.01	0.13	0.00	108	1.14	ns
	4-42	0.13	0.01	0.13	0.00	124	0.53	ns
Live-0	0-42	0.15	0.01	0.15	0.00	140	-0.31	ns
	35-42	0.04	0.04	0.12	0.04	28	1.35	ns
	28-42	0.08	0.02	0.09	0.02	44	0.49	ns
	20-42	0.09	0.01	0.10	0.01	60	0.86	ns
	16-42	0.10	0.01	0.10	0.01	76	0.05	ns
	12-42	0.11	0.01	0.11	0.01	92	-0.04	ns
	8-42	0.12	0.01	0.11	0.00	108	-1.22	ns
	4-42	0.13	0.01	0.12	0.00	124	-2.04	*
Live-1	0-42	0.15	0.01	0.13	0.00	140	-2.32	*
	35-42	0.04	0.04	0.10	0.03	28	1.02	ns
	28-42	0.08	0.02	0.10	0.01	44	0.86	ns
	20-42	0.09	0.01	0.11	0.01	60	1.51	ns
	16-42	0.10	0.01	0.10	0.01	76	0.69	ns
	12-42	0.11	0.01	0.11	0.00	92	-0.21	ns
	8-42	0.12	0.01	0.11	0.00	108	-1.75	ns
	4-42	0.13	0.01	0.11	0.00	124	-2.99	**
Live-2	0-42	0.15	0.01	0.13	0.00	140	-3.04	**
	35-42	0.04	0.04	0.16	0.05	26	1.67	ns
	28-42	0.08	0.02	0.17	0.02	40	2.94	**
	20-42	0.09	0.01	0.15	0.02	55	3.59	***
	16-42	0.10	0.01	0.13	0.01	71	2.34	*
	12-42	0.11	0.01	0.13	0.01	87	1.47	ns
	8-42	0.12	0.01	0.11	0.01	103	-0.97	ns
	4-42	0.13	0.01	0.12	0.01	119	-1.14	ns
Live-4	0-42	0.15	0.01	0.13	0.01	135	-2.19	*

Chapter 6

General discussion

General discussion

Plants can alter the microbial community in their rhizosphere, and in turn, the microbial community influences plant growth and development (Bever 1994; van der Heijden et al., 2008; Hahl et al., 2020). In general, the relationship between plants and soil microbes can be neutral, positive and negative (Nijjer et al., 2007; Bhattacharyya and Jha, 2012). However, often plants grow less well in soil that contains a live microbial community (live soil) than when growing in sterilized soil (Kulmatiski et al., 2008). An overall net effect of soil micorbial pathogenic microbes is one of the many mechanisms behind reduced plant growth in live soils (Cesarano et al., 2017).

In nature, plants have developed many defensive strategies, e.g. via hormone signaling, against microbial pathogens or herbivores. In agriculture, inducing hormonal signaling pathways has become a promising strategy to increase plant resistance against these enemies (Haney and Ausubel, 2015; Yang et al., 2015), and this is now applied to control soil microbial pathogens (Fujita et al., 2006; Yang et al., 2015; Berens et al., 2019). Exogenous application of SA to plant leaves activates systemic acquired resistance in the plant associated with the production of pathogen-related proteins against microbial pathogens (Reymond and Farmer, 1998), while foliar application of JA or MeJA activates induced systemic defenses against herbivores and necrotrophic pathogens (Nahar et al., 2011). If the reduction of plant growth in live soil is caused by an overall pathogenic effect from the soil microbial community, we would expect that plant defense signaling hormones play a role in the interaction between the soil microbial community and plants. In particular, we would expect the negative effect to be mitigated after the application of SA as this would increase the defense of plants against microbes (Maurhofer et al., 1998; Berendsen et al., 2012; Wang et al., 2013).

Although a number of studies assessed the positive effects of hormonal signaling pathways on a plant's immunity against pathogenic microbes, whether and how these hormonal signaling pathways affect the soil microbial community and subsequently plant growth is still poorly understood, and whether activation of SA-induced resistance could potentially select a more beneficial soil microbial community over time is not known.

In this thesis, I examined how harnessing the plants' immune system affects the relationship between plants and the soil microbial community. I tested the hypothesis that the negative effect of live soil on plant growth is due to changes in the microbial community belowground and that activation of SA-induced resistance will potentially mitigate the negative effect of live soil on plant growth through altering the rhizosphere microbial composition and the expression of functional genes. Furthermore, I investigated how long-lasting the effect of the soil microbial community on plant growth is and if the effect changed at different plant growth stages.

In this chapter, I discuss the findings of this thesis and compare them with the results from several recently published studies in this research field, aiming to provide a broader perspective of my research findings within the field of above-belowground plant-soil interactions.

Activation of hormonal induced defenses in plants growing in live soil

Many plant species produce more biomass in sterilized soil than in soil that contains a live microbial community. This could be due to an overall net pathogenic effect of the soil microbial community (Kulmatiski et al., 2008; Miki 2012). In Chapter 2 I studied the effect of live soil on plant growth of four plant species to investigate whether the overall negative soil effect is a common phenomenon among these plant species. Interestingly, the live soil only negatively affected two (*J. vulgaris* and *C. vulgare*) out of the four tested plant species, while for the other two species (*T. repens and D. carota*) we found no effect. This finding is in line with previous studies showing that interactions between plant species and soil microbial communities are highly species-specific (Klironomos, 2002; Joosten et al., 2009; Harrison and Bardgett, 2010; Wang et al., 2019). Plant genotype, diversity and neighboring-species can all influence these interactions, and the soil microbial community (reviewed in Bever et al., 2010), and this effect is mostly likely related to secondary metabolites exuded by the plants (Smith et al., 2018; Zhu et al., 2019; Dror et al., 2020). For example, pyrrolizidine alkaloids (PAs) are a group of secondary metabolites of the species *J. vulgaris* that are known to affect soil microbial pathogens. Genotypes of *J. vulgaris* vary in the concentration of Pas that they contain and presumably exude, and

this can influence soil microbial communities (Kowalchuk et al., 2006; Joosten et al., 2009; Kirk et al., 2010; Kostenko et al., 2012). Other studies have shown that root-emitted volatile compounds influence the composition of soil microbial communities (Delory et al., 2016; Massalha et al., 2017; Bailly 2020).

In addition, I examined if the live soil effect can be altered by the foliar application of SA or JA. Overall, activation of hormonal-induced resistance itself for a plant is costly (Vos et al., 2013). This is exemplified in my work where the foliar application of JA and SA to plant leaves resulted in reduced plant growth when plants were grown in sterilized soil for all four species (Chapter 2). Notably, for the two species in which the live soil had a negative effect on plant growth, we found that this effect was mitigated by the application of SA. In the other two species, the application of SA did not affect plant growth. Plants respond to biotic stresses (i.e., microbial pathogens) through regulation of sophisticated hormonal signaling networks (Fujita et al., 2006; Arnaud and Hwang, 2015). In my study, induced plant defenses triggered by foliar application of the plant hormone SA mitigated the negative effect of live soil, while application of JA did not have a positive effect on plant growth in all treatments and all species. This is probably related to the functions of these hormones; SA-induced resistance targets microbial pathogens (Reymond and Farmer, 1998), while JA-induced resistance targets herbivores and necrotrophic pathogens (Nahar et al., 2011).

For *J. vulgaris*, we then continued by studying the effect of the live soil and SA application during four subsequent generations. The negative effect of the live soil was observed in all generations but did not increase or decline over time (Chapter 2). The mitigating effect of SA on the negative effects of the live soil on plant growth also did not change over generations. The reduced plant growth in live soils can be caused by nutrient competition between plants and soil microbes or by an overall pathogenic effect of soil microbial community (Hodge et al., 2013; Cesarano et al., 2017; Trivedi et al., 2020). However, in our experiment, we fertilized the plants and hence we expect that competition for nutrients was not important and that an overall microbial pathogenic effect is the most likely explanation for the plant growth reduction in live soil. Further, we hypothesized that we can select for a more beneficial community over time because the foliar application of SA mitigates the

negative live soil effect in *J. vulgaris* but we did not observe that the mitigating effect increased over generations.

Little is known about how such hormonal pathways affect the inoculated live soils and how this, in turn, impacts plant growth. Several studies argue that the 'SA-mitigated effect' can be due to (1) a boosted immune system in the plant itself (Chen et al., 2020; Koo et al., 2020), or (2) changes in the plant-microbes interaction (Nishad et al., 2020; Kumar 2020). However, evidence for the second hypothesis in soil environments is still contradictory. For instance, Berendsen et al. (2012) and Doornbos et al. (2011) demonstrated that activation of JA and SA signaling pathways did not affect the resident soil microflora, while a recent study showed that SA modulates colonization of the root microbiome by specific bacterial taxa (Lebeis et al., 2015). In our study, JA application did not affect the relationship between the soil microbial community and plant growth, and this indicates that in our experiments there was no strong cross-talk between SA and JA.

SA-induced defenses and soil microbial composition

Based on the results of Chapter 2 we studied if the SA-mitigated effect on the soil microbial community was accompanied by a shift in the composition of the microbial community. In Chapter 3, we studied the composition of the rhizosphere microbial community of *J. vulgaris* over four generations. We found that the composition of the soil microbial community in the rhizosphere soil changed across generations, but not in a consistent manner. This may have resulted from the experimental design that we selected. For each generation, we used an inoculum, which means that we placed a subset of the microbial community in a sterile background. This may explain why we saw so much variation temporally, as in each generation a different subset of the microbial community may have been activated.

Although we did find an overall effect of SA on the total microbial composition, the direction of these changes was different in each generation. Application of SA selected for different bacterial genera in the rhizosphere soil, but these selected genera differed from generation to generation. This suggests that the effects of SA application to plants on the soil microbial community are not consistent over time. It is also

possible that bacterial microbial community composition is variable over time (Gilbert et al., 2009; Hickey et al., 2013; Lauber et al., 2013; Hannula et al., 2019). The impact of SA-induced resistance on soil microbial communities is still debated. For example, Hein et al. (2008) found that SA-induced resistance in *Arabidopsis* mutants changed the structure of bacterial communities in the rhizosphere. Wang et al. (2015) and Doornbos et al. (2011) both demonstrated that activation of SA-induced resistance did not significantly affect the composition and diversity of the rhizosphere bacterial community. As the SA effect on the microbial composition varied from generation to generation, it is difficult to predict the effects of activation of plant defenses on soil microbes. This may also explain why there was no selection for more beneficial communities over generations.

Application of SA to plants significantly up-regulated genera of *Caballeronia*, unclassified *Cytophagaceae, Crinalium* and *Candidatus Thermofonsia Clade* 2, and down-regulated the genera of *Thermomicrobiales*, unclassified *Rhodobacterales*, *Paracoccus* and *Flavihumibacter*. While the functions of many of these bacteria are poorly understood, bacteria of the genus *Caballeronia* are often reported to play an important role in fixing nitrogen and promoting plant growth, and species in this genus are predominantly endophytic diazotrophic bacteria and N-fixing bacteria (Puri et al., 2018; Padda et al., 2018; Puri et al., 2020). Hence, this suggests that activation of the SA signaling pathway in *J. vulgaris* plants may select for bacterial genera that are beneficial to the plant.

SA-induced defenses and soil microbial functional genes

In Chapter 3, we analyzed the changes of microbial taxonomy in the rhizosphere soil and found that the effects of SA on the rhizosphere bacterial communities of *J. vulgaris* were inconsistent over generations. We hypothesized that we would see a common functional gene expression in the same soil samples, because the functions of the soil microbial community are often distributed across microbial taxa (Burke et al., 2011; Liu et al., 2018; Liu et al., 2020). One of the explanations can be that the composition of the soil microbial community shows a great redundancy concerning the functioning of microbial species and that changes in microbial diversity are not

always consistent with changes in functional gene expression in soil microbial communities.

In our study, we found that the functional genes of rhizosphere microbial communities of *J. vulgaris* were affected by the SA treatment, by generation and by the interplay between SA treatment and generation. However, none of the significantly SA-downregulated genes was present in all four generations, while only one SA-upregulated gene was observed in all four generations. To date, information about the effects of phytohormone application to plants on the functions of rhizosphere microbiomes are limited (Anderson et al., 2004; Carvalhais et al., 2013). To our knowledge, the work presented in this thesis is among the first to study how activation of SA induced resistance affects natural soil microbiomes at the functional gene level. SA induced resistance is often reported to play an important role in resistance to a broad range of microbial pathogens, such as bacteria, fungi and viruses. Concerning viruses, SA has been reported to act as an elicitor in various plant species, such as tobacco, cucumber, Vigna mungo, tomato, sugarcane (Murphy et al., 1999; Gilliland et al., 2003; Mayers et al., 2005; Kundu et al., 2011; Li et al., 2019; Yuan et al., 2019).

A limitation in the current work is that not all detected genes could be annotated with known functions. Interestingly, at a gene ontology level, we found that soil microbial communities in the rhizosphere soil of SA-treated plants utilized several gene ontology processes. For the increased GO terms, they were mostly related to viral RNA genome replication, to interactions with host cells, to organelles of the host cells and to RNA polymerase activities; while for the decreased GO terms, they were associated with processing nitrogen and macromolecules. However, it still remains unproven that if those processes are associated with infection processes of the host plant and are potentially linked to suppression of pathogenic infections. Interestingly, up-regulated GO terms that were involved in viral (RNA) genome replication and viral processes were frequently found in our study in the soil of SA-treated plants. As it is well-reported that viral-phage therapy uses viruses or bacteriophages to control pathogens. A viral phage first attaches to the surface of a pathogenic bacteria, then injects its genome into the cells, self-replicates in the bacteria, and eventually kills the bacteria by causing them to burst or lyse (Duckworth and Gulig, 2002; Svircev et al., 2018; Jamal et al., 2019; Kortright et al., 2019; Rehman et al., 2019). This has recently

been brought up as an alternative for the usage of pesticides to control bacterial pathogens in agriculture (Rehman et al., 2019). Therefore, it is important to note that virus-microbe-plant interactions should be taken into.account in future studies.

Plant growth stages and negative plant-soil effects

In our experiments, we placed a subset of the microbial community in a sterile background and this may have led temporal variation in the soil microbial community in each generation. Most studies on plant-soil-interactions have examined the effect of the soil microbial community on plant mass after a fixed duration of plant growth (Smith and Reynolds, 2012; Hodge and Fitter 2013; Dudenhöffer et al., 2018). However, these interactions may change over time (Bezemer et al., 2018). In Chapter 5, we examined how long-lasting the effect of the soil microbial community on plant growth is and we established relative plant growth rates at different growth stages (early, mid and late plant growth).

We found in all experiments that we carried out to study these temporal effects, that differences in dry plant mass between the plants grown in sterilized soil and inoculated soil (live soil) increased over the course of the experiment. Interestingly, linear regression models with *ln* transformed dry plant mass against time at the early stage and later stage in sterilized soil and live soil, respectively, showed that the relative growth rate of plants in the sterilized soil and live soil only differed in the first weeks and that there were no significant differences in relative growth rates during the late stage. Our study exemplifies that the negative effects of soil inoculation on plant mass can extend over the whole growth period, but that these differences are due to negative effects that occur in the first weeks after planting. This might be because younger plants or seedlings are more vulnerable and susceptible to pathogenic microbes in the soil than older plants with well-developed root systems (Packer and Clay, 2000). Root development plays an important role for plants in suppressing soil microbial pathogens (Watt et al., 2006; Emmett et al., 2014), and is often correlated with soil abiotic or biotic characteristics (Kardol et al., 2013; Arrigoni et al., 2018; Bezemer et al., 2018). Our findings are in line with previous work (Bezemer et al., 2018) on the same plant species, *J. vulgaris*, where seedlings were planted again in soil that had been conditioned by other plants of the same species.

Their findings show that the differences between responses of young and old plants are likely related to the sensitivity of plant stages and not due to temporal changes in the soil community.

Concluding remarks and future perspective

The outcomes of this thesis contribute to our understanding of how harnessing of the plant immune system affects the relationship between plants and the soil microbial community. From this work, we can conclude that the effect of live soil on plant growth is species-specific. Moreover, we conclude that application of SA can mitigate the negative effect of live soil on plant growth and we hypothesize that the negative effect of live soil on plant performance is driven by microbial pathogens in the soil. Further, from the multi-generational experiment, we conclude that activation of SA-associated plant defense pathways alters the composition of soil microbial communities of *J. vulgaris* but that these effects vary over time. We found no evidence that activation of SA signaling pathways in plants results in the selection of bacteria that are more beneficial to plant growth. The functions of the majority of the significantly affected genera by SA-induced resistance in our experiment are not well-known. SA-induced resistance, against soil microbial pathogens in *J. vulgaris* may be through the regulation of virus or viral related pathways. Last but not least, we concluded that negative effects of live soil on plant growth may appear consistent over time, but may only be caused by negative effects on plant growth that occur during the first few weeks. Overall, our study exemplifies that aboveground induction of plant defenses, can lead to complex above-belowground feedbacks.

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Summary

Many plant species grow better in sterilized soil than in soil that contains a live microbial community. One hypothesis to explain this phenomenon is that the overall net pathogenic effect of soil microbial communities reduces plant performance. Induced plant defenses triggered by the application of the plant hormones jasmonic acid (JA) and salicylic acid (SA) may help to mitigate this pathogenic effect. However, little is known about how the activation of SA-induced resistance impacts the microbial composition and the expression of functional genes in the rhizosphere soil.

We manipulated and induced the plant defense system through foliar application of phytohormones (JA or SA), and examined whether the negative effect of live soil on plant growth was reduced. The growth of four plant species (*Jacobaea vulgaris*, *Cirsium vulgare, Trifolium repens* and *Daucus carota*) was affected differently in live soil and by the hormone treatments. Foliar application of SA increased plant growth in live soil for the species, *J. vulgaris* and *C. vulgare,* which were the two species that both produced less biomass in live soil than in sterilized soil, SA application slightly reduced plant growth in live soils for the species *T. repens* and *D. carota* that were not affected in live soil. Application of JA reduced plant growth in live and sterile soil for all species. For *J. vulgaris* the treatments were repeated for three more generations. In each generation, the live soil consisted of a mixture of 10% of soil collected from pots from the previous generation mixed with 90% sterilized soil. In all four generations, plant biomass was measured. The reduction in growth in live soil was consistent in each generation, and in each generation, this negative effect was mitigated by the application of SA to plants. Hence, we found no evidence for an increase in the negative plant-soil feedback over generations, but also no selection effect of SA application over time.

RNA extracted from the rhizosphere soil from each generation was subsequently sequenced. Soil microbial composition at genus level was studied and the expression of functional genes of live soils where plants grown under SA treatments and control were compared. Application of SA to *J. vulgaris* leaves altered the composition of bacterial communities in the rhizosphere soil but only in the second, third and fourth

growth cycle. However, the SA effects on bacterial community composition were small, while there was a substantial temporal effect on rhizosphere bacterial composition. As there were no genera of bacteria that responded to SA application in the first generation this suggests that there are no immediate responses of bacteria in the rhizosphere to SA application to plants.

Subsequently, the effects of the application of SA to *J. vulgaris* on the gene expression and functions of the soil-borne microbial community were examined for each of the four plant generations. Gene expression and functions of the soil-borne microbial community responded to the exogenous application of SA but these effects differed per generation. The number of differentially expressed genes tended to increase over generations, but remarkably there was no overlap for these annotated genes among the four generations. Moreover, we found that foliar application of SA upregulated GO terms of biological processes that were related to viral RNA genome replication, to interactions with host cells, to organelles of the host cells and to RNA polymerase activities. There were six GO terms of which the expression decreased in the second, third and fourth generation, and these were associated with processing nitrogen and macromolecules.

Finally, in a series of experiments, we examined for *J. vulgaris*, how plant responses to live soil changed over time, by repeatedly harvesting plants over time. In all experiments, plant growth was worse in live soil than in sterilized soil and this effect on plant biomass was consistent over time. However, relative growth rates of plants in the sterilized soil and live soil only differed for young plants and a reverse pattern was even observed during the latest stage where relative growth rates were higher for plants in live soil. This shows that while the soil treatment may result in plant biomass being consistently lower, this could have been caused solely by initial effects of the treatment on plant growth. Hence, to better understand plant-soil interactions, it is important to examine not only plant biomass but also plant growth rates. In a third growth experiment, we also examined the effect of the timing of soil inoculation prior to planting on the relative growth rate of *J. vulgaris* plants with four different timing treatments. Biomass was reduced in all inoculated soils and there was a negative relationship between time since inoculation and plant biomass. Again, in all inoculated soils the negative effect of the soil microbial community on plant growth

disappeared two weeks after planting. Overall, these results suggest that young plants or seedlings are most sensitive to soil pathogens.

In conclusion, our research shows that aboveground activation of defenses in the plant affects soil microbial communities and as soil microbes can greatly influence plant performance, this implies that induction of plant defenses, can lead to complex above-belowground feedbacks.

Nederlandse samenvatting

Veel plantensoorten groeien beter in gesteriliseerde grond dan in grond met een levende microbiële gemeenschap. Een hypothese om dit fenomeen te verklaren is dat het algehele netto pathogene effect van microbiële gemeenschappen in de bodem de groei van planten vermindert. Dit pathogene effect kan in theorie verminderd worden door de afweer van planten te induceren met de plantenhormonen jasmonzuur (JA) en salicylzuur (SA). Er is echter weinig bekend over hoe de activering van SA-geïnduceerde resistentie de microbiële samenstelling en de expressie van functionele genen van bacteriën in de rhizosfeerbodem beïnvloedt.

Ik manipuleerde en induceerde de afweer van planten door deze te behandelen met plantenhormonen (JA of SA), en onderzocht of het negatieve effect van levende grond op plantengroei inderdaad verminderd was. De levende grond en de hormoonbehandelingen hadden een verschillend effect op de groei van vier plantensoorten (*Jacobaea vulgaris, Cirsium vulgare, Trifolium repens* en *Daucus carota*). Het behandelen van bladeren met SA verhoogde de plantengroei in levende grond voor *J. vulgaris* en *C. vulgare* ten opzichte van de controle in levende grond. Ook produceerden deze twee soorten minder biomassa in levende grond dan in gesteriliseerde grond. De SA behandeling verminderde de plantengroei in levende grond enigszins voor de soorten *T. repens* en *D. carota* terwijl hun groei niet verminderd was ten opzichte van de groei op gesteriliseerde grond. De behandeling met JA verminderde plantengroei in levende en steriele grond voor alle soorten. Alleen voor *J. vulgaris* werden de behandelingen voortgezet voor nog drie generaties. In elke generatie bestond de levende grond uit een mengsel van 10% grond, verzameld uit potten van de vorige generatie van dezelfde behandeling, gemengd met 90% gesteriliseerde grond. In alle vier de generaties werd de biomassa gemeten. De afname van de groei in levende grond ten opzichte van steriele grond was

consistent in elke generatie en in elke generatie werd dit negatieve effect verminderd door behandeling met SA. Ik vond geen bewijs voor een toename van de negatieve plant-bodem-terugkoppeling over generaties, maar ook geen selectie-effect van SA behandeling in opeenvolgende generaties.

Uit de rhizosfeerbodem van alle behandelingen en generaties van *J. vulgaris* werd RNA geëxtraheerd en gesequenced. De microbiële samenstelling van de bodem werd bestudeerd en de expressie van functionele genen in de SA-behandeling en controle werden vergeleken. Behandeling van *J. vulgaris* bladeren met SA veranderde de samenstelling van bacteriële gemeenschappen in de rhizosfeerbodem, echter alleen in de tweede, derde en vierde generatie. Het effect van de SA behandeling op de samenstelling van de bacteriële gemeenschap was echter klein, terwijl de bacteriële gemeenschap sterk verschilde tussen generaties. Aangezien er in de eerste generatie geen bacterie-genera waren die reageerden op de SA behandeling van bladeren van *J. vulgaris*, suggereert dit dat er geen onmiddellijke reacties zijn van bacteriën in de rhizosfeer op SA-toediening op planten.

Vervolgens werden de effecten van de SA behandeling van bladeren van *J. vulgaris* op de genexpressie en functies van de microbiële gemeenschap in de rhizosfeer onderzocht voor elk van de vier generaties. De exogene toepassing van SA beïnvloedde genexpressie en functies van de microbiële gemeenschap, maar deze effecten verschilden per generatie. Het aantal differentieel tot expressie gebrachte geannoteerde genen nam over generaties toe, maar opmerkelijk genoeg was er geen overlap voor deze genen tussen de vier generaties. Bovendien ontdekte ik dat toediening van SA op het blad de biologische processen die verband hielden met virale RNA-genoomreplicatie, met interacties met gastheercellen, met organellen van de gastheercellen en met RNA-polymerase-activiteiten beïnvloedde. Er waren zes GO-termen waarvan de expressie afnam in de tweede, derde en vierde generatie, en deze waren gerelateerd aan het verwerken van stikstof en macromoleculen.

Ten slotte heb ik in een reeks experimenten onderzocht hoe de relatieve groeisnelheid van *J. vulgaris* planten op levende grond in de loop van de tijd veranderde. In alle experimenten was de absolute plantengroei in levende grond slechter dan in gesteriliseerde grond en dit effect op de biomassa van planten was consistent in de tijd. De relatieve groeisnelheden van planten in de gesteriliseerde grond en levende grond verschilden alleen voor jonge planten en een omgekeerd patroon werd zelfs waargenomen tijdens de latere groeifase waarin de relatieve groeisnelheden hoger waren voor planten in levende grond. Hoewel een levende bodem ertoe kan leiden dat de plantenbiomassa constant lager is wordt dit dus uitsluitend veroorzaakt door een lagere relatieve groeisnelheid op levende bodems in de eerste weken. Om de interacties tussen plant en bodem beter te begrijpen, is het daarom belangrijk om niet alleen de biomassa van planten te onderzoeken, maar ook de relatieve groeisnelheden. In een derde groei-experiment onderzocht ik het effect van de timing van bodeminoculatie voorafgaand aan het planten van zaailingen in de bodem, op de relatieve groeisnelheid van *J. vulgaris* planten. In dit experiment waren er vier verschillende momenten van inoculatie van de bodem voor beplanting. Op alle geïnoculeerde bodems was de biomassa lager dan op steriele bodems en er was een negatief verband tussen de tijd sinds inoculatie en biomassa. In alle geïnoculeerde bodems verdween het negatieve effect van de microbiële bodemgemeenschap op de plantengroei echter al twee weken na het planten. Deze resultaten suggereren dat alleen jonge planten of zaailingen gevoelig zijn voor bodempathogenen.

Concluderend laat mijn onderzoek zien dat bovengrondse activering van afweermechanismen in de plant de microbiële gemeenschappen in de bodem beïnvloedt. Aangezien bodemmicroben de groei en chemie van planten sterk kunnen beïnvloeden, impliceert dit dat inductie van afweermechanismen van planten kan leiden tot complexe bovengrondse-ondergrondse terugkoppelingen.

Curriculum Vitae

Jing Zhang was born in Jinan, Shandoing Province in China on 22nd, May, 1991 (28th April, 1990 on official documents). She finished her bachelor in Geographic Information Systems (GIS) in 2013 at Sichuan Agricultural University in China, then she obtained her MSc degree in Phytopathology in 2015 at the same university. In September 2015, she started her PhD project on "The impact of defense hormones on the interaction between plants and soil microbial community" under the supervision of Prof. Dr. Peter Klinkhamer, Prof. Dr. Martijn Bezemer and Dr. Klaas Vrieling in the group of Plant Sciences, at Leiden university, in the Netherlands.

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