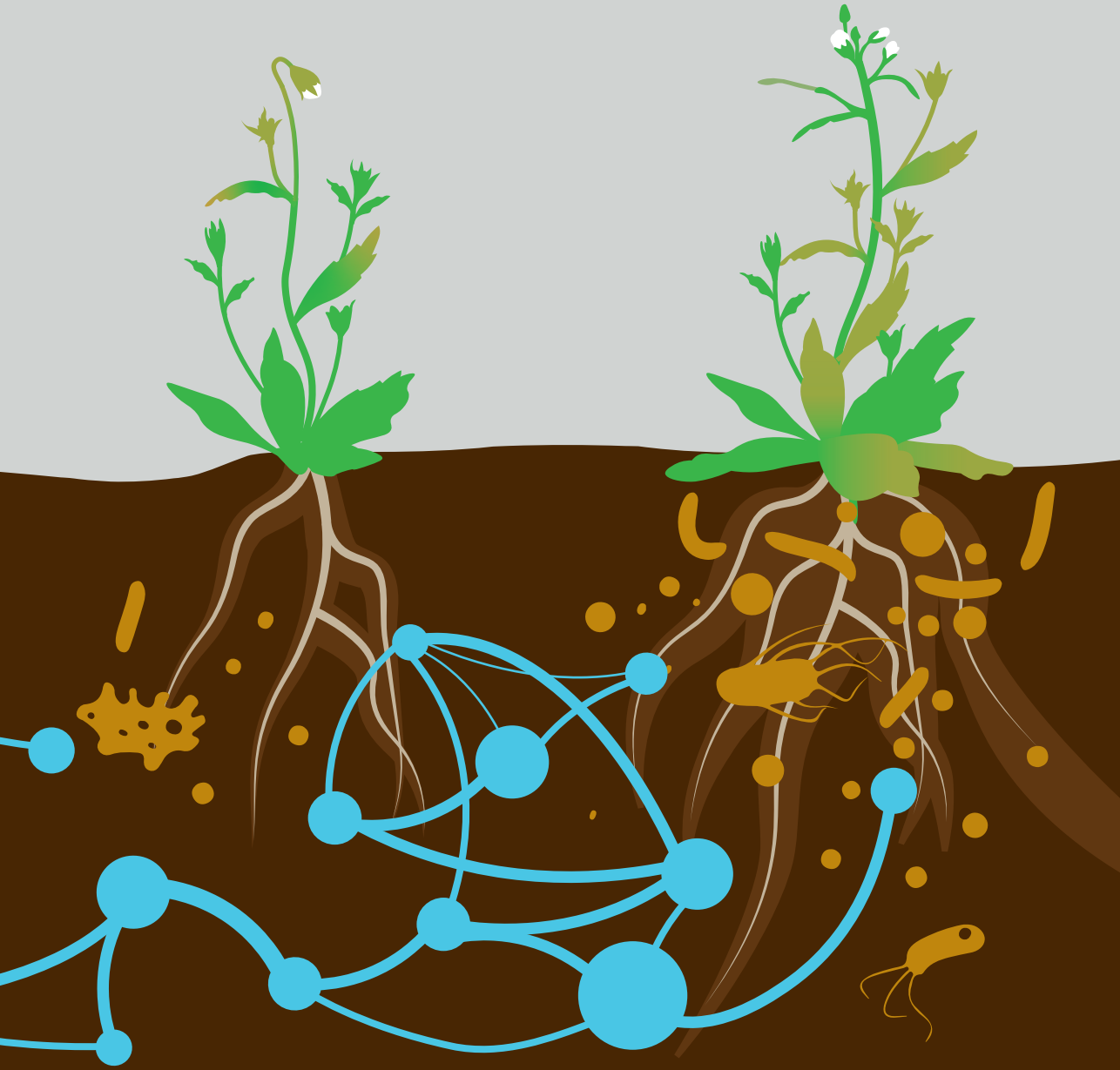
The soil-borne legacy: how plants cry out for help in response to disease



Gilles Vismans

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(met een samenvatting in het Nederlands)

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Gilles Vismans

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Promotor:

Prof. dr. ir. C.M.J. Pieterse

Copromotoren:

Dr. P.A.H.M. Bakker
Dr. R.L. Berendsen

Beoordelingscommissie:

Prof. dr. D.M. Weller
Prof. dr. J.M. Raaijmakers
Prof. dr. E.E. Kuramae
Prof. dr. ir. L. Mommer
Prof. dr. G.A. Kowalchuk

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CHAPTER 1.

General introduction

The root associated microbiome

At first glance plants may not seem interesting to some people. They may appreciate the beauty of flowers or be impressed by huge trees, but they consider plants mostly as sessile organisms that do not do anything. However, the behavior of plants is actually amazing, for example taking into account how they actively explore the soil for nutrients with their fast growing roots (Steffens & Rasmussen 2015), detect light using the phytochrome sensors in the leaves and gravity using statoliths (Correll & Kiss 2002), and even perceive time using their circadian clock (Barak et al 2000). Moreover, a plant does not function as a single entity but it forms complex interactions with other organisms in its environment, of which the interplay with microbes might be the least understood. This entire ecosystem of micro-organisms in, on and around the plant has been called many names. It has been called the second genome and the microbiome (Berendsen et al 2012, Mendes et al 2013), or when the plant and all surrounding microbes are viewed as one organism the holobiont (Vandenkoornhuyse et al 2015). In this thesis we will use "the microbiome" to describe the complex communities of micro-organisms that interact with the plant. Important activities that support plant performance depend on interactions of the plant and its associated microbiome. Most studies have focused on the microbiome of plant roots. Most of the microbes live on the roots and in the thin layer of soil surrounding the root, the rhizosphere (Hiltner 1904). The main source of microbes that associate with plants is the bulk soil. In the rhizosphere, specific microbiota are enriched and in total the microbial population densities are much higher compared to that bulk soil (Foster et al 1983, Hartmann et al 2009). The main reason for this is the deposition of up to 40% of photosynthetically fixed carbon by the plant into the rhizosphere, which makes it a suitable environment for microbes to thrive (Badri & Vivanco 2009). The emphasis of current research is focused on the importance of specific microbiota in nutrient uptake from the soil, tolerance to drought and salt stress, and protection against soil-borne diseases (Beirinckx et al 2020, Berendsen et al 2018, Buee et al 2009, de Vries et al 2020, Yuan et al 2019). As such the root microbiome is an important source for crop improvement (Chen et al 2021, Singh et al 2020). Advances in sequencing techniques and analysis of complex data have boosted microbiome studies (Bakker et al 2020, Berg et al 2020, Fitzpatrick et al 2020, Oyserman et al 2018). This thesis focuses on the root microbiome in relation to plant health, including effects of disease on root microbiome composition and effects of the microbiome on disease severity.

Disease suppressive soils and the soil-borne legacy

The most eminent examples of beneficial effects of the root microbiome on plant health are so called disease suppressive soils. In such soils, susceptible host plants develop no or only minor symptoms in the presence of a virulent pathogen. These type of soils occur most frequently after replanting the same crop for several seasons (Schlatter et al 2017). One of the most well-studied disease

suppressive soils is take all decline (TAD) soil, in which wheat is protected against *Gaeumannomyces tritici* (Hernandez-Restrepo et al 2016), the fungus that causes take all disease (Raaijmakers & Weller 1998). In continuous wheat cultivation, take all disease incidence increases over the years, but steadily declines in the years after a severe disease outbreak (Raaijmakers & Weller 1998). Such a decline in disease is accompanied by an increase in population densities of *Pseudomonas* sp. that produce the antifungal compound 2,4-diacetylphloroglucinol (DAPG) (Raaijmakers & Weller 1998, Weller et al 2002).

Common bean infected with *Fusarium oxysporum* also showed changes in the microbiome. Population densities of families belonging to *Pseudomonadacaea* and *Bacillaceae* were increased (Mendes et al 2018), but also the *Oxalobacteriaceae*, *Burkholderiaceae*, *Sphingobacteriaceae* and the *Sphingomonadaceae* were more abundant in for instance the microbiome of sugar beet infected with *Rhizoctonia solani* (Chapelle et al 2016). The common denominators are the fungal pathogens in the soil and the changes in the microbiome composition. Not only the community compositions change upon pathogen infection, these communities are also enriched in production of phenazine and other antifungal traits (Mendes et al 2018). In a recent study by Carrion and co-workers (2019), sugar beet seedlings were grown in either suppressive or conducive soils infested with *R solani*. Here the enrichment of *Chitinophagaceae, Burkholeriaceae* and *Xanthomonadaceae* also resulted in increased activity of specific biosynthetic gene-clusters aimed at suppressing the pathogen (Carrión et al 2019).

In disease suppressive soils, the pathogen and the root microbiome can interact directly in the rhizosphere. This complicates studies that aim to elucidate the involvement of the plant in effects of disease on the microbiome. In recent studies, the biotic stress factor, either pest insects or microbial pathogens, were kept spatially separated from the root microbiome to avoid a direct impact of the pathogen on the microbiome. For example, whitefly infestation of pepper leaves induced significant changes in the root microbiome (Kong et al 2016), and inoculation of *Arabidopsis thaliana* (Arabidopsis) leaves with *Pseudomonas syringae* pv. *tomato* (*Pst*) resulted in significant changes in the root exudation patterns and the composition of the root microbiome (Yuan et al 2018). These changes in the Arabidopsis microbiome also resulted in protection of a new population of Arabidopsis plants grown on the soils pre-treated with *Pst* infected plants against this bacterial disease. Such transgenerational protection against disease by inheritance of a beneficial microbiome was dubbed the soil-borne legacy (SBL; Bakker et al 2018). Thus plants seem to “cry for help” during biotic stress situations, resulting in the reshaping of the microbiome and supporting suppressive functions (Rolfe et al 2019).

Induced systemic resistance and the microbiome

In the example of take-all decline, *G. tritici* and the microbiome are in direct contact and thus can have a direct impact on the microbiome. The reverse is also true. By producing DAPG, the *Pseudomonas* sp. directly inhibit the growth of *G. tritici* (Weller et al 2012). However, in Arabidopsis DAPG was also demonstrated to prime the immune system of the plant (Weller et al 2012). One of the best studied examples of priming of the plant immune system is induced systemic resistance (ISR) by the plant-beneficial bacterium *Pseudomonas simiae* WCS417 (WCS417) (Pieterse et al 2020). In the primed state, plant defense responses are activated stronger and faster upon pathogen detection (Pieterse et al 2014). To study the involvement of ISR in disease control by beneficial rhizosphere bacteria, the pathogen and the bacteria are introduced spatially separated, respectively on the leaves and the roots, and this separation is maintained throughout the experiment (Pieterse et al 2000, Pieterse et al 1996, Van Wees et al 1999, Zamioudis et al 2015). Colonization of Arabidopsis roots by WCS417 elicits ISR that is effective against a wide range of pathogens (Pieterse et al 2014, Ton et al 2001). WCS417-mediated ISR in Arabidopsis requires the root specific transcription factor MYB72, which through an as yet unknown signal primes the systemic parts of the plant (Pieterse et al 2000, Van der Ent et al 2008, Zamioudis et al 2014). This transcription factor was first identified by studying the root transcriptomic response of roots colonized by WCS417 (Verhagen et al 2004), and localization studies using reporter lines pinpointed the expression to epidermal and cortical root cells in Arabidopsis (Zamioudis et al 2015). Balancing growth and defense is a challenge for plants, since investing energy in defense can result in stunted growth (Huot et al 2014). However, for ISR mediated priming of defense, the defense response is strongly activated only after pathogen attack and there is no growth penalty in the absence of pathogens. Surprisingly, colonization of Arabidopsis roots by WCS417 not only leads to the state of ISR but it can also stimulate plant growth (Pieterse et al 2003). ISR is the result of an intricate interplay of hormones, balancing the correct response. ISR is often dependent on jasmonic acid (JA) and ethylene (ET) but does not result in the accumulating of pathogenesis related proteins (Pieterse et al 1998, Van Wees et al 2008). However, among different bacteria that induce ISR, there are some that do require intact salicylic acid (SA) signaling to function, for example *Bacillus cereus* ART156 and *P. fluorescens* SS101 (Niu et al 2016, van de Mortel et al 2012). Together this shows that different beneficial microbes can induce resistance using different pathways within the plant.

The transcription factor MYB72 has a double role in the plant. Not only is it required for ISR signaling, it is also a key hub in signaling under iron limiting conditions (Verbon et al 2017). Under iron limiting conditions, *MYB72* is activated in the roots of Arabidopsis under regulation of the *bHLH FIT* transcription factor complex. Activation will result in production of phenolic compounds, mainly coumarins. Subsequently, these compounds are secreted by the roots into the rhizosphere and increase iron availability to the plant (Verbon et al 2017). The coumarins suppress the growth of selected soil-borne pathogens and many bacteria, but not that of

WCS417. It is currently hypothesized that the activation of *MYB72* by beneficial microbes creates a positive feedback loop, creating a favorable environment for beneficial bacteria (Stringlis et al 2018, Verbon et al 2017). These beneficial bacteria can in turn assist the plant in taking up iron but also protect the plant against biotic stresses. Thus, the microbiome seems to play a vital role in plant performance and adapts to suit the situation. The intriguing question now is, how does the plant contribute to such changes in the microbiome?

Microbiome modulation through exudation

The composition of the plant root associated microbiome is affected by different stress situations, possibly to alleviate stress effects on the plant. These changes in the microbiome are not random, but orchestrated by the plant through exudation of carbohydrates and metabolites from the roots (Canarini et al 2019, Sasse et al 2018, Stassen et al 2021). One key driver of the root exudate composition is the developmental stage of the plant, reflected in changes in the composition of the plant-associated microbiome (Chaparro et al 2013). Exudation is also important to attract beneficial microbes. The plant hormone strigolactone is a key player in the mutualistic interactions between plants and mycorrhizal fungi. The released strigolactones act as chemo attractants for mycorrhizal fungi, which can improve the phosphate availability to the plant (Akiyama et al 2005). However, this mechanism can be highjacked by parasites such as Striga. These parasitic plants only germinate when exuded strigolactones are detected (Matusova et al 2005). This exemplifies the intricate balance for a plant to attract beneficial organisms and to keep harmful organisms away.

Recently the secondary metabolites classified as coumarins have been the focus of studies in exudate-driven microbiome modulation (Pascale et al 2020). Several studies have shown that coumarin-deficient plant mutants collect different microbes in their root microbiome, and these mutants are more susceptible to foliar pathogen infection under iron limiting conditions (Perkowska et al 2021, Voges et al 2018). In addition, Stringlis et al. (2018) showed that selected beneficial microbes are more resistant against anti-microbial effects of coumarins, compared to selected soil-borne pathogens. If this finding can be generalized, such resilience of beneficial rhizobacteria to coumarins may increase their population densities on the roots and promote priming of the immune system, plant growth and iron uptake, while warding of soil-borne pathogens (Lundberg & Teixeira 2018). Part of the coumarin biosynthesis pathway is dependent on MYB72 and F6'H1 (Clemens & Weber 2016). This transcription factor and enzyme are key tools in experiments that are aimed to better understand the interaction between plants and beneficial microbes. One such study involved a transcriptome analysis of WCS417 under differential coumarin regimes in Arabidopsis root exudates (Yu et al 2021). It was shown that expression of 8% of the bacterial genome was affected by *F6'H1*-dependent root exudates. Noticeable, WCS417 genes involved in transport and metabolism were up regulated, while genes necessary for motility were

downregulated. Also recently, an intriguing role for MYB72 in bacterial evolution became clear in the interaction between Arabidopsis and the beneficial bacterium *Pseudomonas protegens* CHA0. Strain CHA0 was grown in the rhizosphere of gnotobiotic Arabidopsis plants and bacterial populations that developed were transferred to the roots of new gnotobiotic plants. Over time, bacterial populations became more beneficial to the plant in terms of growth promotion, as well as strong inducers of *MYB72* gene expression in the host. Moreover, the resulting offspring of CHA0 was less sensitive to the coumarin scopoletin (Li et al 2021). Thus, coumarins are intricately involved in the shaping of the microbiome under stressful situations for the plant and key players in communication between the plant and its root microbiome.

Thesis outline

Roots are the interface of the plant and the soil in which it grows, not only providing structural support but also a hub for interactions with micro-organisms. These micro-organisms are of vital importance to the plant and beneficial interactions with the so-called microbiome are necessary for a plant to thrive. Accumulation of pathogens in soil can be detrimental to the plant, however, beneficial microbes are also abundant in soil. Disease suppressiveness of soils often results from a severe outbreak of a soil-borne disease and is followed by recruitment of beneficial bacteria that can control the pathogen. Here, we investigated if infection with aboveground pathogens can also lead to recruitment of beneficial microbes. The ecological implications of such a recruitment were also investigated. Moreover, we studied signaling compounds involved in this phenomenon.

The model plant *Arabidopsis thaliana* has been used extensively in microbiome studies (Lundberg et al 2012, Schlaeppi et al 2014, Schneijderberg et al 2020), and it is susceptible to a range of aboveground plant pathogens (Westman et al 2019). In **Chapter 2**, we investigated effects of leaf infection of Arabidopsis plants with either the biotrophic pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) or the necrotrophic fungus *Botrytis cinerea* (*Bc*) on the rhizosphere microbiome. Whereas infection with *Bc* did not result in major shifts in the rhizosphere microbiome, *Hpa* infection resulted in enrichment of three bacterial species. These bacteria were isolated and shown to behave synergistically in protecting Arabidopsis from *Hpa* infection. Moreover, the consortium of the three bacterial species promoted plant growth. In this chapter, we also discovered that a population of Arabidopsis plants grown on soil that was previously conditioned with *Hpa* infected plants was less affected by *Hpa* infection compared to plants grown on a soil previously conditioned with healthy plants. This so-called soil-borne legacy thus seems to protect the offspring of infected plants against new infections.

In **Chapter 3,** we describe the experimental setup to study the soil-borne legacy. In short, Arabidopsis is grown on soils on which previously either healthy or *Hpa*-infected Arabidopsis was grown. This second generation is again infected by *Hpa*

and disease severity is scored by measuring sporulation of the pathogen. In this setup, the soil-borne legacy is observed as a reduced disease severity in a second generation of Arabidopsis grown on soils pre-treated with *Hpa* infected plants as compared to a second generation grown on soils pre-treated with healthy Arabidopsis.

In **Chapter 4**, we delve deeper into the soil-borne legacy using mutants of Arabidopsis that are disrupted in specific signaling pathways. We observed that the coumarin mutants *myb72* and *f6'h1* could not establish the soil-borne legacy when they were used to condition the soil with *Hpa*-infected plants. Moreover, in the rhizosphere of *Hpa* infected wild-type plants, that did establish the soil-borne legacy, we detected a significant increase in the order of *Xanthomonadales.* Such an increase was not observed in the rhizospheres of *Hpa*-infected *myb72* and *f6'h1* mutants. Thus, the *Xanthomonadales* may be essential in establishing the soil-borne legacy in this experimental system. The protective effect of the soil-borne legacy in the second generation of plants appears to depend on SA, since the SA mutants *npr1* and *sid2* were not able to perceive the soil-borne legacy.

In the final experimental chapter, **Chapter 5**, we elaborated on Chapter 2 by investigating similarities and differences in root microbiome assembly of Arabidopsis plants infected with different pathogens. Leaves of Arabidopsis were inoculated with either *Hpa, Pseudomonas syringae* pv. tomato DC3000 (*Pst*), *Xanthomonas campestris* (*Xc*), *Phytophthora capcisi* (*Pc*), or *Bc*, thus covering a broad range of pathogen lifestyles and phylogeny. We observed that especially the two bacterial pathogens had a significant and similar impact on the rhizosphere microbiome.

In **Chapter 6,** the results and hypotheses generated in the experimental chapters are discussed in the context of recent findings in literature and in future research directions.

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CHAPTER 2.

Disease-induced assemblage of a plant-beneficial bacterial consortium

Roeland L. Berendsen[1*], Gilles Vismans[1*], Ke Yu[1], Yang Song[1,2], Ronnie de Jonge[1,3,4], Wilco P. Burgman[1], Mette Burmølle[5], Jakob Herschend[5], Peter A.H.M. Bakker[1], Corné M.J. Pieterse[1]

1: Plant-Microbe Interactions, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands
2: Jiangsu Provincial Coordinated Research Center for Organic Solid Waste Utilization, Nanjing Agricultural University, Nanjing 210095, People's Republic of China.
3: Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium
4: VIB Center for Plant Systems Biology, Technologiepark 927, 9052 Ghent, Belgium
5: Section of Microbiology, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 København, Denmark
*These authors contributed equally

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Abstract

Disease suppressive soils typically develop after a disease outbreak due to the subsequent assembly of protective microbiota in the rhizosphere. The role of the plant immune system in the assemblage of a protective rhizosphere microbiome is largely unknown. In this study, we demonstrate that *Arabidopsis thaliana* specifically promotes three bacterial species in the rhizosphere upon foliar defense activation by the downy mildew pathogen. The recruited bacteria were isolated and found to interact synergistically in biofilm formation *in vitro*. Although separately these bacteria did not affect the plant significantly, together they induced systemic resistance against downy mildew and promoted growth of the plant. Moreover, we show that the soil-borne legacy of a primary population of downy mildew infected plants confers enhanced protection against this pathogen in a second population of naïve plants growing in the same soil. Together our results indicate that plants can adjust their root microbiome upon pathogen infection and specifically recruit a group of disease resistance-inducing and growth-promoting beneficial microbes, therewith potentially maximizing the chance of survival of their offspring that will grow in the same soil.

Introduction

In nature, plants accumulate pathogens in their surrounding soil, which ultimately negatively influences their performance, a phenomenon called negative soil-feedback (Bever et al 2012, Van der Putten et al 1993). Negative soil-feedback mechanisms can promote plant biodiversity by tempering the success of dominant plant species (Klironomos 2002). Such buildup of soil-borne pathogens can be devastating in agricultural monocultures, but is alleviated through crop rotation. Interestingly, continuous cultivation of important crops like wheat and sugar beet can induce soil suppressiveness to disease (Mendes et al 2011, Raaijmakers & Mazzola 2016, Weller et al 2002). In such disease-suppressive soils, plants remain healthy despite the presence of a virulent pathogen. Disease suppressiveness is caused by specific microbes or microbial consortia that inhibit growth and activity of soil-borne pathogens (Mendes et al 2011, Weller et al 2002). This phenomenon is often attributed to the production of antimicrobial compounds that selectively inhibit pathogen growth (Mendes et al 2011, Raaijmakers & Weller 1998). However, stimulation of the host's immune system by protective rhizosphere microbes, a phenomenon called induced systemic resistance (ISR), may also contribute to disease suppressiveness (Pieterse et al 2014).

Buildup of disease suppressiveness in soils typically follows a disease outbreak (Berendsen et al 2012, Weller et al 2002), suggesting that, upon pathogen attack, plants recruit a community of protective microbiota. The capacity of plants to exploit protective benefits from their root microbiome is plant genotype dependent (Haney et al 2015, Pérez-Jaramillo et al 2017, Pieterse et al 2016, Wintermans et al 2016), implying that plants can manipulate protective rhizosphere processes to their advantage. The defense-related phytohormones salicylic acid (SA) and jasmonic acid (JA) are important modulators of the rhizosphere microbiome assembly of *Arabidopsis thaliana* (hereafter called Arabidopsis) (Carvalhais et al 2015, Lebeis et al 2015). SA and JA are major hormonal regulators of the plant immune signaling network in which SA is typically effective against infection by biotrophic pathogens, whereas JA is typically effective against attack by necrotrophic pathogens (Pieterse et al 2012). Because SA or JA systemically accumulates in response to infection by biotrophs or necrotrophs, respectively, and can affect rhizosphere microbiome assembly, we hypothesized that foliar infection by pathogens with these contrasting lifestyles would result in differential stimulation of specific microbiota in the rhizosphere.

To test this hypothesis, Arabidopsis accession Col-0 plants were grown in soil collected from a natural field site at Reijerscamp (the Netherlands) that supports an abundant endemic Arabidopsis population. Five-week-old Arabidopsis plants were leaf inoculated with the biotrophic pathogen *Hyaloperonospora arabidopsidis* (Lapin et al 2012), or the necrotrophic pathogen *Botrytis cinerea* (Coolen et al 2016), or treated repeatedly with 1 mM SA (mimicking biotroph-triggered immunity), or 100 µM methyl JA (MeJA, mimicking necrotroph-triggered immunity), or not treated (control). To identify key bacterial and archaeal rhizosphere community

members upon activation of the different foliar defense responses, DNA was isolated from rhizosphere and bulk soil at one and two weeks after the start of the treatments, and analyzed using a high-density 16S ribosomal DNA (rDNA) oligonucleotide microarray, referred to as the PhyloChip (Hazen et al 2010, Mendes et al 2011, Probst et al 2014). We subsequently characterized the bacteria that were promoted in the rhizosphere upon foliar defense activation and investigated the biological relevance of their promotion.

Materials and Methods

Soil and soil preparation

The soil used in this study was taken from a field in the Reijerscamp nature reserve, the Netherlands (52°01'02.55", 5°77'99.83") in April 2012. An abundant endemic Arabidopsis population was found at the site. The field had been used for crop production for more than 6 decades, before it was given back to nature in 2000. Since then, the site was grazed by free-living cattle and deer and tree seedlings were periodically removed. The soil, a gleyic placic podzol, consisted of coarse sand and gravel covered by a 30-50 cm top layer. The top layer consisted of 88% sand, 8% silt, 2% clay and 2.3% organic matter and had a C/N ratio of 24 at pH 5.4 (soil chemical analysis performed by Eurofins Agro, Wageningen, the Netherlands). The top 20 cm of soil was collected, air dried and sieved (3-mm sieve) to remove plant debris and rocks and subsequently stored at room temperature. To revive the microbial community prior to the experiment, a 5-cm thick layer of soil was placed on a bench in a greenhouse, watered to saturation and sown with Arabidopsis accession Col-0. Seedlings were allowed to grow for three weeks on the soil after which the soil was sieved again to remove plants. The soil was stored at 4°C and was then used within two weeks for the experiment.

Plant growth conditions

Arabidopsis thaliana accession Col-0 was sown on sterilized river sand (Hubun Inc., the Netherlands) saturated with modified ½ strength Hoagland solution (Pieterse et al 1996). After 2 days of stratification in the dark at 4°C, the seeds were allowed to germinate in a growth chamber (21°C, 70% relative humidity, 10 h light/14 h dark, light intensity 100 µmol m^{-2} s^{-1}). After two weeks, single seedlings were transferred to 60-mL pots with approximately 100 g of Reijerscamp soil (described above). Bulk soil pots were left unplanted. Spontaneously developing seedlings from plant seeds that came with the soil were removed with tweezers upon detection. Pots were watered with ½ strength Hoagland every two weeks and watered when required.

Experimental treatments

Five-week-old plants were inoculated with *Hyaloperonospora arabidopsidis* (*Hpa*) strain Noco2 by spraying a spore suspension (10^4 sporangia/mL water) onto all the leaves (Van Damme et al 2005) or with *Botrytis cinerea* (*Bc*) strain B0510 by applying a 5-µL drop of half-strength potato dextrose broth containing 5 x 10^5 spores mL^{-1}

to a true leaf of the rosette (Coolen et al 2016). For hormonal treatments, plants were dipped in a 0.015% (v/v) Silwet L-77 solution with either 1 mM SA or 100 µM MeJA. These concentrations are typically used to mimic activation of the respective hormonal signaling pathways (Vos et al 2015). The hormone treatments were repeated every 4th day until the end of the experiment. Pathogens were inoculated twice with an 8-day interval to ensure continued pathogen-induced signaling to belowground plant parts for the duration of the experiment. Pots were randomly placed in a climate chamber (21°C, 70% relative humidity, 10 h light/14 h dark, light intensity 100 µmol m^{-2} s^{-1}), but were covered with transparent lids to increase humidity for a 72-h period following inoculation of the pathogens. *Hpa* started to sporulate on *Hpa*-inoculated plants 8 days after inoculation. Downy mildew disease symptoms progressed to heavy yellowing of the leaves at day 15. *Bc*-inoculated plants developed lesions on the inoculated leaves, which on primary-inoculated leaves had spread to the leaf edges by day 15.

Plant RNA extraction and qRT-PCR analysis

For RNA extraction, 2 leaves were harvested from each of 4 plants of every treatment at 1, 3, 5, 7, 9, 11, 13, and 15 d after the start of the treatment and snap frozen in liquid nitrogen. Total RNA was extracted from plant leaves and treated with Ambion DNase I (ThermoFischer Scientific). RevertAid H Minus Reverse Transcriptase (Thermo Scientific) was used to convert DNA-free total RNA into cDNA using oligo (dT) primers. Two-step qRT–PCR reactions were performed in optical 96-well plates with a ViiA 7 real time PCR system (Applied Biosystems), using Power SYBR® Green PCR Master Mix (Applied Biosystems) with 10 pmol μL^{-1} primers (Table S1). A standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60 to 95°C with a ramp speed of 1.0°C min^{-1}. Expression levels were normalized to the reference gene *At1g13320*, which encodes PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2A-A3) (Czechowski et al 2005) using the $2^{-\Delta\Delta C}_{T}$ method described previously (Livak & Schmittgen 2001, Schmittgen & Livak 2008).

Microbiome analysis

To analyze the microbiome composition, samples from unplanted soil and plant root systems with adhering soil (50-250 mg) from 16 pots per treatment were harvested at day 8 and at day 15 after the start of the treatment. Pots containing soil and plants were carefully turned over and most soil was gently removed from the roots, keeping roots intact as much as possible. Root systems were then lifted by the shoot and slightly shaken to remove loosely adhering soil. Shoots were subsequently removed with a razor blade and total microbial genomic DNA was isolated from 160 root systems with adhering soil and 32 bulk soil samples using the PowerSoil® DNA Isolation Kit (Mobio). For each microbiome determination, DNA of four samples was pooled to form one replicate. For each treatment and time point, four replicates (with DNA from 4 soil/rhizosphere samples each) were subjected to PhyloChip analysis by Second Genome Inc. (San Francisco, U.S.A.) as described previously (DeSantis et al 2005, Hazen et al 2010, Probst et al 2014, van

der Voort et al 2016). Briefly, PhyloChip analysis is a microarray-based approach that can quantify the relative abundance of over 60.000 microbial taxa. Bacterial and archaeal 16S rRNA gene amplicons were hybridized to the 1,016,064 probes of the PhyloChip G3 spiked with a determined amount of non-16S rRNA genes. Fluorescence intensity (FI) was measured to quantify the hybridization of the amplicons to the probes and scaled to the spiked-in quantitative standards. Fluorescence intensity observed from perfectly matching probes (PM) were compared to mismatching probes (MM) and were considered positive if PM/MM $\geq$ 1.5 and PM-MM $\geq$ 50*N and r $\geq$ 0.95 where N indicates the array specific noise (DeSantis et al 2005) and r represents the response score (Hazen et al 2010). Probes were clustered into probe-sets based on both correlations in FI across all biological samples and taxonomic relatedness (Probst et al 2014). The empirical OTU (eOTU) tracked by a probe set was taxonomically annotated from the combination of the 9-mers contained in all probes of the set. The mean $\log_2$ FI for each eOTU and each sample was calculated and then are referred to as the hybridization score (HybScore) used in abundance-based analysis. eOTUs were considered present if $\geq$80% of their probes were positive. Of 379 eOTUs, 341 achieved this threshold in at least one sample. Principal component analyses were applied to the HybScores of the 75% most abundant eOTUs based on highest HybScore of all biological samples, using XLSTAT Version 2015.6.01.25740 (Addinsoft) add-in for Excel (Microsoft). False discovery rates were determined using the Phyloprofiler tool (Van der Voort *et al.*, 2016).

Selection of bacterial isolates

Rhizospheres of *Hpa*-infected plants were stored in 1 mL of 5 mM $MgSO_4$ with 25% glycerol (v/v) at -80°C at the end of the experiment described above. Rhizospheres were thawed at room temperature and suspended by vortexing for 120 s. In order to maximize the isolation of culturable bacterial species, dilution series were plated on the following media: 1) 1/10 strength tryptic soy agar (TSA; Difco); 2) TSA amended with 10 mg L^{-1} colistin; 3) TSA amended with 5 mg L^{-1} colistin and 10 mg L^{-1} naladixic acid; 4) TSA amended with 5 g L^{-1} NaCl and 64 mg L^{-1} polymyxin B; 5) TSA amended with 5 g L^{-1} NaCl, 64 mg L^{-1} polymyxin B and 10 mg L^{-1} gentamycin; 6) Nutrient Agar (Merck) amended with water yeast agar (per L: 5 g NaCl, 1 g KH_2PO_4, 0.1 g yeast extract (Difco), 15 g agar (Difco)); 7) peptone yeast agar (per L: 10 g peptone (Difco), 2 g yeast extract (Difco), 2 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$ and 15 g agar (Difco)); 8) R2A agar (Difco); 9) *Xanthomonas* selective medium (Kado & Heskett 1970); 10) *Stenotrophomonas* selective medium (Juhnke & des Jardin 1989); and 11) King's medium B agar (King et al 1965) amended with 13 mg L^{-1} chloramphenicol and 40 mg L^{-1} ampicillin. All media were amended with 200 mg L^{-1} Delvocid (DSM; active compound: natamycin) to prevent fungal growth. The plates were incubated for 3-5 days at 20°C. A total of 288 bacterial colonies with unique morphologies were selected and streaked on TSA. Single colonies from pure cultures were inoculated in 1/10 strength tryptic soy broth (TSB; Difco), incubated overnight at 20°C at 180 rpm, and stored at -80°C in 25% (v/v) glycerol. Pure cultures were labelled WCS2014 for "Willie Commelin Scholten" and the year of isolation and numbered.

Identification and characterization of bacterial isolates

A loop of bacterial cells was added to 20 µL of water, incubated for 15 min at 95°C and immediately cooled on ice. This bacterial lysate was diluted 10 times with water and cell debris was removed by centrifugation (1 min, 10.000 *g*). The 16S rRNA gene was amplified with primers F27 and R1492 (Thomas et al 2012) (Table S1). Two µL of the colony lysate was added to a total volume of 50 µL PCR reaction mixture (5 µL 10x Dreamtaq buffer (Thermo Scientific), 1 µL 10 mM dNTP's, 2.5 µL 10 µM forward primer F27, 2.5 µL 10 µM reverse primer R1492, 1 µL Dreamtaq polymerase (Thermo Scientific), 36 µL H_2O). PCR conditions used in the thermocycler (Hybaid, Ashford, UK) were 5 min at 94° C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and a final extension of 10 min at 72°C. The PCR products were checked by electrophoresis on 1.5% agarose gels in 1xTAE buffer (40 mM Tris–acetate/1 mM EDTA, pH 8). PCR products were sequenced by Macrogen (Seoul, Korea). Taxonomy of isolates was determined through the Sequence Match function of the ribosomal database project (Cole et al 2014).

BOX-PCR (Versalovic et al 1994) was performed with primer BOXA1R to determine if the isolated strains were identical on strain level and most likely isogenic (Table S1). For BOX-PCR, bacterial genomic DNA was isolated with the GenElute™ Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's instructions. A volume of 1 µL of the genomic DNA was added to a BOX-PCR-reaction mix (2.5 µL 10x Dreamtaq buffer (Thermo Scientific), 0.5 µL 10 mM dNTP's, 0.25 µL 10 µM primer BOXA1R, 0.125 µL Dreamtaq polymerase (Thermo Scientific), 20.625 µL H_2O) to a total volume of 25 µL. PCR conditions used in the thermocycler (Hybaid, Ashford, UK) were 7 min at 95°C, followed by 30 cycles of 30 s at 90°C, 1 min at 95°C, 1 min at 52°C and 8 min at 65°C, and a final extension of 16 min at 65°C. Amplified PCR fragments were analyzed by electrophoresis on 1.5% agarose gels in 1xTAE buffer.

Genome sequencing and genetic comparison

Whole-genome sequencing libraries for *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 were prepared using the Nextera XT kit (Illumina, USA) according to the manufacturer's recommendations and then sequenced using the Illumina MiSeq (Illumina, USA) technology. The 250bp paired-end reads were cleaned, trimmed and assembled using the A5-MiSeq pipeline (Coil et al 2015) and the assembled draft genomes were uploaded to Integrated Microbial Genomes Expert Review system for gene calling and annotation (Markowitz et al 2012). The genomes are available through the Joint Genome Institute genome portal (https://genome.jgi.doe.gov/; IMG Genome IDs 2747842429, 2747842501 and 2747842428). The genomic distance of the sequenced isolates to sequences of the Refseq database (https://www.ncbi.nlm.nih.gov/refseq/) were estimated using MASH (Ondov et al 2016).

Quantification of biofilm formation

Biofilm formation by the selected bacterial isolates was quantified as described by Ren and coworkers (2015). Briefly, *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 were

inoculated in 5 mL TSB and incubated for 24 h at 20°C at 180 rpm. Dilution series of the stationary phase bacterial cultures were subsequently transferred to 5 mL TSB and again incubated overnight at 20°C at 180 rpm. The exponentially growing dilution with an optical density at 590 nm (OD590) of ca. 0.5 was then selected and diluted to 0.15 in TSB. The bacterial cultures were then added separately or mixed with the other cultures in equal quantities to the wells of Nunc-TSP 96-wells plates (ThermoFisher Scientific, Waltham, USA) and to a total volume of 160 µL. Final bacterial densities in the separate and mixed suspensions were equal. The plates were sealed with Parafilm and incubated for 48 h at 20°C without shaking. Planktonic cells were then washed off the peg lids of the Nunc-TSP plates by transferring the lids successively to microtiter plates containing 200 µL phosphate-buffered saline (PBS). Biofilms formed on the peg lids were then stained for 20 min in 180 µL of aqueous 1% (W/V) crystal violet. The pegs were again rinsed 3 times in PBS and then incubated in 200 µL 96% ethanol to release the crystal violet absorbed by the biofilm. Biofilm thickness was then quantified by measuring the OD590 of the crystal violet-ethanol solution with a plate spectrophotometer (BioTek Synergy HT).

Attraction between colonies

Xanthomonas sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 were inoculated in 5 mL King's medium B and incubated overnight at 20℃ at 180 rpm. The optical density of the bacterial cultures was adjusted to 0.1 at 600 nm. Seven times 1 µL of these dilutions were inoculated in a diagonal row on both sides of a square petri-dish with King's medium B agar with a multichannel pipet, creating a V-shape of increasingly closer inoculation sites (fig. 2). The plates were sealed with Parafilm and incubated for 15 days at 20℃. Colony diameters were measured on a line orthogonal to the line dividing the V-shape.

Selection of rifampicin-resistant mutants

In order to quantify bacterial numbers in soil during plant growth promotion and ISR assays, spontaneous rifampicin-resistant mutants of *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 were obtained as described by Glandorf *et al.* (1992). Briefly, colonies of these strains were transferred to TSA agar plates containing increasing concentrations (50, 100, 150, 200, 250 µg ml^{-1}) of rifampicin. The stability of the rifampicin resistance in the mutants was confirmed and growth rate of the rifampicin-resistant mutants was found to be similar as their respective wild types.

Growth promotion assay

Arabidopsis accession Col-0 was sown on sand and cultivated as describe above. After two weeks, seedlings were transferred to 60-mL pots filled with a potting soil-sand mixture pre-inoculated with *Pseudomonas simiae* WCS417r (Berendsen et al 2015) (formerly known as *Pseudomonas fluorescens* WCS417r (Lamers et al 1988)), rifampicin-resistant mutants of *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 or *Microbacterium* sp. WCS2014-259, or a combination of the

three strains, in all cases to a final density of $1 \cdot 10^8$ cfu/g of soil. Soils were prepared as described by Pieterse *et al.* (1996). The strains were thoroughly washed before being suspended and added to the soil. Initial microbial densities in the soil were determined by suspending approximately 0.1 g of soil of five replicate pots per treatment and plating a dilution series on King's medium B amended with 150 µg mL^{-1} rifampicin. After plants had grown for 4 weeks in microbe-amended soil, the then 6-week-old plants were harvested and shoot fresh weight was determined.

ISR assay

In the ISR assay, plants were grown similarly and on similarly prepared soil as for the growth promotion assay. Assessment of downy mildew disease resistance was performed essentially as described (Van Damme et al 2005). In brief, 5-week-old plants were inoculated with *Hpa* Noco2 as described above. Plants were grown in pots placed in trays and covered with transparent lids to increase relative humidity. Six days after *Hpa* inoculation, the number of *Hpa* spores were determined. To this end, shoots of infected plants were harvested, collected in 15-mL tubes with 3 mL H_2O and vortexed for 30 s. Subsequently, spores were counted using a phase-contrast microscope (Axioskope, Zeiss, Jena, Germany) after which the number of *Hpa* spores produced per plant was calculated. These experiments were repeated with similar results. Analysis of variance was performed with XLSTAT Version 2015.6.01.25740 (Addinsoft) add-in for Excel (Microsoft).

Soil-borne legacy of downy mildew-infected plants

In June 2016, soil was collected from the same site in the Reijerscamp nature reserve after which it was sieved, dried and stored as described above. Eighty 60-mL pots were filled with approximately 110 g of soil watered to field capacity. To prevent growth of moss and algae, the soil surface was covered by a circular plastic cut-out of micro pipette tip holder (Greiner Bio-One, 0.5-10 µL, Item No.: 771280). Arabidopsis Col-0 seeds were suspended in a 0,2% (w/v) water agar solution and imbibed in the dark at 4˚C for 2 days. Approximately 40 seeds were sown on each of 40 pots by pipetting 1-2 seeds in each hole of the cover, the other 40 pots were left unplanted. The pots were placed on small saucers, watered with modified ½ strength Hoagland solution, randomly placed in trays, covered by transparent lids and transferred to a growth chamber (21°C, 70% relative humidity, 10 h light/14 h dark, light intensity 100 µmol m^{-2} s^{-1}). After a week the lids were replaced by lids with a mesh to reduce the humidity in the trays. After two weeks the pots were sprayed with either a *Hpa* spore suspension (50 spores μL^{-1} as described above) or mock-treated by spraying with water. Trays were again covered with transparent lids to increase humidity after which downy mildew infections were allowed to develop for a week. Subsequently, all above-ground parts of the first population of plants were cut off and removed and new seeds were sown on all pots as described above. After two weeks of growth, this second population of plants was either inoculated with *Hpa* or mock treated with water after which downy mildew infections were allowed to develop for 1 week. Downy mildew disease severity was then quantified by determining the number of *Hpa* spores produced per pot on above ground plant tissue as described above.

Results

Root microbiome changes on immune-stimulated plants

In order to investigate the effect of foliar pathogen infection or treatment of the leaves with SA or MeJA on root microbiome assemblage, we monitored the changes in the microbial communities over a period of two weeks after defense activation. PhyloChip analysis of the microbial communities revealed a total of 341 eOTUs, the majority belonging to the phyla *Proteobacteria* (43%), *Firmicutes* (20%), and *Bacteroidetes* (20%) (fig. S1). To identify key drivers of variability in abundance of the eOTUs among samples, we used principal component analyses (fig. 1A). The first principal component (PC1) separates the unplanted soil samples from the rhizosphere samples, confirming the rhizosphere effect for Arabidopsis (Bakker et al 2013, Reinhold-Hurek et al 2015), in which specific microbiota are promoted in the rhizosphere compared to bulk soil. The second principal component (PC2) distinguishes the different plant treatments and separates the rhizospheres of plants infected by *Hpa* from the other rhizospheres. Most eOTUs correlate with PC1 and are either promoted or suppressed by the plant rhizosphere (fig. 1B; fig. S2). Only three eOTUs strongly correlate with PC2 (fig. 1B). These eOTUs, designated eOTU 97, 106 and 107, were significantly more abundant in rhizospheres of *Hpa*-infected plants compared to that of untreated plants at both one and two weeks after inoculation (fig. 1C-E; fig. S3). These eOTUs were recognized as *Xanthomonas, Microbacterium,* and *Stenotrophomonas* sp., respectively. Moreover, at one of the two timepoints eOTU97, 106 and 107 were also significantly more abundant in the rhizosphere of SA-treated plants, albeit at a lower abundance than in *Hpa*-treated plants (fig. 1C-E; fig. S3). In the rhizospheres of *Bc*-infected and MeJA-treated plants, eOTU107 and/or eOTU97 also showed a small but significant increase in abundance at one of the timepoints tested, suggesting that the enhanced abundance of these eOTUs in the rhizosphere is related to the activation of plant defense responses, in particular those induced by *Hpa* infection.

Hpa infection progressed to heavy sporulation in 7 days after inoculation, and triggered SA-dependent defense responses in the leaves, as exemplified by the strongly induced expression of the SA-responsive marker gene *PATHOGENESIS RELATED-1* (*PR-1*) (Pieterse et al 2012) (fig. S4A). The SA treatment also induced *PR-1* (831 fold-induction cumulative over time), but to a lesser extent than *Hpa* (4369 fold-induction cumulative over time). *Bc* infection and MeJA treatment led to a relatively low, but detectable induction of *PR-1.* These results show that the observed increased abundance of eOTUs 97, 106 and 107 in the root microbiome correlated with the activation of the SA response. Although *Bc* infections and repeated MeJA treatment of Arabidopsis leaves caused strong upregulation of the JA-responsive marker genes *PLANT DEFENSIN1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), respectively (Pieterse et al 2012) (fig. S4B-C), significant microbiome changes mediated by these JA-related treatments were not observed.

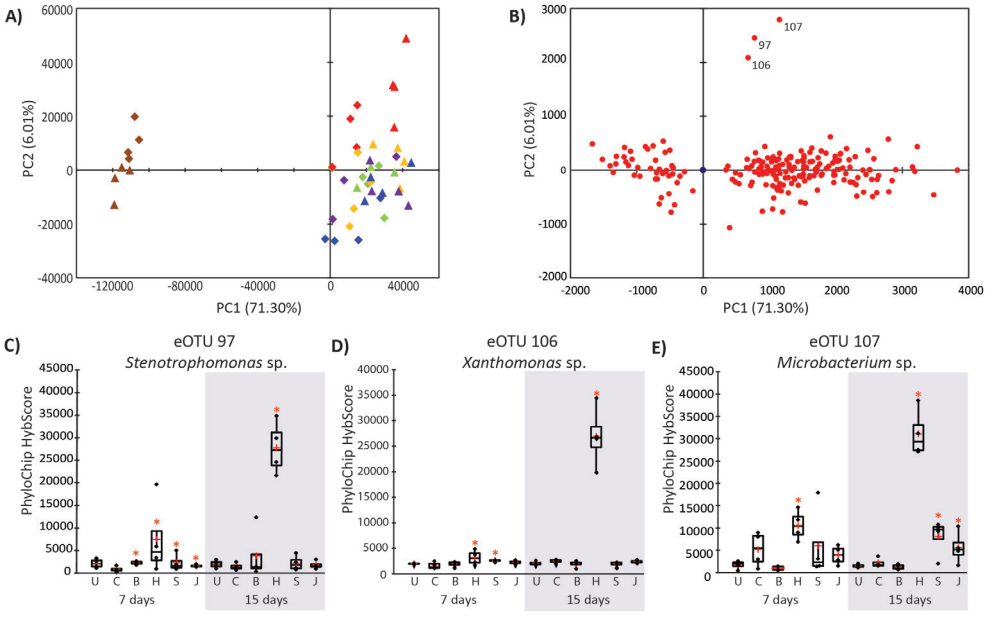

Figure 1. Downy mildew infection promotes growth of specific microbiota in the rhizosphere of Arabidopsis. **A)** Principal component (PC) analysis of microbial communities in unplanted soil and the rhizospheres of pathogen-infected or defense hormone-treated Arabidopsis plants. Microbial communities were isolated from unplanted soil (brown symbols), rhizospheres of untreated control plants (green), rhizospheres of plants of which the leaves were inoculated with the biotroph *Hpa* (red), or the necrotroph *Bc* (blue), or rhizospheres of plants of which the leaves were repeatedly treated with SA (yellow), or MeJA (purple). Microbial communities were analyzed 1 week (squares) and 2 weeks (triangles) after the start of the foliar treatments. Eigenvalues of PC1 and PC2 are expressed on the X- and Y-axis, respectively. **B)** Biplot of eOTU correlations (PC scores) to the same PC1 and PC2. Each red dot represents an eOTU. PC1 separates bulk soil eOTUs (left) from rhizosphere eOTUs (right). Three eOTUs that strongly correspond to PC2 are designated with a number. **C-E)** Quantification of eOTU abundance in the different treatments. Boxplots of PhyloChip HybScore per treatment and time point are shown for **C)** *Stenotrophomonas* sp. eOTU 97, **D)** *Xanthomonas* sp. eOTU 106 and **E)** *Microbacterium* sp. eOTU 107. Black dots represent the values of the 4 replicates per treatment. Red plus signs signify the averages. Red asterisks denotes significant differences from control rhizospheres in the same time point (false discovery rate < 0.05). U, unplanted soil; C, rhizosphere of control-treated plants; B, rhizosphere of *Bc*-inoculated plants; H, rhizosphere of *Hpa*-inoculated plants; S, rhizosphere of SA-treated plants; J, rhizosphere of MeJA-treated plants.

Characterization of recruited rhizosphere microbes of *Hpa*-infected plants

To further characterize eOTUs 97, 106 and 107, we isolated candidate microbes from the rhizosphere of the *Hpa*-infected plants. Using both broad-spectrum media and media selective for *Xanthomonas, Stenotrophomonas,* or *Microbacterium* spp., 279 rhizobacterial strains were isolated and identified based on 16S rRNA gene sequences. The 279 isolates represented 35 genera among which were three isolates belonging to the genus *Xanthomonas*, three to the genus *Microbacterium* and one to the genus *Stenotrophomonas* (Table S2). BOX PCR-generated DNA fingerprints indicated that each of the three representatives of *Xanthomonas* and of *Microbacterium* were identical (fig. S5). The probe sets that defined eOTUs 97,

106, and 107 perfectly matched the 16S rRNA gene sequence of the respective *Xanthomonas*, *Stenotrophomonas* and *Microbacterium* spp. isolates (fig. S6), indicating that we likely isolated the actual eOTUs that were promoted in the rhizosphere by *Hpa* infection.

The *16S rRNA* gene sequences of the isolated *Xanthomonas*, *Stenotrophomonas* and *Microbacterium* spp. did not conclusively distinguish the species level taxonomy of these strains. To further characterize the strains, the genomic distances of *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 were estimated pairwise to all genomes in the Refseq database belonging to respectively the *Xanthomonas*, *Stenotrophomonas* or *Microbacterium* genus (Additional data S3). Average nucleotide identity (ANI) values between 94-96 % have been proposed for defining the boundaries between prokaryotic species (Konstantinidis & Tiedje 2005, Richter & Rosselló-Móra 2009). Based on the ANI, the closest relative to *Xanthomonas* sp. WCS2014-23 in the Refseq database is *Xanthomonas* sp. Leaf148 (97.5% ANI), which has been isolated from *A. thaliana* leaves (Bai et al 2015). All of the genomes in the Refseq database belonging to *Xanthomonas* type strains share 91.5% or less of their ANI, indicating that WCS2014-23 belongs to an undescribed species or a species for which the type strain has not been completely sequenced. The closest relative to *Stenotrophomonas* sp. WCS2014-113 is *Stenotrophomonas maltophilia* strain AA1 (94.5% ANI), a strain isolated from maize roots (Niu et al 2017). However, the ANI shared with the *Stenotrophomonas maltophilia* type strain NBRC 14161 of 90.5% indicates that strain WCS2014-23 is not *Stenotrophomonas maltophilia,* but a new species or a species of which the type strain has not been completely sequenced. The closest neighbor to *Microbacterium* sp. WCS2014-259 was *Microbacterium foliorum* strain 122, an endophyte of *Dactylis glomerata.* The ANI of 88.8 % shared with this strain indicates that none of the *Microbacterium* genomes in the Refseq database derive from the same *Microbacterium* species as WCS2014-259. In conclusion, *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 cannot be taxonomically classified on the species level based on their full genome sequence, but are most closely related to other plant-associated strains of their genera.

The recruitment of the three eOTUs as a consortium suggests that they exist in close association in the rhizosphere. Rhizosphere-colonizing bacteria form biofilms on the root surface in which diverse microbial processes, such as the production of plant growth regulators, defense elicitors, and antibiotics are triggered and may affect both the host and microbes in their surrounding (Beauregard et al 2013). Bacterial interspecific cooperation has been suggested in the synergistic biofilm formation by different bacterial strains isolated from agricultural soil (Ren et al 2015). Whether the three identified eOTUs act synergistically in biofilm formation was investigated in an *in vitro* biofilm formation assay. Indeed, the three bacterial rhizosphere isolates showed synergy in biofilm formation, as the combination of three consistently formed more biofilm than the separate strains (fig. 2A). This suggests that the three rhizobacterial species are synergistically assembled as

a consortium in the rhizosphere of Arabidopsis upon *Hpa* infection of the foliar tissue. In agreement with this, the three strains were found to attract each other on plate (fig. 2B; fig. S7). Colony growth of the *Stenotrophomonas* isolate is stimulated towards both the *Xanthomonas* and the *Microbacterium* isolate. Similarly, growth of the *Microbacterium* colonies is induced in the vicinity of both the *Stenotrophomonas* and the *Xanthomonas* isolate.

To investigate the biological relevance of the promotion of this bacterial consortium, we grew Arabidopsis plants in soils pre-inoculated with the single strains or the three strains together to test for their ability to induce ISR against foliar infection with *Hpa*. *Pseudomonas simiae* WCS417r (hereafter called WCS417r) is a well-studied plant-beneficial rhizobacterium that induces systemic resistance upon colonization of the roots (Berendsen et al 2015, Zamioudis et al 2015). Colonization of the rhizosphere of Arabidopsis plants by WCS417r indeed induced ISR as reflected by the reduced production of *Hpa* spores on downy mildew infected leaves (fig. 3A). None of the single strains reduced the number of *Hpa* spores produced on infected plants significantly. However, the consortium of three strains had a more robust effect than the single strains, as this consortium induced ISR as exemplified by the significant reduction of the number of *Hpa* spores on infected leaves (fig. 3A). Collectively, these results show that the consortium of *Xanthomonas, Stenotrophomonas* and *Microbacterium* isolates that is promoted in the rhizosphere of Arabidopsis upon foliar infection with *Hpa,* can systemically enhance the level of protection against *Hpa*. These results further support the notion that the rhizobacterial consortium act in synergy, resulting in the promotion of plant health.

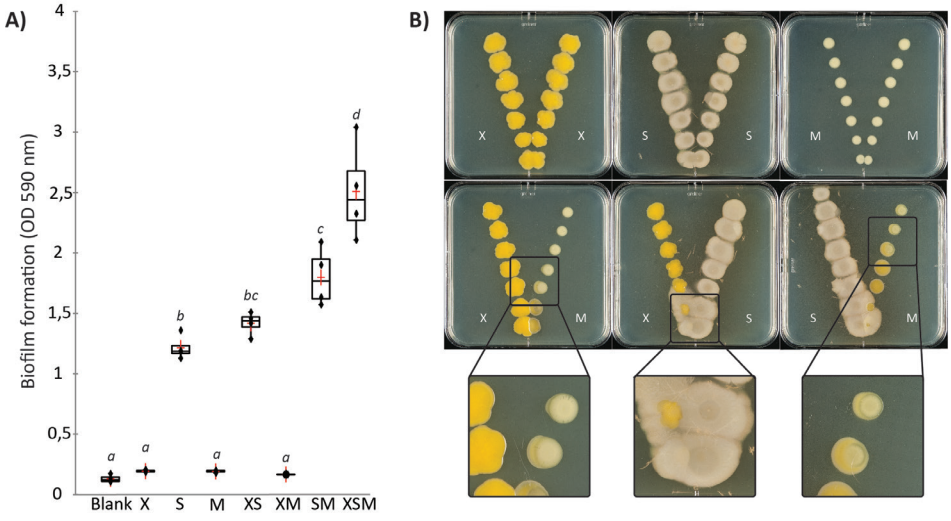

Figure 2. Synergistic interactions between recruited *Xanthomonas, Stenotrophomonas,* and *Microbacterium* spp. strains. **A**) Boxplot of biofilm formation by single *Xanthomonas* sp. WCS2014-23 (X; eOTU 106), *Stenotrophomonas* sp. WCS2014-113 (S; eOTU 97) and *Microbacterium* sp. WCS2014-259 (M; eOTU 107) or the double (XS, XM, SM) and triple combinations (XSM) thereof in Nunc-TSP lid plates. After 24 h of incubation, the biofilm formation was quantified by staining with crystal violet. **B)** Attraction between colonies of X, S and M grown at increasing proximity on King's medium B agar.

Direct activation of defenses by pathogens will generally result in increased level of disease resistance, but comes at a fitness cost for the plant as evidenced by reduced plant growth and seed production (van Hulten et al 2006, Vos et al 2015). ISR, however, as induced by the model strain WCS417r, has limited fitness cost that are mostly outweighed by the plant growth promoting effects of this bacterium (Pieterse et al 2014, Zamioudis et al 2013). We therefore also inoculated soils with the three strains to test their effects on plant growth. None of the bacterial strains, including WCS417r, negatively affected shoot fresh weight (fig. 3B). Moreover, inoculation of the soil with the mixture of the three isolates even increased shoot fresh weight significantly (fig. 3B-C).

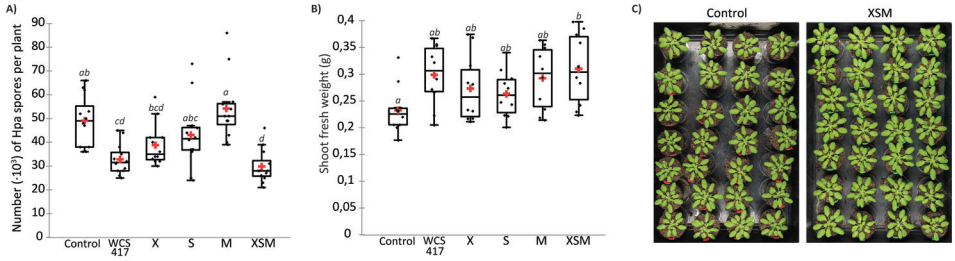

Figure 3. Synergistic effects of recruited rhizobacteria on systemic immunity against *Hpa* and plant growth promotion. **A**) Spore production by *Hpa* on Arabidopsis plants growing on soil pre-inoculated with *Pseudomonas simiae* WCS417r, *Xanthomonas* sp. WCS2014-23 (X; eOTU 106), *Stenotrophomonas* sp. WCS2014-113 (S; eOTU 97) and *Microbacterium* sp. WCS2014-259 (M; eOTU 107or a mixture of X, S and M (XSM). **B)** Boxplot showing shoot fresh weight of 6-week-old Arabidopsis plants grown in soil pre-inoculated with WCS417r, X, S, M, or a mixture of X, S and M. Italic letters depict statistically significant ($P<0.05$) differences according to analysis of variance with Tukey's posthoc test. Black dots represent the respective replicate values. All experiments were repeated at least 3 times with similar results. **C)** Picture showing 6-week-old Arabidopsis plants grown in control soil or soil pre-inoculated with a mixture of X, S and M.

Soil-mediated legacy of *Hpa*-infected plants protects a successive plant population

ISR benefits plants as it primes the plant for enhanced defense against future pathogen or insect attack (Pieterse et al 2014). Nonetheless, it is not likely to rescue an already infected plant. We therefore hypothesized that, in nature, recruitment of beneficial microbes to the root upon pathogen attack benefits a following population of plants, such as the plant's offspring, growing in the same soil. To test this hypothesis, we pre-conditioned the Reijerscamp soil by growing *Hpa*-infected or mock-treated Arabidopsis plants. After removal of this first population of plants, we sowed a second population of naïve Arabidopsis plants on the differently pre-conditioned soils and challenged them with *Hpa* (fig. 4). We included control soils that were left unplanted in the conditioning phase but were otherwise treated the same, including spray treatment with water or *Hpa* spores. In line with our hypothesis, the second population of Arabidopsis seedlings growing in soil pre-conditioned by downy mildew-infected plants were more resistant to foliar downy mildew infection than plants growing in soil pre-conditioned by control plants (fig.

4B). Pre-conditioning of soil by control plants or spraying unplanted soil with *Hpa* spores did not affect resistance of a subsequent population of plants. Together these results suggest that foliar downy mildew infections lead to a soil-mediated legacy that renders a subsequent population of plants growing in the same soil more resistant against downy mildew infection.

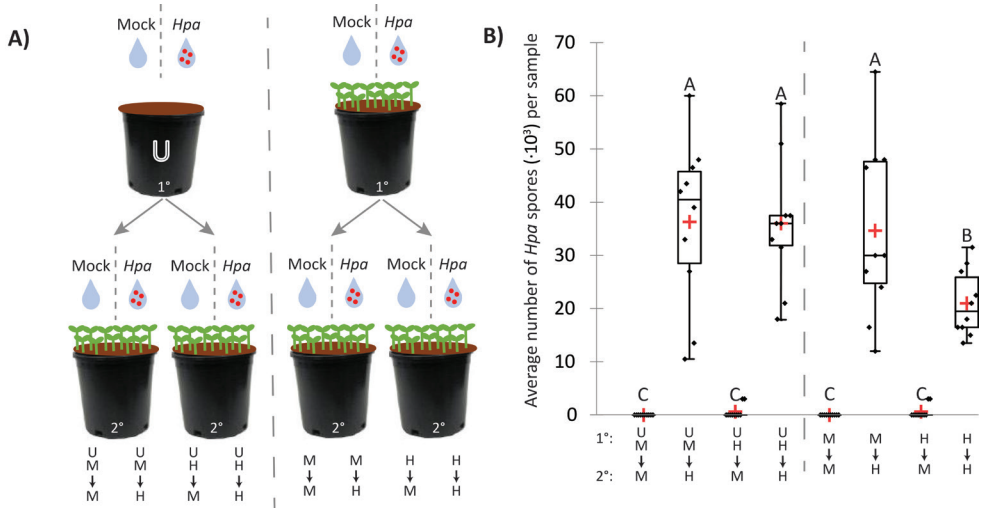

Figure 4. Soil–mediated effects of *Hpa*-infected plants on disease resistance in a subsequent population of plants. **A)** Schematic representation of the experiment. A first population (1°) of Arabidopsis Col-0 seedlings or unplanted soil (U) was inoculated with a *Hpa* (H) spore suspension or mock treated (M). Seven d after inoculation, aboveground plant parts were removed, after which a second population (2°) of naïve Arabidopsis plants were germinated and grown on the remaining soils. After 2 weeks of growth in the pre-conditioned or unconditioned soils, Arabidopsis plants were inoculated or not with *Hpa*. Disease severity was quantified by counting the number of *Hpa* spores that were produced at 7 d after inoculation. **B)** Boxplot showing the number of *Hpa* spores produced on plants growing on the indicated pre-conditioned soils. Capital letters indicate statistically significant ($P<0.05$) differences according to analysis of variance with Tukey's posthoc test. Red plus signs signify the averages of the 10 replicates per treatment. Black dots represent the respective replicate values. The experiment was repeated 4 times with similar results.

Discussion

This study shows that downy mildew infection in Arabidopsis leaves leads to promotion of a bacterial consortium consisting of a *Microbacterium*, a *Stenotrophomonas,* and a *Xanthomonas* sp. in the rhizosphere. Our results suggest that foliar infection with a biotrophic pathogen systemically signals to the roots to promote growth of specific microbial species in the rhizosphere. The promoted microbial species interact synergistically in biofilm formation, suggesting that they are also synergistically assembled at the root-microbiome interface. As a consortium, the increased microbes are beneficial to the plant as together they induce resistance against *Hpa* but also promote plant growth. Together these

findings show that Arabidopsis plants infected with a biotrophic pathogen can promote specific members of their microbiome to aid in their defense. Recent studies with wheat and pepper plants support this finding as pathogen or insect attack resulted in plant-mediated changes in rhizosphere microbial communities (Dudenhöffer et al 2016, Kong et al 2016). Interestingly *Stenotrophomonas* is also one of the few genera that increased in abundance on pepper plant roots upon infestation of foliar tissue by white fly (Kong et al 2016). This shows that this genus can be promoted upon defense activation in various plant species. In contrast to *Hpa,* infection by the necrotrophic fungus *Bc* strongly induced the JA-dependent marker gene *PDF1.2* in above ground plant tissue, but this did not lead to clear promotion of microbes in the rhizosphere. This suggests that different foliar pathogens differently affect the rhizosphere microbiome. A better understanding of the plant genetic basis of disease-induced recruitment of beneficial root-associated microbes could unlock new possibilities for breeding of crop plants that are better able to employ their microbiomes in their defense and have enhanced capacities for controlling disease.

In our study, we used PhyloChip for the analysis of root microbial communities. PhyloChip can more sensitively detect differences in taxon abundance (Miezeiewski et al 2015) and PhyloChip analyses have more reproducible output than sequencing-based approaches (Hazen et al 2010, Wen et al 2017, Zhou et al 2011). However, PhyloChip cannot provide accurate estimates of the relative amounts of a taxon as a proportion of the total community. It is therefore difficult to assess the actual populations of the recruited *Microbacterium*, *Stenotrophomonas*, and *Xanthomonas* spp. in our experiment. For *Pseudomonas* spp., a threshold population level of 10^5 CFU/g root is required for the onset of ISR (Raaijmakers et al 1995). Regardless, it is unlikely that recruitment of ISR-inducing bacteria can arrest or cure an already strongly developed infection. We, thus, tested the hypothesis that recruitment of root-associated protective microbes upon foliar infection by a pathogen benefits the plant's offspring that subsequently germinates the same soil. We demonstrated that downy mildew infections in a first population of plants indeed confer a soil-mediated legacy that provides increased resistance against this pathogen in a subsequent population of naïve plants that grow in the same soil. Together, our results suggest that infection of aboveground plant tissue results in the recruitment of beneficial root-associated microbes that have the potential to protect a subsequent population of plants growing in the same soil against the pathogen that initiated the recruitment.

Acknowledgements

This work was financially supported by ERC Advanced Grant no 269072 of the European Research Council and by the Dutch Technology Foundation STW, which is part of the Netherlands Organization of Scientific Research (NWO) and partly funded by the Ministry of Economic Affairs (Back2Roots Grant 14219) and by a postdoctoral fellowship of the Research Foundation Flanders to R.d.J.

(FWO 12B8116N). We would like to thank Ioannis Stringlis, Marrieke Bos, Iliass Bouallal and Diana van Houten for experimental assistance, Annemiek van Andel for propagation of *Hpa in planta*, Hans van Pelt for taking photographs, Menno van der Voort for help with the Phyloprofiler, Stephen Barnett for advice on the isolation of *Microbacterium* spp. and Alexandre Jousset for his valuable comments on the manuscript. Furthermore, we would like to thank Machiel Bosch and Natuurmonumenten for permitting the sampling of soil in the Reijerscamp reserve.

Author contributions

G.V., P.A.H.M.B., and R.L.B. designed experiments; G.V., R.L.B., K.Y., Y.S., W.P.B., M.B., J.H., performed experiments; G.V., R.L.B., K.Y., Y.S., R.J.,W.P.B., M.B., J.H., analyzed data and G.V., R.L.B., P.A.H.M.B, and C.M.J.P. wrote the manuscript.

Supplemental data

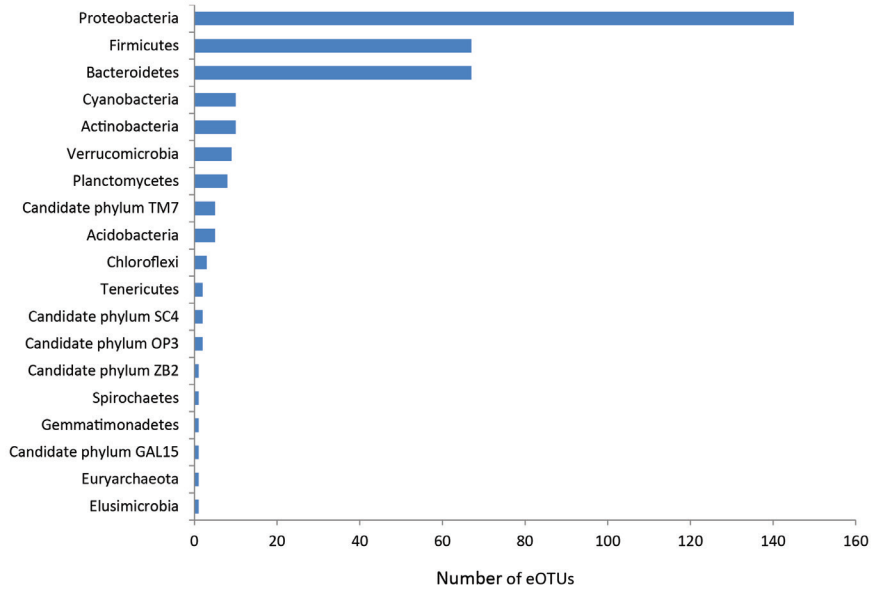

Figure S1. Distribution of the 341 emperical OTUs (eOTUs) that were detected over 18 bacterial and 1 archeal phyla. The number of eOTUs that were discovered through analysis of all 48 samples is shown per phylum

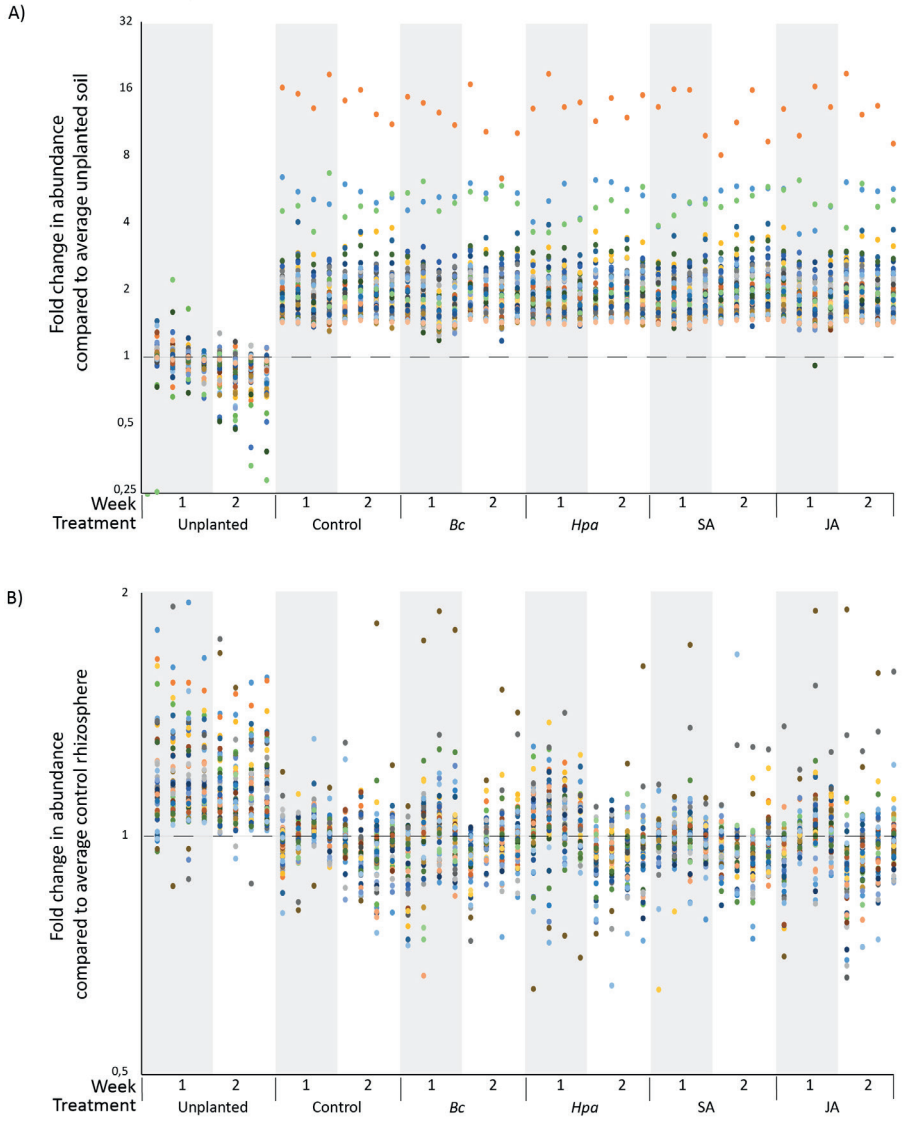

Figure S2. Abundance of eOTUs in rhizosphere and bulk soil. The abundances of eOTUs that are most strongly correlated with PC1 (shown in Fig. 1 in main text) are depicted. **A)** The 50 eOTUs with the strongest positive correlation to PC1 were found to increase in abundance in the rhizosphere relative to the uplanted bulk soil. Abundance is expressed as fold change in HybScore compared to the average HybScore in unplanted soil samples 1 week after the start of treatments. **B)** The 50 eOTUs most negatively correlated to PC1 were found to decrease in rhizosphere samples. Abundance is expressed as fold change in HybScore compared to the average probe intensity in control plant samples 1 week after the start of the treatment. Each color represents a different eOTU. Each dot represents the abundance of an eOTU in a sample. Four replicate samples for each of two time point are shown per treatment. Unplanted, unplanted bulk soil; Control, rhizosphere of control plants; *Bc,* rhizosphere of *Bc*-inoculated plants; *Hpa,* rhizosphere of *Hpa*-inoculated plants; SA, rhizosphere of SA-treated plants; JA, rhizosphere of MeJA-treated plants.

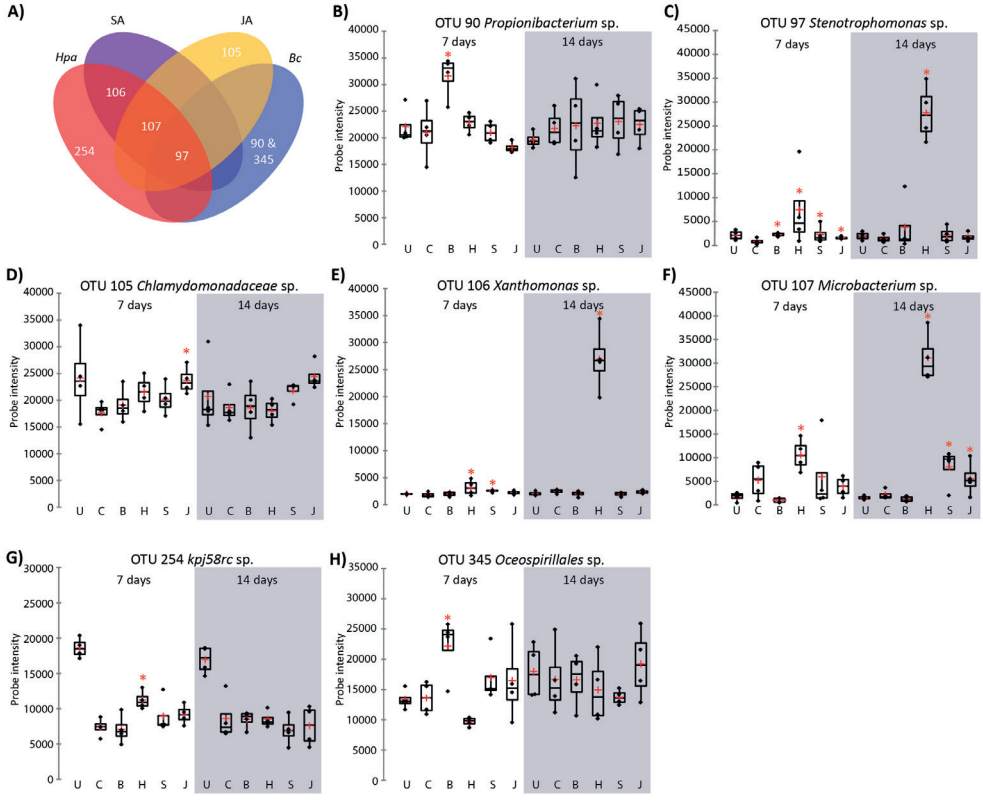

Figure S3. All eOTUs significantly affected by at least one foliar treatment in at least one time point. A) Venn diagram showing overlap between treatments in eOTUs that are significantly different from control rhizospheres in at least one time point (false discovery rate < 0.05 based on average probe intensity). Distinct eOTUs are designated by numbers and correspond to the numbers designated in Supplementary Data 1. **B-H)** Boxplots of PhyloChip HybScore of the indicated enriched eOTUs. Boxes represent median, interquartile range and extremes. Black dots represent the values of the 4 replicates per treatment. Red plus signs signify the averages. Red asterisks denotes significant differences from control rhizospheres in the same time point. U, unplanted bulk soil; C, rhizosphere of control-treated plants; B and *Bc*, rhizosphere of *Bc*-inoculated plants; H and *Hpa*, rhizosphere of *Hpa*-inoculated plants; S and SA, rhizosphere of SA-treated plants; J and JA, rhizosphere of MeJA-treated plants.

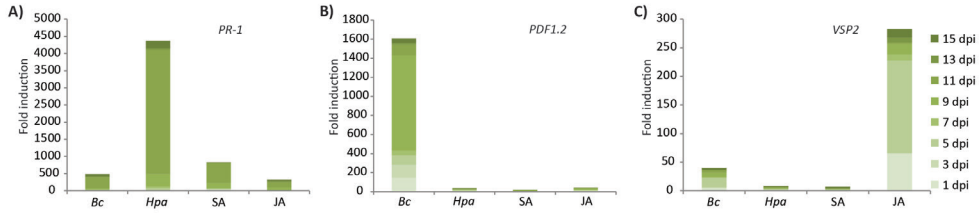

Figure S4. Induction of SA- and JA-regulated defense signaling marker genes. RT-qPCR analysis of **A)** *PR-1* **B)** *PDF1.2* and **C)** *VSP2* gene expression in Arabidopsis Col-0 leaves 1, 3, 5, 7, 9, 11, 13 and 15 days after the start of foliar treatments. Fold induction compared to control plants is shown cumulatively over time. Each bar represents the average of four replicate plants per time point. *Bc,* leaves inoculated with *Bc* on days 0 and 8; *Ha,* leaves inoculated with *Hpa* on days 0 and 8; SA, leaves dipped in 1 mM SA on days 0, 4, 8, and 12; JA, leaves dipped in 0.1 mM MeJA on days 0, 4, 8 and 12.

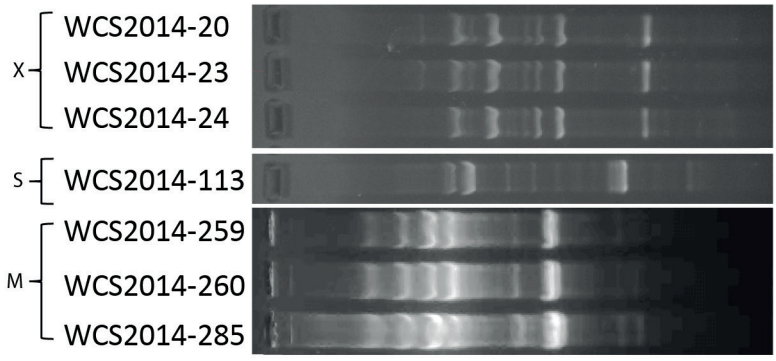

Figure S5. BOX-PCR patterns of isolated *Xanthomonas* sp. (X), *Stenotrophomonas* sp. (S) and *Microbacterium* sp. (M) strains. Indicated strains were isolated from the rhizosphere of Hpa-inoculated Arabidopsis plants growing in Reijerscamp soil during the foliar-defense-activation experiment described in the main text, Fig. 1 and Suppl. Figs. 1-4. Three out of a total of 279 isolated strains were identified as Xanthomonas spp., one as a Stenotrophomonas sp. and three as Microbacterium spp.. All strains within each of these three genera produced the same DNA fingerprint and were concluded to be isogenic. Hence, for each of the three eOTUs that were promoted in the rhizosphere of Hpa-infected plants, a single corresponding strain was isolated.

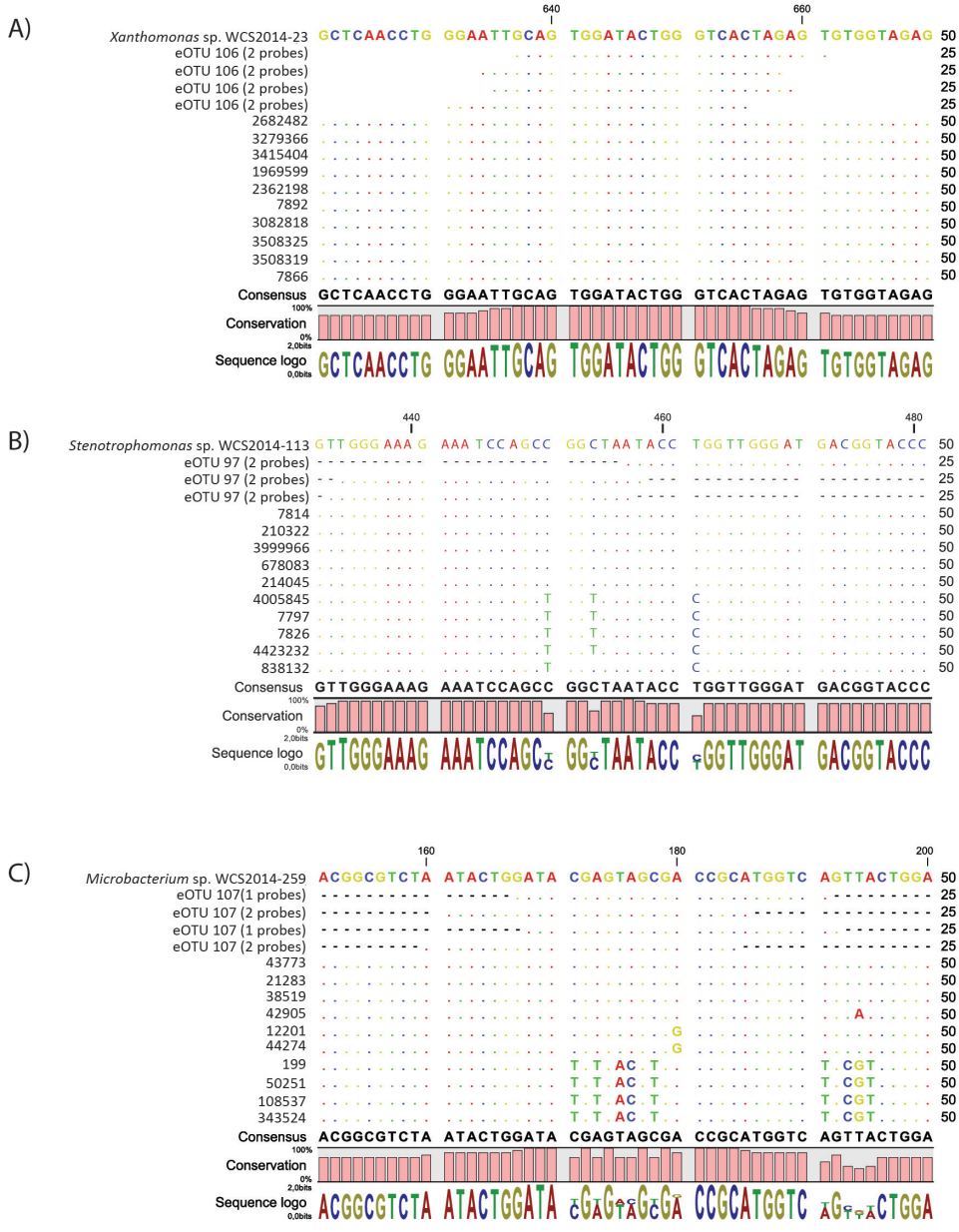

Figure S6. Isolated *Xanthomonas* sp., *Stenotrophomonas* sp. and *Microbacterium* sp. strains are identical to the promoted eOTUs. Alignment of 16S rRNA gene sequences of the isolated *Stenotrophomonas* sp. **(A)**, *Xanthomonas* sp. **(B)** and *Microbacterium* sp. **(C)** with the corresponding PhyloChip probe sequences and 10 Nearest Neighbours in a BLASTn search of the Greengenes database. Matching residues are shown as colored dots and mismatched nucleotides as letters. For each probe it is indicated whether a single strand of the 16S rRNA gene was targeted (1 probe) or that both strands were targeted (2 probes). All probes (6 for eOTUs 97 and 107 and 8 for eOTU 106) were a perfect match for the respective 16S rRNA gene sequences of the isolated strains. Numbers left of sequences indicate the Greensgenes id numbers of the Nearest Neighbours. Oligonucleotide sequences

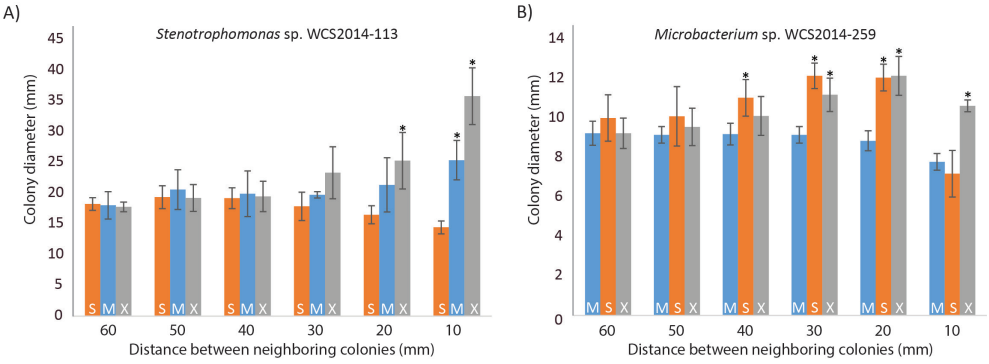

Figure S7. Colony diameters of *Stenotrophomonas* sp. and *Microbacterium* sp. expand at increasing proximity to other consortium members. Bars represent the average colony diameter of **A)** *Stenotrophomonas* sp. WCS2014-113 (S; eOTU 97) and **B)** *Microbacterium* sp. WCS2014-259 (M; eOTU 107) after 15 days of incubation at 20°C on three replicate plates at increasing proximity to colonies of S (orange bars), M (blue bars) and *Xanthomonas* sp. WCS2014-23 (X; grey bars; eOTU 106), Error bars depict standard deviations of the mean. Asterisk denotes statistically significant ($p<0.05$) difference in colony diameter with the diameter of colonies neighbored by self at the same distance determined by a two-tailed Student's *t*-test. All strains limit their own colony size when inoculated 10 mm or smaller distance apart. Colony diameter of M is significantly enhanced in proximity of X and S. Likewise, colonize diameter of S is significantly enhanced in proximity of M and X.

Table S1. Taxonomy of isolates collected from rhizospheres of *Hpa*-infected Arabidopsis plants.

Phylum	Class	Order	Family	Genus	Number of isolates
Actinobacteria	Actinobacteria	Actinomycetales	*Intrasporangiaceae*	*Terrabacter*	3
Actinobacteria	Actinobacteria	Actinomycetales	*Microbacteriaceae*	*Microbacterium*	3
Actinobacteria	Actinobacteria	Actinomycetales	*Micrococcaceae*	*Arthrobacter*	17
Actinobacteria	Actinobacteria	Actinomycetales	*Micrococcaceae*	*Kocuria*	1
Actinobacteria	Actinobacteria	Actinomycetales	*Nocardiaceae*	*Rhodococcus*	1
Actinobacteria	Actinobacteria	Actinomycetales	*Streptomycetaceae*	*Kitasatospora*	1
Actinobacteria	Actinobacteria	Actinomycetales	*Streptomycetaceae*	*Streptomyces*	6
Bacteroidetes	Cytophagia	Cytophagales	*Cytophagaceae*	*Dyadobacter*	1
Bacteroidetes	Flavobacteriia	Flavobacteriales	*Flavobacteriaceae*	*Chryseobacterium*	5
Bacteroidetes	Flavobacteriia	Flavobacteriales	*Flavobacteriaceae*	*Flavobacterium*	39
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	*Chitinophagaceae*	*Chitinophaga*	1
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	*Sphingobacteriaceae*	*Mucilaginibacter*	2
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	*Sphingobacteriaceae*	*Pedobacter*	50
Firmicutes	Bacilli	Bacillales	*Bacillaceae 1*	*Bacillus*	6
Firmicutes	Bacilli	Bacillales	*Paenibacillaceae 1*	*Paenibacillus*	4
Firmicutes	Bacilli	Bacillales	*Planococcaceae*	*Planococcaceae incertae sedis*	1
Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	*Brevundimonas*	4
Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	*Caulobacter*	16
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Bradyrhizobiaceae*	*Bosea*	2
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Brucellaceae*	*Ochrobactrum*	1
Proteobacteria	Alpha-proteobacteria	Rhizobiales	*Phyllobacteriaceae*	*Phyllobacterium*	2
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*	*Rhizobium*	5
Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Sphingobium*	1
Proteobacteria	Betaproteobacteria	Burkholderiales	*Burkholderiaceae*	*Burkholderia*	2
Proteobacteria	Betaproteobacteria	Burkholderiales	*Burkholderiaceae*	*Cupriavidus*	1
Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Acidovorax*	12
Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Pelomonas*	2
Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Polaromonas*	1

Phylum	Class	Order	Family	Genus	Number of isolates
Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Variovorax*	36
Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Duganella*	2
Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Herbaspirillum*	17
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	28
Proteobacteria	Gammaproteobacteria	Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas*	1
Proteobacteria	Gammaproteobacteria	Xanthomonadales	*Xanthomonadaceae*	*Xanthomonas*	3
Proteobacteria	Gammaproteobacteria		*unclassified Gammaproteobacteria*		2
				Total	**279**

Table S2. List of primers used in this study.

Primer	Sequence
PR-1 Fw	5'-CTCGGAGCTACGCAGAACAACT-3'
PR-1 Rv	5'-TTCTCGCTAACCCACATGTTCA-3'
PDF1.2 Fw	5'-CACCCTTATCTTCGCTGCTCTT-3'
PDF1.2 Rv	5'-GCCGGTGCGTCGAAAG-3'
VSP2 Fw	5'-ACGGAACAGAGAAGACCGAC-3'
VSP2 Rv	'5-TCTTCCACAACTTCCAACGG-3'
At1g13320 Fw (Q-PCR Ctrl)	5'-TAACGTGGCCAAAATGATGC-3'
At1g13320 Rv (Q-PCR Ctrl)	5'-GTTCTCCACAACCGCTTGGT-3'
BOX primer BOXA1R	5'-CTACGGCAAGGCGACGCTGACG-3'
16S rRNA forward primer F27	5'-AGAGTTTGATCCTGGCTCAG-3'
16S rRNA reverse primer R1492	5'-GGTTACCTTGTTACGACTT-3'

3

CHAPTER 3.

Methods to study soil-borne legacies of disease in *Arabidopsis thaliana*

Gilles Vismans[1], Jelle Spooren[1], Corné M.J. Pieterse[1], Peter A.H.M. Bakker[1], Roeland L. Berendsen[1]

1: Plant-Microbe Interactions, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands

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Abstract

The rhizosphere microbiome of plants is essential for plant growth and health. Recent studies have shown that upon infection of leaves with a foliar pathogen, the composition of the root microbiome is altered and enriched with bacteria that in turn can systemically protect the plant against the foliar pathogen. This protective effect is extended to successive populations of plants that are grown on soil that was first conditioned by pathogen-infected plants, a phenomenon that was coined “the soil-borne legacy”. Here we provide a detailed protocol for soil-borne legacy experiments with the model plant *Arabidopsis thaliana* after infection with the obligate biotrophic pathogen *Hyaloperonospora arabidopsidis*. This protocol can easily be extended to infection with other pathogens or even infestation with herbivorous insects and can function as a blueprint for soil-borne legacy experiments with crop species.

Introduction

Plants and their microbial communities are closely intertwined in structure and functioning. By depositing large quantities of their photosynthetically fixed carbon into the soil, plants attract and modulate the composition of the microbial community around their roots (Drigo et al 2010, Griffiths et al 2004). In turn, these micro-organisms influence the plant, for instance by providing it with essential nutrients (Berendsen et al 2012, Lugtenberg & Kamilova 2009). The close relationship between plant roots and microbes, coupled with the importance of this so called rhizosphere microbiome for plant health and performance has led to the term "plant holobiont", describing the plant not as a single entity but as the sum of the interactions of the individual species (Vandenkoornhuyse et al 2015). The rhizosphere microbiome can contain both pathogenic and beneficial microbes. Beneficial microbes can control disease by inhibiting the pathogen through antibiosis or competition, but a prominent mode of action is induced systemic resistance (ISR) in the plant. ISR entails systemic priming of defense responses, which enables stronger and faster activation of defense responses upon pathogen infection (Pieterse et al 2014, Walters & Heil 2007). The development of so-called disease suppressive soils, in which a significant outbreak of disease results in accumulation of microbiota that can control the pathogen, is a prominent example of pathogen control by beneficial microbes (Mendes et al 2011, Raaijmakers & Mazzola 2016, Raaijmakers & Weller 1998, Weller et al 2002). Upon encountering stress conditions, such as pathogen attack, plants can change their root exudation profiles (Badri & Vivanco 2009, Carvalhais et al 2015, Yuan et al 2018). The altered rhizodeposition under stress conditions resulting in recruitment of specific beneficial microbes suggested the "cry for help" hypothesis (Bakker et al 2018). Recently it was shown that *Arabidopsis thaliana* recruits three strains of beneficial bacteria into its rhizosphere upon foliar infection with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) (Berendsen et al 2018). The recruited bacteria were isolated in culture and a consortium of a *Xanthomonas,* a *Microbacterium* and a *Stenotrophomonas* sp. was shown to synergistically promote plant growth and induce systemic resistance against *Hpa*. Interestingly, a second generation of Arabidopsis plants grown on soil conditioned with *Hpa*-infected plants showed increased resistance to *Hpa* infection as compared to plants grown on soils conditioned by growing an uninfected first generation of Arabidopsis plants (Berendsen et al 2018). A similar phenomenon has been observed for *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) infected Arabidopsis plants, where pre-treating the soil with *Pst*-infected plants resulted in reduced disease pressure in a subsequent generation of plants growing on the conditioned soil (Yuan et al 2018). The term "soil-borne legacy" was coined in which stress in a first generation of plants results in a microbiome mediated protection against this stress in a subsequent generation (Bakker et al 2018). Here we describe in detail the experimental setup to study the soil-borne legacy in Arabidopsis with *Hpa* as the inducing pathogen.

Materials

Equipment

1. Growth chambers set at 21 °C, 70 % relative humidity and 10-h/14-h day/night cycle with a light intensity during the day of 200 µE/m^2/s provided with LuxLine plus F58W/840 cool white tube lamps (Havells Sylvania, London UK).
2. Small autoclave and autoclavable glassware (250 mL Scott bottle).
3. Containers (30-50 L) for mixing soil and water.
4. Dried and sieved soil, preferably a soil that is easy to handle in terms of planting and watering.
5. Round pots (60 mL) with holes in the bottom, to grow plants.
6. Circular covers of approximately 5 cm in diameter (or to cover the entire pot) are cut from the Greiner bio-one micro-pipette tip 0,5-10 µL holder (Item No: 771280). Make sure approximately 22 holes are included in the soil covers (Figures 2 and 3).
7. Round petri dishes (6 cm) to serve as dishes for small pots.
8. Trays (approximately 45x30x8 cm) to contain small pots. Trays should be able to be covered with transparent lids to achieve 100 % relative humidity.
9. Transparent lids to cover the trays and transparent lids with large holes covered by gauze (Figure 1).
10. Carl Zeiss Microscopy, Standard 25 ICS, Item No. 450815.9902 with 100x magnification.
11. Microscope slides.
12. Falcon™ 15 mL Conical Centrifuge Tubes.
13. Scotch tape.
14. 70 % ethanol.
15. 0-1000 µl and 0-2 µl pipettes and corresponding pipette tips.
16. Balance.

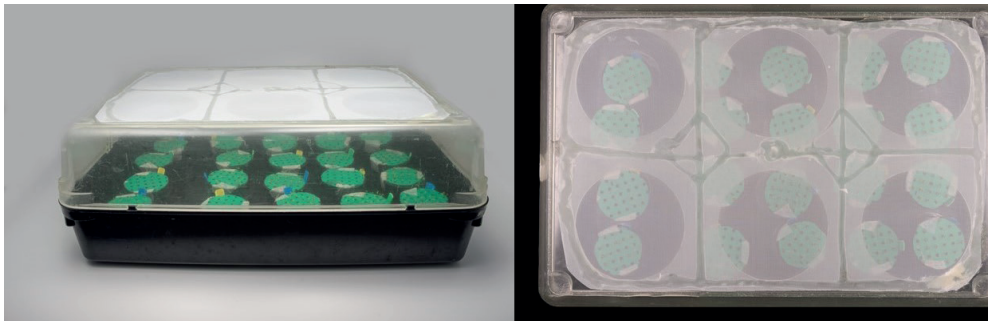

Figure 1. Trays covererd with a gauze lid, to protect the young seedlings, without increasing the relative humidity in the tray. Left side view and right the top view

Buffers, Media and Solutions

Arabidopsis thaliana cultivation

1. Seeds of *Arabidopsis thaliana* accession Col-0.
2. 0,2 % (w/v) Difco bacteriological agar.
3. Half-strength Hoagland solution (Hoagland & Arnon 1950).
4. Plant labels.
5. Seripettor® bottletop dispenser and bottle to water the pots with the same amount each time.

Hyaloperonospora arabidopsidis (Hpa) Noco2 inoculation

1. A continuously maintained culture of *Hpa* Noco2 as source of spores. Every week a spore suspension is needed to infect new plants and propagate *Hpa* for further use.
2. Tweezers.
3. Scissors.
4. Falcon™ 50 mL Conical Centrifuge Tubes.
5. Miracloth (EMD Millipore, Germany).
6. Demi-water.
7. Airbrush, we use a Revell starter kit airbrush connected to the pressurized air of the lab.
8. Recipe's

*Half-strength Hoagland supplementation medium **(note 1)**:*

- 1262 g/5 L (2 mL/L) 5 mM KNO_3
- 680 g/5 L (2 mL/L) 2 mM KH_2PO_4
- 1230 g/5 L (2 mL/L) 2 mM $MgSO_4$
- 28 g/5 L (2 mL/L) 10 µM Fe-EDDHA
- 2950 g/5 L (2 mL/L) 5 mM $Ca(NO_3)_2$
- Micronutrients (2 mL/L)
  - ◊ 3.90 g/5 L H_3BO_3
  - ◊ 9.30 g/5 L KCl
  - ◊ 9.30 g/5 L KCl
  - ◊ 0.85 g/5 L $MnSO_4 \cdot H_2O$
  - ◊ 1.45 g/5 L $ZnSO_4 \cdot 7H_2O$
  - ◊ 0.32 g/5 L $CuSO_4 \cdot 5H_2O$
  - ◊ 0.22 g/5 L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$

Methods

The soil-borne legacy experiment consists of two parts. The first part is the conditioning of the soil with either uninfected or *Hpa*-infected Arabidopsis plants. The second part is assessing of the soil-borne legacy effect with an *Hpa*-infected second generation of plants growing on soil conditioned with healthy plants or *Hpa*-infected plants.

Arabidopsis cultivation and infection

1. Suspend the Arabidopsis seeds in sterile 0,2 % agar in a 15-mL Falcon tube. To calculate the number of Arabidopsis seeds needed, use: '(number of pots x 30) x 2' to make sure there are plenty of seeds to sow. One hundred Arabidopsis seeds weigh approximately 1,5 mg. Add 10 mL of 0,2% agar per 90 mg of seeds. Imbibe at 4 °C in the dark for 2-4 days. **(see note 2)**

2. One day before sowing, mix the soil with the appropriate amount of water or half-strength Hoagland, depending on the type of soil and fill all pots, make sure the pot is filled up to the edge. To assess the soil-borne legacy effect, a minimum of 10 replicates per treatment are needed.

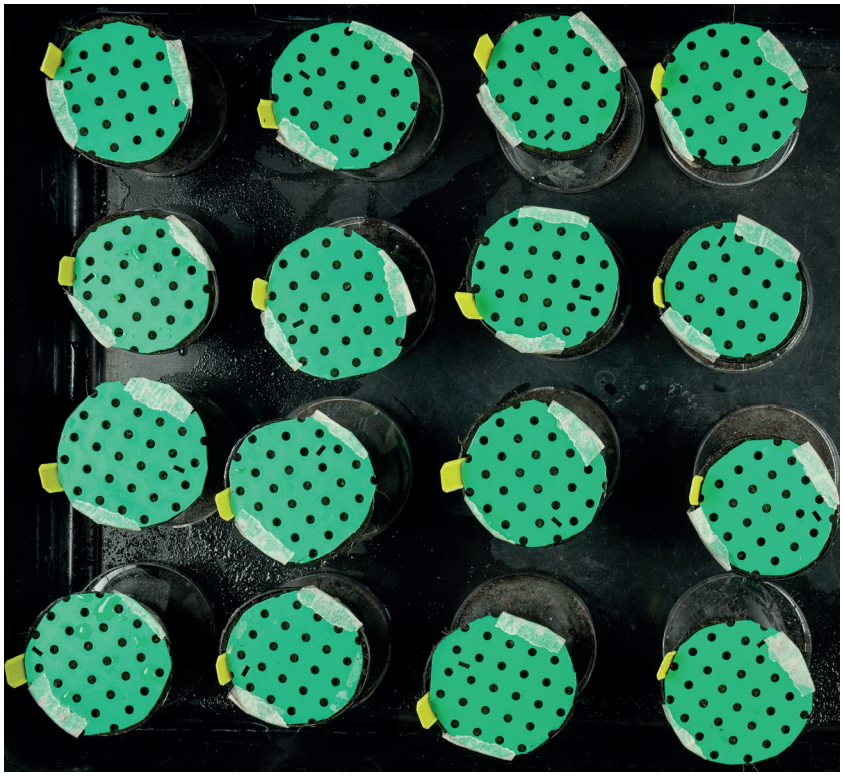

Figure 2. Top view of 5-cm pots right after sowing Arabidopsis seeds in each of the circular holes of the circular soil covers. The soil covers were cut from a Greiner micro-pipette-tip holder and each placed in a 6-cm Petri dish to allow individual watering and to prevent cross contamination between pots

3. Cover the pots with a soil cover (see equipment, item 6) and tape it to the side of the pot (Figure 2). Place each pot in an individual Petri dish bottom or lid to serve as saucers. Store the pots at 4 °C in the dark, until sowing.

4. Sow the seeds on the pot. Using a 0-1000 µL pipette, take up the 0,2% agar with the suspended seeds. Release 1 seed at a time, by turning the volume adjustment of the pipette down slowly. By doing so you are able to control the flow of the pipette better and you are able to sow 1-3 seeds per hole in the cover.

5. Randomize the pots (with the Petri dishes) in the trays (Figure 2). Close the trays with a transparent lid. To make sure the lids are properly closed and to ensure high humidity within the tray, tape the lids to the trays.

6. Move the trays to the plant growth chambers.

7. One week after sowing, change the transparent lids for the lids with gauze to decrease humidity.

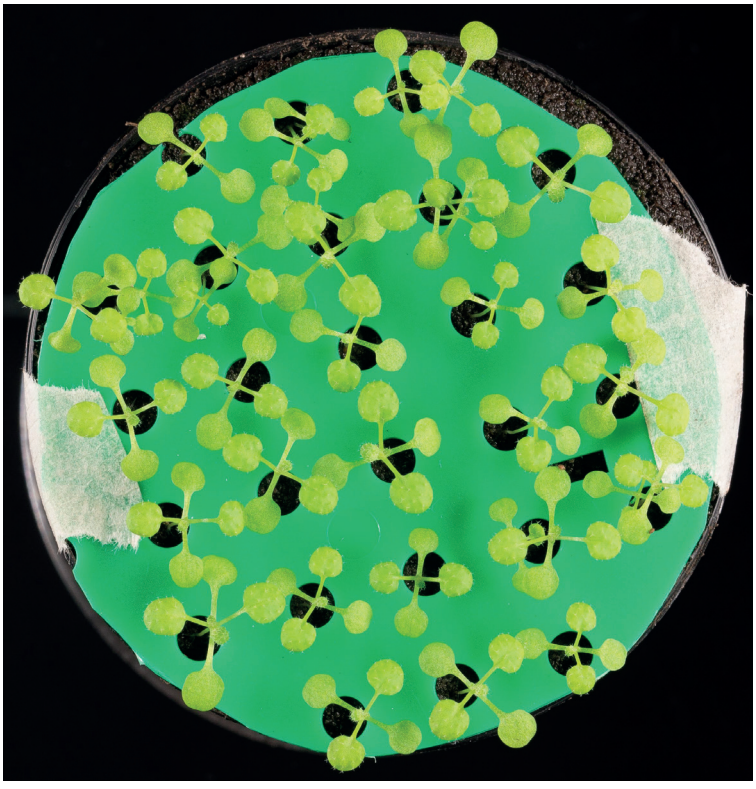

Figure 3. Two-week-old seedlings right before inoculation with *Hpa*. One to two seedlings per hole have grown and all plants are of similar size. Pots with uneven germination or growth are to be removed from the experiment

8. Water the plants by dispensing 5 mL of water into the Petri dishes in which the individual pots are placed. Add more or less water when needed. **(note 3)**.

9. Two weeks after sowing select the plants and pots that are uniform in germination and growth. See Figure 3 for an example of 2-week-old seedlings.

10. Mark the pots and inoculate half of them with an *Hpa* spore suspension according to Asai et *al.* (2015). Mock inoculate the other half of the pots with water. In short, collect sporulating seedlings from a maintenance *Hpa*-infected stock of plants and transfer them to a 50-mL Falcon tube. Add approximately 25 mL of sterile water and shake. After filtering through Miracloth, count the number of spores in three separate 1-µl droplets. Adjust the spore density to 50 spores per µl. By using the airbrush, spray the spore suspension over the plants until tiny droplets start to form on the leaves. Let the droplets dry and proceed with the next step. To treat the control group, do exactly the same, except with sterile water instead of spore suspension.

11. Exchange the lids with gauze for the transparent lids without gauze, to increase relative humidity. Tape the lids to the trays and place back in the growth chambers. Cultivate the inoculated and non-inoculated plants for one week to allow buildup of the soil-borne legacy.

12. One week after *Hpa* inoculation, cut off all above ground plant parts using a razor blade. For a minimum of 10 replicates per treatment, measure the fresh weight of the plants and count the number of spores for a detailed description see the spore counting protocol detailed below.

13. Sow a new generation of seeds on the pots, as described in 1 and 4-8. Water when needed and use half-strength Hoagland solution once during the first week of growing.

14. One week after sowing, exchange the transparent lids for the lids with gauze as in step 7.

15. Two weeks after sowing the second generation of seeds, inoculate all replicates with a *Hpa* spore suspension as mentioned in 9-12.

16. One week after inoculation, determine the disease severity by assessing the number of spores per gram of leaf fresh weight as described in the spore counting step described below.

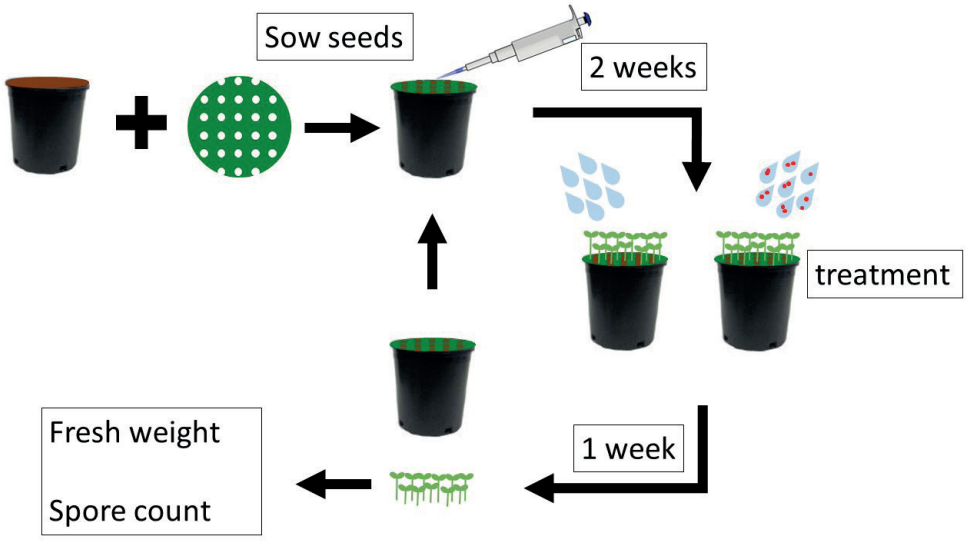

Figure 4. A flowchart depicting the entire workflow of a soil-borne legacy experiment. 1) Fill the pots with soil and cover the soil with a plastic cutout before sowing the seeds for the first generation of plants. 2) After two weeks of growth in the climate chamber, plants are inoculated with either *Hpa* spores or a control solution. During the week that follows the soil microbial community is conditioned by either healthy (uninoculated) plants or by *Hpa*-infected plants. This is the soil-conditioning phase of the experiment. 3) One week after inoculation, the above ground parts of the plants are removed from the pots, and seeds are sown for the second generation of plants. To determine the disease severity in each phase of the experiment, plants are harvested and the number of spores per gram of shoot fresh weight is determined.

A flowchart of these steps is presented in Figure 4.

Hpa inoculation and mock treatment (step 10)

The method for determining the number of spores per gram leaf fresh weight is derived from Asai *et al.* (2015). Since *Hpa* is an obligate biotrophic pathogen it can only be propagated on living plant tissue. The Noco2 isolate of *Hpa* can infect Arabidopsis accession Col-0 **(note 4)**.

1. From a maintenance culture of *Hpa* Noco2 infected Arabidopsis Col-0 plants, cut off the leaves that carry *Hpa* sporangia and collect them in a 50-mL Falcon tube.

2. Add 30 mL sterile water to the tube and shake to release the *Hpa* spores from the leaves.

3. Filter the spore suspension through Miracloth.

4. Assess the spore density by counting the number of spores in three separate 1-µl droplets under a microscope.

5. Dilute the spore suspension to 50 spores/µl.

6. Using the airbrush, spray the spore suspension until tiny droplets start to take shape on the leaves.

7. Leave the droplets to air dry.

8. For the mock treatment, spray sterile water on the plants in the same way as the spore suspension.

9. To ensure proper infection, the pots should be placed in trays with closed lids to increase humidity to 100%. Spray the lid on the inside with some water before closing to increase relative humidity inside the tray.

Spore counting (step 12)
The method for determining the number of spores per gram of leaf fresh weight is derived from Asai *et al.* (2015).

1. Pipette 3 mL of sterile water in the 15 mL Falcon tubes.

2. Balance and tare a 15 mL Falcon tube containing 3 mL of water.

3. Cut off all the aboveground plant parts of one pot using a razor blade and collect them in the tared Falcon tube.

4. Weight the tube to determine the fresh weight of the plant material.

5. Shake the tube containing the plant material.

6. Place three 1-µl droplets from the tube containing the plant material on a microscope slide and count the number of spores in each of the droplets under the microscope. Use the average of the droplets to determine the number of spores per µl.

7. Use the fresh weight and the number of spores per µl to determine the spores per gram of leaf fresh weight.

Notes

1. We usually prepare 5 L stock solutions and use 2 mL/L of these stocks to make 25 L of half-strength Hoagland at a time. Prolonged storage of the half-strength Hoagland solution will lead to precipitation of some of the salts, so don’t make more than you(r lab) will use within 1-2 weeks.

2. The 0,2 % agar makes it easy to distribute the seeds with a pipette. It might take some trial and error to get the concentration of seeds right to consistently pipette 2-3 seeds per hole without pipetting too much agar. It also ensures proper imbibition of the seeds.

3. All pots are watered by adding water or half-strength Hoagland in the Petri dish in which the individual pots are placed, never from the top of the pot.

4. *Hpa* is an obligate biotrophic pathogen and needs to be propagated on living plants. Each week new plants need to be infected with spores from last weeks stock of *Hpa*-infected plants.

Author contributions

G.V., P.A.H.M.B., and R.L.B. designed experiments; G.V., J.S. performed experiments; G.V. and J.S. analyzed data and G.V., P.A.H.M.B, R.L.B., and C.M.J.P. wrote the manuscript.

4

CHAPTER 4.

The involvement of coumarins in the soil-borne legacy of *Hyaloperonospora arabidopsidis*-infected *Arabidopsis thaliana*

Gilles Vismans[1], Pim Goossens[1], Jelle Spooren[1], Sietske van Bentum[1], Basten Snoek[2], Eva Visser[3], Corné M.J. Pieterse[1], Peter A.H.M. Bakker[1], Roeland L. Berendsen[1]

1: Plant-Microbe Interactions, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
2: Theoretical biology and bioinformatics, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
3: University of Copenhagen, Nørregade 10, 1165 Copenhagen, Denmark

Abstract:

Plants deposit photosynthetically-fixed carbon in the thin soil layer directly around the root, the rhizosphere, creating a hospitable environment for microbes. To manage the inhabitants of this nutrient-rich environment, plant roots exude microbe-attracting and repelling compounds. By doing so, plants can fine tune the composition of their root-associated microbial communities, the so-called root microbiome, and profit from their specific functions. Previously, we demonstrated that above ground infection of *Arabidopsis thaliana* by the biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) results in recruitment of specific bacteria on the roots. Moreover, conditioning soil with *Hpa*-infected *A. thaliana* resulted in protection against downy mildew in a subsequent generation of plants growing on such conditioned soil. This phenomenon was dubbed the soil-borne legacy (SBL). In this chapter we provide evidence that the protective effect of SBL results from induced systemic resistance that requires salicylic acid (SA) signaling in the plant, since plant SA signaling mutants *sid2* and *npr1* no longer responded to SBL. For the creation of SBL, coumarins appeared to play a prominent role, since *myb72* and *f6'h1* mutants that are defective in coumarin production, are also defective in creating a *Hpa* infection-induced SBL. The SBL induced by *Hpa*-infected wild-type plants was accompanied by a compositional shift in the root microbiome in which Xanthomonadales and *Fibrobacterales* appear to be specifically recruited.

Introduction:

Microbial communities that associate with the plant root, together referred to as the root microbiome, are the focus of numerous studies that explore their potential for improvement of plant health and plant growth (Arif et al 2020, Berendsen et al 2012, Compant et al 2019, Oyserman et al 2018). Plants sculpt and sustain the root microbiome by depositing large amounts of photosynthetically-fixed carbon in the rhizosphere, creating nutrient-rich conditions for microbial growth and activity (Drigo et al 2010, Griffiths et al 2004). With a punishment and reward strategy, plants can modulate rhizosphere microbiome composition by the exudation of both microbe-stimulatory and -repellent metabolites (Lundberg & Teixeira 2018, Pascale et al 2020, Sasse et al 2018, Stringlis et al 2018). By selectively adjusting the rhizosphere microbiome to favor beneficial microbes, plants can create conditions advantageous to their growth and health.

Previously, it was discovered that plants can dynamically recruit beneficial microbes in response to pathogen attack. Aboveground infection of the model plant *Arabidopsis thaliana* (hereafter: Arabidopsis) with *Hyaloperonospora arabidopsidis* (hereafter: *Hpa*; Chapter 2) or *Pseudomonas syringae pv. tomato* (Yuan et al 2018) led to specific changes in the root microbiome. A subsequent population of plants grown in soils thus conditioned with pathogen-infected plants was more resistant to the aboveground attacker. Moreover, the Arabidopsis root microbiome of *Hpa*-infected plants was enriched for *Xanthomonas, Stenotrophomonas* and *Microbacterium* spp.. These bacteria were isolated and cultured, and only when applied as a consortium, they induced systemic resistance (ISR) against *Hpa* (Chapter 2). Thus, we hypothesize that pathogen infection leads to changes in root exudation profiles that specifically promote beneficial microbes in their root microbiome that in turn induce ISR to fend off subsequent pathogen attack. By increasing the abundance of these beneficial microbes, plants create a soil-borne legacy (SBL) that serves to protect a next generation of plants growing in that same soil. The dynamic recruitment of beneficial microbes is suggested to be important for the buildup of disease suppressiveness in the field. This is exemplified by a study with sugar beet, in which attack by the soil-borne pathogen *Rhizoctonia solani* led to the activation of a disease-suppressive microbial consortium that protected the plant by antagonizing the pathogen (Carrión et al 2019).

It is known that specific beneficial microbes can prime a plant's immune system and bring about a state of (ISR; Pieterse et al 2014, Van Wees et al 2008). In this primed state of ISR, a plant activates defense responses upon pathogen detection much quicker and stronger, but without the plant growth penalty that is often associated with increased resistance (Martinez-Medina et al 2016, van Hulten et al 2006). Root colonization by *Pseudomonas simiae* WCS417r (hereafter: WCS417r) can elicit ISR in Arabidopsis that is effective against a wide range of pathogens (Pieterse et al 1998, Ton et al 2001, Van der Ent et al 2008). WCS417r-mediated ISR is dependent on the transcription factor MYB72 that is expressed in the roots only, but essential for the onset of ISR systemically (Van der Ent et al 2008).

MYB72 is also important in the plant's iron deficiency response as it regulates the biosynthesis and secretion of scopoletin (Stringlis et al 2018, Zamioudis et al 2014), a coumarin involved in mobilizing Fe from the soil (Palmer et al 2013). Moreover, scopoletin has specific antimicrobial activity to which two *MYB72*-inducing *Pseudomonas* spp. were tolerant (Stringlis et al 2018). MYB72 thus seems to serve a double role in signaling the onset of ISR as well as shaping the root microbiome. It was hypothesized that MYB72-dependent coumarin secretion is part of a positive feedback loop that stimulates colonization by *MYB72*-inducing and coumarin-tolerant beneficial microbes (Li et al 2021, Stringlis et al 2018, Voges et al 2018).

In this chapter we set out to investigate whether plants growing on soil pre-conditioned by *Hpa*-infected plants and that are experiencing an SBL, are protected against a subsequent pathogen infection through ISR. We found that these plants are primed for increased defense responses but that their increased resistance relies on SA-dependent signaling and does not require MYB72. MYB72 and the production of coumarins, however, does appear to be essential for the creation of SBL as we discovered that aboveground *Hpa*-infection in coumarin-deficient mutant plants does not result in creation of an SBL.

Materials and methods

Soil and soil conditioning

In this study natural soil collected from the Reijerscamp nature preserve in the Netherlands was used as previously described in Chapter 2

SBL experiments

The SBL setup used in this study was performed as described in detail in Chapter 3 (Vismans et al 2020). For each replicate a 60-ml pot was filled with approximately 120 g of Reijerscamp soil. To prevent algae and moss growth, a non-transparent, perforated plastic cover was placed on the soil. The covers were made by cutting out a circular shape from a micro pipette tip holder (Greiner Bio-One, 0,5-10 µL, Item No.: 771280). *Arabidopsis thaliana* seeds were imbibed by suspending seeds in 0,2% (w/v) water agar in the dark at 4 °C for 2 days. Using a 1000-µl pipette, 1-2 seeds were pipetted in each hole of the cover, resulting in approximately 30 seeds per pot. Each pot was placed on its individual saucer and randomly placed in trays covered by transparent lids. The trays were placed in a climate-controlled plant growth chamber at 21 °C, 70% relative humidity, 10 h light/ 14 h dark light cycles, and at a light intensity of 100 µmol/m^2/s. After one week, the lids were changed for lids with a mesh to reduce humidity. Two weeks after sowing, half of the pots were inoculated with a 50 spores/µL suspension of *Hyaloperonospora arabidopsidis* noco2 (*Hpa)* as previously described (Asai et al 2015). The other pots were sprayed with tap water as a control. The mesh lids were replaced by the transparent lids to increase humidity. *Hpa* symptoms were allowed to develop for one week, after which all aboveground material was cut off using a razor blade. A new generation

of plants was sown and grown as described above. After two weeks of growth of this second population of plants, all plants were inoculated with *Hpa* and symptoms were allowed to develop for one week. Disease severity was measured by determining *Hpa* spore densities normalized for plant fresh weight.

Wild-type plants of the Col-0 accession were used in both the conditioning phase of the experiment (1st population of plants) and in the interrogation phase (2nd population of plants). Depending on the experiment, Col-0 plants were replaced by mutant *myb72-2,* which is impaired in both rhizobacteria-ISR (Van der Ent et al 2008) and in the biosynthesis and secretion of coumarins (Stringlis et al 2018, Zamioudis et al 2014), the coumarin biosynthesis mutant *f6'h1,* which is impaired in the coumarin biosynthesis enzyme Feruloyl-CoA 6'-Hydroxylase1 (Schmid et al 2014), or by SA signaling mutants *npr1-1* (Cao et al 1997) or *sid2-1* (Wildermuth et al 2001). As controls, plants were left unplanted in either the conditioning phase or the interrogation phase of the experiment. All these experiments were repeated at least twice with similar results.

***Hpa* spore production**

To quantify *Hpa* infection, the number of spores produced per g shoot fresh weight was determined. To this end, aboveground plant parts were cut using a razor blade and placed in a 15-ml Greiner tube, containing 3 ml of water, after which shoot weight was measured. After shaking, three separate 1-µL drops were pipetted onto a microscope slide. Spores were counted in each of the individual droplets using a light microscope at a 100x magnification.

Defense priming assay

To test for priming of plant defenses, 2-week-old plants in the interrogation phase of an SBL experiment were dipped in a 1 mM SA solution supplemented with 0.015% Silwet L-77 or in a mock solution containing 0.015% Silwet L-77. Seedlings were harvested at 30 min, four h, and six h after dipping, immediately snap frozen in liquid nitrogen and stored at -80°C until further processing. RNA extraction was performed as described by Oñate-Sánchez (2008) with minor modifications. Quantitative real-time PCR (qPCR) analysis on the defense-related marker gene *PATHOGENESIS-RELATED1* (*PR1*) was performed as described by Van Wees *et a*l. (2013).

Soil-borne legacy microbiome analysis

At the end of the SBL conditioning phase, after the shoots of the first generation of plants were removed, the roots from at least ten replicate pots were collected for each treatment. To this end an entire pot was emptied on a 1-mm^2 mesh sieve and roots were subsequently cleaned under a running water tap. When most of the soil was washed away, the roots were collected using tweezers in Qiagen PowerBead tubes, snap frozen and stored at -80°C until further processing. DNA was extracted using the DNeasy® Powerlyzer® Powersoil® kit (product #: 12855-100). The protocol was followed according to the manufacturer's instructions, with the adjustment of a 10-min incubation step at 60°C after the addition of

solution C1. For the bead beating option in step 4, two times 10 min at 30 Hz in the Qiagen bead beater was used. DNA concentrations were determined using a Thermofisher Nanodrop® 2000 and set to 5 ng/µL. We focused on root samples, because in a pilot experiment in which microbiome composition was determined on soil sampled from the different treatments, no differential microbial communities were detected between planted and unplanted soil, and thus the rhizosphere effect could not be detected (fig. S1, Table S1). Therefor we first isolated the roots from the soil before further processing in all experiments presented in this chapter.

Preparation of 16S library
The *16S rRNA* gene amplicon libraries were constructed using the 16S metagenomic sequencing library protocol for the Illumina Miseq system (Illumina 2013). The protocol was adapted to incorporate 16S V3-V4 phasing primers to increase complexity and decrease the need for PhiX spike (de Muinck et al 2017). Moreover, the barcoded PCR primers described by Baym and co-workers (2015) were used in a total PCR reaction volume of 25 µL instead of 50 µL. Three µL of the purified samples was run on a 1% agarose gel to check the amplicon size and the concentration was determined using the Qubit broad range kit (Thermofisher Scientific). Each sample was adjusted to 2 ng/µL and pooled in a 1:1 ratio. The pooled library was sent for sequencing on a 2x 300 MiSeq at the USEQ sequencing facility (Utrecht University, the Netherlands).

Sequence data pre-processing
The sequence data was analyzed using Qiime2 version 2019.7 (Bolyen et al 2019). Primers were removed using the Cutadapt (Martin 2011) plugin of Qiime2, using the standard settings. Forward and reverse reads were truncated at 260 bp and 215 bp, respectively. Denoising, dereplicating, chimera removal and paired-end joining was done using DADA2 resulting in a final alignment at 100% similarity, giving amplicon sequence variants (ASVs) (Callahan et al 2016). A VSEARCH based consensus taxonomy classifier was used (Rognes et al 2016) with the SILVA database version 128 (Quast et al 2013, Yilmaz et al 2014) for taxonomy assignment. A sequencing bias was observed for the experiment including the *f6'h1* mutant when making a Bray-Curtis-based Principal Coordinate analysis (PCoA). This was identified to be column based during library preparation. Based on this, 6 samples with a sequencing bias were removed from the dataset, resulting in a total of 67 samples with a median number of reads of ~68000 and ~21000 unique ASVs. The top 600 ASVs, based on the average relative abundance, were selected for data analysis.

Sequence data analysis
Rstudioversion 3.5.0 was used for PCoA analysis and statistical analysis of sequence data. Figures have been made in Rstudio version 3.5.0 using phyloseq (McMurdie & Holmes 2013), ggplot2 (Wickham 2016),PRISM8 graph*pa*d and Adobe illustrator CC 2017.

Correlation analysis
The correlation networks were produced in R 3.6 (R core Team 2020), and based on Veen *et al.* (2019) with several adjustments. The Spearman correlations between the parameters were calculated and the pairwise correlations greater than 0.65 were used for the network. For visualization the following functions from the igraph R-package were used: graph_from_dat_frame, simplify, and layout_components (Csardi & Nepusz 2006).

Results

Perception of the soil-borne legacy of *Hpa*-infected Arabidopsis depends on SA and primes foliar defenses
Arabidopsis plants growing on natural Reijerscamp soil that was pre-conditioned by a population of *Hpa*-infected Arabidopsis plants are more resistant to *Hpa* infection than seedlings growing on soil pre-conditioned with healthy plants (Chapter 2). It was hypothesized that the perception of such a SBL is the result of systemic resistance induced by microbes that are recruited by the infected plants. To test this, we first pre-conditioned Reijerscamp soils by growing wild-type Col-0 plants that were either infected by *Hpa* or not. Subsequently, we cultivated wild-type and specific mutants impaired in known defense signaling pathways in the pre-conditioned soils and examined their level of resistance against *Hpa* infection. In the experimental setup, we chose the *myb72* mutant, as this mutant was shown to be blocked in its capacity to develop SA-independent ISR in response to various beneficial rhizobacteria and fungi (Pieterse et al 2014). Because several beneficial rhizobacteria and fungi have been shown to activate an SA-dependent ISR response (also known as systemic acquired resistance, SAR), we additionally used the SA-response mutants *npr1* and *sid2*. It was observed that both wild-type Col-0 plants and mutant *myb72* plants were significantly more resistant when growing on soil that was pre-conditioned by *Hpa*-infected plants than when growing on soil pre-conditioned by healthy plants (fig. 1A). However, no differential *Hpa* spore production for plants growing on soils pre-conditioned by either infected or healthy wild-type plants were observed for both the *npr1 and sid2* mutant plants (fig. 1B). These results suggest that the perception of a SBL of disease involves systemic defense signaling that depends on SA, but does not require MYB72. Moreover, wild-type Arabidopsis plants transcribed significantly more of the SA-responsive marker gene *PR-1* in response to SA treatment when growing on soil pre-conditioned by infected plants than on soil pre-conditioned by healthy plants (fig. 1C). This suggests that defense responses of plants perceiving a *Hpa*-induced SBL are indeed primed for SA-dependent defenses.

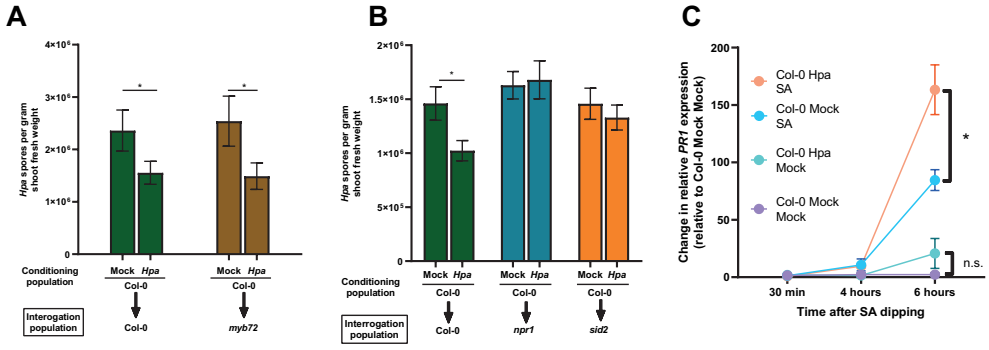

Figure 1. The perception of Hpa-induced SBL is SA dependent and leads to priming of SA-dependent defense responses. A) Disease severity of *Hpa*-inoculated Col-0 or *myb72* plants growing on soil pre-conditioned with healthy or infected Col-0 plants. Bars depict the average spore production per gram of shoot fresh weight of at least 9 replicates. Error bars depict standard error of the mean. Asterisk denotes a significant difference (P<0.05 in Students *t*-test). **B**) Disease severity of *Hpa*-inoculated Col-0, *npr1* or *sid2* plants growing on soil pre-conditioned by healthy or infected Col-0 plants. Bars depict the average spore production per g of shoot fresh weight of 19 replicates with values based on normalized spore counts of 2 independent experiments. Error bars depict standard error of the mean. Asterisk indicates a significant difference (univariate GLM with interaction term, P=0,029, n>19). **C**) *PR-1* expression in leaves of Col-0 plants growing on soil pre-conditioned by healthy or infected Col-0 plants at 30 min, 4 h, and 6 h after dipping in 1 mM SA. *PR-1* expression is expressed as fold-change relative to mock-treated control plants growing on soil conditioned by healthy plants. Asterisk denotes a significant difference as determined by Student's *t*-test (P=0,005).

MYB72 and coumarin biosynthesis are required for the creation of the soil-borne legacy mediated by *Hpa* infected Arabidopsis

Although MYB72 is not required for the perception of the *Hpa*-induced SBL (fig. 1A), this transcription factor may still play a role in the SBL as it regulates the production and secretion of coumarins, most-notably scopoletin. Coumarins are secreted by the roots, have antimicrobial activity and can shape root microbiome assembly (Stringlis et al 2019, Stringlis et al 2018). We therefore hypothesized that MYB72 and coumarin biosynthesis may play a role in the creation of the *Hpa*-induced SBL. This hypothesis was tested by preconditioning Reijerscamp soils with either Col-0, *myb72* or the coumarin-deficient mutant *f6'h1* that were mock inoculated or inoculated with *Hpa*. Moreover, we included non-planted, soil-filled pots that were sprayed with water or *Hpa* spores during the conditioning phase to rule out the possibility that SBL was created via the *Hpa* inoculum. After removal of the first population of plants, Col-0 plants were sown and grown on all pre-conditioned soils and challenge inoculated with *Hpa*. In the inoculated interrogation populations, a significant reduction in *Hpa* spore production was observed for the pre-conditioning treatment with *Hpa*-infected Col-0 plants (fig. 2), confirming the previous findings (fig. 1). Interestingly, pre-conditioning the soil with the coumarin biosynthesis mutants *myb72* and *f6'h1* did not significantly affect *Hpa* resistance in the interrogation population of Col-0 plants (fig. 2). Also, pre-conditioning of unplanted soil with *Hpa* inoculum had no effect on *Hpa* resistance in tester Col-0

plants. We repeated this experiment with similar results (fig. S2 and fig. S3). Our results indicate that the production of root-secreted coumarins is a pre-requisite for the creation of the SBL mediated by *Hpa* infected plants. It also shows that the creation of the SBL is plant mediated.

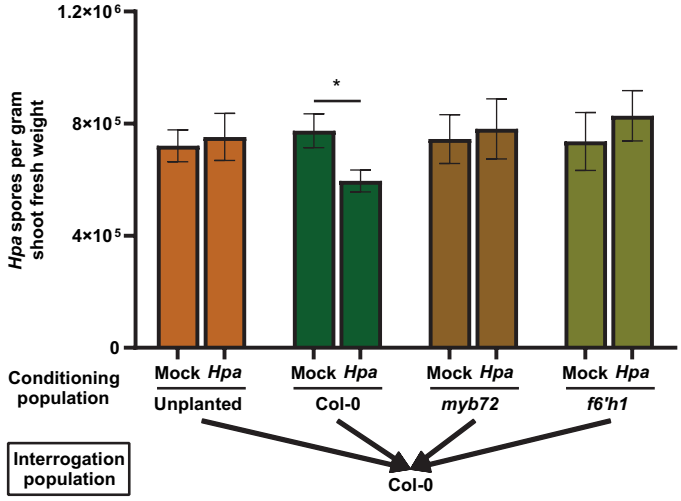

Figure 2. Coumarin biosynthesis is essential for the creation of SBL mediated by Hpa-infected plants. Disease severity of Col-0 plants growing on natural Reijerscamp soil that was pre-conditioned by mock-treated or *Hpa*-inoculated Col-0, *myb72*, or *f6'h1* seedlings. As a control, unplanted pots were inoculated or not with *Hpa*. Bars depict the average spore production per g of shoot fresh weight (n=10). Error bars depict standard error of the mean. Asterisk denotes a significant difference (*P*=0.01 in Students t-test).

Hpa infection results in significant changes in the root microbiome

To investigate the effect of *Hpa* infection on root microbiome assembly, we analyzed the composition of the root microbiomes of the healthy and *Hpa*-infected Col-0, *myb72,* and plants from the above-described experiment (fig. 2). To this end, the roots of Col-0, *myb72,* and *f6'h1* plants were harvested and root-associated microbiota isolated. To investigate changes in the bacterial and fungal communities on the roots, we sequenced the 16S and ITS amplicons derived from these roots and of unplanted soil sampled at the end of the conditioning period. For 16S, the data consisted of 80 samples divided over the treatments (healthy and *Hpa*-infected Col-0, *myb72* and *f6'h1* plants). On average, samples had 107,000 reads representing 21,786 unique ASVs. Plant reads, samples with a sequencing bias, samples with less than 22,000 reads and ASVs represented by less than 258 reads (low abundance ASVs) were removed. These filtering steps resulted in 67 samples with a mean of 105,000 reads and a total of 3175 unique ASVs. For the ITS data we had 80 samples with a total of 7,500,000 reads representing 4244 ASVs. After removal of *Viridiplantae* and *Rhizaria* reads which are not true fungi, samples with less than 7000 reads were removed as well as the ASVs represented by less

than 4292 reads. This resulted in a dataset of 74 samples with a total of 6,700,000 reads and 167 unique fungal ASVs. Both bacterial and fungal communities on the roots of healthy and *Hpa*-inoculated Col-0, *myb72,* and *f6'h1* plants, as represented by 16S and ITS amplicons respectively, showed a clear rhizosphere effect as they were significantly different (adjusted P <0.05 in Adonis test) compared to unplanted soil (fig. 3A, fig. S4, Table S3, Table S1).

Aboveground *Hpa* infection led to a statistically significant shift in the bacterial communities on roots of Col-0, *myb72* and *f6'h1* plants (fig. 3B-D, table S1). Application of a *Hpa* spore suspension on unplanted soils did not result in changes in the microbiome (fig. 3A). In contrast to the bacterial microbiota, fungal root microbiota were not significantly affected by aboveground *Hpa* infection, neither at fungal community level (fig. S4, table S3), nor at individual fungal ASVs abundance level. Thus, the fungal root microbiome is unlikely to play a role in the *Hpa* infection-mediated SBL. Hence, for the further analyses we focused on the *Hpa*-induced changes in the bacterial root microbiome.

Aboveground *Hpa* infection resulted in a significant shift in the bacterial microbiome (adjusted P <0.05 in Adonis test) on the roots of Col-0 and both the coumarin biosynthesis mutants (fig. 3B-D; table S1). However, the bacterial communities on the roots of the mutant plants were significantly distinct from that on the roots of the wild-type mock-treated plants (table S1). The bacterial communities on infected *f6'h1* mutants plants were also significantly different from wildtype plant, although the communities on *myb72* mutant plants were not (P=0.063). As shown in fig. 2, only *Hpa*-infected Col-0 plants created a SBL that increased *Hpa* resistance in the tester population of plants growing in the conditioned soil (fig. 2). Thus, to identify microbiota that are associated with the creation of the disease-induced SBL, we first performed an in-depth analysis of bacterial microbiome changes in the *Hpa*-infected Col-0 plants.

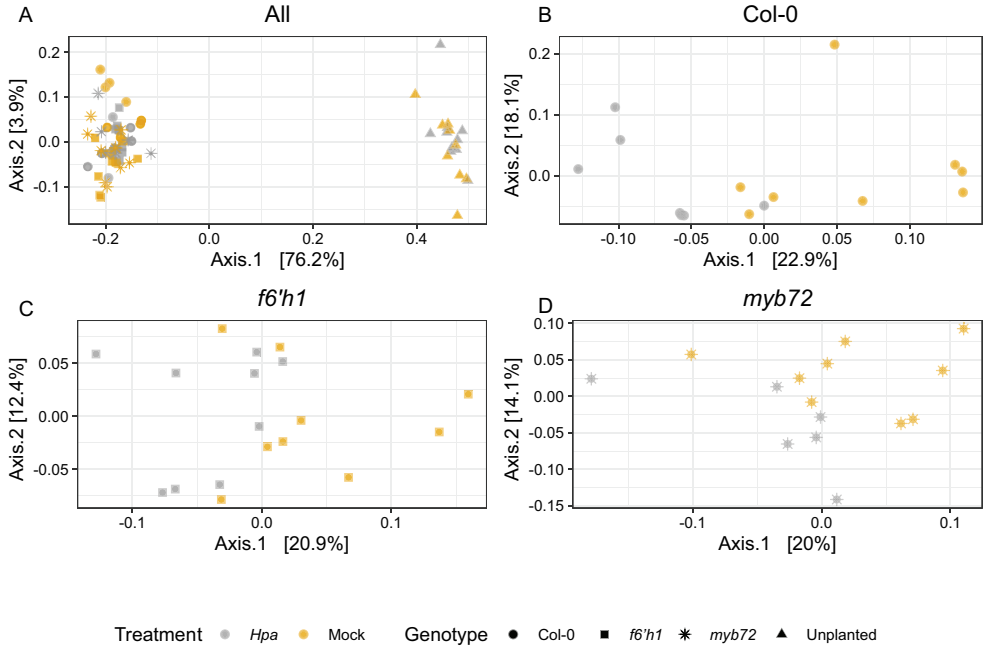

Figure 3. Effect of aboveground *Hpa* infection on root bacterial communities. A) Principal coordinate analysis (PCoA) of bacterial communities in unplanted soil (▲) and the rhizospheres of three-week-old seedlings of Col-0 (●), *myb72* (⁎) and *f6'h1* (■) plants. Samples were taken one week after either mock treatment (yellow symbols) or inoculation with *Hpa* (grey symbols). **B)** PCoA of only the Col-0 samples. **C)** PCoA of only the *f6'h1* samples. **D)** PCoA of only the *myb72* samples.

Co-occurrence network analysis of *Hpa*-induced shift in root microbiome

To decipher the belowground bacterial microbiome response of Col-0 plants to aboveground *Hpa* infection, a co-occurrence network of the top 600 most-abundant ASVs was constructed (fig. 4). We identified 18 clusters of co-occurring ASVs (fig. 4A, S6). One cluster (cluster 2) in particular was strongly and positively correlated with *Hpa* infection (fig. 4B, D). The cumulative relative abundance of this cluster, consisting of 47 ASVs, increased from 10 % on mock-treated plants to 15% on *Hpa*-infected plants (fig. 4E). The ASV constituents of cluster 2 belonged mostly to the orders of *Xanthomonadales* (10 ASVs), *Rhizobiales* (8 ASVs), *Sphingobacteriales* (6 ASVs), *Sphingmomonadales* (2 ASVs) and *Fibriobacterales* (3 ASVs) (fig. 4C, E). Whereas this cluster of ASVs increased in abundance upon *Hpa* infection of Col-0 plants, its abundance remained unaffected in *Hpa*-infected *myb72* and *f6'h1* plants (fig. 4F). Together these data show that there is a group of microbes that change in abundance on the roots of foliarly-infected wild-type plants, while their abundances remain stable on roots of *Hpa*-infected coumarin biosynthesis mutants.

To pinpoint specific microbes that are causative of the *Hpa*-induced SBL, we used Deseq2 and identified 29 ASVs that were significantly affected by *Hpa* infection on the roots of Col-0. The majority of these ASVs significantly increased in abundance (19 ASVs) on the roots of infected plants, whereas 10 ASVs were significantly depleted on the roots of infected plants. Just one of the ASVs with significantly affected abundance on the roots of *Hpa*-infected Col-0, was also significantly affected on the roots of *Hpa*-infected *myb72* plants (fig. S5). This *Rhizobiales* spp. increased in abundance on Col-0 roots upon *Hpa* infection, but decreased in abundance on *myb72* roots of *Hpa*-infected plants.

To test if the 29 ASVs could explain all the variance in the microbiome, we removed the 29 ASVs from the dataset. The removal of the 29 ASVs from the dataset did not remove the community level effect of *Hpa*-infection that we described above (Adonis, P=0.019). Moreover, the above-mentioned cluster analysis indicated that more than 29 ASVs were correlated to aboveground *Hpa* infection. For example, Cluster 2 contained 8 co-occurring *Xanthomonadales* ASVs of which the abundance correlates with *Hpa* infection, whereas Deseq2 only identified 3 *Xanthomonadales* to be significantly enriched. This indicates that there are more, likely taxonomically related, ASVs that are responding to aboveground infection, but that do not meet the rigorous statistical standards of Deseq2. We subsequently used Deseq2 to analyze order-level changes affected by *Hpa*-infection. We found that the relative abundance of *Xanthomonadales* spp*., Cytophagales* spp. *and Fibrobacteriales* spp. increased significantly on the roots of *Hpa*-infected plants, whereas the relative abundance of *Enterobacteriales* spp., *Holophageles* spp. and *Opitutales* spp. decreased significantly following *Hpa*-infection in this experiment (fig. 5).

The SBL is reproducibly generated by *Hpa*-infected plants in independent experiments, and thus it was further investigated if there is consistency in the identity of microbes that are recruited to the roots of infected plants. To test this, we analyzed the root microbiome of an independent experiment that included both Col-0 and *myb72* in the conditioning phase. This experiment was carried out in soil from the same Reijerscamp field but was collected several months after collecting Reijerscamp soil for the first experiment (October 2018 vs April 2019). Also, in this experiment a shift in the bacterial root microbiome was detected in response to *Hpa* infection (fig. S5). In both experiments, *Xanthomonadales* and *Fibriobacterales s*pp. significantly increased in abundance, whereas in the second experiment also *Spingobacteriales, Burkholderiales, Streptomycetales* and *Solibacteriales* spp. significantly increased. The abundance of *Opitutales spp.* decreased significantly in both experiments (fig. 5).

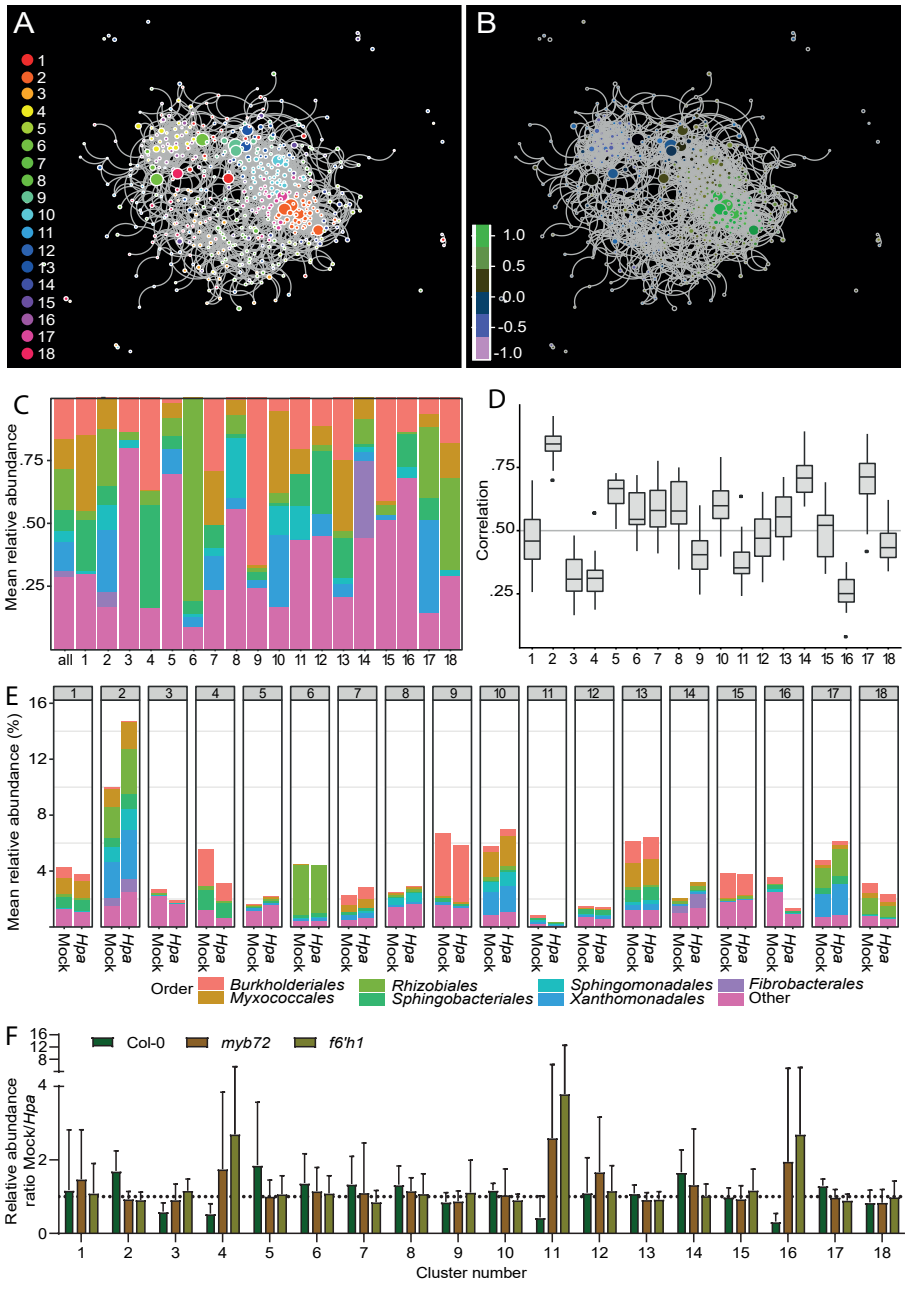

Figure 4. The abundance of co-occurring microbes is correlated with *Hpa* infection. A) Plot of co-occurrence network of the 600 most-abundant ASVs on the roots of healthy and *Hpa*-infected Col-0 plants. Each dot represents one ASV and the size represents their relative abundance. Each color represents a single cluster of co-occurring ASVs. **B)** The co-occurrence plot as in A, colored by relative shift in response to *Hpa* infection (green: more abundant, purple: less abundant). **C)** The community composition of each of the 18 clusters and the overall community, with the colors representing the different taxonomic orders. **D)** Boxplots showing the median (middle line) 1st and 3rd quartile (top and bottom of the box) and the outliers (end of whiskers) of the correlation to *Hpa* infection per cluster.

E) The mean relative abundance of the ASVs for each cluster in both mock treated samples and *Hpa*-infected samples, with the colors representing the different taxonomic orders. **F)** The ratio of the relative abundance of ASVs per genotype for each cluster. The bars show the mean relative abundance for Col-0 (dark green), *myb72* (brown), *f6'h1* (light green) with the error bars representing the standard error of the mean.

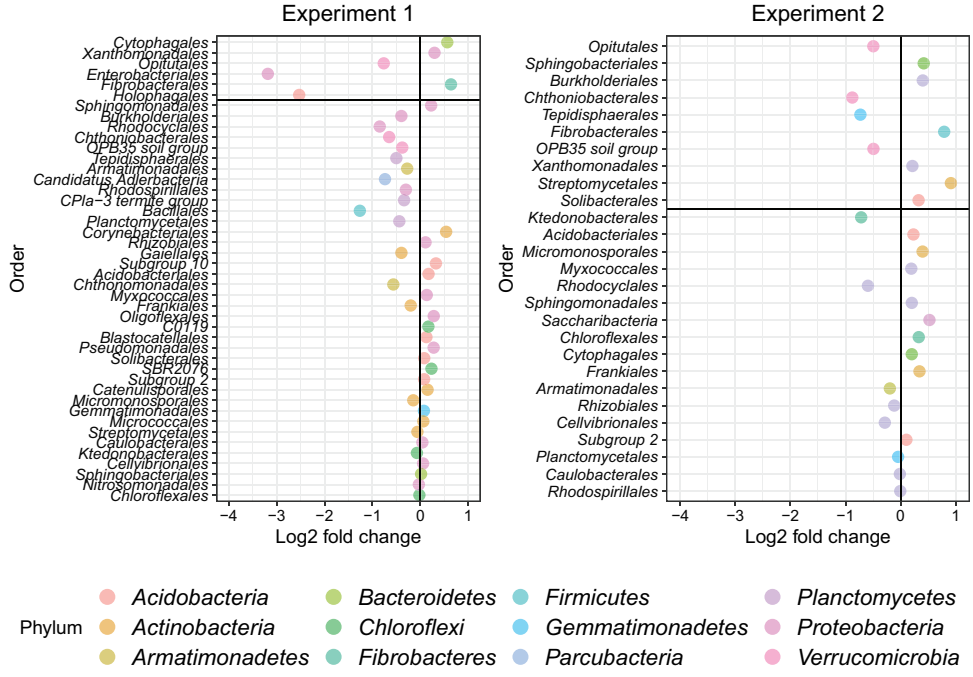

Figure 5. Differential abundance of bacterial orders on the roots of Col-0 plants in response to aboveground *Hpa*-infection in two independent experiments. The Log2 fold change as determined by Deseq2 in two independent experiments. A Log2 fold change greater than 0 represents an increase in *Hpa* infected samples. Each dot represents an agglomeration of all ASVs of the corresponding order. All orders above the horizontal line have an adjusted P-value of less than 0.05. Colors indicate the phyla each ASV belongs to.

To examine whether the same ASVs are associated with the SBL in the two separate experiments, we looked at their change in abundance upon *Hpa* infection as determined by Deseq2 (fig. 6). From a total of 67 ASVs belonging to the *Fibrobacterales* (8 ASVs) or *Xanthomonadales* (59 ASVs), 41 are shared between the two experiments. Two third (26) of these shared ASVs responded similarly to *Hpa* infection in both experiments. The majority of these ASVs (25 out of 26) increased in abundance.

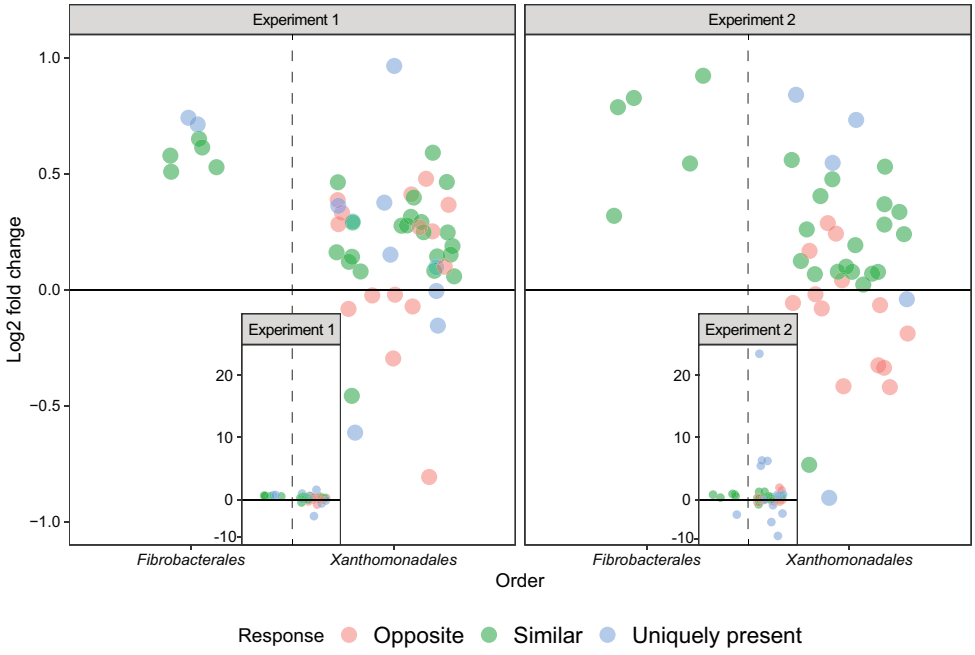

Figure 6. Behaviour of *Xanthomonadales* and *Fibrobacterales* on roots of *Hpa*-infected plants in two independent experiments. All ASVs of the order of *Xanthomonadales* and *Fibrobaterales* in the top 600 ASVs of two independent experiments. Each dot represents one ASV with red dots having an opposite response and the green dots having a similar response in the two experiments. Blue dots represent ASVs unique to that experiment. The insert shows the entire range of Log2-fold change, while the large panel show only ASVs with a Log2-fold change between -1 and 1.

In summary, in both experiments *Xanthomonadales* and *Fibriobacterales* spp. increased in abundance on the roots of Col-0 plants infected by *Hpa*. This is in line with previous results, where we identified and isolated two *Xanthomonadales spp.* (*Xanthomonas* sp. WCS2014-23 and *Stenotrophomonas* sp. WCS2014-113) that notably increased on the roots of 7-week-old Arabidopsis plants following *Hpa*-infection (Chapter 2; Berendsen *et al., 2018*). In the two SBL experiments described here, we did not detect ASVs with sequences matching 100% to the 16S of these two isolates. Whereas the ASVs that were significantly enriched on the roots of *Hpa*-infected plants did not overlap in the two independent experiments, 25 ASVs belonging to the order of *Xanthomonadales* or *Fibrobacterales* increased upon *Hpa* infection in both experiments. Together this indicates an important role for specific *Xanthomonadales* spp. and *Fibrobacterales* spp. in the creation of the *Hpa*-induced SBL.

Discussion

Recent studies showed that plants can create a SBL in response to biotic or abiotic stresses, resulting in microbial communities in the rhizosphere that can act favorably on plant growth and immunity (Bakker et al., 2018). In this chapter, we investigated several aspects of the SBL created by downy mildew-infected Arabidopsis plants. In our study, conditioning a natural soil with *Hpa*-inoculated plants resulted in a consistent reduction of infection in a subsequent *Hpa*-inoculated population of plants growing on the same soil, compared to disease severity in a second population of plants that was grown on soil previously conditioned with healthy plants. To investigate whether protection of the interrogation population of plants against *Hpa* is caused by the induction of ISR by microbiota in the conditioned soil defense-related mutants *myb72, sid2*, and *npr1* were tested for their ability to mount systemic immunity against *Hpa* when growing in soil that was conditions with *Hpa*-infected Col-0 plants. A well-studied beneficial microbe that can elicit ISR in Arabidopsis against a wide range of pathogens, including *Hpa*, is *Pseudomonas simiae* WCS417r (Pieterse et al 2020). Activation of ISR by WCS417r, but also by several other beneficial root-associated microbes, requires the transcription factor MYB72 (Pieterse et al 2014, Van der Ent et al 2008). However, our results show that the SBL-mediated ISR in our study does not require MYB72, but rather depends on SA signaling (fig. 1). For several beneficial microbes it has been shown that systemically induced disease resistance is mediated via a SA-dependent signaling pathway (De Vleesschauwer & Höfte 2009, van de Mortel et al 2012) (Pieterse et al 2020). Hence, the mode of action of the SBL created by *Hpa*-infected plants in Reijerscamp soil may be more similar to the SA-dependent induced disease resistance that is activated by this class of beneficial microbes.

If the induced resistance that is mediated by the SBL of *Hpa*-infected plants is of microbial origin, then a shift in the rhizosphere microbiome of Arabidopsis is expected after foliar *Hpa* infection. We thus hypothesize that aboveground pathogen infection of plants elicits a systemic signaling, that subsequently leads to a change in root secretions that selectively recruit specific microbes that in turn trigger a systemic defense response that provides enhanced protection of the leaves against subsequent *Hpa* infection. Yuan and co-workers (2018) showed that Arabidopsis leaf infections by the bacterial pathogen *P. syringae* can result in a SBL that is effective against this bacterial pathogen and is accompanied by altered root secretions. Thus, changes in the Arabidopsis root microbiome composition appears to be a consistent response to aboveground pathogen infections (chapter 2 Yuan et al 2018). Similarly, a shift in the root microbiome composition of pepper was observed after whitefly infestation (Yang et al 2011), suggesting that changing the root microbiome is a general response to foliar biotic stresses.

In the current study, the downy-mildew-induced shift in root microbiome composition was statistically significant, but more tangled compared to the results in chapter 2, that relied on a Phylochip-based analysis rather than the amplicon

sequencing employed here. The results in this chapter suggest that, although root fungal communities were unaffected, a large number of bacterial ASVs responded to foliar downy mildew infection, but the fold change in abundances was less pronounced compared to those observed in chapter 2. Moreover, none of the ASVs responding to downy mildew infection in this study exactly matched the *16S rRNA* genes of the *Xanthomona*s sp., *Stenotrophomonas* sp. or *Microbacterium* sp. that were identified and isolated in that preceding study. At a higher taxonomic level, we did find that the relative abundance of the bacterial orders of *Xanthomonadales* and *Fibriobacterales* increased significantly in abundance in both experiments. Although not significantly, we also found overlap in the response to *Hpa* infection of identical microbes at the ASV level that were promoted on the roots of infected plants between the two experiments. Not much is known about the small order of the *Fibrobacterales*, except that some members of this order are able to break down cellulose (Ransom-Jones et al 2012) and are found in the bovine digestive tract (Ozbayram et al). The order consists of a single phylum (*Fibriobacter*) and is closely related to the order of *Cytophagales* that was also significantly more abundant on roots of infected plants in one out of the two experiments in the current study. *Xanthomonadales* on the other hand, is a much larger order of often plant-associated bacteria that also comprises the genera *Xanthomonas* sp. and *Stenotrophomonas* sp. that we identified in chapter 2. Together these results indicate that, although the strains that are promoted in response to downy mildew infection are not identical between the three experiments, there is a signal that is picked up by phylogenetically related microbes. Thus, there appears to be a consistent enrichment of specific microbial groups in the Arabidopsis rhizosphere after *Hpa* infection. Future research should identify traits or functions that these microbes share, how they are recruited, and how they affect the plant immune system.

Plants are obviously needed to create the SBL, since inoculating soil with *Hpa* and subsequently leaving it unplanted did not result in protection against *Hpa* in a so-called interrogation population of plants. Although *myb72* mutant plants did respond to the SBL, leading to increased resistance against *Hpa*, MYB72 did prove essential for the creation of the *Hpa* infection-mediated SBL. MYB72 is known to regulate the production of root-secreted coumarins, mainly scopoletin, in response to iron deficiency and upon colonization by plant-beneficial bacteria (Stassen et al 2021, Stringlis et al 2019, Stringlis et al 2018, Voges et al 2018). Coumarins are synthesized in the phenylpropanoid pathway via F6'H1 and indeed, like *myb72*, *f6'h1* mutant plants were unable to create a SBL of disease. Thus, coumarins appear to be involved in the *Hpa*-induced recruitment of bacteria in the rhizosphere that in turn can induce resistance against *Hpa*. In line with this, we found that those bacteria that were promoted in response to infection on wild-type plants, were not promoted on the roots of *myb72* and *f6'h1* mutant plants. However, bacterial communities on roots of healthy *myb72* and *f6'h1* mutant plants were significantly different from those of wild-type plants, making it ambiguous to draw solid conclusions about the observed shifts in the rhizosphere microbiome in relation

to protection against disease. Further study of the rhizosphere microbiome that develops on roots of wild-type plants in the interrogation population may allow a more critical evaluation of the identity of SBL and the role specific microbes play in protection against disease.

Downy mildews are obligate biotrophs and their cultures are maintained by continuous infection cycles on susceptible host plants (Asai et al 2015, Coates & Beynon 2010). The spore suspensions generated to transfer *Hpa* from one Arabidopsis population to the next, in maintenance cultures and in the experiments described here, therefore likely contain many other microbes that have grown in association with *Hpa* for many cycles. Our results show that inoculation of unplanted soils with such *Hpa*-associated microbiomes do not affect the microbial composition of these soils. Moreover, we have identified two mutant Arabidopsis genotypes (*myb72* and *f6'h1*) that are unable to mount a SBL of disease following inoculation with this *Hpa*-associated microbiome. This shows that the buildup of a resistance-inducing SBL requires a plant response to *Hpa* infection and is not the result of co-inoculation of a plant-beneficial microbioate with a *Hpa* spore suspension.

To summarize, we found that downy mildew infection of a first population of plants consistently results in a SBL that in a subsequent interrogation population of plants results in the SA-dependent priming of defenses and increases resistance to *Hpa* infection. Our results indicate that the *Hpa* infection-mediated SBL is conferred by rhizobacteria and consistently find that bacteria of the order *Xanthomonadales* and *Fibrobacterales* are specifically promoted on roots of Arabidopsis in response to downy mildew infection. Future research should pinpoint what exactly is the common genetic denominator amongst the recruited bacteria that enable them to induce resistance. Our results indicate that the production of root-secreted coumarin is important for the creation of the SBL, likely through the directed assembly of a plant-beneficial microbiome, but future research should pinpoint the role of coumarins in the buildup of the SBL.

These findings contribute significantly to our understanding how plants dynamically activate and recruit specific members of their second genome to come to their aid. Comprehension of the mechanisms by which plants create microbiomes that increase their disease resistance would enable breeding or engineering of crop plants that consistently profit from their assembled rhizosphere microbiomes. By developing such elite crops, we could reduce current agricultural dependency on chemical crop protection.

Acknowledgements.

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Author contributions

G.V., P.A.H.M.B., and R.L.B. designed experiments; G.V., J.S. and E.V. performed experiments; G.V., P.G., S.B. and B.S. analyzed data and G.V., P.A.H.M.B, R.L.B., and C.M.J.P. wrote the manuscript.

Supplemental data

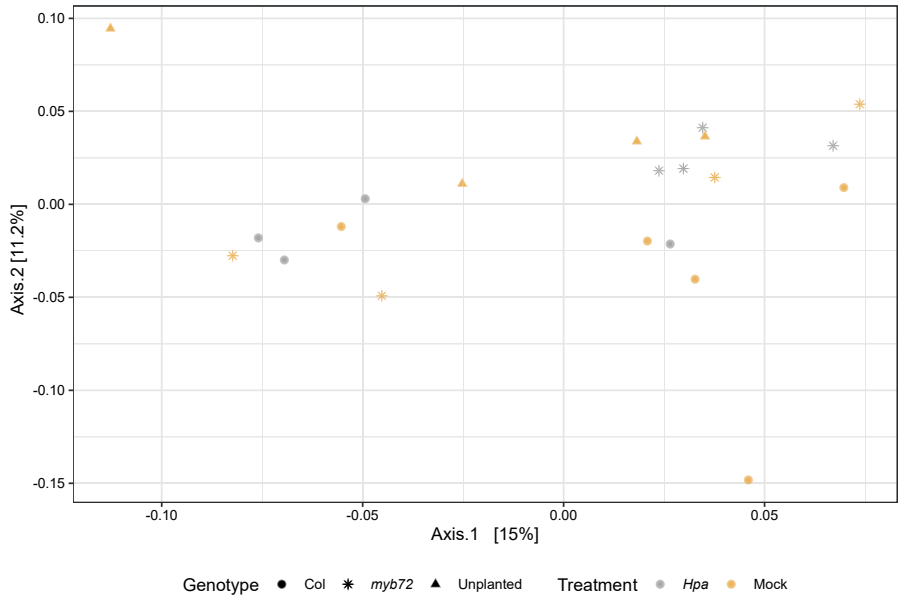

Figure S1. No rhizosphere effect is detected when sampling the first centimeter of topsoil. Effect of aboveground *Hpa* infection on root bacterial communities. A) Principal coordinate analysis (PCoA) of bacterial communities in unplanted soil (▲) and the rhizospheres of three-week-old seedlings of the wild-type accession Col-0 (●) and mutants *myb72* (⁎) and *f6'h1* (■). Samples were taken one week after either mock treatment (yellow symbols) or inoculation with *Hpa* (grey symbols).

Table S1. The R^2 and P-values for treatment (mock or *Hpa*) as by Adonis test corresponding to the PCoA in fig 3

	Col-0 Mock	**Col-0 *Hpa***	***myb72* Mock**	***myb72* *Hpa***	***f6'h1* Mock**	***f6'h1* *Hpa***	**Unplanted Mock**	**Unplanted Hpa**
Col-0 Mock		P = 0,003	P = 0,003	P = 0,02	P = 0,003	P = 0,002	P = 0,002	P = 0002
Col-0 *Hpa*	R^2 =0,17		P = 0,006	P = 0,063	P = 0,031	P = 0,006	P = 0,002	P = 0,002
***myb72* Mock**	R^2 =0,18	R^2 =0,14		P = 0,036	P = 0,087	P = 0,031	P = 0,002	P = 0,002
myb72* *Hpa	R^2 =0,14	R^2 =0,12	R^2 =0,11		P = 0,056	P = 0,436	P = 0,002	P = 0,003
***f6'h1* Mock**	R^2 =0,17	R^2 =0,11	R^2 =0,08	R^2 =0,10		P = 0,011	P = 0,002	P = 0,002
f6'h1* *Hpa	R^2 =0,21	R^2 =0,14	R^2 =0,09	R^2 =0,07	R^2 =0,11		P = 0,002	P = 0,002
Unplanted Mock	R^2 =0,77	R^2 =0,79	R^2 =0,80	R^2 =0,78	R^2 =0,80	R^2 =0,81		P = 0,236
Unplanted Hpa	R^2 =0,80	R^2 =0,82	R^2 =0,83	R^2 =0,80	R^2 =0,83	R^2 =0,82	R^2 =0,06	

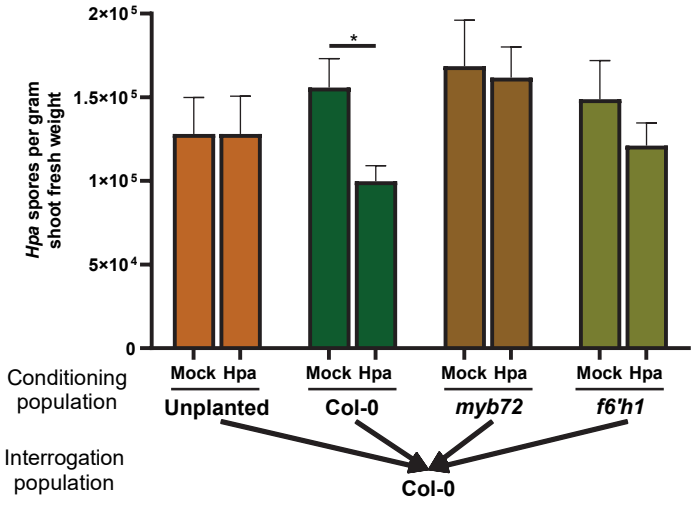

Figure S2. Independent repeat of the SBL experiment.
Disease severity of wild-type Col-0 plants growing on soil pre-conditioned by Col-0, *myb72*, or *f6'h1* seedlings that were either mock-treated or inoculated with an *Hpa* spore suspension. Bars depict the average spore production per gram of shoot fresh weight of 10 replicate pots. Error bars depict standard error of the mean. Asterisk denotes a significant difference ($P < 0.05$ in Students t-test).

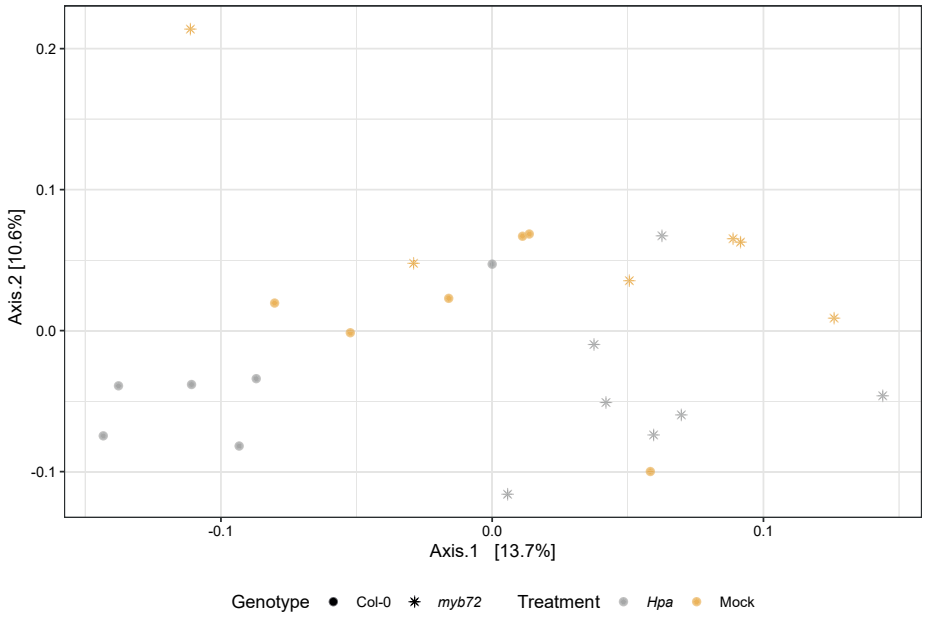

Figure S3. Effect of aboveground *Hpa* infection on root bacterial communities.
Effect of aboveground Hpa infection on root bacterial communities. A) Principal coordinate analysis (PCoA) of bacterial communities in the rhizospheres of three-week-old seedlings of the wild-type accession Col-0 (●) and *myb72* mutants (⁎). Samples were taken 1 week after either mock treatment (yellow symbols) or inoculation with *Hpa* (grey symbols).

Table S2. The R^2 and P-values for treatment (mock or *Hpa*) as by Adonis test corresponding to the PCoA in fig S2

	Col-0 Mock	**Col-0 *Hpa***	***myb72* Mock**	***myb72 Hpa***	**unplanted**
Col-0 Mock		P = 0,35	P = 0,42	P = 0,20	P = 0,14
Col-0 *Hpa*	R^2 =0,14		P = 0,42	P = 0,15	P = 0,32
***myb72* Mock**	R^2 =0,12	R^2 =0,15		P = 0,42	P = 0,42
myb72 Hpa	R^2 =0,12	R^2 =0,22	R^2 =0,14		P = 0,35
unplanted Mock	R^2 =0,02	R^2 =0,17	R^2 =0,15	R^2 =0,17	

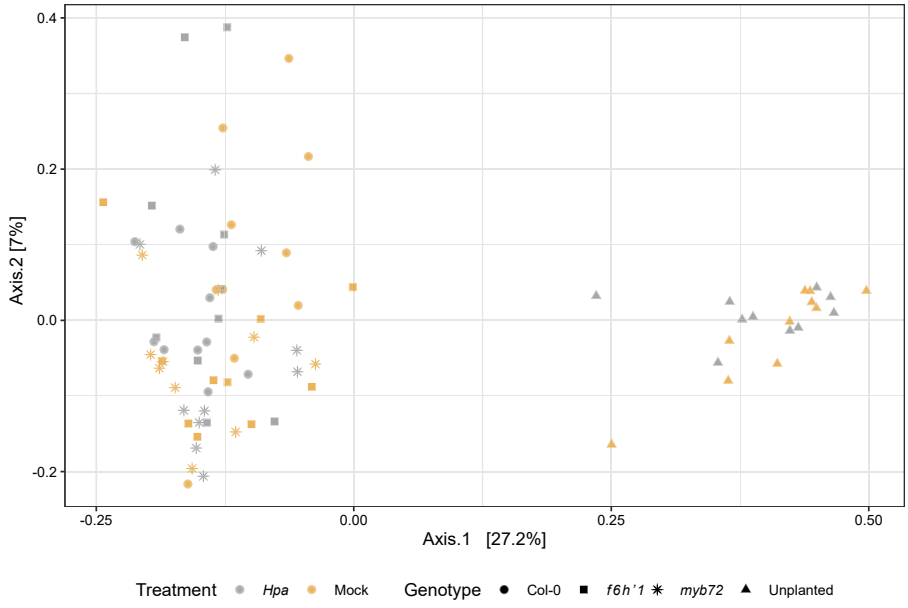

Figure S4. Aboveground *Hpa* infection does not result in changes to the fungal community on the roots.
Principal coordinate analysis (PCoA) of fungal communities in unplanted soil (▲), or the rhizospheres of three-week-old seedlings of the wild-type accession Col-0 (●) and mutants myb72 (*) and f6'h1 (■). Samples were taken 1 week after either mock treatment (yellow symbols) or inoculation with *Hpa* (grey symbols).

Table S3. The R^2 and P-values for treatment (mock or *Hpa*) as by Adonis test corresponding to the PCoA in fig S3

	Col-0 Mock	Col-0 Hpa	myb72 Mock	myb72 Hpa	f6'h1 Mock	f6'h1 Hpa	Unplanted Mock	Unplanted Hpa
Col-0 Mock		*P* = 0,23	*P* = 0,23	*P* = 0,47	*P* = 0,28	*P* = 0,45	*P* = 0,02	*P* = 0,02
Col-0 Hpa	R^2 =0,08		*P* = 0,47	*P* = 0,28	*P* = 0,25	*P* = 0,47	*P* = 0,02	*P* = 0,02
myb72 Mock	R^2 =0,07	R^2 =0,05		*P* = 0,71	*P* = 0,71	*P* = 0,28	*P* = 0,02	*P* = 0,02
myb72 Hpa	R^2 =0,06	R^2 =0,07	R^2 =0,05		*P* = 0,71	*P* = 0,38	*P* = 0,02	*P* = 0,02
f6'h1 Mock	R^2 =0,07	R^2 =0,07	R^2 =0,05	R^2 =0,05		*P* = 0,24	*P* = 0,02	*P* = 0,02
f6'h1 Hpa	R^2 =0,06	R^2 =0,06	R^2 =0,07	R^2 =0,06	R^2 =0,08		*P* = 0,02	*P* = 0,02
Unplanted Mock	R^2 =0,34	R^2 =0,39	R^2 =0,40	R^2 =0,34	R^2 =0,36	R^2 =0,37		*P* = 0,34
Unplanted Hpa	R^2 =0,32	R^2 =0,38	R^2 =0,38	R^2 =0,33	R^2 =0,35	R^2 =0,36	R^2 =0,06	

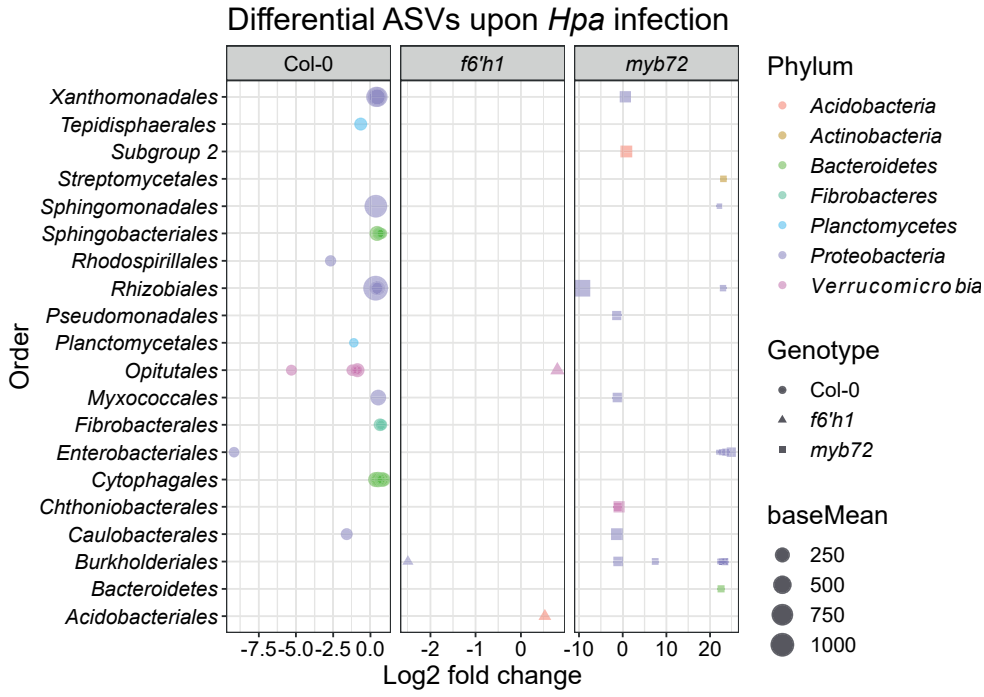

Figure S5. Specific ASVs change in the root microbiome upon foliar *Hpa* infection for Col-0 and coumarin biosynthesis mutants *myb72* and *f6'h1*.
The Log2-fold change in abundance of significantly changing ASVs for accession Col-0 (●) and mutants *myb72* (■) and *f6'h1* (▲). The colors represent the different Phyla, while the size of the symbol represents a measure for abundance.

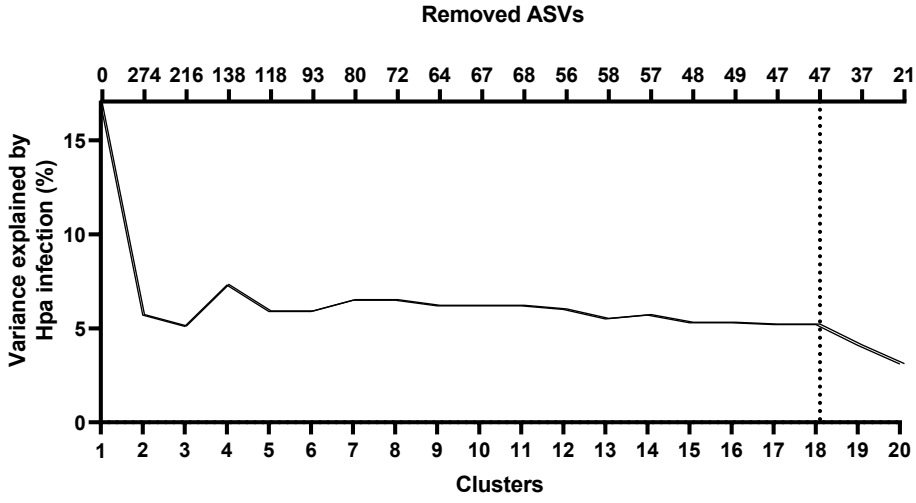

Figure S6. The variance explained by *Hpa* infection (y-axis) by the ASVs (top x-axis) of the most strongly correlating cluster for each number of clusters (bottom x-axis). The number of clusters does not strongly determine the variance explained, while the amount of ASVs explaining the variance decreases as the number of cluster increases. For this reason we selected 18 clusters for further analysis.

5

CHAPTER 5.

Response of the *Arabidopsis thaliana* root microbiome to distinct aboveground microbial attackers

Gilles Vismans[1], Sietske van Bentum, Pim Goossens[1], Eva Visser[1], Corné M.J. Pieterse[1], Peter A.H.M. Bakker[1], Roeland L. Berendsen[1]

1: Plant-Microbe Interactions, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Abstract

The model-plant *Arabidopsis thaliana* is able to respond to aboveground pathogen attack by recruiting microbes to its roots that enhance the plant's immune system and protect against disease. In separate studies carried out in distinct soils, it was found that Arabidopsis recruits a beneficial root microbiome when leaves are infected by distinct attackers, but little overlap was detected in taxonomic identity of the recruited microbes. Here, we studied changes in the Arabidopsis root microbiome composition in response to 5 distinct microbial pathogens representing distinct lifestyles and kingdoms of life in a single soil. Although foliar infection by the fungal pathogen *Botrytis cinerea*, or the oomycete *Phytophthora capsici* only led to small and temporary changes in the root microbiome, infection by the bacterial pathogens *Pseudomonas syringae* pv. *tomato* or *Xanthomonas campestris* more strongly and consistently affected the belowground root microbiome. Interestingly, several microbes that were recruited in response to *P. syringae* also increased in abundance following infection by *X. campestris* inoculation. In contrast to previous findings, we did not see changes in the root microbiome of Arabidopsis infected with the downy mildew *Hyaloperonospora arabidopsidis*. Collectively, these results show that aboveground pathogen attack leads in variable degrees to recruitment of belowground microbes into the rhizosphere. The two bacterial leaf pathogens mediated similar changes in root microbiome composition, indicating a possibility that similar microbes could be recruited to the rhizosphere in response to foliar attack by distinct pathogens.

Introduction

Although most plants are confined to one location during the course of their lifecycle, they are not the only organisms occupying this space. Plants share their surroundings with a plethora of organisms, of which the micro-organisms have been the main focus of study in recent years (Arif et al 2020, Oyserman et al 2018). The microbes that interact with the plant are routinely referred to as "the plant holobiont", "the plant's second genome" and" the plant microbiome" (Berendsen et al 2012, Mendes et al 2013, Vandenkoornhuyse et al 2015). All these concepts emphasize that the plant is not a single entity, but the sum of interactions between the plant and the plant-associated microorganisms. Hence, the performance of a plant is determined by the interactions with its microbiome. Despite such holistic views, it has been abundantly demonstrated that single microbial species within the microbiome can have pronounced effects on plant health. Pathogenic organisms can cause disease and hamper plant growth, whereas beneficial microbes can e.g. provide nutrients or protect against pathogens through competition, antibiosis or induced systemic resistance. Several studies have shown that by secreting metabolites and sugars plants do have a great influence on the composition of their root microbiome (Pascale et al 2020, Sasse et al 2018, Stringlis et al 2019). By secreting antimicrobials or microbial stimulants, plants can create ecological niches that suit specific microbes that in turn can optimize the growth and performance of the plant in a given situation (Hu et al 2018).

Previously, it was found that plants can dynamically alter their root microbiome upon foliar infection by the downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) and this infection resulted in decreased disease incidence in a subsequent population of plants growing on that soil (Chapter 2) (Berendsen et al 2018). Moreover, three bacterial species, a *Xanthomonas* spp., a *Stenotrophomonas* spp. and a *Microbacterium* spp. were found to increase in abundance in response to aboveground *Hpa* infection. These three bacteria were isolated from the roots of *Hpa*-infected plants and together induced systemic resistance to *Hpa* when added to the soil (Chapter 2) (Berendsen et al 2018). Similarly, infection of *Arabidopsis thaliana* (henceforth Arabidopsis) plants by the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) led to a change in root microbiome composition and resulted in increased resistance of a subsequent population of plants growing on that soil (Yuan et al 2018). Together these data suggest that, upon pathogen attack, plants can recruit specific beneficial microbes to their roots and that these microbes function as a microbial soil-borne legacy (SBL; Chapter 4) that increases the resistance of a following generation of plants. However, there was no apparent overlap between the microbes recruited by plants in response to *Hpa* and *Pst* in these two independent studies, possibly because these studies were carried out using different soils.

Here, we investigate the ability of Arabidopsis to modulate the root-associated microbiome upon foliar infection by five distinct pathogens: the oomycetes *Hpa* and *Phytophthora capsici* (*Pc*), the bacteria *Pst* and *Xanthomonas campestris* (*Xc*),

and the fungus *Botrytis cinerea* (*Bc*). We inoculated Arabidopsis plants with these five pathogens and monitored root microbiome composition for three weeks using 16S rRNA amplicon sequencing. We then analyzed this data to see how infections by these different pathogens of plants growing in the same soil affected the root microbiome.

Material and methods

Soil and plant growth conditions

In this study, natural soil collected from the Reijerscamp nature reserve in the Netherlands (Chapter 2) was used. Arabidopsis seeds of accession Col-0 were germinated on river sand that was supplemented with half-strength Hoagland solution, containing 5 mM KNO_3, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 10 µM Fe-EDDHA, 5 mM $Ca(NO_3)_2$, 25 µM H_3BO_3, 50 µM KCL, 0.24 µM $MnSO_4{\cdot}H_2O$, 2 µM $ZnSO_4{\cdot}7H_2O$, 0.5 µM $CuSO_4{\cdot}5H_2O$, and 36 µM $(NH_4)_5Mo_7O_{24}{\cdot}4H_2O$, for 10 days at 100% humidity at 21 °C with 16 h daylight with an intensity of 100 µmol m^{-2} s^{-1}. After 10 days, the seedlings were planted in the middle of a 60-ml pot filled with Reijerscamp soil. The plants were then transferred back to the growth facilities and covered with a transparent lid to increase humidity to 100% for 24 h, after which the lid was removed. Five weeks after sowing, plants were inoculated with one of five pathogens or left untreated. One, two and three weeks after inoculation, a subset of at least 14 replicates per treatment was collected for analysis. The remaining plants were again inoculated with their respective pathogen one day after sample collection.

Cultivation and inoculation of plant pathogens

The obligate biotroph *Hpa* Noco2 was routinely maintained and inoculated on Arabidopsis seedlings as described by Asai *et al.* (2015). Briefly, leaf wash-offs of sporulating plants were diluted in tap water until they contained 100 spores per µl and this spore suspension was subsequently applied by airbrush to the leaves of 5-week-old plants placed in a fume hood, until a thin film of waterdrops was visible on the leaves. The leaves were allowed to dry before plants were returned to the climate chambers.

Pst strain DC3000 (Kunkel et al 1993) was streaked on $^1/_2$-strength King's medium B (KB) broth agar supplemented with 25 mg/mL rifampicin and incubated at 28°C for 24 h. Single colonies from pure cultures were then inoculated in liquid $^1/_2$-strength KB and incubated at 28°C at 250 RPM for 24 h (van Wees et al 2013). *Xc* was streaked on yeast extract dextrose $CaCO_3$ medium plates (YDC) and incubated at 28°C for 48 h. Overnight cultures of *Pst* or bacterial suspension of *Xc* were subsequently centrifuged (4000 *g*, 10 min) after which cells were resuspended in 10 mM $MgSO_4$. The washing procedure was repeated and the final concentration of the bacterial suspensions was set to an OD_{660} of 0.05 and supplemented with 0.02% (v/v) Silwet L77. Leaves of 5-week-old plants were dipped in the bacterial suspension for 3

seconds. Bacterial suspensions were refreshed following the dipping of 30 plants until 50 plants were dipped (van Wees et al 2013).

Pc Lt3112 (Donahoo & Lamour 2008) zoospores were produced by growing a plug of *Pc* inoculum on a fresh V8 medium plate. After 7 days, the Petri-dish containing the colony was soaked in demineralized water to remove nutrients from the medium, to starve the colony and thus stimulate zoospore production. After one h, the demineralized water was replaced and zoospores were allowed to form over 72 h at room temperature. To release the zoospores, the plates were placed at 4°C for 45 min. The zoospore suspension was diluted in demineralized water to a concentration of 50 zoospores per µl and applied to plants using an airbrush until a thin film was visible on the leaves. The plants were allowed to air dry before being put back in the trays.

The fungus *Bc* strain B05.10 (Van Kan et al 2017) was grown for two weeks from a glycerol stock on 0.5 potato dextrose agar (PDA) plates at 22°C. The conidia were suspended in 10 ml of 0.5 PDB by gently scraping off the conidiophores. The suspension was filtered over glass wool and diluted to a density 5 x 10^5 conidia per ml. The conidia were incubated for 2 h at room temperature and two fully matured leaves of a plants were subsequently inoculated with 5 µl of the suspension on and left to dry (van Wees et al 2013). After inoculation, all plants were covered with transparent plastic lids to increase humidity to 100% for 72 h. All plant inoculations were repeated every 8 days.

Plant sampling

Fourteen plants of each treatment were harvested 1, 2 and 3 weeks after the first pathogen inoculation. First, four infected leaves of the plants were harvested and snap frozen in liquid nitrogen for qPCR analysis of the defense-related marker genes *PR1*, *VSP2* and *PDF1.2* according to van Wees *et al.* (2013) with At1g13320 as a reference gene. RNA extraction, cDNA synthesis and qPCR were performed as described in Chapter 2. Subsequently, the plant pot was turned upside down and emptied on a paper towel. Roots were then gently lifted by the shoot and carefully shaken to remove the adhering soil. The shoots were subsequently removed from the roots with a razor blade and the roots were snap frozen in liquid nitrogen until storage at -80°C. DNA of these root samples was isolated as described in Chapter 4.

Preparation of 16S library

The *16S rRNA* gene amplicon libraries were constructed using the 16S metagenomic sequencing library protocol for the Illumina Miseq system (Illumina 2013). The protocol was adapted to incorporate 16S V3-V4 phasing primers to increase complexity and decrease the need for PhiX spike (de Muinck et al 2017). Moreover, the barcoded PCR primers described by Baym and co-workers (2015) were used in a total PCR reaction volume of 25 µL instead of 50 µL. Three µL of the purified samples was run on a 1% agarose gel to check the amplicon size and the concentration was determined using the Qubit broad range kit (Thermofisher

Scientific). Each sample was adjusted to 2 ng/µL and pooled in a 1:1 ratio. The pooled library was sent for sequencing on a 2x 300 MiSeq at the USEQ sequencing facility (Utrecht University, the Netherlands).

Sequence data pre-processing
Qiime2 version 2019.7 was used to pre-process and analyze the data (Bolyen et al 2019). The primers were removed using the Cutadapt (Martin 2011) plugin with default settings. Denoising, dereplicating, chimera removal and end-joining was performed using DADA2 and reads were truncated at 267 and 202 bp for run 1, 265 and 201 bp for run 2, and 261 and 206 bp for run 3, respectively (Callahan et al 2016). The resulting tables were merged and taxonomy assigned using a V-search based consensus taxonomy classifier and the SILVA 128 database as a reference (Quast et al 2013, Rognes et al 2016, Yilmaz et al 2014).

Results

Experimental setup and verification of pathogen efficacy
To trigger defense responses in Arabidopsis leaves, we inoculated leaves of five-week-old Arabidopsis plants with either the oomycete plant pathogens *Hpa* or *Pc*, the fungal pathogen *Bc*, or the bacterial pathogens *Pst* or *Xc*, and left control plants uninoculated. The inoculations resulted in the development of distinct disease symptoms in all inoculated plants. Following *Hpa* inoculation, sporulation was observed already at one week post inoculation (WPI), at which time leaves that were inoculated with *Bc* had already completely died. Yellowing of the leaves in response to *Pst* inoculation was observed from 2 WPI onwards, while inoculation with *Xc* and *Pc* resulted in black spots and white hyphal growth respectively at 3 WPI. To verify activation of defense responses in inoculated leaves, we quantified the expression of the defense-related marker genes *PR1*, *VSP2* and *PDF1.2* by qPCR at 1WPI in four newly formed leaves of each plant that was selected for root harvesting. We found that inoculation with *Hpa* and *Bc* resulted in a mild activation of *PR-1,* whereas plants infected by *Hpa, Xc and Bc* were found to have higher expression of *VSP2*. Inoculation with *Xc* and *Hpa* pathogens induced the expression of *PDF1.2* (fig. 1). This indicates that inoculation with the pathogens elicited a defense response in the leaves.

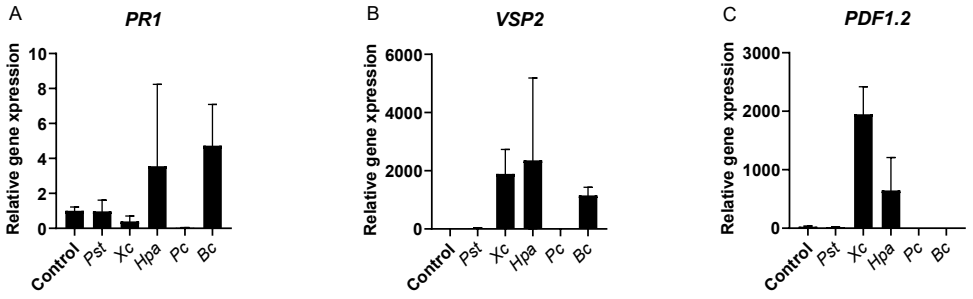

Figure 1. Expression of defense-related genes one week post inoculation of Arabidopsis leaves with 5 distinct pathogens. Quantitative qPCR analysis of **A)** *PR1*, **B)** *VSP2*, and **C)** *PDF1.2* gene expression in Arabidopsis Col-0 leaves one week after inoculation with *Pst, Xc, Hpa*, *Pc* or *Bc*. Bars represent mean relative expression of at least 4 replicate plants. Error bars represent standard deviation.

Distinct but not all foliar infections lead to a dynamic shift in root microbiome composition

We sampled and analyzed the root-associated bacterial microbiome of all plant treatments by sequencing the V3-V4 region of the 16S *rRNA* gene, one, two and three weeks after the initial inoculation. In addition, we sampled and sequenced the microbiome of unplanted soils. A total of 273 samples were generated with a median read depth of 112462 reads per sample. This resulted in a total of 35917 distinct amplicon sequence variants (ASVs). After processing the data and removing samples with low reads (< 20000 reads) and outlier samples based on their composition compared to samples with the same treatment at the same timepoint, we retained a dataset of 195 samples. Of at least 9 replicates per timepoint we analyzed the most abundant ASVs, encompassing 90% of the total number of reads.

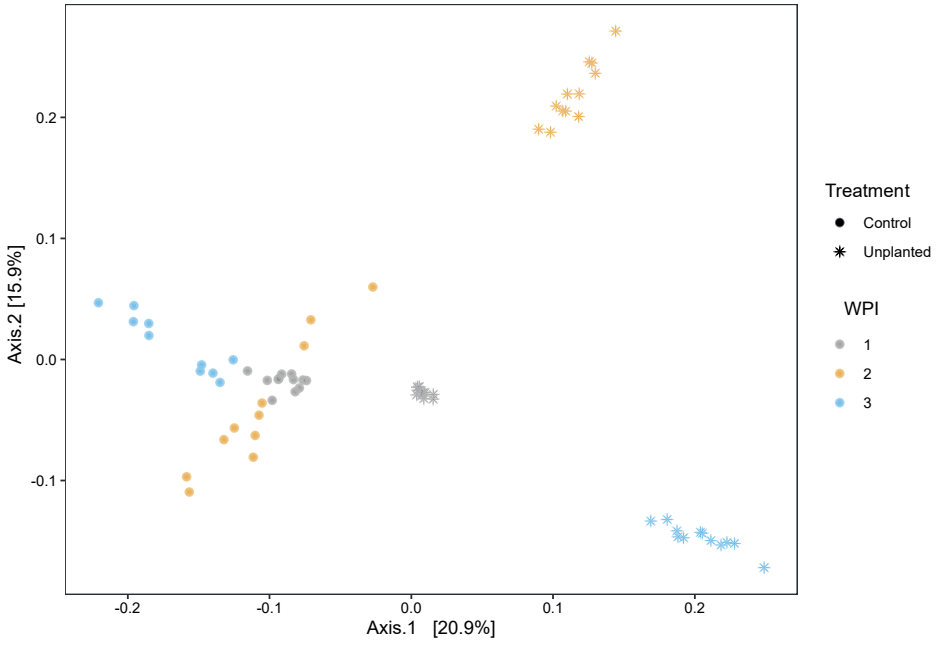

Figure 2. Change in microbial communities in unplanted bulk soil and on roots of uninoculated plants over time. Principal coordinate analysis (PCoA) of bacterial communities on the roots of uninoculated control plants (●) or in unplanted bulk soil (⁎) at 1 (gray symbols), 2 (yellow symbols) and 3 WPI (blue symbols). Eigenvalues of PC1 and PC2 are expressed on the X- and Y-axis, respectively.

We first analyzed the microbial communities in unplanted bulk soil and in the rhizosphere of healthy, uninoculated plants over time. On each of the three timepoints of harvesting, we observed a statistically difference between the microbial communities found in unplanted bulk soil and in the rhizosphere of uninoculated plants significant (Adonis test, $P \leq 0.001$), indicating that plants had selectively assembled a distinct rhizosphere microbiome over time (rhizosphere effect). Moreover, we found that the microbial communities in bulk soil and in the rhizosphere of healthy plants differed significantly between the three timepoints (Adonis test, $P<0.05$). Remarkably, differences in microbiome composition between timepoints appear larger in unplanted bulk soils than in rhizosphere samples (fig. 2). This is substantiated by the fact more of the variance (Adonis test, R^2 values) is explained by the factor "time" in unplanted soils ($R^2 \geq 0.49$), than in rhizosphere samples ($R^2 \leq 0.18$) (table 1).

Table 1. The effect size (R^2) of the factor "time" (WPI) on microbial community composition in unplanted bulk soil samples and samples from the rhizosphere of uninoculated plants.

VS	1 WPI	2 WPI	3 WPI
1 WPI		Unplanted Bulk soil 0.49	Unplanted Bulk soil 0.52
2 WPI	Control plants 0.11		Unplanted Bulk soil 0.51
3 WPI	Control plants 0.18	Control plants 0.17	

We then analyzed the microbial communities in the rhizospheres of plants that were inoculated with one of the five different pathogens and compared them to those of the uninoculated plants. One week after the first inoculation, none of the rhizosphere microbial communities of infected plants were significantly different from the uninoculated control plants (P<0.05 in an Adonis test) (fig. 3A, D). Two weeks after inoculation, plants inoculated with *Pc* (P=0.027), *Bc* (P=0.043) and *Pst* (P=0.029) harbored significantly different microbial communities on their roots compared to uninfected control plants (fig. 3B, D). Three weeks after inoculation, *Pst*-infected plants harbored rhizosphere communities that were even more distinct (P=0.001). Plants inoculated with the second bacterial pathogen, *Xc*, had also assembled a significantly altered root microbiome at this point in time (P=0.001). In contrast, plants infected by the oomycete *Pc* or the fungus *Bc* did no longer harbor a rhizosphere community that was statically distinguishable from control plants at 3 WPI (fig. 3C, D). Thus, over time, an increase in the effect of foliar infection on the rhizosphere microbial population was found in the rhizospheres of plants infected by the bacterial pathogens *Pst and Xc* only, while infection with the fungus *Bc* and oomycete *Pc* seemed to exert just a temporary effect that lasted until two weeks after infection. Surprisingly, we did not find a significant difference between the rhizosphere communities of uninoculated control plants and that of and *Hpa*-infected plants in this experiment, which is in contrast to previous findings (Chapter 2 and 4). The rhizosphere microbial communities of *Pst*- and *Xc*-infected plants were significantly different from that of uninoculated control plants but also of *Hpa-, Bc*- and *Pc*-infected plants (Adonis test P<0.05). However, the rhizospheres of *Pst- and Xc*-infected plants did not differ significantly from each other. This suggest that aboveground infection with the distinct bacterial pathogens *Pst* and *Xc* cause a similar shift in microbiome composition in the rhizosphere.

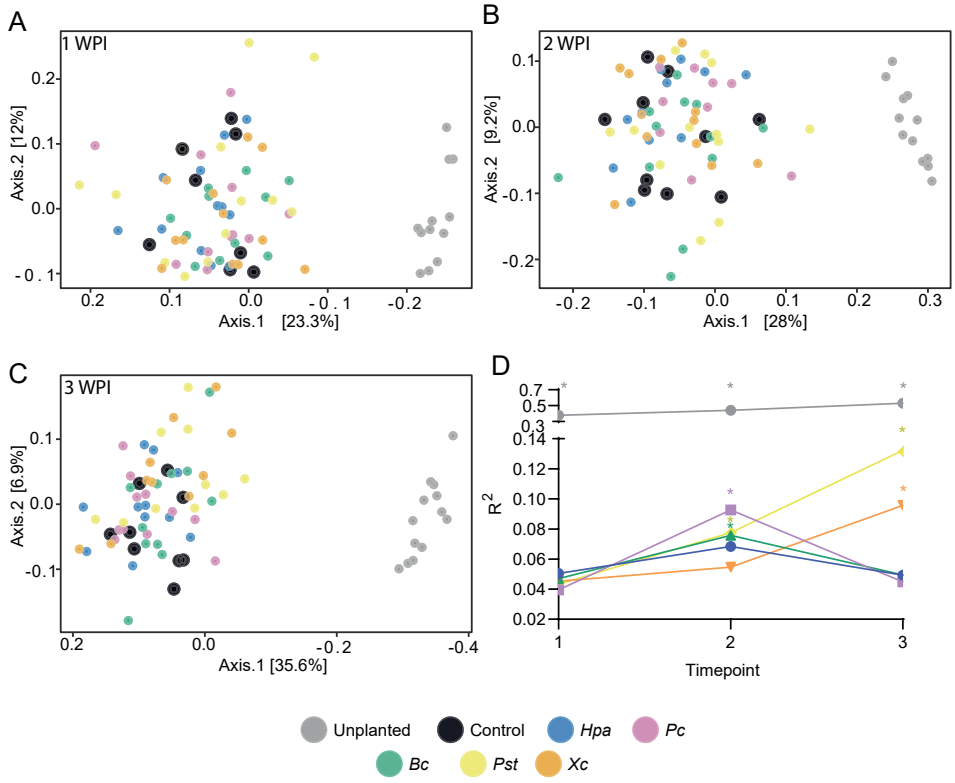

Figure 3. Effect of aboveground pathogen infection on root microbiome composition. Principal coordinate analysis (PCoA) of microbial communities at **A)** 1 WPI, **B)** 2 WPI, or **C)** 3 WPI of unplanted bulk soil (grey), the rhizosphere of uninoculated control plants (black), or the rhizosphere of plants of which the leaves were inoculated with either *Hpa* (blue), *Pc* (purple), *Bc* (green), *Pst* (yellow) or *Xc* (orange). PCoA is based on Bray-Curtis distance matrices. **D)** Adonis effect size (R^2) of each treatment over time. An asterisk represents a significant difference (P<0.05) compared to uninoculated control plants.

Overlap in ASVs responding to infection by distinct pathogens

To enable a detailed comparison of the changes in the root-associated microbial communities induced by the different foliar pathogens, we used the fairly strict Deseq2 method (Love et al 2014) to identify ASVs that are differentially abundant between treatments. In line with the small community level changes describe above, we found no or few ASVs with significantly changed abundance in response to foliar infection by *Hpa, Bc* or *Pc* for each timepoint (fig. S1). However, for *Xc*- (14 ASVs) and especially *Pst*-infected plants (69 ASVs), we found a total of 83 ASVs that had either increased or decreased significantly in rhizosphere abundance compared to uninoculated control plants (fig. 4). Six out of 14 ASVs responding to *Xc* (43%) were also significantly affected by *Pst* infection and always in the same direction (increase or decline) in both treatments. This shows that there are parallels in the belowground microbial response to these two distinct bacterial pathogens.

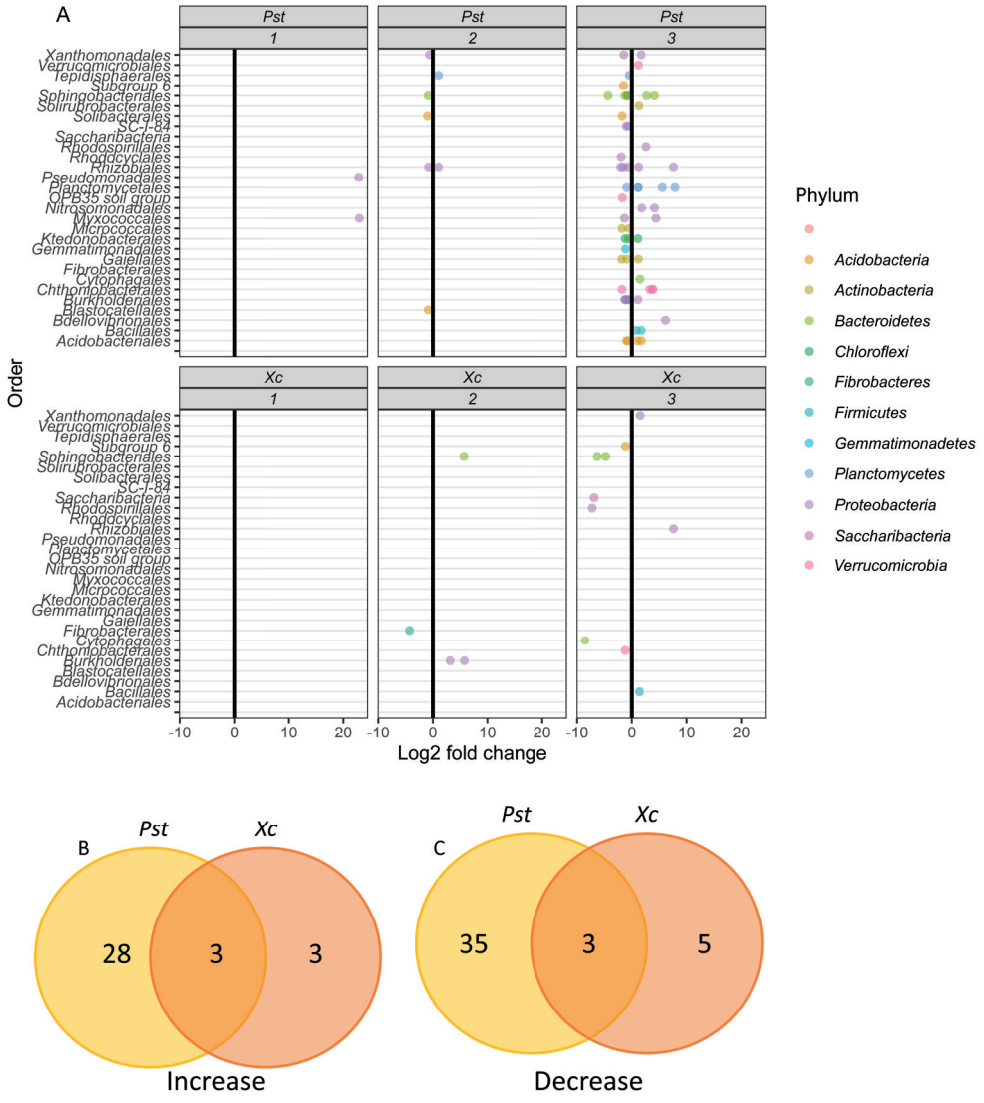

Figure 4. Overlap in ASVs responding in the rhizosphere to foliar *Pst* and *Xc* infection. A) Log2-fold change for all ASVs with significantly different abundance in the rhizosphere ($P < 0.05$ using Deseq2) of *Pst*- and *Xc*-inoculated plants compared to uninoculated control plants. Fold change is shown per timepoint. Each dot represents an individual ASV in a bacterial order (Y-axes). Colors indicate phylum level taxonomy. A positive fold change represents increased abundunce on the roots of inoculated plants vs uninoculated control plants. **B**) Venn diagram expressing the overlap in ASVs with significantly increased abundance on roots of *Pst* and *Xc* infected plants. **C)** Venn diagram expressing the overlap in ASVs with significantly decreased abundance on roots of *Pst*- and *Xc*-infected plants.

Finally, we analyzed how the cumulative relative abundance of the ASVs responding to either *Pst* or *Xc* or responding to infection by both pathogens was affected over all treatments in time (fig. 5). We found that the cumulative relative abundance of the 28 ASVs promoted by *Pst* infection was significantly higher on the roots of *Pst*-infected plants, but also on the root of *Xc*-infected plants 3 WPI (fig 5A). Correspondingly, the cumulative abundance of the ASVs that were reduced in abundance upon foliar *Pst* infection were significantly less abundant in both *Pst*- and *Xc*-infected plants 3 WPI (fig. 5B). Similarly, the cumulative abundance of the ASVs that were reduced in abundance upon foliar infection with *Xc* were also significantly less abundant in both *Pst*- and *Xc*-infected plants 3 WPI. For the three ASVs that, individually, were significantly promoted in response to both *Xc* and *Pst*, logically the cumulative abundance was significantly higher on both *Xc*- and *Pst*-infected plants 3 WPI (fig. 5E). Correspondingly, also the cumulative abundance of the three ASVs that were individually significantly reduced in response to *Xc* and *Pst* infection, was significantly lower in the root samples of all pathogen-inoculated plant treatments 3 WPI (fig. 5F). Together these data suggest that those ASVs that respond to *Xc* and *Pst* infection are similarly promoted or reduced in response to infection by other pathogens, but to a lesser extent and less consistently. In contrast, we found that the cumulative abundance of the 3 ASVs that had significantly increased in response to *Xc* was significantly higher on the roots of *Xc*-infected plants and only 2 WPI (fig 5 left). This suggests that these ASVs are not responsible for the community level changes in response to *Xc* infection 3 WPI and are perhaps coincidentally high at this timepoint.

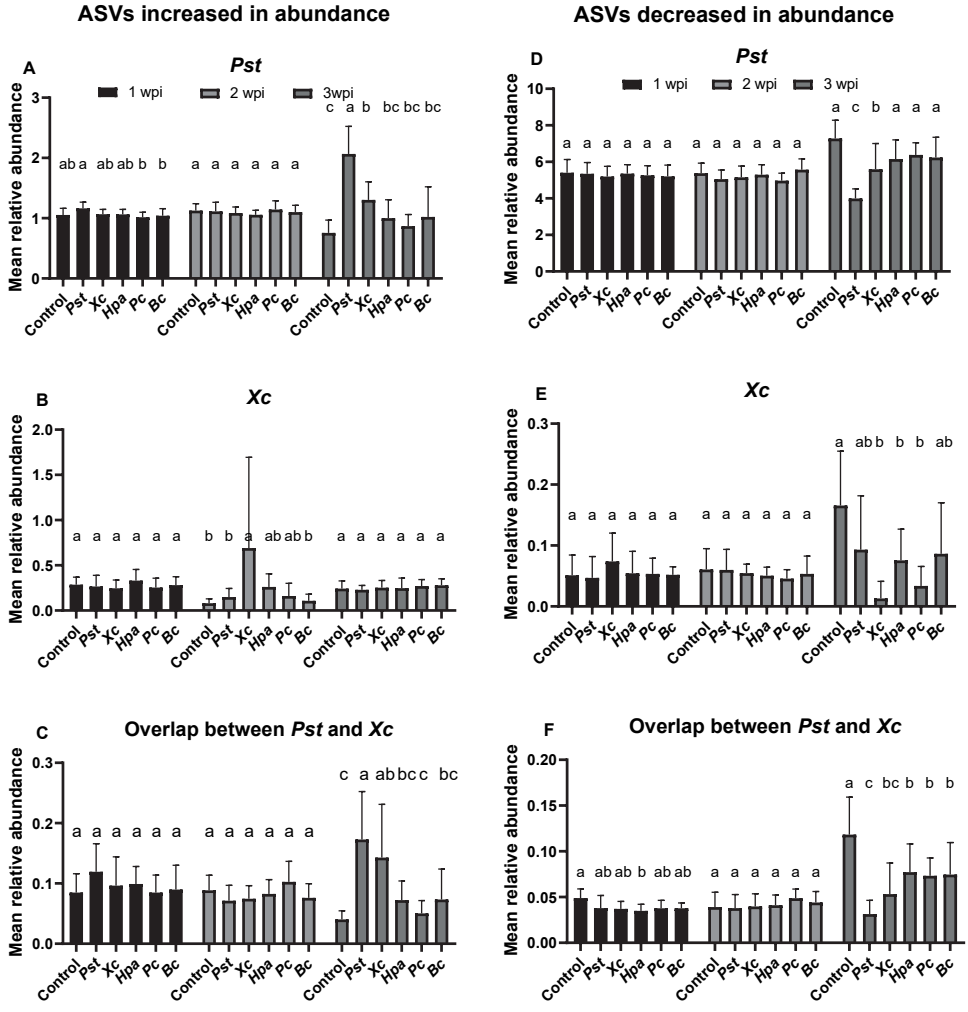

Figure 5. Mean relative abundance of root-associated ASVs responding to aboveground *Pst* and *Xc* infection. Cumulative relative abundance of ASVs that individually significantly (Deseq2, P< 0.05) increase in abundance on roots in response to foliar infection by **A)** *Pst* only (28 ASVs), **B)** *Xc* only (3 ASVs), or **C**) both pathogens (3 ASVs), or that decrease in response to **D)** *Pst* only (35 ASVs), **E)** *Xc* only (5 ASVs), or **F)** both pathogens (3 ASVs). Each bar represents the average abundance of at least 9 replicates. The error bars represent the standard deviation. Different letters represent significances between the treatments within each timepoint (ANOVA with Tukey post-hoc test, *P*<0.05).

Discussion

Aboveground defense activation of plants can induce a plant response that leads to changes in the rhizosphere microbiome (Berendsen et al 2018, Park & Ryu 2021, Yuan et al 2018). These changes can result in a soil-borne legacy of disease that induces resistances in a subsequent population of plants growing in the same soil. Here, we studied how infection of the leaves of Arabidopsis by five distinct foliar pathogens affects the root microbiome. It was shown previously that infection of the leaves of Arabidopsis plants by *Hpa* (Berendsen et al 2018) or *Pst* (Yuan et al 2018) resulted in changes in the root microbiome. In this study, we have shown that in addition to these two pathogens, infection by *Xc*, *Bc* and *Pc* can significantly affect the rhizosphere microbiome. These changes apparently take time to manifest on the roots, as one week after infection, no significant changes of the community as a whole were observed. While the effects of the *Bc* and *Pc* infection were largest at 2 WPI, they were smaller and no longer resulted in a statistically significant differentiation on the community level at 3 WPI. This suggests that although plants were successfully infected by these pathogens, these infections were either halted at some point or the belowground response of the plant were diminished at later timepoints.

Remarkably, we did not find a significant shift in the rhizosphere microbial community of *Hpa*-infected plants, whereas we consistently found this previously (Chapters 2 & 4). Although a small number of ASVs were significantly affected by *Hpa*-infection, the microbiome composition was less affected in this experiment than in previous experiments. The reason for this is not clear, although in a previous experiment (Chapter 2) we found strong upregulation of the *PR-1* defense marker gene, indicative of salicylic acid-dependent responses (Cameron et al 1999). Perhaps specific environmental conditions in the current study led to a defense response to *Hpa* that was divergent from those induced in the previous studies, but such speculations do not explain why *Xc* and *Pst,* which also do not strongly activated *PR-1*, had stronger effects on the rhizosphere microbiome. In the current study, we found that at 1 WPI, *Hpa*-infection most strongly induced the marker genes *PDF1.2* and *VSP2*, indicative of JA-dependent defense responses (Caarls et al 2017). However, the induction of these genes was not statistically significant and unfortunately, we were not able to isolate RNA of sufficient quality 2 and 3 WPI. Our results are therefore inconclusive on the relationship between aboveground defense activation and belowground microbiome modulations.

Regardless, also in this study aboveground infection by pathogens lead to significant changes in the rhizosphere microbiome, which corroborates previous findings (Berendsen et al 2018, Park & Ryu 2021, Yuan et al 2018). *Pst* and *Xc* were the only two foliar pathogens that induced the assembly of rhizosphere microbiomes that were significantly distinct from uninoculated control plants 3 weeks after the first inoculation. Interestingly, although the microbial communities in the rhizospheres of plants infected by *Pst* and *Xct* were distinct from those of uninoculated control plants, the rhizosphere communities of these two pathogen treatments did not

differ significantly from each other. Also, there is overlap in the specific ASVs that responded to these two pathogen treatments. Furthermore, the ASVs that meet the rigorous statistical criteria of Deseq2 to be identified as differentially responsive on the roots to foliar inoculation by the most-impactful pathogen (*Pst*), appeared to be differentially responsive to both bacterial pathogens when studying their combined relative abundance in the rhizosphere. Together the results of this and previous studies (Chapters 2 & 4) suggest that infections by each of the 5 foliar pathogens tested here can result in a statistically significant shift in the microbiome.

Possibly, only those infections that progressively develop over time produce enough continuous signal to effectuate a belowground change in the microbiome. In line with this, we found, here and in Chapter 2, that aboveground infection by *Bc* leads to relatively small changes in the rhizosphere microbiome. One could argue that the necrotrophic lifestyle of this fungus and rapid onset of cell death in the leaves of Arabidopsis following infection result in early termination of the aboveground signal and thus less alterations of the rhizosphere microbiome.

When significant shifts in the rhizosphere microbiome do occur, similar microbes appeared to be promoted and reduced in the rhizosphere in response to distinct foliar pathogens, in this case *Pst* and *Xc*. This suggest that in one soil, different pathogens can cause the creation of similar soil-borne legacies and implies that soil-borne legacies are part of a general response to pathogen attack. It raises the question of whether a soil-borne legacy created in response to one pathogen is effective against another. However, the ASVs that were found to be promoted in response to attack by *Pst* and *Xc* here were not promoted in response to *Hpa* previously (Chapters 2 & 4), even though we used soil from the same field in the Netherlands. If soil-borne legacies are created by infected plants in nature, one would expect that that the microbes involved are taxonomically similar, or at least have a common genetic denominator that enable these microbes to first be recruited by the plant and subsequently enhance the plant's resistance. Understanding what microbial genetics bring about a soil-borne legacy will prove instrumental in comprehending this phenomenon.

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Author contributions

G.V., P.A.H.M.B., and R.L.B. designed experiments; G.V., J.S. and E.V. performed experiments; G.V., P.G., and S.B. analyzed data and G.V., P.A.H.M.B, R.L.B., and C.M.J.P. wrote the manuscript.

Supplemental figure

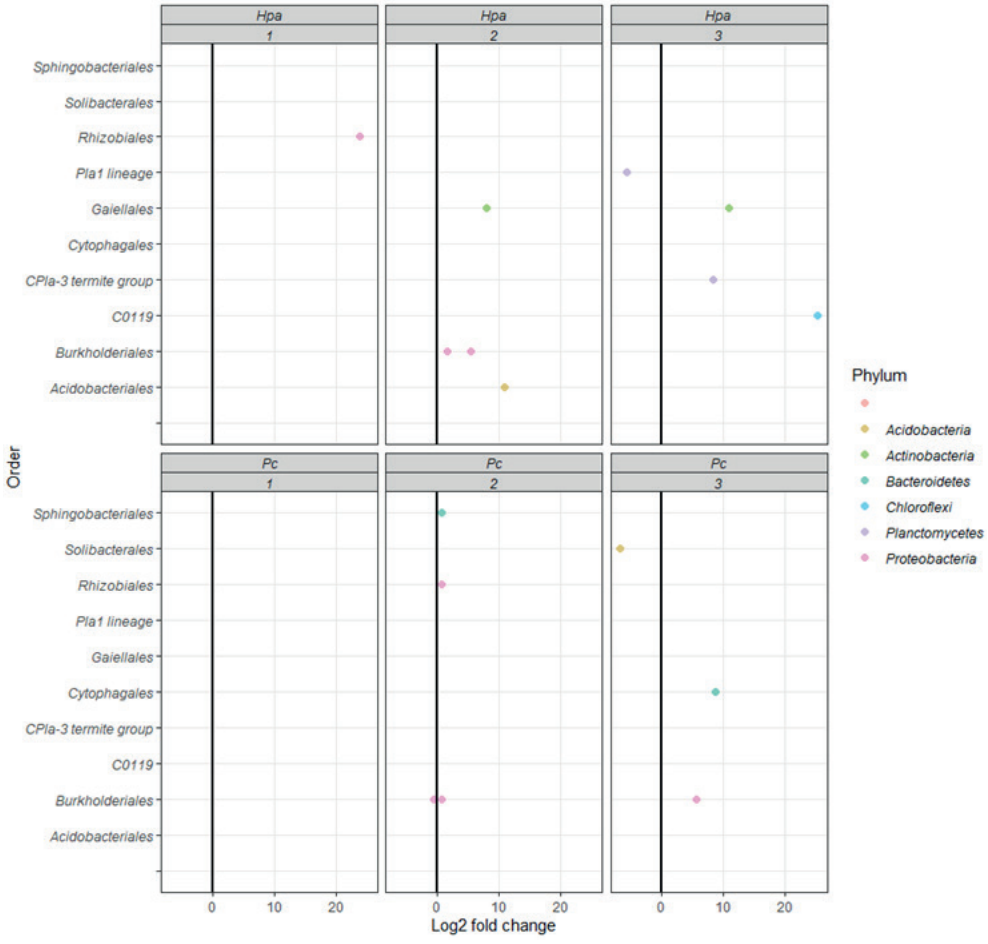

Figure S1) Log2-fold change for all ASVs with significantly different abundance in the rhizosphere ($P < 0.05$ using Deseq2) of *Hpa*- and P*c*-inoculated plants compared to uninoculated control plants. No significant differentially abundant ASVs were detected for *Bc* inoculated plants.

6

CHAPTER 6.

Summarizing discussion

The rhizosphere microbiome and plant performance

There is an urgent need to raise agricultural production to feed the increasing world population. To achieve this, solutions that reduce dependence on agrochemicals and support sustainable agriculture are essential. Thus, studies on plant performance and health have focused on sustainable replacements for pesticides and fertilizers (Berendsen et al 2012, Bulgarelli et al 2013, Raaijmakers & Mazzola 2016, Tkacz & Poole 2015). Modern plant breeding programs aim to increase resistance against diseases and pests and to increase plant performance under abiotic stress conditions (Lee & Tollenaar 2007, Reynolds et al 2011). These efforts not only focus on the visible parts of the plant but also include the plant parts hidden in the soil. The root system of a plant is not only essential for anchoring and for nutrient and water acquisition, but it is also the hotspot for interactions of the plant with the belowground microbiota. A wide range of pathogens, including fungi, viruses, bacteria, and oomycetes are detrimental for plant health and a major threat to agriculture worldwide (Agrios 2005). On the other hand there are plant beneficial microbes that can increase plant growth or suppress plant diseases (Barratt et al 2018, Rouphael & Colla 2020). The fact that soil microbes can have such profound effects on the plant, has made them a valuable target to study. In this thesis, I have focused on the root microbiome in relation to plant health, including effects of disease on root microbiome composition and effects of the microbiome on disease severity. The main research question was if plants under pathogen pressure have an active role in recruiting a specific and protective microbiome and the mechanisms involved in such recruitment.

The effect of the plant on the microbiome

The plant is affected by its microbiome, but vice versa the plant also drives the composition and activities of the microbiome. Bacterial densities in the rhizosphere, the layer of soil directly influenced by the root (Hiltner 1904), is many times higher than in unplanted soil, showing that plants create a favorable environment for microbes to grow. Whereas this so-called rhizosphere effect can vary from plant species to species, it is a general phenomenon (Schneijderberg et al 2020). The main source of microbial diversity is the soil in which plants grow. Soils can differ in many properties, including particle size, structure, organic matter content, nutrient and water availability, and pH, but also in composition and structure of their microbial communities (Köberl et al 2020, Schreiter et al 2014, Seaton et al 2020, Zhao et al 2020). The immense microbial diversity represented by soil is the source from which rhizosphere inhabitants mostly derive, but the plant selectively shapes microbial communities in the rhizosphere (Berg & Smalla 2009). For example, for six different varieties of soybean grown in two soils, both the soil and plant genotype were shown to contribute to microbial community assembly in the rhizosphere (Liu et al 2019). Such effects of genotype on root microbiome structure have also been observed between wild and domesticated species of tomato (Cordovez et al 2021) and common bean (Pérez-Jaramillo et al 2017). In these studies, root microbiomes

were compared between a domesticated variety and a wild relative and found to be different. For lettuce plants, the influence of three different types of soil on the rhizosphere microbiome composition was investigated using a randomized block design. Here operational taxonomic units that (OTUs) in the rhizosphere of lettuce were identified that responded to soil type, showing that the soil is at least partly responsible for the rhizosphere community (Schreiter et al 2014). A large scale ecological study that included different plant species growing in three separate regions in central Europe showed that both plants and soil influence the final rhizosphere microbial community structure (Vieira et al 2020).

Root exudation and recruitment of microbes

Plants actively influence the composition, functioning and activity of their root microbiomes through root exudation of metabolites. By deposition of up to 40% of their photosynthetically-fixed carbon in the narrow zone around their roots, plants create a hospitable environment for microbes in the rhizosphere (Badri & Vivanco 2009). It has been hypothesized that plants can recruit specific microbes to their rhizospheres by adjusting root exudation patterns. Indeed there is evidence that *Arabidopsis thaliana* (Arabidopsis) can shape its microbiome by exuding e.g. coumarins (Stringlis et al 2018), or glucosinolates (Bressan et al 2009). Moreover, maize-root-exuded benzoxazinoids were shown to promote specific *Pseudomonas* species in the rhizosphere (Neal et al 2012). Such specific fine-tuning especially comes into play when a plant encounters biotic or abiotic stress situations (Pascale et al 2020). A well-known example of microbiome shaping under pathogen stress is so-called take-all decline soil. These are soils in which wheat is protected against take-all, a root disease caused by the fungus *Gaeumannomyces tritici*. Take-all decline develops when wheat is grown for multiple years in the same field. Over the years the infection pressure of *G. tritici* increases leading to major disease incidence. After such an outbreak the disease pressure drops and it will stay low if wheat is continued to be grown (Hornby 1998, Weller et al 2002). This drop in disease pressure coincides with increased population densities of 2,4-diacetylphloroglucinol (DAPG) producing fluorescent pseudomonads. The growth of the take-all pathogen is strongly inhibited by DAPG, and thus provides a strong explanation for suppression of disease in take-all decline soils (Raaijmakers & Weller 1998). In this example, it seems that infected plants not only recruit specific microbes, but also a specific function, microbial production of antifungal DAPG, that can protect the plant against the disease. But plants are not only protected against disease by beneficial microbes that can directly inhibit pathogens through antibiosis. Many beneficial rhizosphere microbes can elicit induced systemic resistance (ISR) in plants, a state in which the plant is primed to respond faster and to a greater magnitude upon pathogen attack (Pieterse et al 2014, Van Wees et al 2008). We hypothesized that ISR eliciting rhizosphere microbes are also recruited by roots of plants that are under pathogen attack. To decipher the molecular communication involved in such recruitment, pathogen infection of aboveground plant parts would circumvent complicating direct effects

of the pathogen. In the example of take-all decline, the pathogen may have directly driven recruitment of DAPG-producing pseudomonads making it difficult to assess the role of root exudates. In this thesis, we try to understand the influence of the plant on its microbiome and of the microbiome on the plant in the context of pathogen pressure and plant defense. Thus, we used aboveground infection with leaf pathogens in the model plant Arabidopsis for which an extended molecular toolbox is available.

Microbiome modulation and the involvement of ISR

In Chapter two of this thesis, we studied effects of infection of Arabidopsis with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) on the rhizosphere microbiome. The pathogen infection resulted in recruitment of a consortium of three bacterial strains. A *Stenotrophomonas* sp.*, Xanthomonas* sp.*,* and *Microbacterium* sp. increased in abundance in the rhizosphere of *Hpa*-infected Arabidopsis and they were isolated on culture media. These bacteria were inoculated in soil and, only when applied as a consortium, reduced disease severity in *Hpa*-infected Arabidopsis plants. The spatial separation between *Hpa,* inoculated on the leaves, and the microbial consortium that was applied to the roots suggests that ISR is involved in the protection against disease. Moreover, a population of Arabidopsis seedlings grown on soils that were conditioned with *Hpa*-infected Arabidopsis developed less symptoms after *Hpa* inoculation compared to plants grown on soils in which healthy Arabidopsis was previously grown. This protection of a new generation of plants was dubbed "the soil-borne legacy" (SBL). In Chapter 4, we provide evidence that protection against disease by the SBL is based on induced resistance. Mutants of Arabidopsis defective in salicylic acid (SA) signaling were not responsive to the soil-borne legacy, indicating that the protective effect of the conditioned soil is mediated by the plant. Moreover, we detected primed SA-induced expression of the defense marker gene *PR1* in plants grown in soil that was conditioned with *Hpa*-infected Arabidopsis. Interestingly, the *myb72* mutant of Arabidopsis was fully responsive to the soil-borne legacy, whereas the root specific transcription factor MYB72 is one of the key players in ISR in Arabidopsis. It was shown that upon ISR induction, the transcription of *MYB72* is upregulated. MYB72 is also required for ISR, as *myb72* mutants are unable to induce systemic resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) when beneficial microbes are applied to the roots (Van der Ent et al 2008). Upon colonization of the roots by beneficial bacteria, *MYB72* is activated and the whole plant develops a primed state, preventing severe symptoms from a subsequent pathogen attack (Pieterse et al 2020, Zamioudis et al 2015). This MYB72-dependent ISR requires both jasmonic acid (JA) and ethylene (ET) as the JA mutant *jar1* (Pieterse et al 1998) and the ET mutants *eir1* and *ein2* (Knoester et al 1999) do not exhibit ISR when the beneficial bacterium WCS417r is applied to the soil. Thus, the protective effect of the soil-borne legacy relies on the induction of systemic resistance that requires SA but is independent of MYB72. ISR that relies on SA is not unprecedented, for example ISR elicited by the beneficial rhizobacterial strain

Pseudomonas fluorescens SS101 is dependent on SA signaling. This strain induces systemic resistance in Arabidopsis against *Pst* but does not in the SA-signaling-impaired mutant *npr1* and transgenic *nahG* lines (van de Mortel et al 2012). The systemic resistance induced by the SBL seems to be more similar to the resistance induced by SS101 than to that induced by WCS417r. To summarize, the systemic resistance induced by the SBL is dependent on functional SA signaling in the plant, but is independent of the transcription factor MYB72.

Coumarins are involved in the creation of the soil-borne legacy

Whereas our results suggest that MYB72 is not needed for perception of the soil-borne legacy, this transcription factor is important for several rhizosphere related processes, including modulation of the rhizosphere microbiome (Stringlis et al 2018). The *MYB72* gene was first discovered to be upregulated by root colonization of WCS417r in a transcriptomics study in Arabidopsis (Verhagen et al 2004). As previously mentioned MYB72 is required for the onset of ISR by beneficial *Pseudomonads* (Van der Ent et al 2008), but *MYB72* is also activated during the iron deficiency response (reviewed by Verbon et al 2017). This role for MYB72 in iron acquisition was first discovered using a DNA micro-array to study the iron deficiency response in Arabidopsis (Buckhout et al 2009). Later it was shown that in alkaline soils, where iron is scarcely available, MYB72 and its homolog MYB10 are essential for the uptake of iron and survival of Arabidopsis (Palmer et al 2013). It is now clear that iron limiting conditions in the soil result in activation of *myb72* and the coumarin biosynthesis gene *F6'H1*, which are important for the increase of the production of phenolic compounds called coumarins in the plant. MYB72 is required as a regulator of coumarin production, while F6'H1 works downstream of MYB72 in coumarin biosynthesis (Schmid et al 2014). BGLU42, essential for both ISR and iron uptake, was shown to also work downstream of MYB72 (Zamioudis et al 2014). The β-glucosidase BGLU42 enables these phenolic compounds to be secreted by the root into the rhizosphere (Stringlis et al 2018). Here the coumarins have a double role. For iron acquisition the activation of both *myb72* and *F6'H1* results in an increase of production of the coumarins in the root. After glycosylation by BGLU42 these coumarins are exuded into the rhizosphere, which is mediated by the transporter PDR9. In the rhizosphere the coumarins increase iron availability, as they increase solubility of Fe^{3+} which subsequently can be converted into Fe^{2+} by Ferric reduction oxidase 2 (FRO2) after which it can be taken up into the root by the IRT1 transporter (Verbon et al 2017). The second role is the creation of a niche for beneficial microbes. The antimicrobial activity of coumarins reduces bacterial growth. However, the beneficial bacterium WCS417r is tolerant to coumarins (Stringlis et al 2018). Thus, by exuding coumarins, plants seem to create a niche for coumarin-tolerant beneficial rhizobacteria that induce the MYB72-mediated production of these coumarins.

To study the involvement of coumarins in the creation of the soil-borne legacy, *myb72* and *f6'h1* mutants were included in the conditioning phase of SBL

experiments. Whereas *Hpa*-inoculated wild-type Arabidopsis plants did create a soil-borne legacy, the *myb72* and *f6'h1* mutants consistently did not, suggesting a key role for coumarins in the soil-borne legacy. In these experiments, we included unplanted soil in the conditioning phase to study direct effects of the *Hpa* inoculum. Compared to mock inoculation, spraying soil with the *Hpa* inoculum did not lead to reduced downy mildew severity in the interrogation population. Thus, infected plants are required to create the soil-borne legacy. We also studied the impact of *Hpa* inoculation on bacterial microbiomes of unplanted soil and the rhizospheres of wild type and *myb72* and *f6'h1* mutant plants. In the unplanted soil, *Hpa* inoculation did not affect the bacterial microbiome. The coumarins are a class of antimicrobial phenolic compounds produced by plants (Smyth et al 2009, Yasunaka et al 2005). Recently they have been studied for their communication with the microbiome (Stringlis et al 2019, Stringlis et al 2018, Voges et al 2018). These coumarins are important in shaping the microbiome for increased defense (Lundberg & Teixeira 2018). A current hypothesis for plant protection is that of a positive feedback-loop where the colonization of roots by beneficial bacteria increases the expression of MYB72. This in turn results in increased coumarin biosynthesis and exudation. In the rhizosphere, the beneficial microbes are more resistant to the coumarins, giving them a better opportunity to colonize the roots, which in turn leads to increased *MYB72* expression and so on (Lundberg & Teixeira 2018). Root exudates of Arabidopsis mutants unable to produce coumarins (*f6'h1*) have differential effects on the transcriptome of the beneficial bacteria WCS417r. After exposing WCS417r to root exudates from wild-type or mutant *f6'h1* Arabidopsis, genes related to motility and mobility were suppressed in WCS417r exposed to wild-type exudates, compared to WCS417r exposed to the coumarin mutant (Yu et al 2021). However, differential effects of *Hpa* inoculation on the microbiomes of the wild type and mutant plants were apparent. While the rhizosphere microbiome of the *Hpa*-infected wild type was enriched in *Xanthomonadales* spp., *Cytophagales* spp. and *Fibrobacteriales* spp compared to healthy plants, this was not the case in the rhizospheres of either of the mutants. Thus, the creation of the soil-borne legacy requires *Hpa*-infected plants and coumarins seem to play a pivotal role in assembly of the bacterial rhizosphere microbiome after *Hpa* infection.

The effect of coumarins on the rhizosphere composition is shown by Stringlis and colleagues (2018). Here Arabidopsis plants with different scopoletin exudation patterns under iron limiting conditions resulted in differential abundance of several bacterial and fungal genera in the rhizospheres of Col-0 and *f6'h1* Arabidopsis. Moreover, iron-starved Col-0, which exudes more coumarins than Arabidopsis grown on iron-sufficient medium accommodated higher densities of the beneficial bacterium WCS417r on the roots. This is in contrast to the soil-borne pathogens *Fusarium oxysporum* and *Verticillium dahliae*, which were severely inhibited by scopoletin (Stringlis et al 2018). An effect of coumarins on the functioning of the WCS417r has been shown using a gene expression analysis of WCS417r grown in root exudates of iron starved Col-0 and *f6'h1* Arabidopsis (Yu et al 2021). The iron starvation resulted in differential coumarin exudation between the two conditions and showed that F6'H1-dependent coumarins resulted in changes in

expression of 8% of the total bacterial genome of WCS417r. A large number of genes related to carbohydrate metabolism, amino acid metabolism and nucleotide metabolism were upregulated in a F6'H1 dependent manner, while genes pertaining to motility were downregulated (Yu et al 2021). These results together suggest that coumarins are not only able to shape the microbiome but are also capable to influence the microbiome functioning. The interplay between MYB72, coumarins and beneficial bacteria is not random evolution. In an elaborate study, using several generations of Arabidopsis and the rhizobacterium *Pseudomonas protegens* CHA0 (CHA0), the evolution of this initially detrimental bacterium was studied (Li et al 2021). A gnotobiotic setup was used to grow Arabidopsis in the presence of CHAO. After each of four four-week cycles (16 weeks in total), part of the bacterial populations that developed on the roots were isolated, and a part was transferred to the rhizosphere of new sterile plants. This enabled the researchers to study the evolution of CHAO on the roots of Arabidopsis over time. For several of the evolved and isolated CHA0 bacteria the initial negative effect of CHA0 on plant growth shifted to a mutualistic interaction. The bacteria that showed this mutualistic phenotype exhibited a higher tolerance to scopoletin and a stronger induction of *MYB72* in the plant compared to bacteria that did not show a mutualistic phenotype (Li et al 2021). This shows that even on an evolutionary scale Arabidopsis changes the composition, functioning and beneficial interactions of and with the microbes surrounding its roots.

Many questions regarding the soil-borne legacy remain to be answered, however. First, it is not certain that coumarin exudation is altered in the Arabidopsis rhizosphere in response to *Hpa* infection. The rhizosphere microbiomes of healthy wild-type and coumarin mutants differ also in mock-treated plants in our experiments, which suggests a role for coumarins in maintaining a healthy microbiome under normal conditions. Differences in the starting microbiome also mean that the differences in microbiomes between *Hpa*-infected wildtype and mutants can be due to different starting pools of microbes to select from. This in turn can result in a lack of beneficial functions to select from in the microbiome and no SBL for subsequent generations. Another hypothesis for the lack of SBL in coumarin-deficient plants, is the lack of the positive feedback loop. It can well be imagined that all required microbes and functions are present in the microbiome of all plants but cannot reach the required abundance to induce the SBL without a positive feedback-loop.

Although disease-promoted microbes could protect plants from disease (Chapter 2), a direct effect of coumarins on the plants that perceive SBL has not been irrefutably ruled out and such coumarins might directly affect plant immunity. To untangle direct induction of ISR by coumarins from indirect induction through microbiome modulation, the microbes and the exudates need to be separated. By eliminating microbes from the soil and using a gnotobiotic system, direct effects of coumarins could be tested. The direct application of coumarins in a more in vitro setup could also be used to test the direct effects.

So far, we have considered the rhizosphere and plant root as a homogenous environment, with microbes and exudates evenly distributed over the root system. This is however a poor reflection of reality. Different zones of the root have different exudation rates and exudate composition (Baetz & Martinoia 2014, Weidenhamer et al 2014). In this thesis, we have focused mainly on the role of the coumarins in the shaping of the microbiome, but these are not the only compounds that influence the microbiome under pathogen pressure. Malic acid (Rudrappa et al 2008) and SA (Lebeis et al 2015) have been shown to influence the occurrence of beneficial microbes in the rhizosphere. The inoculation of the leaves of Arabidopsis with *Pst* resulted in an increase of the density of the beneficial rhizobacterium *Bacillus subtilis* on the roots. The same inoculation with *Pst* resulted in an increase of malic acid secretion by the roots, to which *B. subtilis* shows positive chemotaxis (Rudrappa et al 2008). By studying the rhizosphere of wildtype Arabidopsis and a multitude of defense hormone mutants, it emerged that SA was essential to establish a normal root microbiome (Lebeis et al 2015).

Multiple pathogens and their effect on the microbiome composition

The SBL may be a wide-spread phenomenon that can be incited by a range of pathogens. In Chapter 2 we compared the impact of *Hpa* and *Botrytis cinerea* (*Bc*) leaf inoculation on the bacterial rhizosphere microbiome of Arabidopsis. Whereas the biotrophic oomycete pathogen had a significant effect on microbiome composition, the necrotrophic fungus *Bc* did not. Infection of Arabidopsis leaves with the bacterial pathogen *Pst* significantly affected root exudates and microbiome, resulting in a SBL (Yuan et al 2018). In Chapter 5, we investigated effects of a range of Arabidopsis leaf pathogens in an experimental setup, similar to that used in Chapter 2. These pathogens included the oomycetes *Hpa*, an obligate biotrophic pathogen, and *Phytophthora capsici* (*Pc*), a hemi-biotrophic pathogen. Two hemi-biotrophic bacterial pathogens, *Pst* and *Xanthomonas campestris* (*Xc*) and the necrotrophic fungal pathogen *Bc*. In these experiments, persistent changes in rhizosphere microbiomes were only observed for plants inoculated with either *Pst* or *Xc*. Also, for the fungus *Bc* and the oomycete *Pc* leaf inoculation resulted in shifts in the root microbiome, but these did not persist and were exclusively found at two weeks post initial infection. In contrast to the results of experiments described in Chapters 2 and 4, *Hpa* inoculation did not significantly affect the rhizosphere microbiome in the comparative experiments described in Chapter 5. For the bacteria *Pst* and *Xc,* the observed shifts in rhizosphere microbiome composition were similar, suggesting that infection by these pathogens can provoke similar responses in Arabidopsis. Whether in experiments with any of the pathogens used in Chapter 5 a soil-borne legacy was generated remains to be investigated.

Concluding remarks

The soil-borne legacy that is provoked by *Hpa* infection of Arabidopsis is a robust phenomenon, but it is not robustly accompanied by specific shifts in rhizosphere microbial communities. In the present study we did not include the impact of infected plants on functions of the root microbiome. With the use of metagenomics, entire bacterial genomes can be sequenced and identified from rhizosphere samples. With this information the entire suite of possible functions becomes more clear. Even more in depth is the use of meta-transcriptomics, whereby sequencing the transcriptome of the microbiome, the currently active processes in the microbiome could be elucidated. With this information, a clearer picture of the members present in the microbiome, as well as what is happening will develop. Changes in activity, more so than composition in the microbiome would be in line with the findings of Carrión et al (2019), where upon infection specific functions in the rhizosphere increase, and was more apparent than the shift in abundance of specific bacteria.

Another aspect that has not been considered is the migration of microbes from the soil to the shoots and vice versa. We have found that infection with foliar pathogens can change the root microbiome of Arabidopsis and that this can lead to an increase in plant resistance. We did not detect the pathogens in the rhizosphere microbiome and identified Arabidopsis mutants impaired in the creation of SBL, demonstrating that the creation of SBL is mediated by the plant. Vice versa, however, a direct effect soil-borne microbes on the pathogens has not been ruled out as we have not tested if we could find traces of the rhizosphere microbiome back on the leaves. As such, there is a possibility of direct inhibition of the pathogen by the microbiome as part of the protection of the plant. Inoculating the leaves with soil microbes that increase in abundance as a response to pathogen infection of the plant, could give an indication of the possibility of these direct effects. This aspect has been described in relation to coumarins by Stassen and colleagues (2021).

In this thesis, we have shown a proof of concept of microbiome modulation as a response to foliar infection using Arabidopsis. We have shown a multitude of changes in the rhizosphere microbiome upon foliar infection, as well as the induction of systemic resistance by this microbiome, under the influence of coumarin exudation of the plant. These mechanisms of the SBL could be exploited to protect crops in a durable way. First of all, the application of microbes found to be beneficial against a pathogen could be applied either on the seed, in the soil or maybe even topical on the leaves, roots or seeds. One benefit of the microbes described in this thesis is that they work as a consortium. As such, they could be better suited to changing conditions and be more adaptable to a new environment. Not only the microbes themselves could be used in agriculture, but their signal molecules could also prove valuable as well. Direct application of selected microbial compounds could still be an option to either directly protect the plant or induce beneficial changes in the microbiome. A breeding program, specifically

targeted towards optimizing the ability of plants to create SBL's could prove useful in creating crops with a disease resistance inducing microbiome. In general, the breeding for crops with more or better interactions with their root microbiome could be a niche with great potential for yield or (a)biotic stress resistance. To conclude, we have lifted a tip of the veil of the incredibly complex system that is plant microbiome-interactions under pathogen pressure, but by doing so created more questions left to be answered.

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Samenvatting

Op het eerste gezicht zijn planten onafhankelijke organismen die gefixeerd zijn in de bodem en leven door zelfstandig water en nutriënten uit die bodem op te nemen en door zonlicht te benutten via fotosynthese. Onzichtbaar voor het blote oog gebeurt er echter veel meer en zijn planten minder individualistisch dan wordt aangenomen. De plant is gekoloniseerd door miljarden micro-organismen die op, in en rondom zowel de boven- als ondergrondse plantendelen leven. Deze met-de-plant-geassocieerde microbiële gemeenschappen worden aangeduid als het microbioom van de plant. Met name het microbioom van de plantenwortels is van groot belang voor het functioneren van planten. Zo groot zelfs, dat een aanzienlijk deel van de fotosyntheseproducten door de wortels in de bodem worden uitgescheiden om dit wortelmicrobioom te voeden en te cultiveren. Door het uitscheiden van specifieke verbindingen heeft een plant invloed op de samenstelling van het wortelmicrobioom. Hierdoor kunnen specifieke microben worden onderdrukt of gestimuleerd, waardoor de plant het microbioom kan aanpassen ten begunstiging van de eigen groei. In ruil voor de voeding vanuit de plant, helpt het microbioom de plant onder andere met de opname van nutriënten, het verbeteren van de wortelarchitectuur en het stimuleren van het immuunsysteem. Wanneer specifieke microben de plant koloniseren, versterken zij het immuunsysteem van de plant en vergroten zij de resistentie van de plant tegen ziekteverwekkende organismen. Dit stimuleren van het immuunsysteem wordt ook wel geïnduceerde systemische resistentie (*induced systemic resistance*; *ISR*) genoemd. Een plant in staat van *ISR* kan na infectie door een pathogeen het immuunsysteem sneller en heviger activeren, waardoor het pathogeen onderdrukt wordt. Activatie van het afweersysteem heeft duidelijk effect op de groei van ziekteverwekkende organismen, maar ook commensalen worden hierdoor beïnvloed. In deze thesis is onderzocht welk effect pathogeeninfecties van bladeren op het wortelmicrobioom van de plant hebben. Hierbij is een belangrijk deel van het onderzoek gefocust op het belang van signaalmoleculen van de plant in de vorming van een voor de plant gunstig wortelmicrobioom.

In dit proefschrift is er verder gebouwd op kennis over zogenaamde ziektewerende gronden. Alhoewel planten in de meeste gronden vatbaar zijn voor ziekten, zijn er in de literatuur veelvuldig gronden beschreven, waarin planten niet meer vatbaar zijn voor bodempathogenen. Deze ziektewerendheid wordt veroorzaakt door de verhoogde aanwezigheid van specifieke microben die de plant beschermen tegen het pathogeen. Deze gronden ontstaan vaak pas na een hevige uitbraak van een specifieke ziekte waarna een volgende teelt van hetzelfde gewas in hetzelfde veld niet of nauwelijks vatbaar is voor de ziekte. Die waarneming leidde tot de veronderstelling dat planten, wanneer ze worden aangevallen door een pathogeen, beschermende bacteriën kunnen stimuleren die hen helpen zich te verdedigen.

In **hoofdstuk 2** is daarom onderzocht of het inoculeren van de bladeren van de model plant *Arabidopsis thaliana* (hierna: Arabidopsis) met het biotrofe pathogeen *Hyaloperonospora arabidopsidis* (*Hpa*) of het necrotrofe pathogeen *Botrytis cinerea* (*Bc*) een effect heeft op de samenstelling van het wortelmicrobioom. Met name op de wortel van door *Hpa* geïnfecteerde planten werd waargenomen dat drie zogenaamde operationele taxonomische eenheden (*operational taxonomic units; OTUs*) significant toenamen. Deze *OTUs* werden geïdentificeerd als respectievelijke een *Xanthomonas* sp., een *Stenotrophomonas* sp. en een *Microbacterium* sp.. Deze bacteriën werden geïsoleerd en hun effect op plantengroei en infectie door *Hpa* werd onderzocht. Behandeling van grond met het consortium van alle drie de bacteriestammen resulteerde in significante toename van plantengroei en bescherming van Arabidopsis tegen *Hpa*. Dit suggereerde dat Arabidopsis planten die door Hpa worden aangevallen inderdaad een consortium van beschermende microben konden stimuleren in respons op die aanval. Aangezien het rekruteren van een beschermend wortelmicrobioom door ons niet in staat werd geacht reeds zieke planten te genezen, veronderstelden wij dat dit microbioom een rol had in de bescherming van een volgende generatie planten in dezelfde bodem. Deze hypothese werd getoetst door grond te conditioneren met gezonde of *Hpa*-geïnfecteerde Arabidopsis planten, en, na verwijdering van de eerste generatie planten, de vatbaarheid voor *Hpa* van een nieuwe generatie planten op deze grond te bepalen. In deze experimenten bleek dat Arabidopsis planten significant minder ziek werden op door zieke planten geconditioneerde grond in vergelijking met planten op grond waarin eerder gezonde Arabidopsis had gegroeid. Dit fenomeen is de bodemgebonden erfenis (*soil-borne legacy; SBL*) genoemd.

In **hoofdstuk 3** wordt de methode om de *SBL* te bestuderen in detail beschreven. Voor het bestuderen van de *SBL* is gebruik gemaakt van een zanderige bodem, welke per pot, werd bezaaid met ongeveer 25 Arabidopsis zaadjes. Deze zaadjes zijn eerder gesuspendeerd in 0.1% agar oplossing, zodat ze door middel van een pipet evenredig verdeeld konden worden over iedere pot. Om groei van algen en mos tegen te gaan, is een bodembeschermer gebruikt. Deze bodembeschermer is een ronde plastic schijf, met ongeveer 25 gaten evenredig verdeeld over het oppervlak. In elk van deze gaten werd ten minste één Arabidopsis zaadje geplaatst, zodat er een homogene verdeling van Arabidopsis zaailingen op elke pot ontstond. Om de experimentele duur te beperken werd er met zaailingen gewerkt, in plaats van volwassen Arabidopsis. Na twee weken werd de conditionerende populatie geïnoculeerd met *Hpa*, om de *SBL* te induceren. Vervolgens werd deze populatie vervangen door een waarnemende populatie om te kijken of de *SBL* echt was geïnduceerd. Om dit te bewerkstelligen, werd de conditionerende populatie net boven de grond afgesneden en werd de waarnemende populatie gezaaid als eerder genoemd. Vervolgens werd de waarnemende populatie behandeld om een week later te bestuderen of de *SBL* is opgetreden. Dit werd gedaan door de infectie druk van *Hpa* te meten door het aantal sporen op de waarnemende populatie te tellen.

Vervolgens wordt in **hoofdstuk 4** dieper ingegaan op mechanismen achter de totstandkoming van een *SBL* door de conditionerende populatie planten, als ook

de perceptie van *SBL* door de waarnemende populatie. In dit hoofdstuk wordt opnieuw aangetoond dat een infectie door *Hpa* in een conditionerende populatie planten consistent leidt tot verhoogde resistentie van een daaropvolgende populatie planten. Vervolgens toetsten wij de hypothese dat de verhoogde resistentie veroorzaakt door een *SBL* het gevolg is van ISR. Daartoe is gebruik gemaakt van Arabidopsis mutanten die verstoord zijn in de expressie van geïnduceerde resistentie. Enerzijds is daarbij gebruik gemaakt van de *myb72* mutant die verstoord is in expressie van *ISR* zoals dat geïnduceerd wordt door de model bacterie *Pseudomonas simiae* WCS417r, anderzijds van de mutanten *npr1* en *sid2* die verstoord zijn in expressie van salicylzuur- afhankelijke geïnduceerde resistentie. Uit deze experimenten bleek dat de salicylzuur-gecompromitteerde mutanten *npr1* en *sid2* niet beschermd zijn tegen een *Hpa* infectie wanneer ze groeien op grond geconditioneerd door *Hpa*-geïnfecteerde Arabidopsis, terwijl de *myb72* mutant nog wel beschermd is. Daarnaast brachten Arabidopsis planten op grond voorbehandeld met *Hpa*-geïnfecteerde planten significant meer transcripten van het afweermerker-gen *PR-1* tot expressie in reactie op behandeling met een salicylzuur oplossing, dan planten groeiend op grond geconditioneerd door niet-geïnfecteerde planten. Samen wijzen deze resultaten er op dat *SBL* inderdaad leidt tot verhoogde resistentie van de *SBL* waarnemende planten en dat salicylzuur een rol speelt bij de moleculaire totstandkoming van die geïnduceerde resistentie in de plant.

Het feit dat MYB72 geen essentiële rol heeft in de waarneming van de *SBL*, sluit niet uit dat MYB72 een rol speelt bij de creatie van de *SBL*. Eerder onderzoek had namelijk aangetoond dat deze transcriptiefactor ook de biosynthese van coumarines reguleert en dat deze metabolieten, door hun selectieve antimicrobiële werking, de samenstelling van het Arabidopsis wortelmicrobioom kunnen beïnvloeden. In daartoe uitgevoerde experimenten, waarin gebruik werd gemaakt van de coumarine-deficiënte mutanten *myb72* en *f6'h1*, werd aangetoond dat coumarines een belangrijke rol spelen bij de inductie van de *SBL*. Het conditioneren van grond met de door *Hpa* geïnfecteerde mutanten resulteerde niet in verhoogde *Hpa*-resistentie van een waarnemende generatie wildtype Arabidopsis.

Bovendien was aan het eind van de conditioneringsperiode een verschuiving zichtbaar in de samenstelling van het wortelmicrobioom van *Hpa*-geïnoculeerde wild type Arabidopsis ten opzichte van niet *Hpa*-geïnoculeerde Arabidopsis. Deze verschuivingen werden niet waargenomen in het wortelmicrobioom van beide mutanten. Dit alles duidt erop dat de door *MYB72* gereguleerde coumarines essentieel zijn voor het induceren van de *SBL*.

Door de samenstelling van het microbioom vervolgens nauwkeurig te analyseren door middel 16S sequencing werd bepaald welke bacteriën verhoogd aanwezig waren in grond met een *SBL* en dus mogelijk verantwoordelijk voor de inductie van resistentie. Daartoe onderzochten wij het microbioom op de wortels van al dan niet geïnfecteerde planten aan het einde van de conditioneringsperiode in twee onafhankelijke experimenten waarin een *SBL* gevonden werd. Alhoewel

niet exact dezelfde bacteriën duidelijk toenamen in beide experimenten, en we bovendien in beide experimenten ook de drie in hoofdstuk 2 geïdentificeerde soorten niet zagen toenemen, vonden we dat de populatiedichtheid van bacteriën behorend tot ordes *Xanthomonadales* en *Fibrobacterales* significant toenamen in beide experimenten. Ook de in hoodstuk 2 geidentificeerde *Xanthomonas* sp. and *Stenotrophomonas* sp. behoren tot de *Xanthomonadales*. Dit suggereert dat deze verwante bacteriesoorten genetische eigenschappen delen, waardoor ze door de plant consistent geselecteerd worden in respons op infectie door *Hpa*. Bovendien zouden zij verantwoordelijk kunnen zijn voor de verhoogde resistentie van *SBL* waarnemende planten.

In **hoofdstuk 5** is onderzocht of en hoe het wortelmicrobioom van Arabidopsis veranderd na inoculatie met verschillende pathogenen. Daartoe zijn vijf weken oude Arabidopsis planten geïnoculeerd met een van de volgende pathogene microorganismen; de schimmel *Botrytis cinerea* (*Bc*), de oomyceten *Hpa* en *Phytophthora capsici* (*Pc*), en de bacteriën *Pst* en *Xanthomonas campestris* (*Xc*). Vervolgens is na 1, 2 en 3 weken het wortelmicrobioom van de geïnfecteerde planten geanalyseerd. Infectie door alle pathogenen resulteerde in significante veranderingen van het wortelmicrobioom met uitzondering van *Hpa*. Dit laatste is onverwacht gezien de resultaten van de eerdere hoofdstukken. Dit uitblijven van veranderingen in het wortelmicrobioom van *Hpa* geïnoculeerde Arabidopsis kan mogelijk worden verklaard doordat de infectiedruk en daaraan gerelateerde afweerrespons, niet sterk genoeg was om verschuivingen in het wortelmicrobioom teweeg te brengen De veranderingen als gevolg van infectie met *Bc* en *Pc* zijn klein, van korte duur en specifiek voor het pathogeen. Voor *Bc* kan dit te maken hebben met de levensstijl. *Bc* is necrotroof en leeft van dood celmateriaal. Om dit te bewerkstelligen zorgt *Bc* er voor dat de geïnfecteerde delen van de plant afsterven. Een grote infectie kan er voor zorgen dat de plant volledig afsterft voordat er zaden gevormd worden en er dus geen volgende generatie op dezelfde grond zal groeien. Infectie met *Pst* en *Xc* resulteerde in deels overlappende en meer persistente verschuivingen in de samenstelling van het microbioom van de wortel.

In **hoofdstuk 6** worden de resultaten van de experimentele hoofdstukken besproken in de context van bestaande kennis. Het gebruik van voor de mens en milieu schadelijke chemicaliën bij de plantaardige productie in land en tuinbouw moet sterk worden verminderd om op een duurzame manier de wereld te blijven voeden. De resultaten beschreven in dit proefschrift kunnen aan die doelstelling een bijdrage leveren. Wij hebben aangetoond dat infectie met pathogenen in Arabidopsis kan leiden tot veranderingen in het wortelmicrobioom en in het geval van *Hpa* dat dit resulteert in *SBL*. Ook hebben wij aan weten te tonen dat coumarines cruciaal zijn in de tot stand komen van deze *SBL*. Vanuit deze resultaten zijn er verschillende paden te bewandelen naar een toepassing om het gebruik van agrochemicaliën terug te dringen. Ten eerste zijn er bacteriële genera beschreven die de afweer van Arabidopsis tegen pathogenen bevorderen. Dit is in eerder werk ook al aangetoond voor individuele isolaten, maar het feit

dat het hier om een consortium gaat kan mogelijkheden geven voor verbeterde effectiviteit. Hier is aangetoond dat consortia van verschillende bacteriën samen een sterker effect geven dan individuele isolaten. Ook geeft het werk aan coumarines houvast voor verbeterde veredeling strategieën. Planten die beter in staat zijn een sterk microbioom te rekruteren en zo infectie door pathogenen verminderen kunnen van groot belang zijn in het streven naar een duurzame manier van voedselproductie. Verder onderzoek is nodig, naar met name de precieze signalering van de geïnfecteerde plant naar het microbioom en hoe deze signalen worden waargenomen door het microbioom.

List of publications

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Curriculum vitae

Gilles was born on the 24th of February 1990, in Almere The Netherlands. In 2008, Gilles finished his high school education and received his VWO diploma from De Meergronden, Almere, The Netherlands. Following high school Gilles took a gap year in which he travelled to Australia and New Zealand, after which he started his Bachelor's degree in Biology at the Utrecht University in 2009. After his graduation, Gilles started the Master program, Plant Biotechnology at Wageningen University in 2013. Within this Master program, he did a minor internship at the Aarhus University in Denmark for 3 months under the supervision of dr. Claus Krogh Madsen. This internship focused on the nutritional values of grains and the phytase activity in barley which resulted in a technical paper in the journal of microbial methods. Gilles did his major internship at the department of Plant Physiology at Wageningen University under the supervision of Tijs Ketelaar and Sander van der Krol. Here, Gilles looked at the signals needed for stromule formation in Arabidopsis. After a lot of collaborative work, this internship also resulted in a publication. Gilles received his master's degree in plant biotechnology from Wageningen University in 2016 with a Master in Plant Biotechnology.Shortly after, Gilles was hired as a PhD student in the Plant-Microbe Interactions group at Utrecht University. Under the supervision of prof. dr. ir. Pieterse, dr. Bakker and dr. Berendsen Gilles began his work on the Back2Roots program. His research aimed to understand the role of the root microbiome in plant defense, of which this thesis is the end result. After his PhD, Gilles started working at the company KeyGene in Wageningen, where he currently is a patent attorney trainee. Through this job Gilles is training to become a qualified patent attorney and help bridge the gap between useful scientific innovations and its application through industry.