The antagonists and helpers of plant pathogens: unraveling interactions that determine pathogen success

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The antagonists and helpers of plant pathogens: unraveling interactions that determine pathogen success

Antagonisten en helpers van plantpathogenen: Ontrafelen van interacties die het succes van pathogenen bepalen

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1 General Introduction

While agricultural production demands increase, crop loss to plant pathogens is an ever-growing threat to food production. Agricultural intensification has resulted in an increased production of staple crops [1, 2]. However, such practices are unsustainable and rely on a heavy input of industrial synthetic pesticides and fertilizers [3]. This can lead to a range of detrimental effects such as reductions in soil fertility, loss of biodiversity and environmental pollution. Such soil, water and air pollution can not only have negative impacts on a range of organisms in the environment, but can also negatively affect worker and consumer health [4]. Therefore, more sustainable and environmentally friendly methods to control plant pathogens are urgently needed to maintain and improve crop yields, and to ensure future food security for a continuously growing world population. Manipulation of the plant microbiome has been suggested as a viable and sustainable alternative to chemically-based agricultural production [5, 6]. However, the ability of rhizosphere microbial communities to keep diseases under control is influenced by many factors, including the microbial interactions within these communities [7]. Unfortunately, we still have relatively little insight into how microbial interactions affect community assembly and how such interactions eventually impact plant health. This thesis seeks to examine how microbial interactions within the rhizosphere microbiome impact the ability of plant pathogens to proliferate and cause plant disease. To this end, I use bacterial wilt disease in tomato plant, which is caused by pathogen *Ralstonia solanacearum* as a relevant model system.

1. Microbial communities inhabiting soil and rhizosphere environments

Microbial communities living in soil habitats comprise of a vast and versatile range of microbial species [5]. For instance, a gram of soil can contain an estimated 6,000-38,000 bacterial taxa with billions of individual bacterial cells [8] and 200-235 fungal species [9]. Such diverse soil microbial communities are responsible for a range of key ecosystem functions such as decomposition of organic matter and environmental pollutants, recycling of nutrients [9, 10] and supporting plant growth [11].

The rhizosphere, the narrow zone of soil that surrounds and is influenced by plant roots, hosts a high density of microorganisms and invertebrates and is considered to be one of the most dynamic interfaces on Earth [12]. Bacteria, fungi, archaea, protists and viruses are present in high numbers in the rhizosphere (see an example for *Arabidopsis thaliana* root microbiota in Fig. 1), where they are fueled by plant-derived resources such as exudates and dead plant root cells [13]. These complex microbial communities co-exist with animals such as nematodes and arthropods, which connect microorganisms to higher trophic levels [14]. Together with microbes inhabiting plant tissues, these interacting rhizosphere communities can have such large impacts on plant growth and health that they have been referred to as the plant's second genome [15, 16].

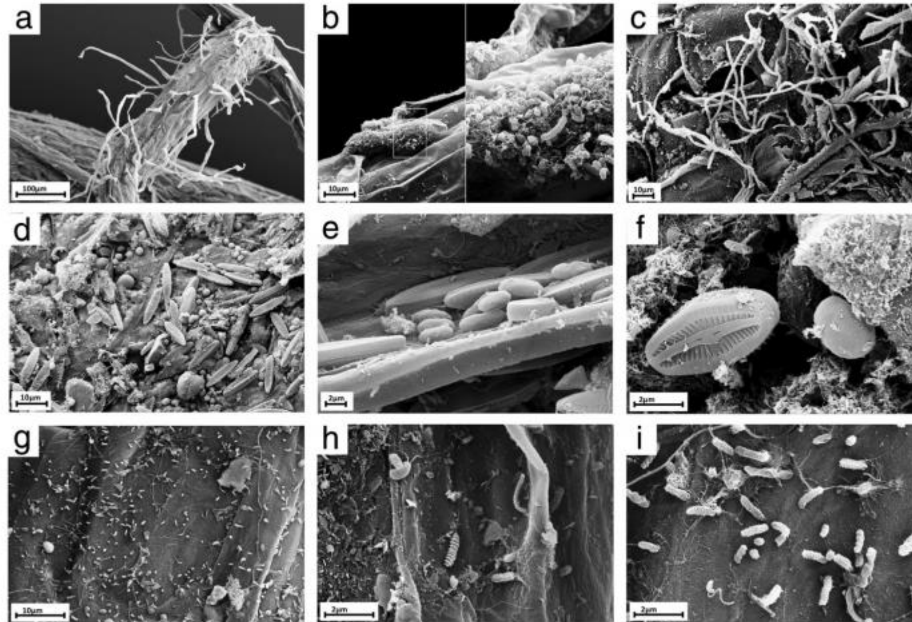

Fig. 1 Microbial consortia naturally formed on the roots of *Arabidopsis thaliana*. Scanning electron microscopy pictures of root surfaces from natural *A. thaliana* populations showing the complex microbial networks formed on roots. (a) Overview of an *A. thaliana* root (primary root) with numerous root hairs. (b) Biofilm-forming bacteria. (c) Fungal or oomycete hyphae surrounding the root surface. (d) Primary root densely covered by spores and protists. (e, f) Protists, most likely belonging to the Bacillariophyceae class. (g) Bacteria and bacterial filaments. (h, i) Different bacterial individuals showing a large varieties of shapes and morphological features [17].

2. Rhizosphere microbial communities and plant health

2.1 Pathogen invasion

Extreme outbreaks of soil-borne diseases are relatively rare in natural habitats [18]. However, plant diseases caused by soil-borne microbial pathogens including fungi, oomycetes, bacteria and nematodes, currently cause heavy losses to a wide range of crops around the world [19, 20]. One of the major factors contributing to this phenomenon is the widespread reliance on intensive agricultural management

practices that have created conditions that are suitable for pathogen infestation, leading to crop losses due to the disease. For example, intensive agricultural practices such as synthetic pesticides not only kill target pathogens, may but also adversely affect many beneficial microorganisms [21]. Such shifts in the functional capabilities of the soil microbiome can for instance lead to decreased resistance to and resilience from pathogen invasion. Intensive agricultural practices also typically involve large inputs of nitrogen, which has linked to more severe damage across a range of crop pathogens, including fungi, bacteria, and viruses [3]. Moreover, continuous cropping with a susceptible plant host generally leads to a build-up of populations of specific plant pathogens [22]. Soil-borne pathogens can also be introduced into local environments by water pollution or other anthropogenic activities [23]. Once in the soil, recruitment from the bulk soil to the rhizosphere is necessary for a pathogen to impact root and plant health [24]. All these factors determine the ultimate ability to the pathogen to enter and proliferate in the rhizosphere, which we refer to as the level of pathogen invasion (Fig. 2).

2.2 Rhizosphere microbial communities can reduce pathogen invasion

The rhizosphere microbial community can function as a first line of defense against pathogen invasion (Fig. 2). Most soil-borne pathogens need to out-compete rhizosphere microbes in order to reach their host or to achieve sufficient density on their host to achieve successful infection of host plant tissue [24]. The phenomenon of disease-suppressive soils clearly demonstrates the potential role of the rhizosphere microbiome in plant disease [25]. Soil transplantation and sterilization studies have

demonstrated that the ability of a soil to suppress the pathogen is of biological nature [21, 25-27]. Furthermore, the use of nucleic acid-based approaches has implicated specific taxa and genes involved in pathogen suppression [21, 28], and cultivation-based studies have yielded disease-suppressive isolates from suppressive soils [25, 29]. A most famous example of natural disease suppressive soil is the development of suppression against the causal agent of take-all disease in wheat [30]. Similar findings have been found for other diseases and crops, such as Fusarium wilt [31], potato scab [32, 33] and bacterial wilt [28]. Plant root-associated microorganisms are increasingly being studied in relation to their ability to help keep plants healthy [34, 35]. However, while some microbiomes are better at preventing pathogen growth than others, it often remains unclear which interactions shape pathogen success.

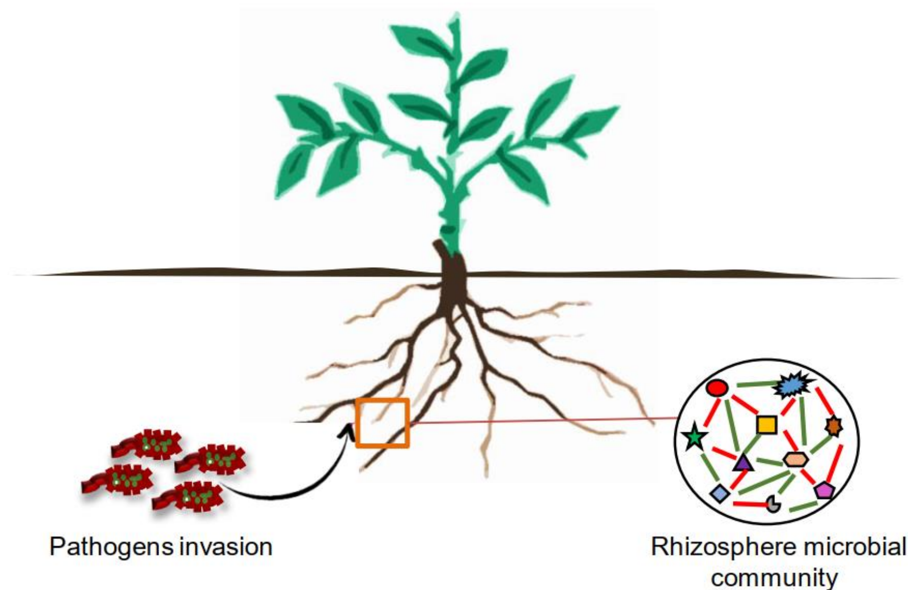

Fig. 2 Rhizosphere microbial communities and plant health. Rhizosphere microorganisms can function as a first line of defense against invading pathogens. The level of this defense is influenced by microbial community composition as well as interactions within the rhizosphere microbial community.

3. Microbial interactions and pathogen invasion

Ecological interactions

Ecological interactions can span a wide range, from antagonistic interactions such as through predation, competition, herbivory and parasitism, to neutral interactions through to beneficial interactions like mutualisms between pollinating and insects [36]. The direction and magnitude of such interactions are critical determinants of ecosystem function and stability [37]. Such interactions form ecological networks in which species are linked together, either directly or indirectly through intermediate species [38]. Therefore, functions of ecological communities are not only based on direct interactions between species, but also based on the indirect interactions that occur via chains of direct interactions or by changing the nature of direct interactions [39, 40]. A similar range and network structure of interactions is also evident in rhizosphere microbial communities, and ecological theory developed in other study systems can help to inform investigations coupling interactions in the rhizosphere with realized functions such as disease suppression.

Ecological interactions in microbial communities

Microorganisms in nature do not exist in isolation but form complex ecological interaction webs [41]. Many types of interactions between bacterial populations occur within the rhizosphere [42]. For instance, multiple species may interact positively via facilitation or negatively through competition (Fig. 3). Also, species can either influence each other directly, where individuals of one species affect the fitness of a second

species, or indirectly, where the impact of one species on the fitness of a second species is realized through direct interactions on other species that subsequently impact fitness (Fig. 3) [43]. Taken together, such interactions are important determinants of community assembly. A tool proposed to visualize such potential interactions is offered by co-occurrence network analysis based on high-throughput sequencing data [41, 44]. Although such approaches provide a means to depict complex correlations in species abundances, it remains unclear to what extent such approaches provide insight into true interactions in the environment. With reference to the rhizosphere, it is clear that correlative network analyses are insufficient to determine the drivers of microbial community assembly and ultimately community resistance to pathogen invasion. Furthermore, the contribution of competitive and facilitative microbe-microbe interactions to the overall community structure remains difficult to evaluate in nature due to strong environmental noise. To overcome these technical hurdles, cultivation-based approaches have been primarily used for assessing possible interactions among microbial isolates and identifying possible molecular mechanisms underlying pathogen inhibition [45-48].

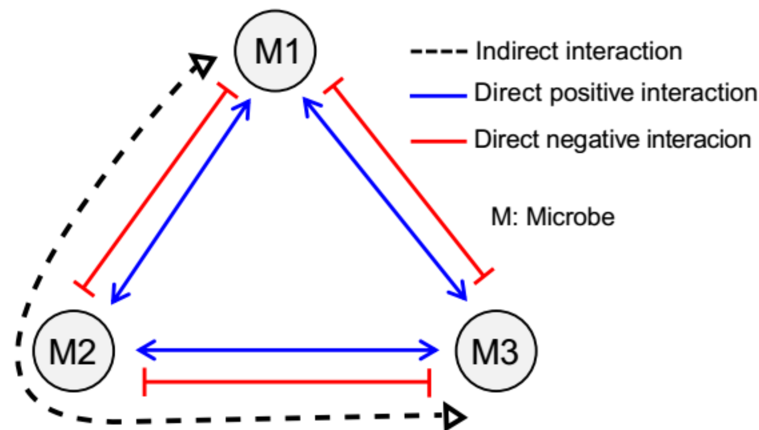

Fig. 3 Conceptual overview of direct, indirect, positive and negative interactions among rhizosphere microbes. There are directly positive (blue arrows) and negative (red stopped lines) interactions between microbes. Microbes can also influence each other indirectly (dotted arrow). For instance, the dotted black curve indicates that microbe 1 (M1) can indirectly influence microbe 3 (M3) through direct interacting with microbe 2 (M2), or vice versa.

3.1 Impact of direct interactions between rhizobacterial species and pathogens on pathogen invasion

Pathogens must first establish themselves in the host-associated microbial communities in order to infect the host and later cause a disease. Many soil-borne microorganisms have been shown to have antagonistic activities toward plant pathogens. Such direct inhibition may thus be an important factor in determining pathogen success and numerous studies have sought to isolate and characterize such pathogen antagonists [25, 49, 50].

a) Effects of direct competitive interactions between rhizobacterial species and pathogens on pathogen invasion

Rhizobacterial species can inhibit pathogen invasion via several different

competitive mechanisms. For instance, some bacteria can produce metabolites, such as antibiotics and enzymes, that exhibit antagonistic activity against plant pathogens [49, 50]. The best-known example of a microbial group with antagonistic members is the fluorescent pseudomonads. Strains from this group have been shown to directly inhibit the growth of the pathogenic fungus *Gaeumannomyces graminis var. tritici* by producing the antifungal metabolite 2,4-diacetylphloroglucinol [25]. Alternatively, rhizobacteria can use indirect mechanisms to compete with the pathogen, such as rapid and efficient utilization of limiting resources. It has recently been shown that resource competition (i.e. similarity in resource preferences between the resident species and the invader) is an important factor explaining the level of success of pathogen invasion in the rhizosphere of tomato plants [51]. Nutrient sequestration is also recognized as an important trait of biocontrol agents to out-compete pathogens [48, 52]. Furthermore, different bacteria can also produce volatile organic compounds (VOCs) that have been shown to inhibit the growth of a broad range of plant-associated pathogenic bacteria [53], fungi and oomycetes [54, 55]. For instance, it has been shown that *Streptomyces* strains isolated from disease-suppressive soils can produce different VOCs with antifungal activity [56].

b) Direct facilitative interactions between rhizobacterial species and pathogens can affect pathogen invasion success

While most studies have primarily focused mainly on the means by which microbes can inhibit target pathogens [26, 57, 58], recent studies have shown that a

significant fraction of plant-associated microorganisms can promote pathogen growth and pathogenicity [59]. Facilitative microbe-microbe interactions are indeed widespread and may emerge for instance as the result of cross-feeding [60], molecular communications such as through quorum sensing [61, 62], or production of public goods such as siderophores [63]. For example, while the inoculation of *Bacillus subtilis* inhibited the growth of the food-borne *Vibrio parahaemolyticus, Pseudomonas putida* promoted this pathogen [64]. Some fungi like Ascomycetes, Basidiomycetes and Zygomycetes can develop chlamydospores, which provide living space for the pathogen *Ralstonia solanacearum* [65]. It has been also shown that toxin production by the bacterial endosymbiont of the plant pathogenic fungus *Rhizopus* is required for successful fungal colonization of rice plants [66], indicating that fungal-bacterial interactions can also promote disease. Facilitation has recently been highlighted as a potential determinant of pathogen success [64]. Therefore, manipulating naturally-occurring pathogens facilitators may provide an alternative means of controlling pathogen development.

3.2 Interactions within resident communities indirectly affect pathogen invasion

The characteristics of both resident communities and the invading species are important for determining the outcomes of biological invasions [67, 68]. From the resident community perspective, species diversity may be considered a shield to invasions and this effect is often attributed to competition for resources [51, 69-71], where highly diverse communities are thought to more completely use available

resources, leaving little free niche space for invaders [72, 73]. In reality, diversity-invasion resistance relationships are more varied, with some studies showing neutral or even negative effects [23, 74, 75]. It has been shown that the nature of species interactions may be more important than the sheer number of interacting species within the community (Wei *et al.* 2015). Invasion resistance may also be mediated to a large extent by specific keystone taxa [76]. However, the type and strength of resident species' interactions, such as competition and facilitation, have often been overlooked in the context of how diversity mediates invasion resistance.

a) Competitive interactions within resident communities indirectly affect pathogen invasion

Competitive interactions among resident species may affect the outcomes of invasions in various ways. For instance, competition is likely to affect the resource availability, and hence the availability of free resource niche space, and the likelihood of invasions [23, 73, 75, 77, 78]. It is predicted that highly competitive resident communities are less prone to invasions if they can efficiently utilize and consume resources that would otherwise be available for invaders [23, 73, 79]. This effect is expected to be especially strong in resident communities that show a high degree of complementarity and hence compete less strongly with each other and collectively more completely with the invader. Furthermore, competing species can inhibit each other directly by producing toxic metabolites, such as antibiotics. Depending on the spectrum of their activity, antibiotic compounds could have negative effects on both

resident community species as well as the invader [80-85]. If the invader is particularly sensitive to toxins produced by the resident community, it is expected that antibiotic-mediated interference competition will constrain invasions. In contrast, if toxins have a disproportionally large negative effect on members of the resident community, such interference competition is expected to promote invasions [83, 86].

b) Facilitative interactions within resident communities indirectly affect pathogen invasion

Invasion resistance has to date mainly been considered from the perspective of competitive interactions such as resource competition, niche preemption and direct antagonism [51, 58, 72, 73]. There is still a conspicuous lack of knowledge regarding how facilitative interactions between resident species influence microbial community establishment and pathogen invasion. Facilitative resident communities might be less efficient at competing for resources with the invader compared to competitive resident communities. Furthermore, facilitative interactions between residents could potentially increase the number of resource niches via production of secondary metabolites or public goods that can also be utilized by the invader, which could promote invasions in the process [87-89]. Moreover, previous studies have demonstrated that bacteria can show preference between different dietary glycans, which can prolong species coexistence in co-cultures [90]. Such dietary preference might leave some resources less utilized, providing an opportunity for invasion [91].

4. Interactions among microbes are sensitive to environmental factors

The microbial interactions described above are sensitive to a range of environmental factors, such as resource availability [76, 92], pH [93] and temperature [94]. Theoretical studies have suggested that certain mutualisms can become competitive under high nutrient conditions [95]. It has also for instance been shown that two yeast strains can interact in at least seven qualitatively different ways depending on the nutrient concentrations encountered [96]. Moreover, the way microbes modify their environment and react to it influences the interactions between different species. For example, it has been found that modifying and reacting to the environmental pH can drive bacterial interactions [93]. Also, negative interactions between microbes have been observed to be mostly driven by competition for resources at low resource availability, but mostly driven by the production of toxic metabolites at high resource availability [97]. Therefore, although the specific environmental conditions may differ widely between habitats, microbes influence their direct environment for instance by consuming resources and excreting metabolites [98]. These environmental changes influence the growth and survival of both the microbes that originally altered the environment as well as other microbial species that are present, whether that be resident organisms or potential invaders. However, although multiple studies have observed a shift in bacterial interactions in response to changes in environmental factors, it is generally not known how shifts in these interactions affect a community's susceptibility or resistance to pathogen invasion.

5. Bacterial wilt disease as a model of soil-borne disease

In this thesis, I use bacterial wilt disease in tomato plant as a model of soil-borne disease, which is caused by pathogen *Ralstonia solanacearum*. *Ralstonia solanacearum* is a soil-borne bacterium that can cause bacterial wilt disease in over 50 plant families and more than 200 plant species, mostly in solanaceous plants [99, 100]. This pathogen causes enormous agricultural and economic losses due to its lethality, persistence, complex subspecies, wide host range and broad geographic distribution [101, 102]. *Ralstonia solanacearum* survives for long periods of time in soil and water and can be transmitted by water, soil particles and infected plants [49]. During the *Ralstonia solanacearum* infestation process, this soilborne pathogen usually enters plant roots, invades the xylem and then spreads quickly to the aerial parts of the plant through the vascular system. With the accumulation of *Ralstonia solanacearum*, the plant becomes stunted and wilted leading ultimately to death [103].

6. Thesis outline

In this thesis, I investigate how bacteria from the tomato rhizosphere interact with each other, and how these interactions affect resident community resistance to pathogen invasion. Furthermore, I examined the extent to which these interactions are influenced by environmental factors such as resource availability (Fig. 4).

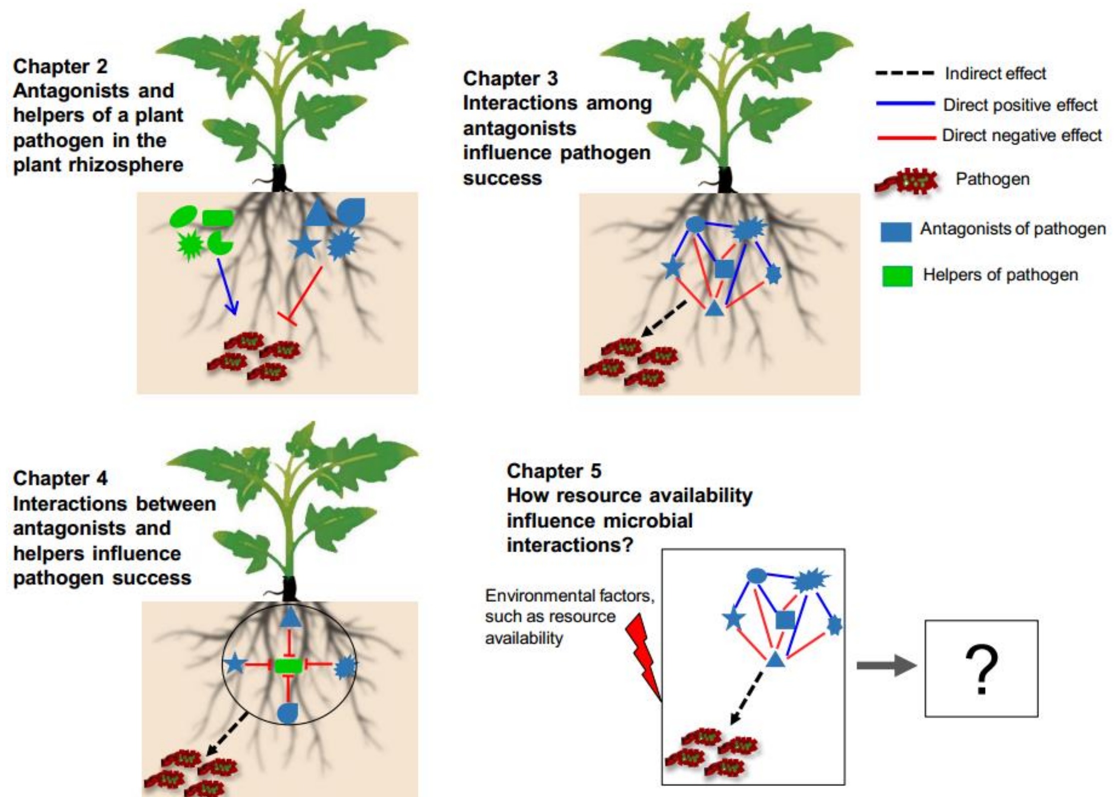

Fig. 4 Overview of the chapters of this thesis.

I start this thesis by presenting a field survey of tomato bacterial wilt disease across six regions in China where *Ralstonia solanacearum* infestation has created a mosaic of disease and healthy tomato plants. As mentioned above, plant health is intimately influenced by its rhizosphere microbiome and their interactions. Although differences can often be detected between the microbiomes of healthy versus diseased plants, the underlying mechanisms driving plant microbiome support of plant health often remain difficult to determine. I addressed this issue by examining soil-borne bacterial communities associated with either healthy or diseased plants across the aforementioned six field locations in **Chapter 2**. To relate community patterns with potential bacterial interactions, I combined direct examination of plant-associated bacterial communities from healthy and diseased soils with interaction studies of

bacterial isolates recovered from these soils. Correlation analyses showed discrepancies between co-occurrence patterns and direct strain interactions. I thus used less complex, yet more controllable synthetic microbial communities in the subsequent studies to investigate the ecological interactions between rhizobacterial isolates and explored how these interactions impact pathogen invasion.

In **Chapter 3**, I tested how antagonistic and facilitative pairwise interactions within resident model bacterial communities may be used to predict invasion by the *Ralstonia solanacearum.* I found that facilitative resident community interactions promoted and antagonistic interactions suppressed invasions both in the lab and in the tomato plant rhizosphere. Crucially, pairwise interactions also reliably explained observed invasion outcomes also in multispecies communities. Mechanistically, this was linked to direct inhibition of the invader by antagonistic communities (antibiosis) and to a lesser degree by resource competition between members of the resident community and the invader.

There were both pathogen-antagonist bacteria and -helper bacteria in our library of rhizobacterial strains isolated from the tomato rhizosphere (**Chapter 2**). However, the importance of the helper bacteria in determining the success of pathogen remains unclear. In **Chapter 4**, I hypothesized that inhibiting such pathogen helpers may help control pathogens indirectly. I examined tripartite interactions between the model pathogen *Ralstonia solanacearum*, two model helper strains and a collection of 46 bacterial isolates recovered from the library of rhizobacterial strains that was built in **Chapter 2**. This setting allowed me to examine the importance of direct (effects of rhizobacteria on pathogen growth) versus indirect (effects of rhizobacteria on helper

growth) pathways on pathogen growth. I found that as compared to direct impacts from rhizosphere isolates on the pathogen itself, the indirect impacts from interactions between rhizosphere isolates and the helper strains were more important determinants of pathogen success *in vitro* and *in vivo.*

In **Chapter 5**, I examined how differences in resource availability impact pairwise interactions within the resident community (the same two-species community combinations as in **Chapter 3**), and how these shifts affect the resident community's ability to resist the invasion of *Ralstonia solanacearum*. Resource availability changed the nature of interactions between resident community members and pathogen invasion. At high resource availability, competitive resident communities produced more antibiotics, making them less susceptible to invasion compared to more facilitative communities. At low resource availability, facilitative communities reached higher productivity, which in turn may be more important for resistance to pathogen invasion than competitive interactions in less productive communities.

In **Chapter 6**, I synthesize the results of this thesis and discuss how the findings of this thesis contribute to the field of microbial ecology. I further expand upon the importance of direct and indirect interactions within rhizosphere microbes with respect to the development of disease protection systems. In addition, future research directions about more holistic understanding of rhizosphere ecology and sustainable control of soil-borne disease are proposed.

Chapter 2 Linking microbial community patterns and pairwise interactions to plant health

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Abstract

Plant disease incidence in the field is influenced by the composition of the rhizosphere microbiome. Although differences can often be detected between the microbiomes of healthy versus diseased plants, the underlying mechanisms driving plant microbiome support of plant health often remain difficult to determine. We addressed this issue by examining soil-borne bacterial communities associated with either healthy or diseased plants across six field locations throughout China. To relate community patterns with potential microbial interactions, we combined direct examination of the plant-associated bacterial communities from healthy and diseased rhizosphere soils (HRS and DRS) with interaction studies of bacterial isolates recovered from these soils. Not surprisingly, the density of the disease-causing agent, *Ralstonia solanacearum*, was significantly higher in DRS as compared to HRS, and distinct microbiome structures and co-occurrence patterns were observed in healthy versus diseased rhizosphere soils. Upon assessing pairwise assays of 515 recovered bacterial isolates with R. solanacearum, we found that HRS yielded a greater proportion of strains that directly inhibited the pathogen as compared to DRS. We then associated these bacterial isolates with 16S rRNA gene sequences obtained from our rhizosphere microbiomes. The relative abundance of OTUs highly related to these isolates were differentially recovered from DRS and HRS samples, with higher abundances of Firmicutes and Actinobacteria in healthy soils and higher abundances of Bacteroidetes and Proteobacteria in diseased soils. Correlation analyses showed discrepancies between co-occurrence patterns and direct strains interactions. Our results may help to guide

efforts for targeted cultivation and application of potential biocontrol agents and offers opportunities for future microbiota manipulation experiments to elucidate the biological mechanisms and interactions driving the observed effects.

Introduction

A variety of soil-borne diseases are increasingly threatening agricultural production around the world [20, 21]. Diverse microbes inhabiting the plant rhizosphere can help plants avoid or limit the damage inflicted by such diseases [13, 25]. Understanding which microbial communities are associated with disease-suppression can thus provide the foundation for soil community manipulation and new opportunities to explore novel strategies to promote plant health in a sustainable way [104]. In line with this aim, a range of disease-suppressive soils has been described in which either specific components or general community action contributes to resistance against soil-borne pathogens [15, 26, 57]. To examine the diversity of plant-associated microbial communities, most studies have been utilized ribosomal amplicon-based approaches [105-108]. One limitation of ribosomal RNA-based root microbiota characterizations is that such approaches only provide indirect information about the functions carried out by its members, based upon taxonomic classification [109]. Culture collections of genetically tractable microbial isolates have been proposed to represent a valuable tool for increasing our understanding of the plant microbiome [110], but these too have inherent limitations. First, the vast majority of known microorganisms are recalcitrant to *in vitro* cultivation [111]. Although it has been shown that relatively large proportions of bacteria of the root microbiome can be isolated [112-

114], it still requires considerable effort including largescale isolation using serial dilutions, multiple cultivation conditions, and subsequent high-throughput taxonomy identification. Second, laboratory cultivation selects isolates that are well adapted to the given medium, temperature and other cultivation parameters, as opposed to the conditions found in the environment of study. Nevertheless, despite the inherent limitations, both cultivation independent and dependent approaches represent valuable tools to our understanding of the role of rhizosphere microorganisms on plant health. For instance, the importance of the rhizosphere microbiome structure on plant health has been documented for disease-suppressive soils [25]. By tracking communities across a range of soil suppressive conditions, microbial community patterns and specific microbial taxa can be identified that are associated with disease suppressiveness. For instance, Gammaproteobacteria, Betaproteobacteria and Firmicutes have been suggested to represent important bacterial phyla associated with disease suppressiveness [21]. Furthermore, inferred microbial co-occurrence networks from community profiling or metagenomic data [41, 115] have suggested potential microbial associations (either positive, neutral, or negative) that may be linked with soil function [106, 116]. However, such correlative approach fail to reveal causal mechanisms through which rhizosphere microorganisms can affect each other, including soil-borne pathogens [110, 117]. For such purposes, microbial strains collections can be used to test interactions between resident populations under laboratory conditions [118, 119], as well as strain-strain interactions that may be important for plant resistance to pathogen infection [17]. For instance, Niu and

colleagues have employed a simplified seven-species synthetic community that was representative of the maize root microbiota to study the role of *in planta* interspecies interactions in altering host health and the establishment of root-associated bacterial communities [120]. A similar approach showed that antagonistic microbial communities were more efficient at suppressing pathogen than facilitative resident communities [121]. These studies strongly suggest that a link can be made from interactions among the microbiota members to impacts on plant health and disease status.

In the present study, we combined cultivation-independent and -dependent approaches to integrate soil microbial community patterns associated with healthy and diseased rhizosphere soils with actual microbial interactions between resident populations. As our model pathogen, we chose *Ralstonia solanacearum,* the causal agent of bacterial wilt disease. This species represents one of the most devastating and globally distributed soil-borne plant pathogens across a range of important crops [100, 122]. Our study targeted six regions across China, where *Ralstonia solanacearum* infestation created a mosaic of disease and healthy tomato plants. We first sampled 139 tomato rhizosphere soils across these six different geographic locations and tested whether healthy and diseased rhizosphere soils differed in pathogen density (using qPCR), bacterial diversity and taxonomic composition as determined by 16S rRNA gene amplicon-based sequencing. We then generated bacterial isolate collections from the aforementioned healthy and diseased tomato rhizosphere soils, characterized their taxonomy and assessed their effects on *Ralstonia solanacearum*'s growth. Furthermore, to relate the result of our cultivation-

independent and dependent approaches, we identified bacterial isolates within microbiomes of the rhizosphere soils, and compared co-occurrence patterns between bacterial community members and *Ralstonia solanacearum* in the field with the effects of matching bacterial isolates on the growth of *Ralstonia solanacearum*.

Materials and methods

Rhizosphere soil sampling

The diseased and healthy rhizosphere soils (Diseased rhizosphere soil: DRS; Healthy rhizosphere soil: HRS) were collected from the rhizosphere of tomato plants in six geographically separated field sites: Changsha of Hunan province (CH), Ningbo of Zhejiang province (NB), Nanchang of Jiangxi province (NC), Nanjing of Jiangsu province (NJ), Nanning of Guangxi province (NN) and Wuhan of Hubei province (WH). For each field, 12 symptomatic (diseased) and 12 asymptomatic plants (healthy) were randomly collected. After the accidental loss of 5 samples, a total of 139 rhizosphere samples were used for further analysis (Table S1). The soil was detached from the roots by gentle shaking, and the remaining soil attached on the surface of roots was considered as the rhizosphere soil [123]. Each rhizosphere soil sample was then divided into two parts: one for isolating bacteria, the other for extracting DNA for bacterial community sequencing and the quantification of pathogen *Ralstonia solanacearum* (Rs) densities using qPCR.

DNA extraction, and quantification of *Ralstonia solanacearum* densities

Soil DNA was isolated from 500 mg of soil using the PowerSoil DNA Isolation Kit

(MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA extracts were quantified using a NanoDrop spectrophotometer (ND2000, Thermo Scientific, DE, USA).

The *Ralstonia solanacearum* densities in the fields were then determined with qPCR using primers targeting the *fliC* gene (Schönfeld et al., 2003), which encodes a flagellar subunit (forward primer: 5′-GAA CGC CAA CGG TGC GAA CT-3′ and reverse primer: 5′-GGCGGC CTT CAG GGA GGT C-3′). The qPCR was carried out on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, CA, USA). We used SYBR Green I fluorescent dye detection in 20 μl volumes containing 10 μl of SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan), 2 μl of DNA template extracted from rhizosphere soil and 0.4 μl of both forward and reverse primers (10 mM each). The qPCR was performed by initially denaturing step for 30 s at 95 °C with subsequent cycling for 40 times with a 5s denaturizing step at 95 °C and a 34s elongation/extension step at 60 °C, and a melt curve analysis for 15 s at 95 °C followed by 1 min at 60 °C and finally for 15 s at 95 °C. Melting curves were obtained based on a standard protocol and used to identify the characteristic peak of PCR product (400 bp) [124]. Three independent technical replicates were performed for each sample.

Bacterial community analysis from diseased and healthy rhizosphere soil samples

To examine bacterial community structure from our 139 tomato rhizosphere samples, environmental DNA extracts were used as template for high throughput 16S rRNA gene tag sequencing as carried out by Shanghai Biozeron Biological Technology Co. Ltd. We amplified the V4 hypervariable region of the bacterial 16S rRNA gene using the primer pair 563F (5′-AYT GGG YDT AAA GVG-3 ′) and 802R (5′-TAC NVG GGT ATC TAA TCC-3′) [125] with an Illumina adaptor (Illumina, CA, USA), and

amplicons were sequenced via an Illumina MiSeq sequencer. Recovered sequences were assigned to each sample using their unique barcodes, and reads were processed with the QIIME open-source bioinformatics pipeline [126]. Filtering of noisy sequences, chimera checking and OTU cutoff was assigned at 97% identity level using USEARCH sequence analysis tool [127]. OTUs were assigned to bacterial taxa using ribosomal Database Project (RDP) database with the online version of the RDP classifier [128].

The relative abundance of a given taxonomic group per sample was calculated as the number of sequences affiliated to that group divided by the total number of sequences recovered from the given sample. The alpha diversity values (including Shannon diversity index, observed OTUs and Pielou evenness) were determined by using the R vegan package after removing sequences identical to *Ralstonia solanacearum* (Dixon, 2003). Principal Coordinates Analysis (PCoA) based on a Bray-Curtis dissimilarity matrix was performed and plotted using the R vegan package to visualize the differences in microbial communities [129]. Permutational multivariate analysis of variance (PERMANOVA) was conducted to examine differences between soil-borne bacterial communities from DRS and HRS across our six field sites by using the R vegan package.

Isolation and identification of rhizobacteria

Isolation. A total of 640 bacterial strains were isolated from the 20 fresh rhizosphere soil samples from the Nanjing field site; half from 10 DRS samples, and the half from 10 HRS samples, according to an established protocol [59]. Briefly, 1 g of each rhizosphere sample was mixed with 9 mL MS buffer solution (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin) in a rotary shaker at 170 rpm min^{-1} for 30 min at 30 °C. After serial dilution in MS buffer solution, 100-μl volumes of the

diluted soil suspensions were plated on 1/10 tryptone soy agar (1/10 TSA, 1.5 g L^{-1} tryptone, 0.5 g L^{-1} soytone, 0.5 g L^{-1} sodium chloride, and 15 g L^{-1} agar, pH 7.0). After a 48-h incubation at 30 °C in the dark, 32 isolates were randomly picked per rhizosphere soil sample. To avoid potential fungal contamination, only highly diluted samples were used for isolation. The isolates were then re-streaked on TSA plates for colony purification. Approximately 5.5% (20 isolates from DRS and 15 from HRS) of the bacterial isolates failed to grow on the TSA plates for unknown reasons when we re-streaked them, resulting in a final collection consisting of 605 bacterial isolates from 20 rhizosphere soil samples (300 strains from DRS and 305 from HRS). All purified isolates were cultured in 100 μl tryptone soy broth (TSB, liquid TSA) on 96-well microtiter plates at 30 °C with shaking (rotary shaker at 170 rpm) for 18 h before freezing and storing at -80 °C in 15% glycerol.

Strain identification. For taxonomic assignment of all 605 rhizobacterial isolates, the full 16S rRNA gene was determined by Sanger sequencing by Shaihai Songon Biotechnology Co., Ltd, Shaihai Station. The PCR (25 μl) was composed of 1 μl of bacterial cells (overnight culture), 12.5 μl mixture, 1 μl of forward (27F: 5-AGA GTT TGA TCA TGG CTC AG-3) and reverse primer (1492R: 5-TAC GGT TAC CTT GTT ACG ACT T-3) each [130] and 9.5 μl of sterilized water. PCR was performed by initially denaturizing at 95 °C for 5 min, cycling 30 times with a 30-s denaturizing step at 94 °C, annealing at 58 °C for 30 s, extension at 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. The taxonomy of 16S rRNA gene sequences were assessed using the RDP classifier against the RDP Bacterial 16S database [131]. A total of 90 bacterial

isolates (70 isolates from DRS and 20 from HRS) that were identical (*i.e.* 100% similar on 1465 bp) to the sequence of *Ralstonia solanacearum* were removed from further analyses. The remaining 515 strains (230 strains from DRS and 285 from HRS) were retained for subsequent analyses.

Effects of rhizobacterial strains on pathogen growth

We used *Ralstonia solanacearum* strain QL-Rs1115 tagged with the pYC12-mCherry plasmid as a model plant pathogen [51, 123]. We first tested the direct effects of our 515 non-*Ralstonia solanacearum* bacterial strains (230 isolates from DRS and 285 from HRS) on the growth of *Ralstonia solanacearum in vitro* by using supernatant assays. Briefly, after 48 h of growth in NB (nutrient broth) medium (glucose 10.0 g l^{-1}, tryptone 5.0 g l^{-1}, yeast extract 0.5 g l^{-1}, beef extract 3.0 g l^{-1}, pH 7.0) on a shaker at 170 rpm, 30°C, all bacterial cultures were filter sterilized to remove living cells (0.22 µm filter). Subsequently, 20 µl of sterile supernatant from each strain's culture and 2 µl overnight culture of the pathogen (adjusted to OD600 = 0.5 after 12 h growth at 30°C with shaking) were added into 180 µl of fresh NB medium (5-times diluted, in order to better reflect the effect of the supernatant). Control treatments were inoculated with 20 µl of 5X diluted NB media instead of the bacterial supernatant. Each treatment was conducted in triplicate. All bacterial cultures were grown for 48 h at 30°C with shaking (170 rpm) before measuring pathogen density as red mCherry protein fluorescence intensity (excitation: 587 nm, emission: 610 nm) using a SpectraMax M5 plate reader [59, 121]. To test for significance of growth promotion or inhibition, *Ralstonia solanacearum* densities were $\log_{10}$-transformed prior to analyses of variance (ANOVA) and Bonferroni t-test to compare mean differences between each rhizobacterial supernatant treatment and the control treatment, with p-values below 0.05 being

considered statistically significant. The effect on pathogen growth was defined as the percentage of improvement or reduction in pathogen growth by the supernatant compared to the control treatment. When the effect on pathogen growth was positive, *i.e.* when the supernatants from strains significantly promoted the growth of the pathogen, they were considered as helpers of the pathogen. If the effect on pathogen growth was negative, *i.e.* when the supernatants from strains significantly inhibited the growth of the pathogen, they were considered as inhibitors of the pathogen.

Phylogenetic tree construction

The 16S rRNA gene sequences of the 515 non- *Ralstonia solanacearum* bacterial strains were aligned using MUSCLE [132]. Sequences in the alignment were trimmed at both ends to obtain maximum overlap using the MEGA X software, which was also used to construct taxonomic cladograms [133]. We constructed a maximum-likelihood (ML) tree, using a General Time Reversible (GTR) + G + I model, which yielded the best fit to our data set. Bootstrapping was carried out with 100 replicates retaining gaps. A taxonomic cladogram was created using the EVOLVIEW web tool (https://evolgenius.info//evolview-v2/). To show the relationship between phylogeny and the effects of rhizobacteria on pathogen growth, we added taxonomic status (phylum) of each rhizobacterial strain and its effect on pathogen growth as heatmap rings to the outer circle of the tree separately, and we added another heatmap ring to show if the strain was isolated from DRS or HRS samples (Fig. 2).

Matching bacterial isolates with OTUs

Because reads from high throughput sequencing were shorter than those produced by Sanger strain sequencing, we first extracted all sequence reads from the dataset of OTUs that were highly similar (>99% over full length of reads) to Sanger 16S rRNA gene sequences using BLAST searches [134]. These reads were then re-

clustered in more stringent OTUs (99% similar over full length of reads) using MOTHUR [135]. New BLAST searches between 99% similar OTUs and full-length 16S rRNA gene sequences from strains were performed to improve similarity matches between OTUs and isolates, and only those that were identical (100% over full alignment) were retained. In total, 23,829 reads could be assigned to 444 isolated strains.

The relative abundance of each strain in the 99%-OTU dataset was then calculated to estimate the relative abundance of each identified bacterial isolate in each rhizosphere samples. Principal Coordinates Analysis (PCoA) based on a Bray-Curtis dissimilarity matrix was performed and plotted using the R vegan package to explore the differences between strain representatives isolated from DRS vs. HRS [129]. Permutational multivariate analysis of variance (PERMANOVA) was conducted to evaluate the significance of this difference using the R vegan package.

Statistical analyses

All statistical tests performed in this study were considered significant at $P < 0.05$. To determine significant differences between health conditions (healthy versus diseased), non-parametric Kruskal-Wallis and post hoc Dunn's tests were performed in R. Testing of linear discriminant analysis effect size (LEfSe) was performed to identify significant differences in bacterial taxa (isolated strains in the 99%-OTU dataset) between healthy and diseased rhizosphere soil (HRS and DRS) samples [136]. Welch's t-test was used to compare mean differences between the relative abundance of screened OTUs (which matched sequences from bacterial isolates) enriched in healthy versus diseased rhizosphere soil samples using STAMP [137].

Spearman's rank correlation coefficients between the relative abundance of OTUs which matched with bacterial isolates and abundance of *Ralstonia solanacearum* were calculated in R software (Version 4.0.2).

Results

Comparison of *Ralstonia solanacearum* density and bacterial community structure between healthy and diseased rhizosphere soil samples

In this study, we recovered bacterial community data from six tomato fields across a wide geographic range where tomato wilt disease was observed (Table S1). Rhizosphere samples were examined from both diseased and healthy tomato plants in each field. For all sites, we detected significantly higher ($p<0.001$) densities of the disease-causing agent, *Ralstonia solanacearum,* in diseased rhizosphere soil (DRS) samples as compared to healthy rhizosphere soil (HRS) samples (Fig. 1A).

To compared bacterial community composition between healthy and diseased rhizosphere samples, HRS and DRS collected from the six sites (Table S1) were subjected to 16S rRNA gen-based amplicon sequencing. Relative abundance analysis indicated that Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Gemmatimonadetes and Firmicutes were most abundant bacterial communities at the phylum level, and the relative abundance of Proteobacteria was higher in DRS than HRS, while the relative abundance of Actinobacteria was higher in HRS than DRS for all six sites (Fig. 1B). Alpha diversity analysis revealed no difference in bacterial evenness or richness indices (Fig. S1). However, principal coordinate analysis, based on the Bray–Curtis dissimilarity index, revealed significant differences between HRS

and DRS samples (p=0.022, Adonis; Fig. 1C), as well as variation explained by the different filed sites (p = 0.001, Adonis; Fig. 1C). A clear difference was observed between HRS and DRS when examining data from each field separately (P < 0.05, Adonis; Fig. S2), with the exception of the Nanchang site (P = 0.425, Adonis; Fig. S2).

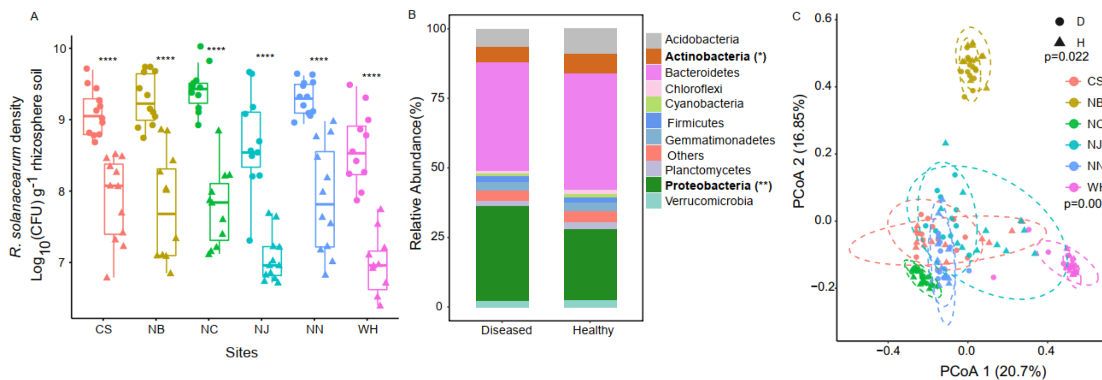

Fig. 1 Differences in *Ralstonia solanacearum* density and bacterial community composition of HRS and DRS samples collected from tomato fields in Changsha (CS), Ningbo (NB), Nanchang (NC), Nanjing (NJ), Nanning (NN) and Wuhan (WH) in China. (A) The population of the bacterial plant pathogen *Ralstonia solanacearum* (Wilcoxon test, mean ± SD, n = 11 or 12; ***P < 0.001), (B) relative abundance of rhizobacteria at the phylum level in HRS and DRS samples collected across the six sites (t-test, mean ± SD, n = 6; **P < 0.01, *P < 0.05), and (C) PCoA analysis with Bray–Curtis distance showing as related to site and plant health status. Indicated p values refer to differences in bacterial community composition between HRS and DRS samples, and between the six field sites (PERMANOVA by Adonis).

Comparison of taxonomic characterization and the effects of bacterial isolates from HRS and DRS on *Ralstonia solanacearum* growth

Our 515 isolates (230 isolates from DRS and 285 from HRS) were classified into

four main phyla, with the following distribution: Proteobacteria 34.4%, Firmicutes 31.7%, Bacteroidetes 17.5% and Actinobacteria 16.5%. This collection contained a total of 26 families and 52 genera (Fig. S3). A total of 37.9% of these isolated rhizobacteria were shown to inhibit pathogen growth, while 53.6% of them significantly improved pathogen growth, and these categories were referred to as pathogen antagonists and helpers, respectively (Fig. S4). Although HRS or DRS both yielded strains with a wide range of effects on *Ralstonia solanacearum* growth, we isolated a higher proportion of *Ralstonia solanacearum* antagonists from healthy rhizosphere soils (40.4%) as compared to from diseased rhizosphere soils (34.7%) ($p=0.016$; Fig. 2A). Conversely, we observed a lower proportion of pathogen helper strains in HRS (51.9%) as compared to DRS (55.7%).

The proportion of strains characterized as either pathogen helpers or antagonists varied across the four phyla represented in our collection (Fig. 2B). For instance, a higher proportion of the isolates affiliated with the Firmicutes inhibited *Ralstonia solanacearum* growth, while more Proteobacteria and Actinobacteria isolates were characterized as helpers, and this held for both DRS and HRS (Fig. 2C). We observed a higher proportion of inhibitors affiliated with the Firmicutes in HRS (67.3%) as compared to DRS (54.2%), while there was a lower proportion of helpers affiliated with the Actinobacteria from HRS (77.5%) than from DRS (89.7%). Moreover, the proportion of isolates identified as Firmicutes was higher for HRS (35.4%) than for DRS (25.6%), while those affiliated with Bacteroidetes were lower for HRS (13.3%) as compared to DRS (22.6%) (Fig. 2C).

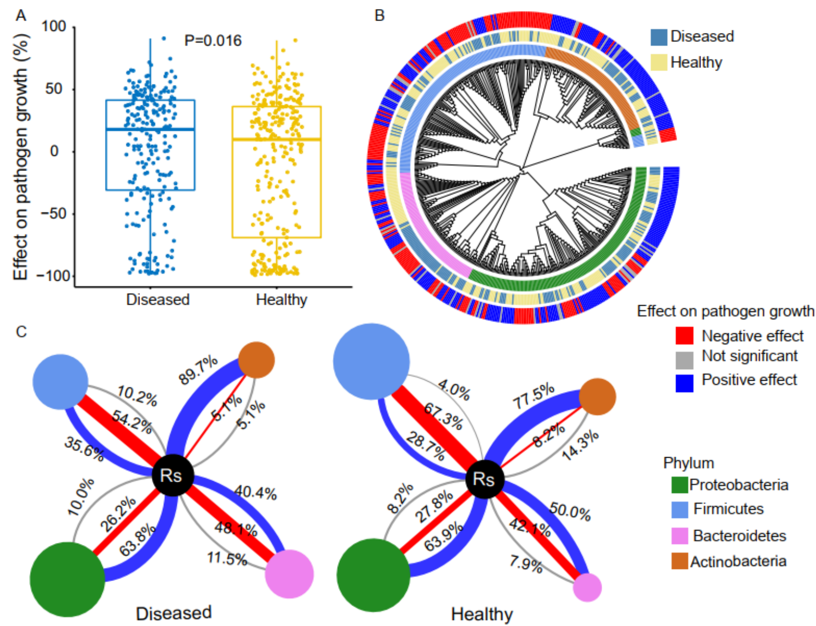

Fig. 2 Taxonomic and functional differences between bacterial isolates from HRS and DRS samples. (A) The effects of bacterial isolates from HRS and DRS on *Ralstonia solanacearum* growth (Wilcoxon test, mean ± SD, n (diseased) = 230 isolates, n(healthy) = 285 isolates). (B) Cladogram depicting the phylogenetic relationship among the 515 isolates based on their full-length 16S rRNA gene sequences. The inner ring shows the four phyla to which the isolates belong. The middle ring indicates whether isolates were recovered from HRS (light yellow) or DRS (light blue) samples. The outer ring depicts the effect of isolate supernatants on *Ralstonia solanacearum* growth: positive effect (blue), negative effect (red) and no significant effect (gray). (C) The proportion of bacterial isolates per phylum whose supernatant showed inhibitory, stimulatory or no effect on *Ralstonia solanacearum* growth from HRS and DRS samples (right and left, respectively). The size of the circles represents the proportion of bacterial isolates identified for a given phylum from HRS and DRS samples. The thickness of lines represents the percentage of bacterial isolates that have the indicated effect on *Ralstonia solanacearum* growth for each phylum.

Linking bacterial isolates with bacterial communities of rhizosphere soils

From our collection of 515 bacterial isolates, 444 (86.2%) were highly similar (100%) to one of 165 OTUs recovered by high-throughput 16S rRNA gene tag sequencing of extracted environmental DNA. The remaining 13.8% (71) of isolates showed no relative OTU in the high throughput dataset. The relative abundance of OTUs highly related to these isolates showed a clear pattern between bacterial strains isolated from DRS and HRS samples (Adonis; p=0.001; Fig. 3A). We further examined the correlations between bacterial OTUs related to the isolated strains, plant health status and the abundance of *Ralstonia solanacearum* in the field. Based on linear discriminant analysis (LDA), 39 rhizosphere bacterial OTUs related to these strains differed between DRS and HRS (Fig. 3B and Table S2). Among these, 10 OTUs were enriched in HRS, while 29 OTUs were enriched in DRS (Fig. 3B). Four OTUs enriched in HRS were significantly and negatively linked with the abundance of *Ralstonia solanacearum* in the field (Spearman's rank correlation analysis, $p<0.05$), and 6 OTUs enriched in DRS were positively correlated ($p<0.05$) with the abundance of *Ralstonia solanacearum*. Other OTUs did not show significant correlations with *Ralstonia solanacearum* in DRS or HRS samples (Fig. 3B and Table S2). Because different isolates could be related to the same OTU, their effects on *Ralstonia solanacearum* growth might not all align with the co-occurrence patterns of this OTU and *Ralstonia solanacearum* (Table S2). For instance, 10 bacterial isolates were closely related to OTU00007, which was enriched in DRS and was positively corelated with *Ralstonia solanacearum*. Of these isolates, 1 inhibited the growth of *Ralstonia solanacearum,* 1

had no effect and 8 facilitated *Ralstonia solanacearum*'s growth in our laboratory assay (Table S2). In addition, of these 39 discriminating OTUs that could be linked with isolates, HRS had higher abundances of Firmicutes and Actinobacteria, while DRS had higher abundances of Bacteroidetes and Proteobacteria (p<0.05; Fig. 3C).

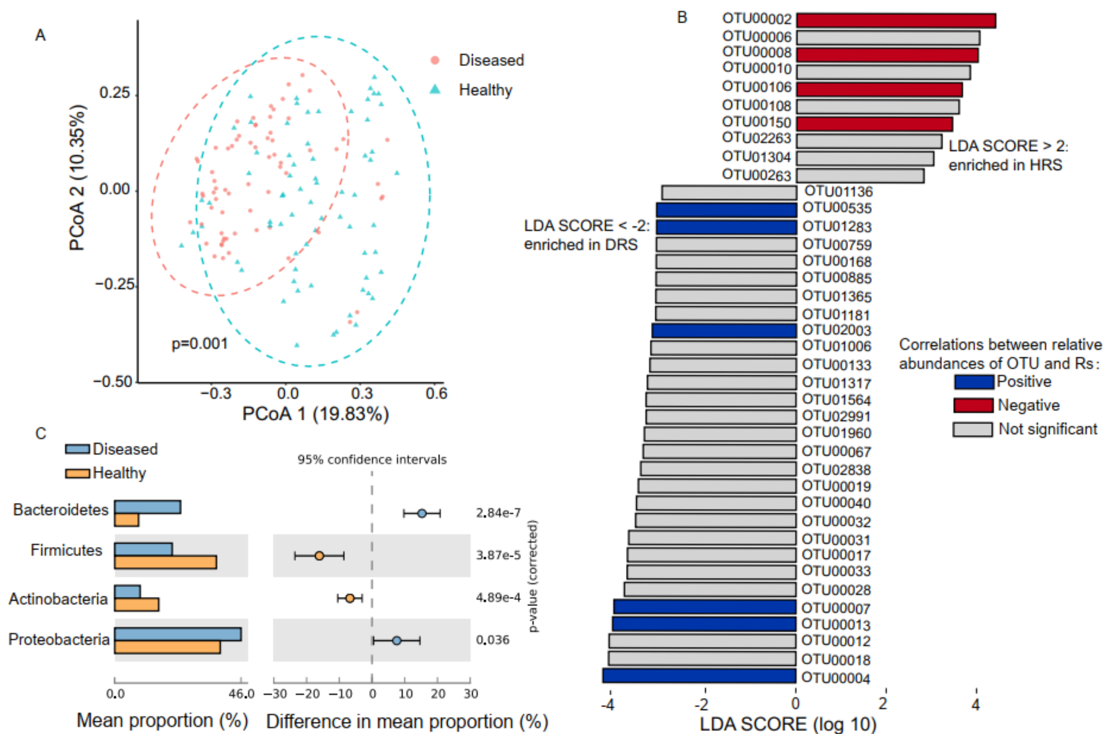

Fig. 3 Differences in bacterial isolates between HRS and DRS as related to sequence-based OTUs. (A) Two-dimensional principal coordinate analysis (PCoA) of bacterial OTUs highly related to strains which were isolated from HRS and DRS samples. Significant differences in bacterial isolates composition in the field were detected between HRS and DRS samples (PERMANOVA by Adonis). (B) Spearman's rank correlations between relative abundance of discriminating OTUs (which were matched with isolates) and the pathogen *Ralstonia solanacearum* (Rs). Discriminating OTUs: enriched in HRS or DRS samples with linear discriminant analysis scores > 2 or < 2. (C) Differences in the abundances of discriminating OTUs (which were matched with isolates) between HRS and DRS samples at the phylum level. P values were calculated using Welch's t-test (P < 0.05).

Discussion

The rhizosphere microbiome is recognized as a major determinant of plant growth and health. Characterizing microbial communities in disease-suppressive soil is a crucial step towards developing management strategies to characterize microbial communities favoring crop health and productivity [32]. In the present study, we have examined bacterial communities occurring in healthy and diseased tomato rhizosphere soils collected from six geographically distant locations, where tomato wilt disease caused by *Ralstonia solanacearum* could be observed. Specifically, we analyzed bacterial community patterns with respect to location and disease status of the plant and tested effects of recovered isolates from diseased and healthy soils on *Ralstonia solanacearum* growth. As expected, diseased rhizosphere soil (DRS) samples harbored significantly higher densities of *Ralstonia solanacearum* as compared to healthy rhizosphere soil (HRS) samples, as previously observed for other soil-borne pathogens and plant species [138, 139]. HRS samples did contain an appreciable density of *Ralstonia solanacearum* (>10^6 CFU/g rhizosphere soil), suggesting that disease development may be impeded by the resident microbial community in the soil [28, 34]. Therefore, not only the absolute pathogen abundance, but also other rhizosphere microbial community feature, may determine the ultimate differences in disease severity in plants.

We found differences in the bacterial taxonomic compositions of HRS versus DRS samples (Fig. 1C and 3A), which is in agreement with previous results related to both plant- and human-associated microbiomes [140, 141]. Actinobacteria and Firmicutes

were two phyla that showed higher abundance in HRS as compared to DRS samples. These two phyla are known to include strains that can produce high levels of secondary metabolites that can act to inhibit plant pathogens [142-144]. Interestingly, the majority of Actinobacterial strains recovered in our study promoted the growth of *Ralstonia solanacearum,* and this was true for both DRS and HRS samples. This apparent discrepancy could relate to the eubiosis of host-associated microbial communities, which can potentially alter disease occurrence [145, 146]. For instance, Lee and colleagues found that higher abundances of Actinobacteria in healthy tomato rhizosphere did not directly antagonize *Ralstonia solanacearum* but instead helped to activate plant immunity, which limited disease development [147]. Geographic location was the greatest determinant of soil-borne microbial community structure (Fig. 1C). This was not unexpected, given the large impact of environmental factors such as soil type and pH on rhizosphere soil microbial communities [116, 148-150]. Despite the large differences across our study locations, we were still able to observe community characteristics specifically related to disease status, which is in line with previous observations relating microbial community patterns to plant as health status [28].

Although more than ten bacterial phyla could be recovered from the tomato rhizosphere samples using cultivation-independent high throughput sequencing, our isolated rhizobacteria were restricted to only four phyla. Disparity regarding the relative recovery across phyla is illustrated by the Acidobacteria, which was one of the top three most abundant phyla as determined by sequencing, but was not represented in our culture collection. In addition, we found 31.7% of our bacterial isolates were belonged

to Firmicutes, while this phylum was much less well represented in our ribosomal amplicon-based dataset. One possible explanation is that these Firmicutes are particularly amiable to the cultivation conditions used in our isolation procedure. Alternatively, Firmicutes may actually constitute a large fraction of the total microbiome, but their detection may be impaired by biases in PCR amplification, for instance due to mismatches within the priming sites used. Such biases related to the completeness and representativeness of strain collections is typically observed, potentially related to numerous factors, such as nutrient availability, oxygen level, temperature, pH, and growth factors [151]. Given these limitations, caution should be exercised when drawing conclusions from purely cultivation-based approaches, and our study could therefore only examine links between cultivation-dependent and -independent approaches for a subset of the total community. Improvements toward attaining more complete and representative strain collections could further help future efforts to link cultivation-independent and -dependent approaches.

Here, by combining both cultivation-dependent and -independent approaches, we linked bacterial isolates with bacterial community in the rhizosphere soils from the Nanjing site. Clear separation of bacterial community compositions between DRS and HRS samples (as assessed by principal coordinate analysis) was observed using either the whole tomato rhizosphere bacterial community (Fig. S2D) or using only OTUs that were highly related to isolates (Fig. 3A). However, as reported recently [152], we observed a discrepancy when comparing co-occurrence patterns of these OTUs with *Ralstonia solanacearum* in the field to the *in vitro* effects of the related bacterial

isolates on growth of *Ralstonia solanacearum*. Because the Illumina high throughput sequencing produced shorter reads than the near full-length Sanger sequences we had for isolates, several isolates could be related to the same OTU. Consequently, different isolates from the same OTU might show differences from each other in their *in vitro* effects on *Ralstonia solanacearum* growth and therefore how these interactions compare to observed co-occurrence patterns with *Ralstonia solanacearum* (Table S2). Our results indeed demonstrate that co-occurrence patterns do not necessarily reflect actual species interactions, as previously observed [153]. Positive or negative links within co-occurrence networks have been shown to be poor predictors of actual interactions upon examination of one-to-one effects interactions [154]. It has also been suggested that co-occurrence may be a result of dispersal limitation [155, 156], or common selection due to specific environmental factors, without actual direct or indirect interaction [152, 157]. Therefore, in our subsequent studies, we will use the library of rhizobacterial strains from this study to investigate the ecological interactions between rhizobacterial isolates and their impacts on pathogen suppression, by constructing lower complexity, higher controllable synthetic microbial communities [158]. In total, our study may help to guide efforts for targeted cultivation and application of potential biocontrol agents, and offers opportunities for future microbiota manipulation experiments to elucidate the biological mechanisms and interactions driving the observed effects.

Acknowledgements

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Supplementary materials

Table S1. Rhizosphere soil collections across China. Sampling locations and experimental design.

Sites	Location	Rhizosphere soil samples	
		Diseased	Healthy
Changsha (CS)	28°11′49"N, 112°58′42"E	12	12
Ningbo (NB)	29°52'00"N, 121°31'00"E	12	12
Nanchang (NC)	28°34'00"N, 115°56'00"E	11	11
Nanjing (NJ)	32°03'00"N, 118°57'00"E	11	12
Nanning (NN)	22°48′00"N, 108°22′00"E	12	12
Wuhan (WH)	30°58′00"N, 114°41′00"E	11	11

Table S2. The link between co-occurrence patterns and ecological interactions. The table shows co-occurrence patterns (Spearman's correlations) between the 39 discriminating OTUs which related to bacterial isolates and *Ralstonia solanacearum* in the field (Figure 3B), and effects of the bacterial isolates on the growth of *Ralstonia solanacearum*.

OTUs which related to isolates	Correlations between relative abundances of OUT and Rs	Effect of each isolate which related to OUT on Rs
Enriched in DRS		
OTU00004	Positive	1 isolate facilitated Rs
OTU00007	Positive	1 isolate inhibited Rs; 1 no effect; 8 facilitated
OTU00012	Not significant	6 isolates facilitated Rs
OTU00013	Positive	1 isolate inhibited Rs
OTU00017	Not significant	1 isolate inhibited Rs
OTU00018	Not significant	7 isolates facilitated Rs
OTU00019	Not significant	1 isolate had no effect on Rs
OTU00028	Not significant	3 isolates facilitated Rs
OTU00031	Not significant	1 isolate facilitated Rs
OTU00032	Not significant	1 isolate facilitated Rs
OTU00033	Not significant	1 isolate facilitated Rs
OTU00040	Not significant	1 isolate inhibited Rs
OTU00067	Not significant	3 isolates facilitated Rs
OTU00133	Not significant	1 isolate had no effect on Rs; 4 facilitated
OTU00168	Not significant	1 isolate facilitated Rs
OTU00535	Positive	1 isolate facilitated Rs
OTU00759	Not significant	1 isolate had no effect on Rs; 2 facilitated
OTU00885	Not significant	1 isolate facilitated Rs
OTU01006	Not significant	6 isolates facilitated Rs
OTU01136	Not significant	1 isolate inhibited Rs; 1 no effect; 8 facilitated
OTU01181	Not significant	1 isolate facilitated Rs
OTU01283	Positive	1 isolate had no effect on Rs; 5 inhibited
OTU01317	Not significant	1 isolate facilitated Rs; 1 no effect; 5 inhibited
OTU01365	Not significant	1 isolate facilitated Rs; 1 no effect; 5 inhibited

OTU01564	Not significant	1 isolate inhibited Rs
OTU01960	Not significant	1 isolate inhibited Rs
OTU02003	Positive	7 isolates facilitated Rs
OTU02838	Not significant	1 isolate inhibited Rs; 4 facilitated
OTU02991	Not significant	1 isolate inhibited Rs
Enriched in HRS		
OTU00002	Negative	2 isolates facilitated Rs
OTU00006	Not significant	2 isolates inhibited Rs; 3 facilitated
OTU00008	Negative	3 isolates inhibited Rs; 1 no effect; 10 facilitated
OTU00010	Not significant	2 isolates had no effect on Rs; 15 facilitated
OTU00106	Negative	26 isolates inhibited Rs
OTU00108	Not significant	1 isolate facilitated Rs
OTU00150	Negative	3 isolates facilitated Rs; 14 inhibited
OTU00263	Not significant	1 isolate inhibited Rs
OTU01304	Not significant	2 isolates facilitated Rs
OTU02263	Not significant	1 isolate facilitated Rs

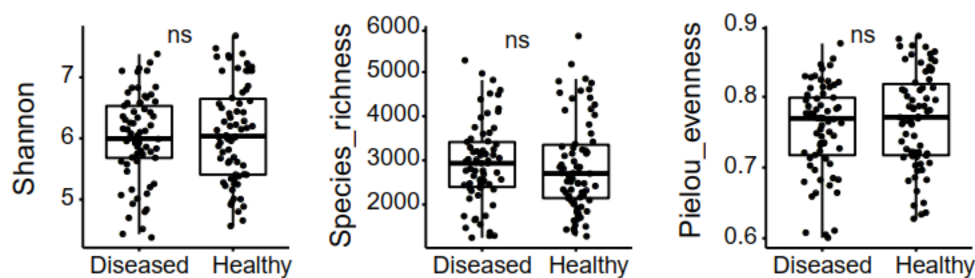

Figure S1. Comparison of the evenness and richness of bacteria in healthy rhizosphere soil (HRS) and diseased rhizosphere soil (DRS) of tomato plants using alpha diversity indices. Alpha diversity analysis of HRS and DRS samples collected from Changsha, Ningbo, Nanchang, Nanjing, Nanning and Wuhan China (Wilcoxon test, mean ± SD, n (diseased) = 69, n (healthy) = 70; ns, nonsignificant).

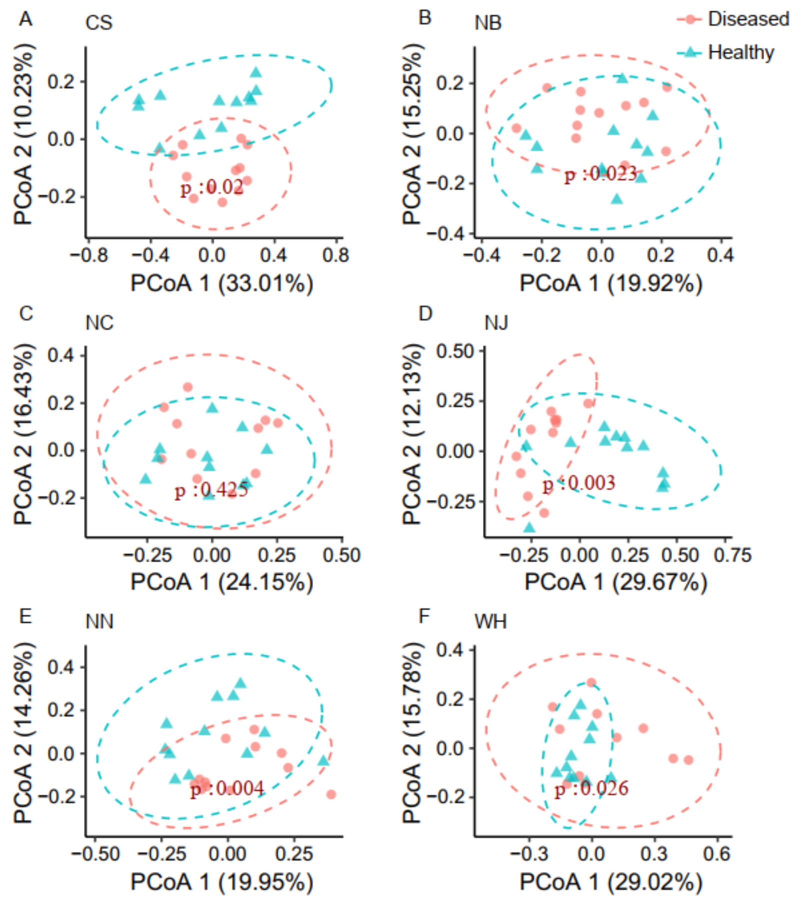

Figure S2. Principal coordinate analysis (PCoA) of bacterial community compositions in tomato rhizosphere in each site. Adonis was used to performed nonparametric multivariate analysis of variance using Bray–Curtis distance matrices for identifying the dissimilarity of bacterial community composition between the rhizosphere soil of diseased and healthy plants in each field. P values are indicated in each panel.

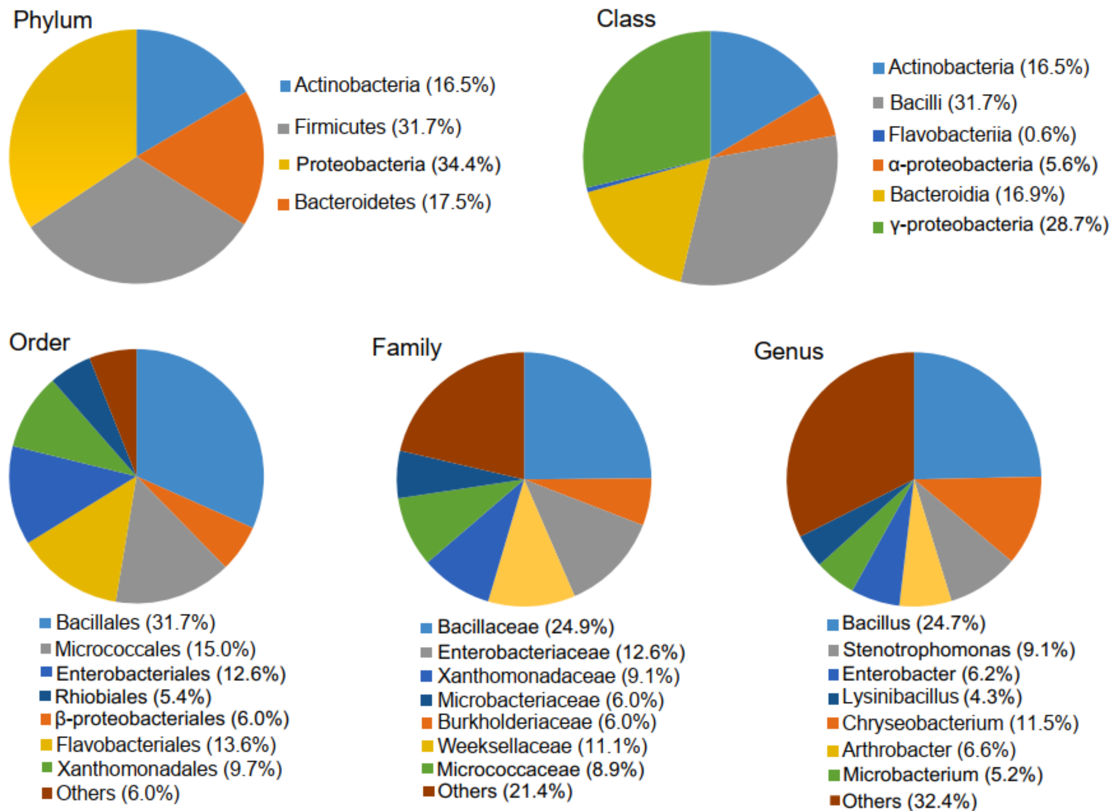

Figure S3. The diversity and taxonomic classification of rhizosphere bacterial isolates. A total of 515 rhizosphere isolates were identified by 16s rRNA sequencing and their closest relatives were determined using the NCBI database. Seven bacterial groups with highest relative abundances at the phylum, class, order, family, and genus levels are shown in the figure, while groups with relatively low abundances were merged and are presented as one group 'Others'. In all panels, percentage (%) values in brackets represent the proportion of each bacterial group of the total isolates (515 bacterial isolates).

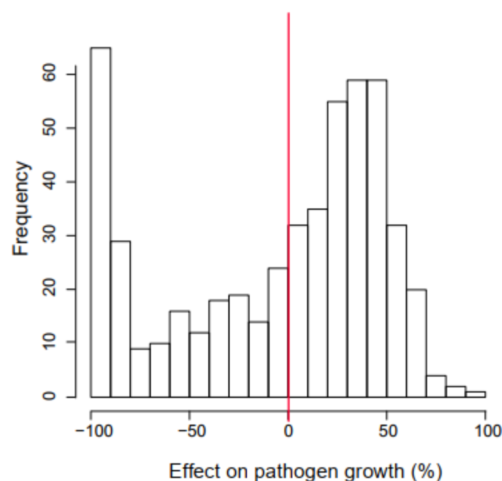

Figure S4. The histograms depict the effects of 515 rhizobacteria on *Ralstonia solanacearum* growth *in vitro*. The red vertical line represents no effect on *Ralstonia solanacearum* growth.

Chapter 3 Facilitation promotes invasions in plant-associated microbial communities

Running title: Facilitation increases invasibility

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Abstract

While several studies have established a positive correlation between community diversity and invasion resistance, it is less clear how species interactions within resident communities shape this process. Here we experimentally tested how antagonistic and facilitative pairwise interactions within resident model microbial communities predict invasion by the plant-pathogenic bacterium *Ralstonia solanacearum.* We found that facilitative resident community interactions promoted and antagonistic interactions suppressed invasions both in the lab and in the tomato plant rhizosphere. Crucially, pairwise interactions reliably explained observed invasion outcomes also in multispecies communities, and mechanistically, this was linked to direct inhibition of the invader by antagonistic communities (antibiosis), and to a lesser degree by resource competition between members of the resident community and the invader. Together our findings suggest that the type and strength of pairwise interactions can reliably predict the outcome of invasions in more complex multispecies communities.

Introduction

The characteristics of both resident communities and the invading species are important for determining the outcomes of biological invasions [67, 68]. From the resident community perspective, species diversity may be considered a shield to invasions and this effect is often attributed to competition for existing resources [51, 69] where highly diverse communities are thought to efficiently use all the available resource niches leaving no free space for invaders [72, 73]. In reality, diversity-invasion resistance relationships are more varied ranging from having neutral to even negative

effects [23, 74, 75] and are sensitive to environmental conditions [79, 88, 159, 160]. Furthermore, it has been shown that trophic network architecture (Wei *et al.* 2015), species identity effects [76] and food web connectance [161] are important predictors of invasions and are often linked with community diversity. For example, how species interact might be more important than the number of interacting species within the community (Wei *et al.* 2015), while invasion resistance may be mediated by certain keystone taxa [76]. However, the type and strength of resident species interactions have often been overlooked in the context of diversity-invasion resistance.

Resident species communities form complex ecological webs where multiple species may interact positively or negatively with each other [162]. Positive interactions between species at the same trophic level can result from facilitation or metabolic cross-feeding, where species benefit from the presence of each other [163]. Negative interactions may result from resource competition [51] or direct interference competition, where species directly suppress each other via antagonism [81-84]. These interactions may affect the outcomes of invasions in various ways. First, facilitation and competition are likely to affect the resource availability, and hence the availability of free resource niche space, and the likelihood of invasions [23, 73, 75, 77, 78]. It is predicted that highly competitive resident communities are less prone to invasions if they can efficiently utilize and consume resources that would otherwise be available for invaders [23, 73, 79]. This effect is expected to be especially strong in the resident communities that show a high degree of complementarity and hence compete less strongly with each other compared with the invader. In contrast, facilitative interactions between residents could potentially increase the number of resource niches via production of secondary metabolites or public goods that can also be utilized

by the invader [87-89]. Furthermore, competing species can inhibit each other directly by producing toxic metabolites, such as antibiotics. Depending on the spectrum of their activity, antibiotic compounds could have negative effects on both resident community species and the invader [80-85]. If the invader is particularly sensitive to toxins produced by the resident community, it is expected that antibiotic-mediated interference competition will constrain invasions. In contrast, if toxins have a disproportionally larger negative effect on the members of the resident community, such interference competition is expected to promote invasions [83, 86]. Resident community species interactions could further affect certain community-level properties such as ecological stability [164], which could have indirect effects on invasions [165].

In the present study, we explored to what extent the type (facilitative vs antagonistic) and strength of two-species resident community species interactions can predict invasions in complex multispecies bacterial communities. Experiments conducted within one trophic level suggest that pairwise bacterial competitions can predict three-species bacterial competitions with as high as 90% accuracy [166]. While predicting competitions in species-rich communities might require additional information about potentially emerging higher-order interactions [167-169], these findings suggest that qualitative information regarding species growth in pairwise co-cultures can be used to predict the competitive outcomes of up to 8-species communities [166]. Here we extend this approach beyond competition to concurrently explore the role of antagonistic and facilitative resident community interactions for biological invasions [170-172]. Our study system consisted of six non-pathogenic bacterial species (resident community), which were isolated from the tomato plant

rhizosphere, and the invader, the plant-pathogenic *Ralstonia solanacearum* bacterium. Specifically, we first characterized antagonistic and facilitative pairwise interactions within model resident bacterial communities and then directly tested how these interactions predict invasions in more complex multispecies communities both *in vitro* and *in vivo* in the tomato rhizosphere. We found that facilitative and antagonistic pairwise interactions reliably predicted invasions: facilitative resident communities were more prone to invasions, while antagonistic resident communities were invaded much less often. Mechanistically, this was linked to direct inhibition of the invader by antagonistic communities (antibiosis), and to a lesser degree by resource competition between the members of the resident community and the invader. Our results suggest that antagonism is an important determinant of community invasion resistance [72, 73], while facilitation might promote invasions by alleviating antagonistic interactions or by releasing vacant niche space for the invader.

Materials and methods

Bacterial strains and the assembly of resident communities

We used *Ralstonia solanacearum* strain QL-Rs1115 tagged with the pYC12-mCherry plasmid [58] as an invading pathogen in our experiments. *Ralstonia solanacearum* is a causal driver of bacterial wilt and capable of infecting various economically important crop species [99]. We set up model resident communities using six bacterial strains isolated from the tomato rhizosphere at the same location as the pathogen (Qilin [118° 57′ E, 32° 03′ N], Nanjing, China). Resident community species listed in Table S1 (*Flavobacterium johnsoniae* WR4, *Chryseobacterium daecheongense* WR21, *Delftia acidovorans* WR42, *Bacillus amyloliquefaciens* T-5, *Lysinibacillus sphaericus* HR92

and *Ralstonia pickettii* QL-A6) have previously been shown to provide protection for associated host plants by inhibiting *Ralstonia solanacearum* pathogen growth via resource competition or direct toxin production (Figure S1). The resident community composition (Table S2) was manipulated using biodiversity-invasion resistance framework where we modulated both resident community diversity (species richness) and composition and then directly tested how this affected community invasion resistance [51]. Invasion outcomes were then explained by interactions 1) within resident communities and 2) between resident community and the invader.

Determining pairwise interactions between resident community species

To quantify the type (facilitative, neutral or antagonistic), strength and direction of each pairwise interaction between resident species, we compared the growth of each species alone and in the presence of each of the other species in two-species co-cultures [173]. All mono-cultures were inoculated with a starting density of 10^5 cells per ml and the co-cultures were inoculated with half of this starting cell density of each species. Resident species were grown for 48h in liquid NA medium (glucose 10.0 g l^{-1}, tryptone 5.0 g l^{-1}, yeast extract 0.5 g l^{-1}, beef extract 3.0 g l^{-1}, pH 7.0) in 48-well microtiter plates (ending volume of 700 µl per well) at 30°C with shaking (170 rpm). Bacterial growth was measured as colony number units (CFU) per ml by serial dilution and plating on NA agar plates after 48h growth. All strains formed distinct colonies on agar plates and could be identified based on colony morphology (Figure S2).

The type of pairwise interaction between two species (here *i* and *j*) was determined by comparing the sum of endpoint of monoculture productivity (population densities) of *i* (MP_i) and monoculture productivity of *j* (MP_j) with the ending productivity of the two-species co-culture (CP_{i+j}). As suggested previously, the density of a species mixture is

expected to be exactly the sum of their growth in the monocultures if species do not interact [173]. Thus, we expected that the interaction between i and j would be facilitative if $CP_{i+j} > MP_i + MP_j$, antagonistic if $CP_{i+j} < MP_i + MP_j$ and neutral if $CP_{i+j} = MP_i + MP_j$.

In order to characterize directionality of pairwise interactions, we compared the ending productivity of each species (CP_i and CP_j) in two-species co-cultures with their ending productivities in monocultures. We then determined the directionality of interaction facilitative if species j had a positive effect on i ($\log_{10}(CP_i / MP_i) > 0$), antagonistic if $\log_{10}(CP_i / MP_i) < 0$ and neutral if $\log_{10}(CP_i / MP_i) = 0$. We also calculated the mean intensity of facilitation (MIF) of co-cultures as an average of $\log_{10}$-transformed pairwise interactions using the following formula: $MIF_{ij} = \frac{1}{2}[\log(CP_i / MP_i) + \log(CP_j / MP_j)]$. The two-species community was defined as facilitative when MIF > 0, antagonistic when MIF < 0 and neutral if MIF = 0.

Predicting resident species interactions in multispecies communities

We simply assumed that pairwise interactions would not change in the presence of additional species and then predicted resident species interactions in multispecies communities using two different indices: by calculating *i)* the proportion of facilitative pairwise interactions of all possible pairwise interactions and *ii)* predicted mean intensity of facilitation (PIF) in a multispecies community. For example, among the total number of all possible pairwise interactions of strains i, j and k, if one of these interactions was facilitative ($CP_{i+j} > MP_i + MP_j$), the proportion of facilitative interactions in this resident community was defined as 1/3. Analogous to MIF, we calculated the predicted intensity of facilitation (PIF) in multispecies co-cultures as the sum of $\log_{10}$-transformed interactions divided by the number of all possible pairwise interactions

within the given community using the following formula: $PIF = \frac{1}{C_n^2}\sum_{1}^{C_n^2} MIF_{ij}$, where MIF_{ij} refers the net intensity of one pairwise interaction between species *i* and *j* in a multispecies community, which has a total of C_n^2 number pairwise interactions. The communities were defined as facilitative when PIF > 0, antagonistic when PIF < 0 and neutral when PIF = 0. PIF thus accounted for both the strength and directionality of all potential pairwise interactions in a multispecies community.

Validating resident species interactions in multispecies communities

To verify resident species interactions in multispecies bacterial communities, we used qPCR to determine the ending densities of each resident species in monocultures and in all possible co-cultures (3, 4, 5 and 6 resident species communities). All communities were assembled in triplicate in liquid NA medium with a starting density of 10^5 cells per ml in monocultures and 33%, 25%, 20% and 16.7% of monoculture densities in 3, 4, 5 and 6 resident species communities, respectively. After 48h in 48-well microtiter plates at 30°C with shaking (170 rpm), bacterial DNA was extracted using e.Z.N.A. The bacterial DNA kit (OMEGA bio-tek) following manufacturer's protocol and extracted DNA was stored at -80°C. Species-specific primers were designed for each resident community member (Table S3, Figure S3) and qPCR analyses were carried out with an Applied Biosystems Step One Plus real-time PCR system using SYBR green I fluorescent dye detection in 20 - μl volumes with 10 μl of SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan), 2 μl of template, 0.4 μl Dye I, 0.8 μl of both forward and reverse primers (10 mM each) and 6 μl sterile water. The PCR was performed by initially denaturizing at 95°C for 30 s, cycling 40 times with a 5-s denaturizing step at 95°C, using a 34-s elongation/extension step at 60°C, and ending with melt curve analysis at 95°C for 15 s, at 60°C for 1 min, and at 95°C for 15 s. Each resident species community

sample was replicated three times.

The observed mean intensity of facilitation (OIF) was calculated using the observed species proportions in the communities based on qPCR data. Similar to PIF, we first determined to what extent the growth of each species was affected by the presence of other species in a given community (growth in the community vs. growth alone). OIF was then calculated according to the following formula: $OIF = \frac{1}{n}\sum \log(CP_i / MP_i)$. Communities were defined as facilitative when OIF > 0, antagonistic when OIF < 0 and neutral if OIF = 0. OIF was calculated only based on *in vitro* data and in the case of MIF, PIF and OIF, antagonism included the effects arising from both resource competition and direct inhibition via toxins.

Measuring resource competition and direct antagonism between the invader and resident community species

All bacteria were first grown to high densities (OD_{600}≈1.0) in liquid NA media overnight at 30°C with shaking (170 rpm), washed three times in 0.85% NaCl, and adjusted to an optical density of 0.5 at 600 nm (OD_{600}) with SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). We then measured the growth of the invader and all six resident community species individually on 48 different single-carbon resources (see Table S4) representative of tomato root exudates (Hu, Wei [84]). When the invader and resident community species both grew on the same resource (OD_{600}>0.05), their niches were considered to overlap regarding that given resource. In contrast, when only one strain grew on a specific resource, the niches were considered not to overlap [51]. This resource competition index estimated the ‘apparent’ resource competition assuming that interacting species would be competing for the same resources even when presented with multiple different resources.

Direct antagonism between the invader and resident community species was measured using supernatant assays [84]. Briefly, after 24h of growth in NA media, all bacterial monocultures were filtered to remove living cells (0.22 µm filter) after 20 µl of sterile supernatant from each resident species culture was mixed with 180 µl of an overnight-grown *Ralstonia solanacearum* culture (OD_{600} = 0.05, five-fold dilution in liquid NA). The control treatments were inoculated with 20 µl of sterile-filtered NA media instead of bacterial supernatant. All bacterial cultures were grown for 24h at 30°C with shaking (170 rpm) before measuring pathogen inhibition as optical density (OD 600 nm). Antagonism was defined as the percentage of reduction in pathogen growth by the supernatant compared to the control treatment for all possible invader-resident species two-species combinations.

Measuring invasion success in multispecies communities

a) Invasion success measured *in vitro*

All possible multispecies resident communities were assembled in triplicate in liquid NA medium with a starting density of 10^5 cells per ml (100%, 50%, 33%, 25%, 20% and 16.7% of monoculture densities in 1, 2, 3, 4, 5 and 6 resident species communities, respectively). Communities were then subsequently exposed to invasion by mCherry-tagged *Ralstonia solanacearum* (10^4 cells per ml) in 96-well plates at 30°C with shaking (170 rpm). After 48h, total bacterial densities were measured as optical density (OD 600 nm) and invasion success measured as the relative invader density to total bacterial densities using red mCherry protein fluorescence intensity (RFP; excitation: 587 nm, emission: 610 nm) with SpectraMax M5 spectrophotometer.

b) Invasion success measured *in vivo*

We used a 50-day-long greenhouse experiment with tomato plants to measure

invasion success *in vivo*. The soil was collected from a rice field in Wuxi (Jiangsu Province, China), sieved at 5 mm and homogenized and sterilized with gamma radiation. Surface-sterilized tomato seeds (*Lycopersicon esculentum*, cultivar "*Micro-Tom*") were germinated on water-agar plates for 3 days before sowing into seedling plates containing cobalt-60-sterilized seedling substrate (Huainong, Huaian Soil and Fertilizer Institute, Huaian, China). *Ralstonia solanacearum* invasion was tested in all possible two-species resident communities, and due to practical reasons, in 18 multispecies resident communities that varied in their predicted mean intensities of facilitation (Table S5).

Three replicates were used for each resident community, and one replicate consisted of a seedling plate that contained six germinated tomato plants (at the three-leaf stage of growth when grown on 700 g sterilized soil). Similar replication was also used for positive (only the invader) and negative (no bacteria) controls. After 3 days of growth on seedling plates, plants were inoculated with assembled resident communities using root drenching method at a final concentration of 10^8 CFU of bacteria g^{-1} soil [174]. Seven days after inoculation of resident communities, *Ralstonia solanacearum* was introduced to the roots of all plants at a final concentration of 10^7 CFU of bacteria g^{-1} soil. Tomato plants were then grown for 40 days in a greenhouse (with natural temperature variation ranging from 25°C to 35°C) and watered regularly with sterile water. Seedling plates were rearranged randomly every two days and disease progression monitored at every seven days. Forty days after inoculation of *Ralstonia solanacearum*, rhizosphere soil was collected from one plant per replicate seedling tray and the abundance of the invader determined with quantitative PCR as the abundance of *Ralstonia solanacearum*-specific *fliC* gene copy numbers [84].

Statistical analyses

To meet assumptions of normality and homogeneity of variance, invader densities measured *in vitro* and *in vivo* were log10-transformed. We first assessed the independent effects of the proportion of facilitative interactions and the mean intensity of facilitation based on pairwise resident community interaction on invasions (pathogen density and disease incidence). The type of interaction between resident community species pairs was included into models as a categorical variable (1= facilitation; 0= antagonism). In the case of multispecies communities, invasions were explained by three quantitative indices, the proportion of facilitative interactions within a community, the predicted mean intensity of facilitation (PIF) and the and observed mean intensity of facilitation (OIF). All indices were fitted as continuous variables and one separate model was used for each index that explained invader densities *in vitro* and *in vivo* and bacterial wilt disease incidence. Additional linear mixed models were used to test invasions as a function of a) niche overlap between resident community and the pathogen (niche preemption by the resident community), b) mean pathogen inhibition by the resident community and c) resident community species identity effects. All analyses were conducted with SPSS (V. 22) and R [175, 176].

Results

(a) Two-species resident species interactions predict invasions *in vitro* and *in vivo*

All species had both negative and positive effects on each other while the magnitude and directionality of these effects varied depending on specific species (Figure 1A). In particular, *B. amyloliquefaciens* was very antagonistic to the other resident community species. (Figure 1A). Furthermore, we found that 9 of the communities showed antagonistic, and 6 facilitative pairwise interactions with each other (Figure 1B, Table

S6). On average, facilitative two-species communities reached higher population densities (R^2=0.79, P<0.001, Figure S4), while antagonistic two-species communities were more inhibitory towards each other (R^2=0.32, P=0.029, Figure S5A). No relationship was found between resident species' resource niche overlap and observed mean intensity of facilitation (Figure S5B), which suggests that facilitation did not arise due to niche complementarity. Together these results suggest that the strength of direct inhibition was more important in explaining the type of pairwise interactions between resident community members compared to resource competition.

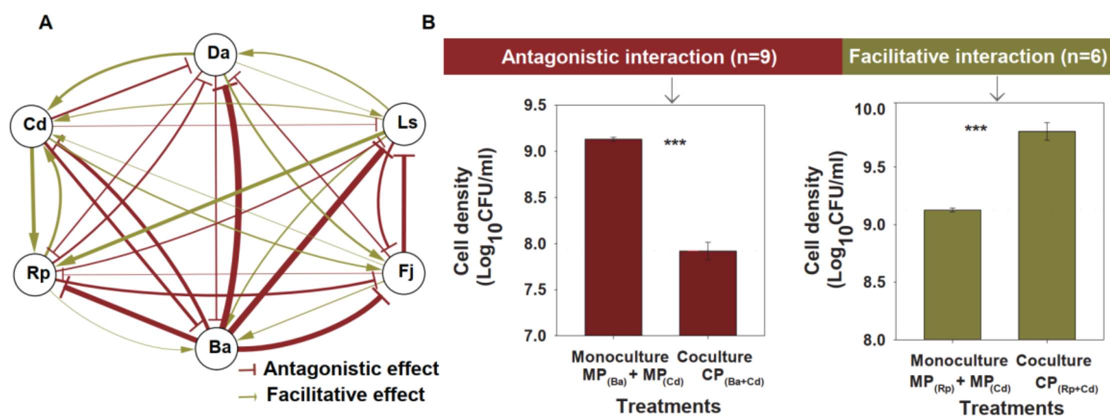

Figure 1. The type and relative strength of resident species pairwise interactions. (A) Network diagram showing the strength and directionality of all pairwise interactions between resident community species. The thickness of lines represents the strength and green and red color the facilitative or antagonistic effects between different species. (B) Nine of the fifteen pairwise interactions were on average antagonistic (co-culture density < monoculture density) and six facilitative (co-culture density > monoculture density). Panels show two examples: Left, antagonism between species Ba and Cd; Right, facilitation between species Rp and Cd. *** denotes for statistical significance at p < 0.001. All error bars denote for ± 1 s.e.m

To link the type of pairwise interaction with the likelihood of invasions, we compared *Ralstonia solanacearum* invasion success in facilitative and antagonistic two-species resident communities. Compared to positive controls (*Ralstonia solanacearum*-only:

red dashed line in Figure 2A-F), pathogen densities were significantly lower in the presence of resident species both *in vitro* and *in vivo*. The intensity of pathogen suppression could be predicted by the type of pairwise interactions between the resident species: pathogen density was significantly higher in facilitative compared to antagonistic communities *in vitro* ($F_{1,43}$=16.02, P<0.001, Figure 2A; R^2=0.49, P<0.0001, Figure 2B) and *in vivo* ($F_{1,43}$=24.40, P<0.001, Figure 2C; R^2=0.26, P=0.0021, Figure 2D). In line with these results, the bacterial wilt disease incidence was also higher in facilitative compared to antagonistic resident communities ($F_{1,43}$=9.03, P=0.004, Figure 2E; R^2=0.14, P=0.013, Figure 2F). Mechanistically, this could be explained by loss of pathogen inhibition as suggested by a negative correlation between the mean intensity of facilitation and direct invader suppression (R^2=0.45, P<0.0001, Figure S6). Together these results suggest that antagonistic two-species resident communities were more inhibitory not only towards themselves but also against the invader.

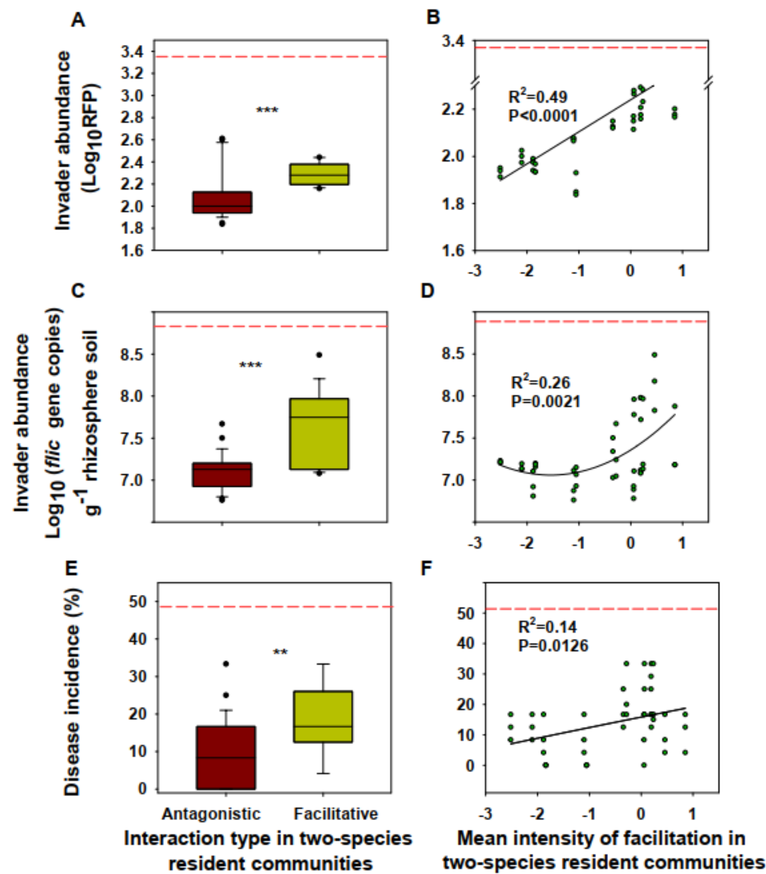

Figure 2. The type of pairwise resident community interactions predicts invasions *in vitro* and *in vivo*. (A) The *Ralstonia solanacearum* invader abundance in antagonistic and facilitative two-species resident communities measured *in vitro.* (B) The relationship between invader abundance and the mean intensity of facilitation in resident communities measured *in vitro*. (C) The relative invader abundance in antagonistic and facilitative two-species resident communities measured in the tomato rhizosphere 40 days after inoculation of the invader. (D) The relationship between invader abundance and the mean intensity of facilitation in resident communities measured *in vivo* in the tomato rhizosphere. (E) The bacterial wilt disease incidence (%) in antagonistic and facilitative pairwise resident communities 40 days after inoculation of the invader. (F) The relationship between disease incidence and the mean intensity of facilitation in resident communities measured *in vivo* in the tomato rhizosphere. In all panels, the red dashed lines show the baseline for positive control treatments (invader-only). In panels, B, D and F, values below and above zero denote for antagonistic and facilitative pairwise resident communities, respectively. Two and three stars denote for statistical significance at $p < 0.01$ and $p < 0.001$ significance levels, respectively. All the bars denote for ± 1 s.e.m.

(b) Predicting and validating invasions in multispecies communities based on pairwise interactions

Interactions within the resident communities could well explain the invader abundance *in vitro* (R^2: 0.45, P<0.0001) and *in vivo* (R^2: 0.28, P<0.0001), and bacterial wilt disease incidence (R^2: 0.18, P=0.0002) *in vivo* (Table 1). The proportion of facilitative interactions were well explained by the increase in invader density in all tested resident communities *in vitro* (R^2=0.35, P<0.0001, Figure 3A). Similarly, both the density of the invader in the tomato rhizosphere (R^2=0.22, P=0.0004, Figure 3B) and bacterial wilt disease incidence (R^2=0.21, P=0.0004, Figure 3C) increased significantly with increasing proportion of facilitative interactions within the resident communities. The predicted mean intensity of facilitation explained well the increase in invader density *in vitro* (R^2=0.45, P<0.0001, Figure 3D) and *in vivo* (R^2=0.21, P=0.0005, Figure 3E) and correlated positively with bacterial wilt disease incidence (R^2=0.19, P=0.0193, Figure 3F). The predicted and observed mean intensities of facilitation correlated positively with each other (R^2=0.44, P<0.0001, Figure S7), demonstrating that pairwise interactions can be used to predict interactions in multispecies communities. As expected, invader densities also increased with increasing observed mean intensity of facilitation both *in vitro* (R^2=0.26, P<0.0001, Figure 3G) and *in vivo* (R^2=0.17, P=0.0019, Figure 3H). However, the observed mean intensity of facilitation did not correlate significantly with bacterial wilt disease incidence (Figure 3I).

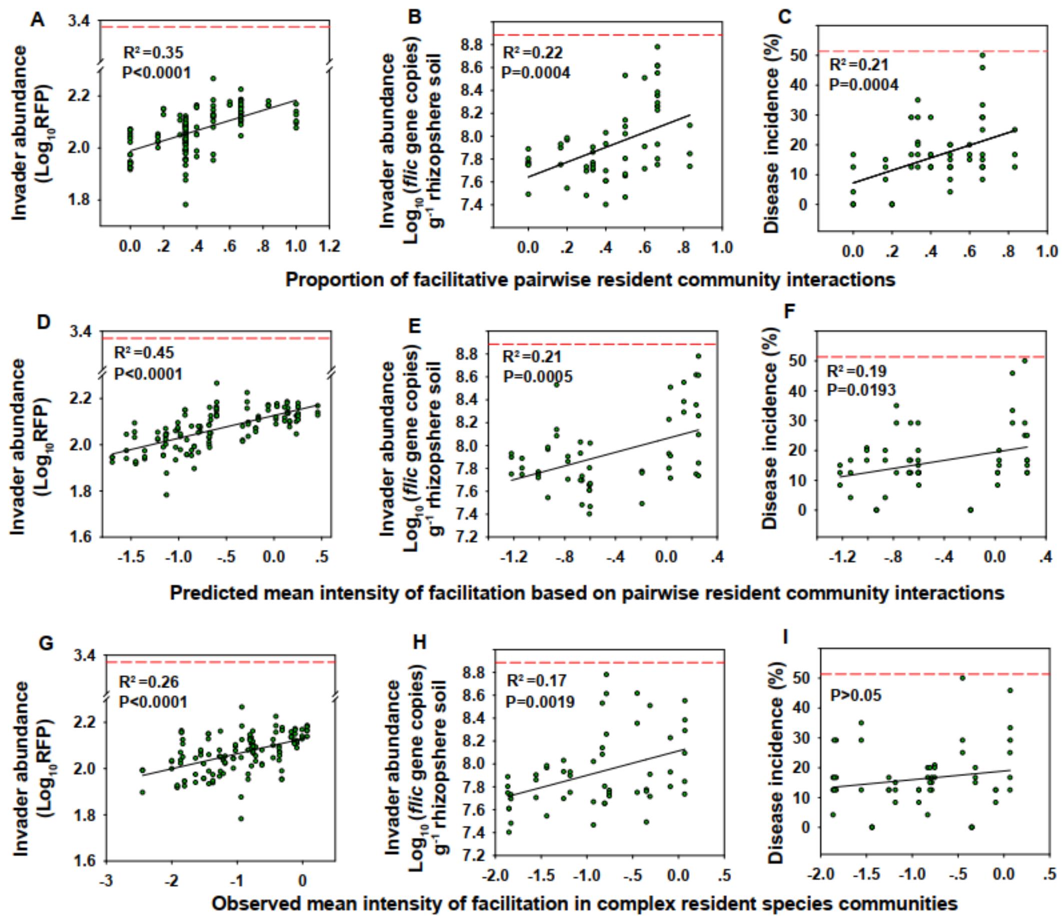

Figure 3. The relationship between invader abundance and disease incidence with predicted and observed mean intensities of facilitation within multispecies communities. (A-B) The relationship between invader abundance and the proportion of facilitative interactions in the resident communities measured *in vitro* and *in vivo*, respectively. (C) The relationship between bacterial wilt disease incidence (%) and the proportion of facilitative interactions in the resident communities. (D-E) The relationship between invader abundance and the predicted mean intensity of facilitation in the resident communities measured *in vitro* and *in vivo*, respectively. (F) The relationship between bacterial wilt disease incidence (%) and the predicted mean intensity of facilitation in the resident communities. (G-H) The relationship between invader abundance and the observed mean intensity of facilitation in the resident communities measured *in vitro* and *in vivo*, respectively. (I) The relationship between bacterial wilt disease incidence (%) and the observed mean intensity of facilitation in the resident communities. In all panels, red dashed lines show the baseline of invader densities in control treatments (invader-only). In panels D-I, values below and above zero denote for antagonistic and facilitative resident communities, respectively.

The low invasion success observed in antagonistic resident communities could be attributed to high levels of direct inhibition of the invader and/or high resource niche overlap between the invader and resident community members. We found that both direct pathogen inhibition and high resource niche overlap reduced invader densities *in vitro* and *in vivo*, while only direct pathogen inhibition significantly reduced the disease incidence (Table 1). Direct pathogen suppression correlated negatively with both predicted and observed mean intensities of facilitation suggesting that antagonistic multispecies communities were more inhibitory to the invader (Figure S8). The species *B. amyloliquefaciens* and *F. johnsoniae* had strong negative effects on pathogen densities *in vitro* and *in vivo* (Table S7). However, only *B. amyloliquefaciens* had a significant negative effect on disease incidence, while species *C. daecheongense* had a slightly positive effect on disease incidence (Table S7). Together these results suggest that pairwise resident community interactions can predict invasions in multispecies communities *in vitro* and *in vivo* and that these effects were primarily linked with direct pathogen suppression.

Table 1 Two different general linear mixed models (GLM) comparing the interactions within resident communities (a), and interactions between community and invader (b) on invader abundance *in vitro* and *in vivo* and disease incidence measured *in vivo*.

	Invader relative abundance *in vitro*			Invader abundance measured *in vivo*			Disease incidence measured *in vivo*		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
(a) Interactions within resident communities									
Proportion of facilitative interactions	1	0.02	0.885	↑ **1**	**11.82**	**0.0009**	↑ **1**	**7.01**	**0.009**
Predicted Mean intensity of interactions	↑ **1**	**129.8**	**<2E-16**	↑ **1**	**14.29**	**0.0003**	↑ **1**	**12.66**	**0.0006**
Observed Mean intensity of interactions	↑ **1**	**8.18**	**0.005**	↑ **1**	**11.24**	**0.001**	1	1.56	0.215
No. of Residuals	167			95			95		
Model summary	R^2: 0.45	AIC: -303.69		R^2: 0.28	AIC: 111.10		R^2: 0.18	AIC: 731.07	
(b) Interaction between community and invader									
Niche breadth									
Niche overlap between the invader and resident communities	↓ **1**	**13.76**	**0.0003**	↓ **1**	**8.62**	**0.004**	1	1.29	0.258
Direct invader inhibition by resident communities	↓ **1**	**79.15**	**8.881E-16**	↓ **1**	**5.24**	**0.024**	↓ **1**	**12.46**	**0.0006**
No. of Residuals	168			96			96		
Model summary	R^2: 0.36	AIC: -277.97		R^2: 0.13	AIC:128.58		R^2: 0.13	AIC: 735.84	

All response variables were treated as continuous variables. The table shows the most parsimonious models selected based on the AIC information. The up and downwards arrows denote for positive and negative effects on response variables, respectively.

Discussion

Here we studied how resident community interactions are linked with invasions in bacterial plant rhizosphere communities. We found that facilitative two- species communities were invaded more easily both in the laboratory and rhizosphere compared to antagonistic resident communities. Furthermore, we could use the pairwise interactions to predict invasion outcomes in multispecies communities containing up to 6 resident species. Specifically, communities characterized by a high proportion of facilitative pairwise interactions, and high predicted and observed mean intensities of facilitation, were more susceptible to invasions. Mechanistically, this was linked to direct inhibition of the invader by antagonistic communities (antibiosis), and to a lesser degree by resource competition between the members of the resident community and the invader. Together these findings suggest that outcomes of relatively simple pairwise interactions can be used to predict invasions in multispecies microbial communities especially when antagonism and facilitation are strongly linked with the resistance to invasion.

Invasion resistance has been thus far mainly considered from the perspective of resource competition and niche preemption [51, 69, 73]. Our results suggest that facilitative interactions should also be considered in the context of invasions. While it is difficult to pinpoint the exact mechanism between facilitation and invasion, the most likely explanation is the loss of pathogen inhibition along with the increase in the mean intensity of facilitation (Figure S6). This is in line with a previous finding where the increase in the antagonistic activity was found to increase the invasion resistance of *Pseudomonas* resident communities [84]. Another explanation could be that facilitative resident communities were less efficient at competing for resources with the invader

compared to antagonistic resident communities. However, this likely played a relatively small role as resource niche overlap had the only significant negative effect on the invader density when measured *in vitro* and *in vivo* but not on disease incidence (Table 1). It is also possible that our resource competition indices measured *in vitro* overestimated the strength of resource competition or underestimated the size of the niche space in the rhizosphere leading to weak correlation with invasions. Furthermore, facilitative interactions could have increased the niche space in the resident communities in favor of the invader, which could have promoted invasions as a side effect [89]. For example, previous studies have demonstrated that bacteria can show diet preference between different dietary glycans, which can prolong species coexistence in co-cultures [90]. Such dietary preference might leave some resources less utilized, providing an opportunity for invasion [91]. It has also been shown that the breakdown of polysaccharides can allow coexistence of species that liberate polysaccharide breakdown products (PBPs), which are consumed by other species that are unable to grow on the polysaccharides alone (recipients) [177]. Facilitative interactions could thus potentially favor the invader if it is unable to grow on the primary substrates on its own [171]. While it is difficult to validate these hypotheses based on our data, we found that facilitative communities were more productive in general and reached higher total cell densities when cultured together compared to alone (Figure S4). This supports the idea that facilitative resident species were benefitting from the presence of each other (for example via cross-feeding), which could also have benefitted the invader by creating vacant niche space. The carrying capacity of resident communities could thus be an important predictor of biological invasions [178].

In addition to within-resident community interactions, the interactions between resident communities and the invader were also good predictors of invasions, albeit to

a lesser extent (Figure 3 and Table 1). While it remains unclear what exact compounds were produced by the resident communities, previous studies have shown that soil bacteria are capable of producing a wide variety of antimicrobials that often suppress *Ralstonia solanacearum* [84, 85]. For example, the *B. amyloliquefaciens* T-5 strain used in this study has been shown to efficiently suppress *Ralstonia solanacearum* both in the lab and plant rhizosphere [85] and this strain also had the greatest negative effect on the pathogen densities and disease incidence in this study (Figure S1). In addition, the strain *F. johnsoniae* had a negative effect on pathogen densities both *in vitro* and *in vivo*. Together these results suggest that pathogen suppression via toxins was likely mediated by the presence of these species.

In general, pairwise resident community interactions predicted well the observed invasion outcomes in multispecies communities (Figure 3, Table 1). However, no correlation was found between the observed mean intensity of facilitation and bacterial wilt disease incidence (Figure 3I). This suggests that while *in vitro* mechanisms (resource competition and antibiosis) can robustly predict invasions in more complex *in vivo* environments [51, 84], they do not account for all aspects of more complex natural environments. There are many potential explanations for these discrepancies that should be validated in future studies. First, investigating the role of microbe-mediated plant immunity is important as both pathogenic and non-pathogenic bacteria can trigger or suppress plant immunity [179, 180]. Furthermore, several bacterial secondary metabolites involved in pathogen suppression also impact plant immunity: for example, 2, 4-diacetylphloroglucinol (DAPG) produced by fluorescent *Pseudomonas* spp. [181] or lipopeptide surfactins produced by *Bacillus subtilis* [182] have a such dual-function. Second, the rhizosphere bacterial communities we used were rather simple, and hence, predictions based on pairwise species interactions

should be tested in more complex multi-trophic communities in the future. Lastly, our predictive indexes only estimated the mean net effects and did not distinguish if both or only one of the species benefitted and vice versa [173]. While this approach seems to be a good predictor of invasion outcomes, accounting for the directionality of interactions and potential emerging higher-order interactions [167-169] is likely to improve these predictions.

In conclusion, our results suggest that qualitative information regarding species growth in pairwise co-cultures can be used to predict the outcomes of invasions in multispecies communities. Even though our results can't be broadly applied across different biological problems, they could offer direct solutions in the context of crop protection. Bacterial pathogens impose an ever-increasing threat for agriculture [183-185] and recent evidence suggests that the rhizosphere microbiome plays an essential role in controlling the onset of disease [15, 186]. Understanding the characteristics that make certain microbiomes more resistant to invasions could potentially allow one to harness beneficial bacterial communities for crop protection. While recent studies have shown that microbial diversity alone may be such important characteristic [51, 84] we here suggest that highly antagonistic microbial communities might also be efficient at constraining pathogen invasions.

Acknowledgements

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to Zhong Wei), and the Qing Lan Project (funding to Yangchun Xu and Zhong Wei). Ville-Petri Friman is supported by the Wellcome Trust [reference no. 105624] through the Centre for Chronic Diseases and Disorders (C2D2) and Royal Society Research Grant (RSG\R1\180213) at the University of York. Alexandre Jousset is supported by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (ALW.870.15.050) and the Koninklijke Nederlandse Akademie van Wetenschappen (530-5CDP18). We thank Jack Law for insightful comments and suggestions for the manuscript.

Supplementary materials

Table S1. Bacterial strains used in this study.

Bacterial strain ID	Taxonomic affiliation (GenBank accession number)	Bacterial abbreviation used in this study	Reference
WR4	*Flavobacterium johnsoniae* (CP000685)	Fj	(Huang et al. 2013)
T-5	*Bacillus amyloliquefaciens* (JF899265)	Ba	(Tan et al. 2013)
HR92	*Lysinibacillus sphaericus* (CP000817)	Ls	(Huang et al. 2013)
WR21	*Chryseobacterium daecheongense* (HQ220102)	Cd	(Huang et al. 2013)
WR42	*Delftia acidovorans* (AM180725)	Da	(Huang et al. 2013)
QL-A6	*Ralstonia pickettii* (HQ267096)	Rp	(Wei *et al.* 2013)
QL- Rs1115	*Ralstonia solanacearum* (GU390462) tagged with red fluorescent marker (PYC12-M plasmid)	Rs	(Wei et al. 2011; Tan et al. 2016)

Table S2. Table showing all possible resident species community combinations in all richness levels (N=64). Table rows show different communities and table columns show the absence (0) or presence (1) of given species within the community.

Community ID	**Species abbreviation**						**Resident community species richness**
	Fj	**Cd**	**Da**	**Ba**	**Ls**	**Rp**	
Control	0	0	0	0	0	0	0
1	1	0	0	0	0	0	1
2	0	1	0	0	0	0	1
3	0	0	1	0	0	0	1
4	0	0	0	1	0	0	1
5	0	0	0	0	1	0	1
6	0	0	0	0	0	1	1
7	1	1	0	0	0	0	2
8	1	0	1	0	0	0	2

9	1	0	0	1	0	0	2
10	1	0	0	0	1	0	2
11	1	0	0	0	0	1	2
12	0	1	1	0	0	0	2
13	0	1	0	1	0	0	2
14	0	1	0	0	1	0	2
15	0	1	0	0	0	1	2
16	0	0	1	1	0	0	2
17	0	0	1	0	1	0	2
18	0	0	1	0	0	1	2
19	0	0	0	1	1	0	2
20	0	0	0	1	0	1	2
21	0	0	0	0	1	1	2
22	1	1	1	0	0	0	3
23	1	1	0	1	0	0	3
24	1	1	0	0	1	0	3
25	1	1	0	0	0	1	3
26	1	0	1	1	0	0	3
27	1	0	1	0	1	0	3
28	1	0	1	0	0	1	3
29	1	0	0	1	1	0	3
30	1	0	0	1	0	1	3
31	1	0	0	0	1	1	3
32	0	1	1	1	0	0	3
33	0	1	1	0	1	0	3
34	0	1	1	0	0	1	3
35	0	1	0	1	1	0	3
36	0	1	0	1	0	1	3

37	0	1	0	0	1	1	3
38	0	0	1	1	1	0	3
39	0	0	1	1	0	1	3
40	0	0	1	0	1	1	3
41	0	0	0	1	1	1	3
42	1	1	1	1	0	0	4
43	1	1	1	0	1	0	4
44	1	1	1	0	0	1	4
45	1	1	0	1	1	0	4
46	1	1	0	1	0	1	4
47	1	1	0	0	1	1	4
48	1	0	1	1	1	0	4
49	1	0	1	1	0	1	4
50	1	0	1	0	1	1	4
51	1	0	0	1	1	1	4
52	0	1	1	1	1	0	4
53	0	1	1	1	0	1	4
54	0	1	1	0	1	1	4
55	0	1	0	1	1	1	4
56	0	0	1	1	1	1	4
57	1	1	1	1	1	0	5
58	1	1	1	1	0	1	5
59	1	1	1	0	1	1	5
60	1	1	0	1	1	1	5
61	1	0	1	1	1	1	5
62	0	1	1	1	1	1	5
63	1	1	1	1	1	1	6

Table S3. Specific qPCR primers designed for each resident bacterial species.

Resident bacterial species	Forward primer (5' - 3')	Reverse primer (5' - 3')
Fj	CACTCCTATGTATAGGAGCTTGACG	AGTATCAATGGCCGTTCCAC
Cd	CGTTTTTGGGTTTTCGGAT	TGGTAAGGTTCCTCGCGTAT
Da	GAAGTTTCCAGAGATGGATTCG	CCACCTATAAGGGCCATGAG
Ba	ACAAGTGCCGTTCAAATAGGG	GCCACTGGTGTTCCTCCAC
Ls	GACATCCCGTTGACCACTG	ATTAGCTCCCTCTCGCGAG
Rp	CTCGAAAGAGAAAGTGGACACAG	GCTTGGCAACCCTCTGTATG

Table S4. List of 48 different carbon resources included in the synthetic plant exudate media, which was used to determine the strength of resource competition (niche breadth and overlap) between the invader and resident community species.

Carbon name and abbreviation	Carbon classification	Carbon ID
Acetic aicd (Ace)	Organic acid	1
L-Alanine (Ala)	Amino acid	2
β-Alanine (Bala)	Amino acid	3
L-Arginine (Arg)	Amino acid	4
Ascorbic acid (Asc)	Organic acid	5
L-Asparagine (Asn)	Amino acid	6
γ-Aminobutyric acid (Ami)	Amino acid	7
Citric acid (Cit)	Organic acid	8
Citrulline (Cin)	Amino acid	9
Ethanolamine (Eth)	Other	10
Formic acid (For)	Organic acid	11
Fructose (Fruc)	Sugar	12
Galacturonic acid (Galac)	Organic acid	13
Glucose (Glu)	Sugar	14
L-Glutamine (Gln)	Amino acid	15
Glutaric acid (Glut)	Organic acid	16
L-Glycine (Gly)	Amino acid	17
Glycolic acid (Glyc)	Organic acid	18
L-Histidine (His)	Amino acid	19
Isoleucine (Iso)	Organic acid	20
Lactic acid (Lac)	Organic acid	21
L-Lysine (Lys)	Organic acid	22
L-Leucine (Leu)	Organic acid	23
Maleic acid (Male)	Organic acid	24

Malic acid (Mal)	Organic acid	25
Malonic acid (Malon)	Organic acid	26
L-Methionine (Met)	Organic acid	27
Myoinositol (Mino)	Other	28
2-Oxoglutaric (Oxo)	Organic acid	29
L-Phenyalanine (Phe)	Organic acid	30
L-Proline (Pro)	Organic acid	31
Pyruvic acid (Pyr)	Organic acid	32
L-Serine (Ser)	Organic acid	33
Succinic acid (Succ)	Organic acid	34
Sucrose (Sucr)	Sugar	35
Tartaric acid (Tar)	Organic acid	36
L-Threonine (Thr)	Organic acid	37
L-Tryptophan (Try)	Organic acid	38
L-Valine (Val)	Organic acid	39
Maltose (Mal)	Sugar	40
L-Arabinose (Ara)	Sugar	41
D-Galactose (Gal)	Sugar	42
D-Mannose (Man)	Sugar	43
D-Xylose (Xyl)	Sugar	44
D-Ribose (Rib)	Sugar	45
D-Mannitol (Mann)	Sugar	46
Inosine (Ino)	Other	47
Oxalic acid(Oxa)	Organic acid	48

Table S5. The predicted mean intensity of facilitation (PIF) in 18 multi-species resident communities used in *in vivo* invasion experiments with tomato. Table rows show different communities and table columns show the absence (0) or presence (1) of given species in these communities. Communities with PIF values below and above zero were defined as antagonistic and facilitative on average, respectively.

Community ID	Species abbreviation						Predicted mean intensity of facilitation
	Fj	Cd	Da	Ba	Ls	Rp	
25	1	1	0	0	0	1	0.2337
28	1	0	1	0	0	1	-0.1915
34	0	1	1	0	0	1	0.2527
40	0	0	1	0	1	1	0.1371
47	1	1	0	0	1	1	0.0201
48	1	0	1	1	1	0	-1.2193
49	1	0	1	1	0	1	-1.1359
52	0	1	1	1	1	0	-0.8643
53	0	1	1	1	0	1	-0.7753
54	0	1	1	0	1	1	0.2524
55	0	1	0	1	1	1	-0.6039
56	0	0	1	1	1	1	-1.0082
58	1	1	1	1	0	1	-0.6632
59	1	1	1	0	1	1	0.0316
60	1	1	0	1	1	1	-0.6759
61	1	0	1	1	1	1	-0.9325
62	0	1	1	1	1	1	-0.5999
63	1	1	1	1	1	1	-0.6054

Table S6. The interaction type and mean intensity of pairwise facilitation (MIF) of six resident bacterial species. Communities with MIF values below and above zero were defined as antagonistic and facilitative on average, respectively.

Community ID (ID shown in Table S1)	Community composition (species abbreviations shown in Table S1)	Interaction type	Mean Intensity of pairwise facilitation (MIF)
15	Cd + Rp	Facilitative	0.85
21	Ls + Rp	Facilitative	0.46
17	Da + Ls	Facilitative	0.24
7	Fj + Cd	Facilitative	0.19
12	Cd + Da	Facilitative	0.19
14	Cd + Ls	Facilitative	0.06
8	Fj + Da	Antagonistic	-0.05
18	Da + Rp	Antagonistic	-0.28
11	Fj + Rp	Antagonistic	-0.34
13	Cd + Ba	Antagonistic	-1.05
10	Fj + Ls	Antagonistic	-1.1
20	Ba + Rp	Antagonistic	-1.84
9	Fj + Ba	Antagonistic	-1.88
19	Ba + Ls	Antagonistic	-2.1
16	Da + Ba	Antagonistic	-2.52

Table S7. General linear mixed models (GLM) comparing the resident community richness and resident species identities on invader abundance *in vitro* and *in vivo* and disease incidence measured *in vivo*.

	Invader relative abundance *in vitro*			**Invader abundance measured *in vivo***			**Disease incidence measured *in vivo***		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Factors									
Resident community richness	Not retained			↑ **1**	**25.87**	**<0.0001**	Not retained		
Ba	↓ **1**	**95.28**	**<0.0001**	↓ **1**	**17.04**	**<0.0001**	↓ **1**	**8.90**	**0.004**
Fj	↓ **1**	**19.96**	**<0.0001**	↓ **1**	**6.99**	**0.01**	Not retained		
Cd	1	2.20	0.14	Not retained			↑ **1**	**5.73**	**0.019**
Rp	Not retained			1	2.1	0.15	Not retained		
Ls	Not retained			Not retained			Not retained		
Da	Not retained			Not retained			1	2.19	0.142
No. of Residuals	185			94			95		
Model summary	R^2: 0.39 AIC: -146.37			R^2: 0.36 AIC:102.34			R^2: 0.15 AIC: 734.91		

All response variables were treated as continuous variables. The table shows the most parsimonious models selected based on the AIC information. The up and downwards arrows denote for positive and negative effects on response variables, respectively. Fj, Cd, Da, Ba, Ls, Rp represent *Flavobacterium johnsoniae, Chryseobacterium daecheongense, Delftia acidovorans, Bacillus amyloliquefaciens, Lysinibacillus sphaericus, Ralstonia pickettii,* respectively.

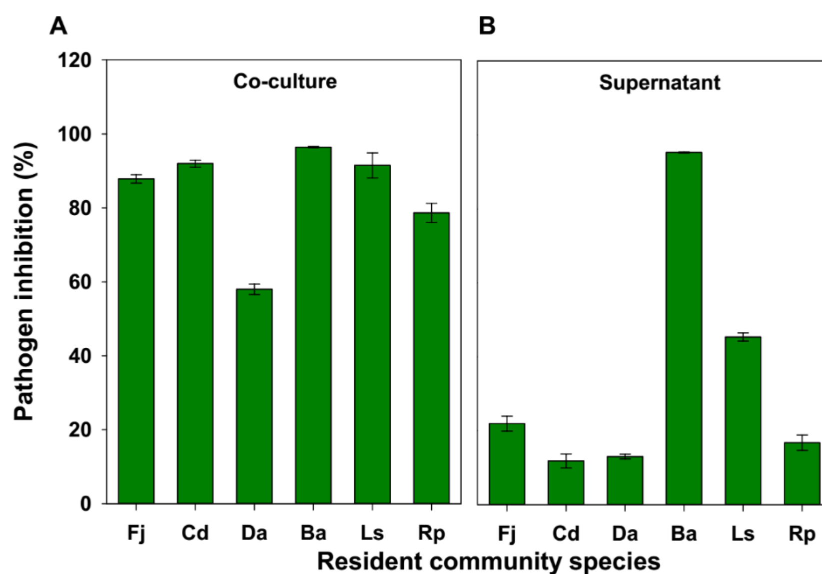

Figure S1. (A) All resident community species inhibited the growth of *Ralstonia solanacearum* in co-cultures. (B) The supernatant of all resident community species inhibited pathogen growth but in general to lesser extent compared to (A). All bars show mean ± SE of three measurement replicates and zero inhibition denotes for pathogen growth alone (A) or in the absence of resident species supernatant (B). Fj, Cd, Da, Ba, Ls, Rp represent *Flavobacterium johnsoniae, Chryseobacterium daecheongense, Delftia acidovorans, Bacillus amyloliquefaciens, Lysinibacillus sphaericus, Ralstonia pickettii,* respectively.

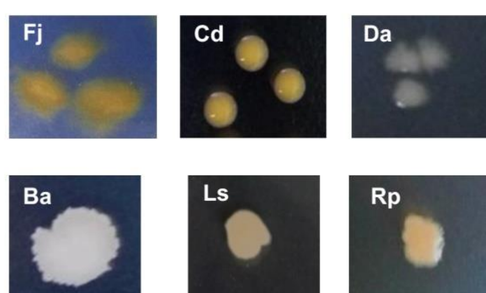

Figure S2. Distinct colonies on agar plates formed by six resident bacterial strains used in this study. Fj, Cd, Da, Ba, Ls, Rp represent *Flavobacterium johnsoniae, Chryseobacterium daecheongense, Delftia acidovorans, Bacillus amyloliquefaciens, Lysinibacillus sphaericus, Ralstonia pickettii,* respectively.

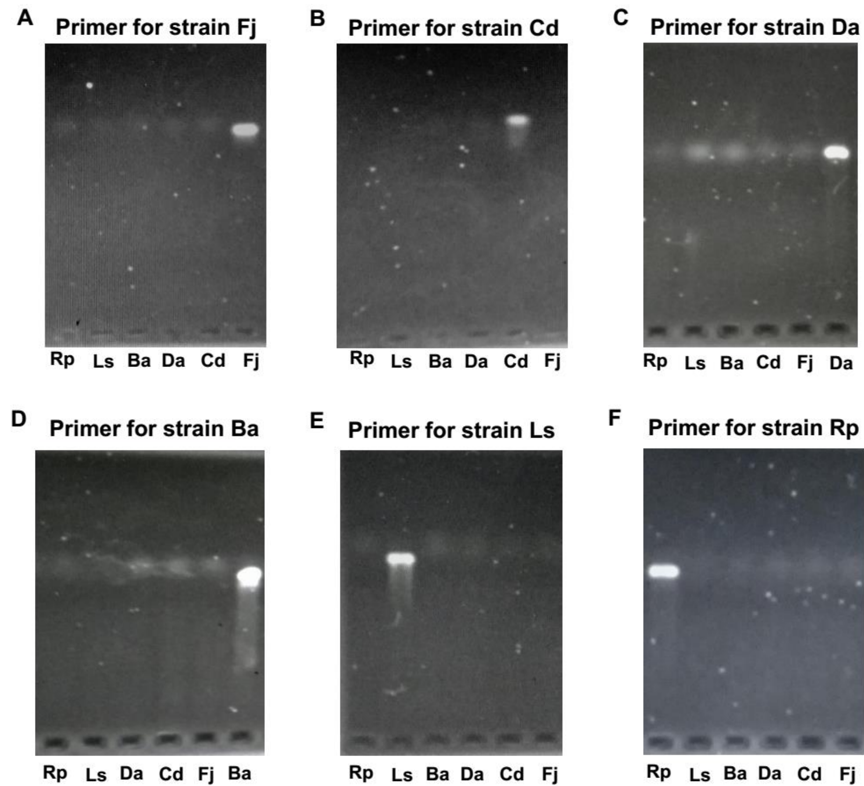

Figure S3. Specificity of specific qPCR primers designed for each resident bacterial species used in this study. Gel electrophoresis picture of six resident bacterial strains amplified by primers of (A) Fj (*Flavobacterium johnsoniae*), (B) Cd (*Chryseobacterium daecheongense*), (C) Da (*Delftia acidovorans*), (D) Ba (*Bacillus amyloliquefaciens*), (E) Ls (*Lysinibacillus sphaericus*), (F) Rp (*Ralstonia pickettii*), respectively.

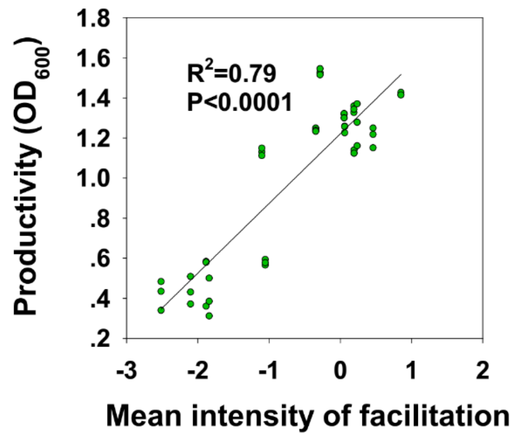

Figure S4. The relationship between productivity (biomass measured as OD_{600} after 48 h growth in NA media) and mean intensity of facilitation (MIF) in two-species resident communities. Communities with MIF values below and above zero were defined as antagonistic and facilitative on average, respectively.

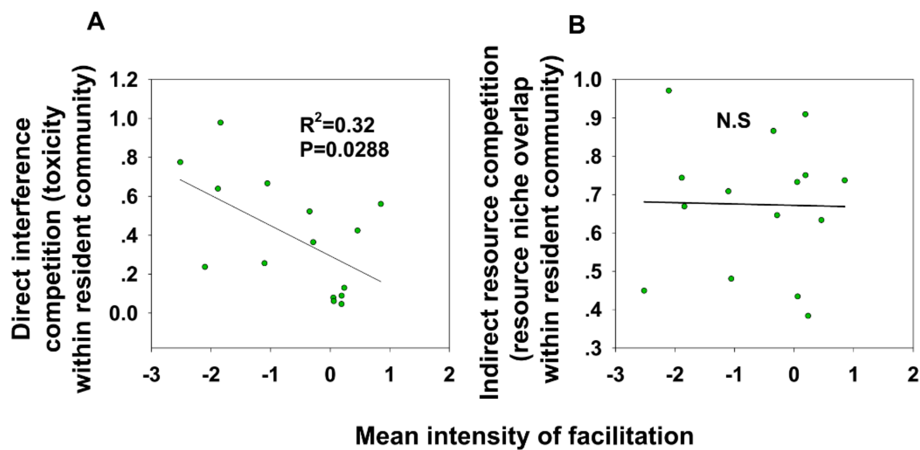

Figure S5. The relationship between mean intensity of facilitation (MIF) and direct interference (antagonism via toxins, A) and indirect resource competition (resource niche overlap, B) in two-species resident communities. Communities with MIF values below and above zero were defined as antagonistic and facilitative on average, respectively.

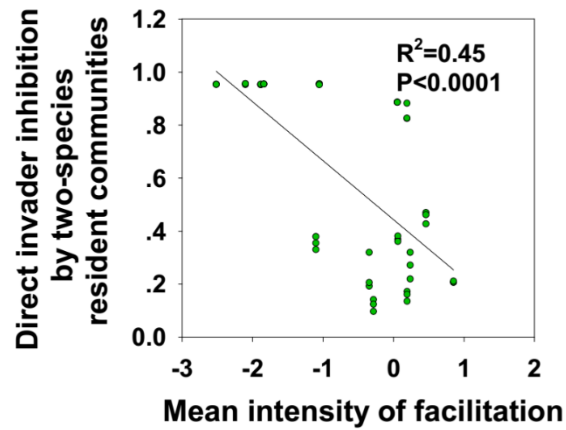

Figure S6. The relationship between direct invader inhibition (antagonism via toxins) and mean intensity of facilitation (MIF) in two-species resident communities. Communities with MIF values below and above zero were defined antagonistic and facilitative on average, respectively.

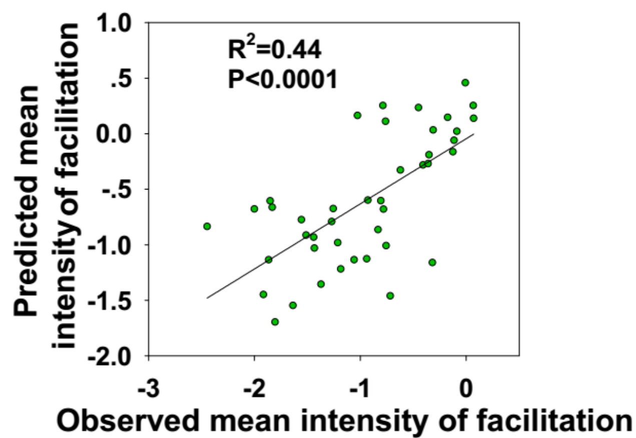

Figure S7. The relationship between observed (OIF) and predicted (PIF) mean intensities of facilitation within resident species communities. Communities with OIF and PIF values below and above zero were defined on average antagonistic and facilitative, respectively.

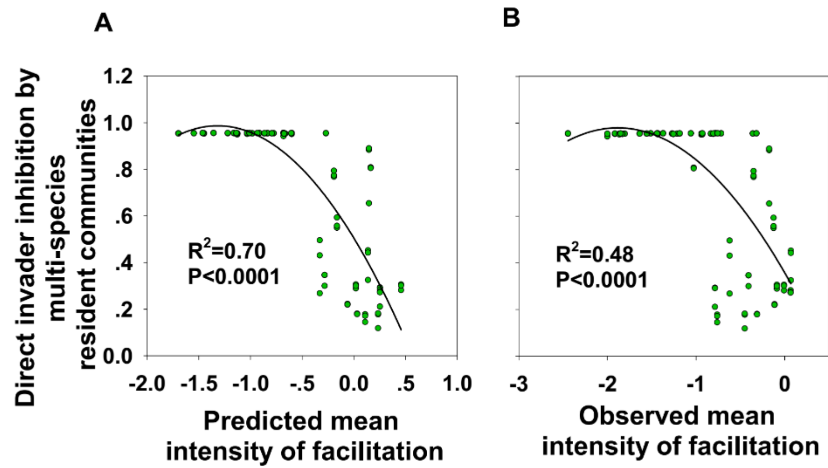

Figure S8. The relationship between direct invader inhibition (antagonism via toxins) and predicted (PIF, A) and observed (OIF, B) mean intensities of facilitation within multi-species resident communities. Communities with PIF and OIF values below and above zero were defined as antagonistic and facilitative on average, respectively.

Chapter 4 Indirect control of *Ralstonia solanacearum* by inhibiting pathogen helpers

Running title: Microbiome-mediated pathogen control

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Abstract

The rhizosphere microbiome forms a first line of defense against soilborne pathogens. To date, most microbiome enhancement strategies have relied on bioaugmentation with antagonistic microorganisms that directly inhibit pathogens. Previous studies have shown that some root-associated bacteria are able to facilitate pathogen growth. We therefore hypothesized that inhibiting such pathogen helpers may help control pathogens. We examined tripartite interactions between a model pathogen *Ralstonia solanacearum,* two model helper strains and a collection of 46 bacterial isolates recovered from the tomato rhizosphere. This setting allowed us to examine the importance of direct (effects of rhizobacteria on pathogen growth) and indirect (effects of rhizobacteria on helper growth) pathways of pathogen growth. We found that the interaction between rhizosphere isolates and the helper strain was the major determinant of pathogen inhibition *in vitro* and *in vivo*. We therefore propose that controlling microbiome composition to prevent the growth of pathogen helpers may become part of sustainable strategies for pathogen control.

Introduction

Plant pathogens have a large negative impact on agricultural production, and there is an urgent need for sustainable strategies to prevent diseases while reducing the environmental footprint of modern agriculture [187]. Plant root-associated microorganisms are increasingly studied in relation to their ability to help keep plants healthy [34, 35]. However, while some microbiomes are better at preventing pathogen growth than others, it often remains unclear which interactions shap pathogen success. To date, most research has focused on pathogen inhibition by some specific plant-associated microorganisms. In line with this logic, most microbiome management strategies have been centered around bioaugmentation with microorganisms that can directly inhibit pathogen growth [15, 84]. These biopesticides represent a promising approach, but are often constrained by the low density that inoculated strains can reach in a multispecies microbiome and the context-dependent success of microbial introductions [123, 188, 189]. These shortcomings are at least partly due to inadequate consideration of the complex microbial interactions that impact pathogen inhibition or proliferation [121, 190].

We propose a new perspective on pathogen ecology by placing focus on microorganisms that promote pathogen growth. Recent studies have shown that a significant fraction of plant-associated microorganisms can promote pathogen growth and pathogenicity [59]. Facilitative microbe-microbe are indeed widespread and interactions may emerge for instance as the result of cross-feeding [60], or production of public goods such as siderophores [63]. Facilitation has been recently highlighted as a potential determinant of pathogen success [64, 121]. We therefore postulate that manipulating naturally-occurring helper bacteria of pathogens may provide a means of

controlling pathogen development without requiring the application of pesticides or biopesticides. To this end, we hypothesized that indirect effects via inhibition of pathogen helpers would have a significant impact on realized pathogen densities.

We first established the prevalence of pathogen-helper bacteria in the rhizosphere by screening a library of rhizobacterial strains isolated from tomato rhizosphere. We specifically tested their pairwise interactions with *Ralstonia solanacearum*, the causative agent of bacterial wilt, a major disease affecting more than 200 crops at a global scale [99, 100]. We observed that a significant fraction of all isolates promoted pathogen growth *in vitro*. We then selected two pathogen helper strains and built tripartite cultivation experiments in which the pathogen was grown together with one of the helper strains and the supernatants of each of the selected bacterial strains that represent a gradient of positive, neutral or negative interactions with the pathogen. Pathogen growth was monitored in each community, both *in vitro* and in the tomato rhizosphere. We then expressed pathogen density as a function of a direct (effect on pathogen) and indirect (effect on the helper strain) pathway for each of these rhizobacteria (Fig. 1). Part of the resulting data was also used to model the relative importance of direct versus indirect effects in determining realized pathogen density. Based upon the results of these experiments, we subsequently discuss the potential utility of bioaugmentation strategies that target pathogen helpers as an element of integrated pathogen control.

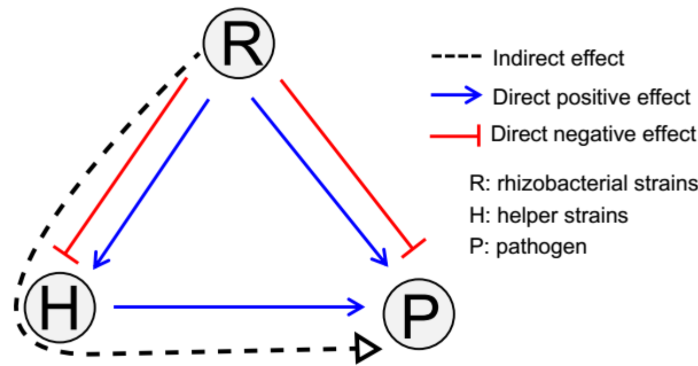

Fig. 1 Conceptual overview of direct and indirect effects of rhizobacterial strains on pathogen growth. In this work, we split the net, apparent effect of single rhizosphere bacterial isolates (R) on pathogen density into direct effects on the pathogen (P) and indirect effects mediated by interactions with helper bacteria (H).

Materials and methods

Assessing strain redundancy among the 515 non-*Ralstonia solanacearum* bacteria

We assessed possible redundancy among 515 strains of the non-*Ralstonia solanacearum* rhizobacteria, which was isolated from tomato rhizosphere (chapter 2 this thesis). To encompass both taxonomic and functional redundancies we considered the 16S rRNA gene sequences as well as the direct effect of their supernatant on *Ralstonia solanacearum*. Self BLAST searches were performed on the full 515 sequence dataset using the *makeblastdb* and *blastn* commands from the BLAST command line tool [134]. Sequences showing >99% identity over >95% of the full length were considered as taxonomically redundant. We then compared the direct effects on pathogen growth of the taxonomically redundant strains, and removed those showing same patterns of interactions (positive, negative or neutral). Accordingly (see the dataset "Library of rhizobacterial strains" in the supplementary information), 355 of the 515 strains (68.9%) were removed from the original dataset for further analyses.

Phylogenetic tree construction

The 16S rRNA gene sequences of the 160 non-redundant bacteria were aligned using MUSCLE [132]. Sequences in the alignment were trimmed at both ends to obtain maximum overlap using the MEGA X software, which was also used to construct taxonomic cladograms [133]. We constructed a maximum-likelihood (ML) tree, using a General Time Reversible (GTR) + G + I model, which yielded the best fit to our data set. Bootstrapping was carried out with 100 replicates retaining gaps. A taxonomic cladogram was created using the EVOLVIEW web tool (https://evolgenius.info//evolview-v2/). To show the relationship between phylogeny and the effects of rhizobacteria on pathogen growth, we added taxonomic status (phylum) of each rhizobacterial strain and its effect on pathogen growth as heatmap rings to the outer circle of the tree separately (Fig.2B).

Effects of pathogen helper strains on pathogen growth *in vivo*

To test the effect of pathogen helper strains on *Ralstonia solanacearum* growth in tomato rhizosphere, we first selected two model helper strains (*Phyllobacterium ifriqiyense* LM1 (Pi) and *Microbacterium paraoxydans* LM2 (Mp)), which showed strong positive effects on the pathogen's growth both in co-culture and in supernatant assays (Fig. S1). Tomato seeds (*Lycopersicon esculentum*, cultivar "*Micro-Tom*") were then surface-sterilized (in 3% NaClO for 5min and in 70% ethyl alcohol for 1min) and allowed to germinate on water-agar plates for 2 days, until the emergence of roots. Germinated seeds were then sown in pots (6 cm × 6 cm × 6 cm) containing 450g soil, which had been collected from Qilin town (118°57' E, 32°03' N), Jiangsu province, China and sterilized by gamma irradiation. Approximately two weeks after seedling transplantation, when tomato plants had reached the three-leaf stage, a cell suspension of either *P. ifriqiyense* (designated LM1) or *M. paraoxydans* (designated

LM2) was inoculated respectively into the soil of the seedlings to a final density of approximately 10^8 cells g^{-1} dry soil. A cell suspension of *Ralstonia solanacearum* strain QL-Rs1115 (10^7 cells g^{-1} dry soil) was inoculated two days later. The control treatment included only the pathogen without the addition of either of the helper strains. Each treatment was replicated three times, and each pot contained 4 tomato plants. Plants were then grown in a growth chamber with a 16:8-h light/dark photoperiod at 30 ± 2 °C to mimic greenhouse conditions. Twenty-seven days after inoculation of *Ralstonia solanacearum*, rhizosphere soil was collected from each replicate pot, resulting in total of 9 rhizosphere soil samples. The rhizosphere soil was collected using previously described methods [84] for determining *Ralstonia solanacearum* population densities (described in detail later in "Quantification of *Ralstonia solanacearum* at the end of the *in vivo* experiment").

Effects of rhizobacteria on pathogen helper strains growth *in vitro*

In a next step, we assessed the potential of different rhizosphere isolates to inhibit helper bacteria. We defined the effect of rhizobacterial strains on the growth of helpers as the indirect effect on *Ralstonia solanacearum* growth. To study these indirect effects, we first chose a subset of 46 rhizobacterial strains covering a gradient of positive, neutral or negative effect on pathogen growth based on supernatant assays (results in x axis of Figure 3C and Figure 4A). We then tested the effects of these 46 rhizobacterial strains on the growth of each of the two helper strains using supernatant assays. Briefly, after 48 h growth in NB media, each of the 46 bacterial monocultures was passed through a 0.22 μm filters to remove living cells. Then 20 μl of sterile supernatant from each strain's culture and 2 μl overnight culture of Pi or Mp (adjusted to OD600 = 0.5 after 12 h growth at 30°C with shaking) were added into 180 μl of fresh NB medium (5-times diluted, in order to better reflect the effect of the supernatant). Control treatments

were inoculated with 20 µl 5X diluted NB media instead of bacterial supernatant. Each treatment was replicated four times. All bacterial cultures were grown for 24 h at 30°C with shaking (170 rpm) before measuring helper density as optical density (OD 600 nm). To test for significance of growth promotion or inhibition, we used analyses of variance (ANOVA) and Bonferroni t-test to compare mean differences of helper density between each rhizobacterial supernatant treatment and the control treatment, with p-values below 0.05 being considered statistically significant. The effect of rhizobacteria on the helpers' growth (results in y axis of Figure 3C and x axis of Figure 4C) was defined as the percentage of increase or reduction in helper growth by the supernatant compared to the control treatment.

In vitro pathogen growth in the presence of a helper strain and supernatant from rhizobacterial isolates

To disentangle the direct effects from the indirect effects of rhizobacteria on *Ralstonia solanacearum* growth, we compared their relative effects using *in vitro* triculture assays comprised of *Ralstonia solanacearum*, one of the two helper strains and supernatant of one of the 46 chosen rhizobacterial strains. Briefly, after 48 h of growth in NB media, each of the 46 bacterial monocultures was passed through a 0.22 µm filters to remove living cells. Then, 20 µl of sterile supernatant from each strain's culture and 2 µl overnight culture of Pi or Mp (densities were adjusted to ~10^7 cells per ml) were added to 180 µl of fresh NB medium (5-times diluted). Each treatment was replicated four times. At the same time, 2 µl overnight culture of mCherry-tagged *Ralstonia solanacearum* (density was adjusted to ~10^6 cells per ml) was added to each treatment in 96-well plates at 30°C with shaking (170 rpm). After 24 h, *Ralstonia solanacearum* density (results in y axis of Figure 4A and 4C) was measured as the red mCherry protein fluorescence intensity (excitation: 587 nm, emission: 610 nm) with a

SpectraMax M5 plate reader.

***In vivo* pathogen growth in the presence of a helper strain and a rhizobacterial strain**

To validate *in vitro* results, we set up greenhouse experiments where plants were inoculated with a bacterial consortium consisting of *Ralstonia solanacearum*, one of the two helper strains and a test rhizobacterial strain. Tomato seeds (*Lycopersicon esculentum*, cultivar "*Micro-Tom*") were first surface-sterilized (in 3% NaClO for 5min and in 70% ethyl alcohol for 1min) and germinated on water-agar plates for 2 days. Seeds were then sown into seedling plates containing 200g of cobalt-60-sterilized seedling substrate (Huainong, Huaian Soil and Fertilizer Institute). At the three-leaf stage, tomato plants were transplanted to nine-cell seedling trays with each cell containing 100g of homogenized, gamma irradiation-sterilized (to avoid potential effects of the resident community) paddy soil collected from Yixing City, Jiangsu Province, China (119°44′E, 31°22′N).

We used 6 well-studied bacterial strains: *Flavobacterium johnsoniae* (Fj), *Bacillus amyloliquefaciens* (Ba), *Lysinibacillus sphaericus* (Ls), *Chryseobacterium daecheongense* (Cd), *Delftia acidovorans* (Da) and *Ralstonia pickettii* (Rp), all isolated from tomato rhizosphere and known to have negative effects of pathogen growth [121]. We tested their direct effects on the pathogen (results in x axis of Figure 4B) again *in vitro*, using the same methods as described above in "Direct effect of rhizobacteria on pathogen growth *in vitro*". We also tested their effects on the growth of each of the two helper strains (results in x axis of Figure 4D) *in vitro*, using the same methods as described above in "Effects of rhizobacteria on pathogen helper strains growth *in vitro*". Each strain was used in combination with each of the two helper strains and *Ralstonia solanacearum* mixture, resulting in a total of 14 treatments (included control and

Ralstonia solanacearum alone) (Table S1). For each treatment, three replicate nine-cell seedling trays were used and each replicate seedling tray contained nine tomato plants. Three days after transplantation, plants of each treatment were inoculated with one helper plus one rhizobacterial strain using the root drenching method at a final concentration of 10^8 CFU of each bacteria g^{-1} soil [174]. Seven days after inoculation of helper plus rhizobacteria, *Ralstonia solanacearum* was introduced to the roots of all plants at a final concentration of 10^7 CFU of bacteria g^{-1} soil. The *Ralstonia solanacearum*-alone treatment inoculated only with the pathogen, and the control treatment was not inoculated with any bacteria. Tomato plants were maintained under standard greenhouse conditions (at natural temperature variation ranging from 25°C to 35°C) and watered regularly with sterile water. Seedling trays were rearranged randomly every two days. Twenty-seven days after inoculation of *Ralstonia solanacearum*, rhizosphere soil samples were collected, using previously described methods [84] from three randomly chosen plants from each replicate seedling tray, resulting in total of 42 rhizosphere soil samples for which *Ralstonia solanacearum* population densities were determined.

Quantification of *Ralstonia solanacearum* at the end of the *in vivo* experiment

We determined *Ralstonia solanacearum* densities using quantitative PCR (qPCR). The *Ralstonia solanacearum* DNA was extracted using a Power Soil DNA isolation kit (Mo Bio Laboratories) following the manufacturer's protocol. DNA concentrations were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and extracted DNA was used for *Ralstonia solanacearum* density measurements using specific primers (forward, 5'-GAACGCCAACGGTGCGAACT-3'; reverse, 5'-GGCGGCCTTCAGGGAGGTC-3') targeting the *fliC* gene, which encodes the *Ralstonia solanacearum* flagellum subunit [191]. The qPCR analyses were carried out

with a StepOnePlus Real-Time RCR Instrument using SYBR green fluorescent dye detection and three technical replicates as described previously [84].

Statistical analyses

To meet assumptions of normality and homogeneity of variance, *Ralstonia solanacearum* densities measured *in vitro* and *in vivo* were log10-transformed. When comparing mean differences between treatments, we used analyses of variance (ANOVA) and the Tukey Test, where p-values below 0.05 were considered statistically significant. *Ralstonia solanacearum* densities were explained by two quantitative indices, the direct effect of rhizobacteria on *Ralstonia solanacearum* growth (the effect of rhizobacteria on *Ralstonia solanacearum* growth) and the indirect effect of rhizobacteria on *Ralstonia solanacearum* growth (the effect of rhizobacteria on helper strains growth). Nonlinear regression analyses (Sigmoidal, Sigmoid, 3 Parameter) were used to analyze the relationship between the direct effect and pathogen density, as well as the relationship between indirect effects and pathogen density in the presence of helper strains *in vitro*, while the relationships between them in the presence of helper strains *in vivo* were analyzed using linear regressions. These analyses were carried out using R 3.6.3 program (www.r-project.org) and SigmaPlot (V.12.5).

To further consider the growth inhibition of *Ralstonia. solanacearum*, we fitted a linear model to estimate the relative importance of direct effects versus indirect effects on the density of *Ralstonia solanacearum in vitro* and *in vivo.* This model considered the interaction scenario where rhizobacterial strains inhibited both the pathogen and its helpers (see the R script “Model” in the supplementary information). These analyses were performed in R version 3.6.3 [175] in conjunction with the package *car, readxl*

and *dplyr,* and *tidyverse* 1.2.1 [192]. Briefly, proportional effects were normalized using a folded cube root transformation as suggested in J.W. Tukey [193] and fitted using a linear model with direct effects, indirect effects, and an interaction between helper strains and indirect effects as fixed factors. Normality of residuals was tested using the Shapiro-Wilk normality test and visual inspection of QQ-plots with studentized residuals. Type-II sum of squares were calculated using the ANOVA function from *car* 3.0-2 [194]. Subsequent visualization of the model outcome (results in Figure 5) showed the predicted *Ralstonia solanacearum* densities for different values of the inhibition via pathogen (Direct) or helper (Indirect) as estimated from the statistical model. For the Direct effect line, the indirect effect is set to be zero, while For the Indirect effect line, the direct effect is set to be zero.

Results

Taxonomic characterization of inhibiting and helping strains of *Ralstonia solanacearum* from the tomato rhizosphere

The 160 non-redundant isolates we examined were classified within four main phyla, with the following distribution: Proteobacteria 33.1%, Firmicutes 25.0%, Bacteroidetes 19.4% and Actinobacteria 22.5%. This collection contained a total of 23 families and 48 genera (Fig. S2). A total of 26.9% of these isolated rhizobacteria were shown to inhibit pathogen growth *in vitro*, while 50.6% of them significantly stimulated pathogen growth. We refer to these two categories as pathogen inhibitors and helpers, respectively (Fig. 2A). Although both helpers and inhibitors were found within each represented phylum, there were clear phylum-level differences with respect to the relative proportion of inhibitors versus helpers (Fig. 2B). For instance, 42.5% of the isolates affiliated with the Firmicutes showed inhibition of *Ralstonia solanacearum* growth, while 49.1% of the Proteobacteria isolates, 51.6% of the Bacteroidetes isolates

and 63.9% of the Actinobacteria isolates were found to act as helpers (Fig. 2C).

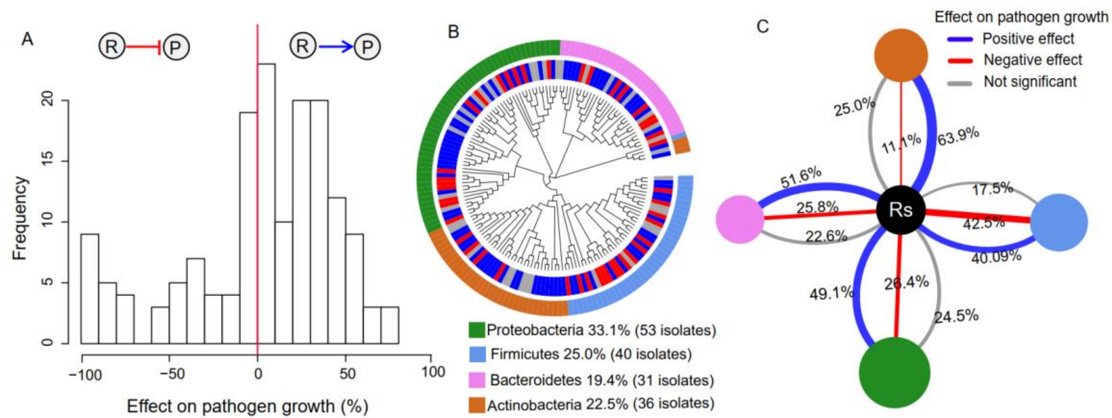

Fig. 2 Taxonomic characterization of rhizobacterial isolates that inhibited or helped the growth of *Ralstonia solanacearum*. (A) Distribution of *in vitro* effects of 160 rhizobacterial supernatants on *Ralstonia solanacearum* growth. The red vertical line represents no effect on *Ralstonia solanacearum* growth. (B) Cladogram depicting the phylogenetic relationship among the 160 isolates based on their full-length 16S rRNA gene sequences. The inner ring depicts the different effect of isolates supernatant on *Ralstonia solanacearum* growth: positive effect (blue), negative effect (red) and no significant effect (gray). The outer ring shows the four phyla to which the isolates belong. (C) The proportion of rhizobacterial isolates per phylum whose supernatant showed inhibitory, stimulatory or no effect on *Ralstonia solanacearum* growth. The size of the circles represents the number of rhizobacterial isolates in the given phylum. The thickness of lines represents the percentage of rhizobacterial isolates that have the indicated effect on *Ralstonia solanacearum* growth in each phylum.

Pairwise interactions between helper strains of *Ralstonia solanacearum* and other rhizobacterial strains

To examine direct versus indirect effects on pathogen growth, we first chose two model helper strains: *Phyllobacterium ifriqiyense LM1* (Pi) and *Microbacterium paraoxydans LM2* (Mp). The supernatant of Pi increased *Ralstonia solanacearum* density by 51.2% *in vitro* (Fig. 3A) and by 139.6% when grown *in vivo* with tomato plants (Fig. 3B). Similarly, Mp increased *Ralstonia solanacearum* density by 39.7% *in vitro* (Fig. 3A) and by 212.9% *in vivo* (Fig. 3B).

We then selected 46 rhizobacterial strains from the full rhizobacterial strain collection to represent a range of positive (50.0%), negative (34.8%) or neutral (15.2%) effects on *Ralstonia solanacearum* growth (Fig. S3). We defined these interactions as the *direct effect* of rhizobacteria on *Ralstonia solanacearum* growth (x axis of Figure 3C and Figure 4A). We tested the effects of the supernatant from each of these 46 rhizobacterial strains on each of the helper strains, Mp and Pi. We found that 10.9% of the strains positively affected the growth of Pi, 82.6% reduced growth and 6.5% had no significant effect (Fig. S3). Following a distinct but comparable pattern, Mp was positively affected by 37.0% of the tested isolates and negatively by 63.0% of them (Fig. S3). We defined these interactions as the *indirect effects* of rhizobacteria on *Ralstonia solanacearum* growth (y axis of Figure 3C and x axis of Figure 4C).

When considering the direct effect of each rhizobacterial strain with their indirect effects on the growth of *Ralstonia solanacearum*, four possible combinations were considered (Fig. 3C) : i) 8 strain combinations showed negative direct effects and positive indirect effects (P^-H^+), ii) 16 strain combinations showed positive direct effects and positive indirect effects (P^+H^+), iii) 30 strain combinations showed negative direct effects and negative indirect effects (P^-H^-) and iv) 38 strain combinations showed positive direct effects and negative indirect effects (P^+H^-). A large majority of strain combinations fell into two of these categories, with 32.6% being P^-H^- and 41.3% being P^+H^-, suggesting that indirect negative effects may be relevant to the control of *Ralstonia solanacearum* growth. We consequently focused the modeling approach, which is described below (in Figure 5 and Table 1), on examining the relative importance of direct effects versus indirect effects on the density of *Ralstonia solanacearum* on these two interaction combinations.

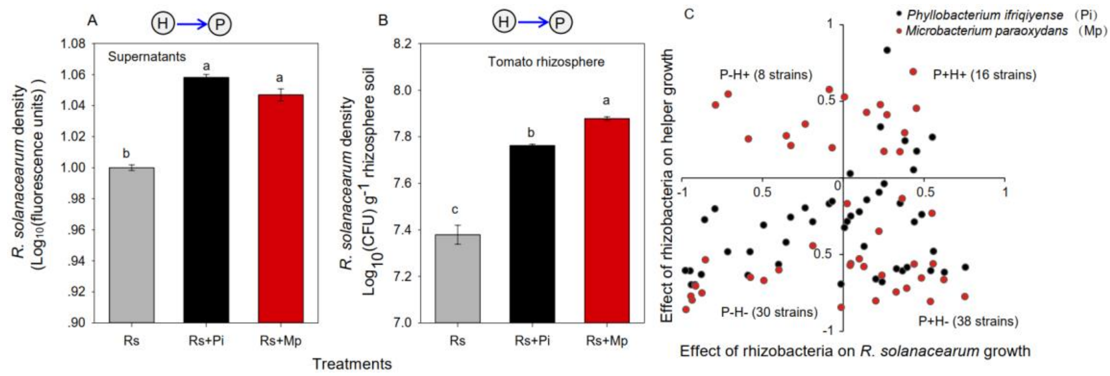

Fig. 3 Effects of the two helper strains *Phyllobacterium ifriqiyense* (Pi) and *Microbacterium paraoxydans* (Mp) on *Ralstonia solanacearum* (Rs) growth *in vitro* (A) and in the rhizosphere of tomato plants (B). Different letters indicate significant differences based on Tukey post hoc test. Error bars show ± 1 SE (n=3). (C) Effects of 46 rhizobacterial strains on the growth of *Ralstonia solanacearum* and the two model helper strains *in vitro*. The x-axis shows the direct effect of each rhizobacterial strain on *Ralstonia solanacearum* growth (data from the experiment in which *Ralstonia solanacearum* was grown in the presence of supernatant from each of the 46 rhizobacterial strains – these are the same data that are presented on the x axis of Figure 4A below). The y-axis shows the effect of each rhizobacterial strain on each of the two helper strains (data from the experiment in which each helper was grown in the presence of supernatant from each of the 46 rhizobacterial strains – these are the same data that are presented on the x axis of Figure 4C below). In panel C, "-1", "0" and "1" on the x-axis denote that *Ralstonia solanacearum* growth is completely inhibited, not influenced or doubled by supernatant from the rhizobacteria, respectively. Similarly, "-1", "0" and "1" on the y-axis signify these same growth effects with reference to growth of the helper strains. Black dots indicate results involving interactions with Pi, and red dots indicate results involving interactions with Mp.

The importance of direct versus indirect effects on *Ralstonia solanacearum* density in the presence of helper strains

To disentangle the direct effects from the indirect effects of rhizobacteria on *Ralstonia solanacearum* growth and subsequently on *Ralstonia solanacearum* density, we examined pathogen growth patterns in a series of tripartite (supernatant of each rhizobacteria + one of the two helpers + pathogen) *in vitro* and *in vivo* assays.

In the presence of the helper strain Pi, *Ralstonia solanacearum* density was positively affected by the direct effects of the rhizobacteria *in vitro* (R^2=0.3066, black line in Fig. 4A) and *in vivo* (R^2=0.0545, P=0.1784, black line in Fig. 4B). When considering the indirect effects of the rhizobacteria, *Ralstonia solanacearum* density increased with the increasing indirect effect both *in vitro* (R^2=0.7522, black line in Fig. 4C) and *in vivo* (R^2=0.4338, P=0.0018, black line in Fig. 4D).

Similarly, when in the presence of the helper strain Mp, *Ralstonia solanacearum* density increased with the increasing direct effect both *in vitro* (R^2=0.3705, red line in Fig. 4A) and *in vivo* (R^2=0.2661, P=0.0165, red line in Fig. 4B). *Ralstonia solanacearum* density also increased with the increasing indirect effect both *in vitro* (R^2=0.7860, red line in Fig. 4C) and *in vivo* (R^2=0.4658, P=0.0011, red line in Fig. 4D). In the presence of either helper, Pi or Mp, the indirect effects explained more of the total variation in *Ralstonia solanacearum* density than the direct effects, with the regression for indirect effects yielding higher r-square values than that for direct effects (Fig. 4A-D). Together, these results demonstrate that inhibition of pathogen helper strains has the potential to limit the growth of *Ralstonia solanacearum*, not only under controlled *in vitro* laboratory assay conditions but also *in vivo*, although it should be noted that the latter was limited to testing of only 6 of the rhizobacterial strains. To gain further insight into the potential prevalence of such a mechanism, we considered this strategy using a modelling approach targeting the relative importance of direct versus indirect effects on pathogen growth.

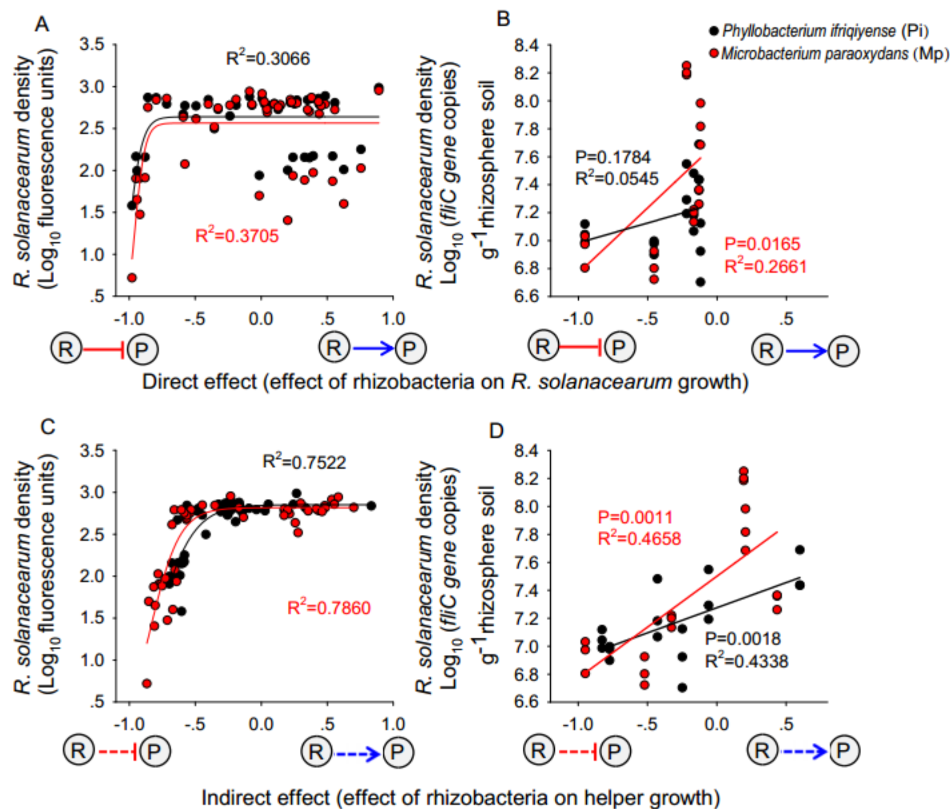

Fig. 4 The importance of direct versus indirect effects on *Ralstonia solanacearum* density in the presence of helper strains. In the presence of helper *Phyllobacterium ifriqiyense* (Pi) or *Microbacterium paraoxydans* (Mp), respectively, the importance of direct effects on the density of *Ralstonia solanacearum* (A) *in vitro* (the data on the x axis are the same data which was presented on the x axis of Figure 3C) and (B) *in vivo* (the data on x axis from the experiment in which *Ralstonia solanacearum* was grown in the presence of supernatant from each of the 6 rhizobacterial strains); the importance of indirect effects on the density of *Ralstonia solanacearum* (C) *in vitro* (the data on x axis are the same data which was presented on the y axis of Figure 3C) and (D) *in vivo* (the data on x axis from the experiment in which each helper was grown in the presence of supernatant from each of the 6 rhizobacterial strains). In all panels, “-1”, “0” and “1” on the x-axis denote that *Ralstonia solanacearum* growth (panels A and B) or helper growth (panels C and D) is completely inhibited, not influenced or doubled by supernatant from the rhizobacteria, respectively.

The relative importance of direct versus indirect effects on *Ralstonia solanacearum* density in the presence of helper strains

To further consider growth inhibition of *Ralstonia solanacearum*, we focused our

modeling approach on the interaction scenarios where rhizobacterial strains inhibited both the pathogen and its helpers (quadrant "H⁻P⁻" in Fig. 3C), and where rhizobacterial strains facilitated the pathogen but inhibited its helpers (quadrant "H⁻P⁺" in Fig. 3C). We constructed a model to predict the direct effects versus indirect effects on the density of *Ralstonia solanacearum* both *in vitro* and *in vivo*. We found that indirect effects provided far better prediction of *Ralstonia solanacearum* density in the quadrant "P⁻H⁻" (Fig. 5A and Table 1) *in vitro* as compared to direct effects on the pathogen. For the results *in vivo*, neither direct nor indirect effects had a significant effect on *Ralstonia solanacearum* density (Table 1), but indirect effects were also the better predictor of *Ralstonia solanacearum* density compared to direct effects on the pathogen in the quadrant "P⁻H⁻" (Fig. 5B). Together, these results suggest that indirect effects of rhizobacteria on the helpers' growth predicted pathogen density far better than direct effects on the pathogen itself.

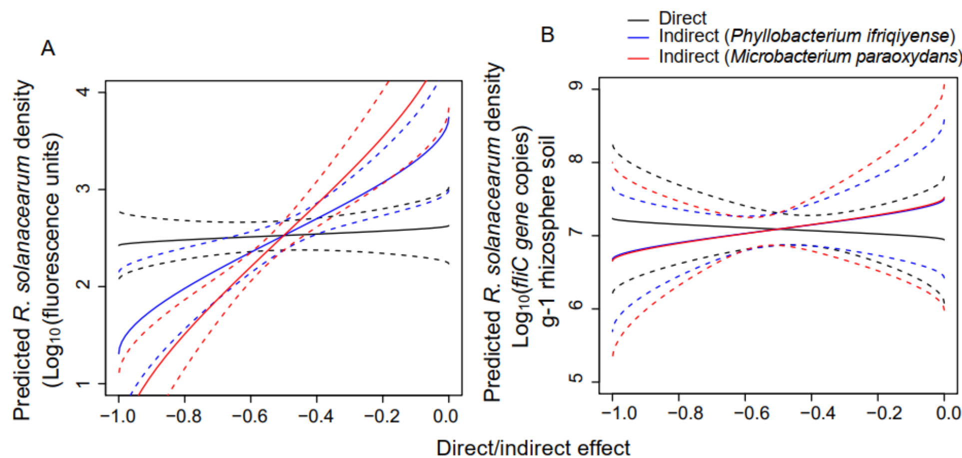

Fig. 5 Relative importance of direct versus indirect effects on *Ralstonia solanacearum* density in presence of helper strains on the interaction scenario where rhizobacterial strains inhibited both the pathogen and its helpers (quadrant "H⁻P⁻" in Figure 3C) both *in vitro* (A) and *in vivo* (B). This shows the predicted *Ralstonia solanacearum* densities for different values of the inhibition via pathogen (Direct) or helper (Indirect) as estimated from the statistical model (Table 1) which with direct effects, indirect effects, and an interaction between helper strains and indirect effects as fixed factors. For the Direct line, the indirect effect was set to zero, while for the indirect line, the direct effect was set to zero.

Table 1 ANOVA table comparing the contribution of direct and indirect effects of the different tested bacterial isolates on the density of *Ralstonia solanacearum in vitro* and *in vivo* on the interaction scenario where rhizobacteria inhibited both the pathogen and its helpers (quadrant "H^-P^-" in Figure 3C).

	Ralstonia solanacearum* density *in vitro			***Ralstonia solanacearum* density *in vivo***		
	df	***F***	***P***	***df***	***F***	***P***
Direct effect	1	2.4521	0.1295	1	0.1916	0.6842
Indirect effect	**1**	**32.9556**	**4.818e-06**	1	1.3481	0.3102
Indirect effect: Mp vs Pi	1	5.0717	0.0330	1	0.0023	0.9639
No. of Residuals	26			4		

Discussion

In this study, we evaluated the prevalence of pathogen-helper bacteria in the rhizosphere microbiome as well as the potential of such helpers as targets for microbiome management strategies aiming to control pathogen growth. As a model pathogen, we used *Ralstonia solanacearum,* a widespread and problematic phytopathogenic bacterium that causes wilt diseases on tomatoes and more than 200 economically important crops and ornamentals [195]. Combining *in vitro* and *in vivo* approaches, we compared the influence of the direct (*i.e.* on *Ralstonia solanacearum* growth directly) vs. the indirect (*i.e.* on the growth of *Ralstonia solanacearum* helper strains) effects of tomato-associated rhizobacteria on the growth of the pathogen. In general, indirect effects, *i.e.* inhibition of helper strains, were the major determinant of pathogen inhibition as compared to direct impacts on the pathogen itself. To our knowledge, this represents the first demonstration of such an indirect strategy for the potential inhibition of pathogen growth.

The isolated rhizobacteria in this study belonged to four major phyla (Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria), which are collectively presumed to be copiotrophic bacteria [196, 197] and are known to be dominant phyla found in the rhizosphere [198]. We found a majority of our isolates (50.6%) promoted

pathogen growth using a supernatant assay. This result adds to the recent realization that many microorganisms may act as helpers of pathogens in the rhizosphere [59]. For example, different bacteria which were affiliated with *bacillus* and *microbacterium* had a mild and overall significant stimulatory effect on the growth of *Ralstonia solanacearum* [59]. Some fungi affiliated with Ascomycetes, Basidiomycetes and Zygomycetes can produce chlamydospores that host *Ralstonia solanacearum* and can facilitate pathogen entry into tomato roots [65]. Moreover, one *Pseudomonas* phylotype was found to exacerbate disease symptoms in tomato plants by establishing commensal interactions with a oomycete pathogen to maximize its access to plant nutrients [199]. Interestingly, most of helpers in our study belonged to the Proteobacteria (49.1%) and Actinobacteria (63.9%), which have often been reported to contain bacteria that are beneficial for plant health [26, 57]. For example, *Cha et al.* reported that Actinobacteria played a key role in the specific suppressiveness of *Fusarium* wilt in strawberry soils [57]. This discrepancy might be due to the fact that their results showed the cooccurrence relation based on high-throughput data, while we tested the real effect of isolated representatives from these phyla. The rhizobacterial collection we isolated in this study does not provide a full taxonomic inventory of the rhizosphere microbiome. For instance, the medium we used most likely selected for copiotrophic strains among all present bacterial strains. Such copiotrophs might have different effects on pathogen growth as compare to more oligotrophic bacteria, as they have higher growth rates and lower substrate affinities than oligotrophic bacteria [200].

Several mechanisms have been put forth to explain mutualism or commensalism among bacteria, mainly as related to the benefits gained from the use of metabolites processed by another member of the community [201]. For instance, peptidoglycan produced by *Bacillus cereus* may promote the growth of several bacterial strains

affiliated with the *Cytophaga-Flavobacterium* group [202]. Siderophores produced by microorganisms can also be accepted as public goods by several other bacterial with siderophore protein receptors to obtain limited iron in the environment to maintain growth and metabolism, hence increasing population biomass [203]. Although beyond the scope of the current study, the promoting mechanisms of the helper strains towards *Ralstonia solanacearum* are most likely related to certain metabolites, as promotion was also observed using supernatant assays (Fig. 1A and 3A).

Similar to the results obtained *in vitro* (Fig. 4A and 4C), both direct inhibition from rhizobacteria on pathogen growth and indirect inhibition from rhizobacteria on its helper growth limited the growth of *Ralstonia solanacearum in vivo*: by a factor of 10, from approximately 10^8 to 10^7 g^{-1} rhizosphere soil (Fig. 4B and D). The reduction in the pathogen growth weakens the ability of the pathogen to infect plants because *Ralstonia solanacearum* virulence gene expression is triggered by quorum sensing, which requires high pathogen population densities [204]. In previous studies, when the pathogen density in the tomato rhizosphere decrease from approximately $10^{7.5}$ to $10^{6.8}$ [59], or $10^{6.2}$ to $10^{5.5}$ [138] g^{-1} rhizosphere soil, the disease incidence significantly decreased. However, as we sampled the plants in an early stage to focus on how microbe-microbe interactions influence the pathogen growth in the rhizosphere, we did not assess actual bacterial wilt disease incidence. Since many potential factors affect plant disease incidence beside pathogen density, we cannot fully claim that the observed patterns of helper inhibition would lead lower disease incidence. For example, plant immunity, and the role of microbe-mediated plant immunity is important as both pathogenic and non-pathogenic bacteria can trigger or suppress plant immunity [205, 206].

The rhizobacterial strains used in this study exhibited a wide range of effects on the pathogen and its bacterial helper strains. Many of them inhibited both *Ralstonia*

solanacearum as well as its helpers. A model on the interaction scenarios where rhizobacterial strains inhibited both the pathogen and its helpers (quadrant "H^-P^-" in Fig. 3C) show that inhibition of the helper strains was a more effective path toward *Ralstonia solanacearum* control than direct inhibition effects on the pathogen itself *in vitro*. The model shows neither direct nor indirect effects have a significant effect on *Ralstonia solanacearum* density in tomato rhizosphere (Table 1). This may be due to the limited number of combinations tested (just from 6 rhizobacterial strains). However, we did observe a trend that the indirect inhibition effect was a better predictor for *Ralstonia solanacearum* density than direct inhibition effect (Fig. 5B). Even if a biocontrol agent is active against *Ralstonia solanacearum* [51, 84], its efficiency in reality may be more due to its interaction with indigenous helpers. We therefore propose that strategies for integrated biological control of the pathogen need to be reconsidered to incorporate indirect effects on pathogen helpers to provide more ecological solutions to combat soil-borne pathogens. Although the underlying mechanisms of helper inhibition still need to be unraveled and our communities here were far less diverse and far simpler than natural communities, our findings contribute to our knowledge of rhizobacteria-pathogen interactions and provide a new potential strategy for efficient and sustainable biological control of soil-borne pathogens.

Acknowledgements

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Supplementary materials

Materials and methods

Measuring the direct effects of rhizobacteria on pathogen growth by co-culture assay *in vitro*

We used *Ralstonia solanacearum* strain QL-Rs1115 tagged with the pYC12-mCherry plasmid as an invading bacterial pathogen [51]. We first tested the effects of 515 bacterial strains on the growth of *Ralstonia solanacearum in vitro* by using co-culture assays. All strains were first grown alone in liquid NA medium (glucose 10.0 g l^{-1}, tryptone 5.0 g l^{-1}, yeast extract 0.5 g l^{-1}, beef extract 3.0 g l^{-1}, pH 7.0) on a shaker at 170 rpm, 30°C overnight. All rhizobacterial strain densities were adjusted to ~10^7 cells per ml and the density of mCherry fluorescence-tagged *Ralstonia solanacearum* QL-Rs1115-RFP to ~10^6 cells per ml. Co-cultures with even starting volumes (50%:50%) were set up in 96-well plates with liquid NA medium. Each treatment was replicated three times. All bacterial cultures were grown for 48 h at 30°C with shaking (170 rpm) before measuring pathogen density as red mCherry protein fluorescence intensity (excitation: 587 nm, emission: 610 nm) using a SpectraMax M5 plate reader [59, 121]. To test for significance of growth promotion or inhibition, *Ralstonia solanacearum* densities (RFP) were log10-transformed prior to analyses of variance (ANOVA) and Bonferroni t-test to compare mean differences between each rhizobacterial supernatant treatments and the control treatment, with p-values below 0.05 were considered statistically significant. The effect on pathogen growth was defined as the percentage of improvement or reduction in pathogen growth by the supernatant compared with the control treatment. When the effect on pathogen growth was positive, *i.e.* when the supernatants from strains significantly promoted the growth of the pathogen, they were considered as helpers of the pathogen. If the effect on pathogen

growth was negative, *i.e.* when the supernatants from strains significantly inhibited the growth of the pathogen, they were considered as inhibitors of the pathogen.

Table S1. Treatments used in *in vivo* experiments with tomato. Table rows show different communities and table columns show the absence (0) or presence (1) of given species in these communities.

Community ID	Species abbreviation								
	Fj	Cd	Da	Ba	Ls	Rp	Pi	Mp	Rs
Control	0	0	0	0	0	0	0	0	0
Ralstonia solanacearum alone	0	0	0	0	0	0	0	0	1
1	1	0	0	0	0	0	1	0	1
2	0	1	0	0	0	0	1	0	1
3	0	0	1	0	0	0	1	0	1
4	0	0	0	1	0	0	1	0	1
5	0	0	0	0	1	0	1	0	1
6	0	0	0	0	0	1	1	0	1
7	1	0	0	0	0	0	0	1	1
8	0	1	0	0	0	0	0	1	1
9	0	0	1	0	0	0	0	1	1
10	0	0	0	1	0	0	0	1	1
11	0	0	0	0	1	0	0	1	1
12	0	0	0	0	0	1	0	1	1

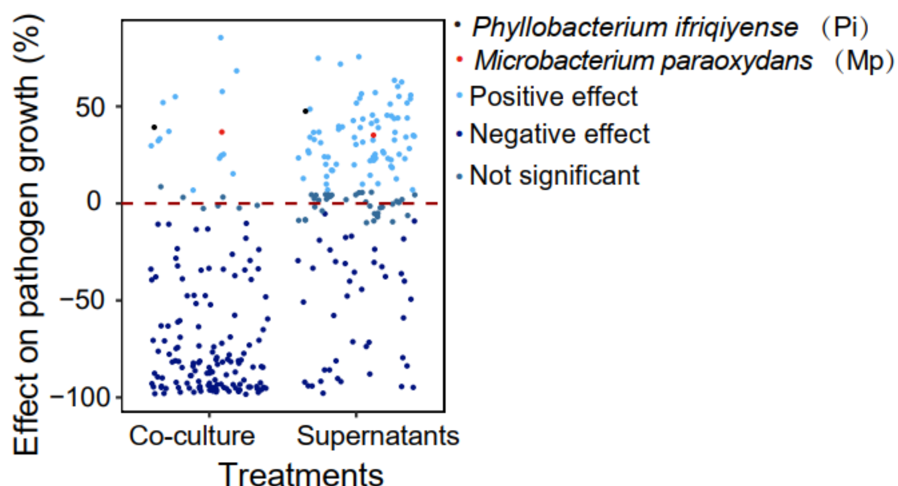

Fig. S1. Effects of 160 rhizobacterial strains on *Ralstonia solanacearum* growth in both co-culture and supernatant assays. *Phyllobacterium ifriqiyense* (Pi) and *Microbacterium paraoxydans* (Mp) were considered helper strains which showed strong positive effects on *Ralstonia solanacearum* growth both in co-culture and supernatant assays. Each dot represents one rhizobacteria in the figure.

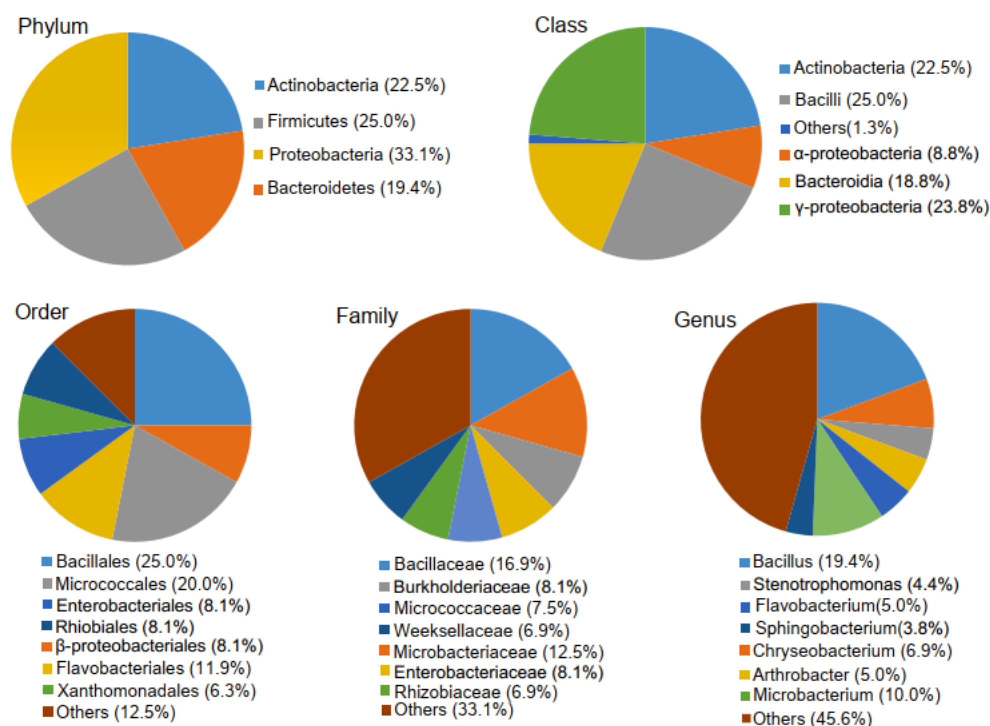

Fig. S2. The diversity and taxonomic classification of rhizosphere bacterial isolates. A total of 160 rhizosphere isolates were identified by 16s rRNA sequencing and their closest relatives were determined using the NCBI database. Seven bacterial groups with highest relative abundances at the phylum, class, order, family, and genus levels are shown in the figure, while groups with relatively low abundances were merged and are presented as one group 'Others'. In all panels, percentage (%) values in brackets represent the proportion of each bacterial group of the total isolates (160 bacterial isolates).

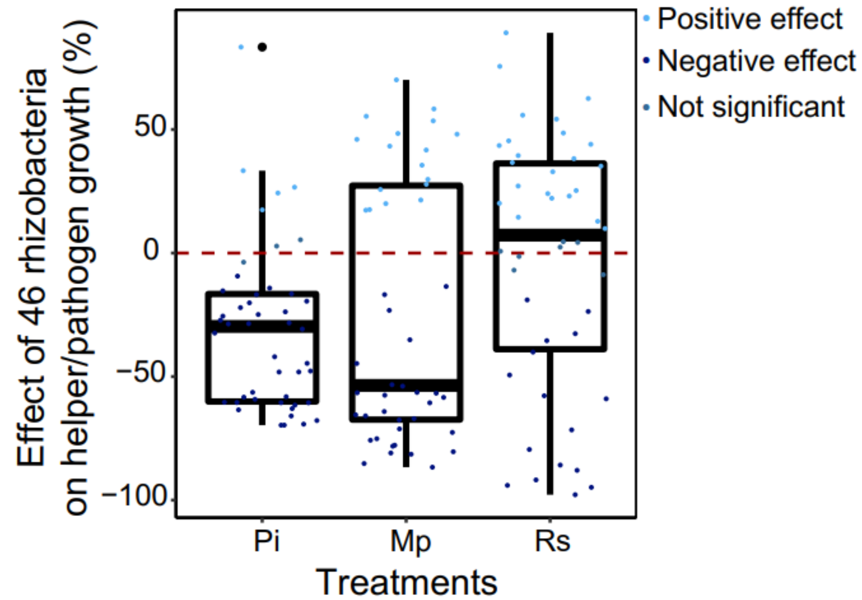

Fig. S3. Effects of 46 rhizobacteria from 160 on helper (*Phyllobacterium ifriqiyense* Pi or *Microbacterium paraoxydans* Mp) or *Ralstonia solanacearum* (Rs) growth in supernatant assay. A subset of 46 rhizobacterial strains covering a gradient of positive, neutral or negative interaction with the pathogen based on supernatant assays was chosen, which also varying in their effect on two helpers. Each dot represents one rhizobacteria in the figure.

Chapter 5 Resource availability alters bacterial pairwise interactions with effects on community resistance to pathogen invasion

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Abstract

Microbes usually exist in communities consisting of a myriad of interacting species. These interactions are sensitive to various environmental factors, such as resource availability. Multiple studies have reported shifts in bacterial interactions in response to varying resource availability levels, yet, how these shifts affect community functions, such as resident community resistance to pathogen invasion, is still poorly understood. In this study, we examined how resource availability shifts the pairwise interactions within the resident community, and how these shifts affect the resident community's ability to resist the invasion of a plant pathogenic bacterium, *Ralstonia solanacearum*. We found that resource availability changed the relationships between interactions within resident community members and pathogen invasion. At high resource availability, interactions between resident bacterial species were mostly driven by the production of secondary metabolites, with direct antagonism as the means of invader inhibition. Therefore, competitive resident communities were invaded to a lesser degree than facilitative communities. At low resource availability, bacteria produced little or no direct antagonist potential, which had little influence on the interactions between them, as well as their collective impact on pathogen inhibition. Rather low resource availability, facilitative communities reached higher community productivity, which in turn showed higher resistance to pathogen invasion than competitive communities. This framework may lay the basis to understand complex microbial interactions and biological invasion as modulated by environmental resource availability.

Introduction

Host-associated microbial communities can function as a line of defense against pathogens, thereby protecting their associated host organism [51, 207]. This process can also be viewed from the perspective of biological invasions where the members of resident communities can impact the ability or inability of invading species to establish in an ecosystem [208, 209]. The characteristics of resident communities are important for determining the outcomes of biological invasions [121, 210]. For example, the interactions between microbes in resident communities can not only influence their survival, growth and contribution to community function [165, 211-213], but also impact the community's resistance to pathogen invasion. Microbes influence their direct environment for instance by consuming resources and excreting metabolites [93]. These changes to the environment influence the growth and survival of both the microbe that originally altered the environment as well as other microbial species that are present, whether that be resident organisms of potential invaders. Facilitative interactions between residents can potentially increase the number of resource niches available via the production of secondary metabolites or public goods that can also be utilized by an invader [87-89]. On the other hand, competing species can inhibit each other, for instance via the production of toxic metabolites, which may have negative effects on both resident and invading species [80, 84].

However, microbial interactions are sensitive to a range of environmental factors, such as resource availability [92]. Theoretical studies have suggested that certain mutualisms can become competitive under high nutrient conditions [95], and it has been shown that two yeast strains can interact in at least seven qualitatively different ways depending on the nutrient concentrations encountered[96]. Although multiple

studies have reported a shift in bacterial interactions in response to changes in resource availability levels, it is generally unknown how shifts in these interactions affect a community's susceptibility or resistance to pathogen invasion. We have previously demonstrated that the interactions within the resident bacterial community can reliably predict pathogen invasion both in lab microcosms and the plant rhizosphere [121]. Facilitative resident communities were more prone to invasions, while antagonistic resident communities were invaded to a lesser extent. In this study, we aimed to specifically explore if these relationships between bacterial interactions and community resistance to pathogen invasion were modulated by resource availability. Higher nutrient concentrations would be expected to allow bacterial populations to metabolize larger amounts of growth substrates, hereby having a larger impact on their surrounding environment [98]. Accordingly, we hypothesized that higher nutrient concentrations would lead to stronger antagonistic interactions and invader inhibition. Especially, competitive communities could also produce higher levels of antagonism than facilitative communities. Competitive communities should therefore provide greater resistance to the invader at high resource availability (Figure 1). As opposed to conditions of high resource availability levels, resident communities at low resource availability conditions might produce lower levels of antagonism. We therefore expect that invasion success may, to some extent be driven by resident community productivity. If facilitative resident community are able to reach higher population densities than antagonistic communities [121], we expect that facilitative communities might better resist to the invader under these conditions (Figure 1), as they may be able to occupy more niche space at low resource availability.

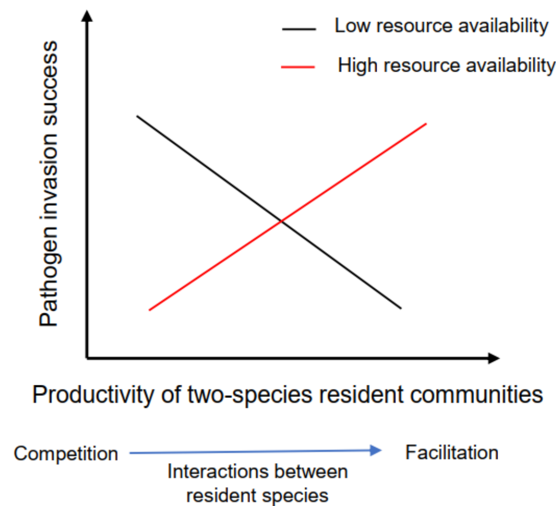

Fig. 1. Schematic figure depicting invasion success as a function of interactions between resident species at low and high resource availability levels. Facilitative communities that reach higher population densities better suppress the invader at low resource availability levels, while competitive communities that reach lower population densities, but produce more direct antagonistic potential, suppress the invader more strongly at higher resource availability levels.

To validate this hypothesis, we examined how resource availability impacted pairwise interactions within the resident bacterial community, and how these changes affected the resident community's resistance to the invasion of the plant pathogenic bacterium, *Ralstonia solanacearum*, the causal agent of bacterial wilt disease [99, 122]. Specifically, we first tested the pairwise interactions between resident bacteria by co-culture (cultured two bacteria together) and supernatant assays (cultured one bacterium in the presence of sterile supernatant from one of the other bacterial strains) at low and high resource availability levels. Two-species communities were then confronted by a *Ralstonia solanacearum* invasion at either high or low resource availability, with invasion success being measured as the resulting density of *Ralstonia solanacearum*. To examine the tested communities toxicity towards the *Ralstonia solanacearum*, we also tested the invasion success in the presence of sterile supernatant from each two-species community at low and high resource availability levels.

Materials and methods

Bacterial strains and the assembly of pairwise resident communities

We used *Ralstonia solanacearum* strain QL-Rs1115 [51] tagged with the pYC12-mCherry plasmid [58] as the model invading pathogen in our experiments. Model resident communities were created using six bacterial strains isolated from the tomato rhizosphere from location from which the pathogen was also isolated (Qilin [118° 57′ E, 32° 03′ N], Nanjing, China). The strain collection used as model communities contained the isolates listed in Table S1 (*Flavobacterium johnsoniae* WR4, *Chryseobacterium daecheongense* WR21, *Delftia acidovorans* WR42, *Bacillus amyloliquefaciens* T-5, *Lysinibacillus sphaericus* HR92 and *Ralstonia pickettii* QL-A6), which have previously been shown to provide protection for associated host plants by inhibiting *Ralstonia solanacearum* pathogen growth via resource competition or direct toxin production [121]. These species can also be differentiated by colony morphology and were used in a previous interaction study [121]. Model resident communities were constructed by using all six resident bacterial strains in all possible one- or two-species combinations (21 communities in total, Supporting Information Table S2). We used a substitutive design so that all communities were set up with the same initial total bacterial density (10^5 cells mL^{-1}) and evenness (i.e. multispecies communities had equal ratios of each species).

Medium and bacterial culture

We set up two different resource availability treatments in this study: 100% NB medium (nutrient broth: glucose 10.0 g l^{-1}, tryptone 5.0 g l^{-1}, yeast extract 0.5 g l^{-1}, beef extract 3.0 g l^{-1}, pH 7.0) providing high resource availability (HRA), and 10% NB medium (by diluting 100% NB medium with sterile water) providing low resource availability (LRA).

Prior to each experiment, one colony of each strain, recovered from -80°C 20% glycerol stocks, was selected and grown in liquid NB with 170 r.p.m. agitation at 30°C for 12 h. Bacteria were then washed three times by centrifugation (5000 rpm, 5 min), resuspended in 0.85% NaCl and adjusted to a density of 10^7 cells mL^{-1} for each resident bacterium and 10^6 cells ml^{-1} for the invading pathogen.

Determining pairwise interactions between resident community species at both high and low resource availability by coculture assay

In order to investigate if and how the resource availability influenced the interaction between bacterial strains, we quantified the strength and direction of each pairwise interaction between resident species at high or low resource availability. To this end we compared the growth of each species alone and in the presence of each of the other species in two-species co-cultures [173]. All mono-cultures were inoculated with a starting density of 10^5 cells per ml, and co-cultures were inoculated with half of this starting cell density for each species. Resident species were grown for 36 h in liquid 100% NB or 10% NB medium in 48-well microtiter plates (ending volume of 700 µl per well) at 30°C with shaking (170 rpm). Bacterial densities of each community were measured as optical density (OD 600 nm) using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Bacterial growth was measured as colony number units (CFU) per ml by serial dilution and plating on NB agar plates after 48 h growth.

To test the significance of the effects of resident bacteria on each other, the productivity of each species (CFU) was log10-transformed prior to a t-test to compare mean differences between each bacterium in co-culture as compared to mono-culture, with p-values below 0.05 being considered statistically significant. The strength of

pairwise interactions between two species (here *i* and *j*) was determined by comparing the final productivity of each species (CP_i and CP_j) in two-species co-cultures with their productivities (MP_i and MP_j) in monocultures ($\log_{10}(CP_i / MP_i)$). We then determined the directionality of interaction. Interactions were considered facilitative if species *j* had a significant positive effect on species *i*, competitive if the effect was significantly negative and neutral if there was not significant effect. We also calculated the mean intensity of interaction (MIF) of co-cultures as an average of $\log_{10}$-transformed pairwise interactions using the following formula: $MIF_{ij} = \frac{1}{2}[\log(CP_i / MP_i) + \log(CP_j / MP_j)]$. The two-species community was defined as facilitative when MIF > 0, competitive when MIF < 0.

Determining pairwise interactions between resident community species at both high and low resource availability by supernatant assay

To assess the impact of resource availability on direct interference competition between resident community species (i.e. toxicity within two-species resident community), we compared the growths of each bacterial strain in the presence of supernatant from each of the other bacteria, which was collected from high resource availability (100% NB) and low resource availability (10% NB) medium, respectively. Briefly, after 36 h of growth in NB or 10% NB medium on a shaker at 170 rpm, 30°C, the 6 resident bacterial monocultures were filter sterilized to remove living cells (0.22 µm filter). Subsequently, 20 µl of sterile supernatant from each strain's culture and 2 µl overnight culture of each resident bacterial strain (density of 10^7 cells per ml) were inoculated into 180 µl of fresh NB medium (10-times diluted, in order to better reflect the effect of the supernatant), respectively. Control treatments were inoculated with 20 µl of 10X diluted NB media instead of the bacterial supernatant. Each treatment was

conducted in triplicate. All bacterial cultures were grown for 24 h at 30°C with shaking (170 rpm) before measuring resident bacterial density as optical density (OD 600 nm) using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

To test the significance of direct toxicity of resident bacteria on each other, we conducted a t-test to compare mean differences between bacterial density (OD_{600}) from the treatment with exposure to bacterial supernatants and the control treatment, with p-values below 0.05 being considered statistically significant. We used the same method as described for the co-culture assay to determine the strength and directionality of direct toxicity of resident bacteria on each other. We also calculated mean intensity of interaction between resident species in supernatant assay (MIF_S) via the method used to calculate the mean intensity of interaction in the co-culture assay. Two-species communities were defined as facilitative when MIF_S > 0, antagonistic when MIF_S < 0.

Measuring resident community toxicity towards *Ralstonia solanacearum* at high and low resource availability

In order to link pairwise interactions between resident species to resident community toxicity towards the pathogen at both high and low resource availability, we quantified pathogen growth in the presence of two-species resident community supernatants, which were collected after growth under the conditions of high (100% NB) or low (10% NB) resource availability, respectively. Briefly, after 36 h of growth in NB or 10% NB medium on a shaker at 170 rpm, 30°C, two-species resident community cultures were filter-sterilized to remove living cells (0.22 μm filter). Subsequently, 20 μl of sterile supernatant from each community's culture and 2 μl overnight culture of *Ralstonia solanacearum* (density of 10^6 cells per ml) were added to 180 μl of fresh NB medium

(10-times diluted, in order to better reflect the effect of the supernatant). Control treatments were inoculated with 20 µl of 10X diluted NB media instead of the community supernatant. Each treatment was conducted in triplicate. Bacteria were grown for 24 h (30°C, 170 rpm) before bacterial densities were measured as optical density at 600 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Pathogen inhibition was defined as the percentage of reduction in pathogen growth compared to pathogen growth in the control treatment.

Measuring invasion success in microcosms at high and low resource availability

Bacterial communities were constructed according to the scheme provided in Supporting Information Table S2. Each resident community was first inoculated into media of the two resource availability treatments (with a starting density of 10^5 cells per ml). The invader *Ralstonia solanacearum* QL-Rs1115 (tagged with the pYC12-mCherry plasmid) was subsequently introduced into all communities (with a starting density of 10^4 cells per ml). Communities were incubated at 30°C with 170 rpm orbital agitation for 36 h (200 µl together with bacteria and medium per well of 96-well plate), a time chosen to allow all communities to reach stationary phase. Invader density was measured as the red mCherry protein fluorescence intensity (excitation: 587 nm, emission: 610 nm) using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Wells contained a total of 200 µl of liquid: 196 µl of media, 2 µl inoculum of constructed resident community and 2 µl inoculum of the invader. Control treatments were inoculated with 2 µl of sterile water instead of the constructed resident community. Each treatment was replicated four times. To control for the auto-fluorescence of resident community, we also grew each community at the two levels of resource availability and subtracted these values from those obtained from co-cultures with the invader.

Statistical analyses

To test how resource availability influences the pairwise interactions between resident species, we used t-tests to compare mean differences between resident bacterial interactions at high and low resource availability levels, with p-values below 0.05 being considered statistically significant. We used linear regression to test whether the secondary metabolites from each resident strain (Mean intensity of interaction between resident species in supernatant) affected the pairwise interactions between resident species (Mean intensity of interaction between resident species in coculture) at high and low resource availability levels.

In order to determine how resource availability-induced changes in pairwise interactions between resident species influence the community resistance to pathogen invasion (Invader density), we used linear regression to analyze relationships between pairwise resident bacterial interactions (Mean intensity of interaction between resident species in coculture) and invader density at low and high resource availability levels. Furthermore, we also used linear regression to disentangle the mechanisms behind the impact of resident bacterial interactions on pathogen invasion at low and high resource availability levels. Specifically, we focused on exploring how changes in the pairwise interactions between resident species, as influenced by resource availability, affected pathogen density via effects on resident community productivity and toxicity towards pathogen. Before all analyses, pathogen density data were log_{10}-transformed to fulfill the parametric model assumptions (i.e. linear regression). All data were analyzed using the R 4.0.2 program (www.r-project.org).

Results

Resource availability influences pairwise interactions between resident bacterial species

Each bacterial species exhibited a different pattern of positive, negative or neutral effects on the other strains, and the magnitude and directionality of these effects were differently influenced by resource availability (Figure 2A, 2B and Figure S1). For example, strain Rp showed a stronger negative effect on strain Ls at low resource availability as compared to at high resource availability, and strain Cd showed a negative effect on strain Fj at low resource availability, but no significant effect on this strain at high resource availability. Overall, resident bacteria produced less or no toxic metabolites in the low-resource availability conditions (Figure 2C), but their interactions were stronger in coculture assays (Figure 2A). For example, the metabolites contained in the supernatant of strain Cd did not significantly inhibit the growth of strain Ba but we observed strong inhibitory effect on strain Ba in coculture (Figure S1). In contrast, supernatants from high-resource media cultures showed stronger negative effects on the growth of other strains (Figure 2D). Moreover, there was a significant relationship between the mean intensity of interaction in coculture and in supernatant at the high resource availability level (R^2=0.59, $P<0.001$, red in Figure 2E), with no relationship being observed at low resource availability (R^2=0.09, P=0.0519, blue in Figure 2E). These results suggest that the pairwise interactions between resident bacterial species were mostly driven by the production of secondary metabolites at high resource availability.

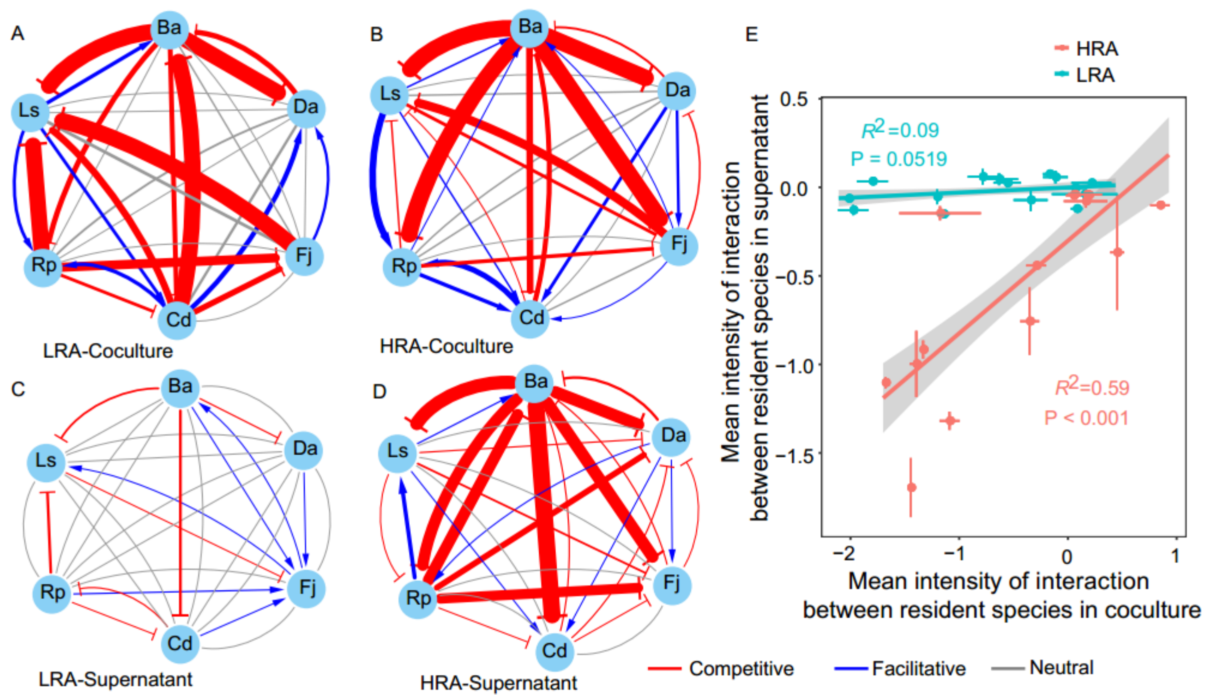

Fig. 2 Resource availability influences pairwise interactions between resident bacterial species. Pairwise interactions at low (A) and high (B) resource availability levels, and toxicity within resident communities at low (C) and high (D) resource availability levels. (E) Relationship between resident pairwise interactions (mean intensity of interaction) in coculture and in supernatant at low and high resource availability levels. Grey shaded areas depict 95% confidence intervals of the logistic regression. Horizontal and vertical lines for each point indicate error bars, which denote mean ± 2 SE. LRA and HRA denote low resource availability and high resource availability, respectively. Coculture refers to the test of pairwise interactions between bacteria in coculture. Supernatant denotes the effects of sterile supernatant from each strain on the other strains.

Changes in resident pairwise interactions due to resource availability impact resident community resistance to pathogen invasion

We examined model resident communities resistance to pathogen invasion as judged by final invader density. We found that higher resource availability led to a reduced density of the invader (Table 1), and there was an interactive effect between resource availability and interaction within the resident community (mean intensity of interaction in coculture) on the density of the invader (Table 1). Invader success correlated

positively with the mean intensity of interaction between resident species at high (R^2=0.35, P<0.001, red in Figure 3) resource availability, but an opposite correlation was observed at low resource availability (R^2=0.4, P<0.001, blue in Figure 3). Compared to positive controls (*Ralstonia solanacearum*-only: red and blue dashed lines in Figure 3), pathogen densities were lower in the presence of resident species at both high and low resource availability.

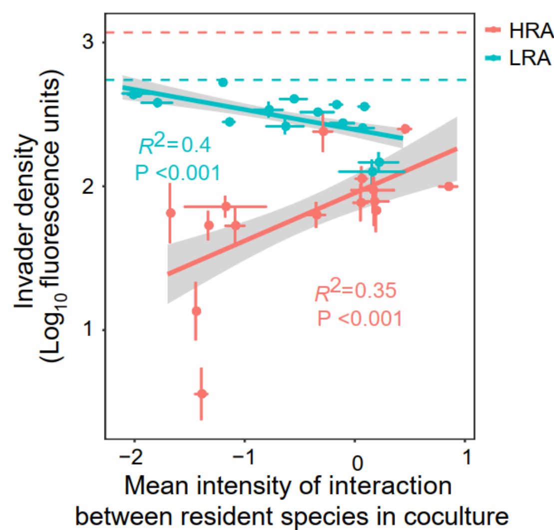

Fig. 3 Resource availability influences the relationship between resident pairwise interactions and resident community resistance to pathogen invasion. The relationship between mean intensity of interaction between resident species in coculture and pathogen invasion success at low and high resource availability, respectively. Red and blue dashed lines show the baseline invader densities in control treatments (invader-only). For the x-axis, values below and above zero denote for competitive and facilitative resident communities, respectively. LRA and HRA denote low resource availability and high resource availability, respectively. Grey shaded areas depict 95% confidence intervals of the logistic regression, and horizontal and vertical lines for each point indicate error bars, which denote mean ± 2 SE.

Table 1. ANOVA table summarizing the interactive effects of pairwise interactions within the resident community and resource availability on the productivity of resident communities, direct pathogen inhibition by resident communities and density of the invader. Significant effects (P < 0.05) are highlighted in bold and the 'up' and 'down' arrows denote positive and negative effects respectively.

	Invader density			**Resident community toxicity towards invader**			**Productivity of resident community**		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Mean intensity of interaction in coculture (MIF)	1	1.21	0.2741	1	7.30	**0.008↓**	1	108.32	**<0.001↑**
Resource availability (RA)	1	145.06	**<0.001↓**	1	65.96	**<0.001↑**	1	230.08	**<0.001↑**
MIF * RA	1	39.32	**<0.001↑**	1	22.55	**<0.001↓**	1	1.72	0.1938
No. of Residuals	86			86			86		

Mechanisms by which resource availability modulates two-species bacterial community resistance to pathogen invasion

To disentangle potential mechanisms underlying the observed patterns of two-species bacterial community resistance to pathogen invasion at low and high resource availability, based on our hypothesis, we focused on exploring how changes in the pairwise interactions between resident species, as influenced by resource availability, affected pathogen density via effects on resident community productivity and toxicity towards pathogen.

We found that higher resource availability led to a greater level of resident community toxicity towards pathogen, and there was an interactive effect between resource availability and interaction within the resident community (mean intensity of interaction in coculture) on resident community toxicity towards pathogen (Table 1). Higher resource availability also led to increased productivity of the resident community, but there was no interactive effect between resource availability and interactions within resident community on the productivity of the resident community (Table 1).

At low resource availability, interactions between resident species (mean intensity of interaction in coculture) had no effect on resident community toxicity towards pathogen (R^2=0.019, P=0.3622, blue in Figure 4A) which negatively influenced invader density (R^2=0.13, P=0.017, blue in Figure 4B). However, interactions between resident species were positively correlated with productivity of the resident community (R^2=0.39, P<0.001, blue in Figure 4C), which was in turn negatively linked with invader density (R^2=0.33, P<0.001, blue in Figure 4D).

In high resource availability conditions, the mean intensity of interaction between resident species in coculture was negatively correlated with resident community toxicity towards pathogen (R^2=0.39, P<0.001, red in Figure 4A) which was negatively

linked with invader density (R^2=0.33, P<0.001, red in Figure 4B). This result suggests that competitive resident communities were also more inhibitory toward the pathogen. Mean intensity of facilitation between resident species was positively correlated with productivity of the resident community (R^2=0.47, P<0.001, red in Figure 4C) which was positively linked with invader density (R^2=0.4, P<0.001, red in Figure 4D).

Moreover, resident community toxicity towards pathogen was negatively correlated with productivity of the resident community at high resource availability (R^2=0.27, P<0.001, red in Figure S2), but not at low resource availability (blue in Figure S2). Together these results suggest that facilitative communities that reached higher total densities suppressed the invader more efficiently in low resource availability conditions. High resource availability conditions yielded a contrasting pattern in which competitive communities that achieve lower total community densities, but produced more direct antagonist compounds, provided a stronger inhibition of the pathogen growth.

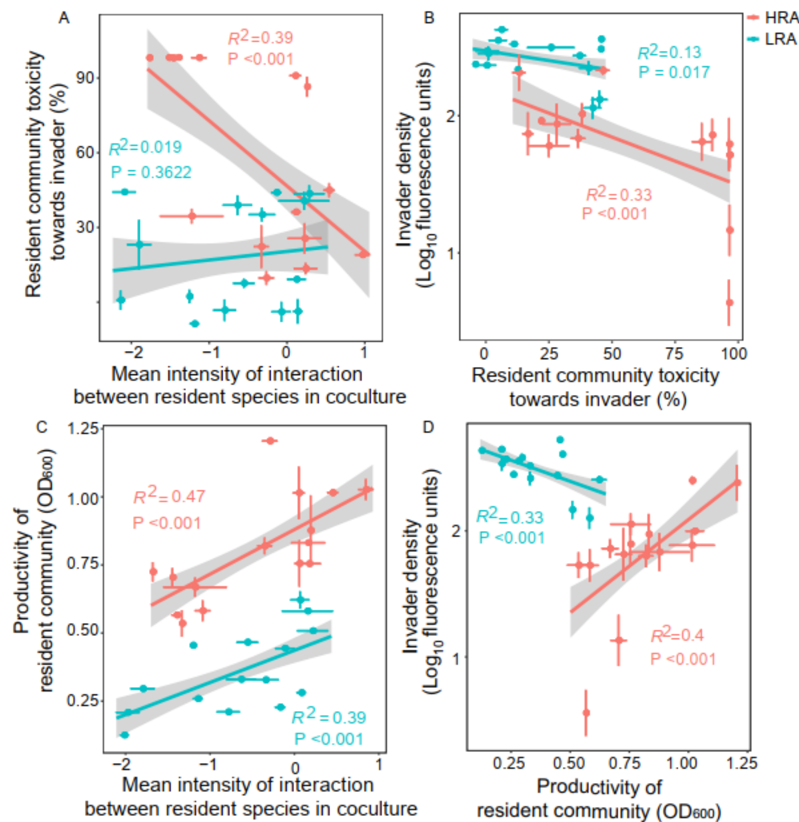

Fig. 4 The relationships between mean intensity of interaction in coculture and (A) resident community toxicity towards pathogen, (C) productivity of two-species resident communities at low and high resource availability. For the x-axis of panel A and C, values below and above zero denote for competitive and facilitative resident communities, respectively. The relationships between invader density and (B) resident community toxicity towards pathogen, (D) productivity of two-species resident communities at low and high resource availability. LRA and HRA denote low resource availability and high resource availability, respectively. Grey shaded areas depict 95% confidence intervals of the logistic regression, and horizontal and vertical lines for each point indicate error bars, which denote mean ± 2 SE.

Discussion

Microbial communities are structured by interactions between their constituent species in the context of their abiotic environment [94]. Interactions within a resident community can have important consequences for ecosystem functions such as the ability of the community to resist biological invasions [121, 210]. For example, it has been observed

that communities with a greater proportion of competitive interactions are better able to constrain invader growth than more facilitative communities [121]. However, the nature of microbial interactions is impacted by environmental conditions such as the level of nutrient availability, and how such impacts are related to community resistance to biological invasion is generally unknown. In this study, we explored how changes in resource availability affect relationships between interactions within model resident communities and their ability to resist pathogen invasion. At high resource availability, we found that more competitive resident communities produced more direct antagonism and subsequently better resisted pathogen invasion than more facilitative communities. At low resource availability, facilitative communities that reached higher total productivity were more resistant to pathogen invasion than more competitive communities. Understanding how resource availability influences the interactions between resident bacteria is thus important for predicting the dynamics and outcomes of biological invasions.

In line with a recent study, we found that interactions between resident bacterial species were mostly driven by the production of secondary metabolites in high resource availability conditions [97]. Given the results of our assays (supernatant assays) involving exposure to spent media, it appeared that the observed microbial interactions involved modification of the environment, for instance via the production of inhibitory compounds. In high resource availability conditions, microbes have sufficient substrates to produce relatively large amounts of inhibitory compounds, thereby leading to a greater level of influence on other microbial strains [214-216]. Interestingly, we found resident bacteria produced little or no toxic metabolites in low resource availability conditions, but there were strong competitive interactions between them in coculture assays. This suggests that interactions at low resource availability

were not driven by the production of toxic metabolites but by competition for resources, presumably because there were not sufficient resources to allow for large investment into the production of such secondary metabolites [217].

Resource availability showed a strong negative effect on the density of the model pathogen invader (Table 1), which contrasts with other studies [88, 159, 218]. For example, Mallon *et al*. found that increasing resource availability may promote *E. coli* invasions due to relaxed resource competition [88]. This discrepancy may be explained by the fact that bacteria were likely able to consume all the available resources during our experiments without addition of new external resources [76]. Furthermore, resident communities produced inhibitory compounds that reduced the growth of invader *Ralstonia solanacearum* under conditions of high resource availability as compared to low resource availability conditions (Table 1 and Figure S3).

We also observed an interactive effect between the pairwise interactions within the resident community and resource availability conditions on invader density. Mechanistically, the greater resistance to pathogen invasion of competitive communities at high resource availability level was linked to direct inhibition of the invader by antagonistic communities (Figure 4A and B). However, facilitative communities were more resistance to pathogen invasion at the low resource availability level, which can be explained by the fact that facilitative communities reached higher total community productivity (Figure 4C and D). Our results suggest that in high resource availability conditions, antagonism is an important determinant of community invasion resistance [97, 121]. This result is also in line with a previous finding, where the increase in the antagonistic activity was found to increase the invasion resistance of *Pseudomonas* resident communities [84]. As our results suggested that interactions in low resource availability conditions were driven by

competition for resources, communities that showed higher productivity may be able to better occupy niche spaces, thereby outcompeting invaders [69, 219, 220].

We conclude that resource availability can modulate bacterial community resistance to pathogen invasion by changing pairwise interactions within the resident community. In many cases, microbial interactions may not be driven by a single parameter — resource availability in our case — but by a set of multiple parameters such as pH, temperature or other environmental factors. However, also in these cases, interactions are mediated by modifying and reacting to the environment [60, 93], which can influence the community functions. Consequently, our framework should be expandable to multivariate systems and may thus lay the basis for understanding how more complex microbial interactions, and the functions they yield at the community level, are impacted by changing environmental conditions.

Acknowledgements

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Supplementary materials

Table S1. Bacterial strains used in this study.

Bacterial strain ID	Taxonomic affiliation (GenBank accession number)	Bacterial abbreviation used in this study	Reference
WR4	*Flavobacterium johnsoniae* (CP000685)	Fj	(Huang et al. 2013)
T-5	*Bacillus amyloliquefaciens* (JF899265)	Ba	(Tan et al. 2013)
HR92	*Lysinibacillus sphaericus* (CP000817)	Ls	(Huang et al. 2013)
WR21	*Chryseobacterium daecheongense* (HQ220102)	Cd	(Huang et al. 2013)
WR42	*Delftia acidovorans* (AM180725)	Da	(Huang et al. 2013)
QL-A6	*Ralstonia pickettii* (HQ267096)	Rp	(Wei *et al*. 2013)
QL-Rs1115	*Ralstonia solanacearum* (GU390462) tagged with red fluorescent marker (PYC12-M plasmid)	Rs	(Wei et al. 2011; Tan et al. 2016)

Table S2. Combinations of resident species community at one- or two-species levels (N=21). Table rows show different communities and table columns show the absence (0) or presence (1) of given species within the community.

Community ID	Species abbreviation						Resident community species richness
	Fj	Cd	Da	Ba	Ls	Rp	
1	1	0	0	0	0	0	1
2	0	1	0	0	0	0	1
3	0	0	1	0	0	0	1
4	0	0	0	1	0	0	1
5	0	0	0	0	1	0	1
6	0	0	0	0	0	1	1
7	1	1	0	0	0	0	2
8	1	0	1	0	0	0	2
9	1	0	0	1	0	0	2
10	1	0	0	0	1	0	2
11	1	0	0	0	0	1	2
12	0	1	1	0	0	0	2
13	0	1	0	1	0	0	2
14	0	1	0	0	1	0	2
15	0	1	0	0	0	1	2
16	0	0	1	1	0	0	2
17	0	0	1	0	1	0	2
18	0	0	1	0	0	1	2
19	0	0	0	1	1	0	2
20	0	0	0	1	0	1	2
21	0	0	0	0	1	1	2

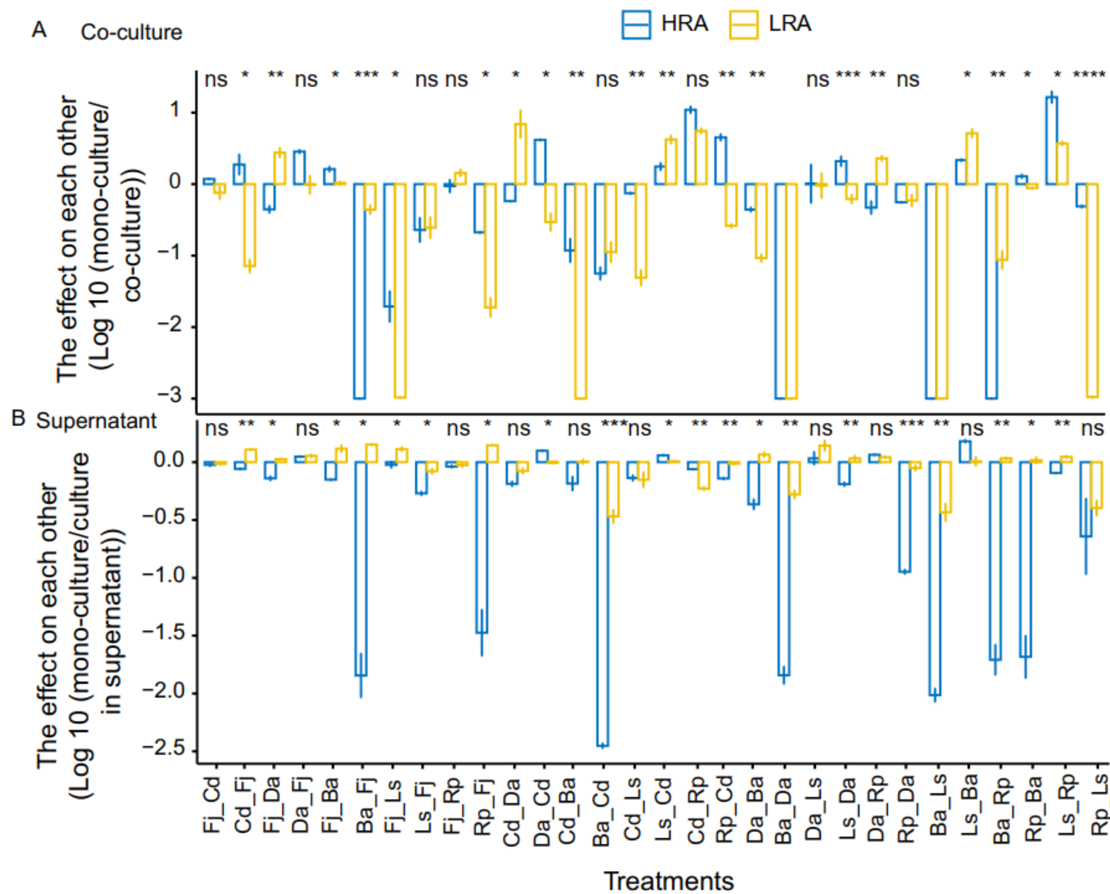

Figure S1. Resource availability influences pairwise interactions between resident bacterial species both in co-culture assay (A) and supernatant assay (B). Fj_Cd means effect of Species Fj on the growth of species Cd. LRA and HRA denote low resource availability and high resource availability, respectively. Bars show mean values ± SE (n = 3). *p<0.05, **p < 0.01, ***p<0.001, and ns denotes no significant difference based on t-test.

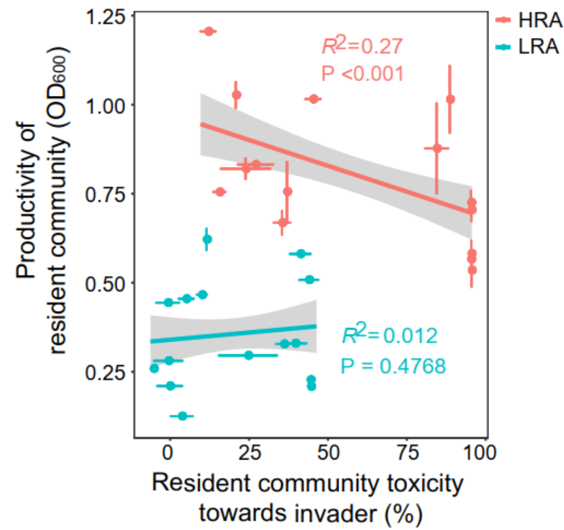

Figure S2. The relationship between direct pathogen inhibition by two-species resident community and productivity of two-species resident community at low and high resource availability, respectively. LRA and HRA denote low resource availability and high resource availability, respectively. Grey shaded areas depict the 95% confidence interval of the logistic regression, horizontal and vertical lines in each dot in the figures are error bars, which denote for ± 2 SEM.

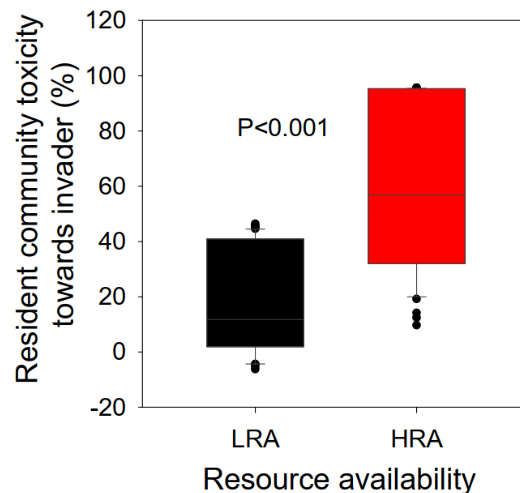

Figure S3. Resource availability influences direct pathogen inhibition by two-species resident communities. LRA and HRA denote low resource availability and high resource availability, respectively. P<0.001 indicates significant differences between LRA and HRA based on Mann-Whitney Rank Sum Test.

Chapter 6 General Discussion

A range of soil-borne diseases are increasingly threatening agricultural production around the world [20, 21]. As production demands increase, there is a pressing need to reduce the use of environmentally unfriendly pesticides and agrochemicals [221]. To achieve this, plant root-associated microbiomes are increasingly seen as a possible driver of natural pathogen resistance and have become a relevant target for innovative strategies aiming at improving crop protection [15, 34, 35]. However, while some microbiomes are better at preventing pathogen growth than others, it often remains unclear which interactions shape actual pathogen success.

With this in mind, this thesis used bacterial wilt disease in tomato, which is caused by pathogen *Ralstonia solanacearum,* as model system, with the aim to investigate plant health-associated bacterial interactions in the tomato rhizosphere. I first sought to assess which microbiome characteristics are important for tomato bacterial wilt disease suppression. In doing so, I have combined direct examination of the plant-associated microbiomes from healthy and diseased rhizosphere soils with interaction studies in the laboratory of bacterial isolates recovered from these soils. Results showed that rhizosphere bacterial communities were one of the important factors influencing the manifestation of disease (**Chapter 2**). However, it still remained unclear which interactions shaped microbial assemblages in this process. Furthermore, the contribution of microbe-microbe interactions to the overall community structure remained difficult to evaluate in the field due to the strong environmental noise encountered. Fortunately, the extensive library of rhizobacterial isolates I recovered from tomato rhizosphere soils provided an excellent resource to further elucidate the specific microbial interactions involved in community resistance to pathogen invasion.

I subsequently used controlled systems with synthetic bacterial communities

which allowed me to disentangle the role of specific organisms and their interactions in the ecological processes that associated with bacterial wilt disease suppression. In these simplified systems, I identified how direct and indirect interactions among rhizobacteria affect community functions, such as resident community resistance to pathogen invasion. In particular, I assessed how interactions among pathogen antagonist strains influence pathogen invasion (**Chapter 3**) and if control of the pathogen can be achieved indirectly though inhibition of pathogen helper strains (**Chapter 4**). Results showed that in addition to the direct interactions between rhizobacterial isolates and the pathogen, indirect effects from interactions among rhizobacterial isolates were more important for determining pathogen success (Figure 1, **Chapter 3 and Chapter 4**). However, microbial interactions are sensitive to a range of environmental factors, such as resource availability [92]. To take this into account, I examined the extent to which microbial interactions were influenced by resource availability and found different levels of resource availability altered bacterial pairwise interactions with effects on community resistance to pathogen invasion (Figure 1 and **Chapter 5**). Taken together, these results form the basis of more informed management strategies based on microbial interactions and community assembly, ultimately aiming to improve soil-borne disease suppressive potential in a targeted fashion without the use of pesticides.

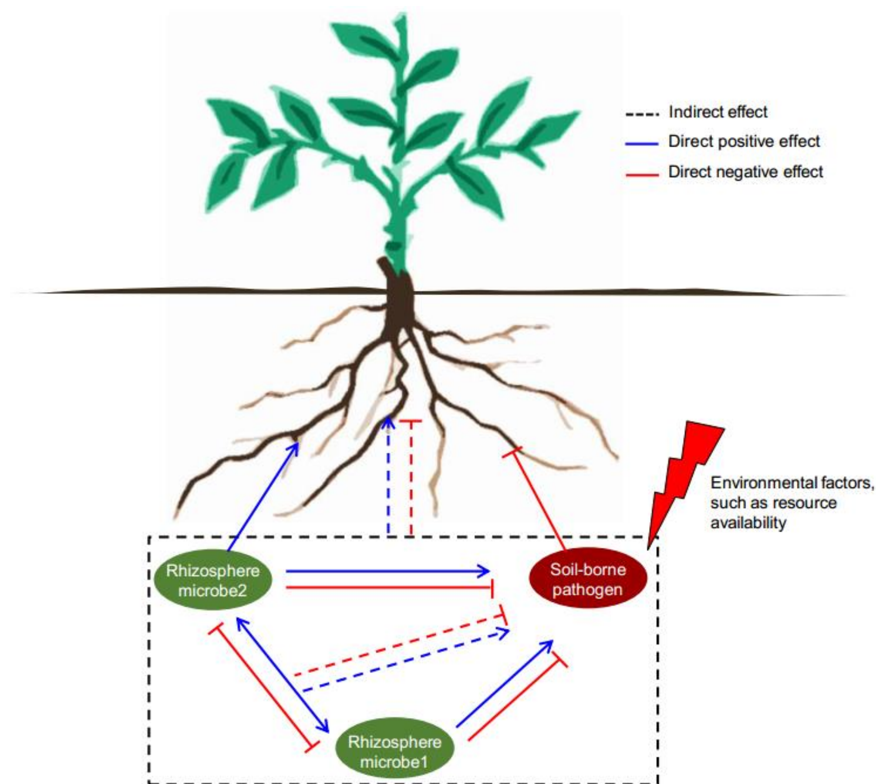

Figure 1. Conceptual overview of direct and indirect effects of rhizosphere microbes and environmental factors on the pathogen and plant growth. Rhizosphere microbes such as pathogens can directly affect the plant growth; both the direct interactions between rhizosphere microbes and pathogen, and the indirect effects from interactions between rhizosphere microbes were important determinants of pathogen success and plant growth. The nature of these interactions is also influenced by environmental factors such as imposed levels of resource availability.

Microbial communities contribute to the suppression of soil-borne plant disease

The rhizosphere microbiome is recognized as a major determinant of plant growth and health. The impact of the rhizosphere microbiome on plant health is demonstrated most clearly by disease-suppressive soils [25]. In these soils, plants are less affected by pathogenic microbes due to protection afforded by their surrounding microbiota. Characterizing microbial communities in such soils therefore is a crucial step towards developing management strategies to developing microbial communities favoring crop

health and productivity [32] and is a necessary first step to develop informed management to control of soil-borne diseases [222]. In **Chapter 2**, I found distinct differences in the microbiomes of diseased versus healthy rhizosphere soil samples. Furthermore, although both diseased and healthy rhizosphere soils harboured relatively densities of *Ralstonia solanacearum* (>10^6 CFU/g rhizosphere soil), diseased soils contained a higher density of the pathogen. These results suggest that the plant-associated microbiome, and interactions amongst its members may play an important role in inhibiting disease development [34, 223]. I therefore further sought to assess how interactions among rhizosphere microbial species were associated with observed reductions in pathogen invasion and disease incidence.

Importance of rhizosphere microbial interactions for plant health

Functions of ecological communities are not only based on direct interactions between species, but also determined by indirect interactions that occur via cascades of direct interactions or by changing the nature of direct interactions [39, 40]. Microbial systems are excellent models for analyzing these types of interactions. Microorganisms in the plant rhizosphere not only interact with each other positively or negatively, but also interact directly and indirectly. These interactions are important for community assembly, which will ultimately affect the community functions, such as community resistance to pathogen invasion and subsequent support of plant health [17].

Interactions between resident communities and pathogens directly affect pathogen success

Pathogens must first establish themselves in the host-associated microbial communities in order to cause a disease. Thus the direct interactions between resident

communities and pathogen are expected to influence pathogen success. The rhizobacterial strains from our collection exhibited a wide range of effects on the pathogen, and supernatants from a sizable proportion of them inhibited *Ralstonia solanacearum* (**Chapter 2**). While it remains unclear what exact compounds were produced in the supernatant by these antagonist strains from our collection, previous studies have shown that soil bacteria are capable of producing a wide variety of antimicrobials that are capable of suppressing *Ralstonia solanacearum* [84, 85]. For example, the *Bacillus amyloliquefaciens* has been shown to efficiently suppress *Ralstonia solanacearum* both in the lab and plant rhizosphere [85] and this species also showed the strongest negative effect on the pathogen densities and disease incidence in this study (**Chapter 3**). While such antagonist strains are acknowledged as important resources for potential biocontrol strategies, recent studies have also shown that some root-associated bacteria are able to facilitate as opposed to antagonize pathogen growth [59, 64] (**Chapter 2**). Two helper strains from our collection whose supernatants showed facilitative effects on the growth of *Ralstonia solanacearum in vitro* also can facilitate *Ralstonia solanacearum* in the tomato rhizosphere (**Chapter 4**), indicating that these helper strains have high potential to promote disease. Several mechanisms have been put forth to explain mutualism or commensalism among bacteria, mainly as related to the benefits gained from the use of metabolites processed by another member of the community [201]. For instance, peptidoglycan produced by *Bacillus cereus* may promote the growth of several bacterial strains affiliated with the *Cytophaga-Flavobacterium* group [202]. Siderophores produced by microorganisms can also be accepted as public goods by several other bacteria with siderophore protein receptors to obtain limited iron from the environment to maintain growth and metabolism, hence increasing population biomass

[203]. Given the fact that the facilitation observed in our studies was stemming from used supernatant assays (**Chapter 2** and **Chapter 4**), it is highly likely that certain metabolites mediate this facilitation. Although beyond the scope of this study, it would be of great interest to determine the exact mechanisms and metabolites involved.

Interactions within resident communities indirectly affect pathogen success

In **Chapter 3** and **Chapter 4**, interactions within resident communities were demonstrated to be good predictors of invasion inhibition. Interestingly, the relative importance of direct interactions between resident community members and the pathogen were found to be less important than the impacts of indirect effects from interactions within resident communities in determining pathogen success. Together, these results suggest that interactions among species within the resident community can be an important determinant of invasion success *in vivo* and *in vitro*, in addition to the previously known direct interactions between resident community members and the invader.

Facilitative resident community interactions promoted, and competitive interactions suppressed, invasions both in the lab and in the tomato rhizosphere. Mechanistically, this was linked to direct inhibition of the invader by antagonistic communities (antibiosis), and to a lesser degree by resource competition between members of the resident community and the invader (**Chapter 3**). Competing species can inhibit each other directly by producing toxic metabolites, such as antibiotics. Depending on the spectrum of their activity, antibiotic compounds could have negative effects on both resident community species and the invader [80-85]. In line with this, our results showed competitive resident communities were more inhibitory not only towards their members, but also against the invader (**Chapter 3**). While it is difficult to

pinpoint the exact mechanism linking facilitation and invasion, the most likely explanation is the loss of pathogen inhibition along with the increase in facilitative interactions (**Chapter 3**). This is in line with a previous finding where the increase in the antagonistic activity was found to increase the invasion resistance of *Pseudomonas* resident communities [84]. Furthermore, I found that facilitative communities were more productive in general and reached higher total cell densities when cultured together compared to alone (**Chapter 3** and **Chapter 5**). This supports the idea that facilitative resident species were benefitting from the presence of each other (for example via cross-feeding or producing public goods), which could have also benefitted the invader by creating additional niche space [89].

In **Chapter 4**, I found that the indirect effects, *i.e.* inhibition of helper strains, were the major determinants of pathogen inhibition as compared to direct impacts on the pathogen itself both *in vitro* and *in vivo*. Even if a biocontrol agent is active against *Ralstonia solanacearum* [51, 84], its efficiency in reality may be more due to its interaction with indigenous helpers. I therefore propose that strategies for integrated biological control of the pathogen need to be reconsidered to incorporate indirect effects on pathogen helpers to provide more ecological solutions to combat soil-borne pathogens.

Context dependency of microbial interactions

The results of this thesis illustrated that bacterial interaction/function relationships are context dependent. As a representative abiotic factor, I investigated different resource availability conditions. Our results showed that while facilitative resident communities were more prone to invasions and competitive resident communities were invaded to a lesser extent in **Chapter 3**, this relationship was shifted by changes in

resource availability (**Chapter 5**). At high resource availability, interactions between resident bacterial species were mostly driven by the production of secondary metabolites [97], with direct antagonism as the means of invader inhibition. Therefore, competitive resident communities were invaded to a lesser degree than facilitative communities. At low resource availability, bacteria produced little or no direct antagonist potential [217], which had little influence on the interactions between them, as well as their collective impact on pathogen inhibition. However, facilitative communities did reach higher community productivity (**Chapter 3** and **Chapter 5**), which in turn may have more resistance to pathogen invasion than competitive communities at low resource availability. Due to this higher productivity, such communities may be able to better occupy available niche space more fully, thereby outcompeting invaders [69, 219, 220]. In many cases, microbial interactions may not be driven by a single parameter — resource availability in our case — but by a set of multiple parameters such as pH [93], temperature [188] or other environmental factors. Therefore, in the study of microbial interactions in the rhizosphere, it is important to consider the effects of relevant environmental factors.

Conclusions and future perspectives

While most previous studies have sought to examine how rhizosphere microbiota can directly impacts pathogen populations, this thesis has demonstrated that the indirect effects from interactions between plant-associated microbial community members can also be strong determinants of disease development. For instance as related to disease suppression, my work showed that in addition to the direct interactions between rhizobacterial isolates and the soil-borne pathogen, the indirect effects from interactions among rhizobacterial isolates were an important determinant

of pathogen success. Therefore, I propose a rethinking of the current approaches used to manage plant diseases in agricultural systems. Instead of a pathogen-focused view, better solutions for controlling plant disease outbreaks may be achieved by managing the composition of the soil microbiome as a whole. For example, instead of attempting to directly reduce pathogen densities, controlling microbiome composition to prevent the growth of pathogen helpers may become part of sustainable strategies of pathogen control.

Organismal interactions are clearly context dependent, and there is a general interest in the broader field of ecology of how pairwise interactions fit into the web of interactions that ultimately determine community function. Within this thesis, I have performed experiments employing both holistic and reductionist approaches to investigate the importance of interactions within rhizosphere microbial communities for plant health. Such approaches allowed me to look at microbial interactions using a large number of isolates under different types of environmental conditions. Due to their short generation times, potential for high levels of reproducibility and manipulations in diverse laboratory environments, microbial systems have distinct advances of such experiments using plants and animals. I believe that such approaches involving microbial communities have the potential to play an important role in more critical testing and development of general ecological theory related to the roles of organismal interactions in community assembly functioning. For instance, it is rather straightforward to test effects of resource availability on interactions between microbes, while this would be much more difficult to test for other organisms such as birds for instance.

It should be noted that natural plant rhizosphere is far more complex than the simple artificial systems I have used in this thesis. In future studies, it would be interesting in moving toward more realistic conditions, increasing environmental complexity and including biotic or abiotic factors in studies on the interaction dynamics of microbes in plant-associated microbial communities. My goal would be to build a more holistic understanding of how all soil microbial taxa interact directly or indirectly, instead of just studying individual groups in isolation (bacterial strains in my case). In this context, various meta-genomic sequencing approaches could also be utilized to further complement the experimental approaches presented in this thesis. With such systematic investigations we may be able to unravel the complex interactions between plants, the environment, and their microbiomes, opening up new possibilities for the development of soil management strategies for sustainable control of soil-borne disease.

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Summary

A range of soil-borne diseases are increasingly threatening agricultural production around the world. As production demands increase, there is a pressing need to reduce the use of environmentally unfriendly pesticides and agrochemicals. To achieve this, plant root-associated microbiomes are increasingly seen as a possible driver of natural pathogen resistance and have become a target for innovative strategies aiming at improving crop protection. However, the ability of rhizosphere microbial communities to keep diseases under control is influenced by many factors, including the microbial interactions within these communities. Unfortunately, we still have relatively little insight into how microbial interactions affect community assembly and how such interactions eventually impact plant health. This thesis seeks examine how microbial interactions within the rhizosphere microbiome impact the ability of plant pathogens proliferate and cause plant disease. To this end, this thesis used bacterial wilt disease in tomato plant, which is caused by pathogen *Ralstonia solanacearum* as a relevant model system.

In **Chapter 2**, I have combined direct examination of the plant-associated bacterial communities from healthy and diseased tomato rhizosphere soils with interaction studies in the laboratory of bacterial isolates recovered from these soils. Results showed that rhizosphere microbial communities were one of the important factors influencing the manifestation of disease. However, correlation analyses showed discrepancies between co-occurrence patterns and direct strain interactions, which suggests that positive or negative links within co-occurrence networks are poor predictors of actual interactions upon examination of one-to-one effects interactions.

I subsequently used controlled systems with synthetic microbial communities which allowed me to disentangle the role of specific organisms and their interactions in the

ecological processes associated with bacterial wilt disease suppression. In these simplified systems, **Chapter 3** assessed how interactions among pathogen antagonist strains influence pathogen invasion and **Chapter 4** tested if control of the pathogen can be achieved indirectly though inhibition of pathogen helper strains. Results showed that in addition to the direct interactions between rhizobacterial isolates and the pathogen, indirect effects from interactions among rhizobacterial isolates were more important for determining pathogen success. These suggest that instead of a pathogen-focused view, better solutions for controlling plant disease outbreaks may be achieved by managing the composition of the soil microbiome as a whole.

Chapter 5 examined the extent to which bacterial interactions were influenced by resource availability and found different levels of resource availability altered bacterial pairwise interactions with effects on community resistance to pathogen invasion. At high resource availability, competitive resident communities produced more antibiotics, making them less susceptible to invasion compared to more facilitative communities. At low resource availability, facilitative communities reached higher productivity, which in turn be more important for resistance to pathogen invasion than competitive interactions in less productive communities. Therefore, in the study of microbial interactions in the rhizosphere, it is important to consider the effects from relevant environmental factors.

Taken together, the results of this thesis form the basis of more informed management strategies based on microbial interactions and community assembly, ultimately aiming to improve soil-borne disease suppressive potential in a targeted fashion without the use of pesticides.

Samenvatting (Dutch summary)

Diverse uit de bodem afkomstige ziekten vormen een steeds grotere bedreiging voor de landbouwproductie over de hele wereld. Omdat de vraag naar productie toeneemt, is er een dringende noodzaak om het gebruik van milieuonvriendelijke pesticiden en landbouwchemicaliën te verminderen. Om hieraan te voldoen worden geassocieerd met plantenwortels microbiomen, steeds vaker gezien als een mogelijke oplossing om de natuurlijke resistentie tegen ziekteverwekkers te vergroten. Bodemmicroorganismen zijn het focus geworden van innovatieve strategieën die gericht zijn op het verbeteren van de gewasbescherming. Het vermogen van microbiële gemeenschappen in de rhizosfeer om ziekten onder controle te houden, wordt echter beïnvloed door vele factoren, waaronder de microbiële interacties binnen deze gemeenschappen. Helaas hebben we nog steeds relatief weinig inzicht in hoe microbiële interacties de samenstelling van de gemeenschap beïnvloeden en hoe dergelijke interacties uiteindelijk de gezondheid van planten beïnvloeden. Dit proefschrift wil onderzoeken hoe microbiële interacties binnen het microbioom van de rhizosfeer van invloed zijn op het vermogen van plantpathogenen om zich te vermenigvuldigen en plantenziekten te veroorzaken. Hiervoor heeft dit proefschrift de bacteriële verwelkingsziekte bij tomatenplanten, veroorzaakt door het pathogeen *Ralstonia solanacearum*, gebruikt als een relevant modelsysteem.

In **Hoofdstuk 2** heb ik onderzoek van de plant-geassocieerde microbiomen uit de bodemrhizosfeer van gezonde en zieke tomaten rechtstreeks gecombineerd met interactiestudies in het laboratorium met bacteriële isolaten die uit deze bodems zijn gewonnen. De resultaten toonden aan dat microbiële gemeenschappen in de

rhizosfeer een van de belangrijke factoren waren die de manifestatie van ziekte beïnvloedden. Correlatieanalyses lieten echter discrepanties zien tussen patronen van co-occurrence bepaald met behulp van moleculaire technieken t.o.v. directe microbiele stam interacties bepaald met kweek analyses. Dit suggereert dat positieve of negatieve verbanden binnen netwerken die samen voorkomen, slechte voorspellers zijn van daadwerkelijke één-op-één interacties van bacteriële populaties.

Vervolgens heb ik gecontroleerde systemen met synthetische microbiële gemeenschappen gebruikt om de rol te ontwarren van specifieke organismen en hun interacties in de ecologische processen geassocieerd met de onderdrukking van bacteriële verwelkingsziekten. In deze vereenvoudigde systemen onderzocht **Hoofdstuk 3** hoe interacties tussen stammen, antagonistisch tegen het pathogeen, de invasie hiervan beïnvloeden. **Hoofdstuk 4** testte of controle over het pathogeen indirect kan worden bereikt door remming van pathogene helperstammen. De resultaten toonden aan dat naast de directe interacties tussen rhizobacteriële isolaten en de ziekteverwekker, indirecte effecten van interacties tussen rhizobacteriële isolaten ook een sterke bepalende factor kunnen zijn voor het succes van ziekteverwekkers. Deze resultaten suggereren dat in plaats van een puur pathogeengerichte visie, betere oplossingen voor het beheersen van uitbraken van plantenziekten kunnen worden bereikt door de samenstelling van het bodemmicrobioom als geheel te beheren.

Hoofdstuk 5 onderzocht de mate waarin microbiële interacties werden beïnvloed door de beschikbaarheid van hulpbronnen. Ik ontdekte dat verschillende niveaus van beschikbaarheid van hulpbronnen de paarsgewijze interacties van bacteriën veranderden met effect op de weerstand van de gemeenschap tegen invasie van

pathogenen. Bij een hoge beschikbaarheid van hulpbronnen produceerden concurrerende de al aanwezige gemeenschappen meer antimicrobiële verbindingen, waardoor ze minder vatbaar waren voor invasie dan in vergelijking met meer faciliterende gemeenschappen. Bij lage beschikbaarheid van hulpbronnen bereikten faciliterende gemeenschappen een hogere productiviteit, wat op zijn beurt belangrijker was voor de weerstand tegen invasie van pathogenen dan de competitieve interacties in dergelijke minder productieve gemeenschappen. Daarom is het bij de studie van microbiële interacties in de rhizosfeer belangrijk om rekening te houden met de effecten van relevante omgevingsfactoren, zoals de beschikbaarheid van voedingsstoffen.

Alles bij elkaar vormen de resultaten van dit proefschrift de basis voor beter geïnformeerde managementstrategieën gebaseerd op kennis van microbiële interacties en gemeenschapsassemblage, met als uiteindelijk doel het ziekteonderdrukkend vermogen van de bodem op een gerichte manier te verbeteren zonder het gebruik van pesticiden.

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Curriculum Vitae

Mei Li (李梅), born on 1 April 1992 in DaLi, Yunnan, China. She has a great interest in biology and nature since she grew up in a small but very beautiful village in DaLi. After attending High School in her hometown, she started her 4-year bachelor study in September 2011 in Nanjing Agricultural University, majoring in Agricultural Resources and Environment. In the last year of her bachelor study, she started an internship in the lab of Prof. Yangchun Xu from Prof. Qirong Shen's research group, to investigate how diversity of bacterial community affects community to suppress soil-borne disease. After her graduation in June 2015, she joined the same lab and directly followed a PhD program which focuses on soil microbial ecology, under the supervision of Prof. Yangchun Xu and Prof. Zhong Wei. In 2019, She obtained a scholarship from China Scholarship Council, which allowed her to continue her PhD project in Ecology & Biodiversity group at Utrecht University, the Netherlands, supervised by Prof. George A. Kowalchuk, Dr. Thomas Pommier (Université Lyon 1) and Dr. Alexandre Jousset. This thesis is the results of her PhD project.